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BIO-HYDROGEN PRODUCTION IN MICROBIAL ELECTROLYSIS CELLS (MECS) WITH ANALYSIS OF MICROBIAL BIOFILMS

Master thesis

In partial fulfilment of the requirements

for the degree of

Diplom-Ingenieurin

submitted by:

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Declaration of Authorship

I hereby declare that I am the sole author of this master thesis and that I have not previously submitted this work on another educational institution on purpose of receiving an academic degree. Sources of other people have been appropriately cited and the data gathered through the methods described have been accurately reproduced.

Vienna, _____ _____

Signature

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Sonia Abreu Dias, BSc.

Abstract

Hydrogen (H_2) is an important renewable fuel and energy carrier of the future. Burning hydrogen does not contribute to greenhouse gas (GHG) emissions, acid rain or ozone depletion since it generates water as a major by-product. But so far, H_2 is mostly produced from fossil fuels. As a result, microbial electrolysis cells (MEC) have emerged as a clean and sustainable alternative of hydrogen production through biological routes of wastewater bacteria. The aim of this work was to construct MECs which use acetate as the sole carbon source. Analytical measurements were done to understand the nature of hydrogen producing communities on an anode biofilm. Since there was the suspicion that hydrogen production correlates with the structure of the formed biofilm, deeper analysis with microscopic methods were performed. We proved that acetate consumption was only possible if an electric current was supplied and no additional H_2 was present in the system. MECs purged with N_2, were able of H_2 production whereas MECs purged with H_2/CO_2 showed the contrary phenomenon. Concerning biofilms, scanning electron microscopy (SEM) was used to observe cell variety, density and the shape of the biofilm. Employing fluorescence *in situ* hybridization (FISH) the species were determined but no ordered shapes on biofilms could be identified, and bacteria showed a thoroughly mixed pattern. Structural behavior could not be properly investigated, and more work must be done in this area. Furthermore, a DNA sequencing method, was used to compare MEC cell cultures to initial inoculum cultures from waste water treatment plant (WWTP) to show the evolution of microorganism communities. This thesis, is a preliminary work to a deeper understanding of biofilms and their spatial and functional phenotypes.

Keywords:

Microbial Electrolysis Cell, FISH, Acetate Oxidizing Bacteria, Biofilms, Confocal Microscopy

Zusammenfassung

Wasserstoff hat großes Potential der universelle Energieträger der Zukunft zu werden, da durch die Verbrennung von H_2 primäre H_2O erzeugt wird. Dadurch trägt die Verbrennung nicht zu Treibhausgasemissionen, saurem Regen oder Ozonabbau bei. Die Herstellung von H_2 wird jedoch meist mit Hilfe fossiler Rohstoffe durchgeführt. Die Produktion von H_2 durch Mikroorganismen in „Mikrobiellen Elektrolysezellen“ (MEC) ist daher eine attraktive Alternative. Diese können mit Hilfe von Abwasserbakterien H_2 über biologischen Wege aus Acetat herstellen. Das Ziel dieser Arbeit war die Konstruktion von MECs und deren Analysen. Um mehr über den Ablauf des Wasserstoffproduktionsprozesses zu erfahren, war es wichtig die Struktur und die Zusammensetzung des Biofilms an der Anode der MEC zu analysieren. Es hat sich herausgestellt, dass Acetatverbrauch nur möglich ist, wenn Strom geliefert wird und kein H_2 im MEC-System vorhanden ist. Befand sich bereits H_2 im System, haben die Zellen wiederum mehr Acetat gebildet.
Bezüglich der Biofilme, wurde Rasterelektronenmikroskopie (REM) verwendet um einen Überblick über die Zellvarietät, die Dichte und die Form des Biofilms zu bekommen. Durch „Fluoreszenz-*in-situ*-Hybridisierung“ (FISH) konnten die Spezies des Biofilms bestimmt werden, jedoch konnte keine geordnete Struktur des Biofilms identifiziert werden. Zusätzlich konnte durch DNA-Sequenzierung, die Bakterienkultur der anfänglichen Inokulums aus der Kläranlage mit MEC-Zellkulturen verglichen werden. Diese Thesis ist eine Vorarbeit zu einem tieferen Verständnis von Biofilmen sowie deren räumlichen und funktionellen Phänotypen.

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Abbreviations

CLSM	Confocal laser scanning microscopy
FA	Formamide
FISH	Fluorescence *in situ* hybridization
GC	Gas Chromatography
H_2	Hydrogen
HPLC	High-performance liquid chromatography
MEC	Microbial Electrolysis Cell
MES	Microbial Electrolysis System
PFA	Paraformaldehyde
WWTP	Wastewater treatment plant

1. Introduction

The work or this thesis was done as part of the H_2.AT project. The project takes place in cooperation with the University of Vienna. The goal is to elaborate an efficient system for complete biological conversion of organic matter, like glucose or cellobiose, to biohydrogen and CO_2.

1.1. General

The use of fossil fuels in recent years has accelerated the depletion of non-renewable resources. Furthermore, the unprecedented increase in greenhouse gas emissions due to combustion of fossil fuels causes global warming and climate change. Sustainable and carbon-neutral energy sources as alternatives to fossil fuels are highly needed to alleviate the global energy crisis and climate change (Rader and Logan 2010; Zhang and Angelidaki 2014). Notably, the production of high-value chemicals such as methane and biohydrogen with the aid of bio-electrochemical methods provides a highly attractive, novel route for the generation of valuable products from wastewater (Bajracharya *et al.* 2016; Kadier *et al.* 2014). It is a novel generation of bioenergy technology which possesses potential for simultaneous wastewater treatment and electric energy generation or valuable chemicals production (Zhang and Angelidaki 2014; Bajracharya *et al.* 2016). In this case, microorganisms, collected from the wastewater treatment plant (WWTP) in Tulln, Austria, promote hydrolysis of organic matter. Samples are screened to find a strain, which produces biohydrogen. Electroactive bacteria are selected and immobilized on a biofilm. Correlations between the formed biofilm, its microorganisms and the produced biohydrogen are examined.

1.2. Microbial electrochemical cells (MECs)

Bio-electrochemical systems (BES) consist of an anode, a cathode and, typically, a separating membrane. These unique systems convert chemical energy into electrical energy (and vice-versa) or other valuable products while employing microbes as catalysts. Sustainable technical solution can be provided by employing two major variants of BES: microbial fuel cell (MFC) and microbial electrolysis cell (MEC).

MECs are used in this work. The groundwork for the MECs used in this thesis was done by Maximilian Schmid during his master thesis (Schmid Maximilian 2017). They use electrochemically active bacteria, which interact electrochemically with the electrodes. In this way, they oxidize organic matter. The bacteria located on the surface of the electrode, work as biocatalysts which transfer electrons to the anode and release protons to the fluid (Rabaey and Rozendal 2010; Logan *et al.* 2008). The electrons then travel through a wire to the cathode and combine with the free protons in solution. However, this does not occur spontaneously. In order to produce hydrogen at the cathode, MEC reactors require an external power supply, but not higher than 0.8 V and 20 mA to avoid direct water electrolysis. Further, the system requires biologically optimal conditions of temperature, pressure and pH. Compared to traditional biological technologies, MECs overcome thermodynamic limitations to achieve high-yield hydrogen production at relatively mild conditions (Zhang and Angelidaki 2014; Bajracharya *et al.* 2016; Kadier *et al.* 2016). Applying MEC technology as an electrically driven hydrogen production technology allows the conversion of organic substrates, such as simple sugars or acetate, into hydrogen (Carmona-Martínez *et al.* 2015; Hu, Fan, and Liu 2008).

In most reported MECs membranes are used to separate the anode and cathode chambers. To reduce the potential losses associated with the membrane and to increase the energy recovery of this process, single-chamber membrane-free MECs present another option (Hu, Fan, and Liu 2008). Schematic diagram of a MEC is shown in Figure 1.

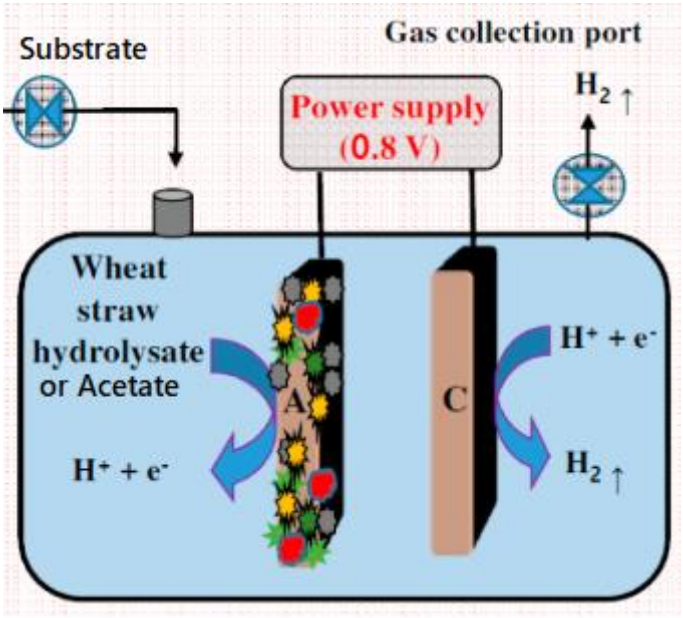

Figure 1: Scheme of single chamber MEC (Kumar et al. 2018)

In case acetate is used as substrate in MEC, electrode reactions are as follows:

Anode:

$$C_2H_4O_2 + 2H_2O \rightarrow 2CO_2 + 8e^- + 8H^+$$

Cathode:

$$8H^+ + 8e^- \rightarrow 4H_2$$

1.3. Microorganisms and anaerobic respiration

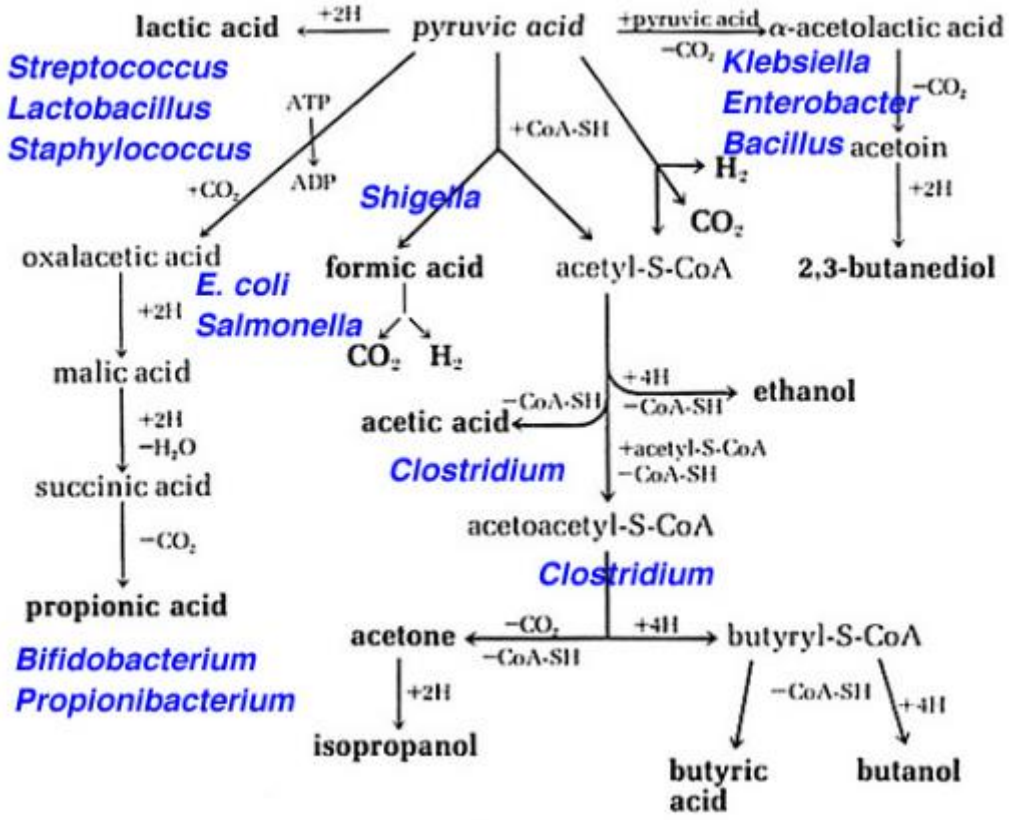

Figure 2: All fermentations in bacteria that proceed through the Embden-Meyerhof pathway with representive bacteria in blue ("Diversity of Microbial Metabolism" n.d.)

Glycolysis oxidizes glucose to two molecules of pyruvate while generating an output of two ATP molecules. Oxidation of glucose also produces two molecules of NADH. These need to be re-oxidized to NAD^+. This can be achieved by producing lactate, succinate, ethanol, butanol, butyrate, etc. However, production of some excreted catabolites, such as acetate, is associated with production of additional energy in form of ATP. An overview of the metabolic pathway in the anaerobic respiration process is shown in Figure 2. Nevertheless, a possible ending of the pathway is acetate, which is considered as a waste product. The question arose, if there is a possibility for further use of this by-product to accomplish a complete conversion into CO_2 and H_2. Production of biohydrogen by biological routes is either done on an anaerobic or enzymatic way or by microbial electrolysis cell (MEC), also called electro-fermentation.

The advantage of MEC is that the energy available in waste streams can be directly recovered as hydrogen (Hallenbeck *et al.* 2009). However, MEC process oxidation of acetate requires incorporation of a moderate external voltage to overcome the thermodynamic limitations of acetate conversion into hydrogen. After all the metabolic pathways involved in MECs are not fully understood yet (Hallenbeck *et al.* 2009). To understand this process, *Geobacter sulfurreducens* and *Shewanella oneidensis* are studied intensively. Those electrochemically active microorganisms are poised to oxidize acetate as an electron donor and effectively couple their metabolism to electrode surfaces (Dopson, Ni, and Sleutels 2016). The electrode operates as external electron acceptor (Hallenbeck *et al.* 2009).

In general, microorganisms which build a biofilm on the anode, act as catalysts and pass electrons to the anode. The transfer of electrons is done either by 'direct' or 'indirect' mechanisms (Dopson, Ni, and Sleutels 2016). Indirect transfer of electrons to the anode involves soluble redox shuttles. Mediators can be either organic (e.g. humic acids) or inorganic (e.g. the SO/H_2S shuttle). Direct transfer of electrons enables electron flow directly from the membrane or pili to the anode (Rabaey and Rozendal 2010).

In this case study, single chamber cells are used. The major issue with the absence of the membrane in MECs is the microbial hydrogen losses to methanogens. Methanogens compete with electrochemically active bacteria for both substrate (CH_3COONa) and product (H_2) (Kadier *et al.* 2014). Bromoethane 2-sulfonate is commonly used to inactivate methanogens.

A further issue of MECs is the potentially production of acetate from CO_2 and H_2 as soon as H_2 is present. This is done by acetogenic bacteria which employ the Wood-Ljungdahl pathway (WLP).

$$2\ CO_2 + 4\ H_2 \rightarrow CH_3COOH + 2\ H_2O$$

Early researches on hydrogen production were typically restricted to the usage of single cultures by feeding with a defined substrate. On the other hand, when carbon-rich wastewater is used as carbon source, mixed culture might be favorable to produce biohydrogen at large scale. Certainly, mixed microbial populations are preferred due to operational simplicity and stability. By using wastewater bacteria a wide variety of substrates can be used as energy source, since bacteria show many different biochemical pathways, and sterilization process can be avoided (Hallenbeck *et al.* 2009).

1.4. *Shewanella oneidensis* and *Geobacter sulfurreducens*

Bacteria of the family *Geobacteraceae* perform complete oxidation of organic substrates with electron transfer directly to the anode. Usually, *Geobacteraceae* utilize simple organic acids as carbon sources and do not utilize sugars (Debabov 2008; Rabaey and Rozendal 2010). Another well-known electro-chemically active bacterium is *Shewanella oneidensis* str. MR-1. For direct electron transfer between bacteria and electrode surface, the electrons should reach the outer membrane of the cell. Direct transfer typically involves at least a series of periplasmic and outer-membrane complexes. In case of *S. oneidensis*, the apparent terminal cell-bound complex is a cytochrome located on the outside of the membrane and capable of donating electrons. For *G. sulfurreducens*, investigations pointed out the importance of conductive pili. A deletion of the pilA gene led to an inhibition of reduction reactions. Furthermore, the formation of pili has to be stimulated for *G. sulfurreducens*. It has been proven that pili formation depends on growth conditions. They formed best in presence of fumarate at suboptimal temperature (25° C) (Debabov 2008). Moreover, both bacteria are, like most electroactive bacteria, proteobacteria which grow in an anaerobic environment. Both are heterotroph, mesophyll and gram negative (Odio, 2011).

1.5. Structured biofilms

A biofilm is a complex microbial community which is surface-attached and growing embedded in a self-produced matrix of extracellular polymeric substances (EPS). It shows properties of a multi-cellular organism forming three-dimensional complex structures depending on the species, the strain and environmental conditions (Pantanella *et al.* 2013). The produced extracellular polymeric substances act as a matrix which protects and renders bacteria

extremely resistant to environmental unfavorable conditions or antibiotics (Nistico 2009). However, for not known reason, a structured biofilm is difficult to cultivate and is therefore poorly investigated. Considering the biological complexity and heterogeneity of the structure, the composition might be linked to the function of the bacteria along with the extracellular electron transfer on the anodes of a MEC (Carmona-Martínez *et al.* 2015).

The range of bacteria capable of biofilm formation on electrodes is relatively broad. Some of the members of these associations are possibly symbionts of electrogenic bacteria and do not participate in direct electron transfer to the electrode. The composition of bacterial associations in the anode chamber depends on the composition of the substrates, wastewater and on the symbiotic relationships within the population. Not all bacteria in the population participate in direct electron transfer to the electrode. Even within the biofilm, not all bacteria can have direct contact with the electrode. This contact may be achieved indirectly via endogenous electron transporters (Debabov 2008).

Figure 3 represents a possible phenotype of bacterial biofilms. Cells of different colors represent different cell lineages. On (a) cells are solitary on a surface. There is no obvious order, bacteria are supposed to bind randomly on a target. (b) When biofilms contain segregated genetic lineages at a high population density, cooperative public goods are often favorable. (c) shows a typical behavior when biofilms contain mixed lineages at high population densities. In this case, interactions are expected to be predominantly antagonistic, although interstrain commensalism or mutualism is possible. (Nadell, Drescher, and Foster 2016)

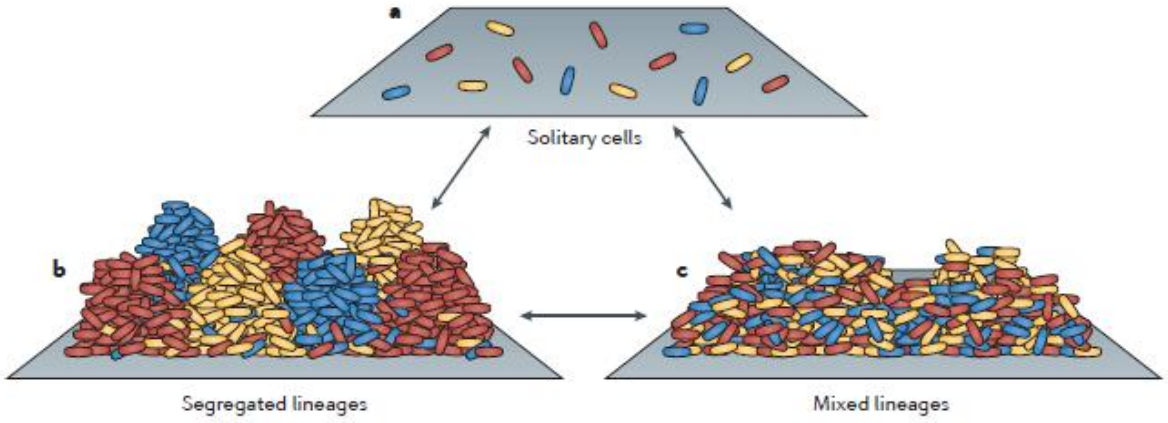

Figure 3: Possible structures in microbial biofilms (Nadell, Drescher, and Foster 2016)

Fluorescence *in situ* hybridization (FISH) and confocal laser scanning microscopy (CLSM), can be used for the simultaneous analysis of the spatial distribution of both Gram-positive and Gram-negative bacteria in biofilms. This technique is a recognized tool for the specific and sensitive identification of target organisms within complex microbial communities. The standard protocol “FISH Protocols, B. FISH with Fluorescently Monolabeled Oligonucleotide Probes,” n.d. (by SILVA ribosomal database project), explains the method generally used for liquid culture. For any application on biofilms, the standard FISH protocol is adapted. Certainly, several sample parameters cause low probe signals, such as penetration problems of the

probe through the cell wall boundary, or low ribosome content (Nistico 2009). However, FISH in combination with CLSM allows the maintenance the biofilm's natural architecture and the investigation of the spatial arrangement of bacteria in a multi-species biofilm.

2. Fundaments and Objectives

The aim is to convert glucose to acetate and then further to H_2 by syntrophic microorganisms. Here, acetate oxidation is a crucial step, which is done in MEC. A voltage of 0.8 V is applied, which is necessary to overcome thermodynamic limitations.

The cathode reaction, for acetate substrate, is expected to be as follows: $8H^+ + 8e^- \rightarrow 4H_2$. To prove the accuracy of this reaction, gas but also cell suspension sampling was done once a day and analyzed. During my master thesis, my tasks also consisted in the growth as well as in the maintenance of the wastewater derived electroactive biofilms. A mixed culture taken as a mixture of anaerobic and aerobic sludge from a wastewater treatment plant (WWTP) served as inoculum for the MECs. Since hydrogen production was proven possible, the MEC design was relatively similar to the study of Hu, Fan, and Liu, 2008.

A further task was the analysis of the biofilm growing on the anode of a MEC. The objective was to visualize three-dimensional structure of the biofilm and to observe any interaction between bacteria species. A further objective was to get a deeper insight into the structure and the settled down bacteria. Some of the members of these associations are possibly symbionts of electrogenic bacteria. Others may not participate in direct electron transfer to the electrode. The idea is that microbial consortium in the structured biofilm will allow a tailored and efficient hydrogen production process.

A major part of this master thesis work is based on microscopic methods. This includes scanning electron microscopy (SEM), to visualize the surface of microcolonies as well as old biofilm and confocal laser scanning microscopy (CLSM) for deeper study of biofilm composition, spatial structure and social interaction. With CLSM it was possible to visualize the topography of different bacteria in a multispecies biofilm. Besides bright field visualization, the focus was on fluorescence *in situ* hybridization (FISH). FISH is a molecular technique for the identification and three-dimensional characterization of microorganisms. The method uses fluorochrome-labeled DNA oligonucleotide probes which bind to targeted sequences of the specific cell and allows *in situ* detection of bacteria (Nistico 2009). These probes are specifically designed to bind to specific cells and adopted for studies on multispecies biofilm. Nevertheless, there are limits related to the complex preparation procedure, the fixation of the sample and the high number of variable parameters. Further, the technique is time consuming (Pantanella *et al.* 2013). Moreover, application of the standard FISH protocols to visualize bacteria in biofilms from a laboratory-scale wastewater reactor produced only weak signals. To increase signal intensity, standard FISH protocol was modified: either a supplement enzymatic pre-treatment of fixed cells was done or the hybridization time of the FISH protocol was adapted (Pavlekovic *et al.* 2009).

