Vol. 23, No. 1(2024) Bio24136
Revista Mexicana de Ingeniería Química

Enhanced denitrification in *Paracoccus denitrificans* PD1222 by electrical field application

Mejora de la desnitrificación de *Paracoccus denitrificans* PD1222 mediante aplicación de campo eléctrico

J.C. Ríos-Guzmán[1], M. Alonso-Vargas[1], A. Cadena-Ramírez[1]*, K. Juárez-López[2], L.A. Portillo-Torres[1]
[1]*Posgrado en Biotecnología, Universidad Politécnica de Pachuca, Carretera Pachuca-Cd. Sahagún, Km 20, Ex-Hacienda de Santa Bárbara, Zempoala C.P. 43830, Hidalgo, México.*
[2]*Posgrado en Ciencias Bioquímicas, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Avenida Universidad 2001, Cuernavaca C.P. 62210, Morelos, México.*
Received: August 25, 2023; Accepted: December 13, 2023

Abstract
Denitrification is an anaerobic respiratory process in which nitrogen oxides, generally nitrates, act as electron acceptors replacing oxygen. Among the many denitrifying bacteria, there is *Paracoccus denitrificans*; a gram-negative, non-fermentative coccoid microorganism whose denitrifying respirome has great similarity to the mitochondrial respiratory chain. The present work evaluated the effect of different electrical potentials imposed on the denitrifying process of *P. denitrificans* using a rotating cylinder electro-bioreactor whose design allowed a homogeneous electrical potential distribution. An electrochemical characterization of the culture medium was carried out to define the potentials where REDOX reactions of an electrochemical nature are despicable (0, 100, 150 and 200 mV), so the results obtained are attributed to the influence of the generated electric field on *P. denitrificans*. The consumption of N and C sources (nitrate NO_3^- and succinate $C_4H_4O_4^=$ respectively), the production of the intermediate (nitrite NO_2^-) and gas (nitrogen N_2) were monitored. The findings suggest membrane and enzymatic alterations that bolster the overall process, with a notable increase in gaseous nitrogen production under the influence of electrical potentials. This novel approach offers promising implications for enhancing biological denitrification processes.
Keywords: *Paracoccus denitrificans*, denitrification, electric fields, bioelectrochemical reactor.

Resumen
La desnitrificación es un proceso respiratorio anaerobio en el que óxidos de nitrógeno, generalmente nitratos, actúan como aceptores de electrones reemplazando al oxígeno. Entre las muchas bacterias desnitrificantes, se encuentra *Paracoccus denitrificans*; un microorganismo cocoide gram negativo, no fermentativo cuyo respiroma desnitrificante posee gran similitud con la cadena respiratoria mitocondrial. El presente trabajo evaluó el efecto de diferentes potenciales eléctricos impuestos al proceso desnitrificante de *P. denitrificans* utilizando un electro-biorreactor de cilindro rotatorio cuyo diseño permitió una distribución del potencial eléctrico homogénea. Se realizó una caracterización electroquímica del medio de cultivo para definir los potenciales donde las reacciones de tipo REDOX de carácter electroquímico (0, 100, 150 y 200 mV) son despreciables, por lo que los resultados obtenidos se atribuyen a la influencia del campo eléctrico generado sobre *P. denitrificans*. Se monitoreó el consumo de las fuentes de N y C (nitrato NO_3^- y succinato $C_4H_4O_4^=$ respectivamente), la producción del intermediario (nitrito NO_2^-) y gas (nitrógeno N_2). Los hallazgos sugieren alteraciones en la membrana y enzimáticas que fortalecen el proceso en general, con un notable aumento en la producción de nitrógeno gaseoso bajo la influencia de potenciales eléctricos. Este enfoque novedoso ofrece implicaciones prometedoras para mejorar los procesos de desnitrificación biológica.
Palabras clave: *Paracoccus denitrificans*, desnitrificación, potencial eléctrico, reactor bioelectroquímico.

*Corresponding author. *E-mail*: arturocadena@upp.edu.mx;
https://doi.org/10.24275/rmiq/Bio24136
ISSN:1665-2738, *issn-e*: 2395-8472

1 Introduction

Currently, it is well-understood that microbial cultures are influenced by physicochemical variables such as pH, temperature, aeration, and nutrient concentration. However, there is another variable that has been studied in biological systems since the 18th century: electric fields (EF) (Paul *et al*., 1993; Vanegas-Acosta, 2015). Electric fields (EFs) are regarded as biophysical factors capable of either stimulating or inhibiting biological responses at various levels, primarily affecting enzymatic activity. (Kolosnjaj-Tabi *et al*., 2019). They exert force by moving a unit charge from one point to another within a specific space. In this process, materials exposed to the field experience forces of attraction and repulsion (Pataro *et al*., 2011; Paul *et al*., 1993).

Evaluating the alterations biological materials undergo when exposed to EF necessitates an understanding of biomolecules like amino acids that possess electrically charged residues. Both these residues and other biomolecules-and by extension, the cells-are influenced by the presence of EF. This is especially true for biochemical processes where electrical potentials naturally exist, such as ATP synthesis (Guerra-Hühne *et al*., 2022). As a result, the energy landscape changes, necessitating adjustments to thermodynamic parameters like redox potential or binding energies. These adjustments can lead to either beneficial or detrimental outcomes by enhancing or inhibiting reaction rates, thereby altering the chemical equilibrium of metabolism (Joy *et al*., 2020; Léonard *et al*., 2021).

The use of electrical potentials (EPs) in microbial cultures hinges on two primary factors: the physicochemical nature of the electrolyte and the geometry of the electrodes. The former directly impacts the process. Specifically, the electric potential that can be applied in a direct current (DC) field diminishes as the conductivity of the medium increases. To enhance this factor, salts are frequently introduced, which in turn improves electron transport in the agitated medium (Giwa *et al*., 2019). Conversely, the configuration of the electrodes differs based on the intended purpose. As the electrode system is pivotal in generating the EF, it is crucial that it does not adversely affect the microorganism's physiology. This is why biocompatible metals like gold, titanium, chrome, nickel, palladium, stainless steel, aluminum, among others, are typically employed (Martínez *et al*., 2023; Giwa *et al*., 2019). If the applied current is intense enough to induce medium hydrolysis, the dissolved components can alter its physicochemical attributes, leading to the emergence of reactive oxygen species such as O_3, H_2O_2, or OH^- ions. Depending on the medium's composition, active species like chlorine or persulfates might also emerge. While these can be detrimental to the microorganism, electrode passivation can transpire, forming a surface film that curtails their electrochemical activity (Feng *et al*., 2016; Giwa *et al*., 2019; van Genuchten *et al*., 2016). The reactor's geometry, as alluded to earlier, plays a vital role. Outcomes can vary based on the microorganism type, its growth phase, field intensity, and the manner of application (Revelo *et al*., 2013; Soto-Vázquez *et al*., 2023). The EF distribution in aqueous solutions is shaped by multiple elements, notably the geometry and positioning of the electrodes. While flat electrodes offer a commendable EF distribution, they are susceptible to edge effects, where disturbances at the electrode edges compromise the EF's uniformity (Griffiths & College, 1999), leading to inconsistent results. Rotating cylinder reactors, like the one highlighted in this study, present advantages. Their electrode surface maintains an equipotential state with uniform current density, minimizing edge effects due to the electrode spacing. This allows for a more consistent EF distribution across the aqueous medium. Moreover, the space between the electrodes ensures a superior EP distribution, yielding a more focused and less dispersed EF (Gabe *et al*. 1983; Walsh, *et al*., 2017). The distribution of an EF in aqueous solutions is influenced by various factors, with the geometry and arrangement of the electrodes being particularly significant. While flat electrodes can offer a uniform distribution of the EF, they are susceptible to edge effects. As the term suggests, disturbances occur at the electrode edges, compromising the EF's uniformity (Griffiths & College, 1999). This can lead to inconsistent and unreliable results.

