Weak electrical stimulation on biological denitrification: Insights from the denitrifying enzymes

Xinyi Dong, Hongbo Liu, Shiping Long, Suyun Xu, Eric Lichtfouse

https://doi.org/10.1016/j.scitotenv.2021.150926

Weak electrical stimulation on biological denitrification: Insights from the denitrifying enzymes

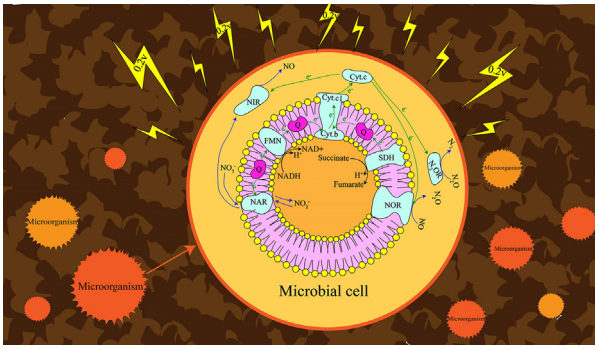
Xinyi Dong [a,1], Hongbo Liu [a,*,1], Shiping Long [b], Suyun Xu [a,*], Eric Lichtfouse [c]

[a] School of Environment and Architecture, University of Shanghai for Science and Technology, 516 Jungong Road, 200093 Shanghai, China
[b] Chongqing New World Environment Detection Technology Co. Ltd., 22 Jinyudadao, 401122 Chongqing, China
[c] Aix-Marseille Univ, CNRS, IRD, INRA, Coll France, CEREGE, 13100 Aix en Provence, France

HIGHLIGHTS

- Weak electrical stimulation was applied to enhance biological denitrification.
- Weak electrical stimulation was found to reduce N_2O production.
- Denitrifying enzyme activity was increased by weak electrical stimulation.
- Weak electrical stimulation accelerates electron transfer.

GRAPHICAL ABSTRACT



ABSTRACT

In order to improve the denitrification efficiency of low carbon to nitrogen ratio (C/N) wastewater, we conducted continuous flow experiments of weakly electrically stimulated denitrification using a direct current output voltage. The results showed that the best denitrification was achieved at a voltage of 0.2 V. The removal of nitrate and total nitrogen was increased by 20% and the production of intermediate greenhouse gas (N_2O) was reduced by 62.6%. We explored the specific pathways involved in the weak electrical stimulated denitrification using enzyme activity as a cut-off point. The enzyme activity analysis and 3D fluorescence spectroscopy revealed that nitrate reductase (NAR) and nitrite reductase (NIR) activities were significantly enhanced by weak electrical stimulation, and the aromatic protein content in extracellular polymers substances (EPS) increased, accelerating electron transfer and promoting the conversion of loosely bound EPS (LB) to tightly bound EPS (TB). The accelerated electron transfer further increased enzyme activity and the metabolic rate of microorganisms. This study indicates that weak electrical stimulation could improve activities of biological enzymes to enhance denitrification efficiency.

Keywords:
Weak electrical stimulation
Denitrification
Enzyme activity
EPS
Electron transfer

1. Introduction

Increasingly prominent nitrogen pollution of water bodies poses a serious threat to human health and ecological functions (Jiang et al., 2017; Guo et al., 2016). Biological nitrogen removal has become one of the cost-effective methods for nitrogen removal because of its high efficiency and low cost. Biological denitrification is completed by aerobic

* Corresponding authors at: 516, Jungong Road, 200093 Shanghai, China.
E-mail addresses: Liuhb@usst.edu.cn (H. Liu), xusy@usst.edu.cn (S. Xu).
[1] Xinyi Dong and Hongbo Liu contribute equally to this work.

nitrification (NH_4^+-N—NO_2^--N—NO_3^--N) and anoxic denitrification (NO_3^--N—NO_2^--N—N_2) using aerobic nitrification bacteria and denitrification bacteria respectively (Ycab et al., n.d.). However, in practical wastewater applications, when the C/N (COD/TN) ratio of wastewater is low, the microorganisms will not be able to perform efficient denitrification due to insufficient carbon source (Zhang and He, 2012). To solve this issue, researchers have combined microbial fuel cells (MFC) with denitrification as an alternative to additional dosage of carbon sources such as methanol and acetate for the treatment of low C/N wastewater (Li et al., 2014; Virdis et al., 2010). In this case nitrate and nitrite acted as electron acceptors in the cathode chamber of MFC. Under the catalytic action of denitrifying microorganisms, the cathode electrode electrons could be used for electrochemical denitrification directly while energy could be recovered (Jin et al., 2014; Zhang and Angelidaki, 2013). Thus in recent years, studies using voltages lower than the hydrolysis voltage (1.23 V) to promote microbial processes have attracted wide attention (Chun et al., 2013).

Studies have shown that electrical stimulation at constant potential shortens the duration of the growth arrest period of denitrifying bacteria cells and significantly increases the denitrification efficiency (Beschkov et al., 2004). At the same time, microbial activity increases with current density (Liu et al., 2015). Higher current intensity promotes denitrification at surface of the MFC cathode, increasing denitrifying bacteria activity, and leading to further denitrification (Huang et al., 2013). When the current intensity exceeds a certain range, it also has an inhibitory effect on microbial activity, which affects the denitrification reaction (Thrash and Coates, 2008). Denitrification is carried out gradually by specific enzymes as shown in Eqs. (1)–(4).

$$2NO_3^- + 4H^+ + 4e^- \xrightarrow{NAR} 2NO_2^- + 2H_2O \quad (1)$$

$$2NO_2^- + 4H^+ + 2e^- \xrightarrow{NIR} 2NO + 2H_2O \quad (2)$$

$$2NO + 2H^+ + 2e^- \xrightarrow{NOR} N_2O + H_2O \quad (3)$$

$$N_2O + 2H^+ + 2e^- \xrightarrow{NOS} N_2 + H_2O \quad (4)$$

Wang et al. (2019) investigated the effect of carbon nanotubes on the denitrification performance of the alkali-producing gene and found that denitrification could be promoted by increasing the activity of NAR, NIR and nitric oxide synthase (NOS). This promotion is directly responsible for the increased nitrate removal efficiency and reduced N_2O production. Previous studies have shown that denitrifying enzyme activity is influenced by electron acceptors (Verbaendert et al., 2011), C/N (Pang and Liu, 2010), pH and other factors (Gao et al., 2012). The decrease in C/N ratio affected synthesis of denitrifying enzymes and hindered the growth of denitrification microorganisms. Low pH and high H^+ concentration caused unstable enzyme structure and inhibited the NAR activity.

