

1 **Contaminants removal and bacterial activity enhancement along**  
2 **the flow path of constructed wetland microbial fuel cells**

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24 **Abstract**

25 Microbial fuel cells implemented in constructed wetlands (CW-MFCs), albeit a relatively new  
26 technology still under study, have shown to improve treatment efficiency of urban wastewater.  
27 So far the vast majority of CW-MFC systems investigated were designed as lab-scale systems  
28 working under rather unrealistic hydraulic conditions using synthetic wastewater. The main  
29 objective of this work was to quantify CW-MFCs performance operated under different  
30 conditions in a more realistic setup using meso-scale systems with horizontal flow fed with real  
31 urban wastewater. Operational conditions tested were organic loading rate ( $4.9\pm 1.6$ ,  $6.7\pm 1.4$   
32 and  $13.6\pm 3.2$  g COD/m<sup>2</sup>.day) and hydraulic regime (continuous vs intermittent feeding) as well  
33 as different electrical connections: CW control (conventional CW without electrodes), open-  
34 circuit CW-MFC (external circuit between anode and cathode not connected) and closed-circuit  
35 CW-MFC (external circuit connected).

36 Eight horizontal subsurface flow CWs were operated for about four months. Each wetland  
37 consisted of a PVC reservoir of 0.193 m<sup>2</sup> filled with 4/8 mm granitic riverine gravel (wetted depth  
38 25 cm). All wetlands had intermediate sampling points for gravel and interstitial liquid sampling.  
39 The CW-MFCs were designed as three MFCs incorporated one after the other along the flow  
40 path of the CWs. Anodes consisted of gravel with an incorporated current collector (stainless  
41 steel mesh) and the cathode consisted of a graphite felt layer. Electrodes of closed-circuit CW-  
42 MFC systems were connected externally over a 220  $\Omega$  resistance.

43 Results showed no significant differences between tested organic loading rates, hydraulic  
44 regimes or electrical connections, however, on average, systems operated in closed-circuit CW-  
45 MFC mode under continuous flow outperformed the other experimental conditions. Closed-  
46 circuit CW-MFC compared to conventional CW control systems showed around 5% and 22%  
47 higher COD and ammonium removal, respectively. Correspondingly, overall bacteria activity, as

48 measured by the fluorescein diacetate technique, was higher (4% to 34%) in closed-circuit  
49 systems when compared to CW control systems.

50

51 **Keywords**

52 Constructed wetlands, urban wastewater, microbial fuel cells, bacterial activity, hydraulic  
53 regime, organic loading rate

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58 **1. INTRODUCTION**

59 Constructed wetlands (CWs) are engineered systems for water and wastewater treatment,  
60 simulating processes occurring in nature (Vymazal, 2011). Treatment in CWs is based on  
61 physical, chemical and biological processes. The treatment beds consist of shallow lined basins  
62 filled with a filter media (generally gravel or sand) and are commonly planted with aquatic  
63 macrophytes (García et al., 2010). CWs treat wastewater from a wide range of sources, such as  
64 domestic, industrial and agricultural wastewater or landfill leachate, in different climate zones  
65 around the world (Langergraber and Haberl, 2001; Molle et al., 2005). These natural systems are  
66 characterized by their low energy demand, comparative low cost, easy operation and  
67 maintenance as well as the possibility to use local materials and labor for their construction.  
68 Hence, they have a strong potential for application as an alternative to conventional systems for  
69 sanitation of small communities, also in rural areas and emerging countries (García, 2001;  
70 Kivaisi, 2001; Puigagut et al., 2007). A disadvantage of CWs is their relatively high area demand  
71 of ca. 1-10 m<sup>2</sup>/p.e. (Kadlec and Wallace, 2009).

72 Microbial Fuel Cells (MFCs) are bioelectrochemical systems that generate current by means of  
73 electrochemically active microorganisms as catalysts (Logan et al., 2006). In a MFC, organic and  
74 inorganic substrates are oxidized by bacteria and the electrons are transferred to the anode  
75 from where they flow through a conductive material and a resistor to an electron acceptor, such  
76 as oxygen, at the cathode (Logan et al., 2006; Rabaey et al., 2007). Compounds oxidized at the  
77 anode are mainly simple carbohydrates such as glucose or acetate that can be already present  
78 in the environment or obtained from the microbial degradation of complex organic substrates  
79 such as organic sediments or wastewater (Min and Logan, 2004; Reimers et al., 2001). Therefore,  
80 MFCs are able to harvest energy in the form of electricity directly from wastewater (Du et al.,  
81 2007; Lefebvre et al., 2011; Min and Logan, 2004).

82 MFC systems can exploit the naturally occurring redox gradient in horizontal subsurface flow  
83 (HF) CWs. The first publication on CWs incorporating MFCs (CW-MFCs) appeared in 2012 and  
84 was published by Yadav et al. (2012). Since then publications on the subject per year are  
85 increasing, resulting in a rough total of around 79 up until March 2018.

86 So far the vast majority of CW-MFC systems investigated are designed as lab-scale systems  
87 working under rather unrealistic hydraulic conditions (up-flow, batch feeding) using synthetic  
88 wastewater (Corbella et al., 2016b; Doherty et al., 2015; Fang et al., 2016; Liu et al., 2012; Oon  
89 et al., 2017; Song et al., 2017; Srivastava et al., 2015; Villaseñor et al., 2013; Wang et al., 2017;  
90 F. Xu et al., 2018; Xu et al., 2017; Zhao et al., 2013).

91 As indicated above, the implementation of MFCs in CWs is a relatively new research field, and  
92 current available information on this topic is mostly focused on optimizing treatment efficiency  
93 and energy production. Conventional MFCs are able to produce up to  $12 \text{ W}\cdot\text{m}^{-3}$  electricity (Logan  
94 and Rabaey, 2012). However, due to high internal resistances the highest reported electrical  
95 output from CW-MFCs is  $2 \text{ W}\cdot\text{m}^{-3}$  (Xu et al., 2017), whereas averages for most systems are even  
96 a magnitude lower. Systems using wastewater reported electricity production of  $9.4 \text{ mW}\cdot\text{m}^{-2}$

97 (Zhao et al., 2013) and  $276 \text{ mW}\cdot\text{m}^{-3}$  (Doherty et al., 2015). In comparison to solar panels with for  
98 example  $175 \text{ W}/\text{m}^2$  (Panasonic HIT® Photovoltaic Module, 2012) it seems that electricity  
99 production alone from wastewater by MFC or CW-MFC technology is currently not a feasible  
100 goal.

101 Besides energy production, CW-MFC systems can also improve the treatment of organic matter.  
102 When comparing closed-circuit (MFC anode and cathode externally connected) and open-circuit  
103 (MFC anode and cathode externally not connected) lab-scale results, Katuri et al. (2011) showed  
104 16-20% higher COD removal for closed-circuit MFC systems. The same tendency was observed  
105 by Srivastava et al. (2015) with 16-20% higher COD removal in closed-circuit compared to open-  
106 circuit CW-MFCs and even 10-31% higher performance compared to conventional CWs (without  
107 anode and cathode). Exemplary COD removal efficiencies in CW-MFC are 75% (Yadav et al.,  
108 2012), 82% (Xu et al., 2018), 76.5% (Zhao et al., 2013) and even up to 100% (Oon et al., 2015),  
109 however the latter used artificial aeration. As mentioned before, most of the systems  
110 investigated so far do not reproduce realistic HF CW conditions due to the flow direction and  
111 geometry of systems (often up-flow in tubular reactors), and smaller internal resistances than  
112 in full-scale implementation due to smaller distances between electrodes and other factors. In  
113 general the presence of an insoluble electron acceptor, i.e. an anode, showed to increase the  
114 metabolic rate of anaerobic bacteria (Fang et al., 2013) and seems to be a beneficial  
115 environment for the growth of bacteria apart from electrogens as well; Xu et al. (2018) found  
116 that the microbial community's richness and diversity is higher in closed-circuit systems and also  
117 Wang et al. (2016b) found higher richness in closed-circuit as compared to open-circuit CW-MFC  
118 systems. Additionally, electroactive bacteria seem to outperform other microbial communities  
119 (Zhang et al., 2015).

120 Apart from organic matter, MFC studies have shown that closed-circuit MFCs show a higher  
121 ammonium treatment efficiency than open-circuit MFCs (Kim et al., 2008; Lu et al., 2009). This

122 increased ammonium removal efficiency could also be observed in CW-MFCs by Corbella and  
123 Puigagut (2018) with ammonium removal efficiencies of  $66\pm 14\%$  and  $53\pm 17\%$  for closed-circuit  
124 and open-circuit mode, respectively.

125 The main objective of this work was to quantify and improve the treatment efficiency of urban  
126 wastewater with CW-MFCs. The effect of hydraulic regime (continuous/intermittent) and  
127 organic loading rate ( $4.9\pm 1.6$ ,  $6.7\pm 1.4$  and  $13.6\pm 3.2$  g COD/m<sup>2</sup>.day) on CW-MFCs performance  
128 and the effect of CW-MFCs on bacterial activity along the flow path of the treatment bed are  
129 also discussed. The authors believe that this work will provide a useful insight into the actual net  
130 contribution of CW-MFCs on the treatment of urban wastewater. In spite of the lack of plants in  
131 the systems, the CW-MFCs used in this research could give additional information on the  
132 pollutant removal in larger scale systems under more realistic CWs design and operation  
133 conditions; also the here used configuration with three MFCs incorporated one after the other  
134 along the flow path of the CWs and the associated measured current along the flow path  
135 together with the measured bacterial activity will help to provide a better insight into the  
136 bioelectrochemical behavior and nutrient removal of CW-MFCs.

