Supplementary Material

S1. Medium preparation:

10 ml/l macro-nutrient solution (containing 28 g/l NH_4Cl, 10 g/l $MgSO_4.7H_2O$ and 0.43 g/l $CaCl_2$), 2 ml/l micro-nutrient solution (containing 2 g/l $FeCl_2.4H_2O$, 1 g/l $CoCl_2.6H_2O$, 1 g/l $NiCl_2.6H_2O$, 0.5 g/l $MnCl_2.4H_2O$, 0.105 g/l Na_2SeO_3, 0.07 g/l $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.05 g/l $ZnCl_2$, 0.05 g/l H_3BO_3, 0.04 g/l $CuCl_2.2H_2O$ and 2 ml/l HCl (35%)) and 1 ml/l vitamin solution (1 g/l pyridoxine hydrochloride, 0.5 g/l nicotinic acid, 0.25 g/l riboflavin, 0.25 g/l thiamine hydrochloride, 0.2 g/l biotin, 0.2 g/l folic acid, 0.01 g/l vitamin B12). The medium was buffered at pH 7 using 50 mM potassium phosphate buffer (containing 2.88 g/l KH_2PO_4 and 5.03 g/l K_2HPO_4).

90% of the H_2O, phosphate buffer were autoclaved in the medium bottles with tubing (upstream of the UV lamp) and glass drip chambers attached for 30 minutes at 121°C. Remaining medium components were dissolved in rest of H_2O and filter sterilised (0.2 µm) into the sterile medium bottle and mixed via N_2 sparging for 10 minutes.

S2. Analytical Methods further details:

BOD_5 test:

1 ml/l of phosphate buffer (pH 7.2, containing 8.5 g/l KH_2PO_4, 21.75 g/l K_2HPO_4, 33.4 g/l Na_2HPO_4 and 1.7 g/l NH_4Cl), $MgSO_4$ (22.5 g/l), $CaCl_2$ (27.5 g/l), $FeCl_3$ (0.25 g/l) and allylthiourea (2 g/l ATU, a nitrification inhibitor) was added to oxygen-saturated, deionised (DI) water and inoculated with settled sewage to prepare dilution water for the test. BOD_5 was calculated as BOD_5 (mg/l O_2) = $((D_1 – D_2) – (S.V_S)) / P$ where D_1 is the initial DO reading

(mg/l), D_2 is the average final DO reading (mg/l), S is Δ DO of the blank (mg/l) / volume of seed added (l), V_S is the volume of seed per bottle (l) and P is 1/dilution factor (APHA, 1999).

COD test:

Chemical Oxygen Demand (COD) was determined from samples digested at 148 °C for 2 hours using a potassium dichromate-based photometric test kit and absorbance values were measured using a Spectroquant Pharo 300 spectrophotometer (Merck Millipore).

DOC test:

Samples which had been passed through a 0.2 µm filter were tested using a TOC 5050A Total Organic Carbon analyser (Shimadzu, Japan) to determine dissolved organic carbon (DOC) concentrations. 7 ml 1/5 diluted samples were added to a vial in an ASI-5000A autosampler and portions were measured simultaneously for total carbon (TC, mg/l) and inorganic carbon (IC, mg/l). Total (dissolved) organic carbon was calculated using TOC, mg/l = TC – IC.

S3. Likely properties of the most abundant OTUs in the multistage MFC biosensor:

Three OTUs of *Geobacter* spp. (MF979786, MF979802 & MF979784) were on average present at 1%, 1% and 45% relative abundance in the flow-mode electrode samples analysed in the present study. *Geobacter* are often identified in acetate-fed MFCs and have been associated with direct electron donation to the electrode via conductive nanowires (Reguera et al., 2006). The three identified *Geobacter* spp. OTUs had > 99% 16S rRNA gene sequence identity to *Geobacter lovleyi* iso10-09 (AB795545.1), which has been described as capable of reducing various metals coupled to acetate oxidation but was unable to oxidise glucose (Sung et al., 2006).