In this study, we used direct current (DC) power supply to study the effect of weak electrical stimulation on the denitrification process by adjusting the C/N ratios and weak electrical stimulation intensity, and investigate the pathway of weak electrical stimulation and its effect on denitrification performance from the perspective of denitrifying enzymes. Components of produced gas during denitrification have been detected together with denitrification efficiency analysis of the process. The effect of weak electrical stimulation on microbial metabolism was explored through denitrifying enzyme activity analysis, EPS content detection and distribution profiling. The mechanism of weak electrical stimulation on microbial denitrification enhancement was further proposed, providing a new tool improving denitrification performance under low carbon source water quality conditions.

2. Materials and methods

2.1. Experimental setup

Fig. 1 shows schematic diagram of the experimental setup. The volume of the experimental set-up in this study was 1.2 L. It was divided into a control group (CK, no electrical stimulation set-up) and an experimental group (EG, electrical stimulation set-up). The electrical stimulation voltage values of 0.2 V (0.567 mA/cm^2), 0.4 V (1.12 mA/cm^2) and 0.6 V (1.74 mA/cm^2) were selected according to results of our previous study (Liu et al., 2021); while the corresponding current intensity was set as the output value of the DC power supply. The experimental electrodes and the usage method are also consistent with the previous study (Liu et al., 2021). In this study, 200 mL of anaerobic denitrification sludge and 800 mL of artificial wastewater were fed to the reactor in a continuous ascending flow with a hydraulic retention time of 12 h. The C/N ratio was set at two values as 4 (COD and NO_3^--N concentrations of 200 and 50 mg/L, respectively) and 3 (COD and NO_3^--N concentrations of 150 and 50 mg/L, respectively).

The main components of the low C/N wastewater are shown in Table 1. The components of the concentrated solution of trace elements are 1.61 g/L $CoCl_2 \cdot 6H_2O$,1.5 g/L $FeSO_4 \cdot 6H_2O$, 0.15 g/L H_3BO_3, 0.18 g/L KI, 0.12 g/L $ZnSO_4 \cdot 7H_2O$, 0.12 g/L $MnCl_2 \cdot 4H_2O$, 0.03 g/L $CuSO_4 \cdot 5H_2O$ and 0.06 g/L $Na_2MoO_4 \cdot H_2O$.

The anaerobic denitrification sludge was obtained from the East Shanghai Wastewater Treatment Plant (31°16′40.50″N, 121°32′39.99″ E) and domesticated using the same conditions as the experimental synthetic wastewater. The sludge was 100% returned and replenished with new sludge every three to five days to maintain a sludge concentration of about 5000 mg/L in the reactor.

2.2. Analytical methods

2.2.1. Analysis of nitrogenous pollutants

Sludge sampling was carried out every 12 h. After adjusting the equipment current intensity and/or C/N ratios and other parameters, it needs to be stabilized for 3–5 days before sampling. The concentrations of NH_4^+-N, NO_2^--N, NO_3^--N and TN were determined by the nano-reagent photometry, N-(1-naphthyl)-ethylenediamine spectrophotometer, ultraviolet spectrophotometry and potassium persulfate to eliminate ultraviolet spectrophotometric determination respectively.

2.2.2. Gas component detection

Gas production was collected continuously for 7 days at an optimum voltage of 0.2 V to analyze the effect of weak electrical stimulation on the denitrification process. The hydrogen, nitrogen and nitrous oxide were measured by the Shimadzu GC-14B gas chromatograph (Japan), Thermo TRACE 1300 gas chromatograph (USA) and Agilent 6890 N gas chromatograph (USA), respectively. The experimental samples were collected using an E-Switch sampling bag, and a 1 mL syringe was used for the measurement. The injection volume of hydrogen, nitrogen and nitrous oxide were 1.0 mL, 0.1 mL and 1.0 mL respectively. A single point calibration was performed using the lowest concentration point when the sample concentration was well below the lowest point of the standard series (The standard curve is shown in Fig. S1). The measuring conditions are listed as in Table 2.

2.2.3. Denitrifying enzymes (NAR, NIR) extraction and activity determination

We investigated the effect of weak electrical stimulation on denitrifying microbial activity under weak electrical stimulation by measuring NAR and NIR activities. The mixed liquid suspended solid from different groups was centrifuged at 5000r for 5 min at 4 °C. The centrifuged precipitate was rinsed with 0.01 M phosphate buffer (PBS) at pH 7.4 for three times, resuspended in 0.01 M PBS buffer at pH 7.4, and sonicated in an ice bath (20 kHz) for 5 min. After sonication, the cell suspension was placed in a centrifuge at 12,000r for 15 min at

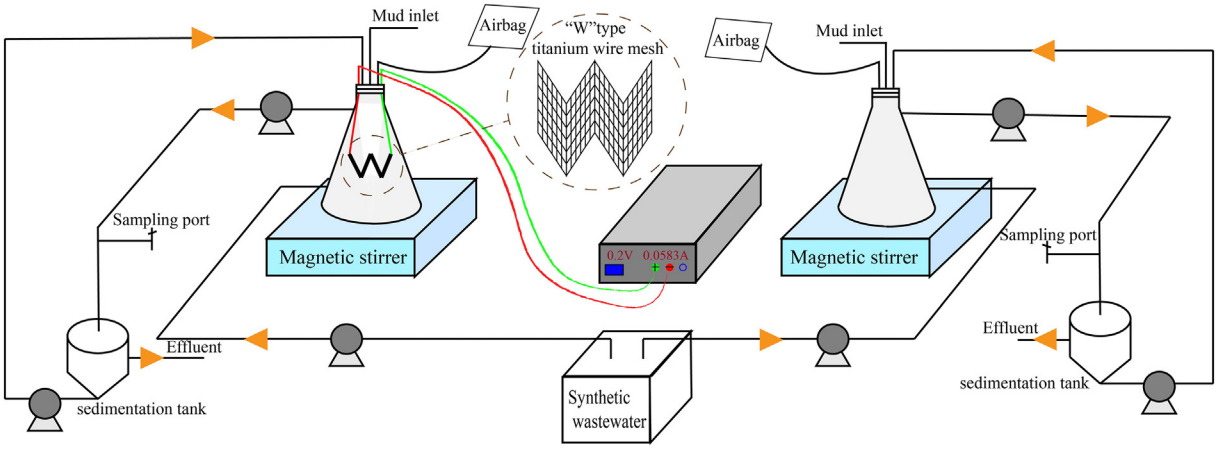


Fig. 1. Continuous flow of weak electrically stimulated denitrification schematic diagram of the experimental setup.

