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Extending the dynamic range of Biochemical Oxygen Demand sensing with multi-stage Microbial Fuel Cells

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Water quality sensing is a promising application for Microbial Fuel Cells (MFCs) as the outputs have been shown to correlate with Biochemical Oxygen Demand (BOD). Nonetheless to date, the upper limit of amperometric linear calibrations from MFC-based BOD sensors have been limited to approximately 250–340 mg/l BOD₅; attributed to substrate saturation of the anodic biofilm.

In this study, an extended detection range was obtained using an array of three MFCs linked hydraulically in series. The sum of the current density generated from each MFC in the array was calibrated against BOD₅ and a linear response was obtained up to 720 mg/l BOD₅ (1175 mg/l COD) with R² of 97%. The dynamic range was double that achieved by the first MFC in the array operating individually. Average response time to reach a stable current for reliable quantification was 2.3 hours, a considerable improvement on the standard 5-day BOD test. The anodic biofilm of the sensor was dominated by *Geobacter* spp. and members of *Porphyromonadaceae* and these bacteria were likely responsible for electricity generation through syntrophy with fermenting bacteria also present in the biofilm.

The dynamic range of the multi-stage MFC sensor could be increased by increasing the hydraulic retention time. The modular mode of operation demonstrated here allows the dynamic range of the sensor to be extended, permitting analysis of BOD concentrations ranging from those typical of municipal wastewater up to those found in certain industrial wastewaters. Critically, sensitive measurement of both low and high levels of organic carbon without requiring amendment or dilution could be achieved online and in real-time with this practical, low-cost MFC configuration.

Water impact

Online monitoring of organic pollution in wastewater could permit real-time process control of the treatment process and prevent effluents being discharged above regulated consent levels unknowingly. With multi-stage MFCs the dynamic sensing range can be significantly increased, compared to that of a single-stage MFC, allowing for monitoring of higher strength industrial and urban wastewaters without requiring dilution nor decreased sensitivity.

1 Introduction

Assessment of wastewater quality is vital for preventing environmental pollution, disease and release of toxic compounds. Biochemical Oxygen Demand (BOD), a measure of the dissolved oxygen (DO) required for microbial oxidation of biodegradable organic matter present in wastewater, is an important property for assessing treatment efficacy¹. BOD has long been quantified by the standard Five Day BOD test (BOD₅),

nonetheless this is a time-consuming (five days), labour-intensive (sample preparation and dilution) and inaccurate (±15%) procedure which could be significantly improved². Because of this 'snapshot' monitoring approach, treatment plants cannot react to sudden changes in BOD which may result in undetected discharges of hazardous wastewater³. More recently, BOD sensors have been developed which allow online monitoring with increased speed and accuracy. However, existing respirometric, photometric and mediated electrochemical sensors⁴ generally exhibit poor stability, low dynamic range and require frequent maintenance; preventing their widespread application⁵.

Microbial Fuel Cells (MFCs) are an emerging technology where the electrical current or charge generated correlates to the concentration of biodegradable organic carbon (e.g. BOD) consumed at the anoxic anode. MFCs show potential for