The unclassified *Porphyromonadaceae* from the present study (MF979795) was found at 5% relative abundance on anodes. The OTU had 99% gene sequence identity to an uncultured

bacterium found in an acetate-fed MEC anode (JX462549.1); indicating it may have a role in electrogenesis. Additionally, the OTU had 98% identity to a bacterial strain of *Petrimonas sulfuriphila* Marseille-P1901 (LT558828.1); reported to perform fermentation of glucose and lactate to acetate using sulfur and nitrate as terminal electron acceptors (Grabowski et al., 2005).

Dysgonomonas spp. is also a member of the *Porphyromonadaceae* family but was observed mainly on first stage anodes and 400 mg/l sludge. The *Dysgonomonas* sp. OTU (MF979788) had 99% 16S rRNA gene sequence identity to *Dysgonomonas oryzarvi* Dy73 isolated from a peptone/starch/fish extract-fed MFC bioanode (Kodama et al., 2012). *D. oryzarvi* has been observed to produce lactate and acetate as major products from glucose fermentation.

Less dominant genera found almost exclusively on the polarised electrodes were unclassified *Comamonadaceae* (3–17%) which also increased down the flow series. The abundant *Comamonadaceae* OTUs (MF979790 and MF979789) were found to be 99% similar to *Acidovorax caeni* T-X2D from a garden pond (KU355878.1), a denitrifying bacterium observed to assimilate glutamic acid and VFAs to products including formic and propionic acids (and tested negative for glucose utilisation; Heylen et al. (2008)).

Anaeromusa spp. was found on polarised electrodes and sludge at relative abundance of 1–7%. *Anaerovibrio burkinabensis* DSM 6283 was a highly similar strain (99%; NR_025298.1) which has been associated with fermenting glutamate and lactate to acetate and propionate (Ouattara et al., 1992); indicative of the methylaspartate-based pathway. *Anaeromusa* spp. is a member of the *Clostridiales* order which have previously been associated with that pathway (Buckel, 2001).

Desulfovibrio spp. were common to both polarised and non-polarised electrodes. The most abundant OTU (MF979796) was similar to an uncultured bacterium from a bioreactor fed glucose (KC179078.1) and *Desulfovibrio simplex* DSM4141 (NR_117110.1); a sulfate-reducing bacterium. Stams and Hansen (1984) described how *Desulfovibrio* spp. were able to consume hydrogen present at low concentrations to enhance the rate of glutamate fermentation by *Acidaminobacter hydrogenoforman* (a member of the *Clostridiales* order) to produce acetate and propionate via a methylaspartate intermediate.

Tolumonas spp. was also found in low relative abundance (1%) on polarised electrodes but at 33 and 3% relative abundance on the 400 mg/l sludge and 2000 mg/l sludge samples respectively. *Tolumonas auensis* DSM 9187 (NR_074805.1) had 99% 16S rRNA gene sequence identity with the OTUs identified in samples from the present study and has been observed to produce toluene. When *T. auensis* was fed with glucose the major fermentation products were acetate, ethanol and formate (Tindall, 1996). This indicated that the genera (*Anaeromusa* and *Tolumonas*) were most likely involved in fermentation of glutamic acid and glucose respectively.

Of the non-polarised samples (sludge), members of the *Enterobacteriaceae* family were prevalent. Seven *Enterobacteriaceae* OTUs were present at more than 2% relative abundance in any sample; one OTU was identified from the genus *Trabulsiella*, another from the genus *Citrobacter* and the remaining five were unclassified. *Citrobacter* sp. (MF979801) and unclassified *Enterobacteriaceae* (MF979798) were found in the 400 mg/l sludge samples, similar (100% and 99% 16S rRNA gene sequence identity respectively) to *Citrobacter freundii* K6 (KX156769.1) and *Citrobacter amalonaticus* 4BeCh (KX355663.1).

Lactococcus spp. completely dominated the 2000 mg/l sludge samples, with relative abundances as high as 95% and was also found at 48% relative abundance in the 400 mg/l

sludge, which was more diverse. In the anode biofilms, *Lactococcus* spp. were observed to decrease in abundance from 19% to 6% down the hydraulic series (consistent with the trends observed in sludge accumulation). *Lactococcus raffinolactis* JCM 5706 (LC071827.1) was identified with 100% 16S rRNA gene sequence identity to the *Lactococcus* sp. OTU (MF979785) from the present study. *L. raffinolactis* has previously been isolated from raw cow's milk and wastewater tanks and is able to convert glucose and other sugars (including lactose and maltose) to lactic acid (Dworkin et al., 2006).

