# Ecophysiology of microorganisms in Microbial Electrolysis Cells

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# Ecophysiology of microorganisms in Microbial Electrolysis Cells

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# Index

# Chapter 1

## Introduction

### 1.1 Energy

All life on earth is dependent on energy: autotrophs (e.g. plants, phototrophic microorganisms) harvest energy from the sun, and heterotrophs (e.g. vertebrates, heterotrophic microorganisms) use organic compounds produced by phototrophs. Alternatively, chemical energy can drive energy conserving mechanisms in microorganisms. Since the origin of life, energy was captured and accumulated on earth as biomass and as carbon rich fossil fuels. Burning of wood and the use of fossil fuels by humans has lead to the release of immense amounts of this captured energy. This energy release allowed development of industries and transportation, but also appeared to be limited and harmful for our own environment. To sustain the use of energy by man, it is essential to look for other usable and transportable forms of energy that can be captured from the sun. Microorganisms have evolved mechanisms to thrive in almost any environment (Canganella & Wiegel, 2011) and gain energy from many different substrates (Lowe *et al.*, 1993). Therefore, exploring the microbial world and understanding the mechanisms that they use to conserve energy seems promising to find alternative ways to produce usable energy. We can use microorganisms to produce energy rich products and to conserve energy from waste streams for production of e.g. biogas (Du *et al.*, 2011). A more recent development is to use microorganisms to produce electrical power from organic substrates and waste streams in bio-electrochemical systems (BES). The principle of 'liberating electrical energy by microorganisms' has been described already in 1911 by Potter (1911). But only since the last decade, BES have more extensively been studied as application for generation and storage of energy (Logan *et al.*,

2006; Rozendal *et al.*, 2006; Grinberg & Skundin, 2010; Lefebvre *et al.*, 2011) and are generally based on the microbial fuel cell.

## 1.2 Microbial Fuel Cell

The Microbial Fuel Cell (MFC) is a two electrode system in which energy in organic compounds is released as electricity by the metabolic activity of microorganisms. The typical MFC consists of an anode and a cathode, separated by an ion exchange membrane and connected to an electrical circuit (Figure 1.1a). At the anode, electrochemical active microorganisms break down organic substrates to protons, electrons and $CO_2$. The anode serves as the electron acceptor for the electrons that are transported to the cathode via the electrical circuit. In the cathode compartment an electron-acceptor is present that is reduced by the electrons from the cathode. The electron flow from anode to cathode can be collected as electrical power. Oxygen or iron(III) (in ferricyanide) are often used as electron acceptors at the cathode to obtain optimal current production. Other conversions that are employed at the cathode, include the use of microorganisms for treatment of pollutants such as nitrate (Clauwaert *et al.*, 2007), cupper(II) (Tao *et al.*, 2011), chromium(VI) (Tandukar *et al.*, 2009) and perchlorate (He & Angenent, 2006; Butler *et al.*, 2010). Because the reaction at the cathode has a higher potential than the reaction at the anode, the electrons will flow from anode to cathode. Typically the MFC consists of two compartments, but other configurations with one compartment, multiple stacked compartments or cylindrical upflow compartments have also been described (Du *et al.*, 2007).

## 1.3 Microbial Electrolysis Cell (MEC)

The microbial electrolysis cell (MEC) has a similar setup as the MFC. However, an applied voltage makes it possible to drive endergonic reactions at the cathode, such as proton reduction (Figure 1.1b). Although there have been attempts to produce several other products at the MEC cathode, the MEC is mostly referred to as a system to produce hydrogen gas ($H_2$) at the cathode (Liu *et al.*, 2005; Rozendal *et al.*, 2006). The advantage of the MEC is that the energy released in the anodic compartment as electrical power can be used directly to produce valuable products, such as $H_2$, at the cathode. Such a reaction can reduce the energy needed to produce $H_2$ about 5 times, compared to $H_2$ production trough

water electrolysis. Microorganisms have been used to catalyze the anodic as well as the cathodic reaction (Rozendal *et al.*, 2008; Jeremiasse *et al.*, 2009a). The MEC has shown potential for the small scale production of $H_2$. The main challenges are the reduction of the material costs and improvement of the cathodic efficiencies (Hamelers *et al.*, 2010; Logan, 2010). Cathodic efficiencies can be increased by the use of good catalysts. A good catalyst for $H_2$ production is platinum, but platinum is very expensive and therefore cheap alternatives are required. A cheap and promising alternative is the biocathode. The biocathode is defined here as a cheap material support (e.g. carbon) cathode at which microorganisms catalyze the reaction (which is $H_2$ formation in the MEC). The biocathode is attractive for technological application and interesting from the scientific point of view because using a cathode makes it possible to explore microbial growth under restricted conditions with regulated available energy in the form of electrical power. In this thesis we studied the microbial communities in MEC systems.

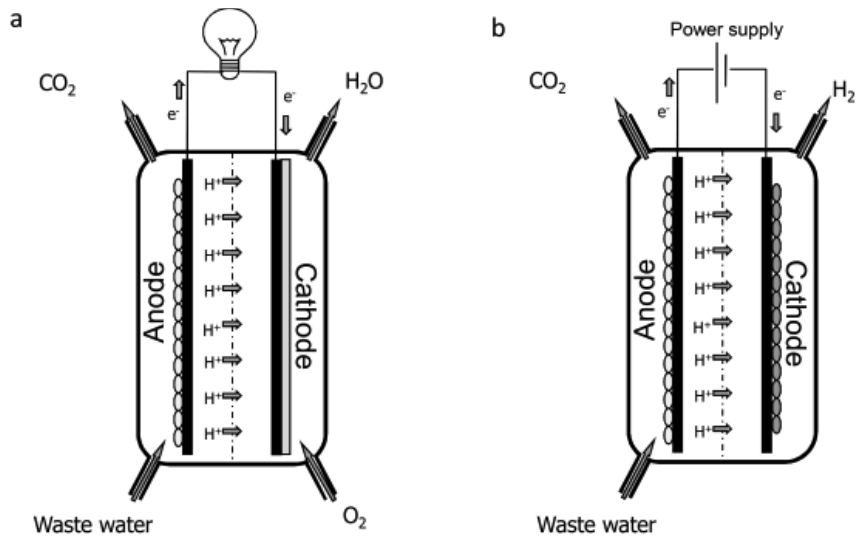


**Figure 1.1** Schematic overview of the MFC and MEC setup a) MFC, b) MEC. Picture kindly provided by René Rozendal.

## 1.4 Hydrogen

$H_2$ is a valuable product. Combustion of $H_2$ delivers clean energy with only water as waste product. Moreover, $H_2$ is an important industrial chemical used in the petroleum industry and in the production of e.g. ammonia, methanol

and hydrocarbons (Ewan & Allen, 2005). $H_2$ can be produced by gasification of fossil fuels or by water electrolysis which requires input of energy from fossil or sustainable sources, such as wind or solar energy. However, expensive catalysts such as platinum are required to catalyze water hydrolysis by sun light and advanced installations are required for wind energy. An alternative that does not require fossil energy, advanced installation or expensive catalysts is biological $H_2$ production. Biological $H_2$ is produced by bacterial fermentation of organic compounds (possible from waste streams). Several bacterial genera like *Clostridium* and *Enterobacter* (Prasertsana *et al.*, 2009) and also others (Hung *et al.*, 2011) are capable of producing $H_2$. However, yields are typically only 10-20% with a maximum of 33% that can be reached by some thermophiles (Benemann, 1996; Logan, 2004; Verhaart *et al.*, 2010). Acetate is an end-product in bacterial fermentations, and cannot be fermented further to $H_2$ and $CO_2$ because of thermodynamic limitations.

## 1.5 Thermodynamics

The energy that is needed to drive a chemical reaction or that can be obtained from a chemical reaction can be calculated with the Gibbs free energy of the reaction ($\Delta G_r$). The available energy depends on the redox potentials of the electron donating reaction and the electron accepting reaction. The sign of $\Delta G_r$ depends on the direction of the equilibrium of the overall reaction. For an exergonic (spontaneous) reaction the $\Delta G_r$ is negative. A spontaneous reaction means that the equilibrium of the reaction lies in the direction of the products. However, often a catalyst is needed to initiate a reaction or to increase the rate of a reaction. The $\Delta G^{o'}_r$ is the $\Delta G_r$ under biological standard conditions (pH 7, 298K, 1M reactants) and is the sum of the $\Delta G^{o'}_f$ (Gibbs free energy of formation) of the products minus the sum of the $\Delta G^{o'}_f$ of the reactants (Logan *et al.*, 2008; Atkins & Jones, 2001).

For example considering the following fermentation reactions under biological standard conditions with $\Delta G^{o'}_r$ ($\Delta G^{o'}_f$ values from: Atkins & Jones, 2001):

$$C_6H_{12}O_6 + 12H_2O \rightarrow 12H_2 + 6HCO_3^- + 6H^+,\ \Delta G^{o'}_r = +3\ kJ/mol, \quad (1)$$

$$CH_3COO^- + 4H_2O \rightarrow 4H_2 + 2HCO_3^- + H^+,\ \Delta G^{o'}_r = +105\ kJ/mol, \quad (2)$$

and

$$C_6H_{12}O_6 + 4H_2O \rightarrow 4H_2 + 2CH_3COO^- + 2HCO_3^- + 4H^+,\ \Delta G^{o'}_r = -206\ kJ/mol, \quad (3)$$

Based on these $\Delta G^{o'}_r$ values, glucose fermentation to 4 $H_2$, 2 acetate and 2 bicarbonate can occur 'spontaneously' (with microorganisms as catalyst), but further fermentation of acetate to $H_2$ cannot occur 'spontaneously' under standard conditions. The fermentation of acetate can only be driven by pulling or pushing the reaction towards product formation by e.g. removing the products such as consumption of $H_2$ causing very low $H_2$ pressure conditions or by addition of energy such as light (phototrophic bacteria).

The $\Delta G'_r$ under specific conditions for the reaction $aA + bB \rightarrow cC + dD$ can be calculated using the equation:

$$\Delta G'_r = \Delta G^{\circ}_r + RT \ln ([C]^c[D]^d/[A]^a[B]^b) \quad (4)$$

In which $\Delta G^{\circ}_r$ is the Gibbs free energy of the reaction at standard conditions (in Joules), R is the ideal gas constant (8.315 J/mol/K), T is the absolute temperature (K), and [A], [B], [C] and [D] are the concentrations of the reactants (in moles per liter).

For electrochemical reactions it is convenient to evaluate the reaction in Volts (V) instead of Joules (J). The relation between $\Delta G_r$ (in J) and $E_r$ (equilibrium potential of the reaction in V) is expressed according to:

$$\Delta G_r = - nF E_r \quad (5)$$

In which n is the amount of electrons transferred (in mol) and F is constant of Faraday (= 96485 Coulomb per mol electrons). Combining equation (4) and (5), results in the Nernst equation:

$$E_r = E^{\circ}_r + (RT/nF) \ln ([C]^c[D]^d/[A]^a[B]^b), \quad (6)$$

$E^{\circ}_r$ is the standard (cell or electrode) potential which can be calculated from the electron donating ($E_{don}$) and electron accepting ($E_{acc}$) reaction according to $E^{\circ}_r = E_{acc} - E_{don}$ or in an MEC, the anode ($E_{an}$) and cathode potential ($E_{cat}$) according to $E^{\circ}_r = E_{cat} - E_{an}$. In contrast to the $\Delta G_r$, the $E_r$ is positive for a spontaneous reaction. For example if we want to evaluate acetate oxidation to $H_2$ and bicarbonate in an MEC, the $\Delta E_r$ for the reaction is calculated with:

Donating reaction at the anode: $CH_3COO^- + 4H_2O \rightarrow 2HCO_3^- + 9H^+ + 8e$ (7)
Accepting reaction at the cathode: $8H^+ + 8e^- \rightarrow 4H_2$ (8)
Overall reaction : $CH_3COO^- + 4H_2O \rightarrow 2HCO_3^- + H^+ + 4H_2$ (9)

Using equation (6) for MEC relevant conditions (pH 7, T=298, $pH_2$=1 bar and 5 mM acetate and 5 mM bicarbonate) gives an anode reaction of $E'_{an}$ = -0.296V and the cathode reaction of $E'_{cat}$ = -0.414V. The overall energy of the reaction is -0.414 - -0.296 = -0.118V (Rozendal *et al.*, 2006). Because this is negative, energy needs to be invested to produce $H_2$ from acetate. By applying a voltage on the MEC, electrolysis reactions become feasible ($E^{o'}_r > 0$). Theoretically only 0.12 V is needed to produce $H_2$ from acetate, but in practice an applied voltage of 0.25V is required because of several losses in potential (Rozendal *et al.*, 2006; Sleutels *et al.*, 2009). In comparison, direct splitting of water without use of microorganisms into $H_2$ and $O_2$ requires a theoretical applied potential of 1.23 V and in practice about 2.0 V (Liu *et al.* 2005). The difference between the theoretical energy needed to drive a reaction and the actual energy that needs to be applied to drive the reaction is called the overpotential (η in Volts). Catalysts can lower the overpotential. In MEC the overpotential is considered as a loss and can be defined as the difference between the theoretical cell voltage and the actual minimal applied voltage needed to form $H_2$ at the cathode (in the example: η = 0.25V -0.12V = 0.13V). Alternatively, the overpotentials of each electrode can be determined separately. In this case, the anode overpotential is the difference between the substrate oxidation potential (e.g. acetate, -0.269V) and the anode potential (as measured), the cathode overpotential is the difference between the cathode potential (as measured) and the $H_2$ formation potential (-0.414V).

In practice the overpotential includes the potential 'loss' in the microorganisms, reaction activation (activation overpotential) and concentration gradients of reactants (concentration overpotential). These different parts of the overpotential can be calculated separately (Jeremiasse *et al.*, 2009b), but, in practice often no details on specific reactions are known and the general calculation of the overpotential as described here is used. From a biological point of view the overpotential (minus the concentration overpotential and activation overpotential) is the maximal potential difference that the micororganisms can use to gain energy. The catalytic ability of microorganisms will also lower the activation overpotential and concentration overpotential.

## 1.6 Microbial metabolism

Microbial metabolism is divided into catabolism and anabolism. During catabolism, metabolic energy is generated by the microorganism which is used in anabolism for building biomass (growth) and for e.g. motility. Typically, microorganisms are

characterized by the primary energy source, the terminal electron acceptor used in catabolism, and the carbon source used in anabolism.

