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Facilitated bio-mineralization of N,N-dimethylformamide in anoxic denitrification system: Long-term performance and biological mechanism

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ABSTRACT

Due to highly recalcitrant and toxicological nature of N,N-dimethylformamide (DMF), efficient removal of DMF is challenging for biological wastewater treatment. In this study, an anoxic denitrification system was developed and continuously operated for 220 days in order to verify the enhanced DMF biodegradation mechanism. As high as 41.05 mM DMF could be thoroughly removed in the anoxic denitrification reactor at hydraulic residence time (HRT) of 24 h, while the total organic carbon (TOC) and nitrate removal efficiencies were as high as 95.7 ± 2.5% and 98.4 ± 1.1%, respectively. Microbial community analyses indicated that the species related to DMF hydrolysis (*Paracoccus, Brevundimonas* and *Chryseobacterium*) and denitrification (*Paracoccus, Arenimonas, Hyphomicrobium, Aquamicrobium* and *Bosea*) were effectively enriched in the anoxic denitrification system. Transcriptional analysis coupled with enzymatic activity assay indicated that both hydrolysis and mineralization of DMF were largely enhanced in the anoxic denitrification system. Moreover, the occurrence of microbial denitrification distinctly facilitated carbon source utilization to produce electron and energy, which was rather beneficial for better reactor performance. This study demonstrated that the anoxic denitrification system would be a potential alternative for efficient treatment of wastewater polluted by recalcitrant pollutants such as DMF.

1. Introduction

N,N-dimethylformamide (DMF) [$(CH_3)_2NCHO$], an anthropogenic organic polar solvent, has been widely employed in chemical, pharmaceutical and textile industries, because it is miscible with water and a wide variety of organic matters (Kong et al., 2018a; Zhao et al., 2018). With the increasing production and consumption of DMF, wastewater containing DMF will be inevitably released into the environment (Välitalo et al., 2017; Torres et al., 2018; Prasse et al., 2015). It was estimated that more than 5000 tons of DMF was discharged or transferred into the environment every year via the discharge of wastewater from related industries (Kong et al., 2018a). DMF displays adverse impacts on human health and environmental quality due to its numerous toxic effects such as embryotoxicity, hepatotoxicity, teratogenicity and carcinogenicity (Swaroop et al., 2009; Kumar et al., 2012). Moreover, DMF is rather persistent in natural environment because of its thermal stability (Kong et al., 2018a). Accordingly, there is an urgent need to develop high-efficient and environmental-friendly methods to remove DMF from wastewater.

Although various physico-chemical methods, such as advanced oxidation process (Sun et al., 2008), photodegradation (Zhao et al., 2018), membrane separation (Das et al., 2006) and physical adsorption (Ye et al., 2013), are available for DMF removal from contaminated wastewater, biological treatment has turned out to be a favorable alternative, because of its merits such as low cost and environmental friendliness (Kong et al., 2018a; Swaroop et al., 2009; Kumar et al., 2012). To date, DMF biodegradation under aerobic condition has been extensively investigated (Swaroop et al., 2009; Zhou et al., 2018). Some species such as *Paracoccus* (Nisha et al., 2015; Zhou et al., 2018), *Ochrobactrum* (Veeranagouda et al., 2006) and *Alcaligenes* (Hasegawa et al., 1997) were found to be capable of degrading DMF under aerobic condition. However, the application of aerobic process was often limited by high aeration costs and disgusting smell caused by bubble aeration. Therefore, DMF

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biodegradation under anaerobic condition has attracted increasing interests in recent years (Kong et al., 2018a and 2018b). Unfortunately, due to the poor biodegradability and high toxicity of DMF (Yang et al., 2014; Rahmaninezhad et al., 2016), the conventional anaerobic process is usually limited by low removal efficiency and poor process stability (Kong et al., 2018a; Hasegawa et al., 1997; Bromley-Challenor et al., 2000b).

Recently, enhanced biodegradation of several recalcitrant pollutants, including dimethyl phthalate (Zhang et al., 2016), phenol (Ramos et al., 2016), triclosan (Wang et al., 2018b) and pyridine (Hou et al., 2018), at the presence of alternative electron acceptor such as nitrate, has been demonstrated. It was well known that the electrons and energy, which could be produced by carbon metabolism, were required in microbial denitrification in order to complete biological reduction of nitrate to N_2 (Zheng et al., 2014). Electron acceptors such as nitrate could act as the electron pools, which might be contributed to the enhanced biodegradation of various organic contaminants (Wang et al., 2019; Su et al., 2019). Electron production and consumption plays a crucial role in both carbon metabolism and denitrification. Therefore, promotion of biodegradation efficiency towards various recalcitrant contaminants in the anoxic denitrification system is attracting increasing attention (Wang et al., 2019; Hou et al., 2018; Shen et al., 2015). In addition, predenitrification based on anoxic and aerobic process (A-O process) was often used for the treatment of chemical wastewater containing various recalcitrant contaminants, while the electron acceptor such as nitrate could be easily formed in the subsequent aerobic nitrification system (Hou et al., 2018; Wu et al., 2019). It would be rather interesting that if the nitrate produced from the aerobic process could be removed through microbial denitrification in the anoxic reactors, with recalcitrant compounds partially removed simultaneously. However, to our best knowledge, information on the feasibility of DMF biodegradation in the anoxic denitrification system, especially in the long-term operation, is still unclear (Bromley-Challenor et al., 2000a). Furthermore, several key insights into anoxic DMF biodegradation, including the underlying metabolic mechanism dominating DMF biodegradation and denitrification, the evolution of community structure, key functional enzymes and electron transport pathway, were deserved to be investigated in order to develop an efficient strategy for the treatment of DMF-containing wastewater.

Therefore, in this study, the promotion of DMF biodegradation at the presence of nitrate was verified during long-term operation. The effects of various operational factors including initial DMF concentration, hydraulic retention time (HRT) and influent pH on reactor performance were investigated. Additionally, the microbial community structures involved in DMF biodegradation were explored to further understand the role of nitrate on the enrichment of specific functional microorganisms. Finally, transcriptional analysis, the key enzymatic activity assays, nicotinamide adenine dinucleotide (NADH) and adenosine triphosphate (ATP) content tests were performed to reveal and verify the proposed microbial metabolic mechanism involving DMF biodegradation and denitrification.

2. Materials and methods

2.1. Reactor setup and synthetic wastewater

In order to verify the positive effect of nitrate towards DMF biodegradation, two identical tubular reactors (Fig. S1) with working volume of 2.0 L were operated in parallel. One of the reactors fed with synthetic wastewater with the addition of nitrate was named after the anoxic denitrification reactor, while the other one fed with synthetic wastewater without the addition of nitrate was named after the anaerobic control reactor. Both reactors were inoculated with the sludge taken from an anaerobic bioreactor treating DMF-containing wastewater, at initial MLSS of 2.0 g L^{-1}. Cylindrical suspended carriers made of high-density polyethylene, with the specific surface area of 820 m^2 m^{-3}, were used for the immobilization of microorganisms in both reactors. Mechanical stirrers were used to ensure the carriers and synthetic wastewater were in full contact. The pH was monitored and kept constant through probes linked to the online real-time control device, with the automatic addition of 1 M HCl solution. Both reactors were maintained at 30±1 °C using water jacket throughout the entire operational period.