4 °C, and the supernatant was then taken and stored. The reaction system consisted of 1.0 mL of crude enzyme extract with 0.01 M PBS (pH = 7.4), 1.0 mM $NaNO_3$, 1.0 mM methyl violet gum and 5.0 mM $Na_2S_2O_4$. The reaction system was placed in a thermostat and shaken for 30 min at 30 °C, and then the nitrite concentration was measured by an ultraviolet spectrophotometer (UV-2600 Shimadzu, Japan). NAR and NIR enzyme activities were expressed as NO_3^--N produced or reduced during the reaction (μg NO_3^--N/min–mg protein) (Wang et al., 2016).

2.2.4. Analysis of extracellular polymers substances (EPS)

The extracellular polymer substances (EPS) was extracted from the sludge by thermal extraction (Chen et al., 2003) after filtered through a 0.45 μm membrane to remove the particulate matter and then analyzed qualitatively using a 3D fluorescence spectrometer F-7000 FL (Hitachi, Japan). The excitation wavelength of the 3D fluorescence spectrometer was set from 200 to 420 nm, and the emission wavelength was set from 250 to 550 nm in increments of 2 nm. The slit widths of both excitation and emission were 2.5 nm, and the scanning speed was 12,000 nm/min. The EPS was also quantified and the protein content in the supernatant of the samples was determined by the Komas Brilliant Blue method (Bradford, 1976).

3. Results and discussion

3.1. Effect of weak electrical stimulation on denitrification efficiency

Fig. 2 shows the variation of NO_3^--N, NO_2^--N, NH_4^+-N and TN concentrations under the influence of different C/N and microbial weak electrical stimulation voltages. By comparing the performances at different voltages in Fig. 2, it can be found that the best effect of microbial weak electrical stimulation is at 0.2 V under both C/N ratios of 3 and 4. The total nitrogen and nitrate in the EG group with the C/N ratio of 3 decreased to about 20 mg/L and 17 mg/L at a weak stimulation voltage of 0.2 V, while the total nitrogen and nitrate in the CK group decreased to about 24 mg/L and 20 mg/L, respectively. The removal rates of total nitrogen and nitrate in the EG group increased both by 8% compared with those in the CK group (Fig. 2a, b). When the microbial weak electrical stimulation voltage was 0.4 V, there was little difference between the degradation in the CK group and the EG group; while at the weak electrical stimulation voltage of 0.6 V, the nitrate removal rate in the EG group was lower than that in the CK group. It could be speculated that under relatively low carbon source conditions, the degradation effect may be negatively correlated with an increase in the weak electrical stimulation, with 0.6 V corresponding to a weak electrical stimulation intensity that is detrimental to microbial metabolisms. The above results are comparable to the results of Prosňanský et al. (2005). The best effect was obtained when the current density was 0.27 mA/cm^2, and there was no difference between the experimental group and the control group when the current density is 0.467 mA/cm^2. The denitrification rate decreased when the current density was greater than 1.2 mA/cm^2, and when the current density exceeded 2 mA/cm^2, the denitrification effect decreased significantly.

Under the condition of an influent C/N of 4, the total nitrogen and nitrate could be reduced to about 11 mg/L and 9 mg/L in the EG group and about 22 mg/L and 18 mg/L in the CK group; meanwhile, the total nitrogen and nitrate removal rates in the EG group were improved by about 12% and 20%, respectively, compared with the CK group (Fig. 2a, b). Microbial heterotrophic denitrification using organic matter or autotrophic denitrification using hydrogen are both alkali-producing processes, and the reduction of 1 mg of nitrate can simultaneously produce 3.57 mg of alkalinity. As shown in Fig. 2e, the alkalinity of the EG effluent was higher than that of the CK effluent, indicating that weak electrical stimulation has a positive effect on the denitrification process.

The variation curves of nitrite nitrogen and ammonia nitrogen in Fig. 2c, d showed that nitrite nitrogen in the effluent of the EG group was lower than that of the CK group under the effect of weak electrical stimulation at 0.2 V, 0.4 V and 0.6 V, while the concentration of ammonia nitrogen showed an opposite trend. There are two main pathways of biological nitrogen cycling associated with nitrate in nature, in addition to biological denitrification, and the dissimilatory nitrate reduction to ammonium (DNRA) (Christensen et al., 2000).

$$NO_3^- + 8e^- + 10H^+ \rightarrow NH4^+ + 3H_2O \quad (5)$$

NH_4^+-N is the end product of the DNRA process, and it can be demonstrated from Fig. 2d that there was an accumulation of NH_4^+-N in the EG group under weak electrical stimulation, partly because the high

Table 1
Composition of artificial simulated wastewater.

Ingredient	Concentration
$C_6H_{12}O_6$	0.0703 g/L (C/N = 3) 0.0934 g/L (C/N = 4)
$C_2H_3NaO_2 \cdot 3H_2O$	0.1594 g/L (C/N = 3) 0.2127 g/L (C/N = 4)
$NaNO_3$	0.3036 g/L
KH_2PO_4	0.0099 g/L
$NaHCO_3$	0.1 g/L
$CaCl_2$	0.01 g/L
$MgSO_4 \cdot 7H_2O$	0.1 g/L
Trace elements	1 mL/L

Table 2
Conditions for determination of hydrogen, nitrogen and nitrous oxide.

Composition	Detector	Temperature	Packed column	Inlet temperature	Column temperature	Carrier gas and velocity
H_2	TCD	120 °C	TDX-02, 2 m * 2 mm	100 °C	100 °C	99.999%Ar, 30 mL/min
N_2	TCD	200 °C	SH-Rt®-Msieve 5A Capillary column, 30 m * 0.53 mm * 50 μm	120 °C	120 °C	99.999%Ar, 1.5 mL/min
N_2O	μECD	100 °C	Propack Q, 2 m * 2 mm	100 °C	70 °C	99.999%Ar, 1.5 mL/min

ammonia nitrogen content of the synthetic water after sludge dilution and partly because DNRA was presumed to have occurred during denitrification. Meanwhile, the degree of accumulation in the EG group increased with the increase of voltage compared to the CK group, indicating that electrical stimulation accelerated the DNRA process. According to Fig. 4, the NIR activity of the EG group was enhanced by weak electrical stimulation, which reduced the accumulation of nitrite due to insufficient carbon source.

The above results showed that the effect of weak microbial electrical stimulation on nitrate-containing wastewater was largely positive at different voltages and C/N ratios. The highest removal efficiencies for both TN and nitrate nitrogen were obtained at 0.2 V. the nitrite nitrogen content was significantly higher in the CK group than in the EG group.

