Comparative metagenomic analysis of electrogenic microbial communities in differentially inoculated swine wastewater-fed microbial fuel cells

Comparative metagenomic analysis of electrogenic microbial communities in differentially inoculated swine wastewater-fed microbial fuel cells

Irina V. Khilyas[1*], Anatoly A. Sorokin[3,4], Larisa Kiseleva[2], David J.W. Simpson[2], V. Fedorovich[2], Margarita R. Sharipova[1], Mami Kainuma[2], Michael F. Cohen[2,5], and Igor Goryanin[2,6,7]

[1]Institute of fundamental medicine and biology, Kazan (Volga Region) Federal University, Kazan, Russian Federation

[2]Biological Systems Unit, Okinawa Institute of Science and Technology, Okinawa, Japan

[3]Institute of Cell Biophysics Russian Academy of Science, Pushcino, Moscow region, Russian Federation

[4]Moscow Institute of Physics and Technology, Dolgoprudnyi, Moscow Region, Russian Federation

[5]Department of Biology, Sonoma State University, Rohnert Park, CA, USA

[6]School of Informatics, University of Edinburgh, Edinburgh, United Kingdom

[7] Tianjin Institute of Industrial Biotechnology, China

*Corresponding author: Tel.: +79372877886 *E-mail address*: irina.khilyas@gmail.com (I. Khilyas)

Key words: metagenomic analysis; microbial fuel cell; agricultural wastes; wastewater treatment; volatile fatty acid removal; bioreactors; microbial inoculum; pig slurry.

Abstract

Bio-electrochemical systems such as microbial fuel cells (MFCs) are promising new technologies for efficient removal of organic compounds from industrial wastewaters, including that generated from swine farming. We inoculated two pairs of laboratory-scale MFCs with sludge granules from a beer wastewater treating anaerobic digester (IGBS) or from sludge taken from the bottom of a tank receiving swine wastewater (SS). The SS-inoculated MFC outperformed the IGBS-inoculated MFC with regard to COD and VFA removal and electricity production. Using a metagenomic approach here we describe the microbial diversity of the MFCs planktonic and anodic communities derived from the different inocula. *Proteobacteria* (mostly *Deltaproteobacteria*) became the predominant phylum in both MFCs anodic communities with amplification the electrogenic genus *Geobacter* being the most pronounced. Eight dominant and three minor species of *Geobacter* were found in both anodic communities of the MFCs. The anodic communities of the SS-inoculated MFCs had a higher proportion of *Clostridium* and *Bacterioides* relative to those of the IGBS-inoculated MFCs, which were enriched with *Pelobacter*. The archaeal populations of the SS- and IGBS-inoculated MFCs were dominated by *Methanosarcina barkeri* and *Methanothermobacter thermautotrophicus*, respectively. Our results thus show a long-term influence of inoculum type on the performance and microbial community composition of swine wastewater-treating MFCs.

Introduction

Livestock farming constitutes an important agricultural sector of many countries but produces considerable amounts of organic wastes that require proper treatment and disposal. The rapidly growing pig farming industry generates high-strength wastewater containing organic compounds, ammonia, phosphates, odorous gases, suspended solids, and pathogens [1]. Treating swine wastewater is especially difficult where land is limited pig farming facilities occur in close proximity to population centers, such as in Okinawa, Japan. The lack of available land for application of swine wastewater

(SW) as a fertilizer and potential for contamination of surface and ground water sources underscores the need to employ thorough treatment of SW.

Common methods of treating SW include aerobic oxidation ponds, lagoons, anaerobic digestion and constructed wetlands [2]. Bio-electrochemical systems such as microbial fuel cells (MFCs) are promising new technologies for efficient removal of organic compounds in wastewaters. Inside the confined anaerobic chamber of an MFC a consortium of bacteria catalyze oxidation reactions, depositing electrons on the anode by a variety of means, including directly via outer membrane proteins or conductive pili or indirectly via secretion and recycling of redox-active molecules [3].

A primary target of SW treatment is a set of volatile fatty acids (VFAs) largely responsible for its noxious odor [4]. The presence of VFAs in an MFC substrate can increase the electrogenic performance of its anodic microbial biofilm [5]. Laboratory-scale single batch-loaded MFCs have been shown to dramatically lower malodorous compounds (primarily VFAs) as well as other constituents present in SW [4].

One important determinant of MFC reactor performance is the composition of the microbial community in the anodic chamber [6]. For obtaining maximal initial power production the anodic biofilm of an existing MFC has been shown to serve as a better inoculum than anaerobic sludge but we know of no study that assesses inoculum performance relative to pollutant removal criteria [7]. To this end, we sought to determine whether a microbial community already familiar with a SW substrate would perform better in an MFC than a distinct beer waste-digesting anaerobic sludge, assessing treatment performance and microbial community composition.

Previous studies have assessed microbial community composition in SW-fed MFCs utilizing denaturing gel gradient electrophoresis while a more recent study has utilized high-throughput amplicon sequencing to examine influences of external resistance and hydrodynamics on the MFC microbiome [8, 9]. Using a metagenomic approach here we describe the microbial diversity of the MFCs planktonic and anodic communities derived from the different inocula. Clustering of microbial

communities based on dominant bacterial genera indicates that the nature of the inoculum is an important influence on the ultimate composition of microbial communities and performance of MFCs.

Materials and Methods

MFC configuration and operation

The internal MFC chamber contained two anodes (approximately 6 x 8 cm), suspended 2-3 mm off the bottom of the chamber, composed of a layer of conductive carbon cloth to which 2 mm average size activated carbon granules were bound with conductive glue to provide more surface area. The granules had been prepared from birch precursor and were pre-treated with a neutral red catalyst. The two cathodes were graphite plates (3 mm thick; 60% porosity) sprayed on the liquid-facing side with an aqueous 5% Fumion membrane polymer (Fumatech, Bietigheim-Bissingen, Germany) while activated carbon granules [treated with iron(II) phthalocyanine] were mechanically pressed to the air-facing side using netting frame. The cathode extended into a bath containing an electrolyte solution (maintained at pH 3 with regular additions of 0.1N HCl).

The anode and cathode electrodes were connected with a multi-channel logger (Graphtech midi LOGGER GL820, Japan) for daily voltage measurements. The corresponding electric current was calculated using Ohm's law (V=IR). Power density was obtained according to $P = \frac{IV}{A}$, where I is the current, V is the voltage, and A is the projected surface area of the cathode. Polarization and power curves obtained by changing external resistances (from 0 Ω to 2100 Ω) in open circuit when the values had stabilized at each resistance indicated an internal resistance of 70 Ω within the MFCs (Fig S1).

For inoculation of the MFCs swine wastewater sludge (SS) containing suspended scrapings from the bottom of the SW holding tank was collected from a local pig farm (Okinawa Livestock and Grassland Centre, Nago, Japan) and industrial granular brewery sludge (IGBS) from a wastewater-treating UASB reactor (Orion Brewery, Nago, Japan). The inocula were not chemically modified or

diluted though the SS inoculum was filtered through a 1 mm stainless steel mesh.

To allow for microbial biofilm formation two MFCs were inoculated with SS and two with IGBS, heretofore referred to as SS-MFC and IGBS-MFC, and allowed to sit for 3 days in open-circuit mode at room temperature (24^{o}C). The MFCs were then provided undiluted SW in fed-batch mode to achieve a 24 h HRT. Regular feeding and monitoring of electrical performance began 13 d following inoculation of the MFCs, coincident with the switch to closed-circuit mode.