3. Material and Methods

3.1. MEC

Media

Medium for MECs

Since bacteria are supposed to use acetate as carbon source; the media is produced with the following substances:

Table 1: Medium for MEC, also called Klasson medium

Substance	Concentration [g/L]
NaH_2PO_4	2.5
Na_2HPO_4	4.6
Pfennig Mineral Solution	50
Vitamins	10
Trace elements	10
Na-Acetate (Carbon source)	6
Sodium 2-bromoethanesulfonate	8

Whereby, vitamins and trace elements are produced as described on Medium 141 of the DSMZ- Site (DSMZ GmbH 2017). Bromoethane-2-sulfonate is used to inhibit methane production.

Table 2: Mineral solution

Pfennig Mineral Solution	Concentration [g/L]
KH_2PO_4	10
$MgCl_2*6H_20$	6.6
NaCl	8
NH_4Cl	8
$CaCl_2*2H_20$	1

At the end the pH is set to 7 and the reactors are sparged either with 100 % N_2 or with an 80% H_2/CO_2 mixture for 2 min to create anaerobic conditions.

Growth Medium for *G. sulfurreducens*

Table 3: Components for growth medium for G. sulfurreducens

Substance	Concentration [g/L]
NH_4Cl	1.50
Na_2HPO_4	0.60
KCl	0.10

Na-Acetate	0.82
Trace element solution	10 mL
$NaHCO_3$	2.50
Na_2-fumarate	8.00
Vitamin solution	10 mL

Medium composition is the same as found on the DSMZ-homepage under number 826. Trace elements and vitamin solution are also produced as described on DSMZ- Site (DSMZ GmbH 2017)

Moreover, Vitamin solution as well as Na_2-fumarate are added after the autoclaving process. Both components are sterile filtered into the medium. The pH is adjusted to 7.

Tryptic Soy Broth (TSB)

TSB, is a universal- enrichment medium from the company Merck with following composition: Casein (pancreatic digest) 17 g/L, Soya peptone (papaic digest) 3 g/L, Sodium chloride 5 g/L, Dipotassium phosphate 2.5 g/L and Dextrose 2.5 g/L. These nutrients support the growth of multiple organisms and are often used for aerobic and facultative anaerobic bacteria. In our case, TSB was used for *S. aureus* (see Biofilm) and *S. oneidensis* cultivation.

Inoculum

A mixed culture taken as a mix of anaerobic and aerobic sludge from a domestic WWTP in Tulln, Austria serves as inoculum for the MECs.

Furthermore, *Shewanella oneidensis* str. MR-1 and *Geobacter sulfurreducens* are used as reference. Both are electroactive bacteria and are able to exchange electrons to the anode. They are important to show if the single-chamber MEC setup is working.

Pure cultures are important to provide well-controlled systems and produce methane-free gases in MECs. Though, *Shewanella oneidensis* MR-1 in a single-chamber MEC, showed much lower H_2 production rate as in mixed cultures. *Geobacter sulfurreducens* strain produced H_2 in MECs at rates and recoveries comparable to mixed cultures.

MEC setup (CELL, pH and Temperature)

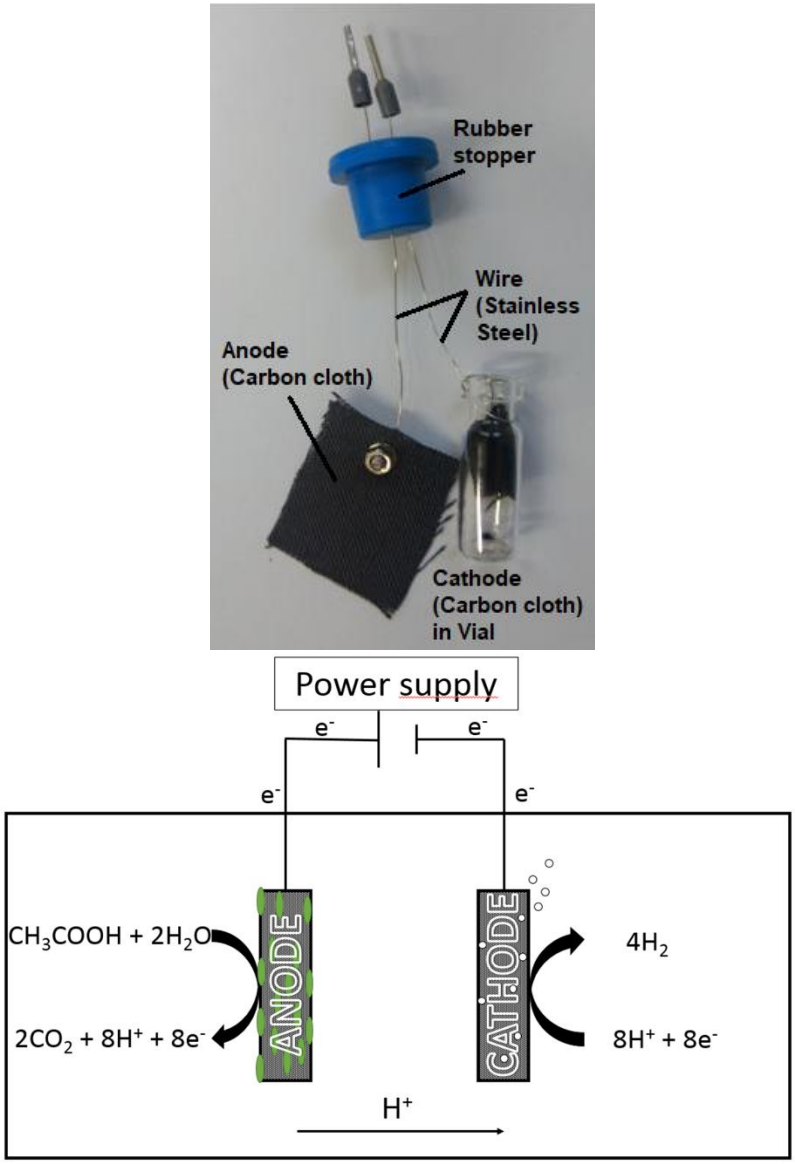

Figure 4: Electrodes used in MEC (left) schematic representation on MEC (right)

MEC reactor serum vials (100 mL) are used, which can be closed airtight with a rubber stopper. The anode (3 x 3 cm) and the cathode (2 x 1 cm) are made of carbon cloth as shown in Figure 4. The cathode is spread with a thin layer of a Platinum-Nafion mixture (15 mg Platinum in 200 µL Nafion) working as catalyst. Since H_2 is produced at the cathode, a vial is used to cover the cathode and to catch formed H_2. In this way, gas formation is easily visible in the vial surrounding the cathode. In the case gas is produced, bubbles appear round the cathode and the media is displaced by the produced H_2.

Figure 5: Experimental setup: MEC in incubator and connected to power supply and computer

Both electrodes are fixed by a metallic stainless wire to keep and stabilize them in the medium. The wires are attached to the rubber stopper and connect the electrodes to an exogenous power supply (Figure 5). Power should not be higher than 0.8 V and 20 mA to avoid direct water electrolysis.

The operation is done in a batch mode. Therefore 45 mL medium is filled into the flasks and closed with the rubber stopper construction. After autoclaving, the MECs are inoculated with 5 mL of bacteria preculture.

Since the system should be completely anaerobic, two MECs are purged with N_2 gas for 2 minutes and the other two MECs are purged with CO_2 and H_2 (20:80) gas for 2 minutes.

Considering that H_2 may escape through the rubber stopper of the reactor, the MECs are turned upside down. Hence, H_2 is prevented to escape easily and it is mostly collected in the surrounding vial. The MECs are fixed on a shaker, as movement provide a greater uniformity on system parameters, such as temperature, mixing, chemical concentration, and substrate concentration (Usack, Spirito, and Angenent 2012).

While slowly shaking, MECs are incubated and maintained at a temperature of 37°C and 50°C.

Analysis of MEC

Measurement of electrical current

Current directly relates to the hydrogen production rate as the electrons that travel to the cathode are eventually converted into hydrogen gas (Kadier *et al.* 2014). A single measurement was taken every minute by the system.

pH

Bacteria just grow in a certain pH range. For this reason, the pH of each media had to be adjusted to 7 and regularly controlled over the experiment. Initial pH adjustment was measured with a pH meter and if necessary adjusted with 0.5 or 1 M NaOH or HCl. Furthermore, pH was monitored with pH paper or a micro pH electrode.

Gas chromatography (GC)

For gas analysis, gas chromatograph « Agilent Technologies 7890A GC System » with a flame ionization detector (FID) is used. In regular intervals of 24 hours, sampling is done by piercing the rubber stopper of the MEC with a gastight syringe (Hamilton Samplelock Syringe). 250 µL gas are taken from the headspace and immediately injected into the CG column. N_2, H_2, O_2, CH_4 and CO_2 are systematically quantified and evaluated using the corresponding « Instrument 1 online » software.

High performance liquid chromatography (HPLC)

Further analysis is done by HPLC to determine volatile fatty acids such as acetic, propionic, butyric, isobutyric, valeric, isovaleric acid and so on. This is necessary to have an exact overview of the oxidized and formed products in the medium. For this reason, sampling is done every 24 hours: 1 mL of medium is taken for Carrez precipitation. Carrez clarification is an essential preparation for the HPLC analysis. This method is used to precipitate proteins, eliminate turbidity-causing materials, or to break emulsions in samples in which the subsequent analysis may otherwise be interfered with as a result of these constituents (Merck 2018). « Agilent Technologies 1260 Infinity II » is the used HPLC. The samples are isocratically pumped with 0.5M H_2SO_4 eluent through the column. The used column “ICSep Ice-300COLUMN ICE-99-9850” separates weak acids by ion exclusion and is specially designed for concise analyses of organic acids. Separated sample components are detected and quantified with a FID and displayed on the software « LCRI2(online)» as chromatogram.

3.2. Biofilm

Inoculum

In a second step, experiments are done to create a structured biofilm. On this purpose, *Staphylococcus aureus*, *Azotobacter vinelandii, Shewanella oneidensis*, *Geobacter sulfurreducens*, *Clostridium acetobutylicum* and as well as an unknown bacterial sample from WWTP have been cultivated.

Media

Tryptic Soy Broth (TSB)

TSB was used for *S. aureus* and *S. oneidensis* cultivation.

Burk Medium

Burk medium is used for the growth of *A. vinelandii*. Following components are used:

Table 4: Burk medium for A. vinelandii

Substance	Concentration [mg/L]
Glucose	10 000
NaCl	100
$CaSO_4$	25
$MgSO_4*7H_2O$	100
$Na_2MoO_4*2H_2O$	1.45
$FeCl_3$	13.5
K_2HPO_4	330
KH_2PO_4	80

In this case, pH is set to 7.

PYG Medium

For *C. acetobutylicum*, medium number 104b on DSMZ site is prepared. Following components are used:

Table 5: PYG medium components for C. acetobutylicum

Substance	Concentration [g/L]
Before autoclaving	
Trypticase peptone	5.0
Peptone from meat	5.0
Yeast extract	10.0
Salt solution	40 mL
After autoclaving	
L-Cys-$HCl*H_2O$	0.5
Glucose	5.0

The pH is set to 7.

A 25x concentrate salt solution is done with following components:

Table 6: Mineral composition for PYG Medium

Substance	Concentration [g/L]
$CaCl_2*2H_2O$	0.25
$MgSO_4*7H_2O$	0.50

K_2HPO_4	1.00
KH_2PO_4	1.00
$NaHCO_3$	10.0
NaCl	2.00

Cultivation of a structured biofilm

S. aureus and *A. vinelandii* are aerobe bacteria with different growth conditions. Whereas *S. aureus* grows best on TSB Medium at 37°C ("Details: DSM-2569" n.d.), while *A. vinelandii* grows best on Burk Medium at 30°C ("Details: DSM-2289" n.d.). Furthermore, the different doubling times, are also taken in consideration. Consequently, it is important to find the optimal growth condition for both aerobe strains.

The same is done for *S. oneidensis*, *G. sulfurreducens* and *C. acetobutylicum*, which are anaerobically cultivated. Also *S. oneidensis* grows best on TSB Medium at 30°C ("*Shewanella Oneidensis* Venkateswaran *et al.* ATCC ® 700550™" n.d.), whereas *G. sulfurreducens* on DSMZ-Medium no. 826 and 30°C. Also *C. acetobutylicum* needs a different medium for best growth conditions.

To perform a structured biofilm bacteria strains are cultivated in pure culture and mixed right before fixation. Alternatively, a bacteria strain is cultivated a few hours in a 24-multiwell plate with carbon cloth, subsequently media is exchanged followed by inoculation with a second bacteria strain. A further experiment consists on letting two bacteria strains grow together. In this way, competitive, cooperative or antagonistic phenotypes patterns can be investigated. Besides, to meet the best growing conditions of two different strains, medium, temperature and incubation period are adapted.

An unknown bacterial biofilm sample from the MECs anode experiment has been investigated. This is necessary, to perform a better understanding of the ecology in microbial communities of the MECs.

Analysis Method 1: Fluorescence in situ hybridization (standard FISH)

The main method to analyze the biofilm on the anode is by fluorescence *in situ* hybridization. This method uses fluorochrome labeled oligonucleotide probes. The fluorescently conjugated 16S rRNA oligonucleotide probes are applied on fixed and permeabilized bacterial cells. The fragile membrane allows dehybridization solution and probes to enter the cells and specifically hybridize their complementary target sequences. If probes bind within the cells, they emit fluorescent signal when excited on confocal laser scanning microscopy (Nistico 2009). A schematic overview of the FISH process is shown in Figure 6.

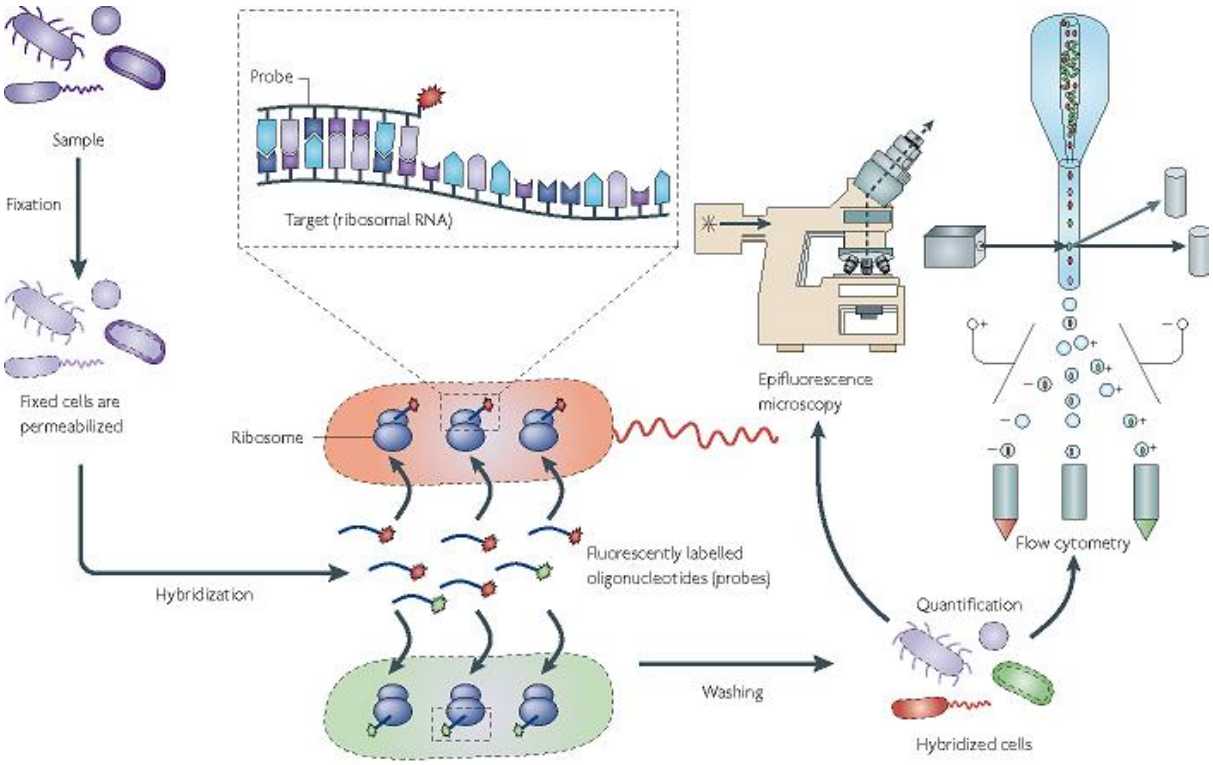

Figure 6: Schematic overview on FISH process (Amann and Fuchs 2008)

Detailed protocol on how to use FISH, is found in Standard FISH protocol, see Annex 1.

In this case, either 1 mL of cell suspension or a 1x1 cm square of carbon cloth with biofilm is investigated.

Conditions are adjusted to the type of cells. This includes the variation of the incubation temperature, the formamide (denaturant) concentration, fixation conditions and permeabilization of cell membrane.

Permeabilization of cell membrane is increased by different enzymatic (lysozyme and protease) pre-treatment procedures on the cell walls prior to the hybridization.

During this thesis, four different probes are used: two probes targeting anaerobic bacteria of *Shewanella* and *Geobacter* genus (SHEW, GEO2+ Helper1+ Helper2), a probe targeting gamma proteobacteria (GAM) and the general reference bacteria probe, consisting out of EUB338-I, EUB338- II and EUB338- III.

The probe sequences and target sites for these probes are listed in Table 12 and Table 13, see Annex 2.

For a better signal, Protease from "*Bacillus licheniformis*" as well as "Lysozyme from chicken egg white" is added to the sample before applying standard FISH protocol.

Microscopy and image analysis

µL-Slides containing 18 wells, from ibidi, are used for suspension cultures. The depression of the well in the slide is suitable for confocal laser scanning microscopy (CLSM). Mounting and procedure are explained in detail in Annex 1. No mounting oil is used. Instead, all the samples are mounted with water prior microscopic observation. For image acquisition, Leica SP8-

gSTED super-resolution microscope with HC PL APO 63x/1.2 Water including Corr CS2, objective is used together with Leica Las X software. Signal intensity is adjusted by changing the gain of the laser power. According to FISH-probes labels, the samples are analyzed in a sequential mode by following channels (max. excitation/emission in nm): Cy3 (548/562) for EUB/ GEO, FITC (490/525) for GAM, Cy5 (650/670) for SHEW, DAPI (405/425) for DAPI signal.

Analysis Method 2: Microbial populations in MEC - Sequencing

Sequencing is used to prove the presence of certain bacteria on the anode biofilm and within the cell suspension community. Therefore, 1 mL of cell suspension is taken from a MEC and filtrated before adding stabilization reagent. Also 1 x 0.5 cm square from the anode is cut out and subsequently treated with DNA Mini Kit (from Qiagen) to extract total nucleic acid. The samples are send to colleagues of the biogas group, within the institute of Environmental Biotechnology for sequencing.

Analysis Method 3: Scanning electron microscope

SEM microscopy was done in the Core Facility of Cell Imaging and Ultrastructure Research - Faculty of Life Sciences - University Vienna. They offer critical point drying service and provide a protocol for pretreatment of bacterial samples:

The pretreatment consists of a fixation with 2.5% glutaraldehyde and an in-depth washing with 0.1M Sodium cacodylate buffer (pH 7). In addition, the sample is dehydrated in increasing ethanol concentrations for several minutes before final storage in 100% ethanol.

Critical point drying is performed by a responsible of the imaging center in the University Vienna. The duration of the procedure depends on the thickness of the samples but takes mostly 1 hour.

The sample is set on a mounting platting and gold coated to achieve a better picture quality. The SEM micrographs are taken with high-vacuum technology and at 20.0 kV.

4. Results

4.1. MEC

There are two approaches to identify H_2 production:

1) appearance of an electric current and

2) gas formation around the cathode which is analyzed by GC along with acetate consumption.

Sample from WWTP

MECs incubated in 37°C

Electric current

MECs have been set up in a 37°C incubator under constant shaking and daily gas flush in addition to gas analysis.

Experiments have been carried on for 72 days. During the first weeks, no clear trend was observable. After day no. 29, electric current appeared on two MECs.

A small batch of four MECs have been analyzed, whereas two have been daily purged with a gas mixture of 20% H_2 and 80% CO_2 and two further with pure N_2 gas. To present an overview of the results, an average of the respectively two samples is calculated and shown in Figure 7.

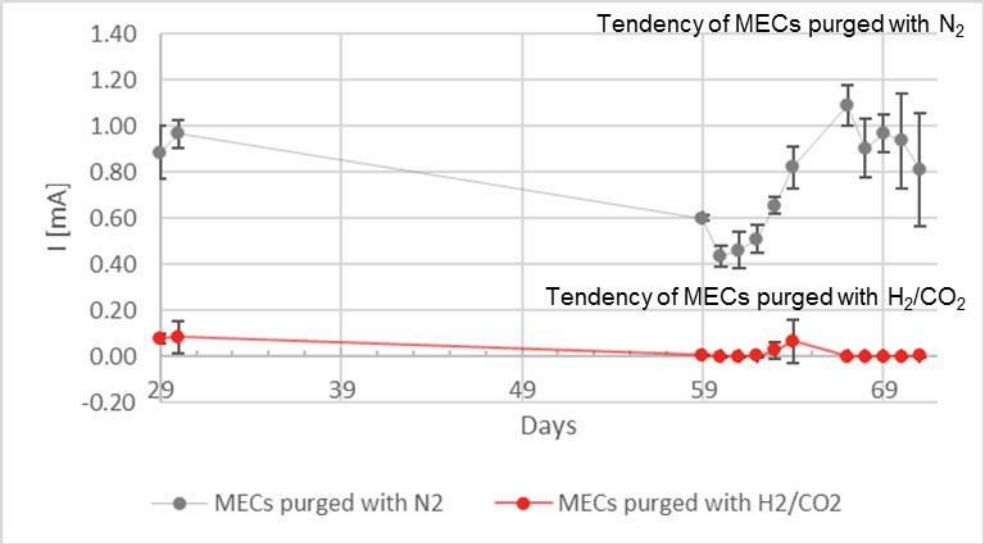

Figure 7: Current flow over the time for MEC purged with N_2 (grey) and MEC purged with H_2/CO_2 gas mixture (red). Average per day is calculated.

