# Weak electrical stimulation on biological denitrification: insights from the denitrifying enzymes

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**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

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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[1, 2]. 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 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[3]. 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[4]. 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[5, 6]. 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[7, 8]. Thus in recent years, studies using voltages lower than the hydrolysis voltage (1.23V) to promote microbial processes have attracted wide attention[9].

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[10]. At the same time, microbial activity increases with current density[11]. Higher current intensity promotes denitrification at surface of the MFC cathode, increasing denitrifying bacteria activity, and leading to further denitrification[12].When the current intensity exceeds a certain range, it also has an inhibitory effect on microbial activity, which affects the denitrification reaction[13]. Denitrification is carried out gradually by specific enzymes as shown in equations 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.[14] 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[15], C/N[16], pH and other factors[17]. 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 (0.567mA/cm$^2$), 0.4 V (1.12mA/cm$^2$) and 0.6 V (1.74mA/cm$^2$) were selected according to results of our previous study[18]; 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[18]. 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 hours. 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).

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

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 $CoCl_2 \cdot 6H_2O$,1.5g/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 $Na_2MoO_4 \cdot H_2O$.

**Insert Table 1. Composition of the artificial simulated wastewater**

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 hours. 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 spectrophotometr, 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

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

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

116 **2.2.3 Denitrifying enzymes (NAR, NIR) extraction and activity determination**

117 We investigated the effect of weak electrical stimulation on denitrifying microbial
118 activity under weak electrical stimulation by measuring NAR and NIR activities. The mixed
119 liquid suspended solid from different groups was centrifuged at 5000 r for 5 min at 4 ℃. The
120 centrifuged precipitate was rinsed with 0.01 M phosphate buffer (PBS) at pH 7.4 for three
121 times, resuspended in 0.01 M PBS buffer at pH 7.4, and sonicated in an ice bath (20 kHz) for
122 5 min. After sonication, the cell suspension was placed in a centrifuge at 12000r for 15 min at
123 4°C, and the supernatant was then taken and stored. The reaction system consisted of 1.0 mL
124 of crude enzyme extract with 0.01 M PBS (pH=7.4), 1.0 mM $NaNO_3$, 1.0 mM methyl violet
125 gum and 5.0 mM $Na_2S_2O_4$. The reaction system was placed in a thermostat and shaken for 30
126 min at 30°C, and then the nitrite concentration was measured by an ultraviolet
127 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)[19] .

**2.2.4 Analysis of extracellular polymers substances (EPS)**

The extracellular polymer substances (EPS) was extracted from the sludge by thermal extraction[20] 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 12000 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[21].

## 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.[22]. 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.

**Insert 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**

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. 2 c, 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)[23].

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

$NH_4^+$-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 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

194 stimulus of 0.2V, we collected gas production fractions from the EG group at 0.2V for
195 comparison with the CK group. Gas production during the denitrification process varies under
196 different C/N conditions[24]. Fig.3a,b and c demonstrated $H_2$, $N_2$ and $N_2O$ contents of the
197 denitrification gas at the optimal weak electrical stimulation intensity (0.2 V) for C/N ratios of
198 3 and 4. The percentage of $H_2$ content in the gas produced by both EG and CK groups was at
199 a lower level at C/N ratios of 3 and 4, but percentage of $H_2$ content in the EG group increased
200 compared to the CK group, presumably because the accelerated electron transfer by the
201 applied voltage promoted the activity of hydrogen-producing bacteria. It is generally believed
202 that autotrophic denitrifying microorganisms use the $H_2$ produced by cathode electrolysis as
203 an electron supply. $H_2$ can replace organic carbon as an electron donor, relieving the challenge
204 of insufficient carbon source, and promoting denitrification, as shown in Fig.2. The $N_2$
205 content in the produced gas was lower in the EG group than in the CK group since the
206 microbial activity of $N_2O$ reductase synthesis was probably inhibited by DOC depletion in the
207 EG group at the late stage of denitrification due to the low carbon source, while the anaerobic
208 ammonia oxidation process could exist in the CK group at the same time, and $NH_4^+$ replaced
209 DOC as an electron donor to undergo redox reactions with $NO_2^-$, and then more $N_2$ was
210 produced[25].

