Water Science & Technology

Water Science & Technology Vol 88 No 8, 2019 doi: 10.2166/wst.2023.334

Nitrogen removal performance of aerobic denitrifying bacteria enhanced by an iron-anode pulsed electric field

Fan Wang[a,b,†], Zhang Bo[a,†], Xiaonan Dong[c,*], Xingxing Zhou[d] and Xiaomin Hu[a]

[a] Institute of Resources and Civil Engineering, Northeastern University, Shenyang 110014, China
[b] Liaoning HaiTianGe Environmental Protection Technology CO., LTD, Shenfu Reform and Innovation Demonstration Zone, Liaoning, 113122, China
[c] Liaoning Municipal Engineering Design h&Research Institute CO., LTD, Shenyang 110006, China
[d] College of Architecture and Environment, Ningxia Institute of Science and Technology, Shizuishan 753000, China
*Corresponding author. E-mail: 2010420@stu.neu.edu.cn
[†]These authors contributed equally to this study.

ABSTRACT

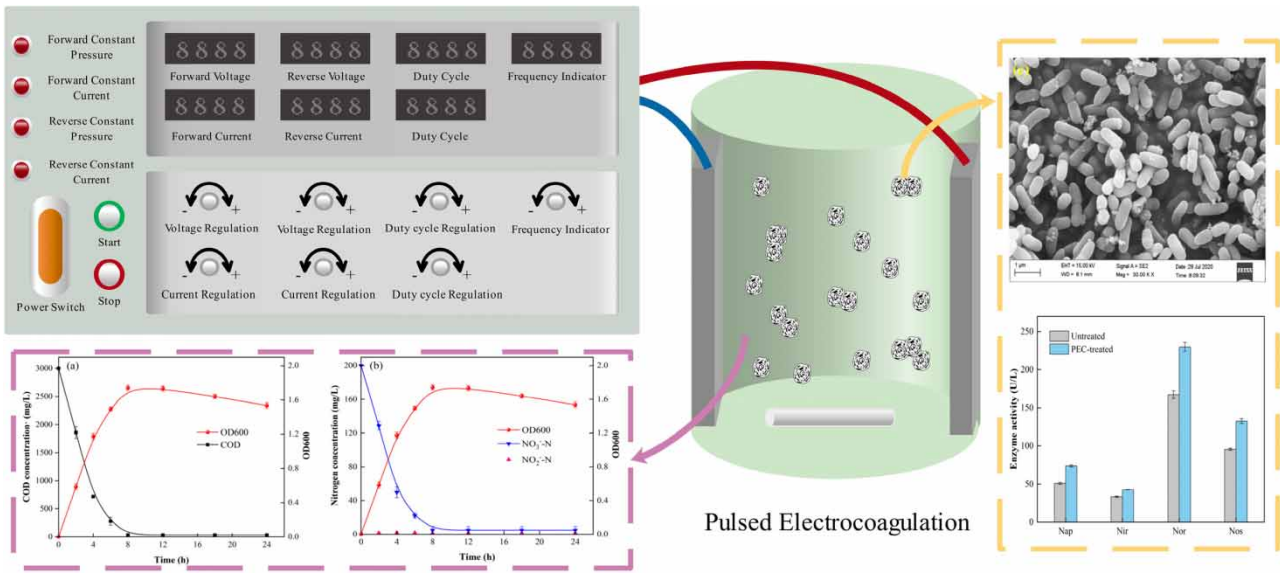
Pulsed electric field (PEF) technology has attracted considerable attention because it can efficiently treat pollutants that are difficult to degrade. In this study, a PEF system using iron as the electrode was constructed to investigate the effect of PEF–Fe on the growth and metabolism of aerobic denitrifying bacteria and the effectiveness of wastewater nitrogen removal. The chemical oxygen demand, NO_3^-–N and nitrate removal rates were 98.93%, 97.60% and 24.40 mg·L^{-1}·h^{-1}, respectively, under optimal conditions. As confirmed in this study, PEF–Fe could improve the key enzyme activities of W207-14. Scanning electron microscopy revealed that the surface of PEF–Fe-treated W207-14 was intact and smooth without any irreversible deformation. Flow cytometry combined with fluorescence staining analysis also confirmed reversible electroporation on the cell membrane surface of PEF–Fe-treated W207-14. Differentially expressed gene enrichment analysis showed that PEF–Fe activated the transmembrane transport function of ATP-binding cassette transporte (ABC) transport proteins and enhanced the cell membrane permeability of aerobic denitrifying bacteria. The significant differential expression of iron–sulphur cluster proteins facilitated the regulation of electron transport and maintenance of the dynamic balance of iron ions within the PEF–Fe system.

Key words: aerobic denitrifying bacteria, cell membrane permeability, high-throughput sequencing, pulsed electric field

HIGHLIGHTS

- A pulsed electric field efficiently removes nitrogen from wastewater.
- Pulsed electric fields can cause reversible electroporation on the surface of microbial cell membranes.
- A pulsed electric field enhances the cell membrane permeability of bacteria.
- A pulsed electric field shortens the growth cycle of aerobic denitrifying bacteria.

GRAPHICAL ABSTRACT



1. INTRODUCTION

With the accelerated modernisation and rapid growth of the urban population, more nitrogenous compounds are being discharged into water bodies, causing various problems (Wang *et al.* 2022; Li *et al.* 2023; Xia *et al.* 2023). For example, excessive nitrates entering water bodies can cause eutrophication, acidification of water bodies, algal blooms, serious damage to local ecosystems and a sharp decrease in available water resources (Yan *et al.* 2022a, 2022b). Upon entering the body, nitrate converts to nitrite, which, in turn, converts the iron centre of haemoglobin from Fe^{2+} to Fe^{3+}, yielding methaemoglobin, which is unable to bind and transport oxygen, ultimately causing methemoglobinemia (Brunato *et al.* 2003). Therefore, nitrogen pollution in water bodies is an important environmental issue that must be addressed urgently.

Currently, biological treatment is widely used for wastewater treatment because of its cost-effectiveness, high efficiency and low pollutant production (Zhang *et al.* 2020). Aerobic denitrification has been proposed as a novel technology for nitrate removal. The discovery of aerobic denitrifying bacteria disproved the conventional theory that denitrification can only occur under anaerobic or anoxic conditions (Ji *et al.* 2015). Aerobic denitrifying bacteria can use both oxygen and nitrate as electron acceptors for denitrification under aerobic conditions, facilitating the completion of the nitrification and denitrification processes in a single reactor, effectively reducing the treatment costs and operational complexity. Therefore, aerobic denitrification is widely used in various fields, such as wastewater treatment systems for domestic, industrial and agricultural wastewater. This technology is also widely used for the remediation of eutrophic water bodies, such as rivers and lakes, as well as for nitrate removal from groundwater (Yao *et al.* 2020).

To date, aerobic denitrification technology has been usually applied by directly injecting a certain amount of aerobic denitrifying bacteria into wastewater or activated sludge (Zhao *et al.* 2022). However, in the actual treatment process, the growth of aerobic denitrifying bacteria is restricted and their reproduction capacity decreases due to the constraints of growth conditions and competition among microbial populations, often rendering them incapable of becoming the dominant population (Bian *et al.* 2022). Meanwhile, in the treatment of high-nitrate-concentration wastewater, the denitrification process is inhibited to a certain extent with an increase in nitrite accumulation, which directly increases the growth time of aerobic denitrifying bacteria and decreases nitrate removal capacity; thus, meeting the long-term demand for high-nitrate-concentration wastewater treatment is difficult (Lan *et al.* 2022). Therefore, developing an efficient aerobic denitrification enhancement technology to fully use its advantages and potential is essential.

In this study, a PEF treatment system with iron as the electrode was developed to investigate the effect of PEF on the growth and metabolism of aerobic denitrifying bacteria. The objectives of this study were to (1) determine the optimal process parameters for stable operation of PEF–Fe treatment, (2) investigate the effect of PEF on the growth and metabolism of the aerobic denitrifying bacteria *Pseudomonas putida* W207-14 (W207-14) and (3) probe the cell membrane permeability of

W207-14 via flow cytometry (FCM) combined with fluorescence staining analysis. The results of this study may provide new strategies for improving the denitrification capacity of PEF in wastewater treatment.

