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Biomass retention on electrodes rather than electrical current enhances stability in anaerobic digestion.

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Abstract

Anaerobic digestion (AD) is a well-established technology for energy recovery from organic waste streams. Several studies noted that inserting a bioelectrochemical system (BES) inside an anaerobic digester can increase biogas output, however the mechanism behind this was not explored and primary controls were not executed. Here, we evaluated whether a BES could stabilize AD of molasses. Lab-scale digesters were operated in the presence or absence of electrodes, in open (no applied potential) and closed circuit conditions. In the control reactors without electrodes methane production decreased to 50% of the initial rate, while it remained stable in the reactors with electrodes, indicating a stabilizing effect. After 91 days of operation, the now colonized electrodes were introduced in the failing AD reactors to evaluate their remediating capacity. This resulted in an immediate increase in CH₄ production and VFA removal. Although a current was generated in the BES operated in closed circuit, no direct effect of applied potential nor current was observed. A high abundance of *Methanosaeta* was detected on the electrodes, however irrespective of the applied cell potential. This study demonstrated that, in addition to other studies reporting only an increase in methane production, a BES can also remediate AD systems that exhibited process failure. However, the lack of difference between current driven and open circuit systems indicates that the key impact is through biomass retention, rather than electrochemical interaction with the electrodes.

1. Introduction

Biorefineries produce sidestreams with high organic content (Verstraete et al. 2005). The success rate of most bio-refineries depends on the full utilization of all resources present in the original biomass, including these sidestreams. In this study, molasses was used to mimic sidestreams originating from bio-refineries. The direct discharge of untreated molasses wastewaters may cause serious environmental issues due to their high concentration of organic matter, high salt content and low pH (Sirianuntapiboon and Prasertsong 2008). Anaerobic digestion (AD) is an established technology and can be considered the first microbial technology to allow energy recovery from complex organic waste streams. AD therefore has the potential to become a key technology to treat these sidestreams and generate heat and electricity for the refinery (Verstraete et al. 2005). AD can also deal with high loading rates, has limited nutrient demands and low operational control and maintenance costs (Mata-Alvarez et al. 2000, Verstraete et al. 2009). Methanogenic archaea are responsible for the final and most critical step of anaerobic digestion, i.e. the production of methane. One of the main drawbacks of anaerobic digestion is a sometimes-observed process failure due to sensitivity of these methanogens to different environmental factors, such as abrupt pH changes, organic overloading and high salt concentrations, leading to the accumulation of volatile fatty acids (VFA) (Ahring et al. 1995, Chen et al. 2008, De Vrieze et al. 2012, Gujer and Zehnder 1983).

Bioelectrochemical systems (BESs) are an alternative technology to anaerobic digestion, capable of directly producing electrical power from liquid organic waste streams. Contrary to AD, very few BES exist beyond the lab-scale, hence their competitiveness with AD remains thus far unproven (Arends and Verstraete 2012, Pham et al. 2006). On the other hand, BESs are highly versatile in terms of potential application, ranging from energy production from organic substrates to product generation and specific environmental niche creation (Arends and Verstraete 2012, Logan and Rabaey 2012, Rabaey and Rozendal 2010). These last two processes are of main interest to AD due to their possible influence on process stability and microbial activity.

It has been postulated that a BES can be used to alter and/or control the main processes in anaerobic digestion (Arends and Verstraete 2012, Sasaki et al. 2010b). Several studies already highlighted that combining anaerobic digesters with a BES resulted in a higher level of biogas production (Rabaey et al. 2005, Sasaki et al. 2011a, Sasaki et al. 2010b, Tartakovsky et al. 2011, Vijayaraghavan and Sagar 2010, Weld and Singh 2011). Different AD-BES configurations, such as the utilization of a BES as pre- or post-treatment device outside the AD reactor or the direct application of a BES in the digester, may lead to enhanced methane production. The introduction of a BES in the recirculation loop of a thermophilic UASB resulted in a higher tolerance of the digester to a severe drop in pH due to the

addition of an acetate pulse to the system (Weld and Singh 2011). The direct application of the cathode in an AD reactor resulted in enhanced COD removal and methane production during anaerobic digestion of filter paper and garbage slurry, respectively (Sasaki et al. 2011a, Sasaki et al. 2010b). The introduction of both the anode and cathode of a BES in the sludge bed of an UASB (Tartakovsky et al. 2011) or in a CSTR (Vijayaraghavan and Sagar 2010) also resulted in increased methane production. A BES can also be used for post-digestion polishing of highly loaded wastewaters, leading to side products such as H_2 (Rabaey et al. 2005).

The objective of this study was (1) to evaluate whether a BES could stabilize anaerobic digestion (AD-BES) of molasses leading to higher COD removal and methane production, and (2) if a BES could remediate systems that have experienced severe process failure and (3) how this influences the microbial community composition of the entire system. The term “stable” was used as long as total residual VFA remained below 1.0 g COD L^{-1} , whereas the term “failure” referred to a 50% decrease in methane production compared to the initial value. To achieve these goals, different lab-scale anaerobic digesters were operated in the presence or absence of a BES to evaluate the stabilizing potential of a BES in AD. The cell potentials were selected to avoid direct electrochemical production of H_2 at the cathode or O_2 at the anode but to potentially stimulate biologically catalyzed H_2 production, which could lead to an increased methane production. The BESs were also introduced in failing AD reactors in order to evaluate their remediating capacity.

2. Material and methods

2.1. Experimental set-up and operation

2.1.1. Reactor set-up

Seven lab-scale continuously stirred tank reactor (CSTR) vessels with a liquid volume of 800 mL were each connected to a gas column to collect the produced biogas (Figure SI1). These reactors are considered reproducible, as indicated in earlier preliminary research (data not shown). Moreover, each reactor was considered a time series in accordance with the research of Wittebolle et al. (2008), Carballa et al. (2011) and Zamalloa et al. (2012). A pair of carbon felt electrodes were introduced in three vessels, each with a surface area of 60 cm² (projected area; BET 2 m² g⁻¹; Carbon felt, 3.18 mm thickness, Alfa Aesar, Ward Hill, MA, USA), which corresponded to a projected surface area to volume ratio of 0.015 m² L⁻¹ reactor. The electrodes were fixed in parallel at a distance of 1 cm. The reactors with electrodes contained a Ag/AgCl reference electrode (MF-2052, BASi, West Lafayette, IN, USA) and were connected to a power source (3030D, Protek, USA) via a stainless steel wire and a 1 Ω resistor. The set-up is described in more detail in the SI (S1).

