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ENVIRONMENTAL BIOTECHNOLOGY

The effects of fulvic acid on microbial denitrification: promotion of NADH generation, electron transfer, and consumption

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Abstract The heterotrophic denitrification requires the participation of electrons which are derived from direct electron donor (usually nicotinamide adenine dinucleotide (NADH)), and the electrons are transferred via electron transport system in denitrifiers and then consumed by denitrifying enzymes. Despite the reported electron transfer ability of humic substances (HS), the influences of fulvic acid (FA), an ubiquitous major component of HS, on promoting NADH generation, electron transfer, and consumption in denitrification process have never been reported. The presence of FA, compared with the control, was found not only significantly improved the total nitrogen (TN) removal efficiency (99.9 % versus 74.8 %) but remarkably reduced the nitrite accumulation (0.2 against 43.8 mg/L) and N_2O emission (0.003 against 0.240 mg nitrogen/mg TN removed). The mechanisms study showed that FA increased the metabolism of carbon source via glycolysis and tricarboxylic acid (TCA) cycle pathways to produce more available NADH. FA also facilitated the electron transfer activities from NADH to denitrifying enzymes via complex I and complex III in electron transport system, which improved the reduction of nitrate and accelerated the transformations of nitrite and N_2O, and lower nitrite and N_2O accumulations were therefore observed. In addition, the consumption of electrons in denitrification was enhanced due to FA stimulating the synthesis and the catalytic activity of key denitrifying enzymes, especially nitrite reductase and N_2O reductase. It will provide an important new insight into the potential effect of FA on microbial denitrification metabolism process and even nitrogen cycle in nature niches.

Keywords Denitrification · Fulvic acid · NADH · Electrons · Promote

Introduction

Nitrogen is a vital element existing in water, soil, atmosphere, and organism, and its cycle is one of the fundamental biogeochemical processes in the biosphere (Erisman et al. 2008). Microbial denitrification, a well-known pathway by which fixed nitrogen such as nitrate returns to the atmosphere from terrestrial and aquatic environments, constitutes one of the main branches of the global nitrogen cycle (Gruber and Galloway 2008; Canfield et al. 2010). However, some undesired intermediates, such as nitrite and nitrous oxide (N_2O), were formed during denitrification process. Nitrite has been proven to be toxic to aquatic organisms, wastewater treatment microbes, and human health (Jensen 2003; Zhou et al. 2011), and N_2O is a potent greenhouse gas with 300-fold higher global warming potential than carbon dioxide (Ravishankara et al. 2009). Therefore, microbial denitrification is closely related to the occurrence of global environmental problems, ranging from aquatic eutrophication to climate change (Wuebbles 2009).

Humic substances (HS), a mixture of different macromolecular organic molecules formed from the decomposition of plant, animal, and microbial cells, are ubiquitous in the environment, such as soils, sediments, and natural waters (Sutton and Sposito

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2005; Janot et al. 2010). It has been widely reported that HS have the ability to act as redox mediators (Lovley et al. 1996; Lovley et al. 1999), thus playing a key role in the transport, fate, and redox conversion of organic and inorganic compounds both in chemically and microbially driven reactions (Watanabe et al. 2009; Borch et al. 2010; Paquete et al. 2014). Further research indicated that within the carbon backbone of HS, electron accepting/donating property is mainly attributed to quinone groups (Cory and McKnight 2005; Jiang and Kappler 2008; Felix et al. 2010). Some humic-reducing microorganisms, such as *Geobacter metallireducens*, *Ralstonia eutropha*, and *Shewanella alga*, can use HS analogs (e.g., anthraquinone-2,6-disulphonate (AQDS)) as electron acceptors by transferring electrons to quinone moieties and reducing it to hydroquinone during anaerobic oxidation of organic compounds (Lovley et al. 1996; Cervantes et al. 2011; Zhang et al. 2012; Martinez et al. 2013). Some microbes capable of dissimilatory HS reduction, like *Geobacter sulfurreducens*, *Geothrix fermentans*, and *Wolinella succinogenes* can also use the reduced form of HS analogs (e.g., anthrahydroquinone-2,6-disulphonate (AH_2QDS)) as electron donors by oxidizing the hydroquinone group for anaerobic growing on fumarate, arsenate, and selenate (Lovley et al. 1999). In addition, it was also found that some denitrifiers, such as *Paracoccus denitrificans* and other mixed denitrifying cultures, despite being incapable of dissimilatory HS reduction, can use AH_2QDS as an electron donor for reducing nitrate and its intermediates including nitrite and N_2O (Lovley et al. 1999; Coates et al. 2002; Aranda-Tamaura et al. 2007).

Is well known that microbial denitrification requires the participation of electrons, which are derived from direct electron donor (usually nicotinamide adenine dinucleotide (NADH)), and the electrons are transferred via electron transport system in denitrifiers and then consumed by denitrifying enzymes. Firstly, denitrifying microbes metabolize carbon sources to produce the direct electron donor, NADH (Young-Ho 2006). When glucose is used as the carbon source, the main diagram for NADH generation is illustrated in Fig. 1; and several enzymes, such as hexokinase (HK), 6-phosphofructose kinase (PFK), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), pyruvate kinase (PK), pyruvate dehydrogenase (PDH), isocitrate dehydrogenase (IDH), a-ketoglutarate dehydrogenase (KGDH), and malate dehydrogenase (MDH) are reported as the key enzymes responsible for glucose utilization and NADH production (Müller et al. 1968; Li et al. 1989; Saltiel and Kahn 2001). Then, the electrons generated from NADH are transferred to denitrifying enzymes via complex I (NADH-ubiquinone oxidoreductase and ubiquinone pool) and complex III (ubiquinol-cytochrome c oxidoreductase (cytochrome bc1 complex)) in denitrifiers (Berks et al. 1995; Chen and Strous 2013). Finally, the denitrifying enzymes, such as nitrate reductase (NAR), nitrite reductase (NIR), nitric oxide reductase (NOR), and nitrous oxide reductase (N_2OR), receive electrons and catalyze the reduction of nitrogen oxides (Berks et al. 1995; Zumft 1997). Despite that the electron transfer ability of HS have been reported, the influences of HS on the metabolism and function of denitrifying bacteria, especially from the aspect of carbon source utilization, electron transfer and enzyme activity, were rarely reported.

Fulvic acid (FA) is a major fraction of humic substances (Janot et al. 2010; Rodríguez and Núnez 2011). It was observed in our current study that FA could positively affect denitrification process by simultaneously promoting NADH generation, electron transfer, and consumption. Thus, the purpose of this study was to report these properties. Firstly, the influence of FA on aqueous denitrification in the absence and presence of FA was compared. Then, the mechanisms for FA significantly increasing the efficiency of denitrification and reducing the accumulations of nitrite and N_2O were investigated from the aspects of carbon source (glucose) metabolism, NADH generation, cell proliferation, electron transfer, denitrifying gene expression, and enzymes' activity.

