

# 1 Urine disinfection and *in situ* pathogen killing using a Microbial Fuel Cell cascade 2 system

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8

## 9 Abstract

10 Microbial Fuel Cells (MFCs) are emerging as an effective means of treating different types  
11 of waste including urine and wastewater. However, the fate of pathogens in an MFC-based  
12 system remains unknown, and in this study we investigated the effect of introducing the  
13 enteric pathogen *Salmonella enterica* serovar *enteritidis* in an MFC cascade system. The  
14 MFCs continuously fed with urine showed high disinfecting potential. As part of two  
15 independent trials, during which the bioluminescent *S. enteritidis* strain was introduced into  
16 the MFC cascade, the number of viable counts and the level of bioluminescence were  
17 reduced by up to  $4.43 \pm 0.04$  and  $4.21 \pm 0.01$  log-fold, respectively. The killing efficacy  
18 observed for the MFCs operating under closed-circuit conditions, were higher by 1.69 and  
19 1.72 log-fold reduction than for the open circuit MFCs, in both independent trials. The results  
20 indicated that the bactericidal properties of a well performing anode were dependent on  
21 power performance and the oxidation-reduction potential recorded for the MFCs. This is the  
22 first time that the fate of pathogenic bacteria has been investigated in continuously operating  
23 MFC systems.

24

25

## 26 **Introduction**

27 A Microbial Fuel Cell (MFC) is a bioelectrochemical reactor in which organic compounds in  
28 the feedstock are oxidised in the anodic chamber to produce carbon dioxide, protons and  
29 electrons (in the form of NADH/NADPH) within the microbial cells. Feedstock fuel  
30 (utilisable substrate) can include a wide range of compounds, from acetate and other low  
31 molecular weight monomers, including sugars right up to particulate macromolecules and  
32 complex real world mixtures such as sludge and urine, under anaerobic conditions in the  
33 anode chamber. Utilisation of complex macromolecules which could be present in urine  
34 (Brooks and Keevil, 1997) implies the abundance of hydrolytic activity around and within the  
35 anodic biofilm. Electrons derived from the NADH redox reactions within the cell are  
36 transported to the anodic electrode (via direct conductance or via chemical redox mediators)  
37 and then travel through the external circuit to the cathode within the cathodic chamber, while  
38 protons generated from the biofilm on the anode pass through a membrane, which separates  
39 both chambers (Bennetto *et al.*, 1983). The use of this technology has attracted increasing  
40 interest in recent years, and several scaled-up applications have been successfully  
41 demonstrated, for treating waste products (as microbial electrolysis cells or MEC) (Cusick *et*  
42 *al.*, 2011; Heidrich *et al.*, 2014) as well as for cleaning waste and producing electricity (as  
43 true Microbial Fuel Cells) (Ieropoulos *et al.*, 2015). The cost-effectiveness of the MFC  
44 technology (Behera *et al.*, 2010; Pasternak *et al.*, 2016a) has also been demonstrated.

45 Although the MFC technology has seen significant scientific development over the last  
46 three decades, implementing this technology in real-world applications requires extensive  
47 studies on health and sanitation hazards. These concerns have rarely been addressed.

48 An important part of the health risk assessment for wastewater treatment technologies  
49 consists of determining the fate of enteric pathogens *in-situ* and *ex-situ* of a treatment  
50 process. Insufficient sanitation, together with unavailability of improved water systems, leads

51 to hundreds of thousands of deaths each year, particularly in Sub-Saharan Africa  
52 (Montgomery and Elimelech, 2007). Therefore, the MFC technology, which could be used in  
53 remote, off-grid areas of Developing World countries, to treat wastewater and generate  
54 electricity (Castro et al., 2014; Yazdi et al., 2015; Hsu *et al.*, 2017), offers great promise.  
55 Although pathogens are likely to be more commonplace in fecal sludge it is common practice  
56 to separate the solid fraction from the top liquid fractions in sludge, and the liquid fraction is  
57 usually dominated by urine. Moreover, the liquid fraction can spread pathogens greater  
58 distances from the source of contamination.