2.1.2. Reactor operation

All seven reactors were inoculated with anaerobic sludge from a municipal sludge digester (Ossemeersen WWTP, Ghent, Belgium). The sludge was diluted with tap water to obtain an initial sludge concentration of 10 g L⁻¹ volatile suspended solids (VSS). All reactors were operated at 34°C in fed batch mode and fed 3 times a week (Monday, Wednesday and Friday) for a total period of 154 days. Fresh feed was prepared for every feeding. A sludge retention time (SRT) of 20 days was maintained. During the start-up phase (Phase 1) waste activated sludge (collected from the Ossemeersen WWTP, Ghent, Belgium) was used as feeding source (Table 1), whereas during Phase 2 and 3 diluted molasses was used as feed (Table 1 and Table SI1). Waste activated sludge was selected to feed the reactor during Phase 1, since this was the same feed that was used in the full-scale reactor from which the anaerobic inoculum sludge sample originated. Phase 1 was considered as an adaptation period for the anaerobic sludge to adapt to laboratory conditions. Molasses was selected as a *proxy* of biorefinery sidestreams, because of its high COD and salt content (Sirianuntapiboon and Prasertsong 2008). Reactor nomenclature was set based on the cell potential during Phase 2 and 3. The first letter (Phase 2) and last letter (Phase 3) of the name show whether a cell potential was applied in the presence of an electrode pair, V(oltage), or whether no electrode pair was present, C(ontrol). The number or letter in the middle of the name indicates the cell potential (1 = 1V cell potential, 0.5 = 0.5V cell potential, O = open circuit potential and N = new electrode pair at open circuit potential) in Phase 2 or 3. During Phase 1 and 2, one reactor (V1C) was operated at a fixed

potential of 1 V, a second reactor (V0.5C) at 0.5 V and a third reactor (VOC) at open circuit potential (OCP). The 4 reactors without electrodes (C1V, C0.5V, COV and CNV) were operated in parallel as control reactors.

Table 1 Operational conditions in the reactors during the different phases of the experiment

Phase	Period	Substrate	OLR (g COD L ⁻¹ d ⁻¹) ¹⁾	Buffer	Electrodes
Phase 1	Day 1-27	Waste activated sludge	1.5 - 2	Yes	V1C, V0.5C and VOC
Phase 2	Day 28-91	Molasses	2	No	V1C, V0.5C and VOC
Phase 3	Day 92-154	Molasses	2	No	C1V, C0.5V, COV and CNV

On day 91 (start of Phase 3), the electrodes were removed from the reactors V1C, V0.5C, VOC and inserted in the reactors C1V, C0.5V, COV, respectively. A piece of 5 cm² was cut from each electrode for molecular and electrochemical analysis. The reactors V1C, V0.5C and VOC were further operated at a liquid volume of 730 mL without electrodes. The content of the four control reactors (C1V, C0.5V, COV and CNV) was mixed and redistributed over the four reactors for a liquid volume of 730 mL per vessel. The liquid volume in the reactors was reduced from 800 to 730 mL to maintain a constant projected surface area to volume ratio of 0.015 m² L⁻¹. The electrode pair previously belonging to V1C was inserted in C1V. The reactor was connected to the power source and operated at 1V for the remainder of the experiment. The electrode pair of V0.5C was inserted in C0.5V and operated at 0.5 V, while the electrode pair of VOC was inserted in COV and operated at OCP. A new electrode pair (projected surface area of 55 cm²) was inserted in CNV at the start of Phase 3 and also operated at OCP. During the entire experiment methane production was measured three times a week and reported at STP (standard temperature and pressure) conditions. Samples were taken three times a week for analysis of pH, VFA and once a week for volatile solids (VS), conductivity and total ammonia nitrogen (TAN).

2.2. Electrochemical characterization

Cell voltages were applied using a portable power supply (3030D, Protek, NJ, USA). Applied cell voltage (dV) and cathode potential (E_{cat}) versus a Ag/AgCl reference electrode were measured continuously at 5 min intervals (34972A, Agilent, MetricTest, CA, USA). The anode potential (E_{an}) was

estimated as $E_{\text{cat}} - dV$. The resulting current was logged as the potential difference over a 1Ω resistor at 5 min intervals. The potentials of the reference electrodes were regularly monitored relative to a calomel electrode (+244 mV vs. Standard Hydrogen Electrode (SHE); QIS, the Netherlands) for correct conversion of the electrode potentials compared to the SHE. Electrochemical calculations were performed according to Logan et al. (2006) and were based on hourly averages. Current and power density are reported normalized to the projected electrode area (60 cm^2 in Phase 1 and 2 and 55 cm^2 in Phase 3). Electrode potentials are reported versus the SHE.

Cyclic voltammetry (CV) of the electrodes was carried out at the end of Phase 2, i.e. after 91 days, and at the end of Phase 3, i.e. after 154 days, of each electrode, as described in the SI (S3).

2.3. Microbial community analysis

Microbial community analysis was applied to the inoculum sludge sample and the planktonic (liquid phase) and electrode biofilm (if present) samples of each reactor (V1C, V0.5C, VOC, C1V, C0.5V, COV and CNV) after 91 and 154 days. The extraction of total DNA from the sludge sample was performed by means of the FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA), following the manufacturer's instructions. The DNA concentration in the extracts was measured with a Nanodrop ND-1000 Spectrophotometer (Isogen Life Science, IJsselstein, the Netherlands). The quality of the extracted DNA was evaluated on a 1% agarose gel. PCR amplification, 16S rRNA gene amplicon sequencing and analysis were carried out as described in the SI (S4). Real-time PCR (qPCR) was performed on total bacteria, total archaea and the methanogenic populations *Methanobacteriales*, *Methanomicrobiales*, *Methanosarcinaceae* and *Methanosaetaceae* as described in SI (S5).

2.4. Analytical techniques

Sludge samples and headspace gas samples were taken three times a week and were analyzed immediately or stored at $-20\text{ }^{\circ}\text{C}$ for further analysis. Biogas composition and VFA were determined as described in the SI (S6). Chemical oxygen demand (COD) was determined using Nanocolor COD 1500 Tube test kits, according to the manufacturer's instructions (Machery-Nagel, Düren, Germany). Total solids (TS), VS, total suspended solids (TSS), VSS, total Kjeldahl nitrogen (TKN) and TAN were determined according to Standard Methods (Greenberg et al. 1992). Anions (PO_4^{3-} , NO_3^- , NO_2^- , SO_4^{2-}) were analyzed using a metrosep A Supp 5-150 column after a metrosep A 4/5 guard column in a 761 Compact IC with a conductivity detector (Metrohm, Switzerland). Potassium was determined using a flame photometer (Eppendorf ELEX6361, Hamburg, Germany). The pH was measured with a C532 pH

meter (Consort, Turnhout, Belgium) and conductivity (EC) was determined by means of a C833 conductivity meter (Consort, Turnhout, Belgium). SEM analysis was carried out as described in S10.

3. Results

3.1. Reactor performance

Methane production values were similar in all seven reactors during the start-up phase (Phase 1). Molasses was used as feed from day 28 on, yet no differences in methane production could be detected up to day 38. The seven reactors produced on average $577 \pm 11 \text{ mL CH}_4 \text{ L}^{-1} \text{ d}^{-1}$ between day 28 and 38 (Figure 1) with an average pH value of 7.67 ± 0.09 on day 38.

Figure 1 Methane production of (a) V1C (◆), V0.5C (■) and VOC (▲) and (b) C1V-CNV (◆), C1V after introduction of the electrodes (■), C0.5V after introduction of the electrodes (▲), COV after introduction of the electrodes (■) and CNV after introduction of the electrodes (■).

