# Temperature, inocula and substrate: Contrasting electroactive consortia, diversity and performance in microbial fuel cells

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ABSTRACT

The factors that affect microbial community assembly and its effects on the performance of bioelectrochemical systems are poorly understood. Sixteen microbial fuel cell (MFC) reactors were set up to test the importance of inoculum, temperature and substrate: Arctic soil versus wastewater as inoculum; warm (26.5 °C) versus cold (7.5 °C) temperature; and acetate versus wastewater as substrate. Substrate was the dominant factor in determining performance and diversity: unexpectedly the simple electrogenic substrate delivered a higher diversity than a complex wastewater. Furthermore, in acetate fed reactors, diversity did not correlate with performance, yet in wastewater fed ones it did, with greater diversity sustaining higher power densities and coulombic efficiencies. Temperature had only a minor effect on power density, ($Q_{10}$: 2 and 1.2 for acetate and wastewater respectively): this is surprising given the well-known temperature sensitivity of anaerobic bioreactors. Reactors were able to operate at low temperature with real wastewater without the need for specialised inocula; it is speculated that MFC biofilms may have a self-heating effect. Importantly, the warm acetate fed reactors in this study did not act as direct model for cold wastewater fed systems. Application of this technology will encompass use of real wastewater at ambient temperatures.

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## 1. Introduction

Bioelectrochemical Systems (BESs) are a suite of technologies that exploit the ability of an anaerobic microbial community to donate electrons from organic matter to a solid anode in an electrical circuit, treating wastewater and generating a current. The formation of a productive anode community able to digest waste organics and liberate electrons is required for successful application. Although community assembly is poorly understood in the microbial world in general and in BES in particular [1] it might be reasonable to assume that it is affected by both the temperature, substrate and inocula [2,3]. These have been studied independently within BES research, but not consolidated within one study where direct comparisons can be made.

Understanding the effects of temperature and feed will be vital for the implementation of this technology. Although most of the putative applications of BES anticipate using a feed composed of complex waste organic matter at ambient temperatures [4], many studies use an acetate feed at controlled and elevated temperatures (typically ~30 °C). These simplified conditions cannot be an accurate reflection of the real life scenarios BES technologies will have to work in to be commercially viable.

Differences in reactor configurations and reporting methods make direct comparison of the performance of wastewater and acetate feeds in different studies difficult, yet wastewater fed reactors typically produce lower power densities (PD) and Coulombic Efficiencies (CE) than those fed with simple substrates [5,6]. In identical reactors, synthetic complex wastewater produced half the power density of acetate [7]. Furthermore, different substrates support very different anodic microbial communities [8,9]. Understanding these differences, and the relative contributions of the hydrolytic, fermentative and electrogenic metabolic stages will be essential in reducing this performance gap.

Studies on the effect of temperature on acetate fed reactors offer insights into electrogenesis only; they have typically found that the maximum power output drops at lower temperatures [10–15]. Cheng et al. [12] and Lu et al. [14] found convincing linear relationships ($R^2 > 0.99$) between temperature (from 4 to 30 °C) and performance: about 33 mW/m$^2$/°C and 4 A/m$^3$/°C implying $Q_{10}$ temperature coefficient values for electrogenesis of 1.5 and 2.0 for power and current respectively. The $Q_{10}$ value is a measure of the rate of change of a biological or chemical system as a consequence of increasing the temperature by 10 °C.

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Studies with more complex wastewaters suggest a degree of temperature sensitivity that is slightly lower than that for electrogenesis. Larrosa-Guerrero et al. [16] using a mixture of domestic and brewery wastewater found an unequivocally linear relationship between temperature and current density and a $Q_{10}$ for power density of 1.6 Ahn and Logan, [17] using domestic wastewater and Wang et al. [18] using brewery wastewater, found a very modest change in performance with temperature, observing $Q_{10}$ values of 1.2 and 1.1 respectively with a change from 30 to ~20 °C. The results of Larrosa-Guerro et al. (2010) are more pertinent as wastewater temperatures in temperate climates vary between 4 and 20 °C. Using domestic wastewater at ambient temperature in a pilot scale reactor with high overpotentials and losses, Heidrich et al. observed that temperature did not have a statistically significant effect [19]. Interestingly, this makes BES compare favourably with analogous studies in methanogenic systems where $Q_{10}$ values range from 2 to 4 [20] and temperature sensitivity can be problematic.

