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Effects of trace elements and current densities on denitrification, microbe growth, ATP generation and enzyme activity in a bio-electrochemical reactor

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This study explored the effects of trace elements and current densities on denitrification, microbe growth, ATP generation, and enzyme activity in a bio-electrochemical reactor (BER). The optimum current density of 200 mA/m^2 and the addition of trace elements significantly improved the nitrate removal efficiency compared with the control (99.9% versus 85.2%) and remarkably reduced the intermediate nitrite accumulation (4.01 ± 0.03 versus 8.25 ± 0.35 mg/L). A microbial study showed that the application of the optimum current density and trace elements promoted the microbial growth, ATP activity, cell membrane permeability and denitrification key enzyme activity. An elemental analysis revealed that iron and molybdenum significantly improved the catalytic activity of nitrate reductase (Nar), while iron and copper enhanced the catalytic activity of nitrite reductase (Nir). This study provides an important insight into the potential effects of using an optimum current density and trace elements on denitrifying microorganisms and the denitrification performance within a bio-electrochemical reactor BER.

Keywords: Bio-electrochemical reactor (BER); Trace elements; Electro-stimulation; Denitrification key enzyme activity; Microbial growth

1. INTRODUCTION

Nitrate (NO_3^--N) contamination of aquatic ecosystems is a serious environmental problem. In recent years, a considerable significant big increase in the nitrogen (N) content of groundwater has occurred due to human, such as the use of animal waste, sewage sludge, and synthetic fertilizers in agriculture, atmospheric N deposition and infiltration from polluted water [1]. NO_3^--N contamination

negatively affects aquatic ecosystems. In addition, at high levels of NO_3^--N in potable water are reduced to nitrite (NO_2^--N) in the digestive system, which can cause conditions such as "blue baby syndrome" and gastrointestinal cancer [2].

Various approaches are already used to treat NO_3^--N from water, including physical-chemical methods and biological methods. Of these, denitrification has been confirmed to provide the most significant reduction in NO_3^--N concentrations. In this process, denitrifying microorganisms use carbon as an electron donor to reduce NO_3^--N to N_2 gas by four steps (NO_3^--N → NO_2^--N, NO_2^--N → NO, NO →N_2O, N_2O → N2) under a given set of conditions [3]. However, the denitrification process is slow and needs a lot of carbon sources. Bio-electrochemical denitrification reactors combine the use of electrochemistry and biological denitrification, and are a promising technology that can be operated with high efficiency at low investment costs [4]. Optimization of the effects of different parameters (such as the C/N ratio, current density, HRT, and pH) of the conditions in bio-electrochemical denitrification reactors has been investigated [5-8]. However, the research focus has been placed mainly on the influence of the operating conditions on the reactor's denitrification performance, whereas the crucially important impacts of the microbial and environmental conditions have usually been overlooked. It is noteworthy that suitable electro-stimulation was found to promote bacterial growth and metabolic activity in a BER, but a high current affected them negatively [9]. Moreover, they also reported that pH had a significant effect on bacterial activity, and pH values of 10.25 and 11.25 at 200 mA/m^2 promoted bacterial inactivation towards the end of the experiment [9]. Furthermore, in the same BER, they explored the influence of current density and different C/N ratios on microbial growth using a modified Gompertz model, and determined that suitable electro-stimulation and a higher C/N could improve microbial growth [10].

Balanced nutrient availability is also important for the growth of denitrifying microorganisms, which in turn affects the bioreactor performance. Previous studies have usually focused on understanding the effects of macronutrients, such as carbon (C), nitrogen (N), and phosphorus (P) on denitrification performance [11]. However, the availability of certain trace elements, such as zinc (Zn), iron (Fe), manganese (Mn), copper (Cu), cobalt (Co), and molybdenum (Mo) also has a significant impact on the denitrification performance. These elements often appear in microbial enzyme systems as a coenzyme and a cofactor, and regulate many reaction pathways [12]. For example, in the first step of the four stages of the denitrification pathway, NO_3^--N is reduced to NO_2^--N and catalyzed by nitrate reductase (NR), which contains Mo in its active site [13]. The second enzyme, Nir, catalyzes reduction of NO_2^--N to NO, and these are either Fe-(NirS) or Cu-(NirK)-containing enzymes [14]. In addition, Co is a constituent of vitamin B_{12}, which is critical for the synthesis of nucleic acids and amino acids [15], and Zn is a bacterial metallic enzyme activator of carbonic anhydrase and carboxypeptidase A, which can stimulate cell growth [16]. Furthermore, Fe and Mn are component parts of the cytochromes involved in metabolic pathways [13]. It is thus important to investigate the influence of trace elements on microbial activity and growth during different levels of electro-stimulation within a BER.

Although bio-electrochemical technology has been employed for decades, further research is still needed on the underlying mechanisms through which electro-stimulation affects the growth of denitrifying bacteria within the BER in the absence and presence of trace elements. Therefore, we aimed to explore the denitrification performance under different current densities in the absence and presence

of trace elements. In addition, the growth curve, activity, membrane permeability and denitrification key enzyme activities of microbes were analyzed to determine the underlying mechanisms of trace elements and the most suitable current density required for achieving an increase in the denitrification efficiency and for reducing NO_2^--N accumulation. Thus, the catalytic activities of Nar and Nir were determined and compared when using a single trace element.

