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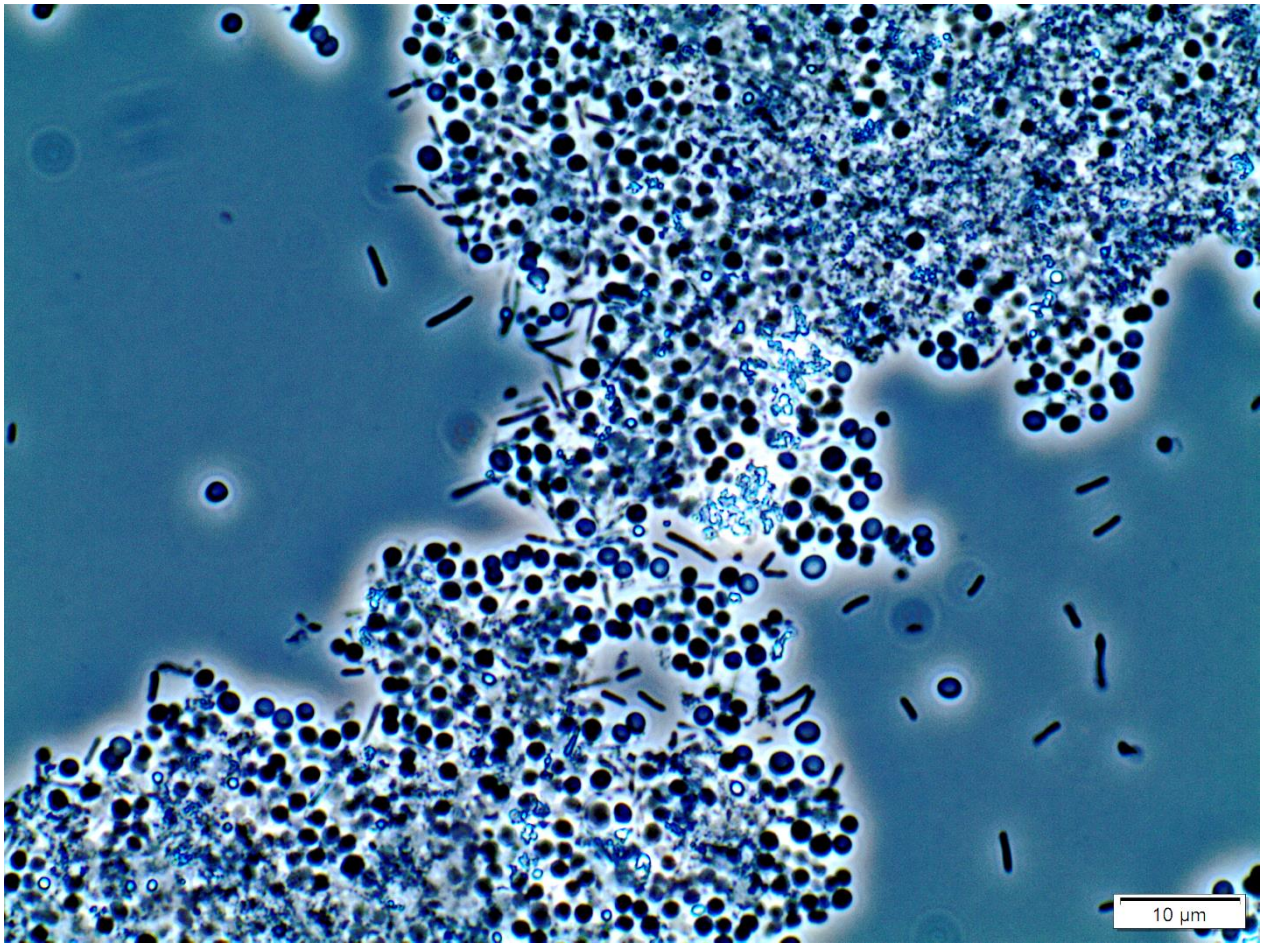

Electrochemical activities of syntrophic acetate oxidizing bacteria and redox mediators:

Usability of methyl viologen and neutral red as electron transmitters

Master's Thesis in the Master's Programmes Infrastructure and Environmental Engineering and Water Resources Engineering and Management

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Master's Thesis No. BOMX02-16-53
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Chalmers tekniska högskola 2016

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Cover:
Microscopic picture of the mixed culture of methanogens and three different strains of SAOB.
Chalmers Reproservice.
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ABSTRACT

The anaerobic digestion process, which uses microorganisms to produce biogas, is common in the treatment of sludge at wastewater treatment plants. Syntrophic acetate oxidizing bacteria (SAOB) play an important role in anaerobic digestion under certain environmental conditions, such as high ammonium concentration. The SAOB oxidize acetate and produce hydrogen, which is consumed by co-existing hydrogenotrophic methanogens. Studying SAOB in isolation is difficult since they are dependent on the methanogenic partner to keep the hydrogen concentration low, which is necessary for anaerobic acetate oxidation to be thermodynamically possible. Electrochemical systems could potentially help in growing SAOB isolated. If the SAOB could direct electrons to the anode instead of hydrogen, growth on acetate could theoretically be possible without a methanogenic partner.

In this study, growth of SAOB in electrochemical reactors in the presence of different redox mediators was investigated. The aim was to find a mediator able to serve as electron shuttle from the bacteria to the electrode of an electrochemical system. Neutral red showed through cyclic voltammetry to be the most promising. Hence SAOB were grown at a constant potential with the possible mediator neutral red. Throughout the experiment the activity of the SAOB was observed through liquid and gas samples.

The activity of the bacteria was high during the experiment and cyclic voltammetry indicated some usage of neutral red. However, only very few electrons were transferred to the electrode. Hence the bacteria cannot use neutral red as mediator to grow at a constant potential. Additionally, the mediator methyl viologen was shown to inhibit growth. Still, there a several more mediators to investigate on the possible use as mediators for SAOB. The formal potentials of humic acid, cysteine, AQDS, resazurin and methylene blue were determined in this thesis.

Contents

List of abbreviations

AQDS	Anthraquinone-2,6-disulfonate
BES	Bioelectrochemical system
C	Counter electrode
CV	Cyclic voltammetry
E_f	Formal potential
E_{Ox}	Oxidation potential
E_R	Reduction potential
E_{rest}	Rest potential of an electrode
E_{set}	Set potential on an electrode
E_0	Standard potential
HPLC	High-performance liquid chromatography
I	Current
MEC	Microbial electrolysis cell
MFC	Microbial fuel cell
R	Reference electrode
SAOB	Syntrophic acetate oxidizing bacteria
SHE	Standard hydrogen electrode
t	Time
V	Voltage
W	Working electrode
2-BES	2-bromoethanesulfonate

1 Introduction

1.1 Background

The ability of bacterial metabolism to create molecules and also to mineralize molecules is widely used in biological wastewater treatment. Biological treatment processes such as activated sludge, trickling filters, and moving bed biofilm reactors are used to oxidize organic pollutants mainly into carbon dioxide, nitrogen and water. Anaerobic treatment on the other hand is commonly used to produce biogas from sludge. Coupling of electrochemical systems to the bacterial metabolism can open new opportunities to treat wastewater. For example energy and chemicals could be produced or energy stored in the form of chemical energy. In microbial bioelectrochemical systems (BESs) the bacteria oxidize and reduce chemical compounds in connection with an electrode (Modin & Gustavsson 2014). BESs generating electric power by the decomposition of organic matter are called microbial fuel cells (MFCs), while BESs producing chemicals through electrolysis are called microbial electrolysis cells (MECs) (Modin & Gustavsson 2014).

For example, one MEC configuration can be used to produce hydrogen from organic matter (Ditzig et al. 2007; Rozendal et al. 2008; Rozendal et al. 2006). The potential for an energy efficient hydrogen production process from wastewater using MECs is very high, with further research and development (Pant et al. 2012; Logan et al. 2008). In addition to the engineering applications, BESs, as an experimental system, offer new opportunities for studying microbial metabolism. Several microorganisms are known to catalyze oxidation and reduction reactions on electrodes. For example *Geobacter sulfurreducens* is well known for its ability to oxidize acetate and transfer electrons to an anode (Bond & Lovley 2003). A group of microorganisms, which has not yet been investigated in BES, is the SAOB. This group of bacteria oxidize acetate under anaerobic conditions and live in close connection to hydrogen utilizing methanogens in their natural habitat (Zinder & Koch 1984). The purpose of this study was to investigate if SAOB could be used in BES and if electrochemical reactors with redox mediators could be used as experimental systems to study SAOB.

1.2 Syntrophic acetate oxidizing bacteria (SAOB)

In biogas processes methane can be produced by acetoclastic or hydrogen-consuming methanogenesis. Hydrogen can be produced by acetate oxidizing bacteria, growing syntrophically with hydrogenotrophic methanogens (Zinder & Koch 1984). These syntrophic acetate oxidizing bacteria (SAOB) occur in biogas reactors as well as in many different anaerobic natural environments (Westerholm et al. 2011). Although oxidation of acetate is energetically unfavorable compared to the acetoclastic reaction, it may be favorable under certain environmental conditions (Hattori 2008). One of these conditions can be high ammonia concentrations (Schnürer et al. 1999). As the concentration of hydrogen is also negatively influencing the SAOB activity, the constant consumption of the hydrogen by the methanogens is a key factor for growth (Hattori 2008). In total, the possible energy gain from the acetate oxidation and methanogenesis is – 31 kJ/mol (Hattori 2008). This equals the possible energy gain from the acetoclastic reaction, but has to be shared by two organisms (Hattori 2008). The chemical reactions of acetoclastic methanogenesis as well as syntrophic methanogenesis are shown in Table 1.

Table 1: Chemical reactions of the acetoclastic and the syntrophic methanogenesis with possible standard Gibbs free energy change (ΔG^0) (Hattori 2008).

Process	Reaction			ΔG^0 (kJ/mol)
Acetoclastic methanogenesis	$CH_3COO^- + H_2O$	→	$CH_4 + HCO_3^-$	-31.0
Syntrophic acetate oxidation	$CH_3COO^- + 4H_2O$	→	$HCO_3^- + 4H_2 + HCO_3^- + H^+$	+104.6
H_2-consuming methanogenesis	$4H_2 + HCO_3^- + H^+$	→	$CH_4 + 3H_2O$	-135.6
Total syntrophic reaction	$CH_3COO^- + H_2O$	→	$HCO_3^- + CH_4$	-31.0

1.3 Redox mediators

For electrochemical systems to work, the microorganisms need to transfer electrons to the electrode. While some bacteria may use direct electron transfer or electron transfer through microbial nanowires, there are also bacteria using a mediated electron transfer (Harnisch & Freguia 2012). For this electron transfer mediators are used as electron shuttles. Mediators are molecules which can take up electrons in their oxidized form (get reduced) and donate the electrons in their reduced form (get oxidized). While bacteria may use natural mediators, there is also the possibility to apply artificial mediators. Electrochemically active molecules like neutral red can be used to carry electrons to the electrode surface (see Figure 1). In bacterial systems many investigations have shown the use of these so called redox mediators for bacteria (e.g. Santos et al. 2003; Aulenta et al. 2010). Using the mediators as transport vehicle for electrons may be used to grow SAOB without methanogens. Electrons normally transferred to hydrogen by SAOB could be transferred to redox mediators instead and following to the electrode. This could avoid the inhibition of SAOB activity by a too large hydrogen concentration.

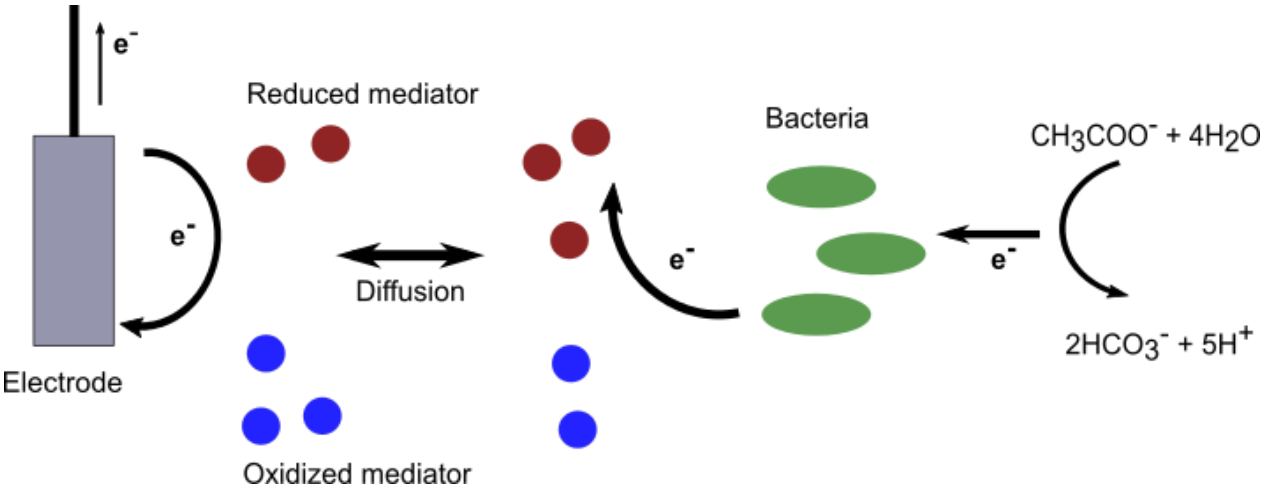

Figure 1: Electron transfer process with mediators from bacterial oxidation of acetate to the electrode.

The different redox mediators used in this study are shown in Table 2. For some mediators a standard potential from the literature is given. This standard potential is in the middle of an oxidation and a reduction potential. If a redox reaction takes place in two steps, two standard potentials are given. For some mediators no standard potential was found in the literature.

*Table 2: Overview of the different used mediators and their standard potential against SHE from the literature. For * no literature value was found.*

Mediator	**Standard potential E^0 (mV)**	**Reference**
Methyl viologen	-446, -760	(Aulenta et al. 2007)
AQDS	-184	(Aulenta et al. 2010)
Cystein	*	
Methylene Blue	*	
Neutral red	-325	(Harrington et al. 2015)
Resazurin	*	
Humic acid	*	

1.4 Cyclic voltammetry

With cyclic voltammetry the redox behavior of molecules and biological material can be investigated (Kissinger & Heineman 1983). The setup up consists of a working electrode, a counter electrode and a reference electrode in an electrolyte solution (Harnisch & Freguia 2012). On the working electrode a voltage is applied and scanned linearly from an initial potential E1 to the switching potential E2 (see Figure 2). At the switching potential the scan is reversed until the initial potential (Mabbott 1983).

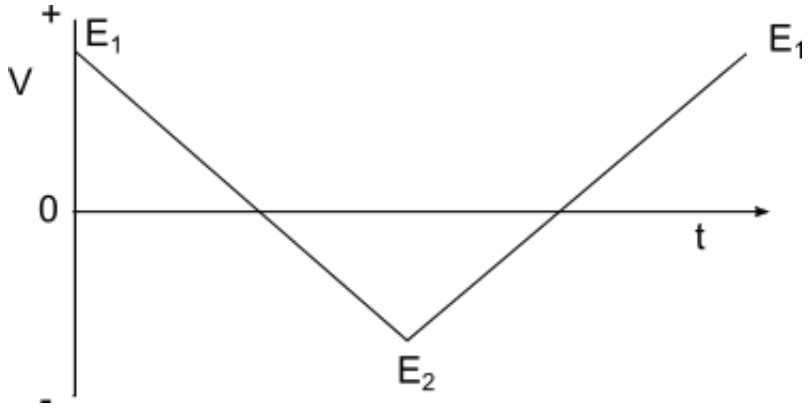

Figure 2: Schematic run of the voltage during cyclic voltammetry.

The responding current (I) to voltage (V) curve for an electrochemical active compound can have a shape as schematically shown in Figure 3. For the oxidation a current peak at a characteristic potential E_{Ox} occurs and for the reduction a negative current peak at a characteristic potential E_R occurs (Harnisch & Freguia 2012).

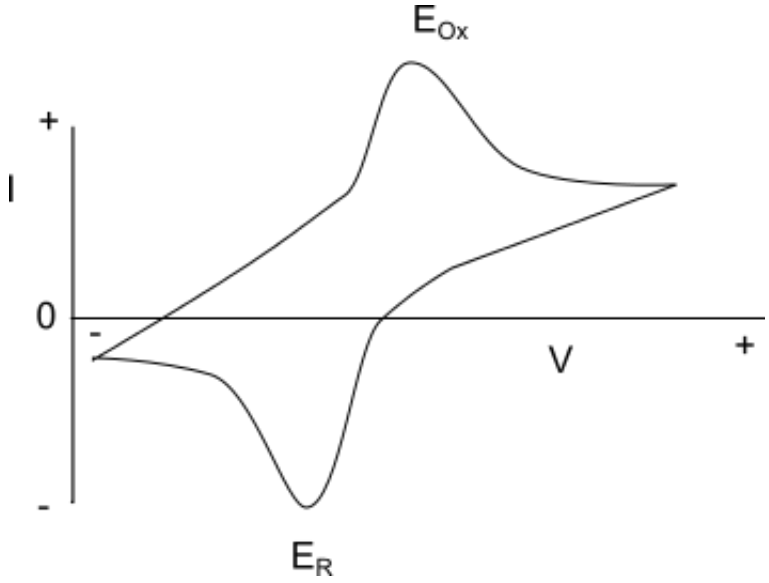

Figure 3: Schematic current/voltage curve for cyclic voltammetry of an electrochemical active compound.

With the arithmetic mean of the oxidation and reduction potential the formal potential E_f can be determined. It replaces the standard potential E_0 of the investigated compound when side reactions are unknown (Harnisch & Freguia 2012).

Scan rates (voltage steps per time) can be varied. This can be used to determine the dominating transport process for the electrochemical active molecule in the media. If the mass transfer to the electrode is dominated by a biofilm or by diffusion can be determined by the relationship between scan rate and peak currents (Harnisch & Freguia 2012). If the peak current is proportional to the square root of the scan rate, the mass transfer is diffusion limited. The biofilm dominates if the peak current is proportional directly to the scan rate.

1.5 Chronoamperometry

A chronoamperometry experiment has also a three electrode setup as in cyclic voltammetry. The potential of the working electrode is set from the rest potential E_{rest} to a set potential E_{set} (Bard & Faulkner 2001). The set potential is kept constant over a determined time until it falls back on the rest potential (see Figure 4).

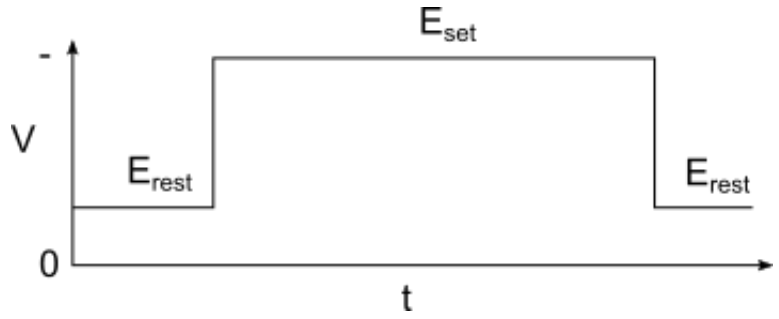

Figure 4: Schematic run of the voltage over time during chronoamperometry.

The responding current from the applied potential may then be used to calculate the electrons which passed through the electrode over the time (Bard & Faulkner 2001).

1.6 Aims of this study

The aim of studying SAOB in combination with redox-mediators is to find a way to grow isolated SAOB on acetate. This way the SAOB can be investigated without the influence of methanogens in further research. Hence after testing different redox-mediators a selection will be made and the chosen mediator tested more detailed with the SAOB. With different experimental steps it will be shown to what extent the bacteria can make use of the mediator and the electrochemical cell.

2 Preliminary tests

Prior to the start of this master thesis project, preliminary experiments with the investigated bacteria were carried out. For all experiments a mixed culture of three SAOB strains and one hydrogen utilizing methanogen was used.

2.1 Experiment 1

In the first experiment, SAOB cultures were cultivated in two 300 ml bottles. A three electrode setup with two 2 x 5 x 0.318 cm carbon felt electrodes and an Ag/AgCl reference electrode was used (see Figure 5). In reactor 3, a potential of 1 V was set on the electrodes, while a potential of 0.5 V was set on the electrodes of reactor 4. The bottles were kept at a constant temperature in a 31 – 32 °C water bath.

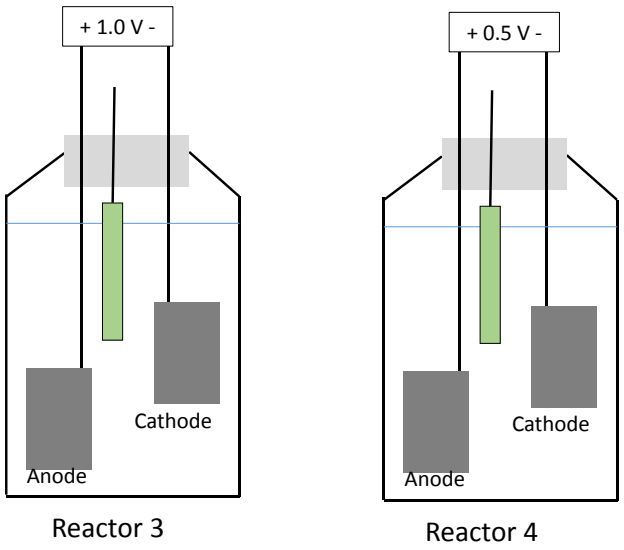

Figure 5: Electrode configuration in the reactor bottles for the first experiment.

The gas phase pressure and acetate concentration were measured over the experimental time (see Figure 6 and Figure 7). Also the current and the anode potential of the reactors were recorded (see Figure 8 and Figure 9). While the gas pressure stays almost constant in reactor 3, reactor 4 seems to produce gas at the end of the experiment. The bacteria are strictly anaerobic (Schnurer et al. 1996). Hence the much higher anode potential in reactor 3 could have a negative effect (see Figure 9), as the bacteria would like to grow at a low redox potential. Promising could be the rise of the current in reactor 3 at the end. However the results are difficult to interpret. The medium contains sulfide, which can be abiotically oxidized on the electrode surface and therefore falsify the current result.