Materials and methods

Denitrifying bacteria and fulvic acid

P. denitrificans (American Type Culture Collection (ATCC) 19367, USA), was used as the model microbe in this study, owing to its wide appearance in the sedimentary environments (Berks et al. 1995). Prior to experiments, the microorganism was grown in Difco nutrient broth at 30 °C in a shaker with constant agitation (160 rpm) for 24 h and harvested in the early stationary growth phase according to our previous publication (Zheng et al. 2014). The cells were then centrifuged at 4500 rpm for 5 min, washed thrice with 0.1 M PBS (pH 7.4), and resuspended in the same buffer. FA was purchased from Aladdin (China). Its spectroscopic and chemical characteristics are detailed in Fig. S1 (Electronic Supplementary Material).

Experiments of the effect of FA on denitrification

The experiments were conducted in serum bottles with the prepared mineral medium. The mineral medium was prepared according to our previous publication with minor modification (g/L): glucose, 4.0; Na_2HPO_4, 2.556; KH_2PO_4, 0.272; $MgSO_4 \cdot 7H_2O$, 0.1; NH_4NO_3, 1.16; KNO_3, 1.45, and trace elements solution of 50 μL/L (Zheng et al. 2014). The trace elements contained (g/L) the following: $FeSO_4$ $7H_2O$, 2.50; $MnCl_2 \cdot 4H_2O$, 0.02; $ZnCl_2$, 0.34; $Na_2MoO_4 \cdot 2H_2O$, 0.242; $CuCl_2 \cdot 2H_2O$, 0.135; and EDTA-Na_2, 7.30. The initial nitrate concentration was 400 mg NO_3^--N/L. The aqueous solution of FA was prepared by dissolving 25 mg of the solid FA in 50 ml of the mineral medium above. The FA concentration of each condition was 0 (control), 10, 20, and 50 mg/L, respectively. It was observed

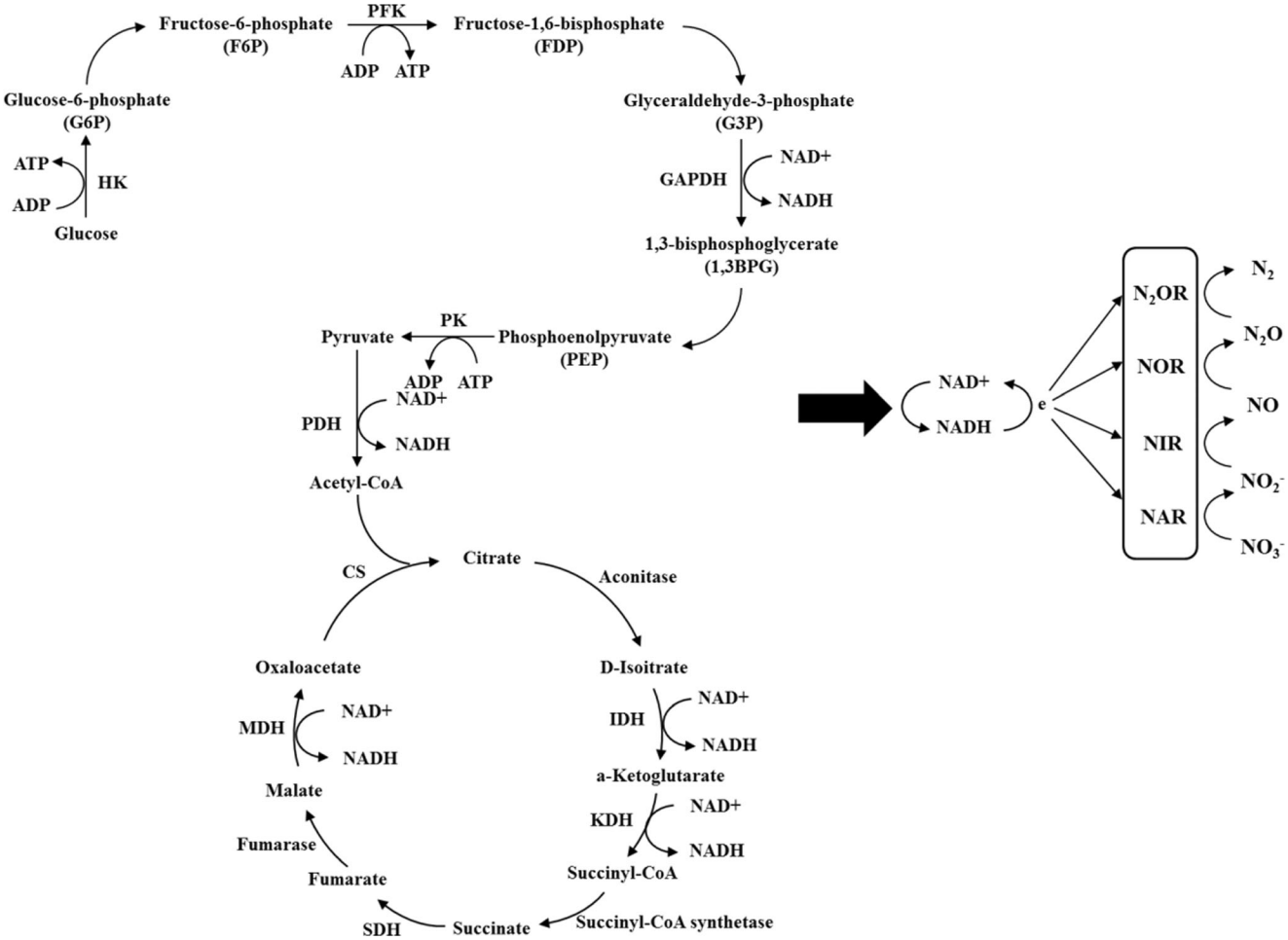

Fig. 1 Schematic diagram of glucose metabolism and its coupling with denitrification. Only the key steps and relevant glycolytic enzymes are labeled. *HK* hexokinase, *PFK* 6-phosphofructose kinase, *GAPDH* glyceraldehyde-3-phosphate dehydrogenase, *PK* pyruvate kinase, *PDH* pyruvate dehydrogenase, *IDH* isocitrate dehydrogenase, *KGDH* a-ketoglutarate dehydrogenase, *SDH* succinate dehydrogenase, *MDH* malate dehydrogenase, *CS* citrate synthase, *NAR* nitrate reductase, *NIR* nitrite reductase, *NOR* nitric oxide reductase, N_2OR nitrous oxide reductase

that the addition of FA did not significantly affect the pH of the mineral medium. Besides, another set of experiment was carried out using the same medium above expect for the addition of glucose and the FA concentration of each condition was 0 (control) and 50 mg/L to evaluate the potential role of FA as carbon source. After the addition of FA bulk solution, liquid bacteria suspension was added to make the initial OD600 value being 0.01 in each bottle. Gas argon was purged into each bottle for 10 min to ensure the anaerobic condition. All bottles were sealed and placed in a shaker (160 rpm) with constant temperature of 30 °C, and the concentrations of NO_3^--N, NO_2^--N, N_2O and glucose were measured during the experiments.

