ACS Sustainable Chemistry & Engineering

Research Article

Cite This: ACS Sustainable Chem. Eng. 2019, 7, 3511−3519

pubs.acs.org/journal/ascecg

Nitrogen Gas Fixation and Conversion to Ammonium Using Microbial Electrolysis Cells

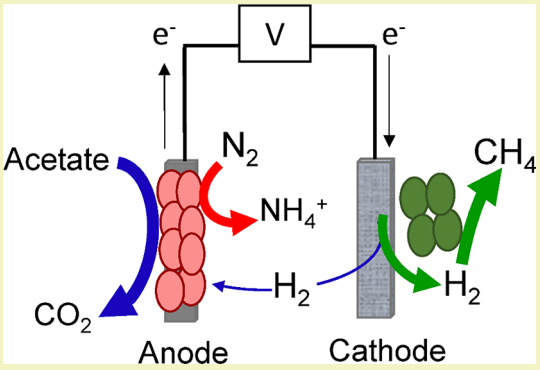
Juan F. Ortiz-Medina,[†] Amy M. Grunden,[‡] Michael R. Hyman,[‡] and Douglas F. Call*[,†]

[†]Department of Civil, Construction, and Environmental Engineering, North Carolina State University, Campus Box 7908, Raleigh, North Carolina 27695, United States

[‡]Department of Plant and Microbial Biology, North Carolina State University, Campus Box 7612, Raleigh, North Carolina 27695, United States

Supporting Information

ABSTRACT: Ammonia (NH_3) is an important industrial chemical that is produced using the energy- and carbon-intensive Haber-Bosch process. Recovering NH_3 from microorganisms that fix nitrogen gas (N_2) may provide a sustainable alternative because their specialized nitrogenase enzymes can reduce N_2 to ammonium (NH_4^+) without the need for high temperature and pressure. This study explored the possibility of converting N_2 into NH_4^+ using anaerobic, single-chamber microbial electrolysis cells (MECs). N_2 fixation rates [based on an acetylene gas (C_2H_2) to ethylene gas (C_2H_4) conversion assay] of a microbial consortium increased significantly when the applied voltage between the anode and cathode increased from 0.7 to 1.0 V and reached a maximum of ∼40 nmol of C_2H_4 min^{-1} mg $protein^{-1}$, which is comparable to model aerobic N_2-fixing bacteria. The presence of NH_4^+, which can inhibit the activity of the nitrogenase enzyme, did not significantly reduce N_2 fixation rates. Upon addition of methionine sulfoximine, an NH_4^+ uptake inhibitor, NH_4^+ was recovered at rates approaching 5.2×10^{-12} mol of NH_4^+ s^{-1} cm^{-2} (normalized to the anode surface area). Relative to the electrical energy consumed, the normalized energy demand [MJ mol^{-1} (NH_4^+)] was negative because of the energy-rich methane gas recovered in the MEC. Including the substrate energy resulted in total energy demands as low as 24 MJ mol^{-1}. Community analysis results of the anode biofilms revealed that *Geobacter* species predominated in both the presence and absence of NH_4^+, suggesting that they played a key role in current generation and N_2 fixation. This study shows that MECs may provide a new route for generating NH_4^+.



KEYWORDS: *Nitrogen fixation, Ammonium production, Haber-Bosch, Microbial electrochemical technology, Geobacter*

INTRODUCTION

Ammonia (NH_3) is a widely used chemical in the industrial, commercial, and agricultural sectors. Increasing from roughly 100 million tons of NH_3 $year^{-1}$ in 2008 to 150 million tons of NH_3 $year^{-1}$ in 2017, NH_3 is the second most produced chemical in the world.[1] Demand for NH_3 is expected to increase as the population grows.[1] The majority (∼80%) of NH_3 produced is used in fertilizers. It is also an ingredient in other products such as explosives, pharmaceuticals, refrigerants, and cleaning products and is receiving growing interest as an alternative transportation fuel.

Most NH_3 is produced through the Haber−Bosch process, an energy- and carbon-intensive technology. In this process, nitrogen (N_2) and hydrogen (H_2) gases are converted into NH_3 under high temperatures (350−550 °C) and pressures (150−300 atm).[2] It consumes 1.5−2.5% of global energy annually and is responsible for roughly 2.5% of global carbon dioxide (CO_2) emissions.[3] Alternatives to Haber−Bosch include a growing list of methods involving electrochemistry, photocatalysis, plasma induction, and metallic complex catalysis.[2] Many of these technologies suffer from low conversion efficiencies, expensive catalysts, and low selectivity.[2] Electrochemical methods in which N_2 is reduced on anode electrodes have been examined for a wide range of electrolytes and catalysts.[4] Finding resilient and selective catalysts remains a challenge for these methods,[4] and high temperatures (which impart an energy penalty) are still preferred because of slow kinetics at ambient temperatures.[4] Bioelectrochemical approaches in which an enzyme (e.g., nitrogenase) is attached to an electrode overcome many of the limitations of abiotic systems. However, the irreversible damage of these enzymes upon oxygen (O_2) exposure, the requirement for soluble electron shuttles such as methylviologen, and long-term enzyme stability are major limitations.[5]

Using whole-cell N_2-fixing microorganisms (called diazotrophs), rather than just the isolated nitrogenase enzyme, is an alternative route to generate NH_3. These naturally occurring microorganisms have nitrogenase enzymes that break the

Received: November 6, 2018
Revised: December 28, 2018
Published: January 1, 2019

DOI: 10.1021/acssuschemeng.8b05763
ACS Sustainable Chem. Eng. 2019, 7, 3511−3519

strong N≡N bond under ambient conditions.[6] Many microorganisms in nitrogen-deficient environments rely on these enzymes to provide fixed nitrogen for growth (e.g., amino acids) or to support the growth of other organisms. Agricultural practices leverage the latter function from symbiotic diazotrophs in the farming of legumes.[7] Free-living diazotrophs, which do not require a symbiont, have been engineered to excrete ammonium (NH_4^+; the protonated form of NH_3; $pK_a = 9.3$) to concentrations as high as 30 mM.[8] One inherent limitation of aerobic diazotrophs, including model *Azotobacter* species, is that their terminal electron acceptor (i.e., O_2) can also inhibit nitrogenase function.[9] Increasing growth and respiration by using higher O_2 concentrations can therefore reduce N_2 fixation activity. Driving high N_2 fixation rates remains a challenge for moving these biological approaches forward.

One possible route to drive the microbial conversion of N_2 into NH_4^+ is to combine the fields of electrochemistry and microbiology. Research in electromicrobiology has shown that electrically active microorganisms, many of which are known diazotrophs, are highly responsive to electrical driving forces.[10] In anaerobic microbial electrolysis cells (MECs), applied voltage drives the conversion of organic material into electrical current by anode respiring microorganisms (exoelectrogens), resulting in the production of energy-rich gases, for example, H_2 and CH_4.[11] Community analyses of anode biofilms have revealed that *Geobacter* species predominate in these systems.[12] These bacteria are also prolific N_2-fixing microorganisms, which is one explanation for their abundance in nitrogen-limited bioremediation sites.[13,14] N_2 fixation has been previously documented in microbial electrochemical technologies (METs),[15−17] but the generation and recovery of NH_4^+ have yet to be shown.

As MEC anodes are typically highly populated with *Geobacter* species, operation occurs under completely anaerobic conditions (where O_2 cannot inhibit nitrogenase function), and applied voltages (>0.2 V) drive anode respiration rates, we hypothesized (1) that we could use voltage to drive N_2 fixation and (2) NH_4^+ could be recovered from N_2-fixing exoelectrogenic communities. To test these hypotheses, we measured the current densities of MECs with only N_2 gas as the nitrogen source at two applied voltages (E_{AP}, 0.7 and 1.0 V). We estimated N_2 fixation rates using the acetylene (C_2H_2) reduction assay, which is a standard method for assessing nitrogenase activity.[18] To determine the impact of NH_4^+, which is known to slow or inhibit N_2 fixation, identical MECs with NH_4^+ and a N_2 headspace were operated in parallel. To explore the possibility of recovering NH_4^+, an NH_4^+ uptake inhibitor was added and the resulting NH_4^+ generation rates were recorded. A microbial community analysis was conducted on the anode biofilms to identify the microorganisms associated with N_2 fixation in the MEC. Finally, an assessment of energy demands to generate NH_4^+ in the MEC was performed.