### 1.6.1 Carbon source

The carbon sources that can be used are organic carbon sources (heterotrophs) or $CO_2$ (autotrophs). Heterotrophs can use a large variety of substrates as carbon source, such as sugars, proteins and fats. Organic substrates are taken up (if necessary after hydrolysis into digestible compounds such as sugars, amino acids and fatty acids) by the microbial cell and broken down to metabolic intermediates (e.g. acetyl-CoA, butyryl-CoA, pyruvate) that are used for cell synthesis (Madigan *et al.*, 2000). Autotrophs fix $CO_2$ to produce such intermediates. Autotrophs can use different mechanisms for $CO_2$ fixation, partly dependent on their exposure to oxygen. For example phototrophs use the so called Calvin cycle for $CO_2$ fixation and acetogens, which produce acetate from $CO_2$ (strict anaerobic Gram-positive bacteria and *Proteobacteria* or Archaea), or methanogens, which produce methane from $CO_2$ and $H_2$ (Archaea), generally use the Wood-Ljungdahl (or reductive acetyl-CoA) pathway. Other pathways that are used for $CO_2$ fixation are the reductive TCA cycle, used by some anaerobes and microaerophiles, the 3-hydroxypropionate/malyl-CoA cycle, used by green nonsulfur bacteria and the 3-hydroxypropionate/4-hydroxybutyrate cycle used by some Archaea (Thauer, 2007).

### 1.6.2 Energy source

Energy can be obtained from chemical conversion (chemotrophs) or from the sun (phototrophs). Chemotrophs are divided into chemoorganotrophs that use organic compounds (e.g. carbohydrates) as energy source or chemolithotrophs that use inorganic compounds (e.g. $H_2$, $H_2S$, $NH_4^+$, etc.) as energy source. Phototrophs are divided into oxygenic phototrophs that use water as electron donor by splitting water into $H_2$ and $O_2$, or anoxygenic phototrophs that use for example sulfur or sulfide as electron donor to reduce $CO_2$ to organic compounds (see Figure 1.2).

Microorganisms conserve the energy from the energy source in ATP. ATP can be synthesized in two ways. First of all, by substrate level phosphorylation (SLP) the formation of ATP is directly connected to a non-membrane-bound biochemical reaction. An energy rich phosphorylated intermediate reacts with ADP to form ATP. The second way of ATP generation is by electron transfer phosphorylation (ETP) when ATP is synthesized via an electrochemical gradient (usually proton gradient) that is build up via membrane bound processes (during aerobic and

anaerobic respiration). This proton gradient can also be created by cytoplasmic proton consumption ($H_2$ formation). ETP is the main mechanism of bacteria that grow chemolithotrophically (Madigan,*et al.*, 2000).

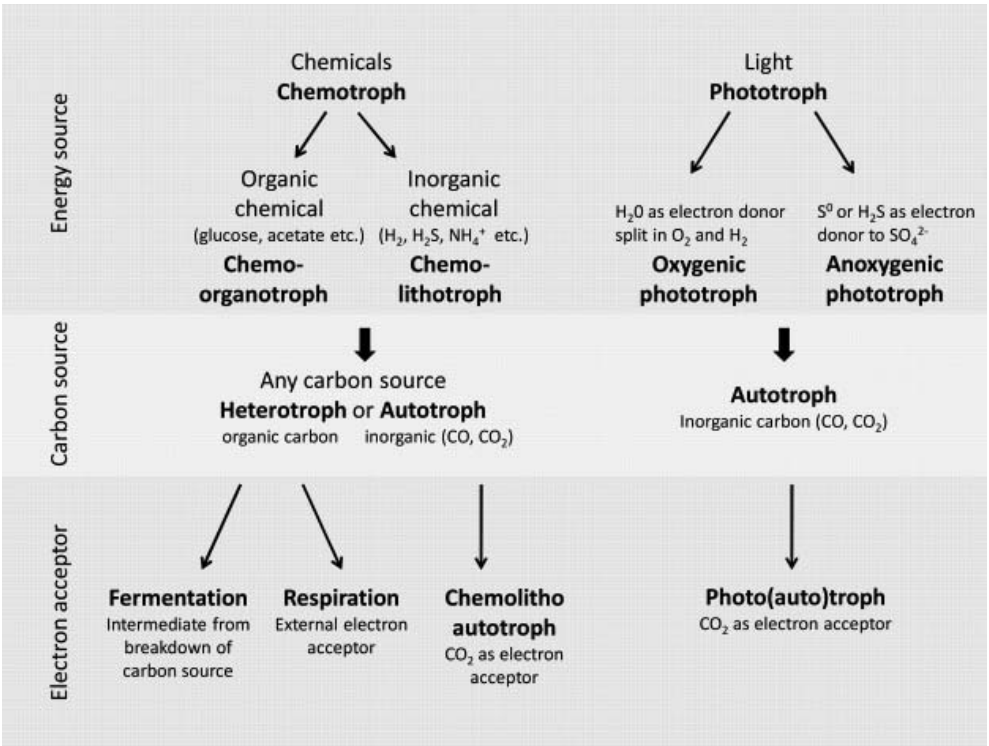


**Figure 1.2** Options for microbial growth separated in energy and carbon source and electron acceptor. Information obtained from Madigan *et al.*, (2000).

### 1.6.3 Electron acceptor

Because microbial ATP formation always involves a sequence of transfer of electrons, the energy source can be termed as electron donor. As a result of this electron transfer, an electron acceptor at the end of the electron flow is essential. In fermentation this is an intermediate from the breakdown of the carbon/energy source itself and therefore the electron donor generates the electron acceptor. The alternative to fermentation is respiration, in which an external electron acceptor is used as terminal electron acceptor such as oxygen for aerobic microorganisms or any other chemical compound that can accept electrons for anaerobic growth. Besides the use of $CO_2$ as carbon source, $CO_2$ can also be used as electron acceptor as is the case for methanogens and acetogens.

To gain energy for either growth (anabolic) or maintenance (catabolic), microorganisms shuttle electrons from electron donating substrates with relatively negative redox potentials to electron accepting substrates with more positive redox potentials. The potential difference between the redox couples determines the energy that can be conserved by the microorganisms. Growth has been observed even under conditions where this difference is close to zero (Dolfing *et al.*, 2008; Kim *et al.*, 2010). In many cases this electron shuttling takes place through NAD(H), FAD(H) or ferredoxin (most common intracellular electron shuttles, also called reducing equivalents), and the electron transfer chain (involving cytochromes and quinones) to any terminal soluble electron acceptor (e.g. oxygen, sulfate, nitrate, or $CO_2$ for several autotrophic microorganisms) (Hamilton, 2003).

However, it has been shown that both the electron donor and the electron acceptor can be solid materials that are reduced or oxidized externally, e.g. when the electron acceptor and donors are solid iron (oxides) or manganese (Nealson & Little, 1997; Stams *et al.*, 2006). A tool to study this so called exocellular electron transfer (EET) is the electrochemical cell (MFC/MEC). It has been shown that an electrode can be used as external electron acceptor (Bond & Lovley, 2003) and also as external electron donor (Gregory *et al.*, 2004). EET can be either direct or indirect (Schröder, 2007). Direct electron transfer is defined as electron transport through cell associated proteins or appendages (e.g. cytochromes or nanaowires) when bacterial cells have direct contact with the electrode. Indirect electron transfer is defined as electron transport through non-cell-associated shuttle compounds such as methyl viologen, humic acid, sulfide, cysteine, riboflavin, phenazine, and quinones (Logan, 2009; Stams & Plugge, 2009). Although electron shuttles in the bulk solution have a large positive effect on the current production in BES (Chen *et al.*, 2010; Watanabe *et al.*, 2011; Yuan *et al.*, 2011), direct electron transfer seems to have many advantages in applications since there is no problem of wash out of shuttle components.

## 1.7 Electron transfer mechanisms in Bio-Electrochemical Systems

### 1.7.1 Phylogeny of the microbial species involved

Microorganisms that can transfer electrons either from or to an electrode are termed electrochemically active microorganisms. Most electrochemically active microorganisms have been described as microorganisms that transfer electrons to the anode of an MFC. Microorganisms of several phylogenetic groups (mostly

from *Proteobacteria*, *Firmicutes*, *Bacteroidetes*) have been detected using molecular biological approaches and of each group representative species have been isolated and tested in an MFC (Phung *et al.*, 2004; Logan, 2009; Nevin *et al.*, 2011). Since the first descriptions of MEC (Liu *et al.*, 2005; Rozendal *et al.*, 2006) only a few studies focused on the microbiology in the MEC, mainly on anode communities (Chae *et al.,* 2008; Call *et al.*, 2009; Cheng *et al.*, 2009; Kiely *et al.*, 2011; Lu *et al.*, 2011; Parameswaran *et al.*, 2011; Thygesen *et al.*, 2011; Torres *et al.*, 2009) compared to microbiological studies in the MFC (854 hits on Scopus for Microbial Fuel Cell and microbial communities).The microorganisms that have been detected in mixed communities of MECs anodes are mainly *Proteobacteria,* but also *Firmicutes* and *Bacteriodetes* were dominant. A Ribosomal Database Project (RDP) search (Cole *et al.*, 2009) for bacterial 16S rRNA genes using the search terms 'MEC' or 'Microbial Electrolysis Cell' gave 275 hits (after filtering for MEC hits that did not mean Microbial Electrolysis Cell) from 7 studies of which 153 *Proteobacteria*, 50 *Bacteroidetes*, 47 *Firmicutes*, 6 *Actinobacteria* and 19 others (see Figure 1.3a). In comparison, a similar search of 'MFC' or 'Microbial Fuel Cell' gave almost 6000 hits with the same dominant phylogenetic groups (Figure 1.3b). Comparative studies (Chae *et al.*, 2008; Kiely *et al.*, 2011) have revealed that *Geobacter* related species are less abundant in MEC than in MFC. However, other *Proteobacteria* seem to be dominant in most experiments in both MEC and MFC. The microbial diversity in MEC biocathodes and catholytes from MECs for $H_2$ production was not studied and in this thesis we describe the first community analyses for MEC biocathodes.

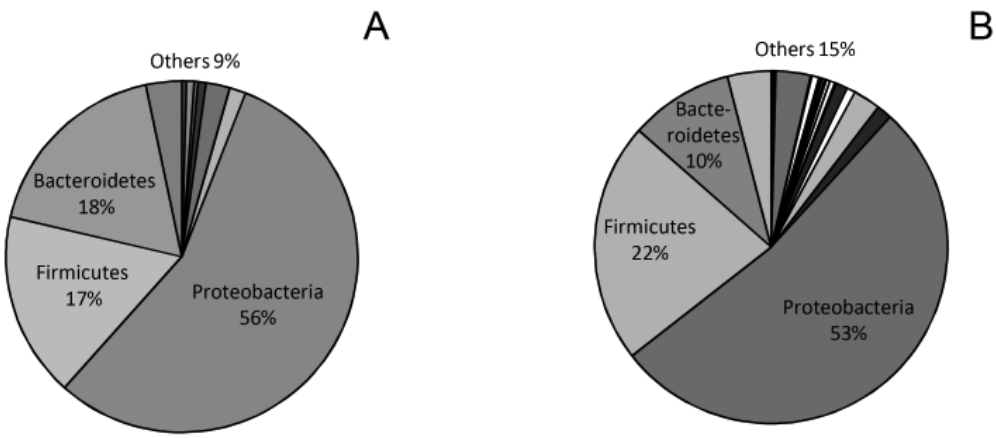


**Figure 1.3** 16S rRNA gene sequences retrieved from RDP related to MEC and MFC. (A) Sequences from 'MEC' and/or 'Microbial Electrolysis Cell'. Total 275 hits from 7 studies were found. (B) sequences from 'MFC' and/or 'Microbial Fuel Cell'. Total 5910 hits were found.

### 1.7.2 Molecular mechanism of exocellular electron transfer and $H_2$ production

The essential properties of microorganisms to function as catalyst at a cathode for $H_2$ production are 1) $H_2$ production ability and 2) uptake of electrons from the cathode (exocellular electron transfer). Cathodic microorganisms have been mainly studied for oxygen reduction (He & Angenent, 2006) in the MFC, but also reduction of other compounds such as metals (Tandukar *et al.*, 2009; Tao *et al.*, 2011), fumarate (Dumas *et al.*, 2008), nitrate (Clauwaert *et al.*, 2007) and chlorinated compounds (Butler *et al.*, 2010) have been studied. Only one study described the $H_2$ production by a pure culture of *Geobacter sulfurreducens* at a cathode (Geelhoed & Stams, 2011) and it has been suggested that *Desulfitobacteria* can produce $H_2$ with electrons derived from a cathode (Aulenta *et al.*, 2008). Coating of electrodes with hydrogenases (Lojou, 2004; Armstrong *et al.*, 2009) or immobilized *Desulfovibrio* cells (Lojou *et al.*, 2002) also catalyzes $H_2$ production, but stability and long term performance of enzyme coatings remained problematic. The catalytic activity of hydrogenases in general has been studied extensively (Vignais & Colbeau, 2004, Armstrong *et al.*, 2009), but the mechanism of exocellular electron transfer towards the microbial cell and the cytoplasm is not yet understood.

### 1.7.3 Hydrogenases

$H_2$ production at a cathode needs a good catalyst to overcome the energy losses due to overpotential. Microorganisms may possess hydrogenases, redox enzymes that catalyze the reversible reaction $2H^+ + 2e^- \leftrightarrow H_2$. Hydrogenases are categorized according to their (redox active) metal site. The three groups of hydrogenases are 1) nickel-iron (NiFe)-hydrogenases of which a sub-group contains also selenium, 2) iron-iron (FeFe)-hydrogenases and 3) iron (Fe) hydrogenases. The catalysis of $H_2$ production is mostly associated with the FeFe-hydrogenases and $H_2$ consumption mostly with NiFe-hydrogenases. Most described FeFe hydrogenases are located in the cytoplasm, but periplasmic FeFe hydrogenases have been described and are thought to be involved in $H_2$ consumption and not production. The NiFe hydrogenases consist of 4 different functional groups, the membrane bound uptake hydrogenases (group 1), the uptake and $H_2$ sensing hydrogenases (group 2), the cofactor F420 and methyl viologen reducing and bidirectional hydrogenases (group 3) and the membrane bound energy converting hydrogenases (group 4) (Table 1.1) (Vignais & Billoud, 2007). Of those, the bidirectional NiFe-hydrogenases can also catalyze $H_2$ production *in vivo* (Vignais & Colbeau, 2004; Burow *et al.*, 2011). The Fe-hydrogenases, which are found in several methanogens, are involved in methane formation from $CO_2$ and $H_2$ rather than in $H_2$ production (Kim & Kim,

2011). Hydrogenases are common enzymes present in microorganisms from almost all phyla, with many different coding genes (Table 1.1). They are involved in transfer of electrons from $H_2$ to an inorganic electron acceptor in aerobic or anaerobic respiration, and in internal cycles of $H_2$. These cycles of $H_2$ oxidation and $H^+$ reduction with several hydrogenases are thought to be a mechanism to conserve energy (hydrogen recycling) (Lupton *et al.*, 1984). In addition, intracellular $H_2$ production or consumption results in changes of the proton gradient across the cytoplasmic membrane (proton motive force). Hydrogenases are not the only enzymes that can catalyze $H_2$ production, also nitrogenases have shown to catalyze $H_2$ production by phototrophic microorganisms (Hillmer & Gest, 1977).