59 There have been some reports already published regarding the disinfection potential of  
60 MFC-driven processes. Nevertheless, these studies were focusing on the disinfecting  
61 properties of the synthesised catholyte, which could be a result of oxygen reduction to H<sub>2</sub>O<sub>2</sub>  
62 (Fu *et al.*, 2010) or electro-osmotic drag occurring in the MFCs. The H<sub>2</sub>O<sub>2</sub> synthesis occurs  
63 spontaneously at the cathode and was successfully employed to disinfect the effluent of a  
64 wetland system. Treating the effluents with H<sub>2</sub>O<sub>2</sub> solution resulted in a significant decrease of  
65 total coliforms (Arends *et al.*, 2014). Another approach for disinfection with the use of  
66 catholyte was described by Jadhav *et al* (Jadhav *et al.*, 2014). The authors supplied the  
67 cathodic chamber with sodium hypochlorite for the simultaneous improvement of power  
68 output and disinfection, which was performed by recirculating the anolyte through the  
69 cathodic chamber. A more recent study showed that highly alkaline catholyte produced in  
70 ceramic MFCs, also possesses strong disinfecting properties; its application resulted in  
71 significant decrease of metabolic activity of *Escherichia coli* (Gajda *et al.*, 2016) chosen as a  
72 representative enteric pathogen . The production rate and properties of the catholyte are  
73 dependent on the properties of the ceramic separator, as well as the power output. It has been  
74 reported that the pH of the catholyte and its disinfection strength is positively correlated with  
75 the ceramic membrane thickness (Merino Jimenez *et al.*, 2016). Moreover, pH alone can be

76 favourable to growth, when at or close to neutral (pH 6.0- 7.5) whilst much higher (>pH 8.0)  
77 or much lower levels (<pH 5.5) may contribute strongly to bacterial survival or killing.

78 The catholyte is one example of the numerous applications that makes MFCs a platform  
79 technology, and one that offers great promise for further investigation. However, the  
80 exposure of MFC anodes - or any bioelectrochemical system - to pathogenic organisms in a  
81 real environment, is a parameter that has not been assessed before, and the hypothesis is that  
82 exogenous microorganisms entering an anodic MFC chamber, will be out-competed by the  
83 established electroactive community, if the latter is thriving under, or close to maximum  
84 power transfer conditions.

85 The aim of this study was to investigate the fate of one of the most important members of  
86 the *Enterobacteriaceae* family, namely *Salmonella enterica* serotype *enteritidis*. This rod-  
87 shaped gram-negative species, which may originate from sewage contamination (Guard-  
88 petter and Guard- Petter, 2001), may cause food-borne diseases.,. This species was  
89 introduced into an MFC cascade system treating human urine, to determine the anodic killing  
90 efficacy when operating in continuous flow conditions. This study used bioluminescent  
91 reporter strains to measure the rate of killing in situ and used viable counts on selective  
92 recovery media to demonstrate that urine can be efficiently disinfected by the MFC cascade  
93 system.

## 94 **Materials and methods**

### 95 **MFC construction and operation**

96 Ceramic earthenware cylinders were used to build open to air cathode, small scale MFCs.  
97 The ceramic (Scientific & Chemical Supplies Ltd, UK) material was used both as the proton  
98 exchange membrane and body of the MFC. Each ceramic cylinder was cut to maintain the  
99 internal volume of empty MFCs equal to 11.4 mL. Carbon fiber veil with a carbon loading of

100 20 g/m<sup>2</sup> was used as the anode (PRF Composite Materials, Dorset, UK). Anodes were cut  
101 into rectangles of dimensions 9×28 cm and folded to obtain a total surface area of 252 cm<sup>2</sup>  
102 and threaded with a plain Ni-Cr wire (Ø0.45 mm, Scientific Wire Company, UK), which was  
103 used for connection. The cathodes were made of conductive graphite paint as described by  
104 Winfield *et al.* (Winfield *et al.*, 2014). In brief, petroleum spirit was used to dissolve the  
105 polyurethane rubber coating (PlastiDip, Petersfield, UK) and mixed with graphite (Fisher  
106 Chemicals, UK) in a 1:3 (plastidip:graphite) ratio. Two layers of conductive paint (carbon  
107 loading of 35.02 mgC/cm<sup>2</sup>) were applied on the surface of the ceramics and supplied with a  
108 nickel-chromium mesh used as a current collector (20x20, 0.18 mm). The total carbon  
109 loading for the cathode was 0.851 gC, and the projected surface area was 24.18 cm<sup>2</sup>.