211 **Insert Fig.3 Denitrification gas production at the optimal weak electrical stimulation**
212 **intensity of 0.2 V; (a) $H_2$, (b) $N_2$,(c) $N_2O$**

213 It was controversial that when the C/N ratio is different, the $N_2O$ content changes in the
214 opposite trend. At a C/N ratio of 3, it was evident that lower levels of $N_2O$ were produced
215 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 $N_2O$[26], and partly due to the weak electrical stimulation, which enhances the denitrifying enzyme activity. Denitrification by microorganisms consists of several stages, such as $NO_3^-$→$NO_2^-$→NO→$N_2O$→$N_2$. 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[27]. 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[28]. 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[16],[29], 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[30], as expressed in formula (6)[31].

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

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

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 0.2V, 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.

**Insert Fig.5 Level abundance of microbial population structure species under weak electrical stimulation**

### 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[32].

Protein is the main component of EPS, in which aromatic protein-like substances have the effect of accelerating electron transfer[33]. The increase of aromatic protein substances promoted electron transfer between electroactive microorganisms and other microorganisms, further enhancing denitrification efficiency.

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

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 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[34]. 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

304 weak electrical stimulation can transform EPS protein from LB to TB and increase the total
305 amount of EPS protein. Overall, weak electrical stimulation enhanced microbial metabolic
306 activity and protein secretion, and the appropriate intensity of weak electrical stimulation
307 enhanced microbial cell-to-cell communication and electron transfer by affecting the
308 distribution of LB and TB and the protein content in EPS.

309 **Insert Fig.7 EPS protein content of weak electrically stimulated denitrification**
310 **continuous flow experimental sludge. (a) COD:TN=3:1, (b) COD:TN=4:1**

311

312 **4. Conclusions**

313 Weak electrical stimulation has a selective effect on the dominant autotrophic
314 microorganisms and enhances denitrification of low carbon source wastewater through
315 enhanced autotrophic denitrification. Appropriate weak electrical stimulation effectively
316 enhanced denitrification by affecting the distribution and content of EPS aromatic proteins
317 with electron transfer function and the activity of denitrifying enzymes NAR and NIR, while
318 reducing the production of the intermediate greenhouse gas $N_2O$. The variation in gas
319 production showed that the various enzymatic activities responded differently to the weak
320 electrical stimuli and that when the microbial weak electrical stimuli were too intense, they
321 inhibited the enzymatic activity and thus affected the denitrification process. Weak
322 electrically stimulated denitrification is a process in which multiple microorganisms work in
323 concert. Based on the pattern of denitrifying enzyme effects, denitrification can be further
324 promoted in the future by detecting and regulating denitrifying bacteria.

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**Figure Captions**

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

**Figure.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

**Figure.3** Denitrification gas production at optimal weak electrical stimulation intensity of 0.2 V; (a) $H_2$, (b) $N_2$, (c) $N_2O$

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

441 **Figure.5** Microbial population structure species level abundance under weak electrical
442 stimulation

443 **Figure.6** EPS three-dimensional fluorescence spectrum of weakly electrically stimulated
444 denitrification continuous flow experimental sludge

445 **Figure.7** EPS protein content of weak electrically stimulated denitrification continuous flow
446 experimental sludge. (a) COD:TN=3:1, (b) COD:TN=4:1