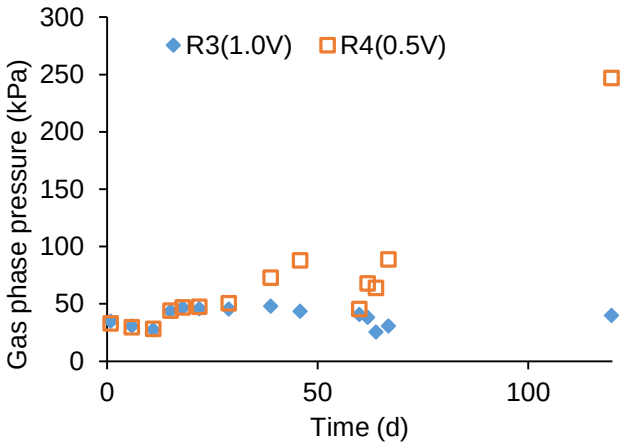

Figure 6: Gas phase pressure in the reactors 3 and 4 over the experimental time.

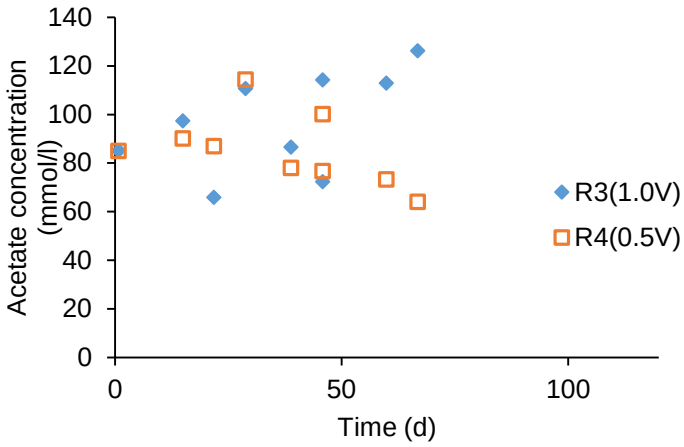

Figure 7: Acetate concentration in the reactors 3 and 4 over the experimental time.

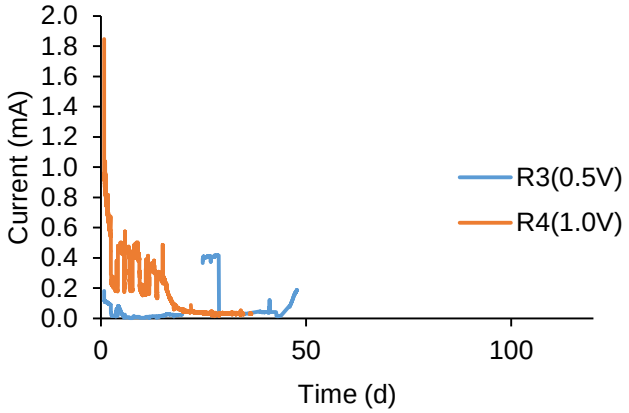

Figure 8: Current in the reactors 3 and 4 over the experimental time.

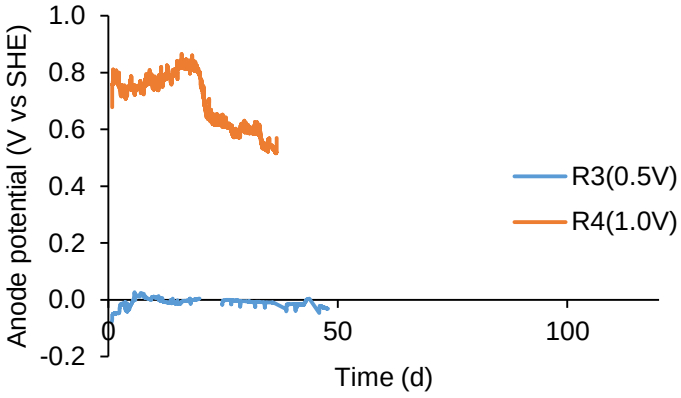

Figure 9: Anode potential in the reactors 3 and 4 over the experimental time.

2.2 Experiment 2

In the next experiment the mixed bacteria culture was grown in a medium without sulfide and with a resazurin concentration of 80 µmol/l. Again the cultures were grown in 300 ml bottles containing 200 ml medium. Two bottles (reactor 5 and 6) were used. Reactor 5 was inoculated with 20 ml of medium from reactor 4 (from experiment 1). Reactor 6 was inoculated with 20 ml of the original culture received from the Swedish University of Agricultural Sciences.

In Figure 10 one can see the production of methane in both reactors. This basically shows a growth of the cultures without sulfide.

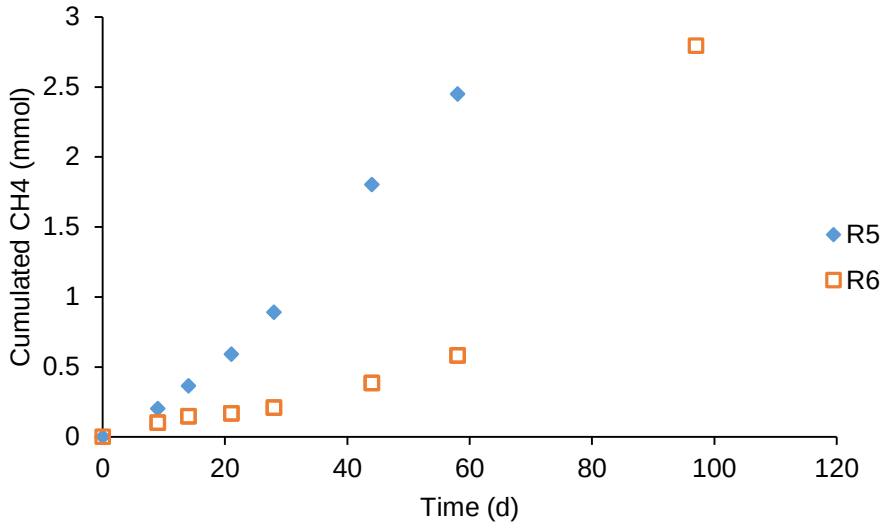

Figure 10: Methane concentrations in reactor 5 and 6 over the time of experiment 2.

2.3 Experiment 3

In experiment 3 a different reactor setup was chosen. A divided electrochemical cell was used with two chambers of 270 ml, containing each an 8 x 3 x 0.318 cm carbon felt electrode. The medium according to experiment 2 was taken and both chambers inoculated with 10 ml of the reactor 4 medium. In the anode chamber an acetate concentration of 100 mmol/l was adjusted, while in the cathode chamber no acetate was added.

The reactors were tested with cyclic voltammetry immediately after inoculation and 76 days after (see Figure 11). Just after inoculation the positive and negative current peaks are very similar. This indicates, that resazurin is oxidized and reduced in the cycle. After 76 days no peak with negative current can be observed, suggesting that resazurin is biologically reduced by bacteria instead of being reduced on the electrode surface.

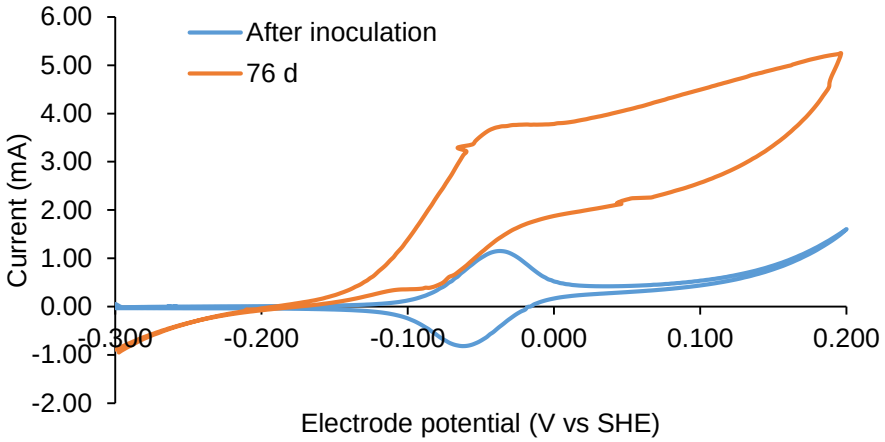

Figure 11: Cyclic voltammogram for the reactor in experiment 2, directly after inoculation and 76 days later.

In Figure 12 the resulting anode potential and current of a scan with varying cell voltage of the reactor can be seen. As the anode potential remained relatively low, but current is generated, a cell voltage of 0.9 V was applied to further grow the bacteria. This may avoid the hampering in the biological activity seen in experiment 1 with an anode potential > 0.5 V vs SHE.

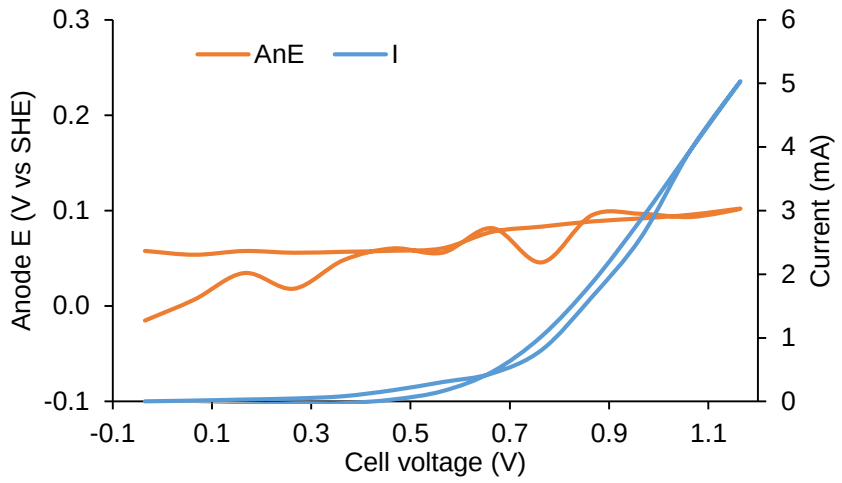

Figure 12: Anode potential and current over varying cell voltage in experiment 3.

88 days after inoculation the control of the cell voltage started. With the cell voltage of 0.9 V a steady state current around 0.05 mA was generated. This would correspond to an acetate consumption rate of 0.0056 mmol/d (concentration change of 0.021 mM/d). The actual acetate consumption rate was 1 mM/d, suggesting very little was used for current generation.

After 193 days of operation, 2-bromoethanesulfonate (2-BES) was added to inhibit the methanogens (see Figure 13). The current dropped to 0.03 mA (see Figure 14), which corresponds to an acetate consumption rate of 0.0034 mmol/d (concentration change of 0.012 mM/d). In the acetate measurements first a decrease and then an increase occurred (see Figure 15). Hence no clear conclusion can be made regarding the acetate consumption. Also the problem of leakage at the reactor occurred.

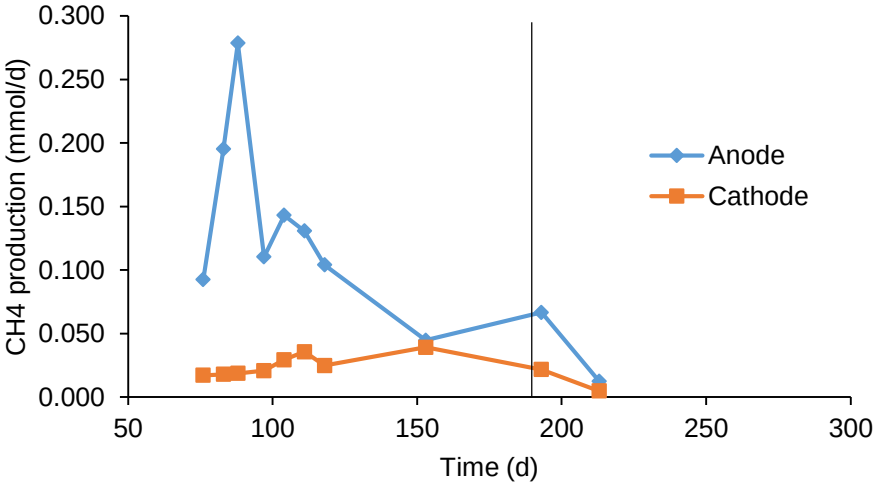

Figure 13: Methane concentration in the reactor over the time of experiment 3. The black line indicates the addition of 10 mM 2-bromoethanesulfinate.

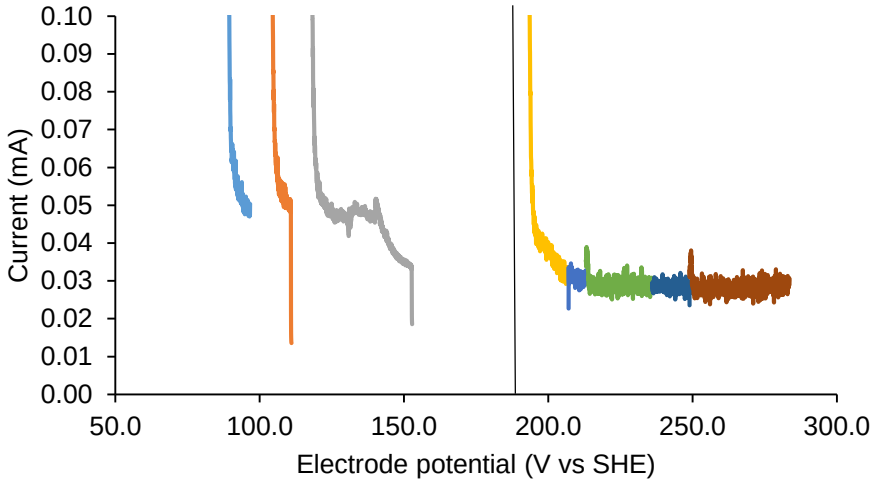

Figure 14: Current in the reactor over the time of experiment 3. The black line indicates the addition of 10 mM 2-bromoethanesulfinate.

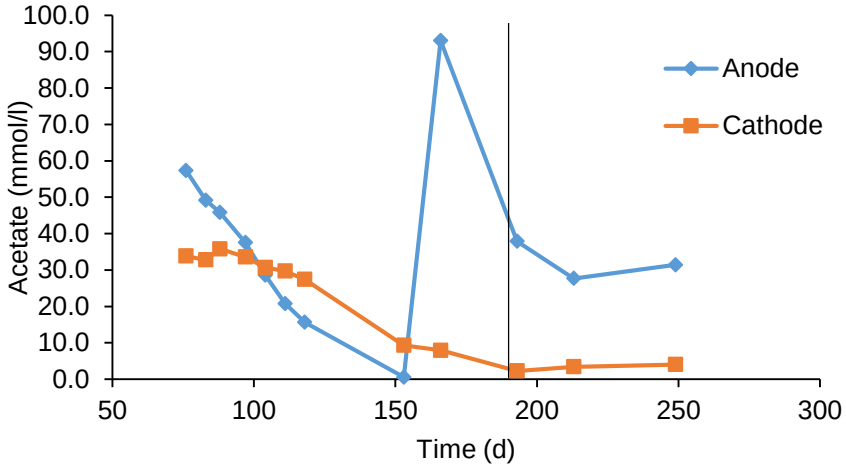

Figure 15: Acetate concentration in the reactor over the time of experiment 3. The black line indicates the addition of 10 mM 2-bromoethanesulfonate.

The results showed a low current generation. This could mean that a small fraction of the oxidized acetate is diverted to current generation, i.e. about 0.3% of the oxidized acetate during normal growth of the culture. After addition of 2-BES to inhibit the methanogen, the current generation dropped even further. This suggests that the bacteria not actually grow by generating current. Instead current generation may be the result of some co-metabolic reduction of the mediator resazurin in the solution.

2.4 Experiment 4

In the fourth experiment, the same medium as in experiment 2 was used in reactors of 300 ml bottles with all electrodes in one bottle. The anode was an 8 x 3 x 0.2 cm carbon felt electrode and the cathode was a 2 cm^2 graphite block, both connected to titanium wires. Acetate was present in a concentration of 100 mmol/l in the medium. To control the temperature the bottle was placed in a 34 °C water bath. In Table 3 the operation procedure is summarized.

Table 3: Operation of the reactor in experiment 4.

Time period (days after inoculation)	**Operation**
0 – 24	No electrochemical control
24	Inhibiting methanogens with 10 mM 2-bromoethanesulfonate
24 – 34	Current controlled at 0.05 mA
34 – 52	Current controlled at 0.4 mA
52 – 57	Current stopped

The initial production of methane stops after the inhibition of the methanogens at day 24 (see Figure 16). Hydrogen production seems to be related to the current flow. The hydrogen concentration increases during the current of 0.4 mA. When the current stops, the hydrogen concentration stays at the same level for 5 days until the end of the experiment. Hence the SAOB are only active when a current of 0.4 mA is applied. This may indicate a use of the electrodes with the applied current by the SAOB.

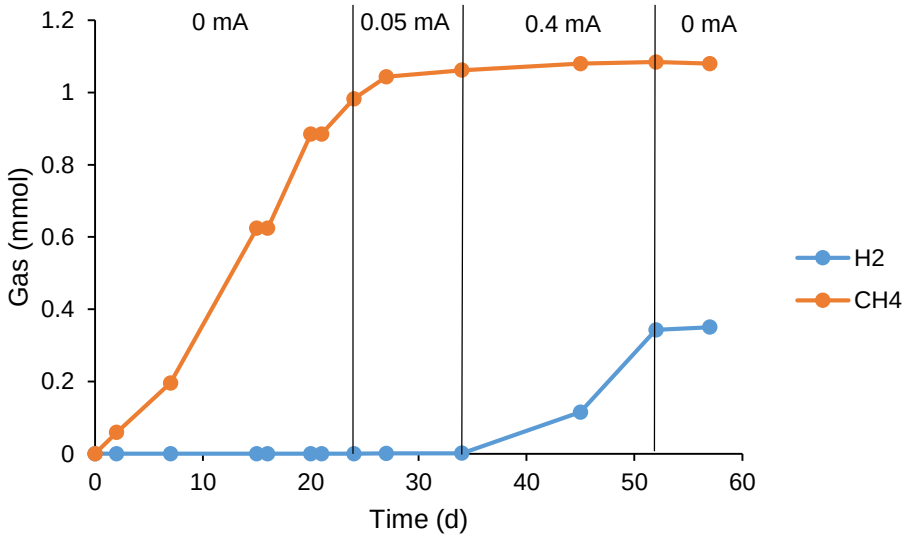

Figure 16: Gas production in the reactor over the time of experiment 4.

Cyclic voltammetry on a scan rate of 1 mV/s on different periods during the experiment gave results shown in Figure 17. It can be seen, that the positive current increases with the time. This suggests an electron transfer from the bacteria to the electrode, possibly via resazurin. However, after the methanogens were inhibited, there is a drop in the responding current. In the final scan (day 52), there is no electrochemical activity anymore.

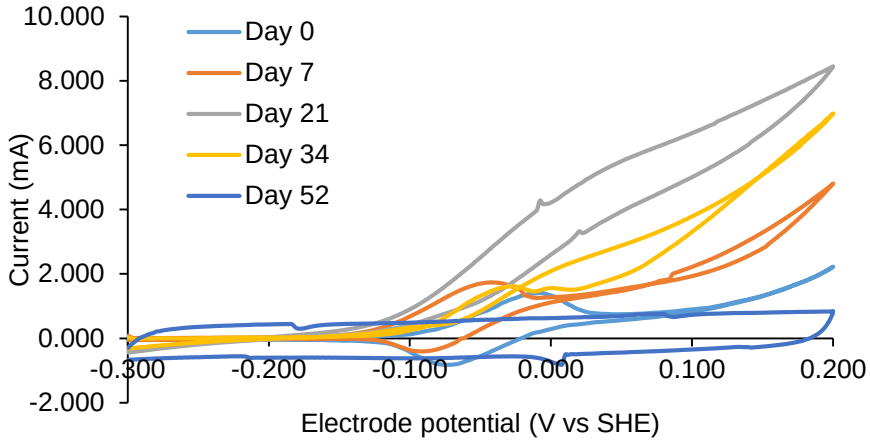

Figure 17: Voltammograms for the cyclic voltammetry tests during experiment 4.

2.5 Experiment 5

In the last preliminary experiment a divided electrochemical cell like in experiment 3 was used. Each compartment contained about 307 ml of medium according to experiment 2. In each chamber was a 9 x 3 x 0.318 cm carbon fiber felt electrode.

After an incubation time of 210 days, the activity was observed in the anode and the cathode compartment. Methane was produced at a rate of 0.75 – 2.2 ml/day. During this time a current of 1 mA was applied between the anode and cathode. This lead to an acetate generation in the cathode compartment (see Figure 18). After the inhibition of the methanogens no acetate production was observed. At this point there were also problems with water migration through the membrane separating the two compartments.

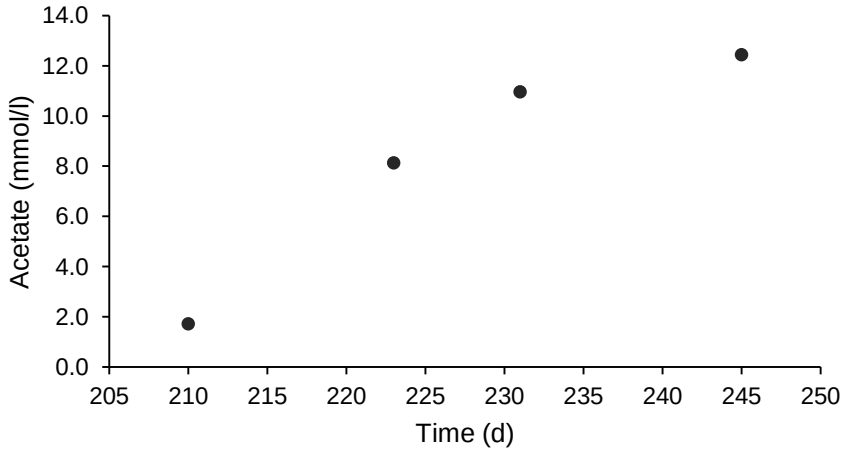

Figure 18: Acetate concentration in the cathode chamber during experiment 5.