The synthetic wastewater was prepared as follows: 7 mM phosphate buffer (KH_2PO_4 and Na_2HPO_4, pH=7.0), 0.05 g L^{-1} $CaCl_2$, 0.1 g L^{-1} $MgSO_4\square 7H_2O$ and 10 mL L^{-1} trace element solution (Wang et al., 2018a). DMF was added at desired concentration and nitrate was added at DMF/nitrate molar ratio of 0.8. Before use, the synthetic wastewater was purged with nitrogen gas for 20 min to remove dissolved oxygen, followed by autoclaving at 121 °C for 20 min.

2.2. Reactor operation

The experimental period of the anoxic denitrification reactor and the control reactor was divided into five phases and the operational conditions for each phase were summarized in Table 1. In phase I (i.e. start-up period), in order to confirm the key role of nitrate in DMF biodegradation, both reactors were operated in parallel for 30 days with influent DMF concentration increased from 4.10 mM to 6.84 mM, while HRT was maintained at 24 h, resulting in DMF loading rate increased gradually from 2.05 mol m^{-3} d^{-1} to 3.42 mol m^{-3} d^{-1}. In order to create denitrification environment, nitrate was dosed into the anoxic denitrification reactor at dosage of 4.40 mol m^{-3} d^{-1}. In phase II, in order to examine the ability of both reactors when suffering from high-strength DMF loading, influent DMF concentration was increased from 6.84 mM to 68.41 mM, while HRT was controlled at 24 h, resulting in DMF loading rate increased from 3.42 mol m^{-3} d^{-1} to 34.20 mol m^{-3} d^{-1}. In phase III, the effect of HRT on reactor performance was investigated with HRT ranged from 96 to 24 h. Influent DMF concentration and pH was controlled at 68.41 mM and 7.0, respectively. In phase IV, influent pH was ranged from 6.0 to 8.0 via adjusting the proportion of KH_2PO_4 and Na_2HPO_4, in order to investigate the effect of pH on reactor performance. The influent DMF concentration, DMF loading rate and HRT were maintained at 6.84 mM, 3.42 mol m^{-3} d^{-1} and 24 h, respectively. In phase V, both reactors were operated at DMF loading rate of 20.52 mol m^{-3} d^{-1} for 60 days to evaluate the systems stability of both reactors, while influent DMF concentration, the influent pH and HRT were maintained at 41.05 mM, 7.0 and 24 h, respectively.

2.3. Analytical methods

Before analysis, all water samples taken from both reactors were immediately filtered through 0.22-μm syringe filters. DMF was identified and quantified using a high performance liquid chromatography (HPLC, DAD-3000, Thermo Scientific, USA). HPLC analysis was performed using a C_{18} column (5 μm, 4.6 × 250 mm) at column temperature of 30 °C and UV–vis wavelength of 205 nm. The mobile phase was 15% methanol and 85% ultrapure water (v/v), which was pumped at the flow rate of 1.0 mL min^{-1}. TOC concentration was determined on vario TOC analyzer (Germany Elementar). NO_3^--N, NO_2^--N and NH_4^+-N concentrations were measured according to previous study (Hou et al., 2018).

High-throughput sequencing analysis was carried out to investigate the microbial community structures in both reactors. After reaching a stable performance during the long-term opera-

Table 1
Operational conditions from phase I to phase V.

Phase	Purpose	Time (d)	pH	DMF (mM)	Nitrate (mM)	HRT (h)
I	Start-up period	0–10	7.0	4.10	5.28	24
		11–30	7.0	6.84	8.80	24
II	DMF concentration	31–90	7.0	6.84–68.41	8.80–87.95	24
III	HRT	91–130	7.0	68.41	87.95	96–24
IV	pH	131–160	6.0–8.0	41.05	52.77	24
V	Long-term period	161–220	7.0	41.05	52.77	24

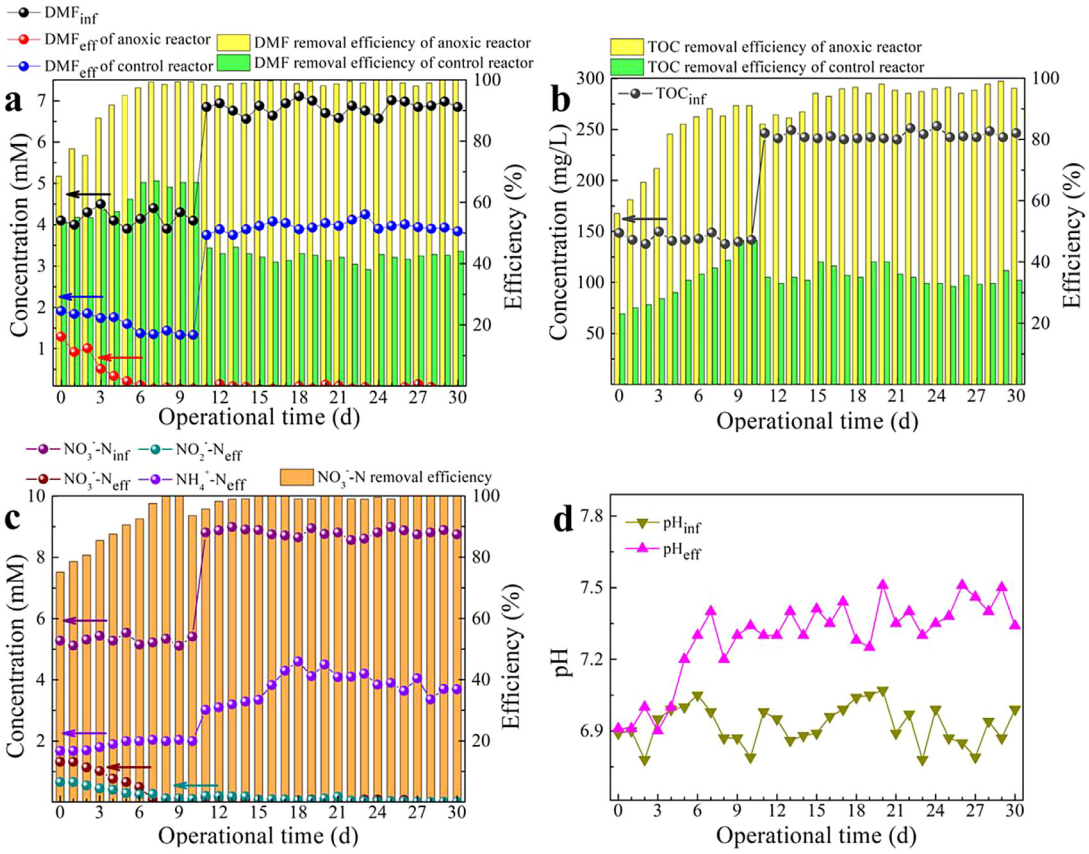

Fig. 1. DMF removal (a) and TOC removal (b) during start-up period in the anoxic denitrification system and control system, nitrate removal (c) and pH variation (d) in the anoxic denitrification system.

tion, water samples were taken from both reactors to analyze microbial community structures and a sample collected from original inoculum for sequencing as a comparison. The bacterial 16S rRNA gene amplification and sequencing were performed in Novogene Bioinforatics Technology Co., Ltd. (Beijing, China). The V4 region of bacterial 16S rRNA gene was amplified using universal primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). PCR product was sequenced on an Ion S5^{TM}XL platform according to standard protocols. The data analysis was performed in accordance to Chen et al. (2019).