Various microorganisms and processes are being studied to understand the effects of applying EF. Among them, one of the most notable is *Paracoccus denitrificans*. This bacterium is a non-fermentative, gram-negative, coccoid α-proteobacteria with significant metabolic versatility. It can thrive in both aerobic and anaerobic environments and shares a notable resemblance to eukaryotic mitochondria (Bergaust *et al*., 2010). *P. denitrificans* is renowned for its ability to carry out biological denitrification, an anaerobic respiratory process. This process parallels that of aerobic microorganisms, with nitrate (NO_3^-) and nitrite (NO_2^-) ions taking the place of O_2 as the final electron acceptors (Olaya-Abril *et al*., 2018; Romualdo-Martínez *et al*., 2022), It is worth noting that not all nitrogen oxidation-reduction processes qualify as denitrification. Only those tied to ATP synthesis are regarded as such (Azziz *et al*., 2016). The necessary electrons for this process derive from the oxidation of organic material or certain compounds acting as electron donors.

Table 1. Composition of the denitrifying culture medium.

Reagent	Concentration	Reagent	Concentration
Na_2HPO_4	29 mM	EDTA	342 μM
NaH_2PO_4	11 mM	$ZnSO_4 \cdot 7H_2O$	15 μM
$CaCl_2$	6.8 mM	$MgCl_2 \cdot 4H_2O$	51 μM
$MgSO_4$	0.4 mM	$FeSO_4 \cdot 7H_2O$	36 μM
$C_4H_4Na_2O_4 \cdot 6H_2O$	50 mg C/L	$Na_2MoO_4 \cdot 2H_2O$	12 μM
$NaNO_3$	37.4 mg N/L	$CuSO_4 \cdot 5H_2O$	13 μM
$CoCl_2 \cdot 6H_2O$	13.5 μM		

P. denitrificans can utilize a broad spectrum of compounds, including alcohols, acetate, humic substances, and others (Cadena-Ramírez *et al*., 2019). The nature of the electron donor also impacts denitrifying respiration. Using succinate ($C_4H_4O_4^=$) as a donor is advantageous because *P. denitrificans* inherently contains the enzyme succinate dehydrogenase. This means that there is no need for the bacterium to express the enzymatic machinery to oxidize succinate. Additionally, during cellular respiration, the electrons generated by the oxidation of succinate are channeled to the respiratory chain (Sazanov, 2007).

The denitrifying respiration process of *P. denitrificans* encompasses four reactions (as shown in equation 1). In this process, nitrate (NO_3^-) is converted to gaseous nitrogen (N_2) through the catalytic action of four metalloproteins. First, nitrate is reduced to nitrite by the enzyme nitrate reductase (Nar). Next, nitrite is transformed into nitric oxide by nitrite reductase (Nir). This nitric oxide is then reduced to nitrous oxide by nitric oxide reductase (Nor). Finally, nitrous oxide is converted to nitrogen gas by the enzyme nitrous oxide reductase (Nos) (Romualdo *et al*., 2022; Galliazzi, 2017; Qian *et al*., 2016; Zhang *et al*., 2023).

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2 \qquad (1)$$

In this study, we explored the impact of electric fields on the metabolism of *P. denitrificans* during the denitrification process.

2 Materials and methods

2.1 Bacterial strain

The *P. denitrificans* PD1222 strain was provided by the Faculty of Chemistry at the Universidad Nacional Autónoma de México (UNAM). It was stored in Luria Bertani (LB) broth with 80% glycerol (1:1 ratio) at -4°C in 2 mL vials until utilized.

2.2 Inoculum and culture media

To cultivate *P. denitrificans*, the contents of a 2 mL vial were transferred to a 500 mL Erlenmeyer flask filled with 250 mL of LB Broth. This broth was supplemented with $CaCl_2$ (10 mM) and $NaNO_3$ (5 mM) to create a modified LB Broth. The flask was then incubated at 28°C with an agitation speed of 200 rpm overnight.

Following incubation, the entire contents of the flask were centrifuged at 10,000 rpm for 12 minutes. The supernatant was discarded, and the biomass was resuspended in another 500 mL Erlenmeyer flask containing 250 mL of the modified LB Broth. This was then incubated at 28°C with an agitation speed of 200 rpm for 14 hours. The composition of the denitrifying culture medium is detailed in Table 1.

Sodium succinate and sodium nitrate were used as sources of carbon and nitrogen, respectively. The C/N ratio employed was 1.34.

2.3 Electrochemical reactor

In this study, we employed a rotary cylinder bioreactor, designed to facilitate the application of an electric potential (EP) to the bacterial culture. The bioreactor features a central rotating cylinder, with a surface area of 534.071 cm^2, that serves both as an anode and a stirrer. This design not only keeps the culture agitated but also promotes even distribution of the EP by maintaining consistent electrolyte (or ion) concentrations. A stainless-steel sheet, with an area of 452.389 cm^2 lining the inner wall of the reactor, acted as the cathode. Both electrodes are arranged concentrically, ensuring that the EF vectors are uniformly distributed throughout the culture medium. A schematic representation of the reactor can be seen in Figure 1.

2.4 Reactor operating conditions

A 2 L rotary cylinder electro-bioreactor with a capacity of 1.5 L was utilized for this study. For batch kinetics, the 14-hour pre-inoculum was centrifuged at 4,000 rpm for 12 minutes. The supernatant was then discarded, and the biomass was rinsed with a sterile saline solution (0.9% NaCl) before being centrifuged

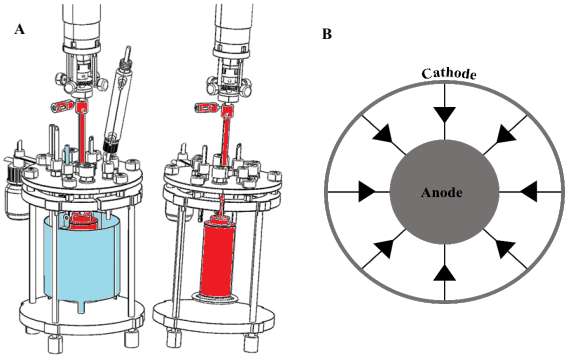


Figure 1. Diagram of the rotating cylinder reactor. (A) Both electrodes are shown, in blue the cathode and in red the rotating cylinder (anode). (B) Both electrodes are equidistant which allows the EF lines (black) to be homogeneous.