2. MATERIALS AND METHODS

2.1. Experimental apparatus

The small BER was the same as previously detailed [9]. The main components of the apparatus were a bench-scale BER (a 1 L conical flask) with an anode (a stainless steel rod) and a cathode (a spiral iron wire), a DC regulated power supply and a magnetic stirrer.

2.2. Inoculum enrichment and groundwater synthesis

Activated sludge collected from an anaerobic tank used to acclimatize heterotrophic denitrifying bacteria [9]. The simulated contaminated groundwater (1 L tap water) contained 0.304 g $NaNO_3$ (i.e., 50 mg-N/L nitrate), 0.044 g KH_2PO_4, and 210 μL of CH_3OH.

In the trace elements experiments, an additional 1 mL of trace element solution was added. The trace element solution was further adjusted on a previous study [17], which contained (g/L): $ZnCl_2$ 1.00; $MnCl_2 \cdot 4H_2O$ 0.10; $FeCl_2 \cdot 4H_2O$ 3.50; $CuCl_2 \cdot 2H_2O$ 0.15; $CoCl_2 \cdot 6H_2O$ 0.50 and $Na_2MoO_4 \cdot 2H_2O$ 0.25. The initial pH was adjusted to 7.25 ± 0.10.

2.3. Experimental start-up

2.3.1. Bio-electrochemical denitrification experiment

The acclimated sludge (4 mL) was added into the synthetic groundwater (1 L) in the BER when the experiment begins. N_2 gas was then purged into each reactor to ensure that the anoxic conditions had been achieved. All the reactors were next sealed and ran for 14 days at room temperature under each of the examined conditions. The current densities were adjusted to 0, 100, 200, 300 and 400 mA/m^2 in the absence and presence of trace elements, respectively.

2.3.2. Effects of single trace element on the activity of denitrifying enzymes

Acclimated sludge (5 mL) was added into the synthetic groundwater (1 L) in the BER at the beginning of the experiment. A treatment without trace elements was employed as the control, whereas in the experimental group the following elements were added Zn (0.48 mg/L), Mn (0.28 mg/L), Fe (1.00

mg/L), Cu (0.05 mg/L), Co (0.15 mg/L) and Mo (0.10mg/L), respectively. Further, N_2 gas was purged into each reactor to ensure that the anoxic conditions. All the reactors were sealed and ran 14 days at each condition, then the activities of denitrifying enzymes were measured at the end of experiments. The single trace element experiment was performed with no electric current.

2.4. Chemical analysis

NO_3^--N, NO_2^--N, and NH_4^+-N were measured by standard methods as previously described every 24 h from each reactor [9].

2.5. Microbiological analysis

Bacterial suspension (1 mL) for determination adenosine triphosphate (ATP) content by ATP fluorescence detector (AF-100, TOA-DKK, Japan). The 3M^{TM} Petriflm^{TM} Count Plate (3M Company, St. Paul, MN, USA) was used for colony counting according to earlier reported manufacturer's instructions.

2.6. Enzyme assays

After 14 d, cells were obtained by centrifugation (6000 rpm for 12 min), and crude cell extracts were prepared for 5 min (20 kHz) by an ultrasonic crusher (Scientz-950E, Ningbo Xinzhi, China), followed by centrifugation (GT10-1, SHKIC, China) at 10000 rpm for 12 min [18]. The measurements of Nar and Nir activities were measured through the consumption of electron acceptor (NO_3^- or NO_2^-) with methyl viologen as an electron donor [18]. The assay mixture contained 1.7 mL of the reaction mixture 10 mM potassium phosphate buffer (pH 7.4), 1 mM methyl viologen, 5 mM $Na_2S_2O_4$, 1 mM electron acceptor ($NaNO_3$ or $NaNO_2$). Then, 300 μL of the cell extract was added. The cell extract was incubated for 30 min, and then used for the determination of NO_3^--N or NO_2^--N. Finally, the specific enzyme activity was calculated.

2.7. Bacterial growth kinetics modeling

The modified Gompertz model was used to represent the kinetics of the bacterial reproduction in this study, using a previously reported formula:

$$\lg N = A + C \times \exp\{-\exp[-B \times (t - M)]\} \quad (1)$$

Maximum growth rate μ_{max} (d_{-1}) and lag time L (d) were also calculated [10, 19]

3. RESULTS AND DISCUSSION

3.1. Nitrate removal

The denitrification performances of a solution containing trace elements and a solution without trace elements (the control) were compared under different current densities. Fig. 1 shows the variations in NO_3^--N, with no current applied, the final NO_3^--N concentration was 10.51 ± 0.67 mg/L in the control, but it was only 4.23 ± 0.02 mg/L in the presence of trace elements. This shows that the addition of trace elements significantly increased the NO_3^--N removal efficiency in the reactor. Similarly, the final NO_3^--N concentration in the presence of trace elements was slightly lower than that of the control at the same current densities. This result suggests that the addition of trace elements could enhance the NO_3^--N reduction efficiency by supplying the essential mineral requirements of the microorganisms.