A gap between day 30 and 59 is due to holiday break. It is shown, that current tendencies remain stable along the batch period. Fluctuations, shown by the error bars, were caused by manipulation of the cells. Measurements presenting a very high value, were excluded, since they have been caused by short-circuit power and would mislead the final results.

It is shown in Figure 7, that those MECs daily flushed with N_2, were able to produce electrical current, whereas the MECs flushed with H_2/CO_2 did not show any significant change.

GC analysis

250 µL gas taken from the headspce of the MEC, were daily analysed by GC.

Figure 8 illustrastes the relation of measured CO_2, H_2 and CH_4. It was expected that H_2 would be around 80%, CO_2 around 20% and CH_4 0%.

Actually, GC analytics shows 64.5 % H_2/21.88 % CO_2 instead of the 80/20 ratio. This could have various reasons:

- Incorrect machine analytics
  To avoid this probleam the calibration was verified using standards
- Loss of H_2 during sampling
  This problem is not totally avoidable, but due to its small influence negligible.
- H_2 was metabolised by the bacteria
  CH_4 metabolism is shut down, since no positive results have been measured. This is due to the supplement of Bromoethane 2-sulfonate. Still, homoacetogenic beactria can utilize H_2 and CO_2 to produce acetate.

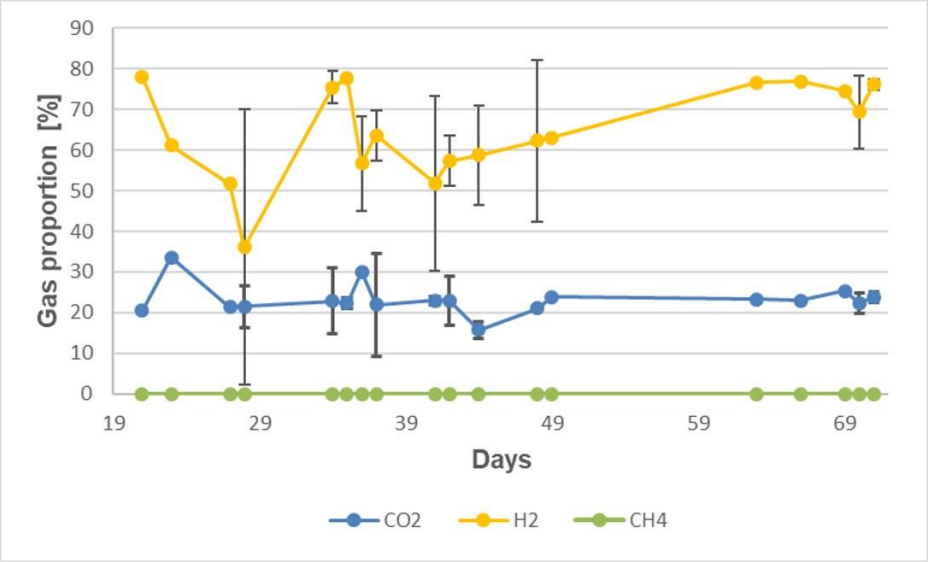

Figure 8: Gas proportion for MECs purged with H_2 /CO_2

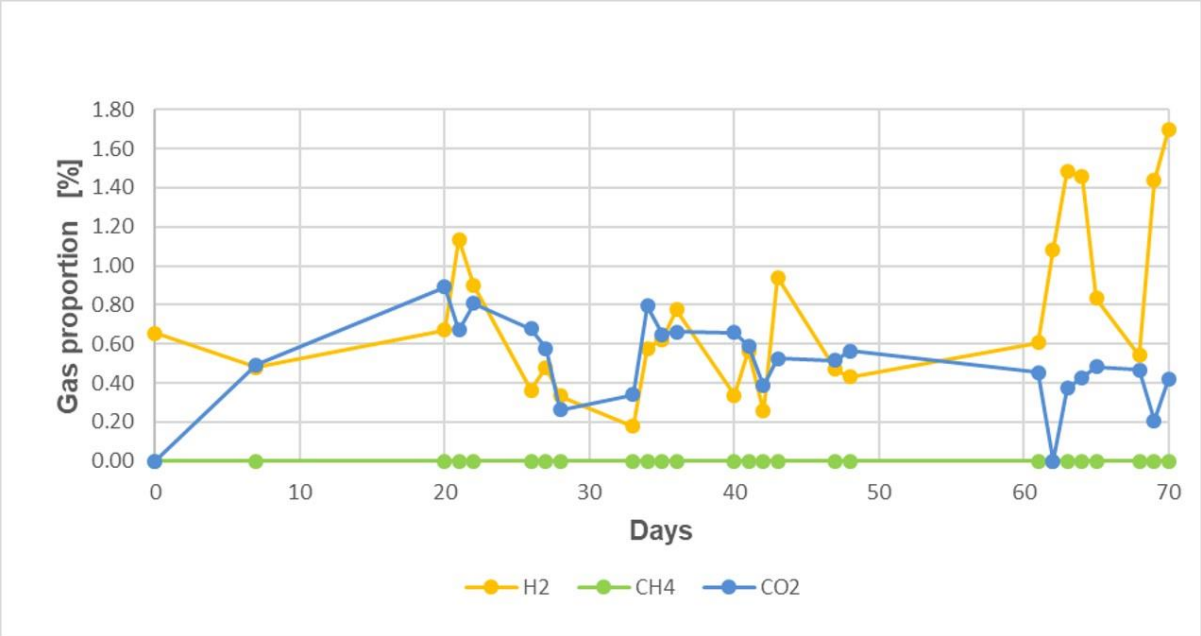

Figure 9: Gas proportion for MECs purged with N_2

MECs have been purged with 100% N_2 but, during the sampling and injecting the sample into the GC, the sample gets in contact with air. This explains the in average quantified 97% N_2 and 1 % O_2 during each measurement. This systematic error is not indicated in the diagrams, since it has no consequences for the results. Important for the experiment is H_2, CO_2 and CH_4 production, shown in Figure 9.

Again like in the previous Figure 9,recorded CH_4 is at 0%. This metbolism is shut down by Bromoethane 2-sulfonate. However, H_2 and CO_2 production is confirmed. Although it is verly low with around 0.6 % for H_2 and CO_2.

For better representation with corresponding error bars, see Figure 10:

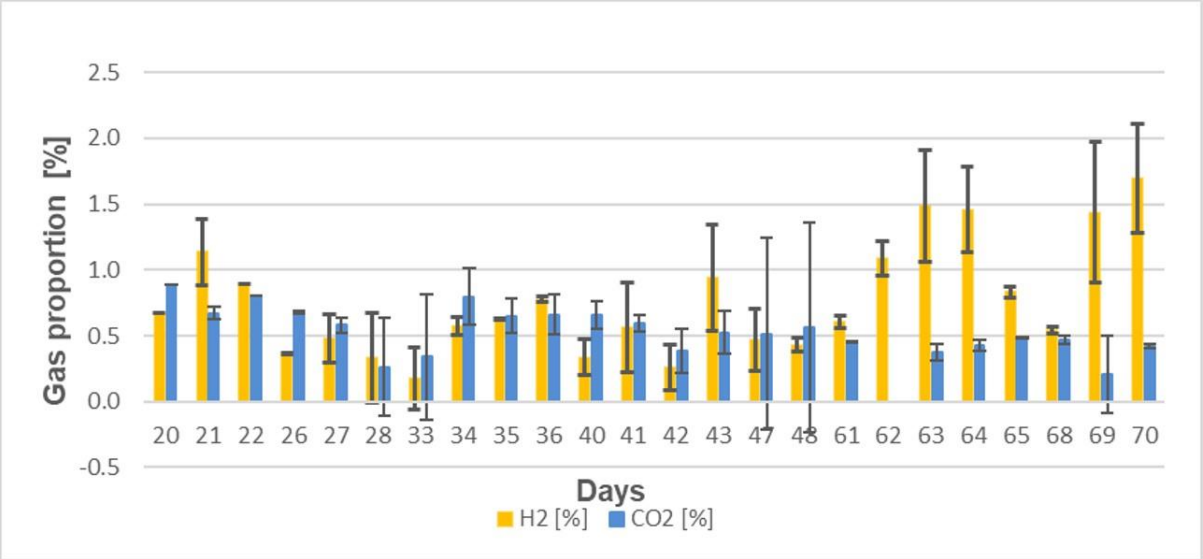

Figure 10: Other representation of the gas proportions H_2 and CO_2 for MECs purged with N_2 with corresponding error bars

HPLC analysis

HPLC analysis revealed the metabolization of acetate. This is shown in Figure 11 for MECs purged with N_2 and in Figure 13 for MECs purged with H_2/CO_2.

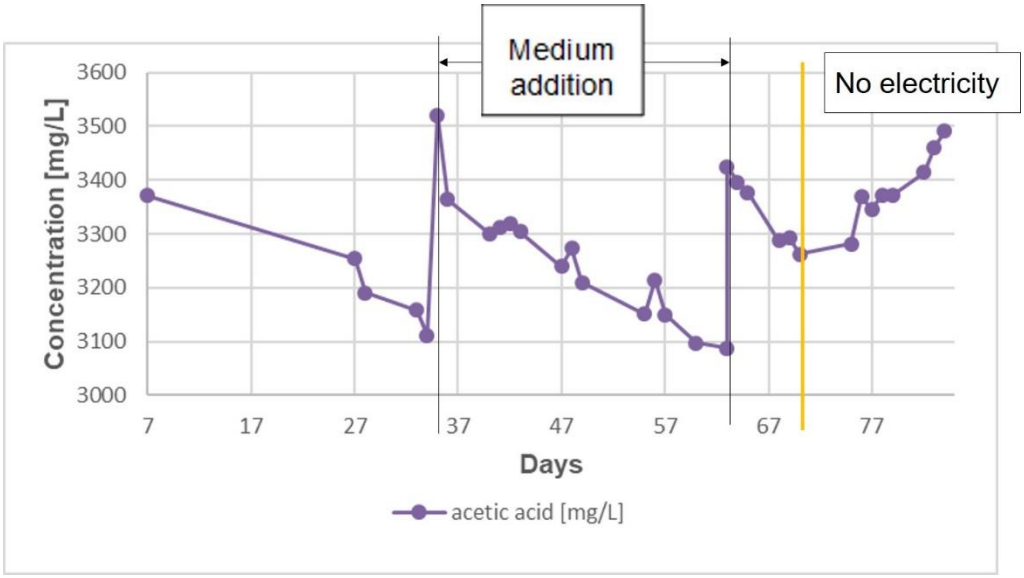

Figure 11: Acetate evolution of MECs purged with N_2. Sudden rise of the acetate concentration due to addition of fresh medium.

Acetate consumption is shown in Figure 11. The peaks appearing on day 35 and 60 indicate a refilling of the medium. A clear trend is observable: the concentration of acetate in the medium is decreasing between day 7 and 34, day 35 and 59, day 60 and 69. During this period, an external voltage of 0.8 mA was supplied. From the first day electricity is shut down, acetate consumption stops, seen on day 70 in Figure 11. In fact, concentration increases rapidly from day 70 on, indicate the production of acetate.

HPLC analysis further revealed the presence of metabolic products like propionic acid, ethanol and 2-propanol as shown in Figure 12.

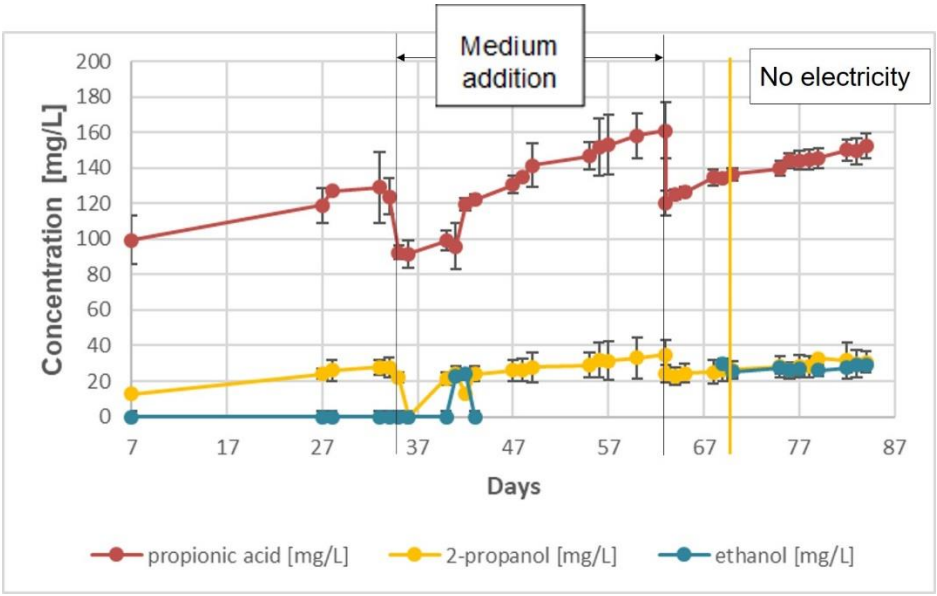

Figure 12: Evolution of detected metabolic products in MECs purged with N_2

In Figure 12 it is shown, that the consumption of acetate correlates with formation of propionic acid.

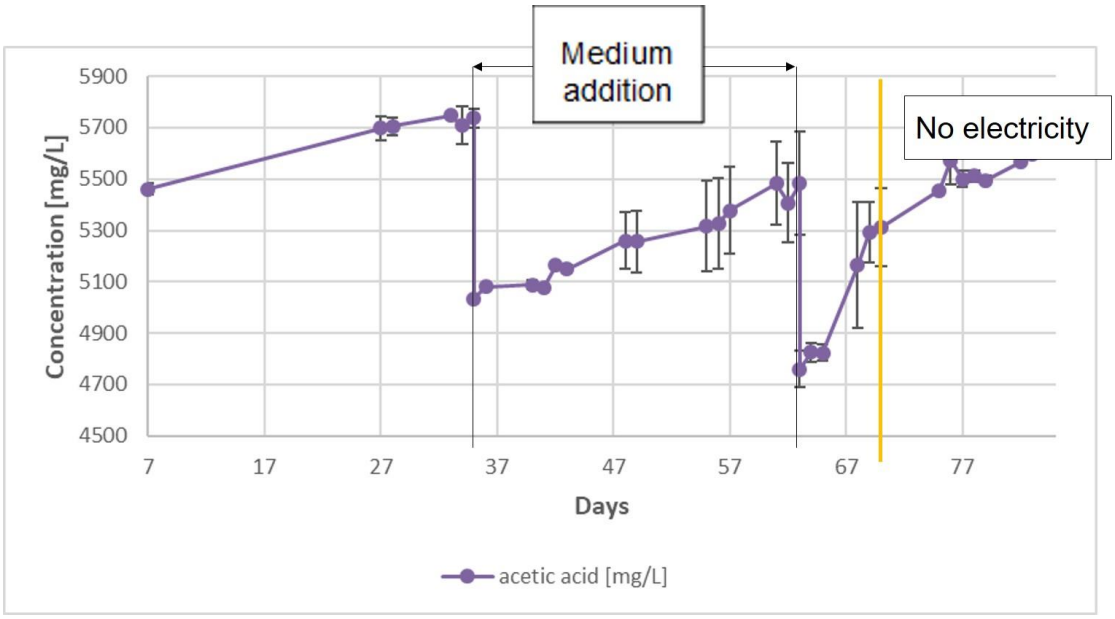

Figure 13: Acetate evolution of MECs purged with H_2/CO_2. Sudden drop of the acetate concentration due to addition of fresh medium

On the contrary to Figure 11, in MEC purged with N_2, a tendency to acetate formation is observed for MECs purged with H_2/CO_2 as shown in Figure 13. Day 35 and 60, medium has been refilled in the MECs. Acetate concentration dropped drastically due to dilution. Even after day 70, when external electricity has been shut down, acetate concentration continued to increase.

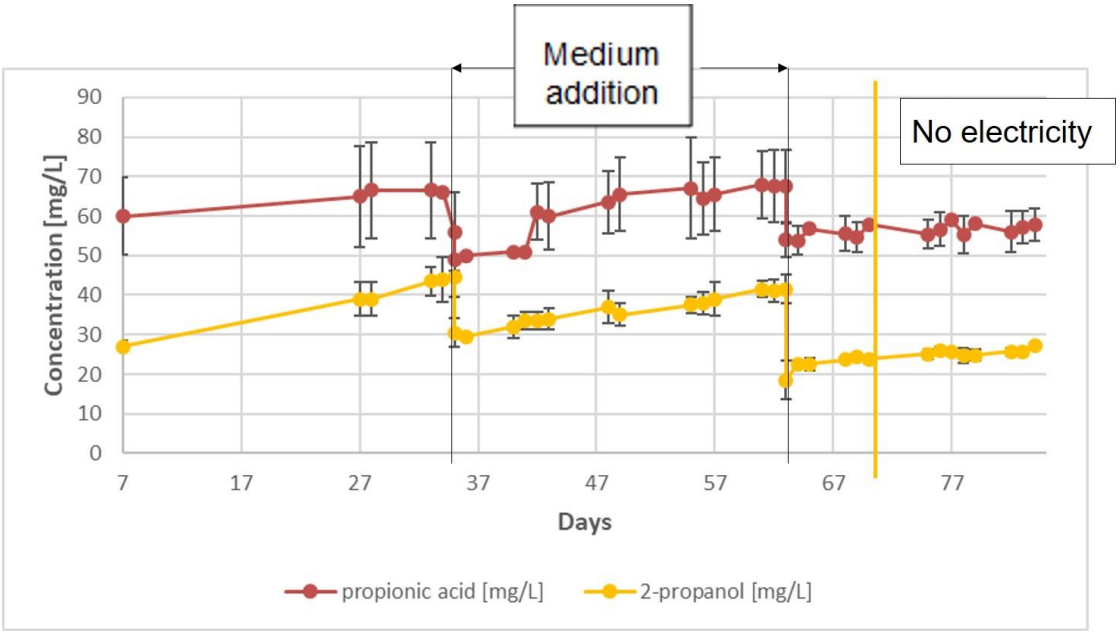

Figure 14: Evolution of metabolic products in MECs purged with H_2/CO_2

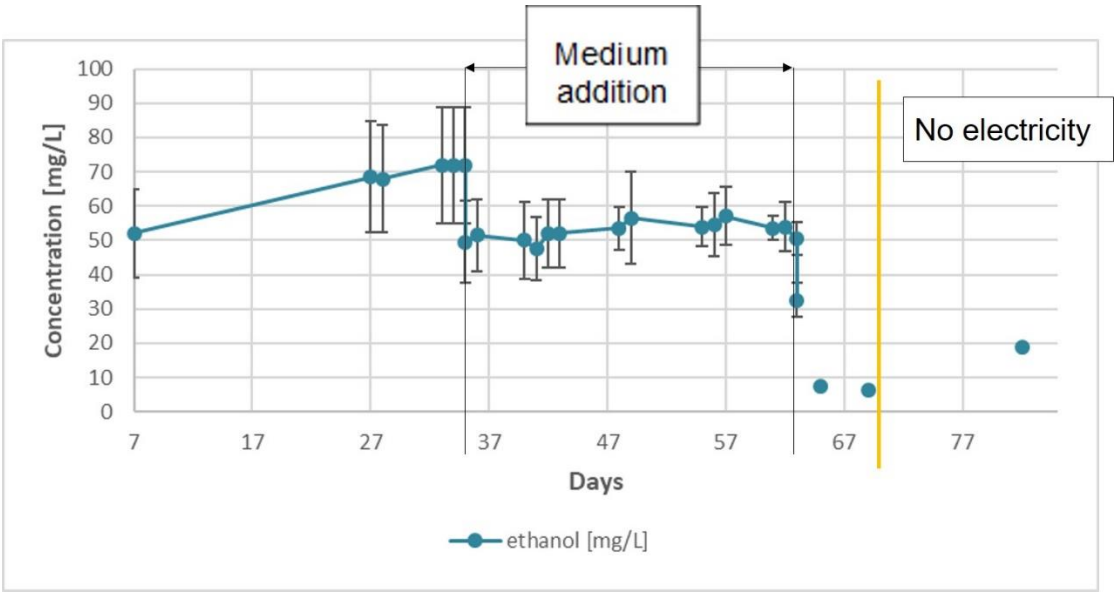

Figure 15: Evolution of metabolically produced ethanol in MECs purged with H_2/CO_2

For a better illustration of the results, propionic acid/2-propanol (Figure 14) and ethanol (Figure 15) formation have been separated into two diagrams. Compared to the results of the MECs purged with N_2, shown in Figure 12, propionic acid concentration is lower, but follows the same trend. 2-propanol behaves the same as on previous experiments. Surprisingly, shown in Figure 15, ethanol concentration in this case is higher, than on MEC purged with N_2.

GC results for MECs running without electricity supply

Note: during this period, no gas exchange was done. Only gas and liquid sampling have been daily taken.

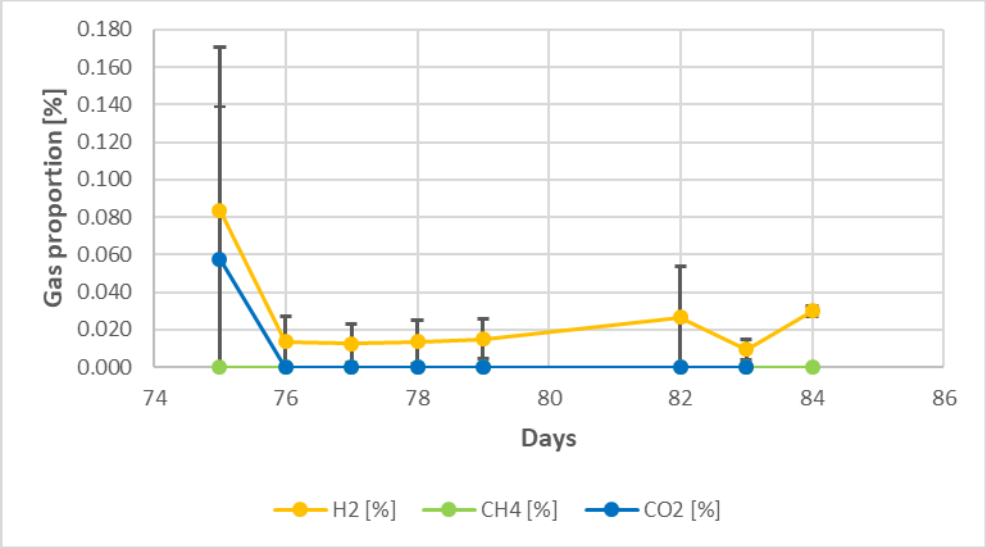

Figure 16: GC Results of MECs without electricity previous purged with N_2

First day, CO_2 and H_2 still high, but without electricity almost instantly drops to zero as shown in Figure 16.