### 1.7.4 Exocellular electron transfer (EET)

From studies in MFC some suggestions have been made on the microbial mechanism for EET as discussed below. The mechanism of direct electron transfer from or to a solid surface is considered to be catalyzed by membrane integrated proteins that can shuttle the electrons from the cytoplasm to the outer surface of the microbial cell. *G. sulfurreducens* is the model organism to study exocellular electron transfer mechanisms because it is a well-known (solid) iron reducer and performs direct electron transfer in anode as well as cathode systems (Lovley *et al.*, 2011). In anode systems the mode of direct electron transfer in *G. sulfurreducens* has been demonstrated with expression and deletion studies of genes involved in the hypothetical electron transfer pathway. Direct exocellular electron transfer involves multicopper proteins (OmpB and OmpC) (Holmes *et al.*, 2008) and several c-type cytochromes (Mehta *et al.*, 2005). Outer membrane cytochrome gene *OmcZ* and pillin structure gene *PilA* were suggested to be essential for high current density and long range electron transfer (Nevin *et al.*, 2009). Outer membrane cytochromes OmcB, OmcE and OmcS appear to be involved but not essential for current production. PilA, a pilin domain protein (also referred to as nanowire), was suggested to have conductive properties and to have an important role in electron transfer within biofilms (Nevin *et al.*, 2009). It has been suggested that the mechanism of electron donation to an electrode involves a series of cytochromes possibly interacting with the intermediate periplasmic quinone pool. Functional analysis of the MEC anodes was done using the Geochip, which was developed for analysis of nitrogen, carbon, sulfur and phosphorus cycling, metal reduction and organic contaminant degradation (He *et al.*, 2007). Geochip based analysis of MEC anodes revealed that multiple cytochromes were involved in EET. Not only cytochromes from *Geobacter* species were abundantly present, but also

**Table 1.1** Classes of hydrogenases with function and genes involved

| Hydrogenase type and group[1] | function | Common gene names | appear in |
|---|---|---|---|
| FeFe | Cytoplasmic, monomeric: $H_2$ production Periplasmic, heterodimeric: $H_2$ uptake | *hydA, hydB, hymC, hupA* | Bacteria and Archaea. Only type also found in eukaryotes |
| Fe only | $CO_2$ reduction with $H_2$ to methane | *hmd* | Methanogens |
| NiFe 1 | Membrane bound $H_2$ uptake | *hupSL, hupAB, hydAB, hyaAB, mbhSL, hybOC, hynSL, hysAB, hoxKG, vhtGA, vhoGA,* | Bacteria and Archaea |
| NiFe 2a | $H_2$ uptake | *hupSL, mbhSL* | Cyanobacteria |
| NiFe 2b | $H_2$ sensing | *hupUV, hoxBC* | Bacteria |
| NiFe 3a | $F_{420}$ -reducing | *frhAG, fruAG, frcAG* | Archaea |
| NiFe 3b | Bidirectinal (NADP) | *hyjSL, hyhSL, hydDA, hyd , cytc3DA, shyDA* | Bacteria and Archaea |
| NiFe 3c | Methyl viologen reducing | *mvhGA, mvhSL, vhcGA, vhuGA* | Archaea |
| NiFe 3d | Bidirectional NAD(P)linked | *hoxYH* | Bacteria |
| NiFe 4 | Membrane bound, energy converting, $H_2$ production | *echFE, ehaNO, ehbMN, cooLH, hycGE, hyfIG* | Bacteria and Archaea |

[1]Classification as described by Vignais & Billoud (2007)

*Desulfovibrio* and *Rhodobacter* cytochromes and low amounts of *Shewanella* and *Anaeromyxobacter* cytochromes possibly involved in electron transfer in MFC were detected (Liu *et al.*, 2010). These findings suggest that the mechanism of electron transfer in MEC anodes is similar to the mechanism in MFC anodes, although differences in species composition has been found comparing MFC with MEC (Torres *et al.*, 2009). Those differences in species composition are most likely due to the differences between MFC and MEC operational conditions such as potential of the electrode or $H_2$ (in MEC) or oxygen (in MFC) leakage from the cathode to the anode, which have consequences for the microbial species that can develop. Previous studies showed that the mechanism of electron donation to an anode did not change when different potentials were applied to a biofilm of *G. sulfurreducens* strain PCA pure culture (Wei *et al.*, 2010). In this study only anodic range of potentials were tested.

Electron uptake from a solid surface, as in cathode systems, has been studied in much less detail. For *G. sulfurreducens* it was shown that gene GSU3274, encoding a putative monoheme c-type cytochrome, was essential for fumarate reduction at a cathode and OmcZ was not involved in electron uptake (Strycharz *et al.*, 2011). This suggests that different mechanisms are involved in electron donation and electron uptake. Differences in mechanisms for electron donation at the anode and electron uptake at the cathode have also been shown for cathodes reducing nitrate or oxygen. In those experiments the anolyte and catholyte of an MFC was analyzed using cyclic voltammetry (Chen *et al.*, 2010). The results showed a large difference between redox activity of the anolyte and the catholyte, which suggests that different reactions occur at the anode and cathode. Other comparable systems where electrons are taken up from an external source can be found in the oxidation of metals (e.g. bio corrosion). For example, sulfate reducing bacteria seem to be able to actively oxidize iron, but evidence of growth with iron as direct energy and electron donor, without chemically produced intermediates such as $H_2$ or Fe(II), still needs confirmation (Hamilton, 2003; Mehanna *et al.*, 2009). The main difference between cathodes and metals is that cathodes are an unlimited source of electrons where metals are oxidized in a terminal process and thus are a limited electron supply. This might promote growth on cathodes while on metal oxides growth is not possible. The exact mechanism of exocellular electron uptake for $H_2$ production is not yet known. A hypothesis has been formulated for the mechanism based on the knowledge from anode systems (Geelhoed *et al.*, 2010; Rosenbaum *et al.*, 2011). They suggest that that the mechanism most likely involves cytochromes for electron shuttling. More ecological studies are needed to understand which microorganisms are important in MEC systems

and physiological and biochemical studies are needed for understanding the mechanisms and improving the MEC performance.

## 1.8 Outline

In this research the microbial communities involved in $H_2$ production in the MEC were investigated. In addition we aimed to get insight in the molecular mechanism of $H_2$ production at the biocathode.

In chapter 2 the microbial community of the first MEC biocathode for $H_2$ production is described. The dominant species that are found in this community are identified and compared with species from the database. A member of this species is used to inoculate a new biocathode.

In chapter 3 the microbial communities of 5 other MEC biocathodes fed with either acetate or bicarbonate are described. Because hydrogenase genes are important enzymes involved in bacterial $H_2$ production, a hydrogenase microarray is used to analyze the hydrogenase genes present in 3 of the biocathodes.

To get a better understanding of the activity and function of microorganisms in the MEC biocathode it is important to study pure cultures in the MEC. In chapter 4 we describe an isolate from a MEC cathode and the reinoculation of this strain in the biocathode compartment. To understand more about the mechanism of electron transfer and $H_2$ production, the biocathode was treated with chemicals that disrupt the membrane potential of the microorganisms.

Further, statistical tools can be helpful to structure large amounts of data. In chapter 5 statistical methods are described that are useful for further studies on MEC. Those methods are applied to find the bands from DGGE profiles from MEC anode samples that were most related to electrochemical performance.

In the final chapter (chapter 6) the relevance of our findings on the microorganisms involved in electron transfer in the MEC is discussed and a mechanism for electron transfer and energy gain with electrons from a solid service is proposed. In addition, recommendations for future research are made that is essential for understanding the mechanisms of electron transfer and the technological challenges for future research on MEC systems.

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# Chapter 2

## Analysis of the microbial community of the biocathode of a hydrogen-producing microbial electrolysis cell

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### 2.1 Abstract

The microbial electrolysis cell (MEC) is a promising system for $H_2$ production. Still, expensive catalysts such as platinum are needed for efficient $H_2$ evolution at the cathode. Recently, the possibility to use a biocathode as an alternative for platinum was shown. The microorganisms involved in $H_2$ evolution in such systems are not yet identified. We analyzed the microbial community of a mixed culture biocathode that was enriched in an MEC bioanode. This biocathode produced 1.1 A $m^{-2}$ and 0.63 $m^3$ $H_2$ $m^{-3}$ cathode liquid volume per day. The bacterial population consisted of 46% *Proteobacteria*, 25% *Firmicutes*, 17% *Bacteroidetes*, and 12% related to other phyla. The dominant ribotype belonged to the species *Desulfovibrio vulgaris*. The second major ribotype cluster constituted a novel taxonomic group at the genus level, clustering within uncultured *Firmicutes*. The third cluster belonged to uncultured *Bacteroidetes* and grouped in a taxonomic group from which only clones were described before; most of these clones originated from soil samples. The identified novel taxonomic groups developed under environmentally unusual conditions, and this may point to properties that have not been considered before.

A pure culture of *Desulfovibrio* strain G11 inoculated in a cathode of an MEC led to a current development from 0.17 to 0.76 A $m^{-2}$ in 9 days, and hydrogen gas formation was observed. On the basis of the known characteristics of *Desulfovibrio* spp., including its ability to produce $H_2$, we propose a mechanism for $H_2$ evolution

## 2.2 Introduction

The high-energy demands of our modern society in combination with the foreseeable depletion of fossil fuels call for the development of sustainable, green forms of energy. Biomass or the organic waste from wastewaters is a source of renewable energy. Recent advances in the use of organic matter for energy production include electricity generation in a microbial fuel cell (MFC) (Logan *et al.,* 2006) and the production of $H_2$ in a microbial electrolysis cell (MEC) (Liu *et al.,* 2005; Rozendal *et al.,* 2006; Logan *et al.,* 2008). These kinds of systems are still under development, but they show great potential for green energy production.

Both MFC and MEC usually consist of two compartments containing an anode and a cathode separated by an ion exchange membrane (Rozendal *et al.,* 2007). The two electrodes are connected through an electrical circuit. At the anode, electrochemically active microorganisms are present that consume organic matter and transfer the electrons derived from metabolic processes to the electrode, either by direct or indirect extracellular electron transfer (Ieropoulos, 2005; Lovley, 2006; Stams *et al.,* 2006; Torres *et al.,* 2009; Lovley & Nevin, 2011). An electron acceptor in the cathode liquid enables a current flow from anode to cathode. Typically, oxygen or Fe(III) is used as the electron acceptor in the MFC (Rabaey & Verstraete, 2005; Logan & Regan, 2006), while in the MEC, protons act as the sole electron acceptor to form $H_2$. For the MEC, a supply of electrical energy is required to make hydrogen gas production possible (Liu *et al.,* 2005; Rozendal *et al.,* 2006).

Acetate is often used as model substrate in MEC systems because it is an end product of fermentation. Theoretically, acetate oxidation yields a potential of −0.29 V (vs. standard hydrogen electrode (SHE), at pH 7, $pH_2 = 1$ bar), while for $H_2$ production from protons, a potential of −0.41 V (vs. SHE, at pH 7, $pH_2 = 1$ bar) is required (Liu *et al.,* 2005). Energy is added by applying enough voltage to render an exergonic reaction. Hence, the theoretically applied voltage required for hydrogen gas production in an MEC fed with acetate is 0.12 V. In comparison, for conventional water electrolysis, the theoretically applied voltage needed is 1.2 V at pH 7 (Liu *et al.,* 2005). The lower energy requirement of the MEC makes it an attractive system

for hydrogen gas production. In practice, however, a minimum applied voltage of 0.25 V is needed because of several potential losses in the system (Rozendal *et al.,* 2006; Sleutels *et al.,* 2009a, b). The total applied voltage demand in practice is for a great part dependent on the overpotential at the electrodes. The use of a good catalyst can decrease the overpotential significantly (Jeremiasse *et al.,* 2009b). Conventionally, platinum is used as a catalyst for hydrogen gas production (Vetter, 1967) and is therefore also applied at MEC cathodes (Rozendal *et al.,* 2006). Because of the high costs and scarcity of platinum, alternative catalysts for $H_2$ production are desirable. Microbial cathodes (biocathodes) form an alternative with great prospectives since they are low cost (both electrode material and catalyst) and self-generating. A biocathode can be defined as an electrode from cheap material (e.g., carbon) with a microbial population present at the electrode or in the electrolyte that catalyzes the cathodic reaction. To act as a biocathode in an MEC, microorganisms need to be able to take up electrons from the electrode material and use these electrons to produce $H_2$.

The uptake of electrons from a solid surface or cathode is known from corrosion studies, where metals (e.g., iron) are oxidized by microorganisms that use the electrons from this reaction for metabolic processes (Dinh *et al.,* 2004; Mehanna *et al.,* 2009). Furthermore, in MFCs, biocathodes have been successfully applied to reduce oxygen, fumarate, nitrate, perchlorate, or chlorinated compounds (Huang *et al.,* 2011).

Microorganisms that can produce $H_2$ are found in a large variety of environments (Schwartz & Friedrich, 2006) and contain hydrogenases that catalyze the reversible reaction $2H^+ + 2e^- \leftrightarrow H_2$. Purified hydrogenases have been successfully used on carbon electrodes as a catalyst for $H_2$ production (Vignais *et al.,* 2001; Lojou & Bianco, 2004; Lojou, 2011; Vincent *et al.,* 2007). The drawback for these systems is that the enzymes are relatively unstable and lose catalytic activity over time. The use of whole cells can help in maintaining enzyme stability. Immobilization of whole *Desulfovibrio vulgaris* cells (well known to contain hydrogenases) on an electrode was successful for $H_2$ production, and the process was more stable than with enzymes only (Guiral-Brugna *et al.,* 2001; Lojou *et al.,* 2002). For continuous $H_2$ production, the challenge is to generate a biocathode with living cells, able to survive and grow.

The microbial uptake of electrons from a cathode for the production of $H_2$ in an MEC was shown for the first time by Rozendal *et al.* (2008). In their study, an MEC half cell with carbon felt electrodes was started up with a biological anode that was initially fed with acetate and $H_2$. Hexacyanoferrate(III) was reduced at the cathode. When stable anodic current was reached, the acetate and $H_2$ supply

was stopped, and the polarities of anode and cathode were reversed, resulting in a biocathode and chemical anode. The cathode potential was poised at –0.7 V vs. SHE resulting in an average current of 1.1 A $m^{-2}$ and production of 0.63 $m^3$ $H_2$ $m^{-3}$ cathode liquid volume per day. A similar setup that was not inoculated served as negative control and produced a current of 0.3 A $m^{-2}$ and 0.08 $m^3$ $H_2$ $m^{-3}$ cathode liquid volume per day. In the present study, we describe the microbial population present on the graphite felt cathode using denaturing gradient gel electrophoresis (DGGE), and cloning and sequencing of 16S ribosomal RNA genes.