Denitrification is a biological process containing several membrane proteins with metal-containing cofactors [20]. Nar belongs to a family of bacterial molybdenum-containing oxidoreductase subunits with a highly conserved structure, all these subunits bind to molybdenum atoms related to molybdenum pterin guanine dinucleotide cofactor (MoMGD) [21]. Moreover, the periplasmic Nar associated with the catalytic subunit has a cytochrome c subunit, and the cytoplasmic nitrate reductase has a [Fe-S] center that can assist the electron transfer between MoMGD and hemoglobin [22]. Thus, the addition of Mo and Fe increased nitrate reductase activity, thereby improving the NO_3^--N removal efficiency.

On the other hand, in both the absence and presence of trace elements, the maximum NO_3^--N removal efficiency was all 99.9% at 200 mA/m^2. Interestingly, lower or higher than this current densities decreased the NO_3^--N removal efficiency. These results are in line with the ones of a previous study, confirming that at an optimum electrical current intensity, denitrification is enhanced via the increased synergistic effect between the applied current and the microbial activities, whereas a large current intensity has negative effects on denitrification efficiency [10].

The NO_3^--N removal rate constants at different current densities in the absence and presence of trace elements solution were also computed (determination coefficients [R^2] > 0.9) following the first-order kinetics model. In the control, the NO_3^--N removal rate constant reached only 0.14 d^{-1} with no current stimulation, whereas that with trace elements reaching 0.16 d^{-1} at 0 mA/m^2. This result indicates that the addition of trace elements enhanced the NO_3^--N removal efficiency as well as significantly increased the NO_3^--N removal rate. However, the NO_3^--N removal rate constant in the control with 200 mA/m^2 reached 0.26 d^{-1}, which shows that the electrical current stimulation had a more remarkable effect than the addition of trace elements alone. Furthermore, the NO_3^--N removal rate constant in the presence of trace elements at 200 mA/m^2 was 0.30 d^{-1}, which further demonstrates the synergy between the optimum current and trace elements and its effect on microbial activity in a BER.

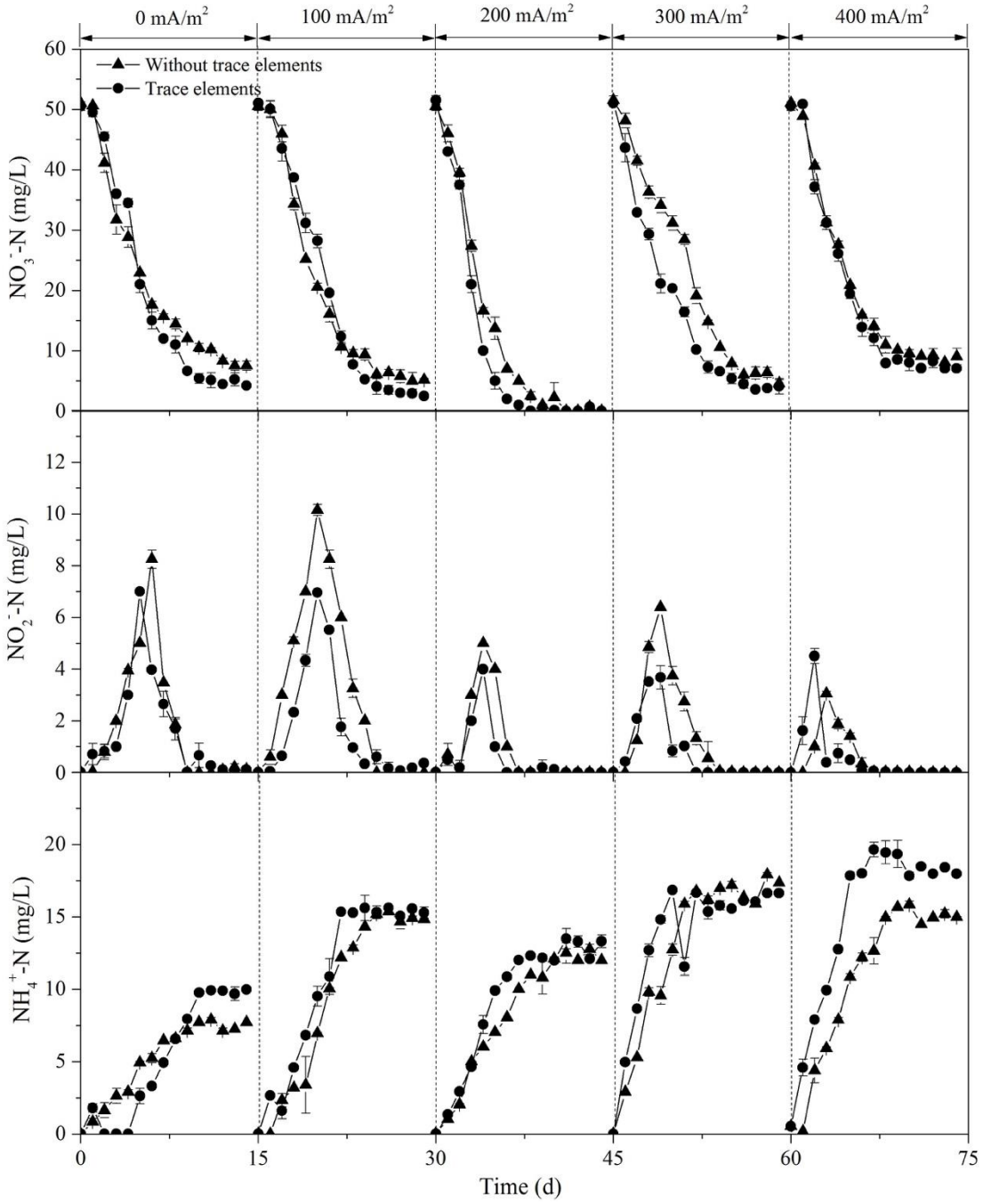

Figure 1. NO_3^--N, NO_2^--N and NH_4^+-N in the absence and presence of trace elements under different current densities.