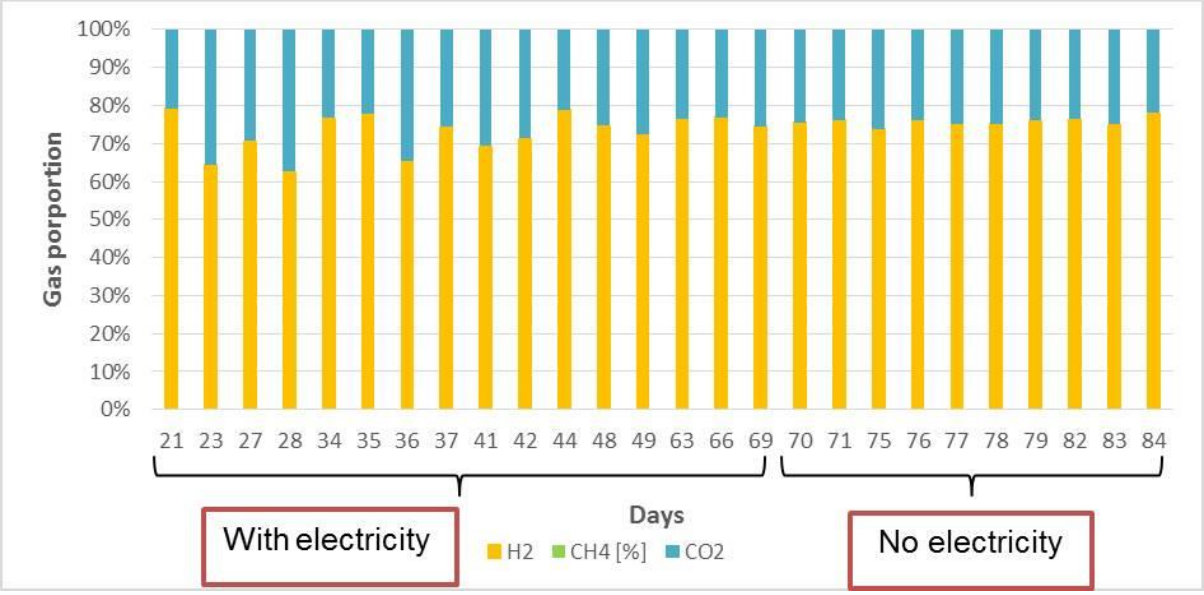

Figure 17: Gas proportion of MECs purged with H_2/CO_2 with (until day 70) and without electricity (after day 70)

N_2 and O_2, coming from ambient air during sampling, have been measured in small amounts. Since these components are coming from ambient air and not important for the experiment, they are not represented in the diagrams. In Figure 17, is it shown, that after shutting off the electric source, still, the gas ratio of H_2 is slightly lower than 80/20. This leads to the assumption of an H_2 uptake from the cells.

Electricity at 50°C

Table 7: Overview of the measurements of MECs in 50°C incubator

Week	Day	Date	I [mA]			
0.0	0	15.03.18	A	B	AA	BB
0.1	1	16.03.18	0	0	0	0
2.6	18	02.04.18	0	0	0	0.5
3.6	25	09.04.18	0	0	0	0.0
4.0	28	12.04.18	0	0	0	5.0
4.9	34	18.04.18	0	0	0	0
5.6	39	23.04.18	0	0	0	0
7.1	50	04.05.18	0	0	0	0
9.4	66	20.05.18	0	0	0	0

In Table 7, an overview of the results of the MECs in the 50°C incubator is shown. The samples A and AA are the same. B and BB are also the same. BB column show two values above zero. Since no other sample showed a change during electricity measurement and since these values are higher than expected, these results do not make sense. A short circuit is the most like cause of this measurements.

Since electricity measurements have not shown any useable results, the GC as well as HPLC analysis were made sporadically.

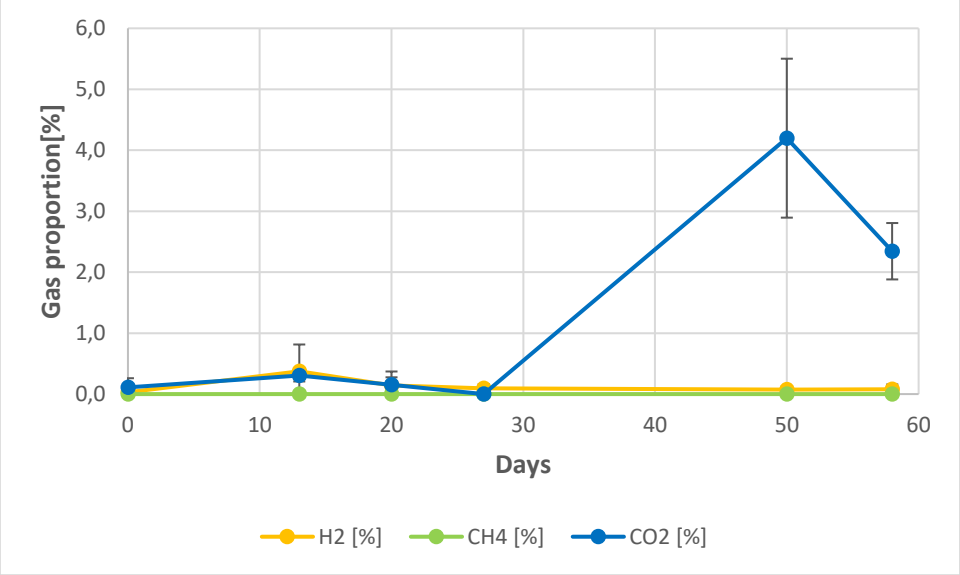

Figure 18: Gas proportion for MECs purged with N_2

MECs have been incubated for almost 60 days in a 50°C incubator. The results of the GC measurements are shown in Figure 18 and Figure 19. No H_2 production is observed in Figure 18 over that period. CO_2 gas proportion was higher on day 50, but since no significant change in H_2 level was observed afterwards, the experiment was stopped.

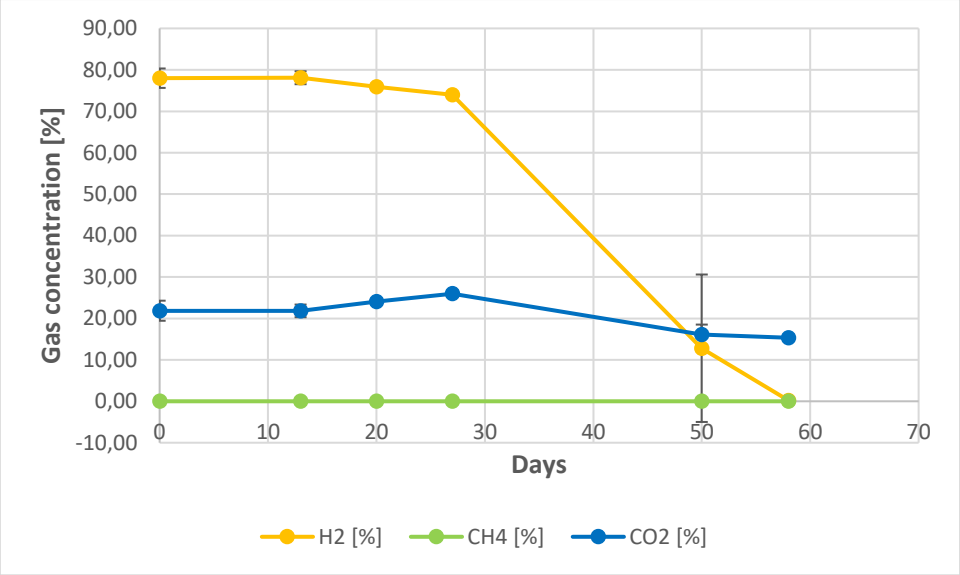

Figure 19: Gas proportion for MECs purged with H_2/CO_2

In fact, MECs have not been gas flushed after day 15, to see if there is any accumulation of produced gases. In Figure 19 a linear decrease on H_2 is observable.

Shewanella oneidensis

Pure cultures of *S. oneidensis* were also used in MECs to produce biohydrogen.

Figure 20 shows the results of HPLC analysis. Propionic acid, ethanol and iso-valeric acid have been as well detected and quantified on HPLC. However, during the whole experiment, the concentration has been stable. Therefore, the trend is not shown here. Moreover, in this context, we are mainly interested on the oxidation of acetic acid.

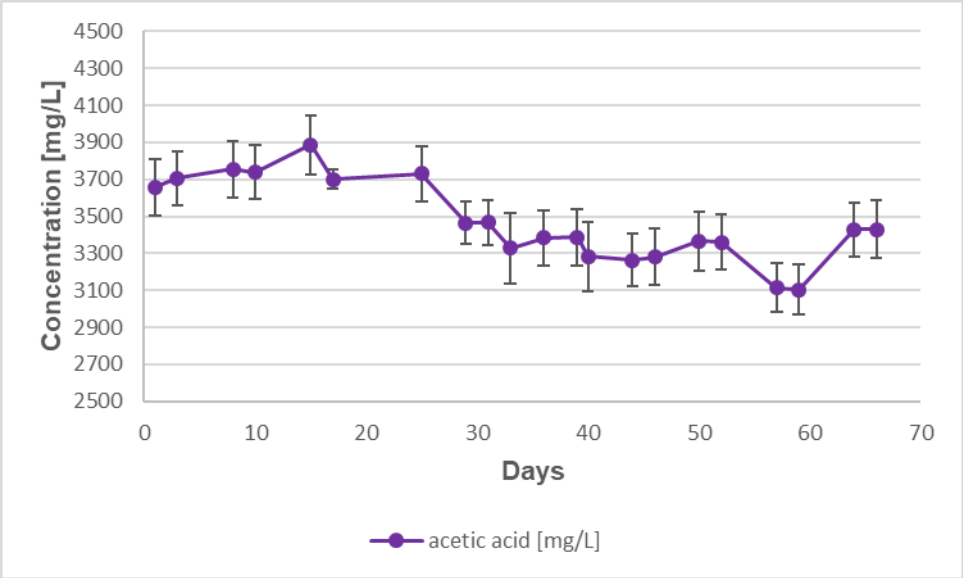

Figure 20: Average change of acetate concentration in S. oneidensis MECs with time

All samples showed approximately the same consumption of acetic acid, indicated by the error bars. Moreover, at the beginning of the batch, a slight increase on acetate concentration was

perceptible. This could be due to metabolization of the remaining lactate of the TSB medium into acetate by the cells.

Considering the gas formation, the results are represented on following diagrams.

Again, to simplify the illustration of the results N_2 and O_2 are not shown in Figure 21.

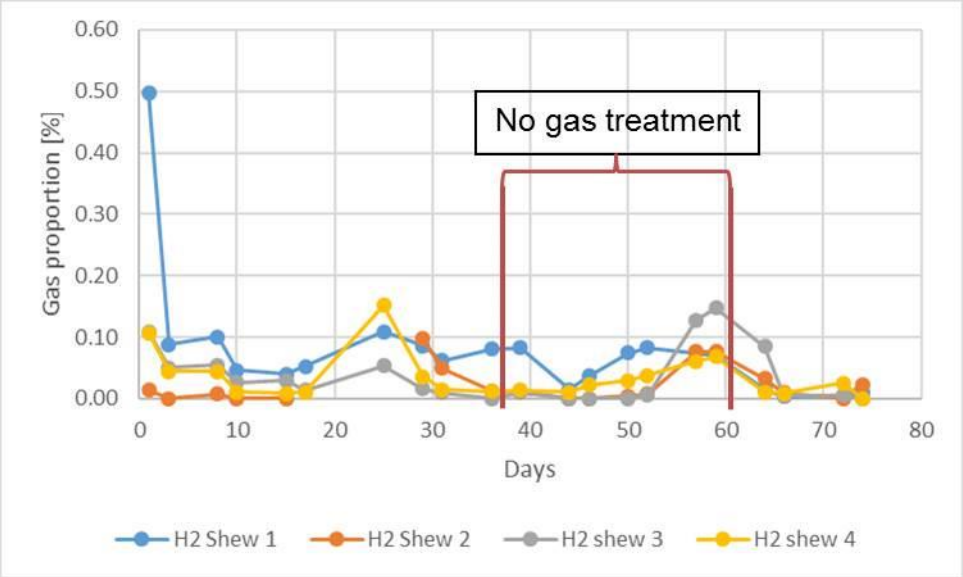

Figure 21: Overview of H_2 production in four different MECs

H_2 production is with a concentration under 0.10 % very low. Moreover, a drastic drop in H_2 as well in CO_2 concentration (see Figure 21) after the first GC measurement is observed.

Considering CO_2, Figure 22 shows the obtained CO_2 concentration of each single *S. oneidensis* MEC. Between day 38 and 58 no N_2 gas treatment was done, to see if there is a possible inhibition by the product.

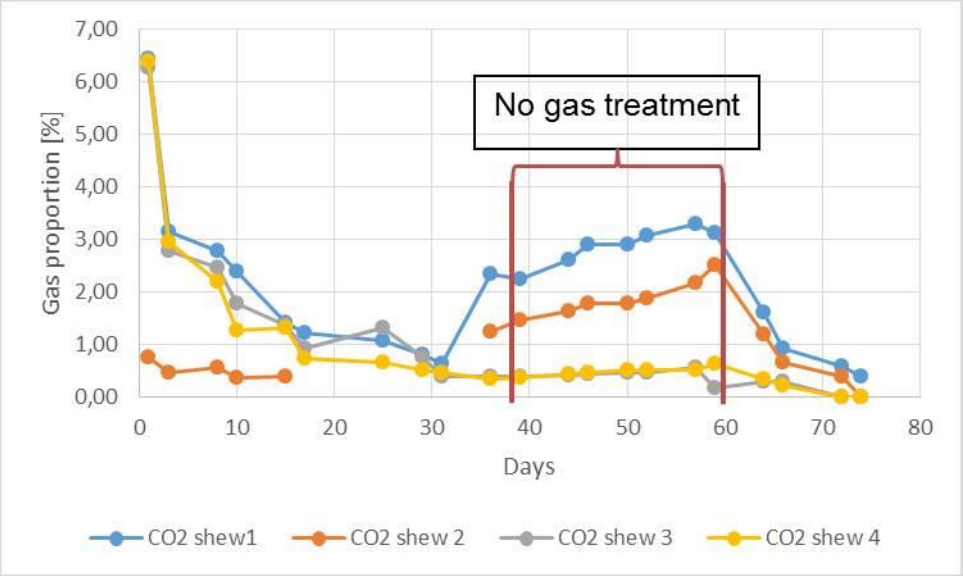

Figure 22: CO_2 generated in S. oneidensis MECs

S. oneidensis MEC no 2 showed a problem during the experiment. After analyzing the cell, a gas leak on the rubber stopper was identified. The measurements between day 15 and day 36 could not be considered, since they were misleading. The stopper robber was changed on day 36 (Figure 22). Subsequent results show the same trend as on the other MEC batches.

Electric current

No electricity trend could be recorded, since the biohydrogen gas production, acetate oxidation and anode oxidation have been too low. The sensitivity of measured current flow is 0.01 mA. A current flow below 0.01 mA probably occurred.

G. sulfurreducens

G. sulfurreducens has not showed representative results yet. In the media composition described by the DSMZ homepage, *G. sulfurreducens* could not grow properly. It turned out, that the media pH dropped dramatically after autoclaving. Originating from pH 6.8 it dropped to 5 and the buffer system also turned out to be unappropriated. For this reason, the decision was taken to change from 0.6M Na_2HPO_4/ 2.5M $NaHCO_3$ to 2.5M NaH_2PO_4/4.6 M Na_2HPO_4. The pH stayed stable after autoclaving and *G. sulfurreducens* grew within 2 days: a flesh-colored turbidity was observed. *G. sulfurreducens* will be used in future experiments.

4.2. Biofilm

Scanning electron microscopy

MEC (no 223) purged with N_2

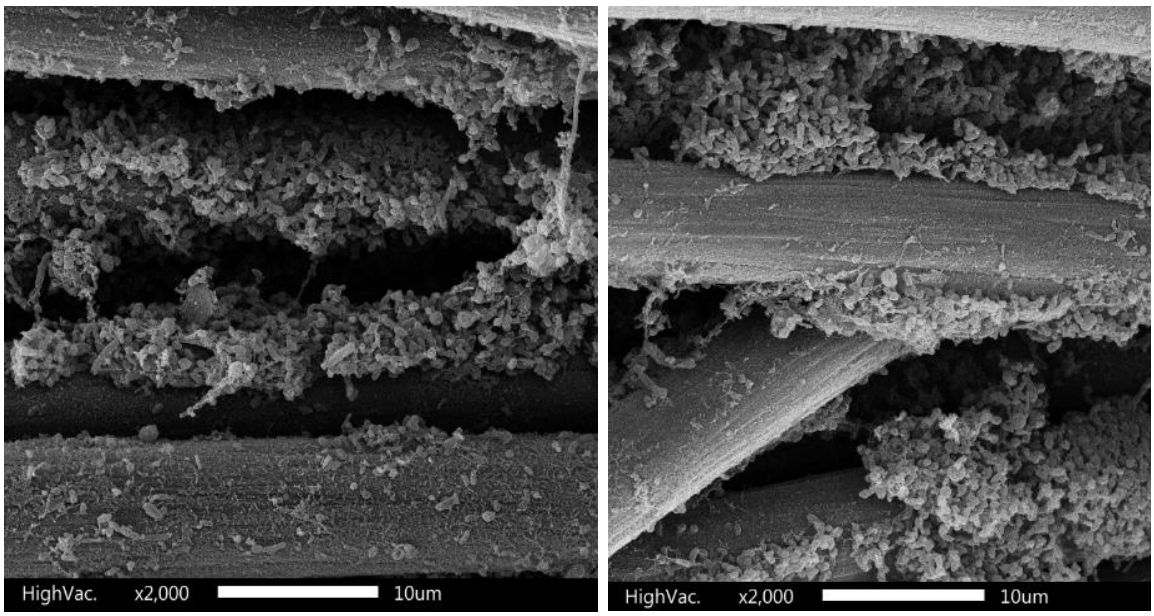

Figure 23: SEM record of anode multi-species biofilm of MEC sample 223 with magnification of 2000x and 20.0 kV

Shown in Figure 23, SEM shows the structure of the biofilm. In Figure 23 an even biofilm formation around most rods is perceptible.

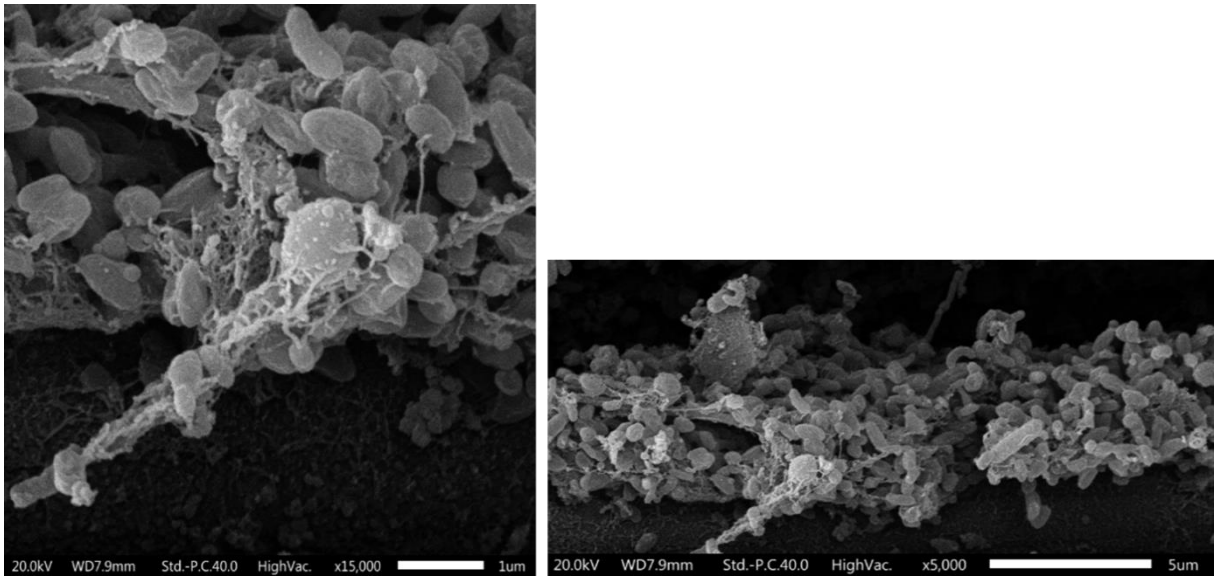

Figure 24: SEM record of anode biofilm of MEC sample 223 with magnification of 15000x/5000 x and 20.0 kV

Clearly identifiable, bacteria are embedded in extracellular matrix. Different shapes of bacteria can be detected, showing the different strains. However, no pili are detected as shown in Figure 24.