2.6 Conclusions for the following experiment

Experiment 1 suggests a possible influence of the sulfide in the media on the current results. This could be avoided by a medium without sulfide. The bacteria culture grows also on a medium without sulfide, as shown in experiment 2. In experiment 3 a possible usage of the redox mediator resazurin is indicated. Though the current generation is probably caused only by a co-metabolic reduction of resazurin.

A low cell potential is already in experiment 1 indicated as better for the bacterial growth. In experiment 3 a cell potential of 0.9 V vs SHE shows a good current production, while the anode potential stays < 0.5 V vs SHE for a good cell growth.

Experiment 4 suggests an electron transfer from the bacteria to the electrode. However, the electrochemical activity drops without methanogens. Hence, the mediator resazurin may not work effectively enough. Also a use of the electrodes is shown in experiment 5. While acetate is produced in the cathode compartment during an applied current, no acetate is produced anymore without methanogens.

Hence, a more effective way to grow the SAOB without methanogens has to be found. The potential below 0.5 V vs SHE seems promising to be used by the bacteria. A use of a redox mediator can be possible, but may work better with a different mediator.

3 Method

3.1 Bacteria source

The bacteria cultures were isolated and provided by Prof. Anna Schnürer at the Department of Microbiology, Uppsala BioCenter, Swedish University of Agricultural Sciences. One culture was a mixed culture of three different SAOB and a methanogen growing on acetate, the other culture was an isolated strain of SAOB growing on glucose. The glucose oxidizing SAOB are closer described as spore forming, rod shaped bacteria *Tepidanaerobacter acetatoxydans* by Westerholm et al. 2011.

3.2 Experimental setup

To realize a closer investigation of the use of redox mediators for SAOB in the limited time frame, the experimental steps are based on each other. The general steps, used methods and gained information are shown in the conceptional model in Figure 19.

First, seven mediators were tested with cyclic voltammetry to gain information about their electrochemical behavior. As the next test with small anaerobic bottles (30 ml) did not work, two promising mediators with clear peaks where chosen. Changing the anaerobic reactors to bigger reactors (340 ml per chamber) these two mediators were tested with cyclic voltammetry on their electrochemical behavior in the final media. One set of reactors with each mediator was inoculated with 20 ml of the mixed bacteria culture and one set was inoculated with 20 ml of the isolated culture. The mixed bacteria culture contained three strains on SAOB and one of methanogens, while the isolated culture was an isolated strain of SAOB, described in section 3.1 above. With cyclic voltammetry the inoculated reactors were tested directly after inoculation, after 2 weeks growing and after 4 weeks growing and at the end of the experimental time (10/11/12 weeks). The whole time gas and liquid samples were taken to examine the activity of the bacteria. One mediator (neutral red) was then chosen to grow the isolated SAOB culture at a constant set potential.

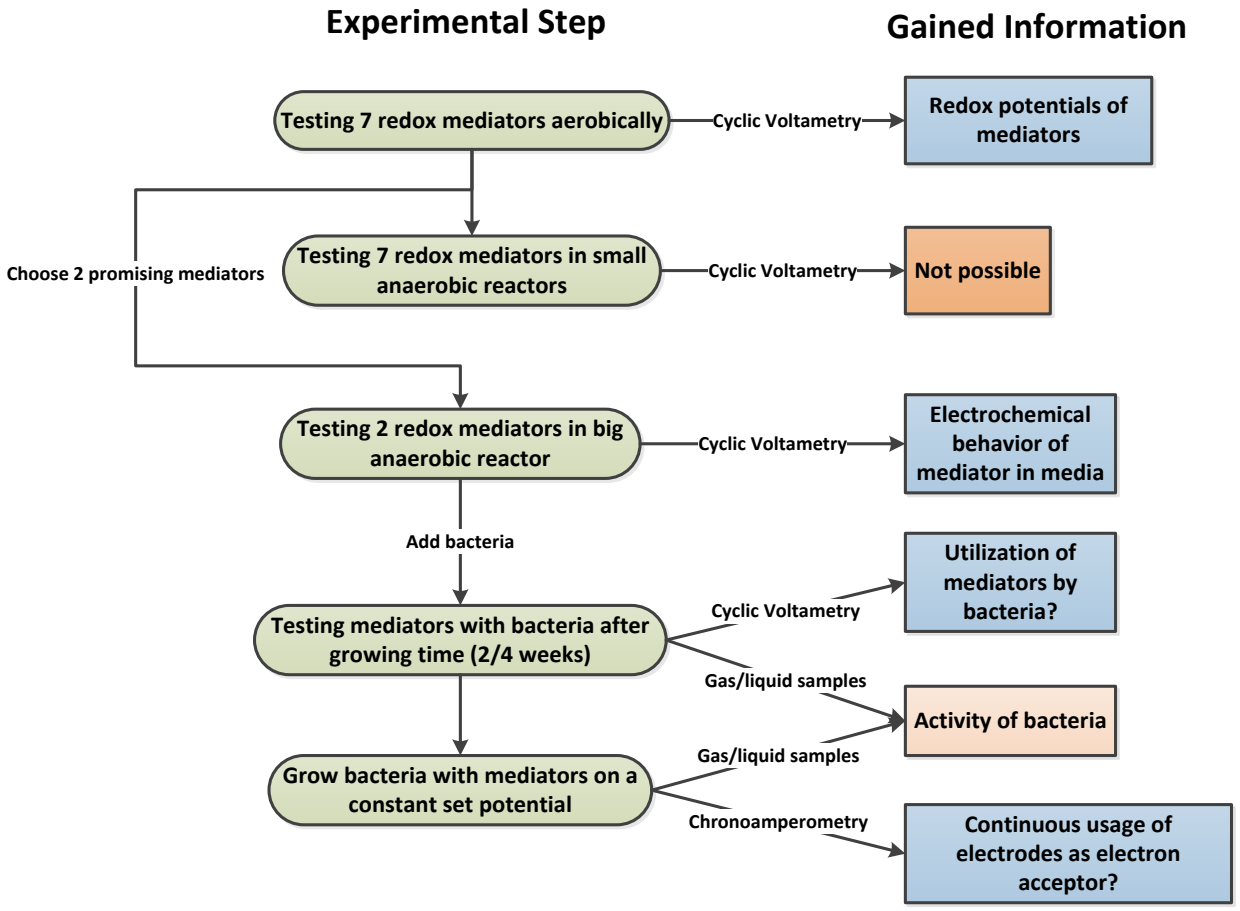

Figure 19: Chronology of the experiments conducted with the information gained from each step.

3.3 Reactor setups

For the first experimental step comparing the 7 mediators, a simple reactor type with two chambers and connection to the atmosphere was used (see Figure 20). The working electrode was made of a titanium wire with a 1 x 2 cm graphite foil (1 mm thick, 97% metals basis, Alfa Aesar) plate. A platinum covered titanium wire was used as counter electrode. As reference electrode served an Ag/AgCl electrode. The two chambers were separated by a 0.18 mm thick membrane (Nafion ® 117, perfluorinated).

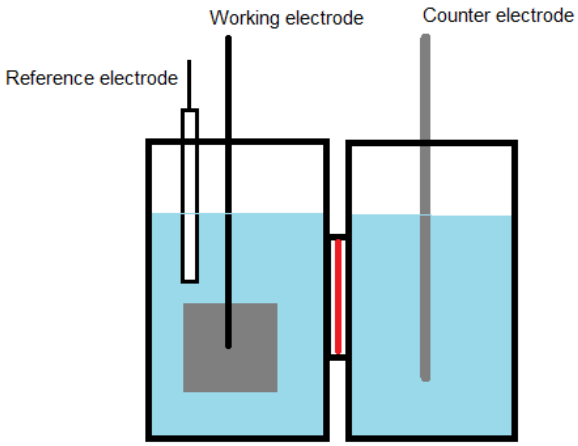

Figure 20: Schematic drawing of the reactor for the cyclic voltammetry testing the mediators. With all electrodes shown and a membrane between the chambers.

Trying to test all seven mediators in anaerobic conditions, 30 ml serum bottles were used as reactors (see Figure 21). For the electrochemical tests two syringes with media were connected over a 1.1 mm thick needle to the reactor media. In one a counter electrode of platinum covered titanium wire was entered and in one an Ag/AgCl reference electrode. The working electrode of titanium wire and 1 x 2 cm graphite foil (1mm thick, 97% metals basis, Alfa Aesar) was permanently installed. The air gas phase was replaced by an Ar/CO_2 gas mixture (82/18, v/v).

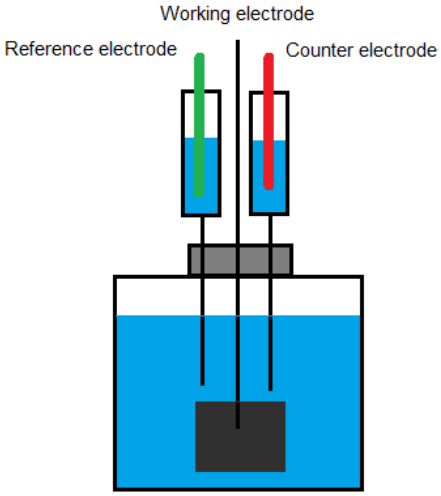

Figure 21: Schematic drawing of the anaerobic reactor for the cyclic voltammetry testing of mediators. With all electrodes shown.

For the anaerobic comparison of two mediators with bacteria a titanium wire with a 3 x 3 cm graphite foil (1 mm thick, 97% metals basis, Alfa Aesar) plate was used as working and as counter electrode. In the chamber with the working electrode an Ag/AgCl reference electrode was located. A schematic drawing of the electrode configuration in the reactor is shown in Figure 22. The two chambers of the reactor were air tightened and separated by a 0.18 mm thick membrane (Nafion ® 117, perfluorinated). In each chamber needles were placed to take gas and liquid samples from the reactor. After every sampling the gas phase was replaced by an Ar/CO_2 gas mixture (82/18, v/v). For a short period the gas phase was replaced by pure nitrogen, due to lack of Ar/CO_2 gas. The temperature was controlled at 40 °C in an incubator.

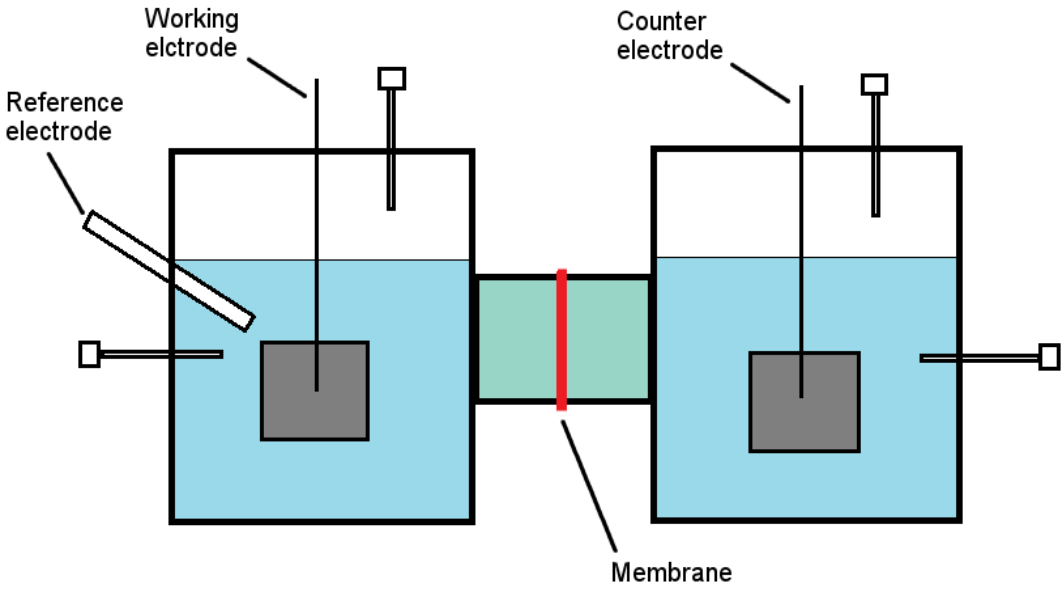

Figure 22: Schematic drawing of the anaerobic reactor for the cyclic voltammetry testing of bacteria and mediators. With all electrodes and the needles for samples from the gas and liquid phase.

3.4 Media

In the first experiment comparing just the mediator without bacteria, just an electrolyte solution of phosphorous buffers (3 mmol/l of KH_2PO_4 and Na_2HPO_4) and deionized water was used.

For the experimental setup with added bacteria a special medium was used. Each reactor chamber was initially filled with 222 ml of medium. The constituents of the medium are shown in Table 4.

Table 4: Starting media before inoculating with bacteria.

Substance	Acetate Media	Glucose Media
KH_2PO_4 Buffer	3 mmol/l	3 mmol/l
Na_2HPO_4 Buffer	3 mmol/l	3 mmol/l
Yeast extract	0.198 g/l	0.198 g/l
Se/Wo solution	1ml/l	1 ml/l
$NaHCO_3$	0.048 mol/l	0.048 mol/l
C1-solution	50 ml/l	50 ml/l
Acetate	0.1 mol/l	-
Glucose	-	0.009 mol/l

A detailed recipe for the Se/Wo solution and the C1 solution can be found in the appendix, section 9.1.

For the added redox mediators a final concentration of 50 mg/l was chosen. Except for methyl viologen in the experimental setup together with bacteria. Here, the final concentration was increased to 250 mg/l to gain a clearer result.

3.5 Cyclic voltammetry

To investigate the electron transfer processes for the experiments a three electrode configuration was chosen. This method is well known and described for example from (Bond et al. 2010). An Ag/AgCl electrode served as reference electrode with a standard potential of +0.2 V vs SHE. Hence to all voltage data 0.2 V is added to have the potential difference related to the standard hydrogen electrode (SHE). The electrode potential was controlled using a potentiostat. To gain a voltammetric cycle, the potential for the experiment was run from -0.8 V to 0.5 V vs SHE and back. As scan rate 1, 5, 10, 20, 50 or 100 mV/s were chosen. The resulting current was recorded with a data acquisition unit.

3.6 Chronoamperometry

With the chronoamperometry a constant potential was set to the reactor electrodes. The potential was adjusted to -0.2 V vs SHE. While applying the voltage for a set time of several days, the current was recorded with a data acquisition unit. The current resulting from the voltage was then related to the electron transfer process of the SAOB.

3.7 Gas chromatography

Hydrogen, methane, oxygen, nitrogen and carbon dioxide concentrations in the gas phase were measured with gas chromatography (Agilent, micro-GC 490). The carrier gases were argon and helium. The columns were a MolSieve 5 Å and a PoraPlot. A thermal conductivity detector was used for detection.

3.8 High-performance liquid chromatography (HPLC)

For analyzing the acetate, glucose, lactic acid and propionate concentrations in liquid samples the HPLC was used with a detection via refractive index. For retention an Aminex HPX-87H (BioRad) column was used. As eluent served 5 mM sulfuric acid at a pumping rate of 0.5 ml/min.

4 Calculations and assumptions

4.1 Cyclic voltammetry

The formal potential E_f of the different mediators was determined by taking the arithmetic mean value between the reduction peak and the oxidation peak from the cyclic voltammogram. If the reaction is taking place in two steps a formal potential was also determined for the second peaks.

4.2 Gas samples

To calculate the molar gas concentration in the reactors, ideal gas conditions were assumed. As room temperature 293.15 K and an atmospheric pressure of 101325 Pa was assumed. As exact volume of the empty reactor chambers 340 ml were assumed. Consequently the ideal gas law was applied.

$$n = \frac{p \cdot V}{R \cdot T}$$

where *n* is the amount of substance, *p* the gas pressure, *V* the gas volume, *T* the temperature in Kelvin and *R* the ideal gas constant ($8.314 \frac{\mathrm{J}}{\mathrm{mol\,K}}$).

From the amount of gas in the reactor, the daily production was calculated by dividing through the days between the gas sampling. After every gas sampling the gas phase was exchanged.

4.3 Dominating transport process

Relating the peak current from the cyclic voltammetry to the scan rate, can give information about the transport mechanisms in the reactor. The transport can be dominated by a biofilm or by diffusion. Is the relation to the square root of the scan rate linear, the transport is diffusion dominated (Harnisch & Freguia 2012). If the relation is linear directly to the scan rate, the electroactive compounds are located on the surface of the electrode, e.g. in a biofilm. Hence the peak currents are plotted against the scan rate and the square root of the scan rate. The linearity can then be determined by the linear correlation coefficient $\rho_{X,Y}$.

$$\rho_{X,Y} = \frac{cov(X,Y)}{\sigma_X \sigma_Y}$$

where *cov* is the covariance function, *X* and *Y* the data series and σ the standard deviation. If $\rho_{X,Y}$ has the value -1 or +1, the relation is completely linear.

4.4 Electron transfer with a constant electrode potential

The amount of electrons transferred in the chronoamperometry experiment are calculated from the resulting current as following.

$$z = \frac{I \cdot t}{F}$$

Here *z* is the amount of electrons, *I* the measured current, *t* the time steps between the measurements and *F* the Faraday constant (96485 C/mol).

The current in the chronoamperometry shows a peak in the beginning and the end of the set potential. It is assumed that this current is only resulting from the change of the potential and is not representative for the long term bacterial activity. Brownson & Banks 2014 mention the charge of the electric double layer as reason for this current peaks. Hence the peaks are cut off like shown in Figure 23. Just the current values between the red lines are taken for the calculation of the bacterial electron transfer over a long time period.

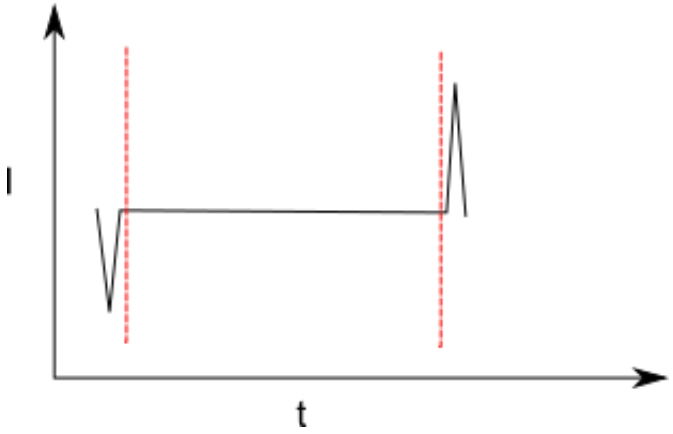

Figure 23: Schematic drawing of a current to time curve in the chronoamperometry with the process of cutting off the peaks.

To monitor the theoretical available amount of electrons in the chambers of the neutral red - glucose reactor, the chemical compounds were converted to electrons available. All organic molecules were calculated as many electrons that could be possibly donated until the total mineralization to CO_2. They were added up together to form an electron balance. This way it can be monitored how many electrons left or entered the system. Table 5 shows the different measured chemical compounds and their counted electrons.

Table 5: Electrons per molecule of the redox active species donated until mineralization to CO_2 and electrons given to hydrogen.

Chemical compound	Formula	Electrons per molecule
Acetate	CH_3COOH	8
Glucose	$C_6H_{12}O_6$	24
Lactic acid	$C_3H_6O_3$	12
Propionate	$C_3H_5O_2$	13
Hydrogen	H_2	2

5 Results

5.1 Cyclic voltammetry – mediators without bacteria

Testing the mediators as well as the pure buffer solution with cyclic voltammetry on a scan rate of 10 mV/s gives the voltammograms Figure 25 to Figure 31. The voltammograms for scan rates of 50 and 100 mV/s can be seen in the appendix.

The redox peaks of humic acid, cysteine and AQDS are hard to exactly locate. In contrast neutral red, methyl viologen, resazurin and methylene blue show clear peaks.

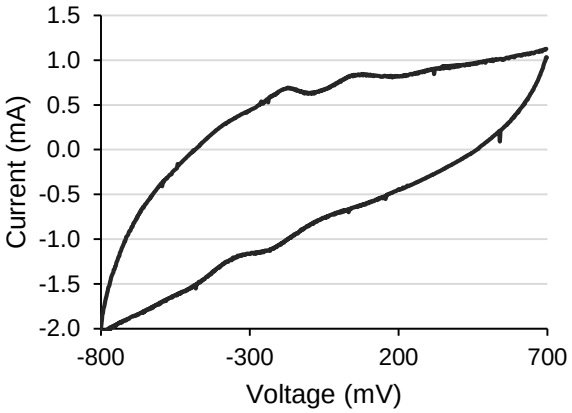

Figure 24: Cyclic voltammogram for the pure buffer solution.

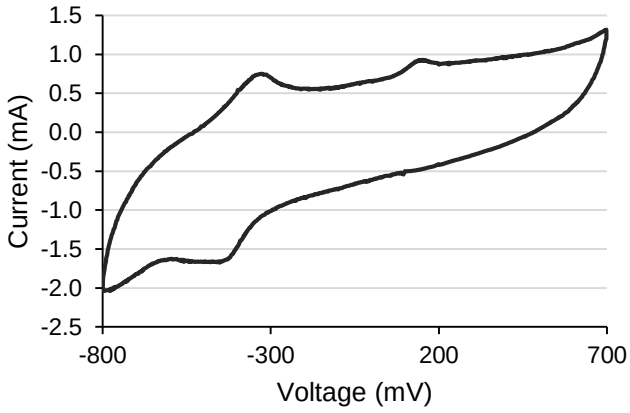

Figure 25: Cyclic voltammogram for neutral red.

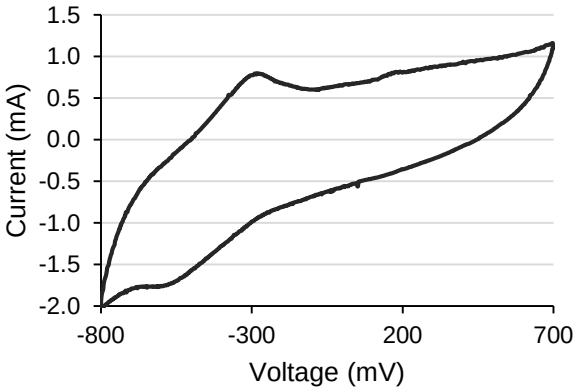

Figure 26: Cyclic voltammogram for methyl viologen.

