Wastewater Treatment and Microbial Communities in an Integrated Photo-Bioelectrochemical System Affected by Different Wastewater Algal Inocula

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Abstract

Integrated photo-bioelectrochemical (IPB) systems are a newly emerging technology for sustainable wastewater treatment through synergistic cooperation between microbial fuel cells (MFCs) and algal bioreactors. This study aimed to advance our understanding of the IPB performance in contaminant removal and bioenergy production and how IPB function is affected by different algal-bacterial inocula. Fed with a synthetic solution, the IPB system could achieve more than 90% removal of solution organic compounds and nearly 100% of ammonium nitrogen. Production of algal biomass was significantly different among three inocula, varying from 5.9 to 53.3 mg L⁻¹. The highest energy production was 0.089 kWh m⁻³, including direct electrical energy of 0.055 kWh m⁻³ and indirect electrical energy of 0.034 kWh m⁻³ from biomass conversion, and a positive energy balance could be achieved. The natural algal inocula resulted in the cyanobacteria *Leptolyngbya* and green alga *Acutodesmus* as dominant photoautotrophs in cathode suspension and biofilms, providing oxygen for MFC function. Differences in IPB efficiency could be related to microbial composition; one inoculum resulted in absence of Xanthomonadaceae bacteria, while another had more γ -proteobacteria. Specific taxa identified could be important for optimizing electricity generation and algal biomass for biofuels production.

Keywords: Photo-bioelectrochemical systems; microbial fuel cells; algal bioreactors; wastewater treatment; microbial community; bioenergy

1. Introduction

Recovering bioenergy from contaminants will promote/facilitate sustainable wastewater treatment, and to achieve this, new technologies such as microbial fuel cells (MFCs) and algal bioreactors are being developed. MFCs are a bio-electrochemical reactor in which exoelectrogenic bacteria oxidize organic compounds and produce bioelectricity [1]. The direct energy recovery from wastewater makes MFCs a promising approach for simultaneous wastewater treatment and bioenergy production. The electricity-generating processes in MFCs promote the oxidation of organic compounds [2]; however, nutrients such as nitrogen and phosphorus are not effectively removed, unless special processes are linked to MFCs [3]. Treatment of wastewater with algae has a long history, especially in removing nutrients [4][5] and wastewater can also be regarded as a resource for growing algal biomass for production of biofuels [6]. Algal bioreactors are typically based on algae using photosynthesis to fix carbon dioxide into organic compounds, resulting in biomass growth and oxygen production. During algal growth, concentrations of dissolved nutrients, e.g., nitrogen (N) and phosphorus (P), are drawn down by algal uptake [7][8]. Combining MFCs and algal bioreactors may complement the functions of these two components to improve the removal of organics (in MFCs) and nutrients (algal uptake), reduce the need of aeration (via algal oxygen production), and harvest bioenergy (bioelectricity in MFCs and biomass in algal bioreactors). While MFC energy production and algal nutrient bioremediation have been studied separately, we lack information about the how different algae and bacteria might affect functions of integrated algal bio-electrochemical reactors.

Algae linked to MFCs has been examined through several approaches [9]. First, algae can provide a substrate for the exoelectrogenic bacteria in the anode of MFCs. Electricity can be successfully produced from the MFC fed with living or dry algal biomass cultivated in a separate photobioreactor [10-12][13]. However, the efficiency of this approach is low, as it still uses two separated reactors and it is hard to transfer chemical energy from algae (via the degradation of the macromolecules in algae) to exoelectrogenic bacteria in the anode of an MFC. Second, algae may be able to catalyze the anode reaction. A study observed electrogenic activity associated with high biomass algal growth and chlorophyll content in a photo-MFC that used light as the input energy, but the dissolved oxygen (DO) produced by algae via oxygenic photosynthesis inhibited the anode process [14]. Third, algae have been cultivated in the cathode to provide DO as an electron acceptor to the cathode reaction, providing DO levels comparable to or better than the mechanic aeration typically required, thereby greatly decreasing energy consumption through eliminating mechanical aeration [15-19]. In addition to providing DO, algae in the cathode compartment could also help improve wastewater treatment through nutrient removal. Therefore, combining algae in the cathode of MFCs provides a promising approach for wastewater treatment.

We have previously developed an integrated photo-bioelectrochemical (IPB) system to synergistically link an MFC with an algal bioreactor for wastewater treatment and bioenergy production [20]. With algae in the cathode, the IPB system effectively removed both organic and nutrient compounds during the one-year operation: more than 92% of organics, 98% of ammonium nitrogen and 82% of phosphate was removed from synthetic wastewater [20]. The analysis of energy balance indicated that the IPB could theoretically produce more energy than it

consumed (related to energy required for mixing of the anolyte and the catholyte), thereby making the treatment process energy neutral or positive. The cathode of the IPB was initially inoculated with green algae laboratory organism *Pseudokirchneriella subcapitata*, and over the operation the cathode microbial community became mixture of both bacteria and algae, both of which contributed critical metabolic activities to the IPB performance. In this previous IPB, we used laboratory cultures of algae to inoculate the IPB. However there is growing interest in using natural algal-bacterial assemblages both for wastewater treatment, but also for production of biofuels [21]. Wastewater contains a range of algal and cyanobacterial taxa which could work in wastewater treatment [22]. It is believed that the source of algal inocula plays a key role in shaping microbial communities in the cathode. However, how different algal-bacterial communities might function together for synergistic MFC function has not been explored.