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development of more viable BOD sensors with low maintenance requirements. However, the upper limit of the dynamic range of MFC-based sensors reported to date has been limited. Jiang et al.⁶ provided a useful overview of the performance of MFC-based BOD sensors to date and a comprehensive evaluation further presented in Section 3.5 of the present study. In search of extended dynamic sensing ranges researchers have employed various methods for single-stage MFCs. Kharkwal et al.⁷ reported an acetate-fed MFC sensor with a MnO_2 cathode catalyst which exhibited a linear amperometric range up to approximately 340 mg/l O_2 validated by comparison with the BOD_5 test. Whereas, Ayyaru and Dharmalingam⁸ reported a linear amperometric range up to 650 ppm for a membrane-optimised MFC, however no validation methods were stated therefore it was attributed to mg/l glucose concentration rather than BOD_5 . Use of different validation methods (e.g. BOD_5 , COD and substrate concentration) hampers direct comparison of data from all published studies. For example, van Haandel and van der Lubbe⁹ found BOD_5/COD ratios of 0.58 and 0.75 for fully biodegradable substrates glucose and acetate respectively, as the BOD_5 test does not determine the total BOD concentration but only a fractional amount determined over five days. Furthermore, there have been reports of coulometric sensors (calibration of the charge passed over a specified time period against BOD) which have a different principle of detection to amperometric sensors (calibration of the current against BOD). Modin and Wilén¹⁰ reported an acetate-fed Microbial Electrolysis Cell (MEC) which was used as a coulometric sensor and exhibited a linear range up to 1280 mg/l validated by BOD_5 . However, this was only achievable by extending the incubation period to 20 hours. Whilst this represents an improvement in response time relative to the five-day BOD test, the large dynamic range is at the expense of near real-time measurement that amperometric sensors enable. When the substrate concentration (i.e. BOD) increases, saturation of the anodic biofilm occurs and the current plateaus, resulting in further increases in concentration yielding no change in current¹¹. This prevents MFC-based sensor application for monitoring higher strength wastewaters such as those from urban and industrial sources¹². Reliance on dilution or amendment using flow injection apparatus is a relatively cost-, labour- and maintenance-intensive approach, compared with the practical and low-cost implementation of an online MFC-based sensor. In addition, such practices reduce detection resolution and sensitivity to low-strength wastewaters. Hydraulically-connected MFCs (serpentine, tubular or multi-staged) possess unique attributes because unconsumed substrate from the first MFC, flows to subsequent cells/electrodes where anodic biofilms acclimated to lower substrate concentrations consume residual substrate¹³. To date, multi-stage MFC configurations have been evaluated with respect to energy recovery¹⁴; but have not been used as sensors. Nonetheless, these studies indicate the potential for improving the range of a BOD sensor with hydraulically-connected MFCs. In this work, multi-stage MFCs were assembled in a single-pass flow system to determine if the BOD sensing range could be

increased. The effect of flow-rate on BOD calibration was determined and a comparative analysis was undertaken of the microbial communities from anodic biofilms and non-polarised biomass collected from the systems in order to elucidate the microbial processes responsible for current/signal generation. The performance of the multi-stage sensor was evaluated with respect to MFC-based BOD sensors previously reported in the literature.

2 Methods

2.1 Synthetic Wastewater Medium

The synthetic wastewater medium used throughout this study contained 10 ml/l macro-nutrient, 2 ml/l micro-nutrient and 1 ml/l vitamin solutions (modified from¹⁵), and was buffered with potassium phosphate buffer (pH 7, 50 mM). The carbon source was the same used in the standard BOD_5 test²; glucose and glutamic acid at a ratio of 1:1 (w/w), at glucose-glutamic acid (GGA) concentrations between 42.5–2000 mg/l (equivalent to 25–1199 mg/l O_2 BOD_5). Full medium preparation details are provided in Section S1, Supplementary material.

2.2 Microbial Fuel Cells

Single Chamber MFCs (SCMFCs) were fabricated in-house using a bespoke CAD design. Nine 10 ml SCMFCs were setup with 11 cm^2 circular carbon cloth anode electrodes (4.91 cm^2 effective area (A_{eff})). Gas diffusion cathodes (11 cm^2) with 0.5 mg/ cm^2 Pt loading were prepared by applying ink containing Pt/C (25 mg, nominally 20% Pt; Alfa Aesar), Nafion perfluorinated resin solution (54 μl , 5 wt.%/45 wt.% H_2O ; Sigma-Aldrich) and ethanol (1.5 ml) to carbon paper (H23C6; Freudenberg). The anode and cathode were separated using Fumapem F-930 cation exchange membrane (FuMA-Tech) and connected using titanium wire and an external resistor ($R_{\text{Ext}} = 953 \Omega$ and later changed to 305 Ω). Each cell had a side medium inlet port and top ports for medium outlet and a Ag/AgCl reference electrode (RE-5B; BASi). Cells were operated independently with no shared electrical connections. The cell and anode potential were logged using a NI-USB-6225 datalogger and LabVIEW SignalExpress (National Instruments). The current was calculated using $I = V/R_{\text{Ext}}$ and current density was calculated by dividing by A_{eff} .