EXPERIMENTAL SECTION

MEC Design and Operation. Serum-bottle MECs were assembled as previously described.[19] Briefly, graphite plate anodes ($A_A = 4.5$ cm^2) and stainless steel mesh cathodes ($A_C = 3.9$ cm^2) spaced 1.5 cm apart were connected to titanium and stainless steel wire current collectors, respectively, and inserted through stoppers. The electrode assembly was installed in glass serum bottles (75 mL), sealed using aluminum caps, and sterilized by autoclaving. The MECs were inoculated with effluent from an active MEC. The growth medium consisted of phosphate buffered medium (PBM) as previously described.[19] Ammonium chloride (NH_4Cl) was not included unless specified. Sodium acetate (1 g L^{-1}) was supplied as the electron donor and carbon source. The MEC headspace was purged with ultra-high-purity N_2 gas prior to each cycle. Two treatments (both operated in triplicate) were examined: (1) MECs without NH_4^+ added and (2) MECs with NH_4^+ (0.31 g L^{-1} NH_4Cl). The MECs were connected to a power supply (3645A DC Power Supply, Circuit Specialists, Inc., Tempe, AZ), and a voltage (E_{AP}) of 0.7 V was applied between the anode and cathode. After 16 cycles, E_{AP} was increased to 1.0 V. Current was determined by recording the voltage over a 10 Ω resistor. The MECs were operated in fed-batch mode, wherein the medium was emptied when the current density (I_A; normalized to A_A) dropped below 0.2 A m^{-2} and then was replaced with new medium. All MECs were operated at 30 °C.

Nitrogenase Activity Assay. Nitrogenase activity was measured by subjecting the MECs to an acetylene gas (C_2H_2) reduction assay (ARA). This method serves as a proxy for N_2 fixation because (1) the nitrogenase enzyme converts C_2H_2 to ethylene gas (C_2H_4), (2) this conversion does not occur abiotically, (3) neither C_2H_2 nor C_2H_4 inhibits nitrogenase activity, and (4) C_2H_4 can be readily quantified by gas chromatography (GC).[18] The ARA was used when current stabilized for both $E_{AP} = 0.7$ V and $E_{AP} = 1.0$ V. After the medium of the MECs was replaced and the MECs were purged with N_2 gas, C_2H_2 (obtained from calcium carbide) was injected into the headspace, giving a final concentration of 10% (v/v gas phase). After the voltage was applied, headspace samples (100 μL) were taken at regular intervals using a gastight syringe (Hamilton, Reno, NV). Samples were injected into a gas chromatograph (Shimadzu GC-14A, Kyoto, Japan) equipped with a flame ionization detector and a stainless steel column [PoraPak N (80/100 mesh); 6 ft × $^1/_8$ in]. The total moles of C_2H_4 were determined from the headspace and dissolved aqueous compositions. Headspace pressure was measured using a pressure gauge and then converted to moles of C_2H_4 using the ideal gas law.

Ammonium Quantification and Inhibition. To determine NH_4^+ concentrations, MEC medium (0.5 mL) was extracted at several points during a batch cycle and filtered (0.22 μm). Dissolved NH_4^+ concentrations were determined using the Low Range AmVer Salicylate Test N'Tube method (Hach Company, Loveland, CO), with a working range of 0.01−2.50 mg of NH_3−N L^{-1}. Accuracy of the method was verified using NH_4^+ standards, which deviated by no more than 0.01 mg of NH_3−N L^{-1}.

To encourage NH_4^+ excretion from the microorganisms, the NH_4^+ uptake inhibitor methionine sulfoximine (MSX) was added. This inhibitor acts on the enzyme glutamine synthetase, which catalyzes the assimilation of NH_4^+ into amino acids. It is commonly used to recover NH_4^+ from free-living diazotrophs.[20] An MSX concentration of 5 mM was selected, which falls within the range used in other studies.[21−23] Once the MEC was filled with fresh, MSX-containing medium, samples (0.5 mL) were taken over the course of a batch cycle at both E_{AP}. In initial tests, MSX generated a previously undocumented false positive for NH_4^+ (~7 mg NH_3−N L^{-1}) with the quantification method stated above. A two-stage trap was therefore used to separate the NH_4^+ from the MSX (full details available in the Supporting Information; Figure S3).

Microbial Community Analysis. To identify the anode microbial community, the anode biofilm was scraped using a sterile razor blade (~200 μg per electrode). Genomic DNA was extracted using a PowerSoil DNA isolation kit (MO BIO Laboratories, Carlsbad, CA) following the manufacturer's instructions. The prokaryotic V3 and V4 regions of the 16S rRNA gene were amplified using polymerase chain reaction, with the forward primer 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTAYGGGRBGCASCAG-3′ and the reverse primer 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACNNGGGTATCTAAT-3′. These primers are based on the sequences used by Yu et al.[24] and included the required Illumina adapters for sequencing. After purification and barcoding, the final amplicons were sequenced using Illumina MiSeq equipment

DOI: 10.1021/acssuschemeng.8b05763
ACS Sustainable Chem. Eng. 2019, 7, 3511−3519

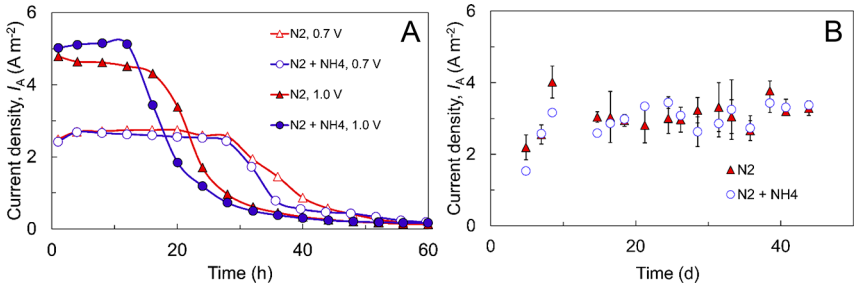


Figure 1. (A) Current densities (I_A; normalized to anode surface area) of the microbial electrolysis cells (MECs) at E_{AP} = 0.7 and 1.0 V under an atmosphere of 100% N_2 with and without NH_4^+ added (5.8 mM) to the medium. A representative cycle (cycle 16; 40 days after inoculation) is shown. Each line represents the average current of triplicate MECs. The error bars are omitted to improve clarity. Abiotic MECs (not shown) did not generate current. (B) Current densities over several cycles at E_{AP} = 0.7 V. Error bars show the deviation of maximum current density per cycle among triplicates.

(Illumina Inc., San Diego, CA) with a paired-end sequencing of 300 base pairs (bp) in length. The sequencing results were further processed and the operational taxonomic units (OTU) assigned using the QIIME software.[25]

Coulombic Efficiency. The Coulombic efficiency (C_E, %) was calculated as

$$C_E = \frac{M\int_0^{t_b} I\,dt}{FnV\Delta S} \quad (1)$$

where the current I (A) is integrated over the duration of the batch cycle (t_b), M represents the molar mass of sodium acetate (82 g mol^{-1}), F is Faraday's constant (96 500 C mol^{-1} e^{-1}), n is the number of electrons per mole of acetate oxidized (8 mol e$^-$ mol^{-1}), V (mL) is the medium volume in the MEC (20 mL), and ΔS (g L^{-1}) is the amount of acetate consumed during a batch cycle. Previous studies using this MEC design have shown that nearly all acetate (1 g L^{-1}) is consumed once the current falls below 0.10 mA (or 0.22 A m^{-2}).[12] All acetate was therefore assumed to be completely consumed by the end of each cycle (ΔS = 1 g L^{-1}).