3.2. Nitrite accumulation

The addition of trace elements and current density also influenced the accumulation of nitrite (Fig. 1). NO_2^--N concentrations increased then sharply decreased, and were undetectable at the end of incubation in all experimental conditions. This trend variation could be attributed to the fact that NO_2^--N is the byproduct of the heterotrophic denitrification process [2, 23]. With no applied current, the NO_2^--N concentration initially reached to 8.25 ± 0.35 and 7.02 ± 0.04 mg/L, and then slowly decreased in

both the absence and presence of trace elements solution, respectively. Similarly, the maximal accumulation of NO_2^--N was reduced by the addition of trace elements solution at 0-300 mA/m^2. Although the addition of trace elements increased the maximal accumulation of NO_2^--N during denitrification at 400 mA/m^2, NO_2^--N still rapidly decreased after the accumulation, and its concentration reached significantly lower values than in the control. These results could be associated with the two distinct classes of Nirs found in bacteria, which either contain Cu (Cu-Nir) or haem (cd1-Nir) as a cofactor [24]. Thus, the addition of Cu and Fe in the trace element solution acted as a cofactor for the NiRs [20], which then improved NO_2^--N reduction. However, regardless of the addition of trace elements, NO_2^--N accumulation at 200-400 mA/m^2 was still remarkably lower than that at 0 and 50 mA/m^2. These results indicate that current stimulation could lead to significant increases in NO_2^--N reduction. Moreover, these data further demonstrate that both electrical stimulation and trace elements can effectively enhance denitrification process, then reducing the harmful accumulation of substances such as NO_2^--N.

3.3. Ammonium accumulation

The initial ammonium was undetectable in both the absence and presence of trace elements at all current densities (Fig. 1). The NH_4^+-N concentrations up to 7.72 ± 0.02 and 9.97 ± 0.36 mg/L, followed by their stabilization in the absence and presence of trace elements at 0 mA/m^2, respectively. At higher current densities, NH_4^+-N showed different levels of accumulation with trace elements or not. The accumulation of NH_4^+-N could be attributed to the action of several factors: (1) dissimilatory nitrate reduction to ammonia (DNRA) could have occurred in the reactor. DNRA is considered to be a counterproductive process for denitrification [25]. Specifically, NH_4^+-N could mainly have been produced through DNRA at 0 mA/m^2. In addition, trace elements are also crucial for the activity of DNRA enzymes, and an increase in NH_4^+-N concentrations can occur under certain conditions. (2) The catalysis of Fe in the cathode could have partly caused NO_3^--N to ammonia via electrochemical reduction [26]. A previous study suggested that NH_4^+-N was mainly formed by microbial action [9]. (3) Large current densities could have caused cell decay [27]. In this respect, the rotting of decaying cells and intracellular materials parts, such as proteins, could have possibly produced smaller part of organic nitrogen in the reactor [28]. This organic-N could then have been produced to NH_4^+-N by microorganisms, which would also have caused NH_4^+-N accumulation [29].

3.4. Variations in ATP content

As can be seen in Fig. 2a, the variations in the ATP content under all experimental conditions first increase then decrease. In the absence of trace elements in the reactor, ATP content increased to 15.99 ± 1.41, 22.56 ± 0.82, 25.81 ± 1.15, 30.49 ± 0.69 and 29.46 ± 0.76 nM, and it then decreased to 7.04 ± 0.06, 9.06 ± 0.12, 3.07 ± 0.10, 3.08 ± 0.08 and 0.14 ± 0.06 nM at 0, 100, 200, 300 and 400 mA/m^2, respectively. The ATP content increased at the beginning of the experiment (100 - 400 mA/m^2), which implies that electrical stimulation enhanced bacterial metabolism and denitrification activity [10].

However, the ATP content rapidly decreased at the end of the experiments using 300 and 400 mA/m^2, which could be attributed to the inhibition (or even death) of the microorganisms due to the continuous application of a high current. The same ATP tendencies were observed in the presence of trace elements, and the ATP content reached as high as 23.56 ± 0.62, 25.27 ± 0.39, 29.11 ± 1.25, 29.88 ± 0.17 and 30.96 ± 1.35 nM, and decreased to 9.63 ± 0.52, 10.16 ± 0.23, 11.01 ± 0.05, 5.16 ± 0.23 and 0.10 ± 0.02 nM at 0, 100, 200, 300 and 400 mA/m^2, respectively.

