

1 The effect of start-up on energy recovery and compositional changes in brewery wastewater in  
2 bioelectrochemical systems

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## Abstract

Start-up of bioelectrochemical systems (BESs) fed with brewery wastewater was compared at different adjusted anode potentials (-200 and 0 mV vs. Ag/AgCl) and external resistances (50 and 1000  $\Omega$ ). Current generation stabilized faster with the external resistances (9 $\pm$ 3 and 1.70 $\pm$ 0.04 A/m<sup>3</sup> with 50 and 1000  $\Omega$ , respectively), whilst significantly higher current densities of 76 $\pm$ 39 and 44 $\pm$ 9 A/m<sup>3</sup> were obtained with the adjusted anode potentials of -200 and 0 mV vs. Ag/AgCl, respectively. After start-up, when operated using 47  $\Omega$  external resistance, the current densities and Coulombic efficiencies of all BESs stabilized to 9.5 $\pm$ 2.9 A/m<sup>3</sup> and 12 $\pm$ 2%, respectively, demonstrating that the start-up protocols were not critical for long-term BES operation in MFC mode. With adjusted anode potentials, two times more biofilm biomass (measured as protein) was formed by the end of the experiment as compared to start-up with the fixed external resistances. After start-up, the organics in the brewery wastewater, mainly sugars and alcohols, were transformed to acetate (1360 $\pm$ 250 mg/L) and propionate (610 $\pm$ 190 mg/L). Optimized start-up is required for prompt BES recovery, for example, after process disturbances. Based on the results of this study, adjustment of anode potential to -200 mV vs. Ag/AgCl is recommended for fast BES start-up.

## Keywords

Adjusted anode potential, brewery wastewater, external resistance, microbial fuel cell, start-up protocol, process recovery

## 1. Introduction

Brewing industry produces on average 5.5 L of wastewater per 1 L of produced beer [1]. As the world wide beer production in 2014 was almost 200 Mm<sup>3</sup>, the volume of produced brewery wastewater was close to 1000 Mm<sup>3</sup> [1,2]. Brewery wastewaters are concentrated (with chemical oxygen demand (COD) of 2000-6000 mg/L) and biodegradable (the ratio of biological oxygen demand (BOD) and COD being ~0.6), with pH close to neutral [3,4]. Brewery wastewaters are traditionally treated using aerobic, energy consuming biological processes such as activated sludge either in centralized municipal wastewater treatment plants or on-site [3,5]. Due to the high chemical energy content of the wastewater, biological treatments for energy recovery are of interest. For example, Chen et al. [6] produced methane (0.31 L CH<sub>4</sub>/g-COD<sub>removed</sub>) from brewery wastewater in a laboratory-scale membrane bioreactor, Zhuang et al. [7] reported 4.1 W/m<sup>3</sup> electricity production in a microbial fuel cell (MFC) with over 85% COD removal, and Estevam et al. [8] hydrogen production of 0.80-1.67 mol-H<sub>2</sub>/mol-glucose.

MFCs are bioelectrochemical systems (BESs) in which organic compounds are oxidized and their chemical energy converted directly to electrical energy [9]. MFCs are promising for the pretreatment of brewery wastewater due to the high carbohydrate and low ammonium concentrations, while meeting the clean-up requirements set for of brewery wastewater likely needs combining bioelectrochemical pretreatment with aerobic or anaerobic post-treatment [3,10]. Recent studies with MFCs indicate that electricity production from wastewaters (0.097

56 kW/m<sup>3</sup>) can partially or fully provide operating energy for the bioelectrochemical wastewater  
57 treatment (0.027 kW/m<sup>3</sup> needed for pumping) [10]. The advantages of MFCs in wastewater  
58 treatment compared to aerobic treatment are the low energy demand and the low excess sludge  
59 production [11]. Unlike anaerobic digestion, MFCs can successfully operate also at low-  
60 temperatures (20 °C and less) [12].

61 Wastewater composition varies highly between breweries [5] and, as brewing is a batch  
62 operation, the wastewater flow and composition fluctuate. Long interruptions in wastewater flow  
63 and washing detergents, for example, may change and inhibit microbial communities of BESs. A  
64 prompt recovery after disturbances is required to meet the overall treatment requirements [13].  
65 However, start-up of BES is often time consuming and for this reason, several approaches for  
66 start-up have been studied. Using bioelectrochemical enrichment culture from a BES operated  
67 under similar conditions transferred as biomass from the biofilm or anolyte solution is  
68 considered as the fastest method for start-up [14–16]. Enrichment cultures can also be stored at +  
69 4 °C as an anticipation for process disturbances [17]. Alternatively, anaerobic digester sludge  
70 [18–20], a combination of anaerobic sludge and enrichment culture [16], wastewater [10,14,21],  
71 rumen contents [22], sediment [22], or even activated sludge [20] can be used as the inoculum  
72 for start-up. The enrichment of electrochemically active microbial cultures can be supported  
73 electrochemically or chemically, e.g., with poised anode potential (stable or varied by maximum  
74 power point tracking method) [23,24] or suppressing methanogenesis by starvation [25], adding  
75 2-bromomethanesulfonate [18], or by inducing oxygen stress [18]. However, the reports of using  
76 poised anode potential are contradictory [14,26]. Some studies report the highest current  
77 densities during the start-up with a more negative anode potentials [14,23] while other BESs  
78 performed best after more positive anode potentials [27,28]. Also, when BESs are started up  
79 using external resistance, some studies suggest that high external resistances (e.g. 1000 Ω) result  
80 in fast current production (compared to 100 Ω external resistance) [21], while others favored  
81 lower resistances (e.g. 5 Ω) to enable higher bacterial growth rates and current densities [29,30].  
82 According to Logan et al. [31] the highest power densities can be obtained when the external  
83 resistance equals the internal resistance of the Ohmic resistance limited MFCs.

84 The efficiency of start-up depends on multiple factors such as the inoculum source, anode  
85 potential or external resistance, substrate, temperature, reactor design and electrode material  
86 [32]. The effect of start-up protocol on electricity generation under equal conditions after the  
87 start-up phase has been studied using acetate [33] or glucose [34] in well buffered media, but not  
88 with real wastewaters. With acetate, it was demonstrated that biofilm of a BES which was  
89 originally operated with a high external resistance, adapted to a low external resistances, which  
90 eliminated power overshoot [33]. With glucose, the start-up with +200 mV vs. Ag/AgCl  
91 accelerated the start-up more than 1000 Ω external resistance [34]. In addition to using direct  
92 electricity precursors (acetate) or other model compounds in start-up, studies with real  
93 wastewaters consisting of complex organic constituents are needed. Anaerobic treatment of real  
94 wastewaters requires sequential and interdependent reactions carried out by diverse anaerobic  
95 microbial community consisting of hydrolytic, fermentative as well as those donating electrons  
96 to the solid anode electrode [35]. Furthermore, start-up should be studied in less buffered  
97 systems. For this reason, the effect of start-up on subsequent electricity generation should be  
98 elucidated also with real wastewaters.

99 The purpose of this study was to optimize the BES start-up by comparing different poised anode  
100 potentials and external resistances, and to evaluate the influences of the different start-up

101 protocols on subsequent power production from brewery wastewater in MFC mode. The changes  
102 in wastewater composition and the biofilm formation on anode electrode were also determined.  
103 To the authors' knowledge, the effects of different poised anode potentials and external  
104 resistances on start-up and subsequent performance under equal conditions have not been  
105 previously studied with real wastewaters.

106

## 107 2. Materials and methods

108

### 109 2.1 BES construction and operation

110 Experiments were conducted in eight air-cathode BESs previously described by Cetinkaya et al.  
111 [19] with anode chamber volume of 123 mL. In this study, cation exchange membranes (CME  
112 7000; 41 cm<sup>2</sup>) were coated with Pt by spraying a mixture of 100 mg 20% Platinum on Vulcan  
113 XC-72R (E-TEK), 1 mL MQ, 3 mL isopropanol, 1 mL propanediol, and 0.807 mL 5% Nafion  
114 (Nafion<sup>®</sup> 117 solution, Aldrich) with an airbrush. This whole mixture was divided on eight  
115 membranes. Two carbon brush electrodes [36] were used as the anodes and two carbon cloth  
116 cathode electrodes (projected areas of 41 cm<sup>2</sup>) were sandwiched between the Pt coated  
117 membranes and supporting frames on the both sides of the anode chamber (Fig. S1). Both anode  
118 electrodes and cathode electrodes were connected in parallel with titanium wires to form a single  
119 circuit. Anolyte with total volume of 500 mL was circulated (80 mL/min) over a recirculation  
120 bottle placed in 37 °C water bath. The recirculation maintained the BES temperature at ca. 29-30  
121 °C. A reference electrode (BASi RE-5B Ag/AgCl) filled with 3 M NaCl (+204 mV vs. SHE at  
122 30 °C) was positioned between the anode electrodes (Fig. S1).

123 The experiments were divided to a start-up phase and to a follow-up operation in MFC mode.  
124 The BESs were inoculated with fresh anaerobic sludge (50 mL) collected from a local municipal  
125 wastewater treatment plant (Viinikanlahti, Tampere, Finland). During the start-up phase (first 42  
126 days), four different operational conditions were compared in duplicate BESs: operation with  
127 either 1000 Ω or 50 Ω external resistance, or anode potential adjusted to either 0 mV or -200 mV  
128 (against Ag/AgCl reference electrode). The BESs are hereafter referred to after the start-up  
129 protocols as: BES<sub>1000Ω</sub>, BES<sub>50Ω</sub>, BES<sub>0mV</sub>, and BES<sub>-200mV</sub>. During the follow-up operation in MFC  
130 mode (days 42-75), a 47 Ω external resistance was connected between anode and cathode in each  
131 of the BESs. The BESs were fed in 6-8 day intervals with diluted brewery wastewater (COD<sub>tot</sub>  
132 1410 ± 60 mg/L) buffered with NaHCO<sub>3</sub> (2 g/L).