<u>*S. oneidensis*</u>

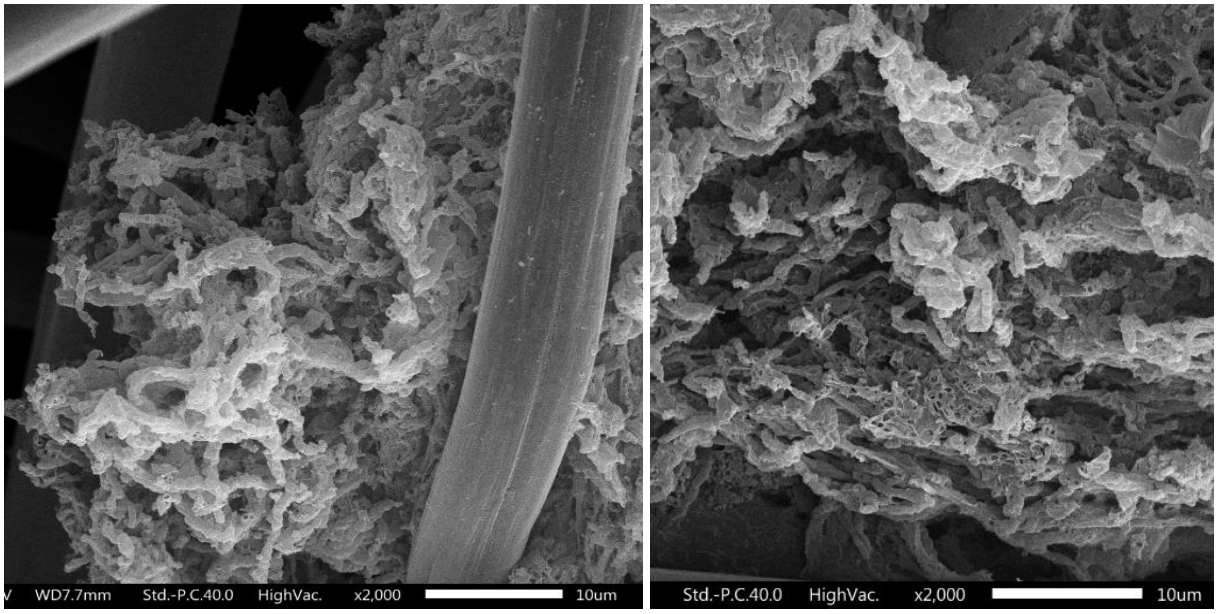

Figure 25: SEM record anode biofilm of S. oneidensis with magnification of 2000 x and 20.0 kV

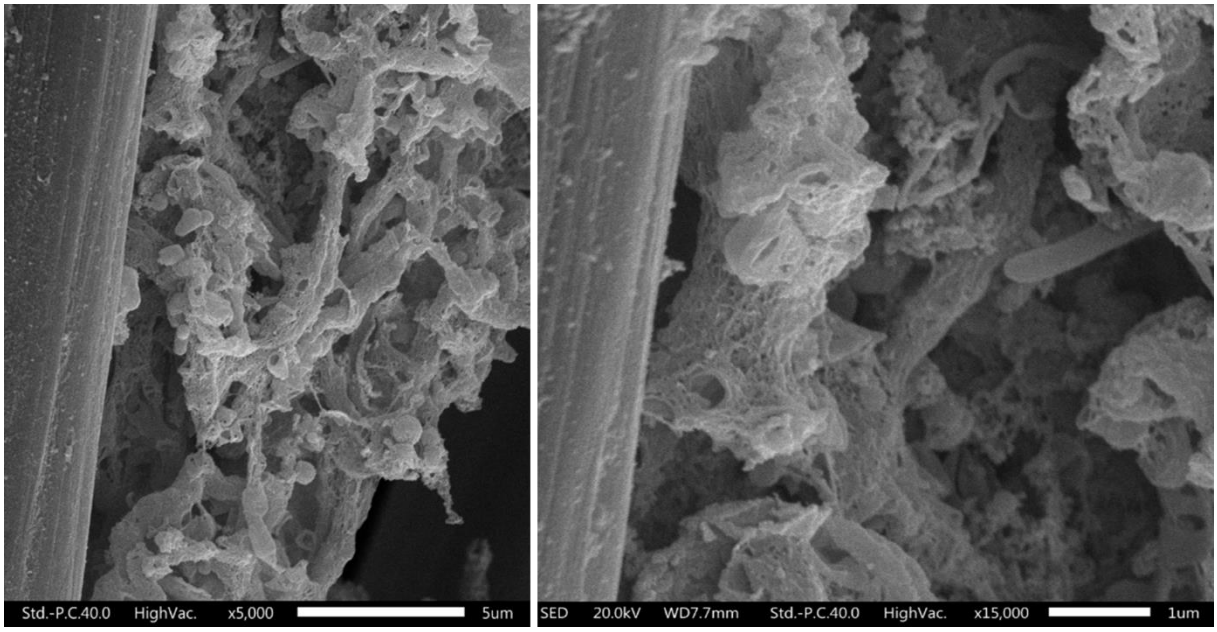

Figure 26: SEM record anode biofilm of S. oneidensis with magnification of 5000x/ 15000x and 20.0 kV

In Figure 26 almost no bacteria can be seen. Nevertheless, the matrix looks like exopolymeric substances (EPS).

In both Figure 25 and Figure 26, it is observable, that beside of EPS, there are many damaged cells. Mostly only the outer shape of the cell can be observed, possible due to the preparation steps.

Cultivation

Cultivation of two different bacteria showed a few surprising results:
For example, it was impossible to grow *A. vinelandii* and *S. aureus* together neither in TSB medium nor in Burk medium. In fact, *A. vinelandii* does not grow in TSB medium neither *S. aureus* in Burk medium. However, mixing the two media together and inoculating it with both bacteria worked well. Both bacteria were able to grow, though *S. aureus* with a higher doubling time.

Most of the time, whenever the first bacteria grew on carbon cloth, the next bacteria could not attach, see Figure 35. For this reason, $CaCl_2$ was added to weaken the negative charge of the membranes of bacteria which already have formed a biofilm.

In the case of anaerobic bacteria, growth rate of *S. oneidensis* and *C. acetobutylicum* were good. *G. sulfurreducens* showed problems while its cultivation was based on the wrong pH and buffering system.

Fluorescence in situ hybridization (FISH)

Essential for FISH is the accessibility of the fluorescently labeled oligonucleotide probes to the binding site. To improve the hybridization results of dense biofilms, an enzymatic pretreatment, prior the hybridization step was sometimes necessary. Lysozyme and Protease have been used in a range of 1 to 10 mg/mL. Fixation and hybridization times were also changed during FISH experiments, to see if the signal could be improved.

A. vinelandii pure culture

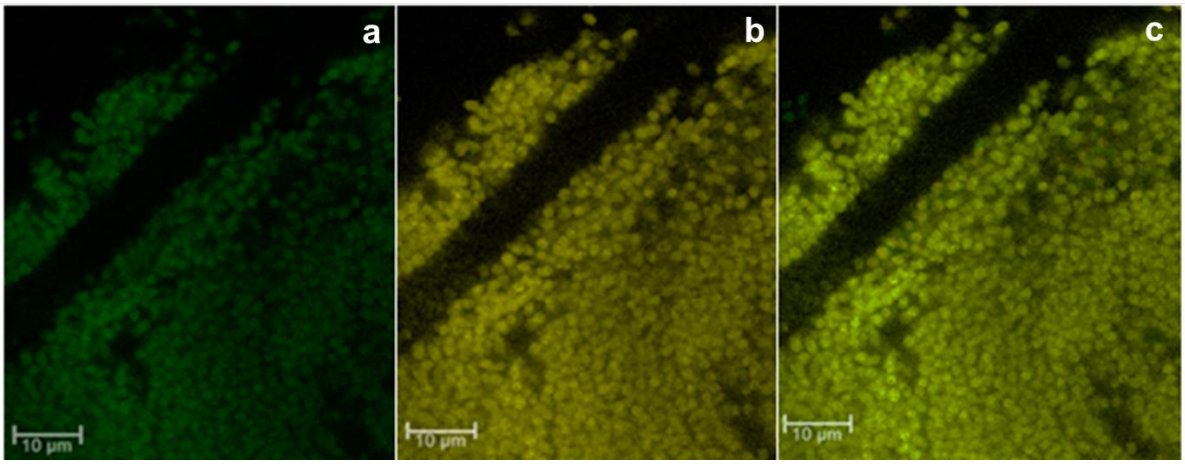

Figure 27: A. vinelandii around carbon cloth rods in all three micrographs (a, b, c). Use of probe "GAM" (green, record a), "EUB" (yellow, record b) and 1 g/L lysozyme, 10 % FA, 4 hours hybridization. Overlay of the images in c. magnification of 63 x

Good binding of the probe "EUB" is visible. "EUB" probe binds to bacteria in general, shown in yellow in Figure 27b and the probe "GAM" for gamma-proteobacteria shown in green in Figure 27a. The signal completely overlaps as shown in Figure 27c.

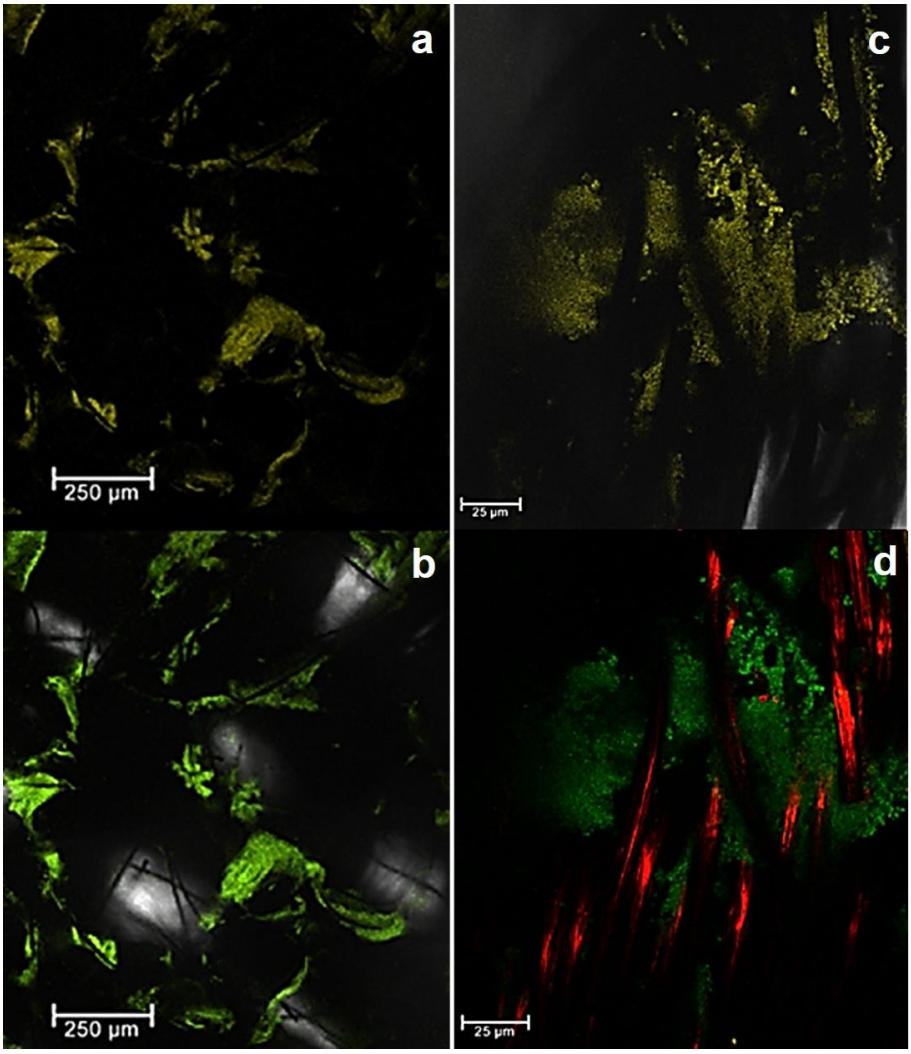

Figure 28: (a) and (b) show, an overview of bacteria cultures on the carbon cloth. Used magnification 20 x. (c) and (d), the same sample with magnification of 63 x. "GAM" and "EUB" probes used. In red (d), the carbon cloth rod.

Again, good results are obtained by using the general, "EUB" (yellow, see a and c) and specific, "GAM" (green, b and d) probe. There is a total overlapping of the results, shown in Figure 28 (b). No enzymatic pretreatment is needed on *A. vinelandii*. Due to reflection, carbon cloth rods shine in red, which makes distinction between culture colonies and carbon cloth easier (Figure 28, (d)).

On the case of *A. vinelandii*, good signal was already obtained with 4 hours of fixation with PFA and 180 min hybridization.

S. aureus pure culture

FISH on *S. aureus* was not so successful as for *A. vinelandii*. It was difficult to get a proper signal. Many variations on FISH parameter as well as enzymatic pretreatments have been done. The micrographs with the best signal are showed in Figure 29 and Figure 30. A general bacteria probe, "EUB", was used. It was expected all bacteria to be stained in yellow. All pictures are taken at a 63x magnification.

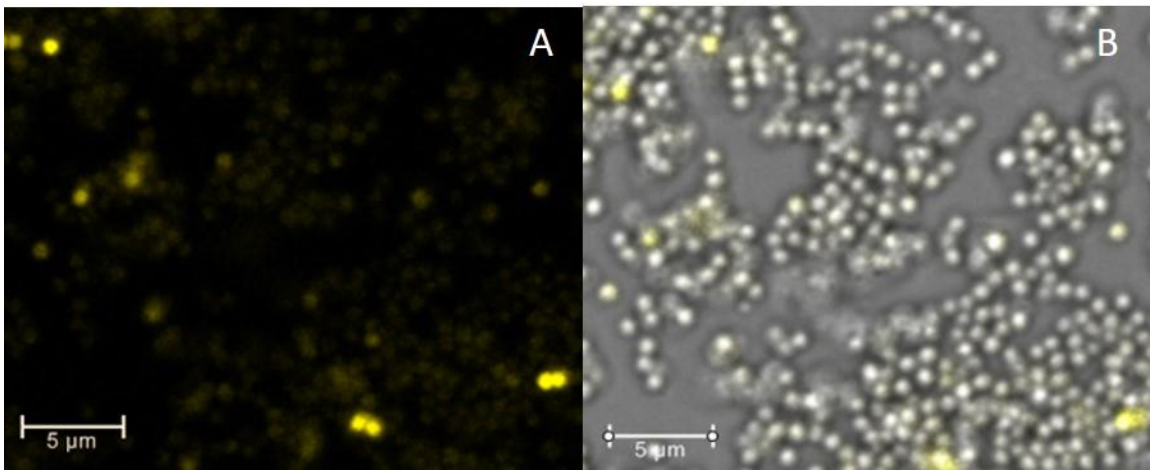

Figure 29: S. aureus in suspension culture, 4h fixation in PFA, 3h hybridization with 10% FA and "EUB" probe. A) shows the EUB probe while in B) the overlay with the brightfield micrograph is shown.

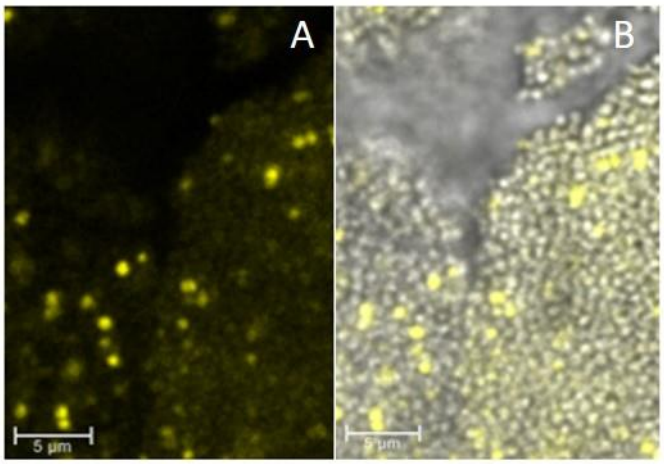

Figure 30: S. aureus in suspension culture, 6h fixation in 50% ethanol, 3h 10% FA hybridization and "EUB" probe. A) shows the EUB probe while in B) the overlay with the brightfield micrograph is shown.

The difference between different fixation methods is shown in Figure 29 and Figure 30. Whereas Figure 29 shows cells treated with PFA, Figure 30 shows cells treated with 50% ethanol. In this case ethanol fixation provided better results. However, this was not the case for *S. aureus* in biofilms.

The use of lysozyme or protease did not show any amelioration of the results on cells in suspension, but it did show better results in the case of biofilm; see Figure 31.

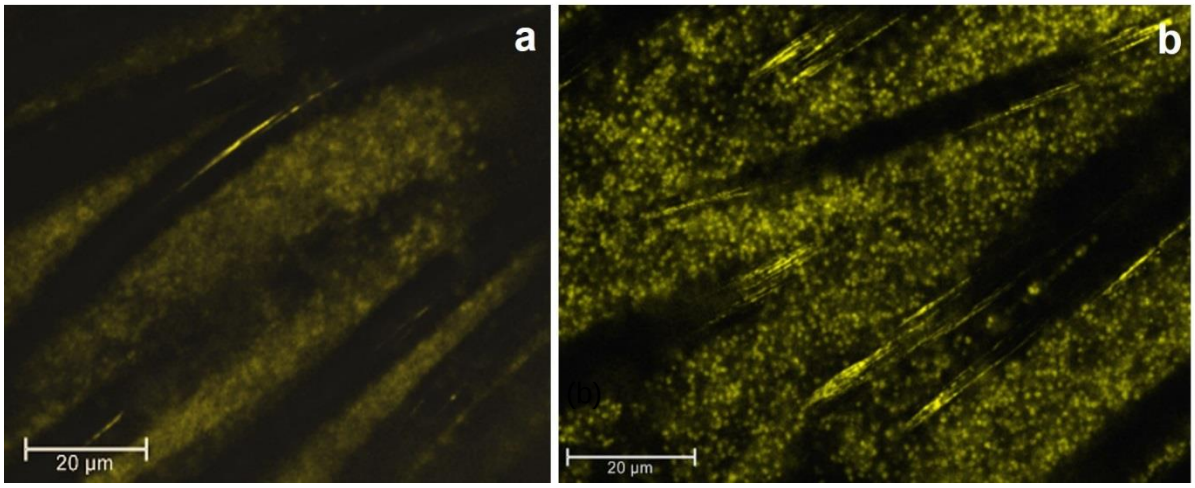

Figure 31: S. aureus on carbon cloth with 1 g/L lysozyme pretreatment in (a) and with 10 g/L lysozyme pretreatment in (b). Used magnitude: 63x. 6h fixation in PFA, 3h 10% FA hybridization and "EUB" probe.

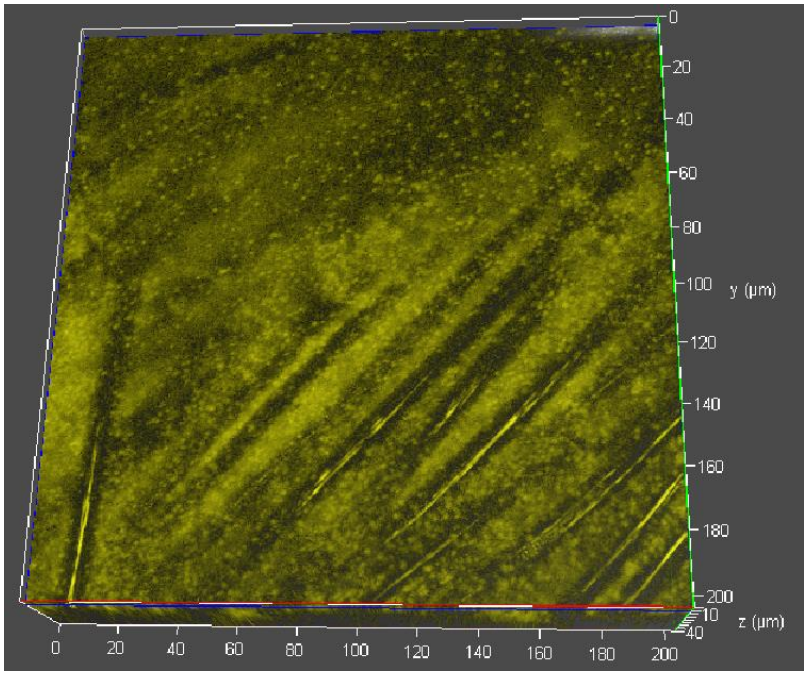

Figure 32: 3D demonstration of S. aureus biofilm on carbon cloth and 10 g/L lysozyme pretreatment. Used magnitude: 63x

As shown in Figure 29 - Figure 32, there is a dense cell population around the carbon cloth strings. Cell culture grew fast, and biofilm formation went well. Fixation could not be done with ethanol and was instead done with 4% PFA. Without enzymatic pretreatment, signal was weak (not shown here), but by increasing lysozyme concentration signal got more intensive and cells better defined. 10 g/L lysozyme seemed to be a good concentration for good fluorescence signal.

***S. aureus* and *A. vinelandii:* two layers and mixed biofilm**

Note: all FISH experiments represented in this chapter are done with 6 hours of hybridization in 4% PFA, 10 g/L lysozyme pretreatment, 3 hours of hybridization in 10% FA, and treated with "EUB" and "GAM" probe.

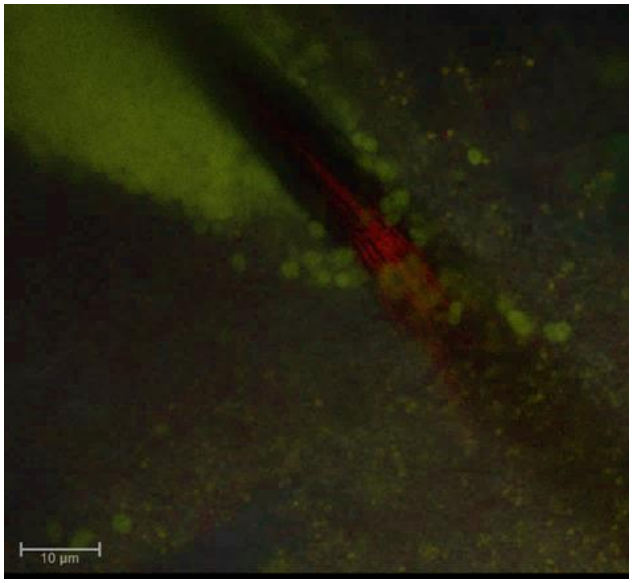

Figure 33: First layer A. vinelandii (rods in green) second layer S. aureus (coccus in yellow). Used magnitude: 63 x

It was difficult to obtain a mixed biofilm, when incubating a bacterium after the other. Once the surface was covered with *S. aureus*, *A. vinelandii* could not bind and vice versa. $CaCl_2$ was added to the medium of the first incubation batch. Ca^{2+} ions were supposed to bind to negative charged membrane and facilitate attachment of new cells on the biofilm. As seen in Figure 33, green fluorescence signal and cell shape are a clear indication of *A. vinelandii* which represents the underground layer. Yellow fluorescence signal and coccus shaped cells are on the other hand a clear indication for *S. aureus*.

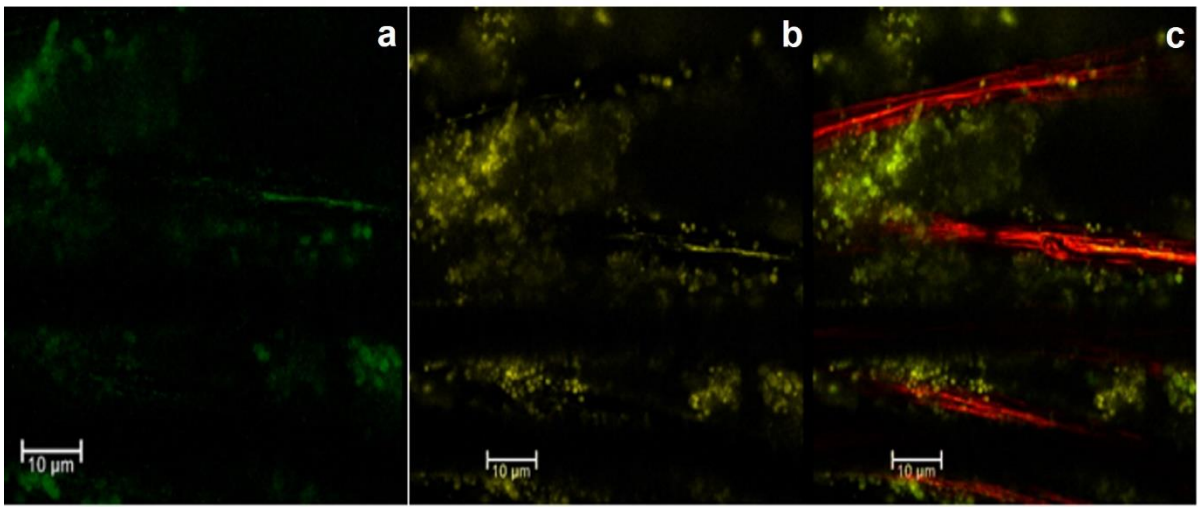

Figure 34: Mixed biofilm of A. vinelandii and S. aureus incubated in the same time. Used magnitude: 63 x.