2. MATERIALS AND METHODS

2.1. Pulsed electric field apparatus

The experimental setup for PEF is shown in Figure 1. The pulsed electric field (PEF) treatment chamber was made of plexiglass with an effective volume of 200 mL, and a pair of test electrodes was placed inside the treatment chamber. The W207-14 in the logarithmic growth phase after activation were added to the treatment chamber at a certain inoculum, and a pulsed power supply (Soyi, China) providing square-wave pulses was used for studying the PEF treatment. During the experiment, the test device was placed in a constant-temperature shaker to ensure the appropriate dissolved oxygen concentration and treatment temperature. Moreover, the pH (Raycom PHS-3E, Shanghai Yidian Scientific Instruments Co.) and conductivity (Raycom DDSJ-308F, Shanghai Yidian Scientific Instruments Co.) of the solution in the system were monitored online in real time.

2.2. Experimental wastewater and methods

The aerobic denitrifying bacteria were isolated through laboratory screening and later identified as W207-14. The C/N ratio, initial pH and temperature of the artificially simulated wastewater (The artificially simulated wastewater contained (per litre): sodium succinate 7.5 g, KNO_3 2.0 g, K_2HPO_4 1.0 g, KH_2PO_4 1.0 g, $MgSO_4{\cdot}7H_2O$ 0.2 g, 2.0 mL trace elements solution. All mediums were adjusted to pH 7.20 $\pm$ 0.01 and autoclaved for sterility at 121 °C for 20 min) were 15, 7.2 and 30 °C, respectively (suitable environment for W207-14 growth). All chemical reagents were of analytical grade. To investigate the effect of PEF on the growth and metabolism of W207-14, experiments were conducted at an initial pH of 7.2, temperature of 30 °C, rotational speed of 160 $r{\cdot}min^{-1}$, different current intensities (0.01, 0.02, 0.03, 0.04 and 0.05 A), different pulse frequencies (100, 500, 1,000, 1,500 and 2,000 Hz), different electrode distances (2, 3, 4, 5 and 6 cm) and W207-14 inoculum of 5% using different iron ion dosing methods (S1 for W207-14, S2 for W207-14 + certain amount of iron ions, S3 for PEF and S4 for W207-14 + PEF). Moreover, optical density at 600-nm wavelength (OD 600) as well as chemical oxygen demand (COD) and NO_3^-–N removal rates, as the evaluation indexes, were used for these experiments. Subsequent experiments were conducted under optimal conditions.

2.3. Analytical methods

The COD was determined via the hydrazine sulphate reduction using an ultraviolet–visible spectrophotometer (DR3900, Hashish Water Analysis Instruments Co., Ltd). Furthermore, NO_3^-–N was determined by the rapid extinction spectrophotometric method using an ultraviolet–visible spectrophotometer (Uvmini-1240, Shimadzu Corporate Management Co., Ltd).

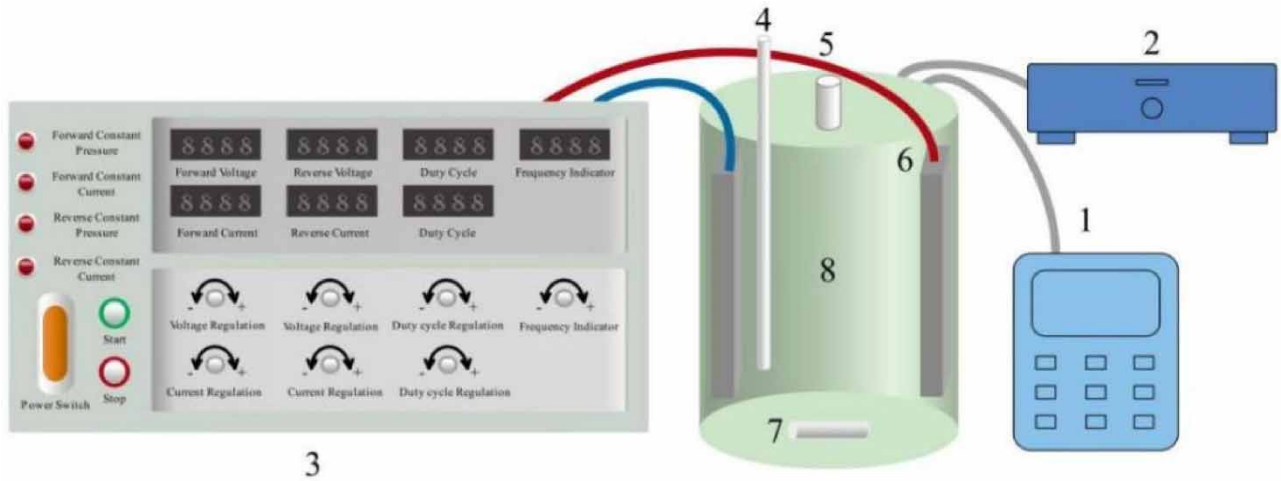


Figure 1 | Schematic of the PEF–Fe treatment system: (1) conductivity meter; (2) pH meter; (3) pulsed power supply; (4) thermometer; (5) inoculation port; (6) electrode; (7) magnetic stirrer; (8) solution to be treated.

The sample morphology was observed using an ULTRA PLUS field-emission scanning electron microscope (SEM). FCM (FACSJazz) was conducted to observe the staining of the samples. Nitrate reductase, nitrite reductase, nitric oxide reductase and nitrous oxide reductase were measured using ELISA Kits 48T. Moreover, Shanghai Bioengineering Co. was commissioned to conduct transcriptome sequencing of W207-14.

2.4. Statistical analysis

Statistical and preliminary analyses were performed using Microsoft Excel 2013. Descriptive statistical analyses were performed using IBM SPSS Statistics 22.0. Figures were created with Origin Pro 9.1 (32-bit). Three copies of each sample were taken for water quality analysis, with at least two biological copies for transcriptome analysis.

3. RESULTS AND DISCUSSION

3.1. Effect of PEF on the aerobic denitrifying bacteria

3.1.1. Current intensity

The experimental results (Figure 2(a)) showed that the current intensity affected the growth and metabolism of W207-14. During the increase in current intensity from 0.01 to 0.04 A, the dissolution rate of the iron anode accelerated, more iron ions were released into the solution for the aerobic denitrifying bacteria to use and the denitrification efficiency gradually increased. At a current intensity of 0.04 A, the OD 600 value and the COD and NO_3^-–N removal rates of the aerobic denitrifying bacteria reached maximum values of 1.772, 98.93% and 97.23%, respectively. As confirmed by previous studies, there exists a critical value of current intensity, beyond which the system has a sufficient amount of metal ions for microbial growth and pollutant removal; therefore, continuously increasing the current intensity will not improve the removal efficiency. Similar conclusions were obtained in this study: When the current intensity was increased to 0.05 A, the growth and metabolism