## 2.3 Material and methods

### 2.3.1 Microbial electrolysis cell operation and sample collection

The setup and operation of the microbial electrolysis cell was described previously (Rozendal *et al.,* 2008). From this experiment, samples were collected for the current study. The inoculum of this setup was a mixed microbial community previously enriched and sequentially transferred over a period of 4 years in MFC and MEC anodes amended with acetate. The original MFC anode was inoculated with anaerobic sludge from a paper mill wastewater treatment plant (Eerbeek, the Netherlands), anodic effluent from a molasses-fed MFC, and *Geobacter sulfurreducens* strain PCA (U. Michaelidou, personal communication). Briefly, the system was started as a two-chamber microbial fuel cell fed with a mixture of acetate and $H_2$ (at the bio-electrode) and with a mixed hexacyanoferrate(II) and hexacyanoferrate (III) solution as catholyte (at the chemical counter electrode). The electrode material was graphite felt of 6 mm thickness. After 8 days, the substrate for the bio-electrode was changed to bicarbonate and $H_2$. Following stable anodic current production at a bio-electrode potential of –0.2 V (vs. SHE), at day 11 the $H_2$ supply was stopped, and the bio-electrode potential was lowered to –0.7 V. This resulted in consumption of electrons and production of $H_2$ at the bio-electrode, now functional as biocathode. The current developed from 0.3 to 1.2 A $m^{-2}$ projected electrode surface area over a period of 13 days. After this period, bicarbonate addition was stopped. In this layout, i.e., a biocathode with a chemical hexacyanoferrate(II) oxidizing anode, the system was run for over 40 days without addition of any carbon source. Subsequently, the system was disassembled, and samples were collected for our study. Graphite felt electrode material with attached biomass was cut into 7-mm-diameter disks originating from four different locations of the electrode (1) influent site: where medium enters the

cathode compartment, (2) middle of the electrode, (3) effluent site: where medium exits the cathode compartment (4) non-flow site: part of electrode which did not have direct contact with the flow path of the medium. Additional liquid samples (2 ml) were taken from the inoculum material (I) and from the effluent (E) at the end of the run. Samples for DNA extraction were stored at −20°C until use.

### 2.3.2 Strains and cultivation

*Desulfovibrio* strain G11 (DSM 7057) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). It was cultured with 10 mM lactate as energy and carbon source and 15 mM sulfate as electron acceptor in anaerobic medium with $H_2$ headspace as additional energy source. The medium consisted of (millimolars) $MgCl_2$, 1; $CaCl_2$, 0.7; $(NH_4)_2SO_4$, 0.4; $NH_4Cl$, 4.7; $KH_2PO_4$, 6; and $Na_2HPO_4$, 9, supplemented with 1 mL $L^{-1}$ trace elements (Zehnder *et al.,* 1980), 1 mL $L^{-1}$ selenite/tungstate solution (Widdel & Bak 1992), 2 mL $L^{-1}$ vitamins (Wolin *et al.,* 1963), and 0.5 mg $L^{-1}$ resazurin. $Na_2S$ (1 mM) was added to reduce the media.

### 2.3.3 *Desulfovibrio* G11 biocathode

One liter of a *Desulfovibrio* G11 culture, grown to the end log phase, was centrifuged (8,000 rpm, 15 min), and the pellet was resuspended in 10 mL anoxic phosphatebuffered saline (pH 7.4). The MEC design was as previously described (Jeremiasse *et al.,* 2010) with the exception that both electrodes consisted of 2.5-mm-thick graphite felt, and the membrane was a Ralex cation exchange membrane. The anolyte (hexacyanoferrate(II)) and catholyte (phosphate-buffered medium, as used in Rozendal *et al.* (2008)) recycle speed was 60 mL $min^{-1}$. The cathode was inoculated with 10 mL of cell suspension. After inoculation the cell suspension was pumped to the electrode compartment and left without pumping for 1 h. The phosphate-buffered medium was supplemented with 2 mM of bicarbonate and 0.1 mL $L^{-1}$ selenium/tungstate solution; selenium is an essential trace element for some hydrogenases in *Desulfovibrio* species (Hensgens *et al.,* 1994; Valente *et al.,* 2006). Temperature was controlled at 303 K, and pH was controlled at 7 by dosing 1 M HCl. The cathode potential was manually adjusted to −0.7 V vs. SHE if needed. The current, pH, anode potential, and cathode potential were logged every 5 min (Memograph M, Endress + Hauser, Naarden, the Netherlands). Cathodic current is measured with a negative sign; all current measurements were multiplied by −1 and thereby indicated with a positive sign. When stable current was reached (day

9 after inoculation), a gas sample was taken from the headspace, and $H_2$, $CO_2$ and $CH_4$ were measured using gas chromatography (Varia CP-4900 microGC, TCD detector, MS5 and PPU columns in parallel).

### 2.3.4 DNA extraction and amplification of 16S rRNA gene

Genomic DNA was extracted from the graphite felt and the liquid samples using the Fast DNA spin kit for soil (Bio101, Vista, CA, USA). Bacterial 16S rRNA genes were amplified with the primers Bact27F and Univ1492R (Lane, 1991). PCR settings were initial denaturation for 2 min at 95°C, followed by 25 cycles of 30 s denaturation at 95°C, 40 s annealing at 52°C, and 1.5 min elongation at 72°C. Post-elongation was 5 min at 72°C. The PCR samples were tested on a 1% agarose gel for amount and size of product. Partial bacterial 16S rRNA genes to be used for DGGE analysis were amplified using primers Bact968F (including GC clamp) and 1401R (Nübel *et al.,* 1996). PCR conditions were as above, except that 35 cycles were applied, and an annealing temperature of 56°C was used.

### 2.3.5 Clone library construction and analysis

For clone library analysis, the electrode sample from the middle of the electrode (2) was used. PCR amplicons of almost complete bacterial 16S rRNA genes were purified using Nucleo Spin Extract II kit (Macherey-Nagel, Düren, Germany) and ligated into pGEM-T easy vector system I (Promega, Madison, WI, USA). After ligation, the vectors were transformed in XL-1 blue competent Escherichia coli cells (Stratagene, Santa Clara, CA, USA) and grown on LB agar containing 100 mg $L^{-1}$ ampicillin, 0.1 mM isopropyl-1- thio-β-D-galactopyranoside, and 40 mg $L^{-1}$ 5-bromo-4-chloro- 3-indolyl-β-D-galactopyranoside (X-gal). Fifty-seven white clones were used for further analysis. Clone inserts from lysed E. coli cells (95°C, 10 min) were amplified with primers T7 and SP6 (Promega, Madison, WI, USA) using 35 cycles and an annealing temperature of 55°C. The PCR products were purified using the DNA clean and concentrator-5 kit (Zymo Research, Orange, CA, USA).

Clone inserts were sequenced by the company Baseclear (Leiden, the Netherlands) using primers T7, SP6, and 1100R (Lane, 1991). The DNA sequences were checked using Chromas (version 2.32, Technelysium Pty. Ltd.), and contigs were constructed from the partial sequences using DNAbaser (version 2.71.0, Heracle Software, Lilienthal, Germany) resulting in a sequence of (at least) the first 1,250 bp of the 16S rRNA gene. The obtained bacterial 16S rRNA sequences were

checked for anomalies using Pintail online software (Ashelford *et al.,* 2005) and compared to the GenBank database using the National Center for Biotechnology Information (NCBI) blastn tool to identify the most closely related sequences. The newly obtained sequences were deposited in the European Molecular Biology Laboratory nucleotide sequence database (accession numbers FR669194-FR669243 and FR675968-FR675974), aligned using the online Silva alignment tool (Pruesse *et al.,* 2007), and merged with the ARB database using ARB software package version 5.1 (Ludwig *et al.,* 2004). A phylogenetic tree was constructed using the ARB Neighbor-Joining Algorithm with bootstrapping (1,000 replicates) and Jukes Cantor correction.

### 2.3.6 Denaturing gradient gel electrophoresis (DGGE)

The bacterial communities populating the graphite electrode on four different spots of the electrode, as well as inoculum and effluent samples, were analyzed with DGGE. Amplicons were run on an 8% polyacrylamide gel containing a formamide and urea denaturant gradient of 30–60%, similar as described by Martín *et al.* (2007). Gels were run for 16 h at 60°C and stained with silver nitrate (Sanguinetti *et al.,* 1994). In addition, all amplicons of the clones were run on DGGE to evaluate their positions on the gel in comparison to the band migration behavior of the total bacterial communities. The bands that were not identified from the clones were excised from the gel. This material was incubated in Tris–EDTA buffer (10 mM Tris and 1 mM EDTA, pH 8) at 4°C for 2 days, and the extract was used for re-amplification with DGGE primers. The PCR product was checked for purity on DGGE, purified and sequenced with primers 968F by Baseclear (Leiden, the Netherlands). The obtained sequences were checked in Chromas and the most closely related relatives identified using the NCBI blastn search tool.

## 2.4 Results

### 2.4.1 Bacterial community on the electrode

During operation the MEC produced 0.63 $m^3$ $H_2$ $m^{-3}$ cathode liquid volume per day or $0.63\times10^{-3}$ $m^3$ $H_2$ $m^{-2}$ electrode surface per day at an applied voltage of –0.7 V vs. SHE, and scanning electron microscopic imaging showed that after more than 80 days of operation, microorganisms were present and attached to the

electrode felt fibers (Rozendal *et al.*, 2008). Analysis of the dominant members of the bacterial population on the graphite felt electrode by DGGE showed a complex pattern which did not differ between the different locations on the electrode (Figure 2.1), but there was a large difference between inoculation material, effluent material, and electrode material. A large number of bands with different intensity were present, presumably reflecting the difference in abundance of different bacterial ribotypes. Subsequent cloning and sequencing of the bacterial community were done with the sample obtained from the middle of the electrode.

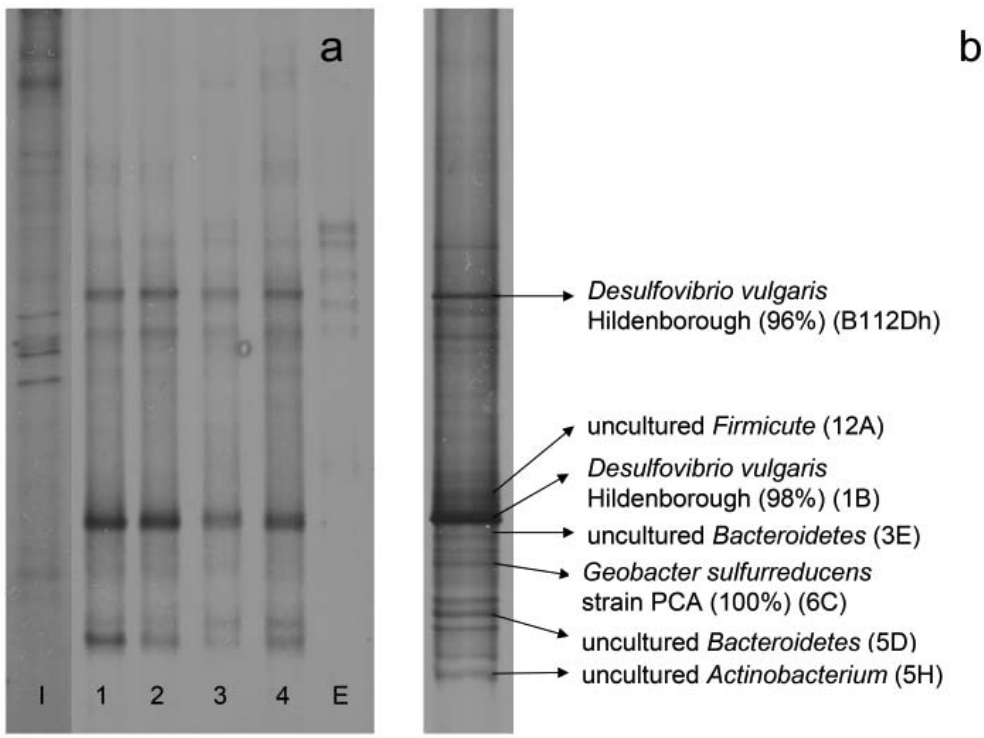



**Figure 2.1** DGGE profile of the total bacterial community on the biocathode. 2a: different locations and samples I: inoculation material; 1: influent site of electrode compartment; 2: middle of electrode; 3: effluent site of the electrode compartment; 4: non flow site of the electrode. E: effluent liquid. 2b: Identification of the bands from sample 2. Identity of the bands is indicated with the matching clone named in brackets, B112Dh (FR669244) was identified by PCR amplification of the band cut from the DGGE profile. The indicated percentages are percentage identity of the found sequence with the indicated closest relatives found in GenBank

### 2.4.2 16S rRNA gene distribution among different phylogenetic groups

The sequences from the 16S rRNA gene clone library (total of 57 clones) were related to seven different phyla (Table 2.1). The majority (88%) of the clones clustered within three phyla, the *Proteobacteria* (26 clones, 46%, FR669218-FR669243), the *Firmicutes* (14 clones, 25%, FR669204-FR669217), and the *Bacteriodetes* (10 clones, 17%, FR669194-FR669203). The other 12% of the clones were phylogenetically related to *Tenericutes* (one clone, 2%, FR675972), *Spirochaetes* (one clone, 2%, FR675968), *Chlorobi* (one clone, 2%, FR675969), *Actinobacteria* (two clones, 3%, FR675973 and FR675974), and unclassified bacteria (two clones, 3%, FR675970 and FR675971).

