Neglected Effects of Inoculum Preservation on the Start-Up of Psychrophilic Bioelectrochemical Systems and Shaping Bacterial Communities at Low Temperature

Sidan Lu1,2†, Binghan Xie1†, Bingfeng Liu1, Baiyun Lu1 and Defeng Xing1*

1 State Key Laboratory of Urban Water Resource and Environment, School of Environment, Harbin Institute of Technology, Harbin, China, 2 Department of Civil and Environmental Engineering, Louisiana State University, Baton Rouge, LA, United States

Bioelectrochemical systems (BESs) are capable of simultaneous wastewater treatment and resource recovery at low temperatures. However, the direct enrichment of psychrophilic and electroactive biofilms in BESs at 4°C is difficult due to the lack of understanding in the physioecology of psychrophilic exoelectrogens. Here, we report the start-up and operation of microbial fuel cells (MFCs) at 4°C with pre-acclimated inocula at different temperatures (4°C, 10°C, 25°C, and −20°C) for 7 days and 14 days. MFCs with 7-day-pretreated inocula reached higher peak voltages than did those with 14-day-pretreated inocula. The highest power densities were obtained by MFCs with 25°C – 7-day-, 25°C – 14-day-, and 4°C – 7-day-pretreated inocula (650–700 mW/m²). In contrast, the control MFCs with untreated inocula were stable at 450 mW/m². The power densities of MFCs with 7-day-pretreated inocula were higher than those obtained by MFCs with 14-day-pretreated inocula. The MFCs with 10°C – 7-day-pretreated inocula and the control MFCs showed higher chemical oxygen demand (COD) removal (90–91%) than other MFCs. Illumina HiSeq sequencing based on 16S rRNA gene amplicons indicated that bacterial communities of the anode biofilms were shaped by pretreated inocula at different temperatures. Compared with the control MFCs with untreated inocula, MFCs with temperature-pretreated inocula demonstrated higher microbial diversity, but did not do so with −20°C-pretreated inocula. Principal components analysis (PCA) revealed an obvious separation between the inocula pretreated at 4°C and those pretreated at 10°C, implying that bacterial community structures could be shaped by pretreated inocula at low temperatures. The pretreatment period also had a diverse impact on the abundance of exoelectrogens and non-exoelectrogens in MFCs with inocula pretreated at different temperatures. The majority of the predominant population was affiliated with Geobacter with a relative abundance of
INTRODUCTION

Temperature is one of the main environmental factors that may potentially influence bacterial activities and wastewater treatment efficiency (Barria et al., 2013). Approximately 80% of natural climates on Earth, including the Arctic Circle and mountain areas, are permanently cold with temperatures below 5°C. Other areas are diurnal or seasonal in low temperatures (De Maayer et al., 2014). For example, the water temperatures in mountain areas are permanently cold with temperatures below 5°C. Other areas are diurnal or seasonal in low temperatures (De Maayer et al., 2014). For example, the water temperatures in mountain areas, are permanently cold with temperatures below 5°C. Other areas are diurnal or seasonal in low temperatures. (De Maayer et al., 2014).

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The possibility of applying BESs at low temperatures has been studied. Furthermore, direct and indirect acclimations have been developed to enrich psychrophilic biofilms at low temperatures (4–15°C). The first investigation reported the direct start-up and stable operation of MFCs and MECs at 4°C when a sequential inoculation method with a mixed-culture inoculum was employed (Lu et al., 2011). The indirect methods first needed to start BESs at a mesophilic condition (mostly 25°C and 30°C), and then the temperatures were decreased to 20°C, 10°C, or 5°C (Catal et al., 2011; Mei et al., 2017). Other methods used psychrophilic microbes as the inocula of BESs to treat wastewater at 15°C (Heidrich et al., 2018; Petropoulos et al., 2019). Although physiological characteristics, microbial growth rate, and microbial activity were reported to be negatively affected by a decreased temperature of wastewater (Zhou et al., 2018), psychrophilic bio-reactors have advantages that include lower operating costs, wide application, and advancement in enriching psychrophilic microbes compared with mesophilic conditions (Petropoulos et al., 2019). A microbial community of the electrode biofilms in BESs were reported to be noticeably shaped by temperature (Mei et al., 2017). Until now, many studies have reported the operation of psychrophilic BESs at 10–15°C, but few of the studies were operated at 4°C. Thus, the exploration of new methods that rely on simple operations to improve the feasibility of psychrophilic reactors is desperately needed. Additionally, a wider range of cold temperatures is also necessary for the application of bio-electrochemical technologies. In this study, the effects of inocula pretreated at different temperatures on the start-up and operation of MFCs at 4°C were estimated, and microbial community structures of the anode biofilms were analyzed using Illumina HiSeq sequencing of 16S rRNA gene amplicon.