Assays of reduction equivalent

The intracellular reduction equivalent (NADH) level was detected according to the literature (San et al. 2002). Amount of 1 mL of the samples was collected at 20 h and centrifuged at 12,000 g for 5 min. After removing the supernatant, 0.2 M NaOH was added to re-suspend the pellets for NADH extraction. The samples were bathed at 50 °C for 10 min afterwards and then cooled down to 0 °C by ice. Thereafter, the extracts were neutralized by adding 300 μL of 0.1 M HCl (for NADH extraction) drop-wise while vortexing. Supernatants were obtained by centrifugation at 15,000 g for 5 min and transferred to new tubes for measurement immediately.

The intracellular NADH concentrations were determined by the enzymatic cycling assay. The mixture of cycling assay consisted of equal volumes of 1.0 M bicine buffer (pH 8.0), ethanol, 40 mM EDTA (pH 8.0), 4.2 mM thiazolyl blue (MTT), and twice the volume of 16.6 mM phenazine ethosulfate (PES), which was then incubated at 30 °C for 10 min. The reaction mixture was prepared as follows: 50 μL neutralized cell extract, 0.3 mL distilled water, and 0.6 mL reagent mixture. The reaction was started by adding 50 μL of alcohol dehydrogenase (ADH, 500 U/mL). The

absorbance at 570 nm was checked every 30 s for 5 min at 30 °C. The concentration of NADH was calibrated with standard solutions of NADH, and the final NADH level was calculated as per unit protein.

Assays of electron transfer system activities during denitrification

To get the crude cell extracts for the electron transfer system activities assays, cells were harvested at reaction time of 20 h and centrifuged at 4000 g for 10 min, which were then washed thrice with 0.1 M PBS (pH 7.4) and resuspended in the same buffer. Thereafter, the suspension was disrupted by sonication at 20 kHz for 5 min, and the cell debris was removed by centrifugation at 14,000 g for 10 min. The above operations were carried out at 4 °C. The crude cell extracts were immediately used for the determination of electron transfer activities, and the protein content was determined according to the reference with bovine serum albumin as standard (Lowry et al. 1951). The activity of complex I of the electron transfer system was assayed according to Humphries and Szweda (Humphries and Szweda 1998) by monitoring the consumption of NADH at 340 nm upon the addition of 100 micromolar concentration (μM) NADH and 50 μM ubiquinone (Aladdin, China) to 300 μL of cell extract. Amount of 5 μM azoxystrobin (Aladdin, China) was added to prevent NADH consumption by complex III of the electron transport chain (Esser et al. 2004). The activity of complex III was measured by the assay kit purchased from Nanjing Jiancheng Bioengineering Institute (China). The complex I and complex III activities were expressed as the consumption of μM NADH/(min mg protein) and μM reduced ubiquinone ($CoQH_2$)/(min mg protein), respectively.

Enzyme assays

The enzyme activity assays were conducted using the crude cell extracts specified in "Assays of electron transfer system activities during denitrification" section. The assays of HK, PFK, and PK activities were according to the literature (Peng and Shimizu 2003). The GAPDH activity was measured by the assay kit purchased from Sciencell Research Laboratories (USA). HK activity was determined by measuring the decrease of NADP at 340 nm. The reaction mixture (a total volume of 1 mL) contained 100 mM Tris-HCl (pH 7.5), 60 mM $MgCl_2$, 1 mM DTT, 0.5 mM $NADP^+$, 2 mM ATP, 15 mM glucose, 2 U glucose-6-phosphate dehydrogenase, and 300 μL of cell extract. PFK activity was assayed by monitoring the decrease of NADH in absorbance at 340 nm. The reaction mixture (1 mL) contained 50 mM imidazol HCl (pH 7.0), 0.05 mM ATP, 5 mM $MgCl_2$, 1 mM EDTA, 0.25 mM NADH, 0.25 mM fructose-6-phosphate (F6P), 0.5 U aldolase, 0.5 U glycerolphosphate dehydrogenase, 0.5 U triosephosphate isomerase, and 300 μL of cell extract. PK activity was measured spectrophotometrically at 340 nm through the oxidation of NADH to NAD^+, and the reaction mixture (1 mL) contained 0.1 M Tris-HCl (pH 7.5), 5 mM ADP, 1 mM DTT, 10 mM KCl, 15 mM $MgCl_2$, 0.5 mM phosphoenolpyruvate, 0.25 mM NADH, 10 U lactate dehydrogenase, and 100 μL of cell extract.

PDH was assayed by measuring the reduction of NAD+ at 340 nm upon the addition of 0.4 mM NAD^+, 0.4 mM TPP, 0.16 mM CoASH, 4.0 mM pyruvate, and 300 μL of cell extract (2 mL) (Humphries and Szweda 1998). IDH activity was measured by the increase of NADPH at 340 nm, and the reaction mixture (2 mL) contained 50 mM PBS (pH 7.5), 5 mM $MgSO_4$, 0.2 mM NADP, 10 mM D,L-isocitrate and 200 μL of cell extract (Müller et al. 1968). KGDH was assayed by measuring the reduction of NAD^+ at 340 nm upon the addition of 0.6 mM NAD^+, 0.1 mM TPP, 0.08 mM CoASH, 4 mM a-ketoglutarate, and 400 μL of cell extract (2 mL) (Humphries and Szweda 1998). MDH activity was determined by measuring the oxidation of NADH at 340 nm, and the assay mix contained 0.2 μM NADH, 1 μmol oxaloacetic acid, 45 μmol K phosphate (pH 7.5), and 400 μL of cell extract (2 mL) (Li et al. 1989). The specific enzyme activities were determined as the gradient of the absorbance variation divided by protein content. The analysis of other enzymes involved in tricarboxylic acid (TCA) cycle was detailed in Supplementary Information.

