



# A two-staged system to generate electricity in microbial fuel cells using methane

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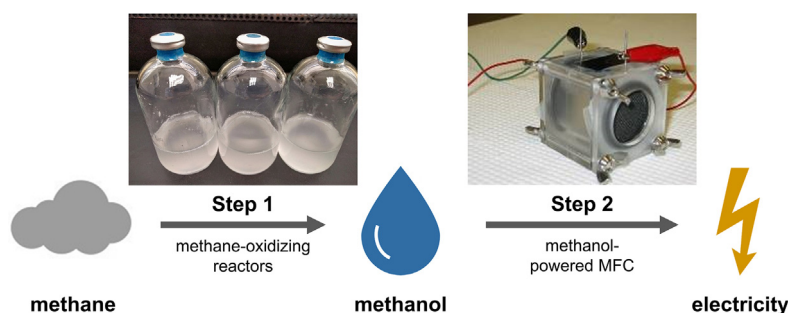
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## HIGHLIGHTS

- A two-step process was used to produce bioelectricity using methane as a substrate.
- In the first step, methane-oxidizing culture oxidizes methane to methanol.
- In the second step, the MFC is supplied with methanol to generate power.
- Acetogens converted methanol into acetate, which was consumed by exoelectrogens.
- Power is generated without the need for engineered strains or aseptic techniques.

## GRAPHICAL ABSTRACT



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## ABSTRACT

Methane is an abundant and inexpensive feedstock that is available as natural gas and renewable biogas. However, methane has not been regarded as a good substrate for microbial fuel cells (MFCs) due to low power densities. To increase power, a two-step strategy was used based on conversion of methane into methanol, followed by electricity generation using methanol as the substrate in the MFC. To produce methanol, a methane-oxidizing culture was grown in a high phosphate buffer resulting in the accumulation of  $350 \pm 42$  mg/L of methanol. The methanol-fed MFC produced a maximum power density of  $426 \pm 17$  mW/m<sup>2</sup>. It was also shown that the methanol-rich medium produced from the first step can directly be supplied to the MFCs, removing the need for purification of methanol. Analysis of the microbial community suggests that acetogens first converts methanol into acetate, which is then consumed by exoelectrogens for power generation.

## 1. Introduction

A microbial fuel cell (MFC) is a technology for harvesting electricity directly from organic matter, and thus it has great potential for treating wastewater economically without the use of energy derived from fossil fuels [1–3]. A variety of substrates can be used in MFCs for electricity production ranging from pure compounds such as acetate [4–7],

propionate [7], butyrate [4,7], glucose [6,8,9], ethanol [10], and xylose [6,11,12], to complex mixtures of organic matter present in wastewater [13–19]. However, few gaseous substrates have been examined other than hydrogen or methane [20–23].

Methane is a readily available from both natural and anthropogenic sources, and is a feedstock that does not compete with food demands [24]. Methane-utilizing bacteria (methanotrophs) have been used to

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convert methane into various bioproducts including biodiesel [25], propylene oxide [26], single cell protein [27,28], extracellular polysaccharides [29], human health supplements [30], and polyhydroxyalkanoate (PHA) bioplastics [31–36]. Use of methane as a substrate for electricity production in MFCs, however, has not been well examined. Electricity was produced first from methane using an uncultured anaerobic methane-oxidizing consortia isolated from oceanic sediment, but the power density was very low ( $0.65 \text{ mW/m}^2$ ) [37] compared to organic substrates such as acetate [38]. A recent study reported that an air-cathode MFC operated in continuous mode on a synthetic, methane-saturated medium generated approximately  $62 \text{ mW/m}^2$  [20]. So far, the highest power density using a methane-powered MFC of  $168 \text{ mW/m}^2$  was obtained using a genetically engineered archaeal strain that was capable of converting methane into acetate, which was then oxidized by exoelectrogens to generate electricity [21].