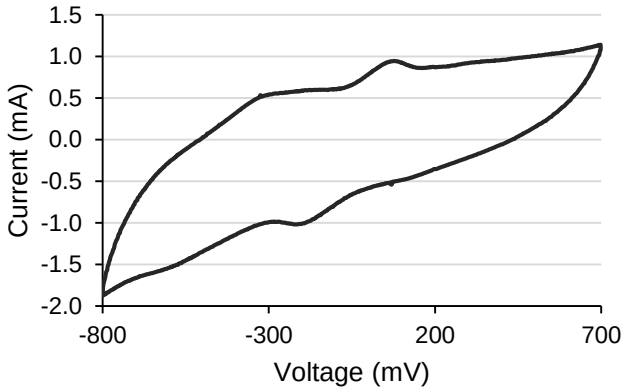

Figure 27: Cyclic voltammogram for resazurin.

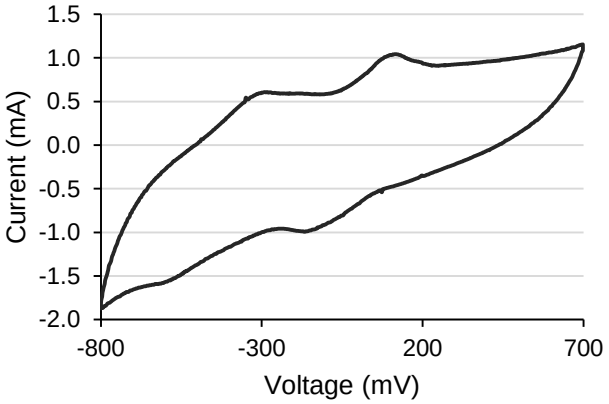

Figure 28: Cyclic voltammogram for methylene blue.

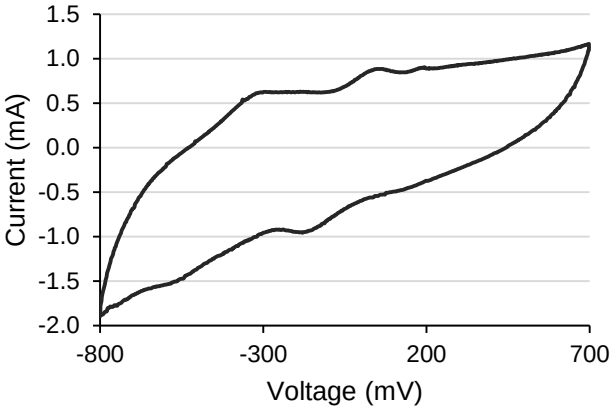

Figure 29: Cyclic voltammogram for humic acid.

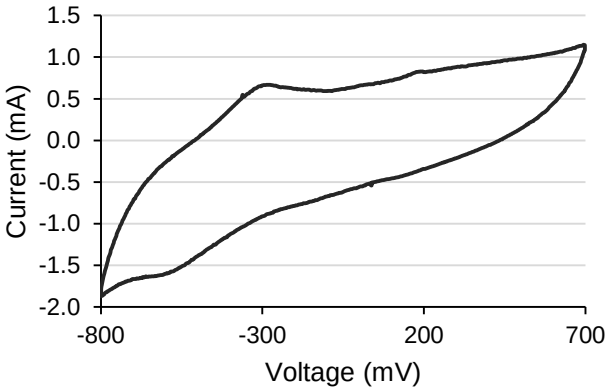

Figure 30: Cyclic voltammogram for cysteine.

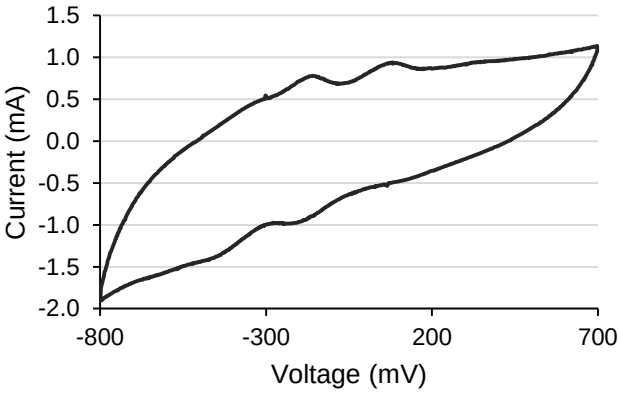

Figure 31: Cyclic voltammogram for AQDS.

The formal potentials E_f for every mediator determined by the peaks in the graph are summarized in Table 6. If a redox reaction takes place in two steps and shows two peaks for reduction/oxidation, two formal potentials are determined.

Table 6: Standard potentials for the different mediators against SHE, determined with cyclic voltammetry.

Mediator	**E_f^1 in mV**	**E_f^2 in mV**
AQDS	-303.5	-82.5
Cystein	-458	
Humic acid	-443.5	-65
Methylene Blue	-449	-26
Methyl Viologen	-437.5	
Neutral Red	-382.5	-143
Resazurin	-452.5	-71

As the redox peaks of neutral red and methyl viologen are clearly determinable, these two mediators were tested further with bacteria in anoxic reactors. The following results concern these reactors, one inoculated with the mixed culture and one with the pure SAOB, for both mediators.

5.2 Gas and liquid samples

The results of all liquid and gas samples are shown ordered to every reactor, sorted to the time scale of the experiment. Every reactor has two chambers, one with the working electrode and one with the counter electrode. From every chamber the result is shown in comparison to the other chamber.

5.2.1 Neutral red – acetate reactor

In the first days the acetate concentration stays at the same level (see Figure 32). After the addition of more inoculum (after 21 days) the consumption seems to increase. Even slightly more in the working electrode chamber than in the counter electrode chamber. The big decrease after 21 days can be explained by the dilution, due to the addition of more bacteria.

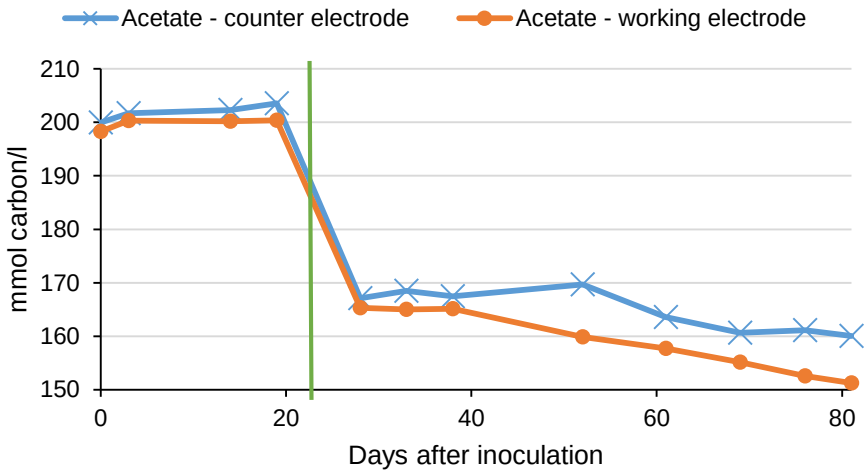

Figure 32: Concentrations found in the liquid samples of the neutral red reactor with acetate over the experiment time. Green line: addition of 20 ml bacteria culture.

Hydrogen is produced constantly over the experiment time in both chambers (see Figure 33). In the counter electrode the production is higher before more bacteria is added to the reactor.

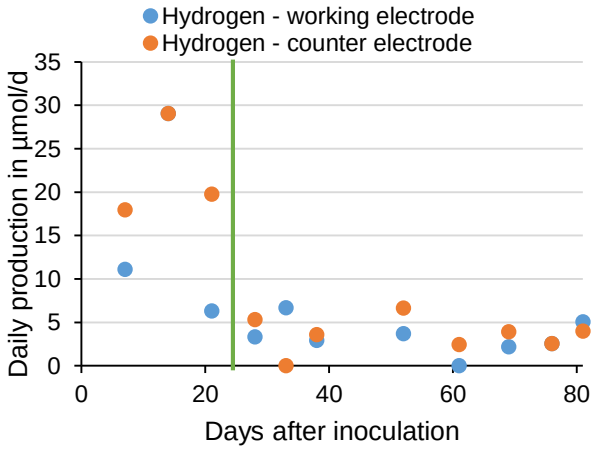

Figure 33: Daily hydrogen production in the neutral red reactor with acetate over the experiment time. Green line: addition of 20 ml bacteria culture

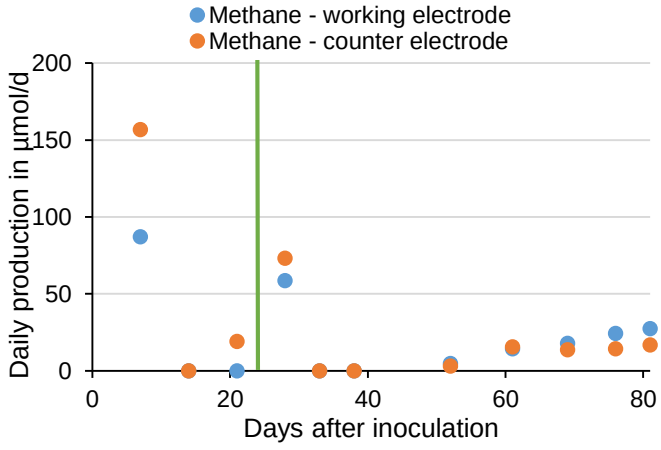

Figure 34: Daily methane production in the neutral red reactor with acetate over the experiment time. Green line: addition of 20 ml bacteria culture.

Also the methane production in Figure 34 is higher at the beginning and drops to almost no methane after the addition of more bacteria. After a longer growing period the methane production increases, slightly more for the working electrode chamber.

5.2.2 Neutral red – glucose reactor

In Figure 35 one can see the concentration of the glucose and its degradation products over the experimental time in the counter electrode chamber. At three points the addition of glucose is visible. While at the beginning the glucose is mostly degraded to lactic acid, later the acetate seems to be the dominating degradation product. Also some propionate is increasingly produced over the time.

In the working electrode the degradation of glucose happens faster (see Figure 36). Especially with the constant set potential after the second glucose addition, the glucose degradation takes only 10 days. Contrary without constant potential after the last glucose addition, the glucose concentration stays almost constant. Lactic acid concentration increases with the degradation of glucose, but also decreases when glucose is completely consumed. Acetate concentration increases over the whole experiment time and slightly more during the lactic acid decrease phases. Also small amounts propionate are produced over the time.

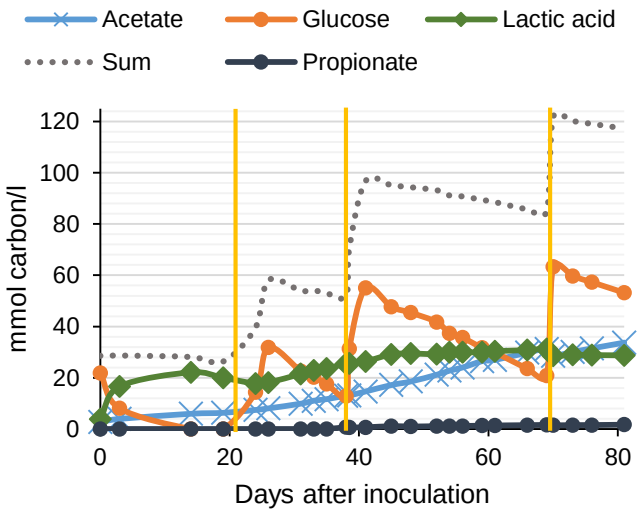

Figure 35: Concentrations in the liquid samples of the counter electrode chamber of the neutral red reactor with glucose. Yellow lines: addition of glucose. Second yellow line: start of constant potential. Third yellow line: stop of constant potential and addition of 20 ml water.

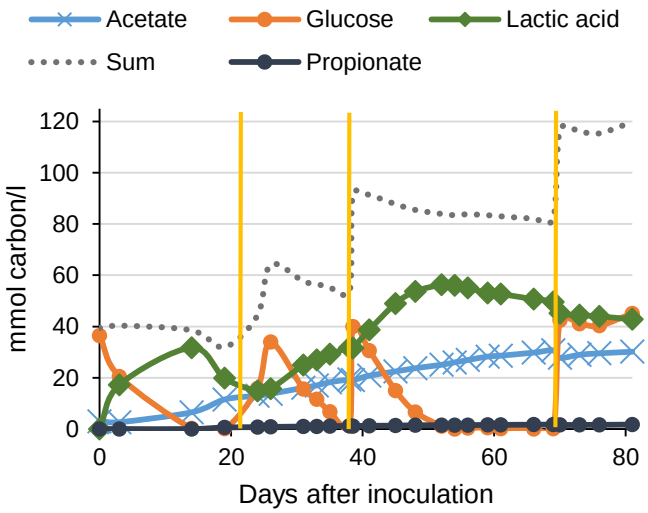

Figure 36: Concentrations in the liquid samples of the working electrode chamber of the neutral red reactor with glucose. Yellow lines: addition of glucose. Second yellow line: start of constant potential. Third yellow line: stop of constant potential and addition of 20 ml water.

The daily hydrogen production (see Figure 37) is increasing after the first addition of glucose (first yellow line). Also at the beginning of the constant potential (second yellow line) the hydrogen production seems to be increased, but decreases simultaneously with the glucose concentration. After the constant potential is shut off (third yellow line), almost no hydrogen is produced, even though glucose was added to the reactor.

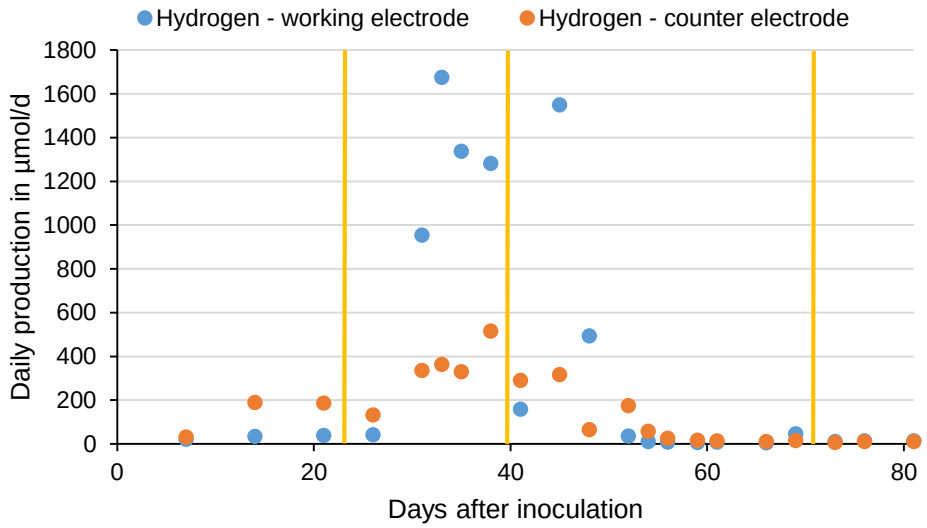

Figure 37: Daily hydrogen production in the neutral red reactor with glucose over the experiment time. Yellow lines: addition of glucose. Second yellow line: start of constant potential. Third yellow line: stop of constant potential and addition of 20 ml water.

5.2.3 Methyl viologen – acetate reactor

The acetate concentration in the methyl viologen reactor with acetate stays the same over the experiment time (see Figure 38). The first decrease is due to dilution by the addition of bacteria culture.

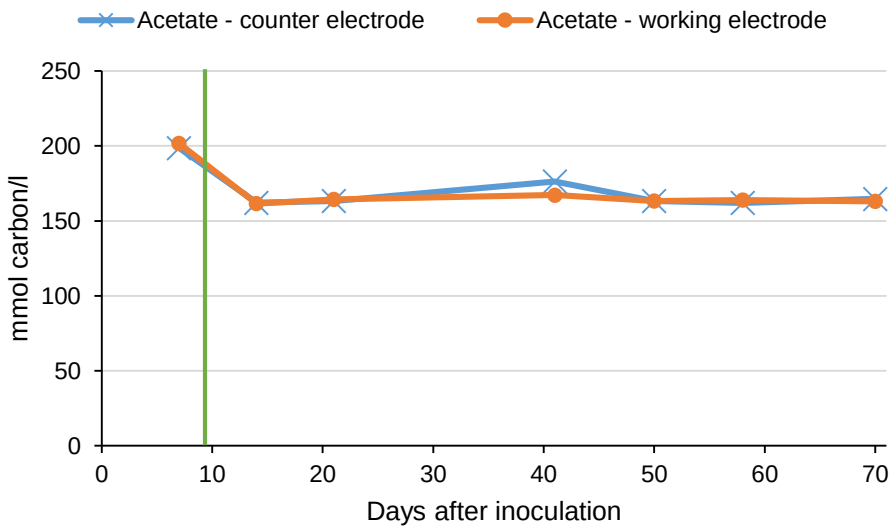

Figure 38: Concentrations found in the liquid samples of the methyl viologen reactor with acetate over the experiment time. Green line: addition of 20 ml bacteria culture.

While at the beginning some methane is produced, the production drops close to zero and stays low until the end of the experiments (see Figure 39).

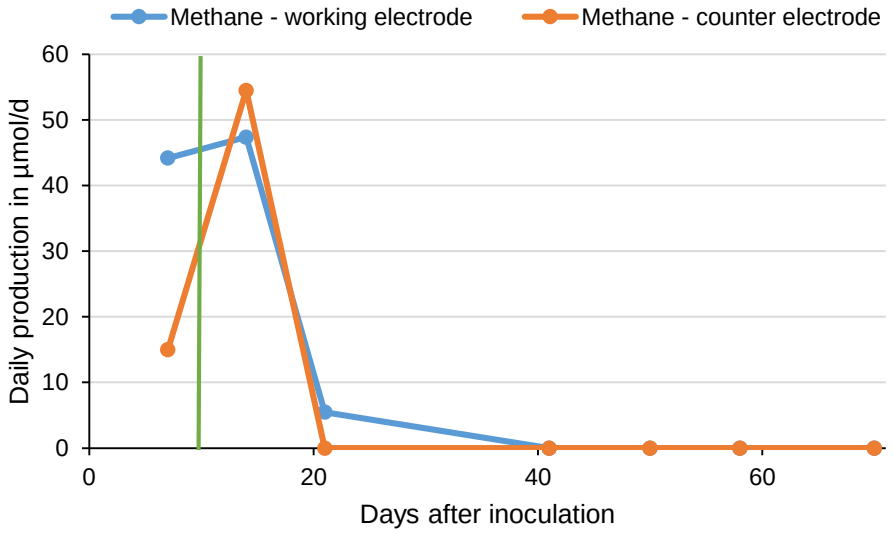

Figure 39: Daily methane production in the methyl viologen reactor with acetate over the experiment time. Green line: addition of 20 ml bacteria culture.

5.2.4 Methyl viologen – glucose reactor

The glucose concentration in the counter electrode chamber stays constant after the first addition of glucose and has just a slight decrease after 52 days (see Figure 40).

In the working electrode a consumption of glucose takes place after 35 days (see Figure 41). By the consumption of glucose a production of lactic acid and acetate takes place.

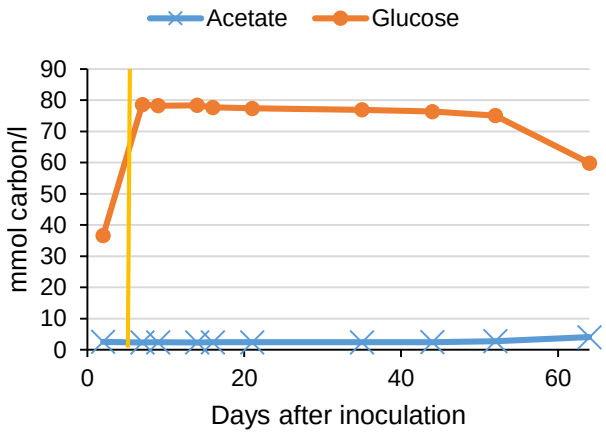

Figure 40: Concentrations in the liquid samples of the counter electrode chamber of the methyl viologen reactor with glucose. Yellow line: addition of glucose.

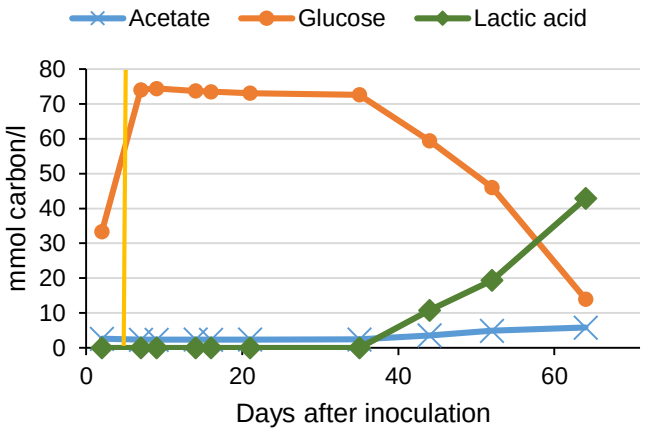

Figure 41: Concentrations in the liquid samples of the working electrode chamber of the methyl viologen reactor with glucose. Yellow line: addition of glucose.

The daily hydrogen production seems not to be related to the glucose consumption and is higher in the beginning and almost totally absent after 21 days (see Figure 42).

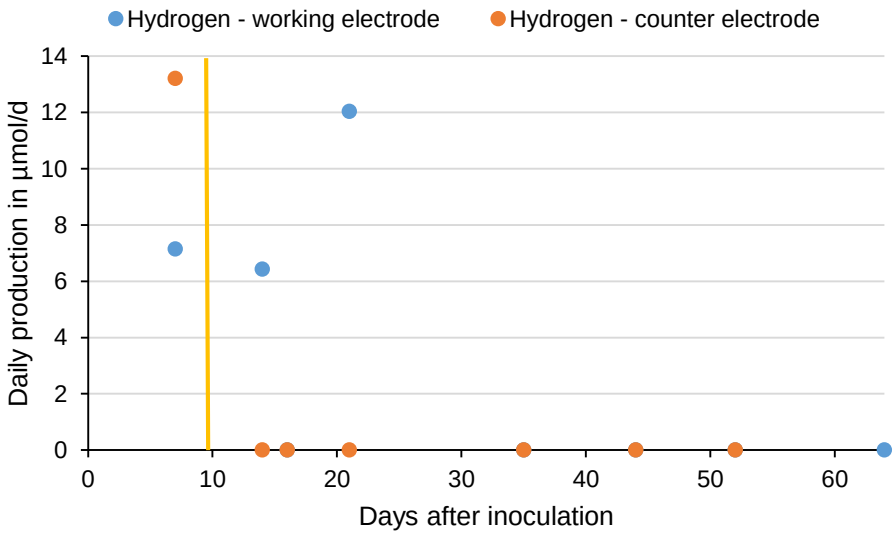

Figure 42: Daily hydrogen production in the methyl viologen reactor with glucose over the experiment time. Yellow line: addition of glucose.

