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Research Article

Effect of C/N ratio on biodegradation of ciprofloxacin and denitrification from Low C/N wastewater by a novel 3D-BER System

Mahdi Hassan [a,b], Guangcan Zhu* [a,b], Zhonglian Yang [a,b], Yongze Lu [a,b], Yan Lang [a,b], Liying Gong [a,b], Shan Huang [a,b]

Mahdi Hassan **E-mail:engrmahdi@gmail.com**

Guangcan Zhu **E-mail:engrmahdi@gmail.com**

Zhonglian Yang **E-mail: yangzhonglian@seu.edu.cn**

Yongze Lu **E-mail: yzlu@seu.edu.cn**

Yan Lang **E-mail: y_yan@seu.edu.cn**

Liying Gong **E-mail: lygong@seu.edu.cn**

Shan Huang **E-mail: 18270911541@163.com**

[a]School of Energy and Environment, Southeast University, Nanjing 210096, Jiangsu, China

[b]State Key Laboratory of Environmental Medicine Engineering of the Ministry of Education, Southeast University, Nanjing 210096, Jiangsu, China

* **Correspondence: gc-zhu@seu.edu.cn; Tel.: + 86-18912966396**

Abstract: Emerging pollutants as pharmaceuticals have been focusing international attention for few decades. Ciprofloxacin (CIP) is a common drug widely found in effluents from hospitals, industrial and different wastewater treatment plants, as well as rivers. In this work, the lab-scale 3D-BER system was established, and more than 90% of the antibiotic CIP removal from the Low C/N wastewater. Best results were obtained with current intensity, and different C/N ratio significantly improve the removal of CIP and nitrates, when the ideal conditions were; C/N = 1.5-3.5, pH =7.0-7.5, and I = 60 mA. The highest removal efficiency of CIP = 94.20 %, NO_3^--N= 95.53 % and total nitrogen (TN) = 84.27 %, respectively. In this novel system, the autotrophic-heterotrophic denitrifying bacteria played vital role for the removal of CIP and enhanced denitrification process. Thus, autotrophic denitrifying bacteria uses CO_2 and H_2 as carbon sources to reduce nitrates to N_2.

This system has the assortment and prosperous community revealed at the current intensity of 60 mA, and the analysis of bacterial community structure in effluent samples fluctuates under different condition of C/N ratios. According to the results of LC-MS/MS analysis, the intermediate products were proposed after efficient biodegradation of CIP. Microbial community on biodegrading was mostly found at phylum, and class level was dominantly responsible for the NO_3^--N and biodegradation of CIP. This work can provide some new insights towards the biodegradation of CIP and the efficient removal of nitrates from low C/N wastewater treatment by the novel 3D-BER system.

Keywords: Biodegradation of Ciprofloxacin; 3D-BER System; Denitrification; Microbial communities; Low C/N Wastewater treatment.

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1. Introduction

1. Introduction

Worldwide, the various kind of emerging pollutants such as pharmaceuticals compounds, anti-inflammatory drugs, antibiotics, beta-blockers [1], and the massive amount of NO3- or NO2- have attracted international attention for the last few decades [2]. The antibiotic ciprofloxacin (CIP) is the 3rd-generation fluoroquinolone group usually used in humans and veterinarians [1,3,4]. The high-proportion of CIP is not fully metabolized in livestock and humans, but evacuated as a parent substance [5]. Hence, CIP can reach the environment through various pharmaceutical industries, sewage treatment plants, livestock activities, landfills, and application of sewage sludge [6], manure, or treated wastewater to agricultural land [3,7,8]. However, due to the potential development and dissemination of antibiotic resistance, this poses a potential threat to the ecosystems and human health [2,3]. Therefore, CIP removal must be considered before being released into the environment [9]. Antibiotic CIP is readily detectable in man aquatic-environment (usually found in surface water at ngL-1 to μg L-1 levels);[7], it occurs at high levels in the effluents of WWTPs (up to 6.55-31 mgL-1) receiving pharmaceutical wastewater and rivers polluted with industrial waste (up to 14 mgL-1) [6,10,11]. During wastewater treatment, 80-90 % of CIP was removed by adsorption to sludge, which stabilizes the substance [3].

Some scientific reports, data has been shown that biodegradation [7], and adsorption process [9] was the highest elimination methods for various kinds of antibiotic in WWTPs [4]. It has been shown in some studies around 50-100% adsorption is the main path of CIP removal by biodegradation in anaerobic sludge system [12,13]. This clearly shows the importance of sludge being released into the environment as a CIP reservoir and developing sludge management strategies [14]. In general, anaerobic digestion was a standard procedure for the stabilization of sludge; it was also meant to extract organic matter without particular regard for the removal of the antibiotics [8]. The biodegradation rate of absorbent CIP durability was 0-40% during sludge treatment [9,15]. A considerable amount of CIP persisted in the digested sludge contained in wastewater treatment plants [10]. Unfortunately, there were no reports on the microbial community structure of CIP degradation [16,17]. The traditional denitrification process depends on four basic denitrifying enzymes [18], including respiratory of nitrate reductase [19] has been shown:

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$$

However, many processes reported in previous studies were CIP effects on the denitrifying enzymes and the activity of existing denitrifying bacteria [19-21]. In recent years, the biofilm electrode reactors (BERs), which combine biological and electrochemical techniques, can efficiently eliminate nitrogen, heavy metal, and antibiotics [10,22]. The autotrophic denitrify bacteria are immobilized on the cathode surface and the hydrogen produced from the water-electrolysis also used as an electron donor in the autotrophic denitrification process [21,22]. Therefore, the anode and cathode materials typically made of carbon and thus provide an inorganic-carbon source that can be used as a pH buffer solution, as shown in Eqs (1, 2 and 3):

$$2H_2O + 2e^- \rightarrow H_2 + 2OH^- \quad (1)$$

$$C + 2H_2O \rightarrow CO_2 + 4H + 4e^- \quad (2)$$

It was shown that, the process of denitrification uses hydrogen instead of an organic carbon source [23], to completely covert nitrate to N_2 as per the following reaction [18,24],

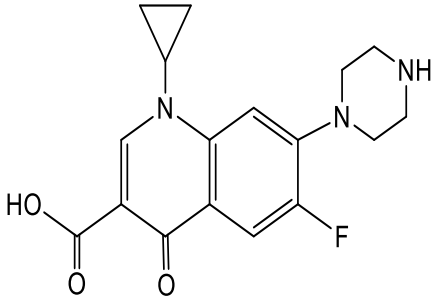
$$2NO_3^- + 5H_2 \rightarrow N_2 + 4H_2O + 2OH^- \quad (3)$$

Since hydrogen is generated from the cathode, it could not cause annoying safety problems, and the dosage is excessive due to the low solubility of hydrogen [18,24]. The BER only requires an inorganic carbon source [25]. From the literature, the organic-carbon also accumulates in the BERs to enhance the elimination of antibiotics [25,26], and nitrogen by the different C/N ratios [7,27]. The simultaneously of heterotrophic-autotrophic bacteria has an improved denitrification process

compared to the traditional BER consumes a large amount of energy and is relatively inefficient [28,29]. We recently introduced a 3D-BER system for the new advance technique in the potential benefits of low currents, and there is the best capability to treat toxic substances. Hence, an effective and economical methods for eliminating CIP antibiotics and nitrate or nitrite were developed. It is also imperious to improve the methodology to remove the residues of CIP antibiotics when high manipulative capacity of the anaerobic process occurred in 3D-BER system.

This study demonstrated that the removal efficiency of antibiotics CIP and nitrogen in the effluent of low C/N wastewater can be improved by a novel 3D-BER system. The major objective of this particular study were; (1) to examine the potential toxicity of ciprofloxacin and nitrogen removal; (2) Comprehensively examine the effect of pH on antibiotics-ciprofloxacin and denitrification; (3) to appraisal the degradation by microbial inhabitants and intermediate product of CIP were investigated. Therefore, this is the first study of the removal of CIP and enhanced the denitrification process for solving many problems in low C/N wastewater. As compared to traditional low C/N ratio in wastewater treatment systems, this technology is also designed to be a more efficient, useful complement or cost-effective alternative.