Energy Demand. To determine the energy demand of NH_4^+ production, the electrical energy consumed, substrate energy consumed, and energy produced as gas (i.e., CH_4) were calculated. The amount of energy added to the circuit by the power source, adjusted for losses across the resistor, W_E, is given by eq 2,

$$W_E = \sum_1^n (IE_{AP}\Delta t - I^2R_{EX}\Delta t) \quad (2)$$

where I is measured at n points over the course of a batch cycle, Δt (s) is the time interval between each measurement, and R_{EX} = 10 Ω is the external resistor. The amount of energy added by the substrate is

$$W_S = \Delta G_S n_S \quad (3)$$

where ΔG_S = 844.1 kJ mol^{-1} is the Gibbs energy of combustion of the substrate (acetate in this case)[26] and n_S is the number of moles of substrate consumed during a batch cycle. The amount of energy available in the produced gas depends on the gas composition. This was determined using a GC (details available in the Supporting Methods). At the end of the experiments, the MECs in this study produced only CH_4. Hydrogen gas, a side product of the nitrogenase, was not detected and was likely consumed by hydrogenotrophic methanogens.[27] The theoretical energy content of the recovered CH_4 was calculated as

$$W_{CH_4} = \Delta G_{CH_4} n_{CH_4} \quad (4)$$

where ΔG_{CH_4} = 817.97 kJ mol^{-1} and n_{CH_4} is the number of moles of CH_4 produced during a batch cycle.[28] The specific energy demand of NH_4^+ production, $E_{NH_4^+}$, is the ratio of the energy inputs (electricity and substrate) and outputs (CH_4) to the moles of NH_4^+ generated, or

$$E_{NH_4^+} = \frac{W_E + W_S - W_{CH_4}}{n_{NH_4^+}} \quad (5)$$

where $n_{NH_4^+}$ is the moles of NH_4^+ recovered in a batch cycle. To determine the electrical energy demand, W_S is removed from eq 5, and for the substrate energy demand, W_E is removed. The amount of usable energy associated with the recovered CH_4 will depend on the conversion efficiency of the technology used to generate electrical energy (i.e., fuel cell versus a combustion engine). In some cases, we adjusted the W_{CH_4} values using a conversion efficiency of 32.9% (an average efficiency of typical biogas converting technologies).[29] Energy demand values that include this conversion efficiency are shown in parentheses next to the nonadjusted values.

RESULTS AND DISCUSSION

Electrical Current Generation during N_2 Fixation. We first determined current density (I_A) and Coulombic efficiency (C_E) when only N_2 was provided as a nitrogen source. After inoculating the serum-bottle MECs containing medium without fixed nitrogen, purging the headspace with N_2 gas and applying a constant whole-cell voltage (E_{AP} = 0.7 V), current generation began within 5 days. Four cycles later (15 days), I_A reached a reproducible maximum of 3.0 ± 0.15 A m^{-2} (Figure 1A). Current densities were stable for more than 10 cycles operated at this E_{AP}, with no significant difference (ANOVA, $p > 0.05$) relative to the average of 3.1 ± 0.27 A m^{-2} over that time period (Figure 1B). This result is consistent with prior reports of METs operating with little to no fixed nitrogen from 15 to 200 days.[15,30] To determine if the lack of a fixed nitrogen source impacted I_A, we operated identical MECs, but with NH_4Cl (5.8 mM NH_4^+) added to the medium. Current generation in those reactors began within 5 days and reached 3.1 ± 0.31 A m^{-2} after stabilizing. Over the course of 12 cycles, I_A in the presence versus absence of NH_4^+ deviated 9.3% on average, but the difference between treatments was not significant (*t*-test, $p > 0.05$). When E_{AP} was increased to 1.0 V, I_A increased in the absence and presence of NH_4^+ to 4.8 ± 0.27 and 5.2 ± 0.27 A m^{-2}, respectively. The lack of a fixed nitrogen source did not negatively impact C_E. With only N_2 present, C_E averaged 89 ± 1%, and was slightly lower (83 ± 1%) with NH_4^+ present at E_{AP} = 0.7 V. At E_{AP} = 1.0 V, C_E decreased relative to E_{AP} = 0.7 V, but remained similar in the absence (71 ± 3%) versus presence (67 ± 2%) of NH_4^+. The near identical I_A and C_E values of the MECs with and without NH_4^+ and similarities to prior reports of MECs (2.1−4.2 A m^{-2} for E_{AP} = 0.7 V)[19,31] highlight that the absence of dissolved NH_4^+ (a condition that

DOI: 10.1021/acssuschemeng.8b05763
ACS Sustainable Chem. Eng. 2019, 7, 3511−3519

encourages N_2 fixation[32]) does not negatively impact current production over time.

N_2 Fixation Rates. To quantify N_2 fixation rates, we conducted the acetylene gas (C_2H_2) reduction assay (ARA). When C_2H_2 was added to the MECs at the start of a cycle, ethylene (C_2H_4) accumulated at a constant rate for the first 50 h at both E_{AP} (Figure 2). At E_{AP} = 0.7 V, the MECs generated

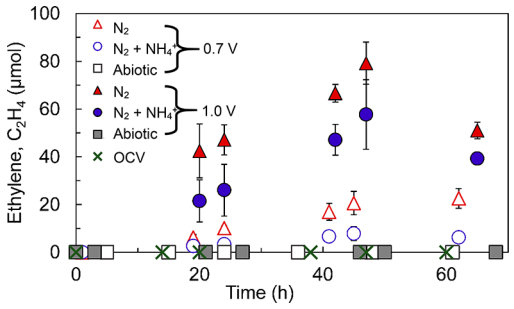


Figure 2. N_2 fixation rates during current generation in the microbial electrolysis cells (MECs) at E_{AP} = 0.7 V and E_{AP} = 1.0 V. To estimate N_2 fixation rates, the acetylene gas reduction assay was performed, wherein the nitrogenase enzyme within the microorganisms reduces acetylene to ethylene gas (C_2H_4; *y*-axis). MECs were purged with 100% N_2 and operated without fixed nitrogen (N_2) or with NH_4^+ added (N_2 + NH_4^+). Results are shown for cycle 17 (E_{AP} = 0.7 V) and 18 (E_{AP} = 1.0 V). Abiotic controls are identical MECs but lack microorganisms, and open-circuit voltage (OCV) controls are biotic MECs without current flow. Errors bars represent the standard deviation of triplicate MECs.

C_2H_4 at a rate of 7.7 ± 1.1 nmol of C_2H_4 min^{-1} in the absence of NH_4^+ and 3.0 ± 0.8 nmol of C_2H_4 min^{-1} when NH_4^+ was added (based on linear regression of C_2H_4 generation over time). These two rates were significantly different (*t*-test, $p < 0.05$). Increasing E_{AP} to 1.0 V increased C_2H_4 production rates in both treatments to 26 ± 2.4 nmol of C_2H_4 min^{-1} (without NH_4^+) and 20 ± 3.7 nmol of C_2H_4 min^{-1} (with NH_4^+). These rates were not significantly different (*t*-test, $p > 0.05$), indicating that NH_4^+ did not have an impact on N_2 fixation. The increase in C_2H_4 production rates between applied voltages was significant (*t*-test, $p < 0.05$) regardless of NH_4^+ presence. The more than threefold increase in C_2H_4 generation rates between E_{AP} = 0.7 V and E_{AP} = 1.0 V was notable considering that I_A increased by less than twofold.

The minimal reduction in C_2H_4 generation rates when NH_4^+ was added was an interesting result because NH_4^+ is known to repress nitrogenase gene expression.[32] Depending on the microorganism and the growth conditions, this threshold can vary. Values as low as 0.2 mM have been reported to completely repress nitrogenase activity in the model microorganism *Azotobacter vinelandii*.[32] For several *Azospirillum* species, concentrations as low as 0.05 mM NH_4^+ reduced nitrogenase activity.[33] In a community with *Geobacter* spp., 0.5 mM NH_4^+ repressed the expression of *nifD*, one of the genes encoding the nitrogenase.[34] Since the N_2 fixation activity in the presence of NH_4^+ was not fully shut down in our MECs, it is possible that the bulk NH_4^+ concentrations could not completely repress the nitrogenase genes. The concentration may have been too low or diffusion limitations may have prevented nitrogenase inhibition by NH_4^+ throughout the entire biofilm. Partial nitrogenase repression has been observed in free-living diazotrophs at varying NH_4^+ concentrations.[33,35] The fact that N_2 fixation occurred in the presence of NH_4^+ may have important implications for wastewater-treating METs. Typical domestic wastewater NH_4^+ concentrations (0.8−3.6 mM NH_4^+)[36] are lower than what was used here. N_2 fixation may therefore be occurring in wastewater-fed METs.