SW for use as MFC feed was stored at 4^{o}C. To remove large particles, the raw SW was sieved through a 0.50 mm mesh (Nylon monofilament). SW feed was diluted with distilled water to adjust the chemical oxygen demand (COD) to 3.5−7.4 g O_2 L^{-1} and the hydraulic retention time (HRT) set to 1 or 2 d over the course of the experiment. Wastewater was added into the MFCs semi-continuously using a peristaltic pump (Masterflex L/S Precision pump, Cole-Parmer, USA) set to a 6 ml min^{-1} flow rate. Operational parameters for the MFCs over the course of the 67-day experiment are summarized in Table S1.

Chemical analyses

Sampling of MFC inflow and outflow was performed every 24 h. COD, volatile fatty acids (VFA), ammonia nitrogen (NH_3-N), and total phosphorus (PO_4^{3-}-P) determinations were measured using the HACH TNTplus Chemistries (HACH Company, Loveland, CO). Total COD of inlet swine wastewater and MFC-treated effluent was measured without filtration. pH was measured with a pH meter (Horiba D-51, Japan).

Chromatography

Specific VFA compounds were quantified using an Agilent 7890A gas chromatograph connected to a LECO Pegasus 4D TOF mass spectrometer. Separation of VFA was performed using a Stabilwax-DA (30 m, 0.25 mm ID, 0.25 μm) column, using helium as carrier gas at 1.11 ml min^{-1} flow for the entire run. Method development was performed using Supelco WSFA-2 Mix to obtain retention index (RI) calibration and quantification calibration curve. Approximately 1 ml of sample was

transferred through a 0.22 µm filter to a glass autosampler vial. A 1:20 split liquid injection (1 µl volume) was injected, with the injection port set at 250ºC, 1 ml min^{-1} septum purge flow. The gradient temperature protocol was 2 min at 100ºC followed by an increase to 145ºC at a rate of 20ºC min^{-1}, holding at 145ºC for 6 min, followed by an increase to 205ºC at 20 ºC min^{-1} and holding this temperature for 4 min. The mass spectrometer was set with 35 to 145 Da mass scan range, 5 spectra sec^{-1} acquisition rate, and -70V electron energy. Ion source and transfer line temperature was 250ºC. Data processing (deconvolution, identification and quantification) was done using LECO ChromaTOF version 4.50.8 software. Acetic acid (99.99% purity), butyric acid (99.5%), 2-ethylbutyric acid (99%), hexanoic acid (99.5%), isovaleric acid (99%), isobutyric acid (99.5%), octanoic acid (99.5%), propionic acid (99.8%), sulfuric acid (99.9%), and valeric acid (99.8%) standards were purchased from Sigma-Aldrich, Japan.

Microbial diversity analysis

DNA was isolated from swine wastewater, inoculum sludges, anodic biofilms (carbon felt and carbon granules) and planktonic samples of each MFC using PowerMax soil DNA isolation kit (MO BIO laboratories, Inc). DNA quality was evaluated by the Agilent 2100 Bioanalyzer system. A DNA library was constructed for shotgun sequencing and a 150 paired-end sequencing reaction was performed on MiSeq platform (Illumina, San-Diego, CA, USA).

The sequencing data were uploaded to the MG-RAST server as FASTAQ files for processing, primary analysis and storage. *Sus scrofa* (pig) genome sequences were marked for exclusion during data submission. Primary submission data and results of the MG-RAST pipeline are available publicly (project mgp19536). The MG-RAST representative hit organism abundances calculation was performed against the SEED database at the level of genera, based on a maximum e-value of 1×10^{-5}, minimum identity cut-off of 60% and minimum sequence alignment of 15. Abundance data were downloaded as TSV files for further analysis. The representative hit data were downloaded from MG-RAST server via MGRASTer package [https://github.com/braithwaite/MGRASTer/] in R 3.1

environment. Abundance analysis was performed in metagenome Seq package [10] and ordination analysis was performed with phyloseq R packages [11]. Krona taxonomic community profiles were built by MG-RAST and stored as an image.

Results

We applied an integrated approach to investigating the effect of two distinct inoculums on performance of MFCs treated SW, comparing source- and site-dependent differences in the diversity of the microbial community, electricity production, and removal of organics.

MFC performance characteristics

The SS-MFC pairs outperformed the IGBS-MFCs pairs in regard to electricity generation (Fig S2) and removal of COD and VFA from the SW feed (Table 1; Fig S3) while operating on a 48 h HRT. Both MFC pairs displayed negligible removal phosphate (Table 1, Fig S4) whereas the SS-MFC performed better than the IGBS-MFC at removing ammonia (Table 1, Fig S5). Over the 67 d course of the experiment the SS-MFC COD removal rate of 2.65 ± 0.11 mg O_2 L^{-1} d^{-1} was slightly but significantly higher than the IGBS-MFC rate of 2.26 ± 0.17 mg O_2 L^{-1} d^{-1} (p = 0.02) while their respective VFA removal rates of 0.76 ± 0.06 mg L^{-1} d^{-1} and 0.66 ± 0.05 mg L^{-1} d^{-1} did not differ significantly (p = 0.27; means ± SE, n = 4). Electrical output of the MFC pairs remained relatively stable over the course of the experiment with the current density of the SS-MFCs (56.6 ± 2.4 mA m^{-2}) being consistently higher than that of the IGBS-MFCs (43.5 ± 6.2 mA m^{-2}) (means ± SD, n = 43; Fig S1).

TABLE 1. Treatment-related characteristics of swine wastewater-fed microbial fuel cells.

Source	COD (mg L^{-1})[a,b]	VFA (mg L^{-1})[a,b]	NH_4^+-N (mg L^{-1})[a,b]	PO_4^{3-}-P (mg L^{-1})[a,b]
SW inflow	6824	1452	365	374
SS-inoculated MFC	1684 (-75.3%)	222 (-84.8%)	286 (-21.6%)	365 (-2.4%)
IGBS-inoculated MFC	2219 (-67.5%)	314 (-78.4%)	327 (-10.4%)	370 (-1.1%)

[a]Results are means of measurements taken of two independently operating MFC for both MFC types, both operating with 100 Ω external resistance sampled at 67 days following initiation of operations.

[b]Percent change in parentheses.

Changes in the concentrations of straight-chain (acetic, propionic, butyric, valeric, hexanoic) and branched chain (isobutyric, isovaleric) VFAs were monitored (Table 2). Of these, propionic acid was found at the highest concentration in the SW. Passage through the SS-MFC removed >90% of all monitored VFAs, except for propionic acid, which was dissipated by 85.9%, and outperformed the removal rate of IGBS-MFC for all VFA tested. Predominance of propionic or acetic acids among VFAs in MFC-treated SW effluent has been previously shown [4, 12]. Several aromatic ring compounds (phenols and indoles) can contribute to the odor of SW [4], however we detected only two aromatic compounds (p-cresol and phenol) at negligible concentrations and indoles were not found (results not shown).

TABLE 2. Removal of selected volatile fatty acids by swine wastewater-fed microbial fuel cells.

	Concentration (mg L^{-1})*						
Source	**Acetate**	**Propionate**	**Isobutyrate**	**Butyrate**	**Isovalerate**	**Valerate**	**Hexanoate**
SW inflow	114.95	425.26	10.40	198.07	81.48	127.26	96.56
SS-inoculated MFC effluent	7.06 ±3.65	59.98 ±51.37	0.90 ±0.78	0.80 ±0.46	5.62 ±4.79	0.98 ±0.88	0.51 ±0.29
IGBS-inoculated MFC effluent	8.78 ±3.72	108.65 ±25.54	6.24 ±1.60	4.50 ±1.12	40.89 ±7.41	12.26 ±3.90	4.87 ±0.83

*± Range of variation between the two MFCs of each type.