2.3 Single-pass, continuous flow system

The continuous flow system was constructed with triplicate feed lines (Cole Parmer) delivering medium to MFCs assembled in a three-stage hydraulic array (Figure 1). Three five litre bottles fitted with 4-port caps were filled with up to 5600 ml sterile synthetic wastewater (providing sufficient feed for 3 days of operation). Medium was drawn by a peristaltic pump (120S/DM3; Watson Marlow) at speeds of 0.52 and 1.24 ml/min and continuously fed through a sterile glass drip chamber and

Figure 1: Setup for the single-pass, continuous flow system with triplicate feed lines (A, B and C) of three stages of MFCs per line connected hydraulically in series (Photograph in Figure S1, Supplementary material).

past a UV lamp (WZ-09815-10; 254 nm; Cole Parmer); to reduce upstream contamination of the feed bottles. At flow-rates of 0.52 and 1.24 ml/min the hydraulic retention time (HRT) was 18.9 and 7.6 minutes for each cell, and 1.82 and 0.73 hours respectively from the pump to the outlet of the third stage MFCs. Each medium bottle was fitted with two 0.2 μ m filters (Fisher Scientific) for nitrogen sparging (10 minutes prior to operation) and gas pressure equalisation. Cells were placed on an aluminium heating platform constructed with heating mats (RS Components) operated at $28.2 \pm 2.0^\circ\text{C}$, to minimise the effect of temperature variation on sensor output. Calibration was achieved by altering the medium GGA concentration. Prior to each medium bottle replacement, accumulated biomass sludge was emptied from the tubing and MFC anode chambers and refilled with fresh medium via the pump.

1.4 Analytical Methods

The BOD₅ test was carried out according to APHA². Four 1/250 diluted replicates per sample were prepared in BOD bottles; the first was opened immediately for initial DO measurement, the remaining three replicates were incubated for 5 days at 20 °C. A 'measured BOD₅' to 'known GGA concentration' ratio of 0.599 was calculated from standard GGA solutions and used to estimate BOD₅ values of prepared solutions. Additional analysis details are provided in Section S2, Supplementary material.

1.5 Biochemical Oxygen Demand Calibration & Normalisation

For each cycle of medium feeding, the average stable current density was defined during the period in which the first derivative of the current density (di/dt) fell below 3% peak current density (to account for signal noise). di/dt approached zero as the current response plateaued (Figure S2). Current densities were 'normalised' to the maximum, average stable current density ($\bar{I}_{Max}$) generated by each cell ($\bar{I}/\bar{I}_{Max} \times 100\%$) to account for performance differences between MFCs and permit sensor comparison. Calibration curves were constructed by plotting current density against BOD₅ and fitted with linear (Equation 1), Michaelis-Menten (Equation 2) and Hill (Equation 3) models.

$$y = mx + c \quad (1)$$

$$y = \frac{v_{Max}x}{K_M + x} \quad (2)$$

$$y = \frac{v_{Max}x^h}{K_M^h + x^h} \quad (3)$$

where y is current density ($\mu\text{A}/\text{cm}^2$ or % for normalised calibrations), x is BOD₅ (mg/l O₂), m is the slope, c is the y -intercept, v_{Max} is the maximum current density, K_M is the Michaelis constant (concentration at half maximal rate) and h is the Hill coefficient (co-operativity of the biofilm-substrate 'binding' reaction).

The coefficient of determination (R^2), residual standard deviation (SD_{res} ; in $\mu\text{A}/\text{cm}^2$ or %) and prediction bands using the 95% confidence intervals of the fitted coefficients were calculated using R¹⁶. Analysis of variance (ANOVA) linear regression models were used to establish p-values (<0.05 deemed as significant correlation), and for non-linear models a lack-of-fit test was performed, where a significant p-value (<0.05) indicated the model 'lacked fit' to the data.

1.6 Microbial Community Analysis

DNA was extracted from biofilm biomass directly on the anode material and non-polarised sludge from the reactor using a PowerSoil DNA kit (Mo Bio Laboratories). For amplification of the V4-V5 region of 16S rRNA genes present in the extracted DNA, barcoded V4f and non-barcoded V5r primers were used with PCR master mix (MegaMix-Blue; Cambio). PCR was performed for one cycle at 95°C (04:00 mins), 30 cycles at 95°C (01:00), 55°C (00:45), 72°C (01:00) and a final cycle at 72°C (10:00). PCR-amplified 16S rRNA gene fragments were purified twice using Agencourt AMPure XP beads following the manufacturer's instructions (Beckman Coulter). A pooled amplicon library was created by combining 26 pmol/dm³ amplified 16S rRNA gene fragments from each sample and clonal amplification on microbeads was achieved by emulsion PCR. Microbeads were loaded onto an Ion 316 microchip and DNA was sequenced on an IonTorrent Personal Genome Machine using a Hi-Q kit (Thermo Fisher Scientific, USA).

