Seasonal variations of pollutants removal and microbial activity in integrated constructed wetland–microbial fuel cell systems

Xiaoou Wang and Yimei Tian

ABSTRACT

This study investigated the seasonal variations of pollutants removal and microbial activity in constructed wetland–microbial fuel cell systems (CW–MFCs). The results showed that the atmospheric temperature significantly influenced the bioelectricity generation and removal of organics and nitrogen in CW–MFCs by primarily influencing the microbial enzymatic activity. The electricity output of CW-MFCs was extremely low below 5 °C, and reached the maximum above 25 °C. The organics and nitrogen removal of closed-circuit CW–MFC reached the highest in summer and autumn, followed by spring, and decreased by an average of 10.5% COD, 14.2% NH_3-N and 10.7% TN in winter, demonstrating smaller seasonal fluctuations compared to open-circuit CW–MFC in which the difference between summer and winter was 13.4% COD, 15.1% NH_3-N and 15.1% TN. Even at low temperatures, the MFC current could enhance the enzymatic activity and stabilize the growth of microorganisms on the electrodes, moreover, the closed circuit operation can promote the bacteria diversity on CW–MFC anodes as well as the abundance of electrogens on CW–MFC anodes and cathodes, and thus reduce the adverse effect of cooling on organics and nitrogen removal in CWs. However, neither MFC nor temperature had a significant influence on phosphorus removal in CW–MFCs.

Key words | constructed wetland, microbial activity, microbial fuel cell, nitrogen, organics, seasonal variation

Xiaoou Wang
Key Laboratory of Clean Energy Utilization and Pollutant Control in Tianjin, School of Energy and Environmental Engineering,
Hebei University of Technology,
Tianjin,
China

Yimei Tian (corresponding author)
School of Environmental Science and Engineering,
Tianjin University,
Tianjin,
China
E-mail: *tian43_b305@126.com*

HIGHLIGHTS

- Temperature significantly influenced CW-MFCs by primarily influencing microbial enzymatic activity.
- Closed-circuit CW-MFC showed smaller seasonal variations of organics and nitrogen removal than open-circuit CW-MFC.
- Closed circuit mode promoted bacteria diversity and electrogens' abundance on CW-MFC anodes, even at low temperatures.
- MFC reduced adverse effects of cooling on organics and nitrogen removal in CWs.

doi: 10.2166/wrd.2021.094

INTRODUCTION

Constructed wetlands (CWs) are an attractive ecological restoration technology, and have been applied in treating various wastewaters worldwide (Wu *et al.* 2011; Wang *et al.* 2017; Cao *et al.* 2019). Pollutants removal in CWs is accomplished through a variety of physical, chemical and biological processes, including sedimentation, interception, precipitation, filtration, adsorption, absorption, volatilization, plant uptake, and microbial degradation (Saeed & Sun 2012). Due to the dependency of biological and biochemical processes on temperature, many studies have reported poorer performance of pollutants removal in CWs under low temperatures. For example, Akratos & Tsihrintzis (2007) reported that the average removal rates of ammonia (NH_3-N) and total Kjeldahl nitrogen (TKN) in a pilot-scale horizontal-flow CW (HFCW) were 37.9 and 58.5% at temperatures of $<15\,^{\circ}C$, and 69.1 and 73.9% at temperatures of $>15\,^{\circ}C$, respectively. Song *et al.* (2006) reported that for a full-scale CW with a total area of 80 ha in Shandong, China, the average removal of 5-day biochemical oxygen demand (BOD_5), chemical oxygen demand (COD), NH_3-N and total phosphorus (TP) was lowest in winter (67.8, 59.4, 32.4 and 28.9%, respectively), and highest in summer (74.1, 66.3, 54.5 and 35.0%, respectively). Sani *et al.* (2013) evaluated the seasonal performance of a vertical-flow CW (VFCW), and significantly higher COD, NH_3-N, and nitrate (NO_{3-}-N) removals were recorded in summer. Saeed & Sun (2012) reported that temperatures between 16.5 and 32 °C favor nitrification in CWs, and nitrification barely occurs at temperatures of $\leq$5–6 and $\geq$40 °C; the suitable temperature for denitrification is 20–25 °C, and, at temperatures of <5 °C, denitrification proceeds very slowly.

Some measures have been taken to mitigate the adverse impacts of low temperature on CWs. For example, Ouellet-Plamondon *et al.* (2006) reported that in a reed CW artificial aeration improved the removal of TKN, NH_3-N and COD in winter by approximately 2.2, 29.4 and 7.5%, respectively. Wu *et al.* (2011) reported that a 0.4 m sawdust layer cover on the CW could keep the temperature inside the wetland constantly above 6 °C, even when the atmospheric temperature dropped to −8 °C during winter, which provided effective system thermal insulation and maintained high pollutants removal (95.0% BOD_5, 84.6% NH_3-N, and 88.2% TP) in the freezing winter period. Kadlec & Wallace (2009) reported that during the winter icing period, wetlands could be operated under a frozen layer by adjusting the water level, and a relatively high water temperature would be maintained inside the wetlands. Wang *et al.* (2017) summarized that hybrid CWs consisting of various types of CWs arranged in series possess higher treatment performance than a single CW in a cold climate.

The interest in integrated constructed wetland–microbial fuel cell (CW–MFC) systems has increased due to their ability to produce electricity and enhance the wastewater treatment efficiency (Doherty *et al.* 2015; Guadarrama-Pérez *et al.* 2019). Fang *et al.* (2013) found that an MFC could improve the decolorization rate and COD removal of a CW by 15 and 12.7%, respectively. Wang *et al.* (2016) reported that the MFC significantly promoted the relative abundance of beta-*Proteobacteria*, nitrobacteria, and denitrifying bacteria in the CW, and thus increased the average COD and NO_3^--N removal by 8.3 and 40.2%, respectively. Srivastava *et al.* (2015) reported that the MFC increased COD removal in a CW by 27–49%. Xu *et al.* (2018) reported that the rates of nitrification and denitrification increased by approximately 82% in a three-biocathode CW–MFC. Yu *et al.* (2020) found that the constructed wetland–microbial electrolysis cell (CW-MES) system successfully enhanced NH_3-N removal at low temperatures (5.6–7.9 °C), while CW-MFC did not exhibit a positive effect. Overall, few of the reported studies have referred to the seasonal variations of CW–MFCs, and the performance of CW–MFCs at low temperature should be further explored.

This study investigated the seasonal variations of pollutants removal and microbial activity in CW–MFCs, aiming to: (1) quantitatively analyze the decreasing degree of CW–MFCs performance caused by low temperature; (2) investigate whether MFC could still help in enhancing the pollutants removal in CWs at low temperature; and (3) investigate the variations of enzymatic activity and microbial community in CW–MFCs under different seasons, with the expectation of offering a reference for enhancing the treatment efficiency of CWs in a cold climate.

MATERIALS AND METHODS

Experimental setup

Two parallel integrated up-flow CW–MFC reactors (closed-circuit CW–MFCc and open-circuit CW–MFCo) were built in the open air. As shown in Figure 1, the wetland reactors were made of Perspex glass, and were 0.7 m long, 0.6 m wide, and 1.0 m high. The substrates (provided by Hebei Yanxi Mineral Processing Factory, China) had four layers: the bottom supporting layer was 20–40 mm gravel (0.15 m deep), topped with 10–30 mm lava (0.30 m deep), and then filled with 5–10 mm gravel (0.05 m deep), and 1–3 mm sand (0.05 m deep). The porosity of the substrates was approximately 0.48. There was a free water surface approximately 0.20 m deep in the reactors. There was a water collection pipe 0.15 m above the substrates' surface through which the effluent was discharged to an effluent water area with a dimension of 0.1 × 0.6 × 1.0 m (length × width × height).

The cathode and anode were graphite plates and self-made carbon fiber brushes, respectively. Four graphite plates (100 × 100 × 8 mm, provided by Tianjin Dongguang Huijin Co., Ltd, China) were evenly placed on the substrates' surface. The carbon fiber brush was 0.7 m long, 0.4 m of which contained the carbon fiber (0.1 m long, provided by Beijing Chuanjing Technology Development Co., Ltd, China). Five carbon fiber brushes were positioned vertically at the four corners and middle of the substrates, and the bottom of the brush was 0.05 m away from the bottom of the substrates. All the graphite plates and carbon fiber brushes were connected by titanium wires as a whole cathode and anode, respectively. The cathode and anode were connected across a variable external resistor (0–9999.9 Ω) by insulated copper wires.