For determining the activities of denitrification reductases (NAR, NIR, NOR, and N_2OR), the assay mixture (1.7 mL) contained 10 mM PBS buffer (pH 7.4), 1 mM methyl viologen, 5 mM $Na_2S_2O_4$, and 5 mM reaction electron acceptor (KNO_3, $NaNO_2$, NO, or N_2O). All the above substances were diluted from stock solution and saturated solutions of NO and N_2O. The saturated solutions (2.0 mM for NO and 25 mM for N_2O) were prepared by purging pure NO or N_2O gas into Milli-Q water continuously for 5 min. It should be concerned that all the operations about NO were under anaerobic conditions or Ar protection for NO being easily oxidized by oxygen. The reaction was started by adding 0.3 mL crude cell extracts into the assay mixture. Then the mixture was immediately settled in a 30 °C incubator, and the data were collected every 10 min. The concentration of NO_2^--N, NO, or N_2O was determined, and the enzyme activity was calculated. In detail, the increased or decreased NO_2^--N concentration was detected by a spectrophotometer for NAR and NIR measurements, and the consumptions of NO or N_2O in mixture were recorded by corresponding microsensors (Unisense, Denmark) for determination of NOR and N_2OR. The activities of NAR and NIR were expressed respectively as the production and reduction of μM nitrite/(min mg protein). For NOR and N_2OR, the units of enzymatic activities were

the consumptions of μM nitric oxide/(min mg protein) and μM nitrous oxide/(min mg protein), respectively.

Gene expression quantification of denitrifying enzymes

The gene expressions of NAR, NIR, NOR, and N_2OR were determined by the quantification of narG, nirS, norB, and nosZ genes via reverse transcriptase quantitative PCR (RT-qPCR) (Philippot et al. 2001). Bacterial cells of model denitrifying bacteria (*P. denitrificans*) were harvested in the exponential growth phase (20 h) by centrifugation (10,000 g) for 10 min at 4 °C and then lysed in TRIzol reagent (Invitrogen) for extraction of total RNA. To avoid DNA contamination, the extracted RNA was treated with DNase I (Ambion) according to the manufacturer's protocol. The extracted total RNA was used to synthesize complementary DNA (cDNA) at 42 °C. Thereafter, cDNA was purified using a QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instruction. The qPCR was performed via a StepOne real-time PCR system (Applied Biosystems, Foster City, USA) in a total volume of 20 μL containing 1 × SYBR Green PCR Master Mix, 0.5 μM of each primer, and 1 μL of cDNA. The primers and amplification conditions are summarized in Table S1. All qPCR assays were performed using three replicates per sample and contained the control reactions without cDNA.

Other analytical methods

The variations of NO_3^--N, NO_2^--N, and total nitrogen (TN) during denitrification were obtained by measuring the supernatant after centrifugation of liquid samples at 12,000 g for 5 min. The assay of N_2O was conducted by a gas chromatograph (GC) (Agilent 7820A, USA) with an electron capture detector (ECD). The N_2O in gas phase was directly sampled, and the N_2O in aqueous phase was detected after using headspace with equilibrium temperature and time of 25 °C for 3 h respectively according to the literature (Zheng et al. 2014). The optical density (OD) at 600 nm was used to evaluate the cellular growth of microbes. The intracellular reactive oxygen species (ROS) production in the absence and presence of FA was determined by fluorescence assay (Su et al. 2015). The assays of FA by Fourier transform infrared (FTIR) and excitation emission matrix (EEM) fluorescence were detailed in the section of Supplementary Material. All other analyses were the same as those described in our previous publications (Zheng et al. 2014). All tests were performed in triplicate, and the results were expressed as mean ± standard deviation. An analysis of variance (ANOVA) was used to test the significance of results, and $p < 0.05$ was considered statistically significant.

Results

Effects of FA on denitrification performance

The denitrification performance in the absence and presence of FA was compared firstly. The data in Fig. 2a showed that the total nitrogen removal efficiency was 74.8 % in the control test, which was remarkably increased to 99.9 % at FA dosages of 10, 20, and 50 mg/L. From the variations of NO_3^--N in Fig. 2b, it can be seen that at any time the nitrate concentration was decreased with the increase of FA from 0 to 50 mg/L. The final nitrate concentration was only around 0.2 mg/L at any FA concentration investigated, but it was 43.8 mg/L in the control. The presence of FA also influenced the accumulation of nitrite (Fig. 2c). The maximal accumulation of nitrite was decreased with the increase of FA, and the nitrite was non-detectable at the end of the tests in all FA experiments. However, the final concentration of accumulated nitrite was 57.4 mg/L in the control test. From Fig. 2d, it was also found that the use of FA remarkably decreased the generation of N_2O during the denitrification. The amount of total N_2O generated in the control test was 0.240 mg N_2O-N/mg TN removed. Nevertheless, in FA tests, the N_2O generation decreased from 0.141 to 0.038 mg, and N_2O-N/mg TN was removed with the increase of FA from 10 to 20 mg/L, and a much lower N_2O generation (0.003 mg N_2O-N/mg TN removed) was observed with further increasing the FA to 50 mg/L. Clearly, the presence of FA not only enhanced the total nitrogen removal greatly but reduced nitrite accumulation and nitric oxide generation remarkably.

Effects of FA on reduction equivalent generation and carbon source utilization

It is well known that NADH is the direct electron donor for denitrification, and the available amount of NADH affects its performance. Figure 3a compared the available intracellular NADH content in the absence and presence of FA. The data in Fig. 3a showed that the available intracellular NADH content was 141 % of the control at 10 mg/L FA. With the increase of FA to 20 mg/L, the available amount of NADH became 163 % of the control. A much higher NADH content (179 % of the control) was obtained, as FA was further increased to 50 mg/L. It can been seen that the presence of FA increased the generation of available NADH.

Several key enzymes involved in the NADH generation during carbon source metabolism, including glycolysis and TCA cycle, were evaluated. From Fig. 3a, the activities of glycolytic enzymes, such as GAPDH, HK, PFK, and PK were increased with the increase of FA. The GAPDH activity was 110 % of the control at FA concentration of 10 mg/L, and 117 % of the control at 20 mg/L reached 129 % at 50 mg/L. The activities of HK and PFK were improved respectively