133 To avoid the presence of inhibiting compounds, the wastewater was collected at the time of tank  
134 emptying from a local brewery. This brewery washes their lines and tanks with reusable acid and  
135 alkaline solutions and disinfect their tanks with peracetic acid, which will be oxidized on the  
136 surface of the tank. For the feedings, wastewater was mixed and divided to smaller batches to be  
137 stored at -20 °C to ensure constant quality at each feeding point. One wastewater batch was  
138 defrosted for every feeding. Wastewater characteristics (Table 1) were measured from one  
139 defrosted wastewater sample. During the start-up phase, feed solution COD was measured at  
140 every feeding point. After dilution with distilled water, the feed solution was flushed with  
141 nitrogen gas for 15 min to remove oxygen, and 330 mL of the buffered solution (NaHCO<sub>3</sub>  
142 dissolved in a mixture containing 70% wastewater and 30% distilled water) was inserted in a  
143 new recirculation bottle to replace the previous bottle to maintain the whole anolyte volume of

144 500 mL. Analyte pH was measured manually twice between feedings and adjusted to 7 with 5 M  
145 NaOH, if pH was below 6.5.

146

147 Table 1. Composition of the brewery wastewater and the BES feed, i.e. diluted brewery  
148 wastewater with NaHCO<sub>3</sub> supplementation.

	Wastewater <sup>a</sup>	Feed solution <sup>b</sup>
pH	7.98	8.6 ± 0.3
COD <sub>tot</sub>	1990 ± 80 mg/L	1410 ± 60 mg/L
COD <sub>s</sub>	1950 mg/L	1370 mg/L
BOD <sub>7</sub>	960 mg/L	670 mg/L
N <sub>tot</sub>	10.3 mg/L	7.2 mg/L
PO <sub>4</sub> <sup>3-</sup> -P	31.3 mg/L	21.9 mg/L
Ethanol	516 mg/L	359 mg/L
Alkalinity	4.7 mM	3.3 mM
Sugars <sup>c</sup>	610 mg/L	430 mg/L
NaHCO <sub>3</sub>		2.0 g/L
Conductivity	n.a.	2.53 ± 0.06 mS/cm

149 <sup>a</sup>Wastewater characteristics are reported as measured values; <sup>b</sup>Feed solution pH, COD<sub>tot</sub> and  
150 conductivity are measured values, NaHCO<sub>3</sub> as added concentration, and others as calculated  
151 values; <sup>c</sup>Sugars are presented as glucose equivalents; n.a. = not analysed

152

## 153 2.2 Analyses and calculations

154

### 155 2.2.1 Electrochemical measurements and calculations

156 Potentiostat (DropSens μSTAT8000) was used for adjusting the anode potential (0 mV or -200  
157 mV vs. Ag/AgCl) and measuring the current at 2 min intervals during the start-up phase so that  
158 anode electrodes were used as working electrodes, cathode electrodes as counter electrodes and  
159 reference electrode as a reference. All the anode potential values in the text are given as mV vs.  
160 Ag/AgCl. Cell voltages and anode potentials of the BESs operated in MFC mode with external  
161 resistances were recorded with Agilent 34970A data Acquisition/Switch Unit (Agilent, USA) at  
162 2 min intervals. Current was calculated according to Ohm's law ( $I=U/R$ ) and normalized against  
163 the anode chamber volume. To compare current production obtained during the different  
164 feedings, average current densities were calculated over each feeding cycle. Coulombic  
165 efficiency (CE) was calculated from the amount of COD (0.5 g) added during each feeding and  
166 electrical current data during the operation in MFC mode (days 56-63) according to Logan et al.  
167 [31]. Fed COD was used for the calculation of CE, as soluble COD was also released from the  
168 anaerobic sludge used as inoculum. Calculating the CE against removed COD would, thus  
169 overestimate the CE.

170 Linear sweep voltammetry (LSV) was done using a potentiostat (Palmsens3, Netherlands) for  
171 anode electrodes after the start-up phase (day 42) and in the end of the operation in MFC mode  
172 (day 71). Scan rate was 0.5 mV/s and the scan ranged from -550 mV to +200 mV (vs. Ag/AgCl).  
173 Measurements were started after 30 min stabilization in open circuit mode one day after the

174 previous feeding. The anodic LSV measurement was followed by whole cell LSV starting from  
175 5-50 mV above open cell voltage towards +5 mV at 0.2 mV/s scan rate. Internal resistance of the  
176 MFC was estimated from the whole cell LSV data ( $R_{\text{internal}} = U/I$ ) based on the current and  
177 voltage at the maximum power point [31].

178

### 179 2.2.2 Chemical analyses

180 Analyte samples were taken for chemical analyses from the circulation bottles three times a  
181 week. Samples were filtered through 0.2  $\mu\text{m}$  polyester filters and stored at  $-20\text{ }^{\circ}\text{C}$  for sugar and  
182 volatile fatty acid (VFA) analyses. Analyte pH and conductivity were measured from fresh  
183 samples with WTW pH 330 meter and WTW InoLab Level 1 Multimeter, respectively. Sugar  
184 concentration was measured as glucose equivalents using phenol-sulphuric acid method [37].  
185 Method was modified by decreasing the sample volume from 2 mL to 1 mL and reagent volumes  
186 from 1 mL to 0.5 mL (5% phenol solution) and from 5 mL to 2.5 mL (sulphuric acid). VFA  
187 (acetate, propionate, butyrate, isobutyrate, and valeric acid) and alcohol (ethanol and butanol)  
188 concentrations were measured with a gas chromatograph equipped with a flame ionization  
189 detector as described by Haavisto et al. [38].

190 Wastewater alkalinity was analysed according to the Finnish standard SFS 3005 by titrating the  
191 sample with 0.10 M HCl to pH 4.5.  $N_{\text{tot}}$  and  $\text{PO}_4^{3-}\text{-P}$  were measured using HACH LANGE kits  
192 (LCK 238 for  $N_{\text{tot}}$  and LCK 349 for  $\text{PO}_4^{3-}\text{-P}$ ) according to the manufacturer's instructions.  $\text{BOD}_7$   
193 was measured with WTW OxiTop measuring system from appropriately diluted samples  
194 according to the manufacturer's instructions (Application report O2 500231). COD was  
195 measured with the dichromate method according to the Finnish standard SFS 5504 (1988).  
196 Soluble COD ( $\text{COD}_s$ ) refers to values measured from filtered (0.2  $\mu\text{m}$ ) samples. Cumulative  
197 effluent acetate and propionate and fed COD (in section 3.3 and Figure S3) were calculated  
198 based on the sum of produced acetate and propionate (as COD equivalents) and sum of COD fed  
199 after the first feeding. Decreased anolyte volumes in the end of the feeding cycles due to  
200 evaporation ( $19 \pm 7\%$  per feeding) were considered when calculating the acetate and propionate  
201 accumulation. Acetate and propionate originated from the degrading organic components of  
202 anaerobic sludge (used as inoculum) and brewery wastewater. Acetate and propionate were  
203 converted to theoretical COD ( $1.0667\text{ gCOD/g}_{\text{acetate}}$  and  $1.5135\text{ gCOD/g}_{\text{propionate}}$ ) according to Van  
204 Haandel and Van der Lubbe [39].

205

### 206 2.2.3 Microbial culture analyses

207 Analyses for total microbial quantity and microbial community composition were conducted  
208 from anode biofilms in the end of the experiment (day 75). Biofilm was sequentially removed  
209 from the anode electrodes in laminar flow hood by mechanically scrubbing the carbon brush  
210 electrodes against each other as long as biomass was visibly detaching to a small volume (10-20  
211 mL) of sterile 0.9% NaCl solution in 50 mL Falcon tube. The overall solution with detached  
212 biomass was collected (80-120 mL) and concentrated by centrifuging ( $5000 \times g$ , 10 min).  
213 Centrifuged biomass samples (15 mL) were divided into microbial community samples (4 mL)  
214 and biomass samples (11 mL) and both samples were frozen ( $-20\text{ }^{\circ}\text{C}$ ). Mechanical scrubbing was  
215 selected as the biofilm separation method based on previous experiments. From the different  
216 biomass detachment methods used in the previous testing mechanical scrubbing resulted in

217 highest quantity of detached biomass and thus allowed the most reliable comparison of  
218 biomasses between different reactors.

219 Frozen biomass samples were freeze-dried (Christ Alpha 1-4 LD plus, Germany) and weighed to  
220 determine the quantity of the biomass. Bio-Rad Protein Assay (based on Bradford method) was  
221 used for measuring the protein mass on anode as bovine serum albumin (BSA) equivalents.  
222 Triplicate samples (approximately 0.01 g of dry sample mixed with 100  $\mu$ L distilled water) were  
223 analyzed according to the manufacturer's standard procedure instructions using UV-VIS  
224 spectrophotometer (Shimadzu UV-1700) for absorbance measurement. For protein mass  
225 calculation, the biomass dry weight was corrected by multiplying the mass with 1.36 to take into  
226 account the share of microbial community samples removed before freeze drying. Then the  
227 protein mass was calculated from the corrected biomass dry weights and the protein  
228 concentrations.