110 The 3D printed Nanocure® RCP30-resin lid designed with inlet and outlet tubes was used  
111 as the front of the MFC, whilst a transparent acrylic lid (3 mm thick) was used to cover the  
112 other side of the chamber. The RCP30-resin lids were designed using SolidWorks 2013  
113 software and printed with Perfactory 4 3D printer (Envisiontec, Germany). Both lids were  
114 assembled with the MFC by a plain nylon screw (Ø3 mm, RS, UK). The detailed schematic  
115 representation of individual MFCs has been described previously (Pasternak *et al.*, 2016b).

116 During the first trial, additional 2 cm<sup>2</sup> of carbon veil wrapped around Ni-Cr wire were  
117 introduced through the channel in the lid to the anolyte chamber. These removable additional  
118 ‘anodes’ had a direct physical and electrical contact with the main anode in the MFCs and  
119 allowed the formation of the anodic biofilm. These removable anodes allowed aseptic  
120 sampling of biofilms (by *in situ* detachment) to monitor the incorporation and survival of *S.*  
121 *enteritidis* cells in the anodic biofilm.

## 122 **Experimental setup**

123 Two individual trials were run over the experimental period. The first trial consisted of a  
124 cascade of 9 MFCs operating in closed circuit (CC) conditions and a separate cascade of 3

125 MFCs operating in open circuit (OC) conditions. Both the CC and OC MFCs were inoculated  
126 with the mixed bacterial community derived from anaerobic activated sludge (Saltford  
127 Scientific Laboratory, Wessex Water, Bristol UK).

128 The second trial consisted of the same two cascades as trial 1, with an additional control  
129 with 3 OC/abiotic control MFCs. This additional control cascade was setup such that its  
130 liquid output was flowing into the input of the OC/biotic control cascade. The abiotic control  
131 MFCs were disinfected prior to the experiment by using 70% ethanol solution, followed by  
132 washing with sterile water and drying at 60°C for 1 hour. All of the MFCs were separated by  
133 physical air gap between the cells to avoid any conductive bridging between the anodes.

134 To estimate the killing potential against *S. enteritidis* and monitor its metabolic activity in  
135 real time, a flow cell supplied with H10720 photosensor module (Hamamatsu Photonics  
136 K.K., Japan) was used. The sensor was introduced after the 9<sup>th</sup> MFC in CC cascade (trial 1)  
137 and after the 3<sup>rd</sup> MFC in the OC abiotic cascade (trial 2).

138 Fresh neat human urine (collected not later than 24 hours before the trial and stored at 4°C)  
139 was used as a fuel and supplied to the cascades by using a multichannel peristaltic pump  
140 (Watson Marlow, USA) at a constant flow rate of 0.90 L/d.

141 The external load connected to each MFC, was 1000  $\Omega$  for the initial 11 days of operation  
142 and 250  $\Omega$  afterwards for the rest of the experiment. The cascade was fed with fresh human  
143 urine as the fuel. Before the trials, the CC and OC MFCs were operated for 167 days in order  
144 to fully allow the maturing of the anodic biofilm and to demonstrate the feasibility of  
145 disinfection in a well-established MFC system.

## 146 **Introduction of *Salmonella enteritidis* strain**

147 The *S. enteritidis* strain was obtained from the collection of the University of the West of  
148 England, the serotype designation was validated by serotyping (Turner, 2013). The strain was  
149 carrying the pBBR1MCS-2 plasmid derivative containing the luxCDABE operon of

150 *Photorhabdus luminescens*. Prior to the experiment, the strain was subcultured in LB media  
151 containing kanamycin (10 µg ml<sup>-1</sup>) as the selective agent and incubated overnight in 37°C.  
152 Subsequently, when the optical density at a wavelength of 600nm (OD<sub>600</sub>) reached 1.0, 15  
153 mL of the culture was centrifuged, washed twice with a 0.9% NaCl solution, re-suspended in  
154 50% glycerol solution, and stored in -20°C until the start of the experiment. To inoculate  
155 urine with *S. enteritidis*, cryopreserved bacterial cultures were centrifuged and re-suspended  
156 in 1 L of neat urine.