3.2. Variation of gas production & compositions under weak electric stimulation

When the denitrification efficiency reached the highest value with a weak electrical stimulus of 0.2 V, we collected gas production fractions from the EG group at 0.2 V for comparison with the CK group. Gas

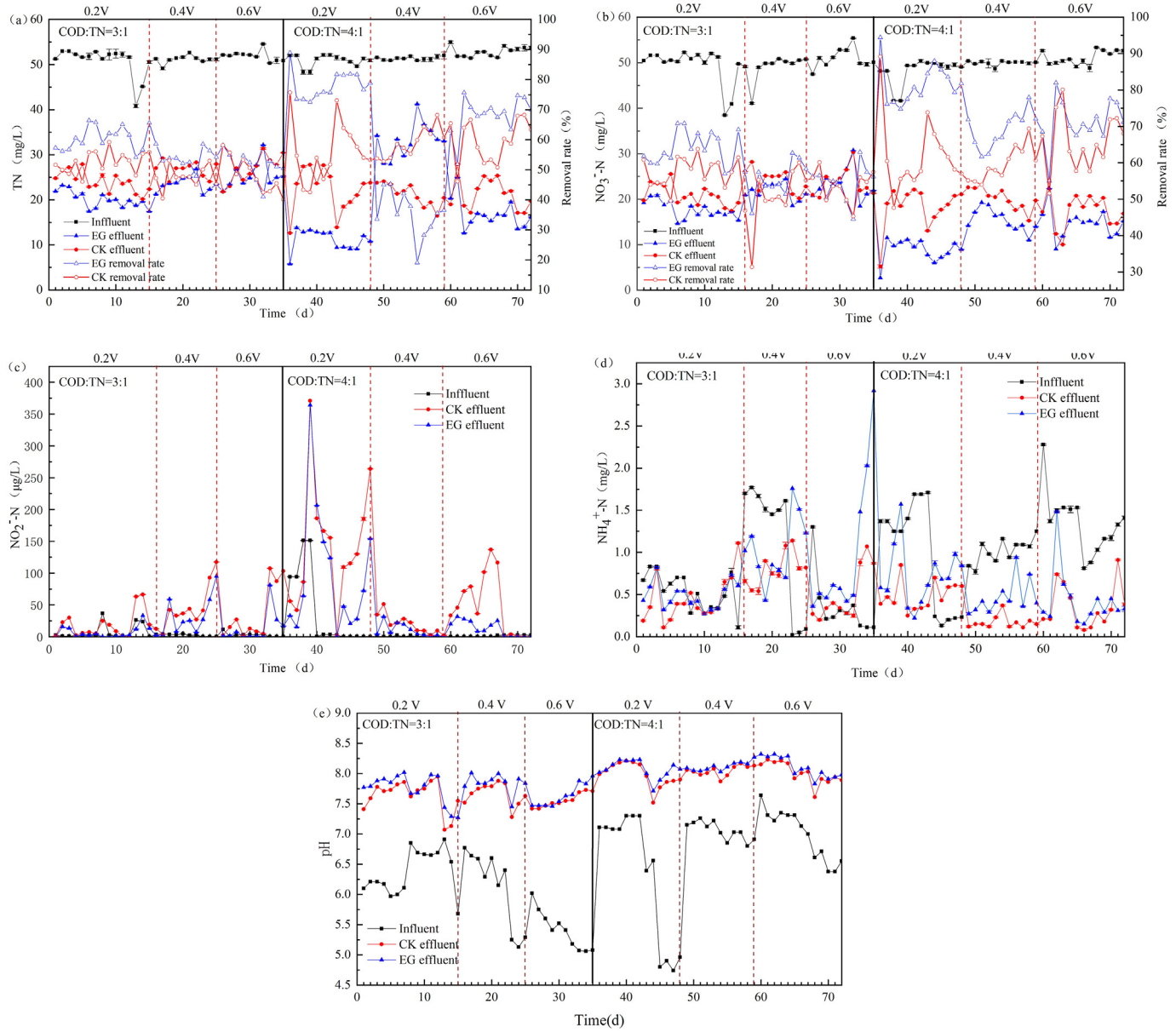


Fig. 2. Removal effect and pH change of weakly electrically stimulated denitrification continuous flow experiment;

(a) TN, (b) NO_3^--N, (c) NO_2^--N, (d) NH_4^+-N, (e) pH.

production during the denitrification process varies under different C/N conditions (Wu et al., 2013). Fig. 3a, b and c demonstrated H_2, N_2 and N_2O contents of the denitrification gas at the optimal weak electrical stimulation intensity (0.2 V) for C/N ratios of 3 and 4. The percentage of H_2 content in the gas produced by both EG and CK groups was at a lower level at C/N ratios of 3 and 4, but percentage of H_2 content in the EG group increased compared to the CK group, presumably because the accelerated electron transfer by the applied voltage promoted the activity of hydrogen-producing bacteria. It is generally believed that autotrophic denitrifying microorganisms use the H_2 produced by cathode electrolysis as an electron supply. H_2 can replace organic carbon as an electron donor, relieving the challenge of insufficient carbon source, and promoting denitrification, as shown in Fig. 2. The N_2 content in the produced gas was lower in the EG group than in the CK group since the microbial activity of N_2O reductase synthesis was probably inhibited by DOC depletion in the EG group at the late stage of denitrification due to the low carbon source, while the anaerobic ammonia oxidation process could exist in the CK group at the same time, and NH_4^+ replaced DOC as an electron donor to undergo redox reactions with NO_2^-, and then more N_2 was produced (Hu et al., 2013).

It was controversial that when the C/N ratio is different, the N_2O content changes in the opposite trend. At a C/N ratio of 3, it was evident that lower levels of N_2O were produced with the 0.2 V weak electrical stimulation, representing a more complete denitrification process. In contrast, at a C/N ratio of 4, the N_2O production was higher with 0.2 V weak electrical stimulation. This may be partly due to the accumulation of nitrite (Fig. 2c), which leads to the production of N_2O (Park et al., 2000), and partly due to the weak electrical stimulation, which enhances the denitrifying enzyme activity. Denitrification by microorganisms consists of several stages, such as $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$. The microorganisms and related enzymes NAR, NIR, NOR and N_2OR in each stage are different, so are the effects of weak electrical stimulation on the activities of different microorganisms and enzymes. Interactions between enzymes can affect the electron supply in endogenous denitrification. Restricted electron supply will lead to a significant accumulation of N_2O in the denitrification phase (Zhou et al., 2012). According to the microbial community detection and analysis results (Fig. 5), it can be concluded that weak electrical stimulation plays a selective driving role for autotrophic and heterotrophic denitrifying bacteria. Under 0.2 V weak electrical stimulation, the genus abundance of microbial communities in the EG group was significantly increased compared to the CK group, especially *Thaurea* (8.2% and 10.4%), *Rhodocyclaceae* (8.0% and 9.1%), *Denitratisoma* (3.1% and 3.4%), *Candidatus Competibacter* (1.3% and 1.5%), and other microorganisms involved in the denitrification process. Most *Candidatus Competibacter* can only undergo NO_3^--N reduction but not NO_2^--N reduction because they form NAR but not NIR (Oehmen et al., 2010). In contrast, *Thauera* was able to determine the reduction of NO_3^--N to nitrogen. From this we speculate that it is the competition for electrons between various enzymes during denitrification that affects N_2O accumulation (Pang and Liu, 2010; Pan et al., 2013), causing both exogenous and endogenous denitrification reactions in different situations. We then tested and analyzed the enzyme activity and EPS of the different groups of sludge.