Canna indica was selected as the wetland plants. After planting, the reactors were submerged in tap water immediately for the plants and microbes to develop. KH_2PO_4, $MgSO_4{\cdot}7H_2O$, NH_4Cl, and $CaCl_2{\cdot}2H_2O$ (in 15 mg/L tap water) were added to the tap water to provide nutrients for plants. After one month, the MFC circuit of both reactors was connected and the external resistors were set to 1,000 Ω to start up the CW–MFCs. During the startup, the inoculum (supernatant (1:1) of aerobic and anaerobic sludge) and carbon source (500 mg/L sodium acetate) were mixed at a ratio of 1:1, and the mixture was continuously pumped into the reactors from the bottom at 0.073 m^3/d. The inoculation supernatant was collected from a municipal wastewater treatment plant in Tianjin, China. Half of the effluent was returned to the reactors to enhance the enrichment of electrogens on electrodes. Additionally, graphite plates were submerged in the mixed solution for 24 hours before starting up the reactors, so as to accelerate the development of microbes on cathodes. Reproducible maximum voltages of 0.73–0.74 V arrived after 10 days of operation, indicating the successful startup of CW–MFCs. Then the external resistance of reactor CW–MFCc was set to 250 Ω, and the circuit of reactor CW–MFCo was disconnected (as control). The influents were then continuously dosed into the reactors at 0.049 m^3/d to begin the experiment, and the effluent reflux ratio was 50% (reflux flow rate was 0.024 m^3/d). The average hydraulic retention time in the reactors was 1.5 days. The influent and reflux flow rate was controlled by a liquid flowmeter installed at the inlet pipe and reflux pipe, respectively. Dissolved oxygen (DO) concentration in the cathode area remained at approximately 1.5 mg/L through continuous aeration by an air compressor. Approximately 1 week after the dosing, the output voltages of CW–MFCc stabilized and sampling began.

Synthetic domestic wastewater was used as influent, and was prepared using analytically pure CH_3COONa, $C_2H_5NO_2$, NH_4Cl, KNO_3, $NaNO_2$, KH_2PO_4, $Na_5P_3O_{10}$, $MgSO_4{\cdot}7H_2O$, and$CaCl_2{\cdot}2H_2O$. The major characteristics of the influents are listed in Table 1.

The whole monitoring period was from September 2016 to August 2017, covering the four seasons, and the CW–MFC reactors stopped operation during the winter icing period (Dec 13, 2016–Feb 13, 2017) because the water in the reactors froze. The 5-day average temperature of spring, summer, autumn and winter is 10–22, $\geq$22, 22–10 and $\leq$10 °C, respectively.

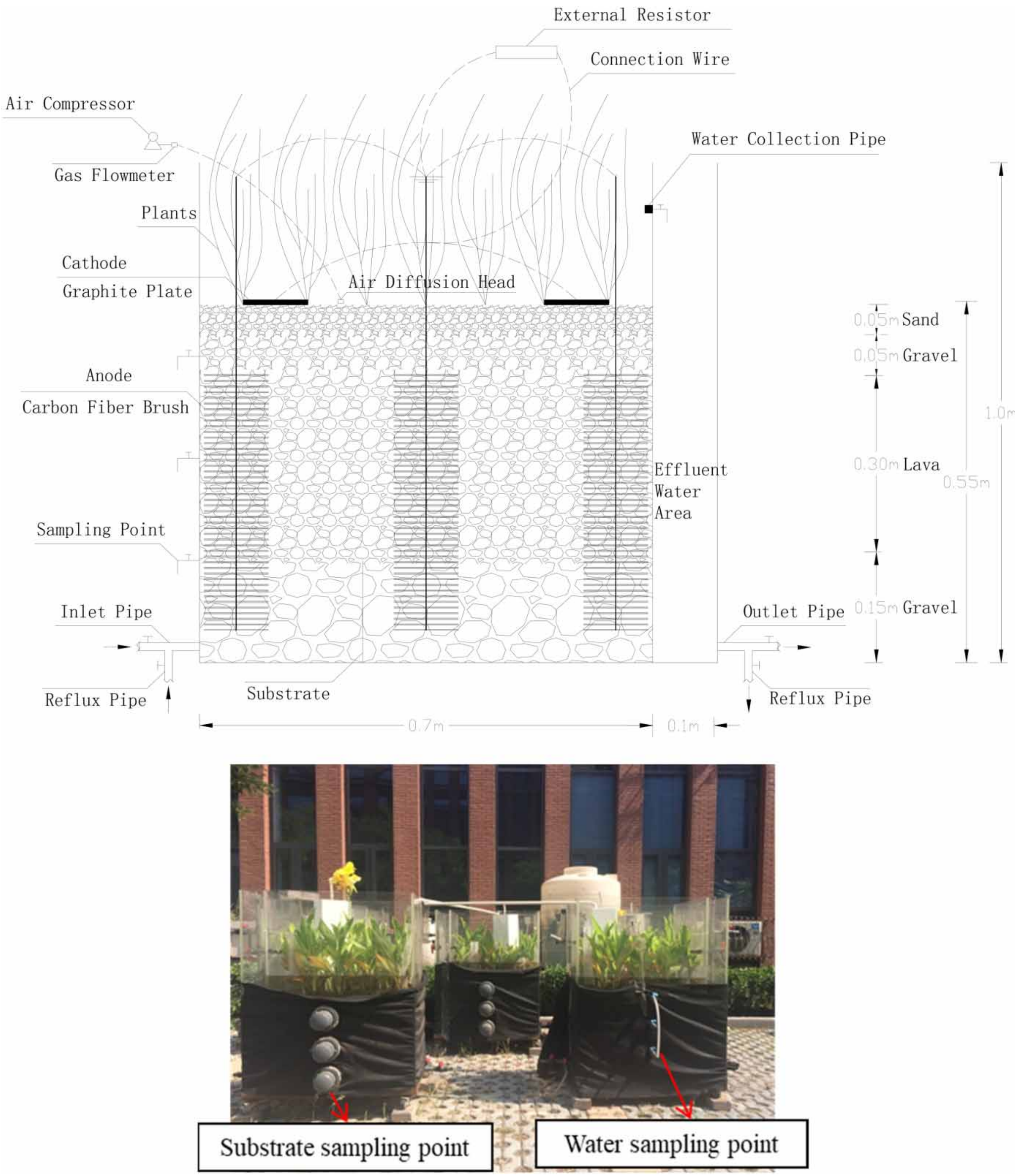

Figure 1 | Schematic diagram of the constructed wetland–microbial fuel cell (CW–MFC) system (above) and a picture of the CW–MFC reactors (below).

Water sampling and analysis

Water samples were collected between 9.00 and 10.00 am every 3 days from the effluent, and were immediately analyzed in the lab for COD, NH_3-N, TN, PO_4^{3-}-P, and TP using a Digital Reactor Block 200 and a HACH DR 2800 spectrophotometer, according to the standard procedure provided by HACH Company, USA. Specifically, COD

Table 1 | Major characteristics of the synthetic influents (mg/L)

pH	COD	NH_3-N	NO_2^--N	NO_3^--N	Org-N	TN	PO_4^{3-}-P	$P_3O_{10}^{5-}$-P	TP
7.0 ± 0.10	200	25	0.1	0.5	15	40.6	4	1	5

NO_2^--N, nitrite nitrogen; Org-N, organic nitrogen; TN, total nitrogen; PO_4^{3-}-P, phosphate; $P_3O_{10}^{5-}$-P, polyphosphate; TP, total phosphorus.

was measured by the quick digestion spectrophotometry method, NH_3-N was measured by the salicylate method, TN was measured by the persulfate digestion method, PO_4^{3-}-P was measured by the molybdovanadate method, and TP was measured by the molybdovanadate method with acid persulfate digestion. The wastewater DO and temperature within the CW–MFCs were measured every 30 minutes by an online DO detector (provided by Hangzhou Sinomeasure Automation Technology Co., Ltd, China). The atmospheric temperature was monitored using a temperature recorder (provided by Hangzhou Sinomeasure Automation Technology Co., Ltd, China), and the data were collected every 30 minutes. The pollutants removal rate (R) was calculated as follows:

$$R = \frac{(C_i - C_e)}{C_i} \times 100\% \qquad (1)$$

where C_i and C_e are the mean influent and effluent concentration (mg/L), respectively.

Bioelectricity measurement and analysis

The output voltage (U) was collected using a multi-channel data logger (Model CT-4008-5v10 mA-164, Shenzhen Neware Electronics Co., Ltd, China) and recorded by a computer at intervals of 30 min. The output current (I) was calculated by Ohm's law. The volumetric power density (P_d) was calculated as follows:

$$P_d = \frac{P}{V} = \frac{U^2}{VR_{ex}} \qquad (2)$$

where P is the power (mW), V is the total volume of the CW–MFC reactor (m^3), U is the output voltage (V), and R_{ex} is the external resistance (Ω).