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448 **Tables and figures**

449 **Table 1. Composition of artificial simulated wastewater**

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

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451 **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℃ | TDX-02，2m*2mm | 100℃ | 100℃ | 99.999%Ar,30 ml/min |
| $N_2$ | TCD | 200℃ | SH-Rt®-Msieve 5A Capillary column, 30m*0.53mm*50μm | 120℃ | 120℃ | 99.999%Ar，1.5 ml/min |
| $N_2O$ | μECD | 100℃ | Propack Q，2m*2mm | 100℃ | 70℃ | 99.999%Ar，1.5 ml/min |

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

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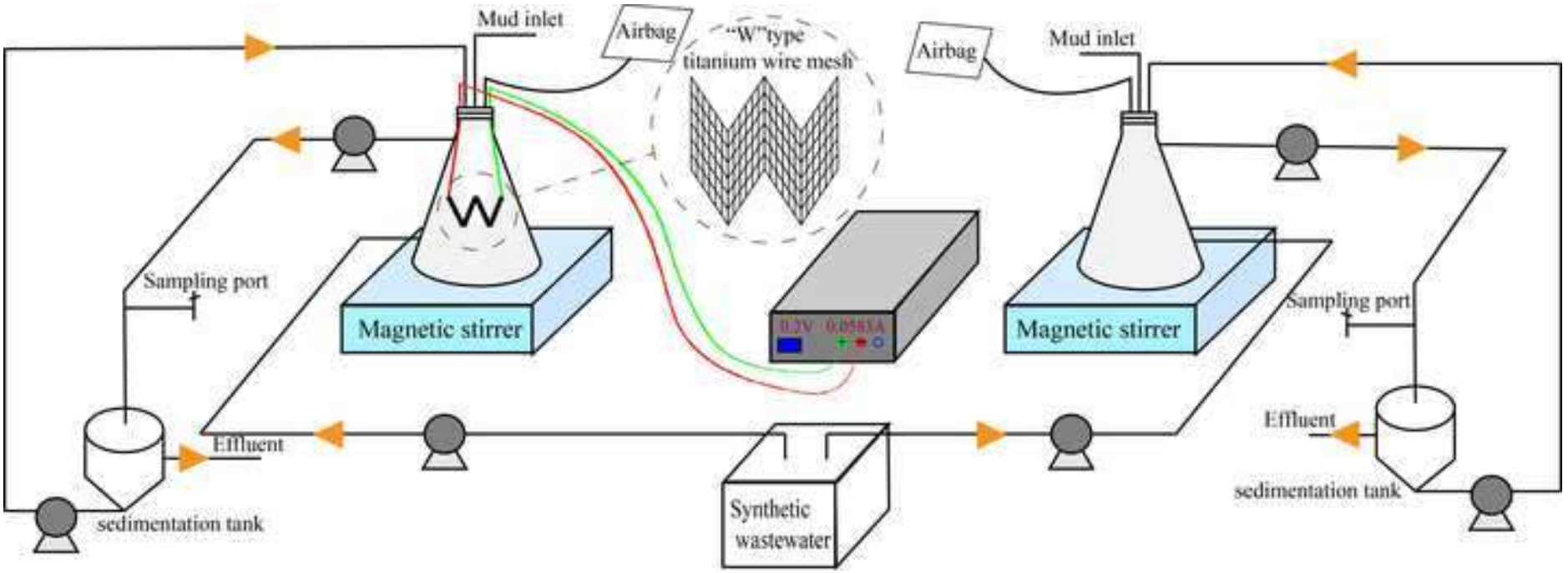

Figure.2 Removal effect and pH change of weakly electrically stimulated denitrification continuous flow experiment;(a)TN, (b)NO3--N, (c) NO2--N, (d) NH4+-N,(e)pH