3.3. Effect of enzyme activity on denitrification process

The denitrification performance is related to the activity of four enzymes NAR, NIR, NOR (nitric oxide reductase) and NOS (Hartzog et al., 2017), as expressed in formula (6) (Zheng et al., 2014).

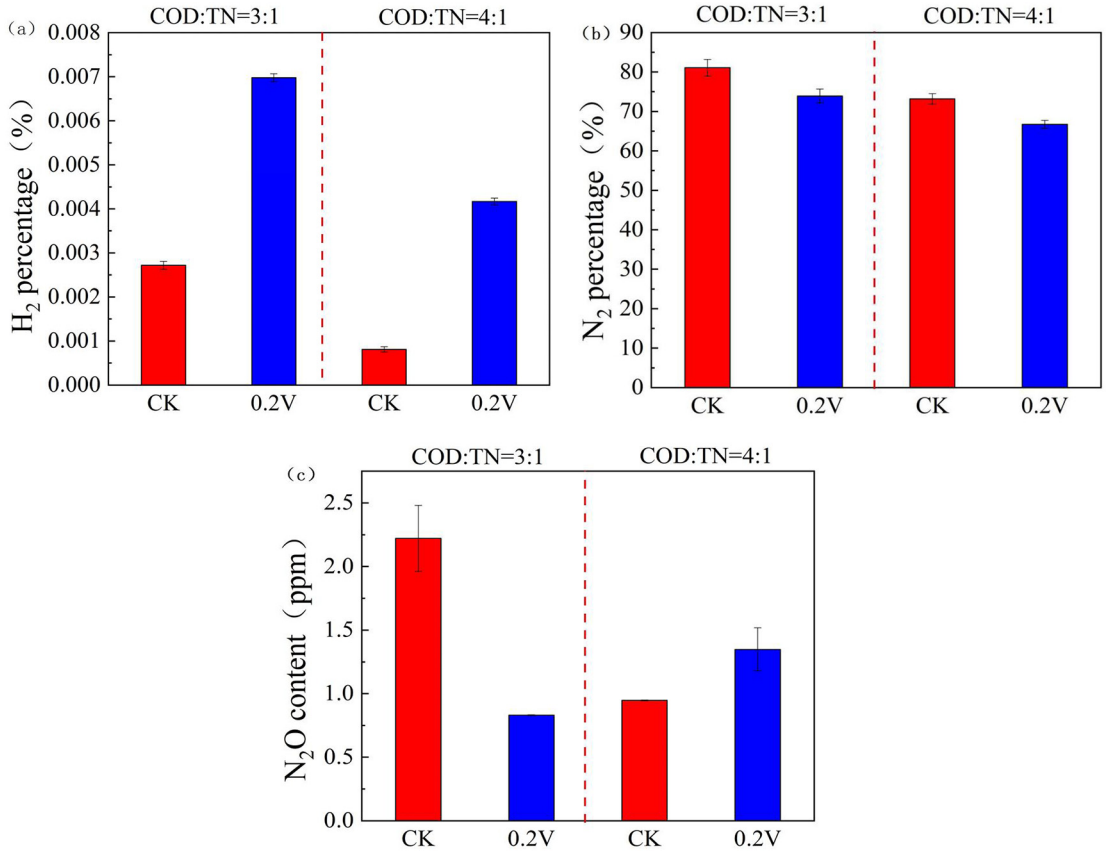


Fig. 3. Denitrification gas production at optimal weak electrical stimulation intensity of 0.2 V;

(a) H_2, (b) N_2, (c) N_2O.

$$NO_3^- \xrightarrow{Nar} NO_2^- \xrightarrow{Nir} NO \xrightarrow{Nor} N_2O \xrightarrow{Nos} N_2 \quad (6)$$

The proceeding of denitrification depends to a large extent on the enzymatic activity, which is significantly influenced by the relevant physicochemical factors. We examined and analyzed the activity of two key enzymes (NAR and NIR) involved in the accumulation of nitrite in sludge. Based on Fig. 4, compared with the CK group, the activities of NAR and NIR in the EG group were significantly enhanced by weak electrical stimulation at both 0.2 V and 0.4 V. Combined with the results in Fig. 2, electrical stimulation can promote denitrification by increasing the activity of NAR and NIR, which is one of the ways to improve NO_3^- removal efficiency and reduce N_2O accumulation. NO_2^--N is the product of the enzymatic reaction of NAR, which in turn is promoted by NIR to reduce NO_2^--N to N_2. In other words, an increase in NAR activity promotes the activity of NIR. The weak electrical stimulation also optimized the microbial population structure (Fig. 5). The proportion of heterotrophic microorganisms such as *Saccharibacteria* and mutualistic microorganisms such as *Smithella* decreased and the proportion of autotrophic microorganisms such as *Thauera* increased under the weak electrical stimulation of 0.2 V, which enhanced the autotrophic denitrification of microorganisms.

The weak electrical stimulation at 0.6 V with low carbon source showed a slight inhibition of NAR and NIR, which confirms that the lack of carbon source cannot be remedied by increasing the voltage stimulation alone. At low C/N ratios, insufficient electron donors affect the ability of less competitive reductases such as NOR and NOS to compete for electrons so that expressed genes related to denitrification are weakened. The carbon source available was unable to synthesize sufficient denitrifying enzymes, which affected the growth of the organism and the removal of nitrate and nitrite nitrogen. Denitrification enzymes require a large number of electrons to operate in the reduction reaction. EPS can act as a transient medium for electron energy transfer (EET) in activated sludge because of the presence of large amounts of protein, which is closely related to the activity of NAR and NIR.

3.4. Effect of extracellular polymers on denitrifying enzyme activity

EPS played crucial roles in the ability of microorganisms to flocculate and in building and maintaining biofilm structure. We carried out a qualitative and quantitative analysis of the EPS from the three-dimensional fluorescence spectrogram (Fig. 6). It can be observed that the fluorescence intensity and area of fluorescence region I (Aromatic protein tyrosine) and II (Aromatic protein tryptophan) of LB three-dimensional fluorescence spectrogram of the EG group were higher than those of the CK group under the effect of weak electrical stimulation of microorganisms, while the corresponding TB showed roughly the opposite trend. The fluorescence intensities and areas of TB and LB in the EG and CK groups were in general consistent with the trends of protein contents in the corresponding TB and LB. EPS played an important role in enhancing intercellular communication as well as electron transfer (Xiao et al., 2017). Protein is the main component of EPS, in which aromatic protein-like substances have the effect of accelerating electron transfer (Shih et al., n.d.). The increase of aromatic protein substances promoted electron transfer between electroactive microorganisms and other microorganisms, further enhancing denitrification efficiency.