The presence of effective electrogens in wastewater is low, in the order of 17 cells per ml [21]. An appropriate choice of inoculum might influence performance in general, and temperature sensitivity in particular. Thus, although wastewater itself is known to be a satisfactory source of inoculum, soils, especially arctic soils, might be superior for systems that are expected to function at low temperatures: soils are inherently more diverse than wastewater [22], and display significant anaerobic activity at low temperatures [23,24]. *Geobacter* species are usually the dominant electrogens in acetate fed reactors [25]. However, in wastewater fed reactors *Geobacter* are typically in a minority, though some of the remaining community may be undiscovered electrogens, non-electrogenic hydrolytic and fermentative organisms must also be present and may be limiting [26,27].

Rather than simply looking at the presence of a single taxon such as *Geobacter*, wider ecological parameters, such as diversity, may prove more useful in understanding the link between performance in BES and community structure. It is hypothesised that higher anode potentials provide the bacteria with more energy and therefore lead to a greater diversity, while low potentials allow only the most capable electrogen, i.e. *Geobacter* [28], to colonise the anode. This would, somewhat counterintuitively, result in lower diversity where there is higher current densities at low electrode potentials. Using 16S rDNA clone library construction and sequencing Torres et al., [28] have observed higher diversity when higher anode potentials were imposed. Conversely Zhu et al. [29] have observed that diversity was not related to imposed anode potentials when measured using 454 pyrosequencing. Both studies used a simple single substrate, acetate, and their conclusions focus on the dominance of Geobacter. Measures of diversity within BES anode samples using DGGE analysis on glucose fed reactors have been shown to correlate positively with power production, with greater power coming from more diverse biofilms [30]. The idea that a wider range of taxa can lead to a more efficient consumption of resource is known as over-yielding and this phenomenon could conceivably affect the performance of BES [31]. The diversity of the biofilm in a BES may be critical to its performance, yet we do not have a conclusive link between the two.

The unprecedented number of sequences analysed per sample by Next Generation Sequencing offers the opportunity to detect some of the less dominant species in the community. However, these techniques still only offer a partial view of diversity in a microbial system, and the diversity observed is still typically a function of the number of sequences analysed [32]. These difficulties can be overcome using Bayesian techniques that infer the underlying species abundance curves which in turn can be used to estimate the total diversity of the sample [33,34]. These techniques have the advantage of using a minimum information criteria that allows for the selection of the best fitting model representing the estimated species abundance curves, whilst also providing confidence intervals for these estimates. This approach removes the effect of sampling depth on the diversity value so that

rational and informed comparisons can be made between samples. Such techniques are still not widely appreciated even within microbial ecology and have never been applied to BES.

This study presents a simple yet rigorous experiment to explore the link between microbial community diversity and BES performance, and how the choice of inoculum, temperature and substrate may affect this. The study focuses on one specific type of BES, the Microbial Fuel Cell (MFC) [1,4–5]. There were two explicit hypotheses: firstly, the use of an arctic soil as an inoculum would enhance the low temperature performance of an MFC relative to one started with a conventional inoculum. Secondly, the communities in the acetate fed anode compartments of these BESs would be a largely electrogenic subset of those found in the wastewater fed reactor (thus the "excess" diversity found in the wastewater fed reactors would be putative hydrolytic and fermentative organisms). All reactor conditions were run in duplicate. The impact of temperature, inoculum and substrate on the microbial communities and total diversity within these reactors was examined by 454 pyrosequencing.

## 2. Materials and methods

### 2.1. Experimental design

The variables examined were: temperature (warm 26.5 °C and cold 7.5 °C); substrate (acetate and wastewater); and inocula (Arctic soil and wastewater). Duplicate reactors were run in parallel to test each of these eight conditions. Once acclimated, the reactors were used to perform three feeding cycles and the performance data was monitored. At the end of these cycles biofilm samples were taken for microbial analysis.

The warm temperature was chosen to represent the typical laboratory temperatures of many MFC studies. The cold temperature represents the lower quartile of the temperatures experienced at a pilot MEC reactor run at ambient temperatures on a wastewater treatment site in the North of England (54°58′N, 01°36′W) [19]. The substrates were acetate, the most commonly used laboratory feed, and sterilised real wastewater. The two different inocula were raw influent wastewater also taken from this site, which has been shown to contain 17 effective electrogens per ml [21], and Arctic soil (see below) which could potentially have more psychrophilic bacteria.