16S rRNA gene sequence data were analysed using the QIIME software package¹⁷. Operational taxonomic units (OTUs) were picked using the uclust method¹⁸ with reference to the Greengenes database (at 97% identity), followed by de novo picking for unmatched sequences. Taxonomic assignment was performed on representative sequences using the RDP classification algorithm¹⁹. Sequences were aligned to a PyNAST template and filtered. The ribosomal RNA operon database (rrnDB) was used to adjust 16S rRNA gene abundance data with respect to 16S rRNA gene copy number in different organisms, to more accurately reflect the relative abundance of the population (>80% confidence²⁰). Unifrac distance matrices

based on community composition were calculated and abundance-weighted for comparative analysis of the microbial communities.

values from each MFC stage and correlated with changes in BOD₅ until the current plateaued (Figure 3A). A linear response was observed in the range 60–360 (n=5), 240–540 (n=6) and 480–840 mg/l O₂ BOD₅ (n=8) for the first, second and third

Figure 2: Average current density and summed current density response of three-stage MFCs to different BOD₅ (estimated from GGA concentrations) at flow-rates of 0.52 ml/min (0–42 days) and 1.24 ml/min (35–62 days). Time 0 days is the beginning of the calibrated period. Shaded bands represent $\pm$ SD from triplicate MFCs per stage.

3 Results and discussion

3.1 Multi-stage MFCs connected hydraulically in series

The triplicate MFCs in the first stage of the hydraulic array (1A, 1B and 1C in Figure 1) were inoculated with effluent from pre-existing batch-fed MFCs; originally inoculated with activated sludge from a wastewater treatment plant (Tudhoe Mill, Northumbrian Water Ltd). Following stable operation, the cells in the second stage (2A, 2B and 2C) and third stage (3A, 3B and 3C) were installed after 154 and 211 days respectively. The experiments reported here were conducted following 691 days of operation. Prior to calibration the external resistance was changed from 953 Ω to 305 Ω corresponding to the resistance at peak power of each MFC (Figure S3). To calibrate the response of the MFC array as a BOD sensor, GGA concentrations were varied between 42.5–2000 mg/l (25–1199 mg/l O₂ BOD₅; Figure 2).

3.2 The sensing range of multi-stage MFC BOD sensors can be modified with flow-rate/HRT

3.2.1 BOD₅ calibration at 0.52 ml/min

Amperometric calibration curves of normalised average stable current densities from each medium cycle were plotted against BOD₅ values estimated from known GGA concentrations (Figure 3; normalised data was used to correct for performance differences between MFCs). A linear model was fitted to

stage MFCs respectively. The linear response of the second and third stages covered higher concentrations as a consequence of receiving residual BOD which had not been consumed in the upstream MFCs. The R^2 values were 0.85–0.97, SD_{Res} of 8–15% of I_{Max} and p-values of 0.0004–0.009 indicating statistically significant correlations.

Typically, the combined electrical output from the MFC array connected in series or parallel (rather than output from each MFC) is reported in application of multi-stage MFCs for electricity generation; as increased current densities have been observed²¹. However, in sensing applications, the output from each individual MFC provides valuable information about substrate consumption through the hydraulic array which would be inaccessible from a combined signal.

When the sum of the normalised current densities from each MFC stage was calculated for each logged data-point (Figure 2, Σ Stages), the fit of the linear model to the summed data exhibited an extended range (60–720 mg/l O₂ BOD₅ (n=12)), compared to the individual MFCs (Figure 3A). The coefficient of determination and precision were also improved (R^2 of 0.97; SD_{Res} of 14%; p of 2.6×10^{-9}), indicating a statistically significant correlation and high degree of linearity between BOD₅ and sensor output. The slope was 0.390%/(mg/l O₂ BOD₅) (% summed current density normalised to a 300% maximum) equating to an analytical sensitivity of 0.553 ($\mu A/cm^2$)/(mg/l O₂ BOD₅).