The effect of weak electrical stimulation on EPS protein was not only in terms of distribution, but also in terms of content. Fig. 7 demonstrated that weak electrical stimulation has a significant effect on the extracellular polymer. It is obvious that the sludge LB protein content in the EG group was lower compared to the CK group, while the TB protein

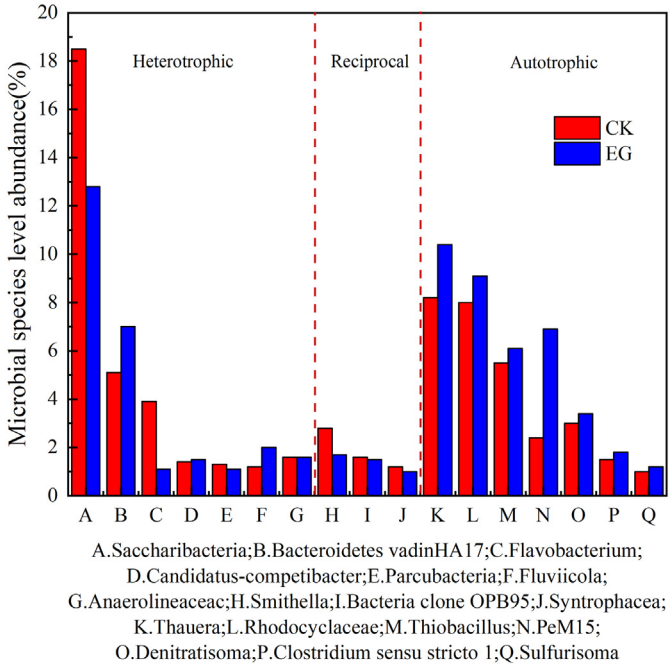


Fig. 5. Microbial population structure species level abundance under weak electrical stimulation.

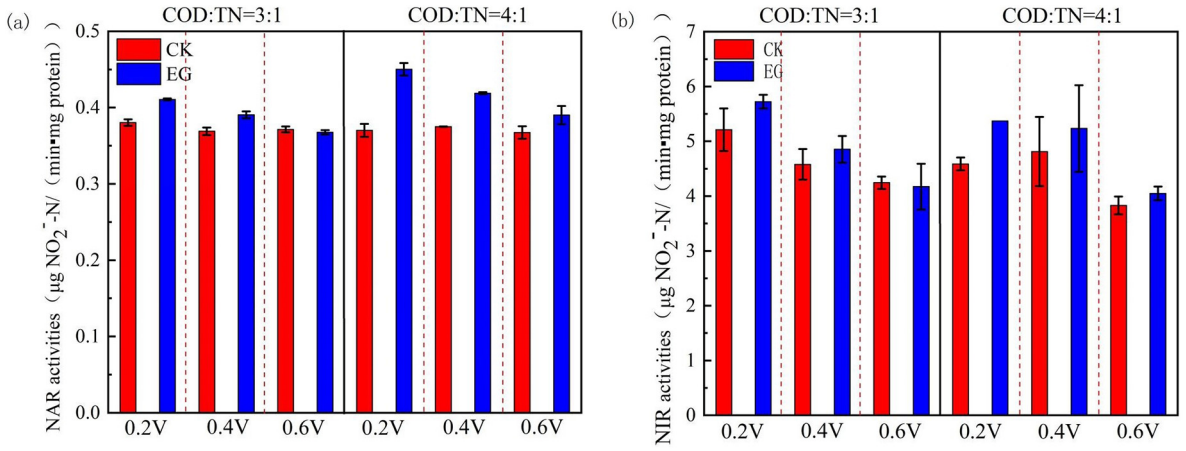


Fig. 4. Weak electrically stimulated denitrification denitrifying enzyme activity; (a) NAR activity, (b) NIR activity.