## 137 **2. MATERIALS AND METHODS**

### 138 **2.1 General design**

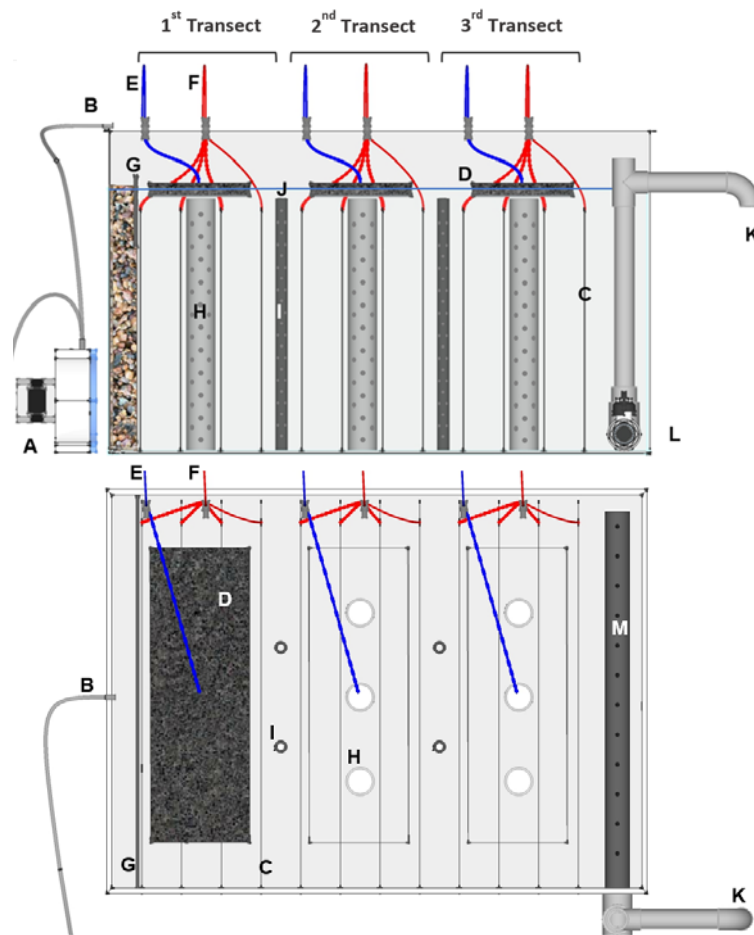
139 For the purpose of this work, eight meso-scale horizontal subsurface flow (HF) CW-MFC systems  
140 consisting of a PVC reservoir of ca. 0.193 m<sup>2</sup> (55 x 35 cm) surface area filled up with 4/8 mm  
141 granitic riverine gravel were constructed. The systems were not planted in order to not add  
142 another influencing parameter and further increase the experiment complexity. Campaigns with  
143 planted CW-MFC duplicates are planned for the future. Wetted depth was set to be 25 cm. At  
144 the inlet and around the drainage of the outlet 7/14 mm granitic riverine gravel was used.

145 The CW-MFCs were designed as three MFCs incorporated one after the other along the flow  
146 path of the CWs. Therefore, the experimental systems were operated as a three-MFC system  
147 (see Figure 1). Each electrode consisted of an anode with four stainless steel mesh rectangles  
148 (Figure 1, C) (SS marine grade A316L, mesh width=4.60 mm,  $\varnothing$ wire=1.000 mm, S/ISO 9044:1999)  
149 in series (4 cm away from each other). Each metal mesh covered nearly the whole cross-  
150 sectional area (0.08 m<sup>2</sup>) of the CW. Each cathode consisted of a carbon felt mat (Figure 1, D)  
151 (1.27 cm thick, with a projected surface of 0.03 m<sup>2</sup>, 99.0% carbon purity). A layer of glass wool  
152 was placed underneath the cathodes in order to avoid any oxygen leaking from the cathode  
153 down to the anode as recommended elsewhere (Venkata Mohan et al., 2008). For the  
154 connected systems (closed-circuit), each electrode's anode and cathode were externally  
155 connected via a 220  $\Omega$  resistance, selected according to results by Corbella and Puigagut (2018).  
156 The voltage across the external resistance for each electrode was continuously monitored by  
157 means of a datalogger (Campbell Scientific CR1000, AM16/32B Multiplexor). For the open-  
158 circuit systems, the anode and cathode were not connected (open-circuit). For the conventional  
159 HF CW control (operated from week 12 to week 23), metal meshes were removed from two of  
160 the systems that were previously operated under open-circuit conditions.

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166 **Figure 1. Section- (top) and plan-view (bottom) of the CW-MFC systems. A: Pump; B: Inflow;**  
167 **C: Anode; D: Cathode; E/F: Anode/Cathode connection to datalogger; G: Inflow barrier to**  
168 **avoid water short-circuiting on surface; H: Gravel core sampling tubes; I: Liquid sampling**  
169 **tubes; J: Water level; K: Standing pipe effluent; L: Drainage; M: Effluent collection tube.**

170

171 Intermediate liquid sampling ports were installed after the first third and second third (Figure 1,

172 I), separating the first, second and third transect of the systems which are basically congruent

173 with the three successive MFCs of the wetland. These sampling ports consisted of two

174 perforated plastic tubes ( $\varnothing=1\text{cm}$ , positioned vertically 5 cm left and right of center). Underneath

175 each cathode three perforated plastic tubes ( $\varnothing=3.2\text{cm}$ , positioned at the center and 8.5 cm left

176 and right of the center) were placed and filled with a plastic mesh “sock” containing the same



177 gravel material as the systems (Figure 1, H). These socks were removable and were used to test  
178 the bacterial activity along the flow path of the wetland.

## 179 **2.2 Operational conditions**

180 All systems received the same primary treated urban wastewater throughout the whole  
181 experimentation period (23 weeks within the period from May until December 2017 excluding  
182 breaks of 8 weeks during summer and the first week of December). Wastewater feeding started  
183 already 6 weeks before the start of experimentation in order to establish the biofilm in the  
184 systems. The wastewater was stored within a reservoir of ca. 180 L that was refilled every  
185 weekday in order to keep the organic matter concentration as stable as possible. Sampling and  
186 analysis were conducted once a week.

187 During the first 10 experimentation weeks (from May to July 2017) the effect of hydraulic regime  
188 and organic loading rate on the treatment performance of closed- and open-circuit systems was  
189 tested. The compared hydraulic regimes were continuous and intermittent feeding. Continuous  
190 flow mode systems received the same flow rate all day long, whereas intermittent flow systems  
191 received alternating 4 hours of double flow and 4 hours of no flow, resulting in the same total  
192 flow as continuous flow systems on a daily basis. The inflow was provided by peristaltic pumps  
193 (Damova MP-3035-6M) controlled by variable frequency drives (VFDs) (Toshiba VF-nC3S).

194 Two different hydraulic loading rates were applied, i.e. 26 and 52 mm/d. The higher rate was  
195 obtained by doubling the flow rate (and thereby dividing the HRT in half) resulting in a  
196 theoretical HRT and average organic loading rate (OLR) of  $3.9 \pm 0.2$  and  $1.9 \pm 0.1$  days and around  
197  $4.9 \pm 1.6$  and  $13.6 \pm 3.2$  g COD/m<sup>2</sup>.day, during low and high loading periods, respectively (the high  
198 OLR is not exactly the double of the low OLR due to natural variations of the urban wastewater  
199 used). During experimentation week 1-5 the eight systems were operated under low OLR, and  
200 during experimentation week 6-10 with high OLR. The parameter OLR was chosen over HRT for

201 comparison of the periods due to the higher reliability in the calculation of the OLR as opposed  
 202 to the HRT which is only a theoretical value and could be different to the real HRT in the systems.  
 203 The other two factors of continuous/intermittent feeding and closed-/open-circuit electrical  
 204 connection led to duplicates of each combination in the first 10 weeks of experimentation (see  
 205 Table 1).  
 206

**Table 1. Operational conditions during the 23 weeks of experimentation concerning organic loading, hydraulic regime and electrical connection within the systems as well as the resulting individual experimental setups of the eight systems**

Experi- mentation Week <sup>a</sup>	Organic loading rate (g COD/m <sup>2</sup> .day)	Hydraulic regime	Electrical connection	Resulting system setup
1-5	Low OLR1 4.9±1.6	Continuous or Intermittent	Closed-circuit or Open-circuit	2x continuous flow / closed-circuit 2x continuous flow / open-circuit
6-10	High OLR 13.6±3.2			2x intermittent flow / closed-circuit 2x intermittent flow / open-circuit
11	Low OLR2 6.7±1.4	Continuous	Closed-circuit, Open-circuit or CW control	4x closed-circuit 4x open-circuit
12-23				4x closed-circuit 2x open-circuit 2x CW control

207 <sup>a</sup> only weeks in which experiments were conducted, i.e. excl. 8 weeks during summer and first week of December  
 208

209 Starting from experimentation week 11 (in September 2017, after 6 weeks of summer break  
 210 during which the systems were fed with water and two weeks of wastewater feeding to restart  
 211 systems), the treatment efficiency experiments were continued (until end of December 2017,  
 212 except for the first week of December), this time only with continuous flow and low HLR (ca.  
 213 26 mm/d) resulting in a theoretical HRT of 3.8±0.3 days and an average OLR of 6.7±1.4  
 214 g COD/m<sup>2</sup>.day. Starting from experimentation week 12 two of the open-circuit CW-MFCs were  
 215 converted to conventional HF CWs by removing the SS mesh anodes, creating a conventional  
 216 CW control duplicate without electrodes, and still leaving two open-circuit CW-MFCs and four

217 closed-circuit CW-MFCs for investigation on solely the impact of the different electrical  
218 connections for the remaining experimentation weeks 12-23 (see Table 1).

### 219 **2.3 Sampling and analysis**

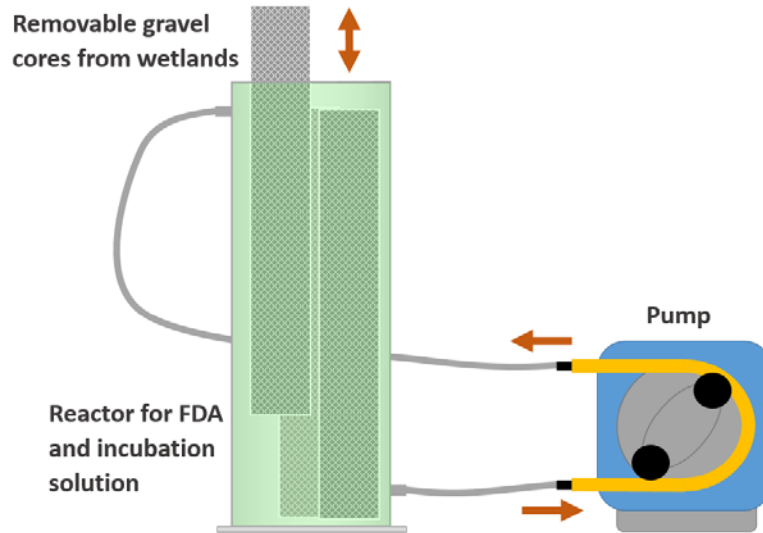
220 Samples were taken weekly from the influent, the intermediate sampling points placed at 1/3  
221 and 2/3 of the wetland length and the effluent of each system. Influent and effluent samples  
222 were grab samples collected from inlet and effluent tubes, respectively. Intermediate samples  
223 were 60 mL composite grab samples (four times 15 mL) extracted from the pairs of sampling  
224 tubes placed after 1/3 and 2/3 from the inlet by means of a syringe. From each tube, two  
225 samples were taken, at 15 and 5 cm depth (i.e., 10 and 20 cm from the bottom of the system).  
226 The parameters total chemical oxygen demand (COD), ammonium -N, nitrate -N, nitrite -N,  
227 sulfate and orthophosphate -P as well as total suspended solids (TSS) and volatile suspended  
228 solids (VSS) were analyzed according to standard methods (APHA-AWWA-WEF, 2005). Physical  
229 parameters such as wastewater temperature, dissolved oxygen (DO) concentration (both;  
230 EUTECH instruments, EcoScan DO 6) and pH (CRISON pH/mV – meter 506) were measured as  
231 well using portable devices. Statistical analysis was conducted using Kruskal-Wallis and Shapiro-  
232 Wilk tests as well as single-factor and two-factor (with replication) analysis of variance (ANOVA).