The Hill model was also fitted to the amperometric data from 0 mg/l up to the maximum BOD₅ concentration at which no

Figure 3: Amperometric calibration curve fitted with (A/C) linear and (B/D) Hill models (up to non-inhibitory substrate concentrations) of 'normalised' average stable current density against BOD₅ (estimated from GGA concentrations) at (A/B) 0.52 ml/min and (C/D) 1.24 ml/min for each stage and the sum of stages in the hydraulic array. Shaded areas represent the 95% prediction interval from model lines and error bars are ±SD from replicate MFCs. K_M in units of mg/l O₂ BOD₅.

decrease in current density was observed (at high BOD₅ concentrations inhibition occurred due to substrate excess). The non-linear range for the first stage MFCs was 60–720 mg/l O₂ BOD₅ ($n=12$); whereas no inhibition was observed with the other stages and the entire calibration range was fitted (60–1199 mg/l, $n=14$). The Hill model increased the sensor range by successfully simulating the non-linear asymptotes of both the initial low signal and saturation parts of the calibration (Figure 3B). The fitted v_{Max} values (100–103%) were close to 100% and the half maximal concentrations (K_M) were 159, 422 and 675 mg/l for the first, second and third stages respectively, concurrent with the trend observed in current generation. Positive Hill coefficients were calculated for each stage with values of 3.2, 7.0 and 8.8. The increasingly positive values indicated an increasingly positive cooperativity in downstream MFCs; logical as the third stage MFCs fed from effluent of the upstream MFCs. In comparison to the Michaelis-Menten (M-M) model (Figure S5), the Hill model calibrations (an extension of M-M model incorporating the cooperativity coefficient, h) had smaller

prediction bands (Figure 3B) and therefore could resolve values more accurately. The greater degree of correlation obtained with the Hill model compared to the Michaelis-Menten model was also evident from the regression statistics (Hill; $R^2 = 0.94$ – 0.97 , $SD_{Res} = 7$ – 11% , lack-of-fit p -values = 0.61 – 0.97 ; Michaelis-Menten; $R^2 = 0.63$ – 0.88 , $SD_{Res} = 12$ – 22% , more significant lack-of-fit p -values = 0.08 – 0.34). The improved fitting with the Hill model indicating positive cooperativity could be explained by the syntrophic relationship between fermentative bacteria (oxidising glucose and glutamic acid) and electrogens (oxidising acetate produced by the fermentative bacteria; discussed further in Section 3.4).

3.2.2 BOD₅ Calibration at 1.24 ml/min Sensor calibration was repeated at a flow-rate of 1.24 ml/min to assess the effect on calibration. At this flow-rate the average step-up response time to reach stable current was 2.3 hours (ranging from 0.9–6.9 hours/1.2–9.5 HRT for increases of 35–1050 mg/l O₂ BOD₅) and average step-down response was 1.7 hours (0.9–3.8 hours/1.3–5.2 HRT for decreases of 120–720 mg/l O₂ BOD₅).

The step-up response time was approximately double the time at 0.52 ml/min and the step-down time was approximately one third the time. However, the response times were observed to be lengthened by previous starvation of downstream MFCs (Figure 2, Stage 3 current density) and additional time was required for recovery of the anodic biofilm's capacity for organic compound oxidation; especially at higher flow-rates due to the shorter HRT.

Maximum average stable current densities were determined as 122 ± 12 , 140 ± 13 and 123 ± 22 $\mu\text{A}/\text{cm}^2$ for first, second and third stage MFCs respectively. These values were used to normalise each dataset to account for electrochemical performance differences between MFCs. Despite the higher flow-rate and thus increased mass-transfer, the maximum current densities were almost identical albeit slightly less (11 – 16 $\mu\text{A}/\text{cm}^2$) than those observed at 0.52 ml/min. Anodic saturation resulted in average limiting anode potentials of -367 ± 23 , -369 ± 34 and -317 ± 35 mV vs. Ag/AgCl for the first, second and third stage cells

The linear models (Figure 3C) correlated well with measured BOD_5 values ($R^2 = 0.94$ – 0.98 , $\text{SD}_{\text{Res}} = 5$ – 20% , $p = 3.82 \times 10^{-14}$ – 1.74×10^{-9}). The slope of the summed current densities ($1.078\% / (\text{mg}/\text{l O}_2 \text{ BOD}_5)$) equated to analytical sensitivity of 1.376 ($\mu\text{A}/\text{cm}^2$) / ($\text{mg}/\text{l O}_2 \text{ BOD}_5$); 2.5-fold greater than that at 0.52 ml/min (a 2.5-times slower flow-rate). The Hill equation also fitted the calibration data ($n=29$; Figure 3D; $R^2 = 0.92$ – 0.97 , $\text{SD}_{\text{Res}} = 6$ – 22% , $p = 0.12$ – 0.76 for lack-of-fit).