Microbial sampling and analysis

Microbial sampling was conducted once during spring, summer, autumn and winter, respectively, between 9.00 and 10.00 am. The anode samples were collected by cutting a small amount of carbon fiber at a depth of 10–15 cm from the substrate surface, and samples from the five carbon fiber brushes were uniformly mixed as one sample. The cathode samples were collected from the biomass attached on the graphite plates, and samples from the four graphite plates were uniformly mixed as one sample. In order to minimize the disturbance of sampling to the cathodes, only 1.0 × 1.0 cm biomass was gently scraped away from the graphite plate, accounting for 1% of the total area of the plate. The substrate (lava) samples were collected at a depth of 10–25 and 25–40 cm, and were uniformly mixed as one sample. During substrate sampling, the influents dosing was stopped and the water in the reactor was drained, and then the substrate sampling holes (10 cm in diameter, Figure 1) were opened to collect substrate samples. Once the sampling was completed, the influents dosing was restarted. All microbial sampling was conducted between 9.00 and 10.00 am.

The enzymatic activity was determined by the analysis of dehydrogenase and catalase activity. Samples were freeze-dried first, and then ground and screened through a 16-mesh sieve for determination of enzymatic activity. Dehydrogenase activity (DHA) was measured with the triphenyl-tetrazolium chloride (TTC) method, for which 1 g (dry basis) of sample was cultured using 1 mL TTC solution (5 g/L) and 0.4 mL glucose solution (0.1 M) in an incubator at 37 °C for 12 h, and the resulting formazan was extracted with toluene and then measured by spectrophotometric quantification at 480 nm. DHA was expressed as μL (H)/g, 12 h, 37 °C for the substrate and μL (H)/cm^2, 12 h, 37 °C for the anodes and cathodes. Catalase activity (CA) was determined by the titration of residual H_2O_2 (3%) with

$KMnO_4$ (0.02 M), and was expressed as mL (0.02 M $KMnO_4$)/g, 1 h for the substrate and mL (0.02 M $KMnO_4$)/cm^2, 1 h for the anodes and cathodes.

The microbial diversity was determined by the Illumina sequencing analysis of the microbial community. Samples were submitted to Novogene Technology Co., Ltd (Beijing, China) to perform DNA extraction, PCR amplification and high throughput sequencing analysis. The specific procedures are provided in the supplementary material.

Data analysis

One-way analysis of variance (ANOVA) was conducted to detect significant differences in the treatment efficiency between the two CW–MFC reactors, followed by a Duncan post hoc test ($P < 0.05$). All of the statistical analyses were conducted using SPSS and Origin software.

RESULTS AND DISCUSSION

As illustrated in Figure 2, during the whole monitoring period (Sep 2016–Aug 2017, except for Dec 13, 2016–Feb 13, 2017), the recorded average daily minimum and maximum atmospheric temperatures were –6 to 27 and 0–36 °C, respectively. Compared to the atmospheric temperature, the wastewater temperature within the CW–MFCs was higher by 1–2 °C in winter and lower by 2–4 °C in summer. Additionally, in both closed-circuit CW–MFCc and open-circuit CW–MFCo, the anode area inside the reactors (0–45 cm from the bottom) remained anoxic/anaerobic (DO < 0.5 mg/L), while the cathode area was under aerobic conditions (DO > 1.0 mg/L) because of aeration.

Bioelectricity generation of the CW–MFC reactor

Figure 3 shows the bioelectricity generation of CW–MFCc with atmospheric temperature. The electricity output of CW–MFCc was quite low when the temperature was

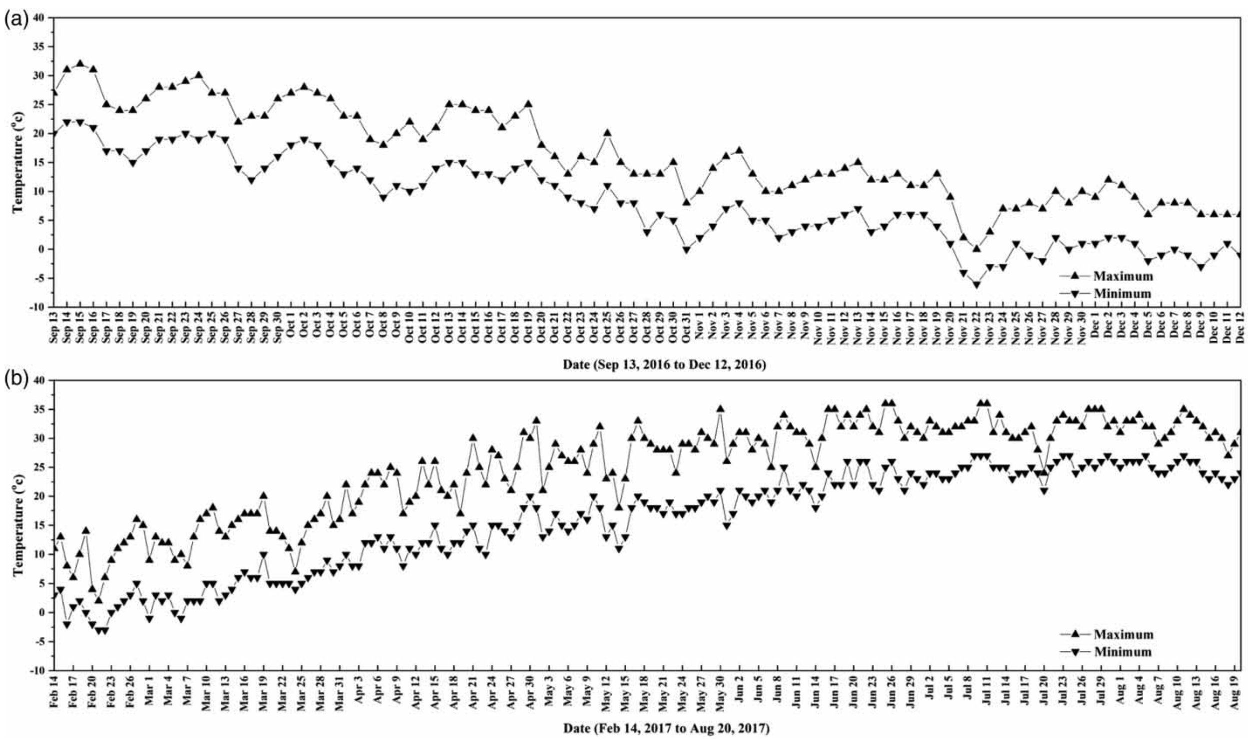

Figure 2 | Atmospheric temperature during the whole monitoring period (Sep 2016–Aug 2017, except for Dec 13, 2016–Feb 13, 2017).

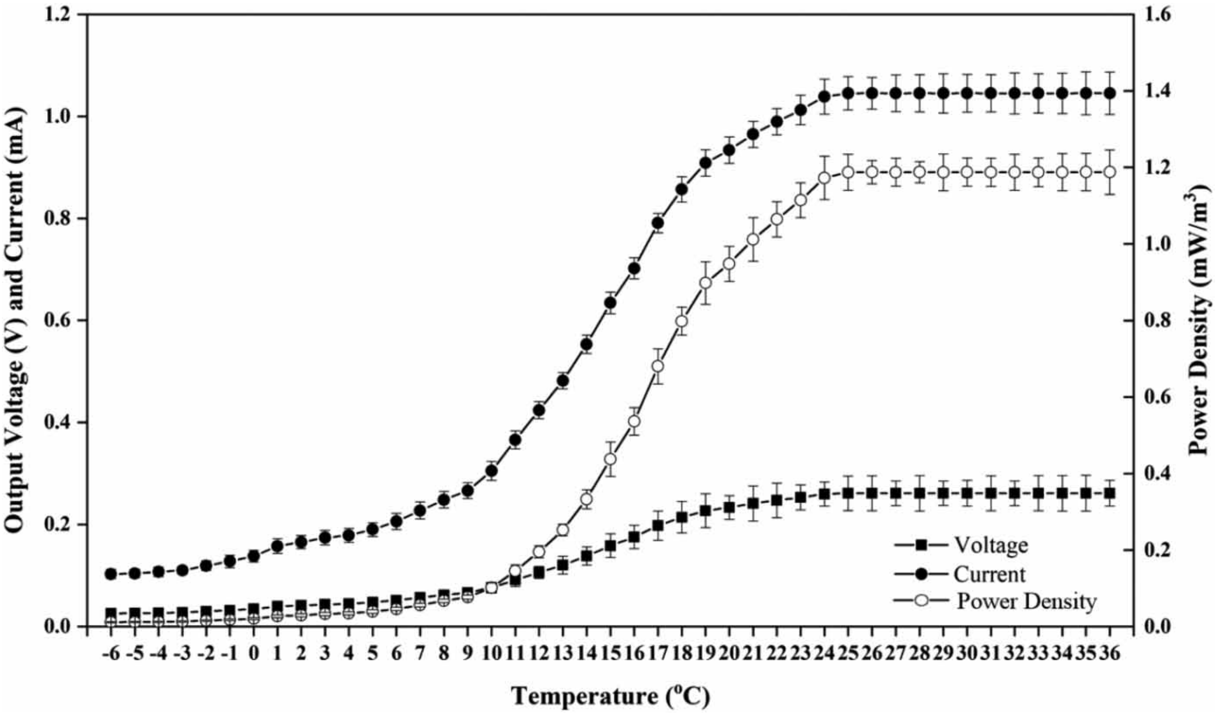

Figure 3 | Bioelectricity generation of the closed-circuit constructed wetland–microbial fuel cell system at various atmospheric temperatures.