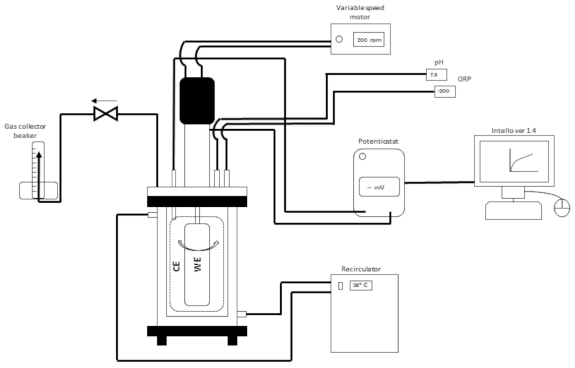


Figure 2. Schematic of the denitrifying rotary cylinder reactor.

again at 4,000 rpm for 12 minutes. After discarding the supernatant once more, the biomass was resuspended in 50 mL of the denitrifying medium. Using a sterile glass funnel, this biomass was introduced into the reactor through one of its ports. Electrodes, calibrated in advance, were connected to monitor pH and ORP (jf LAREN, Mexico). The gas produced by the reactor was measured using a displacement method and collected in a 100 mL beaker filled with a NaCl solution (300 g/L) and methyl red. Additionally, a gas chromatograph was employed for further analysis. The system operated for 12 hours. The reactor's temperature was maintained at 28°C with the aid of a recirculator (Prendo, Mexico), and the agitation was managed using a variable speed motor set to 200 rpm, as illustrated in Figure 2.

Every 2 hours, 3 mL samples were collected using sterile syringes. These samples were then assessed for absorbance at 600 nm using a UV-Vis spectrophotometer (Thermo Fisher Scientific, USA). After that, the samples were centrifuged in 1.5 mL vials at 14,000 rpm for 10 minutes. The supernatant was subsequently filtered through 0.45 nm membranes and stored in refrigerated HPLC vials until further use.

Table 2. Main parameters of the applied electric potential.

Start E	0, 100, 150, 200 mV
Stabilization time	5 s
Step E	0, 100, 150, 200 mV
Duration	12 h

2.5 *Application of electrical potential*

For the application of electrical potential, a VIONIC potentiostat (Metrohm Autolab, Netherlands) controlled by Intello software ver. 1.4 was used, connected in a two-electrode arrangement.

2.6 *Quantification of NO_3^-, NO_2^- and succinate*

The quantification of NO_3^-, NO_2^- (denitrification intermediate product) and succinate was carried out in a UHPLC equipment (Dionex 3000, USA). For NO_3^- and NO_2^-, a WATERS IC-Pak Anion HC 4.6x150 mm column was used with a retention time of 22 min, the sample was read at 214 nm. As mobile phase, the one provided by the manufacturer was used at a flow of 2 mL/min. The sample injection volume was 10 μL.

For succinate, a GRACE Alltech OA-1000 Organic Acids 300mm x 6.5mm column was used with a retention time of 11 min. The samples were acidified with a drop of concentrated phosphoric acid and read at 210 nm. As mobile phase, 25 mM KH_2PO_4 pH 2.5 was used. The sample injection volume was 10 μL.

2.7 *NO, N_2O, N_2 quantification*

For the quantification of N_2, 9 μL were taken directly from the reactor and injected into a gas chromatograph (SRI 310C, USA), equipped with an electrical conductivity detector (TCD) and a ShinCarbon ST, 100/120 mesh column, 2 m, 1/16 in. OD, 1.0mm ID. Helium was used as carrier gas at a flow of 10 mL/min. NO and N_2O could be detected in the same chromatographic column; however, they were not observed in any of the experiments.

2.8 *Kinetic parameters*

The maximum consumption rates were calculated using the slope (the exponential phase) of the corresponding curve (concentration vs time). The experimental yield was calculated with the production of biomass and the consumption of C and N sources, using the following equation:

$$Y = \frac{\Delta x}{\Delta S} = \frac{x_{final} - x_{initial}}{S_{initial} - S_{final}} \quad (2)$$

Table 3. Maximum consumption rates for C and N sources, rate of production for N_2 and N_2 product yield and Consumption efficiency.

	0 mV	100 mV	150 mV	200 mV
$V_{max\,C_4H_4O_4^-}$ (mg C-Succ/L h)	26.62	6.40	20.63	261.99
$V_{max\,NO_3^-}$ (mg N-NO_3^-/L h)	26.41	19.36	22.26	75.47
V_{N_2} (mg N- N_2/L h)	6.02	4.86	8.21	14.25
Y_{N_2}	0.98	0.95	1.00	1.00
$E_{NO_3^-}$	97.304	98.267	98.373	98.994

2.9 Statistical analysis

All experiments were carried out in triplicate. For statistical analysis, a one-way analysis of variance was performed using a Tukey-Kramer multiple comparison test with $\alpha = 0.05$ (NCSS 12).

3 Results

3.1 Denitrifying cultures

In controlled experiments devoid of an electron donor, specifically succinate, the consumption of nitrate was found to be negligible, regardless of the electrical potential (EP) applied. This trend was also evident in control setups lacking the final electron acceptor, nitrate. The denitrification control experiments without EP, as depicted in Figure 3A, demonstrated the depletion of both carbon and nitrogen sources within 6 hours. Notably, a peak accumulation of nitrite (NO_2^-) was observed, reaching 10.70 ± 0.36 mg N-NO_2^-/L at the 2-hour mark. The efficiency of nitrate consumption and the yield of N_2, as detailed in Table 3, suggest that the denitrification process in the control experiment (0 mV) was thorough, with all the nitrogen introduced into the culture being recovered as N_2. This finding is consistent with the biomass behavior, which remained virtually unchanged throughout the cultivation period (Figure 3A). *P. denitrificans* PD1222 exhibited no growth, attributed to the low carbon to nitrogen ratio (C/N) as per stoichiometric equation 9, indicating that the process was solely respiratory and dissimilatory. This was due to the absence of any nitrogen source in the culture medium other than NO_3^-. While such respiratory and entirely dissimilatory behavior has been previously reported in other denitrification studies, primarily involving denitrifying sludge from wastewater treatment facilities (Peña-Calva *et al*., 2004; Martínez-Gutierrez *et al*., 2012; Cadena-Ramírez *et al*., 2007, 2019a, 2019b), it had not been documented in pure cultures like *P. denitrificans* PD1222 until now.