### 233 **2.4 Microbial activity analysis**

234 Microbial activity was determined by means of the fluorescein diacetate (FDA) hydrolysis, a  
235 technique that has shown to correlate well with microbial population and its activity (Adam and  
236 Duncan, 2001). The FDA is a colorless compound which can be hydrolyzed by different enzymes  
237 releasing fluorescein as an end product, which absorbs strongly at 490 nm. For this procedure,  
238 two (out of the four available) closed-circuit systems and two CW control systems were  
239 investigated, using the gravel cores contained within the sampling tubes located in each of the  
240 transects of the systems (see Figure 1). These gravel cores (three for each transect at a time)

241 were introduced into previously constructed reactors of 10 cm diameter and 28 cm height (see  
242 Figure 2).

243



244

**Figure 2.** Microbial activity analysis setup including a reactor for the FDA and incubation solution in which the removable gravel cores (three per transect) from the wetland systems are submerged. The solution is mixed by means of a peristaltic pump.

245

246 At the time the three gravel cores were submerged the reactor already contained a prepared  
247 phosphate buffer at pH 7.6 together with one mL of 0.4 mM FDA (Acros Organics) resulting in a  
248 final concentration of  $8 \cdot 10^{-4}$  mM FDA, following a similar but modified procedure by (Iasur-Kruh  
249 et al., 2010). This solution was recirculated with a pump and after 50 min a 2 ml sample was  
250 taken from the top of the reactor. Fluorescein released was measured using a  
251 spectrophotometer (Spectronic GENESYS 8 Thermo Scientific™) at a wavelength of 490 nm and  
252 then converted to Fluorescein molar mass via a calibration curve. For the purpose of this study  
253 the final Fluorescein molar mass value is then called the microbial activity. Statistical analyses  
254 were conducted using Kruskal-Wallis and Shapiro-Wilk tests as well as single-factor ANOVA.

255 **3. RESULTS AND DISCUSSION**

256 **3.1 Assessment of operational conditions to optimize CW-MFC along the flow path**

257 **3.1.1 Overview**

258 Table 2 shows an overview for COD, ammonium, nitrate, nitrite and orthophosphate removal  
 259 results from inlet to outlet, expressed in total specific mass (g/m<sup>2</sup>.d) for open-circuit and closed-  
 260 circuit CW-MFC systems (see annex Table 4 for removal in percentage). Results are further  
 261 divided into the three different OLR periods (low OLR 1 in first 5 weeks, high OLR in the following  
 262 5 weeks and low OLR 2 in the remaining 13 weeks) and different hydraulic regimes  
 263 (continuous/intermittent) for low OLR 1 and high OLR period and only continuous flow in low  
 264 OLR 2.

265

**Table 2. COD, ammonium, nitrate, nitrite and orthophosphate average mass removal rate (g/m<sup>2</sup>.d) with standard deviation from inlet to outlet for low OLR 1, high OLR and low OLR 2 as well as intermittent or continuous flow hydraulic regime for open-circuit (OC) and closed-circuit (CC) CW-MFC systems**

Removal (g/m <sup>2</sup> .d)		Low OLR 1 (week 1-5) 4.9±1.6 g COD/m <sup>2</sup> .day		High OLR (week 6-10) 13.6±3.2 g COD/m <sup>2</sup> .day		Low OLR 2 <sup>a</sup> (week 11-23) 6.7±1.4 g COD/m <sup>2</sup> .day
		Intermittent flow	Continuous flow	Intermittent flow	Continuous flow	Continuous flow
<b>COD</b> (n=4/5/11) <sup>b</sup>	<b>OC</b>	3.0±1.6	3.0±1.8	8.3±3.5	8.5±3.7	4.6±1.0
	<b>CC</b>	2.8±1.7	3.0±1.8	9.6±3.9	9.6±2.9	4.9±1.1
<b>NH<sub>4</sub> -N</b> (n=4/5/7) <sup>b</sup>	<b>OC</b>	0.2±0.1	0.2±0.1	0.5±0.7	0.6±0.6	0.3±0.2
	<b>CC</b>	0.2±0.1	0.3±0.1	0.7±0.5	0.8±0.4	0.5±0.3
<b>NO<sub>3</sub> -N</b> (n=4/4/8) <sup>b</sup>	<b>OC</b>	-0.009±0.026	-0.013±0.061	0.005±0.014	-0.002±0.018	0.000±0.000
	<b>CC</b>	-0.012±0.035	-0.032±0.064	-0.022±0.033	-0.065±0.042	-0.011±0.012
<b>NO<sub>2</sub> -N</b> (n=4/4/8) <sup>b</sup>	<b>OC</b>	0.023±0.052	0.039±0.078	0.094±0.235	-0.075±0.125	-0.004±0.014
	<b>CC</b>	0.028±0.058	0.058±0.080	0.057±0.114	-0.154±0.046	-0.002±0.020
<b>PO<sub>4</sub> -P</b> (n=4/4/8) <sup>b</sup>	<b>OC</b>	0.02±0.03	0.03±0.01	0.03±0.04	0.03±0.04	0.01±0.01
	<b>CC</b>	0.02±0.02	0.03±0.02	0.02±0.04	0.04±0.06	0.01±0.03

266 <sup>a</sup> Low OLR 2 results are shown in more detail in section 3.2 on the electrical connection effects

267 <sup>b</sup> Some experimentation weeks could not be considered due to highly diluted influent or technical analysis problems

268

269 With regards to different organic loading periods, only continuously fed systems are discussed  
270 and compared for all nutrients, since COD and ammonium treatment, though not being  
271 significantly different, were generally higher in continuously fed systems. In addition,  
272 continuously fed systems showed a very significant higher current density generation within the  
273 first transect (see Figure 3).

274

### 275 **3.1.2 Hydraulic regime effects**

276 In general, closed-circuit and continuously fed systems tended to show higher nutrient removal  
277 efficiencies when compared to the rest of operational conditions tested, although no statistically  
278 significant differences in COD or ammonium removal were found (for details see annex Table 5).

279 When comparing different hydraulic regimes with the same electrical connection, closed-circuit  
280 continuous systems had only 2 and 1% higher COD removal than closed-circuit intermittent  
281 systems during low OLR 1 and high OLR period, respectively. Open-circuit continuous systems  
282 had 2% lower and 4% higher COD removal than open-circuit intermittent systems during low  
283 OLR 1 and high OLR period, respectively. As expected, the majority of COD was removed within  
284 the first transect, since organic matter removal basically follows a first-order degradation  
285 (Kadlec and Wallace, 2009).

286 Ammonium removal rates did not show any significant differences between hydraulic regimes  
287 and electrical connections (for details see annex Table 5) but exhibited the same tendency as  
288 COD but more pronounced, with continuously fed and closed-circuit systems showing higher  
289 removal rates. When comparing different hydraulic regimes within the same electrical  
290 connection, closed-circuit continuous systems showed, in average, 11% and 4% higher  
291 ammonium removal than closed-circuit intermittent systems during low OLR 1 and high OLR  
292 period, respectively. Open-circuit continuous systems had 6 and 12% higher ammonium

293 removal than open-circuit intermittent systems during low OLR 1 and high OLR period,  
294 respectively.

295 Continuously fed systems tended to have a higher nitrate increase throughout all OLR periods,  
296 with (an extremely) significant difference only in the high OLR period, probably caused by the  
297 shortened HRT (for details see annex Table 5). Continuously fed systems showed higher nitrite  
298 removal during low OLR 1 but also nitrite increase in these systems was higher during high OLR,  
299 however, without a significant difference (a significant difference was only found in terms of  
300 electric connection, for details see annex Table 5). The strong nitrite increase in continuously  
301 fed systems in the high OLR period could be a sign of a lack of oxygen and incomplete  
302 nitrification. Dissolved oxygen concentrations in the water column (3 cm and lower below water  
303 level) were below the detection limit of the probe along the whole flow path, i.e. at the inflow  
304 as well as after first, second and last transect.

305 An explanation for the slightly higher COD and ammonium removal in closed-circuit systems  
306 could be that continuous as compared to intermittent flow in HF CWs increases the vertical  
307 redox gradient and thereby provides a higher potential to drive MFC reactions (Corbella et al.,  
308 2014). The insignificance of differences could be partly due to the relatively high standard  
309 deviation, most likely caused by the variation in quality of the used real urban wastewater due  
310 to natural causes like rainfall events or dry periods.

311 Due to the insignificant difference of COD and ammonium removal between hydraulic regimes,  
312 the authors decided to continue operation from week 11 onwards with continuous flow only,  
313 since this is the regular regime for full-scale HF CWs. In addition, intermittently fed systems  
314 showed an extremely significant reduction in current density generation within the first transect  
315 (see Figure 3).

316 Average orthophosphate removal was very similar in the low OLR 1 period and slightly higher in  
317 continuously fed systems during high OLR period, however, without a statistically significant  
318 difference (for details see annex Table 5). A reason for the difference during high OLR period  
319 could be the temporarily (during feeding times) shortened HRT in intermittently fed systems  
320 leading to fewer orthophosphate removal through processes like adsorption and precipitation.