Metagenomic analysis of microbial communities

Inocula

Over 98% and 91% of genes were affiliated with the domain *Bacteria*, and only 2% and 9% of genes were represented by *Archaea* for the SS and IGBS inoculums, respectively (Fig 1A-B). 30% *Gammaproteobacteria* (dominant genus *Enterobacteriaceae* (24%)) were the most abundant in SS

inoculum, whereas 23% *Gammaproteobacteria*, dominated by the genera *Aeromonadaceae* (9%) and *Enterobacteriaceae* (7%), were identified in IGBS inoculum (Fig 1A-B). *Deltaproteobacteria* were represented by the dominant genera *Desulfovibrionaceae* (0.8%) in SS inoculum and *Geobacteraceae* (3%) and *Syntrophaceae* (3%) in IGBS inoculum (Fig 1A-B).

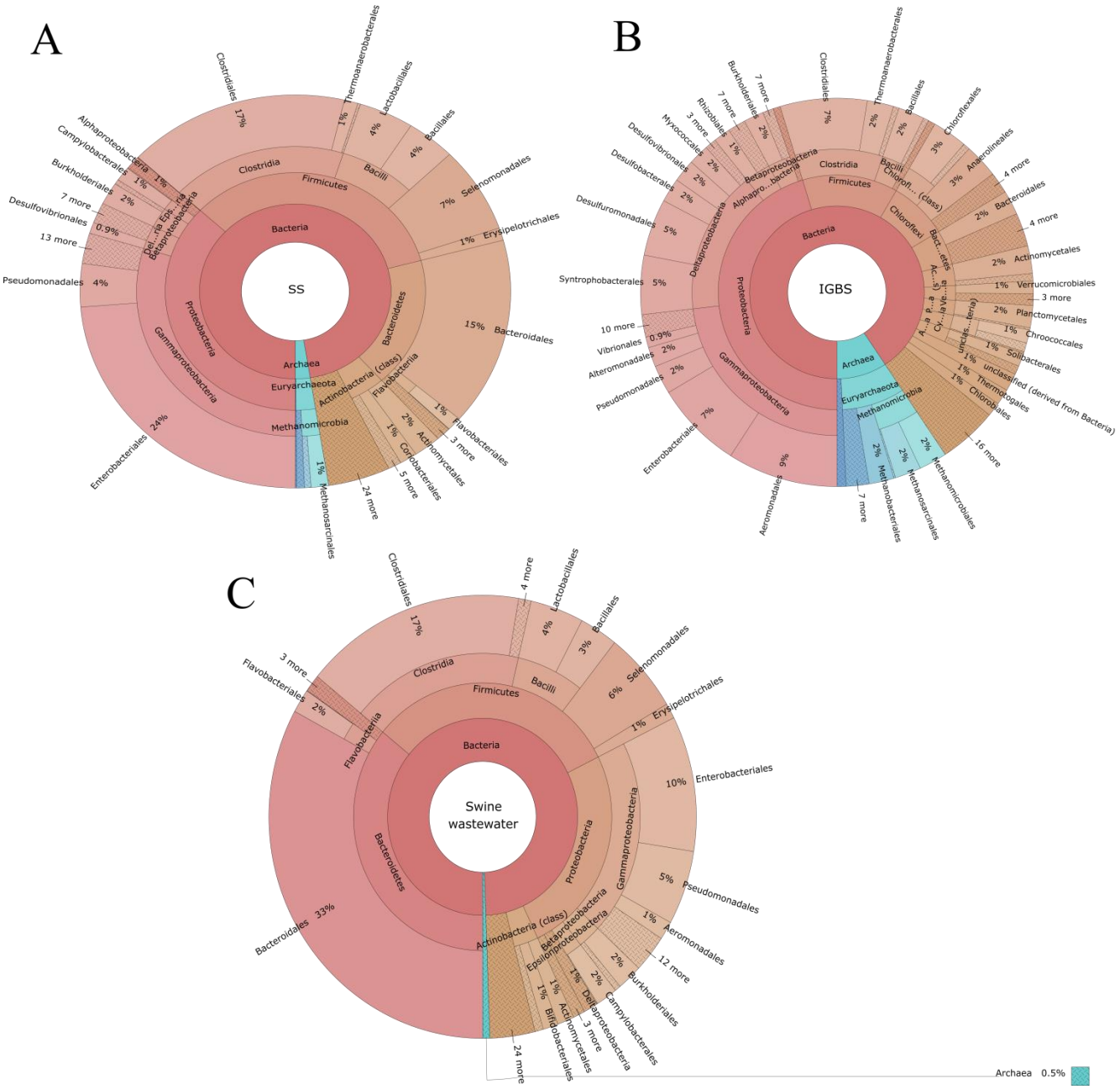

Figure 1. Summary of the microbial community profiles in the multilevel Krona diagrams. Krona plots visualizing taxonomic hierarchies of the microbial communities of (A) swine sludge (SS), (B) industrial granular brewery sludge (IGBS), and (C) swine wastewater.

The phylum *Firmicutes* (34% (SS) and 13% (IGBS)) was represented by *Clostridia* and *Bacilli* classes in both inoculums (Fig 1A-B). The most abundant members of phylum *Bacteroidetes* (18%) were identified as *Prevotellaceae* (8%) and *Bacteroidaceae* (5%) in SS inoculum (Fig 1A). Despite the low content of *Bacteroidetes* in IGBS inoculum (5%) the diversity of bacterial families was similar to that of the SS inoculum (Fig 1B). On the other hand, IGBS inoculum was enriched by *Chloroflexi* (8%), with dominant members *Anaerolineaceae* (3%) and *Chloroflexaceae* (3%) (Fig 1A-B). *Actinobacteria* (*Actinomycetales*) was found to be relatively abundant in both inoculums (Fig 1A-B). *Cyanobacteria* (*Chroococcales*) with abundance >0.5% were detected in SS inoculum, while 2% were identified in IGBS inoculum (Fig 1A-B).

Phylum *Archaea* was more abundant in the IGBS inoculum (9%) compared to the SS inoculum (2%) (Fig 1A-B). Analysis of two inoculums showed that *Euryarchaeota* (*Methanosarcinales* (1%) and *Crenarchaeota* (*Desulfurococcales*) were represented in both inoculums (Fig 1A-B). Thus, two types of inoculums were detailed analyzed aimed to investigation formation of the electrogenic microbial communities of MFCs with a highly effective treatment and degradative ability.

Swine wastewater

The microbial community analysis of SW showed that *Bacteroidetes* (36%), *Firmicutes* (32%), *Proteobacteria* (25%), *Actinobacteria* (3%) and 24 classes of *Bacteria* with relative abundance >1% were present (Fig 1C). The *Proteobacteria* were composed of *Gammaproteobacteria* (19%) (predominantly *Enterobacteriaceae* (10%)), *Epsilonproteobacteria* (2%) (with *Campylobacteraceae* (1%)), *Deltaproteobacteria* (1%) (with *Desulfovibrionaceae* (0.4%)) and *Alphaproteobacteria* (0.9%) (with *Rhizobiales* (0.4%)) (Fig 1C). Phylum *Firmicutes* and *Bacteroidetes* of the SW microbial community had a distribution of dominant members similar to the SS inoculum (Figs 1A-C). Archaeal communities representing 0.5% of the total detected bacteria were dominated by classes of methanogens *Methanomicrobia* and *Methanobacteria* (Fig 1C).

MFC anodic microbial communities

The microbial diversity of anodic communities was similar between the SS-MFCs and IGBS-MFCs at the level of genera (Fig 2). *Deltaproteobacteria* reached up 24% and 31% of the total microbial population on the anodes of SS-MFCs and IGBS-MFCs, respectively (Fig 2C-D). Among the *Deltaproteobacteria* class, *Geobacter* (14% and 20%) was identified as the most highly abundant genus on both MFC anodes.