Table 1. Physical and chemical properties of ciprofloxacin

Name	**Properties**	**Molecular formula**	**structure**
Ciprofloxacin		$C_{17}H_{18}FN_3O_3$	
CAS number	85721-33-1		
* pKa	10.58±0.30 8.71±0.089 6.14±0.13 3.01±0.30		
* Log Kow[b]	0.40		
Molecular weight	331.35 g/mol		

*The pK_a values of ciprofloxacin were obtained from [30], and the value $LogKow^b$ was taken from [31], respectively.

2. Materials and Methods

2.1. Configuration of the 3D-BER system

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The schematic view of the three-dimensional bioelectrochemical reactor system (3D-BERs) was used in this study to evaluate the biodegradation of ciprofloxacin and nitrates removal from Low C/N wastewater (**Figure 1**). The reactor was made of plexiglass cylindrical with a diameter of 10 cm and a height of 20 cm with a total volume of 1.2 L, and had a working volume is 0.785 L. The cathode consisting of eight graphite rods (height of 20 cm and diameter of 0.8 cm) were placed around the periphery of the reactor, while the one graphite rod (height of 20 cm and diameter 1.5 cm), as an anode was fixed in the centre of the reactor and were connected using insulated electrical copper wire by DC-power. A DC-controlled power supply: Model No: HQ3003SIII, supplied by Hansheng Puyuan Technology Beijing Co., Ltd China, ranged (0-3A, 0-30V) was used. Electrolysis of water is the decomposition of water into hydrogen (H_2) and oxygen (O_2) due to the passage of an electric current. Meanwhile, from top to bottom, space was filled with granular activated carbon (GAC) as third particle electrodes (size 1.5-3mm) used for microbial growth purchased from Younga chemical Technology (Jiangsu Co., Ltd). The GAC was the best for the growth of bacteria on cathode and anode on the inner zone and bottom zone in the reactor. The pre-treatment of GAC was washed serval times with deionized water via sulfuric acid solution (0.01M) and then dried for 24-30 h, and finally at 110° C.

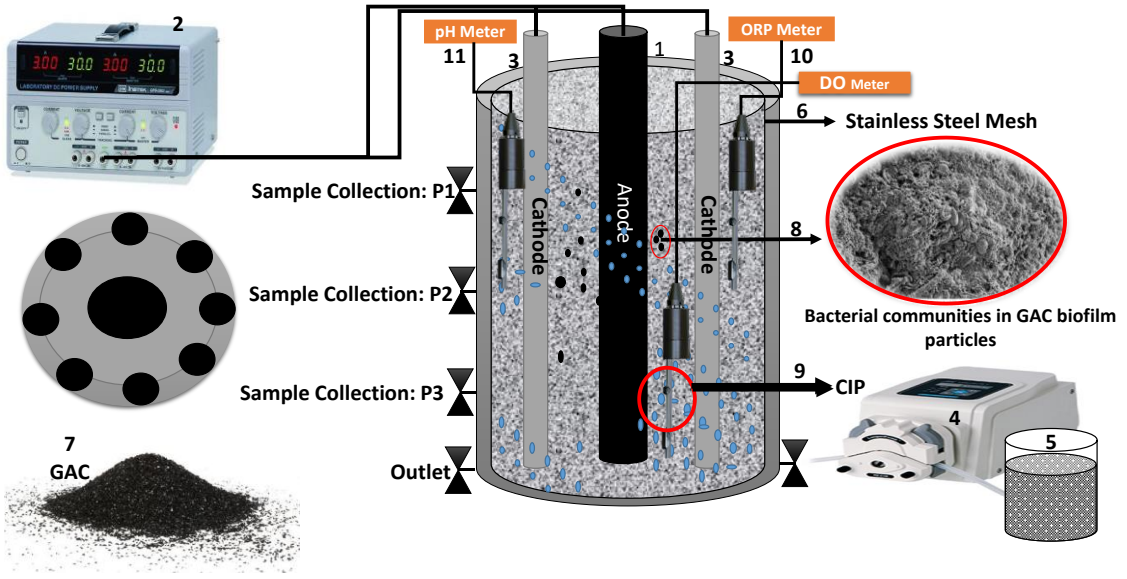

Figure 1. Schematic diagram of the 3D-BERS reactor; (1) Graphite anode rod (×1) Size (200 mm ×15 mm); (2). DC regulated power supply; (3). Graphite cathode rods (× 8) size (200 × 8mm); (4). Peristaltic Pump BT300-2J; (5). Influent water tank; (6). Stainless steel mesh; (7) Granular activated carbon (GAC); (8) Bacterial communities in GAC biofilm particles (9) CIP antibiotics (10) Portable

ORP meter model: pH100; (11). Digital pH meter model: pH100 and Sample Collection Points (P1, P2, and P3).

2.2. Chemicals and Reagents

Ciprofloxacin (Analytical-grade>99%) was obtained from Sigma Aldrich (Munich, Germany). A stock solution of antibiotic CIP (100-500μgL^{-1}) was prepared by a Milli-Q 18 MΩ system (Millipore, Germany). The pH was adjusted using a 1M of H_2SO_4 solution prepared with concentrated sulfuric acid (95-97% purity). High performance liquid chromatography (HPLC) analysis of Merck & Fisher's with acetonitrile (HPLC quality) and O-phosphate acid (85%). Whereas the synthetic wastewater was composed of 30 mgL^{-1} (NO_3^--N), 3.5 mgL^{-1} K_2HPO_4, 4.5 mgL^{-1} NaCl, 5.56 mgL^{-1} KH_2PO_4, 2.75 mgL^{-1} $CaCl_2$, 500 μgL^{-1} $C_{17}H_{18}FN_3O_3$ (ciprofloxacin) and 0.1 mlL^{-1} of concentrated trace elements stock solutions were prepared by dissolving; 1.5g $MgSO_4.7\ H_2O$, 2.2g $MnSO_4.\ H_2O$, 2.2g $ZnSO_4.\ H_2O$, 0.24g $CoCl_2.6H_2O$, 2mg $NiCl_2.6\ H_2O$, 10mg $FeCl_3.6H_2O$, 0.5mg $CuCl_2.2\ H_2O$, 0.5 mg $Na_2MoO_4.\ H_2O$ and 1.5 g $CaCl_2$ into 1 L deionized water [32,33]. Nitrates and antibiotics water effluent samples were filtered by 0.22-micron membrane filters (Millipore, Germany) prior to testing. The pH of simulated influent wastewater is typically 7.5 ± 0.2, and no further adjustment is required.

2.3. Experimental procedure

2.3.1. Bacterial adaptation phase

All experiments runs were inoculated with a mixed culture of acclimated autotrophic-heterotrophic bacteria (**Table 2**). These microbes were enriched from the anaerobic sludge (Mixed Liquor Suspended Sludge (MLSS.100g)) was taken as a source of inoculation from the Nanjing municipal wastewater treatment plant (WWTP). Before cultivation, 1.2 L of anaerobic sludge water was placed in a refrigerator with nutritious material at 4°C for seven-days. While the synthetic water, according to NaAc, Nitrates, and KH_2PO_4, were added into the anaerobic sludge with the ratio of C/N/P = 3:1:0.5. During the first three days, sludge water was circulated by a magnetic pump. The concentration of nitrate-nitrogen in the influent was maintained at approximately 30 mgL^{-1}. After 21 days, anaerobic sludge water was placed in the reactor, and 0.350 L of tap water was added to a total volume of 0.785 L. During the cultivation process, the electrical current was not supplied to the 3D-BER system after two weeks; the direct electrical current was gradually adjusted to 10 mA.

