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Analysis of denitrification performance and microbial community structure in a bio-electrochemical reactor under different current densities with wheat-rice stone powder

Hengyuan Liu[a,*] and Qili Hu[b]

[a] College of Chemistry and Life Sciences, Chifeng University, NO.1 Yingbin Road, Hongshan District, Chifeng 024000, China
[b] College of Ecology and Environment, Chengdu University of Technology, Chengdu 610059, China
*Corresponding author. E-mail: liuhy729@163.com

ABSTRACT

Nitrate widely exists in water pollution and is the most stable form of nitrogen pollution. This study investigated the effect of the current density (CD) and the wheat-rice stone powder (WP) on denitrification performance, microbial diversity and enzyme activity in a bio-electrochemical reactor (BER). It was found that an optimum CD of 200 mA/m^2 and the addition of WP significantly improved the nitrate removal rate constant compared with the control group (12.28 d^{-1} versus 9.75 d^{-1}) and remarkably reduced the intermediate accumulation of nitrite. The application of both optimum CD and WP enhanced the microbial diversity and catalytic activity of nitrate reductase (Nar) and nitrite reductase (Nir). The most dominant microbial taxa in our reactor were *Methyloversatilis*, *Methylotenera*, and an unclassified genus of the family Methylophilaceae. Moreover, WP allowed the denitrifiers to better withstand the stress of high CD. This study presented results supporting the use of an optimum CD and natural mineral addition to improving the performance of the denitrification process within a BER.

Key words: bio-electrochemical reactor (BER), current density (CD), denitrification, microbial community, wheat-rice stone powder (WP)

HIGHLIGHTS

- An optimum CD and WP improved nitrate removal.
- Optimum CD and WP enhanced the microbial diversity and denitrifying enzyme activity.
- WP improved denitrifier resistance to high CD.

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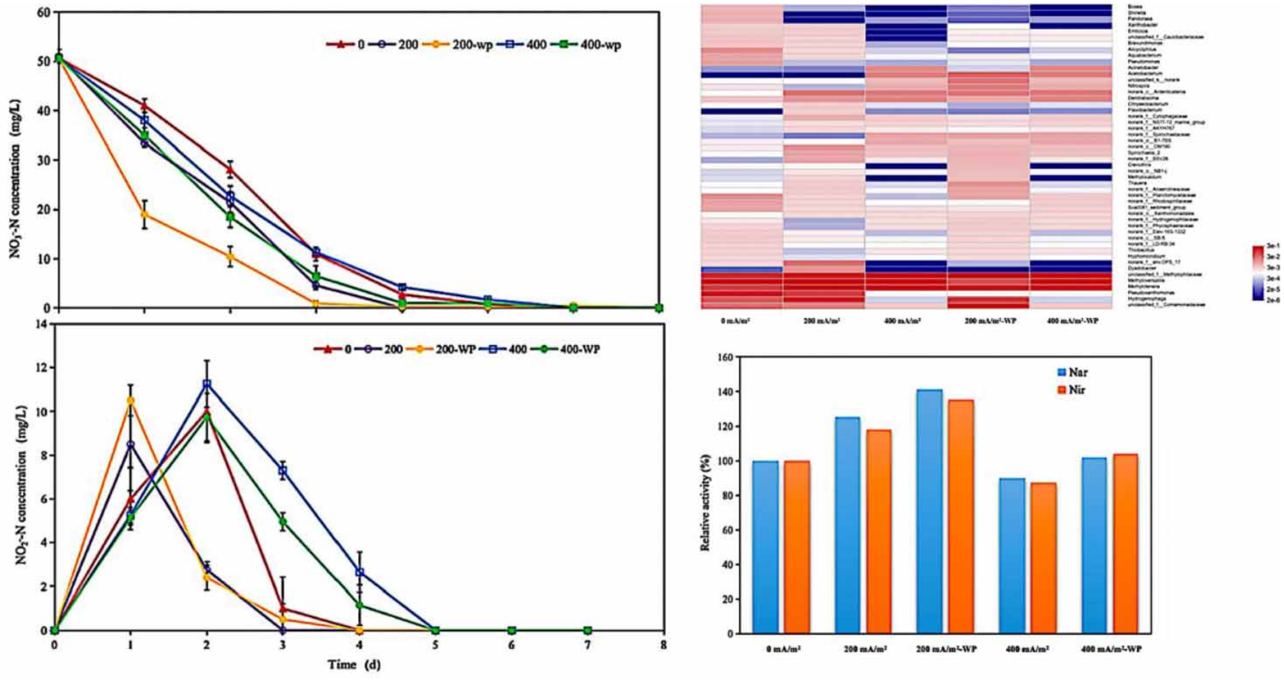
GRAPHICAL ABSTRACT



1. INTRODUCTION

Nitrogen plays a major role in nutrient cycling within a wide variety of global ecosystems, and the nitrogen cycle is an important component of global biogeochemical cycles (Morgane *et al.* 2019). The annual addition of 'active nitrogen' through human activities to the environment has led to imbalances in the global nitrogen cycle, which have resulted in nitrate pollution of groundwater and posed a serious threat to drinking water safety (Hu *et al.* 2019). In the groundwater environment, nitrate sources mainly include atmospheric precipitation, septic systems, agricultural fertilizer, and manure storage (Ma *et al.* 2018; Zhang *et al.* 2020). The pollution has not only affected the water system worldwide (Ballantine *et al.* 2014), but also threatened human health. Nitrate could be transformed in the human body, which may cause blue baby syndrome and increase the risk of gastrointestinal cancer (Garcia-Segura *et al.* 2018). There have been a lot of reports about nitrate pollution in China and abroad, such as in the east and west of Nebraska in the US and the Yellow River Delta in China (Wang *et al.* 2021).