2.4. Transcriptomic sequencing

Transcriptomic analysis was performed to investigate the transcriptional profiles of functional microorganisms in the control rector and anoxic denitrification reactor after the long-term operation. The samples selected for transcriptome sequencing were taken from the control reactor and anoxic denitrification reactor in phase V. Subsequently, all samples were immediately frozen in liquid nitrogen and stored at −80 °C for RNA extraction.

Transcript assembly and annotation. Before proceeding with the assembly, sequenced reads were cleaned up from raw reads by removing reads with (1) sequence adapters (< 50 bp bases in each read), (2) low-quality reads (≤ 20% bases of Q-score), and (3) poly-N containing reads (> 5% of nucleotides in each read). Clean reads were assembled into unigenes by Trinity platform. The assembled unigenes were annotated using BLASTP searching in NR databases at *E*-value < 10^{-5}.

Transcriptomic data analysis. To analyze the differences in gene expression between the control reactor and anoxic denitrification reactor, gene expression levels were analyzed by RSEM using fragments per kilobase per million fragments (FPKM) method. The quantification of differential gene expression was performed by EdgeR software, genes with absolute values of log2 (fold change) > 1 and false discovery rate (FDR) ≤ 0.05 were considered as significantly differential expressed genes (DEGs). Furthermore, functional enrichment analysis, such as GO (Gene Ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes), which was widely used to identify the biological function of DEGs, and the metabolic pathways or signal transduction pathways associated with the DEGs (Wan et al., 2019), was used to the classify function

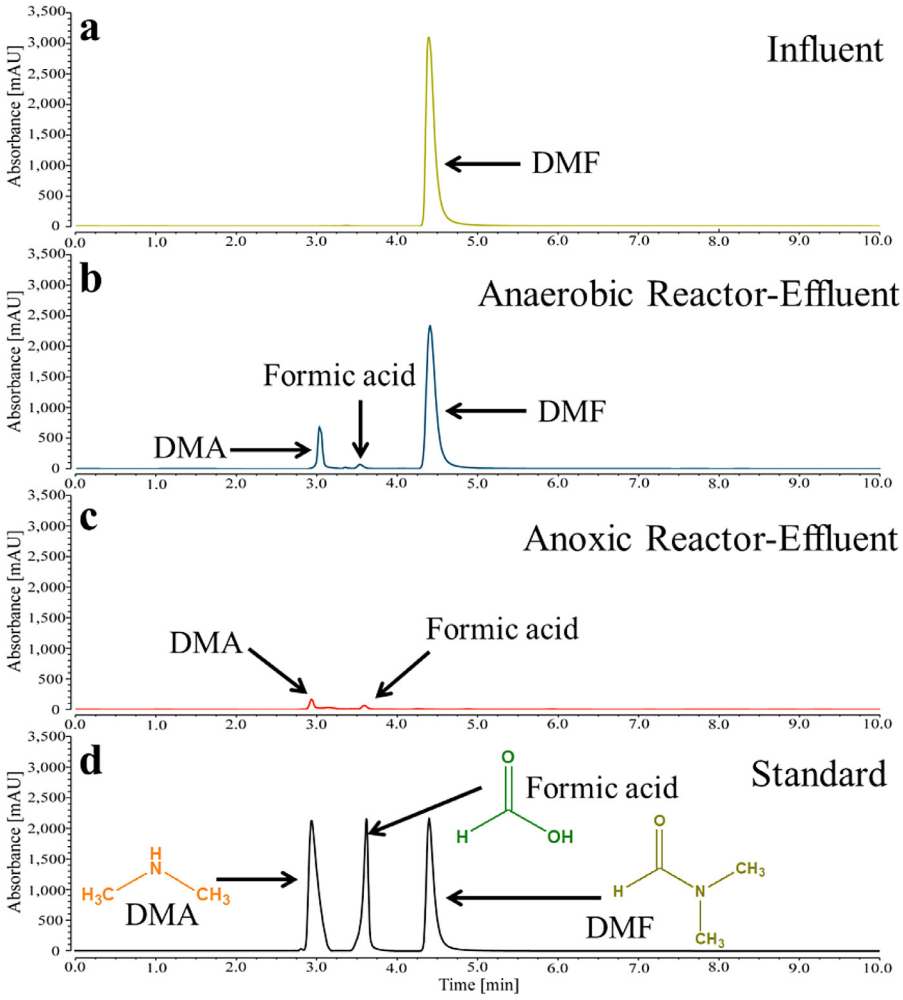

Fig. 2. Intermediates identified during DMF biodegradation in the control system and anoxic denitrification system.

of DEGs, with Q-value $\leq$ 0.05 were considered as significant enriched pathways. Besides, transcriptomic sequences were submitted to NCBI's Sequence Read Archive (SRA) under the BioProject accession number PRJNA628882.

2.5. Key enzyme activity assay, NADH and ATP contents

For the analysis of key enzyme activities, water samples were taken from both anoxic denitrification system and anaerobic control system during long-term period. Water samples were pretreated according to Su et al. (2019) with minor modification. Briefly, 10 mL water sample was harvested by centrifugation at 2000 $\times$ *g* at 4 °C for 5 min. Subsequently, the sediment was washed three times with 10 mL 85% NaCl solution, and then resuspended in 10 mL 85% NaCl solution at 4 °C. For cell breaking, the suspension was further treated by sonication for 10 min at 4 °C. Then, the supernatant was harvested by centrifugation at 10,000 $\times$ *g*, 4 °C for 10 min, and was immediately used to measure key enzymatic activities.