### 2.2. Media and inocula

Autoclaved acetate media [35] containing 1 g/L sodium acetate was compared to wastewater taken from the Cramlington wastewater treatment site (Northumbrian Water Ltd., UK), which was UV sterilised prior to use [20,21]. The cathode chamber was filled with 50 mM pH 7 sodium phosphate buffer, conductivity 8.2 mS/cm. The conductivity of the wastewater was measured using an EC 300 (VWR Ltd., UK) as 1.98 mS/cm at 26.5 °C, and then equalised for the lower temperature and in the nutrient media using phosphate buffer.

The raw wastewater was collected prior to any form of treatment, and was of mixed industrial and domestic origin, COD 0.7–0.8 g/L. Once collected the wastewater was stored at 4 °C in a closed container. The Arctic soil was collected from Ny-Ålesund, Spitsbergen in Svalbard. This was wrapped within three sealed bags, and kept cold in an ice box whilst it was transported to the laboratory where it was stored at 4 °C until used. The inocula of wastewater and soil were measured out to 5 mL or 5 g respectively before being added to the reactors. Samples of each inoculum were preserved in a 50:50 in a mix of ethanol and autoclaved phosphate buffer (1 M, pH 7) in the freezer at −20 °C for microbial analysis.

## 2.3. Reactor design and operation

A standard design of double chamber tubular MFC reactors (78 mL each chamber) with an internal diameter of 40 mm and length of 60 mm were used [36]. The anode was a carbon felt anode (Ballard, UK) with a surface area of 17.5 cm$^2$, the cathode a 2.5 cm$^2$ platinum coated titanium mesh cathode with a surface area 8.13 cm$^2$ (Tishop.com, UK), in 1 M pH 7 phosphate buffer within the cathode chamber. Both electrodes were attached to a stainless steel wire, and placed in a circuit with a 470 Ω resistor, and a multimeter to measure the voltage (Pico ADC-16, Pico Technology, UK). The resistor was chosen to gain a balance between system performance and community development [37]. The membrane between the reactor chambers was Nafion 117, with an area of 12.6 cm$^2$. Reactors were sparged with 99.99% pure N$_2$ in the anode chamber, and air in the cathode chamber for 15 min after every re-fill.

Four reactors were operated in a warm temperature incubator (Stuart Scientific SI 50, UK) set at a temperature of 26.5 °C, the other four reactors were operated in a low temperature incubator (Sanyo MIR-254; Sanyo Biomedical, USA) set at 7.5 °C. The temperature was logged continuously over the experiment using a EL-USB-1 temperature data logger (Lascar Electronics, UK). The reactors were inoculated with either the Artic soil or wastewater, and then filled with either the acetate or sterile wastewater substrate. The substrate was replaced every 5–6 days until a stable power generation was achieved. The reactors were then re-filled and three successive 3 day cycles were run logging the voltage over this time. Chemical oxygen demand (COD) removal during each batch was determined using standard methods (APHA, 1998) and Spectroquant ® test kits (Merck & Co. Inc., USA).

## 2.4. Microbiological techniques

At the end of each experiment a central 6.25 mm$^2$ section the anode was removed from the chamber using aseptic technique and preserved in a 50:50 mix of ethanol and autoclaved phosphate buffer (1 M, pH 7) and stored in a freezer at −20 °C. The DNA was extracted from the inoculum samples and anode samples using a FastDNA Spin Kit for Soil (Qbiogene MP Biomedicals, UK). Extraction was completed as per the manufacturer's instructions. The samples were then pyrosequenced following amplification of the 16 s rRNA gene fragments.

The primers used were F515 (GTGNCAGCMGCCGCGGTAA) and R926 (CCGYCAAT-TYMTTTRAGTTT). Each sample was labelled with a unique eight base pair (bp) barcode connected to a GA linker. Sequencing was completed from the Titanium A adaptor forward only, from the F515, capturing the V4 region and most of the V5 region with a Titanium read of 400–500 bp. Triplicate PCR reactions were carried out using the Roche FastStart HiFi reaction kit (Roche Diagnostics Ltd., UK) and subject to the following optimised thermal cycles: initial denaturation at 95 °C for 4 min; 23 cycles of denaturation at 95 °C for 1 min; annealing at 55 °C for 45 s; extension at 72 °C for 1 min; and final extension at 78 °C for 8 min. An automated thermal cycle Techne TC-5000 (Bibby Scientific, UK) was used, the cycles were optimised to produce enough product with the lowest number of cycles to reduce the impact of chimeras.

