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

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## HIGHLIGHTS

- MPN was used to count effective exoelectrogens in wastewater.
- Current production in microbial fuel cells was used as end point.
- Apparent MPNs were lower for complex substrates than for acetate as test substrate.
- This is because current from complex substrates requires not just exoelectrogens.
- Apparent growth rates were inferred from rates of current production.

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## ABSTRACT

The objective of this work was to quantify the number of exoelectrogens in wastewater capable of producing current in a microbial fuel cell by adapting the classical most probable number (MPN) methodology using current production as end point. Inoculating a series of microbial fuel cells with various dilutions of domestic wastewater and with acetate as test substrate yielded an apparent number of exoelectrogens of 17 per ml. Using current as a proxy for activity the apparent exoelectrogen growth rate was 0.03 $h^{-1}$. With starch or wastewater as more complex test substrates similar apparent growth rates were obtained, but the apparent MPN based numbers of exoelectrogens in wastewater were significantly lower, probably because in contrast to acetate, complex substrates require complex food chains to deliver the electrons to the electrodes. Consequently, the apparent MPN is a function of the combined probabilities of members of the food chain being present.



## 1. Introduction

Microbial Fuel Cells (MFCs) (Logan, 2008) and other microbial electrochemical systems (Heidrich et al., 2014) rely on the growth and function of a group of organisms – known as exoelectrogens – capable of transferring electrons outside their cell walls (Logan, 2009). Although the functions of these bacteria within MFCs are well understood, little is known about their abundance in the wastewaters and sludges used to inoculate these systems, and about their absolute numbers within working systems (Logan, 2009; Torres, 2014).

Growth and kinetics of exoelectrogens in MFCs are not well understood (Torres, 2014), an issue further complicated by uncertainty over the mechanisms of electron transfer (Kumar et al., 2016). Measurements of exoelectrogen numbers, growth rates and growth yields are inherently difficult in MFCs for a series of reasons, which includes a lack of suitable primers and probes, because it is not known which organisms are electrogenic or indeed what makes them electrogenic (see below) and also because sampling of biomass on the electrodes destroys the biofilm required for successful operation (Freguia et al., 2007). Furthermore, because an MFC relies upon a food chain of hydrolytic, fermentative and exoelectrogenic organisms (Lovley, 2008), studies of a single species or trophic level will not necessarily reveal the characteristics of the system as a whole.

Within the field of microbiology new, culture-independent methods for quantification have recently been developed. The most recognised modern technique for defining the absolute quantity of an organism is presently quantitative polymerase chain reaction (qPCR) (Freeman et al., 1999). However, there are several obstacles to using this technique to determine the abundance of exoelectrogenic microorganisms in MFCs, the most important of which is that currently very little is known about their taxonomic

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identities (Huang et al., 2014). Although probes exist for *Geobacter*, the archetypical and apparently most prevalent exoelectrogen, there is no universal "electrogen gene". Thus, qPCR can be used to quantify *Geobacter* specifically but not exoelectrogens generally. The other modern technique for bacterial quantification is fluorescence *in situ* hydridization (FISH) (Baptista et al., 2014), but this again relies on knowing exactly which bacteria to target. Even if this were known, counting cells adhered to a three dimensional black carbon anode structure would also present many challenges. It is therefore not surprising that "knowledge" of the numbers of these bacteria within reactors is based on relatively crude estimates based on surface area (Logan, 2008), turbidity (Rabaey et al., 2003) or substrate removal (Freguia et al., 2007).

Prior to the advent of the modern molecular methods, culture based techniques were used to estimate numbers of bacteria or specific physiological groups (e.g. Toranzos and McFeters (1997)). These techniques have limitations (Toranzos and McFeters, 1997), including the significant fact that the majority of environmental microorganisms cannot be cultured *ex situ*. However, in the case of an MFC, culturing can be done *in situ*, with the presence of an effective exoelectrogen being inferred from electrical activity. This then permits to infer abundance, and possibly growth rates using classical algorithms for estimating the most probable number (MPN) of bacteria and rates of increase in activity. The concept behind the MPN method is to dilute the sample to such a degree that the inoculum in each tube, or in this case reactor, will sometimes, but not always, contain a viable organism, which is able to grow and initiate activity in that tube or reactor. By analyzing and quantifying the results of replicas at different dilutions, the amount of viable bacteria in the original inoculum can be statistically estimated using a Poisson distribution (APHA, 1998).