To address these outstanding issues, the aim of this current study is to examine the IPB system with different sources of algal inoculum, sampled from several wastewater treatment facilities. The present IPB system is different from our previous one mainly in two aspects: first, instead of cation exchange membrane employed previously, an anion exchange membrane is used here to facilitate nitrate movement from the anode to the cathode, and second, algal inocula used are from natural source, rather than laboratory cultures used in the previous study. The specific objectives are: (1) to evaluate the performance of the IPB with algal sources from different wastewaters; (2) to analyze the energy balance and production of algal biomass; and (3) to characterize the microbial community in the cathode related to these different algal sources.

2 Materials and methods

2.1 IPB system setup

The IPB system consisted of a tubular MFC installed in a glass beaker (Figure 1), which functioned as both the cathode compartment and the algal bioreactor. The MFC was constructed in an anion exchange membrane tube (AEM, Ultrex CMI7000, Membranes International, Inc., Glen Rock, NJ, USA) containing an interior supporter of a polyvinyl chloride (PVC) tube with a diameter of 2.54 cm and a height of 24 cm, resulting in an anode liquid volume of about 180 mL. The AEM was used to improve nitrate removal by promoting nitrate migration from the cathode into the anode for heterotrophic denitrification. A 20 cm-long carbon brush (Gordon Brush Mfg. Co., Inc., Commerce, CA, USA) was used as the anode electrode. The carbon brush electrode was pretreated by immersion in acetone overnight and heated to 450 °C for 30 min [23]. The cathode electrode was a layer of carbon cloth with Pt/C as a catalyst that wrapped the AEM tube. To coat the catalyst to the cathode electrode, the Pt/C powder was mixed with Nafion solution, and then applied to the carbon cloth surface with a brush to a final loading rate of ~ 0.5 mg Pt cm^{-2} . The anode and cathode electrodes were connected by copper wires to an external circuit across a 100- Ω resistor. The glass beaker that held the MFC had a diameter of 10 cm and height of 29 cm, with a liquid volume of 1800 mL. A compact fluorescent bulb (32 W, 120 V, Energy Wiser, color temperature 4000 K, Bulbrute Industries, Inc., China) was installed near the cathode to provide the illumination on a 16-h on/8-h off cycle.

2.2 Operating conditions

The IPB system was continuously operated at room temperature of $\sim 20^{\circ}\text{C}$. The anode compartment was inoculated with the anaerobic sludge from a municipal wastewater treatment plant (South Shore, Milwaukee, WI, USA). The cathode compartment was inoculated with algal solutions from one of three different wastewater sources: the algal inocula “Sherwood” and “Francisville” were collected from domestic treatment ponds for small towns in the state of Ohio (Sherwood: 41.2897°N , 84.5511°W) and Indiana (Francisville: 40.9858°N , 86.8839°W), respectively; the algal source “WWExp” was a mixed sample taken from several oxidation ponds of wastewater treatment facilities in Indiana and Ohio, USA. The IPB systems tested with these algal sources were denoted as the IPB-S (Sherwood), IPB-F (Francisville) or IPB-W (WWExp). To reduce the effect of (different) reactors, the same IPB was used for testing all three inocula in a sequential order: after testing one inoculum, the cathode electrode of the IPB was cleaned using bleach before inoculating next algal sample, and data was only collected after the system had been operating with the new inocula for at least one month, which ensured that each IPB system had sufficient time to re-establish microbial communities. The anode compartment was fed with a synthetic solution containing sodium acetate as a carbon source and the following compounds: NH_4Cl , NaCl , MgSO_4 , CaCl_2 , NaHCO_3 , KH_2PO_4 , and trace element solution according to a previous study [24] and as detailed in Table S1. This solution was fed into the MFC anode at a flow rate of 0.4 mL min^{-1} (with an anolyte recirculation rate of 30 mL min^{-1}), resulting in a hydraulic retention time (HRT) of 7.5 h and an organic loading rate of $0.87\text{ kg COD m}^{-3}\text{ d}^{-1}$ in the anode compartment. The effluent of the anode flowed into a 200-mL transitional beaker, which acted as a stabilization unit, and then the liquid in the beaker was pumped into the cathode of the IPB at a flow rate (0.4 mL min^{-1}). The catholyte was mixed by a magnetic stirrer. The theoretical redox reactions based on acetate oxidation in the MFC can be presented as:

2.3 Measurement and analysis

The MFC cell voltage was recorded every 5 min by a digital multimeter (2700, Keithley Instruments, Inc., Cleveland, OH, USA). The concentrations of soluble chemical oxygen demand (COD), ammonium nitrogen, nitrite nitrogen, nitrate nitrogen, and phosphate were measured according to the manufacturer's instructions using a DR/890 datalogging colorimeter (Hach Company, Loveland, CO, USA). The pH, temperature, and dissolved oxygen (DO) were measured using a 556 MPS hand-held multiprobe system (YSI Incorporated, Yellow Spring, OH, USA). The concentration of algal cells was measured using absorbance at 680 nm by an 1100 spectrophotometer (Unico, Dayton, NJ, USA) and converted to biomass according to a calibration curve in the previous study assuming similar absorbance per mass unit by different algal cells [20]. Power density and current density were calculated based on the anode liquid volume, and the normalized energy recovery (NER) was based on the volume of treated water [25].