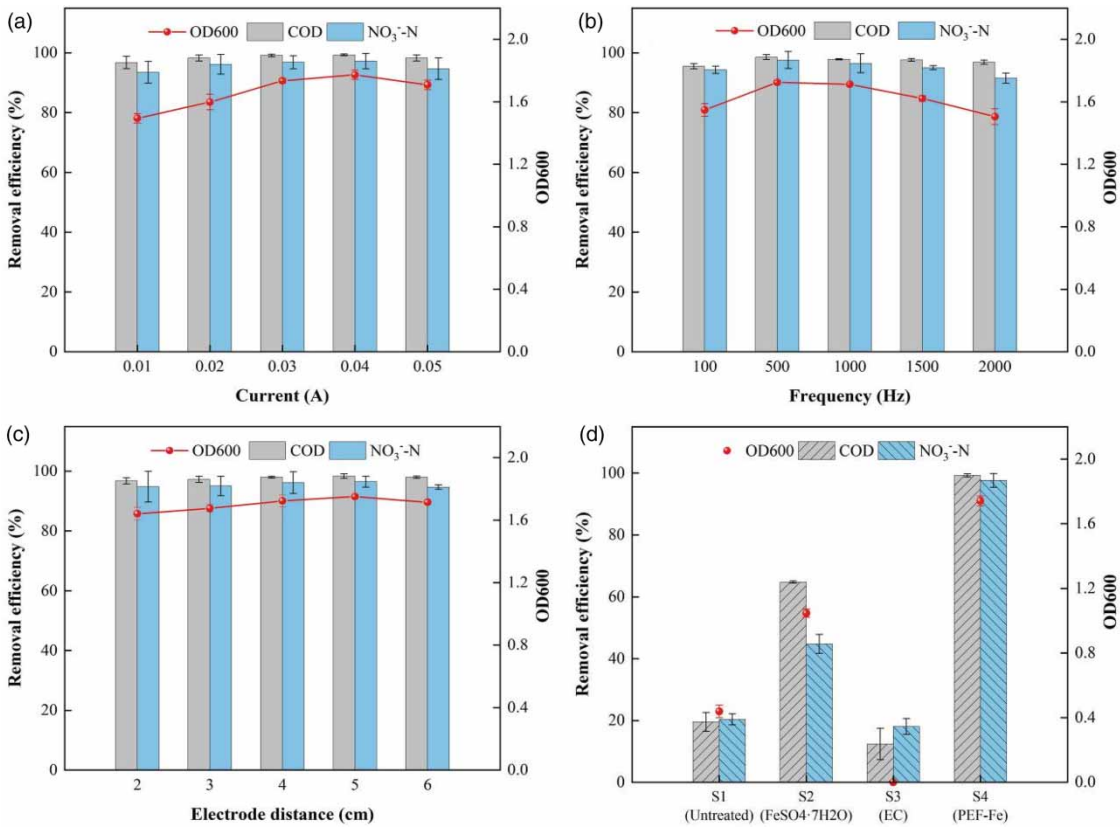


Figure 2 | Effects of (a) current intensity, (b) pulse frequency, (c) electrode distance and (d) iron ions on the growth and metabolism of strain W207-14 treated via PEF.

of W207-14 were inhibited and their OD 600 value, as well as COD and NO_3^-–N removal rates decreased. Therefore, considering the treatment cost as well as the energy and pole plate consumption, selecting a current intensity of 0.03 A for subsequent experiments was appropriate.

3.1.2. Pulse frequency

Under the low-frequency condition, the growth and metabolism of strain W207-14 were not inhibited and were at a high level, and its OD 600 value, COD and NO_3^-–N removal reached the maximum values of 1.724, 98.59% and 97.60%, respectively, at a pulse frequency of 500 Hz. Subsequently, the growth and metabolism of strain W207-14 were inhibited with the increase of pulse frequency to 2,000 Hz, and its OD 600 value, COD and NO_3^-–N removal rates showed a gradual decrease. As a large number of iron ions were released into the solution under the action of PEF–Fe, the phenomenon of agglomeration of some aerobic denitrifying bacteria was observed in the SEM, and at the same time, a small amount of flocs attached to the surface of some of the cells, which together formed a larger volume of 'equivalent cells'. It has been confirmed that larger cells tend to be more tolerant to PEF, and under the condition that the intensity of PEF remains unchanged, a lower pulse frequency is often required to facilitate the accumulation of charge on the cell membrane, thus causing changes in the permeability of the cell membrane (Buchmann & Mathys 2019). Therefore, aerobic denitrifying bacteria subjected to PEF–Fe can withstand lower pulse frequency treatment without causing bacterial inactivation. With the increase of pulse frequency, it is unfavourable to the accumulation of charge on the cell membrane, which may lead to the reduction of the number of reversible electroporation holes on the cell membrane, thus affecting the nutrient uptake by the cells, so the growth and metabolism of strain W207-14 were inhibited (Gui *et al.* 2017). In summary, for aerobic denitrifying bacteria *Pseudomonas putida* W207-14, it is more appropriate to select a pulse frequency of 500 Hz for PEF–Fe treatment.

3.1.3. Electrode distance

Compared with the aforementioned parameters, the electrode distance affected the growth and metabolism of W207-14 to a lesser extent (Figure 2(c)). The OD 600 value as well as the COD and NO_3^-–N removal rates of W207-14 increased and then decreased with increasing electrode distance. When the electrode distance was 5 cm, the OD 600 value and the COD and NO_3^-–N removal rates of W207-14 reached maximum values of 1.750, 98.35% and 96.51%, respectively. When the electrode distance was small, the number of W207-14 directly affected by PEF was small and their growth and metabolism were slow. With the increase in electrode distance, the electrostatic effect between the pole plates weakened and more iron ions were directly used by W207-14, which promoted their growth and metabolism. Subsequently, the growth and metabolism of W207-14 were inhibited when the electrode distance continued to increase to 6 cm. This may be because the mass transfer time of ions increases with an increase in electrode distance when the electrode distance exceeds the optimal distance, which hinders the PEF process (Daccache *et al.* 2020). In summary, an electrode distance of 5 cm was selected for subsequent experiments.

3.1.4. Iron ion dosing mode

The OD 600 values and pollutant removal rates in the S4 treatment were higher than those in the other three treatments (Figure 2(d)). Compared with the S1 treatment, the S2 and S4 treatments provided better absorption and utilisation of iron ions by W207-14, while the S4 treatment enhanced the mass transfer efficiency in the system due to the stimulating effect of the applied electric field and stimulated the activity of W207-14, which could also be reflected through the significant differences in OD 600 values and pollutant removal rates. In the PEF treatment system, nitrate removal mainly occurred via electroflocculation and biological denitrification. In the S3 treatment, pollutant removal in the system was low for a short period of time, with COD and NO_3^-–N removal rates of only 12.37 and 18.03%, respectively, which may be due to the low current intensity and the difficulty for the electrode to directly denitrify H^+ generated from the cathode as an electron donor in the absence of microbial catalysis (Daccache *et al.* 2020); therefore, degradation of pollutants via electroflocculation was considerably limited. The coupling of electroflocculation and biological denitrification in the S4 treatment afforded a significant increase in pollutant removal efficiency, with COD and NO_3^-–N removal rates of 98.93 and 97.60%, respectively. Compared with the OD 600 value of 0.439 in the S1 treatment, the OD 600 value in the S4 treatment was 1.741 for the same period of time, which confirmed that the removal of nitrate in PEF was mainly microbial, i.e. the iron ions precipitated by the iron electrode under the influence of the PEF were mainly absorbed by W207-14 for their growth and metabolism.

Aerobic denitrifying bacteria have heterotrophic nitrification, enabling nitrification and denitrification processes to be completed in one reactor at the same time, and ultimately realising the simultaneous removal of ammonia nitrogen and nitrate

nitrogen (Bao *et al.* 2023). In the actual wastewater treatment process, the nitrification reaction product can directly act on denitrification to improve the system nitrogen removal efficiency, while simplifying part of the nitrogen removal process on the demand for different biological reactors, reducing sludge production and saving costs. In addition, the alkalinity generated in the denitrification process can partially compensate for the alkalinity consumption in the nitrification process, saving energy consumption. Therefore, aerobic denitrification technology has great economic potential and practical application value.

3.2. Growth and metabolism of the aerobic denitrifying bacteria

Figure 3 shows that the growth retardation period of W207-14 under the influence of PEF was considerably short, and these bacteria (i.e. W207-14) rapidly entered the logarithmic growth period, which can significantly reduce operating costs during the actual wastewater treatment process. Moreover, the OD 600 value rapidly increased to 1.494, COD concentration decreased from 3,000 to 278.00 mg·L^{-1}, NO_3^-–N concentration decreased from 200 to 22.22 mg·L^{-1} and corresponding COD and NO_3^-–N removal rates decreased. After 6 h, the growth and metabolism of W207-14 slowed down and entered the growth stabilisation period; additionally, the OD600 value reached a maximum value of 1.741 at 8 h and the COD and NO_3^-–N concentrations decreased to 32.09 and 4.81 mg·L^{-1}, respectively, corresponding to the final COD and NO_3^-–N removal rates of 98.93 and 97.60%, respectively. The nitrate removal rate was 24.40 mg·L^{-1}·h^{-1}. Moreover, during the growth of W207-14 under the influence of PEF, the concentrations of NO_3^-–N were <2 mg·L^{-1} and no significant nitrite accumulation occurred.