The ATP aggregate levels (the absolute area under the ATP curve) clearly reflect the variations in microbial activity in each experimental set-up (Fig. 2b). In our experiment, in the absence of electrical stimulation and trace elements solution (the control), the ATP aggregate level then increased to 180.50 ± 2.12 nM d with the addition of trace elements solution at 0 mA/m^2. This value was approximately 1.13 times that of the control. The ATP aggregate levels also increased with the addition of trace elements solution under the same current densities, which indicates that the trace elements improved microbial activity. Fe is required by the cytochrome for electron transfer, while Mn is essential for electron transfer and Fe replacement [12]. Cu acts as a cofactor for the copper-containing NiR (Cu-NiR) in the process of denitrification, and it can thus contribute to the production of nitric oxide from NO_2^--N [30]. Zn is exist in some kind of enzymes and protohemes and can stimulate cell growth [16]. Mo is the key element in the biological reduction of NO_3^--N to NO_2^--N [14]. As a part of vitamin B_{12}, Co is an essential element for microorganisms that is involved in several significant processes, including the synthesis of nucleic acids, amino acids, and the formation of erythrocytes [15]. In conclusion, the addition of trace elements is crucial for enhancing denitrifying microbial activities within a BER.

However, regardless of the addition of the trace elements, the maximum ATP aggregate levels at 200 mA/m^2 were 226.50 ± 3.53 and 252.04 ± 11.31 nM d, respectively. This result could be attributed to the improved metabolism and denitrification activity of bacteria when suitable current was applied, as high current densities are known to have a negative effect on microbial activity [9]. These data show that suitable electrical stimulation has a more remarkable effect in promoting microbial activity than the addition of trace elements. However, a much higher ATP aggregate level (1.58 times that of the control) was obtained with the addition of trace elements at 200 mA/m^2, which suggests that microbial activity was enhanced via the increased synergy between the suitable current stimulation and the trace elements within the bio-electrochemical denitrification reactor. The ATP content in the presence of trace elements was higher than that in the absence of trace elements at the beginning of the experiment under 300-400 mA/m^2, but the ATP content all decreased below 1 nM and no difference was observed at the end of both types of experiments. These results show that when microbial cells are not damaged, the trace elements enhance microbial activity, but the continuous application of a strong current causes electroporation and electrolysis in the cell wall and membrane and can even cause microorganism death [31]. Therefore, microbial electrical resistance would not be improved, even with the further addition of trace elements.

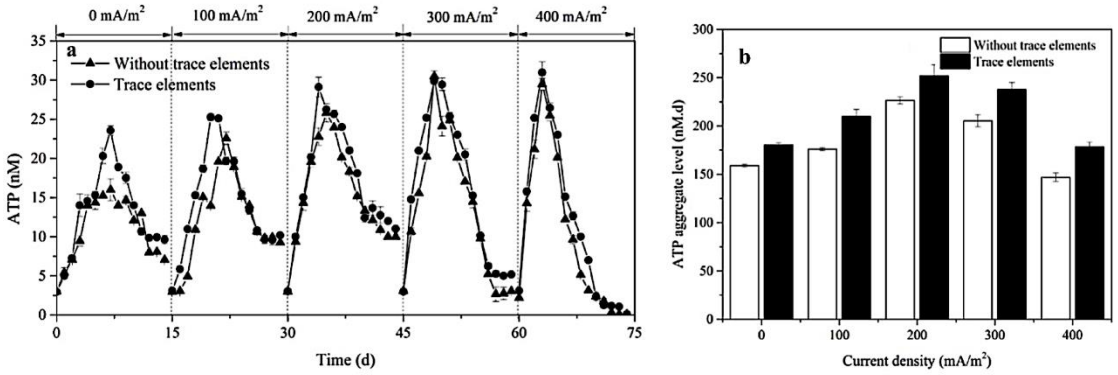

Figure 2. ATP content (a) and ATP aggregate level (b) in the absence and presence of trace elements under different current densities.

3.5. Bacterial growth kinetics within the reactors

Fig. 3 shows the growth curves of bacteria in each group of the BER. The preliminary bacterial colony count changed from 4.99 to 5.16 lg CFU/mL. The results showed that bacterial colony counts in the presence of trace elements solution were slightly higher than those in the control under the same current densities, which suggests that the addition of trace elements promotes the growth of microorganisms, although less significantly ($P>0.05$). A previous study also reported that adding trace elements caused an increase in bacterial metabolism instead of an enhance in bacterial growth [20]. Current stimulation had a more obvious effect on promoting microbial growth than the addition of trace elements, particularly at 200 mA/m^2, where the bacterial counts were higher than other current densities. These results showed that the application of a certain current density led to faster microbial growth, resulting in a higher number of bacteria. However, despite the addition of trace elements, the growth curves showed a decay with time when 400 mA/m^2. This further indicates that large currents are detrimental to microorganisms; they decrease their growth and activity and can even lead to decay [9]. The modified Gompertz model gave a good statistical fit to the observed data and its R^2 values were close to 1.00, which suggested a good fit between the modified Gompertz model and the growth curves in this study (Table S1).