Table 2. The initial concentration of Low C/N wastewater at different C/N ratios

Parameters (mgL^{-1})							
Electric Current (mA)	60	60	60	60	60	60	60
COD	0	15	30	45	75	90	105
NO_3-N	30	30	30	30	30	30	30
C/N	0	0.5	1.0	1.5	2.5	3.0	3.5

2.3.2. Immobilized GAC biofilm at various C/N ratios

The reactor was started for seventy days of batch operations at electric applied current 60 mA. Each cycle of the 3D-BERs (feed, react, settle, decant) was 40 minutes. The dissolved oxygen (DO) in the reactor was kept from 1.25 mgL^{-1} less than 0.2 $mgL^{-1,}$ and the pH was maintained from 7.0±0.5 to 8.0±0.3. The pre-treated sludge was mixing with the ratio of GAC/Sludge: 3:1.5, and introduced into cathode and anode layers of the 3D-BERs. However, the antibiotics CIP concentrations in the influent were 100 to 500μgL^{-1}, respectively. For the batch experiments, the biofilm in the anode/cathode surface, as well as the third electrode GAC, turned into dark grey at room temperature (27 ± 1.5°C). The influent was renewed two times within 24 h to fast growth microorganism in the reactor. In the 3D-BER system, sustain an anaerobic condition to expand denitrifying bacteria. By observing the total nitrogen (TN) concentration in the reactor, when TN was exhausted, the residual substrate was removed, and fresh substrate was fed to the reactor to avoid endogenous metabolism of the microorganism.

2.3.3 Analytical Methods and calculations

On a UV-Visible spectrophotometer (Shimadzu UV-1800, Japan), the nitrate$(N-NO_3^-)$, was measured by an U.V-spectrophotometric method at wavelength λ = 220-275 ×2 nm. Nitrite $(N-NO_2^-)$, was measured by N-(1-naphthyl) ethylenediamine dihydrochloride spectrophotometric method at λ = 540nm, and total nitrogen (TN). $N-NH_4^+$ was measured by Nessler's reagent colorimetric method using a UV−visible spectrophotometer at λ = 420 nm, respectively [34]. The pH and ORP were measured by a pH meter: pH100 delivered by shanghai Yoke Instrument Co., Ltd. China). Although the monitoring of the temperature during the experiments installed an insertion thermometer in the reactor. All samples were stored at 4°C prior to the analysis. At least three samples were taken for each test. Current and voltage were recorded every 60 sec to control the pH, bacterial metabolism, and hydrolysis process. The removal

performance of nitrate and CIP antibiotics was calculated based on the percentages of nitrate reduction and CIP as follows Eqs. (5) and (6):

$$\text{The removal efficiency of Nitrates reduction (\%)} = \frac{\text{Nitrate in} - \text{Nitrate out}}{\text{Nitrate in}} \times 100 \quad (4)$$

Where "in" is the initial concentration of nitrate, and the "out" is the final concentration of nitrate at time t.

$$\text{The removal efficiency of CIP reduction (\%)} = \frac{\text{CIP in} - \text{CIP out}}{\text{CIP in}} \times 100 \quad (5)$$

Where "in" and "out" are indications of CIP antibiotics concentration in the inlet and the outlet of the 3D-BERS, respectively.

2.4. Ciprofloxacin concentrations measurement and by-products identification

CIP detection wavelength was set at 277 nm was determined by high-performance liquid chromatography (HPLC, Tokyo, Japan) [35]. The CIP separation using a Phenomenex column of asymmetry column, C18 (3.5 µm x 4.6 mm x 250 mm). The mobile phase was composed of acetonitrile and 0.01mol.L^{-1} tetra-n-butyl ammonium bromide ($C_{16}H_{36}BrN$) solution, trimethylamine at pH 3.0, and methanol with the ratio of 88:12 v/v at flow rate of 1.0 mL min^{-1}.The amount and processing time of the injection were 10 µL and 7.244 min, respectively. In the procedure, the reaction solution was extracted with a syringe needle, which was then directly determined by HPLC [35]. The biodegradation of CIP was monitored with mass-spectrometry (SCIEX Triple Quad™ 7500 LC-MS/MS: California, USA). The extracts were analyzed by a gradient method (0.3ml.L^{-1}) using an Acclaim^{120} column C18 (2.1 x 150mm x 2 µm) under two mobile phases. In phase A: water containing 0.1% formic acid and in the meantime in phase B-acetonitrile. To assess biodegradation products, thorough analysis of positive and negative ion modes was used. However, the molecular ions observed in positive and negative ions were subsequently applied in electrospray ionization techniques to provide a chromatographic shape of these putative biodegradation products. In order to extract LC-MS/MS spectra of the points determined in the final step, high collision energy was exclusively adjusted, and ion chromatographic response intensity was optimized in the range of 45 eV. From the literature, some researchers confirmed that the BES could improve the mechanism of antibiotic removal from the WWTPs due to adsorption techniques and which is a very economical system. The growth medium of the microbial community utilized the energy, C/N source [36,37]. However, the external calibration curve was used to determine the ciprofloxacin antibiotics concentration with a correlation coefficient (R^2= 0.985).

2.5. Statistical data analysis

All sample data were analyzed using Microsoft Excel (2018) and also used with Origin pro 8.5 and IBM SPSS (v. 20) for the statistical analysis. The final results were expressed as mean ± standard error values, and were considered statistically significant standards of $p<0.05$. For all biological studies, three independent biological tests were performed under similar conditions and replicated appropriately by HPLC: LC-MS/MS.

2.6. DNA Sequencing and PCR amplification

The biofilm samples were collected from the 3D-BERs (anode /cathode) layers (1.0g) and effluent (100 mL) after long-term acclimation at different C/N ratios. The amplicons were isolated from 1.0% agarose gels and purified using DNA-extraction from the PowerSoil DNA® Isolation Kit (Waters, Germany: MOBIO), was analyzed by the Quanti-Fluor™ St-Fluorometer (Madison, WI53711-5399 USA). The amplification of the polymerase chain reaction (PCR) was carried out by Macrogen Inc. (Seoul, Republic of Korea) using standardized prokaryotic primers targeting the V3-V4 region (341F-805R), as previously described [38]. PCR was conducted out in 30μL reaction with 15 μL of Phusion® High-Fidelity PCR Master-Mix (USA); 0.2 μM of forwarding and reverse primers, and about 10 ng template DNA. Initial denaturation at 98°C for 1.5 min, denaturation at 98°C for 30 sec, annealing at 50°C for 30 sec, and 30 extension cycles at 72°C for 30 sec. Build a small fragment library using single-ended sequences that sequence on the IonS5TMXL sequencing platform, then cluster operational taxonomic units (OTUs) by cutting and filtering readings [1,2,39] Species annotation and abundance analysis can reveal the species composition of GAC biofilm samples. Finally, the library was sequenced on the Ion S5TMXL platform to obtain a 400bp-to-600bp single-ended reading. The biofilm samples were analyzed by Beijing Novogene: Genome sequencing company Co., Ltd, China.

2.7. Biofilms GAC samples analysis by SEM

The two biofilms samples of S1 and S2 particle electrodes surface morphology for bacteria in GAC-biofilms were examined by scanning electron microscopy (SEM). Prior to SEM analysis, a series of batch experimental sample processing techniques (i.e., fixed, washed, dehydrated, dried, coating, etc.) was applied. About 0.8 g of GAC biofilm samples were placed in a centrifugal tube (C.T) of 10 mL, and 6000 revolutions/separation 5 for min. Then, the supernatant was removed, then mixed with a sediment sample at the bottom of the C.T was added 7ml of 2.5% glutaraldehyde. Place the C.T in the refrigerator at 4°C for 3 to 4 h, and protected the sample. The tube was centrifuged to centrifuge at 6000 rpm for 5 min, to remove the supernatant after the centrifugation process. Add

about 7 mL of ultrapure water to the GAC biofilm sample left at the bottom of the C.T, then the centrifuge again for 5 min at 6000 rpm, then remove the top liquid. This clean washing was made three times by the centrifugation process [21].