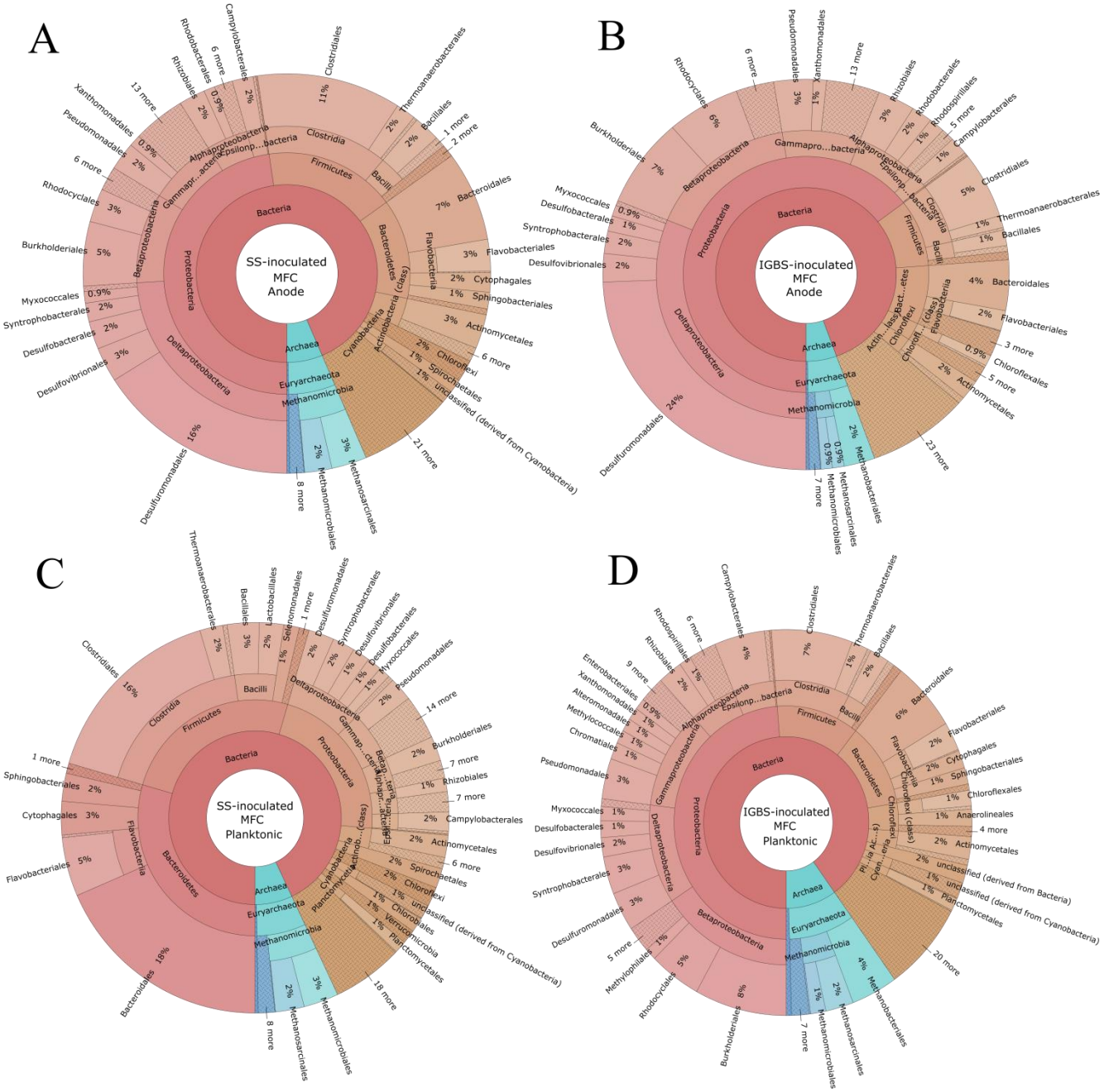

Figure 2. Summary of the anodic and planktonic microbial community profiles in multilevel Krona diagrams. Krona plots visualizing taxonomic hierarchies of the microbial communities of (A) swine sludge-inoculated MFC anode, (B) industrial granular brewery sludge-inoculated MFC anode, and (C) swine sludge-inoculated MFC planktonic contents, and (D) industrial granular brewery sludge-inoculated MFC planktonic contents.

The *Gammaproteobacteria*, common in both inocula (7% of SS and 8% of IGBS) and SW (30%), were substantially less represented on the anodes of both MFCs (Fig 2C-D). Particular declines in the *Enterobacteriaceae* ~~led~~ corresponded with higher relative levels of *Moraxellaceae* (*Acinetobacter*), *Pseudomonadaceae* (*Pseudomonas*) and *Xanthomonadaceae* (*Xanthomonas*) among the *Gammaproteobacteria* in the anodic community of SS-MFCs; and *Pseudomonadaceae* (*Pseudomonas*) and *Xanthomonadaceae* (*Xanthomonas*) in the anodic community of IGBS-MFCs (Fig 2C-D). Known electrogenic bacteria *Shewanella* (*Shewanellaceae*) were found on the anodes of both MFCs (Fig 2C-D).

Phylum *Firmicutes* (*Clostridia* and *Bacillus*) occupied only 13% and 9% of the total microbial population in SS-MFC and IGBS-MFC anodic communities, less compared to the inocula and SW (Fig 2C-D). Slight increases in the proportion of *Bacteroidetes* members (*Bacteroidales* and *Flavobacteriales*) (19%) were observed in the SS-MFC anodic communities compare to the inoculum (Fig 2C-D). Members of phylum *Chloroflexi* (*Roseiflexus* and *Anaerolinea*) were enriched in the population of anodic microbial community of SS-MFC, whereas that of the IGBS-MFC had less *Chloroflexi* (Fig 2). Slight enrichment of facultative heterotrophic *Cyanobacteria* genera (*Cyanothece*, *Synechococcus* and *Nostoc*) on anodes of both MFCs was detected (Fig 2C-D).

Populations of *Archaea* significantly increased only on the anode of SS-MFCs (6%) compare to SS inoculum (2%) and SW (0.5%) (Fig 2C-D). The most abundant genera of *Archaea* were *Methanosarcina* on anode of SS-MFCs and *Methanothermobacter* on anode of IGBS-MFCs (Fig 2C-D).

MFC planktonic microbial communities

Analysis of the SS-MFC planktonic community showed that phyla *Bacteroidetes* (30%), *Firmicutes* (25%), *Proteobacteria* (22%), *Actinobacteria* (3%) and *Archaea* (7%) were highly abundant (Fig 2A). The dominant *Gammaproteobacteria* in inoculum and SW shifted to the *Deltaproteobacteria* in the MFC planktonic microbial communities (Figs 1A-C and 2A). Members of phylum *Archaea* were enriched in the planktonic population similarly to population of MFC anodic surface (Fig 2A-B). The planktonic ~~community~~ communities of the IGBS-MFCs were similar to their anodic microbial ~~community~~ communities (Fig 2B-D). Dominant phyla *Proteobacteria* (49%), *Firmicutes* (12%), *Bacteroidetes* (12%), *Chloroflexi* (4%), *Archaea* (10%) and *Actinobacteria* (2%) were found in the planktonic community of IGBS- MFCs (Fig 2).

Similarity- and phylogeny-based MFC microbial community profiling

To determine the relationship between MFC anodic and planktonic microbial communities, swine wastewater and inocula a two-dimensional ordination plot based on taxonomy was created (Fig 3). Statistically significant dissimilarities were observed across the SW and anodic and planktonic communities of both MFCs.

