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Quantification of effective exoelectrogens by most probable number (MPN) in a microbial fuel cell

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Running title: quantifying electrogens by MPN

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Experimental methods and procedures

Method of substrate sterilisation

Three methods of wastewater sterilisation were compared, these were autoclaving, membrane filtration and UV sterilisation. The UV sterilisation method was optimised, finding that a 5 minute exposure using pumped circulation through a 3.9 lpm ultra violet system UV3.9WL (East Midlands Water, UK) produced the best bacterial kill was determined using Agar enumeration method 9215C with serial dilutions into ¼ strength Ringers sterile diluent (APHA, 1998).

Table E1.

Percentage change of wastewater characteristics caused by the different sterilisation methods

	TCOD	Soluble COD	Total Solids	Bacteria per 0.1ml
Autoclaved (121°C for 15 min)	-15.6% ± 0.9	21.6% ± 0.6	-13.3% ± 5.8	0
Membrane filtered (0.2μm PES)	-61.5% ± 0.5	22.8% ± 1.7	-36.1% ± 11.7	40 ±19
UV sterilised (5 min)	-1.6% ± 0.4	7.2% ± 4.6	-3.3% ± 6.7	0

Bacteria is the average number counted on triplicate plates at zero dilution. All values show mean ± standard deviation (n=3)

UV sterilisation caused the least change in wastewater properties measured and was able to fully sterilise the wastewater.

Reactor set-up

Double chamber tubular design microbial fuel cell reactors (78 ml each chamber) were used, constructed in Perspex, with an internal diameter of 40 mm and length of 60 mm. The anode was a 2.5 cm^2 of 0.2 cm thick carbon felt (Olmec Advanced Materials Ltd, UK), the cathode a 2.5 cm^2 platinum coated titanium mesh with a surface area 8.13 cm^2 (Tishop.com, UK). This gives a ratio of anode to cathode surface area of 1:1.2, well above the ratios estimated to make the reactor anode limited rather than cathode limited (Ishii et al., 2008). The cation selective membrane between the reactor chambers was Nafion$^®$ 117 (DuPont, France), with an area of 12.6cm^2. The electrodes were positioned 1cm apart. The components of the reactor were cleaned and then sterilised using UV light in a Labcaire SC-R microbiological cabinet (Labcaire, UK).

The cathode chamber was filled with 50 mM pH 7 phosphate buffer saturated with air for 20 minutes before being added into the reactors. In the anode chamber three different media were used: acetate solution with added nutrients (Call and Logan, 2008); starch solution with added nutrients (Call and Logan, 2008); primary settled wastewater (Cramlington WWTP, Northumbrian Water Ltd). The quantities of starch and acetate in the nutrient solutions were balanced to give similar total chemical oxygen demand (TCOD) as the wastewater, and were autoclaved (121°C, 15 min) before use. The wastewater was sterilised by circulating the wastewater through a 3.9 lpm ultra violet system UV3.9WL (East Midlands Water, UK). The bacterial kill was determined using Agar enumeration method 9215C with serial dilutions into ¼ strength Ringers sterile diluent (APHA, 1998). The contact time under UV was altered to give effective sterilisation defined as colony free plates in triplicate at zero dilution. This method gave the most successful sterilisation with the least change in chemical composition of

the wastewater (total chemical oxygen demand (TCOD), soluble chemical oxygen demand (SCOD) and total solids (TS)) compared to autoclaving and filtering (Miyahara et al., 2013).

The three media were sparged under sterile conditions for 10 minutes using ultra high purity (UHP) nitrogen (99.998%) (BOC Industrial Gases Ltd., UK), until the dissolved oxygen (DO) as measured with a DO probe, Jenway 970 (Bibby Scientific Ltd, UK), was below the detection limit of 0.01 mg/L.

Unsterilised, screened raw influent wastewater from Cramlington wastewater treatment plant (Northumbria, UK) was used the inoculum for all reactors. This wastewater is a mixture of industrial and domestic origin. Samples were stored anaerobically at 4ºC and used within 24 hours of collection. The inoculum was also sparged with UHP nitrogen before use.