MATERIALS AND METHODS

Electrode Materials and MFC Configuration

The MFCs contained one chamber composed of polycarbonate cubes with an inner cylindrical configuration. The chamber was 3 cm in diameter and 4 cm in length with a volume of 25 mL. An anode and a cathode were placed inside the chamber, and no membrane was used to separate the space (Supplementary Figure S1). The anode was a graphite brush (3 cm in diameter × 3 cm in length) that was placed horizontally inside the left part of the MFC chamber. The anodes were successively washed in 1 M HCl (24 h), 1 M NaOH (24 h), and deionized water (24 h) to remove any possible pollutants. The anodes were later heated in a muffle furnace.
Soluble chemical oxygen demand (COD) was determined using test kits (TNT plus vial test, Hach Co.). The COD removal (%) was calculated as the ratio between the output mass of COD and the input mass in the MFCs. The electrical current was calculated according to $U = IR$. Power density was calculated based on $W = UI/A$ (the electrode surface area, 7 cm$^2$). Coulombic efficiency was calculated as the ratio between the experimental coulombs by integrating the current over time and the theoretical coulombs calculated based on COD changes. The equation for this calculation is $C = F \cdot b \cdot V \cdot ΔCOD$, in which $C$ = Coulombic efficiency, $F = 96485$ s/mole, Faraday constant, $b = 4$, the exchanged number of electrons per mole $O_2$, $V = 25$ mL, the volume of the anode liquid, and $ΔCOD$ = the differences of the COD changed in one cycle.

**RESULTS**

**Electricity Generation by MFCs With Different Pretreated-Inocula**

The MFCs with 7-day-pretreated inocula attained a stable voltage production of $\sim 0.48$ V after operation for 20 days. The voltages of the 10°C – 7-day and 4°C – 7-day MFCs increased faster than did the 25°C – 7-day and −20°C – 7-day MFCs (Figure 1). However, the voltage of the control reactors without pretreatment was very slow to increase, and only reached 0.4 V voltage at the stable stage. The voltages of the 14-day-treated reactors finally
The voltage production of the MFCs with 7-day- and 14-day-pretreated inocula exhibited distinct acclimation, though the control reactors were the slowest and achieved the lowest peak voltage (Figure 1). It can be concluded that both the 7-day- and 14-day-pretreatments in the psychrophilic temperatures had positive impacts on the start-up process, and the MFCs with 7-day-pretreated inocula reached higher peak voltages than did those with 14-day-pretreated inocula. The highest power densities were confined to the MFCs with 25°C – 7-day- and 25°C – 14-day-pretreated inocula (700 mW/m²), while the 4°C – 7-day sample was lower than the 25°C temperatures (650 mW/m²) (Figure 2). In contrast, MFCs with –20°C – 7-day- and –20°C – 14-day-pretreated inocula obtained the lowest power densities of 200–300 mW/m² when the control MFCs were stable at 450 mW/m², indicating that 7-day-pretreated inocula at temperatures that were higher than –20°C were helpful for extracellular electron transfer (EET) in MFCs. In total, the power densities of MFCs with 7-day-pretreated inocula were higher than those with 14-day-pretreated inocula, and the decrease of temperature indicated negative influence on the capacity of EET.

**Effect of Pretreated-Inocula on Organics Removal of MFCs**

Both the pretreatments of 7 days and 14 days at temperatures of 4°C, 10°C, 25°C, and –20°C attained high COD removal rates (73–91%) (Figure 3). The COD removal of MFCs with 10°C – 7-day-pretreated inocula and the control reactors (90–91%) was higher than that of other MFCs, and the MFCs with 4°C – 14-day-pretreated inocula had the lowest COD removal of 73%. The coulombic efficiencies (CEs) of the MFCs fluctuated between 41 and 51%. The 4°C – 14-day-pretreated inocula exhibited the highest CEs, while the 10°C – 7-days and the control reactors (41–42%) exhibited the lowest (Figure 3). The MFCs with 7-day-pretreated inocula had higher COD removal, but lower CEs, than did the 14 days.