321

### 322 **3.1.3 Organic loading effects**

323 Overall, the removal efficiency of COD and ammonium did not depend on the OLR (low period  
324 one  $4.9 \pm 1.6$ , high  $13.6 \pm 3.2$  g COD/m<sup>2</sup>.day and low period two  $6.7 \pm 1.4$  g COD/m<sup>2</sup>.day) and the  
325 thereby reduced HRT, showing no statistically significant differences (for details see annex Table  
326 6). Total COD and ammonium removal on a mass basis was higher during the high OLR period,  
327 due to the higher influent concentrations (see Table 2). Despite the differing OLRs, removal rates  
328 in percentage showed that there were no real differences between OLR periods in COD or  
329 ammonium removal (see Table 4). In fact the removal efficiencies in percentage were rather  
330 increasing a little over time, from around 60% to 70% for COD and from around 25 to 40% for  
331 ammonium, probably due to the maturing of the systems. Both average nitrate and nitrite mass  
332 in closed-circuit systems increased during the high OLR period from in- to outlet. This could be  
333 interpreted as an effect of the observed increased ammonium removal through nitrification.

334 The systems adaptability to fluctuating organic loads illustrates a general asset of CWs; due to  
335 the fact that the majority of treatment happens in the first section of HF CWs, the remaining  
336 part of the system is able to lower the effects of flow and nutrient concentration peaks to a large  
337 degree, given that the systems are not overloaded or clogged (Samsó and García, 2014).

338 For the selection of the optimal OLR in CW-MFC systems it is important to find a good balance  
339 between the provision of sufficient substrate at the anode on the one side and overloading the  
340 system and thereby limiting the cathode functionality through growth of heterotrophic bacteria



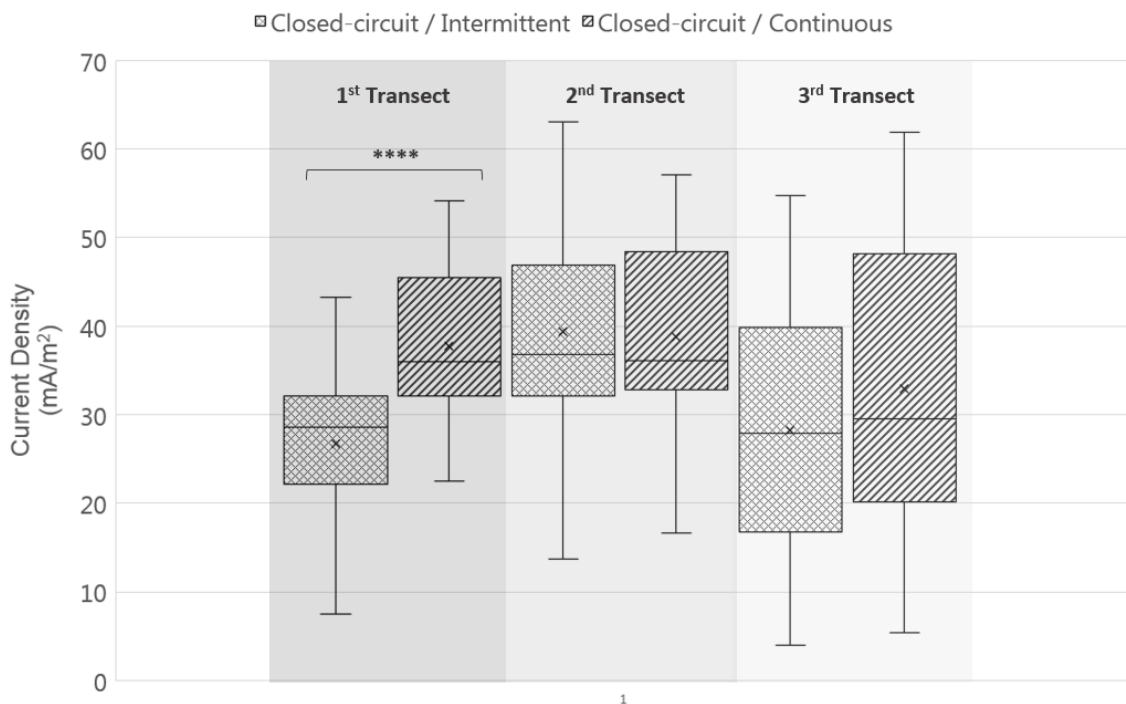
341 on the other (Doherty et al., 2015; Freguia et al., 2008; Villaseñor et al., 2013). Capodaglio et al.  
342 (2015) tested different OLRs in swine manure fed MFCs and found that lower OLR (volumetric  
343 OLR 0.7 kg COD/m<sup>3</sup>.day) advantaged exoelectrogenic bacteria growth and activity over  
344 methanogenics as compared to higher OLR (volumetric OLR 11.2 kg COD/m<sup>3</sup>.day). The highest  
345 OLR chosen in this study (corresponding to 0.06 kg COD/m<sup>3</sup>.day) was governed by the given  
346 strength of the available urban wastewater and the highest hydraulic loading possible for  
347 continuous operation, given the size of the available feeding tank. Since the two tested OLRs in  
348 this study did not show significant differences, it seems they were within the above mentioned  
349 balanced range for the operation of CW-MFC systems, though rather on the very low end  
350 compared to MFC studies which used OLRs of a magnitude higher. However, OLRs in the  
351 presented study are in the range of conventional HF CW OLRs (Vymazal, 2005). Of course the  
352 OLR range for best performance is also dependent on the MFC architecture, e.g. the used anode  
353 with gravel and stainless steel mesh as electron acceptor has to be taken into account as well.  
354 Additionally, by offering a more favorable electron acceptor, MFCs have shown to postpone  
355 methane production, for example in experiments using plant MFCs (PMFC) inside rice  
356 microcosms (Arends et al., 2014) and in CW-MFCs (Fang et al., 2013).

357 With regards to electrical connections, although no significant differences were found within  
358 each of the three OLR periods, there was a slight tendency of increased treatment performance  
359 for closed-circuit systems in high OLR period and low OLR period 2. The authors believe that the  
360 absence of any difference among experimental conditions in continuously fed systems for the  
361 first experimental period (weeks 1-5) was due to the fact that the systems, and therefore the  
362 electrogenic biofilm, was still immature at the beginning of the experimentation, which is also  
363 reflected in the observed current, which was still increasing in all transects at the time (see  
364 Figure 3).

365 Low OLR 1 and high OLR periods had similar orthophosphate mass removal values although the  
 366 influent load was doubled in the latter. Also, removal of orthophosphates in the last low OLR  
 367 period 2 decreased below the levels of low OLR period 1 (see Table 2). These changes were  
 368 probably not due to the different organic loading regimes but more likely due to the fact that  
 369 phosphorus storage in CWs decreases over time due to finite capacity of adsorption sites in the  
 370 biofilm and media (Kadlec and Wallace, 2009). In any case, the organic loading rate seems to  
 371 have had no mentionable effect on orthophosphate removal in open- or closed-circuit systems.  
 372

### 373 3.1.4 Current

374 Figure 3 shows average current densities from the three MFCs corresponding to the three  
 375 transects along the flow path for the intermittently and continuously fed closed-circuit systems.  
 376



\*\*\*\* P-value < 0.0001

**Figure 3. Current density of intermittently and continuously fed closed-circuit systems per electrode and transect along the flow path during the first 10 weeks of experiments**

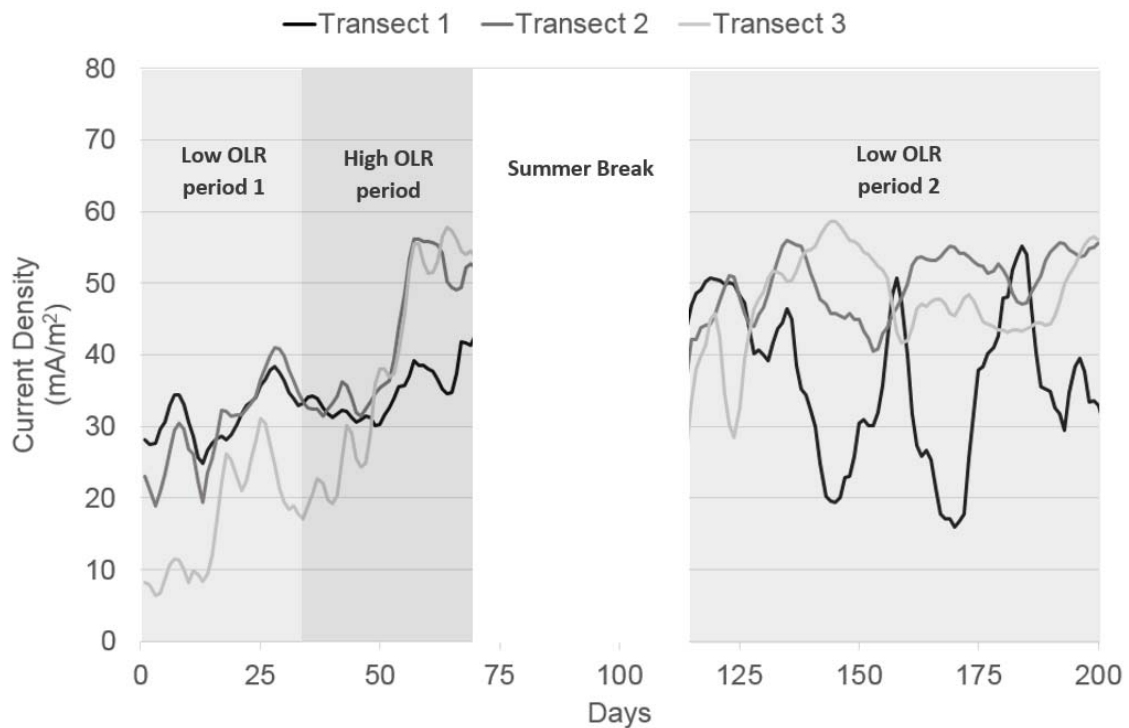
377

378 Average current densities (based on the projected anodic surface area) for closed-  
 379 circuit/intermittent and closed-circuit/continuous systems per transect resulted in  $26.8 \pm 9.4$  and  
 380  $37.7 \pm 8.1$  mA/m<sup>2</sup> for the first electrode,  $39.4 \pm 10.7$  and  $38.8 \pm 10.2$  mA/m<sup>2</sup> for the second  
 381 electrode and  $28.2 \pm 9.4$  and  $32.9 \pm 17.1$  mA/m<sup>2</sup> for the third electrode, respectively. Differences  
 382 among hydraulic regimes were only statistically significant for the first transect ( $p < 0.0001$ ) (F  
 383 (1, 68);  $p = 3E-11$ ), while differences in second (F (1, 68);  $p = 0.73$ ) and third transect (F (1, 68);  
 384  $p = 0.08$ ) were not significant.