Significant changes were observed in the consumption of NO_3^- that were not progressive with the increase in applied EP. Each applied EP exhibited specific characteristics that could not be correlated with its increase. Notably, NO_3^- consumption was impacted at 100 mV (Figure 4B), where the maximum consumption rate decreased by 31.6% for both NO_3^- and succinate compared to 0 mV. However, the overall denitrification process was completed in its entirety, where the efficiency of NO_3^- consumption did not diminish, but the yield of N_2 decreased by only 3%. There was a delay in the process, but it cannot be said to have been affected per se by the yields and efficiencies of substrate consumption. It is possible that at 100 mV, substrate consumption was only affected due to impairments in their transport, which seems to be supported by the fact that NO_2^- accumulated. Another hypothesis is that the expression of denitrifying enzymes was delayed. It is important to remember that the culture of *P. denitrificans* originated from a heterotrophic culture rich in carbon and nitrogen sources and was grown under aerobic conditions. Since denitrifying bacteria are non-fermentative, the initial production of the denitrification proteome depends on energy from aerobic respiration (Hassan *et al*., 2014). Most denitrifying enzymes are not facultative, including NAR, which is denitrifying, and thus needed to be expressed for the process to occur in *P. denitrificans* PD122, including those related to substrate transport (Olaya-Abril *et al*., 2018). This is in line with findings by Cadena-Ramírez *et al*. (2019), who applied a similar EP (104 mV) to denitrifying sludge and observed a delay in denitrification. This decoupling explains the accumulation of NO_2^- due to a deficiency in reductive power. A similar situation was observed in this study, where the lack of reductive power seemingly delayed the process. This is also supported by the behavior of the oxidation-reduction potential (ORP), which clearly showed a delay in the reducing conditions of the culture medium. For the electron source, consumption efficiencies were 100% in all treatments, but CO_2 could not be quantified due to the alkalinity of the medium, where, due to the pH levels, CO_2 was present as dissolved carbonates $(CO_3)_2^-$.

For the EP of 150 and 200 mV (Figures 3C and 3D), the production of nitrogen was expedited, with peak yields for 150 mV reached at 6 hours, and at 200 mV within 4 hours, whereas the control reached its maximum N_2 yield (Y_{N_2}) at 8 hours.

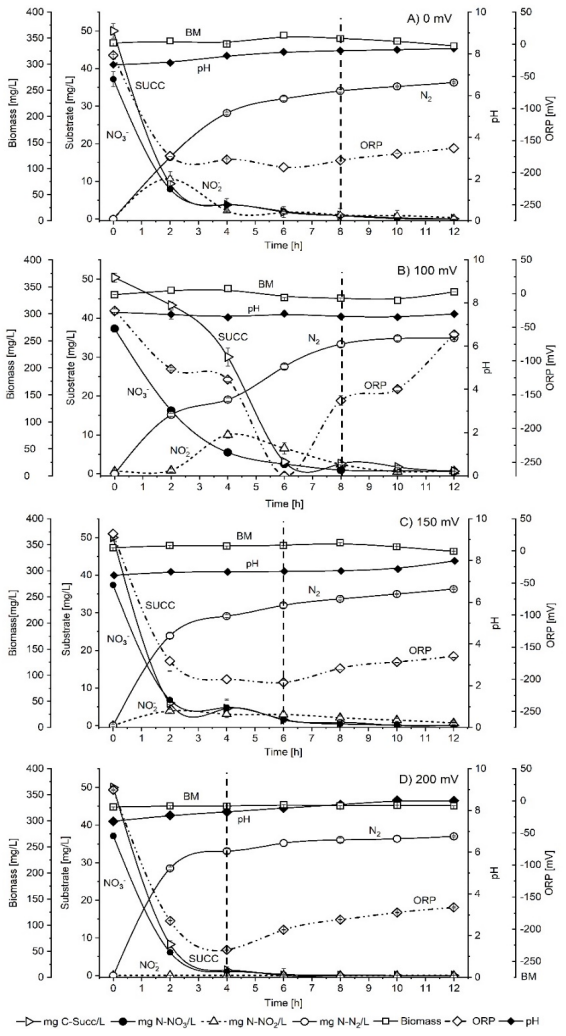


Figure 3. Denitrifying kinetics of *P. denitrificans*. (A) Control, (B) 100 mV EP, (C) 150 mV EP and (D) 200 mV.

There is a clear enhancement of denitrification under the influence of low-intensity EP above 150 mV, which is supported by the nitrogen mass balances. Particularly at 200 mV, the maximum consumption rate (Table 3) increased up to threefold compared to an EP of 0 mV, with a value of 75.47 mg N-NO_3^-/L h. Two possibilities could explain these observations, and they cannot be attributed to changes in substrate transport since the production and consumption behavior of NO_2^- was different, as will be discussed later.

A crucial marker in denitrifying processes is the accumulation of NO_2^-. The accumulation of nitrite in the experiments with EP application differed from the denitrifying control (0 mV) since there was a nitrite maximum (10.00 ± 0.02 mg N- NO_2^-/L) like the control but at 4 h. For the other EPs the accumulation was significantly lower, with the maximum accumulation being observed at 2 h. Values of 3.88 ± 0.09 mg N- NO_2^-/L and 0.04 ± 0.01 mg N- NO_2^-/L were observed for 150 mV and 200 mV respectively. In all experiments the overall NO_2^- was completely converted to N_2. It is worth noting that the peak concentration of NO_2^- at 0 mV occurs when NO_3^- is nearly fully consumed; this observation will be elaborated on later. The accumulation of NO_2^- is noteworthy because its redox potential is lower than that of NO_3^- (420 mV vs. NHE compared to 960 mV vs. NHE) (Berks *et al*., 1995). This serves as an indicator that the electric field (EF) is beneficially altering its transport and biochemical reduction (Nevi *et al*., 2010; Qin *et al*., 2017). Moreover, the rate of N_2 production was enhanced. Considering the redox potentials for each of the intermediates (NO_2^- at +420 mV vs. NHE, NO at +400 mV vs. NHE, and N_2O at +820 mV vs. NHE) (Garsia *et al*., 2012; Stamler *et al*., 2001) it is evident that both NO_2^- and NO have closely aligned redox potentials, whereas N_2O potential is notably higher. Using these values for reference and comparing them to the ORP, one can deduce that both NO and N_2O (which was not observed) were reduced internally in the cell to N_2, especially given the highly reducing properties of the medium. NO_2^- is transported intracellularly through a nitrate reductase subunit. Thus, any alterations in nitrate reductase could influence its transport. Since nitric oxide reductase is membrane-anchored, its behavior might mirror that of nitrate reductase (Tinajero-Vargas, 2020). Furthermore, succinate dehydrogenase from *P. denitrificans*, a constitutive enzyme to the electron transport chain, has its active site facing the cytoplasm and includes a transmembrane portion. All the aforementioned enzymes are metalloenzymes. Therefore, it is entirely feasible for them to respond to EF, either through structural and catalytic modifications or via shifts in their oxidation state, which could ultimately alter their catalytic activity (Albina *et al*., 2019; Tinajero-Vargas, 2020).