5.3 Cyclic voltammetry - mediators with bacteria

The voltammograms of the CV experiments with the scan rates 5, 10, 50 mV/s can be seen in the appendix section 9.3. Following only the lowest scan rate of 1 mV/s and a high scan rate of 20 mV/s will be shown.

5.3.1 Neutral red – acetate reactor

For the reactor with acetate medium and neutral red as mediator initially two oxidation peaks and two reduction peaks can be seen with a scan rate of 1 mV/s (see Figure 43). The initial small oxidation peaks get intensified after the addition of bacteria. The reduction peaks get intensified and shifted to a lower potential. With the growing time of two and four weeks, the first oxidation peak gets intensified while the second reduction peak is lowered again. After 2 and 4 weeks the second reduction peak is even more shifted, while the first reduction peak is not visible anymore. A growing time of 12 weeks shows a slight shift to a higher potential and intensification of the first reduction peak.

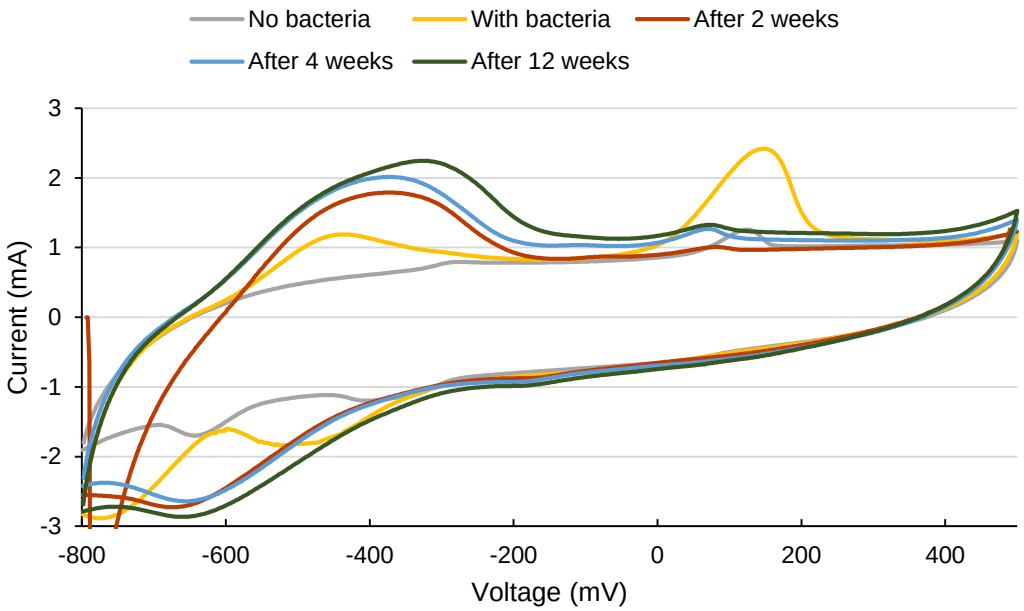

Figure 43: Scan rate of 1 mV/s for the cyclic voltammetry experiment with neutral red as mediator and acetate media.

The scan rate of 20 mV/s shows only one oxidation peak after the addition of bacteria (see Figure 44). This peak has a shift to a higher potential and a higher peak current with bacteria. The shift to the higher potential is even bigger after 2 weeks growing time, while the peak current is lowered. After 4 weeks the peak potential decreases again, together with the peak current. An increase of potential and current of the oxidation peak occurs after 12 weeks growing time. No reduction peak is visible after the addition of bacteria.

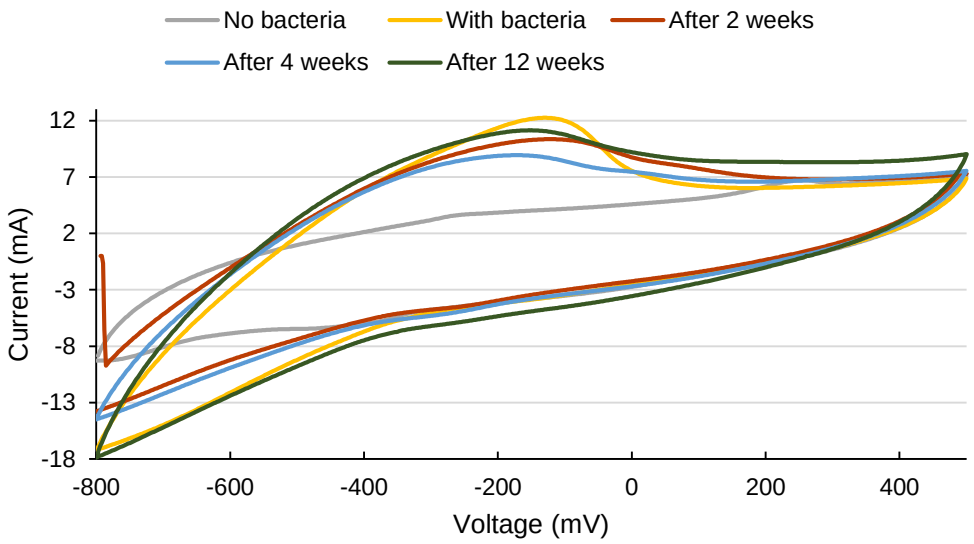

Figure 44: Scan rate of 20 mV/s for the cyclic voltammetry experiment with neutral red as mediator and acetate media.

5.3.2 Neutral red – glucose reactor

Also the reactor with glucose media and neutral red as mediator shows initially two oxidation peaks and two reduction peaks with the scan rate of 1 mV/s (see Figure 45). The oxidation peaks get intensified after the addition of bacteria. The reduction peaks are also intensified and shifted to a lower potential. After 2 weeks growing time only the first intensified oxidation peak is visible and no clear reduction peak can be determined. After 12 weeks growing time the first oxidation peak is shifted to a higher potential, while the second reduction peak is almost not recognizable. Also the reduction peak is almost flattened out.

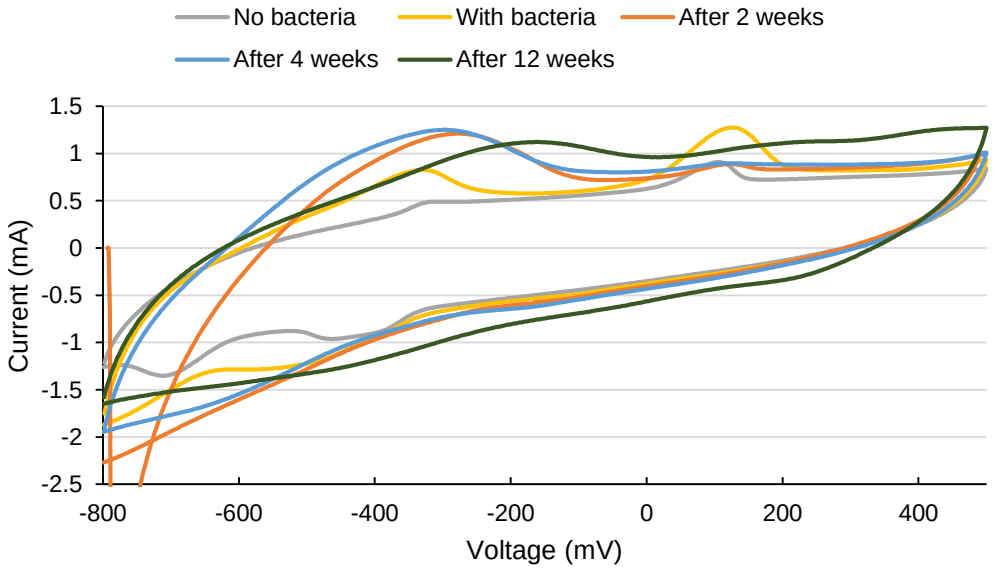

Figure 45: Scan rate of 1 mV/s for the cyclic voltammetry experiment with neutral red as mediator and glucose media.

With the scan rate of 20 mV/s the only visible peak after the addition of bacteria is one big oxidation peak (see Figure 46). While it is intensified and shifted to a higher potential directly after the inoculation, it is more intensified after two weeks growing time. After a growing time of 4 weeks the oxidation peak is flattened and shifted again to a lower potential. A reduction peak is not definable after the addition of bacteria. 12 weeks growing time bring a total flattening of oxidation and reduction peaks.

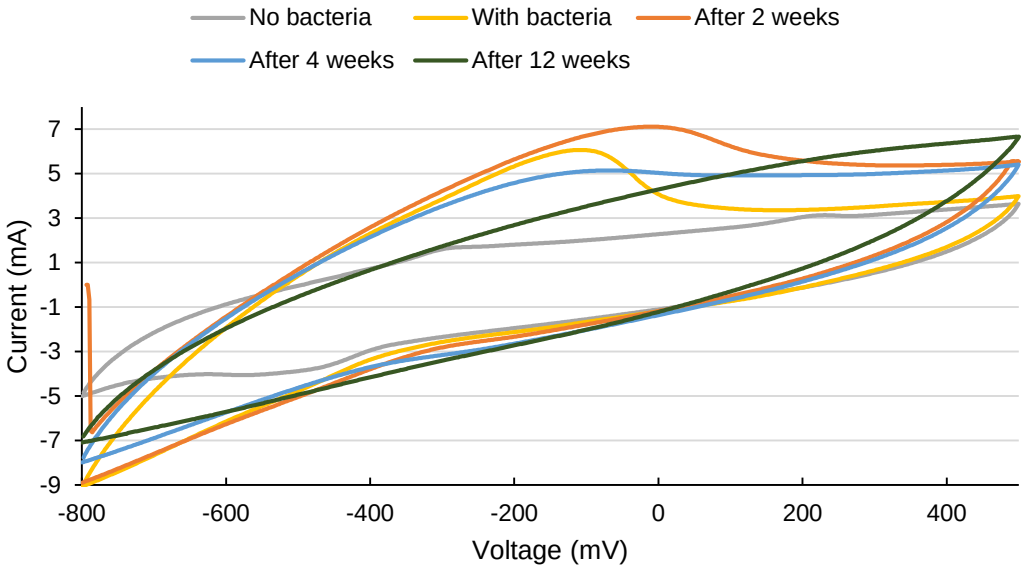

Figure 46: Scan rate of 20 mV/s for the cyclic voltammetry experiment with neutral red as mediator and glucose media.

5.3.3 Methyl viologen – acetate reactor

On a scan rate of 1 mV/s the reactor with acetate media and methyl viologen as mediator has initially two oxidation peaks and two reduction peaks (see Figure 47). The shape of the cyclic voltammogram is approximately the same for the media with and without bacteria. After two weeks growing time, the two oxidation peaks are combined to one and shifted to a higher potential. At the same time the reduction peak is shifted to a lower potential.

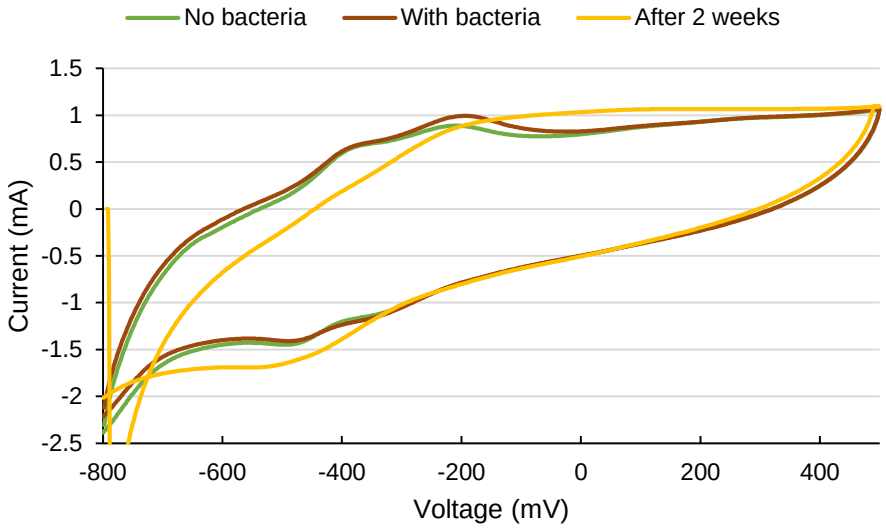

Figure 47: Scan rate of 1 mV/s for the cyclic voltammetry experiment with methyl viologen as mediator and acetate media.

With a scan rate of 20 mV/s a shift to a higher potential and higher current is visible for the oxidation peaks after the addition of bacteria (see Figure 48). The reduction peak is slightly shifted to a lower potential and lower current. After 2 weeks growing time only one flattened oxidation peak and no clear reduction peak is visible.

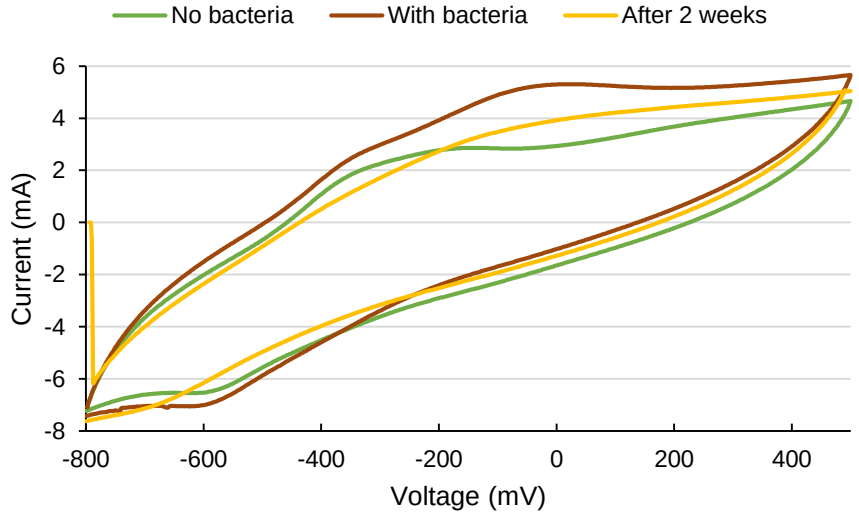

Figure 48: Scan rate of 20 mV/s for the cyclic voltammetry experiment with methyl viologen as mediator and acetate media.

5.3.4 Methyl viologen – glucose reactor

On a low scan rate of 1 mV/s the reactor with glucose medium and methyl viologen as mediator shows one oxidation peak and one reduction peak (see Figure 49). The peaks are very similar before and after the addition of bacteria to the medium. After two weeks growing time the oxidation peak gets slightly flattened and the reduction peaks slightly shifted to a lower potential.

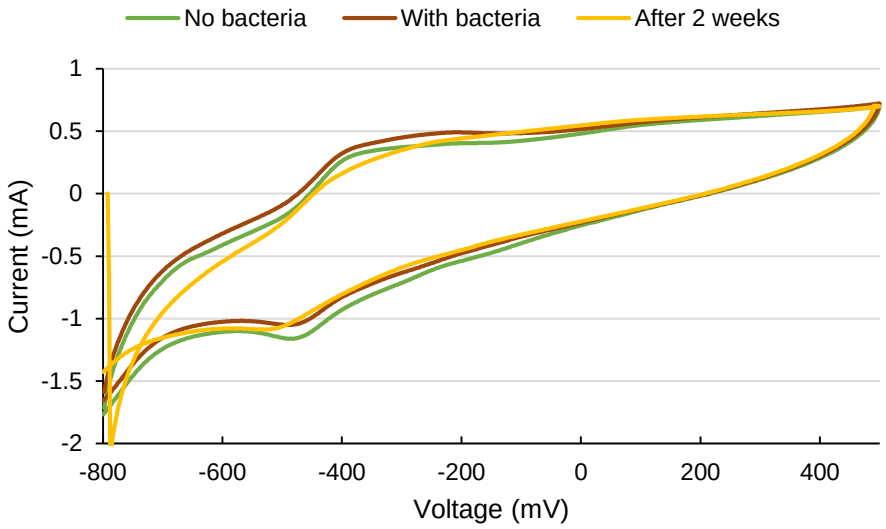

Figure 49: Scan rate of 1 mV/s for the cyclic voltammetry experiment with methyl viologen as mediator and glucose media.

On the scan rate of 20 mV/s the oxidation peak is shifted to a higher potential and current after the addition of bacteria (see Figure 50). After two weeks the oxidation peak is shifted back to the initial potential and current. The reduction peak stays the same after all.

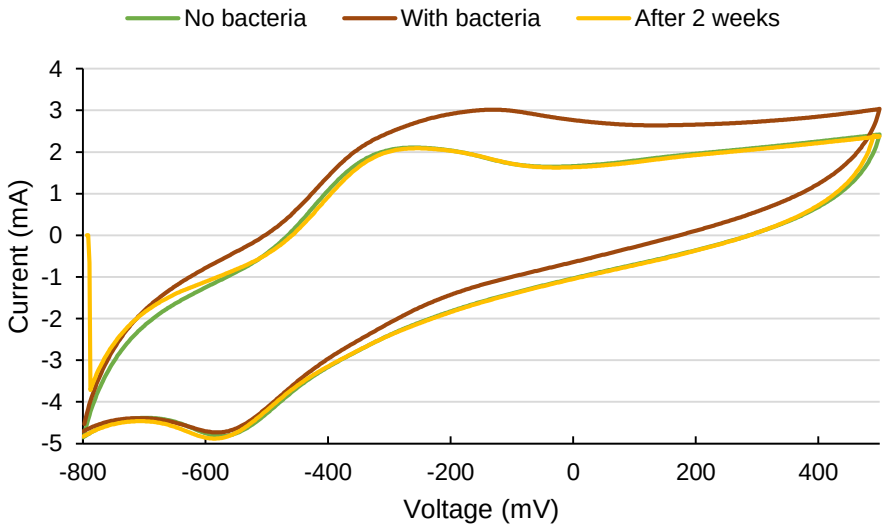

Figure 50: Scan rate of 20 mV/s for the cyclic voltammetry experiment with methyl viologen as mediator and glucose media.

5.4 Dominating transport process

After two weeks growth time the cyclic voltammetry experiments showed a shift of the peak currents depending on the scan rate. In Figure 51 - Figure 58 the relation of the peak currents to the scan rate is compared to the relation of the peak currents to the square roots of the scan rate. To every graph the linear correlation coefficient ($\rho_{X,Y}$) is given. For every reactor the linear correlation coefficient is closer to 1 or -1 with a relation to the square root of the scan rate than with a relation directly to the scan rate. This suggests that diffusion is the major mechanism for transporting the electroactive compounds to the electrode surface.

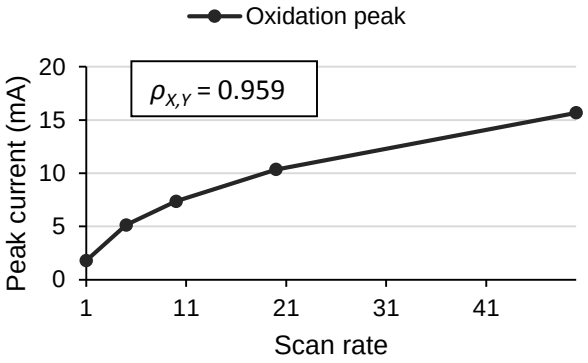

Figure 51: Relation of the peak currents to the scan rate (SR). For the reactor with acetate media and neutral red as mediator after two weeks grow time.

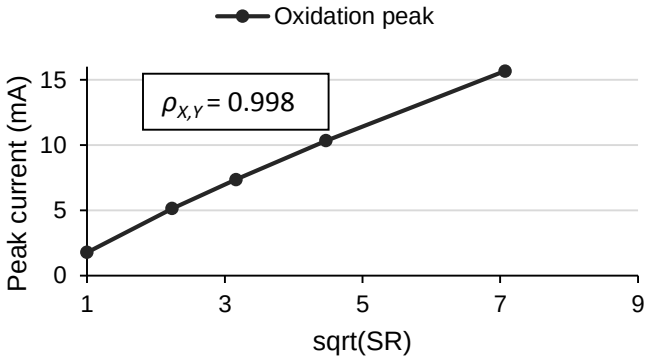

Figure 52: Relation of the peak currents to the square roots of the scan rate (SR). For the reactor with acetate media and neutral red as mediator after two weeks grow time.

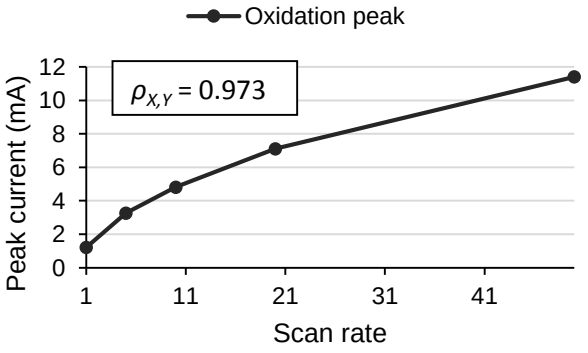

Figure 53: Relation of the peak currents to the scan rate (SR). For the reactor with glucose media and neutral red as mediator after two weeks grow time.

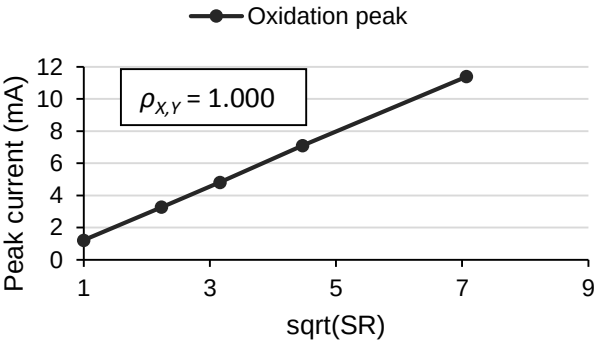

Figure 54: Relation of the peak currents to the square roots of the scan rate (SR). For the reactor with glucose media and neutral red as mediator after two weeks grow time.