229 Microbial community was profiled as previously described by Haavisto et al. [38]. PowerSoil  
230 DNA isolation kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) was used for DNA  
231 extraction followed by partial 16S rRNA gene PCR amplification with GC-BacV3f [40] and  
232 907r [41] primers as described by Koskinen et al. [42]. DNA sequences were separated with  
233 denaturing gradient gel electrophoresis (DGGE) as described by Lakaniemi et al. [43],  
234 reamplified according to Koskinen et al. [42], and sequenced at Macrogen Inc. (Seoul, Korea).  
235 Analyzed sequence data (BioEdit software) was compared to known sequences with BLAST  
236 (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

237

### 238 3. Results and discussion

239

#### 240 3.1 BES start-up

241 BESs were fed in semi-continuous mode with brewery wastewater under the different start-up  
242 conditions and their performances were monitored as current densities (Fig. 1) and as linear  
243 sweep voltammograms (Fig. 2). During the first feeding, high peak currents were detected due to  
244 biological oxidation of organic compounds in the anaerobic sludge used as the inoculum. The  
245 lowest current densities were measured during the 2<sup>nd</sup> feeding, after which the current densities  
246 increased and the highest average current densities were obtained during the last (6<sup>th</sup>) feeding.  
247 The highest average current densities obtained with BES<sub>-200mV</sub> and BES<sub>0mV</sub> were  $76 \pm 39$  and  $44$   
248  $\pm 9$  A/m<sup>3</sup>, respectively (Fig. 1). With BES<sub>50 $\Omega$</sub>  (anode potential  $-463 \pm 14$  mV) and BES<sub>1000 $\Omega$</sub>   
249 (anode potential  $-480 \pm 6$  mV), the highest average current densities were  $9 \pm 3$  and  $1.70 \pm 0.04$   
250 A/m<sup>3</sup>, respectively. According to Wei et al. [44], higher anode potential should increase the  
251 current production because electrochemically active microorganisms gain more energy for  
252 growth, but only if those bacteria are able to utilize the anode at higher potential as an electron  
253 acceptor [26]. This can be affected e.g. by the available substrate [45] and the electron transfer  
254 mechanisms of the bacteria enriched in the biofilm [14,23]. Our results show higher current at  
255 lower anode potential of -200 mV compared to 0 mV. Also Aelterman et al. [46] reported higher  
256 current densities with -200 mV vs. Ag/AgCl adjusted anode potential (compared to 0 and -400  
257 mV) in an acetate-fed BES.

258 The current production stabilized faster in the BESs with the external resistances, but the  
259 obtained maximum average current densities were significantly lower (Fig. 1) than in the BESs

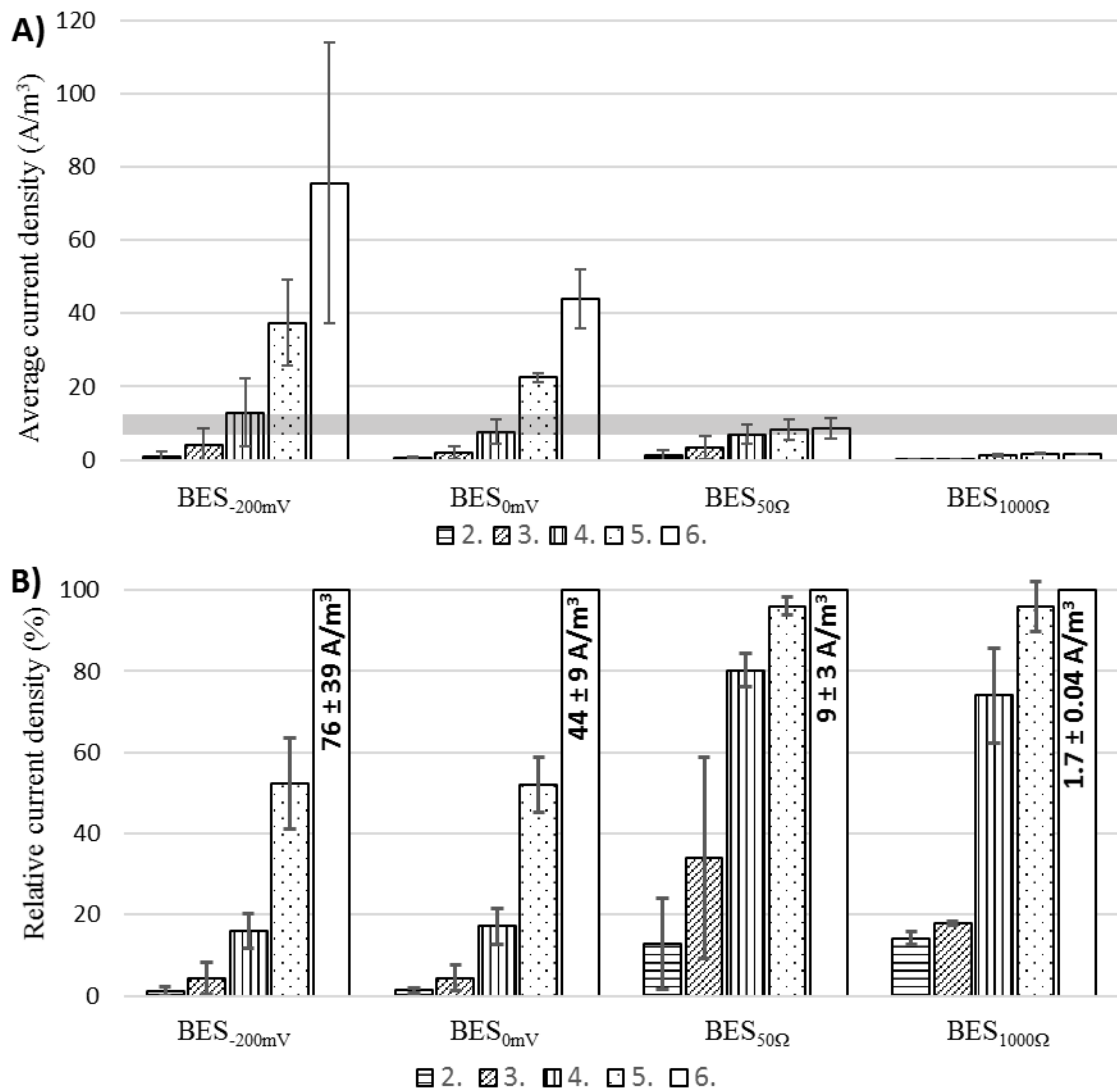
260 with the adjusted anode potential. In the last two feeding cycles, current densities increased by  
261 25 and 38 A/m<sup>3</sup>, 15 and 21 A/m<sup>3</sup>, 1.3 and 0.32 A/m<sup>3</sup>, and 0.37 and 0.068 A/m<sup>3</sup> in BES<sub>-200mV</sub>,  
262 BES<sub>0mV</sub>, BES<sub>50Ω</sub>, and BES<sub>1000Ω</sub>, respectively. This is in contrast to the results of Wang et al. [34]  
263 who reported faster start-up with adjusted anode potential (+200 mV) compared to 1000 Ω  
264 external resistance in terms of current production stabilization. In addition, Hong et al. [33] and  
265 Ahn et al. [21] reported faster current generation stabilization in terms of reproducible current  
266 cycles with higher external resistance, but in this study the current generation stabilization time  
267 was similar with high and low external resistances. The start-up phase was finished in the end of  
268 the 6<sup>th</sup> feeding once the current generation was stabilized with BES<sub>50Ω</sub> and BES<sub>1000Ω</sub> to allow the  
269 comparison of different start-up protocols after minimal start-up duration. The immediate  
270 increase in current density after feeding (Fig. S2) indicates, according to Carmona-Martinez et al.  
271 [47], that the start-up phase was sufficient for the development of a mature electrochemically  
272 active biofilm.

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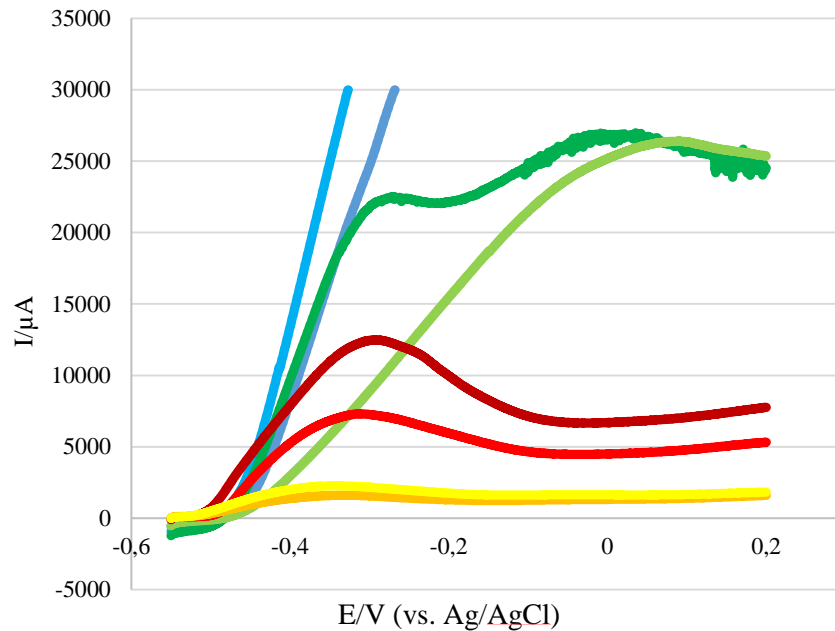
277 Fig. 1. Average current densities during the start-up of BESs fed in semi-continuous mode with  
 278 brewery wastewater. The bars show A) current densities ( $A/m^3$ ) of the feedings 2 – 6 and B) the  
 279 relative current densities (%) of the feedings 2 – 5 calculated against the average current  
 280 densities of the last (6<sup>th</sup>) feeding (=100%) for the different start-up protocols. Standard deviations  
 281 indicate the differences between duplicate reactors. The shaded area of current densities in A)  
 282 represents the stable current density ( $9.5 \pm 2.9 A/m^3$ ) obtained during the subsequent operation in  
 283 MFC mode after the start-up phase.