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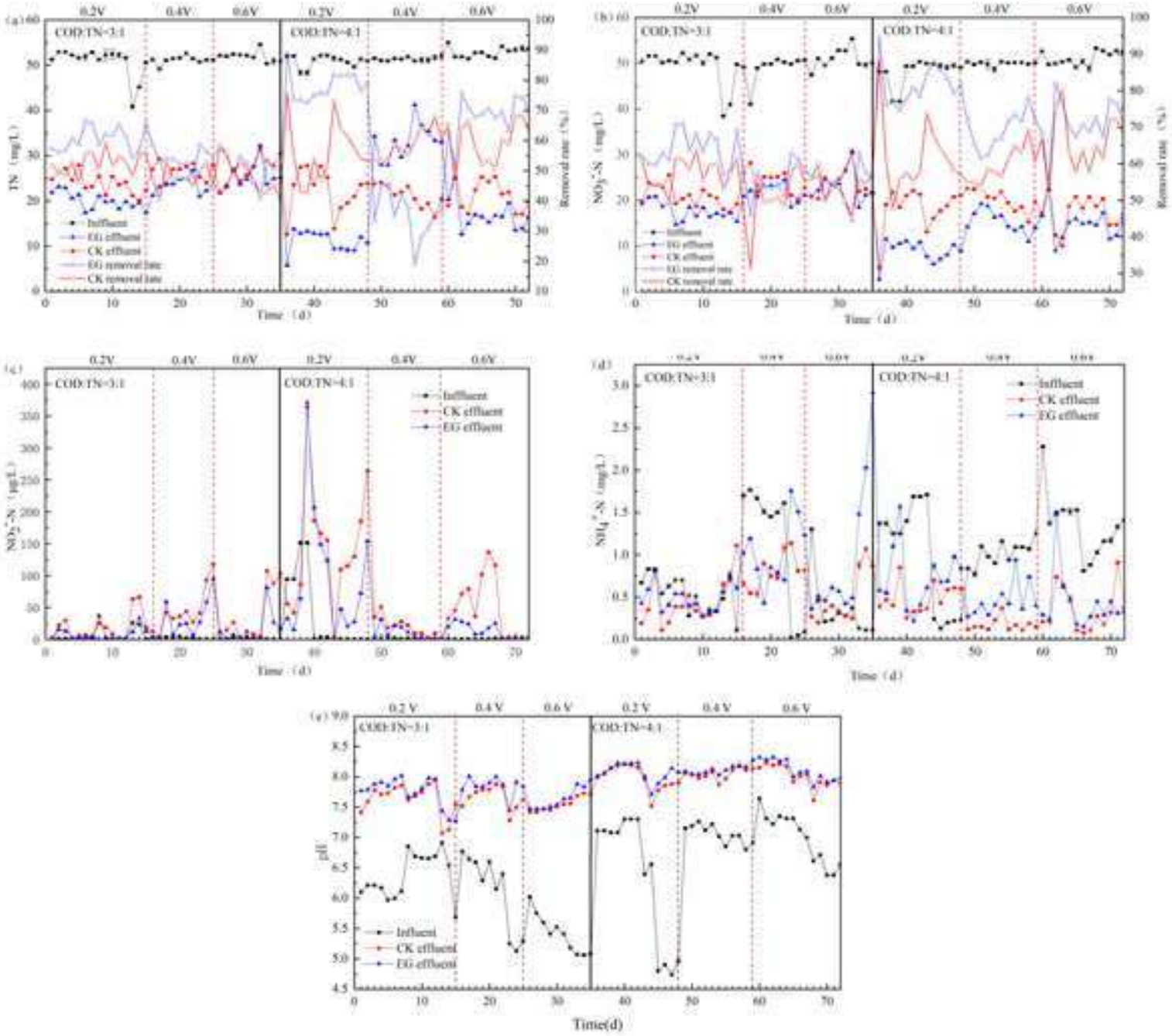

Figure.3 Denitrification gas production at optimal weak electrical stimulation intensity of 0.2 V; (a) H2, (b) N2, (c) N2O