385 Current results show that the hydraulic regime had an extremely significant effect on the first  
 386 third of the systems with higher values in continuously fed systems.

387 With regards to OLR effect, Figure 4 shows the average current densities per transect of the four  
 388 closed-circuit CW-MFC systems during different OLR periods interrupted by the summer break.

389



**Figure 4.** Average current densities from four closed-circuit systems for each transect along time

390

391 Current densities during low OLR period 1 were  $33\pm 6$ ,  $32\pm 9$  and  $16\pm 9$  mA/m<sup>2</sup> for first, second  
392 and third transect, respectively. During the high OLR period current densities increased to  
393  $43\pm 10$ ,  $45\pm 11$  and  $43\pm 13$  mA/m<sup>2</sup> for first, second and third transect, respectively. Finally, during  
394 low OLR period 2 current densities amounted to  $31\pm 15$ ,  $49\pm 9$  and  $50\pm 7$  mA/m<sup>2</sup> for first, second  
395 and third transect, respectively. Current densities in the first low OLR period were generally  
396 lower than in the following high and low OLR period 2. This is probably due to the incomplete  
397 maturity of the systems during the first weeks after experimentation start, rather than due to  
398 OLR effects, since current densities during the second low OLR period are of similar magnitude  
399 than those of the high OLR period.

400

## 401 **3.2 Contaminant removal and microbial activity under different electrical connections**

### 402 **3.2.1 Overview**

403 In this section, contaminant removal efficiency of conventional, open-circuit and closed-circuit  
404 wetlands is addressed from the results obtained during week 12 to 23 of experimentation.  
405 During this period, all systems were operated in continuous flow with an average OLR of  
406  $6.7\pm 1.4$  g COD/m<sup>2</sup>.

407 Table 3 summarizes the results of COD, ammonium, nitrate, nitrite and orthophosphate during  
408 the last 12 weeks of experimentation for all three electrical connections; CW control, open-  
409 circuit (OC) and closed-circuit (CC) CW-MFC systems. The results are shown as average mass at  
410 influent, after first transect, after second transect and effluent as well as removal from influent  
411 to effluent based on the average mass and percentage.

412

**Table 3.** Results for COD, ammonium, nitrate, nitrite and orthophosphate for CW control, open-circuit (OC) and closed-circuit (CC) CW-MFC systems during the last 12 experimentation weeks, expressed as average mass at influent, after first transect, after second transect and effluent as well as removal from influent to effluent based on the average mass and percentage.

		Influent	1/3	2/3	Effluent	Removal from Influent to Effluent	
						(g/m <sup>2</sup> .d)	(%)
<b>COD</b> (n=11) <sup>a</sup>	<b>CW</b>	6.6±1.5	3.3±1.0	2.5±0.6	2.0±1.1	4.5±1.0	69%
	<b>OC</b>	6.4±1.6	3.0±0.9	2.2±0.9	1.8±0.9	4.6±1.0	72%
	<b>CC</b>	6.7±1.5	2.9±1.0	2.1±0.9	1.7±0.9	4.9±1.1	74%
<b>NH<sub>4</sub>-N</b> (n=7) <sup>a</sup>	<b>CW</b>	1.2±0.2	1.1±0.2	0.9±0.2	1.0±0.3	0.3±0.3	19%
	<b>OC</b>	1.2±0.1	1.0±0.2	0.9±0.2	0.9±0.2	0.3±0.2	24%
	<b>CC</b>	1.3±0.1	1.0±0.1	0.8±0.2	0.7±0.2	0.5±0.3	41%
<b>NO<sub>3</sub>-N</b> (n=8) <sup>a</sup>	<b>CW</b>	0.002±0.007	0.000±0.000	0.0041±0.042	0.002±0.005	0.000±0.009	-2%
	<b>OC</b>	0.001±0.004	0.000±0.000	0.031±0.023	0.001±0.004	0.000±0.000	0%
	<b>CC</b>	0.000±0.000	0.001±0.003	0.021±0.017	0.011±0.012	-0.011±0.012	NA <sup>b</sup>
<b>NO<sub>2</sub>-N</b> (n=8) <sup>a</sup>	<b>CW</b>	0.008±0.009	0.003±0.005	0.018±0.026	0.011±0.014	-0.003±0.008	-33%
	<b>OC</b>	0.011±0.017	0.014±0.017	0.034±0.017	0.015±0.019	-0.004±0.014	-40%
	<b>CC</b>	0.014±0.019	0.013±0.011	0.022±0.026	0.016±0.032	-0.002±0.020	-17%
<b>PO<sub>4</sub>-P</b> (n=8) <sup>a</sup>	<b>CW</b>	0.11±0.02	0.11±0.02	0.09±0.02	0.11±0.06	0.00±0.03	1%
	<b>OC</b>	0.11±0.02	0.10±0.02	0.09±0.02	0.09±0.02	0.01±0.01	10%
	<b>CC</b>	0.10±0.02	0.11±0.02	0.09±0.02	0.09±0.03	0.01±0.03	5%

<sup>a</sup> Some experimentation weeks could not be considered due to highly diluted influent or technical analysis problems

<sup>b</sup> Division by zero

413  
414  
415

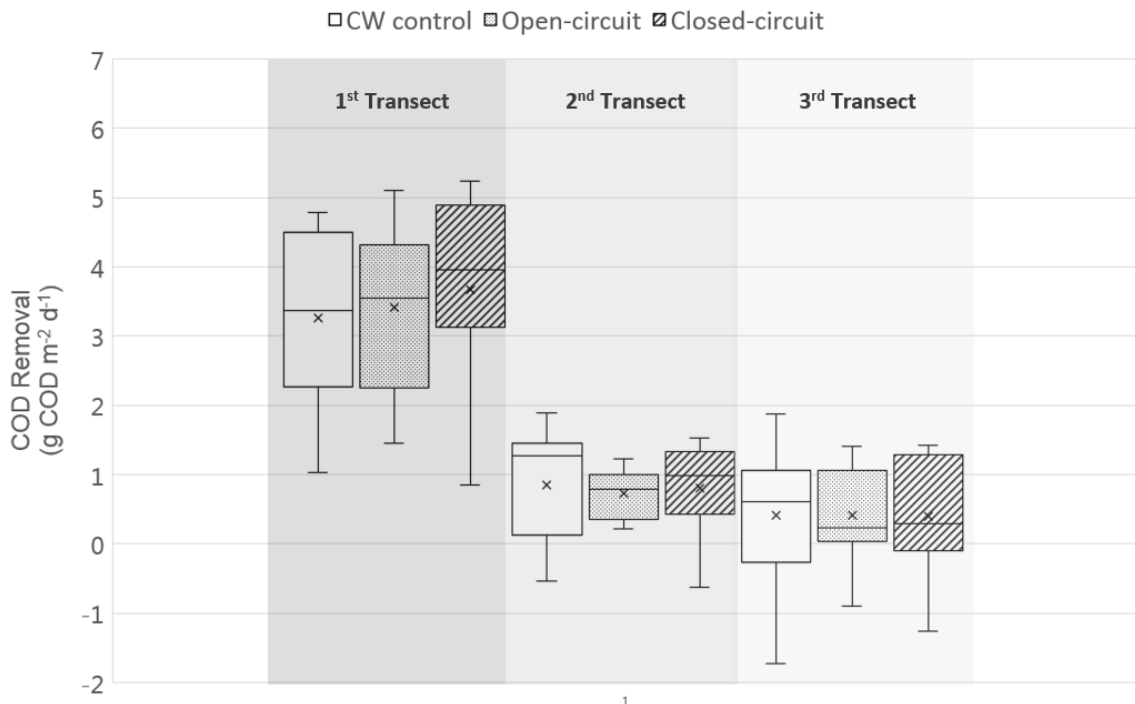
### 416 3.2.2 Electrical connection effect

417 As already previously described, closed-circuit systems on average outperformed open-circuit  
418 system during the first 10 weeks of operation (see Table 3), however, without significant  
419 differences (for details see annex Table 7). COD and ammonium removal from week 11 to 23  
420 showed the same tendency but again without any significant difference. The same is true if  
421 compared with a CW control duplicate (from week 12 to 23) in the way that closed-circuit  
422 systems outperformed open-circuit and CW control systems as well, however, again without any  
423 significant difference. Again, the insignificance of differences, especially in the case of  
424 ammonium, could be partly due to the relatively high standard deviation most likely caused by

425 the variation in quality of the used real urban wastewater due to natural causes like rainfall  
426 events or dry periods.

427 Average COD removal on a mass base in the last 12 weeks of experiments (the time when CW  
428 control was tested as well) in closed-circuit systems was only 2% higher than in open-circuit and  
429 5% higher than in CW control systems (see Table 3). Wang et al. (2016b) found higher  
430 improvement with 8.3% difference in COD removal comparing closed- to open-circuit CW-MFC,  
431 however, using a pH control and vertically batch-fed bench-scale systems. Regardless the  
432 treatment around 75% of the overall COD mass removal was already removed within the first  
433 transect, between 15% and 20% in the second transect and between 5% and 10% in the last (see  
434 Figure 5).