≤5 °C (U < 0.0500 V, I < 0.20 mA, P_d <0.04 mW/m^3), indicating the weak activity of electrogens at temperatures below 5 °C. When the temperature increased from 6 to 10 °C, the electricity output of CW–MFCc began to increase slowly, indicating that the activity of electrogens gradually recovered as the temperature increased. When the temperature exceeded 10 °C, the electricity output of CW–MFCc began to rapidly increase with the increasing temperature. Especially when the temperature was between 14 and 19 °C, the output voltage increased by approximately 0.013–0.022 V as the temperature increased by 1 °C, and the corresponding output current and power density increased by approximately 0.05–0.09 mA and 0.10–0.14 mW/m^3, respectively. As the temperature exceeded 19 °C, the increase in electricity output gradually slowed down with the increasing temperature. When the temperature was above 25 °C, the electricity output of CW–MFCc stopped increasing and remained stable (U ≈ 0.26 V, I ≈ 1.05 mA, P_d ≈ 0.04 mW/m^3), demonstrating that the activity of electrogens had reached the maximum at ≥25 °C.

Temperature is a key factor influencing the MFC performance. Within a certain temperature range the bioelectricity generation in MFCs will increase as the temperature increases (Adelaja *et al.* 2015). This is because increasing the temperature can enhance the electrochemical activity of electrogens (Michie *et al.* 2011) as well as the rate of ion electromigration (Behera *et al.* 2011), which both could reduce the internal resistance of the MFC, thereby increasing its output power. However, microbial proteins will denature at excessively high temperatures, which is detrimental to the MFC performance. Adelaja *et al.* (2015) found that the biodegradation rates of a petroleum hydrocarbon mix (i.e. phenanthrene and benzene) and maximum power density in MFCs were both two times higher at 40 °C (97.10% and 1.15 mW/m^2 anode, respectively) than those at 30 °C, but were four times lower when the operating temperature was raised to 50 °C. It has been reported that the most suitable temperature for the electrochemical activity of anode biofilms is 30–45 °C (Michie *et al.* 2011).

Overall, the atmospheric temperature significantly influenced the electricity generation of CW-MFCs by influencing the electrogens' activity. The electricity output of the CW-MFC system was extremely low when the temperature was $\leq 5\,^{\circ}$C, and reached the maximum at $\geq 25\,^{\circ}$C.

Pollutants removal of the CW–MFC reactor

Pollutants removal in the CW–MFC systems under different atmospheric temperature is provided in Figure 4. The 5-day average temperature varied between 3 and 30 °C. The pollutants removal stabilized at the 5-day average temperature of $\geq 11\,^{\circ}$C in closed-circuit CW–MFCc (84.5–91.0% COD, 83.9–90.8% NH_3-N and 84.0–92.9% TN) and $\geq 14\,^{\circ}$C in open-circuit CW–MFCo (74.5–82.0% COD, 79.5–85.7% NH_3-N and 75.9–81.6% TN), showing significant difference ($P < 0.05$) between CW–MFCc and CW–MFCo. This demonstrated that incorporating MFC could help strengthen the resistance of CWs to lower temperature in terms of organics and nitrogen removal. Considering the dependency of organics and nitrogen bio-reaction kinetics on temperature, it is unsurprising to find the decreases in the removal of COD, NH_3-N and TN at lower temperatures. The maximum difference in the average removal rate under higher ($\geq 11\,^{\circ}$C for CW–MFCc and $\geq 14\,^{\circ}$C for CW–MFCo) and lower (3–6 °C) 5-day average temperature was approximately 12.2% COD, 16.8% NH_3-N, 12.6% TN for CW–MFCc, and 15.6% COD, 19.9% NH_3-N, 16.2% TN for CW–MFCo. Comparing CW–MFCc with CW–MFCo, it can be found that the decreasing degree of organics and nitrogen removal under lower temperatures was smaller in CW–MFCc. However, phosphorus removal in both CW–MFCc (85.2–90.2% PO_4^{3-}-P and 84.4–89.0% TP) and CW–MFCo (84.2–89.2% PO_4^{3-}-P and 83.4–87.4% TP) barely changed with temperature, demonstrating more stable removal than that of organics and nitrogen. Moreover, the removal rates of COD, NH_3-N and TN in CW–MFCc were significantly ($P < 0.05$) higher than that in CW–MFCo by 8.0–15.0%, 2.7–5.9% and 10.2–17.8%, respectively, while there was negligible increase of average removal rates for PO_4^{3-}-P and TP in CW–MFCc (0.81 and 0.87%, respectively).

Pollutants removal in the CW–MFC systems under different seasons is provided in Figure 5. The average removal rates in closed-circuit CW–MFCc under spring, summer, autumn and winter were, respectively, higher than that in open-circuit CW–MFCo by approximately 11.2, 9.4, 10.0 and 12.4% for COD, 4.8, 4.8, 5.1 and 5.7% for NH_3-N, and 12.42, 11.19, 11.60 and 15.6% for TN. It can be found that the gap between CW–MFCc and CW–MFCo in terms of organics and nitrogen removal widened in winter. For closed-circuit CW–MFCc, the organics and nitrogen removal in summer did not significantly ($P > 0.05$) differ from that in autumn, but was significantly ($P < 0.05$) higher than that in spring by an average of 3.2% COD, 4.2% NH_3-N, 3.5% TN, and that in winter by an average of 10.5% COD, 14.2% NH_3-N, 10.7% TN. In the meantime, the average removal rate of COD, NH_3-N and TN in open-circuit CW–MFCo demonstrated a larger difference between summer and winter, which was 13.4, 15.1 and 15.1%, respectively. This further proved that incorporating MFC could still help improve the organics and nitrogen removal in CWs during winter. No significant ($P > 0.05$) difference was observed in phosphorus removal in both CW–MFCc (88.0–88.5% PO_4^{3-}-P and 86.5–87.0% TP) and CW–MFCo (87.1–87.6% PO_4^{3-}-P and 85.5–86.0% TP) under the four seasons.

In CWs, biodegradation is considered as the dominant process responsible for organics removal, and microbial nitrification and denitrification is the primary method of nitrogen removal (Saeed & Sun 2012). Therefore, it is widely acknowledged that temperature significantly influenced the organics and nitrogen removal in CWs by primarily affecting the microbial activities (Yan & Xu 2014). MFC can enhance the anaerobic degradation of organics in the anodes, and thus improve the organics removal of CW–MFCs (Doherty *et al.* 2015). Nitrite and nitrate can be used as electron acceptors in the cathode of an MFC for reducing nitrogen in wastewater while producing bioelectricity (Puig *et al.* 2011). NH_3-N can be used as one of the main substrates for electricity generation and thus its removal was enhanced (Yu *et al.* 2020). Despite the weak bioelectricity generation of the CW–MFC under lower temperature (Figure 3), the presence of MFC reduced the adverse effect of cooling on the organics and nitrogen removal in CWs to a certain extent. As for the similar removal of organics and nitrogen in the CW–MFCs during summer and autumn, this may be because there were only 34 days in the autumn of this study, and 26 of the days had a 5-day average temperature of more than 15 °C.

Many studies have found that the physio-chemical processes of substrates were mainly responsible for P removal