**Microbial Diversity of the Anode Biofilms in MFCs With Pretreated-Inocula**

The observed species and coverage (>99.0%) of each of the MFCs were relatively stable when the sequences number reached 49892–55139, suggesting that the sequencing depths were sufficient to represent the presence of rare OTUs (Figure 4). The OTUs in different samples varied from 702 to 1336. The
estimators of species richness, Chao1 and ACE indices, indicated that MFCs with pretreated inocula had a higher predicted species richness than did the control MFCs with untreated inocula (Table 1). Compared to the control MFCs, MFCs with pretreated inocula exhibited higher Shannon and Simpson values, except for the −20°C pretreated inocula (Table 1). It is obvious that MFCs with 4°C – 7-day- and 10°C – 14-day-pretreated inocula had the highest species richness, while the MFCs with −20°C pretreated inocula had the lowest richness. Thus, pretreatment in psychrophilic temperatures results in different species richness, and pretreatment at 4°C for 7 days and 10°C for 14 days can result in high richness of microbes. When the observed OTUs were similar between 7- and 14-day-pretreatments, the 14-day-pretreatment showed relatively higher diversity indices by enhancing evenness.

Principal components analysis (PCA) indicated that the bacterial communities of the anode biofilms in MFCs were categorized into four clusters based on OTUs (Figure 5). The control MFCs substantially separated from all MFCs with the pretreated inocula. Excluding the bacterial community of the control reactors, the community structures of 4°C – 7 days and 10°C – 14 days were vastly different from the others. There was obvious separation between different pre-acclimated periods at 4°C and 10°C, though not at −20°C and 25°C. The results implied that pretreated inocula at low temperatures (4°C and 10°C) have a substantial impact on the bacterial community structures.
Temperature-Pretreated Inocula Shaped Bacterial Community Structure in MFCs

The heatmap indicated that a difference in predominant populations between the control and the MFCs with pretreated inocula was present; the 4°C – 7-day-pretreated inocula differed from other pretreated MFCs (Figure 6). *Thauera, Thermomonas, Sphingobium, Legionella,* and *Rhodanobacter* were the differential populations in the control MFCs. *Desulfovibrio, Methylobacterium, Nitrospira,* *Candidatus Accumulibacter,* and *Cal'dilinea* were the most differential populations in MFCs with 4°C – 7-day-pretreated inocula, while differential populations in MFCs with 4°C – 14 days belonged to *Devosia* and *Fusibacter.*

The most predominant genera of the MFCs were affiliated with *Geobacter,* *Dechloromonas,* *Limnohabitus,* *Janthinobacterium,* *Arcobacter,* and *Sejongia* (Figure 7). The relative abundance of *Geobacter* varied from 17 to 70% in the bacterial communities of the anode biofilms, which was high in MFCs with −20°C – 7-day- and −20°C – 14-day-pretreated inocula, indicating that *Geobacter* could be effectively enriched using −20°C pretreated inocula. On the contrary, the relative abundance of *Geobacter* was lower in MFCs with both 4°C – 7-day- and 4°C – 14-day-pretreated inocula compared with others, suggesting that a temperature of 4°C may allow more varied communities to grow. The statistical analyses indicated that *Geobacter* was significantly and positively correlated with Shannon and Simpson indices (Table 2). The relative abundance

**Table 2** | Pearson correlations between the predominant genera and the diversity indexes.

| Genera         | Observed species | Chao1 | ACE  | Shannon | Simpson |
|----------------|------------------|-------|------|---------|---------|
| Geobacter      | −0.683           | −0.69 | −0.67 | −0.964**| −0.99** |
|                | 0.058            | 0.069 | 0.069 | 0.000   | 0.000   |
| Dechloromonas   | 0.919**          | 0.910**| 0.907**| 0.783*  | 0.659   |
|                | 0.001            | 0.002 | 0.002 | 0.022   | 0.075   |
| Limnohabitus    | 0.179            | 0.196 | 0.167 | 0.587   | 0.748*  |
|                | 0.887            | 0.641 | 0.693 | 0.126   | 0.033   |
| Janthinobacterium| 0.068            | 0.09  | 0.069 | 0.510   | 0.656   |
|                | 0.873            | 0.832 | 0.872 | 0.196   | 0.077   |
| Arcobacter      | 0.865            | 0.656 | 0.634 | 0.721** | 0.693   |
|                | 0.072            | 0.078 | 0.091 | 0.044   | 0.057   |

Two-tailed. *p < 0.05, **p < 0.01.
of *Dechloromonas* was higher in the 4°C – 7-day-pretreated inocula than in the others, and bacteria was positively connected with richness indices (observed species, Chao1, and ACE). *Limonohabtans* and *Janthinobacterium* were found in larger percentages in the 4°C – 14-day and 10°C – 14-day samples than other reactors, and the amount of *Limonohabtans* was positively correlated with the Simpson index. *Arcobacter* was found to be positively connected with the Shannon index (0.721* naïve*), which had an amount that was also larger in 4°C – 7-day-pretreated inocula.