3.3. Key enzyme activities of the aerobic denitrifying bacteria

The effect of PEF on the nitrate reductase (Nap), nitrite reductase (Nir), nitric oxide reductase (Nor) and nitrous oxide reductase (Nos) activities of W207-14 was investigated; the results are shown in Table S1. Without PEF treatment, W207-14 exhibited Nap, Nir, Nor and Nos enzyme activities of 50.90, 33.41, 167.17 and 95.52 U·L^{-1}, respectively; after PEF treatment, W207-14 exhibited Nap, Nir, Nor and Nos enzyme activities of 73.81, 42.62, 229.68 and 132.71 U·L^{-1}, respectively. The Nap, Nir, Nor and Nos enzyme activities of W207-14 after PEF treatment increased by 45.01, 27.57, 37.39 and 38.93%, respectively. As confirmed in this study, PEF could improve the key enzyme activities of W207-14. Enzymes play a vital role in the life activities of living organisms, and they must be involved in the absorption of nutrients from the environment by microorganisms as well as their utilisation in the body. In the process of biological treatment, the decomposition and transformation of pollutants in wastewater by microorganisms are catalysed by enzymes in a series of complex biochemical reactions (Veneziani *et al.* 2019). Some studies have confirmed that iron ions are an important component of the aerobic respiratory electron transport chain (Simon & Klotz 2013). Additionally, iron ions are common inorganic cationic catalysts for enhancing enzyme activity and are often used as coenzyme catalysts. Furthermore, the denitrification process involves several proteins that require metal ions as cofactors, among which iron ions are required for the cytochrome subunits of nitrate reductase and nitrite reductase. The results of this experiment also confirmed that the PEF action had a significant effect on the key enzyme activity of strain W207-14, which explains, from an enzymatic point of view, that the increase in

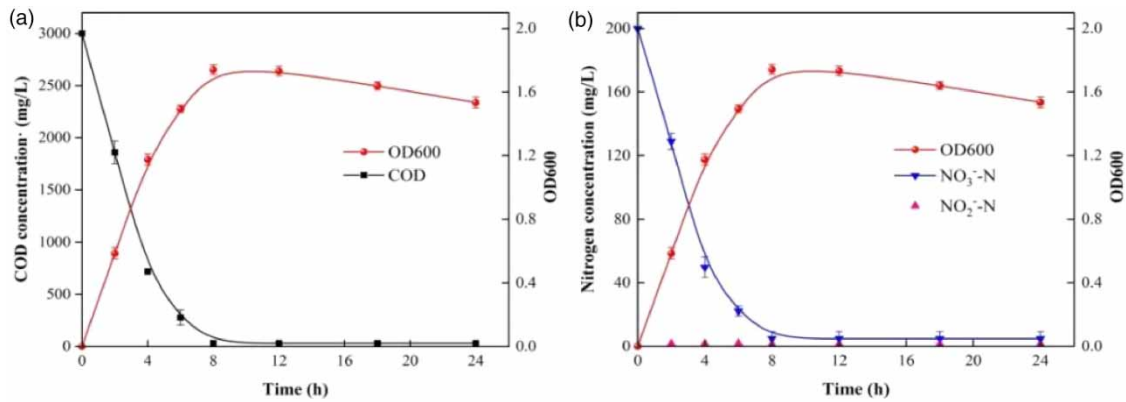


Figure 3 | Growth and metabolism of strain W207-14 treated with PEF: (a) carbon metabolism; (b) nitrogen metabolism.

the activity of the denitrification key enzyme of strain W207-14 after treatment with PEF–C is one of the key reasons for the significant increase in the rate of nitrate removal.

3.4. Morphological characteristics of the aerobic denitrifying bacteria

The effect of PEF on the morphology of W207-14 cells was explored using an SEM (Figure 4). The SEM results showed that the shape of PEF-treated W207-14 was unchanged and rodlike. At the same SEM magnification, the length of the untreated W207-14 bacteriophage was 1.5–2 μm, and its width was ~0.35 μm. The width of the PEF-treated W207-14 bacteriophage did not change considerably, but its length decreased to approximately 0.5–0.75 μm. We speculate that this may be due to the shortening of the life cycle of W207-14 by PEF through the process of inducing cell division, causing a reduction in the critical size of cell division (El Zakhem *et al.* 2006). The treatment time of 8 h was accompanied by further shortening of the cell growth time, and the proliferation rate of the W207-14 cells was significantly accelerated, causing them to divide into two without growing to their original size. From the growth curves, it can be seen that the growth phase of the PEF-treated strain W207-14 was changed, and the shortening of the retardation period caused more cells to enter the logarithmic growth phase earlier, leading to an increase in the number of cells entering the division process, which contributed to the acceleration of the proliferation rate of cells, which caused a decrease in the critical size of cell division. This has obvious advantages in the process of wastewater treatment, because in the traditional wastewater treatment process, aerobic denitrifying bacteria are easy to lose, have poor competitiveness, and difficult to form advantageous flora (Yan *et al.* 2022a, 2022b). This is consistent with the findings that PEF-treated strain W207-14 had higher OD600 values at the same growth time and the growth time required to reach maximum OD600 was significantly shorter. Strain W207-14 is a Gram-negative bacterium with a thin cell wall, which is less tolerant to PEF, but because it divides into smaller cells under the action of PEF, it can still show strong tolerance to the action of PEF of longer duration without easily inducing cell inactivation due to irreversible electroporation in the cell membrane. However, this phenomenon did not affect the aerobic denitrification efficiency of the individual bacteria but rather enhanced the nitrate removal capacity of the individual bacteria under the influence of PEF. At the same time, due to a large amount of iron ions released into the solution via PEF, some W207-14 cells were observed to be agglomerated (Figure 4), whereas a small amount of flocs were attached to the surface of some cells, forming a larger 'equivalent cell', which was more tolerant towards Pulsed electric coagulation, (PEC) and therefore less likely to cause irreversible electroporation on the cell membrane (Guo *et al.* 2018). Therefore, it is less likely to cause cell inactivation due to irreversible electroporation on the cell membrane. Additionally, the surface of PEF-treated W207-14 was observed to be intact and smooth without any irreversible deformation.

3.5. Cell membrane permeability of the aerobic denitrifying bacteria

The effect of PEF on the cell membrane permeability of W207-14 was explored via FCM combined with Propidium Iodide, (PI) and Carboxyfluorescein diacetate, succinimidyl ester, (CFDA SE) fluorescence staining analysis (Figure 5).

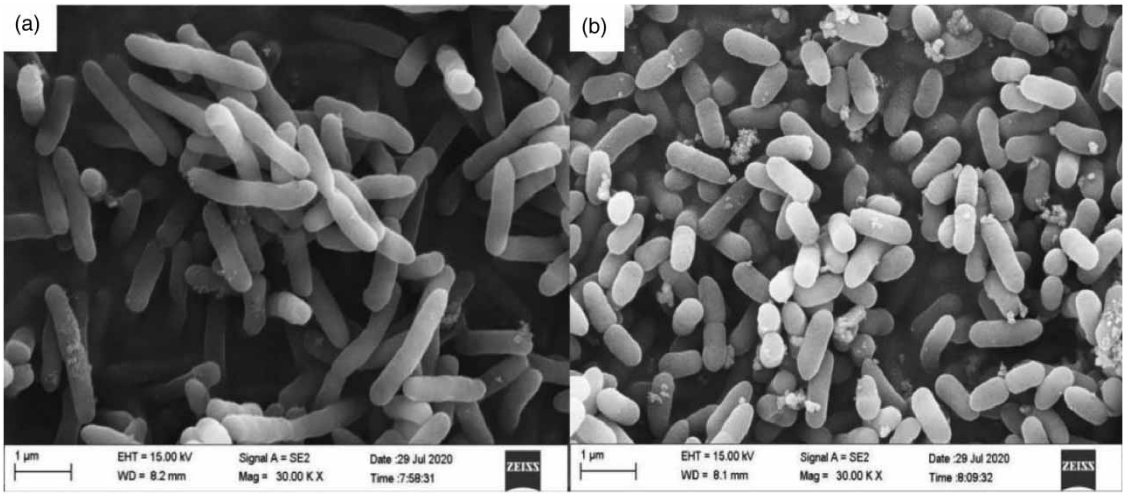


Figure 4 | Effect of PEF on the morphology of strain W207-14: (a) untreated (30,000×) and (b) PEC-treated (30,000×).