Under biological and chemical activities, nitrogen migration and transformation processes occur in water. Denitrification is a crucial process in the nitrogen cycle regulation and key to the biological treatment of groundwater nitrate (Liang *et al.* 2018). Biological denitrification has the advantages of high efficiency and low consumption among many methods; thus, the method of using biological denitrification to remove nitrate from water has been increasingly applied (Zhou *et al.* 2015; Yang *et al.* 2021). Denitrifying microorganisms play a critical role in the successful application of bio-electrochemical denitrification systems to remove nitrogen (as nitrate) from contaminated water (Wang *et al.* 2021). These organisms are central to the entire denitrification process, and can reduce NO_3^--N or NO_2^--N in water to N_2, which is released into the atmosphere, thus reducing the nitrogen-containing pollutant concentrations in water. Other conditions, such as electro-stimulation and natural mineral contents, can considerably enhance microbially mediated biochemical processes. Research has shown that CD substantially impacts microorganism growth and activity in reactors, with optimized CD helping microorganisms achieve their best growth rates and total ATP levels (Liu *et al.* 2017). However, when there is excess current in reactors, microorganism growth, reproduction, and metabolic activity become inhibited, directly affecting NO_3^--N removal and NO_2^--N accumulation in reactors (Liu *et al.* 2015). Wheat-rice stone, a natural mineral with a porous structure, is an appropriate trace element source for bacteria (Liu *et al.* 2020). Wheat-rice stone has been shown to stimulate denitrification in a continuous

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system at different C/Ns and HRT (Hong *et al.* 2012). In addition, the addition of wheat-rice stone could increase the microbial diversity and improve the transcription of Nar and Nir (Liu *et al.* 2020).

Therefore, microorganisms are a major component of BER systems and occupy a key position in the material transformations that constitute denitrification processes. Correspondingly, investigation of the microbial community structure in a reactor operating under different CDs with wheat-rice stone and the characteristics of the microorganisms involved would contribute to understanding bio-electrochemical denitrification systems.

Molecular biological techniques have recently been extended to other disciplines, providing powerful methodologies for exploring the biological treatment processes used in water pollution remediation (Liu *et al.* 2020). Analysis of the DNA sequences of microbial populations improves the grasp of the composition, structure, diversity, and functional characteristics of microorganisms and their populations. Analysis of wastewater biological treatment mechanisms at the biomolecular level could contribute to the development of theoretical and practical application guidelines to improve the relationships between microorganisms and operating conditions, facilitating the optimization of biological treatment systems (Lu *et al.* 2014; Wang *et al.* 2021).

In this study, we hypothesized that the functional microbial community could be enriched by the identification of the optimum current alongside wheat-rice stone addition. Molecular biology techniques (high-throughput 16S rRNA sequencing technology) were used to analyze and compare the microbial communities of microorganisms under no current ($0\ \mathrm{mA/m^2}$), moderate CD ($200\ \mathrm{mA/m^2}$), and high CD ($250\ \mathrm{mA/m^2}$), moderate CD + WP ($200\ \mathrm{mA/m^2}$-WP) and high CD + WP ($400\ \mathrm{mA/m^2}$-WP). These analyses aimed to investigate the differences in denitrification processes, microbial community composition, species, dominance, and relative abundances under different CDs, then elucidate the underlying mechanisms that allow currents and wheat-rice stone addition to influencing microbial community structure. This study can give us a better understanding of the impact of CD and natural minerals on microbially mediated processes in BER of nitrate-contaminated groundwater.

2. MATERIALS AND METHODS

2.1. Experimental reactor

The small BER used in this study is similar to that previously reported by Liu *et al.* (2015). The main components of the apparatus were a BER (a 1 L reagent bottle with rubber stopper) 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. Microbial enrichment culture and groundwater synthesis

Activated sludge collected from a wastewater treatment plant (Chifeng, China) was used to acclimatize heterotrophic denitrifying bacteria (Liu *et al.* 2015). 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 CH_3OH.

2.3. Experimental process

After incubation, the acclimated sludge was added into the synthetic groundwater (1 L) in the BER at the beginning of the reaction. The initial bacterial colony averaged 1.0×10^5 CFU/mL in each reactor (3M™ Petrifilm™ Plates). To ensure anoxic conditions, N_2 gas was purged into each reactor for 5 min. All the reactors were sealed and ran for seven days at room temperature ($25 \pm 2\ ^\circ C$) under each condition. The CD setting range was 0–400 $\mathrm{mA/m^2}$ at 200 $\mathrm{mA/m^2}$ intervals in the absence and presence of trace elements. The activated sludge of each group of experiments was re-added and independent of each other (Liu *et al.* 2015).