The control reactors without electrodes (C1V, C0.5V, COV and CNV) showed decreasing performance from day 40, while the three reactors containing electrodes (V1C, V0.5C and VOC) maintained a similar level of CH₄ production (Figure 1.a). The methane production in the control reactors declined with a simultaneous increase in total VFA concentration and decrease in pH. On day 91, the average methane production in the control reactors decreased to $265 \pm 97 \text{ mL CH}_4 \text{ L}^{-1} \text{ d}^{-1}$ (Figure 1.b), which corresponds to a decrease of 50% compared to the initial methane production during day 28 to 38. Total VFA concentrations on day 91 increased to $11.5 \pm 3.2 \text{ g COD L}^{-1}$ in the four control reactors

(Figure SI2.b), with propionate and acetate as the most important components with 72 ± 14 and $18 \pm 12\%$, respectively. In the three reactors containing electrodes the total VFA concentration remained below $0.32 \text{ g COD L}^{-1}$ (Figure SI2.a). The pH of V1C, V0.5C and VOC increased to 7.85 ± 0.05 on day 91, while the control reactors showed a gradual decrease to 7.42 ± 0.18 .

After 91 days the electrodes were removed from V1C, V0.5C and VOC and inserted in C1V, C0.5V and COV. This led to an immediate 3- to 4-fold decrease in methane production in V1C, V0.5C and VOC and a subsequent rise in total VFA concentrations to values between 2.0 and 2.5 g COD L^{-1} . After the decrease in methane production that followed electrode removal on day 91, reactor V1C (previously at 1 V cell potential) showed an increased methane production rate from day 97 on. Methane production in V0.5C (previously at 0.5 V cell potential) initially decreased but regained from day 105 on. Reactors V1C and V0.5C had a similar methane production profile from day 112 on, i.e. 344 ± 39 and $343 \pm 41 \text{ mL CH}_4 \text{ L}^{-1} \text{ d}^{-1}$, respectively (Figure 1.a). Methane production only partially recovered compared to the average stable methane production of $546 \pm 15 \text{ mL CH}_4 \text{ L}^{-1} \text{ d}^{-1}$ on day 91, with residual VFA levels up to $10.3 \text{ g COD L}^{-1}$ on day 154 in V1C and V0.5C (Figure SI2.a). Propionate was the main component of the total VFA, reaching 86% in V1C and 89% in V0.5C on day 154.

The methane production in VOC decreased further during the entire Phase 3, while the VFA concentration increased to a maximum of $27.0 \text{ g COD L}^{-1}$, which consisted mostly of acetate (44%) and propionate (33%) (Figure 1.a and SI2.a). This build-up of fatty acids was also reflected in the pH values in Phase 3. The pH in V1C and V0.5C was lower compared to Phase 2 (7.47 ± 0.07 and 7.42 ± 0.07 , respectively), yet remained stable from day 105 on, while in VOC the pH decreased from 7.52 on day 91 to 6.16 on day 154.

The reactors C1V, C0.5V and COV showed an increase in methane production and an immediate decrease in VFA levels on day 93, i.e. directly after the introduction of the electrodes. The methane yield was higher than the theoretical maximum of $350 \text{ mL CH}_4 \text{ g}^{-1} \text{ COD}$ in the week following the electrode switch as a result of the removal of residual VFA in the reactors (Figure 1.b and SI2.b). The methane production remained stable for reactor C1V and C0.5V, with values of 501 ± 28 and $571 \pm 20 \text{ mL CH}_4 \text{ L}^{-1} \text{ d}^{-1}$, respectively, from day 119 to 154, until the end of Phase 3, reaching similar stable methane production levels as V1C, V0.5C and VOC in Phase 2. Reactor COV (OCP) showed more variation in methane production in Phase 3 although VFA concentrations remained below detection limit (Figure 1.b). During Phase 3, reactor CNV, in which a new electrode pair was introduced, was also operated under the same conditions as COV. From day 91 to 100, this reactor produced the least methane and showed the highest VFA concentrations ($> 14 \text{ g COD L}^{-1}$, 64% propionate and 25% acetate), compared to C1V, C0.5V and COV. After day 100 the methane production increased and the

VFA content decreased to 6.3 g COD L⁻¹ (88% propionate) on day 154 (Figure SI2.b). From day 105 the performance of the CNV reactor was similar to V1C and V0.5C (Figure 1.b). The methane production rate of CNV did however not reach the same stable levels as C1V and C0.5V.

3.2. Electrochemical performance

The reactors containing an electrode pair were operated at fixed cell potentials (Table 1). The fixed cell potentials resulted in an average current density of $6.8 \pm 2.6 \text{ A m}^{-2}$ and $3.4 \pm 3.4 \text{ A m}^{-2}$ for V1C and V0.5C, respectively, before removing the electrodes (Phase 2). The replacement of the electrodes from V1C and V0.5C to C1V and C0.5V respectively (on day 91), resulted in an average current density of $3.8 \pm 1.8 \text{ A m}^{-2}$ and $6.4 \pm 4.8 \text{ A m}^{-2}$ in C1V and C0.5V, respectively (Table 2). As a result of the applied cell potential of 1 V, potentially a more oxidizing environment at the anode electrode surface was created in V1C and C1V (Table 2) than commonly occurring in anaerobic digestion ($E_h \sim -0.25$ to -0.35 V vs. SHE) (Thauer et al. 1977). The application of a cell potential of 1 and 0.5 V led to more reducing conditions at the cathode than commonly occurring during anaerobic digestion. Cyclic voltammetry results are described in SI (S8)

Table 2 Measured electrical parameters during the experimental phases (Phase 2 and 3). Cell potential (dV), anode (E_{an}) and cathode (E_{cat}) are expressed in volts (V). Electrode potentials are expressed relative to the SHE. Current density (J , $A\ m^{-2}$) is expressed per unit of projected electrode area ($60\ cm^2$ in Phase 2 and $55\ cm^2$ in Phase 3). Input power (P , $mW\ L^{-1}$) is expressed per unit of liquid volume (800 mL in Phase 2 and 730 mL in Phase 3). Theoretical methane production M ($mL\ CH_4\ L^{-1}\ d^{-1}$) is based on measured current and expressed per unit of liquid volume (800 mL in Phase 2 and 730 mL in Phase 3). Solidus: no data since the reactor is operated in open circuit.