In this study, we examined a two-step process to utilize methane as a feedstock for bioelectricity generation based on enriching a natural microbial consortium with aerobic methanotrophs in the first step to oxidize methane to methanol. In the second step, the produced methanol solution was used in an MFC to produce bioelectricity from the methanol using a mixed-culture community. Methanotrophs use methane as a carbon and energy source, but are not known to be capable of electricity generation [39,40]. However, methanotrophs can convert methane to methanol using methane monooxygenase (MMO) enzymes that catalyze the single-step conversion of methane into methanol, which is then metabolized by methanotrophs to formaldehyde using methanol dehydrogenase (MDH), and finally formaldehyde is converted to formate by formaldehyde dehydrogenase. The accumulation of methanol can be achieved using various MDH inhibitors such as phosphate buffer, ethylenediaminetetraacetic acid (EDTA), sodium chloride (NaCl), and ammonium chloride ( $\text{NH}_4\text{Cl}$ ) [41]. Here we examined the use of a phosphate buffer as a simple method to readily convert methane into methanol, with the methanol used in an MFC to produce bioelectricity by a mixed microbial exoelectrogenic and fermentative consortium.

## 2. Materials and methods

### 2.1. Methane-oxidizing cultures

All methane-oxidizing cultures were grown in  $2.38 \text{ g/L}$  ( $25 \text{ mM}$ ) of a phosphate buffer solution (PBS; contained the following chemicals per liter of solution:  $2.283 \text{ g}$   $\text{Na}_2\text{HPO}_4$ ,  $1.226 \text{ g}$   $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ ,  $0.155 \text{ g}$   $\text{NH}_4\text{Cl}$ ,  $0.065 \text{ g}$   $\text{KCl}$ ) amended with  $12.5 \text{ mL/L}$  minerals and  $5 \text{ mL/L}$  vitamins [42]. Activated sludge was obtained from the aeration basin at the Penn State University Wastewater Treatment Plant (State College, PA, USA). Large particles were removed by filtration through a  $100\text{-}\mu\text{m}$  pore-diameter cell strainer (BD Falcon Biosciences, Lexington, TN, USA). The dispersed cells were centrifuged ( $10,000 \times g$ ) for 5 min to produce a pellet, resuspended in  $50 \text{ mL}$  of PBS medium, and then shaken to disperse the cells. Cell suspensions were incubated in  $160 \text{ mL}$  serum bottles (Wheaton, Millville, NJ, USA) capped with thick butyl-rubber stoppers and crimp-sealed under a  $\text{CH}_4\text{:O}_2$  headspace (molar ratio 1:1.5, > 99% purity). Cultures were incubated horizontally on orbital shaker tables at 150 revolutions per minute (rpm) at  $30^\circ\text{C}$ . The headspace of each bottle was flushed daily with a  $\text{CH}_4\text{:O}_2$  mixture (molar ratio of 1:1.5), and every 48 h,  $40 \text{ mL}$  of the suspensions were replaced with  $40 \text{ mL}$  of fresh PBS medium. The methane-oxidizing enrichments were allowed to reach a steady-state condition (based on their maximum cell densities) for the first 16 d, and data were collected starting on day 17. In order to find the concentrations of phosphate and ammonium that resulted in the maximum methanol concentrations, the methane-oxidizing enrichment was subjected to a PBS medium containing different concentrations of phosphate (0, 2.0, 4.0, 6.0, 8.0, 10.0,  $12.0 \text{ g PO}_4\text{/L}$ ) and ammonium (34, 68, 102, 136, 170, 255,  $340 \text{ mg NH}_3\text{-}$

N/L).

### 2.2. MFC construction and operation

MFC tests were conducted in triplicate using single-chamber, cubic-shaped air-cathode MFC reactors containing a cylindrical anode chamber  $4\text{-cm}$  long and  $3\text{-cm}$  in diameter [43]. The graphite fiber brush anode ( $2.5 \text{ cm}$  in both diameter and length) was heat treated at  $450^\circ\text{C}$  in air for 30 min before use and was placed horizontally in the middle of MFC chambers. Cathodes were prepared using a hot-pressing method as previously described [44]. The catalyst layer was prepared by mixing activated carbon (AC, Norit SX plus, Norit Americas Inc., TX, USA) with a 60% polytetrafluoroethylene (PTFE) emulsion (Sigma Aldrich, MO, USA) at a mass ratio of AC:PTFE (6:1). The cathode current collector was a stainless steel mesh ( $42 \times 42$ , type 304, McMaster-Carr, IL, USA). A hydrophobic polyvinylidene fluoride (PVDF) membrane ( $0.45 \mu\text{m}$ , Millipore, MA, USA) was used as a diffusion layer to prevent water leakage. The AC:PTFE, current collector and diffusion layer were pressed at  $3 \times 10^7 \text{ Pa}$  for at least 15 s at  $60^\circ\text{C}$  until the membrane surface became dry [44,45]. The pressed cathodes were then taken out and dried in a fume hood for later use.