284

285 In the end of the start-up phase, the performances of the BESs were compared using anodic  
 286 LSVs (Fig. 2). The maximum current obtained in the LSV decreased in the same order, i.e. from  
 287 BES<sub>-200mV</sub>, BES<sub>0mV</sub>, BES<sub>50Ω</sub> to BES<sub>1000Ω</sub>, as did the highest average current densities obtained  
 288 during the last start-up phase feeding (Fig. 1). The highest current in the BES<sub>-200mV</sub> exceeded the  
 289 maximum current measurable with the potentiostat (30 mA), while with BES<sub>0mV</sub>, BES<sub>50Ω</sub> and  
 290 BES<sub>1000Ω</sub> the highest currents were 26.4, 9.9, and 1.9 mA (215, 80, and 15  $A/m^3$ ), respectively.  
 291 While power overshoot was observed at anode potentials of -340, -300 and 50 mV in the

292 BES<sub>1000Ω</sub>, BES<sub>50Ω</sub> and BES<sub>0mV</sub>, respectively, no power overshoot was detected with BES<sub>-200mV</sub>  
 293 (Fig. 2). Previous studies have shown overshoots at lower anode potentials and current values on  
 294 anodic cyclic voltammetry curves in the BESs operated with high external resistance [33] or  
 295 highly negative anode potential [48] due to the electroactive community not being able to  
 296 produce high currents under given conditions. LSV curves in this study indicate that earlier  
 297 adaptation of microbial cultures to higher current enabled higher current production on varying  
 298 anode potentials.

299



300

301 Fig.2. Anodic linear sweep voltammograms after operating semi-continuous brewery  
 302 wastewater fed BESs with the different start-up protocols for 42 d. The results with different  
 303 colors represent the duplicate reactors with studied start-up protocols: blue for BES<sub>-200mV</sub>, green  
 304 for BES<sub>0mV</sub>, red for BES<sub>50Ω</sub> and yellow and orange for BES<sub>1000Ω</sub>. The current (μA) values of  
 305 BES<sub>-200mV\_1</sub> and BES<sub>-200mV\_2</sub> exceeded the measuring range of the potentiostat (30 mA) with  
 306 anode potentials higher than -326 mV and remained at or above 30 mA at more positive anode  
 307 potentials.

308

### 309 3.2 Performance after start-up

310 After the start-up, all BESs were operated in MFC mode for 28 days with 47 Ω external  
 311 resistance (Table 2), which was close to the internal resistance (59 Ω) of the BES producing the  
 312 highest current (BES<sub>-200mV\_1</sub>) at the end of the start-up. The start-up protocol with adjusted  
 313 anode potential of -200 mV (BES<sub>-200mV</sub>) provided the highest average current density (10.6  
 314 A/m<sup>3</sup>) in the beginning of the follow-up operation period. The current density, however,  
 315 decreased to 9.7 A/m<sup>3</sup> towards the end of the operation (days 63-70). With BES<sub>1000Ω</sub>, after the  
 316 current densities of 1.7 ± 0.04 A/m<sup>3</sup> during start-up, the current density increased to 9.2 A/m<sup>3</sup>  
 317 (Table 2). At the end of the operation, the current densities were similar in all the BESs (9.2-9.7  
 318 A/m<sup>3</sup>) despite the different start-up protocols. The results are in accordance with Wang et al.

319 [34], who showed that after start-up protocols of +200 mV anode potential and 1000  $\Omega$  external  
 320 resistance, the cell voltages at same conditions (1000  $\Omega$  external resistance) became similar (641  
 321  $\pm$  4 mV after 11 days). The highest power density in our study ( $0.51 \pm 0.03$  W/m<sup>3</sup>) was lower  
 322 compared to other results obtained with brewery wastewater (1.3 W/m<sup>3</sup> [49] at pH 7 and 35 °C,  
 323 and 4.1 W/m<sup>3</sup> at 30 °C [7]). This was because our semi-continuous BES with air-cathode was not  
 324 optimal for electricity production, which is also indicated by the high VFA concentrations in the  
 325 effluent (see section 3.3). Higher power densities could likely have been obtained by choosing an  
 326 external resistance close to the internal resistance for each of the BES individually. The electrical  
 327 energy yield was 84 kJ/kg<sub>fed</sub> COD (calculated for 0.51 W/m<sup>3</sup> power density), which is slightly  
 328 lower compared to 110 and 120 kJ/kg<sub>fed</sub> COD obtained by Dong et al. [10] and Lu et al. [50] in 90  
 329 and 20 L brewery wastewater treating MFCs, respectively, but almost tenfold higher compared  
 330 to MFCs fed with liquid fraction of municipal solid waste after solid-liquid separation (8-9  
 331 kJ/kg<sub>removed</sub> COD) [51].

332

333 Table 2. The effect of different start-up protocols on internal resistance, current densities and  
 334 anode potentials. Internal resistance was measured at the end of the start-up phase and after the  
 335 subsequent follow-up operation (calculated from whole cell polarization curves, Fig. S3), and  
 336 average current densities and anode potentials were measured at the beginning (7<sup>th</sup> feeding) and  
 337 at the end (10<sup>th</sup> feeding) of the operation in MFC mode with 47  $\Omega$  external resistance.

	After start-up	In the end of the operation	7 <sup>th</sup> feeding <sup>a</sup>		10 <sup>th</sup> feeding <sup>b</sup>	
	Internal resistance ( $\Omega$ )	Internal resistance ( $\Omega$ )	Avg. current density (A/m <sup>3</sup> )	Avg. anode potential (mV)	Avg. current density (A/m <sup>3</sup> )	Avg. anode potential (mV)
BES <sub>-200mV</sub>	71 $\pm$ 16	86 $\pm$ 15	10.6 $\pm$ 1.1	-460 $\pm$ 12	9.7 <sup>c</sup>	-481 <sup>c</sup>
BES <sub>0mV</sub>	109 $\pm$ 15	140 $\pm$ 27	8.6 $\pm$ 0.7	-452 $\pm$ 4	9.2 $\pm$ 2.1	-469 $\pm$ 19
BES <sub>50<math>\Omega</math></sub>	156 $\pm$ 52	177 $\pm$ 65	7.9 $\pm$ 3.1	-454 $\pm$ 19	9.4 $\pm$ 2.7	-476 $\pm$ 15
BES <sub>1000<math>\Omega</math></sub>	203 $\pm$ 41	121 <sup>c</sup>	6.7 <sup>c</sup>	-437 <sup>c</sup>	9.2 <sup>c</sup>	-466 <sup>c</sup>

338 <sup>a</sup> days 43-50; <sup>b</sup> days 63-70; <sup>c</sup> results from only one replicate due to connection problems

339

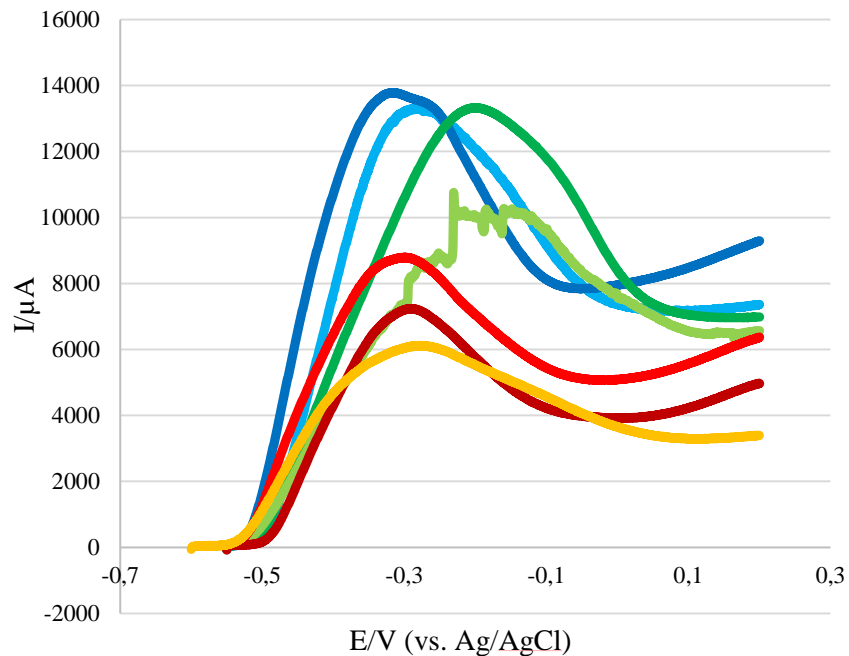
340 In the end of the experiment (day 71), power overshoot was visible in the anodic LSVs (Fig. 3)  
 341 in all BESs at anode potentials between -220 and -300 mV and the shapes of the LSV curves  
 342 resembled those obtained in the anodic LSV of BES<sub>50 $\Omega$</sub>  during the start-up phase (Fig. 2). The  
 343 highest current value on LSV curve with BES<sub>50 $\Omega$</sub>  decreased from 9.9 during start-up (day 42) to  
 344 8.0 mA during the operation with 47  $\Omega$  presumably due to decreased conductivity (from ca. 4-5  
 345 to ca. 3 mS/cm). With BES<sub>1000 $\Omega$</sub> , the highest current in the anodic LSV increased from 1.9 to  
 346 11.7 mA, and with BES<sub>0mV</sub> it decreased from 26.5 to 11.8 mA. The highest anodic LSV current  
 347 of BES<sub>-200mV</sub> reactors was 13.5 mA on day 71. The changes in the anode potentials where the  
 348 peak current is achieved (Figs. 2 and 3) may be due to changes in the quantity or type of redox  
 349 active enzymes synthesized by electrochemically active microbes [33]. In anodic LSVs, the  
 350 higher maximum current (13.5 mA compared to 8.0-11.8 mA) at same or more negative anode  
 351 potential (at -300 mV compared to -300 - -220 mV) demonstrates smaller overpotential with

352 BES- $200\text{mV}$  compared to the other start-up protocols (Fig. 3). However, the differences in  
353 maximum currents on day 71 were small compared to the differences right after the start-up on  
354 day 42 (Fig. 2). Thus, the start-up protocol had an insignificant effect on LSV curve during the  
355 follow-up operation as previously reported by Hong et al. [33].