In Figure 34 a biofilm of *A. vinelandii* and *S. aureus* is shown. Both bacteria have been incubated on a medium mix and could develop well. No ordered structure can be recognized, instead there are mixed *A. vinelandii* and *S. aureus* cell spots around the strings.

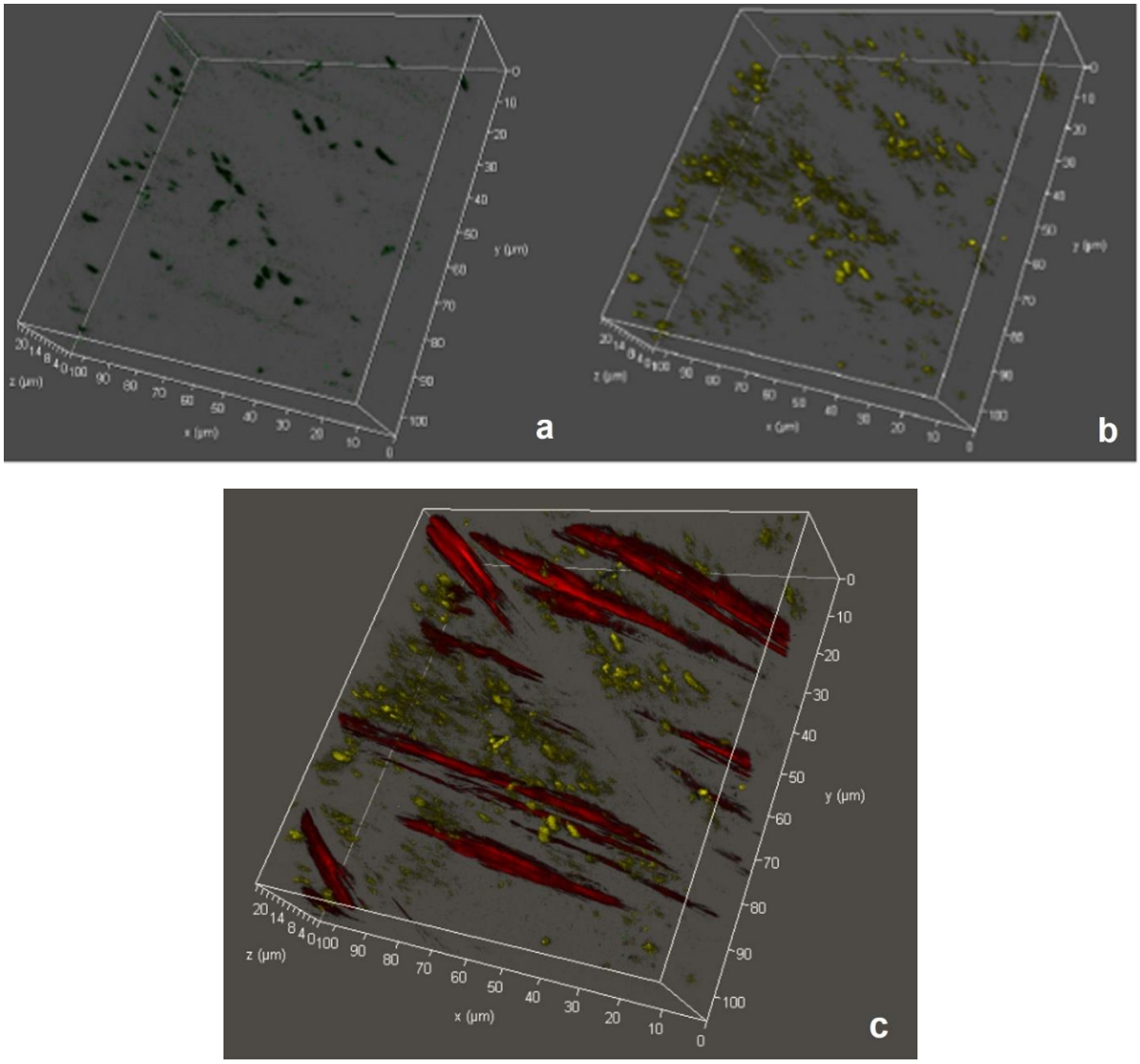

Figure 35: 3D of two layered biofilm with first S. aureus (yellow) and secondly A. vinelandii (green). Used magnitude: 63 x

A thick-layered biofilm of *S. aureus* (yellow, coccus), (c), can be seen within the carbon cloth strings in red, Figure 35. An attempt to grow a second layer of *A. vinelandii* only showed a few attached cells in green, record (a) and as yellow rods on the outer layer in record (c) of Figure 35.

S. oneidensis pure culture

S. oneidensis caused many problems. Besides of no hydrogen production during the MEC experiments (see above), it was also difficult to get any FISH signal. On FISH a general bacterium probe, “EUB” and a specific *Shewanella* strain probe, “SHEW” was used. Figure 36 - Figure 37 are done with 63 x magnification and different zoom.

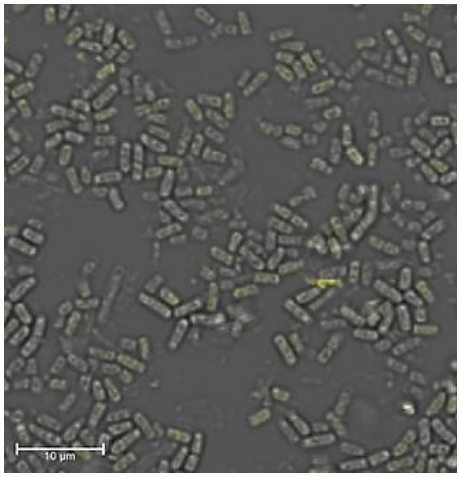

Figure 36: Overlap record of S. oneidensis suspension culture in bright field mode and excited with 548 nm. Magnitude: 63x. "SHEW" and "EUB" probes were used.

Typical for *S. oneidensis* is the shape of thick rods. In Figure 36 the shape is very clear perceptible. However, hardly any cell is stained by FISH probe "EUB" and no signal of probe "SHEW".

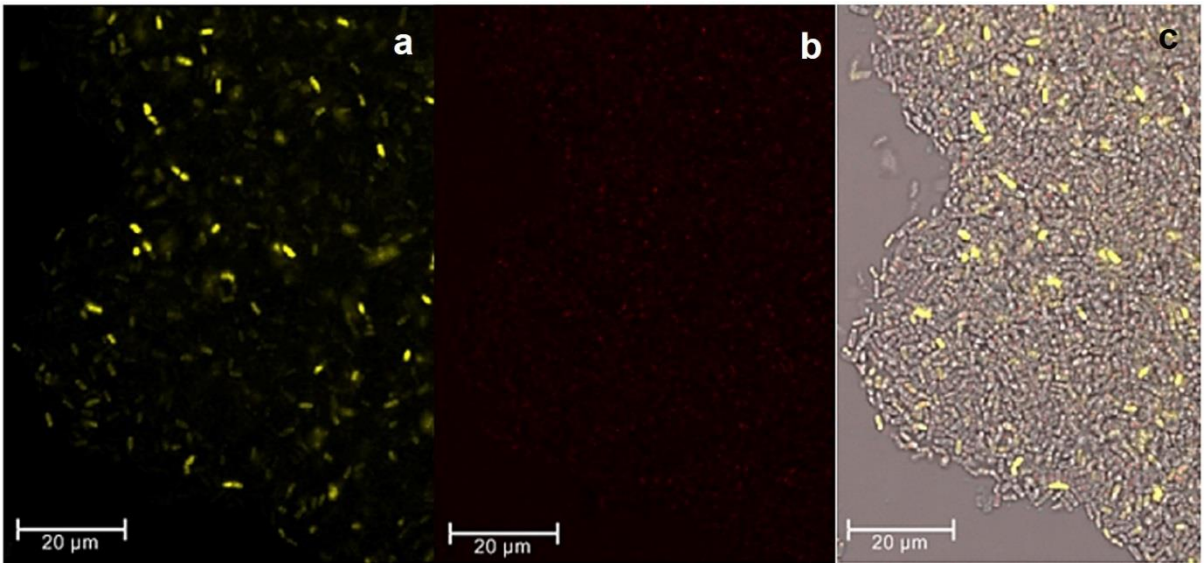

Figure 37: S. oneidensis suspension culture stained with "EUB "(a), with "SHEW "(b) and overlap of prior results and bright film mode (c). Magnitude: 63x.

Figure 37 shows the best results of FISH experiment with *S. oneidensis*. General bacteria probe "EUB" in yellow did not bind well and the signal was very weak, (a). With the specific "SHEW" probe, in Figure 37 (b), the red colored signal was only recorded by intensifying the gain, but no obvious specific signal was obtained. Enzymatic pretreatments did not lead to a better result.

Experiments on biofilm formation on carbon cloth have been even less successful. The cells grew well, but no FISH signal was obtained. For this reason, *S. oneidensis* was not used in the formation of multicellular biofilm.

G. sulfurreducens pure culture

G. sulfurreducens did not grow well. For this reason, few FISH experiments were done. However, the FISH probe “GEO” with two helper probes have been successfully used and good results have been achieved, see Figure 38.

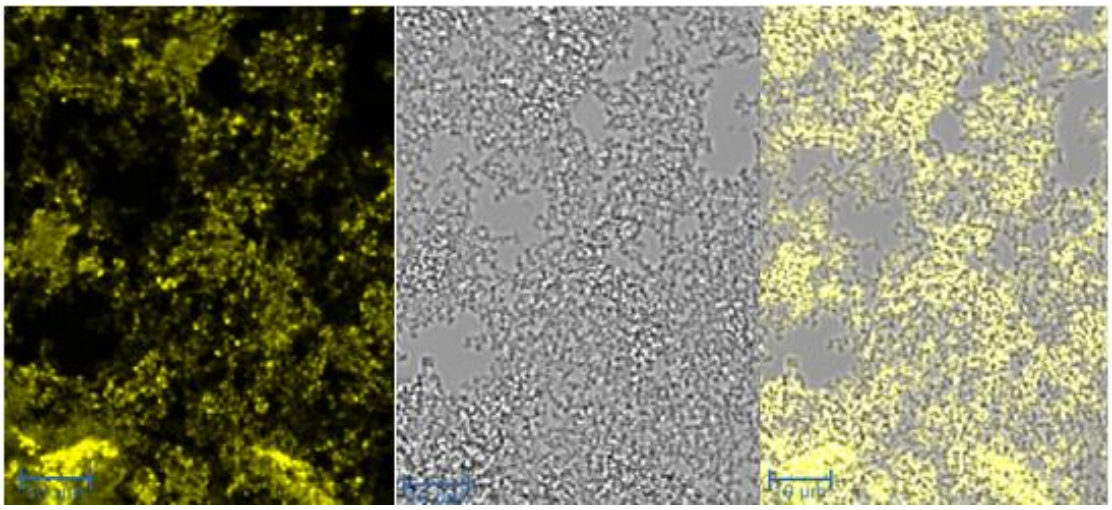

Figure 38: G. sulfurreducens with specific probe. 4h fixation in PFA and 4h in 10% FA hybridization. Magnitude 63x.

Cell density in the suspension culture was very low. FISH probe has bound specifically. 4% PFA fixation with 10% FA hybridization seemed to be the best concentrations. Optimal fixation and hybridization times were found at 4 hours. Longer time showed negative results.

Since *G. sulfurreducens* showed poor growing ability, it was not used for biofilm experiment and it could not be combined with other bacteria.

C. acetobutylicum pure culture

Apart from the two other anaerobic bacteria, *C. acetobutylicum* had a high growth rate. In addition, FISH experiment lead to good results. Since *C. acetobutylicum* is a gram-negative bacterium, fixation with 4% PFA was done, which also caused satisfying results. There was no big difference on using 35% or 10 % FA during hybridization. Worse results have been obtained by doing any pretreatment to the sample.

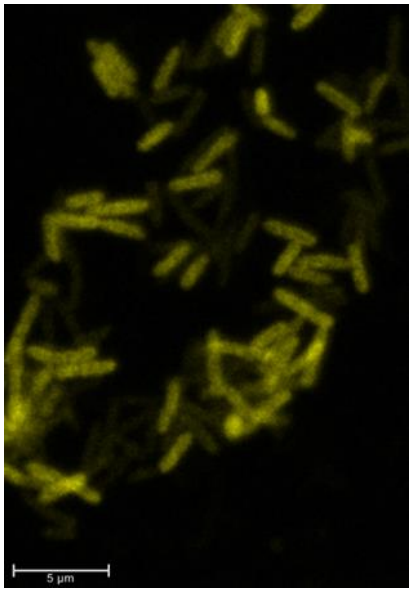

Figure 39: C. acetobutylicum in suspension culture with 63x magnification and stained with general FISH probe "EUB"

Mixed bacteria culture provided from waste water treatment plant and cultivated in MEC

Suspension and biofilm samples from the MECs purged with N_2 have been taken and analyzed by FISH method. Best results have been produced with 2–4 hours of fixation and 3 hours of 10% FA hybridization. For this reason, the same process was applied to the MEC samples with a good outcome.

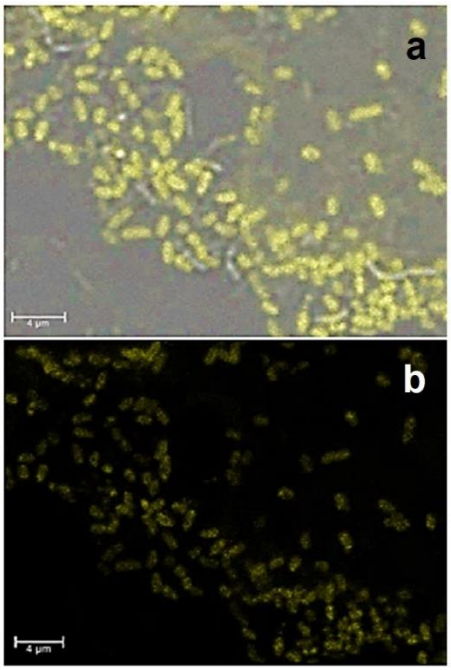

Figure 40: Suspension culture from MEC stained with "EUB" probe (yellow), "SHEW" probe and overlap with bright field. Used magnitude: 63 x

Sample taken for FISH experiment showed a high cell density.

By comparing the two images in Figure 40 it is visible that not all cells are stained by the general FISH probe “EUB” (yellow). Nevertheless, the bright field mode gives a general overview of the present bacteria variety. “SHEW” probe was also used and should bind to *Shewanella* strain. Since no red color can be observed in Figure 41, *Shewanella* strain is not expected in this sample.

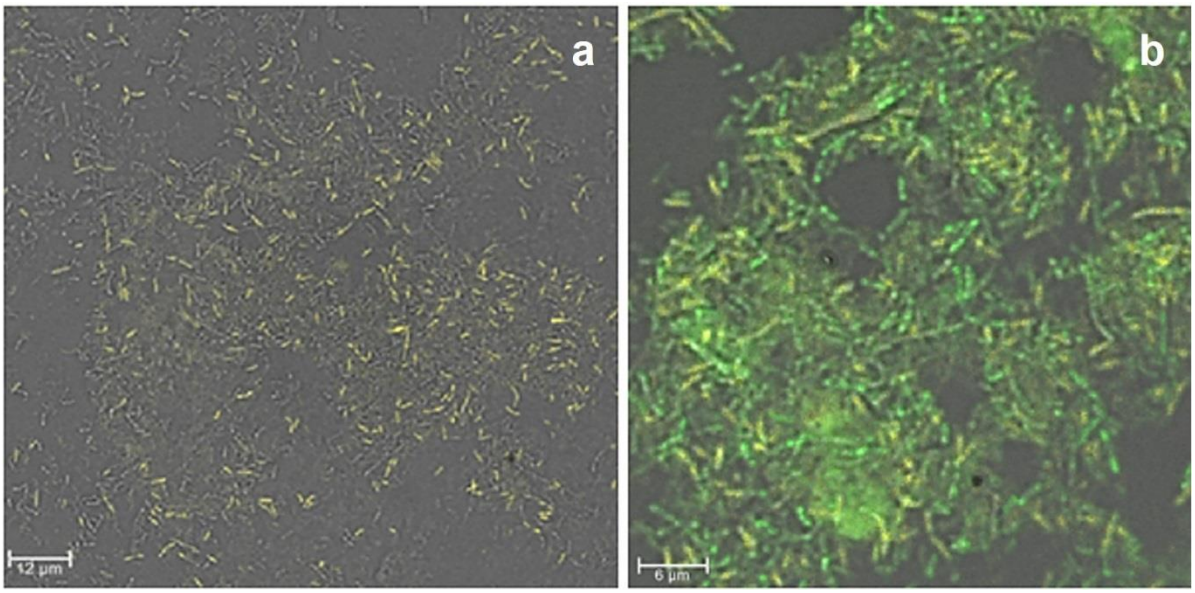

Figure 41: Suspension culture from MEC stained with “EUB” probe (yellow, (a) and (b)), “GAM” probe (green, (b)) and bright field presentation. Used magnitude: 63 x and different zoom

The specific “GAM” probe was able to bind to most bacteria of the sample, Figure 41, (b). Surprisingly, the specific probe shows better results than the general “EUB” FISH probe, (a). On this experiment no pretreatment was used.

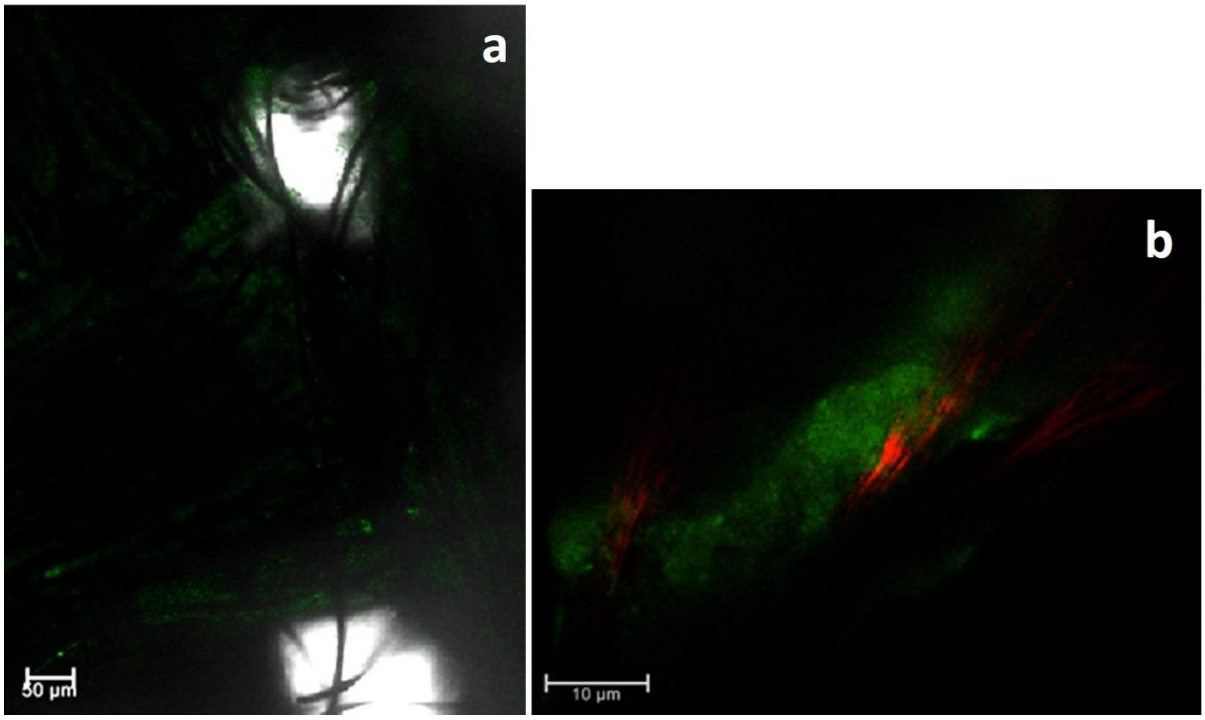

Figure 42: Overview of biofilm on the anode, stained with "GEO", "SHEW" and "GAM" probe (a). Used magnitude: 20x. On (b), Record of biofilm surrounding carbon cloth string (anode in red). Same staining and magnitude 63x

The same sample is used in (a) and (b) of Figure 42 but resolved at different magnitudes. In this case, the sample was taken from a MEC purged with H_2/CO_2 mix. The carbon cloth was processed with 4 hours hybridization on PFA, 1 g/L lysozyme pretreatment and 3 hours of hybridization 10% FA. The shape of the cells is not as well defined as in Figure 40. Moreover, specific "GEO" and "SHEW" probe signal is perceptible in none of the figures. Only "GAM" probe seems to bind on the biofilm.

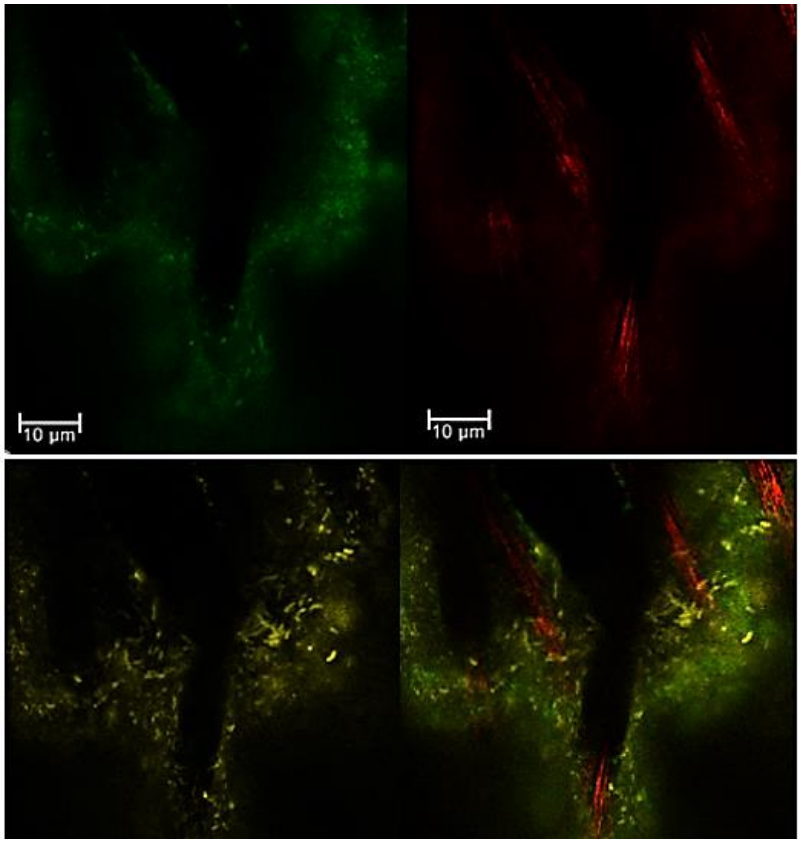

Figure 43: Overview of biofilm on the anode, stained with "EUB", "SHEW" and "GAM" probe. Used magnitude: 63x

In Figure 43 10 g/L lysozyme was used. Like in prior results, "GAM" probe was able to bind specifically and shows intensive signal. "EUB" probe was used in this case and even bound better as "GAM" probe. This is a better result as shown on previous Figure 42. The shape of the cells is well defined and the exact borders from biofilm to the strings are perceptible. Also, "SHEW" probe shows some red signal. On the other hand, the strings are red as well, which indicates poor signal intensity and reflection. Further, "EUB" signal is strong, which could lead to an overlap of signals in other channels. For these reasons, the red signal is misleading and ignored.