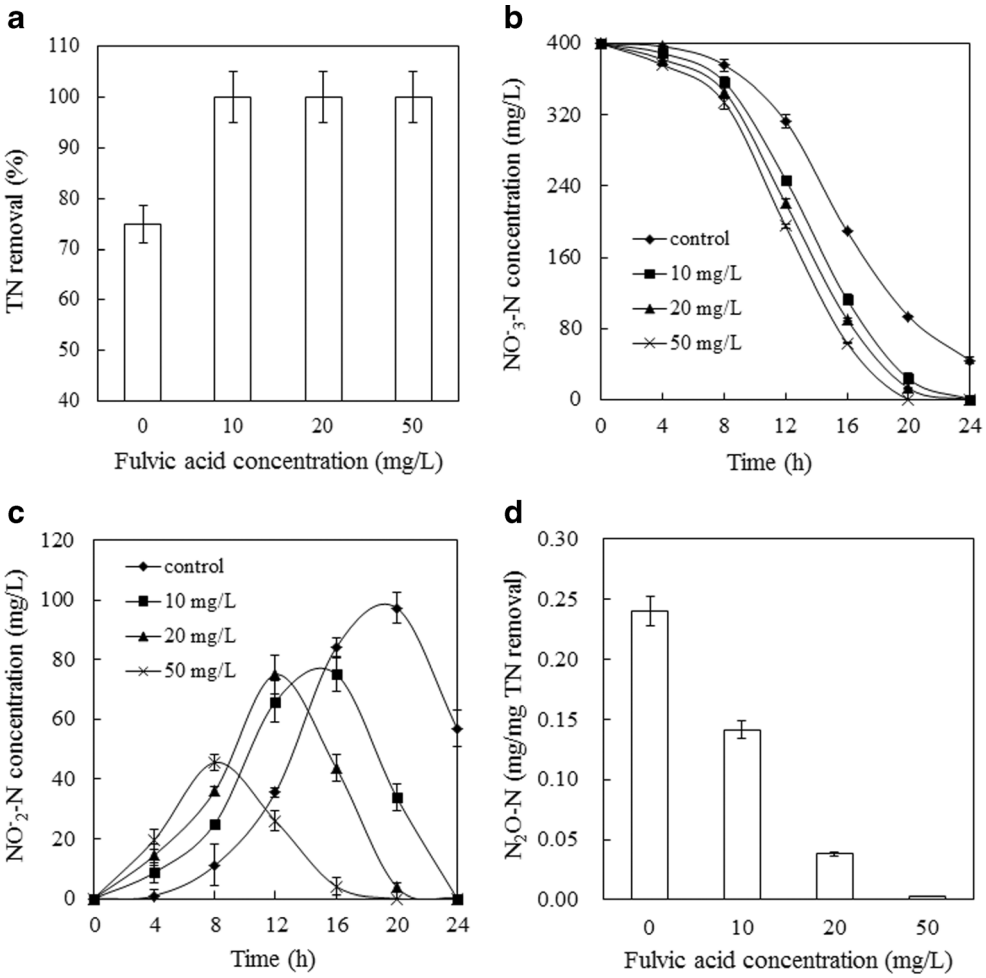

Fig. 2 The effects of FA on the final TN removal efficiency (**a**), time course of NO_3^--N (**b**), and NO_2^--N (**c**), and total liquid and air phase N_2O-N generation (**d**). *Error bars* represent standard deviations of triplicate tests

from 107 and 112 % to 129 and 131 % of the control with the increase of FA from 10 to 50 mg/L. Moreover, the activity of PK was enhanced by FA, which was 127, 132, and 148 % of the control at FA of 10, 20, and 50 mg/L. As illustrated in Fig. 3c, the activities of PDH, IDH, KGDH, and MDH, which are responsible for the NADH production during TCA cycle, were also increased in the presence of FA. The PDH activity increased to about 170 % of the control in all FA concentrations. The activity of IDH was increased from 124 to 132 % of the control with the increase of FA from 10 to 50 mg/L. The relative PDH activity at 10, 20, and 50 mg/L of FA was respectively 115, 120, and 134 % of the control. The MDH activity was increased from 113 to 125 % of the control in all FA dosages. Moreover, it was also observed that the activities of other enzymes involved in TCA cycle, such as citrate synthase, aconitase, succinyl-CoA synthetase, succinate dehydrogenase, and fumarase were increased to some extent in all FA tests (Electronic Supplementary Material, Table S2). All these enzymes involved in carbon source metabolism were enhanced by FA, thus leading to the increased available intracellular NADH content for denitrification. Moreover, the increased glucose consumption and cell growth were also observed (Fig. 3d and Electronic Supplementary Material, Fig. S2).

Effects of FA on electron transfer in denitrification process

In order to investigate the influence of FA on electron transfer in denitrification process, the electron transfer activities of complex I and complex III were studied. The first step of denitrification, i.e., the bio-reduction of nitrate to nitrite, mainly depends on the electron transportation via complex I. It can be seen from Fig. 4b that the activities of complex I was increased with the addition of FA, which were 132, 141, and 155 % of the control at FA concentrations of 10, 20, and 50 mg/L, respectively. The electron transfer via the complex III is also important for the transformation and accumulation of nitrate intermediates during denitrification, such as nitrite and N_2O, by offering electrons directly to NIR and N_2OR (Zumft 1997; Chen and Strous 2013). The data in Fig. 4c also showed that the electron transfer activity of complex III was also enhanced in the presence of FA. At 10 mg/L of FA, the complex III activity was 136 % of the control. The complex III activity was increased to 152 % of the control at 20 mg/L FA