To confirm that anode respiration was linked with N_2 fixation, we also conducted the ARA on the MECs during open-circuit voltage (OCV; no current flow) (Figure 2). No C_2H_4 was detected during the same time period as the MECs in closed circuit mode (60 h). Further evidence that anode respiration was required is that, after approximately 50 h in the closed-circuit MECs (when current generation approached zero), the C_2H_4 generation rate dropped sharply. At that point, acetate was depleted and anode respiration ceased. Outside the electromicrobiology field, some studies have noted correlations between microbial N_2 fixation rates and respiration rates,[37−39] while others have not.[40−42] An inherent challenge when using aerobic N_2-fixing microorganisms is that increasing O_2 concentrations has the dual effect of increasing respiration rates but also potentially inhibiting O_2-sensitive nitrogenases.[38,40−42] Our work here, where O_2 is not present, lends support for the hypothesis that respiration rates (as measured by I_A) are linked to N_2 fixation rates. We also conducted additional tests in which acetate was replaced with H_2 (45% headspace concentration) as the sole electron donor to determine N_2 fixation rates associated with H_2 consuming microorganisms (E_{AP} = 0.7 V; Figure S1). C_2H_4 generation dropped significantly from approximately 20 μmol of C_2H_4 recovered at 45 h with acetate (Figure 2) to below detection with H_2 at the same time point. Current densities also decreased to below 0.1 A m^{-2}. These results suggest that acetate oxidation was the primary source of reducing power for N_2 fixation.

To permit comparison with free-living diazotrophs, we normalized our C_2H_4 generation rates to the total anode protein density (150 ± 3 μg cm^{-2}; protein assay details available in the Supporting Methods). At E_{AP} = 1.0 V, the normalized rate was 39 ± 3.7 nmol of C_2H_4 min^{-1} mg $protein^{-1}$. Free-living, aerobic diazotrophs such as *Azotobacter* species have reached N_2 fixation rates of 35−100 nmol of C_2H_4 min^{-1} mg $protein^{-1}$. Obtaining normalized rates comparable to those of *Azotobacter* species is an important finding because they are some of the best studied free-living diazotrophs. The rates of other microorganisms such as cyanobacteria (e.g., *Anabaena* spp.) and anaerobic free-living bacteria (e.g., *Azospirillum* spp.) range from 0.11 to 6.5 nmol of C_2H_4 min^{-1} mg $protein^{-1}$.[18,43] Regarding species that are similar to exoelectrogenic bacteria, suspended cultures of the anaerobic, metal-reducing bacteria *Geobacter metallireducens* and *Magnetospirillum magnetotacticum* obtained rates of 12 and 22 nmol of C_2H_4 min^{-1} mg $protein^{-1}$, respectively.[44] Therefore, the increase in E_{AP} proved to be an effective way to stimulate N_2 fixation rates that are comparable to many other free-living diazotrophs.

In METs, there are limited reports of N_2 fixation measurements. Wong et al.[15] enriched an exoelectrogenic community on a high-surface-area granular graphite anode poised at +0.2 V vs Ag/AgCl. By making some assumptions about the electrode surface area used in their study (calculation available in the Supporting Information), we estimate an anode normalized rate of 0.40 nmol of C_2H_4 min^{-1} cm^{-2} compared to 5.9 nmol of C_2H_4 min^{-1} cm^{-2} in our MECs at E_{AP} = 1.0 V. One possible explanation for the higher rates reported here is that acetate, rather than glucose, was provided as an electron

DOI: 10.1021/acssuschemeng.8b05763
ACS Sustainable Chem. Eng. 2019, 7, 3511−3519

donor. Acetate-fed METs typically generate higher current than glucose and favor the growth of known diazotrophic exoelectrogens, such as *Geobacter* species.[45] Zhou et al.[16] isolated an exoelectrogenic bacterium belonging to the genus *Azospirillum*, which reached rates of 1.75 nmol of C_2H_4 min^{-1} $(10^8$ cells$)^{-1}$;[16] however, N_2 fixation during anode respiration was not examined.

Ammonium Production. To test the hypothesis that NH_4^+ can be recovered in the MEC, we first measured NH_4^+ concentrations over time while current was generated. In the standard growth medium without NH_4^+ added, little (<10 μM) to no NH_4^+ was detected over the course of a batch cycle (limit of detection = 3 μM) (Figure 3). To eliminate the possibility that NH_4^+ was generated through an abiotic reaction of H_2 and N_2 at the cathode, abiotic reactors were operated at E_{AP} = 1.0 V with a H_2/N_2 [50/50 (v/v)] headspace composition. No NH_4^+ was detected over the same period as the biotic experiments (Figure S2). Abiotic NH_4^+ production was therefore ruled out.

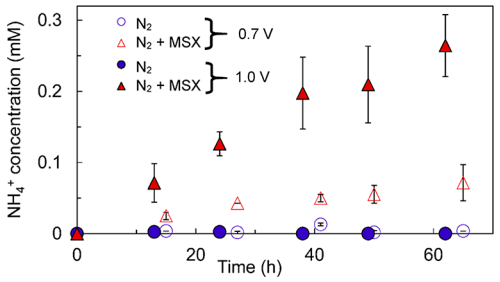


Figure 3. NH_4^+ generation in the microbial electrolysis cells (MECs) at an applied voltage (E_{AP}) of 0.7 and 1.0 V with and without an NH_4^+ uptake inhibitor [methionine sulfoximine (MSX)] added to the medium. All MECs were operated with a 100% N_2 atmosphere and no NH_4^+ added. One complete MEC cycle is shown (~60 h). Error bars show the standard deviation of triplicate MECs.

To encourage NH_4^+ release from the microorganisms during N_2 fixation, we added the NH_4^+ uptake inhibitor methionine sulfoximine (MSX). In the presence of MSX (5 mM), NH_4^+ concentrations increased steadily over time at both values of E_{AP} and reached final concentrations of 70 μM (E_{AP} = 0.7 V) and 260 μM (E_{AP} = 1.0 V). Theoretically, we estimate that 10% of the NH_4^+ that was generated during N_2 fixation was recovered, regardless of E_{AP}. We determined this based on the assumption that 1 mol of NH_4^+ is produced for every 2 mol of C_2H_4 generated during the ARA (a commonly used conversion factor).[46] This would result in up to 3 mM NH_4^+ after 60 h at E_{AP} = 1.0 V and correspond to a rate of around 40 μM NH_4^+ h^{-1}, which is roughly tenfold larger than the actual rate (4.2 ± 0.7 μM NH_4^+ h^{-1}). One explanation for this difference may be that the MSX concentration was too low to fully inhibit the glutamine synthase enzymes. Other studies have used MSX concentrations as low as 0.35 μM or as high as 0.11 M, depending on the microorganism examined.[47,48] Since there are no reports in the literature on the application of MSX to exoelectrogenic biofilms, we used a concentration that was consistent with other studies on free-living diazotrophs such as *Azotobacter chrococcum*.[23] As a reference, using the same chemical inhibitor (0.35−200 μM) with the cyanobacterium *Anabaena*, NH_4^+ generation rates from 10 to 150 μM NH_4^+ h^{-1} were reported.[20,47] In comparison, Ahmad and Hellebust[21] did not notice a significant effect of MSX, suggesting the existence of an alternate NH_4^+ assimilation pathway. The lower NH_4^+ generation rates are therefore likely due to differences in susceptibility to MSX among microorganisms and biofilm growth on the anode that would reduce MSX mass transfer to the microorganisms compared to suspended growth in those other studies.