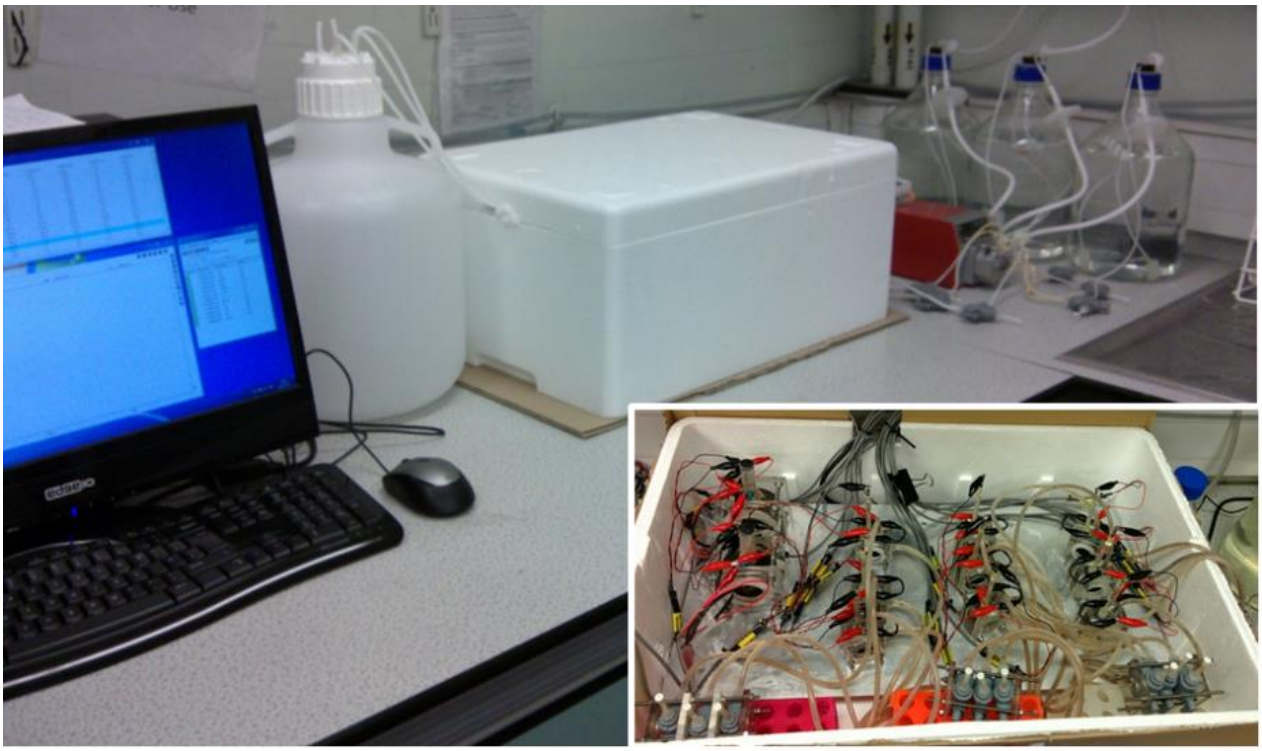

Figure S1: Photograph of multi-stage MFC flow system setup.

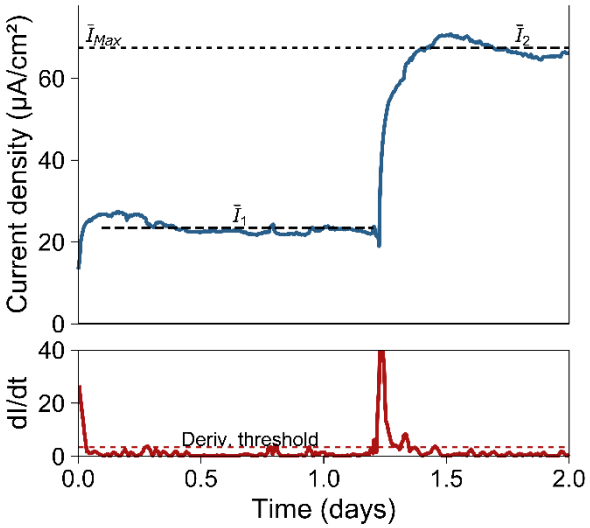

Figure S2: Current response chart annotated with average stable ($\bar{I}$) and maximum average stable ($\bar{I}_{Max}$) current densities, defined during the period in which the 1st derivative (dI/dt) fell below the Derivative threshold (set at 3%).