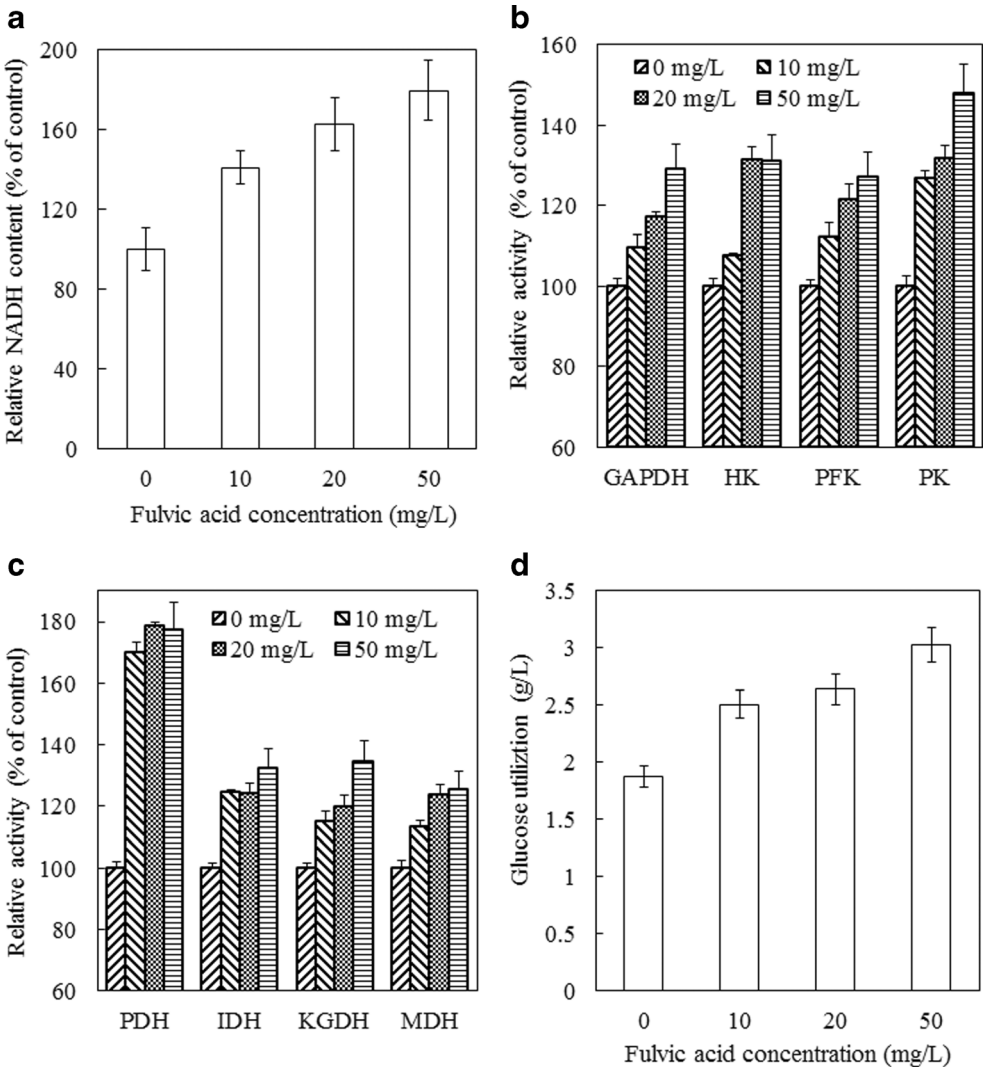

Fig. 3 The effects of FA on NADH generation (**a**), the relative activity of key enzymes involved in glycolysis (**b**), TCA cycle (**c**), and glucose utilization (**d**). *Error bars* represent standard deviations of triplicate tests

and reached 181 % of the control by further increasing the FA to 50 mg/L. It can be indicated that the presence of FA increased the electron transfer via complex I and complex III, thus contributed to the acceleration of nitrate reduction and intermediates transformation.

Effects of FA on synthesis and catalytic activity of denitrifying enzymes

Figure 5 illustrated the effect of FA on gene expression of denitrifying enzymes. It can be seen from Fig. 5 that the expressions of narG and norB, encoding genes for the major subunit of NAR and NOR, were improved slightly and only increased from 115 and 110 % to 176 and 165 % of the control with the increase of FA from 10 to 50 mg/L. On the other hand, the expressions of nirS and nosZ genes, encoding genes for NIR and N_2OR, were improved significantly. The relative expressions of nirS and nosZ were increased from 239 and 118 % to 497 and 508 % of the control with the increase of FA from 10 to 50 mg/L.

The presence of FA also impacted the catalytic activities of denitrifying enzymes. From Table 1, it can be observed that all enzymes investigated in this study were improved by FA. The activity of NAR, NIR, NOR, and N_2OR was respectively 0.131, 0.246, 0.065, and 0.035 (μmol N/min·mg protein) in the control test, which was improved to 0.156, 0.399, 0.095, and 0.060 (μmol N/min·mg protein) at FA concentration of 10 mg/L. With the increase of FA to 50 mg/L, these enzymes activities were further improved to 0.158, 0.433, 0.118, and 0.079 (μmol N/min mg protein). It can be indicated that FA could enhance both synthesis and catalytic activity of denitrifying enzymes.

Discussion

Microbial denitrification requires the participation of electrons. It is well known that NADH is the direct electron donor for denitrification (Berks et al. 1995; Chen and Strous 2013),

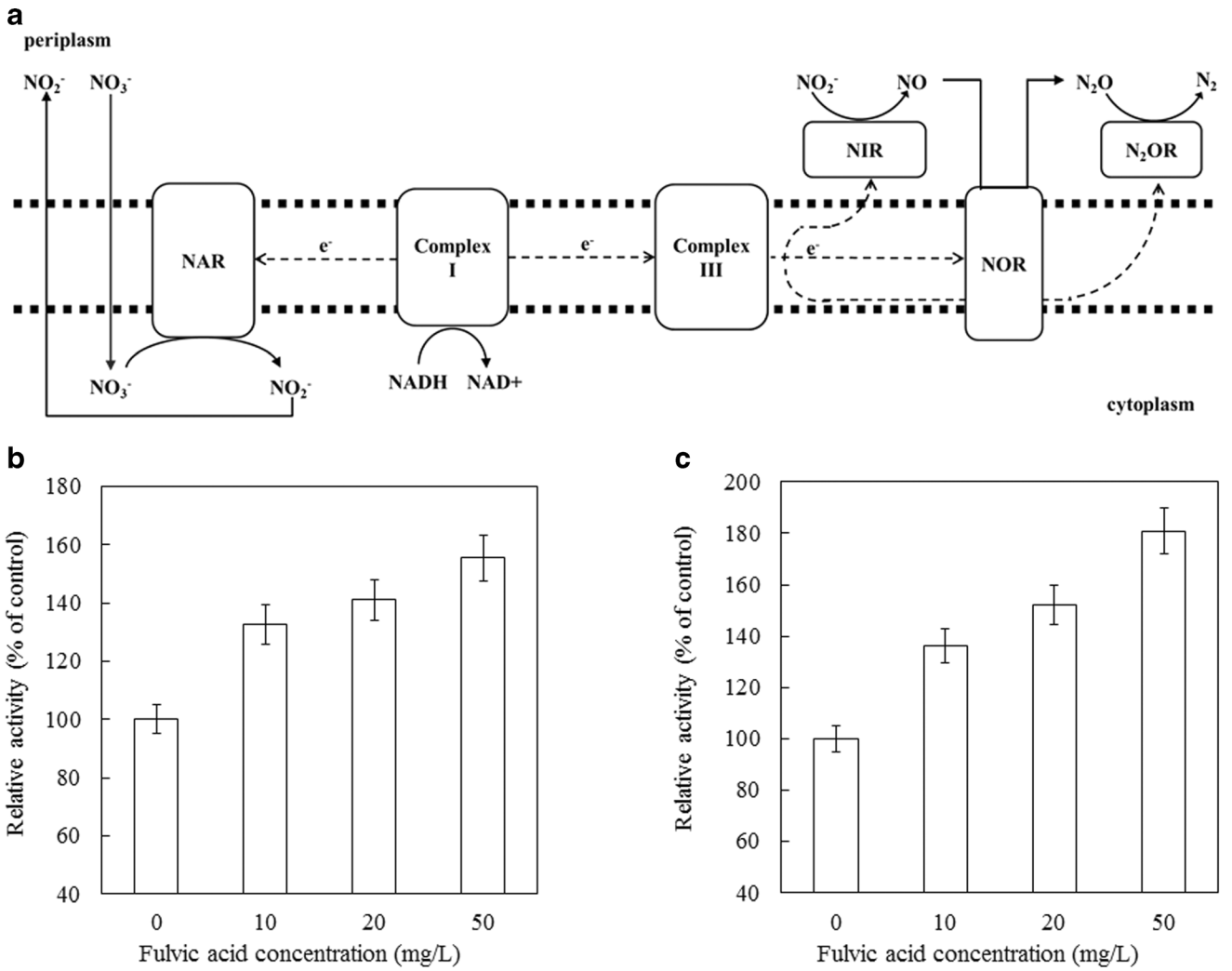

Fig. 4 Schematic denitrification and electron transfer pathway in denitrification (**a**) and the effects of FA on electron transfer activity of complex I (**b**) and complex III (**c**). *Error bars* represent standard deviations of triplicate tests