Next stage, wash the GAC biofilm dry and dwelling 7 mL of 20%, 40%, 50%, 70%, 80% and 90% ethanol for 10 min each. Biofilm samples were dehydrated three times, each time about 7 mL of ethanol (100%) for 15 min. Though, a 7 mL blend of ethanol and acetyl (Ethanol: Isoamyl acetate ratio = 1: 1) was discharged into the C.T for 15 min. Finally, place the 10 mL samples on filter paper and dried in a vacuum dryer (Mecha-Tech system Ltd, UK), for 24 h at a precooling temperature of 10 ^{o}C [23,40]. Then coating (10 nm gold layer) sample was examined with (Hitachi S-4300 FEG-SEM), and the images were collected digitally. The SEM images are shown in **Figure. 2** the morphology of biofilm attached to granular activated carbon (GAC) particle electrodes in the 3D-BERs. The GAC had a high specific area about 450-900 m^2g^{-1} and a porous structure to facilitate the growth of microbial attachment as reported [20,28,41]. The biofilm samples of the S2 were abundant compared to the S1, corresponding to the thicker bacterial community at 60 mA [42]. In the S1 zone, the microbes are less in quantity due to lacking electron donors. It was found that in the 3D-BERs with GAC, biofilm was imbedded in the abundance of microbial activity [41]. It was clearly observed in rod-shaped graphite anode and cathode, which was authentic common in the other denitrification system [20,43]. There also existed huge microbes, which may be hydrogen-heterotrophic denitrifying bacteria.

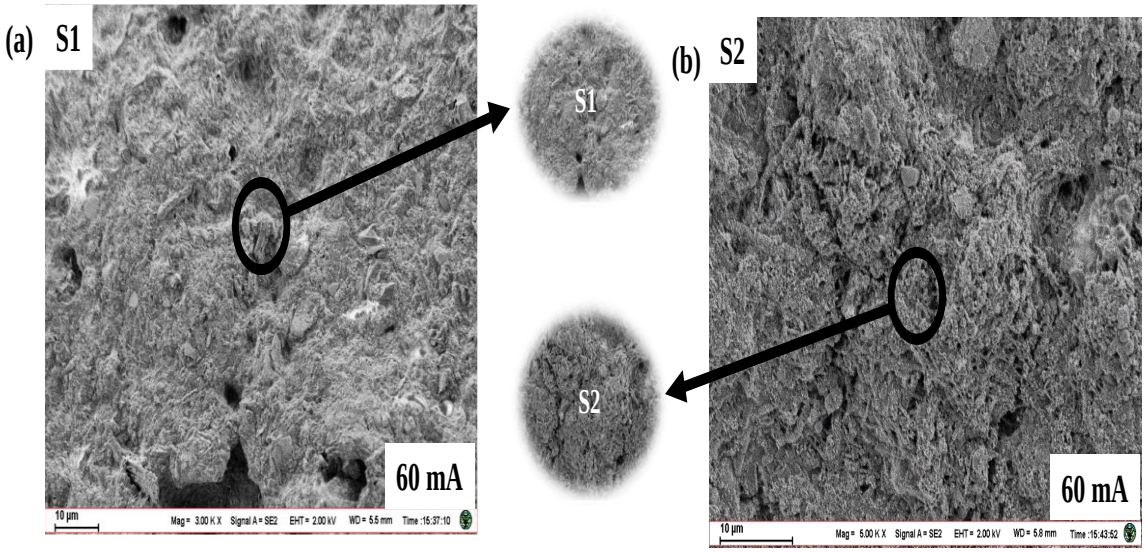

Figure 2. SEM images of S1 (a) and S2 (b) biofilm GAC samples at current intensity 60 mA in the 3D-BERs

2.8. Illumina-MiSeq sequencing

The 16R r DNA gene sequence obtained from Illumina-MiSeq at Novogene Co., Ltd., Beijing, China. Using this program, truncate the adapter with low-quality sequences <Q20 and short <300-bp were trimmed. Disposing of potential chimeric sequences using the Mothur chimeric UCHIME algorithm. The taxonomic classification of the sequences was conducted using the cutting and filtering readings classifier. The observed operational classifiers' richness (OTU), diversity index such as ChaoI and abundance coverage estimator (ACE) are determined by the Mothur function [39,44].

3. Results and discussion

3.1. Removal performance and the effects of C/N ratios

This analysis shows the average nitrogen concentration (i.e., NO_3^--N, NO_2^--N, NH_3-N, and TN) in the effluent under different C/N ratio during the batch experiments (i.e., I = 60 mA and pH 6.0-8.0) as shown in **Figure. 3.** In the influent, the NO_3^--N concentration remains relatively stable at 30 mgL^{-1}. When the C/N ratio increases from 0 to1.5 and 2.5 to 3.5, the average NO_3^--N concentration of the influent falls from 26.09 mgL^{-1} to 0.9177 mgL^{-1} and 11.96 mgL^{-1} to 0.199 mgL^{-1}, respectively. Moreover, the NO_3^--N concentration in the final effluent when the ideal condition of C/N ratio of 1.5-3.5, and the final concentration were 0.916 mgL^{-1} - 0.199 mgL^{-1} as shown in **Figure. 3c** and **Figure. 3g**, respectively. After 30 min, the nitrates concentration dropped rapidly, indicating a significant increase in denitrifying bacteria [19], and the anoxic condition had achieved [43]. It was observed that the removal efficiency of nitrogen increases with the amplified C/N ratios.

In particular, the denitrification performance of 3D-BERs with a current intensity of 60 mA was slightly higher in the system was compared with published previous work, as shown in **Table 3**. When the ratio of C/N increased from 0 to 1.5 and 2.5 to 3.5, the average concentration of total nitrogen in wastewater decreased from 29.26 mgL^{-1} to 0.955 mgL^{-1} and from 17.26 mgL^{-1} to 0.651 mgL^{-1}, respectively. However, TN average removal efficiency of 3D-BERS was 35.60 to 63.90%, 70.59 to 79.58%, 96.81 to 90.83% and 90.83 to 97.82 % when the C/N from 0 to 0.5, 1 to 1.5, 2.5 to 3.0 and 3.0 to 3.5 respectively ($p < 0.05$) [45]. Similar results were also reported [18,28,46]. In 3D-BERS, the average removal efficiency was respectively 96.81 % and 97.82 % when C/N ratio was 1.5 and 3.5. At the same time, as increased the ratio of C/N from 0 to 1.5 and 2.5 to 3.5, the average NO_2^-N concentration decreased from 2.52 mgL^{-1} to 0.44 mgL^{-1} and 1.22 mgL^{-1} to 0.398 mgL^{-1}, respectively.

In addition, the C/N ratio was 1.5 and 3.5; the final effluent concentrations respectively were 0.44 mgL^{-1} and 0.39 mgL^{-1}. However, under these conditions, the initial NO_3^--N loading of denitrification was high, so NO_2^--N in the wastewater remains almost zero and constant [19,45]. In addition, the final effluent of NH_3-N in the 3D-BER system decreased from 4.12 mgL^{-1} to 2.29 mgL^{-1}, 4.06 mgL^{-1} to 1.49 mgL^{-1}, and the different C/N ratios from 0 to 1.5 and 2.5 to 3.5, and the average NH_3-N concentration in the final effluent was 4.06 mgL^{-1} to 1.49 mgL^{-1}, and the C/N ratio was 1.5 to 3.5, compared to other techniques previously studied [27,43,45].