In the present study this insight was used to determine the number of exoelectrogens capable of initiating current in an MFC in a wastewater sample. Specifically, a series of microbial fuel cells was inoculated with various dilutions of wastewater and fed with acetate, starch or domestic wastewater as substrate. Current was continuously recorded over time. Using the mathematical algorithms behind the MPN methodology enabled the use of fewer replicas and dilutions than would be needed if classical MPN tables were used, making the method more practical for in a laboratory setting with limited number of MFC reactors. This set-up allowed estimation of: (i) the apparent number of functional exoelectrogens in the inoculum, by applying the MPN methodology outlined above, and (ii) the growth rate of exoelectrogens, by measuring current production and using the rate of current increase as a proxy for the increase in exoelectrogen biomass.

## 2. Methods

The analytical techniques used and the methods of substrate sterilisation, reactor set-up, and estimates of bacterial abundance are all essentially "standard methods"; their details, including the results of a comparison of three methods of substrate sterilisation, are provided in the Supplementary Data.

### 2.1. Most probable number (MPN) calculations

MPN calculations were made with the original formula developed by Haldane (1939), as presented and discussed by Thomas (1942) and Blodgett (2005). Details are given in the Supplementary Data.

### 2.2. Growth rate

Growth rate of bacteria ($\mu$) is classically calculated by quantifying the change in the number of bacteria over time. In this experiment voltage is deemed to be a suitable proxy for electrogenic bacteria, the rate of voltage rise being equivalent to the rate of growth. It is assumed that each bacterium is capable of donating a specific amount of electrons, therefore an increasing number of electrons are donated to the circuit (i.e. the voltage increases at a constant resistance) as the absolute number of bacteria increases, (it does not represent an increasing ability to metabolise), i.e. voltage is deemed proportional to bacterial number. By plotting current versus time on a log scale, the growth rate of electrogenic bacteria can be estimated form the slope obtained. This gives an overall growth rate for the population of electrogens as whole, not specific species which may be different.

### 2.3. Statistical analyses

All statistical tests were run using Minitab 15 (Minitab Inc., State College, USA). One way ANOVA was used to analyse the difference in growth rates for each substrate. Growth rates were estimated for each reactor where a rise in current was observed (12 samples for acetate, five samples for both wastewater and starch).

## 3. Results and discussion

The present study outlines a method to establish how many exoelectrogens are present in wastewater that can start a microbial fuel cell. By directly measuring current production over time in a series of small scale MFCs inoculated according to the MPN methodology, the number of functional electrode reducing bacteria in wastewater can be established unequivocally, without the use of proxies.

### 3.1. Total number of bacteria in wastewater

Spread plate counts and anaerobic multiple tube counts in rich media with the wastewater used in these experiments gave mean values of $8.3 \times 10^5 \pm 2.4 \times 10^3$ (95% CI) culturable aerobic bacteria, and $6.9 \times 10^4 \pm 1.2 \times 10^2$ (95% CI) culturable anaerobic per ml in this wastewater, which yields a rough estimate of the total number of at most $10^6$ culturable bacteria per ml of wastewater, and agrees well with previous estimates for domestic wastewater (Tchobanoglous and Burton, 1991). The total direct cell count (determined by epifluorescence microscopy) was $1.6 \times 10^8 \pm 6.2 \times 10^6$ (95% CI) cells per ml, implying that 99% of the cells were not readily culturable.

