1 **REVEALING THE PROLIFERATION OF HYDROGEN**

2 **SCAVENGERS IN A SINGLE-CHAMBER MICROBIAL**

3 **ELECTROLYSIS CELL USING ELECTRON BALANCES**

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28　**Highlights**

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30　• The role of H_2 scavengers in the bioelectrochemical H_2 production is quantified

31　• CE and r_{CAT} to estimate MEC performance are not valid under H_2 consumption

32　• Electron equivalent balances help to understand the H_2 fate in single-chamber

33　　MEC

34　• Our approach was experimentally validated with H_2-recycling and

35　　methanogenesis

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ABSTRACT

The bioelectrochemical generation of hydrogen in microbial electrolysis cells (MECs) is a promising technology with many bottlenecks to be solved. Among them, the proliferation of hydrogen scavengers drastically reduces the cell efficiency leading to unrealistic coulombic efficiencies (CE) and cathodic gas recoveries (r_{CAT}). This work provides a novel theoretical approach to understand, through electron equivalent balances, the fate of hydrogen in these systems. It was validated with a long term operated single-chamber membrane-less MEC. In the short term, H_2-recycling (i.e. hydrogen being derived to the anode) resulted in r_{CAT} of only 4% and in CE up to 463%. The 80.5% of the current intensity came from H_2-recycling and only the 19.5% from substrate oxidation. In the long term, methane was produced from hydrogen, thus decreasing r_{CAT} to 0 (r_{CAT} = 94.5% when considering methane production). CE was 74.5% suggesting that H_2-recycling only took place when methanogenic activity was marginal.

KEYWORDS: 2-bromoethanesulfonate, homoacetogens, hydrogen recycling, methanogens, single-chamber membrane-less microbial electrolysis cell

1. INTRODUCTION

Among all the possible renewable energy sources, H_2 gas is one of the most attracting alternatives for the scientific community. It is a clean and renewable energy carrier, without an impact on the greenhouse gas emission at the point of use and a high combustion heat (120 kJ/g) when compared to other possible biofuels (CH_4, 50 kJ/g or ethanol, 26.8 kJ/g) [1]. Moreover, H_2 can be very efficiently converted into electricity by means of chemical fuel cells when compared to biogas [2]. Nowadays, most H_2 is produced by steam reforming of fossil fuels, a non-sustainable technology. For this reason, research is focused on the development of technologies for sustainable H_2 production. Among the different alternatives, the bioelectrochemical generation of H_2 in microbial electrolysis cells (MECs) is a novel technology introduced in Liu et al. [3] with very promising lab results and theoretical higher yields.

MECs take advantage of the capability of the anode respiring bacteria (ARB) of using insoluble electron acceptors in their respiration process and thus, transferring the electrons to a solid anode under anaerobic conditions. Hence, ARB oxidize organic matter and transfer the electrons to the anode, which flow through an external circuit to the cathode. The cathode is also kept under anaerobic conditions and thus, the protons generated in the anode are reduced to form H_2. The global process is not thermodynamically spontaneous and a certain voltage has to be applied to drive the reactions [3]. In any case, the energy contained in the produced H_2 has to be higher than the energy added by the power source in order to make MECs a feasible system.

The use of membranes in MECs to separate the anodic chamber from the cathodic chamber is nowadays a controversial issue. On the one hand, membranes theoretically prevent the diffusion of H_2 from the cathode to the anode and avoid potential problems related to H_2 scavengers and impurities in H_2. On the other hand, membranes are

89   expensive and cause potential losses associated to pH gradients across them [4]. Thus,

90   higher voltages need to be applied for the reactions to take place resulting in a severe

91   decrease of energetic efficiency.

92   Electron flow derived to methanogenesis is one of the major hurdles of

93   bioelectrochemical systems. CH_4 production from organic carbon sources results in a

94   significant decrease of the system efficiency, measured as Couloumbic Efficiency (i.e.

95   ratio of electrons contained in the initial substrate that are converted into current).

96   Avoiding methanogenesis in MECs is not a straightforward issue since these

97   microorganisms are strongly favoured in conventional MEC anodic environments (i.e.

98   anaerobiosis with abundance of electron donors and biofilm formation) and this is why

99   the contamination of H_2 with CH_4 has been widely reported (e.g. [5]). Moreover, when

100   working with fermentable substrates, the H_2 generated in fermentation can be used for

101   methanogenesis as electron donor, which can account for important electron losses at

102   the anodic compartment [6]. This hydrogenotrophic methanogenesis becomes even

103   more important when operating single-chamber systems (i.e. membrane-less), since the

104   H_2 electrochemically formed in the cathode can also be used as electron donor.

105   Nowadays, CH_4 formation is mostly prevented using a chemical inhibitor of

106   methanogenesis (being 2-bromoethanesulfonate, BES, the most common). BES

107   utilisation is practical with short-term lab-scale experiments but it is not economically

108   feasible at a real scale. Other approaches for methanogenesis suppression such as low

109   hydraulic retention times [7], intermittent exposure to air [5], low temperature and pH

110   shocks [8, 9] have not been totally successful yet even at lab-scale conditions.

111   The presence of different H_2 scavengers other than methanogens has also been

112   observed. On the one hand, the effect of homoacetogenic bacteria (e.g. strictly

113   anaerobic bacteria that produce acetate with H_2 as electron donor and inorganic carbon)

114   in two-chamber MECs with fermentable substrates was reported to have a positive

115   effect, since they allow the electron recovery from the produced H_2 in fermentation [6].

116   However, in single-chamber MECs, homoacetogens can have a detrimental effect since

117   they can transform back to acetate the H_2 produced in the cathode. This H_2-acetate loop

118   can result in an increase of the cycles duration and thus, more input energy requirements

119   and lower H_2 recoveries [10]. Nevertheless, the low H_2 recoveries in single-chamber

120   MECs due to H_2-recycling are not only as a result of the homoacetogenic activity, but

121   the use of H_2 as electron donor by ARB has also been reported [11]. In this sense, Lee

122   and Rittmann [7] studied the contribution of H_2-recycling in a continuous single-

123   chamber MEC by minimizing the methanogenic activity, obtaining that from the 62 to

124   the 76 % of the total current intensity was as a result of H_2-recycling. However,

125   methanogenic activity was not completely suppressed and therefore, the contribution of

126   H_2-recycling could have been even higher.

127   A whole understanding of the competition between the different H_2 scavengers in

128   single-chamber MEC systems has not been reported yet, although it was found that

129   methanogenesis inhibition could favour homoacetogenic growth [6]. Lee and Rittmann

130   [7] observed that H_2-recycling and CH_4 production occurred in the system

131   simultaneously. Parameswaran et al. [12] found that homoacetogens could survive in a

132   cell working at low HRT (with high BES concentration) indicating that homoacetogens

133   could compete with hydrogenotrophic methanogens in real systems.

134   This work is the first study where the long term operation of a single-chamber

135   membrane-less MEC with continuous dosage of BES is experimentally assessed. Long

136   and fully monitored cycles and electron equivalent balances are used to understand the

137   existing H_2 losses due to the competition between homoacetogens, ARB and

138   hydrogenotrophic methanogens for H_2.

139

2. MATERIALS AND METHODS

140

141 2.1 Reactor description and operation

142 A single-chamber membrane-less MEC of 1300 mL was used (Figure 1). A carbon fiber

143 brush (PANEX®33 160 K, ZOLTEK) [13] previously inoculated in a microbial fuel

144 cell was used as anode. The cathode was made with carbon cloth coated with carbon

145 powder and platinum suspension on the side facing the anode [14, 15]. Both electrodes

146 were arranged concentrically with the cathode in the outer perimeter, so that all ends of

147 the anode were at the same distance from the cathode. An Ag/AgCl reference electrode

148 (+210 mV vs SHE) was used to monitor the electrode potentials. The reactor operated

149 in batch mode and with constant agitation. A constant voltage of 1.2 V was provided by

150 a power supply (TTI QL355TP). The H_2 produced was collected in a 0.5 L gas sample

151 bag with a twist type valve (Cali-5-Bond, Ritter).