**Table 2.1** Distribution and abundance of 16S rRNA gene sequences in the biocathode clone library within the different phyla. The main cluster within the phylum is indicated for the major groups with the most closely related cultured species and its identity with the specific cluster

| Phylum | % of Total (57 clones) | Cluster of clones (>97% similar to each other) | Similarity of cluster to cultured species |
|---|---|---|---|
| *Proteobacteria* | 46% | 14 clones *Desulfovibrio vulgaris* | > 98% *D. vulgaris* str.Hildenborough |
| *Firmicutes* | 25% | 9 clones of uncultured *Firmicutes* | < 89% *Desulfitobacterium hafniense* DCB-2 |
| *Bacteroidetes* | 17% | 5 clones of uncultured *Bacteroidetes* | < 92% *Rikenella microfusus* ATCC 29728 |
| *Tenericutes* | 2% | | |
| *Actinobacteria* | 3% | | |
| *Chlorobi* | 2% | | |
| *Spirochaetes* | 2% | | |
| *Unclassified Bacteria* | 3% | | |

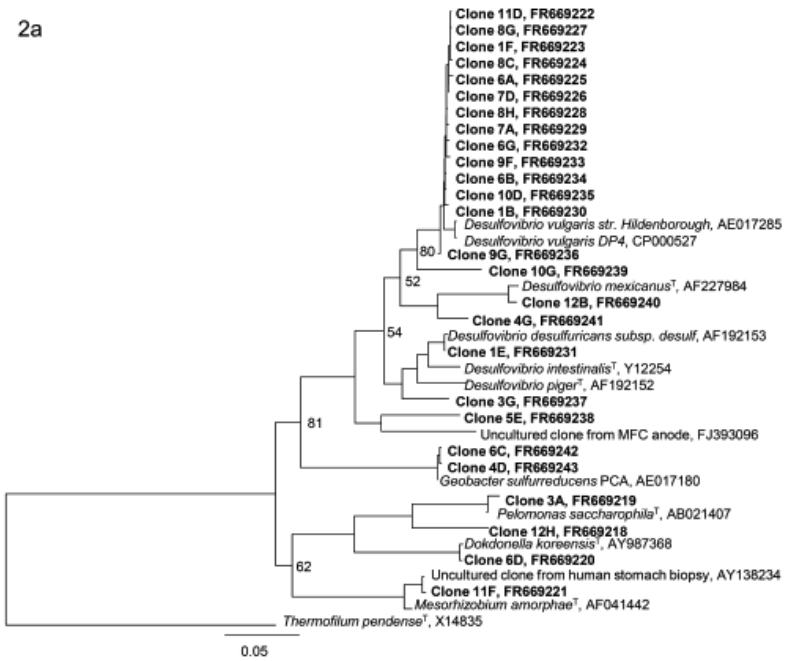
The phylogenetic relation of the clones from our study, including several related uncultured and cultured species from the GenBank database, is shown in Figure 2.2. Within the *Proteobacteria*, 81% (21 clones) belonged to the subclass of the *Deltaproteobacteria* of which 90% (19 clones) of the clones clustered with the genus *Desulfovibrio* (Figure 2.2a). Moreover, 67% (14 clones) showed more than 98% identity to the species *D. vulgaris* Hildenborough, an anaerobic bacterium that is able to use $H_2$, organic acids, or alcohols as electron donor and sulfate as electron acceptor (Postgate & Campbell, 1966). *D. vulgaris* is also known to be able to produce $H_2$ (Carepo *et al.,* 2002). The remaining 10% of *Deltaproteobacteria* (two

clones) showed identical 16S rRNA gene sequences to *G. sulfurreducens* strain PCA, a bacterium which uses $H_2$ or acetate as electron donor to reduce metals (Caccavo *et al.,* 1994). *G. sulfurreducens* is able to use the electrode of an MFC as electron acceptor (Bond & Lovley, 2003) but also as electron donor for fumarate reduction (Gregory *et al.,* 2004; Dumas *et al.,* 2008) and $H_2$ production (Geelhoed & Stams, 2011). The other 19% (four clones) of *Proteobacteria* belonged to the *Alpha-* (one clone), *Beta-* (two clones), and *Gammaproteobacteria* (one clone) and were most closely related to, respectively, *Mesorhizobium amorphae*, *Pelomonas saccharophila*, *Azonexus caeni*, and *Dokdonellala koreensis* (Figure 2.2a).

All 14 clones that clustered in the phylum of *Firmicutes* belonged to the *Clostridia* class. Within the *Clostridia*, one prominent group of nine clones (64% of the total *Firmicutes*) belonged to the family of unclassified *Clostridiales* (Figure 2.2b). The clones in this group showed more than 98% identity to each other but did not belong to any cultured or uncultured genus listed in the GenBank database (using 95% identity in the 16S rRNA gene as the genus delineation value (Rosselló- Mora & Amann, 2001)). The most similar GenBank sequence showed only 90% identity to this cluster of uncultured *Firmicutes* and was obtained from a dechlorinating flow column (FM178833) (Behrens *et al.,* 2008). The most closely related cultured bacterium was *Desulfitobacterium hafniense*, with 88% 16S rRNA gene sequence identity. The other clones within the *Firmicutes* were all related to anaerobic species such as *Clostridium viride*, *Clostridium cylindrosporum*, or uncultured species found in anaerobic environments such as dechlorinating enrichment cultures (EF64459), anaerobic digesters (CU918499 and CR933290), mouse cecum (EU457310), a trichlorobenzene transforming culture (AJ009499), and a denitrifying community inoculated from rice paddy soil (AB486915).

Within the phylum of *Bacteroidetes*, seven clones (70%) belonged to the class of *Bacteroidia*. Within this class, there were five clones that clustered together as unclassified *Bacteroidetes* with more than 99% identity to each other. These five clones did not belong to any cultured genus. In the GenBank database, several sequences were present that clustered in the same taxonomic group (more than 95% identity). These sequences were derived from the following sources: chloraminated drinking water distribution system (EU808333), chromium-contaminated soil (EU037360), and high-carbohydrate and high-pH sludge (FJ5234992) (Figure 2.2c). The closest related cultured microorganism was *Rikenella microfusus* with 92% identity of the 16S rRNA gene. *R. microfusus* is a fermentative bacterium isolated from feces of a Japanese quail (Kaneuchi & Mitsuoka, 1978). The other clones within the class of *Bacteroidia* were related to uncultured species from nitrobenzene polluted river water (EF590019) and a bovine

serum albumin digester (AB175369). The other clones within the *Bacteroidetes* phylum belonged to the class of uncultured *Sphingobacteria* (two clones, 20%) and *Sediminibacteria* (one clone, 10%) and were most closely related to uncultured bacteria from thermophilic anaerobic sludge fed with methanol (AY526509) and *Sediminibacterium salmoneum*, an aerobic bacterium isolated from sediment of the Guanting reservoir in Beijing, China.



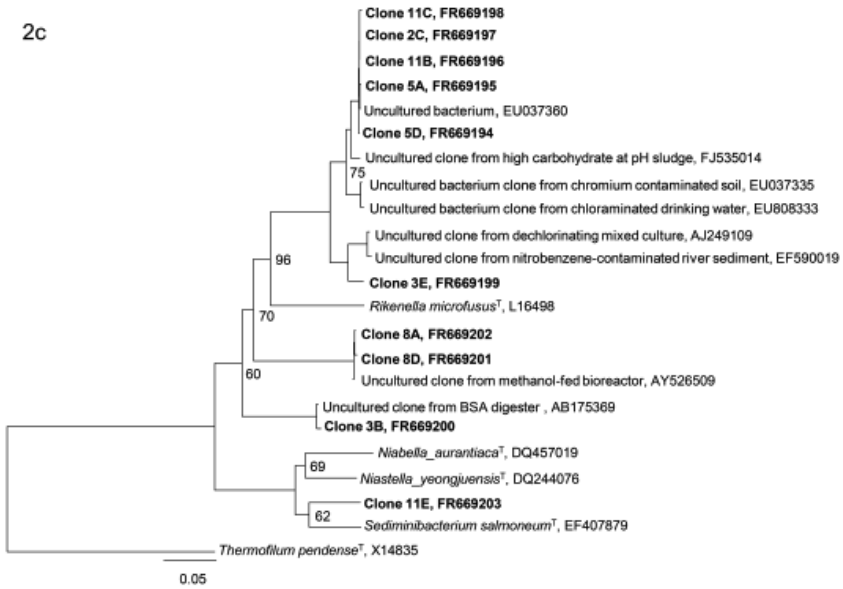



**Figure 2.2** Phylogenetic neighbour joining bootstrap (1000 replicates) consensus tree of the three major phyla detected on the biocathode. A: *Proteobacteria* (accession numbers: FR669218 - FR669243), B: *Firmicutes* (accession numbers: FR669204 - FR669217), C: *Bacteroidetes* (accession numbers: FR669194 - FR669203). The archaeon *Thermofilum pendense* is used as out-group. Clones from our study are indicated in bold and the rest represent 16S rDNA sequences imported from the GenBank database. The sequences in the tree are at least 1250bp long. The scale bar indicates the distance of 0.05 (or 5%) sequence identity

### 2.4.3 Identification of cloned 16S rDNA in the bacterial DGGE profile

Comparison of the sequencing data and the DGGE profiles of the clones and the total bacterial community showed that the most dominant band represented *D. vulgaris* (clone 1B). The other identified bands represented other *Desulfovibrio* sp. (B112Dh, FR669244), *G. sulfurreducens* (clone 6C), uncultured *Bacteroidetes* (clone 3E and clone 5D), an uncultured *Firmicutes* sp. (clone 12A), and an uncultured *Actinobacterium* (clone 5H) (Figure 2.1).

### 2.4.4 A pure culture of *Desulfovibrio* G11 in an MEC

After inoculation of *Desulfovibrio* G11 in the MEC cathode, the current increased from 1.7 (0.17 A $m^{-2}$) to 7.6 mA (0.76 A $m^{-2}$) over a period of 9 days (Figure 2.3). During this period, it was visible that gas accumulated in the system. Analysis of the gas phase on day 9 showed the presence of $H_2$, whereas no methane or carbon dioxide was detected.

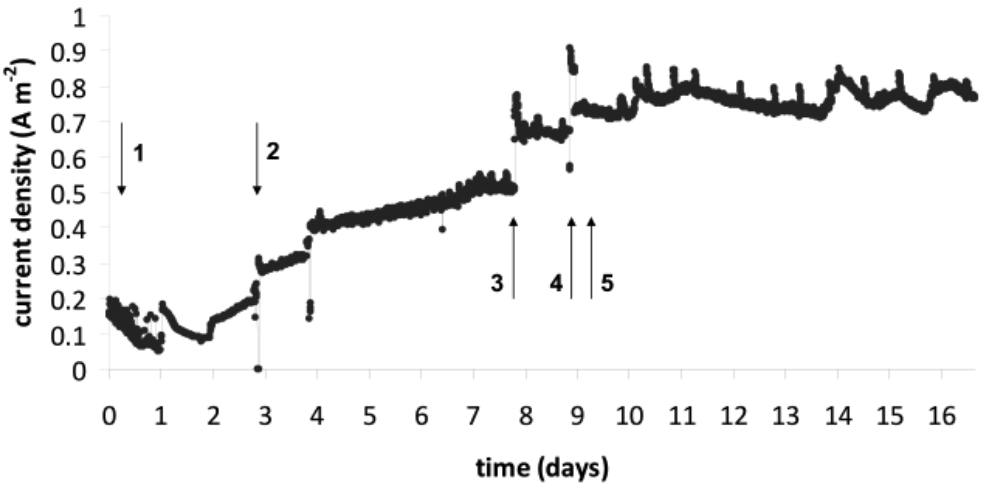



**Figure 2.3** Current production over time after inoculation of the cathode with pure culture of *Desulfovibrio* G11. The current increased from 0.17 A $m^{-2}$ to 0.76 A $m^{-2}$ in 9 days. Arrow 1 indicates the time of inoculation, arrows 2, 3 and 4 indicate an adjustment of the cathode potential to -0.7V vs SHE and arrow 5 indicates the time of gas sampling

## 2.5 Discussion

In previous research, $H_2$ production in an MEC with a biocathode has been shown (Rozendal *et al.,* 2008; Jeremiasse *et al.,* 2009a). Our research gives the first description of a microbial community of a $H_2$ producing biocathode in an MEC. The results showed high bacterial 16S rRNA gene diversity, with the dominant species belonging to the genus *Desulfovibrio*. Two other predominant clusters were found that were related to uncultured *Firmicutes* and uncultured *Bacteroidetes*. In addition to being the dominant ribotype in the MEC biocathode, progression of current was shown after inoculation of an MEC cathode with pure cultures of *Desulfovibrio* G11.

The dominance of *Desulfovibrio* spp. in the biocathode can be reasoned because *Desulfovibrio* species are well known for their ability to produce and consume $H_2$ (Carepo *et al.,* 2002). Coating of an electrode with immobilized *D. vulgaris* cells has been reported to catalyze the evolution of $H_2$ at a cathode (Lojou *et al.,* 2002). However, this catalytic effect occurred only in the presence of the electron shuttle methyl viologen. The application of living *Desulfovibrio* as $H_2$ catalyst at a cathode without an added mediator, as in our system, was not shown before. This is not only fundamentally, but also practically, of great interest because it will allow low-cost and self-maintaining cathode systems for $H_2$ production.

The second major group of bacteria found in this study, the uncultured *Firmicutes*, does not belong to any earlier described genus. Apparently, the conditions in the MEC created an environment in which bacteria belonging to a new taxonomic group were able to develop predominantly. Moreover, it is interesting to note that the closest related genus is *Desulfitobacterium*, of which at least one member, *D. hafniense* strain DCB2, was electrochemically active in an anode from an MFC (Milliken & May, 2007). Furthermore, *Desulfitobacterium* spp. were found as the dominant population in a mixed culture that was producing $H_2$ in a dechlorinating cathode system (Aulenta *et al.,* 2008). Similar to the experiment with immobilized *D. vulgaris*, no $H_2$ was produced in the absence of methyl viologen as a mediator.

The third major group of bacteria in the MEC biocathode belonged to the uncultured *Bacteroidetes* and also constitutes a novel group without cultured relatives at genus level (92% identity). Members of the *Bacteroidetes* phylum are found in a large variety of environments such as soil, sediments, human and animal gut, and seawater. The group of uncultured *Bacteroidetes* clones in our study was most closely related to clones from various environmental samples that presumably all originated from anaerobic sources. The closest related cultured bacterium, *R. microfusus*, grows fermentatively on carbohydrates (Kaneuchi & Mitsuoka, 1978), but no other information about its metabolism is available.