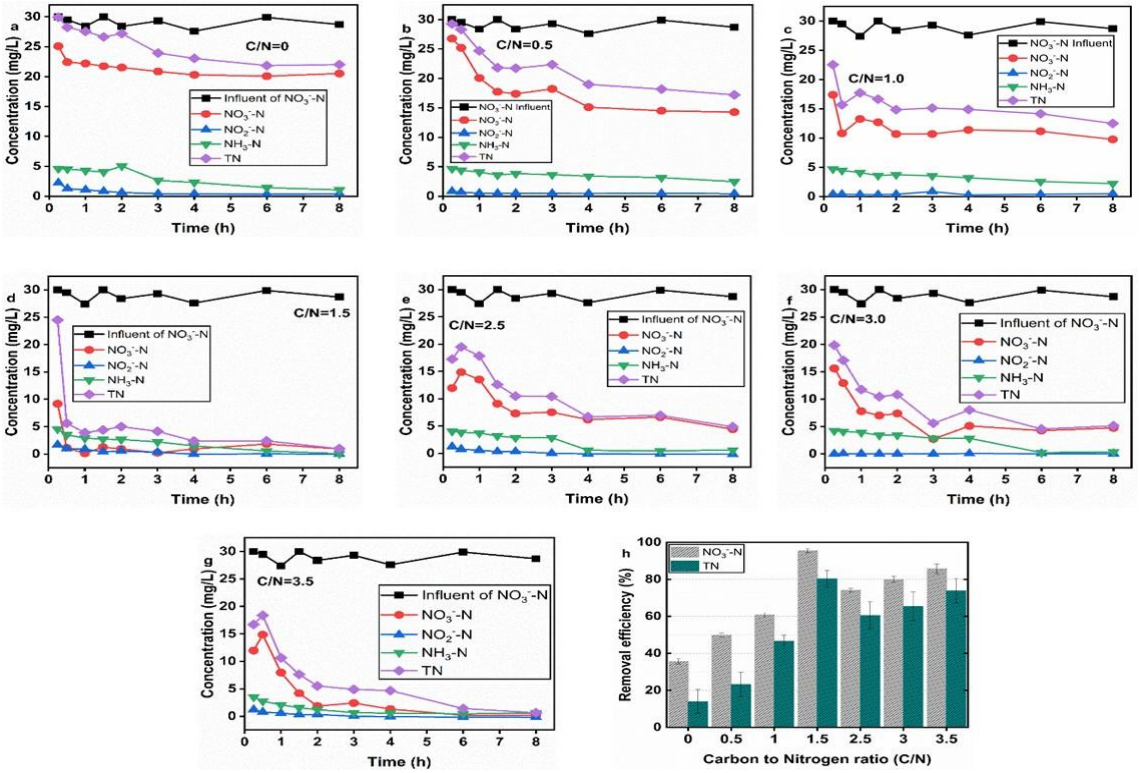

Figure 3. The average effluent concentration of $NO_3^- - N, NO_2^- - N, NH_3 - N$ and TN removal performance and effects on 3D-BERS denitrification at different C/N ratio (i.e., experimental condition: I=60 mA and pH = 5.5-8.0)

Organic carbon sources are known to be limiting factors for the heterotrophic denitrification. The removal of nitrate and total nitrogen increases as the ratio of C/N increases [22]. At maximum C/N ratio endorses the development of heterotrophic-denitrifying microorganisms, allowing denitrification [21,22,47]. If there is a shortage of organic carbon during this period, nitrogen removal will usually decrease, and the NO_2^--N reductase competes with the electron donor nitrate reductase at a low level of C/N ratio. Reductase of nitrite is more complex and

sensitive to the experimental conditions compared to nitrate reductase, resulting in accumulation of NO_2^--N[22].

Autotrophic denitrifiers were dominated by low concentration of C/N ratio required to extended adaptation period with the rapid growth, and had further accumulation of NO_2^--N [41]. As the C/N ratio increased between 0.5 and 1.5, the effluent NO_2^--N reduced rapidly 2.52 mgL^{-1} to 0.242 mgL^{-1}. At ratio C/N=1.5, then NO_2^--N continues to decline at lower levels. Heterotrophic denitrification may play a pivotal role at an advanced level of the C/N ratio, and the rate of denitrification was high. When C/N ratio was 1.5 and 3.5, the removal efficiency increased by 95.53%−85.73% and 80.27%−73.85% for NO_3^--N and TN. However, NH_3-N tends to be opposite to NO_3^--N and TN. These results are in good compared with previous findings [19,21,22]. The NO_3^- was commonly used as an acceptor of electrons and could be reduced to NH_4^+ under anoxic and electron-acceptor conditions [20,43].

The 3D-BER system was an anoxic condition, and the force of the electric field prevents NO_3^- from moving to the surface of the anode, so there may be no electron acceptors near the surface of the cathode. In this study, ratio C/N is very close to the complete denitrification in the range of 1.5 to 3.5 and is established by single heterotrophic denitrification [27,29,48]. Several scientific studies reported on the elimination of nitrogen were conducted by autotrophic denitrification[47,49]. This method, elimination of NO_3^--N, NH_3-N and total nitrogen increased while accumulation of NO_2^--N decreased gradually. In this study, the complete denitrification was obtained in C/N= 1.5 and may be due to system temperature and denitrification removal rate. However, a similar observation was reported by other researchers shown in **Table 3** [41,48].

1

2 **Table 3.** Comparison from the literature of antibiotic Ciprofloxacin removal and denitrification efficiency with previous studies of BER and 3D-

3 BERS

Reactor Type	**Mode**	**H/A Denitrification**	**Treatment Source**	**Effective volume (L)**	**Initial (NO3-N) (mg/L)**	**C/N source**	**Power source**	**Input energy**	**Denitrification removal rate (%)**	**Ciprofloxacin Removal (%)**	**Country**	**References**
Three-dimensional bioelectrochemical reactor system (3D-BERS)	Batch	H&A	Synthetic municipal wastewater	0.738	30 mg/L	CH3COONa	DC	60 mA	95.53	94.20	China	This Study

Bioelectrochemical System (BES),	Batch	H	Synthetic groundwater	1.0	250 g/m^{3}day	CH3COONa	DC	N/A	14.6±0.2 36.2±5.0	N/A	USA	[25]
Three-dimensional biofilm-electrode reactor (3D-BER)	Continuous	H&A	Synthetic municipal wastewater	3.4	30 mg/L	CH3COONa	DC	40 mA	98.3	N/A	China	[18]
A three-dimensional BER	Continuous	H&A	Synthetic groundwater	0.5	20 mg/L	C2H5OH	DC	15mA	100	N/A	China	[22]
A combined single-chamber MFC and BER system	Continuous	H&A	Synthetic ground water	0.480	364 mg/L	CH3OH	MFC	500-700 mV	30	N/A	China	[39]
Bioreactor	Conti	H&A	Synthetic	0.9	30mg/L	C6H12O6	----------	------	N/A	20-22	Singapore	[50]

	nu ous		municip al wastew ater									
A combined BES and sulfur autotrophic denitrificati on system (CBSAD)	Co nti nu ous	A	Syntheti c ground water	33.47	20.9-22.0mg NO3-N/L	Hydro gen	DC	1000 mA	95	N/ A	Chin a	[51]
Fe-C micro-electrolysis reactor and up-flow biological aerated filter (UBAF)	Co nti nu ous	H&A	Syntheti c municip al wastew ater	15	60-100 mg/L	Fe-C micro-electro lysis	N/ A	N/A	N/A	90	Chin a	[52]
Three-dimensional biofilm elec	Bat ch	A	Syntheti c	2	50-100 mg/L	Hydro gen & α-	DC	20-80m A	95 to 98.9	N/ A	Chin a	[20]

trode reacto rs (3D-BERs)		wastew ater	Fe2O3

4 Noted: H/A = Heterotrophic /Autotrophic denitrification; DC= Direct Current; C/N= Carbon to Nitrogen ratio; CIP= Ciprofloxacin

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3.2. Effects of pH on antibiotics CIP, nitrate and total nitrogen removal