2.4 Scanning Electron Microscopy (SEM)

For SEM examination of microorganisms, either 8 mL suspended culture or a piece of carbon cloth with algae attached were added into a mixture (volume ratio 1:1) of 1 mL 25%

glutaraldehyde and 1 mL 1 M phosphate buffer (53 g L⁻¹ KH₂PO₄ and 107 g L⁻¹ K₂HPO₄) overnight, and then were fixed with 1% OsO₄ for one hour. The suspended algae were concentrated by filtration (0.02 µm, Nuclepore filters, Watman Pleasanton, CA, USA). Then the filter with algae was dehydrated with a graded ethanol series: 10%, 25%, 50%, 75%, 95%, 100% and 100%. Samples were critical point dried via liquid CO₂ in a Balzers CPD 020 critical point drying apparatus (Balzers Union, Liechtenstein). After being dried, the samples attached on the specimens were coated with 5.0 nm iridium in the Emitech K575X Sputter Coater (Emitech, Polaron, UK). The same treatment was applied to the carbon cloth with attached algae. The morphology of microorganisms was observed by SEM under 5 kV (S-4800 Hitachi, Japan). Samples collected from the catholyte were also examined using an Olympus BX 41 light microscope and images captured with an Olympus DP70 camera and DP controller software v. 1.2.1.108 (Olympus Optical Co Ltd, Center Valley, PA, USA).

2.5 Microbial and Algal Community Analysis

Sampling and Sequencing: IPBs and algal inocula were sampled for genetic analysis of bacterial and algal community composition. Early light microscopy analysis indicated dominance of cyanobacteria in the ‘algal’ inocula so genetic analysis targeted 16S rRNA to capture cyanobacterial, bacterial and some information about algal chloroplast sequences. Five samples were collected from each of the three IPBs, from the following sites: 1. inocula prior to inoculation; 2. Inocula after the IPB analysis; 3. catholyte suspension; 4. the cathode membrane surface; and 5. the outer wall of the catholyte algal chamber (Figure 1). Microbial cells were collected from the catholyte via filtration of a small volume and from the surface of the cathode

electrode with a sterile spatula, and stored at -80 °C before DNA extraction with a Powersoil kit (MoBio, Carlsbad, CA, USA) according to manufacturer's instructions. DNA from the 15 samples (3 algal sources x 5 sites) were submitted to the University of Wisconsin Madison sequencing center. Samples were prepared as described in the 16S Metagenomic Sequencing Library Preparation Protocol, Part # 15044223 Rev. B (Illumina Inc., San Diego, California, USA) with the following modifications: The 16S rRNA gene V3/V4 variable region was amplified with nested primers (forward primer: 5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCTCCTACGGGNGGCWGCAG-3', reverse primer: 5'-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGACTACHVGGGTATCTAATCC-3') [26], modified with adapter overhang nucleotide sequences added. Library quality was assessed (Agilent DNA1000 chip and Qubit® dsDNA HS Assay Kit) and paired end, 250 bp sequencing was performed using the Illumina MiSeq Sequencer and a MiSeq 500 bp (v2) sequencing cartridge.

Data processing and analysis: The high throughput FASTA sequence files were quality control processed using mothur v. 1.34.3, accessed April 2015, following the standard operating procedure for MiSeq sequencing of 16S rRNA sequences [27]. This included matching paired sequences, removing low quality sequences, trimming and aligning to expected sequence, denoising and chimera removal, and operational taxonomic units (OTUs) identified using neighborhood clustering based on a similarity of 97%. To identify taxa, sequences were aligned against databases Silva v. 119 [28] for bacteria and Greengenes [29] for chloroplast sequences. Sequences identified as chloroplast were removed prior to analysis of bacterial family composition of the 15 libraries, and for non-metric multi-dimensional scaling with a Morisita

index of diversity in PAST v. 2.17c [30]. Chloroplast sequences were examined separately to identify dominant algal taxa. Samples for each IPB were treated as replicates for examination of differences in bacterial composition using one-way ANOVA, performed in Sigmaplot (v. 12.5, Systat Software Inc, San Jose CA, USA).