152 Intensity was calculated from the monitoring of the voltage across an external resistance

153 of 12 Ω by using a 16-bit data acquisition card (Advantech PCI-1716) connected to a

154 personal computer with software developed in LabWindows CVI 2010 for data

155 acquisition.

156 The medium was a 100 mM phosphate buffer with acetate as carbon source prepared as

157 in Parameswaran et al. [10] with the addition of the methanogenic inhibitor BES [16].

158 The acetate concentration in the medium was 235 mg/L (4 mM) and BES concentration

159 was 50 mM except as indicated, where it was increased to 90 and 120 mM.

160

161 2.2 Chemical analyses

162 Acetate was analysed by gas chromatography (Agilent Technologies, 7820-A) using a

163 flame ionization detector (FID) with helium as carrier gas. H_2 production was analysed

164   with the same gas chromatograph using a thermal conductivity detector (TCD) with

165   argon as carrier gas to ensure a good response in H_2 peak.

166

167   2.3 Batch experiments

168   Batch experiments were carried out to assess the cell performance over time. Culture

169   medium was renewed prior to each cycle monitoring. Acetate concentration, gas

170   production/composition and current intensity were measured along the cycles.

171   Obtaining experimental profiles in time and not only start/end measurements was

172   essential for a better understanding of the system.

173   Gas production was calculated as in Ambler and Logan [17]. The same gas composition

174   was assumed in both the headspace and the gas sampling bag and therefore, the final

175   volume of each gas (H_2 and CH_4) was calculated from the total volume (headspace +

176   gas sample bag) and the gas composition of the last analysis of the cycle (equation 1).

177    $V_{i,F} = V_{G,F} \cdot x_{i,F}$ (1)

178   where $V_{G,F}$ is the final volume of gas and $V_{i,F}$ and $x_{i,F}$ are the final volume and final

179   composition of a certain gas, respectively.

180   The moles of H_2 corresponding to that volume were calculated assuming a constant

181   pressure of 1 atm in the reactor-bag system and room temperature.

182

183   2.4 Presence of homoacetogens

184   The presence of homoacetogenic bacteria was tested through an experiment similar to

185   that in Parameswaran et al. [10]. Culture medium was replaced and no acetate, but

186   sodium bicarbonate (3 g/L) was added. The MEC was operated with an applied voltage

187   of 1.2 V. H_2, stored in a gas sampling bag of 1 L, was intermittently sparged from the

188 bottom of the reactor and collected in another gas sampling bag located at the top of the

189 cell. Once the bag at the top was full, the position of the bags was reversed in order to

190 continue sparging H_2 from the bottom of the cell. This operation was repeated nine

191 times between hours 0 and 8 and nine times more between hours 22 and 30 of the

192 experiment.

193

194 2.5 Calculations

195 Coulombic Efficiency (CE) was calculated as in equation 2.

196 $$CE = \frac{\text{Coulombs recovered as current intensity}}{\text{Coulombs in substrate}} = \frac{\int_{t_0}^{t_F} I dt}{F \cdot b_{Ac} \cdot V_L \cdot \Delta c \cdot M^{-1}} \qquad (2)$$

197 where t_0 and t_F are the initial and final times of an experiment, Δc is the acetate

198 concentration change between t_0 and t_F (g acetate/L), M is the molecular weight of

199 acetate (59 g/mol), b_{Ac} is the number of e^- transferred per mole of acetate (8 mol e^-/mol

200 acetate), F is the Faraday's constant (96485 C/mol e^-), I is the current intensity and V_L is

201 the volume of liquid in the reactor.

202

203 Cathodic gas recovery (r_{CAT}) was calculated as in equation 3.

204 $$r_{CAT} = \frac{\text{Coulombs in } H_2}{\text{Coulombs recovered as current intensity}} = \frac{V_{H2,F} \cdot 2 \cdot F \cdot V_m^{-1}}{\int_{t_0}^{t_F} I dt} \qquad (3)$$

205 where V_m is the molar gas volume (24.03 L/mol) at 20 ºC.

206

207 **3. RESULTS AND DISCUSSION**

3.1 CE and r_{CAT} as MEC performance indicators

The performance of a MEC is commonly assessed through the calculation of the coulombic efficiency (CE) and the cathodic gas recovery (r_{CAT}). CE compares the coulombs recovered as current intensity with the coulombs that could be theoretically generated from the substrate oxidation by ARB, while r_{CAT} compares the coulombs consumed in H_2 production with the coulombs arriving to the cathode as current intensity.

However, under certain scenarios, these efficiencies may be misleading and some considerations need to be taken into account when analysing the results.

H_2 is a suitable electron donor and, as such, its presence may induce the growth of hydrogenotrophic bacteria. H_2 is either electrochemically produced at the cathode or appears as a subproduct from the fermentation of organic products. Then, the proliferation of H_2 scavengers in MEC systems is frequent, particularly when operating under single-chamber configuration. The most common scenarios in acetate-fed single-chamber MECs are: i) neither methanogenesis nor H_2-recycling, ii) only H_2-recycling, iii) only methanogenesis and iv) both H_2-recycling and methanogenesis taking place.

In view of simplification, it has been assumed that CH_4 formation comes only from hydrogenotrophic methanogens and thus, acetate is not a carbon source for methanogenesis. This suppression of acetoclastic methanogenesis in single-chamber acetate-fed systems has already been reported and it is justified by the ARB having higher acetate affinity than methanogens [18]. Anyway, the absence of acetoclastic methanogens in our systems was ensured by monitoring acetate concentration in a batch experiment during 70 h without applying any voltage (Figure S1, supplementary data). Acetate concentration remained practically constant indicating that acetate consumption related to non-ARB microorganisms was negligible. The absence of acetoclastic

233   methanogens was also corroborated through advanced microbiological analyses

234   showing that only 2 % of the *Archae*a present in the anode were acetoclastic [19]. It

235   should be noted that if a fermentable substrate different than acetate was used, H_2 from

236   fermentation should be also considered and the system would become much more

237   complex.

238   The utilisation of CE and r_{CAT} to evaluate the MEC performance is not valid when H_2-

239   recycling is occurring. Moreover, r_{CAT} cannot be used when hydrogenotrophic

240   methanogenesis is taking place. In these cases, an extended approach should be used.

241   Nevertheless, obtaining unrealistic CE and r_{CAT} results would be a good indicator of

242   some H_2 being lost: CE higher than 100% suggests H_2-recycling, whereas very low r_{CAT}

243   denotes H_2 losses probably as a consequence of methanogenesis or H_2-recycling.

244

245   3.2   Including H_2-recycling (with or without hydrogenotrophic methanogenesis)

246   When H_2-recycling is taking place the estimated CE values are excessively high (even

247   higher than 100%). Then, the MEC performance becomes much more complex to

248   evaluate and a different approach is needed. In this case, we have used electron

249   equivalent balances (i.e. balances in terms of coulombs) for a better description of the

250   cell performance. As it can be observed in Figure 2, electron equivalent balances are

251   stated for both anodic and cathodic processes, which are linked by the coulombs

252   recovered as current intensity and the coulombs recycled as H_2 by ARB and

253   homoacetogens.

254   Regarding anodic processes, the coulombs recovered as current intensity may come

255   from three different sources: i) the oxidation of the external acetate initially added, ii)

256   the oxidation of the acetate resulting from homoacetogenesis and iii) the oxidation of

257   part of the H_2 produced in the cathode. Moreover, it should be considered that a fraction

258   of this acetate / H_2 is not addressed to current intensity but to the growth of the biomass.

259   The balance in the anodic side can be written as in equation 4.

260     $$C_{CI} = C_{Ac} + C_H' + C_{H2\_r} - C_{Ac}^{ARB} - C_{H2}^{ARB} \qquad (4)$$

261   where C_{CI} are the coulombs recovered as current intensity, C_{Ac} are the coulombs

262   obtained from the oxidation of the external acetate, C_H' are the coulombs obtained from

263   the oxidation of the acetate produced by homoacetogens, C_{H2_r} are the coulombs

264   obtained from the oxidation of the H_2 produced on the cathode by ARB while C_{Ac}^{ARB} and

265   C_{H2}^{ARB} are the acetate and H_2 fractions addressed to biomass growth in terms of

266   coulombs.