which suggests that the denitrification performance is affected by the available amount of NADH. In the current study, the NADH is generated in glucose metabolism. When glucose is supplied as the carbon source for denitrification, series of continuous reactions are involved, and HK, PFK, GAPDH, and PK play vital roles in the generation of NADH via glycolysis (Saltiel and Kahn 2001). Firstly, as seen in Fig. 1, the amount of NADH generated during glycolytic pathway is directly relevant to the activity of GAPDH, which catalyzes the bioconversion of glyceraldehyde 3-phosphate (G3P) to 1,3-biphosphoglycerate (1,3BPG) with the NADH generation. The data in Fig. 3b showed that the GAPDH activity was increased with the increase of FA, and it reached 129 % of the control at FA concentration of 50 mg/L. Thus, more NADH would be generated due to the increased GAPDH activity. Secondly, as illustrated in Fig. 1, the direct substrate for NADH production is G3P, which can be increased by the improvement of HK and PFK. From Fig. 3b, it can be seen that the activities of HK and PFK were improved respectively from 107 and 112 % to 129 and 131 % of the control with the increase of FA from 10 to 50 mg/L. As both HK and PFK were improved, more G3P was formed, which was the second reason for FA increasing glycolytic NADH generation. In addition, Fig. 1 indicates that the generation of NADH via glycolysis is influenced by the conversion of 1,3BPG, which is mainly controlled by the activity of PK. In the current study, the activity of PK was enhanced by FA, which was 148 % of the control at FA of 50 mg/L (Fig. 3b).

Pyruvate was formed at the end of glycolysis pathway and then participated in the TCA cycle (Li et al. 1989). Firstly, four enzymes, PDH (pyruvate dehydrogenase), IDH (isocitrate dehydrogenase), KGDH (a-ketoglutarate dehydrogenase), and MDH (malate dehydrogenase), catalyze the conversions of pyruvate to acetyl-CoA, D-isocitrate to a-ketoglutarate, a-ketoglutarate to succinyl-CoA and malate to oxaloacetate accompanied by the generation of NADH (Fig. 1). As seen from Fig. 3c, the activities of PDH, IDH, KGDH, and MDH were increased with the increase of FA from 10 to 50 mg/L and

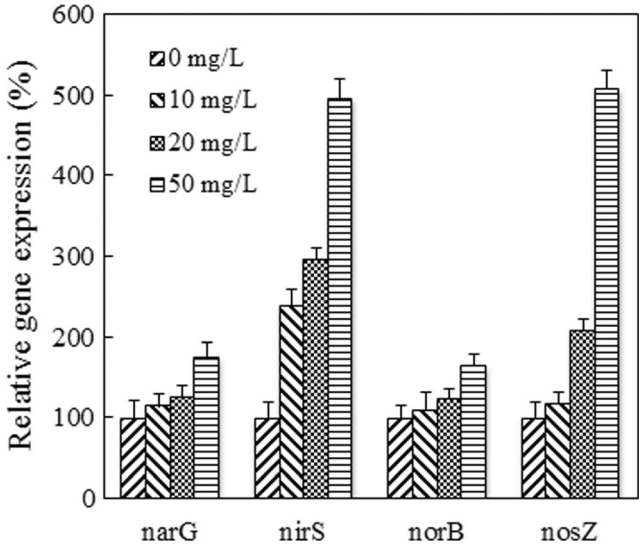

Fig. 5 The effects of FA on gene expressions of denitrifying enzyme genes. *Error bars* represent standard deviations of triplicate tests

reached 177, 132, 134, and 125 % of the control, respectively. The elevated activities of PDH, IDH, KGDH, and MDH lead to the direct increment of available intracellular NADH content generated during TCA cycle pathway. On the other hand, the activities of enzymes like citrate synthase, aconitase, succinyl-CoA synthetase, succinate dehydrogenase, and fumarase that involved in TCA cycle were also observed improved in all FA tests (Table S2). In this way, the stimulation of these enzymes induced more formed intermediates related to NADH generation in TCA cycle, thus also contributing to the available NADH production. Therefore, the presence of FA increased the generation of available NADH, which was an important reason for its enhancing denitrification performance.

It should be noticed that consistent with the literatures (Lovley et al. 1999; Coates et al. 2002), the FA used in this study cannot be utilized as carbon source by *P. denitrificans* for denitrification (Fig. S3). Previous publications found that some intermediates produced in microbial process, such as oxidative stress caused by reactive oxygen species (ROS), could give negative influence on normal cellular metabolism including glycolysis and TCA cycle (Tretter and Adam-Vizi 2000; Su et al. 2015), and humic substances (such as FA) could relieve this side effect by functioning as the free-radical scavenger owing to the presence of simple structure like aliphatic groups (Muscolo and Sidari 2009; García et al. 2012). From the FTIR analysis (Electronic Supplementary Material, Fig. S1), it can be seen that FA had intense bands of 1389 and 1041 cm^{-1}, which indicated the presence of C–H deformation and C–C stretching motions of aliphatic groups (Michael et al. 2007; Li et al. 2011). As a result, lower ROS was observed to be produced in the presence of FA (Electronic Supplementary Material, Fig. S4). Therefore, it can be concluded that the reasons for FA stimulating the available intracellular NADH generation were attributed to its increasing the activities of key glycolysis and TCA cycle enzymes.

As illustrated in Fig. 4a, the NADH generated in the above glucose metabolism is then catalyzed by electron transfer system to produce electron for the reduction of nitrate to nitrite, nitric oxide, nitrous oxide, and final nitrogen gas by electron transfer system (Berks et al. 1995; Zumft 1997). The complex I (NADH-ubiquinone oxidoreductase and ubiquinone pool) and complex III (ubiquinol-cytochrome c oxidoreductase [cytochrome bc_1 complex]) constitute the electron transport chain backbone of *P. denitrificans* (Chen and Strous 2013). One flux of electron is delivered directly to membrane-bound NAR by complex I, while the other one is transferred to NIR, NOR, and N_2OR via complex III. It seems that the performance of these two electron transfer systems may impact nitrate reduction, nitrite accumulation, and N_2O emission. From Fig. 4b, c, the activities of both complex I and complex III were increased in the presence of FA and reached 155 and 181 % of the control at 50 mg/L. The electron transfer improvement of complex I benefited nitrate reduction, and the nitrate concentration in FA tests declined more significantly than the control (Fig. 2b). Moreover, the enhanced electron transfer activity via complex III facilitated the reduction of both nitrite and N_2O reductions, which is consistent with our previous observation (Fig. 2c, d).