The principle of electron uptake from a solid surface has been shown before, but the mechanisms are poorly understood. The reverse process of electron transfer to an anode has been studied in more detail, and those studies provided information on which mechanisms are possible (Rabaey *et al.,* 2004; Lovley, 2008; Nevin *et al.,* 2009). Extracellular electron transfer can take place indirectly using electron shuttles such as methyl viologen, humic acid, sulfide, cysteine, riboflavin, phenazine, and quinones (Stams *et al.,* 2006; Logan, 2009). Membrane-associated proteins such as cytochromes and cell appendages or nanowires have been suggested to be involved in direct electron transfer (Kim, 2002; Mehta *et al.,* 2005; Reguera *et al.,* 2005). For the most extensively studied species *G.*

*sulfurreducens*, expression and deletion studies have shown that direct extracellular electron transfer to an electrode involves multicopper proteins (Holmes *et al.,* 2008), several c-type cytochromes, (Holmes *et al.,* 2006) and pillin structures which most likely are involved in the physical association with the electrode (Nevin *et al.,* 2009). Furthermore, *G. sulfurreducens* can change from electron donating to electron uptake for $H_2$ production after reversing the potential from anodic to cathodic (Geelhoed & Stams, 2011). For extracellular electron uptake from an electrode, several mechanisms have been suggested (Geelhoed *et al.,* 2010; Rosenbaum *et al.,* 2011). Recent findings suggest that *G. sulfurreducens* uses different cytochromes in the pathways for electron donating than for electron uptake (Strycharz *et al.,* 2011). These authors suggest that this might reflect the optimal potential at which specific proteins can accept or donate electrons. With our findings that *Desulfovibrio* spp. are dominant microorganisms at the cathode together with our findings that *Desulfovibrio* G11 is electrochemically active at the cathode, the possible mechanisms of electron transfer and $H_2$ production for this species can be inferred, as discussed below.

The genomes of *Desulfovibrio* species show several c-type cytochromes and multicopper proteins with homology to the proteins involved in electron donation in *Geobacter* species (NCBI search). Similar to the pillin structures in *Geobacter* spp., *D. vulgaris* flagellar appendages (genes *flgC*, *flgB*, and *flgL*) have been associated with physical association during syntrophic growth (Walker *et al.,* 2009) and might also be involved in adherence to electrodes. These similarities suggest that the mechanism of extracellular electron transfer by *Desulfovibrio* spp. could be similar to previously described mechanisms of electron transfer involving extracellular appendage (pilin or flagella)-like structures, cytochromes, or shuttle compounds. The electron transfer from an electrode to the microorganisms can possibly take place by reversed reaction of those previously described mechanisms. More research is needed to understand how electron transfer in cathode systems takes place.

$H_2$ production from protons is energetically costly. For microbial $H_2$ production, energy needs to be added in the form of an electron donor with high energy (e.g., glucose or light) or in the MEC biocathode by the applied voltage. A putative mechanism for the conservation of energy from $H_2$ production at the cathode may be comparable to $H_2$ production from formate in methanogenic co-cultures (Dolfing *et al.,* 2008; Stams & Plugge, 2009). Energy gain and growth from production of $H_2$ have been shown for *Desulfovibrio* G11 grown on formate in coculture with *Methanobrevibacter arboriphilus* AZ (Dolfing *et al.,* 2008). Conservation of energy by *Desulfovibrio* spp. was proposed to involve an energyconserving hydrogenase or

a hydrogenase present at the cytoplasmic side of the membrane. In the genome of *D. vulgaris*, genes coding for both types of hydrogenases are present. The release of protons from formate by a formate dehydrogenase located at the periplasmic side of the membrane combined with proton consumption at the cytoplasmic side results in the generation of a proton gradient over the membrane that can be utilized by a membrane-bound ATPase. It has been suggested that in a similar way energy, could be gained from the production of $H_2$ from electrons derived from a cathode (Geelhoed *et al.,* 2010). Our findings that *Desulfovibrio* species dominate the microbial community of the MEC support the idea that the mechanism of electron transfer from an electrode to the bacterium can take place like suggested before by Dolfing *et al.* (2008) and Geelhoed *et al.* (2010). Comparing MEC experiments to syntrophic growth, Geelhoed *et al.* (2010) calculated that the energy applied to MEC systems is enough to allow energy conservation and growth. However, in those calculations, no energy losses in the system were taken into account. The actual energy available at the cathode can be estimated from the potential of the cathode (–0.7 V vs. SHE) minus the energy needed to form $H_2$ (–0.41 V vs. SHE) which gives the maximum theoretical energy available for the microorganisms (–0.29 V). The cathode losses, expressed as the concentration overpotential, can be calculated as described by Jeremiasse *et al.* (2009b). Under the conditions prevailing in the biocathode system studied here (Rozendal *et al.,* 2008), using a $pKa_2$ of 7.21 for phosphate buffer, the concentration overpotential can be estimated at –0.019 V. Hence, the actual energy available for the microorganisms is –0.29 + 0.019 = – 0.27 V or –52 kJ per mole $H_2$ produced (at pH 7 and $pH_2$ = 1 bar). In comparison, the Gibbs free energy change associated with conversion of formate to $H_2$ and carbon dioxide is –17 to –19 kJ per mole $H_2$ (Dolfing *et al.,* 2008). This shows that for the studied biocathode system, even if the overpotential is taken into account, there is sufficient energy available for the microorganisms to grow. The energetic limits for microbial $H_2$ production and growth in an MEC biocathode still need to be explored.

Our findings that the dominant microorganism in the MEC biocathode is a *Desulfovibrio* sp., together with the knowledge about the $H_2$ metabolism and potential for exocellular electron transfer of *Desulfovibrio* spp., give very strong indications that they are actively involved in the $H_2$ production at the biocathode of the MEC. Since *Desulfovibrio* spp. are also able to consume $H_2$, it can be reasoned that the microbial community on the electrode developed during the anodic phase, in which acetate and $H_2$ were the substrates. However, after switching the polarity, the production of $H_2$ commenced only after several days, suggesting that microbial adaptation and possibly growth were necessary to start $H_2$ production

at the cathode. In addition, the potential electroactivity of *Desulfovibrio* in a cathode was supported by the observed current production and $H_2$ production after inoculation of an MEC cathode with *Desulfovibrio* G11. Besides *Desulfovibrio*, two novel and abundantly present groups of bacteria were present. These bacteria need to be characterized further before their role in an MEC can be inferred.

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# Chapter 3

## Influence of setup design and carbon source on the bacterial community of microbial electrolysis cell biocathodes.

Submitted for publication as: Elsemiek Croese, Adriaan W. Jeremiasse, Ian P.G. Marshall, Alfred M. Spormann, Gert-Jan W. Euverink, Jeanine S. Geelhoed, Alfons J.M. Stams, Caroline M. Plugge. The bacterial community of microbial electrolysis cell biocathodes is more determined by the setup design than by the carbon source.

### 3.1 Abstract

The Microbial Electrolysis Cell (MEC) is an emerging technology for $H_2$ production. The MEC biocathode has shown potential as alternative for expensive metals such as platinum as catalyst for $H_2$ synthesis. In this study we investigated the bacterial communities at the biocathode of 5 $H_2$ producing MECs using molecular techniques. The setups differed in design (large or small) including electrode material and flow path and in carbon source provided at the cathode (bicarbonate or acetate). In addition, we used a hydrogenase gene DNA microarray (Hydrogenase Chip) to analyze the hydrogenase genes present in the 3 large setups. The small setups showed dominant groups of *Firmicutes* and two of the large setups showed dominant groups of *Proteobacteria* and *Bacteroidetes*. The third large setup received acetate but no sulfate (also not as sulfur source). In this setup an almost pure culture of a *Promicromonospora* sp. developed. Most of the hydrogenase genes that were found were coding for bidirectional Hox-type hydrogenases, which have

shown to be involved in cytoplasmatic $H_2$ production. Our results indicate that 1) setup design has a greater influence on the bacterial population than the carbon source, 2) a large variety of species is able to catalyze $H_2$ evolution at the cathode, 3) the Hydrogenase Chip is a useful tool for further studies on MEC biocathodes, and 4) cytoplasmatic $H_2$ production by bidirectional Hox-type hydrogenases rather than energy converting hydrogenases might be a key factor in the mechanisms of microbial $H_2$ production and growth at the MEC biocathode.

## 3.2 Introduction

Hydrogen gas ($H_2$) is a valuable product as a renewable energy carrier and as a reductant in the chemical industry (Lee *et al.*, 2010). $H_2$ can be formed by steam-reforming of natural gas, gasification of fossil or renewable materials and by water electrolysis. Water electrolysis is energetically costly. An interesting alternative is microbial electrolysis. In a microbial electrolysis cell (MEC) microorganisms degrade organic compounds (e.g. acetate) at the anode to $CO_2$, protons and electrons. At the cathode, protons and electrons derived from the anode and energized by a power supply are combined to $H_2$. The anode and the cathode are generally separated by an ion selective membrane (Sleutels *et al.*, 2009a). By using this technology, the fossil energy requirements for $H_2$ production can be diminished more than 5 times compared to $H_2$ production from direct water electrolysis (Liu *et al.*, 2005; Rozendal *et al.*, 2006). At the cathode, platinum is a good catalyst for $H_2$ production but because platinum is expensive and scarce, alternatives are required. A low cost alternative for a platinum cathode is the biocathode. A biocathode can be defined as an electrode made of cheap material (e.g. carbon or graphite) at which microorganisms catalyze the cathodic reaction (in this case: $H_2$ production). In a microbial cell, $H_2$ formation is thermodynamically confined and dependent on the available energy (Geelhoed *et al.*, 2010). Microorganisms can produce $H_2$ through dark fermentation, with a low conversion efficiency of substrate to $H_2$, or by addition of energy in the form of light or heat (Dasgupta *et al.*, 2010; Verhaart *et al.*, 2010). The required energy can alternatively be provided by an electrode. In MEC cathodes inoculated with biomass originated from a wastewater treatment plant, the possibility of electron transfer from an electrode to microorganisms for the production of $H_2$ was demonstrated (Jeremiasse *et al.*, 2009; Rozendal *et al.*, 2008). Little information is available about the types of microorganisms that develop at a biocathode in a microbial electrolysis cell and about the mechanism of electron transfer from the cathode to the microorganism

to produce $H_2$. Biocathode microorganisms have been studied most in cathodes for oxygen reduction, but also fumarate and nitrate reduction, dechlorination and product formation (e.g. methane or acetate) with an electrode as electron donor have been shown (Nevin *et al*., 2011; Rosenbaum *et al*., 2011). Only a few studies describe the microbiology in $H_2$ producing biocathodes (Croese *et al*., 2011; Geelhoed & Stams, 2010; Lojou *et al*., 2002).

Microbial $H_2$ production involves hydrogenases, the enzymes that catalyze the reversible reaction $2H^+ + 2e^- \leftrightarrow H_2$. Hydrogenases are categorized according to their (redox active) metal site. The three groups of hydrogenases are 1) nickel-iron (NiFe)-hydrogenases of which a sub-group contains also selenium, 2) iron-iron (FeFe)-hydrogenases and 3) iron (Fe) hydrogenases. The last group was previously characterized as iron-free hydrogenases because they don't contain a redox active iron. The catalysis of $H_2$ production has been mostly associated with the FeFe-hydrogenases and, $H_2$ oxidation (consumption) mostly with NiFe-hydrogenases. Nevertheless, some NiFe-hydrogenases, mainly the cytoplasmic ones, can also catalyse $H_2$ production *in vivo* (Burow *et al*., 2012; Vignais & Colbeau, 2004). The Fe-hydrogenases, which are found in several methanogens, are involved in methane formation from $CO_2$ and $H_2$ rather than in $H_2$ production (Kim & Kim, 2011). No knowledge is currently available on the hydrogenases involved in $H_2$ production in the MEC biocathode.

For growth, biocathodic bacteria require a carbon source. Carbon dioxide is a low cost carbon source but in lab scale experiments acetate is often used as carbon source because it is the end product of dark fermentation. Acetate seems to be a preferred energy and carbon source for high efficiencies in MFC anodes (Lee *et al*., 2008) and recently it was also shown that in MEC biocathode systems acetate is a preferred carbon source over bicarbonate, for rapid startup of a biocathode (Jeremiasse *et al*., 2012). The effect of different carbon sources on the microbial population of an MEC biocathode has not been studied before.

In the present study 5 MEC biocathode samples from an experiment that was described before by Jeremiasse *et al*. (2012) were analyzed. The microbial communities were determined by 16S rRNA gene analysis and the hydrogenases of 3 of the samples were analyzed by using a Hydrogenase Chip developed previously (Marshall *et al*., 2012). Two small setups were operated of which one was supplemented with acetate (AcS) and the other with bicarbonate (BicS) and three large setups of which one received acetate (AcL), one bicarbonate (BicL) and the third one contained catholyte with acetate but without any sulfate (AcnSL), this to prevent loss of electrons by reduction of (the low amounts of) sulfate

present in the growth medium. We hypothesize that the carbon source will have a major impact on the development and composition of the microbial population.

## 3.3 Material and methods

### 3.3.1 Operational conditions of the microbial electrolysis cell cathode

All setups were operated as described by Jeremiasse *et al*. (2012). In short, two different setups were used. The small setup, described by Ter Heijne *et al*. (2008), consisted of a 22 $cm^2$ graphite paper electrode, and the large setup, described by Jeremiasse *et al*. (2010), consisted of 100 $cm^2$ projected surface area of a 0.25 cm thick graphite felt electrode. The cathode was fed with mineral salts medium containing (g $L^{-1}$) $KH_2PO_4$, 0.68; $K_2HPO_4$, 0.87; KCl, 0.74; NaCl, 0.58; $NH_4Cl$, 0.28; $CaCl_2 \cdot 2H_2O$, 0.1; $MgSO_4 \cdot 7H_2O$, 0.01 and 0.1 mL $L^{-1}$ of a trace element mixture (Zehnder *et al*., 1980), supplemented with either 10 mM sodium bicarbonate or 1 mM sodium acetate. In the anode compartment 100 mM potassium hexacyanoferrate(II) was used. First the optimal potential for operation was determined in two series of small setups with a total cathodic circulation volume of 192 mL which was constantly refreshed with medium at a rate of 36 mL $h^{-1}$. The small setups were inoculated with 10 mL of biomass from the effluent and electrode material of previously operated MEC anodes and cathodes. The two series of setups consisted of 4 MECs that were operated at potentials of -0.5, -0.6, -0.7 and -0.8 V (vs SHE) for more than 60 days. A cathode potential of -0.7 V (vs. SHE) resulted in the highest catalytic activity. After operation, samples were collected from the electrode material of the -0.7 V setups (AcS and BicS). The electrode material including biomass was resuspended in catholyte solution and used as inoculum for the large setups (10 mL per setup). The large setups contained a 100 $cm^2$ (projected surface area) flow-through graphite felt electrode, a total volume of 100 mL and nutrient solution dosed at a rate of 156 mL $h^{-1}$. The large setups were operated at -0.7 V and supplemented with acetate (AcL, inoculated from AcS) or bicarbonate (BicL, inoculated from BicS). A third large setup (AcnSL) was inoculated from the AcL setup (10 mL of electrode biomass resuspended in catholyte) and run without any added sulfate to exclude that sulfate was used as an electron acceptor instead of protons. For this setup the $MgSO_4$ in the nutrient solution described above was replaced by $MgCl_2$. $H_2$ production was determined in a 48 h yield test for the BicL and AcnSL setup and in a 6 h yield test for the AcL setup as described previously (Jeremiasse *et al*., 2012). After operation

1 $cm^2$ electrode material was cut from all 5 cathodes (AcS, BicS, AcL, BicL and AcnSL). Previous work has shown that sampling location at the electrode does not make a difference in DGGE profiles of the microbial population (data not shown). The samples were stored at -20°C for DNA analysis or processed further for SEM imaging.