Reactors were inoculated using anaerobic sludge collected from the Penn State University Wastewater Treatment Plant and operated in batch mode (State College, PA, USA), with a  $1000 \Omega$  resistor in the circuit. The MFCs were emptied and refilled daily with a fresh  $8.0 \text{ g/L}$  PBS medium amended with  $320 \text{ mg/L}$  methanol,  $12.5 \text{ mL/L}$  minerals, and  $5 \text{ mL/L}$  vitamins for 30 d until the reactors reached steady state based on repeatable cycles of voltage production. In some tests, MFCs were refilled with a methanol-rich medium produced from the methane-oxidizing reactors instead of the PBS medium.

Voltage ( $U$ ) across the external resistor in the MFC circuit was measured at 20 min intervals using a data acquisition system (2700, Keithley Instrument, OH, USA) connected to a personal computer. Current ( $I = U/R$ ) and power ( $P = IU$ ) were calculated as previously described [2], and normalized by the projected surface area of the cathode ( $7 \text{ cm}^2$ ). Power density curves were obtained by varying external circuit resistance using the single cycle polarization method, with a single resistor used for a full batch cycle. An Ag/AgCl reference electrode (BASi) was placed in the middle of the MFC chamber to obtain anode potentials (reported versus Ag/AgCl electrode,  $+210 \text{ mV}$  vs. a standard hydrogen electrode), with the cathode potential calculated using the anode potential and the whole cell voltage. Coulombic efficiency ( $\epsilon_c$ ) was calculated by dividing the total coulombs transferred to the anode by the theoretical maximum number of coulombs (total coulombs produced by complete methanol oxidation to carbon dioxide).

### 2.3. Analytical methods

The gas composition of methane-oxidizing reactors were analyzed using a gas chromatograph (SRI Instruments, models 8610B and 310, CA, USA) as previously described [46].

Methanol and acetate concentrations were analyzed using a gas chromatograph (Agilent, model 6890, CA, USA) equipped with a FID and a DB-FFAP fused-silica capillary column with helium as carrier gas (constant pressure of  $103 \text{ kPa}$ ). The oven temperature of the GC was started at  $60^\circ\text{C}$  and programmed at  $20^\circ\text{C/min}$  to  $120^\circ\text{C}$ , and then  $30^\circ\text{C/min}$  to a final temperature of  $240^\circ\text{C}$  held constant for 3 min. The injector and detector temperature were both  $250^\circ\text{C}$  [42].

To analyze total suspended solids (TSS),  $0.5\text{--}5.0 \text{ mL}$  of cell suspension was filtered through pre-washed, dried, and pre-weighed  $0.2 \mu\text{m}$  pore-diameter membrane filters (Pall, Port Washington, NY, USA). The filtered cells and membrane filters were dried at  $105^\circ\text{C}$  for 24 h, then weighed.

For all data, arithmetic mean values and standard deviations were calculated for triplicate samples. Statistical differences between sample

means were tested using the Welch's *t*-test for unpaired samples. The *p*-value was used to evaluate significance, with differences defined as significantly different for  $p \leq 0.05$ .

## 2.4. Microbial community analyses

Microbial communities of methane-oxidizing reactors and methanol-utilizing MFCs were characterized using Illumina sequencing of 16S rRNA genes. DNA was extracted from the liquid suspensions (methane-oxidizing reactors) and biofilm (methanol-utilizing MFCs) using the MO Bio PowerSoil DNA extraction kit (QIAGEN, Germany) following the manufacturer's protocols. PCR was performed on the isolated DNA using the 515F/805R primer set. Amplicon sequences were obtained using Illumina MiSeq and were classified using the Ribosomal Database Project (RDP) at a 95% confidence interval. Relative abundance of each genus was estimated by normalizing the number of reads assigned to each genus against the total reads obtained for that sample. The heatmap was generated using R version 2.11.0 using the heatmap function. The fifteen most abundant classified genera per sample were represented in the heatmap.