267   In the case of cathodic processes, the coulombs recovered as current intensity are all

268   used for H_2 production which, in turn, has four theoretical different endings: i) being

269   captured in the gas bag, the most desirable, ii) being consumed by methanogens, iii)

270   being consumed by homoacetogens, iv) being consumed by ARB. Equation 5 represents

271   the previous processes in terms of coulombs.

272     $$C_{CI} = C_{H2} + C_{CH4} + C_H + C_{H2\_r} \qquad (5)$$

273   where C_{H2} are the coulombs consumed in the production of the measured H_2 and C_{CH4},

274   C_H and C_{H2_r} are the coulombs consumed in the production of H_2 subsequently

275   consumed for the production of CH_4, acetate and current intensity.

276   Although H_2 losses due to leakage (C_{H2_L}) are not considered in equation 5, practical

277   knowledge suggests that, in some cases, they might be required to completely solve the

278   equations system. C_{H2_L} can be taken into account in terms of coulombs by modifying

279   equation 5 as follows:

280     $$C_{CI} = C_{H2} + C_{CH4} + C_H + C_{H2\_r} + C_{H2\_L} \qquad (6)$$

281   Thus, the fate of the electrons would be completely described with equations 4 and 5 (or

282   6). However, each of the parameters in these equations needs to be estimated/measured.

283

284   *3.2.1   Contribution of the growth processes*

285   The fraction of acetate addressed to ARB growth in terms of coulombs, C_{Ac}^{ARB}, can be

286   estimated from equation 7.

287    $$C_{Ac}^{ARB} = Y_{Ac}^{ARB} \cdot (C_{Ac} + C_H') = \frac{100 - CE_{A1}}{100} \cdot (C_{Ac} + C_H') \qquad (7)$$

288   where Y_{Ac}^{ARB} is the biomass/substrate yield of ARB when consuming acetate and CE_{A1} is

289   the real coulombic efficiency of the cell, i.e., the CE of the cell when H_2-recycling does

290   not occur and thus, current intensity is entirely produced from the oxidation of the

291   externally added acetate. Thus, equation 7 calculates the product between the fraction of

292   acetate consumed but not recovered as current intensity and the coulombs obtained from

293   acetate oxidation either from the externally added or the produced by homoacetogens.

294   Note that using either Y_{Ac}^{ARB} or CE_{A1} in the calculation of C_{Ac}^{ARB} implicitly assumes that

295   acetate is only consumed by ARB. Sleutels et al. [20] used CE to assess the competition

296   between ARB and methanogens with acetate as substrate by considering the electrode

297   and methane as the main electron sinks. As previously stated, the presence of

298   acetoclastic methanogens in our system was negligible and therefore, it could be

299   assumed that the acetate not recovered as current intensity was uniquely addressed to

300   ARB growth.

301   The CE_{A1} could be either theoretically estimated or experimentally assessed. For the

302   latter, two additional experiments besides the abovementioned standard monitoring are

303   required. On the one hand, acetate evolution and current intensity are measured in a cell

304   with constant N_2 sparging to evaluate the ARB activity without H_2-recycling

305   (experiment *A1*). The obtained results could be misleading if acetate stripping is

306   simultaneously occurring and this is why the extent of this stripping is evaluated in a

307   second experiment where acetate is monitored with constant N_2 sparging and no applied

308   voltage (experiment *A2*). The experimental estimation of CE_{A1} should be more reliable

309   if it is calculated specifically for each system.

310   Part of the H_2 consumed by homoacetogens (Table 1) is also addressed to biomass

311   growth and can be calculated as follows:

312   $$C_{H2}^{HOMO} = C_H - C_H{}' \qquad (8)$$

313   where C_{H2}^{HOMO} are the coulombs equivalent to the H_2 addressed to homoacetogens

314   growth.

315   Similarly, part of H_2 oxidized by ARB is also consumed for growth and not recovered

316   as current intensity (C_{H2}^{ARB}). Both C_{H2}^{HOMO} and C_{H2}^{ARB} can be also calculated from the

317   biomass/substrate yield as shown in equations 9 and 10.

318   $$C_{H2}^{HOMO} = Y_{H2}^{HOMO} \cdot C_H \quad (9)$$

319   $$C_{H2}^{ARB} = Y_{H2}^{ARB} \cdot C_{H2\_r} \quad (10)$$

320   where Y_{H2}^{HOMO} and Y_{H2}^{ARB} are the biomass/substrate yields of homoacetogens and ARB

321   when consuming H_2.

322   C_{Ac}, C_{H2}, C_{CH4} and C_{CI} can be calculated from off-line/online measurements. The

323   following paragraphs detail how to do so.

324

325   *3.2.2   Coulombs obtained from the oxidation of the externally added acetate, C_{Ac}*

326   The moles of electrons obtained from acetate oxidation are calculated from the amount

327   of the external acetate consumed (Table 1) and converted to coulombs using the

328   Faraday constant (equation 11). The reactor volume remained practically constant

329   during all the experiment (less than the 2 % of the total liquid volume was extracted for

330   sampling).

331    $$C_{Ac} = \Delta c \cdot M^{-1} \cdot V_L \cdot b_{Ac} \cdot F \qquad (11)$$

332

333   *3.2.3   Coulombs consumed in the production of the measured H_2, C_{H2}*

334   C_{H2} is estimated by calculating the moles of electrons consumed during the production

335   of H_2 (Table 1) and converting them to couloumbs (equation 12).

336    $$C_{H2} = n_{H2,F} \cdot b_{H2} \cdot F \qquad (12)$$

337   where $n_{H2,F}$ are the moles of H_2 captured and b_{H2} is the number of e^- transferred per

338   mole of H_2 (2 mol e^-/mol H_2).

339

340   *3.2.4   Coulombs consumed in the production of H_2 converted to CH_4, C_{CH4}*

341   C_{CH4} includes the coulombs consumed in the production of H_2 converted to CH_4 without

342   considering biomass growth (C_{CH4}') and the H_2 consumed for hydrogenotrophic

343   methanogens growth in terms of coulombs (C_{H2}^{MET}). C_{CH4} can be calculated with

344   equation 13.

345    $$C_{CH4} = C_{CH4}' + C_{H2}^{MET} = n_{H2,F}^{CH4} \cdot b_{H2} \cdot F \qquad (13)$$

346   where $n_{H2,F}^{CH4}$ are the moles of H_2 consumed to produce CH_4.

347    $n_{H2,F}^{CH4}$ is calculated from the volume of H_2 consumed to produce CH_4, $V_{H2,F}^{CH4}$, which, in

348   turn, is calculated according to the proper stoichiometry (Table 1) and considering the

349   fraction of H_2 consumed for biomass growth (equation 14).

350    $$V_{H2,F}^{CH4} = 4 \cdot \frac{V_{CH4,F}}{1 - Y_{H2}^{MET}} \qquad (14)$$

351   where $V_{CH4,F}$ is the final volume of CH_4 and Y_{H2}^{MET} is the biomass/substrate yield of

352   hydrogenotrophic methanogens when consuming H_2.

353

354   *3.2.5   Coulombs recovered as current intensity, C_{CI}*

355   C_{CI} is calculated by integrating the current intensity from the initial to the final time of

356   the batch experiment.

357    $$C_{CI} = \int_{t_0}^{t_F} I \, dt \qquad (15)$$

358   Note that being able to calculate C_{Ac}, C_{H2}, C_{CH4} and C_{CI} (equations 11, 12, 13 and 15)

359   we have a system of six linear equations (4, 5, 7, 8, 9 and 10) and six degrees of

360   freedom ($C_H{}'$, C_H, C_{H2_r}, C_{Ac}^{ARB}, C_{H2}^{HOMO} and C_{H2}^{ARB}). Thus, electron equivalent balances

361   can be solved. All the parameters used to calculate the electron equivalent balances are

362   summarized in Table 2.

363   Moreover, two interesting performance parameters, the fraction of the current intensity

364   generated due to the oxidation of the externally added acetate (f_{CI_Ac}) and due to

365   recycled H_2 (f_{CI_H2}), can be also estimated from the parameters calculated by the

366   electron equivalent balances (equations 16 and 17).