The reduction can either enhance or impair nitrate reductase activity due to the electron flow through cytochrome c from the reduction of NO_2^- or N_2O (Kucera *et al*., 1983). To understand how the cytochrome c reduction degree is impacted, it is essential to measure the activity of NAD/$NADH^+$ (which was not done in this study). However, by referencing the calculated denitrification equations (equations 3-7), one can deduce that the reducing potential from the oxidation of the carbon source (equation 8) is being integrated into the respiratory chain.

$$2NO_3^- + 4e^- + 4H^+ \rightarrow 2NO_2^- + 2H_2O \quad (3)$$

$$2NO_2^- + 2e^- + 4H^+ \rightarrow 2NO + 2H_2O \quad (4)$$

$$2NO + 2e^- + 2H^+ \rightarrow N_2O + H_2O \quad (5)$$

$$N_2O + 2e^- + 2H^+ \rightarrow N_2 + H_2O \quad (6)$$

$$2NO_3^- + 10e^- + 12H^+ \rightarrow N_2 + 6H_2O \quad (7)$$

$$C_4H_4O_4^= + 4H_2O \rightarrow 4CO_2 + 12H^+ + 14e^- \quad (8)$$

The general biochemistry for denitrification using

succinate as an electron donor is presented in equation 9.

$$C_4H_4O_4^{=} + 4NO_3^{-} + 4e^{-} + 8H^{+} \rightarrow 4CO_2 + 2N_2 + 5H_2O \quad (9)$$

The figures depict the correlation between the reduction of NO_3^- and the accumulation of NO_2^-. Studies have reported that within *P. denitrificans*, there is an electron competition between NO_3^- and NO_2^-. Moreover, these ions exchange through protein channels, known as antiports, in the membrane. This exchange helps maintain a balance in the concentrations of both ions (Boogerd *et al*., 1983; Glass *et al*., 1998; Thomsen *et al*., 1994). Monitoring NO_2^- during the denitrification process is crucial, as its accumulation can inhibit denitrification at concentrations ranging from 30-250 mg N-NO_2/L (Griffiths and College, 1999). The data presented indicates extracellular concentrations. Notably, in the control scenario, the peak concentration of NO_2^- is attained before NO_3^- hits its lowest levels. The ORP consistently declines as NO_2^- is reduced. Once the ORP achieves its minimum, the reduction rate of NO_2^- appears to quicken. In contrast, when applying a 200 mV EP, the NO_2^- concentration consistently remains low without evident accumulation, while the ORP assumes more negative values.

The analysis indicates a statistically significant difference in the 100 mV and 150 mV treatments for the consumption rates for C and N sources, respectively. These variances in the specific rates across treatments might be attributed to changes in the catalytic activity of succinate dehydrogenase and nitrate reductase, or alterations in their redox states. While succinate dehydrogenase possesses an Fe-S group at its active site, nitrate reductase is more complex, comprising three subunits, each with distinct heme groups and Fe-S bonds. As a result, each subunit exhibits a unique redox potential. Consequently, beyond the structural alterations impacting their catalytic activities, shifts in their redox states emerge as vital considerations (Tinajero-Vargas, 2020; van Spanning *et al*., 2007).

During the application of EP, the ORP value reached more negative values than during the control; this value is usually used as an indicator of the reducing potential of the culture medium. In all the experiments conducted, the ORP value and pH aligned with values typically associated with the denitrification process, indicating that the process was functioning as expected (Venturini *et al*., 2022). The pH value was maintained in a range of 7.5 - 8.5. This alkalization is also an indicator of the evolution of the process. (Gao *et al*., 2003; Olaya-Abril *et al*., 2018). When evaluated together, these two parameters act as indicators of the reduction process experienced by the electron acceptors. However, they should be treated as supplementary indicators since their readings can be influenced by the EP in the range of volts (unpublished data) (Gao *et al*., 2003; Olaya-Abril *et al*., 2018).

Concerning the growth pattern of *P. denitrificans* during the experiments, it was observed that both the lag phase and the acceleration phase were reduced. While some studies have shown that the lag phase diminishes in the presence of an EF (Parvanova-Mancheva *et al*., 2009), the persistent stationary phase throughout our experiment suggests that *P. denitrificans* shifted to a purely dissimilative metabolism. In this mode, all electron flow from the oxidation of the carbon source is funneled into the denitrification respiratory chain, without necessarily contributing to bacterial growth.

Previously, growth kinetics were evaluated using the same denitrifying culture media, which indicated that the lag phase lasted for approximately 3 hours, while the exponential phase concluded after 15 hours. Based on this growth curve, we can deduce that under the experimental conditions tested, the EP influenced the cell division rate of *P. denitrificans*. In research conducted by Cadena-Ramírez in 2019, various EPs were applied to denitrifying sludge, revealing that different EPs exerted distinct biochemical impacts on the denitrification process. However, that study utilized a microbial consortium, complicating the task of pinpointing the precise effects of electron donors on microbial physiology. This contrasts with the current study, which employed an axenic culture, facilitating a more direct correlation of results.

Consequently, there was either an overexpression of denitrifying enzymes, which is necessary for the process to occur (Olaya-Abril *et al*., 2018), or there was a stimulation of the chemical potentials of the denitrifying enzymes, including both nitrate reductase and nitrite reductase, but not of the last two enzymes. It is important to note that although the production of nitric oxide and nitrous oxide was not observed, these compounds were monitored by gas chromatography. Moreover, nitrogen mass balances show that the reduction of nitrite to the other two nitrogen compounds was very rapid, starting from the control at 0 mV. Genetic studies would be required to confirm these hypotheses. Despite these findings, it is clear that low-intensity EPs can affect denitrification more at the physiological level than at the chemical level.

3.2 Cyclic voltammetry

Cyclic voltammetry (refer to Appendix 1) was used to confirm that no electrochemical reactions occurred in the culture medium. Therefore, the results showcased are directly attributed to the interaction between the applied EP and *P. denitrificans*.

Considering the similar rates of consumption for both NO_3^- and $C_4H_4O_4^{=}$ (as shown in Table

3) in assays with and without EP, it seems that the application of EP does not significantly impact metabolism. However, the patterns of accumulation and consumption of NO_2^- indicate that the transport and catalysis of the chemical species may be altered. These alterations could arise from reversible structural changes in the membrane, such as the opening of antiports, shifts in electron flow leading to changes in the reduction level of the terminal cytochrome c in the respiratory chain, or variations in the catalytic activity of denitrifying enzymes (Kucera *et al*., 1983; Ohshima *et al*., 2007; Vaessen *et al*., 2019).