## 157 **Monitoring the disinfection of urine**

158 To estimate the killing potential of the MFCs in real time, the signal obtained from the  
159 photosensor was calibrated with the corresponding signal from the tube luminometer  
160 GLOMAX, 20/20 (Promega, USA). The signal was therefore given in Relative Luminescence  
161 Units (RLU). Moreover, at the end of experiment, the samples were collected from all of the  
162 sampling points and their luminescence was assessed using a standard benchtop luminometer.  
163 In addition, at the end of trial 1, small (2 cm<sup>2</sup>) anode pieces were removed from the anolyte  
164 chambers and analysed in the same manner after resuspending the biofilm by sterile rod and  
165 vortexing (3 min). The quantity of Colony Forming Units (CFU) was assessed using XLD  
166 agar (Oxoid, UK). The pH and ORP were measured with Orion Dual Star pH meter (Thermo  
167 Fisher Scientific, USA).

168 The log reduction (LR) of colony forming units, as well as log reduction of  
169 bioluminescence intensity was calculated using the following formula:

$$170 \quad LR = \log\left(\frac{A}{B}\right)$$

171 Where:

172 A – number of viable microorganisms or bioluminescence intensity before treatment,

173 B – number of viable microorganisms or bioluminescence intensity after treatment.

174 The standard deviation was calculated as described by Zelter *et al.* (Zelter *et al.*, 2001):

$$175 \quad SD_{LR} = [(S_A^2/n_A) + (S_B^2/n_B)]$$

176 Where:

177  $S_A$  and  $S_B$  - the sample standard deviations of the log reduction values for samples before  
178 and after treatment, respectively;

179  $n_A$  and  $n_B$  – number of replicates in population before and after treatment, respectively.

## 180 **Data logging and processing**

181 MFC performances, as well as the signal from the photosensor were recorded using a  
182 Picolog ADC-24 Data Logger (Pico Technologies, UK), with the data logging sample rate set  
183 to 3 minutes. The current was calculated according to Ohm's law:  $I = V/R$ , where  $V$  is the  
184 measured voltage in Volts (V) and  $R$  is the value of the external resistance. The power output  
185  $P$  in Watts (W) was calculated using equation:  $P = I \times V$ . Experimental data were processed  
186 using Microsoft Excel 2010 and plotted by GraphPad Prism 5.0 software.

187

188 **Fig 1. Schematic representation of the experimental setup.**

## 189 **Statistical analysis**

190 The LR data was analysed using Shapiro-Wilk normality test and t-student test ( $\alpha=0.05$ ) to  
191 determine the significance of difference between the means. All statistical analysis was  
192 performed using R statistical environment.

## 193 **Ethics statement**

194 This research involved the use of human urine. The appropriate written consent was given by  
195 all individuals participating in the study. This research was approved by NHS (12/YH/0493)  
196 and University of the West of England Research Ethics Committee (112207).

## 197 **Results and discussion**



198 From previous work, it was established that this type of MFCs could reach power output levels  
199 of the order of  $105.5 \pm 32.2 \mu\text{W}$  (Pasternak *et al.*, 2016b). However, during long-term operation,  
200 undesirable cathodic biofilm formation caused deterioration of performance. Therefore, the  
201 first trial described in the current study, was performed when MFCs produced only  $31.2 \pm 9.2$   
202  $\mu\text{W}$  (Fig 2), which corresponds to 29.5 % of the best performance. During the second trial, the  
203 biofilm formed at the cathodes was removed, thus the power recorded over the experimental  
204 period increased to  $65.3 \pm 9.3 \mu\text{W}$ . In both trials, the power performance recorded for individual  
205 MFCs was stable and indicated that the fuel supplied to the MFCs, at constant flow rate and in  
206 controlled temperature conditions, was utilised at constant reaction rates. The stable power  
207 output was the result of the stable metabolic rate and cell population number (Ledezma *et al.*,  
208 2012) giving constant electrochemical conditions, which in turn helped to stabilise the whole  
209 system, rendering it reliable for investigating the disinfection efficacy of the target reporter  
210 species.