367    $$f_{CI\_Ac} = \frac{\left(1 - Y_{Ac}^{ARB}\right) \cdot C_{Ac}}{C_{CI}} = \frac{\left(1 - \dfrac{100 - CE_{A1}}{100}\right) \cdot C_{Ac}}{C_{CI}} = \frac{\dfrac{CE_{A1}}{100} \cdot C_{Ac}}{C_{CI}} \qquad (16)$$

368    $$f_{CI\_H2} = \frac{\left(1 - Y_{Ac}^{ARB}\right) \cdot C_H{}' + C_{H2\_r} - C_{H2}^{ARB}}{C_{CI}} = \frac{\dfrac{CE_{A1}}{100} \cdot C_H{}' + C_{H2\_r} - C_{H2}^{ARB}}{C_{CI}} \qquad (17)$$

369

370   3.3 <u>Including hydrogenotrophic methanogenesis when no H_2-recycling is occurring</u>

371   The previously developed electron equivalent balances can be used even when no H_2-

372   recycling is occurring but most parameters would be zero. In this sense, the following

373   simplified approach can be more practical. Thus, if hydrogenotrophic methanogens are

374   present in the system, r_{CAT} will be underestimated since the amount of H_2 produced and

375   sequentially diverted to CH_4 would not be considered. Although CE would not be

376   affected, the calculation of r_{CAT} would need a correction by including the H_2

377   theoretically converted into CH_4. Then, the real volume of H_2 produced ($V_{H2,F}^{T}$) would

378   include the measured H_2 and the H_2 converted to CH_4 according to the proper

379   stoichiometry (Table 1). Then, $V_{H2,F}^{T}$ should be used in equation 3 when estimating r_{CAT}.

380    $$V_{H2,F}^{T}=V_{H2,F}+V_{H2,F}^{CH4} \qquad (18)$$

381   where $V_{H2,F}^{T}$ is the total volume of H_2 produced and $V_{F,H2}$ is the measured H_2

382   production.

383

384   3.4 <u>Experimental study: Occurrence of H_2-recycling</u>

385   A 1L MEC was operated for 8 months with BES dosage using an ARB-enriched anode.

386   BES concentration was initially set at 50 mM, a value theoretically high enough to

387   supress methanogenic activity [10]. Under these conditions (i.e. single-chamber

388   membrane-less MEC with BES and under batch operation), methanogenesis could be

389   avoided. However, H_2-recycling was favoured and then, efficient H_2 production was

390   still hindered. Practically from the first days of operation it was observed that the

391   duration of the cycles was not in agreement with the monitored intensity resulting in CE

392   higher than 100 %. Moreover, the highest H_2 production was detected after adding fresh

393   medium in the cell, whereas H_2 concentration in the gas sampling bag was decreasing

394   along the cycle, resulting in r_{CAT} values close to 0 %. Thus, the most plausible option

395   was H_2-recycling either by homoacetogens or H_2-consumers ARB. Figure 3 shows an

396   experiment where sodium bicarbonate and H_2 were added as sole carbon source and

397   sole electron donor, respectively. Acetate concentration was initially zero and it

398   increased over time reaching values of around 70 mg/L. Meanwhile, current density

399   also increased and reached values close to 7 A/m^3. Thus, homoacetogens were present

400   and consumed H_2 and CO_2 to form acetate. Acetate could be subsequently used by ARB

401   to generate current from acetate. However, current intensity due to direct oxidation of

402   H_2 could not be ruled out.

403   Electron equivalent balances were calculated to gain insight on the cell performance

404   under H_2-recycling conditions and hence a cycle was monitored during approximately

405   100 hours.

406   Figure 4 shows the experimental results obtained during the characterisation of the

407   operation with H_2 recycling. As previously detailed, two additional experiments were

408   required for the calculation of C_{Ac}^{ARB}: *A1)* ARB activity was measured in a MEC with

409   continuous N_2 sparging to avoid H_2 utilisation by both homoacetogens and ARB and

410   *A2)* acetate concentration was measured with N_2 sparging but with no applied voltage to

411   estimate acetate stripping. Figure 4A compares the cell current density with (*A1*) and

412   without N_2 sparging (conventional operation). As it can be observed, the duration of the

413   cycle was completely different (in spite of having the same initial acetate

414   concentration): the cycle was completed after 50 hours with N_2 sparging whereas under

415   conventional operation, the current density remained at values around 17 A/m^3 after 100

416   hours. In *A1* H_2 was removed from the system by stripping, while under conventional

417   operation, H_2 was used by homoacetogenic bacteria to produce acetate or by ARB to

418   generate electricity thus, extending the cycles. Regarding acetate measurements, acetate

419 decreased under conventional operation during the first 20 hours of the cycle and

420 remained almost constant during the following 80 hours. In contrast, when N_2 was

421 sparged, acetate was consumed in 50 hours. The decrease in acetate concentration was

422 not related to stripping: Figure 4B shows that when the cell was disconnected and

423 sparged with N_2 (*A2*), acetate concentration did not decrease but slightly increased,

424 probably as a result of water evaporation. Finally, Figure 4C presents the bag

425 composition and shows that the H_2 increased, reached a maximum (100 mL) and then

426 decreased. CH_4 concentration was scarce indicating that H_2 consumption was not

427 addressed to methanogenesis.

428 On the one hand, the CE under conventional operation was, as expected, much higher

429 than 100 % (463 %). However, when N_2 was sparged, C_E decreased to 90.4 %, thus

430 only the 9.6 % of the acetate is consumed for the growth of the biomass (Y_{Ac}^{ARB}).

431 Therefore, CE_{A1} (i.e. the real CE excluding the H_2-recycling effect) was 90.4 %. On the

432 other hand, r_{CAT} was around 4 %. The coulombs generated from acetate oxidation

433 according to the experimental acetate measurements, C_{Ac}, were 1555 C, whereas the

434 coulombs recovered as current intensity, C_{CI}, were 7203 C and the coulombs consumed

435 in H_2 production, C_{H2}, 292 C. For Y_{H2}^{HOMO} and Y_{H2}^{ARB} it was assumed a value of 0.1 mol

436 e^- biomass/ mol e^- substrate, i.e. a value similar to that estimated for ARB when

437 consuming acetate.

438 Substituting the values of C_{Ac}, C_{H2}, C_{CH4}, C_{CI}, CE_{A1}, Y_{H2}^{HOMO} and Y_{H2}^{ARB} in equations 4, 5,

439 7, 8, 9 and 10 it was obtained that:

440 $$5648 = C_H{}' + C_{H2\_r} - C_{Ac}^{ARB} - C_{H2}^{ARB} \qquad (19)$$

441 $$6911 = C_H + C_{H2\_r} \qquad (20)$$

442 $$C_{Ac}^{ARB} = 149.18 + 0.096 \cdot C_H{}' \qquad (21)$$

443 $$C_{H2}^{HOMO} = C_H - C_H' \quad (22)$$

444 $$C_{H2}^{HOMO} = 0.10 \cdot C_H \quad (23)$$

445 $$C_{H2}^{ARB} = 0.10 \cdot C_{H2\_r} \quad (24)$$

446 The equation system (eqs 19 to 24) solution is summarized in Table 3. The fraction of

447 H_2 recycled by homoacetogens, calculated as $C_H/(C_H+C_{H2\_r})$, was 71 %, whereas the

448 fraction of H_2 recycled by the direct oxidation of H_2 by ARB, calculated as

449 $C_{H2\_r}/(C_H+C_{H2\_r})$, was 29 %. Moreover, coulombic losses due to biomass growth were

450 mainly caused by the consumption of acetate by ARB (C_{Ac}^{ARB}) and the consumption of

451 H_2 by homoacetogens (C_{H2}^{HOMO}).

452

453 f_{CI_Ac} and f_{CI_H2} were 19.5 % and 80.5 % respectively (equations 16 and 17), showing

454 that the effect of H_2-recycling can be far from negligible (e.g. in our system, 80.5 % of

455 the current intensity was generated due to H_2-recycling). Moreover, the recycled H_2 in

456 terms of coulombs ($C_H+C_{H2\_r}$) was in just five days around 1.7 times the amount of

457 coulombs that could be generated if all the acetate externally added had been consumed.