### 3.3.2 Scanning electron microscopy (SEM)

Electrode samples were fixed in 2.5% glutaraldehyde (w/v) for 2 h at room temperature and washed twice with 10 mM PBS buffer (pH 7.4). Subsequently, the samples were dehydrated in a graded series of ethanol (10%, 25%, 50%, 75%, 90% and twice in 100% during 20 minutes for each step) and dried in a desiccator. The samples were coated with gold and examined in a JEOL JSM-6480LV Scanning Electron Microscope (acceleration voltage 6 kV, HV-mode, SEI detector).

### 3.3.3 DNA extraction and amplification of 16S rRNA genes

Genomic DNA was extracted from the electrode samples using the Fast DNA spin kit for soil (Bio101, Vista, CA, USA) using the manufacturer's instructions. Bacterial 16S rRNA genes were amplified using the primers Bact27F and Univ1492R (Lane, 1991). PCR settings were: initial denaturation for 2 min at 95°C, followed by 25 cycles of 30 s denaturation at 95°C, 40 s annealing at 52°C and 1.5 min elongation at 72°C. Post-elongation was 5 min at 72°C. The PCR products were tested on a 1% agarose gel for amount and size of the amplicon. For DGGE analysis partial bacterial 16S rRNA genes were amplified using primers Bact968F (including GC clamp) and 1401R (Nübel *et al.,* 1996). PCR conditions were as above, except that 35 cycles were applied and an annealing temperature of 56°C was used.

### 3.3.4 Clone library construction and analysis

For all 5 cathode samples PCR-amplicons of almost complete bacterial 16S rRNA genes were purified using Nucleo Spin Extract II kit (Macherey-Nagel, Düren, Germany) and ligated into pGEM-T easy vector system I (Promega, Madison, WI, USA). After ligation the vectors were cloned in XL-1 blue competent *Escherichia coli* cells (Stratagene, Santa Clara, CA, USA) and grown on LB-agar containing 100 mg $L^{-1}$ ampicillin, 0.1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) and 40 mg $L^{-1}$ 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). After blue-white screening, ninety six white colonies were transferred to 1mL liquid LB medium

with 100 mg $L^{-1}$ ampicillin. After overnight incubation at 37°C all clones were transferred to a GATC 96 well nutrient agar plate with 100 mg $L^{-1}$ ampicillin and sent to GATC (GATC Biotech AG, Konstanz, Germany) for sequencing.

Chromatogram analysis and sequence assembly were performed with DNAbaser version 2.71.0 (Heracle Software, Lilienthal, Germany) and phylogenetic affiliation of the sequences was examined with an NCBI BLAST identity search. All sequences were aligned using the online Silva alignment tool (Pruesse *et al*., 2007) and merged with the ARB database using ARB software package version 5.1 (Ludwig *et al*., 2004). A phylogenetic tree was constructed using the ARB Neighbour Joining Algorithm with Jukes Cantor correction.

The microbial diversity per setup was calculated using Shannon's diversity index (Bianchi & Bianchi, 1982) using:

$$H' = -\sum_{i=1}^{S} p_i \ln p_i$$

where *S* represents the number of ribotypes (defined by >97% gene identity) per sample (richness) and $p_i$ represents the proportion of a specific ribotype relative to the sum of all clones in a sample. Rarefaction curves were created to estimate sampling coverage using DOTUR 1.53 (Schloss & Handelsman, 2005) with identity of 97% or more considered as one operational taxonomic unit. Good's coverage was calculated using the estimate for sampling coverage $\tilde{C}$ described by Good (Esty, 1986; Good, 1953) using:

$$\tilde{C} = 1 - N1/n$$

where $\tilde{C}$ is the sampling coverage of a random sample size n and *N1* is number of classes observed exactly once.

### 3.3.5 Denaturing Gradient Gel Electrophoresis (DGGE)

Partial 16S rRNA genes of the bacterial communities populating the 5 different graphite cathodes were visualized using DGGE. Amplicons were separated on an 8% polyacrylamide gel containing a formamide and urea denaturant gradient of 30-60%, similar as described by Martín *et al*. (2007). Gels were run for 16 hours at 60°C and stained with silver nitrate (Sanguinetti *et al*., 1994) after which the band profiles were compared.

### 3.3.6 DNA Microarray Analysis

The three large setups (AcL, BicL and AcnSL) of this study were further analyzed using a hydrogenase DNA microarray, Hydrogenase Chip version 4.0, designed and synthesized according to the same protocol as Hydrogenase Chip versions 1.0-3.0 (Marshall *et al*., 2012). Hydrogenase gene sequences were taken from Integrated Microbial Genomes and Metagenomes (IMG/M) version 3.4 (Markowitz *et al*., 2008) and several hydrogenase gene clone libraries (Sahl *et al*., 2011; Xing *et al*., 2008). Tiling probes were designed for single-fold coverage of each gene.

DNA was amplified via multiple displacement amplification, labeled with fluorescent Cy3 dye, and hybridized to the Hydrogenase Chip version 4.0 as previously described (Marshall *et al*., 2012). Microarray data analysis was performed using the TilePlot package version 1.3 in R version 2.13.1 (http://www.r-project.org/). Gene presence/absence was determined independently for each sample using the tileplot.single() function. Bright Probe Fraction (BPF) cutoffs for each hybridization were determined using the default method within TilePlot. For each sample, the section of the BPF curve with the sharpest drop-off was used to determine the BPF threshold. Samples BicL and AcnSL were loess-normalized to sample AcL using the tileplot.double() function for quantitative comparisons.

For each gene on the array, a bright-segment length dependent score (BSLDS) was calculated based on the length of each continuous section of the gene with bright probe (or "bright segment length"). The sum of squares of all bright segment lengths for a given gene was said to be the BSLDS. The BSLDS is a method of differentiating results not just based on the fraction of bright probes, but rather on the length of continuous bright segments.

### 3.3.7 Nucleotide sequence accession numbers

All nucleotide sequences obtained in this study were deposited in the European Molecular Biology Laboratory (EMBL) nucleotide sequence database under accession numbers HE582784 to HE583182.

## 3.4 Results

### 3.4.1 Performance of the MEC biocathode

The performance of the biocathodes that were sampled was partly described before (Jeremiasse *et al.*, 2012). The small setups produced 1 A $m^{-2}$ for the acetate setup

and 0.8 A $m^{-2}$ for the bicarbonate setup. $H_2$ was detected but not quantified for the small setups. An un-inoculated control of the small setup produced 0.4 A $m^{-2}$ (Geelhoed & Stams, 2010). The startup time (time till stable current was reached) for the AcS setup was 30 days and for the BicS setup was 60 days. This startup time was similar for the large setups, namely 28 days for the AcL setup, 63 days for the BicL setup and 47 days for the AcnSL setup. The large setups produced an average current of: 2.7 A $m^{-2}$ (AcL), 2.3 A $m^{-2}$ (BicL) and 2.2 A $m^{-2}$ (AcnSL) with a $H_2$ yield of 2.4 (AcL), 2.7 (BicL) and 2.2 (AcnSL) $m^3$ $H_2$ per $m^3$ reactor liquid per day. A control large setup (un-inoculated) produced 0.8 A $m^{-2}$ with 0.32 $m^3$ $H_2$ per $m^3$ reactor liquid per day. The higher current production in the large setups was for a major part explained by the difference in cell design and by the higher surface area of the porous graphite felt electrode (Jeremiasse *et al*., 2012).

### 3.4.2 Microbial community composition

SEM revealed that on all electrode samples microorganisms were attached. The electrode paper from the small setup showed a dense packed biofilm, whereas on the electrode felt from the large setups, which had a much larger surface area available for biofilm attachment, a less dense biofilm was visible (Figure 3.1).

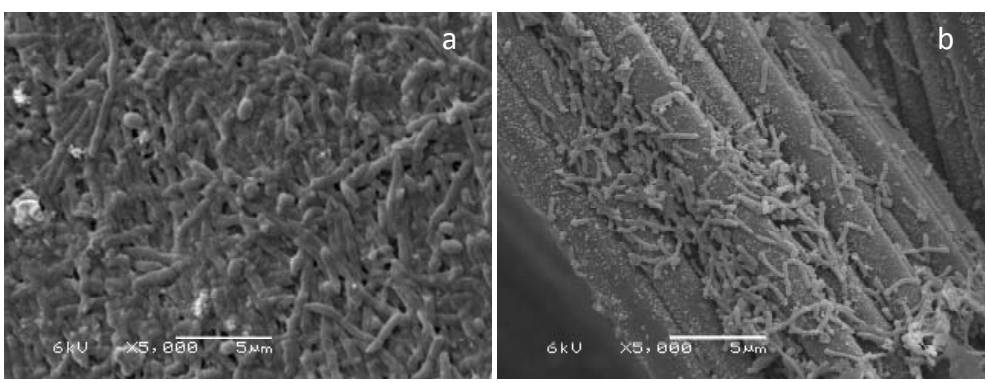


**Figure 3.1** Scanning Electron Micrograph of the paper (a) and felt (b) cathode surface after operation as $H_2$ producing biocathode. All setups were examined and representative pictures are shown here ((a) from BicS, (b) from BicL). Scale bars indicate the actual size of the items on the image.

Denaturing gradient gel electrophoresis (DGGE) analysis revealed that the bacterial communities in the small setups were similar, suggesting that the carbon source has little effect on the community that was enriched. The bacterial communities in the large setups were different from the ones in the small setups, even when supplied with the same carbon source. The DGGE profiles of the large setups

showed two dominant bands that were present in both the AcL and BicL setups, but also several unique bands per setup. The AcnSL setup showed one dominant band that was not abundant in the samples of the other setups (Figure 3.2).

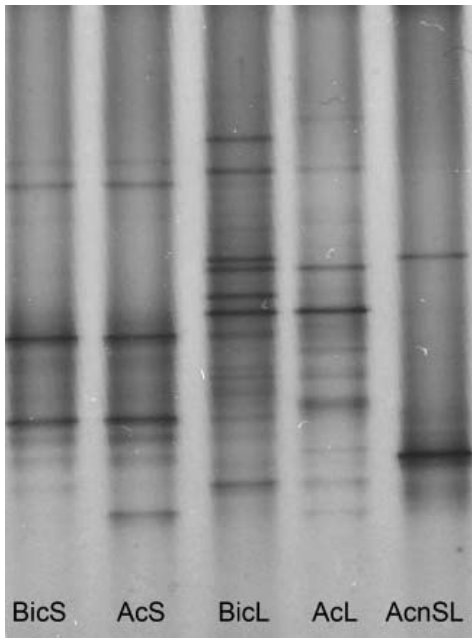


**Figure 3.2** Denaturing gradient gel electrophoresis profiles of bacterial communities on the electrodes from setup BicS, AcS, bicL, AcL and, AcnSL (names as explained in method section).

The 16S rRNA gene clone libraries revealed large bacterial diversity differences in the MEC biocathode samples. However, predominant groups of clones were present in each setup. At the phylum level both small setups consisted of mainly clones affiliated with the *Firmicutes* (BicS: 93%, AcS: 92% of total clones) but for both large setups the majority of the clones affiliated with the *Proteobacteria* (BicL: 91%, AcL: 57%). The AcL setup also contained a predominant group affiliated with *Bacteroidetes* (37%). The AcnSL setup consisted of almost exclusively *Actinobacteria* (98%) (Figure 3.3).

At the species level, the composition of the bacterial communities of the biocathode of the two small setups was very similar, but for the large setups the communities differed more from each other (Table 3.1). The clones obtained from the AcS setup (total 81 clones) consisted of two predominant ribotypes. The first ribotype (32 clones, 40% of total) clustered with uncultured *Clostridiaceae*. The closest related uncultured clone (AY261814) was derived from an UASB reactor (99% identity).

The closest related cultured species was *Clostridium cylindrosporum* (Y18179, 91% identity). The second predominant ribotype (32 clones, 40% of total) belonged to the family of *Peptococcaceae*. The closes related clone (GQ921447, 95% identity) was derived from fracture water from a gold mine. The closest related cultured species was *Desulfotomaculum* sp. Ox39 (AJ577273, 91% identity).

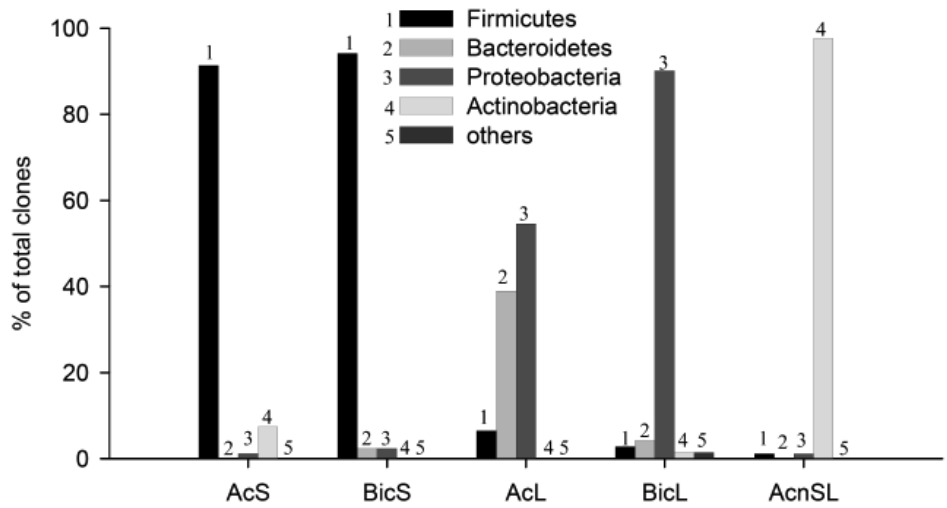


**Figure 3.3** Distribution of different phyla per MEC setup. On the x-axis the different setups are plotted. BicS, AcS, BicL, AcL and AcnSL are as explained in methods section. On the y-axis the percentage of clones within a phylum relative to the total amount of clones in the sample is plotted. (Black (1) indicates the *Firmicutes*, dark gray (3) the *Proteobacteria*, light gray (2) the *Bacteroidetes* and very light gray (4) the *Actinobacteria*. Clones belonging to other groups (only 1% in the BicL setup) are indicated in very dark gray (5)).