Wheat-rice stone was added as a powder to explore the effect of its addition on the denitrification process in the BER. The wheat-rice stone mainly consisted of 65% SiO_2, 15% Al_2O_3, 4% Na_2O, 4% K_2O, 1–2.5% Fe_2O_3, 2–3% CaO, 1–2.5% FeO, and a few MgO, TiO_2, MnO and P_2O_3 (Li *et al.* 2009; Hong *et al.* 2012). All of the WP was sterilized by autoclave at 121 °C for 20 min before use.

2.4. Analytical methods

2.4.1. Chemical analysis

A total volume of 5 mL water sample was taken out from each reactor every 24 h. NO_3^--N, NO_2^--N, and NH_4^+-N were determined using an ultraviolet spectrophotometer (TU-1901, PERSEE, China). The standard deviations were analyzed at a confidence level of 90%.

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The resistance of microbes to the high current stress was calculated for the denitrifying microorganism under each experimental condition. Resistance RS is the ability of a community to remain unchanged and stable when suffering a disturbance (Liu *et al.* 2018). In this study, the resistance of the heterotrophic denitrification process following the disturbance caused by the high current was calculated as follows:

$$RS = 1 - \frac{2|D_0|}{C_0 + |D_0|} \tag{1}$$

C_0 is the response value of the system at the end of undisturbed group interference, t_0 is the response value at the end of disturbing group interference, and d_0 is the difference between them. The formula is standardized through comparison, taking into account the difference between the changes caused by the disturbance. The upper and lower thresholds of the resistance formula are +1 and −1 respectively, +1 means that the disturbance has no impact (maximum resistance), and −1 means that the disturbance has a significant impact (minimum resistance) (Orwin & Wardle 2004).

2.4.2. Microbiological analysis

At the end of each experimental series, liquid and precipitated mixed samples were collected for microbial analysis based on the 16S rRNA gene when NO_3^--N reached a steady-state value. A 10 mL sample was collected and centrifuged at 10,000 rpm for 20 min. A FastDNA® Spin Kit for Soil (MP Bio, USA) was used for DNA extraction of the sediment according to the manufacturer's instructions. The high-throughput sequencing method was employed to detect the 16S rRNA gene of the microorganisms.

2.5. Enzyme assays

After seven days, cells were harvested by centrifugation (6,000 rpm for 12 min), and crude cell extracts were prepared by disrupting cells for 5 min (20 kHz) by ultrasonic crusher (Scientz-950E, Ningbo Xinzhi, China). Then, the treatment and determination methods were based on previous studies (Zheng *et al.* 2014). The assay mixture contained 1.7 mL of the reaction mixture and 300 μL of the cell extract. The cell extract was incubated for 30 min and then applied to determine the $NO3^-$-N or $NO2^-$-N concentration and to calculate the specific enzyme activity.

3. RESULTS AND DISCUSSION

3.1. Heterotrophic denitrification

Figure 1 shows the NO_3^--N removal efficiencies finally increased 100% at all conditions, but the removal rate constants were different (9.75, 11.35, 12.28, 9.67 and 10.88 d^{-1} for '0', '200', '200-WP', '400' and '400-WP', respectively). The nitrate-removal procedures of experiments '200' and '200-WP' therefore represented the fastest denitrification rates. However, there was a clear decline when CD reached 400 mA/m^2. In other words, compared with the condition with no or moderate CDs, those with high CD required a long time to complete conversion of the NO_3^--N, indicating that a high CD obstructed the NO_3^--N removal process. The minerals could improve the NO_3^--N conversion even under the negative effect of high CD. This shows that the addition of WP and appropriate electrical stimulation significantly increased the NO_3^--N removal rate constant in the reactor, though it was still lower than 0 mA/m^2 and 200 mA/m^2. Denitrifying bacteria could utilize trace metals from wheat-rice stones for metabolizing (Liu *et al.* 2020). Previous studies have shown that the denitrification rates were accelerated by the release of trace metals from wheat-rice stones (Hong *et al.* 2012).

However, the maximum nitrate removal rate constants were obtained at 200 mA/m^2 with or without WP. More interestingly, CDs lower or higher than this value was all coupled with a decreased nitrate removal rate constants. These results were consistent with previous research, explaining that under the optimum current intensity, the synergistic effect between applied current and microorganisms was enhanced, while higher current intensity had an adverse effect on denitrification (Liu *et al.* 2017).

The addition of trace elements and CD also influenced the accumulation of nitrite (Figure 1). Nitrite concentrations first increased and then sharply decreased, and were undetectable at the end of incubation under all experimental conditions. The intermediate product of heterotrophic denitrification accumulates because of the inhibition of the enzyme responsible for NO_3^--N to NO_2^--N reduction (Zhao *et al.* 2011). With no applied current, the NO_2^--N initially reached 6.05 ± 1.32 mg/L and then decreased. According to the nitrate reduction pathway, both the Nar and Nir were inhibited by current intensity.