Duplicate reactors were inoculated with differing volumes of wastewater (1 ml, 10 ml, 25 ml and 50 ml). The anode compartment was then filled with the sterile substrates. An inverted 50 ml syringe filled with UHP nitrogen was placed into the refilling port on top of the anode chamber to provide an anaerobic headspace. The cathode chamber once filled was left open allowing the diffusion of oxygen into the liquid. Both electrodes were attached to stainless steel wire, and placed in a circuit with a 470 Ω resistor. It is acknowledged that the external resistor has an impact on the anode potential, which in turn affects the activity of the biomass (Aelterman et al., 2008) and therefore the current produced. However even at wide ranging anode potentials 0 to -400 mV vs Ag/AgCl (Aelterman et al., 2008), and external resistance 10 to 1000 Ω (Zhang et al., 2011) activity is initiated within the MFC's, the different resistance or anode potential then results in varied performance. The 470 Ω resistor was chosen to be in the mid-range of resistance typically used in MFC systems, and to be representative of what

may be used in the application of this technology. A data logging multimeter (Pico ADC-16, Pico Technologies, UK) was attached to record voltage output every 30 minutes. Reactors were allowed 800 hours at room temperature (20-25 °C) to show acclimatisation before the experiment was ended. The use of triplicate reactors may have been better than using duplicates, but some compromise had to be made due to the limitations of equipment and practicality. In order to attain that the reactors were started at the same time with the same inoculum, and to make sure that the spread of inoculum amount was sufficient to cause the desired MPN dilution out effect (see below), this was a compromise that had to be made. With the acetate fed experiment an additional, second set of reactors was run separately with lower inocula of 0.01 ml, 0.1 ml, and 1ml, and with 25 ml as a positive control. Control 'reactors' (using no inoculum) were run during each test.

The total chemical oxygen demand (TCOD), volatile fatty acids and anions of the media, inocula, and the reactor effluent once the current had dropped to zero, were measured. TCOD was measured in duplicate according to standard methods (APHA, 1998) and (Spectroquant ® test kits, Merck & Co. Inc., USA) colorimetric reagent kit. Volatile fatty acids of the media and inocula were measured in duplicate using an ion chromatograph (IC) Dionex ICS-1000, with an Ionpack ICE ASI column, and heptafluorobutyric acid as the eluent and tetrabutylammonium hydroxide as the regenerant. Anions were measured using ion chromatograph (IC) Dionex ICS-1000, with an Ionpack AS 14A column, with carbonate as the eluent. Coloumbic efficiency was expressed as the percentage of coloumbs recovered from the total coloumbs of substrate used (Logan, 2008).

Estimates of bacterial abundance

The total number of aerobic culturable bacteria present in the wastewater samples used for inoculation was approximated using spread plate method 9215C (APHA, 1998), with peptone based nutrient agar (Lab M Ltd, UK). Serial dilutions were undertaken into sterile ¼ strength ringers solution, with each dilution plated in triplicate. Plates were incubated at 37 °C for 48 hours. Anaerobic bacteria were enumerated using an autoclaved (121 °C for 15 min) basal salts medium (Shelton and Tiedje, 1984) with 1 g/L of both yeast extract and glucose as a carbon source, sparged with sterile UHP nitrogen for 20 minutes. A volume of 9 ml was then added to sterilised Hungate tubes, 1 ml of wastewater was then added to five tubes, and dilutions made down to 10^{-12} with five replicates at each dilution. The headspace of the tubes was sparged with nitrogen, and the tubes incubated at 37 °C for two weeks. The number of anaerobic bacteria was determined using the MPN methodology (APHA, 1998), and added to the number of aerobic bacteria to give an estimated total number of bacterial cells.

A further estimate of the total number of cells was made using epifluoresence microscopy using SYBR gold nucleic acid stain (Invitrogen Ltd., UK). A 100x dilution was made with sterile phosphate buffer, and the cultures were stained for 30 minutes with light excluded. Aliquots of 1 ml were filtered onto 0.2-µm-pore-size black polycarbonate filters (Merck Millipore, USA) which were placed onto slides, and mounted with Citifluor antifadent (Citifluor Ltd., UK). A coverslip was placed over the resultant preparation, and nail varnish was used as a sealant. Cell counts on 10 random fields of view were performed using a Nikon Eclipse Ci-L Fluorescence Microscope (Nikon Ltd, UK).

Most Probable Number (MPN) Calculations

Pre-calculated MPN tables (APHA, 1998) are used to give a value of the most probable number of bacteria within a sample. These tables are based on standard sets of dilutions and typically three biological replicates. However, when the experimental data is not available to use these standard tables, the MPN can be calculated using the original formula used to produce these tables, and 95% confidence limits can be calculated using Haldane's formula (Haldane 1939). Thus by calculating the MPN and confidence limits from first principles, statistically valid results can be achieved where the data set is imperfect due to a non-standard number of replicas or dilutions.