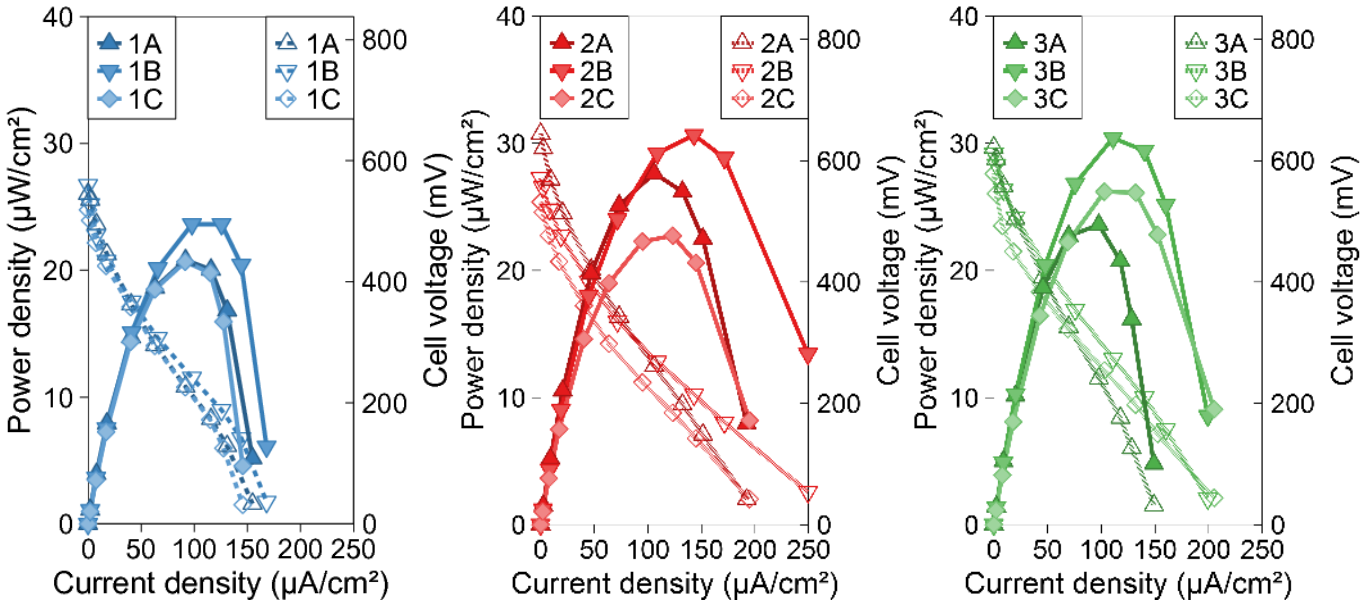

Figure S3: Polarisation and power density curves recorded on cells using 480 mg/l O_2 BOD_5 medium.