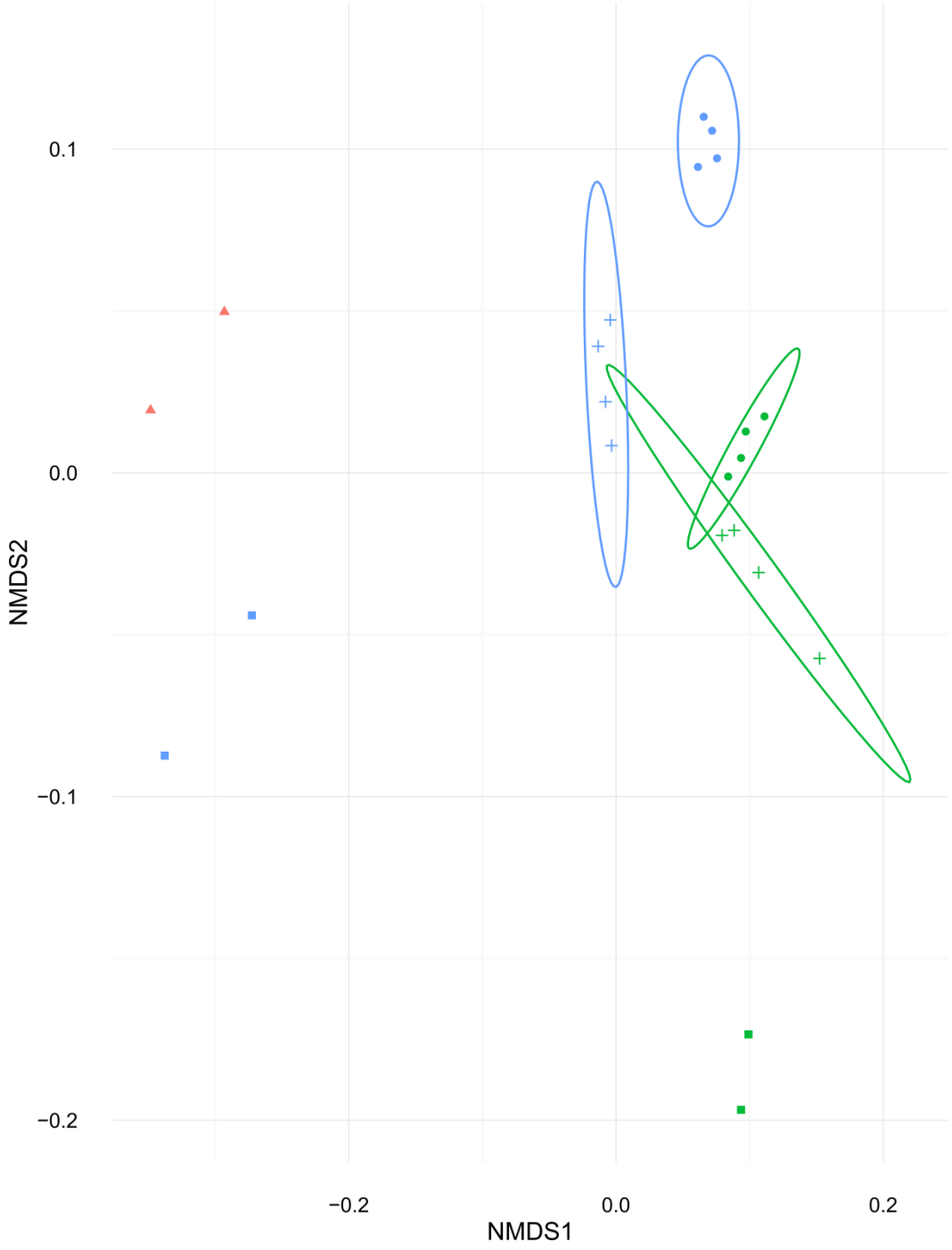

Figure 3. Ordination plots of a non-metric multidimensional scaling (NMDS) for the microbial communities from SW (inflow), SS- and IGBS-inoculated MFCs. Blue color indicates microbial communities derived from SS, green color indicates microbial communities derived from IGBS, red color indicates microbial communities derived from SW (circles, anodic microbial communities; crosses, planktonic microbial communities; squares, microbial communities of inoculums; triangles, SW microbial community). NMDS was based on Bray-Curtis distances of prokaryotic species abundance.

Each sample type can be seen to form a distinct cluster with the IGBS-MFC anodic and planktonic communities overlapping and the SS-MFC anodic and planktonic communities in close proximity. The SS and IGBS inoculum communities are well separated from each other. Thus, anodic and planktonic communities of IGBS-MFCs and SS-MFCs clustered close to one another, while SW samples did not (Fig 3).

A heat map of dominant bacterial genera based on a hierarchical clustering analysis was created to confirm the similarity and differences between the MFC anodic and planktonic microbial communities, swine wastewater and inocula (Fig 3). The planktonic MFC communities have a high similarity with their inoculum communities. These planktonic-inoculum clusters form a secondary cluster with each other. The MFC anodic communities form their own distinct cluster which contains *Geobacter* spp., a well-known genus of electrogenic bacteria. The SW community differed from all microbial communities and formed a separate cluster (Fig 3). Clustering of microbial communities based on dominant bacterial genera indicates that the electrogenic communities in the MFC developed from their inocula.

Diversity of dominant microbial species in MFC anodic microbial communities

Detailed analyses revealed five abundant genera of *Proteobacteria* enriched on the anodes of MFCs. The genus *Geobacter* was represented by eight predominant and three minor species in both MFC anodic communities (Fig 4). Highly abundant *Pelobacter* genus (*Pelobacter propionicus*, *Pelobacter carbinolicus*) were identified in both anodic communities of MFCs (Fig 4). The diversity observed within the genus *Desulfovibrio* was significant (Fig 4). *Gammaproteobacteria* were represented by six dominant bacterial genera in the anodic communities of the SS-MFCs and IGBS-MFCs. The *Acinetobacter* genus was represented by four abundant species in anodic microbial communities of both MFCs. Diversity of *Pseudomonas* members associated with MFCs anodes was tremendous. Among them *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* were the most

abundant species in the anodic biofilms of the IGBS-MFCs and SS-MFCs, respectively. The *Azotobacter* genus was dominated by *Azotobacter vinelandii* on anodes of both MFCs. Six abundant species of *Xanthomonas* genus (dominant *Xanthomonas campestris*) were identified in the anodic biofilms of both MFCs. Twenty different members of *Shewanella* genus were found in anodic biofilms of both MFCs and *Shewanella baltica* was the most abundant specie among them. One member of *Methylobacter* genus (*Methylobacter tundripaludum*) was enriched on anodes of both MFCs (Fig 4).