Relative to other bioelectrochemical technologies at ambient conditions, the MEC NH_4^+ generation rates are quite comparable. Normalized to the anode surface area, the MEC rates increased from 1.3 × 10^{-12} mol of NH_4^+ s^{-1} cm^{-2} at E_{AP} = 0.7 V up to 5.2 × 10^{-12} mol of NH_4^+ s^{-1} cm^{-2} at E_{AP} = 1.0 V. Knoche et al.,[10] in the only study to date that reports bioelectrochemical NH_4^+ excretion, immobilized cells of *Anabaena variabilis* SA-1 onto indium tin oxide electrodes and applied cyclic voltammetry, producing up to 4.7 μM NH_4^+ at a rate of 2.6 × 10^{-12} mol s^{-1} cm^{-2}.[10] Milton et al.[5] used a fuel cell with electrode-immobilized nitrogenase and hydrogenase enzymes instead of whole-cell microorganisms. Their system generated 2.3 × 10^{-11} mol of NH_4^+ s^{-1} cm^{-2} after applying 60 mC of charge over 2 h. However, an electron mediator (methyl viologen) was required to shuttle electrons to/from both enzymes. Recently, Rago et al.[17] enriched a biocathodic microbial community to perform N_2 fixation at a fixed applied potential of −0.7 V vs SHE. While this is a more direct approach for N_2 conversion to NH_4^+, the maximum current density detected in their system was around 0.01 A/m^2, at least two orders of magnitude lower than the current generated in this study. Since our work shows a link between N_2 fixation rates and current density, it is unlikely that biocathode-driven N_2 fixation was a major contributor to our observed N_2 fixation rates.

Microbial Community Analysis. To identify the microorganisms in the MECs, genomic DNA from the anode biofilms was extracted and the 16S rRNA genes were amplified and sequenced. Among the bacteria, *Geobacter* was the most prevalent genus with a relative abundance of 43.7 ± 2.3% in the absence of NH_4^+ (Figure 4). This genus is frequently detected in exoelectrogenic biofilms that use acetate as the electron donor.[12,49] Many *Geobacter* spp. are also diazotrophs, including *G. sulfurreducens*, *G. metallireducens*, and *G. uraniireducens*.[13,14,44] In the presence of NH_4^+, *Geobacter* spp. abundance was similar (43.5 ± 6.8%; Figure 4), suggesting that the requirement for N_2 fixation did not negatively impact their ability to colonize the anode. Except for *Methanocorpusculum*

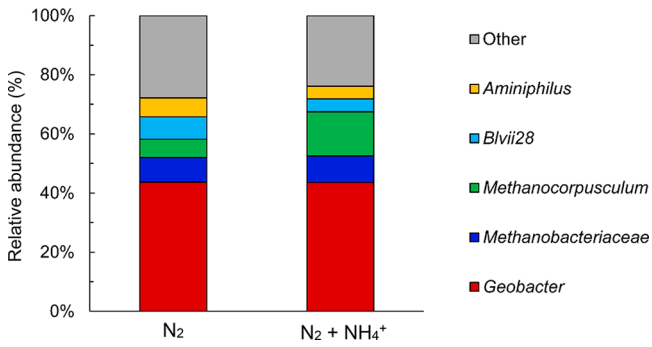


Figure 4. Microbial community composition of anode biofilms in the presence and absence of NH_4^+. Percentages show the average relative abundance of genera from triplicate anode biofilms. Dominant biofilm genera (relative abundance >5%) are shown individually, while the rest are grouped as "Other".

DOI: 10.1021/acssuschemeng.8b05763
ACS Sustainable Chem. Eng. 2019, 7, 3511−3519

spp. (discussed below), all genera with an average relative abundance >5% did not significantly vary in abundance in the presence versus absence of NH_4^+ (*t*-test, $p > 0.05$). This finding is supported by the relatively high N_2 fixation rates recorded in the presence of NH_4^+ (Figure 2) and another study that showed *Geobacter*-rich environments maintained similar transcript levels of key N_2 fixation genes when NH_4^+ concentrations increased from 0 to 350 μM.[34]

Other bacteria identified in the anode include species belonging to the genus *Aminiphilus*, as well as the *Blvii28* group. The former genus is known to ferment several amino acids, generating products such as acetate.[50] This function suggests a possible syntrophy with *Geobacter* spp., as *Aminiphilus* spp. are frequently found in acetate-fed METs[51] with relative abundances as high as 43%.[51] The latter has also been found in wastewater-inoculated systems such as upflow anaerobic sludge beds, anaerobic biofilm membrane bioreactors, and anaerobic digesters, where they likely ferment carbohydrates and generate H_2.[52−54] Only a single member of this group, *Acetobacteroides hydrogenigenes*, has been successfully isolated.[55] For both genera, the diazotrophic and exolectrogenic capabilities remain unknown.

Within the archaea, methanogenic microorganisms including *Methanocorpusculum* spp. (6.2 ± 3.4%) and an unknown genus belonging to the family *Methanobacteriaceae* were detected (8.3 ± 2.8%) in the absence of NH_4^+. These genera contain hydrogenotrophic methanogens that require H_2 and CO_2 to generate CH_4.[56,57] Their presence explains why CH_4 was recovered instead of H_2, which commonly occurs in single-chamber MECs.[27] Some members of the *Methanobacteriaceae* have been reported to fix N_2.[58] It is plausible that they contributed to the observed N_2 fixation rates and NH_4^+ production in the MEC. Methanogen N_2 fixation rate estimates reported in the literature, which range between 0.7 and 15 nmol of C_2H_4 min^{-1} mg $protein^{-1}$,[59] would account for only about 1.8−38% of our normalized rates. Coupling this estimate with the significantly lower C_2H_4 generation rates when we supplied the MECs with H_2 (Figure S1) suggests that methanogens were not the primary N_2 fixers in the MECs. For *Methanocorpusculum* spp., nitrogenase-like gene sequences, but with no experimentally established diazotrophic behavior, have been reported.[60] The significant decrease (*t*-test, $p < 0.05$) in abundance of this genus when NH_4^+ was absent suggests that these microorganisms may not grow as well when required to fix N_2. Elucidating the contribution of *Geobacter* spp. and methanogens to N_2 fixation in METs is warranted.

Energy Demand. The energy needed to drive NH_4^+ production in the MECs consists of electrical and substrate (i.e., acetate in this study) inputs. It is important to note that these inputs also produce energy-rich gases (H_2 and CH_4) in the MEC.[26,27] This is recoverable energy that can be used to offset some of the energy demands. The MECs in this study generated CH_4 (no H_2 detected), which is due to methanogenic consumption of cathodic electrons and/or H_2.[27] The MECs generated 0.27 ± 0.02 mmol of CH_4 per cycle, regardless of E_{AP}. The energy recovered was 136−162 MJ mol^{-1} (NH_4^+) at E_{AP} = 0.7 V (45−53 MJ mol^{-1} when adjusted for CH_4 conversion to electricity) and 37−47 MJ mol^{-1} (12−15 MJ mol^{-1}) at E_{AP} = 1.0 V. Considering only the electrical energy input, the energy demand ranged from −92 to −65 MJ mol^{-1} (E_{AP} = 0.7 V) and from −14 to −7.1 MJ mol^{-1} (E_{AP} = 1.0 V) because the recovered energy was greater than the consumed electrical energy. These values become positive if adjusted to account for CH_4 conversion into electricity (16−25 MJ mol^{-1} for E_{AP} = 0.7 V and 14−17 MJ mol^{-1} for E_{AP} = 1.0 V). Conventional and emerging N_2 fixation technologies do not generate energy. Recovering energy-rich gas in MECs may therefore impart an energy advantage over other approaches. Relative to only the substrate energy, the energy demands ranged from −0.041 to 0.051 MJ mol^{-1}, indicating that the majority of the substrate energy was converted to CH_4. The substrate energy is an important factor that increases the net energy demand (around 150 MJ mol^{-1} at E_{AP} = 0.7 V and 37 MJ mol^{-1} at E_{AP} = 1.0 V). This energy expenditure can be reduced or eliminated by utilizing waste sources low in nitrogen such as industrial wastewater (e.g., pulp and paper wastewater).[15] Taking into account both the electrical and substrate energy inputs, the energy demands were 51−84 MJ mol^{-1} (166−175 MJ mol^{-1}) at E_{AP} = 0.7 V and 24−34 MJ mol^{-1} (55−58 MJ mol^{-1}) at E_{AP} = 1.0 V. The decrease in demand when E_{AP} increased can be explained by a larger increase in NH_4^+ production rates relative to the increase in energy input. It may be possible to obtain higher generation rates at higher voltages, but voltages above 1.2 V should be avoided to minimize unwanted abiotic water electrolysis. Another consideration is the requirement to separate and recover NH_4^+ from the MEC. While the evaluation of NH_4^+ extraction technologies is outside the scope of the present study, there are several methods that could be used. For example, driving NH_4^+ transport across a cation-exchange membrane has already been shown in METs. Alternatively, NH_4^+ conversion to NH_3 gas in a separate alkaline cathode chamber may also be a possibility.[61,62]

Relative to other NH_3 generation technologies, the MEC energy demand was large because only small amounts of NH_4^+ were recovered when the inhibitor was used. On the basis of the N_2 fixation rate estimates reported here, if 100% recovery was achieved (assuming NH_4^+ is the main product), the energy demand could be lowered by an order of magnitude to about 3.2 MJ mol^{-1} at E_{AP} = 1.0 V (~5.6 MJ mol^{-1} if CH_4 conversion to electricity is considered). Engineering high NH_4^+-producing exoelectrogenic strains similar to the approach used by Barney et al.[8] to develop an aerobic *Azotobacter* spp. that accumulated up to 30 mM NH_4^+ at a rate of 200 μM h^{-1} may be an approach to further reduce energy requirements.[8,63,64] Additionally, optimizing the MEC design and materials could also help reduce the energy demand through, for example, using large surface area electrodes, minimizing electrode spacing, and optimizing N_2 gas transfer to the biofilm.