The triplicate samples were then pooled and cleaned using QIAquick PCR Purification Kit (Qiagen, UK). The DNA concentration was quantified by UV–Vis spectrophotometry using a Nanodrop 2000 (Thermo scientific, USA). The individual samples were pooled to give equal concentrations of all reactor samples, and double concentrations of the wastewater and arctic soil inocula. Sequencing was carried out by the Centre for Genomic Research (University of Liverpool, UK) using the Roche 454 sequencing GS FLX Titanium Series.

## 2.5. Data analysis

The pyrosequencing data set was split as per the barcodes and unassigned sequences were removed. The flowgram files were cleaned using a filtering software Ampliconnoise Version 1.25 [34] to give the filtered flowgram file. Filtering at a minimum flowgram length of 360 bp including the key and primer before the first noisy signal, all flowgrams were then truncated to 360 bp. A pairwise distance matrix was then calculated using the Pyronoise algorithm [34]. This uses an iterative Expectation-Maximization algorithm which constructs denoised sequences by clustering flowgrams using the initial hierarchical clusters generated in the previous step and the filtered flowgram file. The cut-off for initial clustering was set at 0.01 and the cluster size was 60, as recommended by Quince et al. [34]. The flowgrams were then denoised.

PCR errors were removed using the Seqnoise algorithm [34], generating a distance matrix by application of the Needleman-Wunsch algorithm [38] for pairwise alignment. The optimal parameters used were: the cut-off for initial clustering of 0.08, and cluster size of 30. Chimera removal was completed using the Perseus algorithm [39] which for each sequence searches for the closest chimeric match using the other sequences as possible parents. The sequences are then classified and the good classes filtered at a 50% probability of being chimeric, producing the final FASTA file which is denoised and chimera free ready for downstream bioinformatics analysis.

A QIIME (v1.4.0) bioinformatics pipeline [40] was used to provide the following analysis: taxonomy assignment using Greengenes (http://greengenes.lbl.gov) at the 97% similarity level; Operational Taxonomic Unit (OTU) table creation; classification using the RDP classifier; summary of taxonomic data from classification; rarefaction data generation of the diversity in a reactor; and calculation of the differences between the reactors. The dissimilarity of the community structure between duplicates was examined using both a weighted (relative abundance) and unweighted (presence/absence) phylogenetic diversity metrics using UniFrac, giving a distance matrix containing a dissimilarity value for each pairwise comparison. The raw OTU table generated was used to produce the species abundance pattern (with the log abundance normalised to the number of sequences in each sample) and the rank abundance curves, (where percentage abundance is used to normalise samples). In total 19 samples were analysed. The number of sequences per sample ranged from 8112 to 77,436 with a total number of observations of 549,178.

An estimate of the total diversity for each sample was calculated using a Bayesian modelling approach as described by Quince et al. [33], where the 'posterior distribution' of the taxa area curve is estimated from the known distribution of the data gathered in the sequencing. Three distributions are modelled: Log-normal; Inverse Gaussian; and Sichel, and the Deviance Information Criterion (DIC), as described by Spiegelhalter et al. [41] is used to compare the fit from each model. The lower the deviance or DIC values the better the model fit and those models within six of the best DIC values can be considered as a plausible fit. Using the fitted abundance distributions, the sampling effort required to capture 90% of the taxa within that sample is estimated.

The Minitab 15 (Minitab Inc., State College, USA) statistical software was used to run analysis of variance (ANOVA) tests on the experimental data, and t-tests on the distance matrix data for the sequences samples. Data were checked for normality prior to completing ANOVA, and if necessary the Box-Cox transformation was used. The performance of the MFC reactors were analysed on the basis of three variables: % COD removal; coulombic efficiency (CE); and power density (mW/m$^2$).

## 3. Results

### 3.1. Reactor performance

All 16 reactors produced current. The cold reactors took a long time to acclimatise, up to 840 h, compared to up to 280 h for the warm reactors. However, once stable the reactors three successive feeding cycles were performed and the results analysed (Table 1). Wastewater fed reactors had a significantly higher COD removal and coulombic efficiency than acetated fed reactors ($p < 0.001$ ANOVA), while their

**Table 1**
Performance data for each of the conditions tested, values represent duplicate reactors averaged over three cycles, diversity estimates use the Log of the log normal diversity estimate. ANOVA *p* values are given representing the statistical significance of the difference between each of the factors.