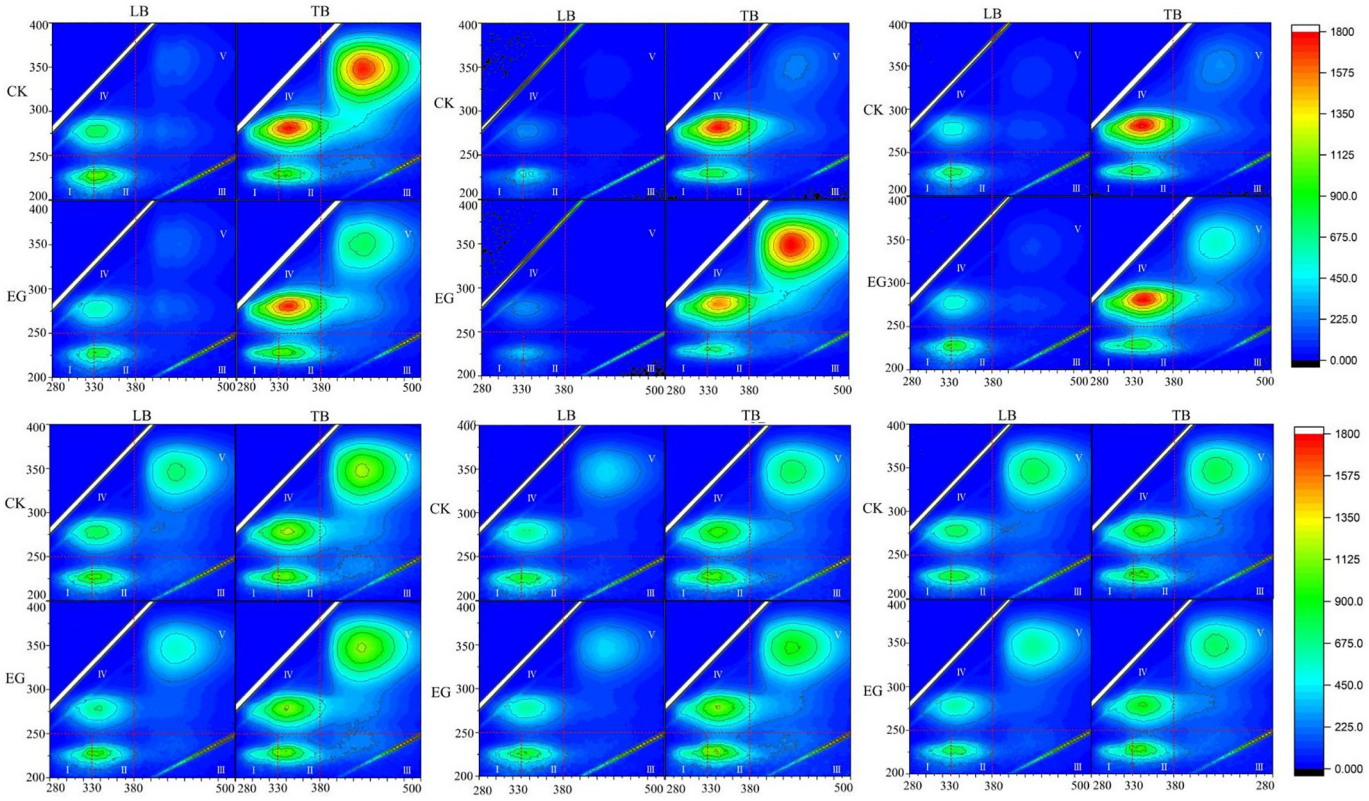


Fig. 6. EPS three-dimensional fluorescence spectrum of weakly electrically stimulated denitrification continuous flow experimental sludge.

content was higher than the CK group, with a tendency for the total EPS protein to increase. LB-EPS was located in the outer layer, so the increase of its content can increase the zeta potential of the cells and/or flocs. According to the DLVO theory, the electrostatic repulsion between the cells and flocs increases, which hindered the flocculation effect and had an impact on SVI (Sludge Volume Index). TB-EPS is located in the inner layer, which binds tightly to the cell surface, stably adheres to the cell wall, and has little effect on the sludge (Yu et al., 2008). The LB of the EG group was much less than that of the CK group, which was consistent with the removal performances of total nitrogen and nitrate nitrogen. With the decrease of LB, the degree of sludge flocculation increases, and the adsorption and removal efficiency of pollutants was better. When the intensity of weak electrical stimulation was 0.6 V, the protein content of LB and TB in the EG group was lower than that of CK, and the total amount of EPS protein decreased, which was obviously inhibited. It was speculated that the appropriate intensity of weak electrical stimulation can transform EPS protein from LB to TB and increase the total amount of EPS protein. Overall, weak electrical stimulation enhanced microbial metabolic activity and protein secretion, and the appropriate intensity of weak electrical stimulation enhanced microbial cell-to-cell communication and electron transfer by affecting the distribution of LB and TB and the protein content in EPS.

4. Conclusions

Weak electrical stimulation has a selective effect on the dominant autotrophic microorganisms and enhances denitrification of low carbon source wastewater through enhanced autotrophic denitrification. Appropriate weak electrical stimulation effectively enhanced denitrification by affecting the distribution and content of EPS aromatic proteins

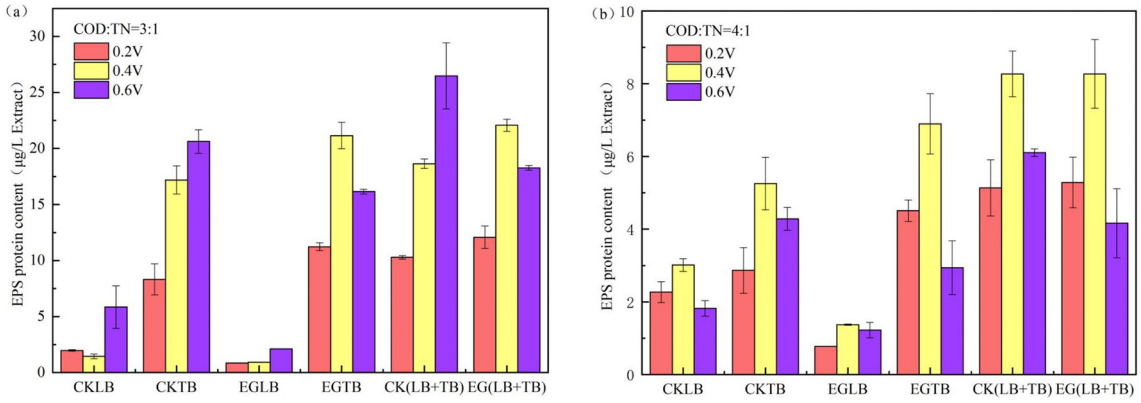


Fig. 7. EPS protein content of weak electrically stimulated denitrification continuous flow experimental sludge.

(a) COD:TN = 3:1, (b) COD:TN = 4:1.

with electron transfer function and the activity of denitrifying enzymes NAR and NIR, while reducing the production of the intermediate greenhouse gas N_2O. The variation in gas production showed that the various enzymatic activities responded differently to the weak electrical stimuli and that when the microbial weak electrical stimuli were too intense, they inhibited the enzymatic activity and thus affected the denitrification process. Weak electrically stimulated denitrification is a process in which multiple microorganisms work in concert. Based on the pattern of denitrifying enzyme effects, denitrification can be further promoted in the future by detecting and regulating denitrifying bacteria.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.150926.

CRediT authorship contribution statement

Xinyi Dong: Lab investigations and part of the pilot investigations, mechanism modelling. **Hongbo Liu:** Funding acquisition, co-conceptualization, co-project administration, co-supervision, draft manuscript. **Shiping Long:** Methodology, data curation. **Suyun Xu:** Co-project administration, co-supervision. **Eric Lichtfouse:** Review & editing.

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.

Acknowledgments

The authors have stated that there was no conflict of interest. We gratefully acknowledged the co-funding of this work by the National Natural Science Foundation of China (No. 52070130) and the Shuguang Project of Shanghai (Education and Scientific Research Project of Shanghai, 18SG45).

References

Beschkov, V., Velizarov, S., Agathos, S.N., Lukova, V., 2004. Bacterial denitrification of waste water stimulated by constant electric field. Biochem. Eng. J. 17, 141–145.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Chen, W., Westerhoff, P., Leenheer, J.A., Booksh, K., 2003. Fluorescence Excitation—Emission matrix regional integration to quantify spectra for dissolved organic matter. Environ. Sci. Technol. 37, 5701–5710.

Christensen, P., Rysgaard, S., Sloth, N., Dalsgaard, T., S.J.A.M.E. Schw?Rter, 2000. Sediment mineralization, nutrient fluxes, denitrification and dissimilatory nitrate reduction to ammonium in an estuarine fjord with sea cage trout farms. Aquatic Microbial Ecology 257, 73–84.

Chun, C.L., Payne, R.B., Sowers, K.R., May, H.D., 2013. Electrical stimulation of microbial PCB degradation in sediment. Water Res. 47, 141–152.

Gao, Y., Cornwell, J.C., Stoecker, D.K., et al., 2012. Effects of cyanobacterial-driven pH increases on sediment nutrient fluxes and coupled nitrification-denitrification in a shallow fresh water estuary[J]. Biogeosciences 9 (7).