Tetramethylbenzidine (TMB) chromogenic method was used to determine DMFase activity by measuring the absorbance of TMB at 450 nm after chromogenic reaction, which showed positive correlation with the activity of DMFase. Similarly, dimethylamine dehydrogenase (DADH), methylamine dehydrogenase (MADH), formaldehyde dehydrogenase (FDH) and formic acid dehydrogenase (FADH) were analyzed based on the corresponding enzyme-linked immunoassay kit (96T, JingKang biological, China). The activities of four denitrifying enzymes namely nitrate reductase (NAR), nitrite reductase (NIR), nitric oxide reductase (NOR) and nitrous oxide reductase (N_2OR) were analyzed using the corresponding denitrifying enzyme-linked immunoassay kit (96T, JingKang biological, China). Lactate dehydrogenase (LDH) activity, NADH (electron carrier) and ATP (energy source) contents were determined by the corresponding enzyme-linked immunoassay kits (48T, JingKang biological, China).

3. Results and discussion

3.1. Enhanced DMF biodegradation in denitrification system

During 30 days' start-up period, DMF removal efficiency in the anoxic denitrification system rapidly increased from 75.3 $\pm$ 2.3% on day 3 to 99.3 $\pm$ 1.5% on day 8 at DMF loading rate of 2.05 mol m^{-3} d^{-1} (Fig. 1a). Thereafter, DMF removal efficiency was kept higher than 98% in the following 22 days, despite of further increase of influent DMF concentration up to 6.84 mM. However, the removal efficiency of DMF in the control system was always in the low range of 45%–60%, even at the low influent DMF concentration of 4.10 mM. In addition, more than 95% of TOC could be removed in the anoxic denitrification system while it was only around 40% in the control reactor (Fig. 1b), suggesting almost complete mineralization of DMF could be achieved in the anoxic denitrification system. Similar phenomenon was also observed in

previous study, where the biodegradation of recalcitrant pyridine could be enhanced in the anoxic system at the presence of nitrate (Shen et al., 2015). Wang et al. (2019) found that the supplementation of nitrate into anoxic system resulted in complete removal of 5 mM N-methylpyrrolidone by Paracoccus within incubation time as short as 11 h, as compared to the removal efficiency as low as 24% in the anaerobic control system in the absence of nitrate.

Specially, NO_3^--N was well removed along with DMF biodegradation in the anoxic denitrification system, as indicated by effluent NO_3^--N concentration decreased gradually from 0.66±0.18 mM on day 5 to 0.08±0.12 mM on day 9, and then kept below 0.04 mM in the following 21 days (Fig. 1c). NO_2^--N concentration within the first 5 days was much higher than those in the following 25 days, probably due to the unavailable of efficient electron donor (Li et al., 2017). The formation of NH_4^+-N was a key evidence for DMF mineralization, since previous studies demonstrated that nitrogen in nitrogen-containing compounds was usually transformed into NH_4^+-N during biodegradation process (Kong et al., 2018a; Wang et al., 2018a; Jiang et al., 2018). However, NH_4^+-N concentration in the effluent after day 17 only accounted for 62.9 ± 4.1% of the nitrogen in influent DMF, probably due to the consumption of nitrogen in DMF as nutrient substance for cell growth during microbial metabolism (Wang et al., 2019). Additionally, both NH_4^+ release and microbial denitrification were often accompanied by the increase of pH values (Fig. 1d) (Qian et al., 2019). In order to keep pH constant, HCl solution should be added into the anoxic denitrification system continuously, confirming the occurrence of basicity producing reactions such as microbial denitrification and NH_4^+ release.

In this study, DMF biodegradation products in both anoxic denitrification system and anaerobic control system were identified using HPLC analysis, as shown in Fig. 2. For the anaerobic control system, two new peaks were observed at retention time of 3.023 min and 3.527 min (Fig. 2b), which could be assigned to dimethylamine (DMA) and formic acid, respectively, through comparison with the standards (Fig. 2d). In the effluent of the anaerobic control system, the corresponding concentrations of DMF, DMA and formic acid were 3.76±0.25 mM, 1.84±0.16 mM and 0.27±0.18 mM, respectively, indicating the obvious accumulation of intermediates such as DMA and formic acid. However, only trace DMA and formic acid could be observed for the effluent of anoxic denitrification system, with their concentrations well below 0.25 mM and 0.15 mM, respectively (Fig. 2c). Compared to the anaerobic control system, not only DMF depletion but also intermediates removal was substantially enhanced in the anoxic denitrification system, due to the key role of nitrate as the electron acceptor (Wang et al., 2019). Kong et al. (2018a) and Sanjeevkumar et al. (2013) also found that DMA and formic acid were the main intermediates involved in DMF biodegradation process. Therefore, it could be speculated that hydrolysis was the initial step in DMF biodegradation process, with DMA and formic acid formed through this hydrolysis reaction. DMA could be converted to the downstream intermediate namely mono-methylamine (MMA), which could be further transformed to formic acid (Zhao et al., 2018; Sanjeevkumar et al., 2013; Kong et al., 2018a). NH_4^+ could be released from MMA via the cleavage of C–N bond, with the simultaneous generation of formaldehyde (Zhao et al., 2018). However, formaldehyde was not observed in this study, probably due to its fast transformation into formic acid. The intermediates such as formaldehyde and formic acid could be well utilized as the electron donors for microbial denitrification. Eiroa et al. (2005 and 2007) found that simultaneous formaldehyde biodegradation and denitrification could be achieved in anoxic batch assays and continuous anoxic reactor, even at different formaldehyde loading rates. Smith et al. (2001) found that formic acid could serve as the electron donor to remediate nitrate contaminant under anoxic environment, with the produced nitrite completely removed in the denitrification process.

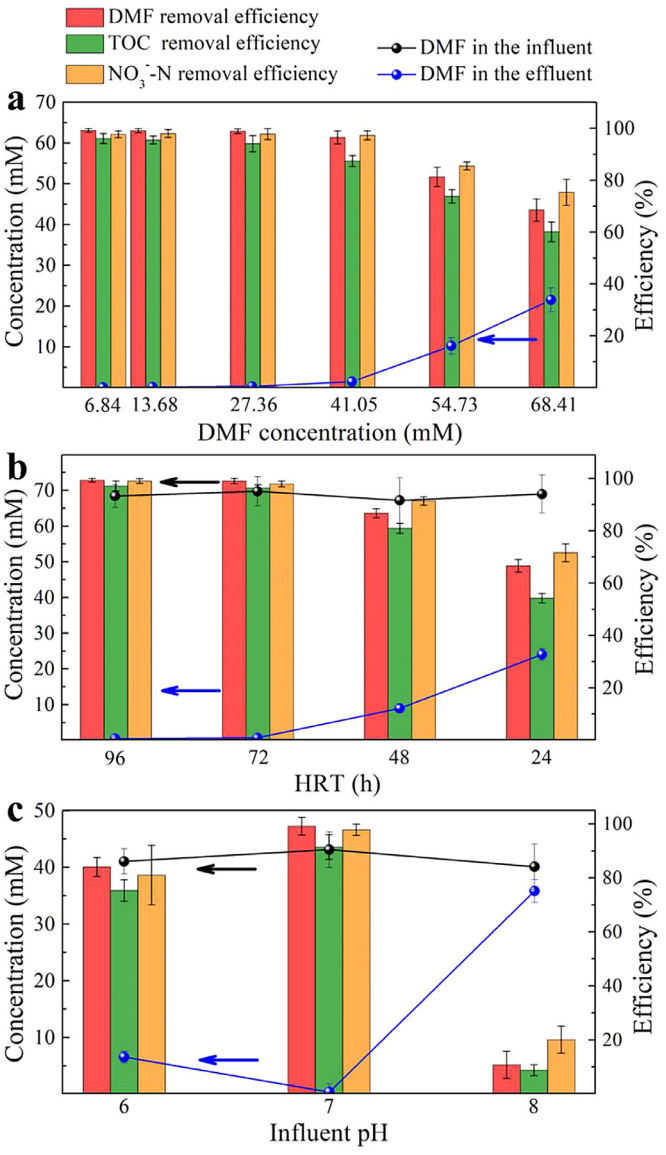

Fig. 3. Effect of influent DMF concentration (a), HRT (b) and influent pH (c) on reactor performance in the anoxic denitrification system.