|  | Coulombic efficiency (%) | Power density (mV/m²) | COD removal (%) | Log diversity estimate |
|---|---|---|---|---|
| Acetate feed | 54.5 | 5.0 | 19.4 | 4.6 |
| Wastewater feed | 10.7 | 4.5 | 62.1 | 3.0 |
| *p* value | <0.001 | 0.880 | <0.001 | <0.001 |
| Warm | 31.7 | 6.3 | 45.9 | 3.6 |
| Cold | 33.4 | 3.1 | 38.2 | 4.1 |
| *p* value | 0.244 | 0.017 | <0.001 | 0.157 |
| Arctic soil inoculum | 36.4 | 4.8 | 42.5 | 4.2 |
| Wastewater inoculum | 28.7 | 4.7 | 41.6 | 3.4 |
| *p* value | 0.009 | 0.556 | 0.690 | 0.036 |

power densities were comparable. The acetate fed reactors had a statistically significantly higher diversity than the wastewater fed reactors ($p < 0.001$ ANOVA).

Temperature had a modest but statistically significant effect on COD removal ($p < 0.001$ ANOVA) and a notable, but statistically insignificant effect on power density. The high variance of power density (coefficient of variance 17.3) within the acetate samples will have weakened the power of the ANOVA in this study. The $Q_{10}$ value for power density with all the reactors combined was 1.7, when the different substrates were considered the $Q_{10}$ was 2 for acetate and 1.2 for wastewater. There was no significant relationship between temperature and diversity. The choice of inoculum had little discernible effect on COD removal or power density and only a modest effect on coulombic efficiency and diversity.

There is an apparent correlation between the CE (positive) and COD removal (negative) in the reactors with the Quince log normal diversity estimates ($p = 0.002$, Spearman Rho correlation). However, this is due to the large difference between the acetate and wastewater samples, i.e. the acetate reactors have a high diversity, high CE and low COD removal. When separated into the two substrate types, only the wastewater fed reactors have a positive correlation between the Quince log normal diversity estimate with CE, (0.810, $p = 0.015$) and power density (0.857, $p = 0.007$).

When all the performance criteria were combined the acetate and wastewater fed reactors separated into two distinct groups (Fig. 1).

### 3.2. Microbial diversity

A species abundance curve plotted from the OTU table (SI) shows that diversity was highest in the Arctic soil inoculum, then in the wastewater inoculum, and lowest in the reactors. The diversity in the reactors fed acetate was higher than in those fed wastewater. This difference is also observed with the Chao ($p = 0.003$) and Shannon ($p = 0.000$) estimates.

A nested ANOVA on total diversity estimates also shows that the acetate fed reactors have a significantly higher diversity than the wastewater fed reactors irrespective of the model used (Log-normal $p = 0.001$; Inverse Gaussian $p = 0.000$; and Sichel $p = 0.027$). Inoculum type and temperature have a secondary effect, but only when the reactors are fed acetate (Fig. 2). The Arctic soil inoculum gives rise to higher diversity than the wastewater inoculum (Log-normal $p = 0.006$), and lower temperatures give higher diversity than warmer ones (Log-normal $p = 0.037$).

### 3.3. Microbial community composition

Despite the different inocula used, the dominant microorganisms found enriched within all reactors were *Geobacter* (Fig. 3). Within the acetate fed reactors *Geobacter* accounted for on average 47% of the microbial population though this was highly variable (st. dev. 23%); in the wastewater fed reactors this was 21% (st. dev. 10%). There was no significant relationship between the percentage of *Geobacter* and reactor performance.

The organisms found to be present in the reactors can be separated into three groups: those present only in the acetate fed reactors, those present only in the wastewater fed reactors, and organisms found in both (Fig. 4). The putative fermenting and hydrolysing organisms found only in the wastewater fed reactors form the smallest group. The putative electrogens, found in both the acetate and wastewater fed reactors form a larger group, but the largest group by far is the group of organisms found exclusively in the acetate reactors. Of the ten most dominant species found in both reactors, all have previously been observed in MFC reactors [42–44]. Those which dominate within
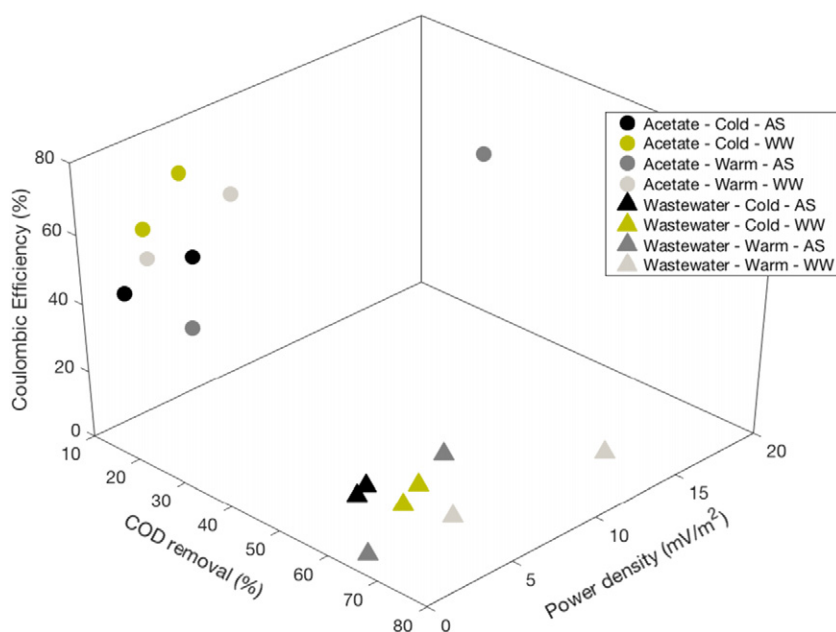