435



**Figure 5. COD removal for each transect for CW control, open-circuit and closed-circuit systems (n=11, CW control duplicate started in week 12 and experimentation week 19 could not be used due to a highly diluted influent)**

436

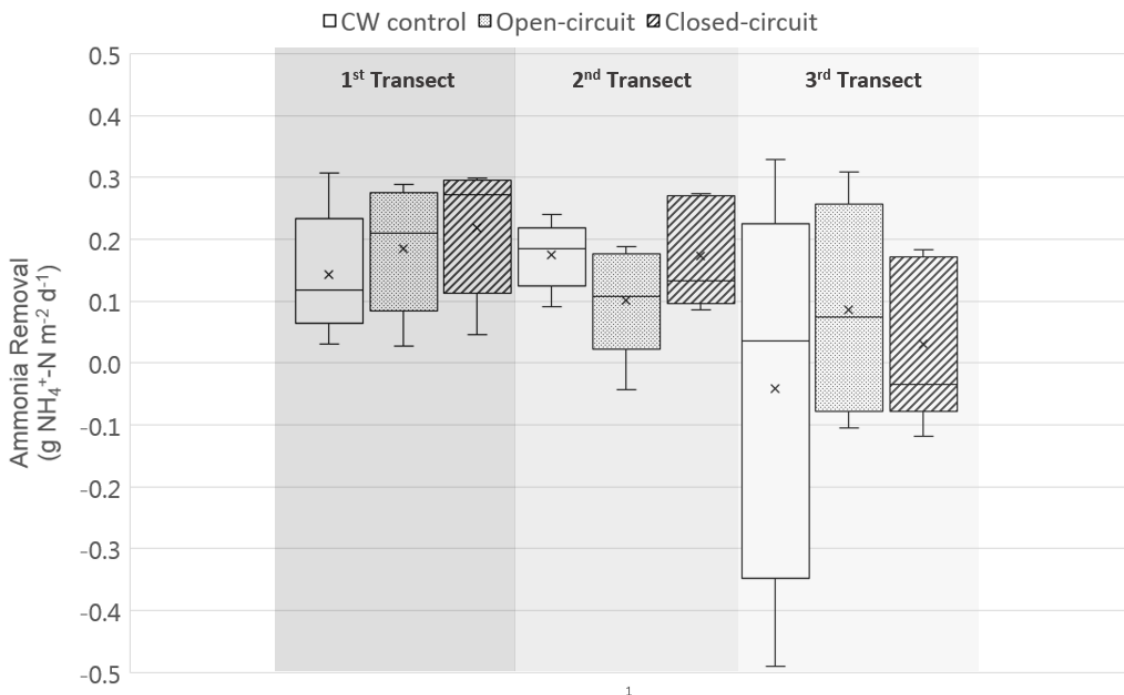
437 The overall COD removal of 74% in closed-circuit systems is comparable to earlier CW-MFC  
438 studies, with 75% (Yadav et al., 2012), 82% (Xu et al., 2018) and 76.5% (Zhao et al., 2013). In this  
439 regard, the presented study confirms results of these CW-MFC systems which were less  
440 representative for real situations; e.g. all mentioned above were in bench-scale, up-flow  
441 hydraulic regime, fed with synthetic or modified wastewater. Yadav et al. (2012) used very fine  
442 gravel (2-4 mm), only Xu et al. (2018) used a continuous flow but had a sand media and Zhao et  
443 al. (2013) used artificial aeration at the cathode. Some of these factors might influence  
444 treatment behavior, long term operation (e.g. clogging due to fine media) and possibly present  
445 up-scaling problems (e.g. flow direction, artificial wastewater). In comparison to full-scale HF  
446 CW systems the presented COD treatment efficiencies are not outstanding, but authors believe  
447 that the reason could be that meso- as well as lab-scale systems often have unfavorable  
448 hydraulic conditions due to the smaller scale, resulting in a lower HRT than the calculated  
449 theoretical HRT. An additional reason could be the lack of development of plants, which have  
450 shown to provide a significant positive wastewater treatment effect in subsurface flow CWs  
451 (Tanner, 2001).

452 Zhang et al. (2015) found indications through CE calculations in wastewater fed MFC systems  
453 (comparing closed- and open-circuit), that electrogenic bacteria outcompeted other microbial  
454 degradation pathways, while Fang et al. (2013) showed that electrogenic bacteria such as  
455 *Geobacter sulfurreducens* and *Beta Proteobacteria* inhibited the growth of *Archaea* at the  
456 anode. Although the difference in COD removal in the presented study is very low, the more  
457 competitive electroactive pathway and potential inhibition of non-electroactive bacteria could  
458 have been the reason for the increased COD removal in closed-circuit systems.

459 Average ammonium removal on a mass base in the last 12 weeks in closed-circuit systems was  
460 17% higher than in open-circuit systems and 22% higher than in CW control (see Table 3) but  
461 not statistically different (for details see annex Table 7). Average ammonium removal in

462 transects was not as homogeneous across treatments as for COD; in closed-circuit systems the  
 463 majority was removed in the first and second transect and only a small portion in the last, in  
 464 open-circuit systems the majority was removed in the first and the rest in even parts in second  
 465 and third, and in CW control basically the whole treatment took place in the first and second  
 466 transect (see Figure 6).

467



**Figure 6. Ammonium removal per transect for CW control, open-circuit and closed-circuit systems (n=7; CW control duplicate started in week 12 and five experimentation weeks could not be used due to technical analysis or influent dilution problems due to rainfall)**

468

469 The high variability in the last transect of CW control is remarkable and could indicate that it was  
 470 more unstable than in open- or closed-circuit systems. Nitrate and nitrite effluent levels were  
 471 generally very low during the time of electrical connections comparison (only week 11 was  
 472 unusually high, but probably due to the start-up after summer). Both parameters increased a  
 473 little in the second transect across all treatments and dropped again in the last (see Table 3).  
 474 The only statistically significant difference between electrical connections occurred for nitrate



475 when looking at the removal from inlet to outlet (for details see annex Table 7). Table 3 shows  
476 that the average nitrate level in closed-circuit CW-MFC systems was actually very similar after  
477 the first transect and even lower after the second transect as compared to CW control and open-  
478 circuit CW-MFC systems. Only in the last transect nitrate levels only dropped by nearly half in  
479 closed-circuit CW-MFC while they went close to the initial influent concentration in the other  
480 electrical connections.

481 The observed average ammonium removal of 41% in closed-circuit systems was rather low  
482 compared to preliminary results of Zhao et al. (2013) with an average of 77%, however, as  
483 mentioned above, the system had an artificially aerated cathode. In terms of improvement of  
484 efficiency compared to a control, Wang et al. (2016b) reported a 40% improvement of nitrate  
485 removal in closed-circuit CW-MFCs compared to open-circuit, however, with a pH control. Most  
486 other works on CW-MFCs were rather focused on organic matter and not on nitrogen removal.  
487 Xu et al. (2018) recently observed an average of 82% total nitrogen removal, however, the  
488 systems were continuously up-flow fed bench-scale systems with a tubular shape. Furthermore,  
489 Xu et al. (2018) did a functional analysis of the microbial community, comparing a closed-circuit  
490 CW-MFC with a CW control system, showing that (1) diversity and richness were higher in CW-  
491 MFC, (2) in the CW-MFC anode compartment the most common microbial functional groups  
492 were ammonia oxidizing bacteria (AOB), nitrite-oxidizing bacteria (NOB) and anaerobic  
493 ammonium oxidation (anammox) bacteria, with NOB and anammox being significantly higher  
494 than in the control and (3) in the CW-MFC cathode compartment the microbial functional groups  
495 denitrifying bacteria (DNB), dissimilatory nitrate reduction to ammonium (DNRA), and  
496 electroactive bacteria were significantly higher than in the control. In another microbial  
497 community analysis in CW-MFC systems, Wang et al. (2016b) found that anodes of closed-circuit  
498 as compared to open-circuit systems had a significantly improved richness in electroactive  
499 bacteria, nitrobacteria and DNB. Corbella et al. (2015) also found that *Geobacter* and

500 methanogenic populations were significantly higher in closed-circuit when compared to open-  
501 circuit CW-MFC.

502 Of course the microbial community will also be dependent on the used materials for filter media,  
503 anode, cathode etc.; Wang et al. (2016a) found a significantly different distribution of microbial  
504 communities depending on the used CW-MFC anodes, comparing carbon fiber felt, graphite  
505 rods, foamed nickel and stainless steel mesh. Stainless steel mesh, the material used in this  
506 experiment, and foamed nickel had significantly lower relative abundance of *Proteobacteria*  
507 than carbon fiber felt and graphite rods, which was related to a lower power production.  
508 However, reported voltage outputs by Wang et al. (2016a) using stainless steel mesh reached  
509 averages from ca. 17 to 41 mV, which was by far surpassed in the presented systems with  
510 averages of  $304\pm 96$ ,  $462\pm 33$ , and  $457\pm 50$  mV for first, second and third transect, respectively.

511 The above described enrichment in anammox bacteria was already indicated in earlier research  
512 on MFC systems; Di Domenico et al. (2015) observed that MFC mode provides conditions  
513 favoring the cultivation of anammox in the anodic compartment of the anaerobic digestate fed  
514 systems used, without inoculating anammox bacteria at any point (only electroactive bacteria  
515 *G. sulfurreducens* were inoculated). In another bench-scale MFC experiment, Li et al. (2015), this  
516 time using synthetic wastewater, were able to prove higher abundance of anammox bacteria  
517 and associated higher nitrogen removal in closed-circuit MFC systems (open-circuit as control).  
518 However, these were inoculated with anammox bacteria in advance. Anammox bacteria were  
519 detected in conventional HF CW systems without MFC systems as well, however, Coban et al.  
520 (2015) could not detect any anammox activity in HF CWs inferring that the process is of low  
521 importance in the nitrogen removal of conventional CW systems.

522 Another possible ammonium removal pathway could be volatilization due to proton loss at the  
523 cathode and associated locally elevated pH, which cannot be excluded since the authors did not

524 have the capability to measure pH on a micro-scale at the cathode, e.g. by using microprobes  
525 (Kim et al., 2008).

526 In MFC systems designed for nitrogen removal, simultaneous nitrification and denitrification  
527 (SND) could be accomplished; Viridis et al. (2008) observed that although oxygen was present at  
528 the cathode, biofilm stratification at the cathode allowed nitrifying bacteria in the outer layer  
529 and putative denitrifying bacteria were found in the inner layers in a micro-anoxic environment.  
530 However, large amounts of oxygen around the cathode would inhibit the bioelectrochemical  
531 denitrification (Kelly and He, 2014), which is the case for the presented systems, and again there  
532 would have been no possibility to measure SND in the presented experimental setup.

533 Conventional nitrification through supply with oxygen could have only happened at the systems  
534 very surface since DO measurements in the influent, effluent and the water column were always  
535 below detection limit, and therefore oxygen could have only partly been responsible for  
536 ammonium removal, which still could not have explained the differences between treatments.  
537 Xu et al. (2018) also described how, even in separator-less (e.g. without a membrane or glass-  
538 wool between anode and cathode) CW-MFCs, like the ones presented here, unwanted oxygen  
539 diffusion to the anode is inhibited by microorganisms which deplete the oxygen before it can  
540 reach further down, forming a so-called “microbial separator”. This separator maintained also  
541 anaerobic conditions for the anode with just 2 cm distance from the cathode which showed the  
542 highest maximum power density compared to higher distances and systems with a separator.  
543 This distance is comparable to the distance between cathode and beginning of the anode (which  
544 extends vertically nearly until the bottom) in the presented work.