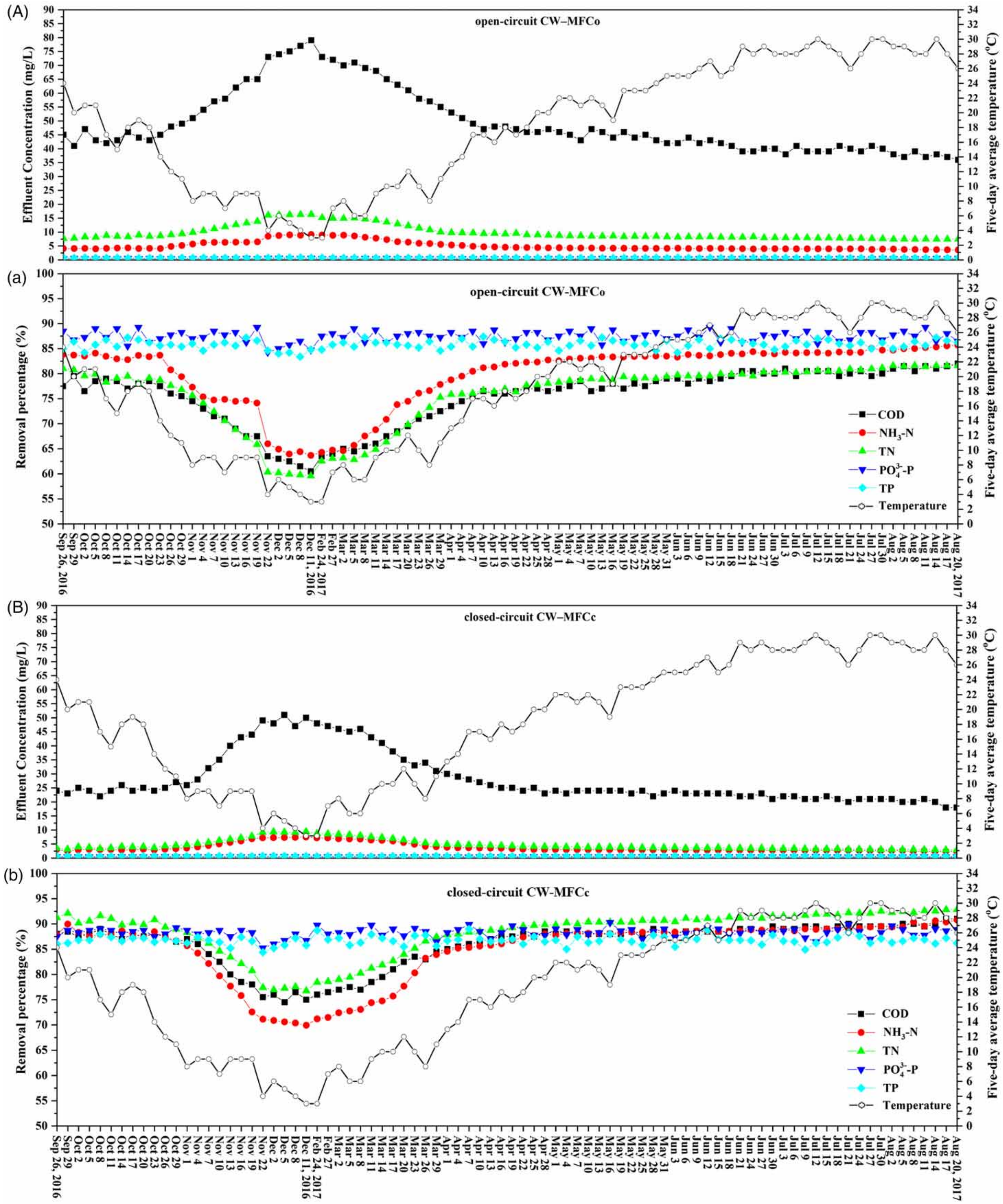

Figure 4 | Pollutants removal of the constructed wetland–microbial fuel cell systems at various atmospheric temperatures.

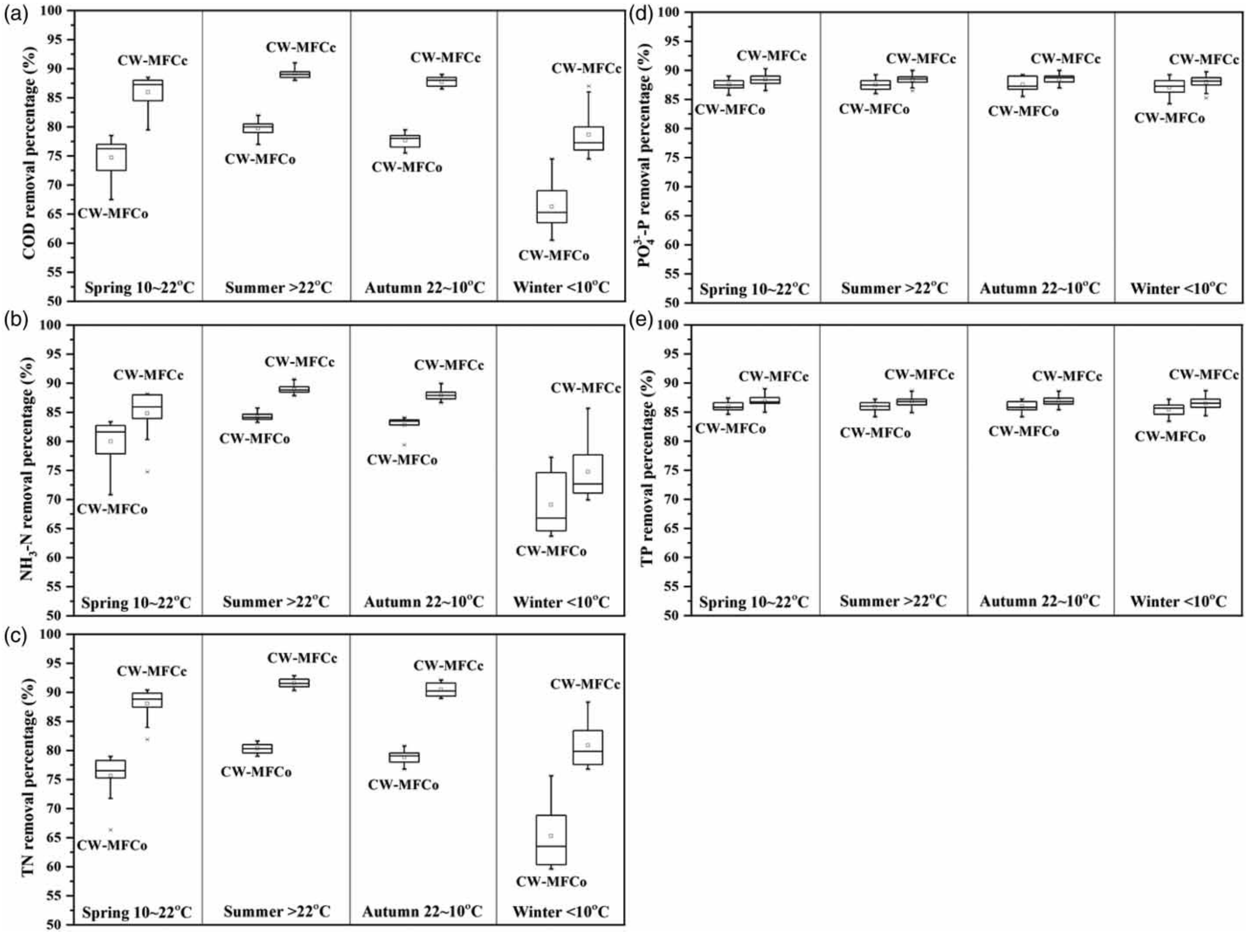

Figure 5 | Pollutants removal of the constructed wetland–microbial fuel cell systems under different seasons. Error bars are ±1 standard deviation.

in CWs, including sedimentation, filtration, interception, adsorption, absorption, precipitation, ion exchange, and complexation reactions (Lan *et al.* 2018). From the results of this study, it can be summarized that the primary method of P removal in CW–MFCs was also the physio-chemical processes of substrates.

Overall, the MFC not only significantly ($P < 0.05$) improved the organics and nitrogen removal but also mitigated the negative effects of lower temperature on organics and nitrogen removal in CWs. However, both MFC and temperature had no significant influence on phosphorus removal in CW–MFCs.

Enzymatic activity

As an intermediate carrier of hydrogen, DHA can reflect the microbial oxidative capability during the organics degradation, therefore it is often used to measure microbial activity (Barrena *et al.* 2008). As shown in Figure 6(a) and 6(b), DHA of the anodes was higher than that of the cathodes by approximately 3.2–4.6 times in closed-circuit CW–MFCc and 3.3–5.2 times in open-circuit CW–MFCo, respectively. This may be owing to the much higher COD concentration in the wetland interior, since the influents were dosed into the reactors from the bottom. Catalase is an oxidoreductase mainly associated with the activity of aerobic microorganisms. As shown in Figure 6(a) and 6(b), CA of the cathodes was significantly ($P < 0.05$) higher than that of the anodes by approximately 23.7–35.6% in closed-circuit CW–MFCc and 16.7–33.1% in open-circuit CW–MFCo, respectively, which resulted from the aerobic environment (DO > 1.0 mg/L) in the cathode area. Compared to open-circuit CW–MFCo, enzymatic activity of the anodes and cathodes in closed-circuit CW–MFCc under