Based on the above analysis about DMF depletion, TOC decrease, NO_3^--N depletion, NO_2^--N formation, NH_4^+ release, pH increase, as well as the identification of biodegradation intermediates during simultaneous DMF biodegradation and denitrification, it could be inferred that DMF could be mineralized in the anoxic denitrification system.

3.2. The effect of key operational parameters

3.2.1. Influent DMF concentration

As shown in Fig. 3a, with the increase of influent DMF concentration from 6.84 mM to 13.68 mM, DMF and TOC removal efficiencies at HRT of 24 h and DMF/nitrate molar ratio of 0.8 were as high as 99.2 ± 2.0% and 96.1 ± 1.9%, respectively, confirming the excellent DMF removal performance in the anoxic denitrification system. However, as influent DMF concentration further increased to 27.36 mM, both DMF removal and TOC re-

moval efficiencies were firstly slightly decreased, but then gradually recovered to 96.5 ± 3.2% and 87.4 ± 2.1% after day 85, even when influent DMF concentration further increased to 41.05 mM. However, when influent DMF concentration further increased to 54.73 mM and 68.41 mM, DMF removal efficiency was sharply dropped to 81.3 ± 3.7% and 68.5 ± 2.3%, respectively, which could be attributed to the toxicity exerted by DMF at high concentration. Similarly, NO_3^--N reduction efficiency was always above 98% with the increase of influent DMF concentration from 6.84 mM to 41.05 mM, but then decreased to 75.3 ± 5.2% when the influent DMF concentration increased to 68.41 mM. Hou et al. (2018) also indicated that both pyridine removal and denitrification were severely inhibited at high pyridine loading rate, which could be attributed to microbial growth and activity repressed by the toxic substrate at high concentration.

3.2.2. HRT

It was well known that HRT is an important parameter for the bioprocess because the removal efficiency of various pollutants is associated with HRT closely (Wu et al., 2018). As shown in Fig. 3b, the anoxic denitrification reactor showed a stable performance in terms of DMF and TOC removal efficiencies when HRT was varied within the range of 96–72 h. However, with the decrease of HRT to 48 h, both DMF removal and TOC removal efficiencies were sharply declined to 87.2 ± 3.1% and 81.4 ± 2.2%, respectively. Further decrease of HRT to 24 h caused a significant deterioration of reactor performance in the anoxic denitrification system in terms of DMF removal and TOC removal. Correspondingly, with the decrease of HRT from 96 to 48 h, NO_3^--N removal efficiencies decreased from 99.3 ± 2.1% to 90.1 ± 3.4%. At short HRT of 24 h, abundant residual of NO_3^--N was observed in the effluent. This phenomenon could be attributed to the low denitrification rate when recalcitrant substrate was used as the sole electron donor (Shen et al., 2015). Therefore, for the operation of the anoxic denitrification system, relatively long HRT should be adopted when low-quality carbon source such as DMF was used.

3.2.3. Influent pH

For the biodegradation of various xenobiotics such as DMF under anoxic condition, pH value in the incubation system plays an important role, as both contaminant biodegradation and denitrification could be dramatically affected by incubation pH (Shen et al., 2015). During phase IV, in order to investigate the effect of influent pH on DMF biodegradation, the influent pH was ranged from 6.0 to 8.0. As shown in Fig. 3c, DMF removal and TOC removal efficiencies were increased from 84.0 ± 2.5% and 75.3 ± 1.9% to 99.1 ± 3.3% and 91.4 ± 4.6% with the increase of the influent pH from 6.0 to 7.0, but were sharply decreased when the influent pH value further increased to 8.0. Obvious deterioration of the reactor performance was observed when the influent pH value was maintained at 8.0 for 10 days, which could be attributed to the inhibition of the bacterial community under the alkalescent condition (Wu et al., 2019; Nisha et al., 2015). In addition, under the alkalescent condition, the occurrence of basicity producing reactions would be inhibited, including DMF biodegradation and denitrification. Similarly, optimal NO_3^--N removal was also achieved at pH of 7.0, with the residual NO_3^--N well maintained below 0.02 mM. From these results, it could be concluded that DMF biodegradation, TOC removal and denitrification were optimal at neutral condition, which was also observed in tricyclazole biodegradation system (Wu et al., 2018).

3.3. Long-term stability

Long-term performance is one of the major concerns regarding the practical application of the anoxic denitrification system developed for the treatment of DMF wastewater (Kong et al., 2019a). To assess the stability of the anoxic denitrification system during the long-term operation, the anoxic denitrification reactor was operated continuously from day 161 to day 220 at high DMF concentration of 41.05 mM. As shown in Fig. S2, complete removal of DMF was maintained in the anoxic denitrification reactor, while TOC removal efficiency increased gradually from 90.1 ± 2.5% on day 161 to 98.2 ± 2.6% on day 220. Correspondingly, NO_3^--N removal efficiency was well above 98% from day 161 to day 220, indicating the stable performance of microbial denitrification. Similar to phase I (i.e. start-up period), NH_4^+-N concentration in the effluent was always below 27 mM, only accounting for 65.1 ± 3.5% of the nitrogen in DMF in the influent, probably due to the consumption of NH_4^+-N in biosynthetic process (Wang et al., 2019). These results further confirmed that the stable long-term performance could be achieved in the anoxic denitrification reactor, even under high DMF loading rate, suggesting that the practical application of the anoxic denitrification system would be technically feasible for the treatment of DMF-containing wastewater.

3.4. Microbial community analysis

High-throughput sequencing technology was employed to analyze the microbial community diversity and structure after the long-term operation. Table S1 listed the parameters associated with microbial community diversity. Compared to the original inoculum sludge, long-term adaption to high-strength DMF-containing wastewater caused obvious decrease in microbial richness and diversity, which was reflected by the lower Chao and Shannon index. This result indicated that the metabolism of bacterial community sensitive to the toxicity of DMF could be inhibited (Chen et al., 2019). In addition, the principal component analysis (PCA) based on the sequencing data indicated that the microbial populations have been significantly changed in both control reactor and anoxic denitrification reactor after the long-term operation (Fig. S3), compared with the initial inoculum. Kong et al. (2019a) also indicated that the presence of recalcitrant substrates such as DMF induced microbial selection in the anaerobic treatment system, resulting into the reduced community diversity.