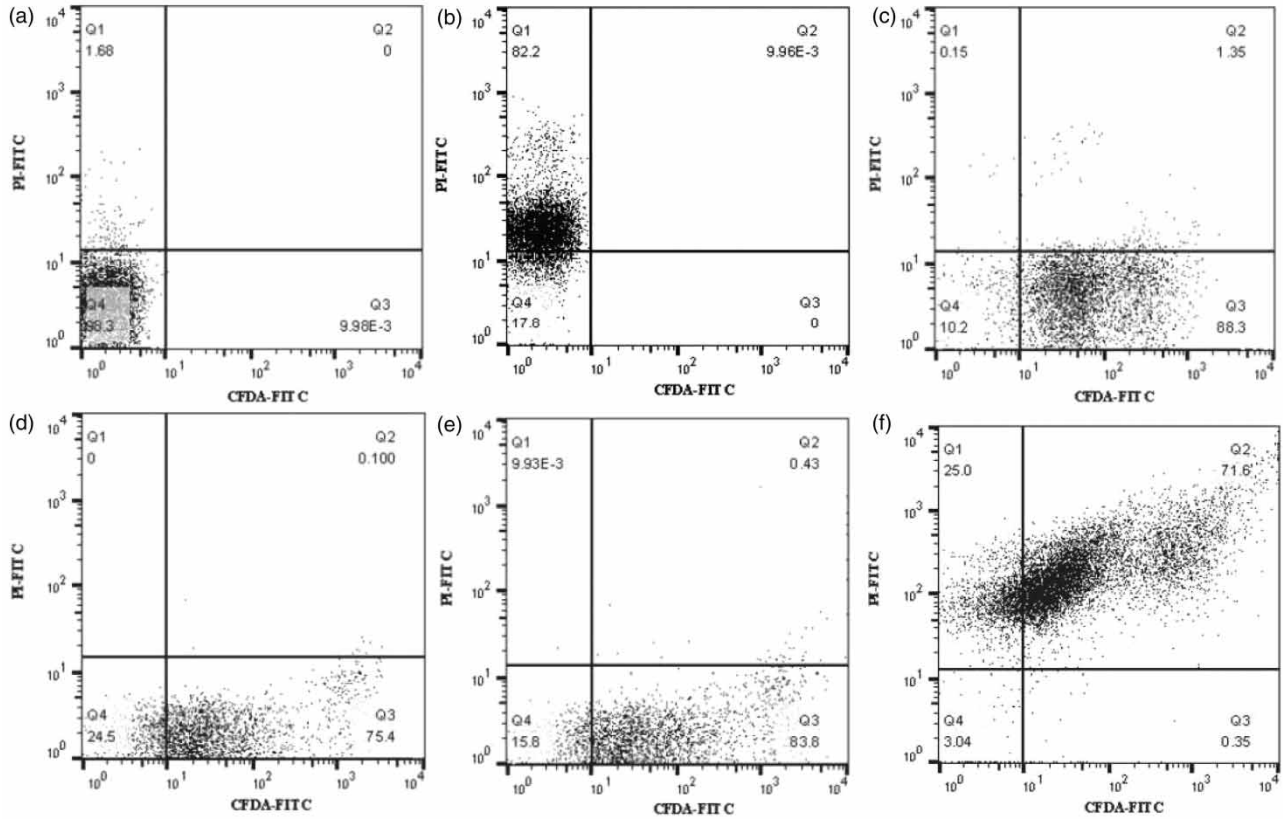


Figure 5 | Flow cytometry (FCM) dot plots of strain W207-14 cells after staining: (a) PI staining of untreated W207-14; (b) PI staining of pulsed electrocoagulation (PEF)-treated W207-14; (c) CFDA SE staining of untreated W207-14; (d) CFDA SE staining of PEF-treated W207-14; (e) PI–CFDA SE double staining of untreated W207-14; (f) PI–CFDA SE double staining of PEF-treated W207-14.

PI is a membrane-impermeable dye that cannot enter cells with intact membranes; however, when cell membranes are damaged or perforated, PI can enter cells and bind to nucleic acids showing red fluorescence (Davey & Hexley 2011) (Figure 5(a) and 5(b)). Almost all cells without PEC treatment before PI staining were located in the Q4 quadrant and the percentage was ~98.3%, indicating that W207-14 without PEF treatment had intact cell membranes and did not exhibit cell membrane breakage or perforation. The majority of the cells after PEF treatment moved to the Q1 quadrant and the proportion of cells in the Q1 quadrant increased to 82.2%, with 17.8% of cells still located in the Q4 quadrant, indicating that the cell membrane of W207-14 after PEF treatment underwent electroporation, which led to PI entering through the damaged cell membrane and binding to nucleic acids. The proportion of cells showing red fluorescence via PI staining significantly increased; thus, most cells moved from the Q4 quadrant to the Q1 quadrant. Therefore, PEF induced the change in the cell membrane permeability of W207-14.

Because the survival status of cells that can be stained by PI is still controversial, this experiment introduced another staining reagent, CFDA SE, to confirm the physiological status of the cells (Zhou *et al.* 2020). The hydrolysis product Crystal Field Splitting Energy, (CFSE) accumulates and appears fluorescent only in cells with esterase activity, whereas dead cells are not stained (Zhao *et al.* 2011) (Figure 5(c) and 5(d)). The majority of the cells without PEF treatment before CFDA SE staining were located in the Q3 quadrant, with a proportion of ~88.3%, indicating that most W207-14 cells had high esterase activity and could be identified as viable cells and only a small proportion of W207-14 was unstained or lost esterase activity. After PEF treatment, 75.4% of the cells were still located in the Q3 quadrant, which was slightly lower than the percentage of the untreated cells. The results indicate that PEF did not cause widespread inactivation of W207-14 and most W207-14 cells were able to survive and maintain high physiological activity in the presence of PEF.

Because the staining information obtained via PI or CFDA SE single staining is relatively single, FCM combined with PI–CFDA SE double staining can more visually observe the changes in cell membrane permeability and esterase activity

of the aerobic denitrifying bacteria treated with PEF at the same time (Figure 5(e) and 5(f)). The majority of the cells without PEF treatment before PI–CFDA SE double staining were located in the Q3 quadrant, with a proportion of ~83.8%, indicating that most W207-14 cells without PEF treatment had high esterase activity while the cell membrane was intact. The majority of the cells treated with PEF moved towards the Q2 quadrant and the proportion of cells in the Q2 quadrant increased to 71.6%, with another 25% moving to the Q1 quadrant, indicating that the PEF-induced cell membrane damage did not cause widespread inactivation of W207-14. Moreover, this result indicated that most of the W207-14 cells maintained high physiological activity despite changes in cell membrane permeability and PEF was similarly able to induce reversible electroporation on the cell membrane. We hypothesised that the damage to the cell membrane caused via PEF-induced reversible electroporation is recoverable sublethal damage. The mechanism of microbial inactivation via PEF is based on the leakage of components caused via irreversible electroporation on the cell membrane. Moreover, during recovery of reversible electroporation at low electric field intensity, the improvement in cell membrane permeability promoted the uptake of nutrients in the medium by microorganisms (Johnson *et al.* 2010).

3.6. Transcriptomic study of the aerobic denitrifying bacteria

The changes in the transcriptome of W207-14 before and after PEF treatment were explored at the same growth time (8 h) during the growth stabilisation period, where sample A represents W207-14 without PEF treatment and sample D represents W207-14 with PEF treatment. Principal component analysis (Fig. S1) showed that the biological replicates within the respective groups of samples A and D were close to each other with high reproducibility, reflecting the reliability of the transcriptome sequencing test results (Xu *et al.* 2022). Meanwhile, the distances between the samples under different treatment conditions between the groups of samples A and D were large and there was a large variability, indicating that PEF-induced substantial transcriptional changes in W207-14.

A total of 834 genes were screened for significant differential expression, including 553 upregulated genes and 281 downregulated genes (Figures 6 and S2). The close branching distance and similar gene expression colours between biological replicates under the same treatment conditions within each group of samples A and D indicated that the gene expression patterns were similar and highly correlated among the biological replicates under the same treatment conditions; the farther branching distance and significant difference in gene expression colour among samples under different treatment conditions between groups of samples A and D indicated that the gene expression pattern was changed via PEF treatment (Siavoshi *et al.* 2022). Clustering analysis of the 834 significantly differentially expressed genes can reflect more intuitively the differential expression among the same genes of W207-14 before and after PEF treatment and provide a reference for the subsequent functional analysis of genes corresponding to differential genes.