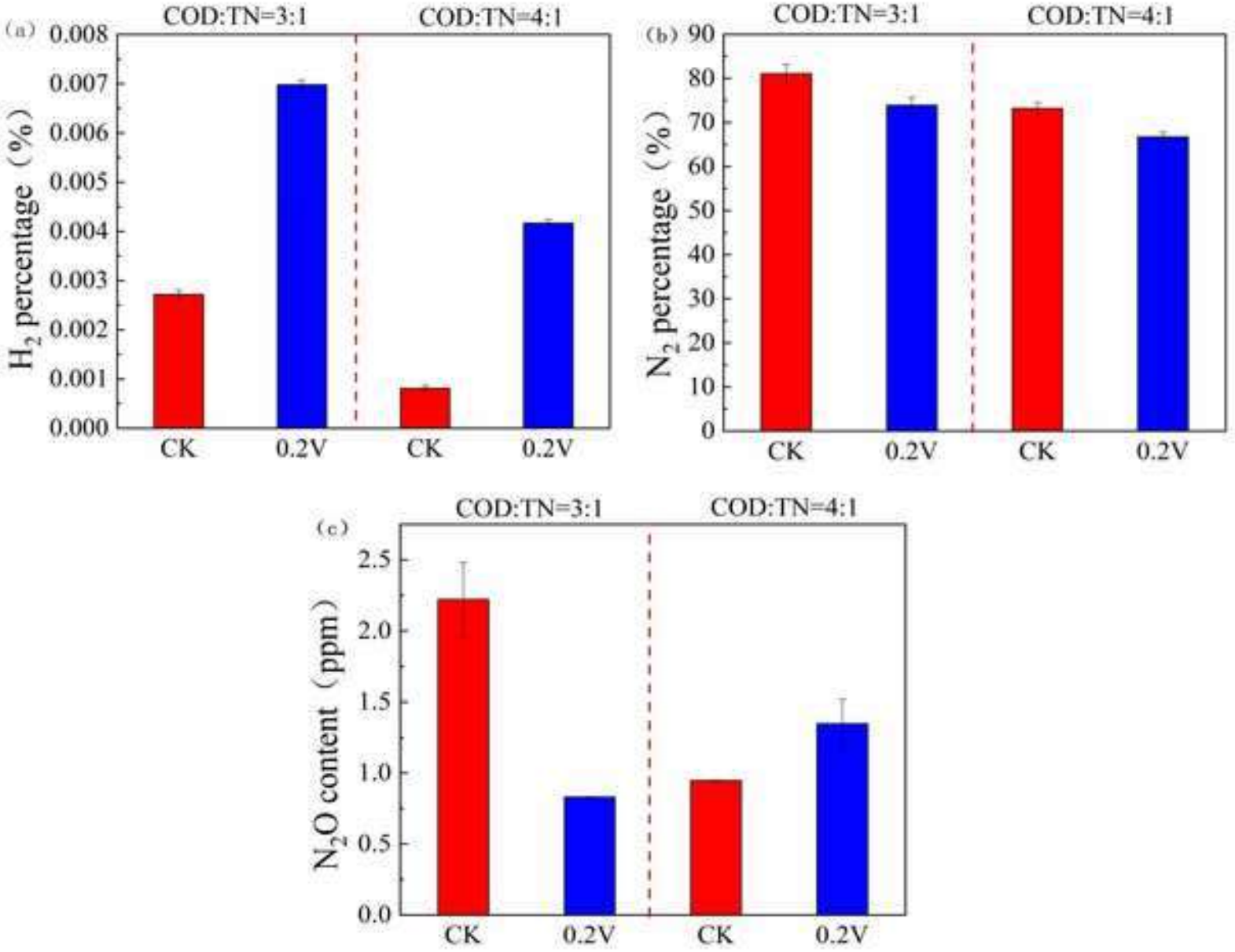

Figure.4 Weak electrically stimulated denitrification denitrifying enzyme activity; (a)NAR activity, (b) NIR activity