458

459 3.5 Experimental study: Presence of methanogens

460 At week 9 of operation, batch experiments suggested growth of methanogens even

461 though there was a BES concentration of 50 mM. It was increased to 90 and

462 subsequently to 120 mM and, surprisingly, CH_4 formation was detected even at those

463 high concentrations. Our results suggest that methanogens grew in the MEC even at

464 higher BES concentrations, either as a result of a too thick biofilm preventing BES to

465 penetrate inside or as a result of a development of BES resistance by methanogens [21].

466　Figure 5 shows the evolution of the methanogenic activity during the cell monitoring

467　performed at different weeks of operation. At weeks 9-10, the ratio $H_2/(H_2+CH_4)$ only

468　started to decrease (i.e. CH_4 was formed) approximately 70 hours after the renewal of

469　the medium. At week 16, $H_2/(H_2+CH_4)$ decreased to 35 % in just 45 hours. BES

470　concentration was increased to 120 mM at week 19 and although methanogenic activity

471　was reduced, it was far from supressed. At week 22 of operation, BES concentration

472　was decreased to 50 mM to obtain results comparable to the literature. Under these

473　operational conditions, most of the H_2 produced was converted to CH_4 at the end of the

474　monitoring, as shown in Figure 5 for week 34. Thus, it was observed that BES may not

475　be an adequate long term solution for methanogenic inhibition when H_2 is widely

476　available (i.e. batch conditions with high retention time).

477　Figure 6 shows an example of the monitoring of a cycle (week 34) where methanogenic

478　activity was significant. As it can be observed the cycle lasted approximately 50 hours,

479　during which acetate concentration was decreasing (Figure 6B). Regarding gas

480　production, H_2 reached a maximum volume between hours 3 and 4 of monitoring and

481　then it started decreasing. In contrast, CH_4 production was increasing during all the

482　cycle.

483　The CE of the cell was 74.5 %, whereas the r_{CAT} if only comparing the coulombs

484　recovered as H_2 to those recovered as current intensity was 0. A much more realistic

485　r_{CAT} value of 94.5% was calculated by computing CH_4 into the balance, assuming that

486　all CH_4 produced came from H_2 [22] and transforming moles of CH_4 into moles of H_2

487　by considering a Y_{H2}^{MET} of 0.1 mol e⁻ biomass /mol e⁻ substrate (equations 14 and 18).

488　Acetate-driven methanogenesis could be discarded since it would have resulted in a

489　much lower CE. These results show that when methanogenesis became important, H_2-

490   recycling, if still occurring, lost importance since only the 5.5 % of the coulombs

491   recovered as current intensity were not subsequently recovered as H_2 or CH_4.

492   As previously stated, the electron equivalent balances can also be used to describe the

493   behaviour of the cell under methanogenesis conditions. In the presented case, the

494   calculated CE suggested that H_2-recycling was not occurring, thus C_H, C_H' and C_{H2_r}

495   could be neglected. Therefore, the previous system of equations (equations 4, 5, 7, 8, 9

496   and 10) could be reduced to only three linear equations:

497    $C_{CI} = C_{Ac} - C_{Ac}^{ARB}$ $\qquad$ (25)

498    $C_{CI} = C_{H2} + C_{CH4}$ $\qquad$ (26)

499    $C_{Ac}^{ARB} = \dfrac{100 - CE}{100} \cdot C_{Ac}$ $\qquad$ (27)

500   Note that CE_{A1} was replaced by CE in equation 27 since CE did not need to be

501   corrected by H_2-recycling. According to the measurements/calculations, C_{Ac} was 3378

502   C, C_{CI} was 2518 C, C_{H2} was 0 and C_{CH4} was 2379 C. Substituting these values into

503   equations 25, 26 and 27 it was obtained:

504    $-860 = -C_{Ac}^{ARB}$ $\qquad$ (28)

505    $2518 = 2379$ $\qquad$ (29)

506    $C_{Ac}^{ARB} = \dfrac{100 - CE}{100} \cdot 3378$ $\qquad$ (30)

507   As it can be observed, to solve the system C_{H2_L} had to be included in equation 29 as

508   follows:

509    $2518 = 2379 + C_{H2\_L}$ $\qquad$ (31)

510   However, as deduced from equation 31, the value of C_{H2_L} was very low and can be

511   assumed as experimental error. Table 4 summarizes the results of the CE, r_{CAT} and

512   electron equivalent balances calculations. The use of electron equivalent balances gives

513   similar information to that provided by CE and r_{CAT}, but returns the values of C_{Ac}^{ARB} and

514   C_{H2_L} in terms of coulombs.

515   The results so far suggest that H_2-recycling took place when the methanogenic activity

516   was not important. Moreover, the CE evolution showed that CE was higher than 100 %

517   when methanogens were not dominant. CE decrease to values around 75 % was

518   proportional to the methanogenic activity increase. Results could also suggest that CE

519   was decreasing as a consequence of acetate consumption by methanogens. However,

520   this was ruled out taking into account results in the literature and our own results in the

521   CE and r_{CAT} calculations.

522   Thus, if working with single-chamber MECs, the most feasible strategy to avoid H_2

523   scavengers would be preventing H_2 to be available for the microorganisms. Some

524   options would be the use of membranes or using reactors with architectures for a fast H_2

525   separation in order to make H_2 unavailable for the microorganisms [11]. On the other

526   hand, other possible strategies based on the selective inhibition of methanogens would

527   not be useful in a system with these characteristics, since H_2-recycling would not be

528   avoided.

529

530   **4. CONCLUSIONS**

531   In membrane-less single-chamber MEC, the presence of H_2 scavengers is a significant

532   hurdle in view of its real application. Under these conditions, the classical indexes CE

533   and r_{CAT} calculated to estimate its performance are no longer valid.

534   When methanogens are present, r_{CAT} should be calculated estimating the amount of H_2

535   converted to CH_4.

536   When methanogens are selectively inhibited, H_2-recycling (due to homoacetogenic

537   bacteria or due to direct H_2 oxidation) is very likely to occur, causing large deviations in

23

538 the estimated CE and r_{CAT} values. A different approach based on electron equivalent

539 balances is presented in this work which, through a better understanding of the process

540 occurring in the cell, results in the calculation of two new parameters, f_{CI_Ac} and f_{CI_H2},

541 which are much more realistic indicators of the real cell performance.

542 Two experimental studies under different scenarios (proliferation of homoacetogens or

543 methanogens) were presented. The proposed approach based on balances was

544 successfully applied and under H_2-recycling conditions the estimation of the MEC

545 performance was much more accurate.

546 Moreover, electron balances showed that H_2-recycling could be an issue as important as

547 CH_4 generation, since the H_2-acetate loop increases the operating costs and makes

548 infeasible the production of H_2 in MECs.

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621 **Table captions**

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625 **Table 1** Stoichiometry of the possible reactions occurring in a MEC.

626

627 **Table 2** Nomenclature and description of parameters.

628

629 **Table 3** Summary of the electron equivalent balances during a cycle with H_2-recycling.

630

631 **Table 4** Summary of the results in a cycle with methanogenic activity.

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634 **Table 1** Stoichiometry of the possible reactions occurring in a MEC.

Reaction / Microorganisms	Stoichiometry
Acetate oxidation / ARB	$CH_3COO^- + 4H_2O \rightarrow 2HCO_3^- + 9H^+ + 8e^-$
CH_4 formation / Hydrogenotrophic methanogens	$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$
Acetate formation / Homoacetogens	$4H_2 + 2CO_2 \rightarrow CH_3COO^- + H^+ + 2H_2O$
H_2 oxidation / ARB	$H_2 \rightarrow 2H^+ + 2e^-$
H_2 formation / chemical reaction	$2H^+ + 2e^- \rightarrow H_2$

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Table 2 Nomenclature and description of parameters.