	Phase 2			Phase 3			
	V1C	V0.5C	VOC	C1V	C0.5V	COV	CNV
dV (V)	0.97 ± 0.12	0.51 ± 0.03	0.00 ± 0.00	0.98 ± 0.14	0.51 ± 0.04	0.01 ± 0.01	0.00 ± 0.01
E_{an} (V)	0.14 ± 0.24	-0.24 ± 0.10	-0.30 ± 0.03	-0.09 ± 0.08	-0.26 ± 0.03	-0.29 ± 0.02	-0.06 ± 0.04
E_{cat} (V)	-0.84 ± 0.22	-0.75 ± 0.10	-0.30 ± 0.02	-1.06 ± 0.06	-0.76 ± 0.04	-0.30 ± 0.01	-0.06 ± 0.03
J ($A\ m^{-2}$)	6.78 ± 2.58	3.36 ± 3.44	/	3.80 ± 1.75	6.44 ± 4.77	/	/
P input ($mW\ L^{-1}$)	50.2 ± 21.5	12.8 ± 11.1	/	13.4 ± 0.0	24.1 ± 16.4	/	/
M ($mL\ CH_4\ L^{-1}\ d^{-1}$)	127.5 ± 48.6	65.5 ± 64.8	/	71.8 ± 33.1	111.1 ± 82.2	/	/

3.3. Microbial community analysis

3.3.1. Qualitative microbial community composition based on 16S rRNA gene sequences

The microbial community was characterized at two time points to compare community diversity (Figure 2 and SI4). Averaged over both time points and all reactors, the number of observed OTUs (operational taxonomic units) was lower in the planktonic samples compared to the anodes ($P = 0.003$) and cathodes ($P = 0.004$). The relative abundance of methanogens was higher on the anode and cathode (30 ± 10) compared to the planktonic samples (5 ± 4). The methanogenic community was dominated by *Methanosaeta* (relative abundance of up to ~29%).

Figure 2 Heatmap representing all OTUs present at a relative abundance $\geq 5\%$ in at least one of the samples. The color scale ranges from 0 to 40% relative abundance. Planktonic (P), anodic (A) and cathodic (C) samples are presented at the end of Phase 2 and 3, i.e. after 91 and 154 days, respectively. Taxonomy is shown at the phylum level (left column) and at the lowest determined level, i.e. order or genus (right column).

The dominant bacterial populations belonged to the orders *Actinomycetales*, *Lactobacillales*, *Clostridiales* and *Sphaerochaetales*. Visualization of the variability in community composition between samples using principle component analysis (Figure SI5) and Tukey HSD tests indicated that there was a significant difference in composition between day 91 and day 154 (PC1 scores: $P < 0.001$, PC2 scores: $P = 0.033$). At both time points, the microbial planktonic communities of the reactors with and without electrodes were similar ($P > 0.100$; Figure SI4). There were no significant

differences between the communities associated with the anode and cathode (Day 91: $P = 0.670$, day 154: $P = 1.000$). The community profile of the planktonic samples in both the reactors with and without electrodes differed significantly from the anodic and cathodic samples (Day 91: $P < 0.040$, day 154: $P < 0.003$). The applied potential did not have a significant effect on the composition of the microbial communities. Environmental parameter fitting showed a correlation between higher methane yield and the surface-attached communities in the reactors with electrodes, and more specifically with a higher abundance of *Methanosaeta*, at day 91 ($P = 0.001$). Higher VFA and TAN concentrations were correlated to populations in the planktonic samples of reactors without electrodes, such as *Trichococcus* and *Peptoniphilus*, at days 91 and 154 (VFA: $P = 0.001$, TAN: $P > 0.018$).

3.3.2. Quantitative analysis of the methanogenic community

The qPCR results revealed a diverse methanogenic community in the planktonic phase, as well as on the anodes and cathodes (Figure 3). The inoculum sludge sample consisted of a diverse methanogenic community, yet dominated by *Methanosaetaceae* at a concentration of $4.0 \times 10^9 \pm 2.9 \times 10^8$ copies g^{-1} sludge. Hence, acetoclastic methanogenesis in the inoculum sample was most likely already dominated by the *Methanosaetaceae*. The *Methanosaetaceae* remained the dominant acetoclastic methanogenic population in all samples in Phase 2 and 3, with the exception of the planktonic sample in V0.5C after 154 days. In contrast to the planktonic acetoclastic methanogens, that were dominated by *Methanosaetaceae*, the planktonic hydrogenotrophic methanogens were represented in both the *Methanobacteriales* or *Methanomicrobiales* groups throughout all samples. After 91 days of operation, there was a clear increase in *Methanobacteriales* copy numbers in V1C to a value of $2.5 \times 10^9 \pm 3.9 \times 10^8$ copies g^{-1} sludge in the planktonic phase. This was in contrast to VOC, which showed a value of only $6.8 \times 10^7 \pm 5.2 \times 10^6$ copies g^{-1} sludge in the planktonic phase. The anode in V1C showed a 100-fold higher concentration of *Methanosaetaceae*, *Methanomicrobiales* and *Methanosarcinaceae* compared to the cathode on one hand. A 10-fold higher concentration of *Methanosaetaceae*, *Methanobacteriales*, *Methanomicrobiales* and *Methanosarcinaceae*, was observed in the anode in V1C compared to the anode in ϕ VOC on the other hand.

After 154 days, the *Methanosarcinaceae* copy numbers reached values of $1.4 \times 10^8 \pm 1.7 \times 10^7$, $1.8 \times 10^8 \pm 3.3 \times 10^7$ and $8.4 \times 10^5 \pm 8.2 \times 10^4$ copies g^{-1} sludge in the planktonic phase of V1C, V0.5C and VOC, respectively, on day 154, thus revealing higher *Methanosarcinaceae* concentrations in V1C and V0.5C compared to VOC. In contrast, VOC was clearly dominated by the hydrogenotrophic *Methanobacteriales*, with a value of $2.5 \times 10^8 \pm 2.5 \times 10^7$, $7.5 \times 10^7 \pm 6.7 \times 10^6$ and $5.4 \times 10^9 \pm 1.1 \times 10^9$

copies g⁻¹ sludge in V1C, V0.5C and V0C respectively. There was no difference between the anode and cathode in C1V, C0.5V, C0V and CNV after 154 days, as well as between the anodes and cathodes of the different reactors. Overall, all anodes and cathodes were clearly dominated by *Methanosaetaceae* at similar copy number levels of around 5.0×10^{11} copies cm⁻².

Figure 3 Real-time PCR results of the *Methanobacteriales* (■), *Methanomicrobiales* (■), *Methanosarcinaceae* (■) and *Methanosaetaceae* (■) after (a) 91 days and (b) 154 days of operation.

4. Discussion

Anaerobic digestion of molasses in the presence of a BES resulted in stable methane production at both a fixed potential and at OCP. The allocation of pre-inoculated electrodes to failing digesters resulted in immediate process remediation irrespective of the previously applied cell potentials, yet pre-inoculation of the carbon felt electrodes was crucial to regain stable operation. Overall, it appears that retention of biomass is a critical factor towards the remediation, rather than current.

The decrease in methane production and pH in the four control reactors (C1V, C0.5V, COV and CNV) in Phase 2 indicates that the methanogenic process was disturbed, which could be a consequence of the high concentration of potassium, i.e. 6.90 g L^{-1} , in the molasses feed (Table SI1) (Ahring et al. 1995). A concentration of 5.85 g L^{-1} is assumed to cause 50% inhibition of acetoclastic methanogens due to a neutralization of the cell membrane potential from the passive influx of ions (Appels et al. 2008, Chen et al. 2008). Hence, molasses are often diluted prior to their treatment to avoid digester failure (Satyawali and Balakrishnan 2008).