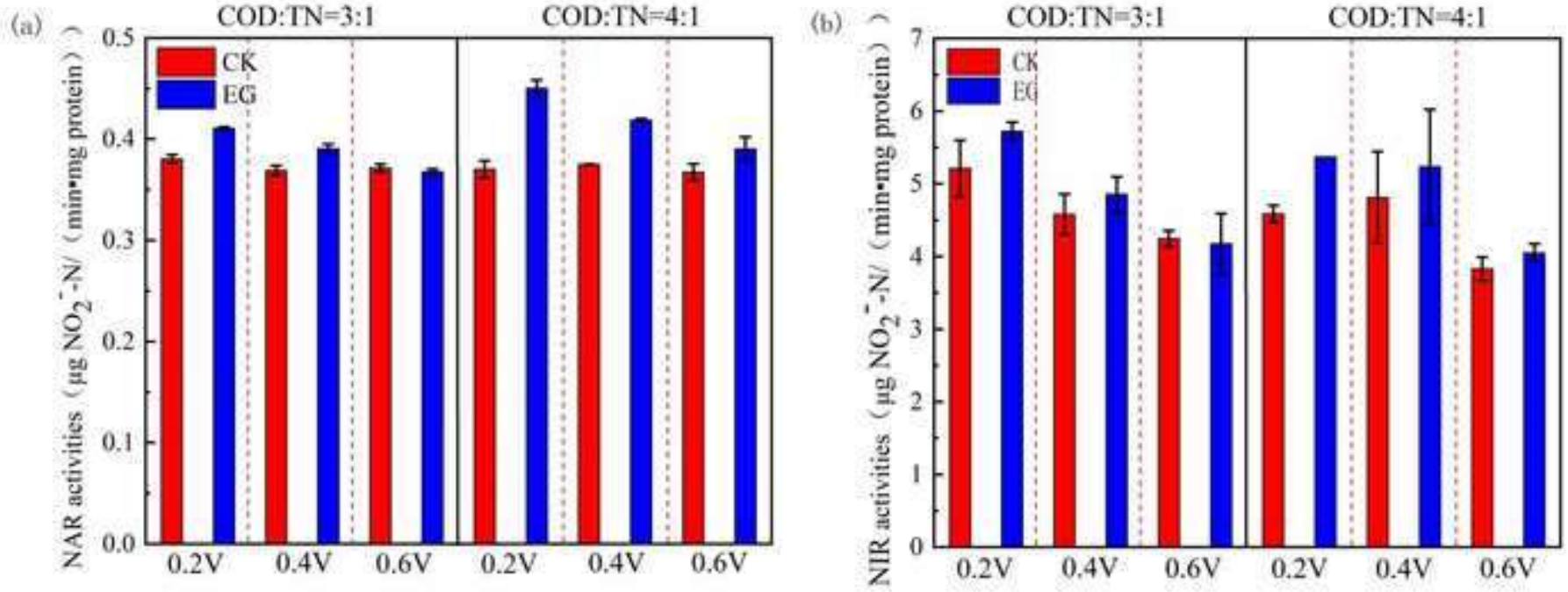

Figure.5 Microbial population structure species level abundance under weak electrical stimulation