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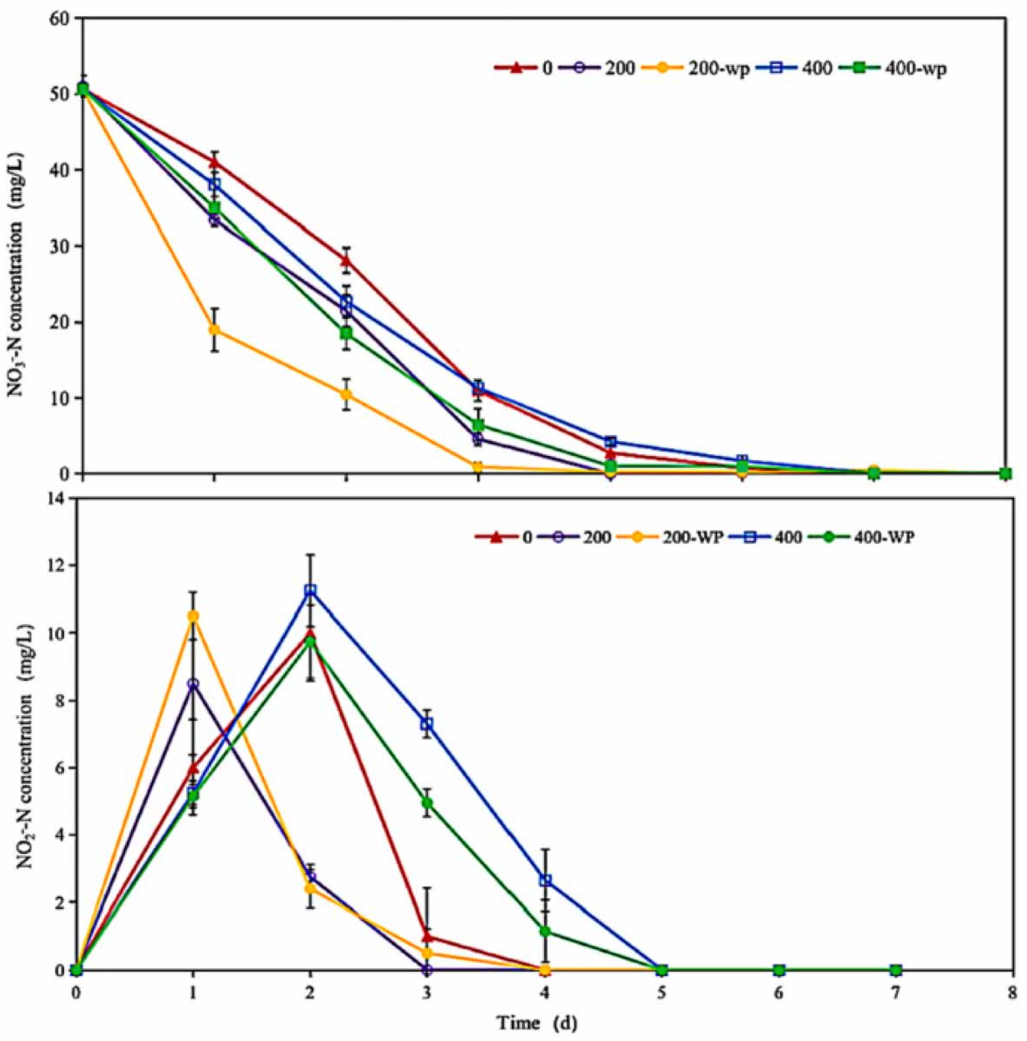

Figure 1 | Concentrations of NO_3^--N and NO_2^--N at different current densities during the batch experiments (-WP: with wheat-rice stone powder).

The NO_2^--N with higher accumulation and has noticeable hysteresis occurred at higher CD. However, minor NO_2^--N was measured at the same CD following with WP, showing that natural minerals may be beneficial to reduce the accumulation of nitrite in the process of denitrification.

3.2. Analysis of microbial community diversity

In this study, 16S rRNA high-throughput sequencing was performed on post-experimental sludge under no current (0 mA/m^2), moderate CD (200 mA/m^2), high CD (400 mA/m^2), moderate CD + WP (200 mA/m^2-WP), and high CD + WP (400 mA/m^2-WP). The table of microbial community diversity indexes offers a clear comparison of the differences in microbial diversity between the reactors operating under different experimental conditions (Table 1). The coverage of all sequenced samples was >99.7%, which indicated that the results accurately reflected the microorganisms in the samples.

The Sobs index is the number of observed operational taxonomic units (OTUs), and the Chao index is the number of estimated OTUs. Both indices can be used to characterize microorganism community richness, with a higher value indicating that more species were present in a given community (Wang *et al.* 2020). Based on the data in Table 1, at the moderate CD of 200 mA/m^2, the Sobs and Chao indexes were 520 and 573, respectively, both much higher than those of the no current (0 mA/m^2) and higher CD (400 mA/m^2). This suggests that the moderate current stimulation considerably increased the number of species within the microbial community in the reactors, whereas a higher current application caused certain

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Table 1 | Diversity index of the microbial community (-WP: with wheat-rice stone powder)

	Sobs	Chao	Shannon	Simpson	Coverage
0 mA/m^2	342	354	3.21	0.16	0.9981
200 mA/m^2	520	573	3.71	0.14	0.9988
400 mA/m^2	321	352	3.03	0.20	0.9987
200 mA/m^2-WP	652	701	4.02	0.12	0.9979
400 mA/m^2-WP	395	414	3.25	0.18	0.9984

species to die off. Furthermore, mineral additions are beneficial to improve microbial diversity. There was a substantial increase in the Sobs and Chao indices, reaching 652 and 701, respectively. However, the CD was more influential in shaping microbial communities than WP addition. The higher the microbial diversity, the higher the stability of the BER.