**DISCUSSION**

**Current Generation by BES With Temperature-Pretreated Inocula**

The preservation procedure of the inoculum has an important influence on the performance of psychrophilic MFCs. However, the procedure has been naturally neglected and very few studies have completely analyzed the mechanisms. In this study, the MFCs attained high COD removal rates of 73–91%, and the peak voltages all reached 0.48 V. The maximum power density attained by MFCs was 630–712 mW/m² by inocula pretreated at 25°C. The power densities of the other MFCs in decreasing order were attained by inocula pretreated at 4°C, 10°C, and −20°C, with values of 526–650 mW/m², 497–533 mW/m², and 404–437 mW/m², respectively. The power densities are higher than those found in a previous study that treated domestic wastewater at 23–30°C. The coulombic efficiencies of 422 mW/m² at 0°C to 1,260 mW/m² at 30°C, and found that the MFCs initially operated at 15°C hardly generated electricity, while the reactors that first started up at 30°C were effective in power generation when the temperatures decreased to 4°C or 10°C (Cheng et al., 2011). The coulombic efficiencies were higher than those in a study that examined the influences of low temperatures, indicating that the coulombic efficiency varied from 24 to 38% at 20°C and 4°C, with power densities ranging from 486 mW/m² to 602 mW/m². Moreover, there was a confusing phenomenon in which, even though the reactor conditions were the same, some MFCs could be easily started while others were unable to start. Our results indicated that this might be attributable to the fact that some inocula were used once they were collected, but other inocula were unintentionally kept in refrigerators for some days before inoculation. Although the MFCs were initially operated at 4°C, the power densities were even higher than in a previous study of MFCs (422 mW/m²) that treated domestic wastewater at 23–30°C (Ahn and Logan, 2010). The reason for the contradiction in the results might be the influences of inocula preservation. Moreover, the present coulombic efficiencies (41–51%) were approximately two times higher than those found in a study that obtained coulombic efficiencies of 24–38% at 4°C, which might also be attributable to an effect of the inocula preservation.

**Enrichment of Psychrophilic Geobacter at Low Temperature**

The mesophilic *Geobacter* species has been widely investigated as a typical exoelectrogen (*Geobacter sulfurreducens* and *Geobacter metallireducens*) (Holmes et al., 2016; Tian et al., 2017). In this study, the enriched psychrophilic *Geobacter* species differed phylogenetically from mesophilic *Geobacter* species. The relative abundance of psychrophilic *Geobacter* in MFCs with inocula pretreated at 10°C and 25°C decreased with an increase of pretreatment period from 7 to 14 days, and the diversity and relative abundance of non-exoelectrogenic bacteria increased correspondingly. In contrast, there was not a large difference in the relative abundance of psychrophilic exoelectrogens at −20°C, because the growth and reproduction of exoelectrogens and non-exoelectrogens was presumably inhibited at −20°C. PCA analysis also revealed that the bacterial communities of MFCs with −20°C – 7-day- and −20°C – 14-day-pretreated inocula were similar. This suggests that the pretreatment period had a complicated impact on the enrichment of exoelectrogens and non-exoelectrogens at different pretreated temperatures. MFCs with 10°C – 14-day-pretreated inocula exhibited a lower relative abundance of *Geobacter* than MFCs with 10°C – 7-day-pretreated and untreated inocula, though they had a higher power density. The community structure