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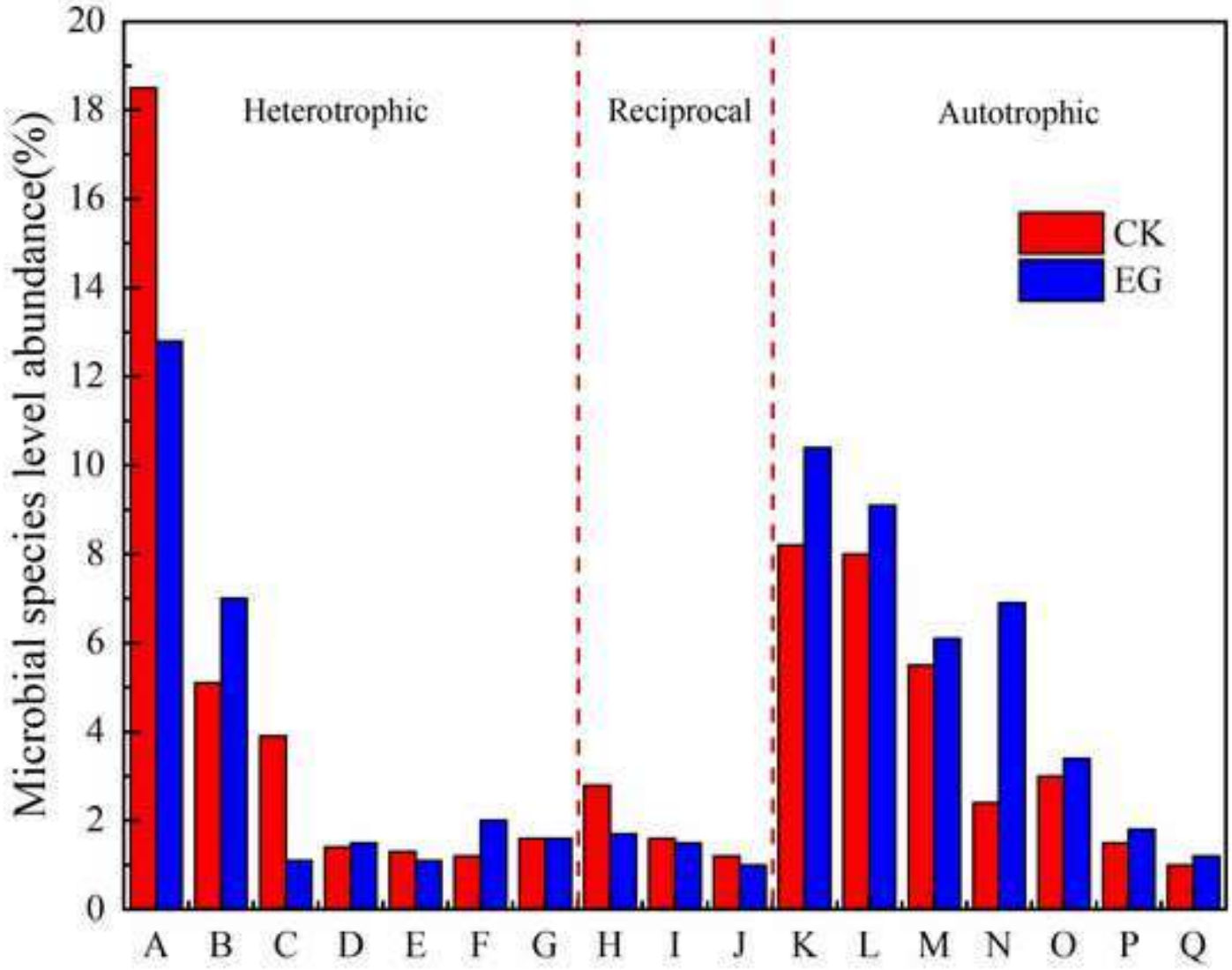


A.Saccharibacteria;B.Bacteroidetes vadinHA17;C.Flavobacterium;
D.Candidatus-competibacter;E.Parcubacteria;F.Fluviicola;
G.Anaerolineaceac;H.Smithella;I.Bacteria clone OPB95;J.Syntrophacea;
K.Thauera;L.Rhodocyclaceae;M.Thiobacillus;N.PeM15;
O.Denitratisoma;P.Clostridium sensu stricto 1;Q.Sulfurisoma