211

212 **Fig 2. Temporal power performance of the individual MFCs in the cascade system**  
213 **observed during the first (a) and the second (b) trial.**

214

215 The luminescence observed for the whole experimental period did not exceed  $3.93 \times 10^3$   
216 RLU. In contrast, a strong signal reaching  $1.73 \times 10^6$  RLU was recorded when the photosensor  
217 was introduced to the abiotic control (Fig 3). The immediate response of the sensor was  
218 observed after the second hour of the experiment, when all of the MFCs in the triplet were  
219 fully filled with urine, allowing the treated urine to pass through the sensor chamber.  
220 Therefore, the real-time monitoring of bioluminescence intensity with the photosensor  
221 introduced in two different sampling points, revealed that the environment within the closed  
222 circuit MFCs, was indeed hostile to the pathogens, suppressing microbial activity of

223 exogenous bacteria. The bioluminescence reaching up to  $1.73 \times 10^6$  RLU in the case of the  
224 abiotic control, indicated that none of the materials used to construct the MFCs, nor the  
225 conditions occurring in abiotic OC MFCs were toxic against the *S. enteritidis* strain.  
226 Although the data from the photosensor were generated from two cascades with a different  
227 number of units, the luminescence results were confirmed by analyzing the samples collected  
228 from both cascades (3<sup>rd</sup> unit in each cascade), using the benchtop luminometer (Fig 4).

229

230 **Fig 3. Real-time bioluminescence recorded for the closed-circuit MFCs (after 9<sup>th</sup> MFC**  
231 **in the cascade) and abiotic open circuit control MFCs (after 3<sup>rd</sup> MFC in the cascade).**

232 Monitoring the microbial activity and viability in all of the sampling points, as well as  
233 during the inoculation of the MFCs with *S. enteritidis* allowed the determination of the log-  
234 reduction (LR) values for the above-mentioned parameters. In both trials, a positive effect  
235 (negative LR values) on microbial viability (CFU) and metabolic activity (reflected by RLU  
236 – bioluminescence being dependent on metabolic rate) was observed as a result of 18 hours  
237 of incubation in batch culture (bottle  $t_x$ ). Moreover, only negligible difference was observed  
238 when comparing RLU LR values of batch culture (bottle  $t_x$ ) and the inlet to the cascade (inlet  
239  $t_x$ ). The differences recorded in the CFU LR values were probably the result of experimental  
240 variance in sampling bacterial cells undergoing sedimentation in batch culture. This  
241 demonstrated that there was no disinfection effect prior to entering the cascade system, that  
242 might have been caused by mechanical pressure derived from peristaltic pump or redox  
243 reactions occurring during the residence within the silicon tubing. Similarly, only negligible  
244 negative effects (positive LR values) were observed, when the pathogenic cells were  
245 processed through the abiotic OC cascade (ac3). The retention of pathogenic cells in abiotic  
246 MFCs may have initiated the adsorption, sedimentation and biofilm formation mechanisms.

247 It is known, that *Salmonella* species are able to form a biofilm structure on various types of  
 248 substrata (Jones and Bradshaw, 1996; Giaouris and Nychas, 2006), thus some positive effect  
 249 (decrease of viability) may have been caused by the accumulation of its metabolic by-  
 250 products, or simply by attachment of the dead or dying bacterial cells to the biofilm. On the  
 251 other hand, when the biofilm was removed from the 2cm<sup>2</sup> of anode at the end of trial 1, none  
 252 of the viable *Salmonella sp.* cells were detected neither in open circuit nor closed circuit  
 253 MFCs (Table 1). Similarly, the observed LR values for luminescence were indicating that  
 254 none of the *Salmonella enteritidis* cells were incorporated to the matured biofilm.

255 **Table 1. Monitoring of viability and luminescence of *S. enteritidis* on the biofilm surface.**

256 \*the LR result is shown based on the calculation that 1 CFU would give 7.09 LR. The LR  
 257 values cannot be calculated when the CFU=0, na – not applicable.

