Seasonal variations of pollutants removal and microbial activity in integrated constructed wetland-microbial fuel cell systems
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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% NH3-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% NH3-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

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.

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 (NH₃-N) and total Kjeldahl nitrogen (TKN) in a pilot-scale horizontal-flow CW (HFCW) were 37.9 and 58.5% at temperatures of <15°C, and 69.1 and 73.9% at temperatures of >15°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 (BOD₅), chemical oxygen demand (COD), NH₃-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, 66.5, 54.5 and 35.0%, respectively). Sani et al. (2015) evaluated the seasonal performance of a vertical-flow CW (VFCW), and significantly higher COD, NH₃-N, and nitrate (NO₃-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 ≤5-6°C and ≥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, NH₃-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% BOD₅, 84.6% NH₃-N, and 88.2% TP) in the freezing winter period. Kadlec & Wallace (2000) 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. (2015) 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 NO₃-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 NH₃-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

Figure 1 | Schematic diagram of the constructed wetland–microbial fuel cell (CW–MFC) system (above) and a picture of the CW–MFC reactors (below).
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 cath-
ode 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. KH2PO4,
MgSO4·7H2O, NH4Cl, NaNO2, KNO3, Na2HPO4, Na3P2O10,
MgSO4·7H2O, and CaCl2·2H2O. 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, ≥22, 22–10
and ≤10 °C, respectively.

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

| pH   | COD  | NH3-N | NO2-N | NO3-N | Org-N | TN    | PO4−-P | PO1−-P | P1−-P | TP |
|------|------|-------|-------|-------|--------|-------|-------|--------|--------|-----|----|
| 7.0 ± 0.10 | 200  | 25    | 0.1   | 0.5   | 15     | 40.6  | 4     | 1      | 5      |

NO2-N, nitrite nitrogen; Org-N, organic nitrogen; TN, total nitrogen; PO4−-P, phosphate; P1−-P, polyphosphate; TP, total phosphorus.

Water sampling and analysis

Water samples were collected between 9.00 and 10.00 am
every 5 days from the effluent, and were immediately anal-
alyzed in the lab for COD, NH3-N, TN, PO4−-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
was measured by the quick digestion spectrophotometry
method, NH3-N was measured by the salicylate method, TN
was measured by the persulfate digestion method, PO4−-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:

\[ R = \left( \frac{C_i - C_e}{C_i} \right) \times 100\% \]  

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 (Pd) was calculated as follows:

\[ P_d = \frac{P}{V} = \frac{U^2}{VRex} \]  

where \( P \) is the power (mW), \( V \) is the total volume of the CW–MFC reactor (m³), \( U \) 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)/cm², 12 h, 37 °C for the anodes and cathodes. Catalase activity (CA) was determined by the titration of residual H₂O₂ (3%) with KMnO₄ (0.02 M), and was expressed as mL (0.02 M KMnO₄)/g, 1 h for the substrate and mL (0.02 M KMnO₄)/cm², 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 ≤5 °C (U < 0.0500 V, I < 0.20 mA, Pd < 0.04 mW/m³), 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/m³, 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, Pd ≈ 0.04 mW/m³), 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/m² 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 ≤5 °C, and reached the maximum at ≥25 °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 ≥11 °C in closed-circuit CW–MFCc (84.5–91.0% COD, 83.9–90.8% NH₃-N and 84.0–92.9% TN) and ≥14 °C in open-circuit CW–MFCo (74.5–82.0% COD, 79.5–85.7% NH₃-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, NH₃-N and TN at lower temperatures. The maximum difference in the average removal rate under higher (≥11 °C for CW–MFCc and ≥14 °C for CW–MFCo) and lower (3–6 °C) 5-day average temperature was approximately 12.2% COD, 16.8% NH₃-N, 12.6% TN for CW–MFCc, and 15.6% COD, 19.9% NH₃-N, 16.2% TN for
Figure 4 | Pollutants removal of the constructed wetland–microbial fuel cell systems at various atmospheric temperatures.
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$_4^{3-}$/C0$_7$-P and 84.4–89.0% TP) and CW–MFCo (84.2–89.2% PO$_4^{3-}$/C0$_7$-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$_4^{3-}$/C0$_7$-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 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 in CWs, including sedimentation, filtration, interception, adsorption, absorption, precipitation, ion exchange, and complexion 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–MFCs 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 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
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.
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

| 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 | 1,780,950 | 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 | 7.619 | 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 | 1,925,959 | 0.986 | 95.599 |
| 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 | 1,952,238 | 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 | 65.445 |
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.
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 electrogener 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 5% 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 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% NH3-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% NH3-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 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.

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**DATA AVAILABILITY STATEMENT**

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

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