## 3. Results and discussion

### 3.1. Step 1: Conversion of methane to methanol

Methanol accumulated up to  $322 \pm 10$  mg/L in the methane-oxidizing reactors after a 72-h cycle when the concentration of phosphate was 6.0 g/L (Fig. 1). The rate of methanol accumulation decreased over time, likely due to either enzymatic degradation of methanol or product inhibition slowing down production. During the initial 24 h of the cycle, the average volumetric production of methanol was  $10 \pm 0.9$  mg/L-h. The maximum specific methanol production was  $9.8 \pm 1.0$  mg/g TSS-h, which was comparable to or higher than previously reported values [47,48].

The final concentrations of methanol after a 72-h cycle depended upon the initial concentrations of phosphate present in the PBS medium (Fig. 2a). For phosphate levels  $< 8.0$  g/L, the final concentrations of methanol increased with initial phosphate concentrations. At higher levels, the final concentrations of methanol stabilized at approximately  $350 \pm 42$  mg/L. This result suggests that phosphate is an effective inhibitor for methanol oxidation by MDH, and MDH activity could be

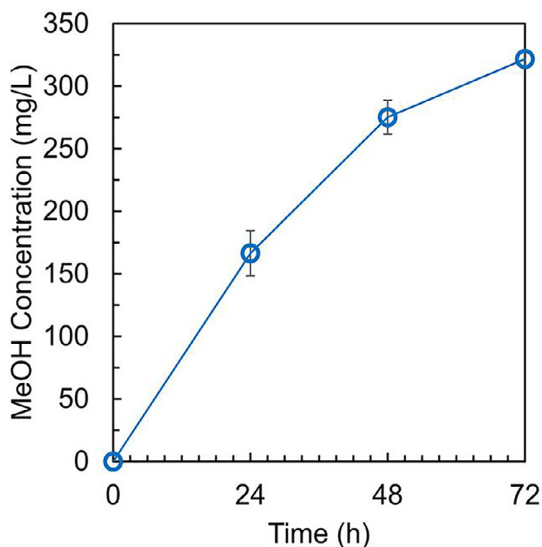


Fig. 1. Concentrations of methanol (mg/L) in methane-oxidizing reactors monitored over a 72-h cycle, using an initial phosphate concentration of 6.0 g/L.

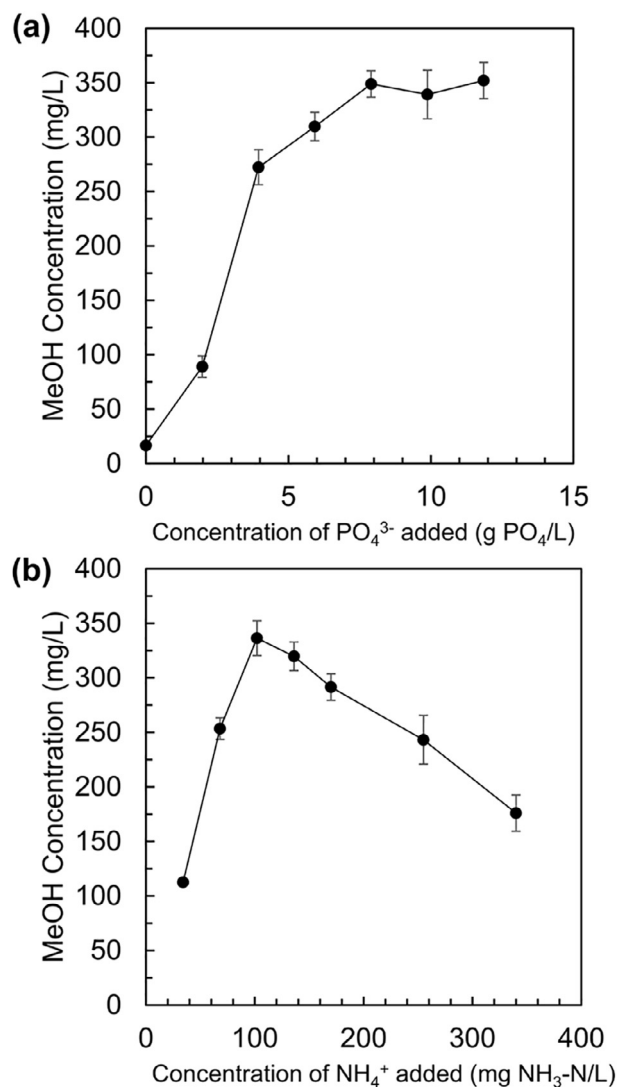


Fig. 2. Final concentrations of methanol (mg/L) accumulated after a 72-h cycle with respect to initial concentrations of (a) phosphate ( $\text{PO}_4^{3-}$ ) and (b) ammonium ( $\text{NH}_4^+$ ) present in PBS media.

effectively inhibited at approximately 8.0 g/L (84.2 mM) of phosphate.