It was well known that microbial community structure played an important role in developing a successful biological system for treatment of various contaminants (Wu et al., 2019). As shown in Fig. S4, the community structure analysis at the class level showed that *Gammaproteobacteria, Alphaproteobacteria, Bacteroidia, Bacilli, Planctomycetacia, Ignavibacteria* and *Clostridia* were the major classes in the inoculum sludge, accounting for 55.13%, 22.31%, 15.99%, 1.76%, 1.16%, 1.09% and 0.51%, respectively. After the long-term acclimatization of high-strength DMF, the microbial community structure was varied obviously. The abundance of *Alphaproteobacteria* (anoxic denitrification reactor, 68.15%; control reactor, 78.39%) was significantly increased in both reactors, indicating the enrichment of the bacteria within this class. However, the relative abundance of *Gammaproteobacteria*, the most dominant member in the inoculum, sharply dropped to 21.11% in the anoxic denitrification reactor and 4.71% in the control reactor, respectively, indicating the inhibition of *Gammaproteobacteria* by the toxicity of DMF. The abundances of the classes namely *Bacteroidia, Bacilli, Planctomycetacia* and *Ignavibacteria* also obviously decreased under the selective pressure caused by the recalcitrant DMF.

The bacterial community at genus level indicated that *Paracoccus* (18.40%), *Arenimonas* (15.93%), *Hyphomicrobium* (14.41%), *Aquamicrobium* (12.50%), *Brevundimonas* (7.72%), *Methylopila* (5.47%), *Chryseobacterium* (5.02%), *Bosea* (3.26%) and *Brucella* (2.40%) were dominant in the anoxic denitrification reactor, com-

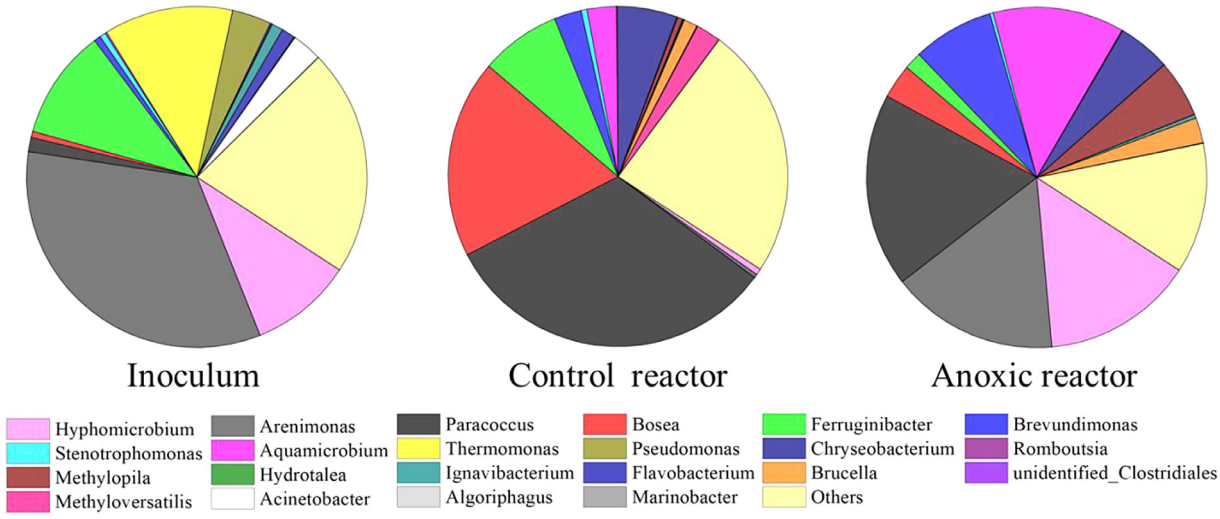

Fig. 4. The classification of microbial community in inoculum, control system and anoxic denitrification system at genus level.

pared with the control reactor (Fig. 4). *Paracoccus*, which was the most abundant species in the anoxic denitrification reactor, could degrade DMF efficiently by producing the hydrolytic enzyme (Zhou et al., 2018; Kong et al., 2019a, 2019b). Hou et al. (2018) and Liu et al. (2012) found that *Paracoccus* was capable of denitrifying using recalcitrant contaminants as the electron donor. The genus *Arenimonas* was an excellent denitrifier, which was documented to has ability of degrading refractory organics with carbon dioxide as the major product in the denitrification system, even under a low COD/N condition (Xing et al., 2020; Zhang et al., 2019). The high abundance of *Arenimonas* in the anoxic denitrification reactor might be account for the enhanced bio-mineralization of DMF. *Hyphomicrobium* was reported to be a typical methylotrophic bacterium capable of denitrifying, could use nitrogenous pollutants such as dimethyl sulfoxide as the sole carbon source supporting microbial denitrification (Martineau et al., 2015; Murakami-Nitta et al., 2002; Tairo et al., 1996; Sperl et al., 1971). *Aquamicrobium* has reported to be involved in the oxidation of low-quality alkanes in the presence of high-saline and high concentration of nitrate (Chang et al., 2015; Wang et al., 2015a). *Brevundimonas*, a member of the class *Alphaproteobacteria*, was reported to be mainly responsible for biotransformation of various complex substrates via hydrolysis (Xu et al., 2019; Lee et al., 2010; Liang et al., 2010). *Methylopila*, which was a methylotrophic species, might be responsible for the oxidation of DMF via demethylation pathway (Wang et al., 2015b). *Chryseobacterium* was found to be involved in the hydrolysis of highly toxic acyl-containing contaminants, while the intermediates could be further degraded during biodegradation process (Guo et al., 2008; Gurav et al., 2013; Zhao et al., 2016; Rashid et al., 2011). The genus *Brucella* could metabolize various complex substrates (Muthukumar et al., 2003). *Bosea* was frequently observed in sewage effluent contaminated by hydrocarbons, showing excellent performance in terms of efficiently remove nitrate and organics simultaneously in the denitrification system (Lu et al., 2017; Al-Mailem et al., 2014; Zeng et al., 2019).

In comparison to the anaerobic control reactor, the growth of hydrolytic species (*Paracoccus, Brevundimonas* and *Chryseobacterium*) and denitrification related species (*Paracoccus, Arenimonas, Hyphomicrobium, Aquamicrobium* and *Bosea*) was distinctly promoted in the anoxic denitrification reactor after the long-term acclimatization of high-strength DMF. The enrichment of these functional species would be contributed to the enhanced DMF biodegradation, mineralization and nitrate reduction in the anoxic denitrification reactor.