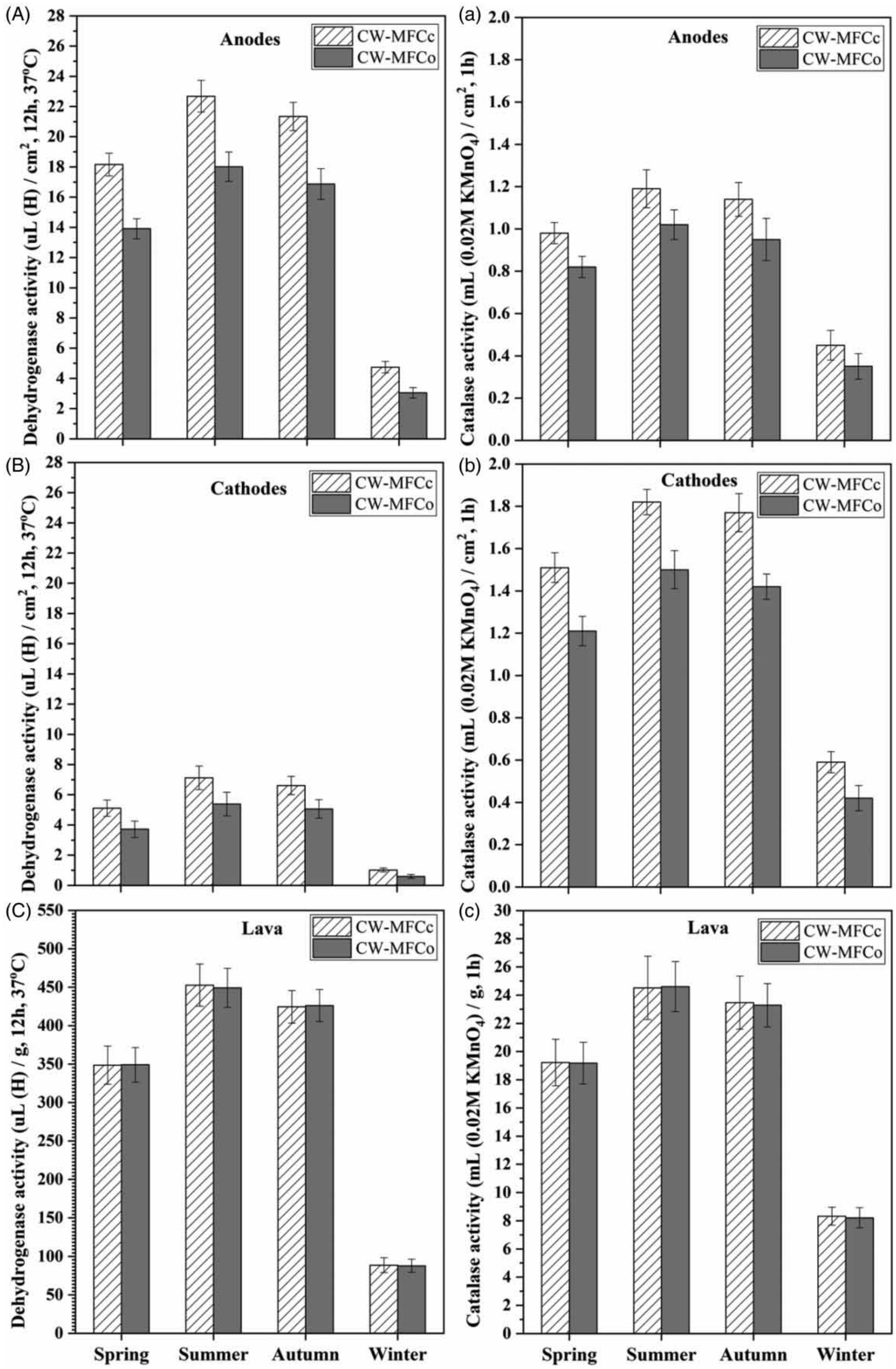

Figure 6 | Enzymatic activity of dehydrogenase and catalase in the constructed wetland–microbial fuel cell systems under different seasons. Error bars are ±1 standard deviation.

the four seasons was significantly ($P < 0.05$) higher by 25.9–55.4% and 30.6–72.9% for DHA, and 16.7–28.6% and 21.3–40.5% for CA, respectively, while DHA and CA of the substrate were similar under the same season. This proved that the MFC current could enhance the growth of microorganisms on the electrodes. It was obvious that DHA and CA of CW–MFCs reached the highest in summer and autumn, followed by spring, and the lowest in winter, which was consistent with the seasonal variations of organics and nitrogen removal (Figure 5) as well as the variations of electricity generation with temperature (Figure 3). In closed-circuit CW–MFCc, enzymatic activity of the anodes, cathodes and substrate during winter, respectively, significantly ($P < 0.05$) dropped by 79.1, 85.7 and 80.4% for DHA, and 62.2, 67.6 and 66.1% for CA, when compared to summer. As has been noted, the wastewater temperature within the CW–MFCs was higher than the atmospheric temperature by 1–2 °C in winter. Since the anodes were installed inside the substrate, while the cathodes were placed on the substrate's surface, the lower temperature resulted in the larger decrease of the enzymatic activity in the cathode area. Moreover, the decrease of enzymatic activity in the anodes and cathodes during winter was smaller in closed-circuit CW–MFCc than that in open-circuit CW–MFCo. This demonstrated that the MFC current could also stabilize the growth of microorganisms on the electrodes at lower temperatures to a certain extent.

Microbial diversity

Table 2 shows the observed microbial species and alpha diversity indices of anodes, cathodes and lava in open-circuit CW–MFCo and closed-circuit CW–MFCc under the four seasons. The Shannon and Simpson index was used to identify community diversity, the Chao1 and ACE estimator was used to identify community richness, and the Good's coverage was used to characterize sequencing depth. It was obvious that the microbial species and diversity in both CW–MFCo and CW–MFCc declined in winter when compared to summer and autumn. For the anodes, the observed species and alpha diversity indices in CW–MFCc were much higher than in CW–MFCo, especially in winter. This result indicated that the closed circuit mode promoted the bacteria diversity on MFC anodes, which was consistent with the study by Li *et al.* (2019). For the cathodes, it was only in summer that the bacterial community diversity in CW–MFCc was higher than that in CW–MFCo. This may be because the effect of temperature on microbes was stronger than the stimulation of MFC, since the cathodes were placed on the substrate's surface where they were directly exposed to the exterior atmosphere. For lava, the bacteria diversity in CW–MFCc and CW–MFCo was similar, which demonstrated that the enhancement of bacteria diversity by MFC was limited to the electrode surface. This was because the electricity generation in CW–MFCs was by direct transfer of electrons to the electrode by electrogens growing on the electrode (Liu *et al.* 2005).

The reported electrochemically active bacteria (EAB) in CW–MFCs includes *Proteobacteria*, *Firmicutes*, *Bacteroidetes* and *Acidobacteria*, among which *Proteobacteria* and *Firmicutes* are the main ones (Wang *et al.* 2016; Li *et al.* 2019). As shown in Figure 7, the bacterial community of the anodes, cathodes and lava samples at the phyla level was primarily composed of 10 phyla, and the dominant microbes were phyla *Proteobacteria*. For anodes, the closed circuit mode significantly promoted the abundance of *Firmicutes* and *Bacteroidetes*. The result that phyla *Bacteroidetes* was enriched on an MFC anode was consistent with previous studies (De *et al.* 2010; Li *et al.* 2019). For cathodes, *Proteobacteria* and *Firmicutes* were enriched in closed-circuit CW–MFCc, while there was approximately 7.5–14.7% *Cyanobacteria* on the cathodes of open-circuit CW–MFCo. As shown in Figure 8(b), the typical EAB at the genera level in closed-circuit CW–MFCc mainly included *Pseudomonas*, *Rhodoferax*, *Clostridium*, *Escherichia*, *Enterobacter*, *Shewanella*, *Desulfovibrio* and *Geobacter*. In CW–MFCc, the proportion of the eight genera EAB was approximately 16.7–17.5% on anodes and 10.1–10.5% on cathodes in spring and winter, while it decreased to 13.5–13.8% on anodes and 8.2–9.0% on cathodes in summer and autumn. In open-circuit CW–MFCo, the eight genera EAB were less than 3% on both anodes and cathodes (Figure 8(a)). This further proved that the closed circuit operation contributed to the accumulation of EAB on MFC electrodes at low temperatures in winter.