Parameter	Description	Dimension
b_{Ac}, b_{H2}	Number of e⁻ transferred per mole of acetate (8 mol e⁻/mol Ac) and H_2 (2 mol e⁻/mol H_2)	mol e⁻/mol substrate
C_{Ac}	Coulombs obtained from the oxidation of the initially added acetate	C
C_{CH4}	Coulombs consumed in the production of H_2 converted to CH_4	C
C_{CH4}'	Coulombs consumed in the production of H_2 converted to CH_4 (without considering hydrogenotrophic methanogens growth)	C
C_{CI}	Coulombs recovered as current intensity	C
C_H	Coulombs consumed in the production of H_2 converted to acetate by homoacetogens	C
C_H'	Coulombs obtained from the oxidation of acetate produced by homoacetogens	C
C_{H2}	Coulombs consumed in the production of the measured H_2	C
C_{H2_L}	H_2 losses due to leakage	C
C_{H2_r}	Coulombs obtained from the oxidation of H_2	C
C_{Ac}^{ARB}	Acetate consumed for ARB growth in terms of coulombs	C
C_{H2}^{ARB}	H_2 consumed for ARB growth in terms of coulombs	C
C_{H2}^{HOMO}	H_2 consumed for homoacetogens growth in terms of coulombs	C
C_{H2}^{MET}	H_2 consumed for hydrogenotrophic methanogens growth in terms of coulombs	C
CE	Coulombic efficiency	-
CE_{A1}	Coulombic efficiency in experiment A1 (no H_2- recycling)	C
Δc	Acetate concentration change over t_F and t_0	g Ac/L
F	Faraday constant (96485 C/mol e⁻)	C/mol e⁻
f_{CI_Ac}	Fraction of the current intensity generated due to the oxidation of the external acetate initially added	-
f_{CI_H2}	Fraction of the current intensity generated due to H_2-	-

Parameter	Description	Dimension
	recycling	
I	Current intensity	A
M	Molecular weight of the acetate (59 g/mol)	g/mol
$n_{H2,F}$	Moles of H_2 at the end of a batch experiment	mol
$n_{H2,F}^{CH4}$	Moles of H_2 converted to CH_4 at the end of a batch experiment	mol
r_{CAT}	Cathodic efficiency	-
t, t_0 and t_F	Time / Initial and final times of the batch experiments	s
$V_{G,F}$	Final volume of gas	L
$V_{H2,F}$	Final volume of H_2	L
$V_{i,F}$	Final volume of the gas i	L
V_L	Volume of liquid in the reactor (1.3 L)	L
V_m	Molar gas volume (24.03 L/mol at 20ºC)	L/mol
$V_{H2,F}^{CH4}$	Volume of the H_2 consumed to produce CH_4	L
$V_{H2,F}^{T}$	Volume of H_2 produced including that consumed to produce CH_4	L
$x_{i,F}$	Final composition of the gas i	-
Y_{Ac}^{ARB}	Biomass/substrate yield for ARB when consuming acetate	mol e⁻ biomass/mol e⁻ substrate
Y_{H2}^{ARB}	Biomass/substrate yield for ARB when consuming H_2	mol e⁻ biomass/mol e⁻ substrate
Y_{H2}^{HOMO}	Biomass/substrate yield for homoacetogens when consuming H_2	mol e⁻ biomass/mol e⁻ substrate
Y_{H2}^{MET}	Biomass/substrate yield for hydrogenotrophic methanogens when consuming H_2	mol e⁻ biomass/mol e⁻ substrate

641

642 **Table 3** Summary of the electron equivalent balances during a cycle with H_2-recycling.

Parameter	Normal operation	With N_2 sparging
CE	463 %	90.4 %
r_{CAT}	4 %	--
C_{CI}	7203 C	2989 C
C_{Ac}	1555 C	3306 C
C_{H2}	292 C	--
C_{CH4}	0 C	--
C_H	4893 C	0 C
C_H'	4403 C	0 C
C_{H2_r}	2018 C	0 C
C_{Ac}^{ARB}	572 C	317 C
C_{H2}^{HOMO}	489 C	0 C
C_{H2}^{ARB}	202 C	0 C
f_{CI_Ac}	19.50 %	100 %
f_{CI_H2}	80.50 %	0 %

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645   **Table 4** Summary of the results in a cycle with methanogenic activity.

Parameter	Value
CE	74.5 %
r_{CAT}	0 %
r_{CAT} (considering CH_4)	94.5 %
C_{CI}	2518 C
C_{Ac}	3378 C
C_{H2}	0 C
C_{CH4}	2379 C
C_H	0 C
$C_H{}'$	0 C
C_{H2_r}	0 C
C_{Ac}^{ARB}	860 C
C_{H2}^{HOMO}	0 C
C_{H2}^{ARB}	0 C
C_{H2_L}	139 C
f_{CI_Ac}	100 %
f_{CI_H2}	0 C

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649 **Figure captions**
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653 **Figure 1** (A) Schematic diagram and (B) image of the MEC used in this study.

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656 **Figure 2** Reaction pathways and parameters of electron equivalent balances in an

657 acetate-fed single-chamber MEC.

658

659 **Figure 3** Batch experiment with the addition of sodium bicarbonate and H_2 sparging

660 (A) Acetate concentration and (B) Current density over time. Current density is shown

661 from time 5 hours due to monitoring problems.

662

663 **Figure 4** Monitoring of the MEC with H_2-recycling (A) Current density under

664 conventional operation (solid) and with N_2 sparging (experiment A1) (dashed), (B)

665 Acetate concentration under conventional operation (●), with N_2 sparging (experiment

666 A1) (△) and with N_2 sparging and no applied voltage (experiment A2) (□) and (C) Gas

667 production under conventional operation: H_2 (◆) and CH_4 (▽).

668

669 **Figure 5** Methanogenic activity vs time represented as the ratio H2/H2+CH4 at

670 different weeks of operation. Week 9 (●), week 10 (✖), week 16 (○), week 19 (△),

671 week 29 (◆) and week 34 (▼) of operation. Concentration of BES: 90 mM (solid), 120

672 mM (dashed) and 50 mM (dash-dotted).

673

674 **Figure 6** Monitoring of the MEC with the presence of methanogens (A) Current

675 density, (B) Acetate concentration and (C) Gas production: H_2 (◆) and CH_4 (▽). Note