RLU				CFU			
Average	SD	Average	SD	Average	SD	Average	SD
179.3	10.5	5.03	0.02	0	0	>7*	na
228.7	42.6	4.93	0.06	0	0	>7*	na
323.0	58.8	4.78	0.05	0	0	>7*	na
336.7	61.8	4.76	0.06	0	0	>7*	na

258

259 **Fig 4. Changes in bioluminescence intensity, bacterial viability and physical-chemical**  
 260 **parameters of anolyte. Log reduction (LR) was calculated using Inlet t<sub>0</sub> as the reference**  
 261 **point. LR datasets are represented by an average of 3 replicates ±SD. Closed circuit MFCs**  
 262 **were marked by gray circles. The star \* symbol indicates the first trial, while labels without**  
 263 **symbol indicate the second trial. The data between oc3 and k3 are shown disconnected, since**  
 264 **the two cascades were independent (see Fig.1), i.e. the effluent from oc3 did not flow into k3.**

265 ORP data are shown for Trial 2 only, due to technical problems of measurement during Trial  
266 1.

267

268 The log-reduction of RLU value calculated for the abiotic OC cascade (ac3) was 3 orders  
269 of magnitude lower than that from the closed circuit MFCs, which is also consistent with the  
270 results obtained from the real time luminescence monitoring. Nevertheless, a significant  
271 ( $p < 0.05$ ) disinfecting effect was observed for the biotic OC control (oc3). The RLU LR  
272 values reached  $3.13 \pm 0.02$  LR and  $3.64 \pm 0.04$  LR in two individual trials, while the CFU LR  
273 observed for Trial 2 was equal to  $2.71 \pm 0.05$  LR and  $0.16 \pm 0.15$  LR for Trial 1. In all cases, the  
274 LR values were still lower when compared to those observed from the closed circuit MFCs,  
275 showing that the killing potential of OC MFCs was much lower than that of the CC MFCs.  
276 The difference in CFU LR values when comparing both trials indicates that perhaps the weak  
277 disinfecting properties observed from the biotic OC control, could have been the result of  
278 lytic or hydrolytic biochemical reactions, which were dependent on the length of time that  
279 individual MFCs were running for. Trials 1 and 2 were performed in two different periods of  
280 time, resulting in two different power performance levels (as shown on Fig 2). The cathode  
281 regeneration procedure (Pasternak *et al.*, 2016b), was not carried out for the OC MFCs, thus a  
282 significant deterioration of the electrodes could have affected the overall OC MFC  
283 environment and biofilm metabolism. The ORP values, reaching  $-490.7$  mV (vs SHE), which  
284 is the highest negative ORP observed in this study, indicated good dynamic/electrochemical  
285 conditions within the OC MFCs (Fig 4). The sub-optimum power output conditions occurring  
286 in the CC MFCs ( $31 \mu\text{W}$  Trial 1 &  $65 \mu\text{W}$  Trial 2 vs  $105 \mu\text{W}$  max recorded [15]) may have  
287 allowed antagonistic (to electron transfer) fermentation processes. Such conditions could  
288 have a negative effect on the killing efficacy, suppressing the overall capabilities of the  
289 MFCs.

290 Although the killing effect on *S. enteritidis* was observed for the biotic OC control, the  
291 CFU LR values observed for the corresponding closed circuit MFCs were significantly higher  
292 ( $p < 0.05$ ). Moreover (with one exception), the LR values were increasing after the treatment  
293 in each triplet of CC MFCs, reaching up to  $4.43 \pm 0.04$  LR at the end of the cascade. The pH  
294 of urine increased from pH 6.8 (when fresh) to as high as pH 8.94-9.59 thus helping to  
295 antagonize the growth of *Salmonella* in all of the inoculated, biotic MFCs, while the ORP,  
296 monitored for Trial 2 indicated a highly reducing environment. Moreover, a linear decrease  
297 of ORP was noticed along the CC cascade. These values of CFU LR are in agreement with  
298 those observed when the hydrogen peroxide derived from cathodic reactions was used against  
299 coliforms (Arends *et al.*, 2014).