The μ_{max} were calculated from the modified Gompertz model under all experimental conditions (Fig. 4a). We observed that the μ_{max} increased with current densities up to 200 mA/m^2, but it started to decrease beyond this value, even when trace elements were added. The μ_{max} was 0.18 d^{-1} in the absence of electrical stimulation and trace elements solution (the control). With the addition of trace elements in the reactor, the μ_{max} reached 0.19 d^{-1} at 0 mA/m^2 (an increase of only 1.05 times that of the control). In contrast, when only the 200 mA/m^2 current density was applied in the reactor without trace elements, the μ_{max} reached 0.37 d^{-1}, which was 2.05 times higher than the value of the control. These results further indicated that suitable electro-stimulation substantially improves microbial growth rate, as compared to the addition of trace elements alone. Furthermore, at the simultaneous application of the optimal current density (200 mA/m^2) and trace elements, the μ_{max} reached 0.40 d^{-1} (2.22 times that of the control). This

shows the synergistic effects of the application of optimal electro-stimulation and the addition of trace elements within the bio-electrochemical denitrification reactor, resulting in the stimulation of microbial growth. A quadratic function model was used to describe the change in μ_{max} with increasing current density. Adj.R^2 values were 0.946 and 0.952 in the absence and presence of trace elements, respectively, which suggests that the quadratic function provided a good statistical fit to the observed data (Table S2).

As visible in Fig. 4b, the lag time (L) initially decreased but then stabilized with the increase in the current densities with or without added trace elements. The lag time (L) was 4.74 d for the control in the absence of current within the reactor. However, when trace elements were added, the lag time (L) became 4.19 d in the absence of electrical stimulation. When the 200 mA/m^2 was applied in the reactor without trace elements, the lag time (L) further decreased to 2.43 d. This confirms that suitable electro-stimulation had a greater favorable effect on the multiplication of cells at the lag time than trace elements. In addition, the lag time (L) was shortened to 2.12 d at 200 mA/m^2 and added with trace elements in the reactor, which was only 0.42 times that of the control. A previous study established that the reduction in the lag phase during exposure to electric fields owing to the membrane permeability [33]. Microbial membrane permeability is promoted by suitable electro-stimulation [10], facilitating the penetration of trace elements into cells. These results imply that the application of optimal electro-stimulation in the presence of trace elements shortens the time for microbial adaptation to the environment, thus contributing to an improvement in denitrification performance. An exponential equation was used to describe the lag time change with the increase in the current density with or without added trace elements. The strong positive correlations (Adj.R^2 = 0.989 and 0.992) show that the data of the models are well fitted with the measured data (Table S2).

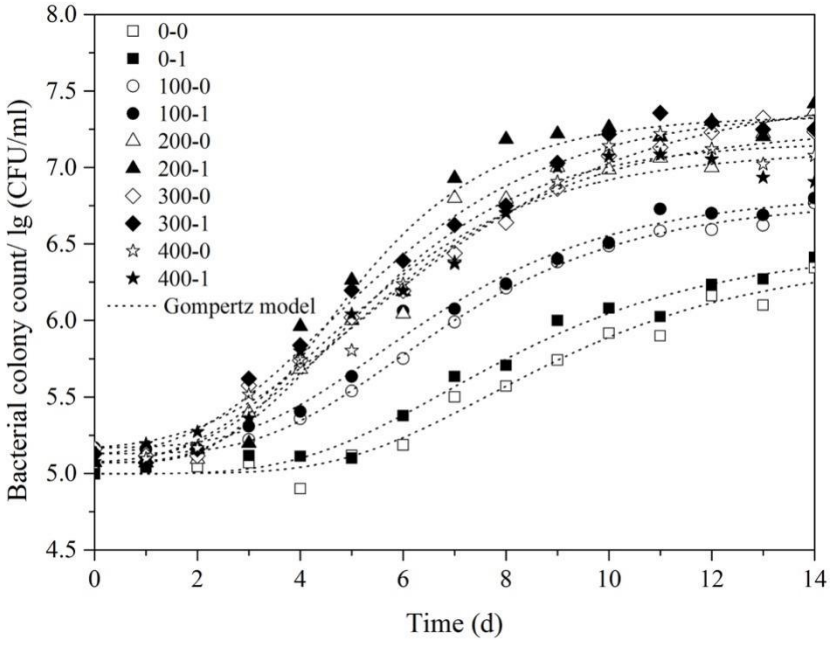

Figure 3. Microorganism growth curves in the absence and presence of trace elements under different current densities (-0: without trace elements; -1: with trace elements)

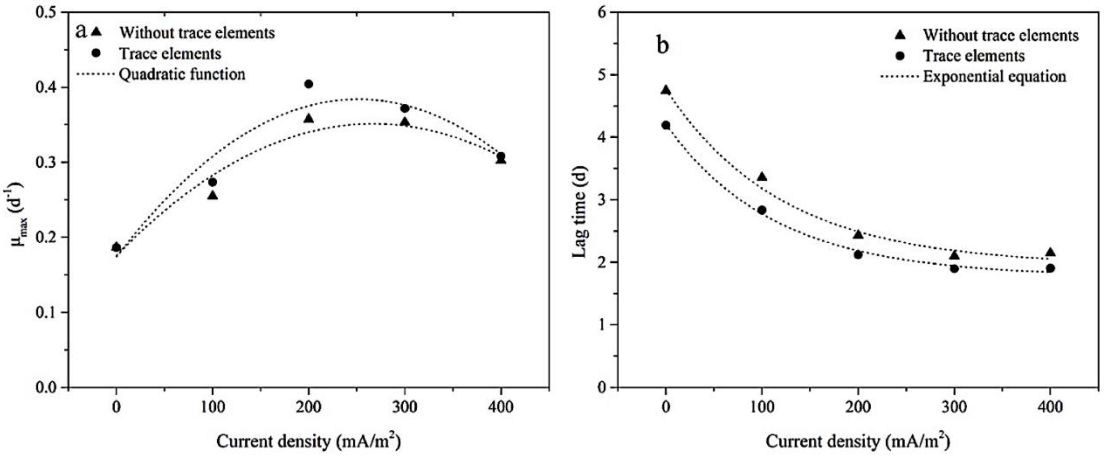

Figure 4. The maximum growth rate μ_{max} (a) and the lag time (b) in the absence and presence of trace elements under different current densities.