676 the different scales in (C).
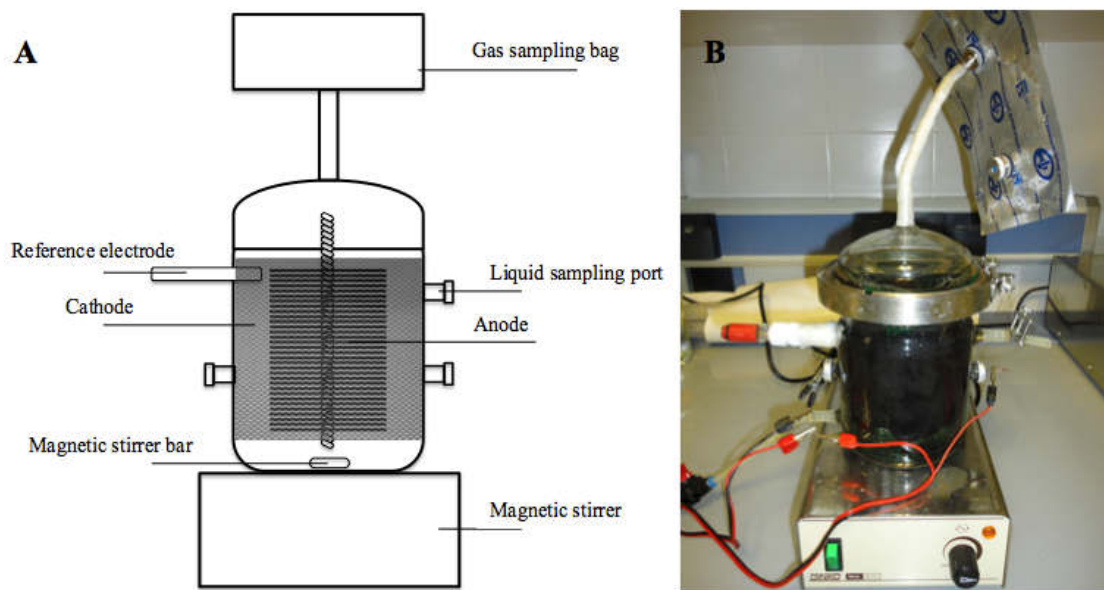
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678   **Figure 1** (A) Schematic diagram and (B) image of the MEC used in this study.
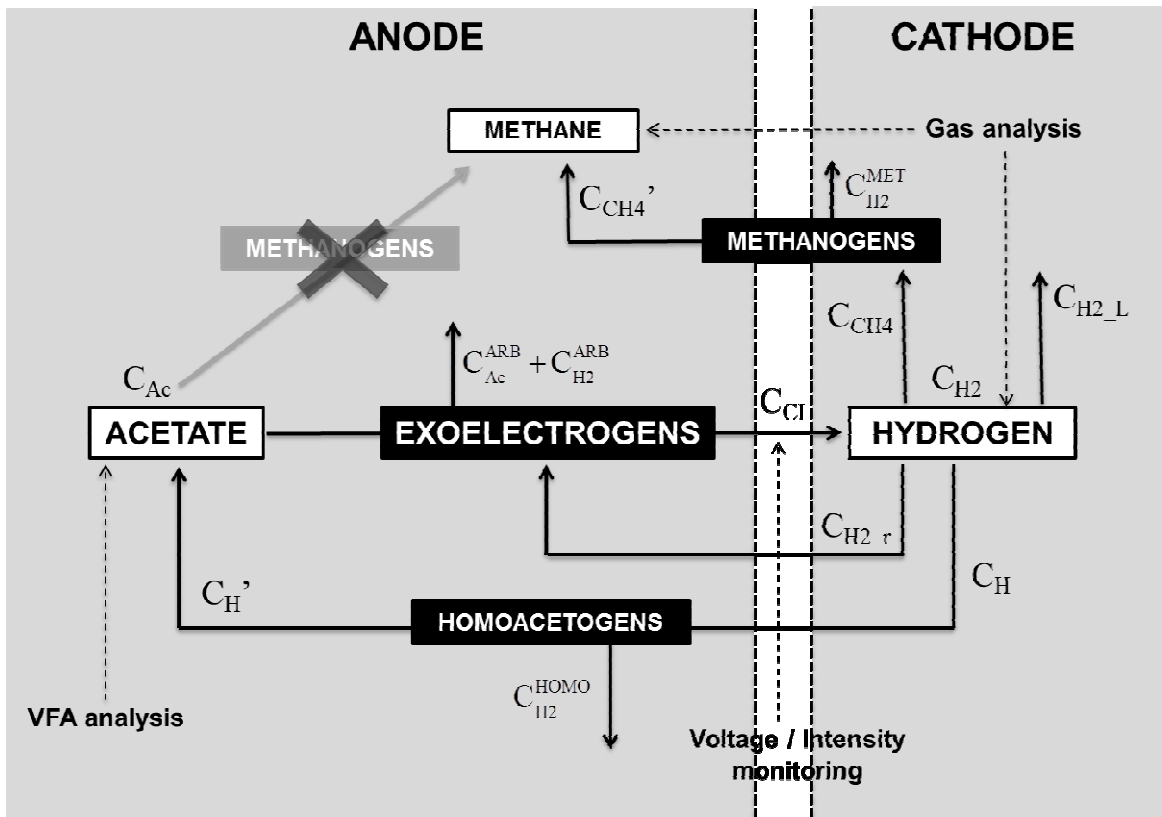
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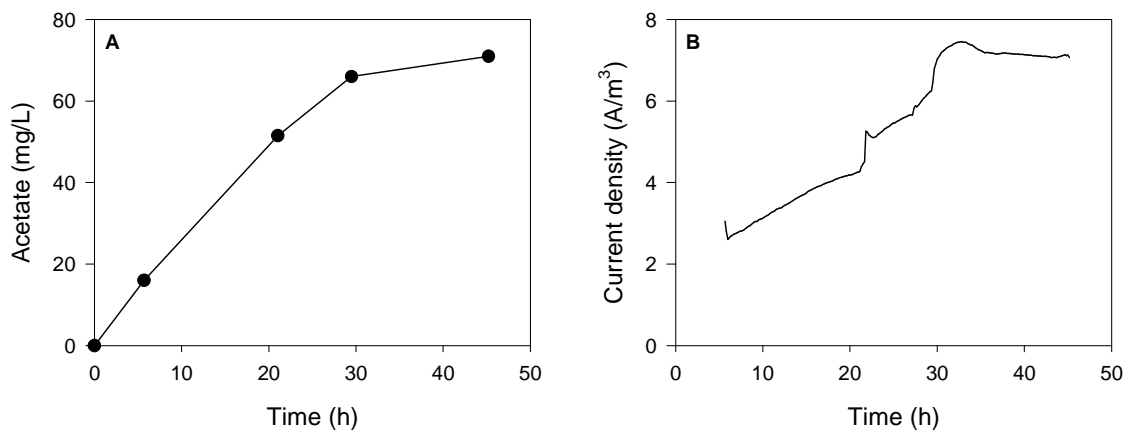685

Figure 2 Reaction pathways and parameters of electron equivalent balances in an acetate-fed single-chamber MEC.

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694 **Figure 3** Batch experiment with the addition of sodium bicarbonate and H_2 sparging

695 (A) Acetate concentration and (B) Current density over time. Current density is shown

696 from time 5 hours due to monitoring problems.

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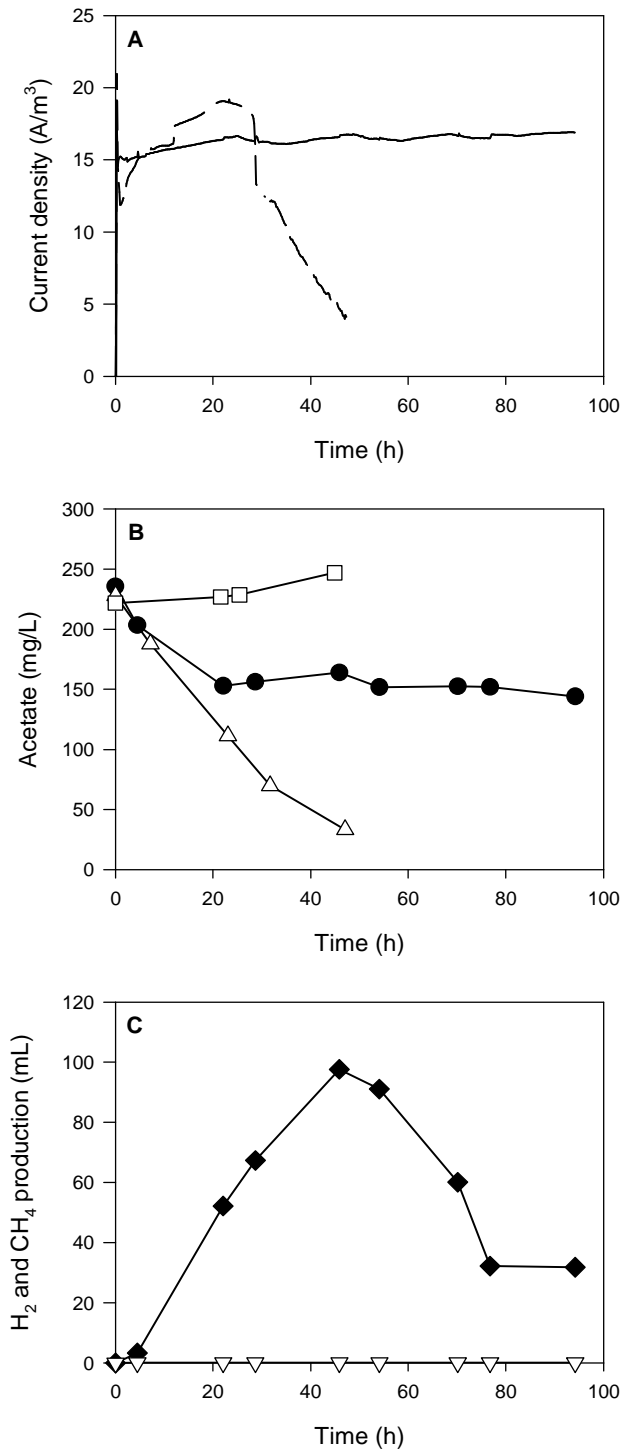
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Figure 4 Monitoring of the MEC with H_2-recycling (A) Current density under

conventional operation (solid) and with N_2 sparging (experiment A1) (dashed), (B)