The linear BOD_5 detection ranges were 25–180 ($n=14$), 60–240 ($n=14$) and 120–420 $\text{mg}/\text{l O}_2$ ($n=16$) for the first, second and third stages respectively, and 60–300 $\text{mg}/\text{l O}_2 \text{ BOD}_5$ ($n=16$) for the summed current density. The corresponding Hill-modelled K_M values were 102, 174 and 264 mg/l for the MFC stages 1–3. The correlations indicated that decreasing HRT decreased the linear detection range of the MFC sensor array. The fitted Hill coefficients were $h = 3.12$, 5.06 and 5.28; less than the corresponding values at 0.52 ml/min, indicating that the dependence of the downstream MFCs was reduced.

Figure 4: Predicted BOD_5 against estimated BOD_5 (from known GGA concentration calibrated to BOD_5 test values) for values predicted by the linear models using current densities obtained during calibration at (A) 0.52 ml/min and (B) 1.24 ml/min. A linear regression line and 95% prediction band is shown for the 'Σ Stages' predicted values. $y = x$ is shown as the 'ideal' prediction. Error bars represent standard error of prediction.

respectively (0 – 25 mV more positive than at 0.52 ml/min; Figure S4). ANOVA comparing average stable values from each stage at each flow-rate indicated there were statistically significant differences between the maximum current densities (p -values = 0 – 10^{-109}), whereas the differences between anode potentials were less/not significant (p -values = 10^{-83} – 0.95). These differences could therefore potentially be attributed to cathodic catalyst degradation and/or membrane biofouling, which occurred over the 5 months since installation. It is known that catalyst degradation and membrane biofouling can limit the maximum current which can be generated in a MFC²².

At 1.24 ml/min and BOD_5 concentration of 1199 $\text{mg}/\text{l O}_2$ (2000 mg/l GGA), current decrease due to substrate excess inhibition was observed in every MFC (Figure 2, day 59). Simultaneously, biomass sludge was observed to accumulate and samples were collected for microbial community analysis. With the same BOD_5 concentration at 0.52 ml/min, current decreases resulting from substrate excess were only observed in the first stage MFCs (Figure 2, day 24), indicating that higher flow-rates exposed downstream MFCs to excessive amounts of substrate.

3.3 A multi-stage MFC biosensor estimates BOD_5 with greater accuracy and precision than the standard BOD_5 test

At 0.52 ml/min, with 300 mg/l GGA (measured BOD₅ of 179.9 ± 9.4 mg/l O₂) the average stable current densities were 74.2, 85.2 and 3.0 µA/cm² for the sequential MFC stages with a summed current density of 85.9 µA/cm². Thus, the second and third

exhibited higher sensitivity (steeper linear fits; Figure 3C), resulting in a more accurate BOD₅ prediction from input current densities. The linear prediction model (Figure 4, purple lines) for both flow-rates was close to ideal ($y = x$) with $m = 1.000$ (0.52

Figure 5: (A–C) Weighted Unifrac principal coordinate analysis of communities analysed with polarised (purple) and non-polarised (yellow) samples highlighted. (D) Copy number-corrected, relative abundance of bacterial genera obtained from Ion Torrent sequencing of 16S rRNA genes from MFC Stage 1, 2 and 3 anode biofilms and sludge taken from first stage anode chambers fed 400 and 2000 mg/l GGA medium.