**Fig. 1.** 3D plot showing reactor performance in terms of Coulombic efficiency (CE), COD removal and power density (PD) for the different reactor conditions (AS is Arctic soil inoculum, WW is wastewater inoculum).
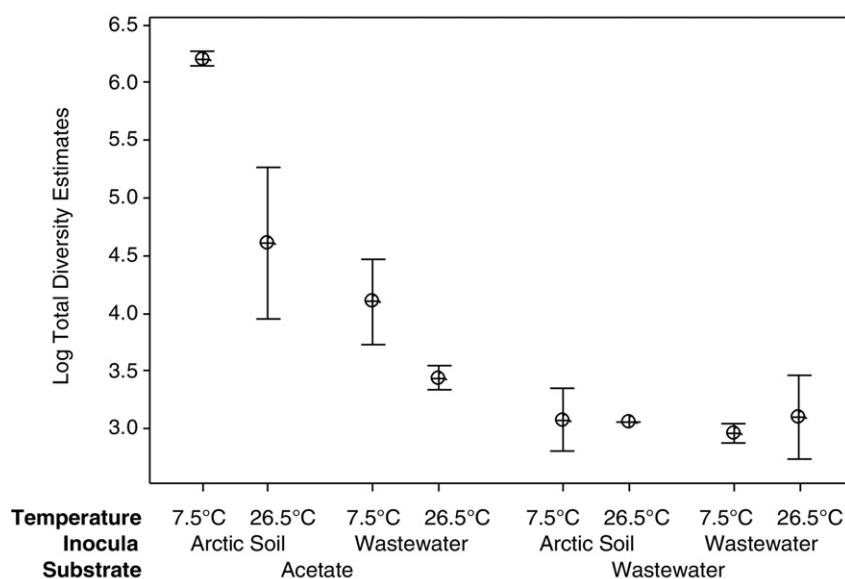
**Fig. 2.** Estimates of total diversity for each set of reactor conditions using the Log normal diversity estimate; lines indicate one standard error of the mean.

the wastewater fed and within the acetate fed reactors only are mostly Proteobacteria, of the Beta, Gamma and Alpha classes, which are largely facultative or obligate anaerobes.

### 3.4. Similarity between replicas

The reactor performance varied considerably between replicate reactors, especially in the warm temperature reactors. Despite this, the Unifrac dissimilarity matrix (data not shown) showed that the duplicate reactors fed with acetate were indistinguishable ($p = 1$) and clustered based on temperature, with both weighted and unweighted analysis. The wastewater fed duplicate reactors on the other hand were typically different, and did not cluster either by temperature or inoculum.

## 4. Discussion

Realistic temperatures and substrate quality are currently two of the most important discrepancies between prevailing laboratory research and plausible pilot scale BES reactors pre-requisite to the application of BES technologies.

It appears that BES may be less temperature sensitive than anticipated. The effects of temperature on COD removal were modest, and effects on coulombic efficiency undetectable: only power density dropped appreciably with temperature in this study. Crucially, wastewater fed reactors were less temperature sensitive than acetate fed reactors. This is the first controlled demonstration of this phenomenon, which was previously only hinted at by studies of acetate and wastewater in isolation.