Overall, in closed-circuit CW-MFCc, the microbial community composition at the phyla level on anodes and cathodes was similar under the four seasons. The closed

Table 2 | Microbial diversity indexes in the constructed wetland–microbial fuel cell systems under different seasons

		Observed-species	Shannon	Simpson	Chao1	ACE	Good's coverage	PD_whole tree
Open-circuit CW–MFCo								
Anode	Spring	928	4.421	0.713	1,219.572	1,246.234	0.990	66.801
	Summer	1,538	7.339	0.964	2,013.814	2,067.515	0.984	97.745
	Autumn	1,272	5.875	0.852	1,737.153	1,736.001	0.986	86.046
	Winter	734	3.694	0.648	932.985	973.350	0.993	57.067
Cathode	Spring	1,215	7.373	0.985	1,749.989	1,780.930	0.986	84.120
	Summer	1,382	7.798	0.986	1,796.299	1,858.841	0.986	91.862
	Autumn	1,220	7.421	0.986	1,733.890	1,747.039	0.986	84.788
	Winter	1,172	7.102	0.979	1,814.247	1,782.391	0.986	81.463
Lava	Spring	1,209	6.619	0.945	1,632.460	1,687.157	0.987	86.788
	Summer	1,578	7.319	0.956	2,132.064	2,073.974	0.985	111.167
	Autumn	1,485	7.726	0.984	1,652.602	1,767.321	0.989	98.682
	Winter	917	5.279	0.838	1,168.487	1,182.301	0.991	71.241
Closed-circuit CW–MFCc								
Anode	Spring	1,440	7.555	0.974	1,625.902	1,738.800	0.989	94.789
	Summer	1,880	8.950	0.995	2,029.463	2,090.419	0.990	121.148
	Autumn	1,442	7.633	0.984	1,853.157	1,925.595	0.986	93.599
	Winter	1,408	7.687	0.978	1,799.267	1,796.147	0.987	97.101
Cathode	Spring	1,138	6.012	0.935	1,601.580	1,649.927	0.987	77.823
	Summer	1,553	8.049	0.990	1,810.973	1,932.238	0.987	100.648
	Autumn	1,262	6.771	0.964	1,747.763	1,890.075	0.985	85.998
	Winter	1,035	5.853	0.932	1,231.711	1,329.222	0.991	72.464
Lava	Spring	979	6.193	0.956	1,278.640	1,312.090	0.990	71.804
	Summer	1,487	6.445	0.903	1,994.040	2,013.345	0.984	102.439
	Autumn	1,429	7.928	0.987	1,639.218	1,679.065	0.990	99.017
	Winter	860	4.487	0.730	1,077.600	1,106.129	0.992	63.445

circuit mode can promote the bacteria diversity on CW–MFC anodes as well as the abundance of EAB on CW–MFC anodes and cathodes, even at low temperatures, and thus enhance the organics and nitrogen removal of CW–MFCs in winter.

CONCLUSIONS

The atmospheric temperature significantly influenced the bioelectricity generation and removal of organics and nitrogen in CW–MFCs by primarily influencing the microbial enzymatic activity. The electricity output of CW-MFCs was extremely low below 5 °C, and reached the maximum above 25 °C. The organics and nitrogen removal of closed-circuit CW–MFC reached the highest in summer and autumn, followed by spring, and decreased by an average of 10.5% COD, 14.2% NH_3-N and 10.7% TN in winter, demonstrating smaller seasonal fluctuations compared to open-circuit CW–MFC in which the difference between summer and winter was 13.4% COD, 15.1% NH_3-N and 15.1% TN. Even at low temperatures, the MFC current could enhance the enzymatic activity and stabilize the growth of microorganisms on the electrodes. Moreover, the closed circuit mode can promote the bacteria diversity on CW–MFC anodes as well as the abundance of EAB on

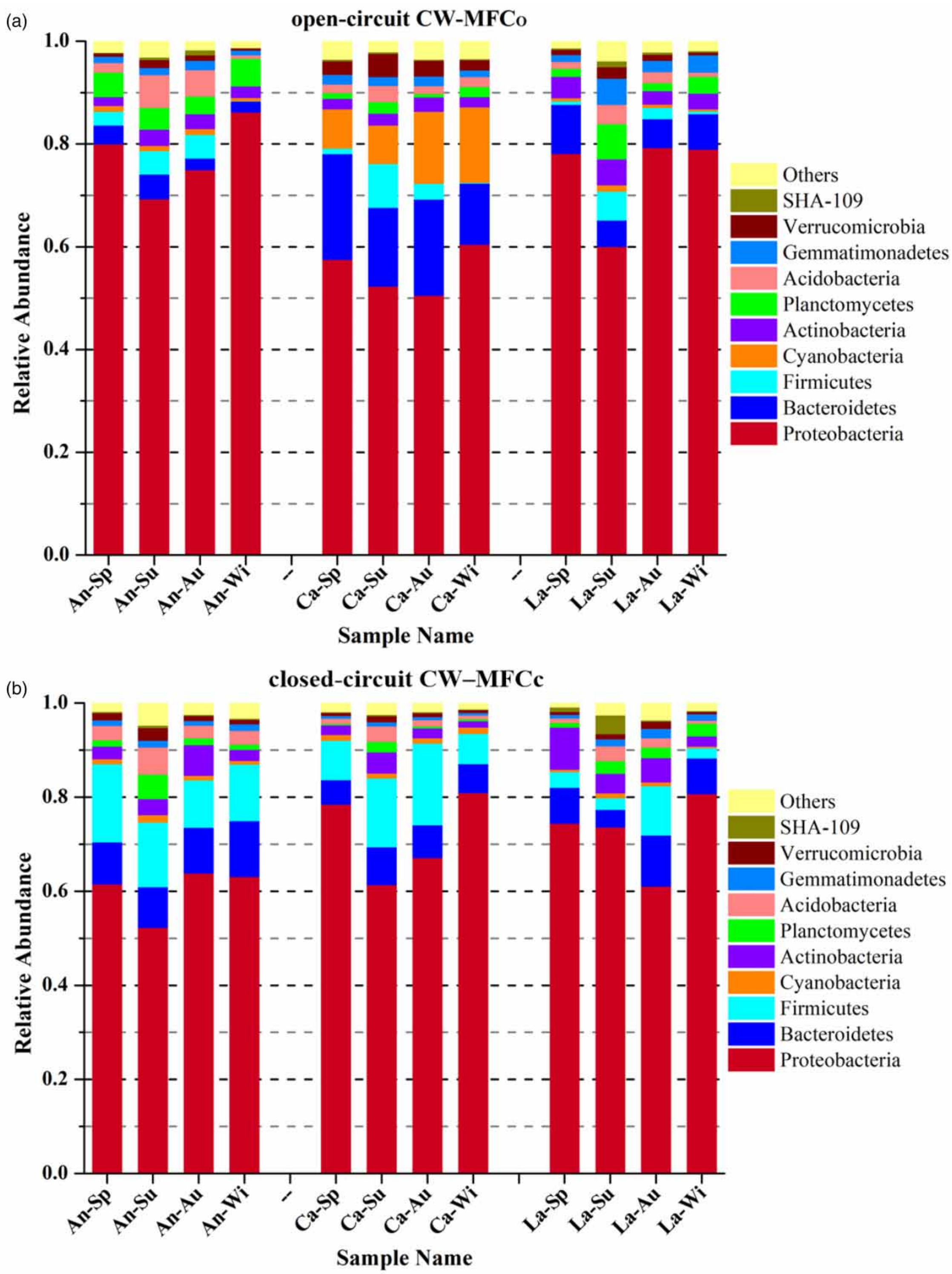

Figure 7 | Relative abundance plot at phyla level based on sequencing results of 16s rRNA. An: Anode; Ca: Cathode; La: Lava; Sp: Spring; Su: Summer; Au: Autumn; Wi: Winter.