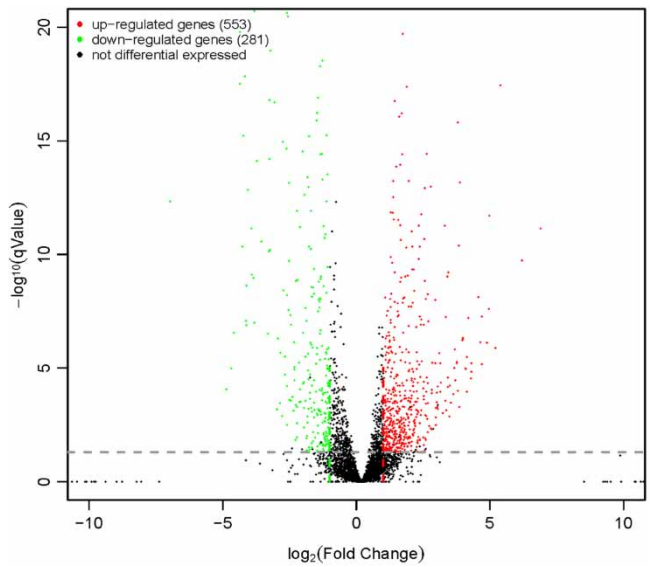


Figure 6 | Volcano map of the significantly differentially expressed genes.

All differentially expressed genes of W207-14 before and after PEF treatment were classified into three major functional categories: biological processes, cellular components and molecular functions (Fig. S4). Among them, in the biological processes category, differentially expressed genes are commonly found in several GO Terms such as cellular process (GO:0009987), metabolic process (GO:0008152), biological regulation (GO:0065007), stress response (GO:0050896) and biological process regulation (GO:0050789). In the cellular components category, differentially expressed genes are commonly found in several GO Terms such as cell (GO:0005623), cellular fraction (GO:0044464), cell membrane (GO:0016020), cell membrane fraction (GO:0044425) and protein complex (GO:0032991). Furthermore, in the molecular function category, differentially expressed genes are commonly found in several GO Terms such as catalytic activity (GO:0003824), binding (GO:0005488) and transport activity (GO:0005215).

The results indicate that PEF induced different levels of stress responses in the growth and metabolism of W207-14. The PEF-treated W207-14 maintained normal life activities through the regulation of genes involved in cellular and metabolic processes and other related genes to adapt to the changes in the growth environment created via PEF. Moreover, to obtain sufficient energy for growth and metabolism under the influence of PEF, W207-14 needs to enhance its uptake of nutrients and its ability to bind to different ions by regulating the expression of genes related to catalytic activity, binding and electron transfer (Chueca *et al.* 2015). The abundant changes in the expression of genes related to the cell membrane in the cell fraction category confirm that the cell membrane is one of the main targets of PEF action on microorganisms.

3.7. Transcriptomics-based enhancement mechanisms

The research investigated the transcriptomics-based PEF–Fe enhancement mechanism using significant differentially expressed gene enrichment analysis. The 30 GO Terms with the highest enrichment and the strongest significance were selected from the differentially expressed genes of the PEF–Fe-treated strain W207-14 to plot the scatter plot of the significantly enriched GOs of differentially expressed genes (Figure 7).

The results showed that PEF–Fe action could influence the metabolic process of strain W207-14. The highest percentage of GO Term related to metabolism was found among the top 30 significantly differentially expressed genes GO Term. It was confirmed that strain W207-14 must regulate the expression of metabolism-related genes in different degrees to maximise the amount of energy from organic matter for growth and metabolism in a stressful environment. At the same time, cell membrane repair must be completed to adapt to the stimulation from the PEF–Fe environment and maintain normal life activities without being inactivated. The effect of PEF–Fe can affect the aerobic respiration of strain W207-14. Aerobic denitrifying bacteria use the same fundamental mechanism of aerobic respiration via the aerobic respiratory electron transport system (respiratory chain) for both aerobic respiration and denitrification processes (Yang *et al.* 2020). The enhanced expression of these genes located near aerobic cellular respiration helps facilitate electron transfer during aerobic respiration when subjected to PEF–Fe treatment, providing sufficient energy for the vital functions of strain W207-14 (Hao *et al.* 2022). The use of PEF–Fe can influence the redox process of strain W207-14, which is a crucial aspect of conventional biological denitrification. In this process, NH_4^+-N is oxidised to NO_3^--N and gradually reduced to N_2 under aerobic conditions. The nitrogen metabolism process of strain W207-14 can be performed smoothly under a PEF–Fe stress environment by differentially regulating the expression of redox-related genes under the action of PEF–Fe, which can affect the iron–sulphur cluster binding of strain W207-14. The iron–sulphur cluster is a fundamental redox centre that has been present in all living organisms for a long time and plays a critical role in cellular metabolism (Braymer *et al.* 2021). Aerobic denitrifying bacteria often use periplasmic nitrate reductase (Nap) containing an [4Fe–4S] iron–sulphur cluster as a marker for aerobic denitrification (Pandey *et al.* 2020). Strain W207-14 can regulate the expression of genes related to the iron–sulphur cluster and redox enzymes in conjunction with PEF–Fe treatment, enabling aerobic denitrification. The action of PEF–Fe improves the efficiency of the aerobic denitrification process.

Figure S4 and Table S2 show directed acyclic plots of the significant GO Term under the functional classification of cellular fraction GO for the PEF–Fe-treated strain W207-14, which was selected for enrichment correlation analysis in conjunction with the previous effect of PEF–Fe on strain W207-14.

Under the functional classification of cellular components, the GO Term branch of the cell membrane (GO: 0016020) has a significantly differentially expressed gene, the membrane protein complex (GO: 0098796). Among the 20 upregulated genes in the membrane protein complex GO Term, 10 encode ABC transporter proteins. These proteins are widely found in various organisms and can use Adenosine triphosphate,(ATP); hydrolysis-generated energy to transport organic substances bound to

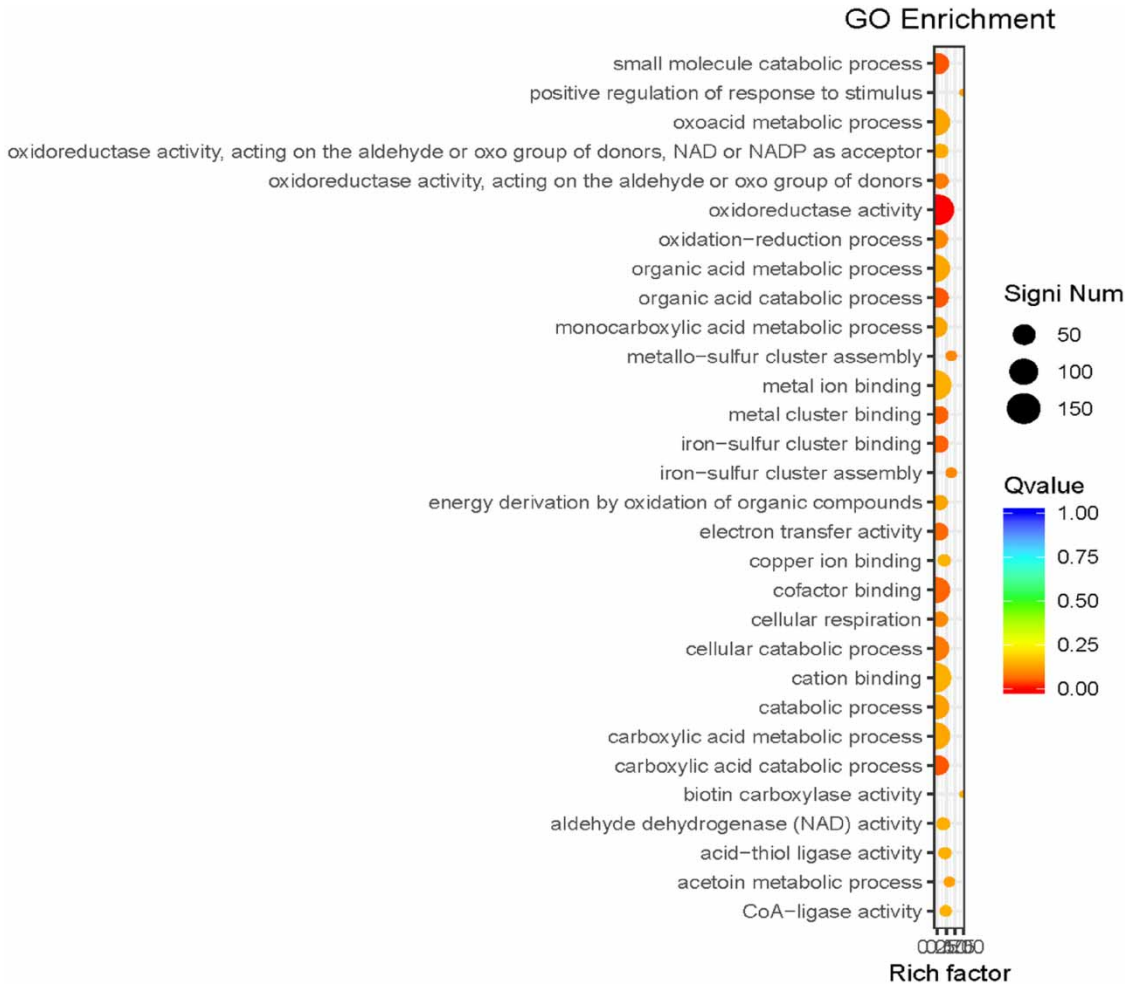


Figure 7 | Scatterplot of significant enrichment GO functions of differentially expressed genes.