These findings are potentially significant because the ability to sustain treatment through cold periods is essential if BESs are to be adopted for wastewater treatment at low temperatures. Moreover this may give BESs a "comparative advantage" over conventional methanogenic systems where $Q_{10}$ values can be as high as 4 [20] compared to 1.2 in wastewater fed BES. There is no obvious explanation for this difference in temperature sensitivity. It could reflect differences in the anaerobic "food chain" in BESs and methanogenic technologies. We know that growth yields are lower in electrochemical systems than in methanogenic controls [37]. If some of that missing energy was dissipated as heat there could a very slight warming of the reactor. As fanciful as this may sound, self-heating is an established microbial phenomenon (composting) [45]. A statistically significant 0.9 °C increase in reactor temperature over the influent water temperature (several degrees higher than the outside air temperature) was observed at pilot scale [46].

No special inoculum was required to obtain enhanced performance at low temperatures. The bacterial species present in the biofilms were similar regardless of inoculum or temperature. The only benefit of the arctic soil inoculum was a reduced start up time at low temperatures.
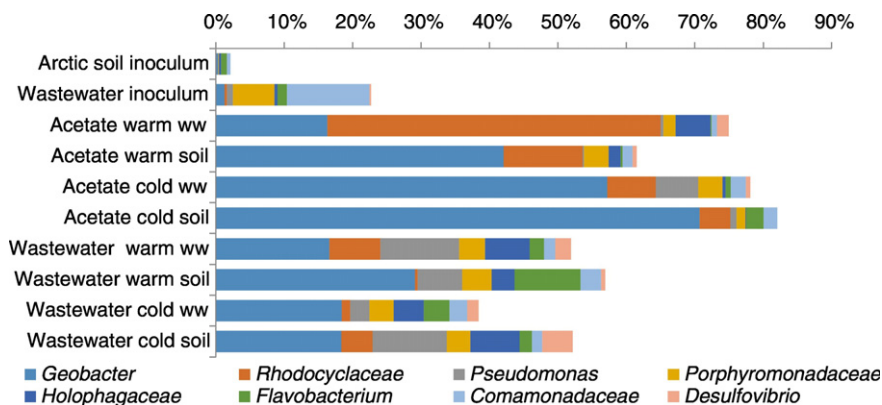


**Fig. 3.** Relative abundance of the eight most dominant genera found in the reactors (average; $n = 2$); where the genus name was not given by the classification database the family name is used.
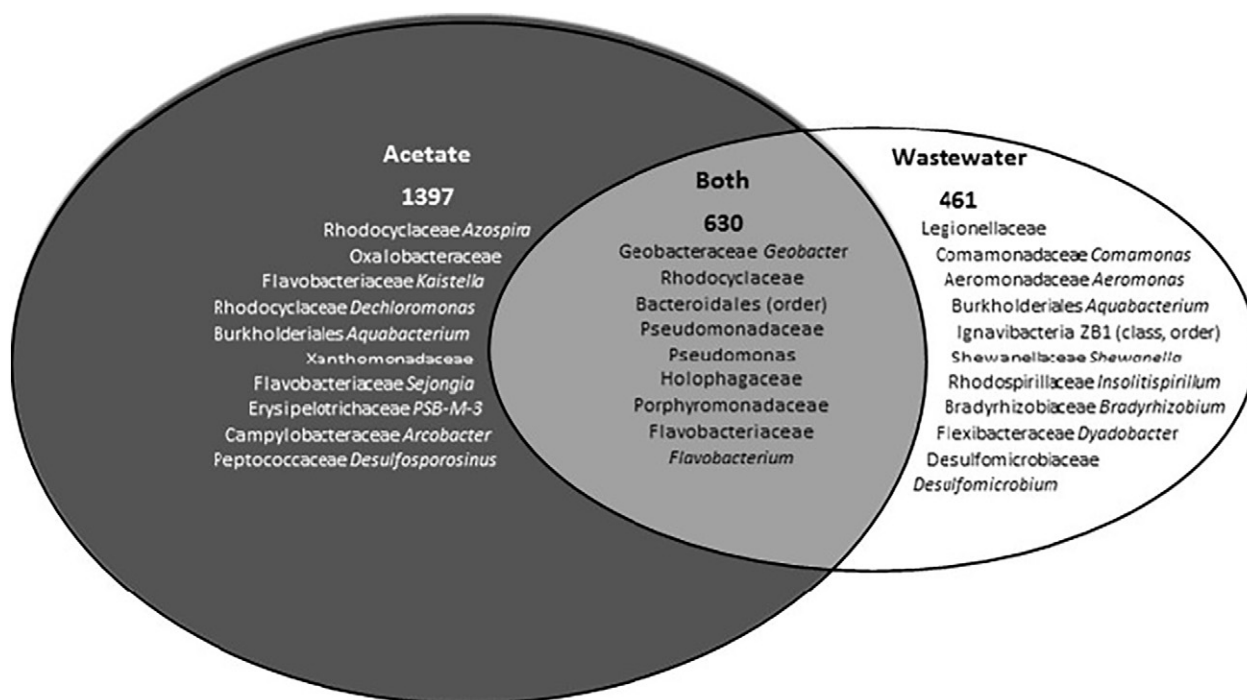
**Fig. 4.** Venn diagram showing the proportions of species found only in the acetate fed reactors, in the fed wastewater reactors, and of those found in both. The numbers given at the top are the number of different OTUs found in these groups. The lists show the top 10 most dominant organisms within each group listed at the family and genus level; where this data was unavailable a higher taxonomic level is given.