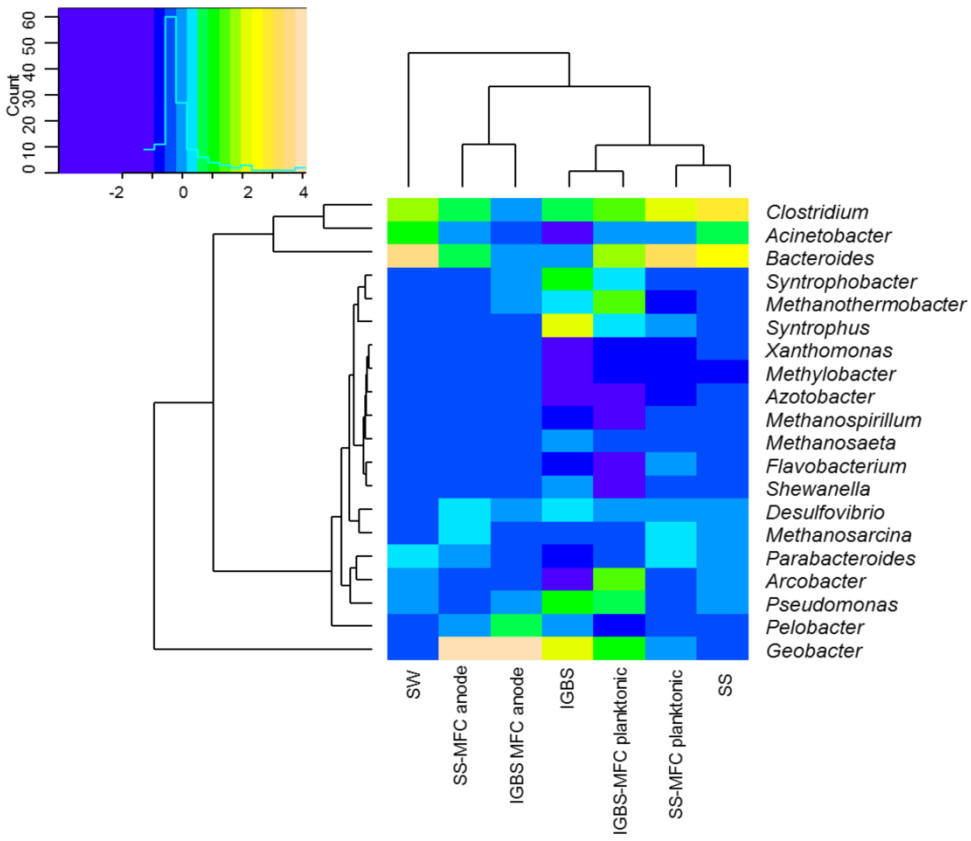

Figure 4. Heatmap diagram visualize the dominant bacterial and archaeal genera in the microbial community profiles. Bottom represents the different samples.

Among *Firmicutes*, over 50 species of the genus *Clostridium* were identified from the MFC anodes, including 31 abundant species (dominant *Clostridium thermocellum*) and 9 relatively abundant species. *Flavobacterium johnsoniae* and *Flavobacterium psychrophilum*, *Bacteroides fragilis* and *Parabacteroides distasonis* were the three dominant bacterial species among *Bacteroidetes* in the anodic communities of both MFCs. Acetoclastic methanogens (*Methanosarcina barkeri* and

322 *Methanothermobacter thermautotrophicus*) belonging to the domain of *Archaea* were identified in
323 MFC anodic and planktonic populations (Fig 4).

324 Discussion

325 This study demonstrated the compositions and phylogenetic distributions of SW, inocula, anodic
326 and planktonic microbial communities in SS- and IGBS-inoculated MFCs. The results showed
327 insignificant differences in bacterial richness and diversity between microbial communities both
328 MFCs, while SW differed significantly.

329 MFCs treatment efficiency of SW

330 Treatment of SW using MFCs inoculated with two different inoculums achieved substantial
331 COD removal rates. A previous study found that a single-chambered MFC with a working volume 28
332 mL removed only 27% of the COD in SW having a high initial COD of 8,320 mg L^{-1} after 44 h (Min
333 et al., 2005) whereas we found 76.4% and 65.7% removal of COD from SW by the SS-MFCs and
334 IGBS-MFCs, respectively, after 48 h (data not shown). The average current density of the MFCs (Fig
335 1, Table 1) were within the range reported for other wastewater-fed MFC systems [5]. Consistent with
336 other reports [13, 14], differences in external resistance within the range we tested (10 -1000 Ω) did
337 not notably alter the performance of the MFCs (data not shown).

338 Swine wastewater is characterized by high content of VFAs although their initial concentration
339 in raw swine wastewater across different farms can vary substantially. Our results are consistent with
340 others demonstrating that MFC treatment of SW largely eliminates VFAs, which are largely
341 responsible for the SW odor [4]. Importantly, the SW feed in our experiments was approximately 5-
342 fold higher strength and the HRT less than five times that utilized by Kim et al. [4] and yet the MFCs
343 still performed well at removing the VFAs.

In summary, use of SS as an anodic inoculum resulted in superior treatment performance of the MFCs over the 67 d course of the experiment compared to IGBS inoculum. This may indicate a more general tendency of pre-adapted inocula to perform better at degrading the substrate [15].

The microbiome of electrogenic of anodic biofilms and planktonic populations of MFCs

We used metagenomic analysis to explore the whole taxonomic diversity of the SS- and IGBS inoculums, SW, anodic and planktonic microbial communities of MFCs. *Proteobacteria* (mostly *Deltaproteobacteria*) became the predominant phylum in both MFCs anodic communities, while *Firmicutes* and *Bacteroidetes* decreased. The planktonic community of the IGBS-MFCs showed notable variation in relative abundance and became more similar to their anodic communities. In contrast, the planktonic communities of the SS-MFCs were intermediate between the SW and anodic communities. A previous study of a distillery wastewater-treating pilot-scale MFC inoculated with IGBS showed that the dominant anodic phyla (*Proteobacteria, Bacteroidetes* and *Firmicutes*) were similar to that of the IGBS inoculum [16].

Previous studies have shown that SW could be used as a suitable inoculum for electricity production using MFCs, distinguished by the chamber and cathode types [4, 17]. Analysis of the anodic microbial communities in the SS-MFCs mainly showed that dominant species belonged to three major phyla *Proteobacteria, Bacteroidetes* and *Firmicutes* [9, 12]. Results of metagenomics analysis in our study are in good agreement with results in the literature [9, 12].

Detailed analysis of the dominant anodic bacterial species in SW-treating MFCs showed high diversity in members of the *Deltaproteobacteria*, *Gammaproteobacteria*, *Firmicutes, Bacteroides* and *Archaea.* Among all *Deltaproteobacteria, Geobacter metallireducens, Pelobacter propionicus, Desulfovibrio vulgaris, Syntrophobacter fumaroxidance* and *Syntrophus aciditrophicus* were found to be the most abundant in the anodic microbial communities of both MFCs. The well-known electrogenic *Geobacter sulfurreducens,* dominant in the MFC microbial biofilms, generates a current via membrane c-type cytochromes (*omcZ)* and secretion of pili encoded by the *pilA* gene [3, 18, 19]. In

contrast, anoditrophilic Fe(III)-reducing *Pelobacter carbinolicus* was characterized as a non-electrogenic symbiotic bacterium responsible only for converting of substrates to acetate and hydrogen for use by *G. sulfurreducens* [3, 20]. Cytochrome *c* localized on the outer cell membrane of *Desulfovibrio desulfuricans* contributed to the electron transfer in an electricity-generating MFC [21].

Members of *Deltaproteobacteria* might contribute to VFA degradation. Our data demonstrate a relative abundance of *Syntrophobacter fumaroxidance* and *Syntrophus aciditrophicus* on the MFC anodes, which may aid in metabolism of propionic and butyric acid in the SW. Pure culture experiments with *Geobacter* species isolated from swine wastes were examined the ability to biodegrade individual and mixtures of VFAs [22]. It was shown that *G. metallireducens, G. humireducens* and *G. grbiciae* consume VFAs and stimulate VFAs oxidation depending on availability of Fe (III).

This study demonstrates that *Acinetobacter baumannii* and *Pseudomonas fluorescens* belonging to *Gammaproteobacteria* were prevalent members in the anodic community both MFCs. The *Gammaproteobacteria* possess diverse metabolic capabilities involved in a breakdown of different substrates and production of soluble redox active compounds, resulting in current generation in MFCs [3, 23, 24]. *Acinetobacter* species dominating in the microbial community of MFCs fed with fermentable substrates were able to produce electricity [25]. Production of *pili*-like structures encoded by *csuC* and *csuE* genes in *A. baumannii* influences the colonization of different abiotic surfaces [26]. We found a considerable number of *Pseudomonas* species in both MFCs types. The ability of *Pseudomonas* to consume various carbon sources is known. Moreover excretion of soluble electrochemically redox mediators participating in the electricity production in MFCs has been observed [23, 27]. Thus, dominant *Pseudomonas fluorescens* might be responsible for COD removal from the SW and the excretion of redox mediators contribute to the observed electricity generation of the MFCs.