Methane production remained stable in the reactors with electrodes (V1C, V0.5C and VOC). The presence of the carbon felt electrodes served as carrier material for the formation of a biofilm, that could be detected with SEM (Figure SI6). Anaerobic digesters with biomass attached on carrier material were reported to show better performance when digesting difficult substrates, such as cellulose rich organic waste (Held et al. 2002, Sasaki et al. 2010a, Yang et al. 2004). Based on biofilm properties described by Arends and Verstraete (2012) and VS-measurements, the calculated amount of biofilm for the reactors V1C, V0.5C and VOC would account for only 0.22% of the total biomass in the reactor (SI11). However, this biomass was protected from potential inhibiting components in the molasses due to the biofilm organization. A similar concept takes place in sludge granules where microorganisms in the center of the granule undergo less negative influence of toxic waste streams (Bae et al. 2002). The presence of the carbon felt electrodes had a positive influence on the process stability in all three experimental reactors, regardless of the applied potential.

The removal of the electrodes from V1C, V0.5C and VOC and subsequent insertion in C1V, C0.5V and COV, respectively (Phase 3) resulted in immediate removal of VFA, showing the importance of the biofilm on the carbon felt electrodes to remediate the digesters. The performance of CNV decreased further during Phase 3, until 15 days after the introduction of the electrodes methane production increased again. This indicates that over time an active methanogenic biofilm developed on the bare carbon felt, allowing protection of sensible microorganisms, especially methanogens. This is in accordance with the findings of Lalov et al. (2001), who detected biofilm growth on the carrier material between 10 to 20 days after start-up in a digester treating vinasses.

The reactor COV (operating on OCP after the electrode switch) did not reach stable methane production during Phase 3, in contrast to C1V and C0.5V. This can be due to free ammonia toxicity, since the pH of the reactor increased from 7.87 to 8.12 four days following the electrode switch. The pH in the biofilm was probably higher than in the bulk liquid due to methanogenic activity, leading to further local increase of the pH. An average ammonium concentration of 1200 mg TAN L⁻¹ was detected in COV in Phase 3, hence a pH of 9 in the biofilm would result in a free ammonia concentration of 500 mg L⁻¹, which can be considered toxic to methanogens (Chen et al. 2008). The increase in methane production in reactors C1V and C0.5V was more gradual and the pH remained lower, decreasing the impact of free ammonia toxicity.

In Phase 3, the methane production of VOC decreased to a value below 10 mL CH₄ L⁻¹ d⁻¹, indicating reactor failure. V1C and V0.5C partially recovered, as a stable, yet lower amount of methane was produced and residual VFA levels were present at concentrations up to 12.6 and 14.4 g COD L⁻¹ in V1C and V0.5C, respectively. This state of “inhibited steady state”, as previously described for free ammonia toxicity (in this work most likely caused by the high potassium concentration in the case of V1C and V0.5C) can therefore be applicable in this case (Angelidaki and Ahring 1993). The difference in behavior between the two reactors previously operated at a fixed cell potential and the reactor at OCP suggests a certain impact of the applied potential, however none of the measured physicochemical parameters could account for this. The difference in VFA concentrations between V1C and V0.5C, and VOC, is more likely a consequence of process failure, rather than the immediate cause (Appels et al. 2008).

The measured current densities can be correlated to the occurrence of various processes in the AD-BES configuration. Interestingly, the current density was higher in the system that was operated at 0.5V cell potential (C0.5V) compared to the 1 V system (C1V) in Phase 3. Liquid conductivity was similar in all reactors so a change in current based on this can be ruled out. Visual observation did not reveal any differences between the various reactors at the end of the experiment, hence an explanation for this phenomenon is lacking at present. Indications for catalysis were only found on the anodes of V1C and V0.5C on day 91, while only irreversible redox behavior was observed for the cathodes of V1C and V0.5C on day 91 and both the anodes and cathodes in C1V and C0.5V on day 154 (S8). The produced current could have resulted in a theoretical methane production at the cathode of 128 ± 49 and 65 ± 65 mL CH₄ L⁻¹ d⁻¹ for V1C and V0.5C, respectively in Phase 2 and 72 ± 33 and 111 ± 82 mL CH₄ L⁻¹ d⁻¹ for C1V and C0.5V, respectively in Phase 3 (Table 2). These values could amount to maximum 20 % of the measured total methane production in V1C, V0.5C and C1V and C0.5V. The source of the current at the anode remains to be elucidated as the abundance of known

current generating microorganisms such as *Geobacter sulfurreducens* (Bond and Lovley 2003) on the anode is relatively low in comparison to other microorganisms. No H₂ was detected in the biogas, which coincides with the fact that the cathode potentials were not low enough to generate H₂ gas directly. However, biological H₂ production linked to hydrogenotrophic methanogenesis can be an alternative route for enhanced biogas production as detected in the systems with electrodes. Biologically catalyzed H₂ production on cathodes has been shown to yield current densities, during polarization, of up to 3.8 A m⁻² for mixed culture biocathodes at applied cathode potentials of -0.8 vs. SHE (Rozendal et al. 2008). Direct methanogenesis on electrodes by hydrogenotrophic methanogens has also been suggested, however intermediate H₂ production in the biofilm or at the electrode surface cannot be ruled out in mixed cultures (Villano et al. 2010).

The applied cell potentials and the resulting electrode potentials in this study show remarkable differences with other work concerning (B)ESs in anaerobic digestion. Tartakovsky et al. (2011) observed improved AD performance based on applied potentials of 2.8-3.5 V with dimensionally stable electrodes in a UASB reactor. The improvement was attributed to enhanced hydrolysis due to micro-aerobic conditions at the anode and additional H₂ input for methanogenesis and improved biogas quality at the cathode. However, the mentioned study was lacking an adequate control, as there was no open circuit system present. The potentials in the current work (Table 2) likely do not give rise to micro-aerobic conditions, and would not result in H₂ production at the carbon electrodes. Therefore any enhancement or stabilization of the AD process in this work that could be attributed to the introduced (bio)electrochemical environment must be due to direct stimulation of the (attached) microbiota or due to a purely electrochemical reactions. Zamalloa et al. (2013) observed the precipitation of various metal salts when operating stainless steel electrodes at a 2V applied potential in an anaerobic septic tank. A higher applied voltage was used, likely leading to higher cathode pH and anodic iron dissolution, causing the precipitation of various salts as well as sulfide. The possibility of sulfur cycling in BESs has indeed already been demonstrated (Dutta et al. 2009, Rabaey et al. 2006). However, in the current study, species involved in the sulfur cycle could not be detected by means of 16S rRNA gene amplicon sequencing (Figure 2) and the potential (bio)electrochemical oxidation of sulfur could not explain the stable operation of V1C, V0.5C after removal of the electrodes (Phase 3).

Sasaki et al. (2010b) placed a carbon electrode (75 cm² L⁻¹ projected surface) in a methanogenic reactor operated on a complex feed, i.e. artificial garbage slurry. Enhanced methanogenesis was shown at potentials of -0.6 and -0.8 V vs. Ag/AgCl which is in contrast to the present study, where there was no difference in methane production between the three different cathode potentials. Moreover, Sasaki et al. (2010b) added an artificial electron mediator (0.2 mM AQDS; E₀' = -184 mV),

described by Benz et al. (1998), that might have obscured the effect of the biofilm development on the electrodes by shuttling electrons from low potential cathodes to the bulk solution.