3 Results and discussions

3.1 Contaminant removal in the IPB systems

The IPB system with anion exchange membrane (AME) and integrating different natural wastewater algal inocula could effectively remove both organic compounds and nutrients from a synthetic wastewater (Figure 2), confirming the findings from previous research of an IPB operating with a cation exchange membrane and with laboratory algal cultures [20]. The IPB system removed more than 90% of soluble COD in the anode, for all three of the cathode algal sources. However, the COD in the catholyte increased relative to the influent (anode effluent) (Table 1), resulting in high anode COD removal but lower net COD removal for the whole IPB (Figure 2). This may be for several reasons: first, the AME might promote transport of anions (e.g., acetate ions) from the anolyte through the AME into the catholyte, thereby increasing the organic concentration in the catholyte; second, algal photosynthesis produced organic compounds, some of which were released from healthy cells into the soluble fraction; and third, unhealthy or dying algal cells might be degraded by heterotrophic bacteria in the cathode releasing organic compounds. Our previous work demonstrated that in the absence of algal photosynthesis, the cathode of an AEM-MFC could well polish the anode effluent via aerobic

treatment to achieve a lower COD concentration than that in its anode effluent [31]. This therefore suggests that it was the algal photosynthesis and possible organic release from cells and/or cell degradation that resulted in elevated COD in the catholyte, and not the AEM function. The increased COD concentration in the catholyte creates challenges for direct discharge of the treated effluent (even after harvesting algal cells), and post treatment will be required to decrease the COD. Inclusion of mixotrophic algal taxa may also help with organic removal while generating algal biomass [32].

Both nitrogen and phosphorus nutrients were removed in the IPB system, with some differences in the AEM function from a CEM-based IPB system, and also some performance differences among three algal sources. Unlike the previous CEM-IPB system where anode effluent contained less than 40% of ammonium nitrogen [20], this AEM-IPB system retained more than 80% of ammonium nitrogen in its anode effluent, because AEM prevented ammonium migration. The cathode achieved an effective ammonium removal such that no ammonium nitrogen was detected in the cathode effluent (Table 1) demonstrating algal ammonium uptake in the catholyte. Likewise, nitrite was below the detection limit ($< 0.3 \text{ mg L}^{-1}$) in the catholyte. Nitrate, on the other hand, exhibited a concentration varying between 5 and 13 mg L^{-1} , although lower than $\sim 20 \text{ mg L}^{-1}$ reported in a previous CEM-based IPB system [20]. This improved nitrate removal performance was related to the use of AEM, which promoted movement of nitrate ions from the catholyte to the anolyte where heterotrophic denitrification occurred; there was also possibility that algal uptake of nitrate was enhanced, but differentiation of nitrate removal by different mechanisms would be very challenging in the present system. The anode effluent contained $0.1 - 0.7 \text{ mg L}^{-1}$ nitrate that was remained from nitrate migrated from the cathode across the AEM (Table 1), indicating effective denitrification in the anode. Despite high dissolved oxygen due to

algal photosynthesis, denitrification was also expected in the cathode, via heterotrophic denitrification (using organics as an electron donor) and/or bioelectrochemical denitrification (using the cathode electrode as an electron donor) [3]. The concentration of nitrate in the effluent of the IPB-S was more than double that in the IPB-F or the IPB-W (Table 1); given the similar performance of electricity generation (that is the driving force for nitrate migration across the AEM), this difference is likely related to the difference in cathode denitrification that needs to be further investigated for the presence and function of denitrifying organisms in the cathode. As a result, the removal efficiency of total inorganic nitrogen in the IPB-S was 75%, significantly lower than 88% obtained from the IPB-F and the IPB-W achieved ($p < 0.001$, ANOVA) (Figure 2). Phosphate removal in the present IPBs was much lower than that in the previous CEM-based IPB; the highest phosphate removal efficiency was only 44% in IPB-F and IPB-W (Figure 2), which was a little more than half of that in the previous CEM-IPB system based on green algae from laboratory inocula. The decreased phosphate removal was not likely due to the use of AEM, because phosphate removal in an IPB system relies on microbial uptake, by algal and bacterial cells [33-34]. Thus, it is possible that the use of different algal inocula and the resulting bacterial communities resulted in this difference. Optimizing IPB function for nutrient removal may include selection of algal and bacterial assemblages.

3.2 Electricity generation

Electricity generation is a key output from the IPB system, and can be presented as either power production or energy recovery [35]. Power production is commonly used in the MFC studies, but it does not reflect energy issues [36]; thus, both power density and energy recovery was

presented here. The power densities of the three IPB systems are shown in Figure 3A. The highest power density of $8.70 \pm 0.17 \text{ W m}^{-3}$ was produced by the IPB-F during the light period, while the other two generated slightly lower power densities ($8.44 \pm 0.20 \text{ W m}^{-3}$ for IPB-S, and $8.40 \pm 0.13 \text{ W m}^{-3}$ for IPB-W). When the power data are converted into energy data including the time factor, the NER of the IPB-F ($0.061 \pm 0.001 \text{ kWh m}^{-3}$) was also higher than that from the IPB-S ($0.055 \pm 0.001 \text{ kWh m}^{-3}$) or the IPB-W ($0.056 \pm 0.002 \text{ kWh m}^{-3}$). Compared with the CEM-IBP system in our previous study [20], these IPB systems produced more than 3.5 times the maximum power density and more than 4.5 times the NER. This improved electricity generation in the present IPB systems was related to the optimized MFC configuration, especially a smaller diameter of the tubular reactor, which shortens the distance between the anode electrode and cathode electrode, thereby decreasing the internal resistance [25].