356

357 Both the average current densities (Table 2) and the LSV curves (Fig. 3) showed that the start-up  
358 protocol did not significantly affect the current production in BESs after 30 d operation with  
359 selected external resistance after the start-up phase. Thus, the start-up phase should aim mainly at  
360 accelerating the biofilm development rather than producing maximum current.

361



362

363 Fig. 3. Anodic linear sweep voltammograms measured in the end of the experiment (day 71)  
364 after operating the BESs in MFC mode (with  $47\ \Omega$  resistance) for 29 days. The results with  
365 different colors represent the duplicate reactors with studied adjusted anode potentials or external  
366 resistances used as start-up protocols in the start-up phase (days 0-42): blue for BES- $200\text{mV}$ , green  
367 for BES $0\text{mV}$ , red for BES $50\Omega$  and orange for BES $1000\Omega$ . Due to connection problems, the results of  
368 BES $1000\Omega_2$  are not shown.

369

### 370 3.3 Transformation of wastewater organics during and after start-up

371 Sugars and alcohols were almost completely removed ( $>96\%$  sugar removal and no alcohols  
372 were detected in the effluent) from the diluted brewery wastewater during both the start-up and  
373 the subsequent follow-up operation phases. During the start-up phase, VFA accumulated in BESs  
374 during all the feedings (Figs. 4. and S3). The highest concentrations were detected at the end of  
375 the 2<sup>nd</sup> feeding after which VFA concentrations decreased towards the end of the start-up phase  
376 (Table 3). VFA accumulation was similar with all start-up protocols in the end of both the start-  
377 up and the follow-up operation (Table 3) with acetate and propionate contributing the most of the

378 VFAs, whilst also butyrate ( $\leq 321$  mg/L) and valerate ( $\leq 251$  mg/L) were detected in some  
 379 samples. In the end of the start-up phase, the acetate and propionate concentrations were in the  
 380 range of 1100-1300 mg/L and 630-680 mg/L, respectively. Highest acetate (2800-4700 mg/L)  
 381 and propionate (1200-2200 mg/L) concentrations at the end of 2<sup>nd</sup> feeding of the start-up phase  
 382 likely originated from degradation of the anaerobic sludge used as inoculum. The anaerobic  
 383 sludge inoculum (10% v/v) attached to the carbon brush anode electrodes. The sludge was  
 384 partially hydrolyzed during the incubation especially during the start-up phase (first five batch  
 385 cycles), increasing the soluble COD concentrations. Although the COD from the anaerobic  
 386 sludge hydrolysis likely affected the BES performance, it did not compromise the comparison of  
 387 the different start-up protocols. During the follow-up phase, Coulombic efficiency (CE)  
 388 calculated for the fed COD was  $12 \pm 2\%$  (on days 56-63), which is of the same order as results of  
 389 Wen et al. ( $<10\%$ ) and Zhuang et al. (6.3-7.6%; calculated for removed COD) reported for  
 390 brewery wastewater [7,52].

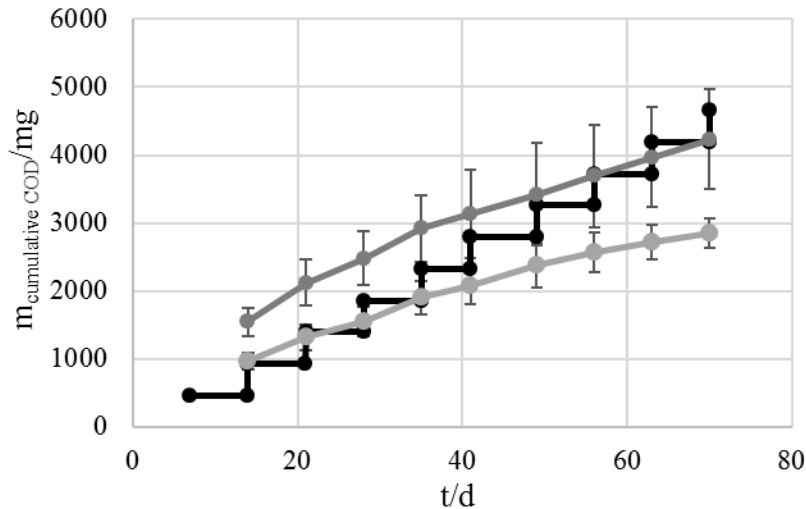
391

392 Table 3. The organic compounds present in the diluted brewery wastewater (feed solution) and  
 393 BES effluents after the start-up phase (day 42) and the follow-up period (day 70). Standard  
 394 deviations stand for differences between duplicate BESs.

Sample	Start-up protocol	Acetate (mg/L)	Propionate (mg/L)	Sugars (mg/L)	Ethanol (mg/L) <sup>a</sup>
Feed solution		0	0	430	360
Effluent from the end of the start-up phase	BES <sub>-200mV</sub>	1100 ± 600	680 ± 80	10.9 ± 1.1	n.d. <sup>b</sup>
	BES <sub>0mV</sub>	1120 ± 140	670 ± 150	9.7 ± 1.0	n.d.
	BES <sub>50Ω</sub>	1200 ± 200	630 ± 50	9.3 ± 1.1	n.d.
	BES <sub>1000Ω</sub>	1290 ± 120	650 ± 150	13 ± 6	n.d.
Effluent from the end of the follow-up operation	BES <sub>-200mV</sub>	1170 ± 70	420 ± 140	10 ± 3	n.d.
	BES <sub>0mV</sub>	1100 ± 200	500 ± 200	14 ± 3	n.d.
	BES <sub>50Ω</sub>	1400 ± 200	480 ± 130	12.2 ± 1.1	n.d.
	BES <sub>1000Ω</sub>	1270 ± 50	400 ± 100	13.4 ± 1.5	n.d.

395 <sup>a</sup> Detection limit for ethanol was 80 mg/L; <sup>b</sup> n.d. not detected

396



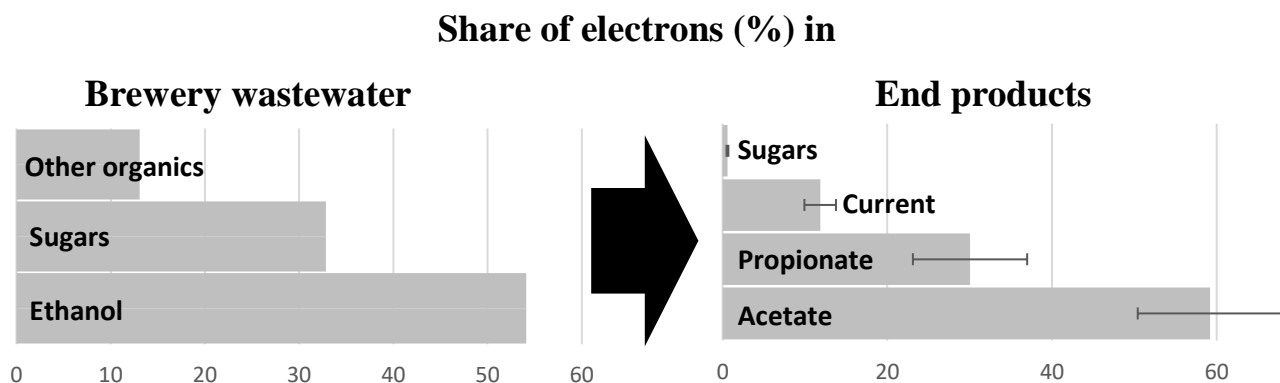
397

398 Fig. 4. Cumulative acetate (grey) and propionate (light grey) formation as COD equivalents in  
 399 BES.200mV after the first feeding. Cumulative fed COD (measured average feed solution COD) is  
 400 shown in black as a reference. Higher concentrations of acetic acid and propionic acid compared  
 401 to the fed COD are due to acetate and propionate production from the anaerobic sludge used as  
 402 inoculum. COD accumulation with the other start-up protocols is shown in Fig. S4.

403

404 The low COD removal (Fig. 4) and current densities (Table 2) demonstrate that the studied BESs  
 405 were not optimal for current production, but the efficient conversion of organic compounds from  
 406 the wastewater to simple VFAs (Fig. 5) indicate that BESs are promising for pretreatment of  
 407 brewery wastewater due to simultaneous degradation of organic compounds and current  
 408 production. The highest COD removal efficiency reported from brewery wastewater has been  
 409 95% [50] showing that high COD removal from brewery wastewater is attainable with process  
 410 optimization. In addition, optimizing the BES for current production would likely enable more  
 411 efficient removal of VFAs and higher production of current. The accumulation of VFAs in  
 412 MFCs was also reported by Chandrasekhar & Mohan [53] during bioelectrochemical  
 413 pretreatment of food waste for dark fermentation. During the follow-up operation in MFC mode  
 414 in this study, almost 90% of the effluent COD was acetate and propionate in all the BESs (Fig.  
 415 5). This would be suitable influent composition for anaerobic digestion as the VFA  
 416 concentrations remained well below inhibitory to methanogens (13 g/L acetate and 3.5 g/L  
 417 propionate) [54].