Conclusions

The present investigation elucidated the intricate interplay between imposed electric potentials and the denitrification kinetics of *Paracoccus denitrificans*. Utilizing a rotating cylinder electro-bioreactor, a uniform electrical potential distribution was achieved, ensuring controlled experimental conditions. Electrochemical characterization of the culture medium was instrumental in discerning potentials devoid of electrochemical REDOX reactions, thereby ascertaining that observed phenomena were attributable to the EFs direct influence on *P. denitrificans*.

Systematic monitoring of N and C source consumption, alongside intermediate NO_2^- and gaseous nitrogen production, indicated pronounced membrane and enzymatic alterations under varying EPs. The enzymes discussed in this study, have demonstrated the potential of the EFs to induce structural and catalytic modifications; these alterations have the capacity to significantly impact the catalytic activity of the enzymes under investigation. Significantly, the imposition of EP increased gaseous nitrogen evolution, highlighting its potential as a key parameter for optimizing denitrification processes. Referring that, the variations observed with the EP imposed, can be attributed to changes in the catalytic activity of the enzymatic complex that forms the electron transport chain as well as the denitrifying process.

This study offers a foundation for future endeavors aimed at harnessing EPs to enhance biological denitrification, with implications for advanced wastewater treatment methodologies. Further research is warranted to explore the broader implications of these findings and their potential applications in biotechnology and environmental science.

Acknowledgment

The first author thanks the support to Consejo Nacional de Humanidades, Ciencias y Tecnologías (CONAHCyT) for the scholarship awarded to obtain the master's degree in biotechnology on the postgraduate program of Universidad Politécnica de Pachuca, included in the National Quality Postgraduate Program. This research was funded by, CONAHCyT-Ciencia de Frontera 2019, number 58540.

Nomenclature

ATP	Adenosinetriphosphate
DC	Direct current
EF	Electric Field
E_{NO_3}	Nitrate consumption efficiency
EP	Electric Potential
H_2O_2	Hydrogen peroxide
mg C-Succ/L	Succinate-carbon miligrams per liter
mg N-NO_2^-/L	Nitrite-nitrogen miligrams per liter
mg N-NO_3^-/L	Nitrate-nitrogen miligrams per liter
NAR	Nitrate reductase
N- NO_2^-	Nitrite-nitrogen
N- NO_3^-	Nitrate-nitrogen
ORP	Oxidation-Reduction Potential
V_{max}	Maximum consumption rates
Y_{N_2}	Yield for the generation of molecular nitrogen with respect to the nitrogen source

References

Albina, P., Durban, N., Bertron, A., Albrecht, A., Robinet, J.C., and Erable, B. (2019). Influence of hydrogen electron donor, alkaline pH, and high nitrate concentrations on microbial denitrification: A review. *International Journal of Molecular Sciences 20*, 5163. https://doi.org/10.3390/ijms20205163

Azziz, G., Illarze, G., and Irisarri, P. (2016). Heterotrophic denitrification and *Paracoccus spp*. as tools for bioremediation. In: *Microbial Models: From Environmental to Industrial Sustainability. Microorganism for Sustainability* (S. Castro eds.), Pp. 209-216. Springer, Singapore.

Bergaust, L., Mao, Y., Bakken, L. R., and Frostegard, A. (2010). Denitrification response patterns during the transition to anoxic respiration and posttranscriptional effects of suboptimal pH on nitrogen oxide reductase in *Paracoccus denitrificans*. *Applied and Environmental*

Microbiology 76, 6387-6396. https://doi.org/10.1128/AEM.00608-10

Berks, B. C., Ferguson, S. J., Moir, J. W. B., and Richardson, D. J. (1995). Enzymes and associated electron transport systems that catalyse the respiratory reduction of nitrogen oxides and oxyanions. *Biochimica et Biophysica Acta (BBA) - Bioenergetics 1232*, 97-173. https://doi.org/10.1016/0005-2728(95)00092-5

Boogerd, F. C., Van Verseveld, H. W., and Stouthamer, A. H. (1983). Dissimilatory nitrate uptake in *Paracoccus denitrificans* via a ΔgmH+-dependent system and a nitrate-nitrite antiport system. *Biochimica et Biophysica Acta (BBA) - Bioenergetics 723*, 415-427. https://doi.org/10.1016/0005-2728(83)90049-X

Cadena-Ramírez, A., Texier, A. C., González, I., Cervantes, F. J., and Gomez, J. (2007). Qualitative and quantitative determination of a humic model compound in microbial cultures by cyclic voltammetry. *Environmental Technology 28*, 1035-1044. https://doi.org/10.1080/09593332808618862

Cadena-Ramírez, A. (2018). Efectos del Potencial Redox en la Oxidación de Compuestos Fenólicos por un Lodo Desnitrificante. Tesis de doctorado en Biotecnología, Universidad Autonóma Metropolitana, México.

Cadena-Ramírez, A., Texier, A. C., Martínez, I. G. and Hernández, J. G. (2019a). Inhibitory effects of quinoid redox mediators on a denitrifying culture. *Environmental Technology 40*, 1306-1315. https://doi.org/10.1080/09593330.2017.1421264

Cadena-Ramírez, A., Texier, A. C., González, I. and Gómez, J. (2019b). Implications of electric potentials applied on a denitrifying process. *Environmental Technology 40*, 2747-2755. https://doi.org/10.1080/09593330.2018.1450447

Feng, Y., Yang, L., Liu, J., and Logan, B. E. (2016). Electrochemical technologies for wastewater treatment and resource reclamation. *Environmental Science: Water Research & Technology 2*, 800-831. https://doi.org/10.1039/C5EW00289C

Gabe, D. R. and Walsh, F. C. (1983). The rotating cylinder electrode: a review of development. *Journal of Applied Electrochemistry 13*, 3-21. https://doi.org/10.1007/BF00615883

Galliazzi, A. (2017). Aislamiento y caracterización de bacterias desnitrificantes antárticas con potencial uso en biorremediación de diésel. Tesis de ingeniería en biotecnología, Universidad ORT, Uruguay.

Gao, D.W., Peng, Y.Z., Liang, H., and Wang, P. (2003). Using oxidation-reduction potential (ORP) and pH value for process control of shortcut nitrification-denitrification. *Journal of Environmental Science and Health, Part A 38*, 2933-2942. https://doi.org/10.1081/ESE-120025842

Garsia, M. M., and Maruthamuthu, S. (2012). Redox potential measurements in microbial electrochemistry. In: *Microbial Electrochemical and Fuel Cells* (H. Scott and E. Hao, eds.), Pp. 167-191. Woodhead publishing, Reino Unido.

Giwa, A., Dindi, A., and Kujawa, J. (2019). Membrane bioreactors and electrochemical processes for treatment of wastewaters containing heavy metal ions, organics, micropollutants and dyes: Recent developments. *Journal of Hazardous Materials 370*, 172-195. https://doi.org/10.1016/j.jhazmat.2018.06.025

Glass, C., and Silverstein, J. (1998). Denitrification kinetics of high nitrate concentration water: pH effect on inhibition and nitrite accumulation. *Water Research 32*, 831-839. https://doi.org/10.1016/S0043-1354(97)00260-1

Griffiths, D. and College, R. (1999). *Introduction to Electrodynamics*. Prentice Hall. Estados Unidos.