Implications. On the basis of the obtained results, the use of an MET offers an alternative to NH_4^+ using diazotrophic, exoelectrogenic bacteria. Compared to other methods that require expensive materials (e.g., platinum, gold, and ruthenium), high temperatures and/or pressures, and sunlight or rely on nonrenewable catalysts, the MEC used here generates NH_4^+ under ambient conditions, with inexpensive electrode materials (e.g., graphite and stainless steel) and self-renewing microbial catalysts.[11,26,45] Our findings suggest that multiple N_2 fixation pathways may occur in the MEC (Figure 5). Diazotrophic exoelectrogenic bacteria fix N_2 while consuming organic matter and respiring on the anode. The electrons transferred to the anode result in the cathodic generation of H_2 that can be further converted to CH_4 via methanogenesis. Methanogens may also fix N_2, although likely to a lesser degree than the exoelectrogens. To further optimize

DOI: 10.1021/acssuschemeng.8b05763
ACS Sustainable Chem. Eng. 2019, 7, 3511−3519

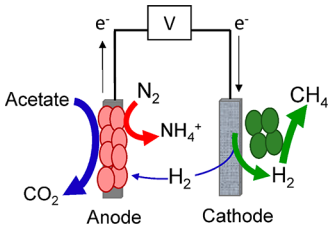


Figure 5. Graphical representation of N_2 fixation in the microbial electrolysis cell (MEC). Oxidation (consumption) of acetate provides the required electrons (e^-) for N_2 fixation by the exoelectrogenic bacteria (pink circles). The exoelectrogens transfer the electrons to the anode during respiration. An external voltage (V) drives electrons to the cathode where they produce H_2 gas. H_2 can be consumed by the exoelectrogens to provide electrons for further N_2 fixation and/or converted to CH_4 by methanogens (green circles).

the technology, identifying and engineering promising N_2-fixing exoelectrogens will be needed, rather than relying on chemical inhibitors. We justify the use of an NH_4^+ uptake inhibitor here to explore the potential for NH_4^+ generation from the MEC, but it is not a long-term, sustainable approach. There are promising diazotrophic exoelectrogens (e.g., *G. sulfurreducens*) that may yield new NH_4^+-excreting, engineered strains.[13,14,44] On the basis of the high relative abundance of *Geobacter* spp. in our MEC anode biofilms, we hypothesize that these microorganisms were the primary contributors to the high current and N_2 fixation rates in the MECs.[15,30] Further studies must assess the capability of N_2 fixation and NH_4^+ production by pure cultures of *Geobacter* species, as well as determine the N_2 fixation capabilities of other microorganisms such as methanogens.

CONCLUSIONS

This study explored the possibility of converting N_2 gas into NH_4^+ using single-chamber microbial electrolysis cells (MECs). We showed that MEC N_2 fixation rates can approach those reported for model aerobic diazotrophs such as *Azotobacter* species. Increasing the electrical input from an applied voltage from 0.7 to 1.0 V resulted in a significant increase in N_2 fixation rates. The addition of an NH_4^+ uptake inhibitor resulted in generation rates up to 5.2×10^{-12} mol of NH_4^+ s^{-1} cm^{-2} (normalized to the anode surface area). While the energy demands of this proof-of-concept study were larger than commercially available processes (i.e., Haber–Bosch), the possibility of recovering multiple products, including H_2, CH_4, and NH_4^+, may provide a unique niche for this technology.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssuschemeng.8b05763.

Additional figures showing current density and C_2H_4 production with H_2 as the sole electron donor, NH_4^+ production in abiotic MECs with H_2 present, and a schematic of the acid trap used to separate NH_4^+ from the MSX inhibitor; additional methods, including a description of the acid trap, protein measurements, MEC gas headspace analysis, and calculation of N_2 fixation rates from the literature (PDF)

AUTHOR INFORMATION

Corresponding Author

*E-mail: dfcall@ncsu.edu (D.F.C.).

ORCID

Douglas F. Call: 0000-0002-8279-9551

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

Next Generation (Illumina) sequencing of microbial communities was performed by the Genomic Sciences Laboratory at North Carolina State University. Special thanks to Mark Poole, Ling Wang, Joe Weaver, and Christy Smith for their help with the MEC operation, sequencing analysis, and analytical instrumentation. This work was supported by funding from North Carolina State University and the National Science Foundation through CBET-1840956.

REFERENCES

(1) Erisman, J. W.; Sutton, M. A.; Galloway, J.; Klimont, Z.; Winiwarter, W. How a Century of Ammonia Synthesis Changed the World. *Nat. Geosci.* **2008**, *1* (10), 636–639.

(2) Cherkasov, N.; Ibhadon, A. O.; Fitzpatrick, P. A Review of the Existing and Alternative Methods for Greener Nitrogen Fixation. *Chem. Eng. Process.* **2015**, *90*, 24–33.

(3) Zhou, F.; Azofra, L. M.; Ali, M.; Kar, M.; Simonov, A. N.; McDonnell-Worth, C.; Sun, C.; Zhang, X.; MacFarlane, D. R. Electro-Synthesis of Ammonia from Nitrogen at Ambient Temperature and Pressure in Ionic Liquids. *Energy Environ. Sci.* **2017**, *10* (12), 2516–2520.

(4) Giddey, S.; Badwal, S. P. S.; Kulkarni, A. Review of Electrochemical Ammonia Production Technologies and Materials. *Int. J. Hydrogen Energy* **2013**, *38* (34), 14576–14594.

(5) Milton, R. D.; Cai, R.; Abdellaoui, S.; Leech, D.; De Lacey, A. L.; Pita, M.; Minteer, S. D. Bioelectrochemical Haber-Bosch Process: An Ammonia-Producing H 2 /N 2 Fuel Cell. *Angew. Chem., Int. Ed.* **2017**, *56* (10), 2680–2683.

(6) Saikia, S. P.; Jain, V. Biological Nitrogen Fixation with Non-Legumes: An Achievable Target or a Dogma? *Curr. Sci.* **2007**, *92* (3), 317–322.

(7) van Kessel, C.; Hartley, C. Agricultural Management of Grain Legumes: Has It Led to an Increase in Nitrogen Fixation? *F. Crop. Res.* **2000**, *65* (2–3), 165–181.

(8) Barney, B. M.; Plunkett, M. H.; Natarajan, V.; Mus, F.; Knutson, C. M.; Peters, J. W. Transcriptional Analysis of an Ammonium-Excreting Strain of Azotobacter Vinelandii Deregulated for Nitrogen Fixation. *Appl. Environ. Microbiol.* **2017**, *83* (20), 1–22.

(9) Gallon, J. R. The Oxygen Sensitivity of Nitrogenase: A Problem for Biochemists and Micro-Organisms. *Trends Biochem. Sci.* **1981**, *6* (C), 19–23.

(10) Knoche, K. L.; Aoyama, E.; Hasan, K.; Minteer, S. D. Role of Nitrogenase and Ferredoxin in the Mechanism of Bioelectrocatalytic Nitrogen Fixation by the Cyanobacteria Anabaena Variabilis SA-1 Mutant Immobilized on Indium Tin Oxide (ITO) Electrodes. *Electrochim. Acta* **2017**, *232*, 396–403.