The Shannon and Simpson indexes reflect microbial community diversity, with a higher Shannon (Simpson) index indicating higher (lower) community diversity. At a CD of 200 mA/m^2, the Shannon index recorded was 3.71, which was higher than those calculated at both 0 mA/m^2 (3.21) and 500 mA/m^2 (3.03). After adding wheat-rice powder, the Shannon index further increased to 4.02 and 3.35 at 200 mA/m^2-WP and 400 mA/m^2-WP, respectively. The Simpson index was calculated to be 0.12 at 200 mA/m^2-WP, lower than the results calculated for the experimental conditions. The microbial community diversity of mixed systems is complex (Yang *et al.* 2020), explaining that systems have strong adaptability and high stability, which is conducive to denitrification.

These results support the hypothesis that a suitable CD and WP addition are together conducive to increasing the diversity of microorganisms in a reactor, facilitating enhanced nitrate removal in BER.

3.3. Analysis of microbial community structure

A multi-level species Sunburst diagram using concentric circles was generated to facilitate visualization of the correspondence between samples and species. Ratio structures were displayed at multiple levels, and relationships between different levels of data can be clearly seen. The distribution ratios of dominant species among different samples can also be seen in Figure 2. The upper part illustrates the composition of species abundance in the 0 mA/m^2, 200 mA/m^2, 400 mA/m^2, and 200 mA/m^2-WP treatments.

The four samples showed similar microbial community compositions, with different proportions of each phylum detected under the different experimental conditions. Members of the phylum Proteobacteria accounted for the highest proportion among the samples. In the 200 mA/m^2 and 200 mA/m^2-WP treatments, there was elevated microbial activity, faster growth and reproduction, and greater uniformity in the microbial community structure. Moreover, there was a reduction in the proportion of the dominant phylum compared with the other samples, which may be because the current stimulus led to a corresponding increase in the growth and activity of other taxa, resulting in a more affluent microbial community. Proteobacteria are generally divided into five classes: Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria, and Epsilonproteobacteria (Chung *et al.* 2006; Liu *et al.* 2018). Among these, many Betaproteobacteria genera are present in anaerobic environments and can digest organic nutrients, as well as hydrogen, ammonia, nitrogen, methane, and volatile fatty acids (Xia *et al.* 2010). Many common denitrifying bacteria are found in this class, including *Hydrogenophaga*, *Alcaligenes*, and *Dechloromonas* (Chakraborty & Picardal 2013; Liu *et al.* 2020). Several Gammaproteobacteria genera are chemoorganotrophs and facultative anaerobes, with common ammonia-oxidizing bacteria or nitrifying bacteria and various denitrifying bacteria found in this class. Since the phylum Proteobacteria contains many microorganisms associated with denitrifying taxa, it has been reported as the dominant reactor phylum in many previous studies of bio-electrochemical denitrification systems (Hao *et al.* 2013; Ailijiang *et al.* 2016; Chen *et al.* 2016).

Under the studied CD, Bacteroidetes and Chloroflexi were the other two dominant phyla, and were more strongly affected by current. Most Bacteroidetes were chemoorganotrophs, and their relative abundances increased with CD and were not inhibited by the higher CD (400 mA/m^2). In contrast, the relative abundance of the Chloroflexi was highest at the optimal CD of 200 mA/m^2, and decreased at 400 mA/m^2.

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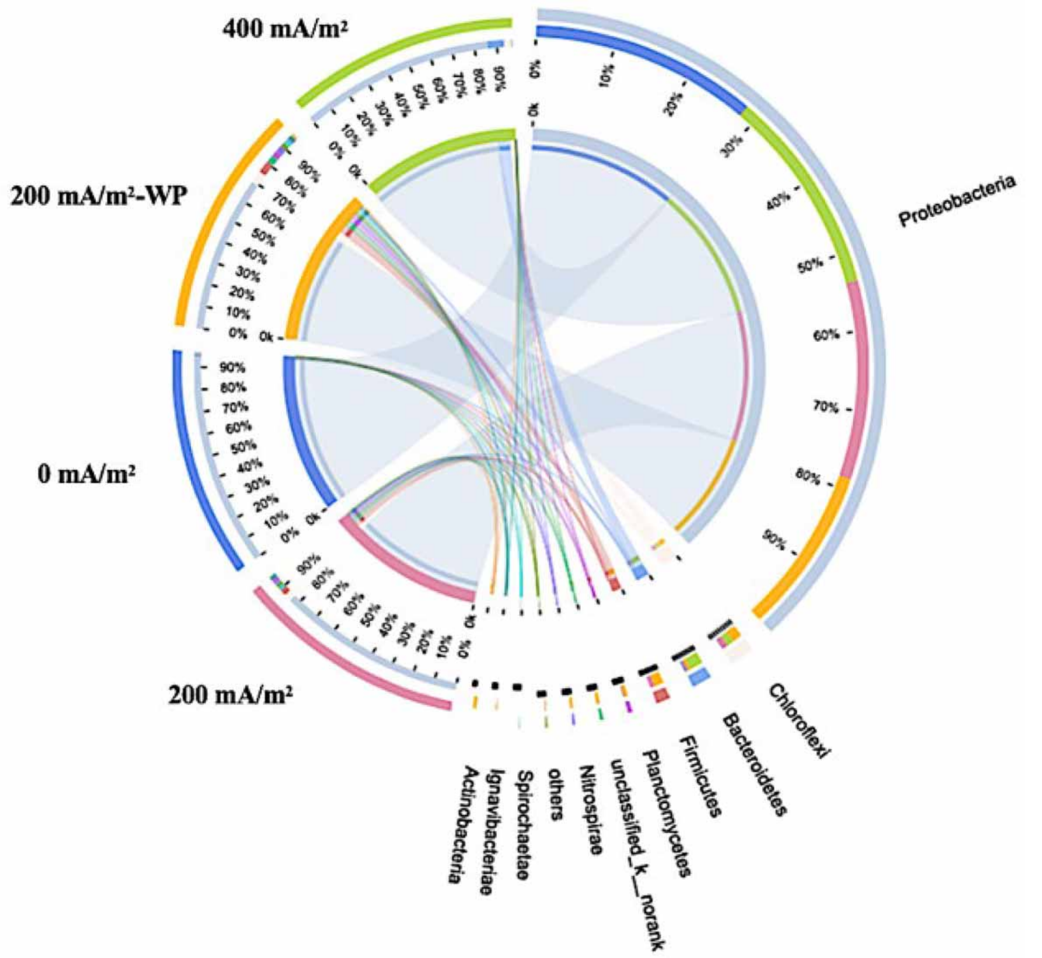