Ammonium also had an effect on the final concentrations of methanol (Fig. 2b). The amount of methanol that accumulated increased with ammonium concentrations up to approximately 100 mg  $\text{NH}_3\text{-N/L}$ , but decreased for higher ammonium levels. Ammonium concentrations are known to impact maximum cell concentrations in methanotrophic culture [49]. While ammonium is a necessary nitrogen source for cell synthesis, high levels of ammonium can be toxic for methanotrophs as it is a competitive inhibitor of methane oxidation by MMO [50].

### 3.2. Step 2: Methanol-powered MFC

Electricity generation steadily increased over the 45-d acclimation period in a single-chamber, air-cathode MFC following initial inoculation with anaerobic sludge and then replacement with fresh methanol and PBS medium every 2 d (Fig. 3). The maximum voltage obtained after 45 d was approximately 0.5 V with an external resistance of 1000  $\Omega$  (Fig. 3). Based on polarization tests taken after 45 d, the maximum power density was  $426 \pm 17$  mW/m<sup>2</sup> (Fig. 4a).

During MFC operation, methanol was rapidly consumed, with a final concentration of  $51 \pm 4$  mg/L after 24 h (Fig. 4b). During this 24 h cycle, the concentration of acetate in solution slowly increased to a

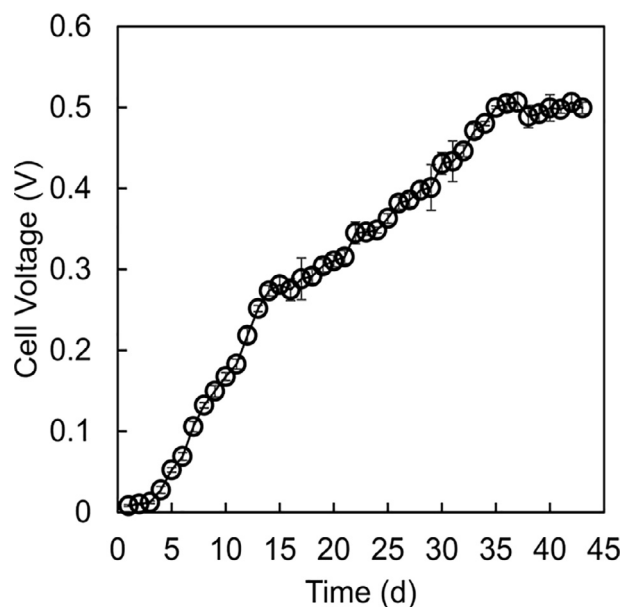


Fig. 3. Voltage generation in triplicate single-chamber MFCs using 320 mg/L of methanol during start up.

maximum of  $55 \pm 12$  mg/L at 12 h, and then decreased to a final value of  $16 \pm 3$  mg/L after 24 h. The production of acetate in a methanol-fed MFC indicated that acetate is a byproduct of methanol oxidation and/or fermentation, with acetate likely used for current generation. The coulombic efficiency ( $\epsilon_c$ ) calculated for a cycle was  $22 \pm 3\%$ .

### 3.3. Integration of step 1 and step 2

In the tests described above, the two reactors were fed separate solutions. To demonstrate that the two reactors could be coupled together, the methanol-rich solution from the methane-oxidizing reactors (step 1) was directly used in the MFCs acclimated to methanol (step 2). The maximum power density produced using the methanol reactor effluent was  $398 \pm 15$  mW/m<sup>2</sup>, which was only slightly less than that produced using fresh PBS medium amended with 320 mg/L of methanol. This indicated that the methanol produced from the first reactor did not need to be purified before being supplied to the MFCs.