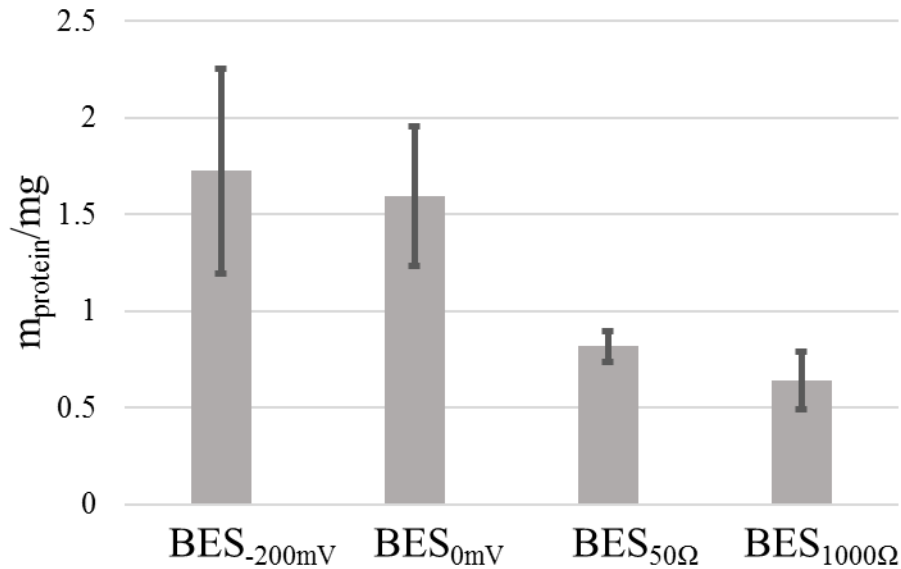
418



419  
 420 Fig. 5. Fate of electrons (%) in BES fed with brewery wastewater in the end of the BES  
 421 operation in MFC mode. The bars represent the COD components of the brewery wastewater (on  
 422 the left), and the average values of transformation products in the effluents of all BES on day 63  
 423 (on the right). The term current indicates the share of electrons recovered as electrical current.

424  
 425 **3.4 Biomass accumulation and microbial community composition on the BES anodes**  
 426 Biomass accumulation and community composition on the BES anodes was determined on day  
 427 75 at the end of the operation of the BESs in MFC mode (Fig. 6, and Fig. S6), because it was not  
 428 possible to take biofilm samples from the carbon brush anodes during the operation. The highest  
 429 protein quantity of 1.6-1.7 mg/BES was detected from BES anodes started up with the adjusted  
 430 anode potentials (BES<sub>0mV</sub> and BES<sub>-200mV</sub>). Approximately 50% less protein (0.6-0.8 mg/BES)  
 431 accumulated on the anodes of the BESs started up with the fixed external resistances. Higher  
 432 anode potentials (at certain potential range, which is dependent on e.g. microbial community and  
 433 substrate) are associated with higher biomass yields due to more energy available for microbial  
 434 growth [27], which is in agreement with our study (anode potentials in BES<sub>50Ω</sub> and BES<sub>1000Ω</sub>  
 435 were between -463 and -480 mV). To evaluate the effect of average current density on biomass,  
 436 the biomass was illustrated as a function of the average current densities calculated over the  
 437 feeding cycles 2-10 (Fig. S5), which clearly shows that anodic biomass increased with average  
 438 current densities. The higher biomass production accompanied with higher current densities was  
 439 well in accordance with the results by Aelterman et al. [46] reporting approximately 50% higher  
 440 mass of biofilm with 0 and -200 mV anode potentials than with -400 mV (vs. Ag/AgCl) anode  
 441 potential on day 22.

442



443

444 Fig. 6. Protein mass (mg) as bovine serum albumin (BSA) equivalents on an MFC anode  
 445 electrodes (2x carbon brush: Length 5.5 cm, diameter 2.5 cm). Standard deviations show the  
 446 differences between duplicate BESs.

447

448 Anaerobic sludge from municipal wastewater treatment plant is a rich source of microorganisms  
 449 capable of utilizing a complex range of different substrates [55]. It is also known to contain  
 450 electrochemically active microorganisms [20]. Anaerobic sludge was, therefore, used as  
 451 inoculum in this study to support the conversion of different brewery wastewater constituents to  
 452 electricity. As indicated by functional changes in electricity generation, phylogenetically very  
 453 diverse microbial communities were enriched differently during the start-up under the different  
 454 selection pressures of BES<sub>-200mV</sub>, BES<sub>0mV</sub>, BES<sub>50Ω</sub> and BES<sub>1000Ω</sub>. This resulted in considerably  
 455 different process and start-up performances.

456 The microbial communities were analyzed on day 75 after operating the BESs in MFC mode for  
 457 33 days with the same external resistance. Based on qualitative PCR-DGGE analysis (Table S1),  
 458 in the end of the follow-up operation the anode electrodes were occupied by several known  
 459 electrochemically active bacteria: *Klebsiella* sp., *Citrobacter* sp., *Azonexus* sp., *Escherichia coli*,  
 460 and *Geobacter* sp. [35,56–58]. The fermentative bacteria of the biofilm samples included *E. coli*  
 461 and *Selenomonas* sp. [59]. *E. coli* and *Citrobacter* sp., as facultative anaerobes, consumed  
 462 oxygen potentially penetrating through CEM of the air-cathode reactor [57]. Only minor  
 463 differences in community compositions were seen both between the duplicate MFCs and  
 464 between different start-up protocols. Thus, no significant effects on the final anodic microbial  
 465 community could be associated with the different start-up protocols (Fig. S6).

466 Microbial communities in anaerobic sludge remain viable and diverse for long periods [60].  
 467 Electricity generation in this study was different in the end of the studied start-up protocols  
 468 indicating different community profiles. However, the operation under equal selection pressure  
 469 (i.e. external resistance of 47 Ω) after the start-up period for 33 days resulted in enrichment of  
 470 microbial communities into similar direction. For example, Ishii et al. [61] have reported



471 variation in the abundance of the bacteria in different classes during the long term BES  
472 operation, which supports the conclusion that in this study the similar selection pressure in BESs  
473 after the start-up affected the final microbial communities at anodes.

474

#### 475 4. Conclusions

476

477 Among the studied start-up protocols (-200 mV and 0 mV vs. Ag/AgCl adjusted anode potentials  
478 and 50  $\Omega$  and 1000  $\Omega$  external resistances), -200 mV accelerated the current production most in  
479 air-cathode BES fed with brewery wastewater, with the highest average current density of  $76 \pm$   
480  $39 \text{ A/m}^3$ . Adjusted anode potentials of 0 and -200 mV enhanced the anode biofilm growth more  
481 than external resistances of 50 and 1000  $\Omega$ . High volatile fatty acids accumulation and low ( $12 \pm$   
482 2%) Coulombic efficiency show that further optimization of the BES reactor configuration is  
483 needed to enhance the conversion of the organic content of the brewery wastewater into current.  
484 The results indicate that of the tested methods, the most optimal for efficient BES start-up is  
485 adjusted anode potential of -200 mV. The start-up conditions did not affect electricity production  
486 or microbial community composition in long-term BES operation, but are considered critical for  
487 fast recovery after process disturbances.

488

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490

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493

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495

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687 The effect of start-up on energy recovery and compositional changes in brewery wastewater in  
688 bioelectrochemical systems  
689 Johanna M. Haavisto<sup>1,\*</sup>, Marika E. Kokko<sup>1</sup>, Aino-Maija Lakaniemi<sup>1</sup>, Mira L. K. Sulonen<sup>1,#</sup>,  
690 Jaakko A. Puhakka<sup>1</sup>