Inoculum sizes in the range of 50–0.01 ml per reactor allowed observation of the desired MPN dilution effect, with a positive result being current production in a reactor, and a negative result being the absence of current production. No current was produced in non-inoculated control reactors. The number and distribution of positive and negative outcomes of the tests (Table 1) indicated an MPN of effective exoelectrogens of 17 per ml of wastewater (Table 2). This number is significantly lower than the previous esti-

**Table 1**
The number of positive outcomes for each inoculum size out of the total number of reactors run with that inoculum size.

| Inoculum size (ml) Test substrate | 50 | 25 | 10 | 1 | 0.1 | 0.01 |
|---|---|---|---|---|---|---|
| Wastewater | 2/2 | 2/2 | 0/2 | 1/2 | – | – |
| Starch | 2/2 | 2/2 | 1/2 | 0/2 | – | – |
| Acetate | 2/2 | 4/4 | 2/2 | 3/4 | 1/3 | 0/2 |

**Table 2**
The MPN of electrogens per ml of wastewater. Numbers in brackets indicate the upper and lower bounds at 95% confidence. The probability of presence in wastewater was calculated from the total count of viable bacteria per ml.

| Substrate | MPN calculated after Blodgett (2005) | MPN estimated after Thomas (1942) |
|---|---|---|
| Wastewater | 0.6 (0.3–2.5) | 0.8 (0.3–2.5) |
| Starch | 1.0 (0.3–3.2) | 1.1 (0.3–4.0) |
| Acetate | 17.0 (5.5–52) | 17.6 (6–51.5) |

mate of $1.0 \times 10^3$–$7.5 \times 10^5$ (Yang et al., 2016). This suggests that not all exoelectrogens present in wastewater are capable of initiating current production in reactors, or that there was indeed a large difference in the number of exoelectrogens in the samples. Chinese wastewater treatment systems with steel pipes may allow the growth of more exoelectrogens than UK systems (Yang et al., 2016). A direct comparison of the two methods would make an interesting study, and verify if the rapid and easier $WO_3$ method did in fact reveal the numbers of exoelectrogens that are able to make an MFC work.

### 3.2. MPN of exoelectrogens in wastewater as measured with wastewater or starch as test substrate

The MPN of exoelectrogens in wastewater as measured with starch or UV-sterilized wastewater as test substrate (see SI for experimental details) was lower than when measured with acetate (Table 2). To appreciate this result it should be noted that MPNs found with starch or UV sterilized wastewater as substrate are the products of two or more probabilities. Wastewater and starch are complex feeds, requiring a series of steps in their breakdown (Lovley, 2008), with each step likely mediated by different microorganisms. The electrons originating from these complex substrates are passed down this "food chain", culminating in the final step of being donated to the electrode (Kiely et al., 2011). Thus the MPN of the wastewater and starch fed reactors is a composite of the MPN of the acetate fed reactors (the number of exoelectrogens) multiplied by the probabilities of the presence of organisms able to complete the upstream steps. In the acetate fed reactors such a chain is not apparent: electrons from acetate can be directly donated to the electrode by acetate consuming exoelectrogens (Dolfing, 2014). This is why "MPN" was low for one trophic level (acetate), and lower still for a relatively simple starch based chain and lower still for wastewater.

### 3.3. Growth rates of exoelectrogens

Traditionally, the growth rate of bacteria and archaea is determined by quantifying the change in the number of organisms over time; however, this is not practicable within a working MFC. This lead to the hypothesis that voltage rise can be used as a proxy for growth, based on the assumption that each exoelectrogen is capable of donating a specific amount of electrons to the electrode, that is that current, and therefore voltage, is proportional to the number of active exoelectrogens. The average apparent growth rates thus obtained with acetate, starch and wastewater as growth substrates, ranged between 0.023 and 0.035 $h^{-1}$ (Table 3). Contrary to previous work (Velasquez-Orta et al., 2011), these values were not significantly different from each other ($p = 0.282$, $F = 1.36$, one way ANOVA), which would indicate that in this work the growth of the exoelectrogens was the limiting factor during exponential growth. Interestingly, the values estimated with this methodology were similar to the growth rate of *Geobacter sulfurreducens*, a known exoelectrogen, reported in the literature (Table 3), and to those of typical fermenting organisms. Although it cannot be excluded that the voltage rise on which these estimates are based was caused by an increasing capability of bacteria to donate electrons, rather than by an increase in bacteria numbers, or that electrochemical factors such as the properties of the electrodes themselves were a limiting factor rather than the bacteria on them, the agreement between reactors/substrates, as well as the agreement with growth rates for specific exoelectrogens found in other studies (Table 3), suggest that voltage rise is a reasonable proxy for bacterial growth within MFCs.