them across membranes (Wu *et al.* 2023). The ABC transporter proteins can transport various substances across membranes using two mechanisms: inward and outward transport. Based on the transport mechanism of these proteins, it is hypothesised that under the action of PEF–Fe, reversible electroporation occurs on the cell membrane surface of aerobic denitrifying bacteria. This improves the cell membrane permeability, allowing the transporter protein to facilitate the inward transport of nutrients such as sugars and amino acids from the extracellular environment to the intracellular matrix (Elbourne *et al.* 2019). This promotes cell growth and metabolism. Additionally, the outward transport process can also aid in 'detoxification' by transferring substances that are harmful to the growth of W207-14 cells out of the cells. This helps to keep the non-essential exogenous substances or secondary metabolites at a low concentration within the cells, reducing growth pressure and maintaining normal cell growth, which greatly improves the survival rate of the cells.

Studies have identified the following three strategies for regulating cell membrane permeability: (a) lipid-mediated membrane permeability regulation; (b) regulation of membrane protein function; and (c) regulation of the intracellular membrane energy system (Choudhuri & Klaassen 2018). The ABC transporter protein is a membrane channel protein that regulates the entry and exit of substances into and out of the membrane using the energy generated by ATP hydrolysis in the cytoplasm. This protein can influence both the function of membrane protein and the intracellular membrane energy system. PEF–Fe treatment activates the transmembrane transport function of ABC transporter protein and enhances cell membrane permeability by regulating membrane protein function and the intracellular membrane energy system. This ultimately leads to a significant increase in the growth rate and nitrate removal rate of strain W207-14, providing a molecular biological explanation for the previous findings.

Figure S5 shows that significant GO Term directed acyclic plots of strain W207-14 after PEF–Fe treatment under molecular functional GO functional classification. Table S3 presents the significant differentially expressed genes. Iron–sulphur clusters play an important role in the intracellular energy regulation of W207-14 in the PEF–Fe system. The primary function of the iron–sulphur cluster is electron transfer, which is involved in intracellular energy transfer as a cofactor group of electron transfer proteins (Pauleta *et al.* 2023). The iron–sulphur cluster uses electrons generated by PEF–Fe interaction to convert iron ions from the oxidised state to the reduced state, followed by the transfer of electrons to other receptor proteins and the return to the initial oxidised state, thus starting a new electron transfer. Through the energy regulation of the iron–sulphur cluster, the strain W207-14 within the PEF–Fe system is always in a suitable energy state. The iron–sulphur cluster has a certain catalytic effect and can participate in the system's binding and activation of substrates (Boncella *et al.* 2022). For instance, periplasmic nitrate reductase (Nap), a hallmark of aerobic denitrification and an iron–sulfur cluster in the form of [4Fe–4S] in Nap could better facilitate the process of aerobic denitrification (Flamholz & Newman 2022). The PEF–Fe system precipitates many Fe ions, and the iron–sulphur cluster can participate in intracellular iron storage and maintain the dynamic balance of intracellular iron ions so that the growth metabolism of strain W207-14 can better use iron ions. Compared with the control group, a further significant increase in the growth rate and nitrate removal rate of strain W207-14 under the action of PEF–Fe was inextricably linked to the regulation of iron–sulphur clusters in the aerobic denitrification process.

4. CONCLUSIONS

The growth and metabolism of W207-14 were enhanced through PEF treatment. The growth time required for W207-14 to achieve the maximum OD 600 value was reduced by five times and the nitrate removal rate increased by 599.14% after PEF treatment compared with those of untreated W207-14. Using SEM, a smaller size was observed after PEF treatment. Moreover, FCM combined with fluorescence staining analysis revealed that the PEF-treated cells maintained higher physiological activity while the cell membrane was damaged, confirming the reversible electroporation of the membrane surface of the PEF-treated W207-14 cells. Differentially expressed gene enrichment analysis indicated that PEF–Fe treatment activated the transmembrane transport function of the ABC transport proteins, which led to enhanced cell membrane permeability in aerobic denitrifying bacteria. Additionally, the significant differential expression of iron–sulphur cluster proteins facilitated the regulation of electron transport and maintenance of the dynamic balance of iron ions within the PEF–Fe system.

ACKNOWLEDGEMENTS

The research was supported by the Liaoning Provincial Science and Technology Plan Project 'Application Research on Solidification and Resourcing of Domestic Waste Incineration Fly Ash Based on Geopolymerization Theory' (2020JH2/10300094) and the Liaoning Provincial Central Leading Local Science and Technology Development Special Project 'Application Research on Solidification and Resourcing of Waste Incineration Fly Ash and Waste Concentrate' (2021JH6/10500217). Special thanks are due to the instrumental or data analysis from Analytical and Testing Center, Northeastern University, China and Shiyanjia Lab (www.shiyanjia.com).

AUTHORSHIP CONTRIBUTION STATEMENT

F. W. investigated the article, experimented he data, conceptualised the whole article, conducted formal analysis, and rendered support in data curation; B. Z. conducted formal analysis, wrote the original draft, wrote the review and edited the article; X. D. reviewed the article; X. Z. reviewed the article; X. H. reviewed and supervised the article, administered the project.

DATA AVAILABILITY STATEMENT

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

CONFLICT OF INTEREST

The authors declare there is no conflict.

REFERENCES

Bao, H., Wu, M., Meng, X., Han, H., Zhang, C. & Sun, W. 2023 Application of electrochemical oxidation technology in treating high-salinity organic ammonia-nitrogen wastewater. *Journal of Environmental Chemical Engineering* **11**, 110608.

Bian, X., Wu, Y., Li, J., Yin, M., Li, D., Pei, H., Chang, S. & Guo, W. 2022 Effect of dissolved oxygen on high C/N wastewater treatment in moving bed biofilm reactors based on heterotrophic nitrification and aerobic denitrification: Nitrogen removal performance and potential mechanisms. *Bioresource Technology* **365**, 128147.

Boncella, A. E., Sabo, E. T., Santore, R. M., Carter, J., Whalen, J., Hudspeth, J. D. & Morrison, C. N. 2022 The expanding utility of iron-sulfur clusters: Their functional roles in biology, synthetic small molecules, maquettes and artificial proteins, biomimetic materials, and therapeutic strategies. *Coordination Chemistry Reviews* **453**, 214229.

Braymer, J. J., Freibert, S. A., Rakwalska-bange, M. & Lill, R. 2021 Mechanistic concepts of iron-sulfur protein biogenesis in Biology. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* **1868**, 118863.

Brunato, F., Garziera, M. G. & Briguglio, E. 2003 A severe methaemoglobinemia induced by nitrates: a case report. *European Journal of Emergency Medicine* **10** (4), 326–330.

Buchmann, L. & Mathys, A. 2019 Perspective on Pulsed Electric Field Treatment in the Bio-based Industry. 7.

Choudhuri, S. & Klaassen, C. D. 2018 8.12 - Molecular Biology of ABC Transporters. *In:* MCQUEEN, C. A. (ed.) *Comprehensive Toxicology (Third Edition).* Oxford: Elsevier.

Chueca, B., Pagán, R. & García-gonzalo, D. 2015 Transcriptomic analysis of Escherichia coli MG1655 cells exposed to pulsed electric fields. *Innovative Food Science & Emerging Technologies* **29**, 78–86.