Algae can also affect electricity generation, mainly through DO supply. All three cathodes algal chambers could provide substantial DO, reaching over 25 mg L^{-1} in IPB-S (Figure 3B). There were some differences between the IPB, with lower DO reached in the IPB-W during illumination, which may relate to algal cell density in the catholyte. However the lowest algal biomass was in the IPB-F (Table 1) which had higher DO (Figure 3B), so this difference may relate to the photosynthetic capacity of the distinct algal species in the cathode. Given similar performance of electricity generation among three IPB systems, the differences in DO were clearly not limiting factors for MFC function. It was noted that during the dark, the DO was not depleted to zero; instead, the lowest DO concentration was about 5 mg L^{-1} , similar for all three IPB systems (Figure 3B). This is likely due to a smaller diameter of the IPB reactor in this study which allowed a larger water/air interface for oxygen exchange.

The catholyte pH is another key factor affecting electricity generation and may be influenced by both microbial and electricity-generating activities. The pH of the catholyte increased under illumination, as photosynthetic oxygen production resulted in more electricity generation (more oxygen reduction consuming more protons) along with more CO_2 or HCO_3^- uptake by algae, both of which can lead to a higher pH. During the dark, the decreased electricity generation (Figure 3A) and no photosynthesis resulted in relatively lower pH. Two processes were presented to buffer the high pH; one was externally added bicarbonate (in the synthetic solution), which did not seem to be as effective as CO_2 addition in our previous study, and the other was naturally occurring nitrification in the presence of DO in the cathode that released protons. The use of bubbling with CO_2 provides inorganic carbon for algae photosynthesis but also provides pH buffering [37], so can be a valuable addition to a wastewater treatment plant, where there is typically a readily available source of CO_2 from combustion of biogas generated from anaerobic digestion of primary sludge [38].

3.3 Biomass production

Algal biomass can be another important output from the IPB system and use of algal biomass produced for biofuels synthesis will make the IPB process for wastewater treatment more sustainable and economically feasible [6]. Biomass production was evaluated by measuring the biomass concentration (suspended biomass) in the catholyte effluent, but biofilm attached to the cathode electrode was difficult to remove and thus not quantified. The highest biomass concentration of $53.3 \pm 8.9 \text{ mg L}^{-1}$ (or $15.3 \pm 2.6 \text{ mg L}^{-1} \text{ d}^{-1}$) ($n = 5$) was calculated for the IPB-S,

while the lowest concentration of $5.9 \pm 1.2 \text{ mg L}^{-1}$ (or $1.7 \pm 0.3 \text{ mg L}^{-1} \text{ d}^{-1}$) ($n=3$) was in the IPB-F (Table 1). Although the IPB-F produced a smaller density of suspended biomass, its DO production, electricity generation and nutrients removal was comparable to the other two systems, possibly due to functional cells in the biofilms. One can observe from the SEM pictures (Figure 4) that the dominant algal species in those three systems exhibited different sizes. The algal cells in the IPB-S (Figure 4A) had the largest size among the three, in an ovoid shape with a length of $72.3 \pm 7.7 \text{ }\mu\text{m}$ and a diameter of $52.0 \pm 7.8 \text{ }\mu\text{m}$ ($n>8$ cells). The IPB-F had the smallest size cells in a round shape with a diameter of $24.6 \pm 5.4 \text{ }\mu\text{m}$ (Figure 4B); the dominant algal cells in the IPB-W were also in a round shape with a diameter of $33.9 \pm 2.9 \text{ }\mu\text{m}$ (Figure 4C). The order of algal size in three IPBs follows the reverse order of their suspended biomass concentration, indicating that the size of algal cells might have affected their attachment to carbon fibers (diameter of $82.3 \pm 6.7 \text{ }\mu\text{m}$) of the cathode electrode. It is likely that the larger cells (e.g., those in the IPB-S) tend not to attach to carbon fibers, while the smaller algae (e.g., those in the IPB-F) are easier to aggregate and form biofilm. As a result, most of the larger cells ended up in the catholyte as suspended biomass, while the smaller cells were largely present in the biofilms on the beaker wall and on membranes. The light microscopy images and genetic analysis (see below) also suggests dominance of filamentous cyanobacteria, some of which may do well in suspension, while others readily form biofilms on the IPB wall or membranes. The aggregation of algal cells can also relate to extracellular polymeric substances (EPS) excreted from algal and bacterial cells, but this was not investigated here.