As shown in **Figure. 4**, the influences of various pH values on the average concentration of nitrogen and ciprofloxacin (Total nitrogen, NO_3^--N, and CIP) during the C/N ratio in batch process from 0 to 3.5 and electrical current intensity was I= 60 mA. A series of batch experiments were conducted in 3D-BERs for seventy days of operation in different C/N ratios to evaluate the NO_3^--N and CIP removal. In the novel 3D-BERs, the elimination of NO_3^--N decreased from 25.07 mgL^{-1} to 9.18 $mgL^{-1}$and 4.41 mgL^{-1} to 0.199 mgL^{-1}, and for CIP reduced from 377.5 μgL^{-1} to 106.36 μgL^{-1} and 115.9 μgL^{-1} to 106.3 μgL^{-1} while the C/N between 0 to 1.5 and 2.5 to 3.5 at the pH range of 6.0 to 7.5 [19,20,41]. Under those conditions, the maximum removal efficiencies of NO_3^--N, TN, and CIP were found to be 35.66 to 95.55%, 13.93 to 80.27%, and 12.59 to 94.20 %, while the C/N ratio between 0 to 1.5 and 2.5 to 3.5 at electric current intensity 60 mA, respectively [19,47,53]. The maximum antibiotic CIP removal efficiency could be reached 84.61% and 94.20% when C/N was 3.5 and 1.5 at the pH value of 7.0 to 7.5. In this system the highest removal rate was at pH 7.5 as compared to pH value of 6.7 to 8 .0 [39,41]. However, pH played a pivotal role in ciprofloxacin degradation and gradually NO_3^--N removal. It was observed that the average concentration of CIP in influent was decreased from 377 to 106.36 μgL^{-1} and 115.9 to 116.99 μgL^{-1} while the C/N ratio different from 0 to 1.5 and 2.5 to 3.5. At the C/N = 1.5 and 3.5, the final effluent of CIP concentrations was 106.36 μgL^{-1} and 116.99 μgL^{-1}, respectively as shown in **Figure. 5** [48,54,55]. It was described that denitrification inhibited with CIP and development more obvious during the prolonged microbial cultivation period was reported [6,56,57].

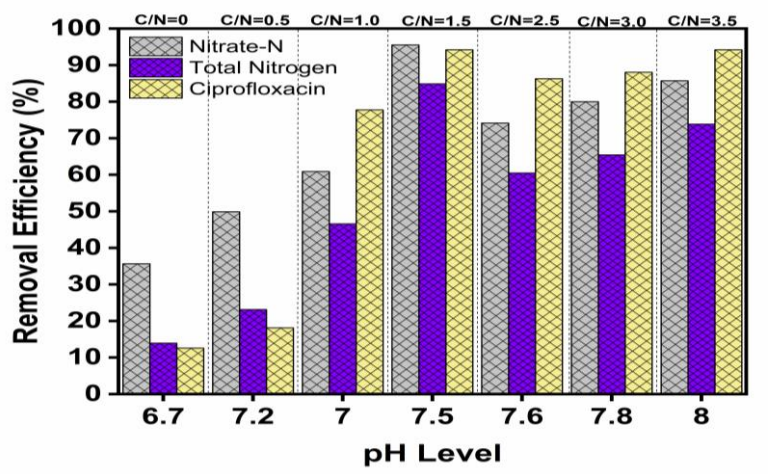

Figure 4. Effects of pH on antibiotics ciprofloxacin and nitrates removal under different pH levels and C/N ratios in the 3D-BER System.

Though, the pH was an essential factor in the denitrification process and removal of antibiotics. For most denitrifying bacteria, the ideal pH was 7.0-8.2, and previous researchers reported that pH below 6.0 or 9.0 could be immobile heterotrophic and autotrophic denitrification [39,47]. Although pH=7.5, antibiotic CIP, NO_3^--N, and total nitrogen (TN) achieved 106.36 μgL^{-1}, 0.917 mgL-1, and 0.024 mgL^{-1}, respectively, and the final effluent amount was lowest level. The CIP was entirely removed within 16 h of batch experiments at the average removal efficiency of 94.20 ± 0.5% was reported [10] and observed in this study. Furthermore, the highest average removal efficiency of CIP, TN, and NO_3^--N reached 94.20 %, 80.27 %, and 95.53 %, and the enhanced removal efficiency was obtained in 3D-BERS.

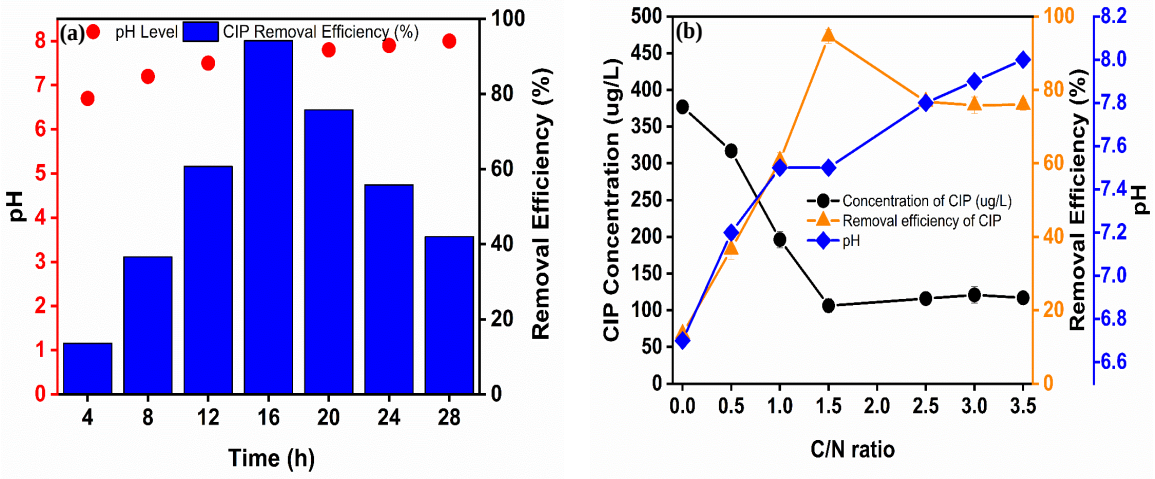

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Figure 5. The removal efficiency of (a) CIP antibiotics, and (b) concentration in the 3D-BERS (i.e. experimental conditions: C/N=0−3.5, pH=6.0−8.2)

3.3. Effect of biodegradation mechanism of antibiotic ciprofloxacin

Generally, the ideal pH for Autotrophic-Heterotrophic denitrification systems was chosen to be 7.5, because recent reports have shown that increasing antibiotics CIP levels can significantly reduce the performance of autotrophic denitrification systems [6,57]. For another factor that was selected as the ultimate circumstance, the final result under optimal conditions was pH = 7.5. Therefore, the latest novel 3D-BER system was introduced to enhance the denitrification process and achieves the highest removal efficiency of nitrogen. However, **Eq.1** and **Eq.3** were initiated to indicate that the pH illustrates by the OH^- produced during the denitrification process and water electrolysis. In our research work, we found that the pH level of the final effluent maintained at 7.5 ± 0.2[46,49]. Moreover, it indicates that CO_2 produced by the graphite anode is not only dissolved in the carbonate but also dissolved in the hydrogen carbonate, as shown in **Eq. 3**, and reacts with OH^- so that the pH in following equations shows:

$$CO_2 + H_2 \rightarrow H_2CO_3 \quad (6)$$

$$H_2CO_3 + OH^- \rightarrow H_2O + HCO_3^- \quad (7)$$

$$HCO_3^- + OH^- \rightarrow H_2O + CO_3^{2-} \quad (8)$$

The pH of the system was always in a favourable condition for the biological activity of the denitrifying bacteria. Some scientific research suggests that CIP was predominantly eliminated by adsorption during biological wastewater treatment [7,13]. Previous publications demonstrated that CIP is difficult to operate in the environment and is hardly degraded by biological processes [9]. Sludge adsorption is the main route to remove ciprofloxacin in wastewater treatment [8,26,50]. According to novel research by Li and Zhang et.al shown that the initial CIP concentration of 100 μgL^{-1} after 48 h of incubation with a mixture collected from the aeration tank of a biological wastewater treatment plant treated with saline sewage and a removal capacity of about 32.2% [26]. However, some work on the biodegradation of CIP by biological wastewater treatment processes is significant. The effect of degradation mechanism of antibiotics ciprofloxacin by changes in the

microbial community [9,50,57]. For this research work, the CIP biodegradation was investigated by a series of batch experiments using nitrified sludge anoxic sludge, and the operating system was 8 h at different C/N ratios. Although the anaerobic sludge is very effective for the biodegradation of CIP, with a concentration of 500 μgL^{-1} in the 3D-BER system.