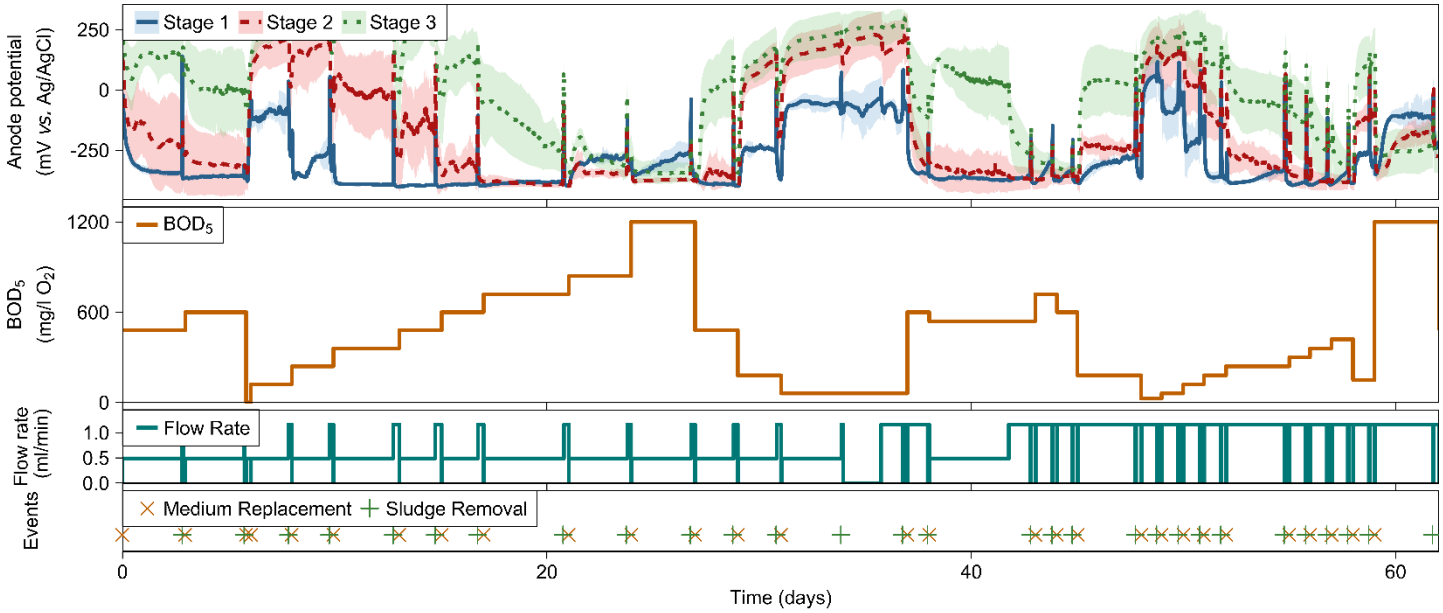

Figure S4: Average anode potential response of three-stage MFCs to different BOD_5 (estimated from GGA concentrations) at flow rates of 0.52 ml/min (0–42 days) and 1.24 ml/min (35–62 days). Time 0 days is the beginning of the calibrated period. Shaded bands represent ±SD from triplicate MFCs per stage. Events indicate occasions when medium bottles were replaced and sludge was removed from anodic chambers.