stages were inaccurate at predicting the BOD₅ value as the current was so low (the calibration models were unable to predict values below 240 and 480 mg/l O₂ respectively, Figure 4A). The linear models calibrated to the first stage and summed current density predicted BOD₅ values with mean percentage error (the difference between actual and fitted values as a percentage of actual BOD₅) of +2% and +4% respectively (+4 and +7 mg/l O₂; Figure 4A). For the same analyte at 1.24 ml/min, the sensor generated average stable current densities of 122.0, 58.0 and 10.5 µA/cm² for first, second and third stage MFCs and 190.4 µA/cm² for the sum of stages. The mean percentage error of prediction was -7% (-13 mg/l O₂; Figure 4B) for the summed current density. Therefore, the calibrated MFC response achieved better accuracy than the accepted standard deviation of the BOD₅ test (±15% (30 mg/l O₂)). Above the concentration at which the current densities plateau the linear prediction models were incapable of estimating BOD₅ values accurately. Larger errors of prediction were observed for calibrations based on the first, second and third stage MFCs at 0.52 ml/min as the models were calibrated with a smaller number of observations ($n < 12$). Moreover, calibrations at a higher flow-rate (which incidentally had $n > 12$ observations)

ml/min) and 0.957 (1.24 ml/min). The Hill model provided a convenient three-parameter description of calibrations but did not have the predictive power of the linear model. As current densities approached the plateau of V_{Max} the error of prediction by the Hill model increased as only small changes in current density (µA/cm²) separated large concentration differences (g/l) outside of the linear range (Figure S6).

3.4 Microbial communities from anodic biofilms and non-electrode associated biofilms indicate that the biosensor signal is generated from syntrophic interactions between fermenting bacteria and electrogens

Differences between communities from anodic biofilms from each MFC (sampled after 757 days of operation) and biomass from the non-anode associated sludge that accumulated (*i.e.* non-polarised) were evaluated by principal coordinate analysis (PcoA) and the relative, copy number-corrected abundance of bacteria at genus level was determined (Figure 5). The first three Principle Coordinates explained 93.1% of the total variance between samples. There was close clustering of replicate samples within two broad clusters representing the polarised and non-polarised samples; reflecting compositional

446 differences between the anode-associated communities and
 447 sludge. Along Principle Coordinate 1, the biofilm samples were
 448 positioned in order of MFC stage, with the third stage having
 449 the greatest distance from the non-polarised samples (Figure
 450 5B).
 451 A large proportion (24–50%) of the anode bacterial community
 452 comprised *Geobacter* spp. Organisms from an unclassified

genus within the *Porphyromonadaceae* increased from 5–12%
 through MFC stages 1–3 (Figure 5D). Almost no *Geobacter* spp.
 or *Porphyromonadaceae* (< 0.2% and 1% respectively) were
 detected in the non-polarised samples, indicating these
 organisms were the likely electrogens responsible for MFC
 current generation²³. The anodic bacterial community also
 contained sugar fermenters (*Dysgonomonas* spp., *Lactococcus*

Figure 6: Comparison of linear range upper measurement limits reported in the literature of amperometric MFC-based sensors fed carbon-containing substrate to the anodic chamber (in units of mg/l of respective validation method; C for DOC, O₂ for BOD₅ and COD, or Sub (substrate)). Bars for each study are coloured by the validation method(s) employed by each author. The carbon-containing substrate used for each calibration is listed in square brackets at the end of each bar. Full corresponding data table is Table S1 in Supplementary material.

spp. and members of *Enterobacteriaceae*) and amino acid fermenters (*Anaeromusa* spp., unclassified *Comamonadaceae* and *Tolomonas* spp.) which were likely responsible for the degradation of glucose and glutamic acid to substrates utilised by the electrogens^{24,25}. Some of the fermentation products (including lactate, acetate, propionate and ethanol) could be further degraded (by *Anaeromusa* spp. and *Desulfovibrio* spp.) to produce additional acetate^{26,27}. This indicated there were syntrophic interactions between a range of fermentative bacteria and electrogens on the MFC anode. A more complete discussion of the anodic microbial communities is presented in Supplementary material (Section S4 and Figure S7).

The 400 mg/l GGA sludge had a high relative abundance of *Lactococcus* spp. and *Tolomonas* spp. which were likely responsible for fermentation of glucose and glutamic acid respectively^{28,29}. The 2000 mg/l GGA sludge was completely dominated by *Lactococcus* spp. (>88% relative abundance) indicating a high occurrence of glucose-to-lactate conversion. At this concentration, the pH 7-buffered medium was observed to decrease but only to pH 6.46. Interestingly, when *Lactococcus* spp. dominated, there was a decrease in MFC current output, which may have been due to substrate excess inhibition of the anodic biofilm. Alternatively, the non-anode associated fermenters may have been more effective competitors for glucose than the electrogenic biofilm.