Figure 2 | Collinearity relationship of microbial samples under different current densities (phylum level) (-WP: with wheat-rice stone powder).

3.4. Analysis of the dominant microorganisms in the reactor

Genus-level microorganism heatmaps at the end of the 0 mA/m^2, 200 mA/m^2, 400 mA/m^2, 200 mA/m^2-WP, and 400 mA/m^2-WP experiments in the BER are presented in Figure 3. Heatmaps use color gradients to represent data in a two-dimensional matrix or table, making them very useful for visually comparing similarities in multiple sample communities at each taxonomic level. In these samples, we noted that the most dominant microbial taxa were *Methyloversatilis, Methylotenera*, and an unclassified genus of the family Methylophilaceae, which contains a variety of microbial genera, including *Methylophilus*, *Methylobacillus*, *Methylovorus*, and *Methylotenera*, that use methanol as an organic carbon source for denitrification (Doronina *et al.* 2013; Zhang *et al.* 2015). The different experimental CDs changed the proportions of each microorganism. For example, the higher CD strongly inhibited the *Methylophilaceae* while facilitating the growth of *Methyloversatilis*. Heterotrophic *Methyloversatilis* could reduce NO_3^--N to NO_2^--N. Members of this genus are the main microbial taxa in heterotrophic denitrification systems. They use methanol as their carbon source and can utilize monomethyl compounds as both carbon and energy sources (Baytshtok *et al.* 2009; Liu *et al.* 2016). *Methyloversatilis* species can adapt to a wide range of electric currents, and were found to maintain high community proportions in the current study, even under high CD conditions. Members of the genus *Methylotenera* are also common denitrifying bacteria, and play essential roles in the methanol denitrification metabolic pathway (Mustakhimov *et al.* 2013). We found that the proportion of dominant denitrifying bacteria increased under the optimal CD, thereby reducing nitrate and nitrite.

The microbial communities under the three CDs also included a genus of the class *Ardenticatenia*. In this genus, the *Ardenticatena maritima* 110S sequence is capable of encoding complete denitrification-related enzymes, such as Nar (NapA), Nir (NirK), nitrous oxide reductase (Nor), and nitric oxide reductase (NosZ) (Hemp *et al.* 2015). Other genera

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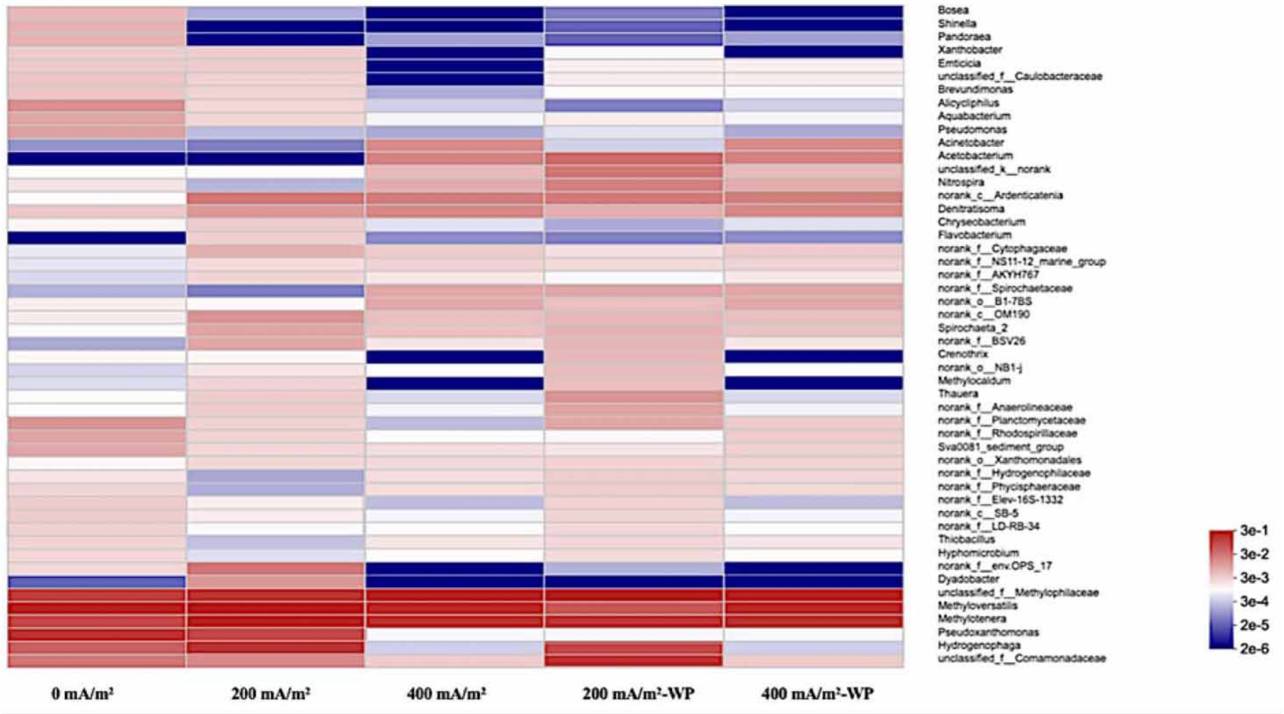