3.4 Microbial community in the cathode

This study contributes to the small number of genetic profiling studies of bacteria associated with algae growing in wastewater [21]. After quality control and paired matching of the FASTA sequences, there were 6,270 - 221,667 sequences per sample library (median 9316). Of the OTUs identified to taxa, the dominant classes of bacteria identified in all samples were cyanobacteria (29% of total), α -, β - and γ -proteobacteria (17%, 13% and 12% respectively), Bacteroidetes (14%), Planctomycetes (4.3%), Acidobacteria (3%) and Actinobacteria (2.2%) (Figure S1). In a study of bacteria associated with algae growing in wastewater, similar classes were also found though they found more sequences and taxa within the γ -proteobacteria in exponential phase cultures than in later cultures [21]. In all 5 samples for each IPB, WWExp had significantly higher proportion of γ -proteobacteria sequences than the other two ($p < 0.003$; one-way ANOVA). Bacterial family sequence numbers were adjusted to the median sequence number for the 15 samples and dominant families identified were Cyanobacteria Family I (9.2%), Rhodobacteraceae (2.9%), Rhodocyclaceae (2.8%), Acidobacteriaceae (2.4%), Comamonadaceae (2.3%), and Chitinophagaceae (2.1%) (Figure 5). Among the Rhodobacteraceae (especially in the IPB-F), sequences were identified as *Rubrimonas cliftonensis*, a chemoheterotrophic bacterium which could contribute to nitrate reduction [39]. Other notable taxa identified (Table S2) were *Sphingopyxis*, known to degrade organics in wastewater [40], *Aquimonas* and *Hydrogenophaga* common in freshwater ecosystems including wastewater [20, 41]. Bacterial taxa with potential negative effects on algal growth, e.g. *Pseudomonas* and Bdellovibrionaceae [21], were not identified even in the sample with the highest sequencing depth. There was evidence for *Leadbeterella* and *Rhodopirellula* - taxa with cellulase activity, possibly involved with breakdown of green algal cell walls [42]. The common wastewater bacterial family Shewanellaceae was also not identified. IPB-W samples lacked

Xanthobacteraceae and IPB-F samples lacked Xanthomonadaeae which was significantly higher in IPB-W and IPB-S samples ($p < 0.04$). Notable Xanthomonadaeae species identified in IPB-S and IPB-W samples included the common aquatic bacterium *Aquimonas voraii*, *Lysobacter brunescens*, which has extracellular phosphatase activity [43], which could improve phosphorus availability for algae, and *Silanimonas lenta*, strains of which may tolerate high pH [44], which could arise due to algal photosynthetic CO₂ uptake.

There were also clear differences in bacterial family composition shown in the non-metric multidimensional scaling plot (Figure 6). In the IPB-S, the cathode had distinctly different composition to the other samples suggesting conditions on the cathode surface promoted different bacteria. The composition in samples collected from the IPB-S and IPB-W catholyte chamber wall were very similar to the initial inoculum, while the suspension was distinct and similar to the final inoculum source. In the IPB-F, each sample was distinctly different, suggesting greater divergence of bacterial growth and conditions from the initial inoculum. The lack of Xanthomonadaeae taxa and this change in composition within the IPB-F may have contributed to the lower performance.

Algae and cyanobacteria were also important components of an IPB (Figure 4) providing photosynthetic oxygen production and both algae and cyanobacteria can contribute to wastewater nutrient remediation. The dominant photoautotrophs identified to genus or species included the cyanobacteria *Leptolyngbya*, also identified microscopically as forms containing phycoerythrin (Figure 4D) or phycocyanin (Figures 4E and F) as previously described [45]. *Leptolyngbya* were 1-11% of sequences on the cathode surface and suspension and 12-13% of sequences on the wall

of all three IPBs. Another cyanobacterium, *Merismopedia glauca*, was up to 1.1% of sequences (Table S1). Chlorophytes were the eukaryotic algae identified from chloroplast sequences, with *Acutodesmus obliquus* (syn. *Scenedesmus obliquus* (Turpin) Kützing) representing minor components in the IPB-W, 1-2% in the IPB-F but 19% in the IPB-S, including 70% of sequences in suspension (and possibly as ovoid cells in Figure 4A). *A. obliquus* sequences were cross-checked on NCBI via BLAST and confirmed as plastid sequences from *Acutodesmus* or the closely-related *Scenedesmus*. *A. obliquus* has been demonstrated to be useful in wastewater nutrient remediation, and produces fatty acids useful for biofuel production [46], and similar taxa were dominant in algal wastewater communities [21]. Other green algae were evident in IPB-W and IPB-F (Figures 4D and E), with chlorophytes including the family Chlamydomonaceae contributing 3-64% of sequences in the three IPB sampling sites (possibly as the larger cells in Figure 4C). Taxa from other algal divisions were not identified in sequence matches in greengenes or SILVA, and green algae are often found to dominate wastewaters [47]. However, algal reference sequences are not typically as well represented as bacteria in 16S rRNA sequence databases but even more careful targeting (e.g., 18S rRNA), can lead to large proportions of unidentified sequences [21]. Differences in oxygen production and pH maintenance (Figure 3) may relate to these differences in algal and cyanobacterial composition, and the performance of different taxa under variable light conditions within the IPB. Some taxa, such as *A. obliquus*, were dominant in suspension, while *Leptolyngbya* dominated on the catholyte wall surface, and often form biofilms [48]. While both attached and suspended algae can contribute to remediation [49], suspended cells may be easier to harvest for potential biofuels processing, so selection of photoautotrophs for IPB might target desirable taxa for harvesting that do well in suspension.