3.4. Identification of degradation pathways by LC-MS/MS

After solid-phase extraction (SPE in Germany), biodegradation experiments of antibiotic ciprofloxacin were performed by LC-MS/MS. Nevertheless, the LC-MS/MS identified a total of four CIP biotransformation products by (LC-MS/MS: LCQAD-6460, Agilent Technologies, Santa Clara, CA 95051, USA) (i.e., the molecular structure, intermediates products were revealed in **Table S1**, and the total ion chromatogram (TIC) was revealed in **Figure. S1**). During the several batch experiments, the four possible biodegradation pathways of antibiotic ciprofloxacin were suggested, as shown in **Figure. 6**. Moreover, in this pathway, C_2H_2 having a piperazinyl substituent of CIP is removed by demethylations to generate CIP-BBP1 at the pathway point "S" and transferred to CIP-BBP4 at the conduit point "Q" by loss of the C_2H_5N fragment of CIP-BBP1 (Table S1). While the biodegradation of CIP intermediates products was CIP-BBP1 and CIP-BBP4 previously reported [16,58], but almost similar products were also identified in 3D-BERS. In the first time of this study, these products were investigated and have not been listed in the literature. In pathway at point "S," CIP-BBP1 is shaped via hydroxylation (-OH) at the CIP-BBP1-S position, and again CIP-BBP4 via hydroxylation at the CIP-BBP4-Q position by replacing the piperazinyl group of CIP-BBP1-Converted to CIP-BBP4 as presented in **Table S1**. In P and Q-points pathways, CIP-BBP2 and CIP-BBP4 were instigated by substituting the fluorine at the P-position of CIP [15] and the carboxyl group at the Q-position of CIP by hydroxylation, respectively. Meanwhile, the biodegradation of CIP by acetylation of the NH group of piperazine rings having inactivation of enzyme culture, i.e., the CIP-acetylation process [59]. Therefore, the 4-pathways is divided into two main reactions: Piperazinyl-substituent decomposition reactions (a), and hydroxylation reactions (b). **Figure S1.** indicated that the biodegradation mechanism of antibiotic CIP was proposed in the 3D-BER system. Almost CIP are converted to four products mainly by degradation and hydroxylation of the piperazinyl substituent.

According to Pearson's bivariate correlation analysis, CIP biodegradation was significantly correlated ($p < 0.01$, $R^2 = 0.996$). To better describe the CIP metabolic degradation pathways [4,23] at the gene and predominant enzyme levels, superior isolates of ciprofloxacin in anaerobic sludge can be used by transcriptomics and metagenomics studies [31,59]. The information obtained from LC-MS/MS was insufficient for the fully characterize and the chemical structure of these metabolites. Whereas, the realistic standards will be used in future work to confirm the formation of Ciprofloxacin biotransformation pathways.

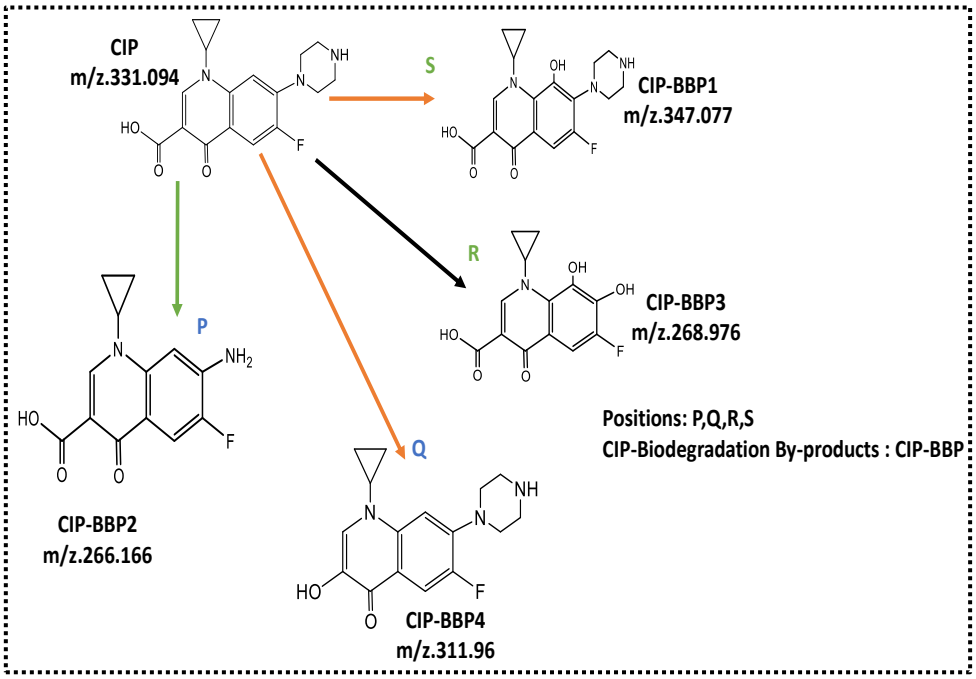

Figure 6. Proposed chemical structure of intermediate products and possible pathways of CIP biodegradation in the novel 3D-BERS by LC-MS/MS

3.5. Bacterial diversity and community composition

Evolution of ciprofloxacin degradation and denitrifying bacteria under long-term acclimation from low C/N wastewater. Summarizes in **Table 4** the microbial species richness and diversity indices for these two samples. The high-throughput sequence analysis of 63,376 classifiable sequences obtained from S2 samples belonged to 35 class, 21 phyla, and 205 genera. The 73,382 classification sequences in S1 samples were 39 class, 23 phyla, and it belonged to 195 genera. **Figure 7.** illustrates the phylogenetic classification of phylum and class level sequences for two main samples at different batch operations. *Proteobacteria, Bacteroidetes,* and *Firmicutes* were identified as predominant phyla in

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S2 and S1, but their relative abundance was different in each sample (**Figure 7a**). Although in the samples S2 and S1, bacteria dominated, the bacteria in the sample S2 were more abundant than the S1 samples. According to the contract, the number of S1samples of *Firmicutes* (1.8%) was obtained in less abundant [15], than in sample S2 (5.6%). In the taxonomic classification, the relative abundance of class levels was shown in **Figure 7b**.

Table 4. Diversity indices of bacterial communities in S1 and S2 sludge samples of 3D-BERS at different C/N ratio and applied electric current 60 mA

Sample ID	No.of reads	No.of OTUs	ACE	Shannon	Simpson	Chao1	Coverage
S1	73282	302	330.705	4.367	0.838	319.22	0.999
S2	62324	353	363.642	5.247	0.936	361.571	0.999

However, the average richness of sample S1 is less than 2.0% for the classes of *Gammaproteobacteria* (50.93%), *Alphaproteobacteria* (24.24%), *Bacteroidia* (3.46%), *Bacillus* (2.51%), *Deltaproteobacteria* (4.17%) and Clostridia (1.31%), whereas, those in S2 sample were *Alphaproteobacteria* (29.37%), *Gammaproteobacteria* (70.84%), *Bacteroidia* (1.87%), *Deltaproteobacteria* (17.52%), *Bacilli* (1.22%) and *Clostridia* (11.47%) [2,59]. It was evident that the distribution of the main classes in these two samples was significantly different. Nevertheless, the amount of *Gammaproteobacteria* present in sample S2 was more significant than that of sample S1 compared to the distribution of *Alphaproteobacteria*, *Bacteroidia*, *Deltaproteobacteria*, *Bacillus*, *Actinobacteria* and *Clostridia* [1,3], in the S1 sample was relatively low compared to the sample S2 anaerobic sludge from the 3D-BER system.