The 16S rRNA gene amplicon sequencing results revealed that community richness, i.e. the number of OTUs, was higher on the anodes and cathodes, compared to the planktonic phase. Yet, no specific conclusions could be drawn concerning differences in the bacterial community in the reactors, except for the apparent dominance of the *Lactobacillales* and *Clostridiales*. The higher richness in the biofilms that were developed on these electrodes could be explained by the fact that (1) activated carbon fiber is a suitable carrier for microbial biofilm development and (2) microorganisms that decreased in abundance in the planktonic phase, because of the changing conditions in the reactor system, maintained stable growth in the biofilm (Fernandez et al. 2008, Gong et al. 2011). The higher richness in the biofilm that had formed on the electrodes is in correlation with the stable methane production in these reactors, as is the case in anaerobic biofilm reactors (Fernandez et al. 2008). The retention of an active methanogenic community in such a biofilm is most often the crucial factor to maintain stable operation. Indeed, amplicon sequencing revealed a high relative abundance of *Methanosaeta* in the biofilm as the main acetoclastic methanogen. The presence of carbon fibers in the electrodes could lead to high methane production efficiencies by protecting the methanogenic community from high levels of residuals VFA and salts, which may act as stressors (Sasaki et al. 2009, 2011b). The correlation between a higher methane yield and the higher abundance of *Methanosaeta* is to be expected, since an OLR of only 2 g COD L⁻¹ d⁻¹ was applied, leading to circumstances that favor *Methanosaeta* over *Methanosarcina* (De Vrieze et al. 2012, Ribas et al. 2009). However, as there was no significant difference in community composition between anode and cathode biofilm, nor was there any difference between the planktonic phase in the different reactors, with or without electrodes, it can be confirmed that there was no effect of the applied cell potential and that biofilm development was the crucial factor to obtain stable methane production.

Quantitative real-time PCR analysis confirmed the overall dominance of *Methanosaeta* seen in the amplicon sequencing. *Methanosaeta* copy numbers were up to a 1000 times higher on the electrodes, compared to the planktonic phase, indicating the important contribution of the biofilm to methane production, in spite of the fact that this accounted for only 0.22% of total biomass.

However, *Methanosaeta* copy numbers were a 100-fold higher on the anode compared to the cathode in reactor V1C after 91 days. Yet, not only *Methanosaeta*, but also the other methanogenic groups were more abundant on the anode compared to the cathode in reactor V1C, indicating that conditions were more favorable for methanogenic growth at the anode, compared to the cathode.

The positive charge of the anode, thus attracting the negatively charged bacteria, may be an explanation for this, in contrast to the negatively charged cathode. In the failing reactors, an

evolution from a *Methanosaeta* to a *Methanosarcina* dominated methanogenic community was expected in the planktonic phase because of deteriorating conditions, yet this shift only took place in V1C and V0.5C after 154 days of operation and not in any of the failing reactors after 91 days (C1V, C0.5V, COV and CNV) nor in VOC after 154 days. These results indicate that a preceding applied cell potential catalyzes a transition from a *Methanosaeta* to a *Methanosarcina* dominated methanogenic community. This transition can be directly associated with the partial recovery of methane production in V1C and V0.5C, compared to VOC showing complete failure, yet residual VFA concentrations remained high. The results confirm that *Methanosarcina* was responsible for the partial recovery of methane production in V1C and V0.5C, however, at the cost of higher residual VFA concentrations, as stated by Conklin et al. (2006) and De Vrieze et al. (2012).

5. Conclusions

Anaerobic digestion of molasses in a reactor in which a carbon felt electrode pair was introduced maintained stable methane production, while the control reactors (no electrode pair) failed. There was no direct effect of the applied cell potential on methane production, although a hysteresis effect could be observed after removal of the electrodes. Introduction of pre-inoculated electrodes in failing reactors resulted in immediate process recovery, indicating the remediating capacity of pre-inoculated electrodes. *Methanosaeta* was the dominant acetoclastic methanogen on the electrodes, irrespective of the applied cell potential. This study demonstrated that the main mechanism behind the stabilizing effect of a BES in anaerobic digestion appears to lie in biomass retention, rather than (bio-)electrochemical stimulation. This is in contrast to several other studies that however lacked a suitable control treatment to distinguish the effect between biomass retention and (bio)electrochemical stimulation.

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Supporting Information

Specific details concerning the reactor set-up, molasses characterization, the cyclic voltammetry analysis (CV), the amplicon sequencing analysis, the real-time PCR analysis and biogas and VFA analysis can be found in SI. VFA concentration profiles, CV results and discussion, RDA and PCA analysis results, SEM analysis results of the electrodes and a calculation of the biomass fraction in the biofilm on the electrodes are also included in SI.

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Supporting Information

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S1. SCHEMATIC OVERVIEW OF THE REACTOR SET-UP

In Figure S11 the experimental set-up of the anaerobic digestion reactors containing electrodes is depicted. The reactor itself was a glass Schott Bottle (Duran Group GmbH, Mainz, Germany), closed with a rubber stopper and containing two carbon felt electrodes (a), each with a surface area of 60 cm² (projected area; BET 2 m² g⁻¹; Carbon felt, 3.18 mm, Alfa Aesar, Ward Hill, MA, USA), connected to a power source (c) and a 1 Ω resistor (d) by means of a stainless steel wire. A Ag/AgCl reference electrode (b) (MF-2052, BASi, West Lafayette, IN, USA) was used. Volumetric biogas production was evaluated by means of water displacement (e). Mixing of the reactors was carried out using a magnetic stirrer (f).

Figure S11 Schematic overview of the reactor set-up.

S2. MOLASSES CHARACTERISTICS

Table SII Characteristics of the molasses feed applied during Phase 2 and 3 (n=3). All analyses were carried out in triplicate, except for the K^+ analysis.

Parameter	Value
pH	5.44 ± 0.10
Conductivity ($mS\ cm^{-1}$)	14.7 ± 0.3
Total COD ($g\ L^{-1}$)	44.7 ± 0.9
Total solids ($g\ L^{-1}$)	47.7 ± 5.6
Volatile solids ($g\ L^{-1}$)	33.6 ± 5.2
Total ammonia nitrogen, TAN ($mg\ L^{-1}$)	122 ± 1
Kjeldahl nitrogen, TKN ($mg\ L^{-1}$)	2746 ± 25
Acetate ($mg\ L^{-1}$)	173 ± 1
PO_4^- ($mg\ L^{-1}$)	797 ± 51
Cl^- ($mg\ L^{-1}$)	805 ± 127
NO_3^- ($mg\ L^{-1}$)	398 ± 93
SO_4^- ($mg\ L^{-1}$)	1034 ± 136
K^+ ($g\ L^{-1}$)	6.90
COD:N ratio	16.3 ± 0.4
COD:P ratio	56.1 ± 3.8
TS:VS ratio	1.4 ± 0.3
COD:VS ratio	1.3 ± 0.2

S3. CYCLIC VOLTAMMETRY – MATERIAL AND METHODS

Cyclic voltammograms of the electrodes were recorded with a potentiostat (VSP, Biologic, France). A random section of felt was cut from the electrode with sterile scissors and used as working electrode. The counter electrode was a Pt-wire (BASi, West Lafayette, IN, USA) and a Ag/AgCl reference electrode was used (+197 mV vs. SHE, BASi, West Lafayette, IN, USA).