The relatively high abundance of *Shewanella baltica* in the anodic microbial communities provides evidence of their importance in the conversion of COD into electricity. Previous studies have

showed that electrogenic *Shewanella* species might transfer electrons to the anodes of MFCs either through nanowires or excretion of redox active second metabolites [28, 29].

Our study demonstrates a relative abundance of bacteria related phyla *Firmicutes* and *Bacteroidetes*. It is well known that *Clostridium* species participate in fermentation processes and conversion of organic substrates to VFAs and hydrogen and that they are indigenous microbiota of the swine gastrointestinal tract and manure [12]. *Bacteroidetes* are widely recognized as the intestinal microflora associated with fermentation of carbohydrates and utilization of nitrogenous compounds, as well odor production [30]. We found that the remaining dominant bacteria, *Flavobacterium johnsoniae* and *Bacteroides fragilis* became even more abundant in the planktonic populations of both MFCs.

16S rRNA sequence analysis of a SW-treating MFC microbial community showed that two members of *Firmicutes,* a gram-positive *Turicibacter* sp. and *Sedimentibacter* spp., were the dominant genera on the anodes of a MFC having a maximum power point tracking system [9]. Earlier studies demonstrated reduction of VFAs level depending on a seasonal shift of *Bacteroidetes* members in an anaerobic lagoon used for swine waste treatment [12].

In our study, two archaea species *M. barkeri* and *M. thermautotrophicus* increased in the bacterial communities of SS-MFCs and IGBS-MFCs, respectively. Rotaru et al. established that the acetoclastic methanogen *M. barkeri* in association with electrogenic bacterium *G. metallireducens* participates in direct interspecies electron transfer (DIET) [31]. We found a potential for a DIET-type bacterial association between *M. barkeri* and *G. metallireducens* in the anodic microbial community of the SS-MFCs; possible association between *M. thermautotrophicus* and *G. metallireducens* was found in the anodic microbial community of IGBS-MFCs.

Taken together, the profiling of microbial community diversity based on similarity and phylogeny supports a model for development of electrogenic biofilm in MFCs from their inocula.

Conclusion

This research demonstrates the importance inoculum source on the electrogenic and degradative activities and ultimate microbial community composition of SW-treating MFCs. MFC treatment of SW is a potentially more environmentally friendly alternative to energetically costly aerobic treatment or odorous space-demanding anaerobic lagoons. Our comprehensive analysis of SS- and IGBS-inoculated MFCs treating SW revealed stable electricity production by both types of MFCs with the SS-MFC showing the highest current density. Both MFC pairs displayed moderate removal of ammonia, did not remove phosphate, and were highly efficient at removing COD and odiferous VFAs. Analysis of microbial communities of both MFCs showed that MFC anodic communities form their own distinct cluster which contains *Geobacter* spp., represented by eight predominant and three minor species, in both MFC anodic communities. Clustering of microbial communities based on dominant bacterial genera indicates that the electrogenic communities in the MFCs developed from their inocula. The MFC environment enriched for a spectrum of bacterial genera, including *Pelobacter*, *Pseudomonas*, *Arcobacter*, *Syntrophus*, *Syntrophobacter*, *Bacteroides*, *Clostridium* as well as two acetoclastic methanogens (*Methanosarcina* and *Methanothermobacter*).

Acknowledgements

Funding was provided by the Okinawa Institute of Science and Technology. Dr. I.V. Khilyas was supported by Russian Government Program of Competitive Growth of Kazan Federal University and RFBR grant No 16-34-60200. The authors are grateful to Dr. Alejandro Villar-Briones (Biology Resources Section, Research Support Division Okinawa Institute of Science and Technology Graduate University) for carrying out GC analysis of VFAs, the OIST DNA Sequencing Section for sequencing support, Dr. Sasaki Toshio for performing SEM, and the Okinawa Prefecture Livestock and Grassland Research Center (OPLGRC), Nakijin for providing swine wastewater.

Conflict of Interest

The authors declare no conflict of interest.

References

1. M. B. Vanotti, P. D. Millner, P. G. Hunt, and A. Q. Ellison, "Removal of pathogen and indicator microorganisms from liquid swine manure in multi-step biological and chemical treatment," *Bioresource Technology,* vol. 96, pp. 209-14, 2005.
2. F. Humenik, J. Rice, C. Baird, and R. Koelsch, "Environmentally superior technologies for swine waste management," *Water Science and Technology,* vol. 49, pp. 15-22, 2004.
3. D. R. Lovley, "Electromicrobiology," *Annual Review of Microbiology,* vol. 66, pp. 391-409, 2012.
4. J. R. Kim, J. Dec, M. A. Bruns, and B. E. Logan, "Removal of odors from swine wastewater by using microbial fuel cells," *Applied and Environmental Microbiology,* vol. 74, pp. 2540-3, 2008.
5. G. Sun, A. Thygesen, M. T. Ale, M. Mensah, F. W. Poulsen, and A. S. Meyer, "The significance of the initiation process parameters and reactor design for maximizing the efficiency of microbial fuel cells," *Applied Microbiology and Biotechnology,* vol. 98, pp. 2415-27, 2014.
6. B. E. Logan, "Exoelectrogenic bacteria that power microbial fuel cells," *Nature Reviews Microbiology,* vol. 7, pp. 375-81, 2009.
7. J. R. Kim, B. Min, and B. E. Logan, "Evaluation of procedures to acclimate a microbial fuel cell for electricity production," *Applied Microbiology and Biotechnology,* vol. 68, pp. 23-30, 2005.
8. J. Ma, H. Ni, D. Su, and X. Meng, "Bioelectricity generation from pig farm wastewater in microbial fuel cell using carbon brush as electrode," *International Journal of Hydrogen Energy,* vol. 41, pp. 16191-16195, 2016.
9. A. Vilajeliu-Pons, S. Puig, N. Pous, I. Salcedo-Davila, L. Baneras, M. D. Balaguer, and J. Colprim, "Microbiome characterization of MFCs used for the treatment of swine manure," *Journal of Hazardous Materials,* vol. 288, pp. 60-8, 2015.
10. J. N. Paulson, O. C. Stine, H. C. Bravo, and M. Pop, "Differential abundance analysis for microbial marker-gene surveys," *Nature Methods,* vol. 10, pp. 1200-1202, 12//print 2013.
11. P. J. McMurdie and S. Holmes, "phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data," *PLoS One,* vol. 8, p. e61217, 2013.
12. K. L. Cook, M. J. Rothrock, Jr., N. Lovanh, J. K. Sorrell, and J. H. Loughrin, "Spatial and temporal changes in the microbial community in an anaerobic swine waste treatment lagoon," *Anaerobe,* vol. 16, pp. 74-82, 2010.
13. K. P. Katuri, K. Scott, I. M. Head, C. Picioreanu, and T. P. Curtis, "Microbial fuel cells meet with external resistance," *Bioresource Technology,* vol. 102, pp. 2758-66, 2011.
14. D. Y. Lyon, F. Buret, T. M. Vogel, and J. M. Monier, "Is resistance futile? Changing external resistance does not improve microbial fuel cell performance," *Bioelectrochemistry,* vol. 78, pp. 2-7, 2010.
15. A. S. Mathuriya, "Inoculum selection to enhance performance of a microbial fuel cell for electricity generation during wastewater treatment," *Environmental Technology,* vol. 34, pp. 1957-64, 2013.
16. L. Kiseleva, S. K. Garushyants, H. Ma, D. J. Simpson, V. Fedorovich, M. F. Cohen, and I. Goryanin, "Taxonomic and functional metagenomic analysis of anodic communities in two pilot-scale microbial fuel cells treating different industrial wastewaters," *Journal of Integrative Bioinformatics,* vol. 12, p. 273, 2015.
17. B. Min, J. Kim, S. Oh, J. M. Regan, and B. E. Logan, "Electricity generation from swine wastewater using microbial fuel cells," *Water Research,* vol. 39, pp. 4961-8, 2005.