(11) Logan, B. E.; Rabaey, K. Conversion of Wastes into Bioelectricity and Chemicals by Using Microbial Electrochemical Technologies. *Science* **2012**, *337* (6095), 686–690.

(12) Zhu, X.; Yates, M. D.; Hatzell, M. C.; Ananda Rao, H.; Saikaly, P. E.; Logan, B. E. Microbial Community Composition Is Unaffected by Anode Potential. *Environ. Sci. Technol.* **2014**, *48* (2), 1352–1358.

(13) Methe, B. A.; Webster, J.; Nevin, K.; Butler, J.; Lovley, D. R. DNA Microarray Analysis of Nitrogen Fixation and Fe(III) Reduction in Geobacter Sulfurreducens. *Appl. Environ. Microbiol.* **2005**, *71* (5), 2530–2538.

DOI: 10.1021/acssuschemeng.8b05763
ACS Sustainable Chem. Eng. 2019, 7, 3511–3519

(14) Holmes, D. E.; O'Neil, R. A.; Chavan, M. A.; N'Guessan, L. A.; Vrionis, H. A.; Perpetua, L. A.; Larrahondo, M. J.; DiDonato, R.; Liu, A.; Lovley, D. R. Transcriptome of Geobacter Uraniireducens Growing in Uranium-Contaminated Subsurface Sediments. *ISME J.* **2009**, *3* (2), 216−230.
(15) Wong, P. Y.; Cheng, K. Y.; Kaksonen, A. H.; Sutton, D. C.; Ginige, M. P. Enrichment of Anodophilic Nitrogen Fixing Bacteria in a Bioelectrochemical System. *Water Res.* **2014**, *64* (1), 73−81.
(16) Zhou, S.; Han, L.; Wang, Y.; Yang, G.; Zhuang, L.; Hu, P. Azospirillum Humicireducens Sp. Nov., a Nitrogen-Fixing Bacterium Isolated from a Microbial Fuel Cell. *Int. J. Syst. Evol. Microbiol.* **2013**, *63* (Pt 7), 2618−2624.
(17) Rago, L.; Zecchin, S.; Villa, F.; Goglio, A.; Corsini, A.; Cavalca, L.; Schievano, A. Bioelectrochemical Nitrogen Fixation (e-BNF): Electro-Stimulation of Enriched Biofilm Communities Drives Autotrophic Nitrogen and Carbon Fixation. *Bioelectrochemistry* **2019**, *125*, 105−115.
(18) Hardy, R. W. F.; Burns, R. C.; Holsten, R. D. Applications of the Acetylene-Ethylene Assay for Measurement of Nitrogen Fixation. *Soil Biol. Biochem.* **1973**, *5* (1), 47−81.
(19) Call, D. F.; Logan, B. E. A Method for High Throughput Bioelectrochemical Research Based on Small Scale Microbial Electrolysis Cells. *Biosens. Bioelectron.* **2011**, *26* (11), 4526−4531.
(20) Colnaghi, R.; Green, A.; He, L.; Rudnick, P.; Kennedy, C. Strategies for Increased Ammonium Production in Free-Living or Plant Associated Nitrogen Fixing Bacteria. *Plant Soil* **1997**, *194*, 145−154.
(21) Ahmad, I.; Hellebust, J. A. Effects of Methionine Sulfoximine on Growth and Nitrogen Assimilation of the Marine Microalga Chlorella Autotrophica. *Mar. Biol.* **1985**, *86* (1), 85−91.
(22) Ronzio, R. A.; Rowe, W. B.; Meister, A. Studies on the Mechanism of Inhibition of Glutamine Synthetase By Methionine Sulfoximine. *Biochemistry* **1969**, *8* (3), 1066−1075.
(23) Cejudo, F. J.; de la Torre, A.; Paneque, A. Short-Term Ammonium Inhibition of Nitrogen Fixation in Azotobacter. *Biochem. Biophys. Res. Commun.* **1984**, *123* (2), 431−437.
(24) Yu, Y.; Lee, C.; Kim, J.; Hwang, S. Group-Specific Primer and Probe Sets to Detect Methanogenic Communities Using Quantitative Real-Time Polymerase Chain Reaction. *Biotechnol. Bioeng.* **2005**, *89* (6), 670−679.
(25) Caporaso, J. G.; Kuczynski, J.; Stombaugh, J.; Bittinger, K.; Bushman, F. D.; Costello, E. K.; Fierer, N.; Peña, A. G.; Goodrich, J. K.; Gordon, J. I.; et al. QIIME Allows Analysis of High-Throughput Community Sequencing Data. *Nat. Methods* **2010**, *7* (5), 335−336.
(26) Logan, B. E.; Call, D.; Cheng, S.; Hamelers, H. V. M.; Sleutels, T. H. J. A.; Jeremiasse, A. W.; Rozendal, R. A. Microbial Electrolysis Cells for High Yield Hydrogen Gas Production from Organic Matter. *Environ. Sci. Technol.* **2008**, *42* (23), 8630−8640.
(27) Call, D.; Logan, B. E. Hydrogen Production in a Single Chamber Microbial Electrolysis Cell Lacking a Membrane. *Environ. Sci. Technol.* **2008**, *42* (9), 3401−3406.
(28) Villano, M.; Monaco, G.; Aulenta, F.; Majone, M. Electrochemically Assisted Methane Production in a Biofilm Reactor. *J. Power Sources* **2011**, *196* (22), 9467−9472.
(29) U.S. Environmental Protection Agency. Combined Heat and Power Partnership. Opportunities for Combined Heat and Power at Wastewater Treatment Facilities: Market Analysis and Lessons from the Field. 2011. https://www.epa.gov/chp/opportunities-combined-heat-and-power-wastewater-treatment-facilities-market-analysis-and.
(30) Belleville, P.; Strong, P. J.; Dare, P. H.; Gapes, D. J. Influence of Nitrogen Limitation on Performance of a Microbial Fuel Cell. *Water Sci. Technol.* **2011**, *63* (8), 1752−1757.
(31) Ren, L.; Siegert, M.; Ivanov, I.; Pisciotta, J. M.; Logan, B. E. Treatability Studies on Different Refinery Wastewater Samples Using High-Throughput Microbial Electrolysis Cells (MECs). *Bioresour. Technol.* **2013**, *136*, 322−328.
(32) Laane, C.; Krone, W.; Konings, W.; Haaker, H.; Veeger, C. Short-Term Effect of Ammonium Chloride on Nitrogen Fixation by Azotobacter Vinelandii and by Bacteroids of Rhizobium Leguminosarum. *Eur. J. Biochem.* **1980**, *103* (1), 39−46.
(33) Hartmann, A.; Fu, H.; Burris, R. H. Regulation of Nitrogenase Activity by Ammonium Chloride in Azospirillum Spp. *J. Bacteriol.* **1986**, *165* (3), 864−870.
(34) Mouser, P. J.; N'Guessan, A. L.; Elifantz, H.; Holmes, D. E.; Williams, K. H.; Wilkins, M. J.; Long, P. E.; Lovley, D. R. Influence of Heterogeneous Ammonium Availability on Bacterial Community Structure and the Expression of Nitrogen Fixation and Ammonium Transporter Genes during in Situ Bioremediation of Uranium-Contaminated Groundwater. *Environ. Sci. Technol.* **2009**, *43* (12), 4386−4392.
(35) Bottomley, P. J.; Grillo, J. F.; Van Baalen, C.; Tabita, F. R. Synthesis of Nitrogenase and Heterocysts by Anabaena Sp. CA in the Presence of High Levels of Ammonia. *J. Bacteriol.* **1979**, *140* (3), 938−943.
(36) Tchobanoglous, G. *Wastewater Engineering. Treatment, Disposal and Reuse*, 3rd ed.; McGraw-Hill, 1991.
(37) Hurek, T.; Reinhold-Hurek, B.; Turner, G. L.; Bergersen, F. J. Augmented Rates of Respiration and Efficient Nitrogen Fixation at Nanomolar Concentrations of Dissolved O2 in Hyperinduced Azoarcus Sp. Strain BH72. *J. Bacteriol.* **1994**, *176* (15), 4726−4733.
(38) Linkerhägner, K.; Oelze, J. Cellular ATP Levels and Nitrogenase Switchoff upon Oxygen Stress in Chemostat Cultures of Azotobacter Vinelandii. *J. Bacteriol.* **1995**, *177* (18), 5289−5293.
(39) Linkerhägner, K.; Oelze, J. Nitrogenase Activity and Regeneration of the Cellular ATP Pool in Azotobacter Vinelandii Adapted to Different Oxygen Concentrations. *J. Bacteriol.* **1997**, *179* (4), 1362−1367.
(40) Zhang, Y.; Pohlmann, E. L.; Roberts, G. P. Effect of Perturbation of ATP Level on the Activity and Regulation of Nitrogenase in Rhodospirillum Rubrum. *J. Bacteriol.* **2009**, *191* (17), 5526−5537.
(41) Kuhla, J.; Oelze, J. Dependence of Nitrogenase Switch-off upon Oxygen Stress on the Nitrogenase Activity in Azotobacter Vinelandii. *J. Bacteriol.* **1988**, *170* (11), 5325−5329.
(42) Post, E.; Kleiner, D.; Oelze, J. Whole Cell Respiration and Nitrogenase Activities in Azotobacter Vinelandii Growing in Oxygen Controlled Continuous Culture. *Arch. Microbiol.* **1983**, *134* (1), 68−72.
(43) Fay, P. Oxygen Relations of Nitrogen Fixation in Cyanobacteria. *Microbiol. Rev. Rev.* **1992**, *56* (2), 340−373.
(44) Bazylinski, D. A.; Dean, A. J.; Schuler, D.; Phillips, E. J. P.; Lovley, D. R. N2-Dependent Growth and Nitrogenase Activity in the Metal-Metabolizing Bacteria, Geobacter and Magnetospirillum Species. *Environ. Microbiol.* **2000**, *2* (3), 266−273.
(45) Logan, B. E.; Regan, J. M. Electricity-Producing Bacterial Communities in Microbial Fuel Cells. *Trends Microbiol.* **2006**, *14* (12), 512−518.
(46) Staal, M.; Lintel-Hekkert, S. te; Harren, F.; Stal, L. Nitrogenase Activity in Cyanobacteria Measured by the Acetylene Reduction Assay: A Comparison between Batch Incubation and on-Line Monitoring. *Environ. Microbiol.* **2001**, *3* (5), 343−351.
(47) Ramos, J. L.; Guerrero, M. G.; Losada, M. Sustained Photoproduction of Ammonia from Dinitrogen and Water by the Nitrogen-Fixing Cyanobacterium Anabaena Sp. Strain ATCC. *Appl. Environ. Microbiol.* **1984**, *48* (1), 114−118.
(48) Gordon, J. K.; Brill, W. J. Derepression of Nitrogenase Synthesis in the Presence of Excess NH4+. *Biochem. Biophys. Res. Commun.* **1974**, *59* (3), 967−971.
(49) Torres, C. I.; Krajmalnik-Brown, R.; Parameswaran, P.; Marcus, A. K.; Wanger, G.; Gorby, Y. A.; Rittmann, B. E. Selecting Anode-Respiring Bacteria Based on Anode Potential: Phylogenetic, Electrochemical, and Microscopic Characterization. *Environ. Sci. Technol.* **2009**, *43* (24), 9519−9524.
(50) Diaz, C.; Baena, S.; Fardeau, M.-L.; Patel, B. K. C. Aminiphilus Circumscriptus Gen. Nov., Sp. Nov., an Anaerobic Amino-Acid-Degrading Bacterium from an Upflow Anaerobic Sludge Reactor. *Int. J. Syst. Evol. Microbiol.* **2007**, *57* (8), 1914−1918.