300 It is therefore hypothesised that the cascade effect was creating favourable conditions for  
301 the removal of pathogenic species from urine by the sequential increase of the reducing force,  
302 along with the increase of the pH. The good decrease of the ORP force and increase of the  
303 pH, together with the lower LR observed for the OC biotic control (oc3) suggest that ORP  
304 and pH were two important factors influencing the killing potential of MFCs. However,  
305 although direct effect of ORP and pH on pathogen inactivation was observed, the LR values  
306 achieved for closed circuit MFCs were higher than the open circuit controls in all trials. The  
307 closed circuit MFCs reached higher LR values in comparison to OC MFCs for which the  
308 recorded pH was comparable or even higher (Trial 1 – Figure 4) than for the closed circuit  
309 MFCs. Therefore, the results suggest that the increase of pH, caused by the urea hydrolysis  
310 was not the only factor contributing to the inactivation of the pathogens. It is assumed that  
311 the bioelectrochemical reactions generating electric current were introducing additional stress  
312 mechanisms against the pathogenic cells, thus increasing the disinfection effect during the  
313 treatment of human waste in MFCs.

314

315 **Fig 5. Relationship between power and oxidation-reduction potential with killing**  
316 **efficacy.**

317

318 To further investigate this hypothesis, linear regression models were tested for ORP, power  
319 and LR variables (Fig 5). The highest correlation coefficients were calculated when the effect  
320 of power generated by the MFCs on CFU:LR and ORP on RLU:LR were investigated,  
321 respectively. These results indicate that although both factors were well describing the LR  
322 variables, the change in ORP had a more marked effect on the metabolic activity of the  
323 pathogens, whilst power output had higher impact on the viability of pathogenic bacteria. It is  
324 therefore concluded that both of these factors played a role in creating a hostile environment  
325 for the pathogenic bacteria. The results also indicate that oxidising the urine constituents in  
326 current-generating pathways have induced a killing effect when compared to the non-current  
327 generating pathways occurring in both types of OC controls.

328 The negative ORP was driven by the current-producing reactions. The decrease of  
329 metabolic activity observed by the decrease of the bioluminescence was followed by a  
330 decrease of viability. Such a decrease in viable counts of *E. coli* was also observed, when the  
331 negative potential was artificially supplied to the carbon fiber electrode, resulting in over 3  
332 log-fold reduction (Matsunaga *et al.*, 1994). It is therefore assumed, that the negative anodic  
333 potential created in MFCs treating urine may lead to the inhibition of the respiratory chains of  
334 bacteria and consequently, death of the cells.

335 The decrease of the viability of the representative serovar of *Salmonellae* observed in this  
336 study was of a higher magnitude, when compared to conventional wastewater treatment  
337 plants. In a comprehensive study reported by Koivunen *et al.* the authors have identified 32  
338 different *Salmonella* serovars and recorded removal efficiency of 2 and 3 log units during  
339 treatment in biological-chemical reactors and tertiary filtration units (Koivunen *et al.*, 2003).

340 Increased killing efficiency was observed when UV or ozone were used to treat wastewater  
341 (Gehr *et al.*, 2003). The disinfection efficiency observed in this study is similar to the more  
342 recently described method of electroporation using a conductive nanosponge (Boehm and  
343 Cui, 2013). These authors recorded a disinfection efficiency reaching up to 6 log-fold  
344 reduction of enteric bacteria (including *Salmonella enterica* serovar *typhimurium*).  
345 Nevertheless, the voltage externally applied to the anode was 2 orders of magnitude higher  
346 than that produced by the MFCs used in this study and an additional disinfectant was used to  
347 induce the killing process. It is possible that the power production in MFCs may have a  
348 similar effect on pathogenic bacteria. The electrochemical process described herewith, may  
349 lead to the increased uptake of ionic species to the interior of bacterial cells.

350 It is also assumed that the kill rates observed in this study may have also resulted from the  
351 formation of ionic-redox chemical species that led to negative ORP. In addition, the role and  
352 contribution of other potential bacteriocidal mechanisms (e.g. lytic enzymes, antibiotics,  
353 bacteriocins or other toxic molecules) has not been investigated in the present study.  
354 Considering that closed circuit MFCs have shown significantly higher killing efficiency, it is  
355 concluded that the production of electric power resulted in changing both the physico-  
356 chemical parameters of urine and influenced the integrity of the bacterial cells, leading to a  
357 high killing efficacy in a continuously operating MFC system.

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362

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Fig. 1