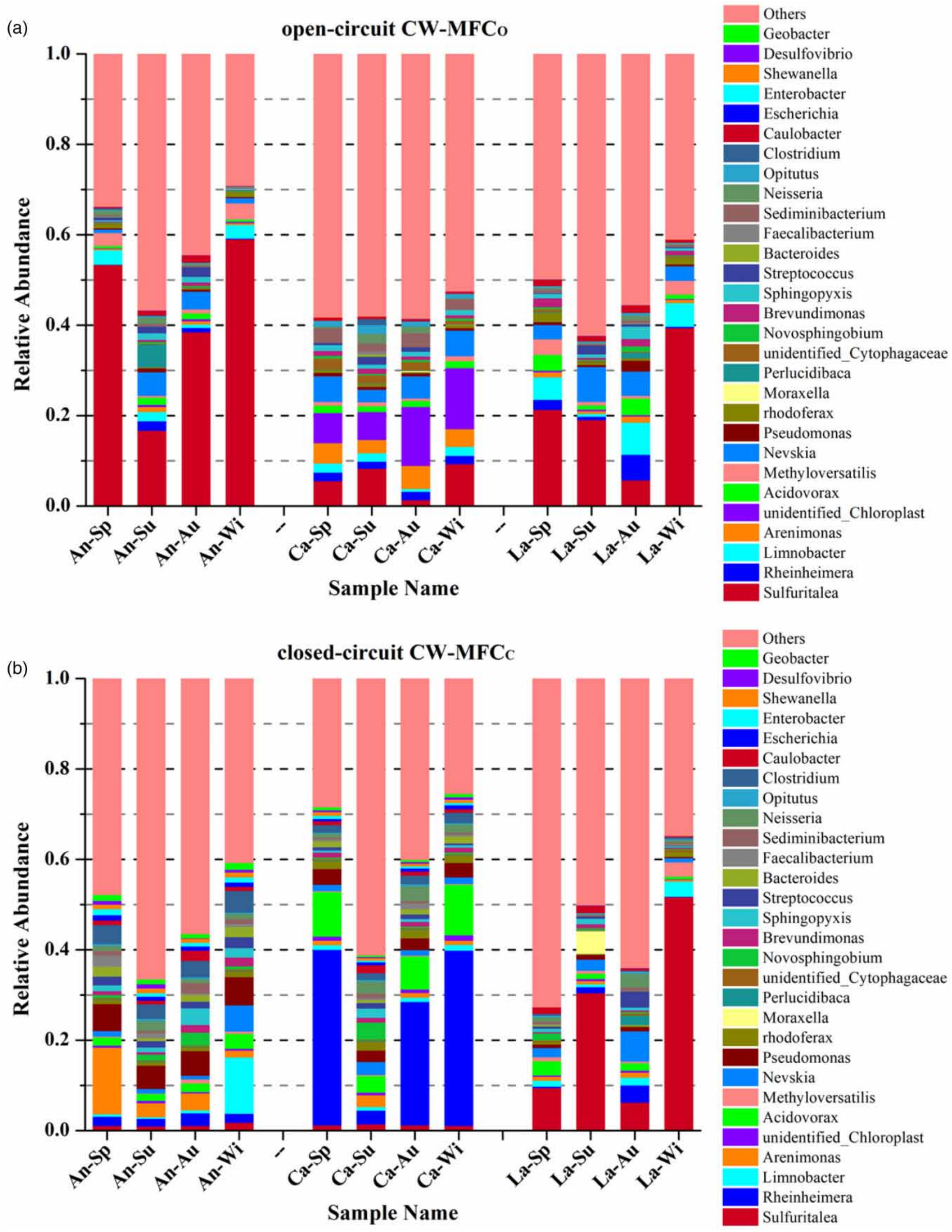

Figure 8 | Relative abundance plot at genera level based on sequencing results of 16s rRNA. An: Anode; Ca: Cathode; La: Lava; Sp: Spring; Su: Summer; Au: Autumn; Wi: Winter.

CW–MFC anodes and cathodes, and thus reduce the adverse effect of cooling on the organics and nitrogen removal in CWs. However, both MFC and temperature did not significantly influence the phosphorus removal in CW–MFCs.

ACKNOWLEDGEMENTS

This work was supported by The Key Special Program on the S&T for the Pollution Control and Treatment of Water Bodies of China (2014ZX07203-009).

DATA AVAILABILITY STATEMENT

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

REFERENCES

Adelaja, O., Keshavarz, T. & Kyazze, G. 2015 The effect of salinity, redox mediators and temperature on anaerobic biodegradation of petroleum hydrocarbons in microbial fuel cells. *Journal of Hazardous Materials* **283**, 211–217.

Akratos, C. S. & Tsihrintzis, V. A. 2007 Effect of temperature, HRT, vegetation and porous media on removal efficiency of pilot-scale horizontal subsurface flow constructed wetlands. *Ecological Engineering* **29** (2), 173–191.

Barrena, R., Vázquez, F. & Sanchez, A. 2008 Dehydrogenase activity as a method for monitoring the composting process. *Bioresource Technology* **99**, 905–908.

Behera, M., Murthy, S. S. R. & Ghangrekar, M. M. 2011 Effect of operating temperature on performance of microbial fuel cell. *Water Science and Technology* **64** (4), 917–922.

Cao, S. W., Jing, Z. Q., Yuan, P., Wang, Y. & Wang, Y. 2019 Performance of constructed wetlands with different substrates for the treated effluent from the municipal sewage plant. *Journal of Water Reuse and Desalination* **9** (4), 452–462.

De, S. L., Cabezas, A., Marzorati, M., Friedrich, M. W., Boon, N. & Verstraete, W. 2010 Microbial community analysis of anodes from sediment microbial fuel cells powered by rhizodeposits of living rice plants. *Applied & Environmental Microbiology* **76**, 2–8.

Doherty, L., Zhao, Y. Q., Zhao, X. H., Hu, Y. S., Hao, X. D., Xu, L. & Liu, R. B. 2015 A review of a recently emerged technology: constructed wetland-Microbial fuel cells. *Water Research* **85**, 38–45.

Fang, Z., Song, H. L., Cang, N. & Li, X. N. 2013 Performance of microbial fuel cell coupled constructed wetland system for decolorization of azo dye and bioelectricity generation. *Bioresource Technology* **144** (6), 165–171.

Guadarrama-Pérez, O., Gutiérrez-Macías, T., García-Sánchez, L., Guadarrama-Pérez, V. H. & Estrada-Arriaga, E. B. 2019 Recent advances in constructed wetland–microbial fuel cells for simultaneous bioelectricity production and wastewater treatment: a review. *International Journal of Energy Research* **43**, 5106–5127.

Kadlec, R. H. & Wallace, S. D. 2009 *Treatment Wetlands*, 2nd edn. CRC Press, Taylor and Francis Group, Boca Raton, FL, USA.

Lan, W., Zhang, J., Hu, Z., Ji, M. D., Zhang, X. W., Zhang, J. D., Li, F. Z. & Yao, G. Q. 2018 Phosphorus removal enhancement of magnesium modified constructed wetland microcosm and its mechanism study. *Chemical Engineering Journal* **335**, 209–214.

Li, H., Zhang, S., Yang, X. L., Yang, Y. L., Xu, H., Li, X. N. & Song, H. L. 2019 Enhanced degradation of bisphenol A and ibuprofen by an up-flow microbial fuel cell-coupled constructed wetland and analysis of bacterial community structure. *Chemosphere* **217**, 599–608.

Liu, H., Cheng, S. A. & Logan, B. E. 2005 Production of electricity from acetate or butyrate using a single-chamber microbial fuel cell. *Environmental Science & Technology* **39** (2), 658–662.

Michie, I. S., Kim, J. R., Dinsdale, R. M., Guwy, A. J. & Premier, G. C. 2011 Operational temperature regulates anodic biofilm growth and the development of electrogenic activity. *Applied Microbiology and Biotechnology* **92**, 419–430.

Ouellet-Plamondon, C., Chazarenc, F., Comeau, Y. & Brisson, J. 2006 Artificial aeration to increase pollutant removal efficiency of constructed wetlands in cold climate. *Ecological Engineering* **27**, 258–264.

Puig, S., Serra, M., Vilar-Sanz, A., Cabré, M., Bañeras, L., Colprim, J. & Balaguer, M. D. 2011 Autotrophic nitrite removal in the cathode of microbial fuel cells. *Bioresource Technology* **102** (6), 4462–4467.

Saeed, T. & Sun, G. Z. 2012 A review on nitrogen and organics removal mechanisms in subsurface flow constructed wetlands: dependency on environmental parameters, operating conditions and supporting media. *Journal of Environmental Management* **112**, 429–448.

Sani, A., Scholz, M. & Bouillon, L. 2013 Seasonal assessment of experimental vertical-flow constructed wetlands treating domestic wastewater. *Bioresource Technology* **147**, 585–596.

Song, Z. W., Zheng, Z. P., Li, J., Sun, X. F., Han, X. Y., Wang, W. & Xu, M. 2006 Seasonal and annual performance of a full-scale constructed wetland system for sewage treatment in China. *Ecological Engineering* **26** (3), 272–282.

Srivastava, P., Yadav, A. K. & Mishra, B. K. 2015 The effects of microbial fuel cell integration into constructed wetland on the performance of constructed wetland. *Bioresource Technology* **195**, 223–230.

Wang, J. F., Song, X. S., Wang, Y. H., Abayneh, B., Li, Y. H., Yan, D. H. & Bai, J. H. 2016 Nitrate removal and bioenergy production in constructed wetland coupled with microbial fuel cell: establishment of electrochemically active bacteria community on anode. *Bioresource Technology* **221**, 358–365.

Wang, M., Zhang, D. Q., Dong, J. W. & Tan, S. K. 2017 Constructed wetlands for wastewater treatment in cold climate – A review. *Journal of Environmental Sciences* **57**, 293–311.

Wu, S., Austin, D., Liu, L. & Dong, R. 2011 Performance of integrated household constructed wetland for domestic wastewater treatment in rural areas. *Ecological Engineering* **37** (6), 948–954.

Xu, L., Zhao, Y. Q., Wang, X. D. & Yu, W. Z. 2018 Applying multiple bio-cathodes in constructed wetland-microbial fuel cell for promoting energy production and bioelectrical derived nitrification-denitrification process. *Chemical Engineering Journal* **344**, 105–113.

Yan, Y. J. & Xu, J. C. 2014 Improving winter performance of constructed wetlands for wastewater treatment in northern China: a review. *Wetlands* **34**, 243–253.

Yu, B., Liu, C. L., Wang, S. Y., Wang, W. D., Zhao, S. Y. & Zhu, G. B. 2020 Applying constructed wetland-microbial electrochemical system to enhance NH+ 4 removal at low temperature. *Science of the Total Environment* **724**, 138017.

First received 16 October 2020; accepted in revised form 17 January 2021. Available online 2 March 2021