545 Orthophosphate removal during the first 10 weeks of operation differed only very slightly  
546 between treatments, again with higher rates in closed-circuit and continuously fed systems with  
547 a removal of up to 29% in closed-circuit continuous (see Table 3). Differences were not

548 statistically significant (for details see annex Table 7). Ichihashi and Hirooka (2012) observed  
549 phosphate removal of 70-82% in closed-circuit MFC systems, with 4.6–27% in form of  
550 precipitation on the cathode, mainly in the form of struvite. While Corbella and Puigagut (2018)  
551 also found 15 % higher  $\text{PO}_4^{-3}$  removal, comparing closed- to open-circuit CW-MFC systems, they  
552 also found white precipitation on the cathode which was not struvite but mostly Calcite ( $\text{CaCO}_3$ )  
553 and Halite ( $\text{NaCl}$ ). However, maybe the conditions for struvite crystal precipitation were not met,  
554 i.e.  $\text{Mg}^{2+}$ ,  $\text{NH}_4$ , and  $\text{PO}_4^{-3}$  should exceed the solubility limit. Struvite solubility decreases with  
555 increasing pH (Doyle and Parsons, 2002). In addition, Zhang et al. (2012) found that biological  
556 phosphorus uptake, rather than chemical precipitation, can be increased in low current (smaller  
557 than 10 A) bioelectrochemical systems which is the case for the study of Corbella and Puigagut  
558 (2018) with ca. 1.45 mA and also the presented study with an average of ca. 1.48 mA across all  
559 three transects in the first 10 weeks. In any case, in the presented study no white precipitation  
560 was found on the cathodes.

561 Orthophosphate concentrations in the last 12 weeks basically stayed the same along the flow  
562 path across all three treatments. As described earlier it seems that adsorption sites already got  
563 limited in that period, since removal rates were higher in the first 10 weeks of experiments. In  
564 general, phosphorus storage in subsurface flow CWs takes place in plant biomass, bed media or  
565 accretion sediments and has a finite capacity (Kadlec and Wallace, 2009).

566 During the time of electrical connection comparison, from week 12 to 23, average voltages in  
567 the closed-circuit CW-MFC systems for the three transects amounted to  $304 \pm 96$ ,  $462 \pm 33$  and  
568  $457 \pm 50$  V. Average current densities during the electrical connection comparison, from week 11  
569 to 23, were  $31 \pm 15$ ,  $49 \pm 9$  and  $50 \pm 7$   $\text{mA/m}^2$  for transects 1, 2 and 3, respectively. These results  
570 are in the range of current densities in earlier CW-MFC experiments, with averages of 22.3  
571  $\text{mA/m}^2$  by Villaseñor et al. (2013) and 70  $\text{mA/m}^2$  by Yadav et al. (2012). Polarization curves help  
572 to electrochemically characterize MFC systems and are shown for a closed-circuit CW-MFC

573 replicate in the annex (see Figure 8). The resulting maximum power densities and corresponding  
574 current densities amounted to 6.7 mW/m<sup>2</sup> and 27.3 mA/m<sup>2</sup> in the first transect, 36.6 mW/m<sup>2</sup>  
575 and 92.8 mA/m<sup>2</sup> in the second transect and 35.9 mW/m<sup>2</sup> and 92.8 mA/m<sup>2</sup> in the third transect.  
576 The estimated internal resistances derived from the polarization curves were around 215 Ω, 100  
577 Ω and 100 Ω for first, second and third transect, respectively. Principally, the potential maximum  
578 power is achieved when internal and external resistances are close to each other (Lefebvre et  
579 al., 2011). Therefore, it seems that the external resistance of 220 Ω fits very well for the first  
580 transect. According to the results, the second and third transect could potentially perform better  
581 with a lower external resistance around 100 Ω, however, it was decided to keep the same  
582 external resistance for all three transects for this experiment. The lower maximum power  
583 density in the first transect could be due to the higher organic loading in the first transect as  
584 compared to the second and third, which could a) potentially cause a clogging in the carbon felt  
585 cathode, limiting its potential and/or b) as also mentioned above in the discussion on the OLR,  
586 it was found that, in MFC systems, lower OLR benefited exoelectrogenic bacteria growth and  
587 activity over competing methanogenics (Capodaglio et al., 2015).

588 Coulombic Efficiency (CE) is the proportion of the produced charge to the carbohydrates which  
589 are theoretically derived from oxidation, indicated by the change of COD from transect to  
590 transect (Scott, 2016). The CEs over the whole time period in the three consecutive transects  
591 ranged from 0% to 8%, -34% to 46% and -89% to 93%, with averages of 1±3%, 10±17% and  
592 2±34%, respectively. Earlier reported CW-MFC CEs range from 0.05-0.06% (Yadav et al., 2012)  
593 up to 2.8-3.9% (Liu et al., 2014). However, the authors believe that the parameter CE is not very  
594 useful for describing a CW-MFC's electric efficiency, especially if expressed per transect, since  
595 not only organic matter from the influent can contribute to the MFC signal but also accumulated  
596 organic matter within the gravel bed is a fuel source for MFC (Corbella et al., 2016a). This is  
597 probably the reason why the CE could reach high levels in the second and third transect; due to

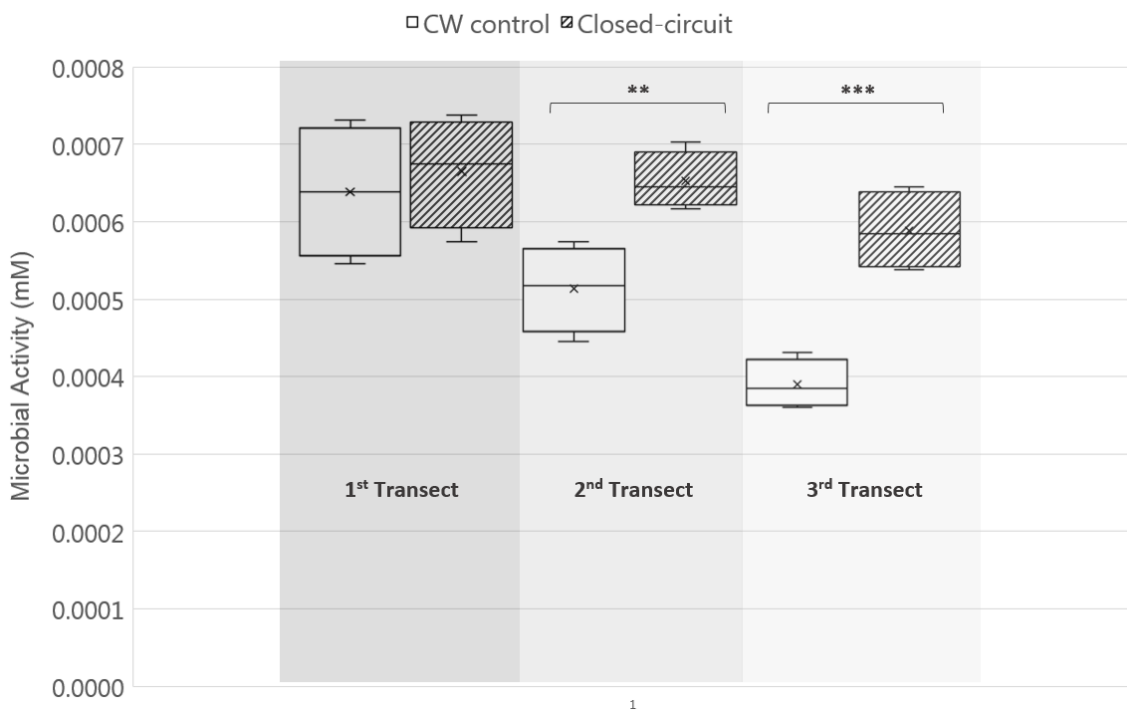
608 little COD removal and currents similar to the first transect it appears like a high current was  
 609 produced with only little input. Therefore, the reported high positive CE values in this paper,  
 610 especially in the second and third transect, are most likely overestimated. The second and third  
 611 transect CE even reached negative values due to eventually increasing COD concentrations  
 612 within the wetland caused by changes in influent wastewater quality.

603

604 **3.2.3 Microbial activity**

605 Figure 7 shows microbial activity, determined through the FDA experiment, along the flow path  
 606 of the CW control systems and closed-circuit CW-MFC systems (all continuously fed).

607



608

**Figure 7. Microbial activity along transects for control CW and closed-circuit continuously fed systems**

609

610 Generally, the activity was highest in the first transect, both in the closed-circuit and in CW  
 611 control systems (activity analysis was not performed for open-circuit systems), and the activity  
 612 stayed on a higher level in the closed-circuit as compared to the CW control systems. Differences

613 between average microbial activities of closed-circuit and CW control systems were not  
614 statistically significant in the first transect ( $F(1, 4); p = 0.65$ ), but statistically very significant in  
615 the second transect ( $p < 0.01$ ) ( $F(1, 4); p = 0.006$ ) and extremely significant in the third transect  
616 ( $p < 0.001$ ) ( $F(1, 4); p = 0.0006$ ).

617 The higher microbial activity within the first transect, irrespective of the treatment, is probably  
618 due to the higher availability of organic matter as a substrate, favoring the growth of  
619 microorganisms (Wu et al., 2014), with a subsequent decrease in microbial activity along the  
620 flow path, which has been observed already before in vertical and horizontal sequential CW  
621 systems (He et al., 2014). This decrease in activity is also reflected by the decrease in ammonium  
622 and COD removal along the systems flow path. Closed-circuit CW-MFC showed higher activity  
623 than CW control systems in all three transects. In percentages the microbial activity in closed-  
624 circuit systems was 4%, 21% and 34% higher than the control in first, second and third transect,  
625 respectively. Xu et al. (2018) analyzed diversity and richness (activity was not measured) of  
626 microbial communities in CW-MFC and CW control systems and found higher diversity and  
627 richness in closed-circuit CW-MFC systems. Also Wang et al. (2016b) found higher richness in  
628 closed-circuit as compared to open-circuit CW-MFC systems. Hence, in the presented systems a  
629 higher diversity and richness in closed-circuit CW-MFCs could have contributed to the measured  
630 higher activity. Corbella et al. (2015) also found that *Geobacter* and methanogenic populations  
631 were significantly higher in closed-circuit when compared to open-circuit CW-MFC.