**Table 3**
Average apparent growth rates of electrogens – as estimated by plotting current versus time on a log scale, and taking the slopes of the resulting plots – compared to growth rates of known electrogens.

| | Average growth rate ($h^{-1}$) | Reference |
|---|---|---|
| Wastewater fed community | 0.028 ± 0.013 | This work |
| Starch fed community | 0.023 ± 0.005 | This work |
| Acetate fed electrogens | 0.035 ± 0.020 | This work |
| *Geobacter sulfurreducens* | 0.023–0.099 | Cord-Ruwisch et al. (1998) |
| *Geobacter sulfurreducens* | 0.04–0.09 | Esteve-Nunez et al. (2005) |
| Fermenting micro-organisms | 0.05 | Lovley (2008) |

### 3.4. COD removal and coulombic efficiency

The coulombic efficiency of the reactors was low (Table S1), as would be expected during acclimatisation.

### 3.5. Alternative MPN based methods

Having developed an elegant plate based approach where electrochromic alteration of tungsten trioxide ($WO_3$) is used as an indication of exo-electrogenesis, Yang et al. (2016) have recently coupled this $WO_3$ method with the MPN method to count the number of exoelectrogens in wastewater, which yielded an estimate of $1.0 \times 10^3$–$7.5 \times 10^5$ per ml (Yang et al., 2016). Unfortunately a verification that all organisms counted with the $WO_3$ method are capable of – and well positioned to – initiating current in a reactor is missing. Indeed, the MPN estimate of 17 exoelectrogens per ml of wastewater is significantly lower than their estimate. Rotaru et al. (2015) have shown that there is no direct correlation between Fe (III) oxide reduction rates and current produced with a series of *Geobacter* species. This suggests that not all exoelectrogens present in wastewater are capable of initiating current production in reactors, or alternatively that there was indeed a large difference in the number of exoelectrogens in the two wastewaters. Chinese wastewater treatment systems with steel pipes may allow the growth of more exoelectrogens than UK systems (Yang et al., 2016). A direct comparison of the two methods will make an interesting study, and verify if the rapid and easier $WO_3$ method did in fact reveal the numbers of exoelectrogens that are able to make an MFC work. An improved understanding of exoelectrogens, especially the mechanisms behind exoelectrogenesis – what makes an exoelectrogen a competent exoelectrogen capable of initiating current – , and the ensuing development of genetic markers for exo-electrogenesis, resulting in the development of tests for the presence and expression of the genes for exo-electrogenesis could ultimately enable the development of more direct methods for measuring the abundance of competent exoelectrogens, that is exoelectrogens that are able to initiate current production in a bioelectrochemical system.

The reactors in the system used here did not have their potential poised, and used a resistance allowing for reasonable current production. The simple methodology outlined here could be used in future work at different potentials or resistances to produce an understanding how these factors affect apparent MPNs of competent exoelectrogens.

## 4. Conclusions

This study shows that MPN methodology can be used to estimate the number of effective exoelectrogens in wastewater. Key findings are that voltage can be used to estimate cell numbers, and that the apparent number of exoelectrogens detected with acetate as test substrate is higher than the number detected when more complex substrates are used. This is plausible because the methodology is based on statistics. Current generation from complex substrates requires a "food chain" rather than an exoelectrogen per se, and the chance to have several organisms in one sample is necessarily smaller than having just one organism in that sample.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2016.06.066.

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