However additional algal or cyanobacterial biofilms on IPB surfaces might be useful for maintenance of oxygenation even with regular depletion of suspended cells related to harvesting.

3.5 Energy balance in the IPB systems

Energy consumption (input) and production (output) for the three IPB systems with different algal and microbial compositions was summarized in Figure 7. All three IPB systems had the same energy input (consumption), because the flow rates of the anode feeding and the anolyte recirculation pumps were fixed. Among the total input of 0.068 kWh m^{-3} (per volume of the treated water), the feeding pump consumed 0.011 kWh m^{-3} and the anolyte recirculation pumping required 0.057 kWh m^{-3} . The energy consumption by the IPB system was significantly lower than that of aerobic treatment that requires about 0.6 kWh m^{-3} [50], likely benefiting from the omission of aeration, which was unnecessary because of algal oxygen production. The three systems had different calculated energy production or total output, mainly due to difference in biomass production. The IPB-S had the highest energy output of 0.089 kWh m^{-3} , in which direct electrical energy was 0.055 kWh m^{-3} and indirect electrical energy from biomass conversion was 0.034 kWh m^{-3} . The IPB-F and IPB-W generated comparable direct electrical energy at 0.056 - 0.061 kWh m^{-3} ; however, the energy obtained from biomass conversion in the IPB-W was only 55% of that in the IPB-S, and the IPB-F produced as low as 0.004 kWh m^{-3} . If the produced energy could be used to offset energy input, the direct electrical energy in the IPB-F could support 89.7% of the energy consumption, higher than the IPB-S (80.9%) and the IPB-W (82.4%). This is a significant improvement from our previous IPB system, which generated direct electrical energy for 14-29% of the energy consumption [20]. If the indirect energy from

biomass was included (based on the assumption that biomass contains 20% oil that can be converted to biodiesel with $E_{\text{biodiesel}}$ of $\sim 37\,800\text{ MJ ton}^{-1}$ and conversion efficiency of 30% from diesel to electricity), both the IPB-S and the IPB-W could theoretically achieve a positive energy balance, while the IPB-F would have a negative energy balance.

The energy analysis demonstrates that the IPB system is an energy-efficient process for wastewater treatment, and has a potential to be an energy neutral technology, as well as providing essential nutrient and organic bioremediation services. Further research is needed to clarify outstanding issues and optimize the energy benefits of the IPB. First, energy consumption needs to be further decreased and the major target should be to reduce the intensity of the anolyte recirculation, which is the major energy consumer (84% of total energy input). Using only periodic anolyte recirculation could significantly decrease energy demand but as energy production may also be reduced due to insufficient mixing, this needs to be carefully tested and there may be an optimal point at which the difference between energy production and consumption reaches the maximum. Secondly, energy production needs to be further improved, possibly through further optimization of reactor configuration, materials, and operation conditions. This study illustrates significant improvements from the previous CEM-IPB [16], so further optimizing should be possible. This study has demonstrated that algal community composition can affect IPB function and we need to improve our understanding of how specific algal species affect the system performance via DO supply, interaction with key bacteria for nutrient removal, and influence of biofilm on the cathode electrode. Thirdly, energy balance of the IPB system needs to be examined at a larger scale, e.g., $> 100\text{ L}$, in order to investigate the

issues associated with system scaling up, which is a key challenge for the development of bioelectrochemical systems such as MFCs.

4. Conclusions

This study has advanced our understanding of integrated photo-bioelectrochemical systems in the aspects of treatment performance and microbial community affected by algal inocula.

Organic removal was less influenced by the algal and microbial composition related to the distinct algal inocula, because of the anode treatment and sufficient supply of dissolved oxygen in all three systems. Energy production, in the forms of both electricity and biomass, was significantly affected by algal sources, and two algal inocula resulted in positive energy balance in the IPB. Natural algal assemblages offer robust inocula for algal bioreactors integrated with MFCs, and algal and cyanobacterial taxa offer oxygenation, nutrient uptake and options for biomass production for biofuels production. Specific algal taxa and/or algal wastewater sources may provide optimum composition of heterotrophic bacteria for promoting electricity generation.

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Table 1. Characteristics of the synthetic wastewater solution flowing through the compartments of the IPB system; Values are means of the measurements (5 measurements for IPB-S, 3 measurements for IPB-F, 4 measurements for IPB-W) $\pm$ standard deviation. Algal biomass was calculated from absorbance at 680 nm using a conversion from a previous study [20].