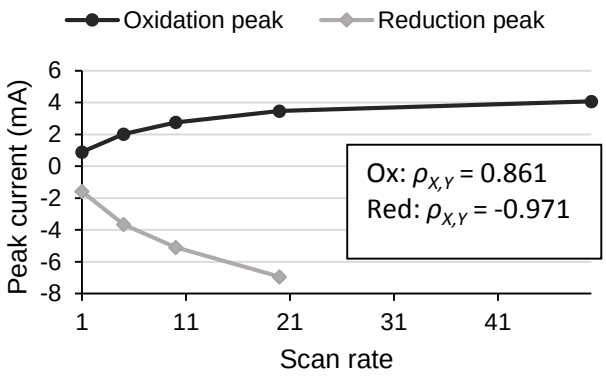

Figure 55: Relation of the peak currents to the scan rate (SR). For the reactor with acetate media and methyl viologen as mediator after two weeks grow time

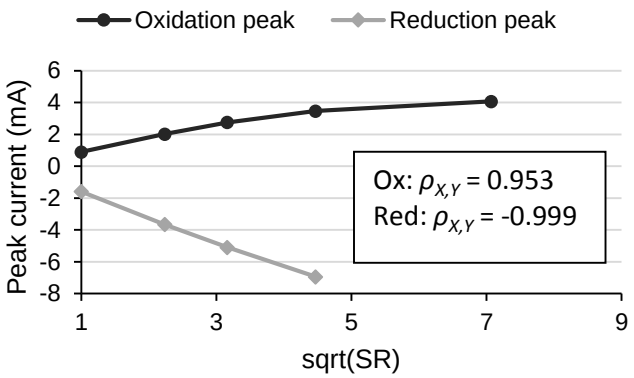

Figure 56: Relation of the peak currents to the square roots of the scan rate (SR). For the reactor with acetate media and methyl viologen as mediator after two weeks grow time.

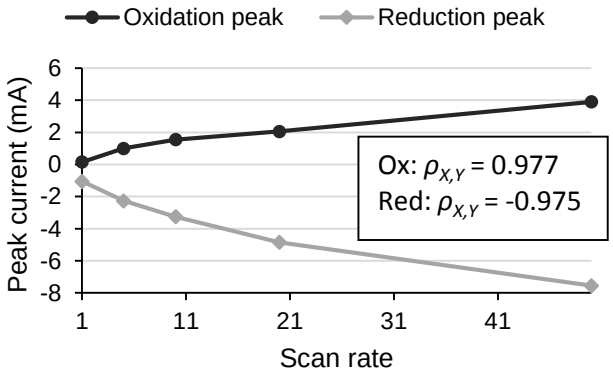

Figure 57: Relation of the peak currents to the scan rate (SR). For the reactor with glucose media and methyl viologen as mediator after two weeks grow time.

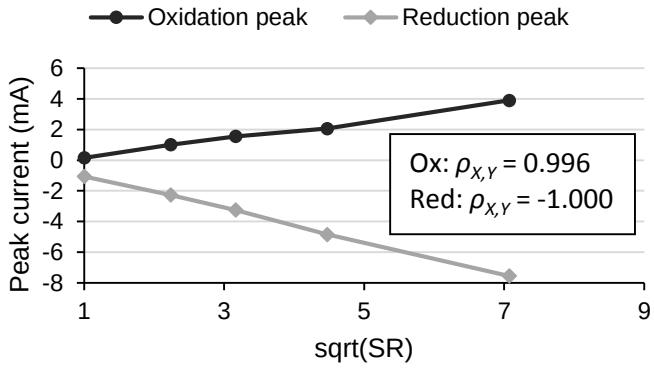

Figure 58: Relation of the peak currents to the square roots of the scan rate (SR). For the reactor with glucose media and methyl viologen as mediator after two weeks grow time.

5.5 Electron transfer with a constant electrode potential

The chronoamperometry experiment with constant set potential gave a resulting current over the time. Only the reactor with glucose medium and neutral red as mediator was investigated with chronoamperometry.

As example the resulting current curve of the first chronoamperometry experiment, 38 days after inoculation, is shown in Figure 59. In the beginning and at the end, a positive respectively a negative current peak occurs. These peaks show up shortly after the potential change and at the moment the set potential stops. In the meantime the current is almost constant and close to 0 A.

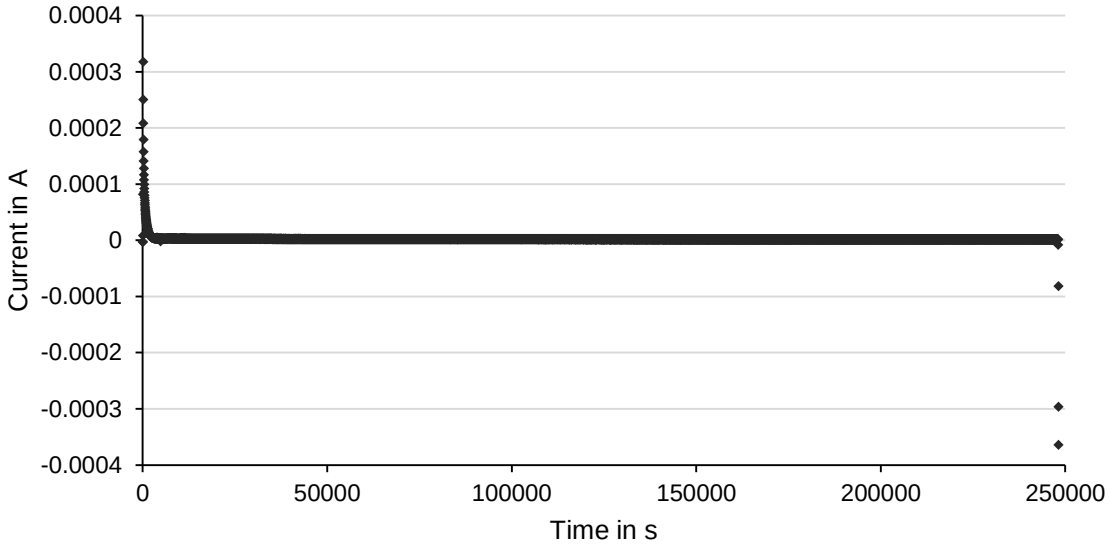

Figure 59: Resulting current during the chronoamperometry with -0.2 V vs SHE, 38 days after inoculation.

After cutting off the initial and final current peak, the current values are used to calculate the equivalent amount of electrons. Table 7 shows the electrons transferred with an almost constant current. The amount of transferred electrons is very low and ranges from -0.48 to 4.45 µmol.

Table 7: Electrons transferred to the electrode during the experiments with constant set potential of -0.2 V vs SHE. Initial and final current peak cut off.

Starting day - Days after inoculation	Duration in s	Total transferred electrons in µmol	‰ of electrons available from glucose
38	245000	4.45	0.159
41	326000	0.53	0.011
48	47300	-0.21	0.005
49	247000	0.44	0.011
54	152000	0.12	0.004
56	252000	-0.01	0.000
59	160000	-0.48	0.018
62	330000	0.94	0.037
66	245000	-0.04	0.002

The experimental time, during which a constant potential was applied, lasted from day 38 to 69 after inoculation. In the first period from day 38 to 48 a consumption of almost all the glucose took place. From day 48 to 69 only very small amounts of glucose were left in the working electrode chamber (see section 5.2.2). Calculating the electrons consumed by the glucose degradation and the electrons which went to degradation products, give the electrode balance in Table 8. The electron balance shows a negative value indicating the loss of some electrons to different paths than the measured degradation products. In the first period this electron loss is bigger than in the second period. The electrons which went to the electrode are very few compared to the missing electrons from the balance.

Table 8: Electron balance (in mmol electrons) for the working electrode chamber of the glucose - neutral red reactor.

Compound	**Period 1 (day 38 – 48)**	**Period 2 (52 – 69)**
Glucose	-30.49	-0.92
Acetate	3.90	3.25
Lactic acid	19.28	-8.74
Propionate	0.29	0.11
Sum	-7.02	-6.30
To electrode	$-5.0 \cdot 10^{-3}$	$-0.8 \cdot 10^{-3}$

Also in the counter electrode chambers the electrode balance shows a negative sum (see Table 9). In the second period way more electrons left the system than it was the case in the first period.

Table 9: Electron balance (in mmol electrons) for the counter electrode chamber of the glucose - neutral red reactor.

Compound	**Period 1 (day 38 – 48)**	**Period 2 (52 – 69)**
Glucose	-9.50	-19.75
Acetate	4.68	7.22
Lactic acid	3.03	0.02
Propionate	0.33	0.37
Sum	-1.46	-12.13

5.6 Microscopic pictures

5.6.1 Neutral red – glucose reactor

Observing liquid samples from the reactor with glucose medium and neutral red as mediator, pictures were taken through a microscope. From the inoculation with the stock culture only one SAOB strain should be present. As described from Westerholm et al. 2011, the SAOB should be rod shaped and spore forming.

In the working electrode chamber the only bacteria form found with the microscope was round shaped and grouped as shown in Figure 60.

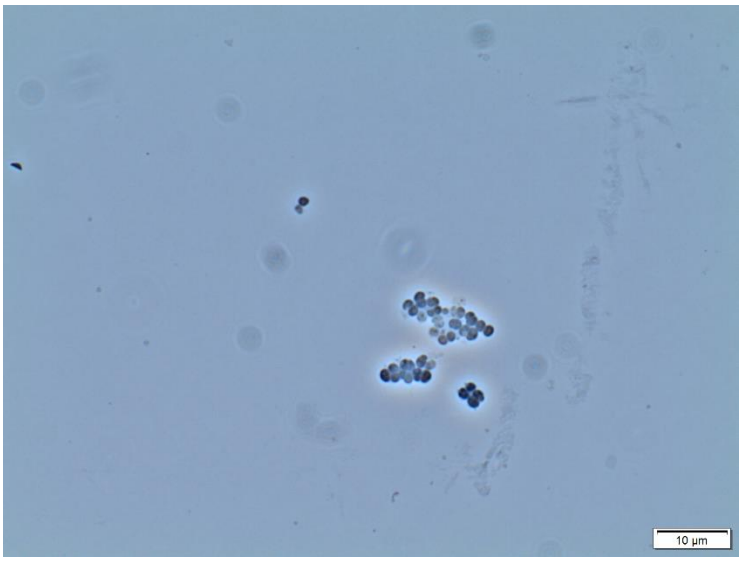

Figure 60: Spores or coccoid bacteria in the working electrode chamber of the neutral red - glucose reactor.

Rod shaped bacteria (see Figure 61) as well as round shaped bacteria or spores (see Figure 62) are found in the counter electrode chamber.

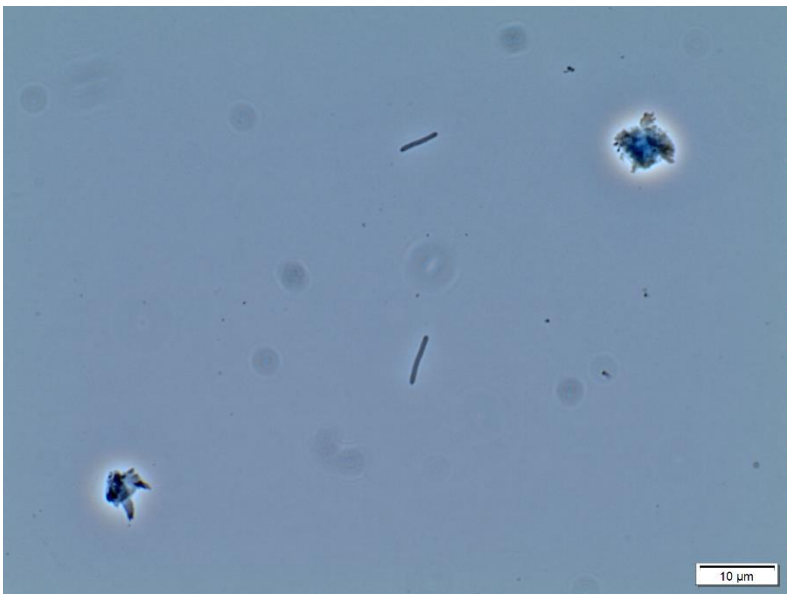

Figure 61: Rod shaped bacteria in the counter electrode chamber of the neutral red - glucose reactor.

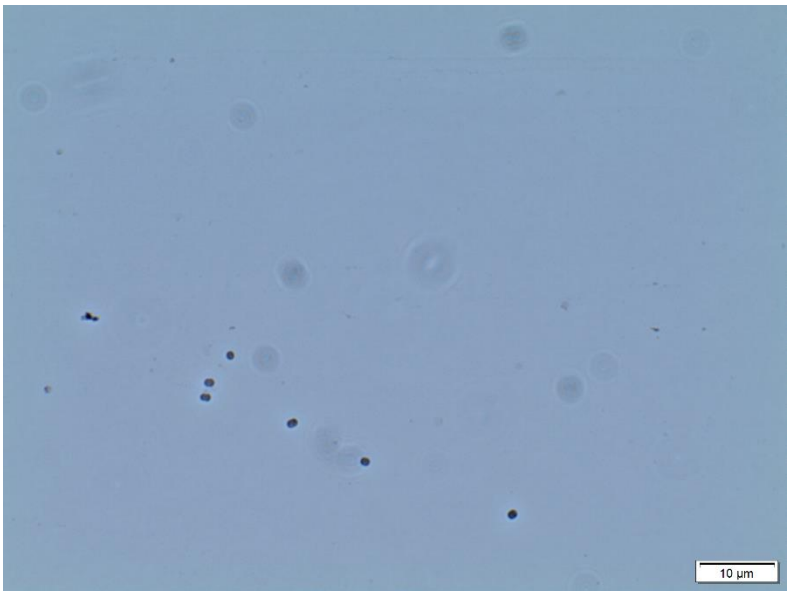

Figure 62: Spores or coccoid bacteria in the counter electrode chamber of the neutral red - glucose reactor.

5.6.2 Mixed stock culture

In the mixed stock culture three different SAOB strains and one methanogen strain should be present. In Figure 63 different shapes of bacteria can be seen. Coccoid as well as rod shaped bacteria are visible. The rod shaped bacteria form chains (green circle) and even stick in chains with round shaped objects (red circle). This objects could be either coccoid bacteria or spores formed by the rods.

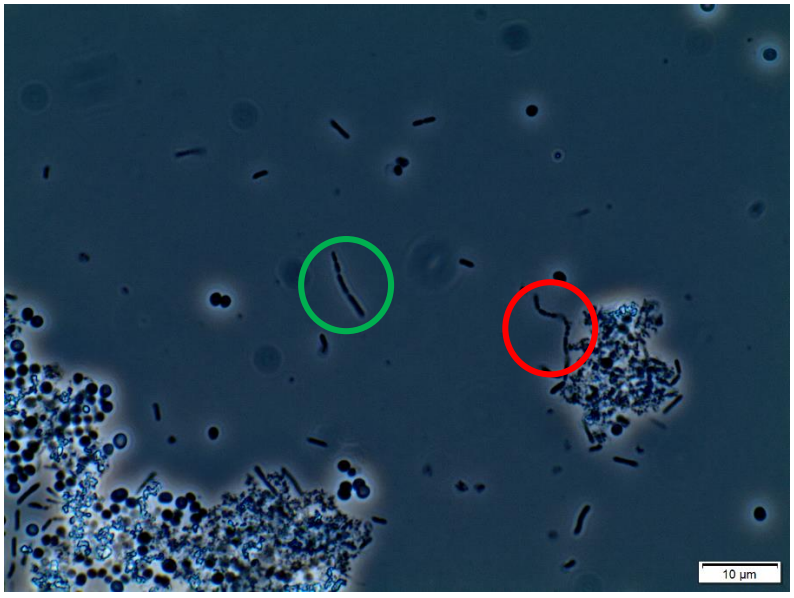

Figure 63: Different bacteria in the mixed culture medium with three different SAOB and a methanogen strain.

5.7 Color change in the neutral red reactor

In the reactors with neutral red the color of the medium changed with the time of the experiment. In Figure 64 and Figure 65 the change in the reactor with glucose medium is visible, which is similar in the reactor with acetate media. Neutral red works as pH and redox indicator. Hence the color change from red to light yellow could indicate a change in pH or redox status.

Figure 64: Reactor with glucose medium and neutral red mediator 3 days after inoculation.

Figure 65: Picture of reactor with glucose media and neutral red mediator 54 days after inoculation.

6 Discussion

6.1 Neutral red reactors

The reactors with neutral red as redox mediator changed the media color during the experiment. In 1972, Bishop described the dependency of the color of neutral red on its redox state. If neutral red is in reduced state it is colorless, if in oxidized state it is reddish-violet. In section 5.7 the color change from red to light yellow is visible, as well as darker flocs on the ground. Hence the remaining neutral red in the media is all in reduced form after the experimental time. The darker flocs may indicate an uptake of the oxidized form of neutral red by the bacteria. At the same time the color of neutral red is pH dependent (Bishop 1972). In a lower pH the color is bluish-red and changes to orange yellow at high pH. Hence the lighter color in the media could be also explained by an increasing pH, while the pH close to the bacteria would be lower. Probably both mechanisms have some influence, even though the media is well buffered and should not change easily in pH.

6.1.1 With acetate media

In the reactor with acetate medium and neutral red as mediator, the consumption of acetate is steady but low throughout the 81 days. From the acetate concentration it seems like the activity of the bacteria increases after more bacteria are added to the reactor. Contrary the methane production is decreasing after the addition. The hydrogen and methane production gives the impression that the bacteria are more active directly after the inoculation. After an inactive state the methane production increases after 60 days. The bacteria may require some time to adopt to the neutral red media and get active in the new reactor.

After the addition of bacteria to the reactor the cyclic voltammetry with a scan rate of 1 mV/s shows a big current increase in the second oxidation peak. It may be caused by some compound added to the media with the bacteria. The following weeks the large second oxidation peak has disappeared, indicating the absence of the compound again. A current drop at low voltage occurs after two weeks growing time. This may be caused by hydrogen evolution on the electrode surface. In section 5.2.1 it can be seen, that the bacteria produce hydrogen at this time. However the first oxidation peak increases in current with the growing time. The increase is slightly bigger than for the corresponding reduction peak. If bacteria use the mediator, there would be increasingly more reduced neutral red in the medium than oxidized neutral red. Hence an increasing oxidation peak compared to the reduction peak shows a usage of the mediator in the medium. Still it is unclear if the two reduction peaks merged to one peak, after 2 weeks growing time. All reduction peaks could be also shifted towards a lower potential. This would mean only the second reduction peak is visible. With the second reduction peak no clear statement could be made, as it does not correspond to the first oxidation peak.

With a higher scan rate of 20 mV/s the oxidation peaks are flattened and the reduction peak is not even clearly determinable anymore. This may be caused by the bacteria using the mediator. A scan rate this high may not get the full amount of neutral red in one sharp peak. The bacteria could be simply not fast enough to supply the reduced neutral red in this time.

Especially on this high scan rate a shift to lower currents is clearly visible for the lowest potential at -0.8 V vs SHE with the addition of bacteria. This indicates a hydrogen production on the electrode. SAOB using enzymes for the hydrogen production, like hydrogenase, may catalyze this reaction. This would cause the negative shift and also makes it harder to distinguish the reduction peak.

6.1.2 With glucose media

A high activity of the bacteria can be seen in the reactor with glucose medium and neutral red as mediator. In the working electrode chamber a fast consumption can be seen. In the period with a constant potential the glucose is also consumed very fast, but no glucose is consumed after the constant potential. This may indicate a use of the electrode potential. But as the glucose was also consumed before the constant potential, it is likely the bacteria became inactive. After 14 days of constant potential almost no glucose is left anymore and so the bacteria may just minimalized their consumption. In the counter electrode chamber the glucose consumption is lower and only in the beginning the glucose is completely consumed. The lower consumption may be an indication of less activity due to the missing anode potential. But as the consumption is also slightly lower without constant potential, this cannot be said with certainty. Interesting in both chambers is the degradation of lactic acid if no glucose is available anymore. While the other metabolic products acetate and propionate are just accumulating, lactic acid seemed to be used in case of missing glucose.

The hydrogen production for both chambers increased between 31 and 39 days after inoculation, which may be explained by the increasing activity after some growing time. After the start of the constant potential, the hydrogen production is high, but decreases related to the glucose concentration in the working electrode chamber. As in both compartments hydrogen is produced, it is likely that the bacteria did not use the electrodes. With an optimal use of mediator and electrodes, the hydrogen production should be mainly on the counter electrode chamber. Remarkable is the fact, that even though glucose is consumed in the counter electrode chamber, no hydrogen is produced after day 56.

In the cyclic voltammetry on a low scan rate of 1 mV/s the second oxidation peak is increased directly after inoculation. As it is flat afterwards, it might be also caused by a compound from the added bacteria liquid, like in the acetate reactor. Also the current drops for the glucose reactor the same way as it does for the acetate reactor discussed before. It may be also caused by hydrogen production in the first two weeks growing time. An increase in the oxidation peak current is visible with the growing time, even though in the measurement after two weeks almost no glucose is left in the media. The reduction peaks are hard to determine, but could be in the same range of increase. This would mean limited use of the mediator by the bacteria. However the peaks shift to a higher potential after 12 weeks growing time and are flattened. Suggesting a slow reaction of the inactive bacteria at this time.

On a higher scan rate of 20 mV/s the current of the oxidation peak increases much. Even more after two weeks of growing, even though the glucose is almost completely consumed in the media. At the same time, the reduction peak is impossible to determine. It could be an indication for only reduced neutral red in the media. This would indicate a bacterial usage. Contrary the oxidation peak current decreases again after 4 weeks and glucose addition.