632 As discussed in the section on COD removal comparing electrical connections, electrogenic  
633 bacteria in MFCs outcompeted other microbial communities and were also able to inhibit  
634 growth of *Archaea* at the anode (Fang et al., 2013; Zhang et al., 2015). This advantage in  
635 competition could be another factor responsible for the increased activity in the studied CW-  
636 MFC systems. Also, as mentioned above in the discussion on the OLR, it was found that, in MFC  
637 systems, lower OLR benefited exoelectrogenic bacteria growth and activity over competing

638 methanogenics (Capodaglio et al., 2015). Therefore, a possible explanation for the varying  
639 differences in microbial activity between closed-circuit and CW control systems along the flow  
640 path could be that the decreasing OLR from transect to transect is leading from an insignificant  
641 difference in the first to a very significant difference in the second and extremely significant  
642 difference in the third transect. However, in comparison to the mentioned MFC studies, even  
643 the higher OLR at the influent of the presented study is already quite low (around a magnitude  
644 lower as in the MFCs), but in the range of OLRs in conventional HF CWs (Vymazal, 2005).  
645 Therefore, the presented results could give an indication that even a further decrease in OLR,  
646 from an already relatively low level, still causes a recognizable advantage to the exoelectrogenic  
647 over the methanogenic pathway.

648 MFCs have also been used for monitoring of microbial activity, in low contaminated  
649 environments like groundwater (Tront et al., 2008) or monitoring of anaerobic digestion  
650 processes (Liu et al., 2011).

#### 651 **4. CONCLUSIONS**

652 The different tested organic loading rates and hydraulic regimes had no significant effect on  
653 treatment efficiency of COD or ammonium in the examined meso-scale horizontal-flow CW-MFC  
654 systems, but continuously fed systems showed slightly better treatment performance than  
655 intermittently fed systems. In addition, intermittent flow significantly decreased current  
656 production in the first transect of closed-circuit CW-MFC systems when compared to continuous  
657 flow.

658 In terms of electrical connection, closed-circuit CW-MFC systems were able to enhance  
659 treatment efficiency in comparison to open-circuit CW-MFC and CW control systems, however,  
660 again without significant differences, which might be due to the use of real urban wastewater  
661 which varied in strength over time due to natural causes like rainfall events or dry periods.



662 Microbial activity clearly decreased along the flow path, as did ammonium and especially COD  
663 removal. Microbial activity was higher in all three transects in closed-circuit mode when  
664 compared to control conditions, which could be one of the reasons for the observed  
665 enhancement of treatment performance. Differences between closed-circuit and control  
666 systems were not significant in the first transect but very significant in the second and extremely  
667 significant in the third, possibly indicating that the lower organic load along the flow path  
668 benefited the activity of electrogenic bacteria over competing non-electrogenic bacteria.

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872

**Table 4.** COD, ammonium and orthophosphate mass based average removal rate in percentage from inlet to outlet for low OLR 1, high OLR and low OLR 2 as well as intermittent or continuous flow hydraulic regime for open-circuit (OC) and closed-circuit (CC) CW-MFC systems.

Removal (%)		Low OLR 1 4.9±1.6 g COD/m <sup>2</sup> .day		High OLR 13.6±3.2 g COD/m <sup>2</sup> .day		Low OLR 2 <sup>a</sup> 6.7±1.4 g COD/m <sup>2</sup> .day
		Intermittent flow	Continuous flow	Intermittent flow	Continuous flow	Continuous flow
COD (n=4/5/11) <sup>b</sup>	OC	58%	56%	58%	62%	72%
	CC	56%	58%	68%	69%	74%
NH <sub>4</sub> -N (n=4/5/7) <sup>b</sup>	OC	23%	29%	18%	30%	24%
	CC	27%	38%	35%	39%	41%
NO <sub>3</sub> -N (n=4/4/8) <sup>b</sup>	OC	-95%	-110	44	-24	0%
	CC	-186	-290	-539	NA <sup>c</sup>	NA <sup>c</sup>
NO <sub>2</sub> -N (n=4/4/8) <sup>b</sup>	OC	71%	71%	67%	-78%	-40%
	CC	67%	83%	48%	-314%	-17%
PO <sub>4</sub> -P (n=4/4/8) <sup>b</sup>	OC	21%	29%	10%	11%	10%
	CC	21%	29%	10%	16%	5%

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<sup>a</sup> Low OLR 2 results are shown in more detail in the section 3.2 on the electrical connection effects

<sup>b</sup> Some experimentation weeks could not be considered due to highly diluted influent or technical analysis problems

<sup>c</sup> Division by zero

**Table 5.** Two-factor ANOVA (with replication) results for the comparison of the factors hydraulic regimes (intermittent vs. continuous) and electric connections (open-circuit vs. closed-circuit) as well as the interaction between the two factors, separated in low OLR 1 and high OLR periods.

Two-factor ANOVA			Hydraulic Regime	p-value	
				Electric Connection	Interaction
Low OLR 1	COD	F (1, 4)	0.94	0.93	0.87
	NH <sub>4</sub> -N	F (1, 4)	0.51	0.53	0.98
	NO <sub>3</sub> -N	F (1, 4)	0.67	0.64	0.75
	NO <sub>2</sub> -N	F (1, 4)	0.74	0.52	0.84
	PO <sub>4</sub> -P	F (1, 4)	0.66	0.85	0.86
High OLR	COD	F (1, 5)	0.45	0.96	0.94
	NH <sub>4</sub> -N	F (1, 5)	0.43	0.71	0.85
	NO <sub>3</sub> -N	F (1, 4)	0.0007 ***	0.03 *	0.10
	NO <sub>2</sub> -N	F (1, 4)	0.44	0.02 *	0.78
	PO <sub>4</sub> -P	F (1, 4)	0.86	0.62	0.69

\* significant difference (p < 0.05)

\*\* very significant difference (p < 0.001)

\*\*\* extremely significant difference (p < 0.001)

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**Table 6.** One-factor ANOVA (with replication) results for the comparison of low OLR 1 and high OLR periods (considering only continuously fed closed-circuit CW-MFC systems) based on removal percentages ( $\text{NO}_3^-$ -N and  $\text{NO}_2^-$ -N could not be calculated due to division by zero)

One-factor ANOVA	p-value
COD F (1, 4)	0.39
$\text{NH}_4$ -N F (1, 4)	0.84
$\text{PO}_4$ -P F (1, 4)	0.35

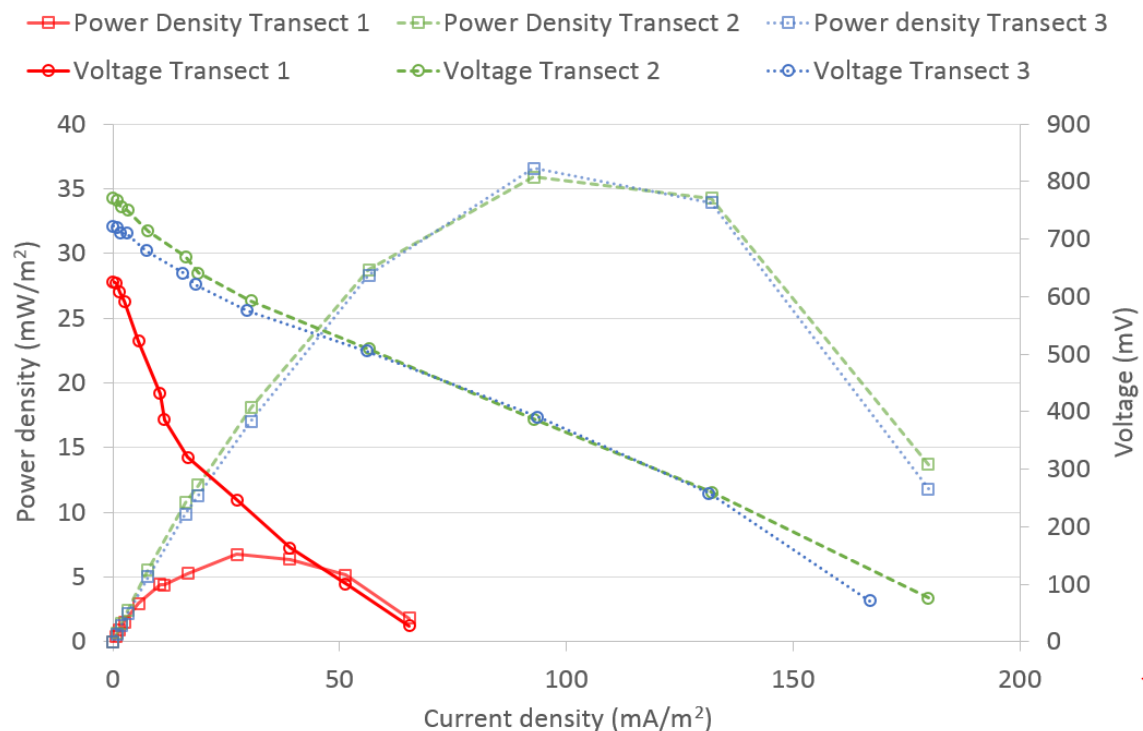
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**Table 7.** One-factor ANOVA (with replication) results for the comparison of the electric connections during the low OLR 2 period, for the total system from inlet to outlet and each of the three transects separately.

One-factor ANOVA	p-value	Electric Connection (low OLR 2 period)			
		Inlet-Outlet	Transect 1	Transect 2	Transect 3
COD F (2, 11)	0.73	0.77	0.91	0.99	
$\text{NH}_4$ -N F (2, 7)	0.16	0.55	0.29	0.67	
$\text{NO}_3$ -N F (2, 8)	0.03*	0.35	0.38	0.21	
$\text{NO}_2$ -N F (2, 8)	0.74	0.33	0.73	0.71	
$\text{PO}_4$ -P F (2, 8)	0.84	0.72	0.27	0.14	

\* significant difference ( $p < 0.05$ )

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**Figure 8.** Power density and polarization curves for each transect of one of the closed-circuit CW-MFC replicates measured during sampling week 10