### 3.4. Microbial community analysis

Analysis of the microbial communities developed in the suspended consortia in methane-oxidizing reactors indicated the predominance of a Type I methanotrophic genus (*Methylococcobium*) and methylotrophic genera (*Methylobacillus* and *Methylophilus*) (Fig. 5a). Pure cultures of the genus *Methylococcobium* have previously been reported to efficiently convert methane to methanol [51]. The dominance of methylotrophic genera indicated that methanol secreted by methanotrophs is taken up by methylotrophs even with the presence of high levels of phosphate, which is an MDH inhibitor [41,52]. This suggests that further optimization targeted towards increasing the ratio of methanotrophs to methylotrophs might be needed to increase the methanol production yield and the overall efficiency of the process.

The three major genera found in methanol-fed MFCs were *Methylophilus*, *Arcobacter*, and *Acetobacterium* (Fig. 5b). It is possible that the dominance of *Methylophilus* was affected by the microbial community in methane-oxidizing reactors (Fig. 5a), which was also dominated by *Methylophilus*. The presence of *Methylophilus* also suggests that some methanol can be oxidized using diffused oxygen as an electron acceptor through *Methylophilus* activity. *Arcobacter* is a known microaerobic exoelectrogen commonly found in acetate-fed MFCs

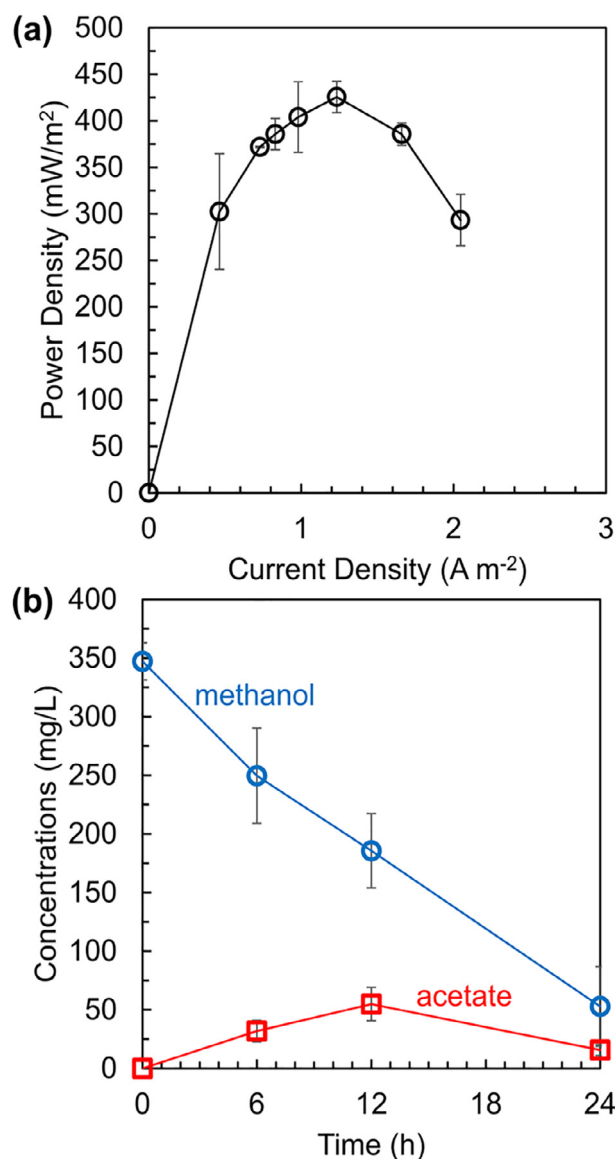


Fig. 4. (a) Power density curve of methanol-fed MFCs taken after 45 days of operation. (b) Concentrations of methanol (blue circle) and acetate (red square) monitored over a 24-h operation of MFCs.

[53,54]. *Acetobacterium* is an acetogenic genus known to convert methanol into acetate through acetogenic fermentation [55,56]. The presence of both *Arcobacter* and *Acetobacterium* suggests that the primary mechanism of generating bioelectricity from methanol was acetogenic fermentation of methanol into acetate, followed by acetate utilization by the exoelectrogens. This conversion route would be consistent with the measurement of acetate in the solution of the MFC over the 24 h fed batch cycle [57]. Power production using acetate is reduced at acetate concentrations below  $\sim 150$  mg/L. The power densities measured here were therefore understandably lower than that possible in this type of MFC using higher acetate concentrations ( $\sim 1$  g/L) [38]. It is not clear why *Geobacter* was not detected in the MFCs, as opposed to a previous study stating that *Geobacter* represented a substantial portion of the bacterial community only in the anode of methanol-fed MFCs [58]. It is possible that the initial seeding of the reactors affected the resulting microbial communities and the primary mechanism for conversion of methanol into bioelectricity, but this would need to be specifically addressed in a future study.