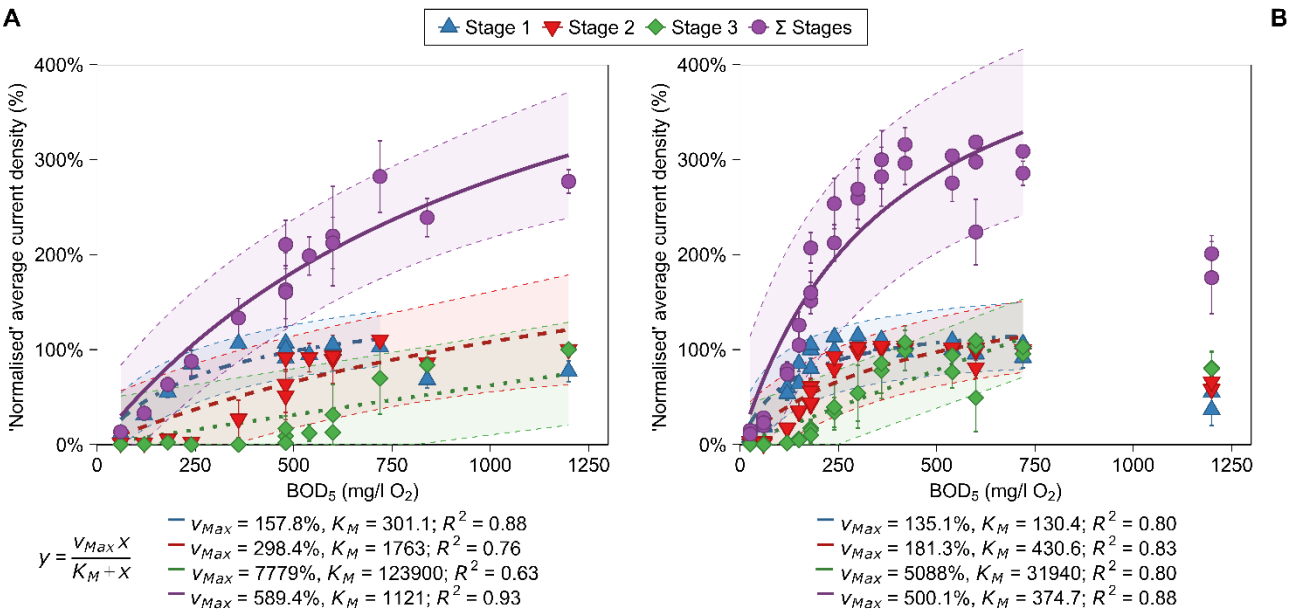

Figure S5: Calibration curve (poorly) fitted with Michaelis-Menten model of 'normalised' average stable current density against BOD_5 (estimated from GGA concentrations) at (A) 0.52 ml/min and (B) 1.24 ml/min for each stage and the sum of stages in the hydraulic array. Shaded bands represent the 95% prediction interval from model lines and error bars are ±SD from replicate MFCs.

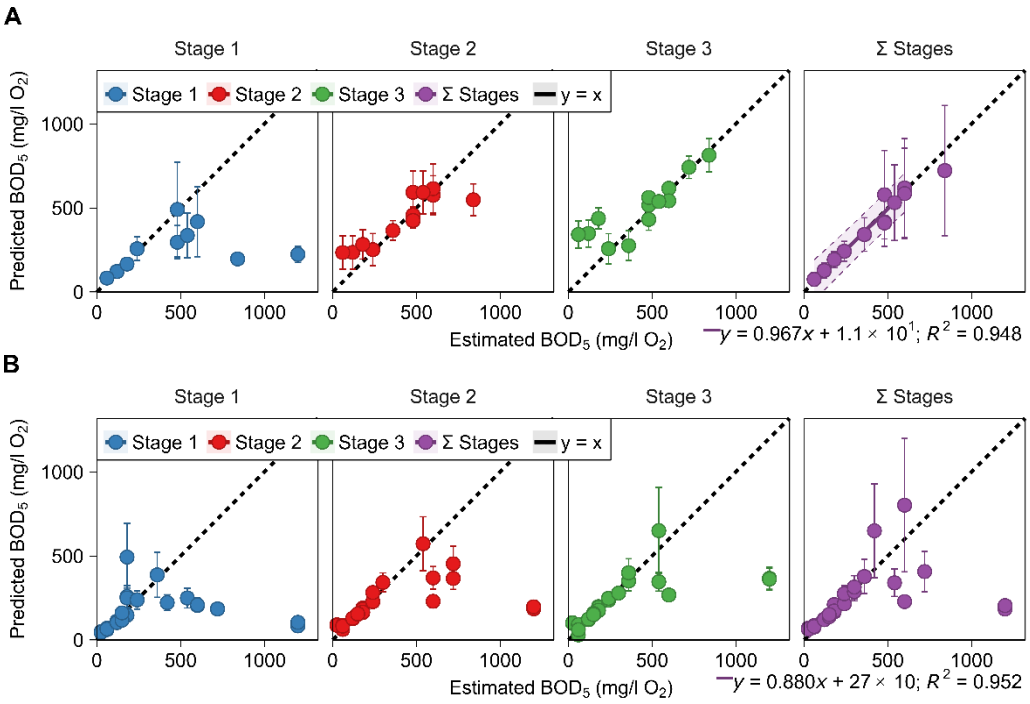

Figure S6: Predicted BOD_5 plotted against estimated BOD_5 (from known GGA concentration calibrated to BOD_5 test values) for values predicted by the Hill models using current densities obtained during calibration at (A) 0.52 ml/min and (B) 1.24 ml/min. A linear regression line and 95% prediction band is shown for the 'ΣStages' predicted values. $y = x$ is shown as the 'ideal' prediction. Error bars represent mean percentage error. Outliers with range between lower and upper prediction bounds above 1000 mg/l O_2 were removed as the error bars were outside the limits of the model.