3.5. Transcriptomic analysis and enzyme activity assay

In order to explore the effect of microbial denitrification on DMF metabolism, the differential genes expression in the anoxic denitrification system and control system were analyzed by RNA sequencing. Compared with the control system, a total of 1127 differential expressed genes (DEGs) in the anoxic denitrification system were significantly upregulated (Fig. S5). Significantly enriched GO terms (Q-value $\leq$ 0.05) were related to carbohydrate transport, carbohydrate transmembrane transporter activity, cellular aldehyde metabolic process, hydrolase activity, organic acid metabolic process, small molecule metabolic process and transferase activity (transferring nitrogenous groups) (Fig. S6). The upregulated expression of various transmembrane transporters and transferase might be beneficial for DMF diffusion into intracell during DMF metabolism, since most of transmembrane transporters could transport various substrate-binding proteins such as ATP-binding cassette transporter (Lu et al., 2019; Chang et al., 2009). Through KEGG analysis, enrich of these upregulated DEGs were related to microbial metabolism and energy metabolism, including carbon metabolism (ko01200), amino sugar and nucleotide sugar metabolism (ko00520), fatty acid degradation (ko00071), biosynthesis of unsaturated fatty acids (ko01040), nitrogen metabolism (ko00910), fatty acid metabolism (ko01212) and ribosome (ko03010) (Fig. S7). These results indicated that substantial stimulation on cellular regulation and microbial metabolism could be achieved in the anoxic denitrification system.

Interestingly, we also found that the key genes encoding lactate dehydrogenases (LDH), which was reported as an indicator of cell structure damage (Wan et al., 2016), were significantly downregulated in the anoxic denitrification system compared to the control system without nitrate (Fig. 5a). Importantly, the result of enzymatic activity assay was perfectly agreed with the gene expression level, where the catalytic activity of LDH in the control reactor was 58% higher than that in the anoxic denitrification reactor (Fig. 5b). Upregulated genes responsible for the release of LDH in the control reactor suggested that the high-strength DMF loading had significant effects on cell structures, thereby possibly causing the death of partial cells. High-strength organic solvents such as 1–methoxy-2-propanol and methanol would result into the reduced extracellular polymeric substances in bio-aggregate and the disintegration of granules, which was adverse for the stability of biological wastewater treatment system (Lafita et al., 2015; Lu et al., 2015). In this

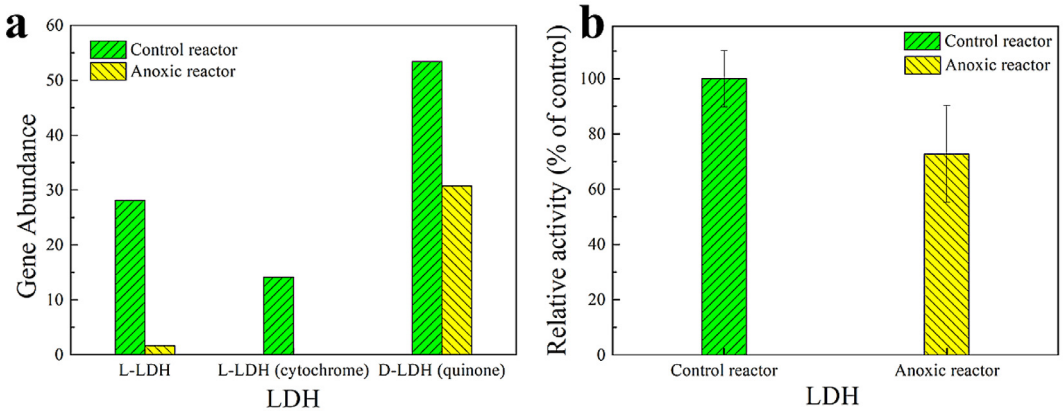

Fig. 5. Gene abundance (a) and relative activity (b) of LDH in the control system and anoxic denitrification system.

study, the poor performance of the control reactor could be partially attributed to the damage of cell structure and sludge structure at the presence of more residual DMF, considering the fact that DMF was poorly removed in the control reactor (Torres et al., 2018). On the contrary, the upregulation of the genes responsible for DNA repair was observed in the anoxic denitrification reactor (Fig. S6&Fig. S7), which was beneficial for the maintenance of long-term operation stability.

3.6. Mechanism dominating DMF biodegradation and denitrification

In this study, the highly toxic and recalcitrant DMF was efficiently utilized as the electron donor for microbial denitrification, even at high DMF loading rate. Microbial denitrification needs to consume electrons to accomplish thoroughly reduction of NO_3^--N to N_2, providing the electron pools for the biodegradation of organic matters such as DMF. The available of electron acceptor such as nitrate would be contributed to the enhanced biodegradation of organic contaminants through the rapid consumption of electrons (Wan et al., 2016). For the first time, the present study proved that simultaneous DMF biodegradation and microbial denitrification was technically feasible.

It was well known that biological denitrification occurs intracellularly, through the sequent biological reductions of NO_3^--N to NO_2^--N and finally to N_2 (Fig. 6a), which were catalyzed by four essential denitrifying enzymes, namely nitrate reductase (NAR), nitrite reductase (NIR), nitric oxide reductase (NOR) and nitrous oxide reductase (N_2OR) (Wan et al., 2016; Wang et al., 2019). By transcriptional analysis, we found that the gene expression level involved in these denitrifying enzymes including quinone nitrate reductase [EC:1.7.5.1], periplasmic nitrate reductase NapA [EC:1.7.99.4] and nitrite reductase [EC:1.7.2.1] were significantly upregulated in the anoxic denitrification reactor, whereas no significant variation was observed for NOR and N_2OR (Fig. 6b). Su et al. (2019) has reported that both NOR and N_2OR were freely distributed in periplasm, which were more easily affected by the toxic DMF as compared to NAR and NIR. Moreover, it should be noted that nitrate/nitrite transporter (nrt) had a much higher expression level (Fig. S8), which was rather beneficial for denitrification. The results of enzymatic activity assay illustrated in Fig. 6c verified our speculation, where the catalytic activities of NAR and NIR in the anoxic denitrification system were higher than those in the control system.