The electrolyte was sterile 0.1 M phosphate buffer solution (PBS) ($8.8 \text{ g L}^{-1} \text{ K}_2\text{HPO}_4$, $6.8 \text{ g L}^{-1} \text{ KH}_2\text{PO}_4$, $9.5 \text{ g L}^{-1} \text{ NaCl}$). Immediately after harvesting the electrode, a turnover CV was performed, with the following CV settings: scan rate of 1 mV s^{-1} , (1) 60 s open circuit; (2) scan towards negative vertex potential; (3) scan towards positive vertex potential; (4) repeat (2) and (3). Before CV analysis, the electrode section was washed three times with electrolyte to remove sludge particles not directly associated with the electrode. The electrode was subsequently stored overnight in 0.1 M PBS at 34 °C. The next day non-turnover cyclic voltammetry (scan rates: 50, 25, 5, 1 mV s^{-1}) was performed under the assumption that in case any substrate was left in the biofilm, it would have been consumed. Vertex potentials were chosen well above and below the measured operating potentials during incubation in the reactors. A non-used piece of carbon felt was incubated for 24 h in 0.1 M PBS at 34 °C and used for a blank scan. Current densities are reported normalized to the projected area of the analyzed section of the electrode.

S4. PCR AMPLIFICATION, 16S rRNA GENE AMPLICON SEQUENCING AND ANALYSIS

PCR amplification of the universal 16S rRNA genes was carried out according to the protocol as described by Dennis et al. (2013). The PCR reaction mixture (50 µL) contained 20 ng of template DNA, 5 µL 10x buffer, 1 µL dNTP mix (10 mM each), 4 µL 25 mM MgCl₂, 0.2 µL *Taq* polymerase, 1.5 µL BSA (Invitrogen, US) and 8 µM of each of the primers 926F (5'-AAACTYAAAKGAATTGACGG-3') and 1392R (5'-ACGGGCGGTGTGTRC-3') modified on the 5' end to contain the 454 FLX Titanium Lib L adapters B and A, respectively (Engelbrektson et al. 2010). The reverse primers also contained a 5–6 base sample unique bar-code. The PCR protocol consisted of an initial denaturation step of 95°C for 3 min, followed by 30 cycles of 95 °C for 30 s, 55 °C for 45 s and 72 °C for 90 s, with a final elongation step of 72 °C for 10 min. Amplifications were performed using a Veriti® 96-well thermocycler (Applied Biosystems). 16S rRNA gene amplicons were sequenced using the Roche 454 GS-FLX Titanium Platform at the Australian Centre for Ecogenomics (ACE).

Amplicon sequences were quality filtered, trimmed to 250 base pairs and dereplicated using the QIIME pipeline (Caporaso et al. 2010). Chimeric sequences were removed with UCHIME (Edgar et al. 2011) and homopolymer errors were corrected using Acacia (Bragg et al. 2012). The number of sequences per sample was normalized to 2100 (minimum number of sequenced per sample) to allow comparison of diversity without bias from unequal sampling effort. CD-Hit OTU (operational taxonomic unit) was used to cluster sequences at 97% similarity (Wu et al. 2011) and cluster representatives were selected. GreenGenes taxonomy (DeSantis et al. 2006) was assigned to each cluster representative based on BLASTn comparison (Altschul et al. 1990). A table with the abundance of different operational units (OTUs) and their taxonomic assignments in each sample was generated. The number of OTUs observed at equal number of sequences between samples (richness) and Simpsons Diversity Index (evenness) were calculated.

All statistical analyses were implemented using R Studio (version 2.15.0) and R packages vegan (Oksanen et al. 2012) and RColorBrewer. The effects of position within the reactor (planktonic, anode, cathode) on richness and evenness were investigated using Tukey Honestly Significant Differences tests (TukeyHSD). The effects of position within the reactor on the community composition were determined using Permutational Multivariate Analysis of Variance on Hellinger transformed OTU abundances. Relative abundances of OTUs were used to generate a heatmap.

S5. REAL-TIME PCR ANALYSIS

Real-time PCR (qPCR) was performed on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Carlsbad, CA). Triplicate samples of a 100-fold dilution of the DNA-samples were analyzed for total bacteria, total archaea and the methanogenic populations *Methanobacteriales*, *Methanomicrobiales*, *Methanosarcinaceae* and *Methanosaetaceae*. Total archaea can be considered a valid estimation of total methanogens in AD, because of the highly unfavourable conditions of non-methanogenic archaea in AD (Raskin et al. 1995, Woese et al. 1990). To quantify total bacteria the general bacterial primers P338F (5'-ACTCCTACGGGAGGCAGCAG-3') and P518r (5'-ATTACCGCGGCTGCTGG-3'), as described by Ovreas et al. (1997), were used. The primer sets used for total archaea (ARC) and the methanogenic populations *Methanobacteriales* (MBT), *Methanomicrobiales* (MMB), *Methanosarcinaceae* (Msc) and *Methanosaetaceae* (Mst) were previously described by Yu et al. (2005). The reaction mixture of 20 µL was prepared by means of the GoTaq qPCR Master Mix (Promega, Madison, WIS, USA) and consisted of 10 µL of GoTaq® qPCR Master Mix, 3.5 µL of nuclease-free water and 0.75 µL of each primer (final concentration of 375 nM) and 5 µL of template DNA. The qPCR program was performed in a two-step thermal cycling procedure which consists of a predenaturation step for 10 min at 94 °C, followed by 40 cycles of 15 s at 94 °C and 1 min at 60 °C for total bacteria. The qPCR program for total archaea, *Methanobacteriales*, *Methanomicrobiales*, *Methanosarcinaceae* and *Methanosaetaceae* consisted of a predenaturation step for 10 min at 94 °C, followed by 40 cycles of 10 s at 94 °C and 1 min at 60 °C. The qPCR data were represented as copies per gram of wet sludge or per square centimetre of carbon felt.

S6. BIOGAS AND VOLATILE FATTY ACID ANALYSIS

Biogas composition was analyzed with a Compact GC (Global Analyser Solutions, Breda, The Netherlands), equipped with a Porabond precolumn and a Molsieve SA column. Concentrations of CH₄, CO₂ and H₂ were determined using a thermal conductivity detector with a lower detection limit of 1 ppmv for each gas component. The VFA were extracted with diethyl ether and measured in a GC-2014 gas chromatograph (Shimadzu, 's-Hertogenbosch, the Netherlands), which was equipped with a capillary fatty acid-free EC-1000 Econo-Cap column (dimensions: 25 mm x 0.53 mm, film thickness 1.2 µm; Alltech, Laarne, Belgium), a split injector and a flame ionization detector. The lower detection limit for VFA analysis was 2 mg L⁻¹.