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

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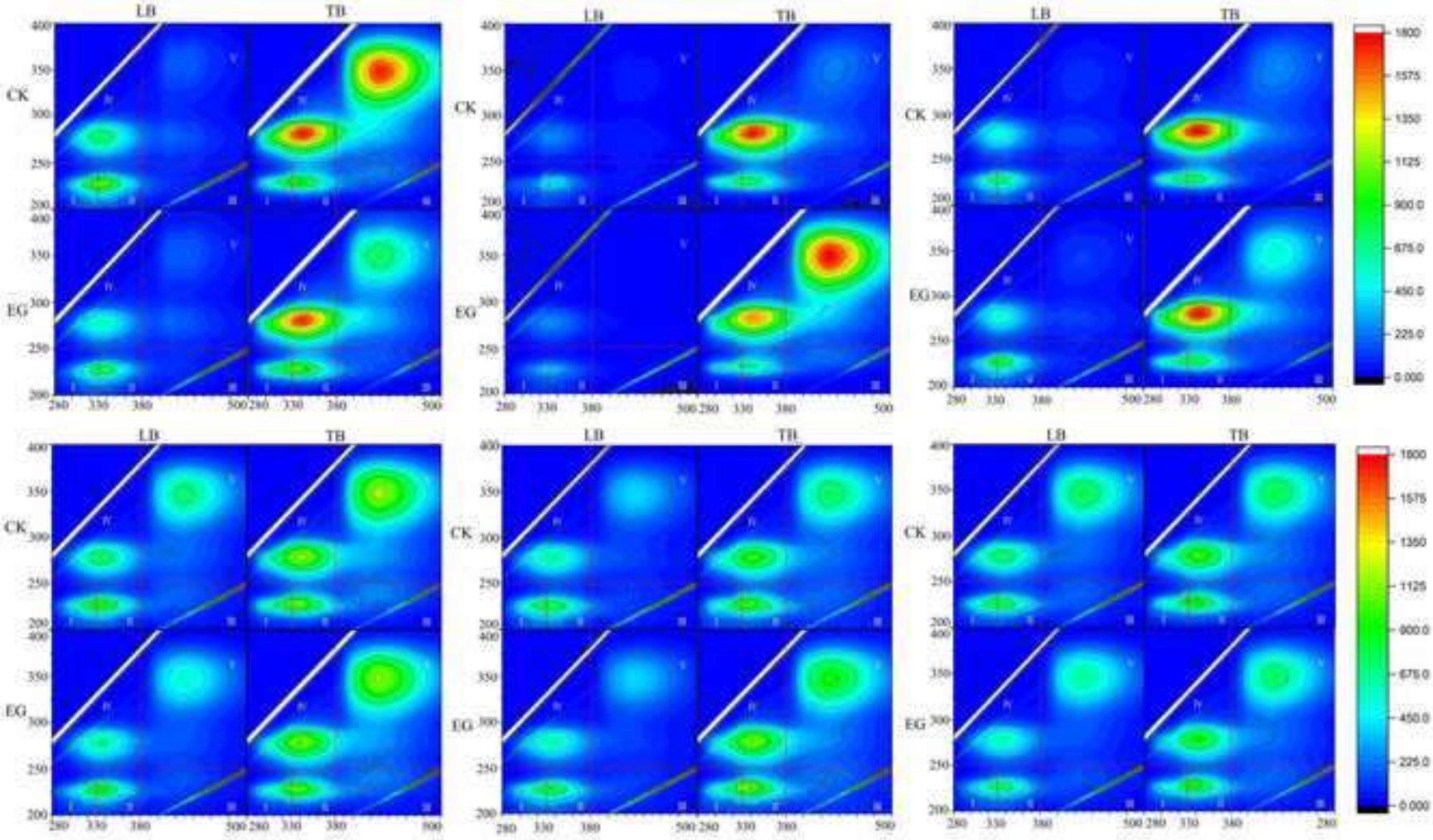

Figure.7 EPS protein content of weak electrically stimulated denitrification continuous flow experimental sludge. (a) COD:TN=3:1, (b) COD:TN=4:1

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