Nothing in our findings would justify the use of an external inoculum to improve reactor performance. BES reactors acclimated on wastewater are able to operate at low temperatures with a minor drop in COD removal, and a tolerable drop in power density. A seasonal drop in power with relatively sustained effluent quality would probably more acceptable to water utilities for whom the overwhelming priority will be effluent quality.

The use of acetate conferred a modest advantage on MEC performance, the most obvious improvement being the enhanced coulombic efficiency seen. We speculate that this difference may be more pronounced in a better design of reactor. Curiously, though all reactors received the same COD loading, COD removal was actually poorer in the acetate fed reactors than in the wastewater fed reactors. COD removal in acetate fed reactors is typically good, though, the explicit comparison of wastewater and acetate removal in reactors is rare. We are therefore reduced to speculation that rapid growth of bacteria in the anolyte on the readily degradable acetate ("acetate weeds") may have led to the formation of biomass that was itself difficult to degrade and so subsequently contributed to the COD. The exceptionally high diversity in the biofilm of the anode of the acetate fed reactor compared to wastewater fed reactors suggests that the microbial ecology of the two reactors were extremely different.

It was expected that reactors fed wastewater would contain groups of hydrolytic and fermenting bacteria able to digest the longer and more complex organic food molecules, plus a group of electrogenic organisms able to perform the final step of electron donation to the anode. It was also expected that acetate fed cells would contain only this final subset of bacteria - those able to perform the electrogenic step. This turned out not to be the case; not only was the diversity in the acetate fed reactors far higher than in the reactors fed real wastewater, but established electrogenic species such as *Shewanella* were present in the wastewater fed reactors while absent in the acetate fed ones. The microbiology of acetate and wastewater systems appears to be relatively distinct.

The controversy over how diversity relates to performance in acetate fed BESs has been documented [29]. Some of the difficulty no doubt arises because of the difficulty of authoritatively measuring microbial diversity caused by variations in sample size and sequencing error. We have sought avoid these pitfalls by the rigorous use of the appropriate protocols. The method of Quince et al., 2008 determines the community, not the sample diversity and the method of Quince et al., 2009 compensates for homopolymer based sequencing error. Both methods are well cited and rarely used (though in fairness the second methods paper was primarily applicable to a particular sequencing platform).

No correlation was observed with either diversity or the dominance of *Geobacter* and performance within the acetate fed reactors. However, within the wastewater fed reactors a significant correlation between performance and diversity was observed (higher diversity resulting in improved performance). This would be expected as the wider bacterial community may be more able to provide an effective food chain to break down the complex waste. Electrode potentials were not imposed, but allowed to develop naturally and to be dictated by the environmental conditions. This is more like the pilot scale reactors we built [46] and the full scale reactors we envision. Though there may indeed be advantages to controlling the potential at full or pilot scale: it is not clear how this would be achieved in practice.

In summary: this study rigorously evaluated the effects of temperature, inoculum, and substrate on MFC reactor performance and biofilm diversity. It was anticipated that simple relationships would exist, whereby each factor would affect both performance and diversity, and that acetate would be a simple model for real wastewater. However, complex and counterintuitive patterns emerged. Temperature was observed to have less effect in BES than it does in other anaerobic systems, and particularly when complex real wastewaters were used. Working reactors with real wastewaters can be productively operated at low temperatures without the need for a specialised inoculum. Substrate is observed to have the most significant effect on both reactor performance and diversity.

These findings are important. Most BES research is still carried out in warm laboratories using simple substrates. It is tacitly assumed that these simple and easy to manage systems will be a realistic model for the real-world applications that we aspire to. However, this study has shown we need to exercise caution with this approach: an acetate fed reactor is not necessarily a simple version of a wastewater fed reactor.

## Acknowledgments

## Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bioelechem.2017.07.006.

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