Figure 3 | Heatmap of a major genus under different current densities (-WP: with wheat-rice stone powder).

highly abundant at the 200 mA/m^2 and 200 mA/m^2-WP included *Acetobacterium*, *Denitratisoma*, and *Acinetobacter*, which were also common genera of denitrifying bacteria (Liu *et al.* 2016; Lyu *et al.* 2017). Moreover, various strains under the genus *Acinetobacter* can achieve aerobic and anaerobic denitrification at low temperatures. Low nitrite accumulation under all experimental conditions could be attributed to the high percentage of *Denitratisoma*, which contains/facilitates Nir to mediate the reduction of NO_2^--N. (Xu *et al.* 2018). Combined with reports in the literature, the effects of current on nitrate reduction and accumulation, as well as the microorganism activity, growth, and reproduction, indicated that a CD of 200 mA/m^2 and 200 mA/m^2-WP promoted the growth and metabolism of microorganisms, while giving rise to consistently high diversity levels in the microbial community structures. A few dominant species accounted for high community proportions at this optimized CD, and individual taxa growth rates were enhanced.

Moreover, *Hydrogenophaga* spp., from the Comamonadaceae family of the Betaproteobacteria, was capable of autotrophic growth using hydrogen (Xu *et al.* 2019). At higher CD, large amounts of hydrogen, which accumulated as the reactions progressed, were produced by the cathodic electrolysis of water in the bioelectrochemical reactors. The result also suggested that the inorganic carbon produced by the heterotrophic denitrification process provided a carbon source for the growth of hydrogen-oxidizing. These autotrophic denitrifying bacteria also facilitated the growth of *Hydrogenophaga* spp. in our reactors. At the genus level, the structures of the 200 mA/m^2 and 200 mA/m^2-WP microbial communities were similar. This indicated that at the moderate CD, the addition of WP did not strongly affect the microorganism community structure in the reactors, but only changed the proportions of specific genera. However, the community structure of the 400 mA/m^2 and 400 mA/m^2-WP treatment samples was significantly different from that in the 200 mA/m^2 experiments, owing to the former having a greater proportion of genera that were minimally present or even absent in the latter. Some genera were found in all the studied denitrification systems and seemed to be more tolerant to CD than the dominant genera in our samples, with higher currents appearing to facilitate their growth and reproduction. This finding can provide guidance for future bio-electrochemical denitrification system operations under high currents, as injection of an enriched culture of these current-tolerant bacteria into such a reactor would improve the current tolerance of its microbial community. Moreover, there is little correlation among *Methyloversatilis, Methylotenera*, and *Methylophilaceae*, and the proportion of one of them is not affected by the other two genera. *Denitratisomas* is another kind of important bacteria. The bacteria are negatively correlated with many bacteria, which may be explained by the substrate competition between them.

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In addition, the relatively uniform microbial structural composition under the 200 mA/m^2 and 200 mA/m^2-WP treatments indicated that an optimized current would increase microbial community uniformity, which in turn would improve the buffering capacity of the system in terms of its ability to withstand environmental stresses. The increase of denitrification microbial diversity and uniformity is helpful to maintain the stability of the reactor (Wang *et al.* 2021). This is an important point in the practical application of BER.

3.5. Catalytic activity of denitrifying enzymes

Microbial denitrification largely depends on the expression and activity of denitrifying enzymes such as nitrate reductase (Nar) and nitrite reductase (Nir), and is affected by many other factors. It is evident in Figure 4 that Nar was improved by 200 mA/m^2 with and without WP. The activity of Nar was reached 125 and 141% compared to the control group in the absence and presence of WP, respectively. Similarly, the activity of Nir increased from 118 to 135% compared to the control group with and without WP. These results indicated that both suitable electro-stimulation (200 mA/m^2) and the addition of trace elements improved the catalytic activities of denitrifying enzymes. Nar and Nir are key enzymes responsible for denitrification. These enzymes accept electrons to reduce nitrate and nitrite (Liu *et al.* 2020). Therefore, an appropriate level of current stimulation might positively affect denitrifying enzymes at the gene level, thereby improving the activity of denitrifying enzymes. In this case, the addition of WP in the reactor also increased the activity of Nar and Nir. The results of catalytic activity of denitrifying enzymes further confirmed the kinetic results. In short, WP can improve the catalytic activity of the Nar and Nir to raise the denitrification rates.