Daccache, M., Koubaa, M., Maroun, R. G., Salameh, D., Louka, N. & Vorobiev, E. 2020 Pulsed electric field-assisted fermentation of Hanseniaspora sp. *yeast isolated from Lebanese apples.* Food Research International **129**, 108840.

Davey, H. M. & Hexley, P. 2011 Red but not dead? Membranes of stressed Saccharomyces cerevisiae are permeable to propidium iodide. *Environmental Microbiology* **13**, 163–171.

Elbourne, L. D. H., Hassan, K. A., Ren, Q., Cameron, A. D., Henderson, P. J. F. & Paulsen, I. T. 2019 Microbial Solute Transporters⋆. *In:* SCHMIDT, T. M. (ed.) *Encyclopedia of Microbiology (*Fourth Edition*).* Oxford: Academic Press.

El zakhem, H., Lanoisellé, J. L., Lebovka, N. I., Nonus, M. & Vorobiev, E. 2006 Behavior of yeast cells in aqueous suspension affected by pulsed electric field. *Journal of Colloid and Interface Science* **300**, 553–563.

Flamholz, A. I. & Newman, D. K. 2022 Microbial communities: The metabolic rate is the trait. *Current Biology* **32**, R215–R218.

Gui, M., Chen, Q. & Ni, J. 2017 Effect of sulfamethoxazole on aerobic denitrification by strain Pseudomonas stutzeri PCN-1. *Bioresource Technology* **235**, 325–331.

Guo, J., Dang, J., Wang, K., Zhang, J. & Fang, J. 2018 Effects of nanosecond pulsed electric fields (nsPEFs) on the human fungal pathogen Candida albicans: an in vitro study. *Journal of Physics D: Applied Physics* **51**, 185402.

Hao, Z.-L., Ali, A., Ren, Y., Su, J.-F. & Wang, Z. 2022 A mechanistic review on aerobic denitrification for nitrogen removal in water treatment. *Science of The Total Environment* **847**, 157452.

Ji, B., Yang, K., Zhu, L., Jiang, Y., Wang, H., Zhou, J. & Zhang, H. 2015 Aerobic denitrification: A review of important advances of the last 30 years. *Biotechnology and Bioprocess Engineering* **20**, 643–651.

Johnson, P. E., Van Der Plancken, I., Balasa, A., Husband, F. A., Grauwet, T., Hendrickx, M., Knorr, D., Mills, E. N. C. & Mackie, A. R. 2010 High pressure, thermal and pulsed electric-field-induced structural changes in selected food allergens. *Molecular Nutrition & Food Research* **54**, 1701–1710.

Lan, M., Yang, P., Xie, L., Li, Y., Liu, J., Zhang, P., Zhang, P. & Li, B. 2022 Start-up and synergistic nitrogen removal of partial nitrification and anoxic/aerobic denitrification in membrane aerated biofilm reactor. *Environmental Research* **214**, 113901.

Li, L., Ma, L., Zhang, H., Wu, J., Naqvi, S. M. Z. A., Li, Z., Wei, W., Awais, M., Li, S., Ewulo, B. S. & Hu, J. 2023 Development of improved flow analysis prototype method for measuring and understanding agricultural non-point source (NPS) nitrogen (N) and phosphorus (P) pollution. *Computers and Electronics in Agriculture* **210**, 107894.

Pandey, C. B., Kumar, U., Kaviraj, M., Minick, K. J., Mishra, A. K. & Singh, J. S. 2020 DNRA: A short-circuit in biological N-cycling to conserve nitrogen in terrestrial ecosystems. *Science of The Total Environment* **738**, 139710.

Pauleta, S. R., Grazina, R., Carepo, M. S. P., Moura, J. J. G. & Moura, I. 2023 2.06 - Iron-sulfur clusters – functions of an ancient metal site. In: REEDIJK, J. & POEPPELMEIER, K. R. (eds.) *Comprehensive Inorganic Chemistry III (Third Edition).* Oxford: Elsevier.

Siavoshi, A., Taghizadeh, M., Dookhe, E. & Piran, M. 2022 Gene expression profiles and pathway enrichment analysis to identification of differentially expressed gene and signaling pathways in epithelial ovarian cancer based on high-throughput RNA-seq data. *Genomics* **114**, 161–170.

Simon, J. & Klotz, M. G. 2013 Diversity and evolution of bioenergetic systems involved in microbial nitrogen compound transformations. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* **1827**, 114–135.

Veneziani, G., Esposto, S., Taticchi, A., Selvaggini, R., Sordini, B., Lorefice, A., Daidone, L., Pagano, M., Tomasone, R. & Servili, M. 2019 Extra-Virgin Olive Oil Extracted Using Pulsed Electric Field Technology: Cultivar Impact on Oil Yield and Quality. *6.*

Wang, J., Chen, P., Li, S., Zheng, X., Zhang, C. & Zhao, W. 2022 Mutagenesis of high-efficiency heterotrophic nitrifying-aerobic denitrifying bacterium Rhodococcus sp. *strain CPZ 24.* Bioresource Technology **361**, 127692.

Wu, J., Zhao, R., Zhao, L., Xu, Q., Lv, J. & Ma, F. 2023 Sorption of petroleum hydrocarbons before transmembrane transport and the structure, mechanisms and functional regulation of microbial membrane transport systems. *Journal of Hazardous Materials* **441**, 129963.

Xia, Q., He, J., He, B., Chu, Y., Li, W., Sun, J. & Wen, D. 2023 Effect and genesis of soil nitrogen loading and hydrogeological conditions on the distribution of shallow groundwater nitrogen pollution in the North China Plain. *Water Research* **243**, 120346.

Xu, X., Lin, L., Yang, J., Qian, W., Su, R., Guo, X., Cai, L., Zhao, Z., Song, J. & Yang, C. 2022 Simultaneous single-cell genome and transcriptome sequencing in nanoliter droplet with digital microfluidics identifying essential driving genes. *Nano Today* **46**, 101596.

Yan, L., Jiang, J., Liu, S., Yin, M., Yang, M. & Zhang, X. 2022a Performance and mechanism of nitrate removal by the aerobic denitrifying bacterium JI-2 with a strong autoaggregation capacity. *Bioresource Technology* **265**, 128111.

Yan, L., Zheng, Y., Chen, W., Liu, S., Yin, M., Jiang, J. & Yang, M. 2022b Step feed mode synergistic mixed carbon source to improve sequencing batch reactor simultaneous nitrification and denitrification efficiency of domestic wastewater treatment. *Bioresource Technology* **358**, 127440.

Yang, J., Feng, L., Pi, S., Cui, D., Ma, F., Zhao, H.-P. & Li, A. 2020 A critical review of aerobic denitrification: Insights into the intracellular electron transfer. *Science of The Total Environment* **731**, 139080.

Yao, Z., Yang, L., Wang, F., Tian, L., Song, N. & Jiang, H. 2020 Enhanced nitrate removal from surface water in a denitrifying woodchip bioreactor with a heterotrophic nitrifying and aerobic denitrifying fungus. *Bioresource Technology* **303**, 122948.

Zhang, H., Li, S., Ma, B., Huang, T., Qiu, H., Zhao, Z., Huang, X. & Liu, K. 2020 Nitrate removal characteristics and 13C metabolic pathways of aerobic denitrifying bacterium Paracoccus denitrificans Z195. *Bioresource Technology* **307**, 123230.

Zhao, W., Yang, R., Zhang, H. Q., Zhang, W., Hua, X. & Tang, Y. 2011 Quantitative and real time detection of pulsed electric field induced damage on Escherichia coli cells and sublethally injured microbial cells using flow cytometry in combination with fluorescent techniques. *Food Control* **22**, 566–573.

Zhao, Q., Peng, Y., Li, J., Gao, R., Jia, T., Deng, L. & Du, R. 2022 Sustainable upgrading of biological municipal wastewater treatment based on anammox: From microbial understanding to engineering application. *Science of The Total Environment* **813**, 152468.

Zhou, J., Wang, T. & Xie, X. 2020 Locally Enhanced Electric Field Treatment (LEEFT) Promotes the Performance of Ozonation for Bacteria Inactivation by Disrupting the Cell Membrane. *Environmental Science & Technology* **54**, 14017–14025.

First received 24 May 2023; accepted in revised form 8 October 2023. Available online 18 October 2023