**Inoculum Influences Start-Up of Psychrophilic BES**

Performances of MFCs are mainly determined by electrode biofilms capable of EET (Logan et al., 2006; Malvankar et al., 2012). Direct start-up of BESs at temperatures below 5°C is difficult because of the slow growth of the electrode biofilm-composed psychrophilic exoelectrogens. The performance of MFCs decreased because of the decline of temperatures (Cheng et al., 2011). Various strategies have been used to enrich exoelectrogenic bacteria and overcome the problem of temperature (Lu et al., 2011, 2012b; Shrestha et al., 2019). Only one previous investigation reported for the first time the successful direct start-up and stable operations of MFCs and MFCs at 4°C via a sequential inoculation approach with a mixed-culture inoculum (Lu et al., 2011). Our study reports that the inoculum preservation approach can enhance the power output of MFCs at 4°C. The recent studies indicated that inocula from different sources influence the electricity generation of MFCs and the community structure of the anode biofilms at mesophilic conditions (Mei et al., 2015; Do et al., 2018). The current study explored the effects of the pretreated inocula of activated sludge from a wastewater treatment plant (WWTP) on the performance of MFCs at 4°C, but the inocula from other sources should be estimated in the psychrophilic BESs after pretreatment at different temperatures. The MFCs obtained higher power density after being preserved at 25°C, indicating that exoelectrogenic microbes pre-enriched at a mesophilic temperature successfully adapted to 4°C. The psychrotolerant bacteria isolated at 10°C was able to grow at temperatures as low as 4°C (Holmes et al., 2004). However, the electricity generation by a pure culture in BESs at 4°C has not yet been reported.
and the relative abundance of Geobacter in MFCs with inocula pretreated at 20°C were similar, but the power densities were substantially different with different pretreatment periods. Therefore, the higher relative abundance of Geobacter is not the reason for a higher power density. Presumably, DNA from dead cells at 14 days preservation was amplified and sequenced, resulting in the overestimation of the relative abundance of Geobacter. These results imply that this phenomenon may be derived from the differences in species and the activity of EET of Geobacter. To estimate the EET activity of putative psychrophilic exoelectrogens, the electrode biofilms in MFCs with the inocula pretreated at different temperatures require further investigation using metatranscriptomic or metaproteomic approaches.

Moreover, because of short sequencing reads, it is worth mentioning that the next-generation sequencing technologies primarily determined OTUs at the genus-level. The real condition of the taxonomic level of species for Geobacter might be different depending on whether or not the bacterial proportions were the same in each sample. As far as we know, there are a number of different Geobacter species (Zhou et al., 2017), and the ability of EET is different. Single-molecule sequencing technology with long reads will provide a powerful tool in distinguishing Geobacter spp. at the species-level.

**Shaping Bacterial Community Structures by Temperature-Pretreated Inocula**

Anaerobic psychrophilic microbes have only been discovered in natural environments (Letttinga et al., 2001). The discovery of groups of microbes that naturally prefer low temperatures or adapt quickly to psychrophilic temperatures is helpful for the advancement of biological technologies (Ahn and Logan, 2010). Mesophilic bacteria enriched in MFCs require longer periods of time to adapt in low temperatures, thereby causing the changes of community structures to be uncertain when the operation time is relatively short. Our study indicated that the response of exoelectrogens and non-exoelectrogens to temperature is substantially different, and so far only a putative psychrophilic exoelectrogen, Geobacter spp., has been identified in BESs (Lu et al., 2011, 2012a). A recent study has reported that psychrophilic Geobacter was enriched in MFCs at 7.5°C using arctic soil as the inoculum (Heidrich et al., 2018). PCA determined the bacterial communities of MFCs with inocula pretreated for 7 days and 14 days at 25°C were similar, but they were clearly separated at 4°C and 10°C for different pretreatment periods. Our study also provides another new approach for the fast start-up of psychrophilic BES at 4°C using preservation inocula beyond the sequential inoculation method.

**CONCLUSION**

This study describes the effect of inocula pretreated at different temperatures on the electricity generation and microbiome in MFCs at low temperatures. Inocula pretreated at both psychrophilic (−20°C, 4°C, and 10°C) and mesophilic (25°C) temperatures were observed to enable fast start-up of MFCs at 4°C. After the start-up, the psychrophilic MFCs obtained the power densities of 404–712 mW/m², the COD removals varied from 73 to 91%, and the coulombic efficiencies were 41–51%. Moreover, different pretreatment temperatures and periods both impacted the electricity generation and bacterial communities of the anode biofilms of MFCs. The pretreatment period had different effects on the enrichment of psychrophilic exoelectrogens and non-exoelectrogens in MFCs with inocula pretreated at different temperatures. In terms of power output of MFCs, the 7-day-pretreatments were generally more beneficial than were those of 14 days. In terms of temperature, all psychrophilic and mesophilic pretreatments achieved the similar peak voltage of 0.48 V. The majority of psychrophilic populations demonstrated the potential of the enrichment of Geobacter in MFCs with pretreated inocula. This study provides a simple method to start up psychrophilic MFCs, which have been understandably neglected by previous studies. The inocula pretreatment method can also be applied in the start-up of other psychrophilic BESs.

**AUTHOR CONTRIBUTIONS**

SL, BX, and DX designed the study and wrote the manuscript. SL, BX, BFL, BYL, and DX conducted the data analysis. All authors read and approved the final manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00935/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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