However, Nar decreased to 90 and 102% compared to the control at 400 mA/m^2 with WP or not, respectively. Nir also decreased to 87.1% and 104.3% compared to the control at 400 mA/m^2 with and without WP. These results suggested that the high CD could inhibit the inhibition in the catalytic activities of Nar and Nir, which finally induced a NO_3^--N and NO_2^--N reduction. Similar to the NO_3^--N reduction kinetics (Figure 1), high CD significantly reduced the catalytic activity of Nar and Nir in the 400 mA/m^2. These results suggested that 400 mA/m^2 strongly inhibited NO_3^--N and NO_2^--N reductase.

3.6. The resistance of denitrifying bacteria against high CD

Environmental stress refers to the disturbance caused by the environment to the living state of organisms or the environmental impact that has a restrictive effect on the development of the ecosystem (Liu *et al.* 2020). As far as the bioelectrochemical denitrification system, the CD in the reactor is one of the most important environmental stresses to

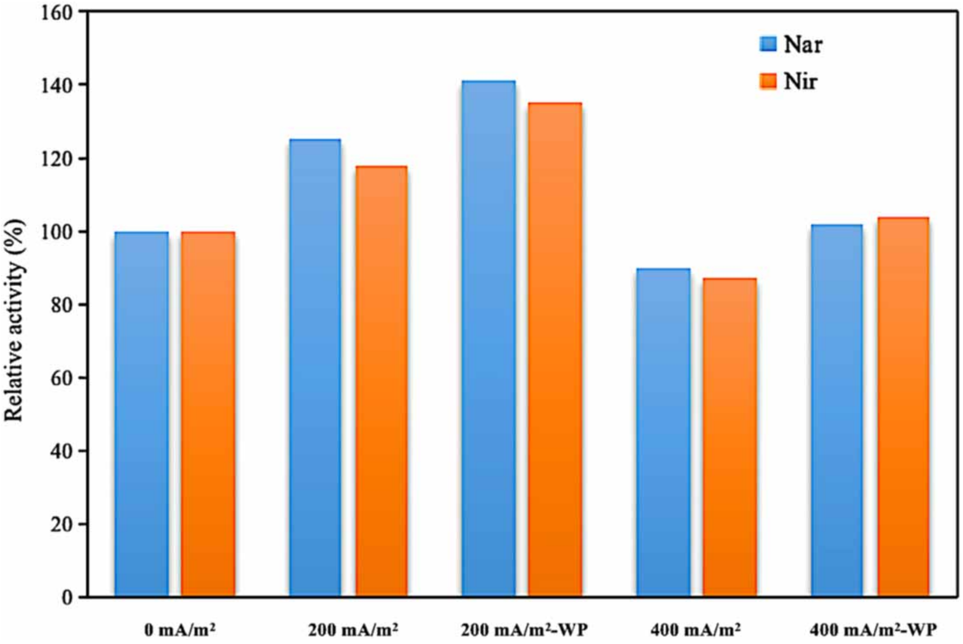

Figure 4 | The relative activities of Nar and Nir at the end of each experiment under different current densities (-WP: with wheat-rice stone powder, control: 0 mA/m^2 without trace elements).

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microorganisms. Hence, it is important to investigate the change of microbial resistance to high CD (400 mA/m^2) in the absence and presence of WP. To explore the microbial resistance ability, the nitrate-reducing reaction constant was induced as a response variable and the optimal CD (200 mA/m^2) without the addition of WP system as the undisturbed control (C_0). The resistance of microorganisms under different conditions was then calculated according to Equation (1). A significant disturbance was found under the larger CD, as the resistance decreased from 1 to 0.79 at 400 mA/m^2. In contrast, the resistance value of 400 mA/m^2-WP increased to 0.91. The addition of WP enhanced the resistance of denitrifying bacteria to the high current because it contained various metal elements necessary for microorganism growth. As a result, it synergistically promoted microorganism growth and produced an antagonistic effect to counteract the influence of high currents. This shows that the internal environment of the reactor balanced by minerals might improve microbial resistance to harmful substances, reduce system disturbance and improve the stability of the reactor system (Liu *et al.* 2020).

4. CONCLUSION

In this study, nitrate and nitrite concentration changes were determined to quantify denitrification performance. High-throughput sequencing was conducted to analyze the diversity, similarity, difference, and structure of microbial community at different CD with WP and investigate the dominant strains present in different samples. Both the appropriate electrical stimulation (200 mA/m^2) and WP were able to reduce the NO_3^--N and NO_2^--N, inhibit the NO_2^--N accumulation, and elevate the stress-resistant ability of the denitrifiers. Moreover, the most dominant microbial taxa were *Methyloversatilis*, *Methylotenera*, and an unclassified genus of the family Methylophilaceae in our reactor. After WP addition, the microbial diversity and catalytic activity of denitrifying enzymes increased, so did the resistance of denitrifying bacteria at high CD. These findings are expected to contribute to improving the efficiency of bio-electrochemical reactors used in the removal of nitrate pollution.

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CONFLICT OF INTEREST STATEMENT

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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