DOI: 10.1021/acssuschemeng.8b05763
ACS Sustainable Chem. Eng. 2019, 7, 3511−3519

(51) Lesnik, K. L.; Liu, H. Establishing a Core Microbiome in Acetate-Fed Microbial Fuel Cells. *Appl. Microbiol. Biotechnol.* **2014**, *98* (9), 4187−4196.
(52) Wang, Y.; Wang, Q.; Li, M.; Yang, Y.; He, W.; Yan, G.; Guo, S. An Alternative Anaerobic Treatment Process for Treatment of Heavy Oil Refinery Wastewater Containing Polar Organics. *Biochem. Eng. J.* **2016**, *105*, 44−51.
(53) Li, N.; He, L.; Lu, Y.-Z.; Zeng, R. J.; Sheng, G.-P. Robust Performance of a Novel Anaerobic Biofilm Membrane Bioreactor with Mesh Filter and Carbon Fiber (ABMBR) for Low to High Strength Wastewater Treatment. *Chem. Eng. J.* **2017**, *313*, 56−64.
(54) Latif, M. A.; Mehta, C. M.; Batstone, D. J. Enhancing Soluble Phosphate Concentration in Sludge Liquor by Pressurised Anaerobic Digestion. *Water Res.* **2018**, *145* (60), 660−666.
(55) Su, X.-L.; Tian, Q.; Zhang, J.; Yuan, X.-Z.; Shi, X.-S.; Guo, R.-B.; Qiu, Y.-L. Acetobacteroides Hydrogenigenes Gen. Nov., Sp. Nov., an Anaerobic Hydrogen-Producing Bacterium in the Family Rikenellaceae Isolated from a Reed Swamp. *Int. J. Syst. Evol. Microbiol.* **2014**, *64* (Pt 9), 2986−2991.
(56) Oren, A. The Family Methanocorpusculaceae. In *The Prokaryotes: Other Major Lineages of Bacteria and The Archaea*; Rosenberg, E., DeLong, E. F., Lory, S., Stackebrandt, E., Thompson, F., Eds.; Springer: Berlin, 2014; pp 225−230.
(57) Oren, A. The Family Methanobacteriaceae. In *The Prokaryotes*; Rosenberg, E., DeLong, E. F., Lory, S., Stackebrandt, E., Thompson, F., Eds.; Springer: Berlin, 2014; pp 165−193.
(58) Leigh, J. Nitrogen Fixation In Methanogens: The Archaeal Perspective. *Curr. Issues Mol. Biol.* **2000**, *2* (4), 125−131.
(59) Lobo, A. L.; Zinder, S. H. Nitrogen Fixation by Methanogenic Bacteria. In *Biological Nitrogen Fixation*; Wiley: 1992; pp 191−211.
(60) Dos Santos, P. C.; Fang, Z.; Mason, S. W.; Setubal, J. C.; Dixon, R. Distribution of Nitrogen Fixation and Nitrogenase-like Sequences amongst Microbial Genomes. *BMC Genomics* **2012**, *13* (1), 162.
(61) Zhang, C.; Ma, J.; He, D.; Waite, T. D. Capacitive Membrane Stripping for Ammonia Recovery (CapAmm) from Dilute Wastewaters. *Environ. Sci. Technol. Lett.* **2018**, *5* (1), 43−49.
(62) Tarpeh, W. A.; Barazesh, J. M.; Cath, T. Y.; Nelson, K. L. Electrochemical Stripping to Recover Nitrogen from Source-Separated Urine. *Environ. Sci. Technol.* **2018**, *52* (3), 1453−1460.
(63) Bageshwar, U. K.; Srivastava, M.; Pardha-Saradhi, P.; Paul, S.; Gothandapani, S.; Jaat, R. S.; Shankar, P.; Yadav, R.; Biswas, D. R.; Kumar, P. A.; et al. An Environmentally Friendly Engineered Azotobacter Strain That Replaces a Substantial Amount of Urea Fertilizer While Sustaining the Same Wheat Yield. *Appl. Environ. Microbiol.* **2017**, *83* (15), 1−14.
(64) Bali, A.; Blanco, G.; Hill, S.; Kennedy, C. Excretion of Ammonium by a *nifL* Mutant of *Azotobacter vinelandii* Fixing Nitrogen. *Appl. Environ. Microbiol.* **1992**, *58* (5), 1711−1718.

DOI: 10.1021/acssuschemeng.8b05763
ACS Sustainable Chem. Eng. 2019, 7, 3511−3519