However it is unlikely, that the bacteria can reduce all mediator in the short time on a high scan rate if they cannot on a low scan rate. After 12 weeks, no peaks are visible anymore. The bacteria are inactive this time and may have also bound the neutral red. This would lead to absence of neutral red and indicate an uptake by the bacteria.

On the low as well as on the high scan rate it can be seen, that the negative potential at -0.8 V vs SHE lead to negative current decreasing with the addition of bacteria. This may be caused by the hydrogen production catalyzed by the bacteria, as already discussed for the neutral red – acetate reactor. Also here the reduction peaks could be simply hidden by the hydrogen production.

In the constant potential experiment the resulting current showed, that almost no electrons were transferred to the electrodes. The missing electrons from glucose reduction, which did not go to acetate, lactic acid or propionate, can be assumed to be gone to hydrogen or for assimilating carbon into new microbial cells. As the gas measurements are not as precise as the liquid measurements, they cannot be compared directly. The amount of electrons gone to the electrode is very small.

6.2 Methyl viologen reactors

The glucose and the acetate medium reactors with methyl viologen as mediator did both not show a high activity. At the beginning there is some small gas production in both reactors, which decreases to basically zero after twenty days. In the acetate reactor, the acetate is not consumed. After 35 days a consumption of glucose take place in the working electrode chamber of the glucose reactor. But also this seems not related to the hydrogen production. Hence there is no activity of the bacteria concerning hydrogen production.

Egnchi et al. 1985 used methyl viologen as an inhibitor for methanogens. This inhibiting effect is confirmed by the low to absence activity of the tested bacteria in this study. As there is also no hydrogen production after the initial time, methyl viologen could also be an inhibitor for SAOB. Wolin et al. 1964 state an inhibition of methane production together with an increase in hydrogen production for the bacteria *Methanobacillus omelianskii* after the addition of methyl viologen. Hence the inhibition of SAOB would be a different process than for *Methanobacillus omelianskii*.

In the cyclic voltammetry at 1 mV/s the addition of bacteria makes no difference and flattens out after 2 weeks. This may indicate a reduction in the concentration of methyl viologen through degradation, adsorption or uptake by the bacteria.

6.3 Dominating transport process

The peak current showed a linear correlation to the square root of the scan rate after two weeks growing time. This linear correlation indicates diffusion as the limiting transport factor to the electrode (Harnisch & Freguia 2012). All reactors showed this linear correlation. Hence no biofilm process seems to have a great influence in any reactor.

From an optical point (see section 5.7) the bacteria seem to settle down on the ground in flocs. This also confirms, that no biofilm is forming on the electrode surface.

6.4 Method and sources of errors

The microscopic pictures show rods as well as round shape objects for the neutral red – glucose reactor. In the reactor should be only one form of bacteria. Westerholm et al. 2011 show that the rod shaped SAOB also form round spores. Hence, the round object could be spores and the reactor not contaminated with different bacteria. Also in the microscopic picture of the mixed bacteria culture rod bacteria in connection with round objects can be seen. This could confirm spore formation of the rod bacteria. However, it is not possible to say this with absolute certainty. There could be some contamination of the reactor. As the medium with glucose, as well as the medium with acetate on 40 °C provide optimal conditions for bacterial growth, every small contamination may overgrow the investigated bacteria. This would lead to a falsification of the result. A different bacteria could increase the glucose/acetate consumption without leading to hydrogen production or without leading to a current on the electrode. As the glucose consumption is always coupled to hydrogen production it is unlikely that other bacteria consume all the glucose. Also the acetate reactor shows methane and hydrogen production till the end. Therefore, it seems unlikely, that another microorganism have completely displaced the initial bacteria mix. On the other hand, the glucose consumption was coupled to lactic acid formation. This could be indicating different microorganisms coexisting.

Initially, the reactor setup was chosen to be a small serum flask with a needle connection to syringes with the counter and reference electrode. These setup did not work, as the electrodes were not properly connected to the medium through the needles. Most likely small air bubbles clogged the needles. As the needles were already 1.1 mm thick, a connection through needles is in general not recommendable. The following reactor setup with bigger reactors and electrodes directly in the media worked well. Hence a bigger scale for electrochemical reactors can be recommended. Though it definitely limits the amount of possible different experiments.

With the bigger reactor setup some air intrusion happened. During the incubation time the intrusion has been probably very small, while the main leakage happened during gas sampling. This was confirmed by directly sampling the reactors after gas exchange, when no intrusion other than through sampling was possible. The intrusion was highly dependent on the sampling access to the gas phase. While the neutral red reactors had a slide closure, the methyl viologen reactors had caps to lock the needle for gas sampling. The air intrusion while sampling the methyl viologen reactors was remarkably higher. Through this intrusion the gas samples got falsified and uncertain. Hence, it is suggested to improve the sealing of the sampling openings at the reactor.

For some time nitrogen gas had to be used instead of the Ar/CO_2 gas mixture. This can potentially lead to a change in the buffer system. However, the media is buffered very well and should not change the pH fast.

In the cyclic voltammetry some peaks are maybe unseen due to the scan range. To get a complete view over the redox peaks, also with highly shifting peaks, a bigger scan range is required. Especially the reduction peaks were shifted to a lower potential, hence first a lowering of the negative voltage border is recommended.

7 Conclusion

Aim of this study was to investigate if SAOB can use methyl viologen and neutral red as redox mediator to grow in an electrochemical reactor. With the results it is possible to answer this question and even more information can be found.

The methyl viologen reactors do not show much activity. Hence methyl viologen it is not suitable as redox mediator for SAOB. More likely methyl viologen is an inhibitor, not only for methanogens but also SAOB.

In the reactors with neutral red a high activity was observable, especially for the glucose media. It is not certain if there is a connection to the neutral red as mediator. The cyclic voltammetry experiments may indicate a usage of the neutral red by the bacteria. However, it is visible in the liquid samples, that the bacterial activity is independent from the electrode potential. An electron transfer through the mediator neutral red to the electrode did not happen to a great extent. Through the chronoamperometry it is visible, that not many electrons were transferred with a constant potential on the electrode. Reasons could be either the potential or the mediator. The potential is higher than the formal potential of neutral red and is also not directly stopping the activity of the SAOB. This should lead to constant oxidation of neutral red through the electrode without inhibiting the bacteria. Hence the potential can be excluded and the not working mediator is the reason for the missing electron transfer by the bacteria. However, the darker color of the bacteria flocs may indicate an uptake of neutral red for other reasons.

In future experiments, a wider range of mediators should be investigated. As some transformations of neutral red could be interpreted in the cyclic voltammetry, some other mediator may also be used in a long term test with a constant potential. The cyclic voltammetry tests with seven mediators in the beginning, show resazurin and methylene blue also as mediators with clear peaks. Hence they could be investigated in future experiments.

The number of tested mediators always depends on the effort for the experiment and hence on the size of the reactors. It will be hard to decrease the size of the reactors. One reason is the good connection of the electrodes with the media. On the other hand a frequent sampling also requires a certain volume of the media. In general the closures of the reactor can be improved. To avoid gas intrusion during sampling every opening should have a slide closure.

Even though precautions were taken to prevent bacterial contamination of the reactors, some bacterial contamination is very likely according to the results. This may have led to a falsification of the results such as a too high consumption of glucose or acetate. Hence further improvements in the experimental setup are necessary. Seals and closures could be tested for their impermeability and changed if required. Furthermore all work and sampling on the reactors could be done under a laminar flow cabinet with a germicidal lamp. This could decrease the risk of contamination especially from the surrounding air.

SAOB growing on glucose showed a high activity, depending on the glucose concentration. At the same time the mixed SAOB/methanogens growing on acetate took a long time to become active. Hence longer time periods are suggested for future experiments with the mixed culture on acetate.

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9 Appendix

9.1 Recipe for the solutions added to the media

Table 10: Ingredients of the Se/Wo solution, added 1 ml/l to the media.

Compound	Concentration (mM)
Na_2SeO_3	0.1
$Na_2WO_4*2H_2O$	0.1

Table 11: Solutions mixed to C1 solution, 50 ml/l added to the media.

Compound	Volume (ml)
Trace element solution	1
Vitamin solution	1
Salt solution	12.5
Water	35.5

Table 12: Ingredients of the trace element solution.

Compound	Concentration	Unit
$FeCl_2*4H_2O$	2	g/l
H_3BO_3	0.05	g/l
$ZnCl_2$	0.05	g/l
$CuCl_2$	0.03	g/l
$MnCl_2*4H_2O$	0.5	g/l
$(NH_4)Mo_7O_{24}*4H_2O$	0.05	g/l
$AlCl_3$	0.05	g/l
$CoCl_2*6H_2O$	0.05	g/l
$NiCl_2$	0.05	g/l
EDTA	0.5	g/l
HCl conc.	1	ml

Table 13: Ingredients of the vitamins solution.

Compound	Concentration (g/l)
Pyridoxamine	0.25
Nicotinic acid	0.1
Nicotinamide	0.1
DL-panthothenic acid	0.05
Vitamin b12	0.05
P-aminobenzoic acid	0.05
Pyridoxine HCl	0.1
Biotin	0.02
Thioctic acid	0.05
Folic acid	0.02
Riboflavin	0.05
Thiamine HCl	0.1

Table 14: Ingredients of the salts solution.

Compound	Concentration (g/l)
NH_4Cl	24
NaCl	24
$CaCl_2*2H_2O$	8.8
$MgCl_2*6H_2O$	8

9.2 Cyclic voltammetry – mediators without bacteria

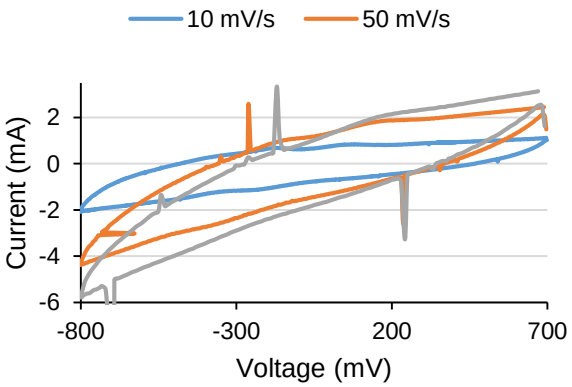

Figure 66: Cyclic voltammogram for the pure buffer solution.

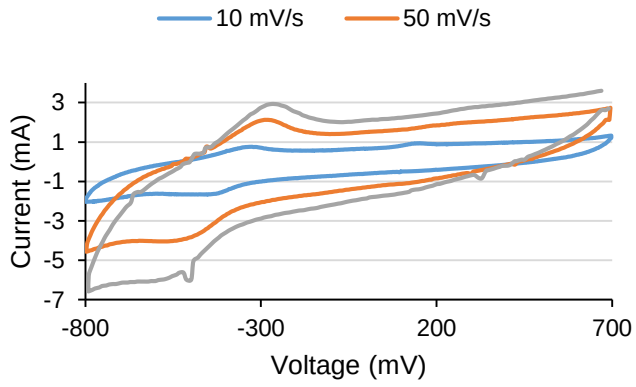

Figure 67: Cyclic voltammogram for neutral red.

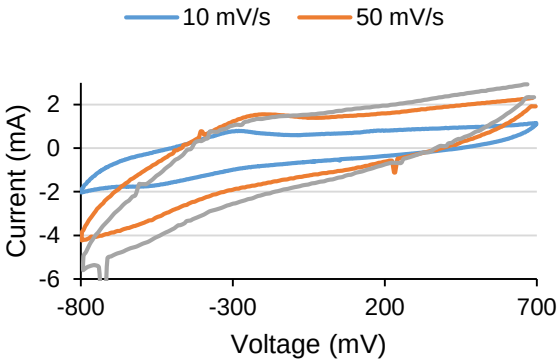

Figure 68: Cyclic voltammogram for methyl viologen.

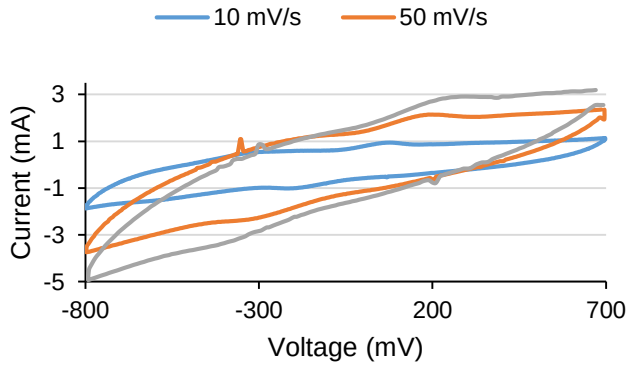

Figure 69: Cyclic voltammogram for resazurin.

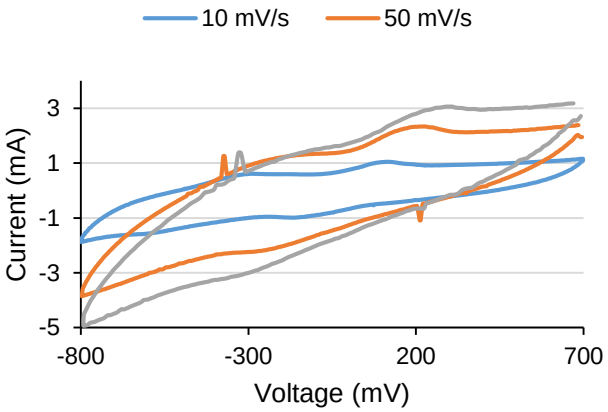

Figure 70: Cyclic voltammogram for methylene blue.

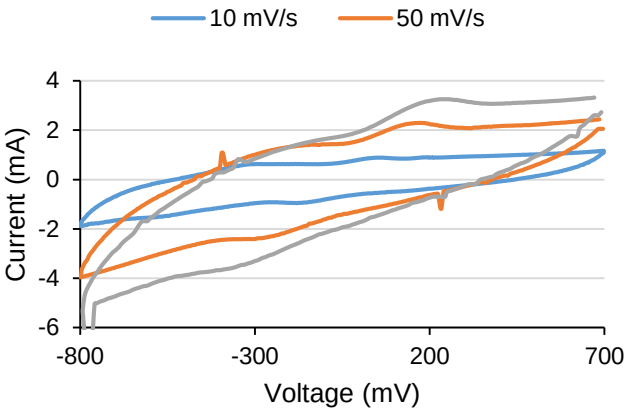

Figure 71: Cyclic voltammogram for humic acid.

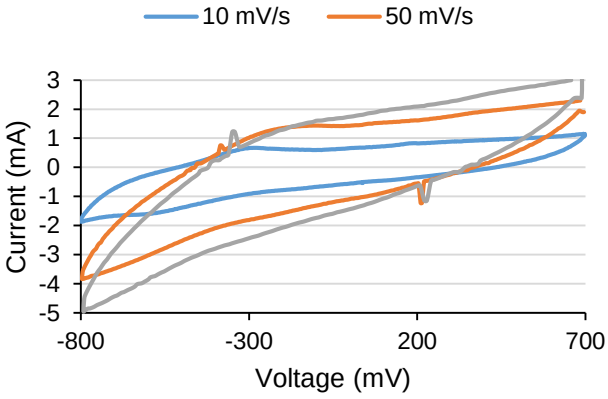

Figure 72: Cyclic voltammogram for cystein.

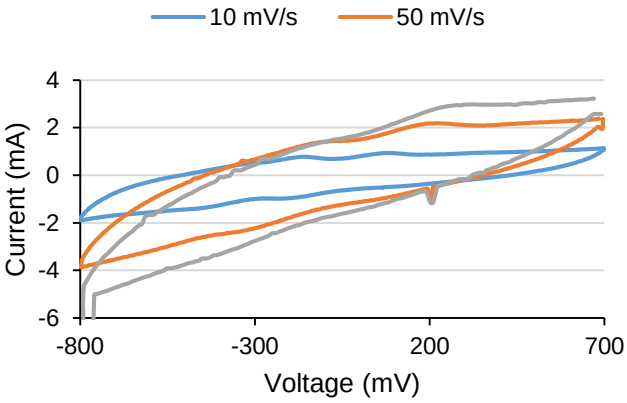

Figure 73: Cyclic voltammogram for AQDS.

9.3 Cyclic voltammetry – mediators with bacteria

9.3.1 Base media – acetate

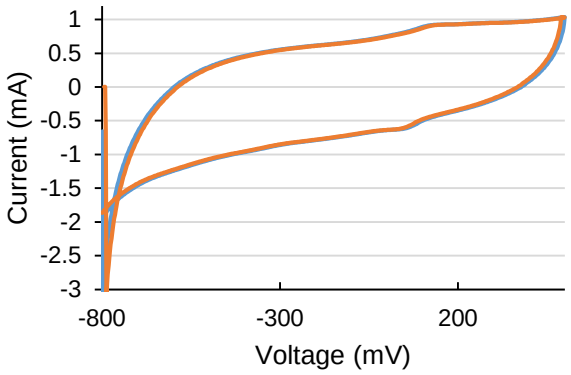

Figure 74: Cyclic voltammogram for the base media with acetate on a scan rate of 1 mV/s.

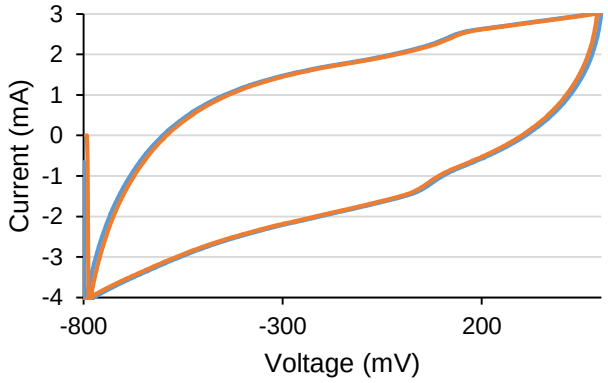

Figure 75: Cyclic voltammogram for the base media with acetate on a scan rate of 5 mV/s.

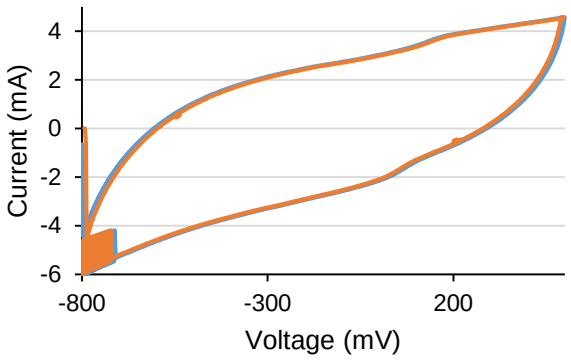

Figure 76: Cyclic voltammogram for the base media with acetate on a scan rate of 10 mV/s.

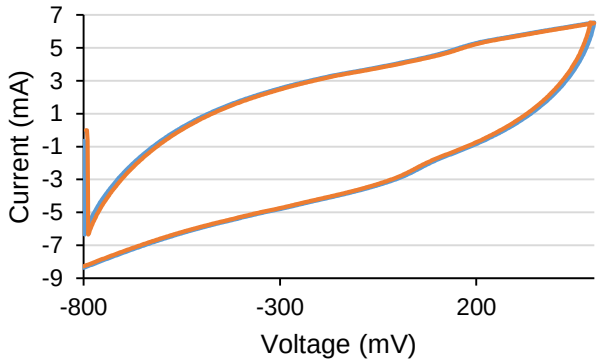

Figure 77: Cyclic voltammogram for the base media with acetate on a scan rate of 20 mV/s.

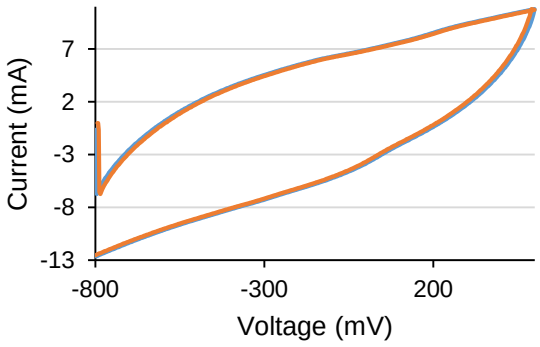

Figure 78: Cyclic voltammogram for the base media with acetate on a scan rate of 50 mV/s.

9.3.2 Base media - glucose

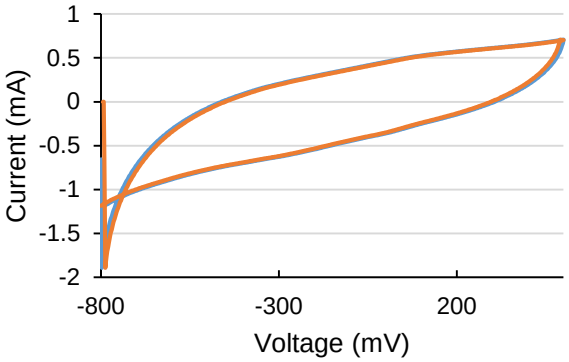

Figure 79: Cyclic voltammogram for the base media with glucose on a scan rate of 1 mV/s.

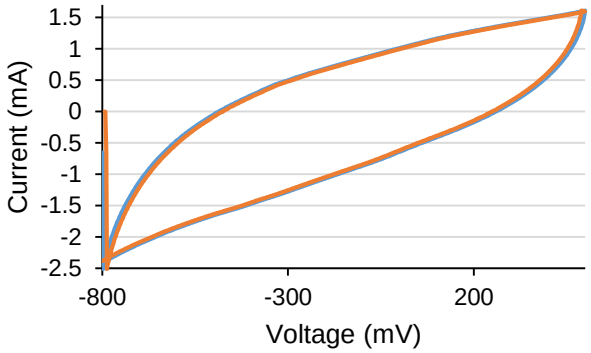

Figure 80: Cyclic voltammogram for the base media with glucose on a scan rate of 5 mV/s.

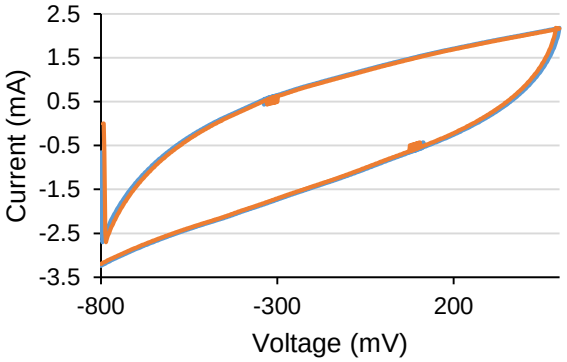

Figure 81: Cyclic voltammogram for the base media with glucose on a scan rate of 10 mV/s.