3.6. Catalytic activity of denitrifying enzymes

Denitrification is highly rely on the expression and activities of essential denitrifying enzymes such as Nar and Nir. From Fig. 5, it is evident that Nar was improved by 200 mA/m^2, regardless of the presence of trace elements. The activity of Nar was increased from 102% to 113% and 107% to 121% compared to the control with the elevation from 0 to 200 mA/m^2 in the absence and presence of trace elements, respectively. Similarly, the activity of Nir rose from 105% to 120% and from 110% to 126% compared to the control with the augmentation in 0 to 200 mA/m^2 in the absence and presence of trace elements, respectively. These results indicate that both suitable electro-stimulation (200 mA/m^2) and the addition of trace elements improve the catalytic activities of denitrifying enzymes. Nar and Nir are important key enzymes responsible for denitrification. These enzymes reduce nitrates and nitrites [13]. The key encoding gene for Nar is *narG*, and those for Nir are *nirK* and *nirS* [13]. The synthesis of these denitrifying enzymes depends on the transcriptional expression of their encoding genes. Therefore, optimal electro-stimulation might exert a positive effect on denitrifying enzymes at the gene level, thereby improving the activity of denitrifying enzymes. A previous study reported that the denitrifying enzymes (such as Nar and Nir) are metalloenzymes containing Fe, Cu or Mo ions [18]. Therefore, the addition of trace elements in the reactor would also increase the activity of Nar and Nir. However, the application of optimal electro-stimulation (200 mA/m^2) and the addition of trace elements caused obvious improvements in Nir activity, which could have been related to the membrane-bound status of Nar, whereas Nir is located in the periplasm [34], the periplasm-located Nir is more readily promoted by environmental influences.

However, Nar decreased to 73.5% and 79.4% compared to the control as the current density increases to 400 mA/m^2 in the absence and presence of trace elements, respectively. Nir also decreased to 68.3% and 67.5% compared to the control as the current density increases to 400 mA/m^2 in the absence and presence of trace elements, respectively. These results suggest that the high current density could

have inhibited the inhibition in the catalytic activities of Nar and Nir, which finally induced a reduction in NO_3^--N and NO_2^--N reduction. In addition, the catalytic activities of Nar and Nir with trace elements slightly were higher than those in the control at 400 mA/m^2. These findings indicate that the addition of trace elements improved microbial electrical resistance in this study, although this only played a minor role.

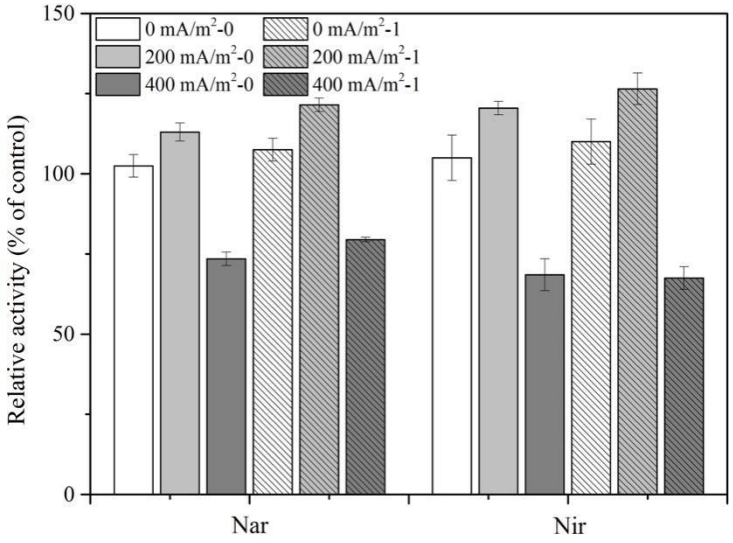

Figure 5. The relative activities of Nar and Nir at the end of each experiment in the absence and presence of trace elements under different current densities (-0: without trace elements; -1: with trace elements, control: 0 mA/m^2 without trace elements).