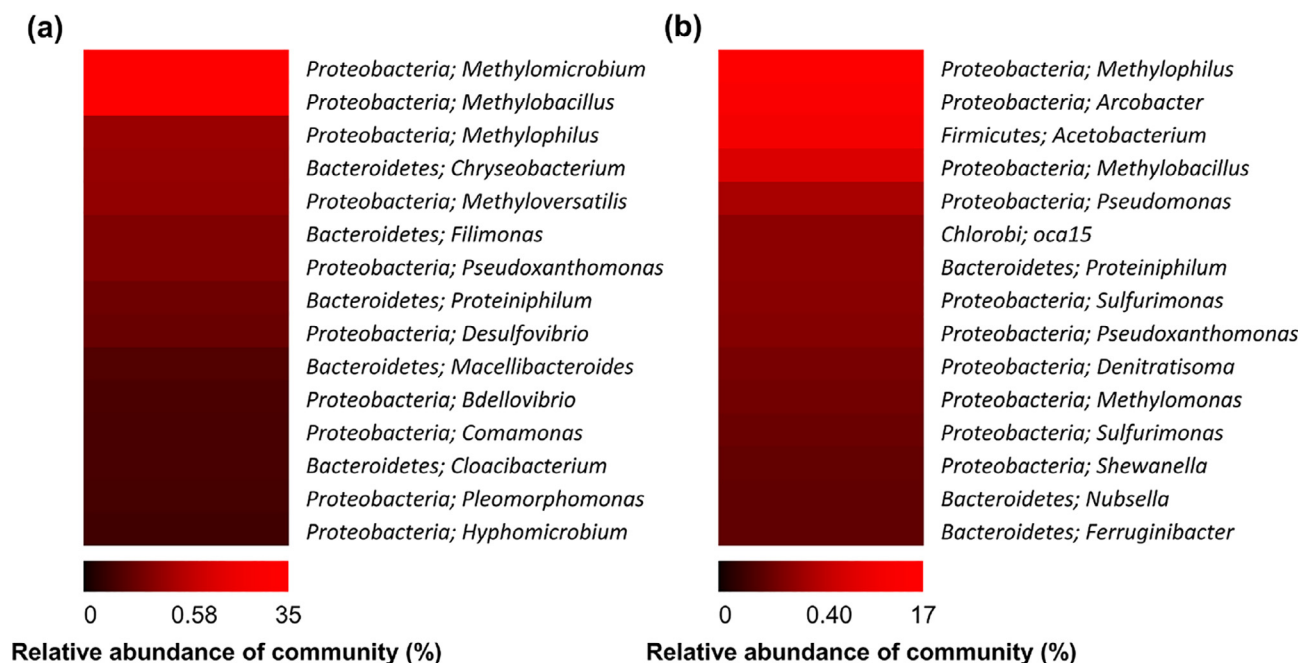


Fig. 5. Microbial communities developed in the suspended consortia in (a) methane-oxidizing reactors; and (b) methanol-fed air-cathode MFCs. The heatmap was created using 15 most abundant classified genera selected from each sample. The color intensity of scale demonstrates the relative abundance of each genus. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

#### 4. Conclusions

A two-step process was developed to produce bioelectricity from methane, based on conversion of methane into methanol in the first reactor, followed by electricity generation using methanol in the MFC. In the first reactor, methane-oxidizing reactor consisting primarily of methanotrophs and methylotrophs released high concentrations of methanol due to the use of a high concentration of phosphate, a known MDH inhibitor. The maximum concentration of methanol was  $350 \pm 42$  mg/L when the phosphate concentration was 8.0 g/L. In the second step, the microbial community consisted of acetogenic bacteria known to convert methanol into acetate and known exoelectrogens. The methanol-fed MFC produced a maximum power density of  $426 \pm 17$  mW/m<sup>2</sup>. This two-step process enabled bioelectricity generation derived from methane, without the need for engineered bacterial strains or aseptic techniques.

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