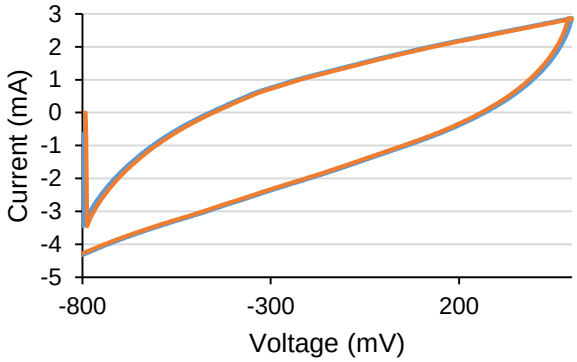

Figure 82: Cyclic voltammogram for the base media with glucose on a scan rate of 20 mV/s.

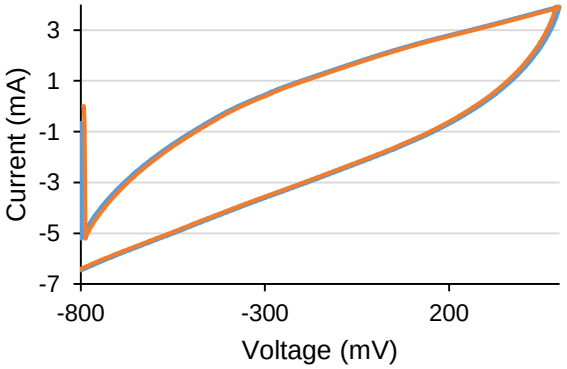

Figure 83: Cyclic voltammogram for the base media with glucose on a scan rate of 50 mV/s.

9.3.3 Neutral red – acetate reactor

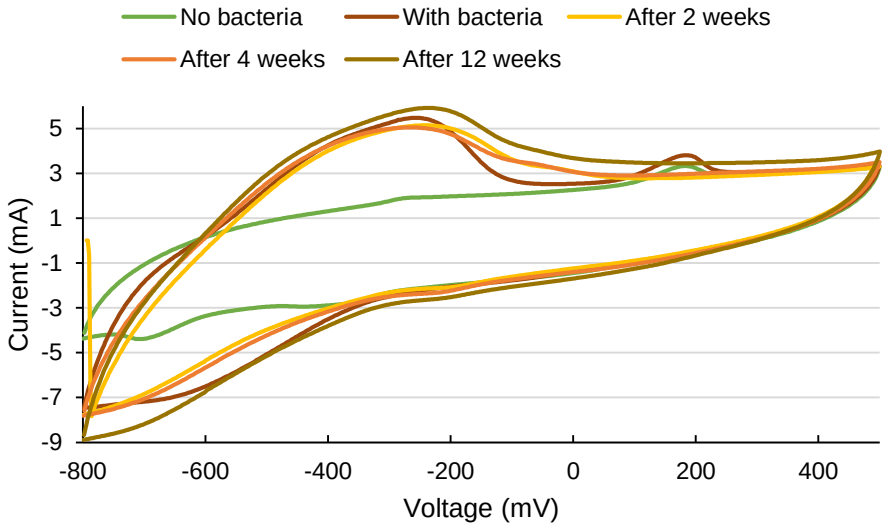

Figure 84: Scan rate of 5 mV/s for the cyclic voltammetry experiment with neutral red as mediator and acetate media.

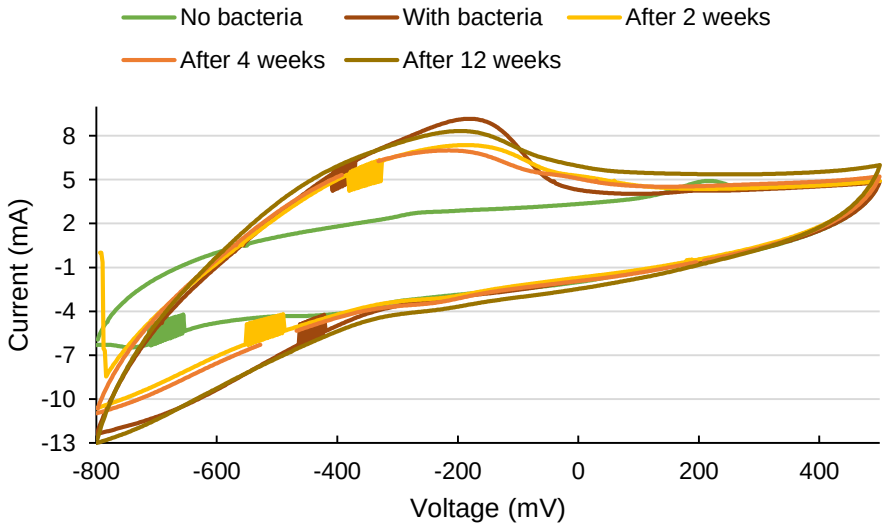

Figure 85: Scan rate of 10 mV/s for the cyclic voltammetry experiment with neutral red as mediator and acetate media.

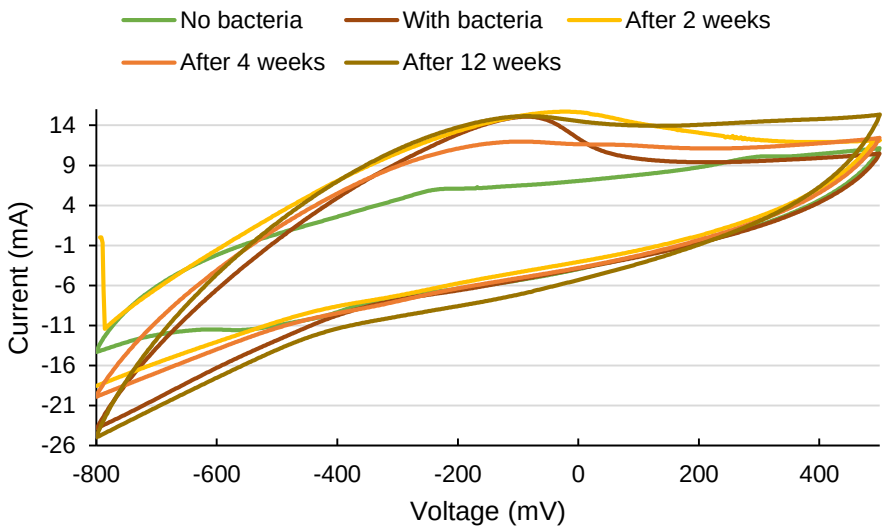

Figure 86: Scan rate of 50 mV/s for the cyclic voltammetry experiment with neutral red as mediator and acetate media.

9.3.4 Neutral red – glucose reactor

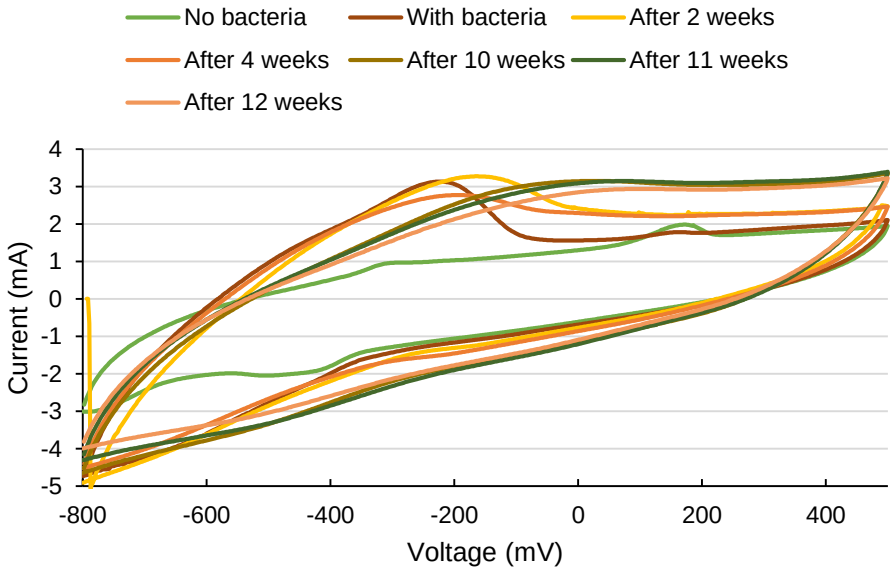

Figure 87: Scan rate of 5 mV/s for the cyclic voltammetry experiment with neutral red as mediator and glucose media.

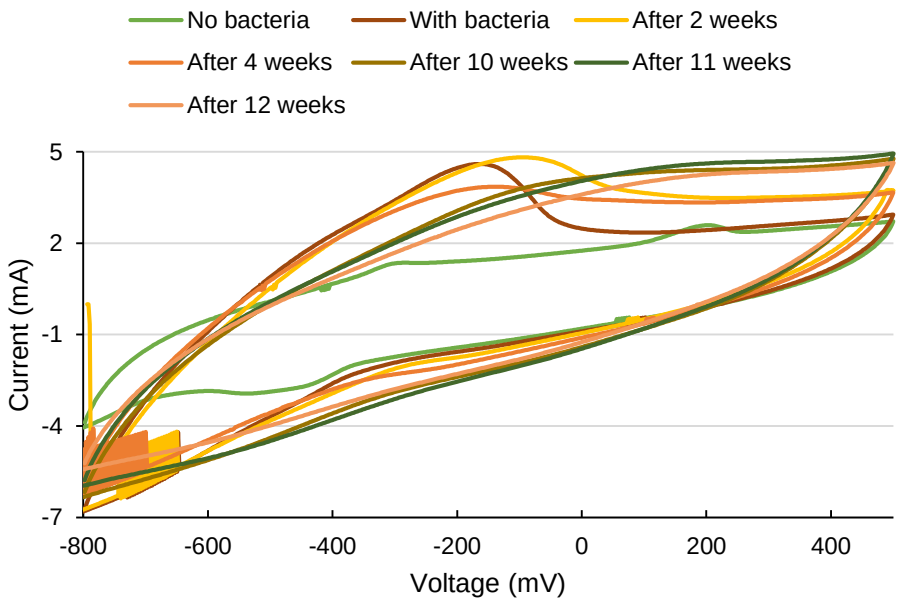

Figure 88: Scan rate of 10 mV/s for the cyclic voltammetry experiment with neutral red as mediator and glucose media.

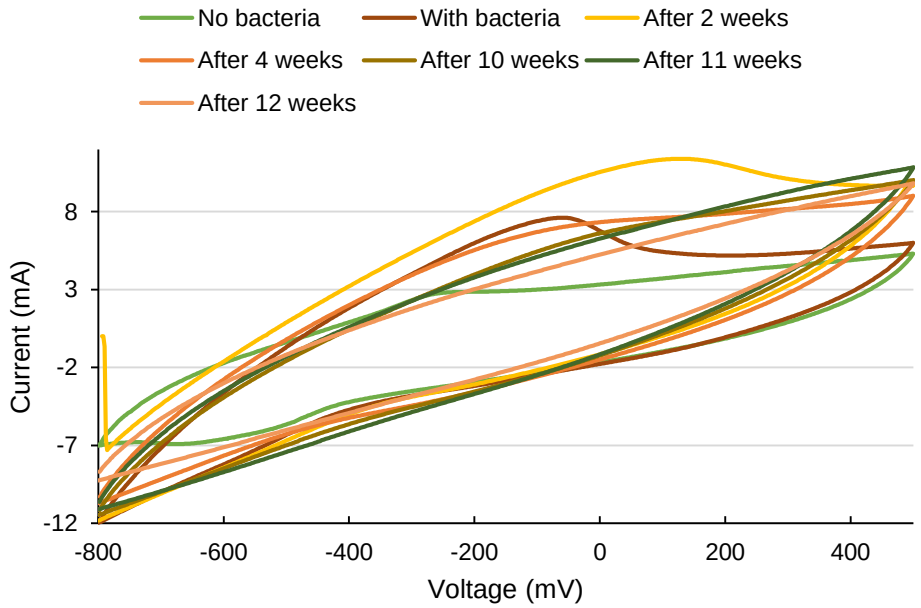

Figure 89: Scan rate of 50 mV/s for the cyclic voltammetry experiment with neutral red as mediator and glucose media.

9.3.5 Methyl viologen – acetate reactor

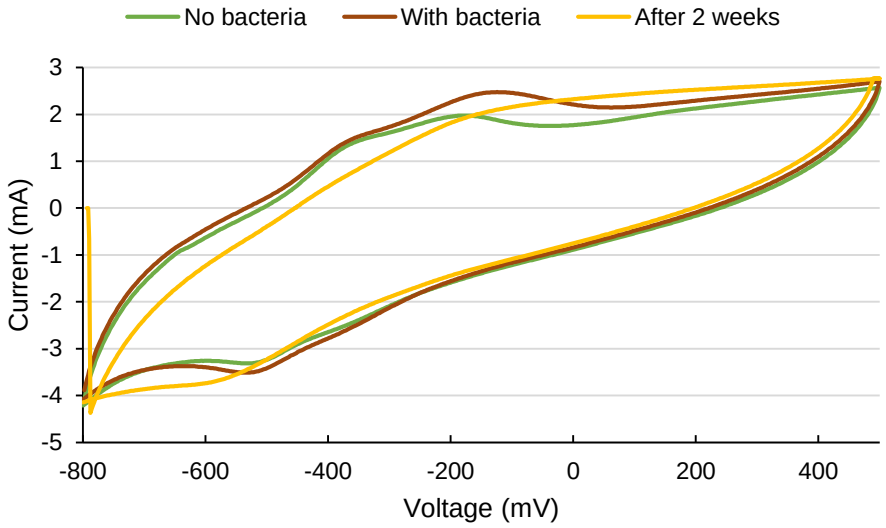

Figure 90: Scan rate of 5 mV/s for the cyclic voltammetry experiment with methyl viologen as mediator and acetate media.

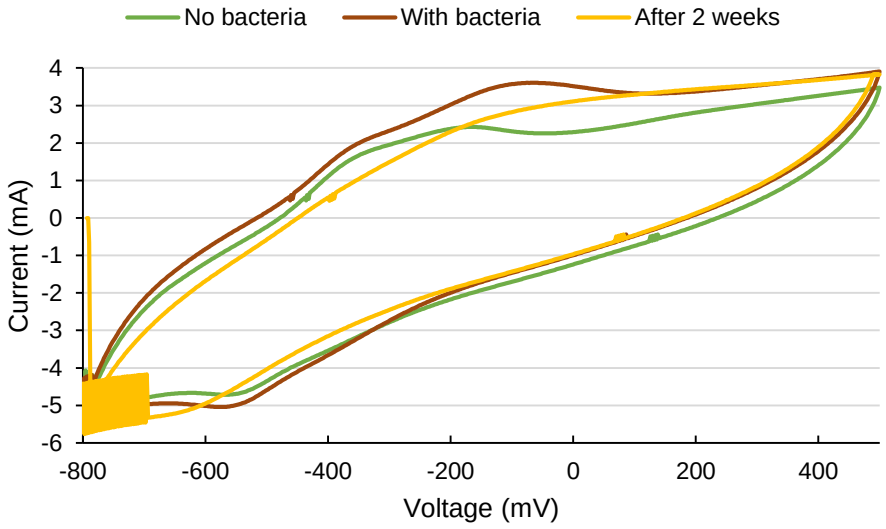

Figure 91: Scan rate of 10 mV/s for the cyclic voltammetry experiment with methyl viologen as mediator and acetate media.

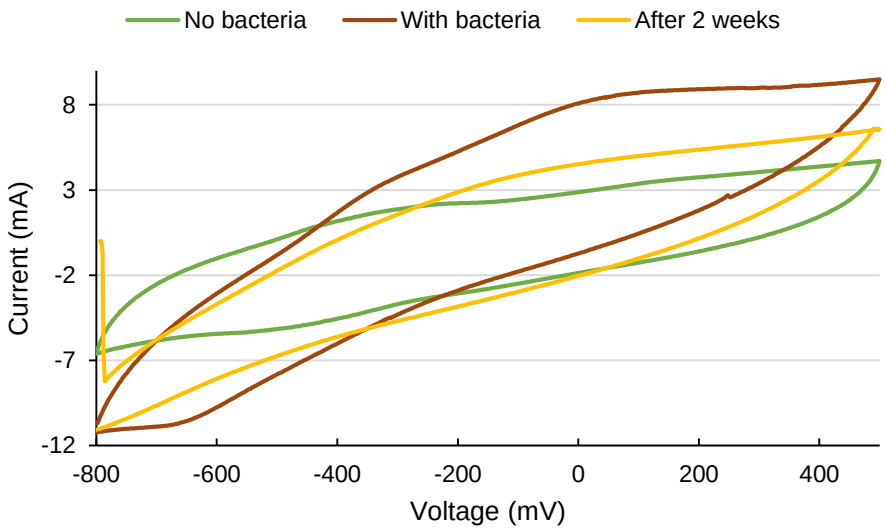

Figure 92: Scan rate of 50 mV/s for the cyclic voltammetry experiment with methyl viologen as mediator and acetate media.

9.3.6 Methyl viologen – glucose reactor

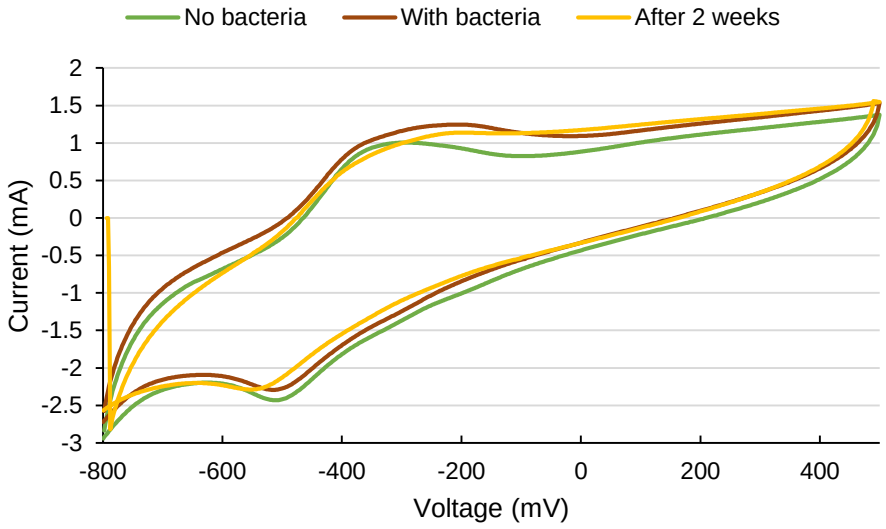

Figure 93: Scan rate of 5 mV/s for the cyclic voltammetry experiment with methyl viologen as mediator and glucose media.

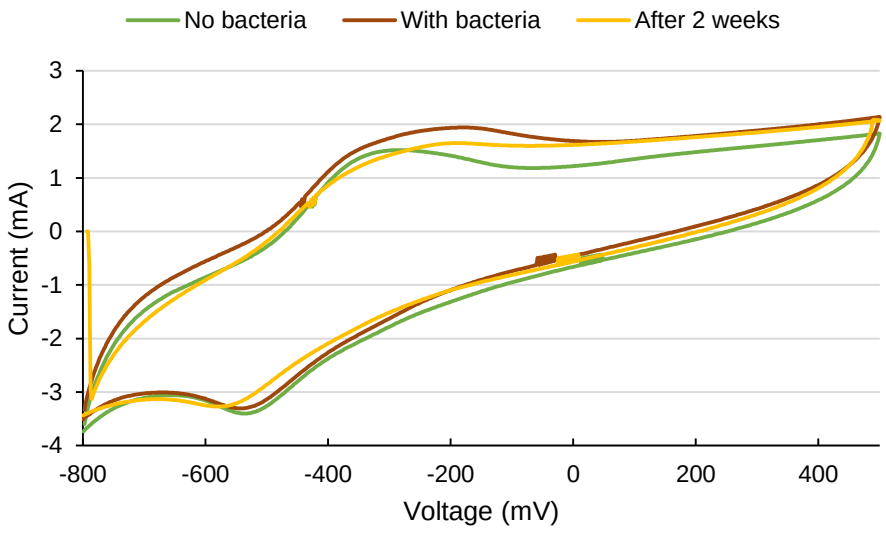

Figure 94: Scan rate of 10 mV/s for the cyclic voltammetry experiment with methyl viologen as mediator and glucose media.

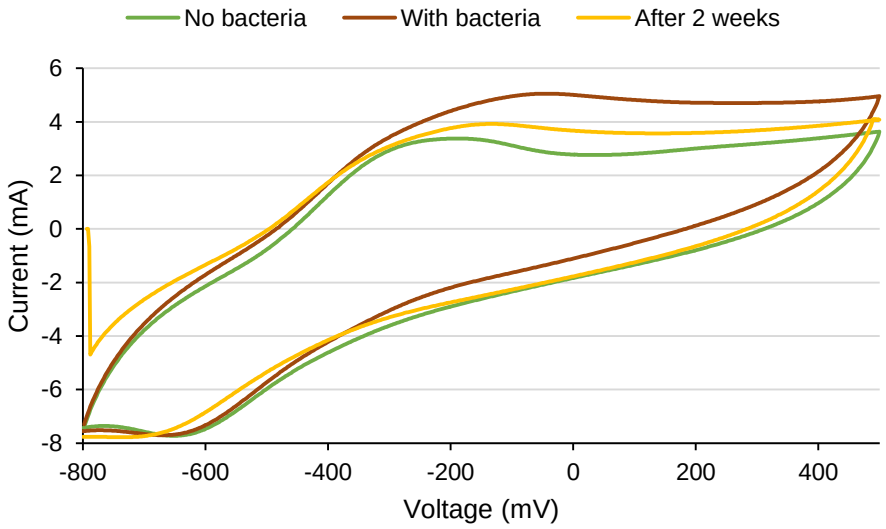

Figure 95: Scan rate of 50 mV/s for the cyclic voltammetry experiment with methyl viologen as mediator and glucose media.