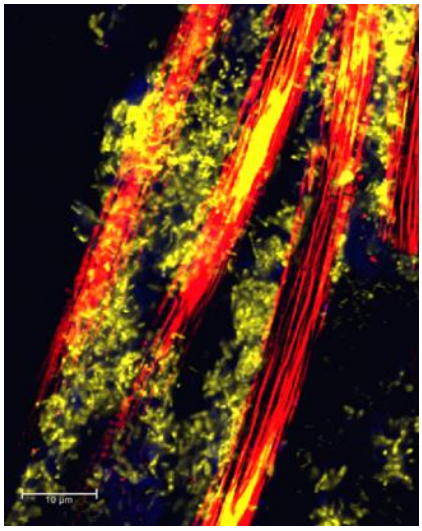

Figure 44: Overview of biofilm on the anode, stained with "EUB", "SHEW" probe and 10 g/L lysozyme. Used magnitude: 63x.

No bright field record could be taken. However, most of the cells showed autofluorescence signal when exciting them with 405 nm light. The blue signal is weak but perceptible. In Figure 44 a ratio between stained and not stained bacteria is visible.

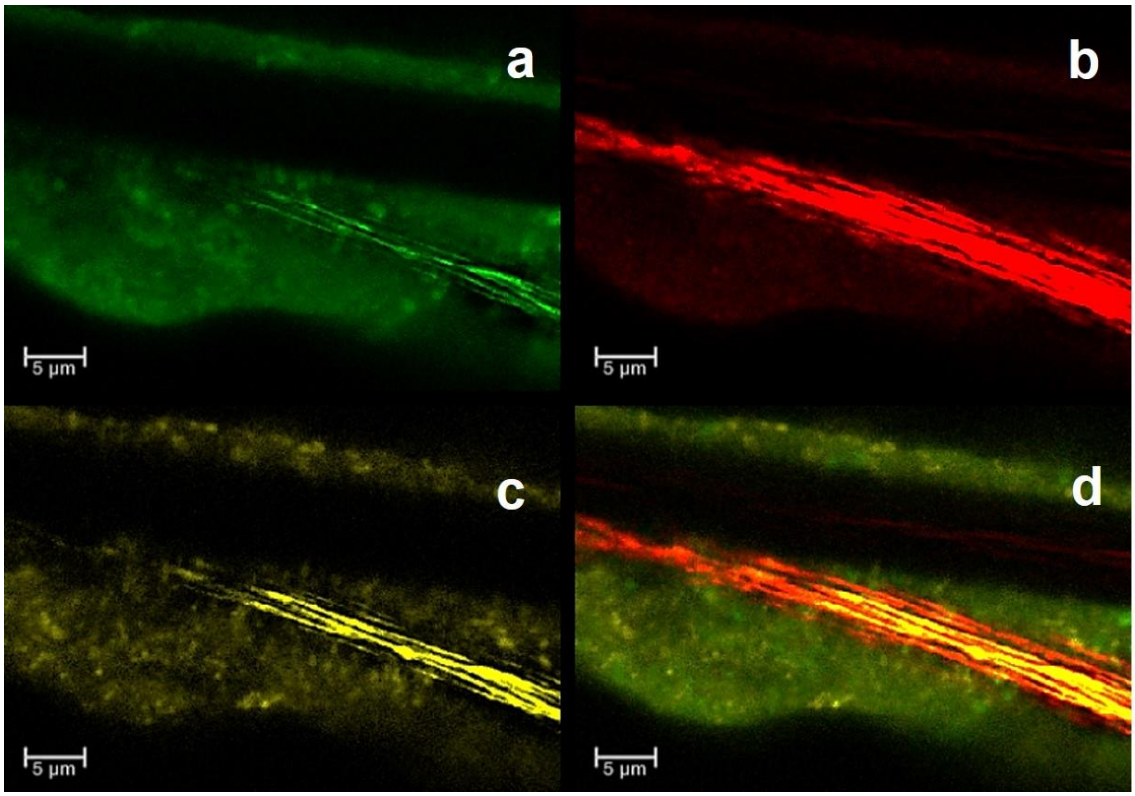

Figure 45: Overview of biofilm on the anode, stained with "EUB", "SHEW" and "GAM" probe. Used magnitude: 63x

Figure 45 shows FISH results without lysozyme or other pretreatments. "GAM" and "EUB" could bind to the sample, since there is a green and yellow signal. In this case again, red

signal just highlights the string. In comparison with Figure 44, the shape of the cells is not well defined.

Sequencing

Table 8: Overview of the bacteria found by sequencing analysis on 2 MECs purged with N_2

Sample	Proportion [%]	Class	Genus	Species
Biofilm 1	77.6	*Deltaproteobacteria*	*Geobacter*	*Geobacter anodireducens*
	11.5	*Spirochaetia*	*Sphaerochaeta*	
	6.6	*Clostridia*		
	3.7	*Anaerolineae*		
	0.3	others		
	0.2	*ARCHEA*		
Biofilm 2	46.4	*Spirochaetia*	*Sphaerochaeta*	
	38.3	*Deltaproteobacteria*	*Geobacter*	*Geobacter anodireducens*
	9.2	*Clostridia*		
	6.0	*Anaerolineae*		
	0.2	*Thermotogae*	*Mesotoga*	*Mesotoga infera*
	0.0	others		
	0.0	*ARCHEA*		
Inoculum	38.0	*Anaerolineae*		
	17.5	*Methanomicrobia*	*Methanoculleus*	*Methanoculleus receptaculi*
	4.1	*Thermotogae*	*Mesotoga*	*Mesotoga infera*
	1.0			*Candidatus Cloacamonas*
	0.4	others		
	39.0	*ARCHEA*		
Suspension culture 1	69.7	*Clostridia*		
	18.3	*Deltaproteobacteria*	*Geobacter*	*Geobacter anodireducens*
	5.0	*Anaerolineae*		
	3.5	*Thermotogae*	*Mesotoga*	*Mesotoga infera*
	2.8	*Spirochaetia*	*Sphaerochaeta*	
	0.3	others		
	0.4	*ARCHEA*		
Suspension culture 2	43.2	*Clostridia*		
	25.0	*Deltaproteobacteria*	*Geobacter*	*Geobacter anodireducens*
	22.5	*Spirochaetia*	*Sphaerochaeta*	
	4.9	*Thermotogae*	*Mesotoga*	*Mesotoga infera*
	3.7	*Anaerolineae*		
	0.5	*Actinobacteria*		
	0.2	others		

	0.0	*ARCHEA*		

Biofilm as well as suspension culture was analyzed. Moreover, initial inoculum was also analyzed to see how cell populations evolved during the experiment. Each sample showed a high variety of microorganism, but due to a very low percentage, they were not suspected to contribute to the system. For this reason, low to zero matching percentage (less than 0.2 %) were excluded from the results in Table 8.

Pie charts see Figure 46 - Figure 48, show a better overview and visualization of bacteria population listed in Table 8. In Table 8 all details of found microorganisms are listed, whereby in Figure 46 - Figure 48 only the biological classification is represented.

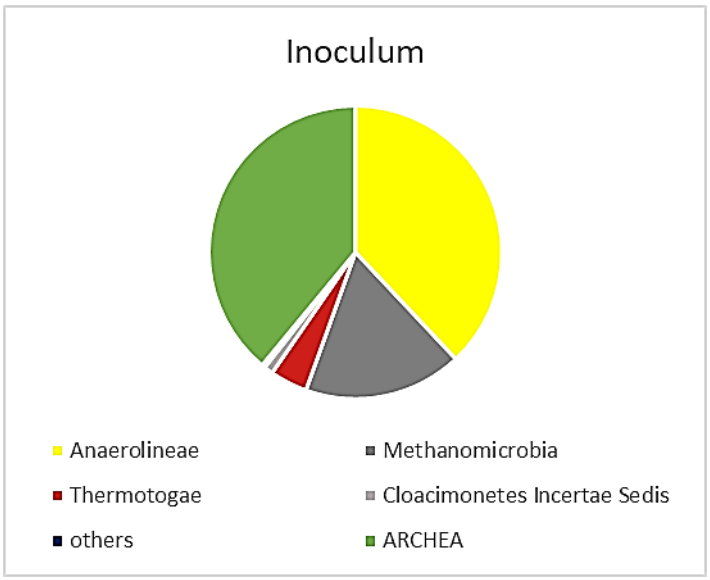

Figure 46: Pie chart representation of microorganism found in inoculum suspension

The MEC batches have been inoculated with a sample taken from the WWTP in Tulln. Figure 46 shows a pie chart, representing the microorganism composition of an used inoculum for MECs. The inoculum comprises mixed sludge; aerobically stabilised sludge (activated sludge) and anaerobically stabilised sludge. A composition of mainly three different microorganisms were detetected: Anaerolineae with 38 %, *Methanoculleus receptaculi* (*Methanomicrobia*) with 17.5 % and *Archea* with 39 %.

Further two MECs have been analyzed by sequenzing. They have been operated with bacteria sample from the inoculum shown in Figure 46. Figure 47 and Figure 48, represents how microorganisms population changed during the experiment.

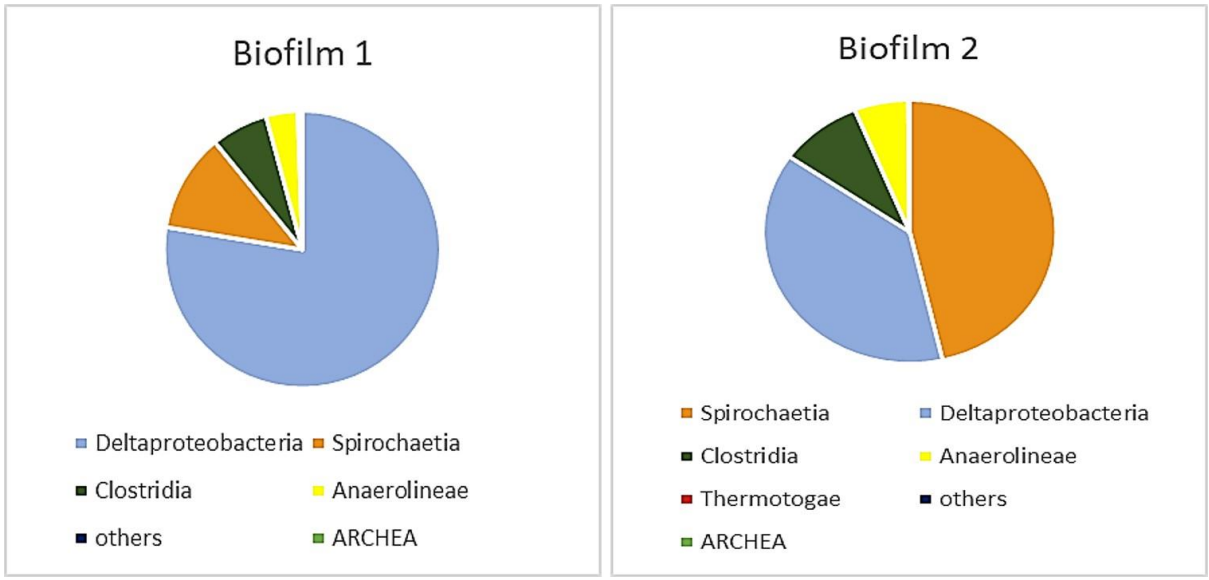

Figure 47: Pie chart representation of microorganism found on the biofilm of MEC 1(left) and MEC 2 (right)

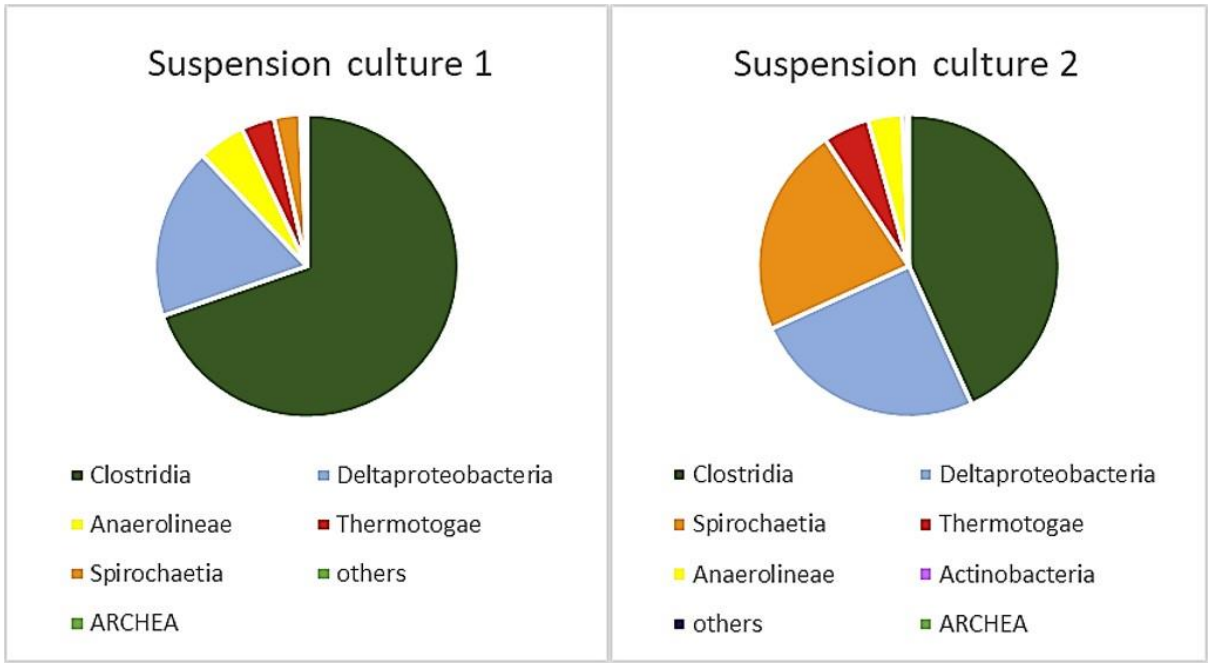

Figure 48: Pie chart representation of microorganism found on the suspension culture of MEC 1 (left and MEC 2 (right)

Note: “Biofilm 1” and “Suspension culture 1” represent MEC 1. “Biofilm 2” and “Suspension culture 2” represent MEC 2.

As seen in Figure 46 to Figure 48, almost the same bacteria strains have evolved during MEC experiment. On the biofilm, *Geobacter anodireducens* (*Deltaproteobacteria*) is the most dominant bacteria with almost 60%. In addition, *Sphaerochaeta* (*Spirochaetia*) plays an important role on biofilm formation. It is the second biggest group on the biofilm with 11.5 % for MEC 1 and 46.4% for MEC 2. *Archea*, methanogens have almost vanished and *Anaerolinea* fraction is drastically reduced to around 5%. Moreover, bacteria from the class of *Clostridia* developed well in MEC conditions, since it is found in the biofilm and as well in the suspension culture. In fact, it is the most abundant bacteria found in the suspension with

69.2 % in MEC 1 and 43.2 % in MEC 2. The proportion of *Clostridia* increased radically, since in the inoculum almost no *Clostridia* was identified.

Again, seen in Figure 47 -Figure 48 *Archea* is practically non-existent anymore and also here, *Anaerolinea* fraction has dropped, but *Mesotoga infera* (*Thermotogae*) managed to stay stable in suspension culture. Compared to the biofilms, suspensions display the same bacteria strain, but in different propotions. *Geobacter anodireducen*s (*Deltaproteobacteria*) plays a much smaller role in the cell suspension with only 20% in contrast to the biofilm (60%).

5. Discussion

5.1. MEC

Sample from WWTP, Tulln

A single chamber has been set up, because it is easier to build as double chamber MECs and works well, see Figure 4 to Figure 21. According to Liu *et al.*, 2004, and Rozendal *et al.* 2007, this modification can be achieved without affecting the electrochemical performance and volumetric hydrogen production rate theoretically doubles. Although, the presence of a membrane is essential for the purity of the hydrogen that is produced at the cathode. Without the membrane the produced hydrogen may be polluted with gaseous metabolic products from the anode chamber (e.g. CO_2, CH_4, H_2S) (Rozendal *et al.* 2007).

Turning around the MECs and equipping the cathode with a vial, hinders produced H_2 to mix up with CO_2 or other metabolic products. In Figure 10 H_2 production was shown possible, however in low proportions (< 1%).

If no daily gas treatment was performed, no H_2 gas accumulation was observed. This led to the presumption, that microorganisms in MEC may metabolize H_2 in a further step. For this reason, two MECs have been treated with H_2/CO_2 gas mix of 80/20 percent ratio. In GC analysis, the 80% of H_2 portion was never measured. Since, H_2 gas is very volatile, it is difficult to perform any analysis. This is indicated by the range of the error bars in Figure 8. GC analytics, for instance, showed 64.5 % H_2 /21.88 % CO_2 instead of the 80/20 ratio. This could mean that either the analysis is wrong – which can be excluded, since calibration is periodically done. Or that H_2 slipped away while taking sample – which is a possible cause. Another possible root cause could be that H_2 is actually metabolised by the microorganisms. Nevertheless, none of the results show a value higher than 80% H_2. This leads to the assumption, that no H_2 is produced.

Jadhav, Ghosh Ray, and Ghangrekar, demontrasted the ability of acetogenic bacteria, including *Clostridium aceticum* to reduce CO_2 to acetate, 2-oxobutyrate and formate, in 2017. According to the equation: $2CO_2 + 8e^- + 8H^+ \rightarrow C_2H_4O_2 + 2H_2O$, CO_2 and H_2 is needed for the formation of acetate. Nonetheless, MECs purged with H_2/CO_2, showed a clear decrease in H_2, shown in Figure 8. CO_2 gas proportion, in contrary, showed a higher percentage as the theoretical 20%. This could be expected, since the formation of one mole acetate needs only 2 moles of CO_2 but 8 moles of H_2. With other words, H_2 is consumed faster, than CO_2, which leads to its higher gas percentage proportion.

Concerning the HPLC analysis, an exogenous power supply, within an acceptable range of electric current, is important to improve the benefits of MECs. This was also confirmed by Dhar, 2016. In MECs treated with N_2 gas, shown in Figure 11, clear acetate consumption is observable as long as an electric current is provided. Contrary, a linear increase of acetate concentration was demonstrated as soon as electricity was shut off.

In MECs treated with H_2/CO_2 gas mixture, no acetate oxidation is observed. Quite the contrary, instead acetate concentration increased during the experiment, see Figure 13. External electricity supply did not contribute to a change within the system. Acetate concentration did not rely on the presence of an exogenous power supply, since the same trend was observable after shutting down the electricity.

This leads to the conclusion, that acetate consumption is only possible if electricity is supplied and almost no H_2 is present in the MEC system. Due to the presence of acetogens, acetate is generated as soon as H_2 reached a threshold value.

Also, other metabolic products have been produced along the experiment. This is shown in Figure 12, Figure 14 and Figure 15. Since they are not important for the case study, the evolution of the metabolic products is captured for other future experiments.

Moreover, in the experiment carried out at 50°C, no notable results have been obtained. H_2 production was also after 50 days at 0% and CO_2 proportion did not show any considerable change. The experiments done at 50° C clearly showed, that the samples taken from WWTP in Tulln, have no thermophile bacteria able of H_2 production out of acetate.

Shewanella

Precultures of *S. oneidensis*, which grew on specific grow medium, TSB, showed high turbidity and high content of H_2 and CO_2. However, the concentrations dropped drastically after changing media from TSB to Klasson media. Since, TSB is based on lactate and Klasson on acetate, the bacteria metabolism had difficulties with this drastic modification. For this reason, only the first GC measurement in Figure 21 showed a higher H_2/CO_2 concentration. Besides, in the case of acetate concentration in Klasson medium, a slightly increase in acetate concentration was perceptible in Figure 20. This could be also due to the remaining lactate of the TSB medium being metabolized into acetate by the cells. Only low consumption of acetic acid is ascertainable during the whole experiment.

This could also be the reason of low H_2 production, which was under 0.1 % in the headspace gas content. Since *S. oneidensis* is known for its high potential of H_2 production, more H_2 was expected. However, it was not possible to exactly quantify the gas volume. For this reason, an exact concentration in composition was impossible to calculate.

Furthermore, between day 38 and 58 no N_2 gas treatment was done, to see if there is a possible inhibition by the product. Since there is a slightly increase in the gas proportion of component H_2 and CO_2 during these 20 days, production inhibition could be excluded. In fact, *S. oneidensis* in the experimental setup could not produce more H_2 as 0.06 % per day.

Summarizing it can be said that biohydrogen production by *S. oneidensis* is possible, but very low in the present experimental setup. Higher titers could be achieved by changing the carbon source (lactate) in the growth medium. It was observable in Figure 21 that high concentrations of the components were caused by remaining lactate in the medium and cells. This caused a longer lag phase.

At some point, there was also the assumption, that the *Shewanella* strain might be the wrong one, since we expected higher H_2 production. Also, during FISH experiments, *S. oneidensis* specific probe was the only one which could not bind to the bacteria.

5.2. SEM

S. oneidensis cells seem to be damaged. The fixation steps before SEM may have been too harsh. However, the fixation step should preserve the form of the microorganisms. In the case of the SEM record for MEC biofilm, the cells are well defined, no damage is observed, and fixation worked well. For this reason, there is the assumption that *S. oneidensis* cells have

been dead already before fixation. This would explain the high cell density in growth medium, the low biohydrogen yield in MEC and the unsuccessful FISH experiment.

MEC purged with N_2 shows an even biofilm formation. A high variety of cells is observable, indicating many microorganism species. This was expected; however, SEM does not show which bacteria participate in direct electron transfer to the electrode. It was also expected to see pili on some bacteria or at least in *S. oneidensis*. This is not observed in any SEM micrographs. Pili are important in some cells for direct electron transfer, especially for *Geobacter* species. The lack of pili on the cells on SEM micrographs may be caused by harsh fixation condition.

5.3. FISH

FISH experiment with *A. vinelandii* worked well. *A. vinelandii* belongs to the class of gamma-proteobacteria. Accordingly, probe “GAM” bound specifically. Shown in Figure 27 and Figure 28, it can be seen, that all bacteria labelled with the general bacteria probe were also labelled with the specific probe. The results have been very good, and no problems have showed up while performing FISH in suspension culture neither on biofilm. The use of lysozyme did not show better results.