Based on the analysis of DMF biodegradation products, hydrolysis and oxidation were the major processes account for DMF metabolism. In the anoxic denitrification system, the abundance of the gene responsible for DMF hydrolysis was 5.6-fold of that in the control system (Fig. 6d), indicating that the presence of nitrate could significantly fastened the hydrolysis of DMF, which was reported to occur intracellularly (Lu et al., 2019). The gene expression of formate dehydrogenase (FDH) was remarkably upregulated in the anoxic denitrification reactor as compared to the control reactor. Correspondingly, the activities of several key functional enzymes in the anoxic denitrification system, including N,N-dimethylformamidase (DMFase) [EC:3.5.1.56], dimethylamine dehydrogenase (DMDH) [EC:1.5.8.2], formaldehyde dehydrogenase (FADH) [EC:1.2.1.46] and formate dehydrogenase (FDH) [EC:1.2.1.2], was obviously higher than those in the control system (Fig. 6e). During DMF biodegradation, the step of DMF to DMA catalyzed by DMFase was rather critical, because it directly linked to DMF utilization rate. The dehydrogenases such as FDH was rather crucial for the oxidative degradation of the intermediates such as DMA, MMA, formaldehyde and formic acid, which was beneficial for the providing of electrons for microbial denitrification. Particularly, FDH was responsible for catalyzing formic acid into CO_2 and H_2O, with electron and energy produced simultaneously (Wang et al., 2019). Both high gene expression and high FDH activity demonstrated that DMF could be oxidized into CO_2 to provide electron and energy for microbial denitrification, since formic acid was the main intermediates during DMF biodegradation in the anoxic denitrification system. These results further indicated that the occurrence of denitrification not only enhanced DMF hydrolysis but also facilitated DMF bio-mineralization, which was consistent with the high TOC removal and NH_4^+-N release.

NADH, which is generated during the biodegradation of various organics, can be used as the direct electron donor for biological reduction reactions including microbial denitrification (Su et al., 2019). In organisms, tricarboxylic acid (TCA) cycle is the final pathway for the decomposition of major nutrients such as carbohydrates, lipids and amino acids (Zhang et al., 2018). In general, more ATP could be produced in denitrification bioprocess than anaerobic bioprocess, due to the fact that organic substrate could be more effectively metabolized by denitrifier to provide electron and energy for nitrate reduction (Wan et al., 2019). In this study, the contents of NADH and ATP in the anoxic denitrification reactor were much higher than those in the control reactor, as shown in Fig. 6f. However, it was found that the upregulation of the expression level of the gene responsible for TCA cycle were insignificant in the anoxic denitrification reactor (Table S2). Since the gene expression variation of key enzymes related to conventional energy metabolism was negligible, nitrate might play a key role in enhancing the catabolism of DMF and its derivatives to provide electron and energy for supporting microbial denitrification, rather than the conventional TCA cycle pathway in this study.

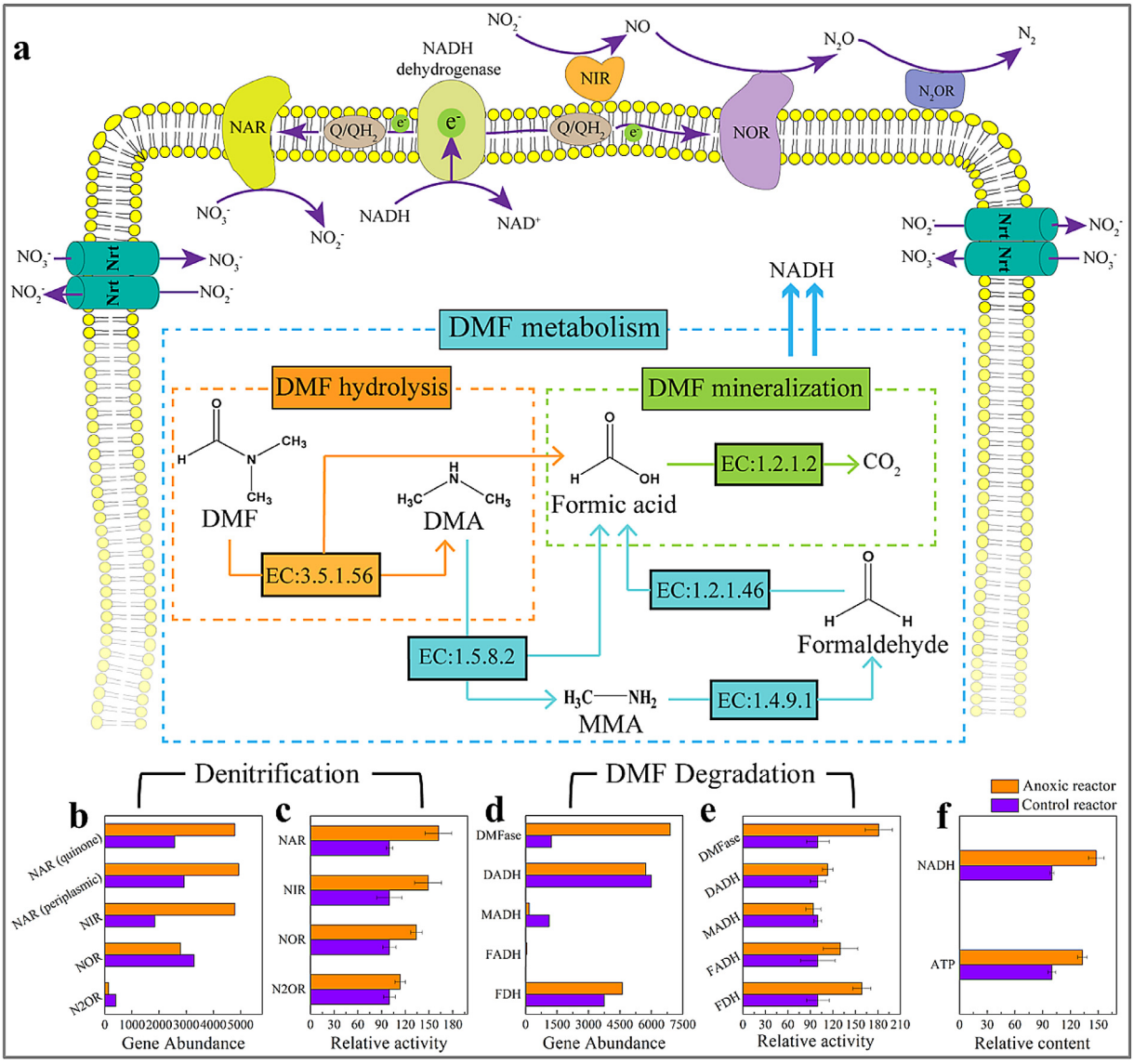

Fig. 6. Microbial metabolic mechanism in the anoxic denitrification system: schematic microbial denitrification and DMF metabolism pathway (a); key gene expression (b) and relative activities of NAR, NIR, NOR and N_2OR (c); key gene expression (d) and relative activities of DMFase, DADH, MADH, FADH and FDH (e); relative contents of NADH and ATP (f).

4. Conclusion

This study demonstrated the feasibility of the anoxic denitrification system for the treatment of high-strength DMF containing wastewater. DMF could be completely removed at DMF-loading rate as high as 20.52 mol m^{-3} d^{-1}. Our results found that the occurrence of denitrification not only improved the biodegradation capability of specific functional microorganisms but also promoted carbon source utilization to produce electron and energy. In summary, this is the first study for exploring the effect of microbial denitrification on DMF biodegradation at microbial metabolism and transcriptional level, which provides a new insight for developing efficient and economic approach to enhance high-strength DMF removal from wastewater.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2020.116306.

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