Acetate concentration under conventional operation ($\bullet$), with N_2 sparging (experiment

A1) ($\triangle$) and with N_2 sparging and no applied voltage (experiment A2) ($\square$) and (C) Gas
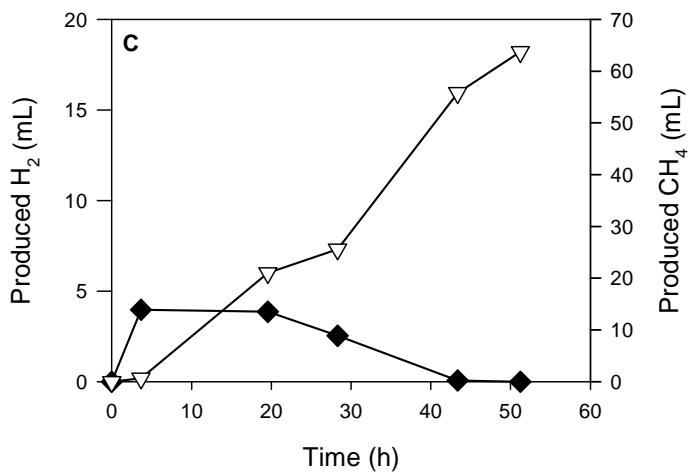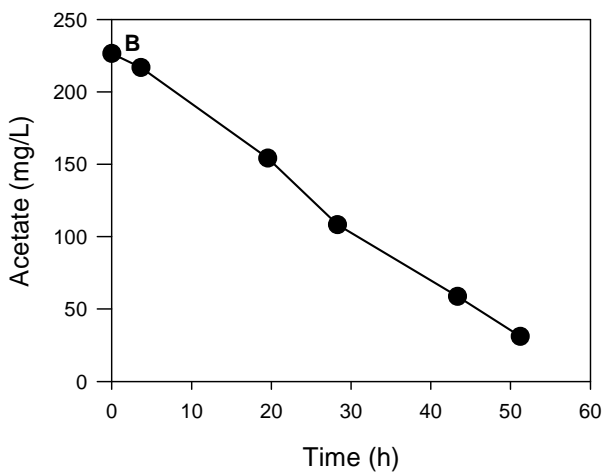
production under conventional operation: H_2 ($\blacklozenge$) and CH_4 ($\triangledown$).
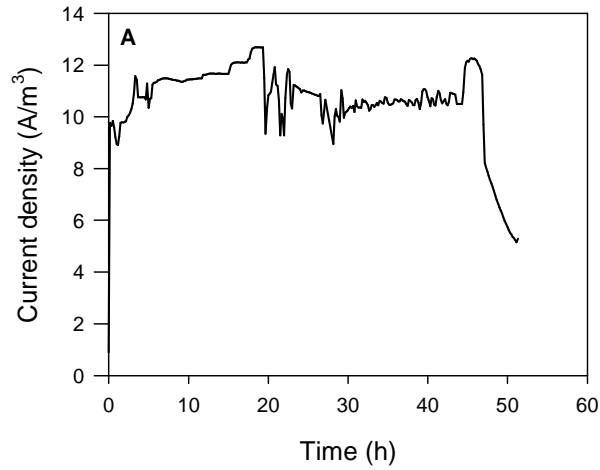
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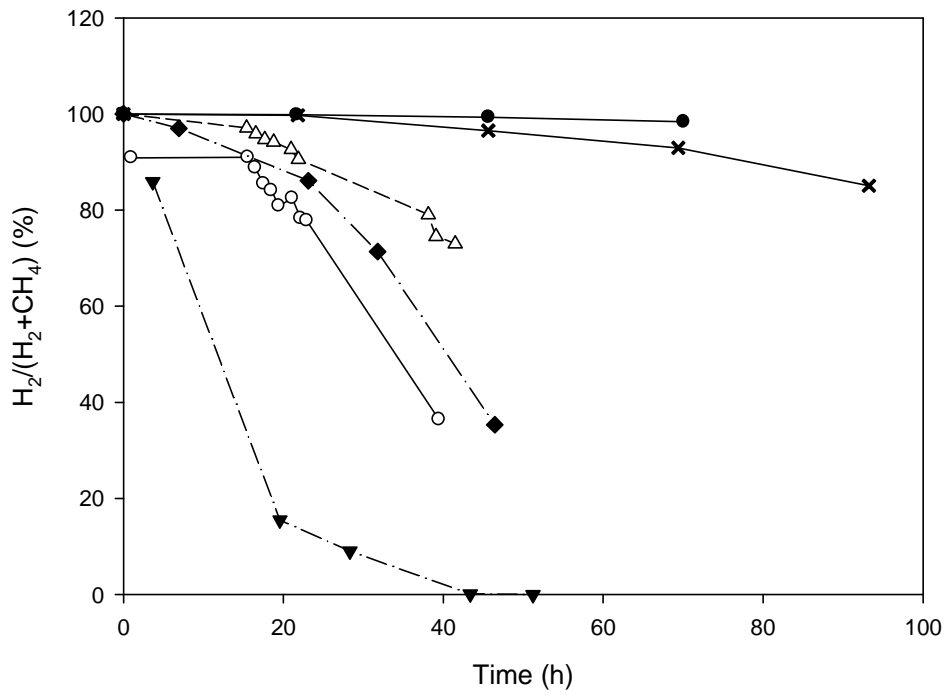
Figure 5 Methanogenic activity vs time represented as the ratio H_2/H_2+CH_4 at different

weeks of operation. Week 9 (●), week 10 (✕), week 16 (○), week 19 (△), week 29

(◆) and week 34 (▼) of operation. Concentration of BES: 90 mM (solid), 120 mM

(dashed) and 50 mM (dash-dotted).

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Figure 6 Monitoring of the MEC with the presence of methanogens (A) Current

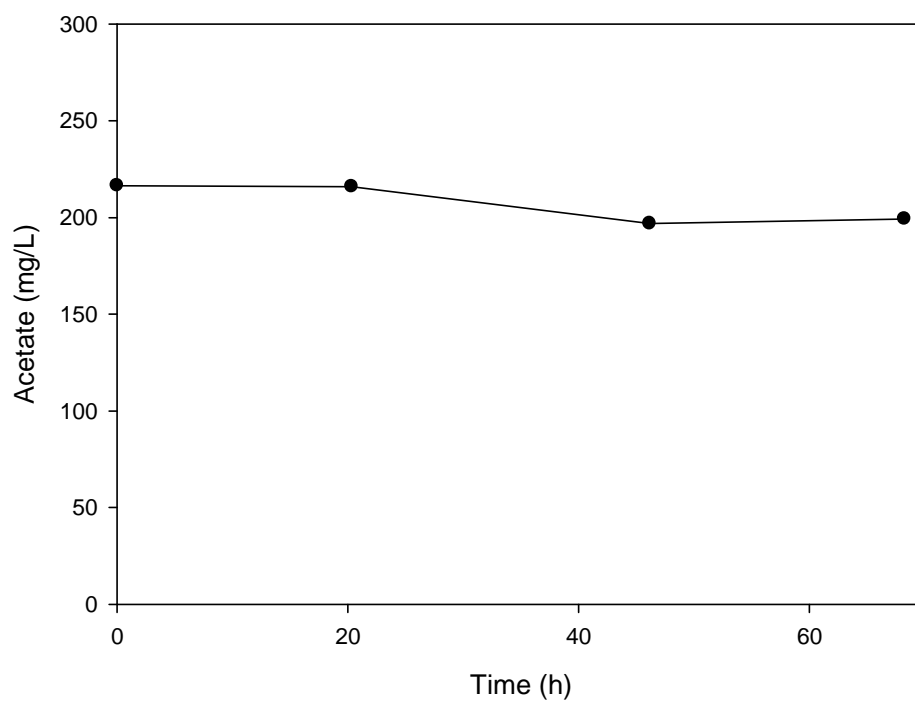density, (B) Acetate concentration and (C) Gas production: H_2 ($\blacklozenge$) and CH_4 ($\triangledown$). Note

the different scales in (C).

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Supplementary data

Figure S1 Acetate concentration versus time in the MEC without applied voltage.