FISH signal for *S. aureus* was bad. In the beginning, 35-45 % FA was used, as described on most FISH protocols, for example as described by Thurnheer, Gmür, and Guggenheim, 2004. However, no results were obtained. Neither by changing fixation method nor by using enzymatic pretreatment. Since *S. aureus* has short doubling times and showed high cell density, dilution of the sample was done right before FISH experiment. The results were better, since the probe could bind to most cells. Nevertheless, the signal was weak.

S. aureus is a gram-positive bacterium and therefore ethanol fixation is recommended. Best results have been achieved by applying ethanol fixation and 10 % formamide, as described in the publication of Nistico, 2009 and Cardinale *et al.* 2017.

After the formation of a pure culture biofilm a second cell culture does not attach spontaneously to the first layer. To create a mixed biofilm, all cells have to be inoculated and grown simultaneously in the same medium next to a surface to adhere. Moreover, attention should be paid to the doubling times of the bacteria strains. As an example, in Figure 35 *S. aureus* was incubated as long as *A. vinelandii*. However, doubling time of the *S. aureus* is much shorter than the doubling time of *A. vinelandii*. As a result, the biofilm for *S. aureus* is too thick and only a thin layer for *A. vinelandii* was given. It would be better, to grow the biofilm in a mixed medium and adding bacteria strains after each other. So, the bacteria with the highest doubling rate are added first. Another possibility is to add bacteria at the same time to the media.

Besides, of growing well, it was impossible to stain *S. oneidensis* with FISH probes. Assuming, the wrong bacteria strain or the wrong specific FISH probe, “EUB” probe was still supposed to bind on the sample. Nevertheless, even the general FISH probe could not bind. There is the assumption that *S. oneidensis* membrane may not be permeable to the probes. Neither enzymatic pretreatments nor a higher SDS percentage (4% instead of 2%) could achieve better results.

There was no penetration of the probes with the biofilm sample. This is probably due to the exopolymeric substances (EPS) surrounding the cells. The same problem was already

observed by Pavlekovic *et al.* 2009. In future, harsher pretreatments should be done for *S. oneidensis* to achieve a better permeability of the membrane.

C. acetobutylicum was easy to cultivate and to work with in FISH. Since, *S. oneidensis* and *G. sulfurreducens* had some problems; no further work was done with these strains. Consequently, experiments where done with the mixed culture of the MECs.

Suspension culture was analyzed, but not all cells could be stained by FISH probes. Surprisingly, the specific probe “GAM” showed good results. In fact, most of WWTP bacteria are of the class of gamma- proteobacteria.

In addition, the same results are observable on bacteria on the anode of the MEC. Where no signal of “GEO” and “SHEW” could be found, “GAM” showed an intense signal, leaving no doubt, that bacteria on the biofilm are mostly gamma-proteobacteria.

Enzymatic pretreatment did cause better results in the case of biofilm bacteria: the cells are better defined.

With FISH experiment, there is no need to combine different staining techniques, like RNA/DNA and EPS staining. Localization of the species should to be revealed by specific FISH probes.

Table 9: Overview of the FISH results and used parameters

	S. aureus	**C. acetylbutylicum**	**G. *sulferreducens***
Parameter	**Intensity**	**Intensity**	**Intensity**
PFA	similar results	good results	no results yet
EtoH		not done	
Lys 1 g/L	better results in biofilm	not done	
Lys 10 g/L		not done	
Protease	no amelioration	not done	
Hybridization 35%	bad results	not done	
Hybridization 40%	bad results	not done	
Hybridization 10%	good results	good results	
Hybridization Duration	2 -3 hours	2 hours	
	A. vinelandii	***S. oneidensis***	**MEC Sample**
Parameter	**Intensity**	**Intensity**	**Intensity**
PFA	good results	poor results	best results
EtoH	bad results	poor results	fair results
Lysozym 1 g/L	no amelioration	poor results	no need
Lysozym 10 g/L	no amelioration	poor results	no need
Protease	no amelioration	poor results	no need
Hybridization 35% FA	good results	poor results	-
Hybridization 40% FA	good results	poor results	-
Hybridization 10% FA	best results	poor results	best results
Hybridization Duration	2 hours	no difference	3-4 hours

5.4. Sequencing

Biofilm and cell suspension culture from previous MECs experiments were analyzed. Moreover, initial inoculum was also analyzed to see how cell populations evolved during the experiment. Each sample showed a high variety of microorganism, but due to a very low percentage, they were not suspected to contribute to the system. For this reason, low to zero matching percentage (less than 0.2 %) were excluded from the results in Table 8.

It is obvious that the structure would change over the period which is due to the adaptation of the community structure from complex wastewater environment to acetate as sole carbon and energy source. The determined microorganism communities in the MECs differed strongly from that of the inoculum (see Figure 46 to Figure 48).

Anaerolineae, *Methanoculleus receptaculi* (*Methanomicrobia*), *Archea* and *Mesotoga infera* (*Thermotogae*) were the three main microorganisms in the inoculum sample. They are aerotolerant organisms and typical for the wastewaters of the area. However, many others were found, but showed low presence (< 0.2%). During MEC experiment, *Archea* and *Methanoculleus receptaculi* (*Methanomicrobia*) almost vanished (< 0.2%).

The DNA PCR products of the two-investigated pH 7 anode biofilms were sequenced and resulted in the identification of the genus *Geobacter* (39% - 78%) and *Sphaerochaeta* (11.5% - 46.4%). *Geobacter species* are most abundant in MEC reactors, implying that they play some role in MEC operation. It is found in a higher percentage on biofilm, than the suspension culture. As a matter of fact, *Geobacter anodireducens* is the closest known relative being to *G. sulfurreducens* (98% similarity). Similar to other members of the genus *Geobacter*, *G. anodireducens* is able of electron transfer coupled with the oxidation of acetate (Sun *et al.* 2014). The dominance of *G. anodireducens* for the respective conditions is well in line with previous studies (Carmona-Martinez 2015; Dhar 2016).

Sphaerochaeta genus is the second most abundant species in MEC biofilms (see Figure 47). However, these strains may contribute insignificantly to hydrogen recovery in MEC reactors, since no information was found about their electroactivity. The *Sphaerochaeta* genus does not seem to be an antagonist for biofilm formation. It is either a symbiont or it plays a neutral role. This could only be assessed, by reconstructing a biofilm without this genus and observing the phenotype as well as the functional difference.

Anaerolineae ratio dropped drastically compared to the inoculum sample, but it is the only genus that has the same ratio on biofilm as well as on suspension culture. Different as expected, *Anaerolineae* seems also able to oxidize acetate.

Bacteria strains like *Clostridia* and *Mesotoga infera* (*Thermotogae*) are especially found in the suspension culture. *Clostridium* is a typical wastewater bacterium responsible for degradation of organic substance. *Mesotoga infera*, in the other hand is able of electron transfer, but does not produce biohydrogen. Both bacteria do not contribute to MEC system. However, sequencing has shown, that microbial community responded sensitively to changes in its microenvironment.

Sequencing combined with FISH allows visualization of biofilm communities, clearly defining bacteria species and the three-dimensional structures present in the sample. Manipulation of individual bacteria strain, may clarify its function in the biofilm. In addition, further analysis is necessary for profoundly understanding of functional and spatial phenotype of biofilms. FISH method can also be enhanced, by using more and different probes to reveal exact localization of the species in question.

6. Conclusion

MEC is becoming popular because microorganisms can be used as catalysts and wastewater can be effectively used as potential substrate for hydrogen production. Single chamber set up is an easy construction easy to build and works well. It was shown that H_2 production is possible, however in low proportions (< 1%).

On the other side, no H_2 is produced in MECs treated with H_2/CO_2 gas mixture. Small amounts of CO_2 and H_2 may be used in a further step for acetate production, since acetate concentration is increasing during the experiment. It has been demonstrated, that this process is independent from electricity supply. In contrast, acetate consumption is only possible if electricity is supplied and almost no H_2 is present in the MEC system. A system to instantaneously remove H_2 would probably maximize H_2 production. For this reason, a new MEC design and a change of MEC parameters should be considered.

Unlike written in most publications, methanogenesis was no problem. The use of Bromoethane 2-sulfonate did hinder the formation of methanogens. *S. oneidensis* was used as a reference electroactive bacterium. Normally, it is able to produce H_2 at high rates. During this experiment production of H_2 was possible, but at a very low rate (<0.1 %). Moreover, FISH analysis did not work. Specific probes could not bind. This lead to the assumption, that the wrong strain was present. For this reason, a sample will be sent for sequencing. Besides, SEM analysis showed many damaged cells. There was another assumption, that the cells might be damaged due to the change of the growth medium to Klasson medium. If further work is done with this strain, it might be advantageous to change growth medium composition and to use harsher methods while FISH experiment. For future work the *Geobacter* bacteria is important, since it is the main bacterial colony in the anode biofilm and the most important community for electron transfer.

Concerning biofilm analysis, SEM proved to be a good method to get a view on cell variety, density as well as on the shape of the biofilm. Nevertheless, there is no information about bacteria identity. For this reason, FISH method was used. Fast reliable results about microorganism communities and biofilm phenotypes were expected. Yet, manipulation was not easy and there were many frameworks to consider.

For instance, *S. aureus* demonstrated that a moderate cell number is primordial for successful FISH. Therefore, dilution or concentration of a sample should always be considered prior FISH experiment. Whereas some bacteria, like *A. vinelandii* or *C. acetobutylicum* did not show any difficulties, others did. Use of harsher chemicals or longer hybridization times was the consequence. Biofilms are more difficult work with, since they are protected by an external matrix which renders penetration of the probes sometimes impossible.

Nevertheless, good results on biofilms were achieved. Concerning its phenotype, no ordered shape could be identified, and bacteria showed a thoroughly mixed pattern. Structural behavior in biofilm could not be properly investigated and more work has to be done in this area. Comparing to PCR sequencing, it is a cheap method, which is able to show results within a day. This makes FISH a good and fast method for investigation of microorganisms. In addition, some results on biofilm formation were obtained. Due to FISH, it could be found out, that whenever a biofilm is already formed, no second biofilm could attach on a later point. On the other hand, if Ca^{2+} ions are added, a second layer of bacteria can be attached to a first

layer. Further, it could be shown, that whenever different bacteria grow in the same medium, they are able to attach randomly to the surface. Further investigation is needed in this domain.

Sequencing also lead to interesting results. Bacterial composition of biofilms differ from bacterial composition in suspension culture. On the anode biofilm of a previous MEC experiment, *G. anodireducens* and *Sphaerochaeta* genus were the most abundant bacteria, whereas *Mesotoga infera* (*Thermotogae*) and *Clostridia* the most abundant bacteria in the cell suspension. Moreover, there was the possibility to compare MEC cell culture to initial inoculum culture from WWTP. This demonstrated the microorganism communities have evolved drastically in MECs.

To profoundly study biofilm phenotypes, a combination of different analysis methods is necessary. For future experiments, live dead assay is highly recommended. However, the combination of SEM, sequencing and FISH did show an overview on the spatial phenotype of an anode biofilm.

To take full advantage of MEC, it is very important to understand which intracellular and extracellular factors influence the metabolic rate. It is also necessary to understand biofilm interactions. H_2 production with wastewater is only possible, if electroactive bacteria are promoted. There are also many limitations like methanogenesis, low H_2 yields, expensive bioreactor design materials, energy input and the lack of efficient bacterial strains. A lot of effort has to be done before biological hydrogen production reaches an economical level worth to be industrially produced. However, MEC technology is still at its infancy and it has great potential to become a key process in future biohydrogen production cheaper and a more sustainable alternative to electrolysis.

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8. Annex

8.1. FISH Protocol

FISH Protocol

Materials

For Sample Fixation

1. Sterilized Phosphate- Buffered saline (PBS)

   Prepare stock solution: 500 mL MQ Water with 145 mM NaCl, 1.4 mM NaH_2PO_4, 8 mM Na_2HPO_4. Adjust pH 7.4 and autoclave.
2. 4 % Paraformaldehyde (PFA) (if 37 % PFA, dilute 108 µL of it into 892 µL PBS). Store at RT
3. Ethanol solution of 96%

For Immobilization

1. Multi-well plate for biofilm staining and coated slides from ibidi with 18 wells form suspension culture.
2. (Optional for gram-positive bacteria): Lysozyme working solution of 0.5 -10.0 mg/mL in a 0.05 M EDTA and 0.1 M Tris-HCl buffer.
3. Ethanol solutions of 50 %, 80 %, 96 % in 50 mL tubes. Keep them stored at -20°C

For Hybridization and further Washing

1. 5M NaCl stock solution in 0.5 L MQ Water. Adjust pH to 8, autoclave and store at RT.
2. 1M Tris/HCl in 200 mL MQ Water. Adjust pH to 8 with 1 M HCl, autoclave and store at RT.
3. 10% (w/v) Sodium dodecyl sulfate (SDS), which is sterilized by filtration and stored at RT.
4. 0.5 M EDTA in MQ Water. Adjust pH with crystallized NaOH to a pH of 8. Autoclave and store at RT.
5. Formamid (FA), as pure as possible! Since FA is toxic, precautions must be taken.
6. MQ Water 200 mL
7. FISH probes, fluorescently labelled oligonucleotides. They are diluted in PCR water to 30-50 ng/µL. Light and thawing should be avoided as much as possible. It is advisable to prepare stock solutions out of the stock. Store the stock solutions at -20°C.

Optional

1. DAPI staining (4′,6-Diamidin-2-phenylindol)
2. Alexa Fluor 350

Procedure

Sample Fixation

A good fixative should preserve the cell morphology while concomitantly permeabilizing all cells for the labelled oligonucleotide. Standard fixatives are aldehydes and alcohols.

The process is adjusted to cells in suspension culture or cultivated as biofilms. Furthermore, we distinguish between gram-positive and gram-negative cells.

For gram-positive bacteria it is not advisable to use PFA, because the thick cell wall may become completely impenetrable, otherwise.

Liquid bacterial culture	Biofilm
gram-negative cells	**gram-negative cells**
1. Take of a sample of 1 mL and centrifuge 10 min at 6000 rpm 2. Discard supernatant and resuspend pellet in 892 µL PBS 3. add 108 µL of 37 % PFA (1-4% final concentration) 4. Incubate (for thick walled cells) 1 - 24 h (for fragile cells) at 4°C 5. Centrifuge 10 min. at 6000 rpm and discharge supernatant 6. Thoroughly resuspend fixed cells in 1000 µL PBS and centrifuge 7. Repeat washing step 8. Resuspend cells after second washing step in 500 µL PBS and add 500 µL absolute ethanol 9. Samples can be stored at -20°C for several months	1. Place a 3x3 mm square of tissue sample into a 1 mL tube 2. Add 892 µL PBS and 108 µL 37 % PFA (1-4% final concentration) 3. Incubate 1 - 24 h at 4°C, depending on tissue thickness 4. Discard PFA carefully and add 1 mL PBS 5. Discard PBS and add 1 mL of fresh PBS 6. Remove PBS and add 0.5 mL PBS and 0.5 mL 99% ethanol 7. Store at -20°C
gram-positive cells	**gram-positive cells**
1. Take of a sample of 1 mL and centrifuge 10 min at 6000 rpm 2. Wash pellet in 1000 µL PBS 3. Discard supernatant and resuspend pellet in 500 µL fresh PBS 4. add 500 µL absolute ethanol 5. stored at -20°C	1. Place min a 3x3 mm square of tissue sample into a 1 mL tube 2. Add 1000 µL PBS 3. Gently shake the tube and discard PBS 4. Remove PBS and add 0.5 mL PBS and 0.5 mL 99% ethanol 5. Store at -20°C

Immobilization

Liquid bacterial culture	**Biofilm**
1. Pipette 10 µL sample in the wells 2. Let air dry	1. Place tissue into a 96 multi-well plate
Additional permeabilization of gram-positive or unknown bacteria (optional) Add 20 µL lysozyme solution to sample on objective slide or 100 µL to tissue. Incubate for 10 min – 3 hours at 37 °C. Rinse twice with ice cold PBS.	
3. Dip slides into 50-70-96% ethanol solution series for 3 min each 4. Let it air dry	2. Apply 50-70-96% ethanol solution series for 3 min each by exchanging the solutions in the same well 3. Let it air dry

Hybridization

Note: The formamide concentration is dependent on the probe used and determines the stringency of the hybridization. For most probes a concentration of 30-40% FA is recommended. However, it is better to try different FA concentrations while keeping hybridization temperature constant.

➔ Before starting fixation, prepare hybridization buffer (HB) and 100-500 µL probe dilutions.

Table 10: Composition of hybridization buffer to final volume of 2 mL (for slide, adapt volume for tissue sample)

Components	**Volume [µL]**								
Formamide %	**0**	**10**	**20**	**25**	**30**	**35**	**40**	**45**	**50**
5 M NaCl	360	360	360	360	360	360	360	360	360
1 M Tris / HCl	40	40	40	40	40	40	40	40	40
MQ Water	1598	1398	1198	1098	998	898	798	698	598
Formamide	0	200	400	500	600	700	800	900	1000
10% SDS	2	2	2	2	2	2	2	2	2

Note: add SDS last to avoid precipitation

Liquid bacterial culture	Biofilm
1. Prepare HB and probe dilutions 2. Pipette 20 µl HB on each well 3. Add 2 µl of probe dilution to the samples in each well 4. Prepare hybridization humidity chamber by placing folded paper towel approximately the size of a slide into a 50 mL a polyethylene tube. Soak tissue with remaining HB. 5. Place the slide into the polyethylene tube. 6. Cover tube with tin foil 7. Place tube horizontally in a hybridization oven for 100-180 min at 46°C	1. Prepare HB and probe dilutions 2. Pipette 50 µl HB on each well 3. Add 5 µl of probe dilution to the samples in each well 4. Pure remaining HB in wells around the sample and close plate with the lid 5. Cover with tin foil 6. Incubate in a hybridization oven for 100-180 min at 46°C

Washing

- ➔ place 50 mL MQ water on ice
- ➔ prepare 50 mL washing buffer and preheat it to 46 °C

Note: The stringency in the washing buffer is achieved by adjusting the NaCl concentration.

Table 11: Composition of washing buffer

Components	**Volume [mL]**								
Formamide %	**0**	**10**	**20**	**25**	**30**	**35**	**40**	**45**	**50**
5 M NaCl	9	4.5	2.15	1.49	1.02	0.7	0.46	0.3	0.18
1 M Tris / HCl	1	1	1	1	1	1	1	1	1
0.5 EDTA	0	0	0.5	0.5	0.5	0.5	0.5	0.5	0.5
MQ Water	40	42.7	46.35	47.01	47.48	47.8	48.04	48.2	48.32

Liquid bacterial culture	Biofilm
1. Take slide out of the hybridization chamber and put it into the washing buffer tube for 10 min 2. Dip slide shortly into ice-cold MQ water 3. Let it air dry	1. Work in a preheat water bath (48°C) 2. Eliminate HB and probes 3. Rinse sample twice with washing buffer 4. Add washing buffer and incubate for 10-15 min 5. Replace washing buffer by ice- cold MQ water 6. Remove MQ water 7. Let it dry with compressed air

DAPI / Alexa Fluor 350 (optional)

Liquid bacterial culture	**Biofilm**
1. For counterstaining, cover each well with 20 µl of a 1 µg mL-1 DAPI/ Alexa Fluor 350 solution 2. Incubate for 10 minutes in the dark 3. Replace working solution by 20 µL PBS and incubate for 5 min 4. Remove PBS and let it air dry	1. Add 25 µL of a 1 µg mL-1 staining solution 2. Incubate for 10 minutes in the dark 3. Wash it with PBS 4. Let it dry with compressed air

Microscopy

Before to microscope, add a droplet of water or mounting oil on each well.

Biofilms tissue is placed on a normal microscope glass-slide, wettened with some drops of water and covered by coverslip.

8.2. FISH Probe details

Table 12: Details of used FISH probes (1)

	GEO	GEO 1	GEO2	GAM
Sequence (5'->3')	[CY3] GAA GAC AGG AG G CCC GAA A	GTC CCC CCC TTT TCC C GC AAG A	CTA ATG GTA CGC GGA C TC ATC C	[FITC] GCC TTC CCA CAT CGT T T
Physical Property	Length : 19bp Weight :6521.6g/mol T_m :58.8°C GC :57.9%	Length : 22bp Weight :6567.2g/mol T_m :65.8°C GC :63.6%	Length : 22bp Weight :6695.4g/mol T_m :62.1°C GC :54.5%	Length : 17bp Weight :5640.3g/mol T_m :52.8°C GC :52.9%
Purification:	HPLC	HPLC	HPLC	HPLC
Synthesis Scale:	1.0 µmol	0.20 µmol	0.20 µmol	0.20 µmol
Delivery Format:	Conc. Adjusted (H_2O) Conc (pmol/µl) : 100 Vol (µl) : 150 Aliquotes :5 Ali. Vol. (µl) :25	Conc. Adjusted (H_2O) Conc (pmol/µl) : 100 Vol (µl) : All Aliquotes :5 Ali. Vol. (µl) :25	Conc. Adjusted (H_2O) Conc (pmol/µl) : 100 Vol (µl) : All Aliquotes :5 Ali. Vol. (µl) :25	Conc. Adjusted (H_2O) Conc (pmol/µl) : 100 Vol (µl) : All
Quality Control:	MALDI	MALDI	MALDI	MALDI

Table 13: Details of used FISH probes (2)

	EUB338	EUB338 II	EUB338 III	SHEW
Sequence (5'->3')	[CY3] GCT GCC TCC CG T AGG AGT	[CY3] GCA GCC ACC CGT AGG TGT	[CY3] GCT GCC ACC CGT AGG TGT	[CY5] AGC TAA TCC CAC CTA G GC WTA TC
Physical Property	Length : 18bp Weight :6108.2g/mol T_m :60.5°C GC :66.7%	Length : 18bp Weight :6117.3g/mol T_m :60.5°C GC :66.7%	Length : 18bp Weight :6108.2g/mol T_m :60.5°C GC :66.7%	Length : 23bp Weight :7589.7g/mol T_m :60.6°C GC :47.8%
Purification:	HPSF	HPSF	HPSF	HPLC
Synthesis Scale:	1.0 µmol	1.0 µmol	1.0 µmol	0.20 µmol
Delivery Format:	Conc. Adjusted (H_2O) Conc (pmol/µl) : 200 Vol (µl) : All	Conc. Adjusted (H_2O) Conc (pmol/µl) : 200 Vol (µl) : All	Conc. Adjusted (H_2O) Conc (pmol/µl) : 200 Vol (µl) : All	Conc. Adjusted (H_2O) Conc (pmol/µl) : 100 Vol (µl) : All
Quality Control:	MALDI	MALDI	MALDI	MALDI