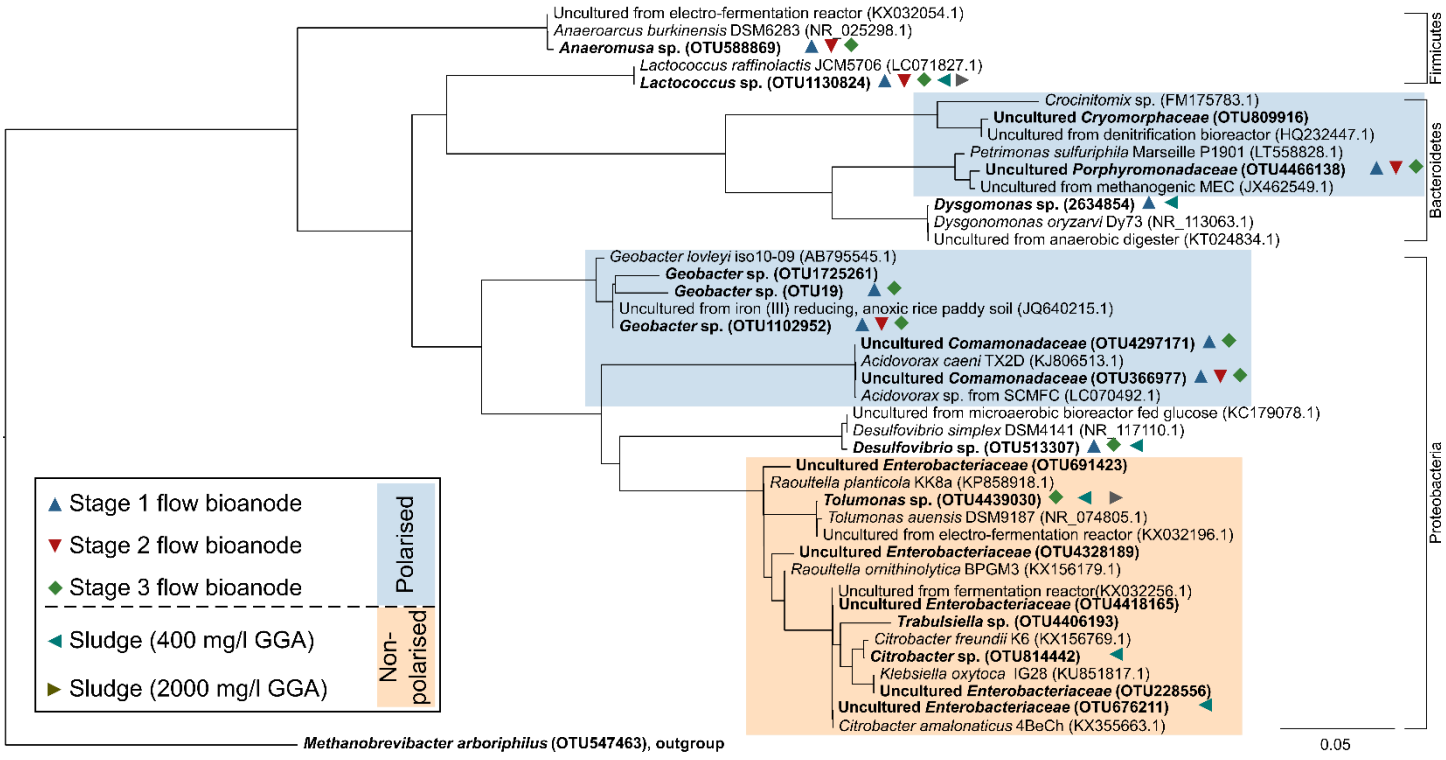

Figure S7: Phylogenetic tree of 16S rRNA gene sequences extracted from this study (bold) for electrode and sludge samples from multi-stage SCMFCs (symbols). Only sample OTUs which represented at least 2% of the total relative abundance are shown. Additional high-similarity

sequences are from the NCBI Nucleotide database collection (Accession number in brackets). Sequences exclusively (>2%) from polarised (blue) and non-polarised (orange) samples are highlighted. *M. arboriphilus* was used as an archeal outgroup. The scale bar indicates the number of nucleotide position changes.

Table S1: Summarised table of data from linear amperometric sensor upper detection limits reported in the literature represented in Figure 6.