## 691 **SUPPORTING INFORMATION**

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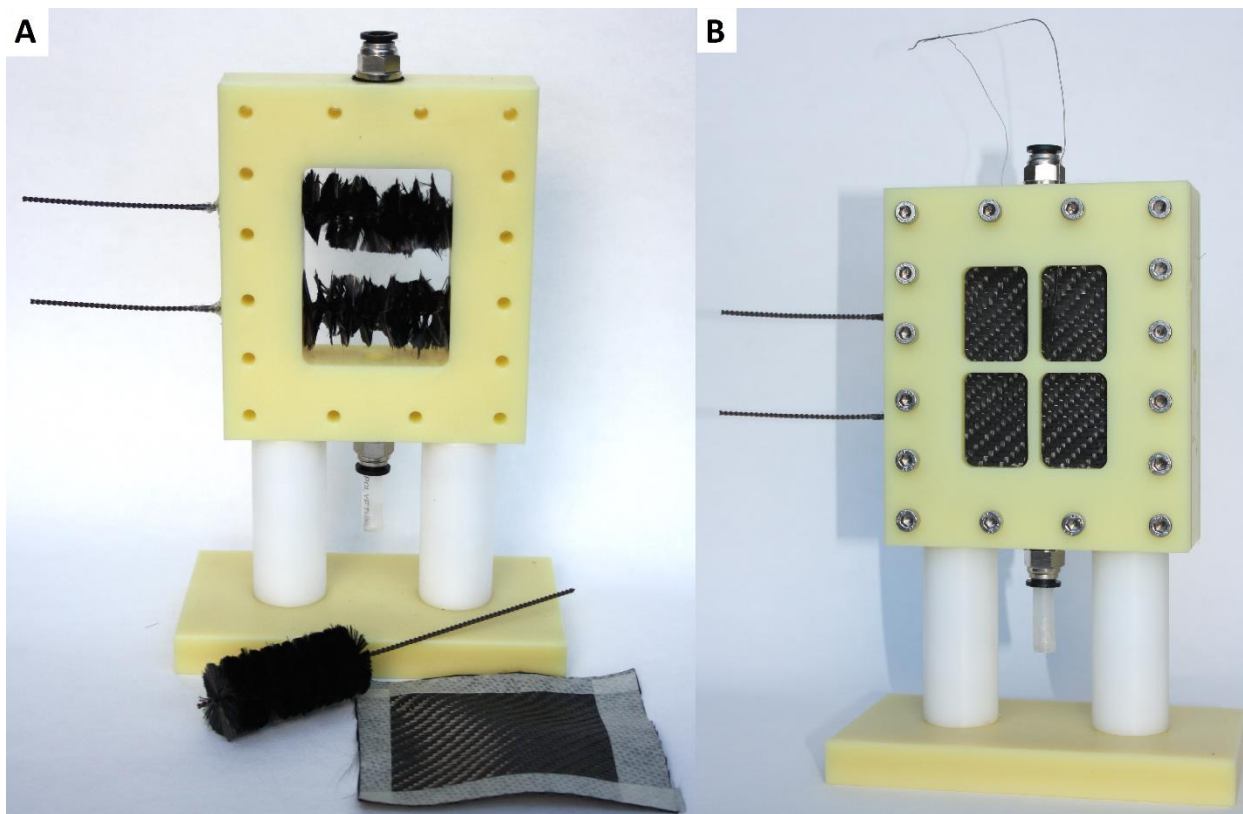
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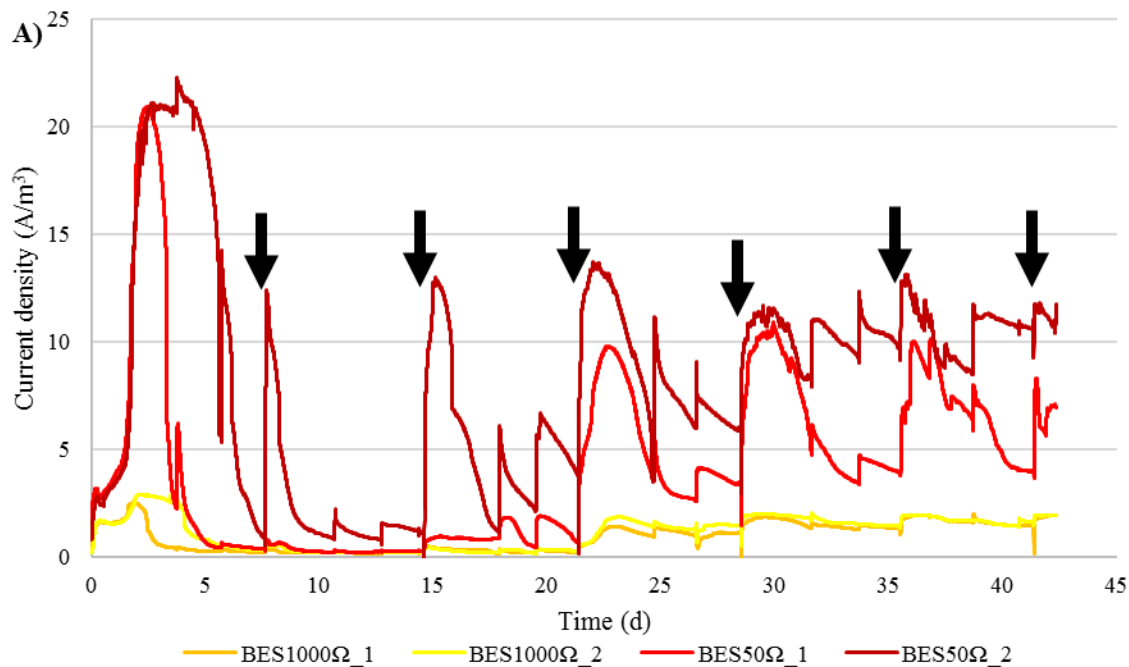
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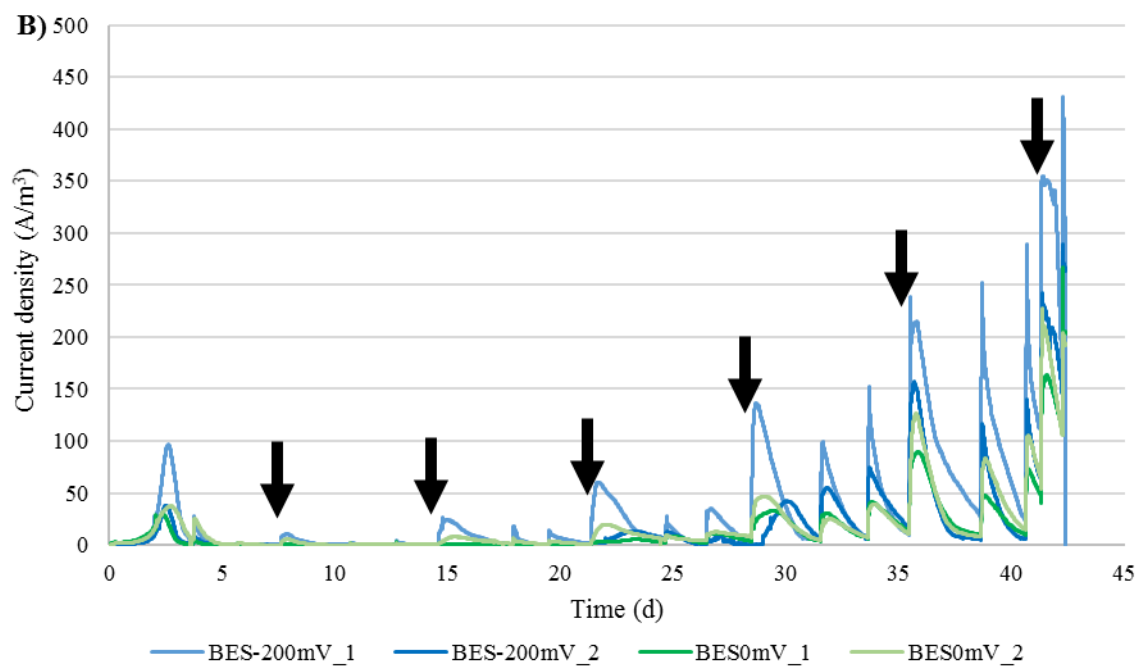
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703 **Fig. S1.** Photographs of the bioelectrochemical systems with air cathodes A) anode electrode  
704 positions and anode (carbon brush) and cathode (carbon cloth) electrode materials and B)  
705 bioelectrochemical system with air-cathodes assembled on both sides of the anode chamber.  
706 Metallic recirculation tube connections are shown below and above the anode chamber frame in  
707 both photos. Reference electrode was positioned in the middle of the anode chamber parallel to  
708 the anode electrodes.





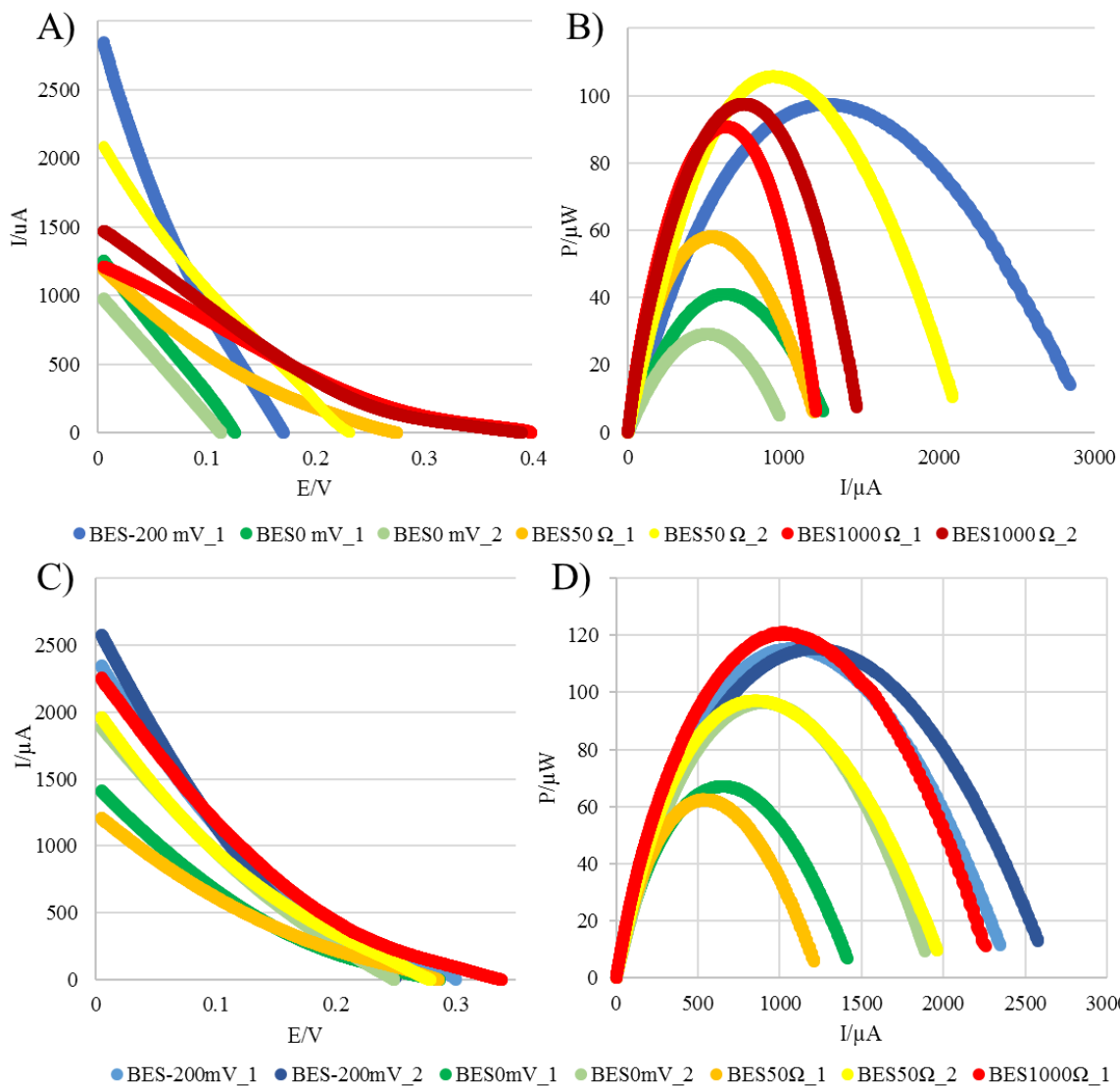
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711 **Fig. S2.** Current densities of the BESs with A) external resistances and B) adjusted anode  
 712 potentials during the start-up phase. Black arrows represent the feeding points.