Guerra-Hühne, J., Bola, S., Calzia, D., Alexopoulou, D., Funk, R. H. W., Mühlen, S. and Roehlecke, C. (2022). Effect of direct current electric fields on cone like retinal photoreceptor cells. *Frontiers in Biosciences (Landmark Ed) 27*, 273. https://doi.org/10.31083/j.fbl2709273

Hassan, J., Bergaust, L. L., Wheat, I. D. and Bakken, L. R. (2014). Low probability of initiating nirS transcription explains observed gas kinetics and growth of bacteria switching from aerobic respiration to denitrification. *PLoS Computational Biology 10*, e1003933. https://doi.org/10.1371/journal.pcbi.1003933

Kolosnjaj-Tabi, J., Gibot, L., Fourquauz, I., Golzio, M. and Rols, M.P. (2019). Electric field-responsive nanoparticles and electric fields: physical, chemical, biological mechanisms and

therapeutic prospects. *Advanced Drug Delivery Reviews 138*, 56-67. https://doi.org/10.1016/j.addr.2018.10.017

Joy, J., Stuyver, T., and Shaik, S. (2020). Oriented external electric fields and ionic additives elicit catalysis and mechanistic crossover in oxidative addition reactions. *Journal of the American Chemical Society 142*, 3836-3850. https://doi.org/10.1021/jacs.9b11507

Kucera, I., Dadak, V., and Dobry, R. (1983). The distribution of redox equivalents in the anaerobic respiratory chain of *Paracoccus denitrificans*. *European Journal of Biochemistry 130*, 359-364. https://doi.org/10.1111/j.1432-1033.1983.tb07161.x

Léonard, N. G., Dhaoui, R., Chantarojsiri, T., and Yang, J. Y. (2021). Electric fields in catalysis: From enzymes to molecular catalysts. *ACS Catalysis 11*, 10923-10932. https://doi.org/10.1021/acscatal.1c02084

Martínez-Gutierrez. E., González-Márquez, S., Martínez-Hernández, S., Texier, A. C.; Cuervo-López, F. M. and Gómez, J. (2012) Effect of phenol and acetate addition on 2-chlorophenol consumption by a denitrifying sludge. *Environmental Technology 33*, 1375-1382. https://doi.org/10.1080/09593330.2011.627882

Martínez, J. M., Abad, V., Quílez, J., Raso, J., Cebrián, G. and Álvarez-Lanzarote. (2023). Pulsed Electric Fields (PEF) applications in the inactivation of parasites in food. *Trends in Food Science & Technology 138*, 470-479. https://doi.org/10.1016/j.tifs.2023.06.030

Nevi, K. P., Woodard, T. L., Franks, A. E., Summers, Z. M., and Lovely, D. (2010). Microbial electrosynthesis: Feeding microbes electricity to convert carbon dioxide and water to multicarbon extracellular organic compounds. *MBio 1*. https://doi.org/10.1128/mbio.00103-10

Ohshima, T., Tamura, T., and Sato, M. (2007). Influence of pulsed electric field on various enzyme activities. *Journal of Electrostatics 65*, 156-161. https://doi.org/10.1016/j.elstat.2006.07.005

Olaya-Abril, A., Hidalgo-Carrillo, J., Luque-Almagro, V. M., Fuentes-Almargo, C., Urbano, F. J., Moreno-Vivián, C., Richardson, D. J., and Roldán, M. D. (2018). Exploring the denitrification proteome of *Paracoccus denitrificans* PD1222. *Frontiers in Microbiology 9*. https://doi.org/10.3389/fmicb.2018.01137

Parvanova-Mancheva, T., and Beschkov, V. (2009). Microbial denitrification by immobilized bacteria *Pseudomonas denitrificans* stimulated by constant electric field. *Biochemical Engineering Journal 44*, 208-213. https://doi.org/10.1016/J.BEJ.2008.12.005

Pataro, G., Ferrari, G., and Donsi, F. (2011). Mass transfer enhancement by means of electroporation. In: *Mass Transfer in Chemical Engineering Processes* (J. Markos, ed.), Pp. 151-176. IntechOpen. Reino Unido.

Paul, R., Kaler, K. V. I. S., and Jones, T. B. (1993). A nonequilibrium statistical mechanical calculation of the surface conductance of the electrical double layer of biological cells and its application to dielectrophoresis. *Journal of Physical Chemistry 97*, 4745-4755. https://doi.org/10.1021/j100120a600

Peña-Calva, A., Olmos-Dichara, A., Viniegra-González, G., Cuervo-López, F. M., and Gómez, J. (2004). Denitrification in presence of benzene, toluene, and m-xylene: Kinetics, mass balance, and yields. *Applied Biochemistry and Biotechnology 119*, 195-208. https://doi.org/10.1007/s12010-004-0002-0

Qian, J., Zhou, J., Zhang, Z., Liu, R. and Wang, Q. (2016). Biological nitrogen removal through nitration coupled with thiosulfate-driven denitrification. *Scientific Reports 6*, 27502. https://doi.org/10.1038/srep27502

Qin, S., Zhang, Z., Yu, L., Yuan, H., Clough, T. J., Wrage-Mönnig, N., Luo, J., and Zhou, S. (2017). Enhancement of subsoil denitrification using an electrode as an electron donor. *Soil Biology and Biochemistry 115*, 511-515. https://doi.org/10.1016/j.soilbio.2017.09.020

Revelo, D. M., Hurtado, N. H., and Ruiz, J. O. (2013). Celdas de combustible microbianas (CCMs): Un reto para la remoción de materia orgánica y la generación de energía eléctrica. *Información Tecnológica 24*, 17-28. https://doi.org/10.4067/S0718-07642013000600004

Romualdo-Martínez. I. V., Hernández, R. A. I., González, B. G., and Beristain, C. R. (2022). Metabolic and Kinetics changes of activated sludge because of failures in the aireation system in a WWTP. *Revista Mexicana de Ingeniería Quimica 21*, IA2914. https://doi.org/10.24275/rmiq/IA2914

Sazanov, L. A. (2007). Respiratory complex I: Mechanistic and structural insights provided by the crystal structure of the hydrophilic domain. *Biochemistry 46*, 2275-2288. https://doi.org/10.1021/bi602508x

Stamler, J. S., and Meissner, G. (2001). Physiology of nitric oxide in skeletal muscle. *Physiological Reviews 81*, 209-237. https://doi.org/10.1152/physrev.2001.81.1.209

Soto-Vázquez, A., Sánchez-Galindo, P., Barraza-Madrigal, J. A. and Guzmán-Castañeda, J. I. (2023). Electrocoagulation as a possible treatment for wastewater polluted with industrial lubricant oils. *Revista Mexicana de Ingeniería Quimica 22*, IA2313. https://doi.org/10.24275/rmiq/IA2313

Thomsen, J. K., Geest, T., and Cox, R. P. (1994). Mass spectrometric studies of the effect of pH on the accumulation of intermediates in denitrification by *Paracoccus denitrificans*. *Applied and Environmental Microbiology 60*, 536-541. https://doi.org/10.1128/aem.60.2.536-541.1994.