Reference	MFC Type	Calibration Substrate	Validation Method	Upper detection limit (mg/l)
(Gil et al., 2003)	DCMFC	Starch WW	COD	50
(Kim et al., 2003)	DCMFC	Starch WW	BOD_5	25
(Kang et al., 2003)	DCMFC	GGA	COD	6
(Chang et al., 2004)	DCMFC	GGA	COD	100
(Moon et al., 2004)	DCMFC	GGA	COD	200
(Chang et al., 2005)	DCMFC	GGA	COD	113.5
(Moon et al., 2005)	DCMFC	GGA	BOD_5	20
(Kumlanghan et al., 2007)	DCMFC	GGA	Sub.	25,000*
(Di Lorenzo et al., 2009a)	SCMFC	Glucose	COD	350
(Di Lorenzo et al., 2009b)	SCMFC	Glucose	COD	250
(Peixoto et al., 2010)	SBMFC	Municipal WW	BOD_5	78
(Liu et al., 2011)	DCMFC	AD WW	COD	200
(Peixoto et al., 2011)	SBMFC	Municipal WW	BOD_5 / COD	78 / 118
(Zhang and Angelidaki, 2011)	SBMFC	Acetate	BOD_5	250
		Glucose	BOD_5	250
		Municipal WW	BOD_5	250
(Feng et al., 2013)	SCMFC	Acetate	COD	150
(Yang et al., 2013)	SCMFC	GGA	BOD_5	120
(Di Lorenzo et al., 2014)	SCMFC	Acetate	COD	164
(Ghangrekar, 2014)	DCMFC	Acetate	COD	212
(Hsieh and Chung, 2014)	DCMFC	Municipal WW	BOD_5	240
(Quek et al., 2014)	DCMEC	Acetate	Sub.	3
(Ayyaru and Dharmalingam, 2014)	SCMFC	Glucose	Sub.	750
(Tian et al., 2014)	DCMFC	Wastewater	BOD_5	50
(Liu et al., 2014)	DCMFC	OECD WW + Glucose	COD	100
(Quek et al., 2015a)	DCMEC	Yeast Extract	COD / Sub.	64 / 100
(Wu et al., 2015)	DCMFC	Acetate	COD / Sub.	200 / 275
(Quek et al., 2015b)	DCMFC	Acetate	DOC / Sub.	4 / 12
(Hsieh et al., 2015)	DCMFC	Glucose	BOD_5	235
		Methionine	BOD_5	235
		Acetate	BOD_5	235
		Glycerol	BOD_5	235
(Jia et al., 2016)	SBMFC	Starch	COD	3,000*
(Kretzschmar et al., 2016)	SCMFC	Acetate in WW	Sub.	410

Reference	MFC Type	Calibration Substrate	Validation Method	Upper detection limit (mg/l)
(Schievano et al., 2016)	SCMFC	Municipal WW	COD	25
(Li et al., 2016)	DCMFC	GGA	BOD_5	30
(Jiang et al., 2016)	DCMFC	Acetate	Sub.	57
(Jin et al., 2017)	DCMEC	VFAs	Sub.	8,804**
(Tardy et al., 2017)	DCMFC	Acetate	DOC	16
		Peptone	DOC	22
(Anam, 2017)	DCMFC	GGA	BOD_5	250
(Jiang et al., 2017)	DCMFC	Acetate	Sub.	820
(Kharkwal et al., 2017)	SCMFC	Acetate	BOD_5	343
		Municipal WW	BOD_5	178
(Franzetti et al., 2017)	SCMFC	Acetate	COD	100
(Spurr et al., 2017), *present study*	SCMFCs	GGA	DOC / BOD_5 / COD / Sub.	504 / 760 / 1,175 / 1,250

DC – Double Chamber; SC – Single Chamber; SB – Submersible; MFC – Microbial Fuel Cell; MEC – Microbial Electrolysis Cell.

* The reported ranges of Kumlanghan et al.(2007) and Jia et al. (2016) did not account for dilution due to injection of samples into large volume reactors and therefore were omitted from the figure.

**The reported range of Jin et al. (2017) was the nominal VFA concentration fed to the cathode chamber. However, < 1.5 mM VFA (2.5%) entered the anodic chamber of an abiotic control with nominal concentration of 60 mM VFA in the cathode chamber. Thus, only anode chamber-fed sensors were included in the figure to enable valid comparisons.

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