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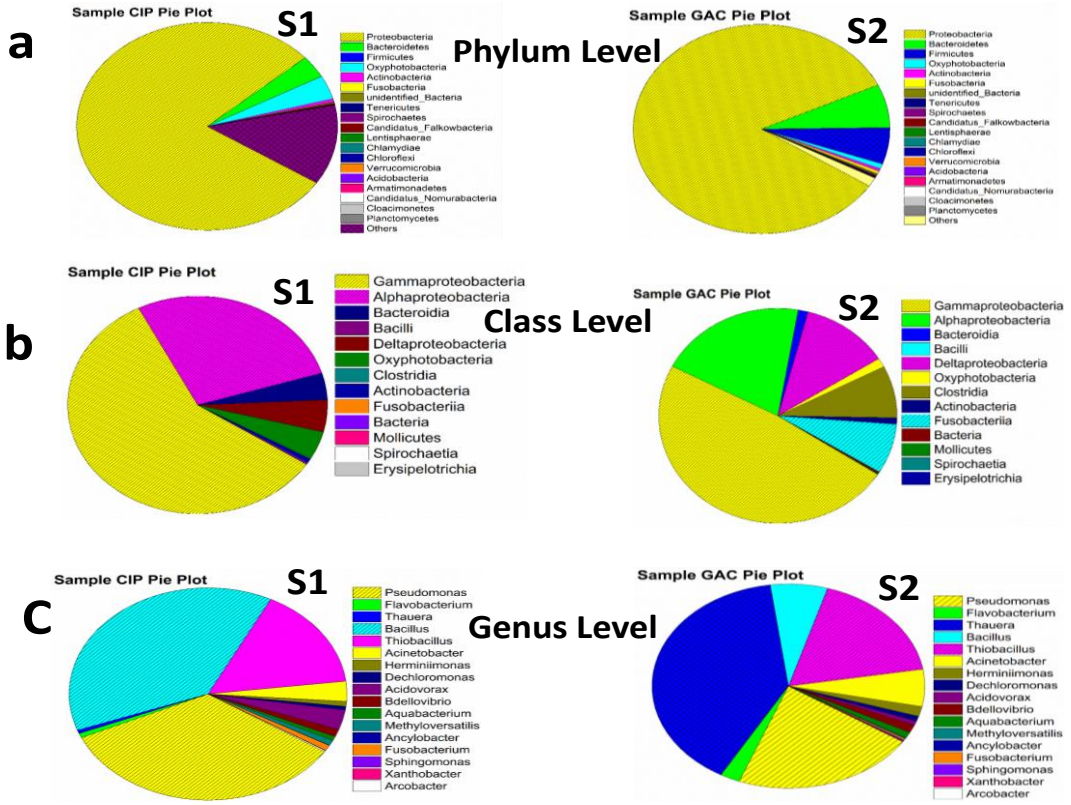

Figure 7. Bacterial community composition of relative abundance at (a) phylum, (b) class, and (c) genus levels at different C/N ratios and constant electric current I= 60 mA in the 3D-BER System

For the understanding of the similarities and some variations between sample S1 and S2, shown in **Figure 7c** that the genus levels of more than 65 abundant genera. In the abundant genus with a relative richness of more than 2.5%, the abundance of *Pseudomonas* was significantly higher in the sample S1 (50.93%) [9], while the relative abundance of the sample S2 was only 35.44% [4,57]. In general, it has been widely found in previous studies (such as *Pseudomonas aeruginosa*), especially in terms of the possibility of denitrification [19,60]. Also, *Pseudomonas* stutzeri [41,61], and the certain type of *Pseudomonas* bacteria belonging to the genus *Pseudomonas sp. C27* have autotrophic and heterotrophic denitrifiers that utilize an assortment of electron donors *[40]*. As the C/N ratio increases, the impact on bacteria output involved in autotrophic denitrification, the microbial growth yield of denitrifying autotrophic microbes is smaller than that of heterotrophic bacteria. Despite a gradual increase in the C/N ratio, the denitrifying autohydrogenotrophic bacteria may have been gradually domesticated to heterotrophic denitrifying bacteria for effective nitrates and antibiotic CIP

elimination. Therefore, *Pseudomonas* abundance in sample S1 indicates that overall similarity to autotrophic and heterotrophic denitrifying species is related to *Pseudomonas* in the occurrence of organic substance and applied electrical currents. Moreover, significantly higher amounts of *Thauera* were detected in sample S2 (64.66%) compared to sample S1 (0.80%). In contrast to S2 samples, *Bacillus* (11%), *Thiobacillus* (28.8%), *Flavobacterium* (3.81%) [4] and *Acinetobacter* (9.59%) are the most important genus of S2 samples, but the abundance of S1 example was *Thiobacillus* (23.34%), *Bacillus* (5.6%), *Acinetobacter* (4.53%) and *Flavobacterium* (0.99%) [2,52]. *Thiobacillus* has been identified as a widespread autotrophic bacterium in recent years, closely related to the oxidation of nitrates to nitrogen.[40,49]. In recent years, several spices belonging to the genus *Thiobacillus*, *Bacillus,* and *Thauera* have been suggested to play an important role in antibiotic CIP and removal of nitrates [57]. Whereas, *Thiobacillus* and *Thauera* have the capability to transfer electron directly from the carbon electrode and promote nitrate reduction [16,59]. The richness and abundance of *Pseudomonas* (35.4%), *Thiobacillus* (28.88%), and *Thauera* (64.66%) [24,62] were enriched in the 3D-BER system. The removal of CIP and nitrates was due to the distinction between the dominant *Thauera, Pseudomonas,* and *Thiobacillus,* which helped to merge heterotrophic denitrification and autotrophic denitrification processes. In conclusion, the main bacteria enhanced in the third electrode (GAC) will continue in both heterotrophic-autotrophic denitrifications, whereas enriched bacteria in S1 are primarily involved in autotrophic denitrification. Therefore, further research is needed to characterize the denitrifying bacteria in the 3D-BER system.

4. Conclusion

In this study, the simultaneous removal of ciprofloxacin and nitrates from Low C/N wastewater by a novel 3D-BER System. It has proved to be more economically and technically attractive than traditional denitrification process due to the more than 90% reduction in antibiotics, 55% sludge production. However, the removal performance increased significantly with increasing C/N ratios and maximum removal efficiency of antibiotics CIP, NO_3^--N and TN were obtained from the ranged 12.59% to 94.20%, 35.66% to 85.73% and 13.93% to 73.85% % at different condition of pH 6.7 to 8.0, *I*=60 mA, C/N 0 to 3.5, respectively. Perhaps some resistant bacteria could be used antibiotic CIP or dead biomass as an organic substrate for the better growth of microbial activities

in an anaerobic condition. MiSeq-Illumina throughput pyrosequencing was used to analyse the microbial community of samples S1 and S2 under mixotrophic conditions. The S2 sample was enriched with a high richness of *Pseudomonas* (35.44%), capable of autotrophic-heterotrophic denitrification, although it contains *Thiobacillus* (28.82%) and *Bacillus* (11%) predominant autotrophic bacteria. It could be concluded that the novel 3D-BER system was a feasible technology to exacerbate antibiotic CIP biodegradation and low denitrification impact of low C/N wastewater treatment. The structure of CIP-degrading microbial communities at different C/N ratios, obviously varied with CIP biodegradation. However, future research should involve the appropriate, efficient removal of nitrogen and antibiotic CIP biodegradation mechanism in the novel 3D-BER system.

Declaration of Competing Interest

The authors declare no conflict of interest.

Author Contributions:

Mahdi Hassan: Formal analysis, Data curation, Investigation, Methodology, Writing-original draft, Writing-review & editing. **Guangcan Zhu:** Funding acquisition, Supervision, Project administration, Investigation, Resources. **Yongze Lu:** Project administration, Validation. **Yuan Lu:** Investigation, Writing-review & editing. **Yan Lang:** Revised it critically for important intellectual facts. **Liying Gong:** Investigation, Data curation, Review & editing. **Shan Huang:** agreed to be accountable for all aspects of the work and also revised the manuscript.

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