		COD	$\text{NH}_4^+ \text{-N}$	$\text{NO}_3^- \text{-N}$	$\text{NO}_2^- \text{-N}$	$\text{PO}_4^{3-} \text{-P}$	Algal biomass (mg L^{-1})	Algal biomass productivity ($\text{mg L}^{-1} \text{d}^{-1}$)	Electricity consumption (kWh m^{-3})		Electricity production (kWh m^{-3})
Supply rate ($\text{mg L}^{-1} \text{d}^{-1}$)		76.8	15.1	< 0.1	< 0.1	1.8			feeding	mixing	
Anode influent (mg L^{-1})		266.7	52.3	< 0.2	< 0.3	6.2					
IPB-S (mg L^{-1})	Anode effluent	24.0 $\pm$ 8.1	44.3 $\pm$ 2.0	< 0.2	< 0.3	5.5 $\pm$ 0.5	53.3 $\pm$ 8.9	15.3 $\pm$ 2.6	0.011	0.057	0.055 $\pm$ 0.001
	Cathode effluent	32.5 $\pm$ 8.5	< 0.4	12.9 $\pm$ 0.6	< 0.3	4.4 $\pm$ 0.4					
IPB-F (mg L^{-1})	Anode effluent	22.7 $\pm$ 4.0	42.3 $\pm$ 1.2	< 0.2	< 0.3	5.8 $\pm$ 0.9	5.9 $\pm$ 1.2	1.7 $\pm$ 0.3	0.011	0.057	0.061 $\pm$ 0.001
	Cathode effluent	39.0 $\pm$ 7.7	< 0.4	5.9 $\pm$ 1.8	< 0.3	3.5 $\pm$ 0.9					
IPB-W (mg L^{-1})	Anode effluent	22.0 $\pm$ 7.5	45.3 $\pm$ 1.0	0.7 $\pm$ 0.4	< 0.3	5.6 $\pm$ 0.6	30.2 $\pm$ 6.1	8.7 $\pm$ 1.8	0.011	0.057	0.056 $\pm$ 0.002
	Cathode effluent	45.2 $\pm$ 5.4	< 0.4	5.1 $\pm$ 1.6	< 0.3	3.5 $\pm$ 0.7

Figure 1. Schematics of the integrated photo-bioelectrochemical (IPB) system consisting of an MFC with anion exchange membrane (AEM) and an algal bioreactor. Synthetic wastewater (anode influent) is fed into the anode compartment, where organic compounds are biologically degraded for generating electrons. Electrons move from the anode electrode (carbon brush) to the cathode electrode (carbon cloth), where oxygen reduction occurs to complete the electrical circuit. The treated wastewater (anode effluent) is discharged into in a transitional beaker, and this solution then is supplied to the cathode compartment (algal bioreactor), where algae grow and produce dissolved oxygen for supporting the cathode reaction. The final effluent from the cathode compartment is discharged containing suspended algal cells. Samples for microbial community analysis were collected from the cathode membrane (A), the suspension (B) and the algal bioreactor wall (C). The algal bioreactor was mixed with a magnetic stirrer and provided with a light source.

Figure 2. Summary of removal efficiencies of organic matter (as COD) and macronutrients in the three IPB systems based on different wastewater algal inocula: S- Sherwood, F – Francisville, W-WWExp (see Material and Methods for details). Bars are means of the measurements (5 measurements for IPB-S, 3 measurements for IPB-F, 4 measurements for IPB-W) and the error bar is standard deviation. Inorganic nitrogen is ammonium plus nitrate and nitrite.

Figure 3. The performance and operating parameters of three IPB systems based on the different wastewater algal sources (see Fig. 2) over selected three day-night cycles: power density (A), dissolved oxygen (B), and catholyte pH (C). The white indicates the illuminated period (16 hours) and the shadow indicates the dark period (8 hours).

Figure 4. Images of microbial communities collected from cathode electrode membrane with Scanning Electron Microscopy (SEM) (A-C) and of source inocula with light microscopy (D-E). Paired images of are from the IPBs IPB-Sherwood (A, D), the IPB-Francisville (B, E), and the IPB-WWExp (C, F). Round to elliptoid cells and tapered cells are evident as are cyanobacterial filaments (black arrowheads in C). In SEM images, carbon fiber strands of the cathode membrane (*) can be seen with resident bacterial cells (white arrowheads).

Figure 5. Bacterial families identified from 16S rRNA gene sequences in samples from Sherwood (S), Francisville (F) and WWExp (W) IPB systems. Stacked bars represent sequences identified to bacterial families found in each sample: initial inoculum (inoc1) and inoculum at the end of the IPB run (inoc 2), suspended in the catholyte (susp), on the cathode membrane (cath) and on the algal cathode chamber outer wall (wall) (see Fig. 1). Only families which were represented by sequences accounting for > 0.1% of the total sequences in all samples are shown and sequence numbers are adjusted to the median sequence coverage over the 15 samples. Taxa are from the bottom in order of decreasing abundance across all samples.

Figure 6. Non-metric multidimensional scaling of bacterial family composition in the 15 sequenced samples, grouped for each IPB-S (green), IPB-F (red) and IPB-W (blue) with labels for individual samples as for Figure 5. Sheppard plot (right) showed a stress of 0.1935. Polygons for each IBP show distinct differences but also clear overlap in bacterial family composition between the three IPBs.

Figure 7. Energy input (consumption), output (production), and the net energy balance in the three IPB systems based on different wastewater algal inocula: S- Sherwood, F – Francisville, W-WWExp. Energy output consists of two parts, indirect electric energy from biomass conversion (the hatched part) and direct electric energy (non-hatched part). The values are means of the measurements (5 measurements for IPB-S, 3 measurements for IPB-F, 4 measurements for IPB-W).

Graphical Abstract