18. K. P. Nevin, B. C. Kim, R. H. Glaven, J. P. Johnson, T. L. Woodard, B. A. Methe, R. J. Didonato, S. F. Covalla, A. E. Franks, A. Liu, and D. R. Lovley, "Anode biofilm transcriptomics reveals outer surface components essential for high density current production in *Geobacter sulfurreducens* fuel cells," *PLoS One,* vol. 4, p. e5628, 2009.
19. L. V. Richter, S. J. Sandler, and R. M. Weis, "Two isoforms of *Geobacter sulfurreducens* PilA have distinct roles in pilus biogenesis, cytochrome localization, extracellular electron transfer, and biofilm formation," *Journal of Bacteriology,* vol. 194, pp. 2551-63, 2012.
20. H. Richter, M. Lanthier, K. P. Nevin, and D. R. Lovley, "Lack of electricity production by *Pelobacter carbinolicus* indicates that the capacity for Fe(III) oxide reduction does not necessarily confer electron transfer ability to fuel cell anodes," *Applied and Environmental Microbiology,* vol. 73, pp. 5347-53, 2007.
21. C. S. Kang, N. Eaktasang, D. Y. Kwon, and H. S. Kim, "Enhanced current production by *Desulfovibrio desulfuricans* biofilm in a mediator-less microbial fuel cell," *Bioresource Technology,* vol. 165, pp. 27-30, 2014.
22. J. D. Coates, K. A. Cole, U. Michaelidou, J. Patrick, M. J. McInerney, and L. A. Achenbach, "Biological control of hog waste odor through stimulated microbial Fe(III) reduction," *Applied and Environmental Microbiology,* vol. 71, pp. 4728-35, 2005.
23. K. Rabaey, N. Boon, S. D. Siciliano, M. Verhaege, and W. Verstraete, "Biofuel cells select for microbial consortia that self-mediate electron transfer," *Applied and Environmental Microbiology,* vol. 70, pp. 5373-82, 2004.
24. S. Ishii, S. Suzuki, T. M. Norden-Krichmar, T. Phan, G. Wanger, K. H. Nealson, Y. Sekiguchi, Y. A. Gorby, and O. Bretschger, "Microbial population and functional dynamics associated with surface potential and carbon metabolism," *ISME Journal,* vol. 8, pp. 963-78, 2014.
25. J. Yu, Y. Park, H. Cho, J. Chun, J. Seon, S. Cho, and T. Lee, "Variations of electron flux and microbial community in air-cathode microbial fuel cells fed with different substrates," *Water Science and Technology,* vol. 66, pp. 748-53, 2012.
26. A. P. Tomaras, C. W. Dorsey, R. E. Edelmann, and L. A. Actis, "Attachment to and biofilm formation on abiotic surfaces by *Acinetobacter baumannii*: involvement of a novel chaperone-usher pili assembly system," *Microbiology,* vol. 149, pp. 3473-84, 2003.
27. T. H. Pham, N. Boon, P. Aelterman, P. Clauwaert, L. De Schamphelaire, L. Vanhaecke, K. De Maeyer, M. Hofte, W. Verstraete, and K. Rabaey, "Metabolites produced by *Pseudomonas* sp. enable a Gram-positive bacterium to achieve extracellular electron transfer," *Applied Microbiology and Biotechnology,* vol. 77, pp. 1119-29, 2008.
28. L. T. Angenent, K. Karim, M. H. Al-Dahhan, B. A. Wrenn, and R. Domiguez-Espinosa, "Production of bioenergy and biochemicals from industrial and agricultural wastewater," *Trends in Biotechnology,* vol. 22, pp. 477-85, 2004.
29. Y. A. Gorby, S. Yanina, J. S. McLean, K. M. Rosso, D. Moyles, A. Dohnalkova, T. J. Beveridge, I. S. Chang, B. H. Kim, and K. S. Kim, "Electrically conductive bacterial nanowires produced by *Shewanella oneidensis* strain MR-1 and other microorganisms," *Proceedings of the National Academy of Sciences,* vol. 103, pp. 11358-11363, 2006.
30. F. De Filippis, N. Pellegrini, L. Laghi, M. Gobbetti, and D. Ercolini, "Unusual sub-genus associations of faecal *Prevotella* and *Bacteroides* with specific dietary patterns," *Microbiome,* vol. 4, p. 57, 2016.
31. A. E. Rotaru, P. M. Shrestha, F. Liu, B. Markovaite, S. Chen, K. P. Nevin, and D. R. Lovley, "Direct interspecies electron transfer between *Geobacter metallireducens* and *Methanosarcina barkeri*," *Applied and Environmental Microbiology,* vol. 80, pp. 4599-605, 2014.

542

Supporting Information

Fig. S1. Cell voltage and power density vs. current density (cell polarization) of MFCs (A) inoculated with swine wastes; (B) inoculated with brewery sludge. Open circles, voltage; closed circles, power density.

Fig. S2. Current generation during swine wastewater treatment by MFCs inoculated with swine waste sludge and brewery sludge. Mean data from duplicate experiments; error bars indicating ±SD to not exceed the diameter of the data point symbols. Open boxes, SS-inoculated MFC; open diamonds, IGBS-inoculated MFC.

Fig. S3. Total COD concentrations in SW feed and within MFCs inoculated with swine waste sludge and industrial granular brewery sludge. Squares, SS-inoculated MFC; circles, IGBS-inoculated MFC; Mean data from duplicate experiments.

Fig. S4. Change in total phosphorus (PO_4^{3-} -P) in inflow and outflows of MFCs inoculated with swine waste sludge and industrial granular brewery sludge. Mean data from duplicate experiments with error bars (±SD).

Fig. S5. Changes in ammonia (NH_4-N) in inflow and outflows of MFCs inoculated with swine wastes sludge and brewery industrial granular sludge. Mean data from duplicate experiments with error bars (±SD).

Fig. S6. SEM images of the anodic biofilms the MFCs inoculated with (A) swine waste and (B) brewery sludge. Samples of anode surfaces (activated carbon granules and fiber) the MFCs were taken after 67 days of swine wastewater treatment upon disassembling the MFCs. Slices of anode electrodes (1 cm^2) were briefly rinsed with deionized water and fixed in 2.5% glutaraldehyde for 2 h, further in 1% osmium tetroxide. Dehydration of microbial biofilms was carried out using a series of ethanol–water solutions (25, 50, 75, 95, 100%). After gold coating, the obtained specimens were observed using a Focused Ion Beam Scanning electron microscope (Helios NanoLab 650, USA). High

resolution images were acquired using an accelerating voltage of 20 kV at a working distance of 3.1–6.5 mm.

Table S1. Summary of MFC operation modes.

Table S2. Concentration of VFAs in the SS-inoculated MFC.

Table S3. Concentration of VFAs in the IGBS-inoculated MFC.

Table S4. Diversity of dominated species in the SW, inocula, anodic and planktonic microbial communities of SS- and IGBS-inoculated MFCs.