3.7. Catalytic activity of denitrifying enzymes with single trace element

The effects of individual elements in the trace elements solution on the catalytic activity of Nar and Nir were investigated separately. As shown in Fig. 6, Mo remarkably increased the catalytic activity of Nar, and the value was approximately 134% compared to the control. Nar enzymes are known to contain active sites, with Mo as a cofactor or Mo-bis-MGD as a factor, in which a Mo atom is bound to molybdopterin guanine dinucleotide [24]. Thus, the addition of Mo improved the activity of Nar and enhanced NO_3^--N removal. The addition of Fe increased the relative activity of Nar increased to 112%. This is due to the fact that Fe is a necessary trace element for microbial growth, participates in a variety of biochemical reactions in cells, as a metal cofactor of various enzymes, and is also an important electronic carrier on Nar [20]. Moreover, the catalytic activity of Nir was increased to 122% and 124% with the addition of Fe and Cu, respectively. As discussed in 3.2, Nir divided contains homotrimeric Cu-containing enzymes and haem-containing enzymes. Thus, the Cu and Fe acting as a cofactor for the Nir could prompt the catalytic activity [35]. Zn and Co also slightly increased the catalytic activity of Nar and Nir. This further suggests that Zn and Co as the enzyme activators could improve the activity of denitrifying enzymes. These results provide further opinions into the importance of selecting Mo, Cu, and Fe ores, as well as other active minerals, for improvements in the denitrification performance in a

range of practical applications.

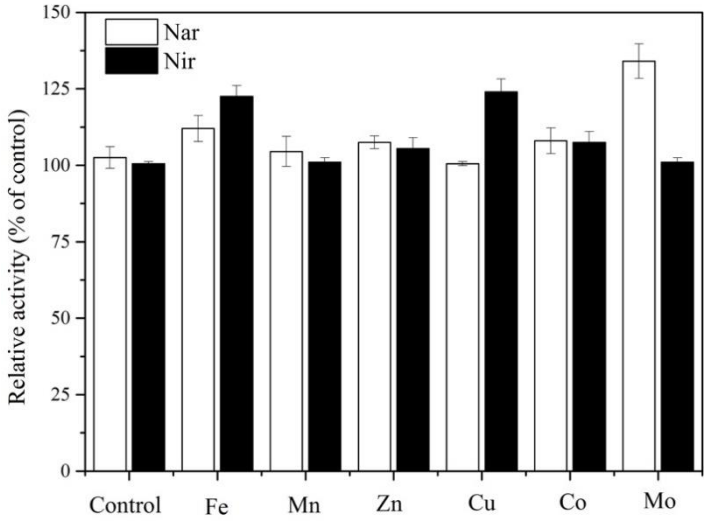

Figure 6. The relative activities of Nar and Nir at the end of each experiment under different trace elements (control: 0 mA/m^2 without trace elements).

4. CONCLUSIONS

The largest NO_3^--N removal efficiency was obtained at an optimum current density (200 mA/m^2) with trace elements or not, and the maximum ATP aggregate level and bacterial counts were get at this current density. An optimum current density and adding trace elements improved the μ_{max} and shortened the associated lag time. Furthermore, the optimum current density and the addition of trace elements increased the catalytic activity of both (Nar and Nir) examined denitrifying enzymes. This work contributes to better understanding the mechanisms through which current density and trace elements influence denitrification performance, which has important implications for the remediation of groundwater in bio-electrochemical reaction systems.

DECLARATIONS:

AUTHORS CONTRIBUTIONS

Hengyuan Liu: Conceptualization, Investigation, Formal analysis, Writing - original draft, Writing - review & editing. Qili Hu: Investigation, Formal analysis, Writing - original draft, Writing - review & editing. Nan Chen: Writing - review & editing. Chuanping Feng: Conceptualization, Writing - review & editing.

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AVAILABILITY OF DATA AND MATERIAL
All data generated or analyzed during this study are included in this article.

CONFLICTS OF INTEREST
The authors declare no conflicts of interest.

SUPPORTING INFORMATION:

Table S1. Parameters and fitting results using the modified Gompertz model under different experimental conditions.

Running conditions		A	B	C	M	Adj. R^2	RMSE
Current density (mA/m^2)	Trace elements						
0	absence	5.0430	0.3431	1.3997	7.6888	0.9718	0.0795
100	absence	5.1290	0.3868	1.6502	5.8401	0.9780	0.0712
200	absence	5.0700	0.4631	2.0981	4.5706	0.9971	0.0138
300	absence	5.1600	0.3201	2.3193	5.2240	0.9884	0.0565
400	absence	5.0700	0.3766	2.1825	4.7211	0.9812	0.1550
0	presence	4.9970	0.3442	1.4708	6.8483	0.9735	0.2736
100	presence	5.1680	0.3828	1.6589	5.4977	0.9913	0.0722
200	presence	5.0700	0.5332	2.2641	4.3514	0.9902	0.0940
300	presence	5.1200	0.3307	2.4785	4.7928	0.9813	0.1487
400	presence	5.0700	0.4109	2.0379	4.3398	0.9766	0.1641

Table S2. Effect of current densities on the maximum growth rate (μ_{max}) and lag time (L) in the absence and presence of trace elements.

	Maximum growth rate (μ_{max})			Lag time (L)		
	Adj. R^2	**RMSE**	**Quadratic function**	**Adj. R^2**	**RMSE**	**Exponential equation**
Absence of trace elements	0.880	0.0012	$y=-0.000002x^2+0.0013x+0.1757$	0.989	0.0255	$y=1.9521+2.8107\times0.9917^x$
Presence of trace elements	0.896	0.0022	$y=-0.000003x^2+0.0017x+0.1739$	0.992	0.0139	$y=1.7722+2.4332\times0.9911^x$

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