Guo, Y., Peng, Y., Wang, B., Li, B., Zhao, M., 2016. Achieving simultaneous nitrogen removal of low C/N wastewater and external sludge reutilization in a sequencing batch reactor. Chem. Eng. J. 306, 925–932.

Hartzog, P.E., Sladek, M., Kelly, J.J., Larkin, D.J., 2017. Bottle effects alter taxonomic composition of wetland soil bacterial communities during the denitrification enzyme activity assay. Soil Biol. Biochem. 110, 87–94.

Hu, Z., Lotti, T., van Loosdrecht, M., Kartal, B., 2013. Nitrogen removal with the anaerobic ammonium oxidation process. Biotechnol. Lett. 35, 1145–1154.

Huang, B., Feng, H., Ding, Y., Zheng, X., Wang, M., Li, N., Shen, D., Zhang, H., 2013. Microbial metabolism and activity in terms of nitrate removal in bioelectrochemical systems. Electrochim. Acta 113, 29–36.

Jiang, C., Yang, Q., Wang, D., Zhong, Y., Chen, F., Li, X., Zeng, G., Li, X., Shang, M., 2017. Simultaneous perchlorate and nitrate removal coupled with electricity generation in autotrophic denitrifying biocathode microbial fuel cell. Chem. Eng. J. 308, 783–790.

Jin, L., Zhang, G., Tian, H., 2014. Current state of sewage treatment in China. Water Res. 66, 85–98.

Li, W., Zhang, S., Chen, G., Hua, Y., 2014. Simultaneous electricity generation and pollutant removal in microbial fuel cell with denitrifying biocathode over nitrite. Appl. Energy 126, 136–141.

Liu, H., Tong, S., Chen, N., Liu, Y., Feng, C., Hu, Q., 2015. Effect of electro-stimulation on activity of heterotrophic denitrifying bacteria and denitrification performance. Bioresour. Technol. 196, 123–128.

Liu, H., Ouyang, F., Chen, Z., Chen, Z., Lichtfouse, E., 2021. Weak electricity stimulates biological nitrate removal of wastewater: hypothesis and first evidences. Sci. Total Environ. 757, 143764.

Oehmen, A., Carvalho, G., Lopez-Vazquez, C.M., van Loosdrecht, M.C.M., Reis, M.A.M., 2010. Incorporating microbial ecology into the metabolic modelling of polyphosphate accumulating organisms and glycogen accumulating organisms. Water Res. 44, 4992–5004.

Pan, Y., Ni, B.-J., Bond, P.L., Ye, L., Yuan, Z., 2013. Electron competition among nitrogen oxides reduction during methanol-utilizing denitrification in wastewater treatment. Water Res. 47, 3273–3281.

Pang, D.H., Liu, Z.F., 2010. The Mechanism of aerobic denitrification and the outlook of dealing with NO_x waste gas. Pollut. Control Technol. 23 (3), 74–77 84.

Park, K.Y., Inamori, Y., Mizuochi, M., Ahn, K.H., 2000. Emission and control of nitrous oxide from a biological wastewater treatment system with intermittent aeration. 90, 247–252.

Prosňanský, M., Watanabe, T., Kuroda, M., 2005. Comparative study on the bioelectrochemical denitrification equipped with a multi-electrode system. Water Sci. Technol. 52, 479–485.

C. Shih A.K. Museth M. Abrahamsson A.M. Bianco-Rodriguez A. Bilio J. Sudhamsu B.R. Crane K.L. Ronayne M. Towrie A.V.J.S. Jr , Tryptophan-accelerated electron flow through proteins, Science ,320.

Thrash, J.C., Coates, J.D.J.E.S., 2008. Technology, review: direct and indirect electrical stimulation of microbial metabolism. Environ. Sci. Technol. 42, 3921–3931.

Verbaendert, I., Boon, N., De Vos, P., Heylen, K., 2011. Denitrification is a common feature among members of the genus bacillus. Syst. Appl. Microbiol. 34, 385–391.

Virdis, B., Rabaey, K., Rozendal, R.A., Yuan, Z., Keller, J., 2010. Simultaneous nitrification, denitrification and carbon removal in microbial fuel cells. Water Res. 44, 2970–2980.

Wang, S., Gao, M., Li, Z., She, Z., Wu, J., Zheng, D., Guo, L., Zhao, Y., Gao, F., Wang, X., 2016. Performance evaluation, microbial enzymatic activity and microbial community of a sequencing batch reactor under long-term exposure to cerium dioxide nanoparticles. Bioresour. Technol. 220, 262–270.

Wang, Z., Chen, C., Liu, H., Hrynshpan, D., Savitskaya, T., Chen, J., Chen, J., 2019. Effects of carbon nanotube on denitrification performance of Alcaligenes sp. TB: Promotion of electron generation, transportation and consumption. Ecotoxicology and Environmental Safety 183, 109507.

Wu, G., Zhai, X., Jiang, C., Guan, Y., 2013. Effect of ammonium on nitrous oxide emission during denitrification with different electron donors. J. Environ. Sci. 25, 1131–1138.

Xiao, Y., Zhang, E., Zhang, J., Dai, Y., Yang, Z., Christensen, H.E.M., Ulstrup, J., Zhao, F., 2017. Extracellular polymeric substances are transient media for microbial extracellular electron transfer. Sci. Adv. 3, e1700623.

C. Ycab Z.D. Rui C. Qsa C. Trab C. Ywa D. Xc D. Jr C. Dya C. Mca C.J.B.T. Twab , Coupling anammox with denitrification in a full-scale combined biological nitrogen removal process for swine wastewater treatment, Bioresour. Technol., 329.

Yu, G.-H., He, P.-J., Shao, L.-M., He, P.-P., 2008. Stratification structure of sludge flocs with implications to dewaterability. Environmental Science & Technology 42, 7944–7949.

Zhang, Y., Angelidaki, I., 2013. A new method for in situ nitrate removal from groundwater using submerged microbial desalination-denitrification cell (SMDDC). Water Research 47, 1827–1836.

Zhang, F., He, Z., 2012. Simultaneous nitrification and denitrification with electricity generation in dual-cathode microbial fuel cells. J. Chem. Technol. Biotechnol. 87, 153–159.

Zheng, X., Su, Y., Chen, Y., Wan, R., Li, M., Wei, Y., Huang, H., 2014. Carboxyl-modified single-walled carbon nanotubes negatively affect bacterial growth and denitrification activity. Sci. Rep. 4, 5653.

Zhou, Y., Lim, M., Harjono, S., Ng, W.J., 2012. Nitrous oxide emission by denitrifying phosphorus removal culture using polyhydroxyalkanoates as carbon source. J. Environ. Sci. 24, 1616–1623.