3.5 Improved performance of a multi-stage, MFC-based BOD sensor relative to previously reported single-stage sensors

30 11 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 8 48 49 50 51 52 53 54 55 56 57 58 59 60 7 61

An external validation method must be used to confirm that a sensor can accurately measure BOD. It is noted that previous cross-study comparisons of MFC-based BOD sensors have been conducted without full consideration of the validation methods used⁶². It is evident that the BOD₅ test was used for the sensor validation in less than 50% of the sensor articles published (Figure 6). This was likely due to the time-consuming nature of measuring samples; the very reason these sensors are being developed. Validation was also done by calibrating against the COD, dissolved/assimilable organic carbon (DOC/AOC) or substrate concentration; measurements which, to a varying degree dependent on substrate, did not provide an accurate representation of the BOD₅ value. This discrepancy is further complicated by authors using the term “BOD concentration” to reference substrate or COD concentrations interchangeably with BOD₅ values in unspecified units of mg/l.

The widest amperometric range of a MFC sensor previous to this study had an upper limit of approximately 340 mg/l BOD₅, although the variety of validation methods makes valid cross-study comparisons difficult. It is shown in Figure 6 that once the validation method is taken into account (e.g. based on assumed BOD₅/COD ratio of 0.5⁶³) a large number of studies with single-stage MFC sensors achieve approximately the same amperometric upper limit of around 250 mg/l O₂ BOD₅ and recent developments have done little to advance this. Compared to previously reported single-stage sensors it is evident that the novel multi-stage sensor presented in this study is capable of measuring organic loading to significantly

higher concentrations up to 720 mg/l BOD₅ (504 mg/l DOC, 1175 mg/l COD and 1250 mg/l GGA) (Figure 6).

It is also apparent that an additional benefit of multi-stage sensors over single-stage sensors is the potential to distinguish a signal decrease caused by a BOD decrease from that caused by the presence of toxic chemicals. When BOD decreases, the signal decrease occurs in the final MFC in the array first, whereas a toxicant presence would affect the first MFC before those downstream. Thus, the ordered response could distinguish toxic events from a decrease in BOD.

4 Conclusions

This study has demonstrated a proof-of-concept configuration for extending the dynamic range of MFC-based BOD sensors. With the GGA-based synthetic wastewater medium employed in the present study anode biofilms were enriched with electrogenic bacteria, *Geobacter* spp. and organisms from the *Porphyromonadaceae*. Range extension was achieved as the multi-stage array enabled utilisation of unconsumed substrate in downstream MFCs. With a three-stage MFC array the range was extended approximately 2 times further than was possible with a single-stage MFC, with linear calibration achieved up to 720 mg/l O₂ BOD₅. The biosensor range could be ‘tuned’ by increasing the flow-rate resulting in an increased BOD sensitivity at the expense of decreased detection range (desirable for low strength effluents). Whereas, variations in the operational current density were normalised to the maximum current density (under high BOD conditions) which did not alter the dynamic sensing range and enabled cross-comparisons between MFCs. Compared to the standard five-day BOD test MFC-based BOD sensors permit a step-change improvement with response detected in minutes and stabilised in less than 2.5 hours.

Future improvements which benefit single-stage MFCs could easily be applied to multi-stage MFCs and the modular design facilitates hydraulic optimisation because additional MFC stages can be connected to fully consume the substrate. Further optimisations could include development of an enhanced cell architecture for sensing that overcomes issues with sludge accumulation. In addition, the reactor volume could be investigated to determine the optimum electrode area: volume ratio favouring increased consumption by the upstream MFCs resulting in further extension of the sensing range. There is an opportunity to use multi-stage MFCs as BOD sensors for monitoring high strength wastewaters (until substrate becomes inhibiting), without requiring amendment, dilution or aeration. However, when fed real wastewater the sensor performance will be affected by conductivity, temperature, pH, dissolved oxygen and the composition of the BOD. These factors will all need to be accounted for when applying MFC-based BOD sensors in the field.

Conflicts of interest

There are no conflicts to declare.

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