Tinajero-Vargas, S. A. (2020). Estudios estructurales de ATP sintasa de *Paracoccus denitrificans*. Tesis de Maestría en ciencias, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, México.

Vaessen, E. M. J., Timmermans, R. A. H., Tempelaars, M. H., Schutyser, M. A. I., and den Besten, H. M. W. (2019). Reversibility of membrane permeabilization upon pulsed electric field treatment in *Lactobacillus plantarum* WCFS1. *Nature 9*. https://doi.org/10.1038/s41598-019-56299-w

van Genuchten, C. M., Bandaru, S. R. S., Surorova, E., Amrose, S. E., Gadgil, A. J., and Peña, J. (2016). Formation of macroscopic surface layers on Fe (0) electrocoagulation electrodes during an extended field trial of arsenic treatment. *Chemosphere 153*, 270-279. https://doi.org/10.1016/j.chemosphere.2016.03.027

van Spanning, R. J. M., Richardson, D. J., and Ferguson, S. J. (2007). Introduction to the Biochemistry and Molecular Biology of Denitrification. In: *Biology of the Nitrogen Cycle*, (H. Bothe, S. J. Ferguson and W. E. Newton, eds.), Pp 3-20, Elsevier. Estados Unidos.

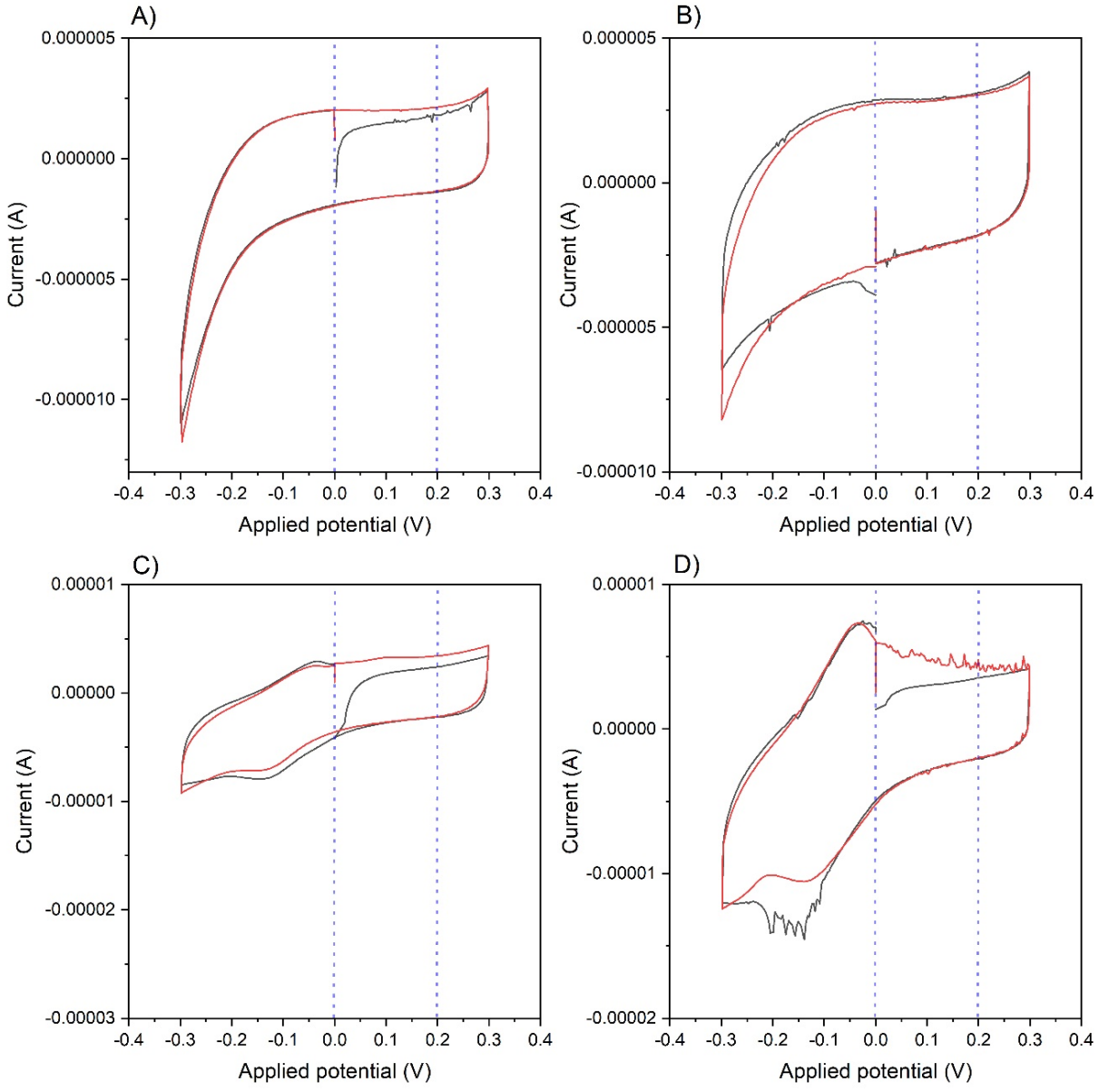
Vanegas-Acosta J. C. (2015). Electric fields and biological cells: numerical insight into possible interaction mechanisms. Tesis de doctorado en ingeniería eléctrica. Eindhoven University of Technology, Alemania.

Venturini, M., Rossen, A., Bucci, P. and Silva-Paulo, P. (2022). Applying the Nernst equation to control ORP in denitrification process for uranium-containing nuclear effluent with high loads of nitrogen and COD. *Water 14*, 227. https://doi.org/10.3390/w14142227

Walsh, F. C., Kear, G., Nahlé, A. H., Wharton, J. A. and Arenas, L. F. (2017). The rotating cylinder electrode for studies of corrosion engineering and protection of metals-An illustrated review. *Corrosion Science 123*, 1-20. https://doi.org/10.1016/j.corsci.2017.03.024

Zhang, Y., Dong, W., Li, C., Wang, H., Wang, H., Ling, Y., Yan, G. and Chang, Y. (2023). Effects of antibiotics on corncob supported solid-phase denitrification: Denitrification and antibiotics removal performance, mechanism, and antibiotic resistance genes. *Journal of Environmental Sciences 130*, 24-36. https://doi.org/10.1016/j.jes.2022.10.020

Appendix



Appendix 1. Voltammograms obtained during electrochemical characterization of the medium compounds. (A) Carbon source, (B) Nitrate, (C) Medium without sources of C or N and (D) medium with sources of C and N.