S7. TOTAL VFA CONCENTRATION PROFILES IN THE DIFFERENT REACTORS

Figure SI2 Total VFA concentration of (a) V1C (♦), V0.5C (■) and VOC (▲) and (b) C1V-CNV (♦), C1V after introduction of the electrodes (■), C0.5V after introduction of the electrodes (▲), COV after introduction of the electrodes (■) and CNV after introduction of the electrodes (■).

S8. CYCLIC VOLTAMMETRY – RESULTS AND DISCUSSION

Although no direct conclusions can be drawn from the CV scans presented here, some interesting observations can be made that require further investigation to understand the specific mechanisms of adding electrodes to anaerobic digestion.

The CVs of all cathodes at day 91 as well as the anode of V0.5C and VOC showed an irreversible oxidative peak at 0.05 V vs. SHE, indicating the possible release of an adsorbed component from the electrode (Figure SI3.a and b). The same behaviour was noticed at day 154 for the cathodes of C0.5V, COV, CNV and the anodes of C0.5V and CNV (Figure SI3.c and d). The maximum current density increased from 0.2 to 0.6 mA cm⁻² from day 91 to 154 for the anode that was positioned in V0.5C and C0.5V. In all other instances no increase of peak height was observed. At day 154 two other irreversible oxidative peaks were noticed at 0.36 V vs. SHE for the anode of C0.5V and at 0.30 V vs. SHE for the anode of C0.5V, cathode of COV and anode of COV, respectively. This indicates there was another component adsorbed to the carbon electrodes and the biofilm, which was released under oxidative polarization conditions during the CV. Carbon felt electrodes were able to sorb at least 2 different components that were irreversibly released under oxidative conditions in the CVs. The peak at 0.30 V vs. SHE was not noticed for the electrodes placed in CNV. This indicates that for noticeable sorption of this particular component a longer timeframe is needed than 63 days.

Next to these irreversible peaks, a set of reversible peaks can be observed at day 91 (before the switch) in the scans of the anode of V0.5C and V1C with an approximate midpeak potential of -0.22 V vs. SHE. This can be related to the presence of a *Geobacter* population as detected by the amplicon sequencing on these anodes (Figure 2). The slightly different midpeak potential and changed shape of the CV can be related to the different anode potentials that were maintained (Zhu et al. 2012). Members of the *Geobacter* genus were also detected on the anode of C1V at day 154. The anode of C1V during Phase 3 was the same anode of V1C in Phase 2 days, however the specific peak pattern of day 91 was not observed on day 154 (Figure SI3.e). This can indicate that the *Geobacter* population was still present but not active anymore.

In all scans no active catalysis was observed except for the scan from the cathode of C0.5V on day 154 where the onset of H₂ evolution reaction increased from -0.8 to -0.6 V vs. SHE compared to the blank scan (Figure SI3.g). This cathode was the only cathode at day 154 that showed the presence of the hydrogenotrophic methanogenic genus *Methanobrevibacter* (Figure 2). The cathode of C0.5V also had of all electrodes the highest abundance of *Methanobacteriales*, as determined by qPCR

analysis, with a value of $2.4 \times 10^{10} \pm 9.5 \times 10^8$ copies cm^{-2} (Figure 4). This relates to the observation made by Villano et al. (2010) who also observed a shift in HER (H_2 evolution reaction) potential.

The non-turnover scans did not yield any noticeable (ir)reversible redox components (data not shown).

Figure SI3 Overview of the different CVs: (a) anode CVs at day 91, (b) cathode CVs at day 91, (c) anode CVs at day 154, (d) cathode CVs at day 154, (e) anode CVs day 154 scaled to compare with day 91, (f) cathode CVs day 154 scaled to compare with day 91 and (g) blank electrodes at day 91 and 154. For all CVs, the first and second cycle are shown.

S9. REDUNDANCY ANALYSIS (RDA) AND PRINCIPLE COMPONENT ANALYSIS (PCA).

Figure SI4 Redundancy analysis showing the microbial community composition at the OTU level (Hellinger transformed) constrained by type of reactor (with/without BES), potential (no potential, 0.5V, 1.0V and OCP) and type of sample (planktonic ($\circ$), anode ($\diamond$) and cathode ($\square$)). The arrows indicate correlations to performance data. This analysis was performed using the following samples: (a) after Phase 2 on the planktonic, anodic and cathodic samples of V1C ($\bullet, \blacklozenge, \blacksquare$), V0.5C ($\bullet, \blacklozenge, \blacksquare$), VOC ($\bullet, \blacklozenge, \blacksquare$) and the control reactors C1V, C0.5V, COV and CNV ($\bullet, \blacklozenge, \blacksquare$) and (b) Phase 3 on the planktonic, anodic and cathodic samples of C1V ($\bullet, \blacklozenge, \blacksquare$), C0.5V ($\bullet, \blacklozenge, \blacksquare$), COV and CNV ($\bullet, \blacklozenge, \blacksquare$) and the control reactors V1C, V0.5C and VOC ($\bullet, \blacklozenge, \blacksquare$). The black crosses represent the individual OTUs and the taxonomy of those contributing most to the variability between samples is given.

Figure SI15 Principle component analysis (PCA) showing the microbial community composition at the OTU level (Hellinger transformed). PCA was carried out on the inoculum sample, after Phase 2 (●) and Phase 3 (●) on the planktonic (●), anodic (◆) and cathodic (■) samples of all reactors.

S10. SEM ANALYSIS OF THE ELECTRODES

In order to obtain a clear image of the biofilm that was developed on the electrodes, SEM analysis was carried out on the anodes and cathodes in V1C, V0.5C and VOC after Phase 2. A piece of 1 cm² was cut from each electrode at the end of Phase 2. Before SEM analysis, samples were coated with a thin gold layer with a SCD005 Sputter Coater (Bal-Tec AG, Principality of Liechtenstein). The samples were subsequently studied by means of a FEI XL30 scanning electron microscope (FEI, The Netherlands) equipped with a LaB₆ filament. The SEM images revealed that there was a clear biofilm development on the different electrodes, yet with a limited thickness of 20 µm (Figure S15).

Figure SI6 SEM images of (a) the anode of V1C, (b) the cathode of V1C, (c) the anode of V0.5C, (d) the cathode of V0.5C, (e) the anode of VOC and (f) the cathode of VOC at the end of Phase 2.

S11. CALCULATION OF THE BIOMASS FRACTION IN THE BIOFILM ON THE ELECTRODES

The total surface area of the electrodes was $0.0327 \text{ m}^2 \text{ L}^{-1}$ of reactor. Assuming a uniform biofilm thickness of $20 \text{ }\mu\text{m}$, the total volume of the biofilm would be $6.54 \times 10^{-7} \text{ m}^3 \text{ L}^{-1}$. A biofilm typically has a VS-concentration of 20 kg VS m^{-3} biofilm (Arends and Verstraete 2012), resulting in 0.0131 g L^{-1} extra biomass in the reactor, while the reactors V1C, V0.5C and VOC had an average VS concentration of 6.0 g VS L^{-1} on day 91 of the test. The biofilm thus accounted for a maximum of 0.22% extra biomass in the reactor.

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