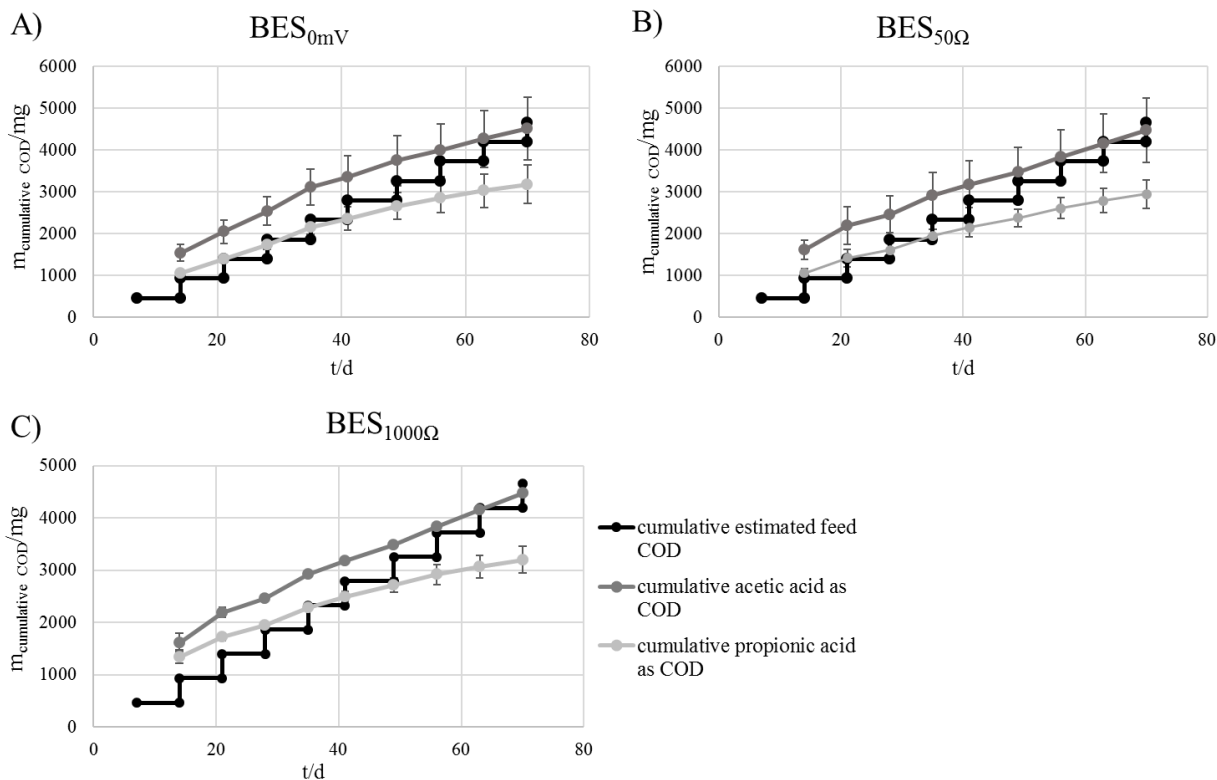
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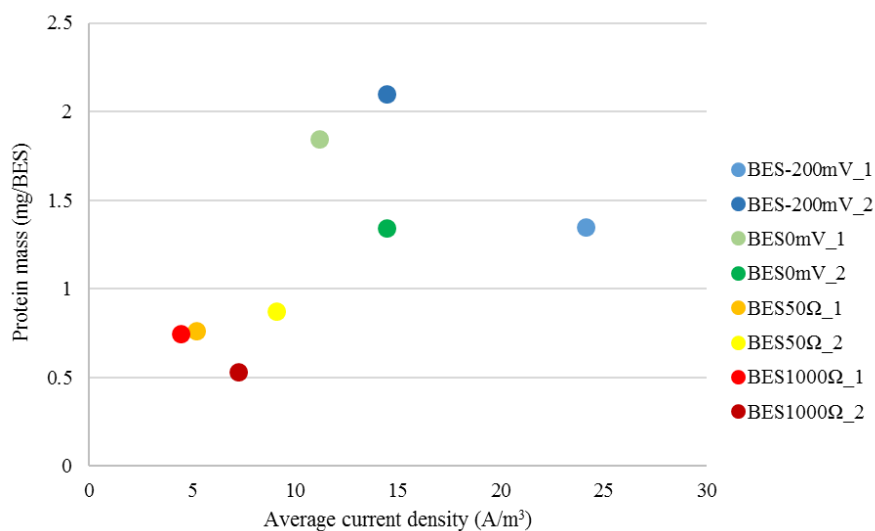
715 **Fig. S3.** Whole cell linear sweep voltammetry polarization and power curves of the studied BESs  
 716 in the end of the start-up phase (A and B) and in the end of the experiment (C and D). Due to  
 717 connection problems, the results of BES-200mV\_2 are not shown in A and B and BES1000 $\Omega$ \_2 in C  
 718 and D.

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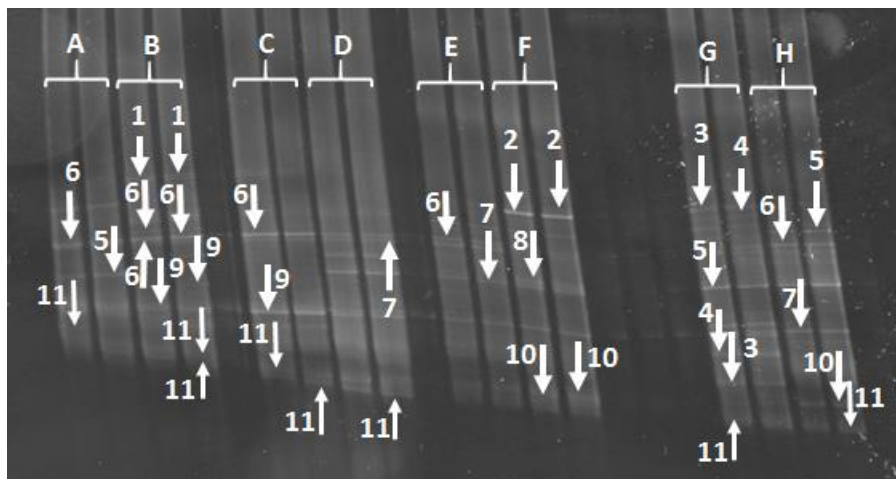
720

721 **Fig. S4.** Cumulative COD in feed, and acetic acid and propionic acid formation as COD  
 722 equivalents after the first feeding in A) BES<sub>0mV</sub>, B) BES<sub>50Ω</sub> and C) BES<sub>1000Ω</sub>. Added COD in  
 723 feed is shown in black as a reference. Higher concentrations of acetic acid and propionic acid  
 724 compared to fed COD are due to the COD content of anaerobic sludge inoculum.



725

726 **Fig. S5.** Dependence of anodic biofilm protein mass on average current density. Average current  
 727 densities were calculated as mean values of feeding cycles 2-10 between days 7 and 70.



729

730 **Fig. S6.** Denaturing gradient gel electrophoresis profiling of the bioelectrochemical system  
 731 anode biofilms. Duplicate samples were prepared from each BES: A) and B) BES<sub>-200mV</sub>, C) and  
 732 D) BES<sub>0mV</sub>, E) and F) BES<sub>50Ω</sub>, and G) and H) BES<sub>1000Ω</sub>. Bands with same number have similar  
 733 affiliation (Table S1).

734

735 **Table S1.** Affiliations of the micro-organisms detected from the anode biofilms of brewery  
 736 wastewater-fed bioelectrochemical systems. The identification was conducted by using  
 737 polymerase chain reaction denaturing gradient electrophoresis (PCR-DGGE) followed by  
 738 sequencing. Identification of multiple bands with similar affiliation caused variation in sequence  
 739 length and similarity presented in the table.

Band label	SL	Sim (%)	Affiliation (acc)	Class/Family	Origin of the sample
1	303-304	98.7-99.0	uncultured bacteroidetes bacterium (JF681276.1)	-/-	thermophilic anaerobic reactor
2	449-472	100	uncultured beta proteobacterium (AB635880.1)	Betaproteobacteria / -	groundwater from deep tube well
3	285-410	98.6-100	<i>Klebsiella</i> sp. (MF442345.1)	Gammaproteobacteria / <i>Enterobacteriaceae</i>	roots of Brazil nut tree
4	288-400	99.0-99.8	<i>Citrobacter</i> sp. (LT556085.1)	Gammaproteobacteria / <i>Enterobacteriaceae</i>	
5	463	98.5	uncultured bacterium clone (KC551588.1)	-/-	activated sludge
		97.8	uncultured <i>Azonexus</i> sp. (LC001033.1)	Betaproteobacteria / <i>Azonexaceae</i>	microbial fuel cell
6	299-469	98.2-100	<i>Selenomonas</i> sp. (AB717126.1)	Negativicutes / <i>Selenomonadaceae</i>	microbial fuel cell
		98.2-100	<i>Schwartzia</i> sp. (GQ332209.1)	Negativicutes / <i>Selenomonadaceae</i>	grease hat within grease trap
7	392-417	99.5-99.8	<i>Escherichia coli</i> (CP022414.1)	Gammaproteobacteria / <i>Enterobacteriaceae</i>	
8	418	99.5	<i>Geobacter</i> sp. (JF736650.1)	Deltaproteobacteria / <i>Geobacteraceae</i>	microbial fuel cell
9	286-464	96.9-100	<i>Azospira</i> sp. (JF736645.1)	Betaproteobacteria / <i>Rhodocyclaceae</i>	microbial fuel cell
10	377-399	98.4-99.0	<i>Desulfovibrio marrakechensis</i> (KX261411.1)	Deltaproteobacteria / <i>Desulfovibrionaceae</i>	Sludge and beet sugar industrial wastewater
		99.5-100	uncultured bacterium clone (KU589101.1)	-/-	UASB reactor
11	350-402	96.9-100	uncultured bacterium clone (EF515442.1)	-/-	microbial fuel cell

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