The MNP is calculated through a series of iterations based on a Poisson and binomial distributions (Blodgett, 2005) using the following formula, solving λ for the concentration:

$$\sum_{j-1}^{k} \frac{g_j m_j}{1 - \exp\left(-\lambda m_j\right)} \;=\; \sum_{j-1}^{k} t_j m_j \tag{1}$$

Where: k = the number of dilutions; g_j = the number of positive (or growth) tubes in the jth dilution; m_j = the amount of the original sample put in each tube in the jth dilution; t_j = denotes the number of tubes in the jth dilution (i.e. the number of biological replicates).

A probability is assigned to each possibility of the number of bacteria based on the outcome at each dilution, a positive outcome being current produced by the reactor. The number with the highest probability is given as the MPN. Using the spreadsheet developed by Blodgett to make these iterative calculations, the most probable numbers of electrogens per 100 ml of wastewater can be calculated (Garthright and Blodgett, 2003).

Thomas' simple formula which is based on the same principles as the full test, but has a simpler algorithm to solve, can also be applied to the data set; this formula has been shown to be in substantial agreement with actual data (Thomas, 1942). Using the data from the range of dilutions where at least one of the replicates is positive to where at least one of them is negative, the following calculation can be made:

$$MPN/100\ ml\ =\ \frac{no.positive\ tubes\ \times\ 100}{\sqrt{(ml\ sample\ in\ negative\ tubes)\times(ml\ sample\ in\ all\ tubes)}} \tag{2}$$

The approximate confidence limits of this calculation at the 95% level can be estimated from the standard error of $\log_{10}$ (MPN), a method presented by Haldane (Haldane, 1939):

m_1, m_2, m_3 ……. denotes inoculation amounts ranging from the largest to the smallest of the chosen dilutions,

g_1, g_2, g_3 ……. denotes the number of positive tubes at the corresponding dilutions.

Firstly T is calculated using the first dilution, this is repeated for the number of dilutions used:

$$T_1 = \exp(-\ MPN\ \times\ m_1)\ , T_2 = \exp(-\ MPN\ \times\ m_2)\ ...\ ...\ ...\ etc \tag{3}$$

The term B can then be calculated:

$$B\ =\ [g_1\ \times\ m_1\ \times\ m_1\ \times\ T_1\ /((T_1\ -\ 1)^2)] + [g_2\ \times\ m_2\ \times\ m_2\ \times\ T_2\ /((T_2\ -\ 1)^2)] + [g_3], [\ g_4]\ ....\ etc. \tag{4}$$

Then the standard error of $\log_{10}$ (MPN)

$$Standard\ Error\ of\ \log 10\ (MPN) = 1/\big(2.303\ \times\ MPN\ \times\ (B^{0.5})\big) \tag{5}$$

95% confidence intervals (CI) are given by:

$$Log_{10}\ (MPN) \pm 1.96\ \times\ Standard\ Error \tag{6}$$

The MPN method is based on the assumptions that: bacteria are distributed randomly within the sample; they are separate, not clustered together; they do not repel each other; and every

reactor whose inoculum contains at least one viable organism will produce detectable growth or change; and the reactors are independent (Blodgett, 2009).

Supplementary results

Table S1. COD removal and Coulombic efficiencies of all reactors fed on the different substrates. The values in grey are the reactors where acclimatisation did not occur

Inoculum (ml)	0.01	0.01	0.1	0.1	0.1	1	1	1	1	10	10	25	25	25	25	50	50
COD removal (%)																	
Acetate	85.6	77.1	85.4	80.3	80.5	86.7	95.6	92.4	82.1	87.3	79.9	84.7	86.6	80.2	77.6	79.0	77.3
Wastewater						60.8	41.9			59.1	69.8	68.1	70.5			80.3	62.5
Starch						88.2	84.5			88.2	86.4	89.4	81.7			80.8	90.5
Coulombic efficiency (%)																	
Acetate	0.1	0.1	1.8	0.5	0.8	4.6	5.3	1.8	0.1	6.7	7.5	10.5	8.9	10.1	9.2	9.1	0.8
Wastewater						3.6	0.1			0.3	0.2	9.4	12.5			10.4	7.4
Starch						1.3	0.78			0.82	13.4	12.5	16.9			17.4	1.6*

*Unrepresentative value, data logging equipment failed after the point of acclimation.

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