The FTIR analysis (Electronic Supplementary Material, Fig. S1) indicated that there were aromatic C=C, carboxyl C=O, and COO− groups in FA (1593 cm^{-1}), which had been reported to be the major components of the quinone structure

Table 1 The effects of FA on the activity of key denitrifying enzymes[a]

Fulvic acid concentration (mg/L)				
Item	0	10	20	50
NAR	0.131 ± 0.005	0.156 ± 0.003	0.157 ± 0.004	0.158 ± 0.003
NIR	0.246 ± 0.011	0.399 ± 0.013	0.418 ± 0.021	0.433 ± 0.025
NOR (N1)	0.065 ± 0.009	0.095 ± 0.010	0.107 ± 0.005	0.118 ± 0.007
N_2OR (N2)	0.035 ± 0.002	0.060 ± 0.005	0.069 ± 0.005	0.079 ± 0.006
N1/N2	1.857 ± 0.007	1.588 ± 0.009	1.549 ± 0.010	1.487 ± 0.007

[a] The unit is μmol N/(min·g protein) except N1/N2. The data are the averages and their standard deviations of triplicate tests

(Cory and McKnight 2005; Jiang and Kappler 2008). It can be suggested that FA can function as electron shuttles conferring the capacity to serve as electron carriers in denitrification process. The humic analog, anthraquinone-2,6-disulfonate (AQDS), had been observed to have the ability to act as electron shuttle during denitrification by *Paracoccus versutus* sp. GW1, but it mainly enhanced the bioconversion of nitrate to nitrite and showed little influence on nitrite bio-reduction, thus resulted in the accumulation of nitrite (Xi et al. 2013). In this study, besides the function as an electron shuttling similar to AQDS, FA also induced much higher electron transfer activities of both complex I and complex III in electron transfer system of *P. denitrificans*. As a kind of low molecular weight hydrophilic humic substances, FA has the ability to reach the cell periplasm or membrane (Klein et al. 2014). In this condition, FA is presumably associated with the membrane-bound electron transfer chain, thus improving the performance of electron transportation from NADH to denitrifying key enzymes, which contributed to the remarkable increase of total nitrogen removal and decreases of nitrite accumulation and N_2O generation.

Nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase are the well-known key enzymes responsible for microbial denitrification. These enzymes receive the electrons via electron transportation chain and reduce nitrate, nitrite, nitric oxide, and nitrous oxide to nitrogen gas finally (Berks et al. 1995; Zumft 1997). The key encoding genes for NAR, NIR, NOR, and N_2OR are respectively narG, nirS, norB, and nosZ genes (Philippot et al. 2001), and the synthesis of these denitrifying enzymes depends on the transcriptional expression of these encoding genes. Analysis by RT-qPCR assay targeting narG, nirS, norB, and nosZ indicated that the gene expression of these enzymes was enhanced by the presence of FA to different extent. Among them, the level of nirS and nosZ gene expression was elevated much higher than that of narG and norB, and their relived expressions were 497 and 508 % of the control, respectively (Fig. 5). It can be seen that the presence of FA poses positive effects on denitrifying enzymes from gene level.

The gene expression not only influences the synthesis of denitrifying enzymes but affect the catalytic activities of these enzymes. From Table 1, all activities of denitrifying enzymes were observed improved by FA and increased with the increment of FA addition. The activities of NAR and NIR were increased from 0.156 to 0.157 and 0.399 to 0.418 (μ mol N/min·mg protein) with the increase of FA from 10 to 20 mg/L. The NAR and NIR activities were further improved to 0.158 and 0.433 (μ mol N/min mg protein) with the increase of FA to 50 mg/L. It can be calculated that compared with the control (i.e., without FA addition), the increase of NIR was respectively 1.62-, 1.70-, and 1.76-fold of the control at FA of 10, 20, and 50 mg/L, while that of NAR was 1.19-, 1.20-, and 1.21-fold of the control, suggesting that FA induced a much faster nitrite reduction than nitrate, which was an important reason for less nitrite accumulation observed in Fig. 2c.

The levels of nitrite in biological nitrogen removal process have been reported to significantly influence the accumulation of N_2O, and the increase of nitrite concentration can cause the increase of N_2O emission due to the bioconversion of N_2O to N_2 being readily inhibited by the toxicity of nitrite (Zhou et al. 2008; Yang et al. 2009). Owing to the higher activity of NIR than NAR induced by the presence of FA, the nitrite accumulation was obviously lower than that in the control (Fig. 2c), which was one reason for lower N_2O generated in all FA tests. In addition, the observed N_2O accumulation in denitrification process is the balance of its production (i.e., from nitric oxide to nitrous oxide via the activity of NOR) and consumption (from nitrous oxide to nitrogen gas via N_2OR), which has been reported to be positively correlated with the ratio of NOR activity/N_2OR activity (N1/N2) (Zhu and Chen 2011). The data in Table 1 showed that compared with the control, the ratio of N1/N2 decreased from 1.588 to 1.487 with the increase of FA from 10 to 50 mg/L. The N1/N2 ratio in all FA tests was lower than that of the control, which agreed with lower N_2O emission being observed in the presence of FA (Fig. 2d).

The effects of humic substances on denitrifying enzymes activities were seldom reported. Yin et al. found that some quinone compounds can increase the activity of NAR and NIR of denitrifying bacteria (Yin et al. 2014); however, its mechanism has not been explained. It was widely reported that NAR and NOR of *P. denitrificans* are membrane-bound while NIR and N_2OR are periplasm-located (Berks et al. 1995; Zumft 1997; Chen and Strous 2013). When FA reaches the cell periplasm or membrane (Klein et al. 2014), these can interact with these denitrifying enzymes. In the literature, HS have the ability to positively influence the denitrifying enzymes via facilitating the enzyme–substrate interaction by forming protective complexes (Benitez et al. 2005; Li et al. 2013), which might also be the reason for the increased enzyme activities observed in the presence of FA.

FA is ubiquitous in the environment, such as wastewater, groundwater, river, sediment, and soil. Denitrifying microbes have also been widely reported in these circumstances. Although humic substances had been reported to have the ability to transfer electron, this study showed that during aqueous denitrification, FA could remarkably enhance the generation of NADH and promote the transfer and consumption of electrons by stimulating the metabolism of carbon source via glycolysis and TCA cycle pathways and the activities of electron transport system and key denitrifying enzymes, which resulted in the increase of total nitrogen removal with lower nitrite accumulation and less N_2O emission. It will provide an important new insight into the potential effect of FA on microbial denitrification metabolism process and even nitrogen cycle in nature niches.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval This article does not contain any studies with human participants performed by any of the authors.

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