The microbial community of the BicS biocathode (total 85 clones) consisted of the same predominant ribotypes as the AcS setup, but in different proportions. 19% (16 clones) belonged to the uncultured *Clostridiaceae* with 100% identity to the clones from the AcS setup. In addition 59% (50 clones) belonged to the family of *Peptococcaceae* again with 100% identity to this ribotype from the AcS setup.

In the AcL setup most clones affiliated with the *Proteobacteria*, and the dominant ribotype was found in the *Bacteroidetes*. 23% (18 clones of a total of 77 clones) belonged to the species *Kaistella koreensis* (AF344179, 99% identity), an aerobic, non-spore forming rod, isolated from industrial phenolic compound-degrading waste water (Kim *et al*., 2004). Further, 4 clones were related to *Leptothrix* sp. MOLA

523 (AM990747, 99% identity) isolated from *Petrosia ficiformis* sponge. 36% of the *Proteobacteria* belonged to the *Gammaproteobacteria* of which most clones (12 of total 18 clones) were related to several *Pseudomonas* species. *Pseudomonas* species have shown to be electrochemically active in MFCs (Logan, 2009; Raghavulu *et al*., 2011). The other clones affiliated to the *Proteobacteria* in this sample clustered with several different genera and did not group within predominant ribotypes of more than 4 clones.

For the BicL setup, the predominant ribotypes within the *Proteobacteria* all belonged to the *Betaproteobacteria*. Of the total 71 clones, 13% (9 clones) grouped with the genus *Hydrogenophaga*. Closest related was an uncultured *Hydrogenophaga* sp. (GU560177, 99% identity) detected in a biofilm of a reactor for treatment of pharmaceutical wastewater. The closest related cultured species was *Hydrogenophaga flava* strain 2 (NR_028718, 97% identity). *Hydrogenophaga* ssp. are Gram-negative, aerobic bacteria that grow chemolithoautotrophically or chemoorganotrophically with $H_2$ (Willems *et al*., 1989). Another 13% (9 clones) grouped with *Desulfovibrio vulgaris* Miyazaki F (NC_011769, 99% identity). *Desulfovibrio* spp. are sulfate-reducing species well known to consume and produce $H_2$ (Postgate & Campbell, 1966; Walker *et al*., 2009). A further 13% (9 clones) grouped with the genus *Azonexus*, with the closest related clone (AJ009452, 99% identity) that was derived from a trichlorobenzene-degrading microbial consortium. The closest related cultured species was *Azonexus caeni* (AB166882, 97% identity) a denitrifying bacterium isolated from a wastewater treatment plant (Quan *et al*., 2006). 7% (5 clones) grouped with the species *Azospira oryzae* (NR_024852, 99% identity) a Gram-negative, highly motile, nitrogen-fixing bacterium isolated from anaerobic soil (Reinhold-Hurek & Hurek, 2000).

The AcnSL setup showed a biocathode microbial community of almost exclusively *Actinobacteria* of which 96% (82 of the 85 clones) belonged to one ribotype and showed 99% identity with the species *Promicromonospora* sp. CPCC100077 (FJ529706). *Promicromonospora* spp. are Gram-positive, spore forming bacteria. Strain CPCC100077 was isolated from soil at 3000 m altitude at the Qinghai–Tibet plateau in China (Zhang *et al*., 2010). For more detailed information on the less dominant ribotypes found in the clone library we refer to the phylogenetic tree in the Appendix A.

### 3.4.3 Bacterial diversity and sampling coverage estimation

Statistical analysis of the clone libraries showed a lower diversity for the small setups with a Shannon's diversity index of 1.41 for AcS and 1.23 for BicS and a

**Table 3.1** Overview of the dominant ribotypes on the different MEC biocathode samples with the closest relatives and the closest cultured species as found in GenBank. BicS, AcS, BicL, AcL and AcnSL as explained in methods section.

| Ribotype | MEC | #clones (total) | Closest relative (GenBank accession #, identity) | Closest cultured (GenBank accession #, identity) |
|---|---|---|---|---|
| AcS 1 | Small acetate | 32(81) | uncultured *Clostridiaceae* clone derived from UASB reactor (AY261814, 99%) | *Clostridium cylindrosporum* (Y18179, 91%) |
| AcS 2 | Small acetate | 32 (81) | Clone derived from a gold mine (GQ921447, 95%) | *Desulfotomaculum* sp. Ox39 (AJ577273, 91%) |
| BicS 1 | Small bicarbonate | 16 (85) | uncultured *Clostridiaceae* clone derived from UASB reactor (AY261814, 99%) | *Clostridium cylindrosporum* (Y18179, 91%) |
| BicS 2 | Small bicarbonate | 50 (85) | Clone derived from a gold mine (GQ921447, 95%) | *Desulfotomaculum* sp. Ox39 (AJ577273, 91%) |
| AcL 1 | Large acetate | 18 (77) | *Kaistella koreensis* (AF344179, 99%) | |
| BicL 1 | Large bicarbonate | 9 (71) | uncultured *Hydrogenophaga* (GU560177, 99%) | *Hydrogenophaga flava* strain 2 (NR_028718, 97%) |
| BicL 2 | Large bicarbonate | 9 (71) | *Desulfovibrio Miyazaki* F (NC_011769, 99%) | |
| BicL 3 | Large bicarbonate | 9 (71) | Clone from trichlorobenzene-transforming microbial consortium (AJ009452, 99%) | *Azonexus caeni* (AB166882, 97%) |
| BicL 4 | Large bicarbonate | 7 (71) | *Azospira oryzae* (NR_024852, 99%) | |
| AcnSL 1 | Acetate no sulfate | 82 (85) | *Promicromonospora* sp. CPCC100077 (FJ529706, 99%) | |

higher diversity for the large setups with a Shannon's diversity index of 2.91 for AcL and 2.65 for BicL. The diversity of AcnSL was very low (0.19). The Goods coverage ranged from 77% to 96% (see Table 3.2) and rarefaction curves showed levelling off of the curves which indicated that sufficient samples were taken to be able to draw conclusions on which ribotypes were dominantly present in the samples.

**Table 3.2** Good's coverage and Shannon's diversity index of the bacterial communities of 5 different setups.

| Setup | No. of sequenced clones | Good's coverage (%) | Shannons diversity index |
|---|---|---|---|
| AcS | 81 | 96 | 1.41 |
| BicS | 85 | 93 | 1.23 |
| AcL | 77 | 77 | 2.91 |
| BicL | 71 | 85 | 2.65 |
| AcnSL | 85 | 96 | 0.19 |

### 3.4.4 Hydrogenase Chip

Several different hydrogenase genes were detected in the samples from the large setups. 41 of 2275 genes on the array showed BPF values above the identified thresholds (0.958, 0.917, 0.979, for samples AcL, BicL, and AcnSL respectively) in at least one of the samples analyzed. Of those 41 genes with above-threshold BPF values, 8 were from genome sequences of bacterial isolates and 33 were from metagenomic sequences. Of these 33 genes from metagenomes above the BPF threshold, only 4 had a BSLDS greater than 100 (equivalent to 10 bright probes across a contiguous section of the gene). Those 4 genes with a high BSLDS values were considered the most likely to be truly present, rather than the result of cross hybridization to the relatively small number of probes targeting most metagenomic genes.

Within the used detection limit 6 hydrogenase genes were considered present in the AcL setup (Table 3.3) of which 4 were from bacterial isolates namely: 1) NiFe hydrogenase large subunit (Lcho_1459) from *Leptothrix cholodnii* SP-6, 2) FeFe hydrogenase (DMR_02480) from *Desulfovibrio magneticus* RS-1, 3) NiFe hydrogenase large subunit (Anae109_4306) from *Anaeromyxobacter* sp. Fw109-5 and 4) NiFe hydrogenase large subunit (RPC_3774) from *Rhodopseudomonas palustris* BisB18. Furthermore, two coenzyme F420-reducing hydrogenases (the

Table 3.3 Hydrogenase genes identified as being present using the Hydrogenase Chip. The function and extra information (remarks) were obtained from the IMG database.

| Setup | Function | Locus tag and species or IMG identifier | Remarks | Present in 16S rRNA gene clone library? |
|---|---|---|---|---|
| AcL | NAD(P)$^+$ dependent, NiFe hydrogenase (Hox type) | Lcho_1459 from *Leptothrix cholodnii* SP-6 | Bidirectional, cytoplasmic, involved in both $H_2$ consumption and production | Yes (4/77 clones) |
| AcL | NAD(P)$^+$ dependent FeFe hydrogenase | DMR_02480 from *Desulfovibrio magneticus* RS-1 | Cytoplasmic, involved in $H_2$ production | Yes (1/77 clones) |
| AcL | nickel-dependent (NiFe) hydrogenase large subunit NAD$^+$-reducing hydrogenase subunit (Hox type) | Anae109_4306 from *Anaeromyxobacter* sp. Fw109-5 | Bidirectional, cytoplasmic, involved in both $H_2$ consumption and production | |
| AcL | nickel-dependent (NiFe) hydrogenase, large subunit | RPC_3774 from *Rhodopseudomonas palustris* BisB18 | Cytoplasmic, membrane bound $H_2$ase. Has interaction with cytochrome b and can also act on soluble electron donors. | |
| AcL | Coenzyme F420-reducing hydrogenase | Metagenome data IMG identifier 2000494750 and 2006298366 | Coenzyme F420-reducing $H_2$ases can act in a similar way as NAD(P)$^+$-dependent hydrogenases | |
| BicL | nickel-dependent (NiFe) hydrogenase large subunit (hya/hyb type) | DvMF_1733, 0270 and 0273 from *Desulfovibrio vulgaris* Miyazaki F | No evidence that hya/hyb type $H_2$ases are involved in $H_2$ production. | Yes (9/71 clones) |
| AcnSL | nickel-dependent (NiFe) hydrogenase large subunit NAD$^+$-reducing hydrogenase subunit (Hox type) | Anae109_4306 from *Anaeromyxobacter* sp. Fw109-5 | Same as in sample AcL. Bidirectional, cytoplasmic, involved in both $H_2$ consumption and production | |
| AcnSL | probable ferredoxin NiFe hydrogenase, large subunit | az03808 from *Azoarcus* sp. BH72 | Homology to hoxC which is a $H_2$ sensor | |
| AcnSL | iron only (FeFe) hydrogenase large subunit, C-terminal domain | Metagenome data IMG identifier 2004316449 and 2004332277 | Not related to anything known but in general FeFe $H_2$ases are involved in $H_2$ production | |

distribution, flow path, electrode material or local $H_2$ partial pressure. The different setup designs are referred to as large and small setup, with this naming we do not tend to imply that the actual volume has the largest influence. Our results showed that on both small setups mainly *Firmicutes* and on both large setups mainly *Protebacteria* were enriched on the cathodes. Because the results of the AcnSL setup are very different from all the other setups this setup is discussed separately. The detectable bacterial diversity was much lower in the small setups than in the large setups. Since the large setup was inoculated with biomass from the small setup fed with the same carbon source, one would expect selection for species able to use the specific carbon source (acetate or bicarbonate) and thus a decrease or no change in bacterial diversity. However, as indicated by Shannon's diversity index, the bacterial diversity is higher in both large setups compared to both small setups. This also indicates that the cell design has a greater influence on the development of the microbial community than the carbon source. In contrast, when we compare the microbial populations in the same size setups, the Shannon's diversity index shows higher values for the setups fed with acetate which can be used by (facultative) heterotrophic microorganisms, than setups fed with bicarbonate, which can only be used by autotrophic growing microorganisms. This was found for both the small and the large setups. On species level the two small setups were very similar, whereas the AcL and BicL setups showed more diversity of species. This difference was confirmed with the Hydrogenase Chip data which showed that in the AcL sample $NAD(P)^+$ dependent NiFe, FeFe hydrogenases, and a coenzyme F420-reducing hydrogenase were found to be abundantly present. In the BicL sample, three different NiFe hydrogenases from *D. vulgaris* Miyazaki F were abundantly present. A semi quantitative analysis of the samples showed that the NiFe hydrogenase from *D. vulgaris* Miyazaki F was present in higher abundance in sample BicL than in any of the other samples. These differences between samples AcL and BicL suggests that the carbon source does have some effect on the microbial population as was suggested by Jeremiasse *et al*. (2012). Nevertheless, no clear conclusions on the effect of the carbon source can be made, as no exclusive autotrophic bacteria were identified in the bicarbonate setups or heterotrophic species in the acetate setups. Although no acetate was measured in the bicarbonate systems (data from Jeremiasse *et al.* (2012)), it cannot be excluded that acetogenic bacteria produce acetate from bicarbonate (Nevin *et al*., 2011) and that this acetate is used by other microorganisms for heterotrophic growth. Also decaying biomass could have been used as a carbon source for heterotrophic growth.

The major effect of the setup design on the microbial population might be caused by several components in the setup. First of all, the electrode material was different. In both types of setups the electrode material was graphite, but the shape and surface area, flat paper versus three dimensional felt, was different and could have influenced the attachment of bacteria to the electrode. This could also explain the differences in species diversity of the large compared to the small setups. The relatively small and flat electrode surface of the small setups might cause only attachment of the initial biomass to the electrode (Torres *et al*., 2009) where in a later stage no new biomass can attach. In the large setups there is sufficient surface available for attachment of other bacteria that are slower in colonization of the electrode. This was supported by the SEM images, because in the small setup cathodes the whole surface was colonized by biomass and in the large setups parts of the electrode surface were not covered with microorganisms. The current production in the large setups with the graphite felt electrodes reached a steady state which indicates that the microbial coverage was not the limiting factor for current production. However, these differences in microbial coverage of the cathode observed with SEM, could also be caused by imperfect nutrient distribution in the felt electrodes.

The differences in flow path of the nutrient solution might have influenced the microbial population because of differences in mixing, mass transfer in the electrode compartment and shear forces at the electrode surface. Poor mixing and mass transfer limitation might create local high concentration of metabolic or electrochemical products (e.g. $H_2$ pressure or pH) or gradients in nutrient availability that will influence the growth of microorganisms. Shear forces at the electrode surface remove microorganisms that are not able to attach sufficiently strong to the electrode.

The bacterial community of the AcnSL setup was very different from all the other setups. At the electrode of the AcnSL setup an almost pure culture of *Promicromonospora* sp., a member of the *Actinobacteria*, was found to be dominant. The sulfate, although only present in trace amounts, was initially left out from the medium to make sure no electrons were lost in dissimilatory sulfate reduction. However, sulfur is an essential element and although very little sulfur is needed to sustain growth (Sievert *et al*., 2007) the lack of sulfur might limit growth of a diversity of bacteria. Interestingly, after startup, this setup performed similar as the large setups fed with additional sulfate as source of sulfur. In the other setups no clones related to *Promicromonospora* spp. were detected. The lack of sulfate clearly has a major influence on the type of microorganisms that grow at the cathode.

Limiting factors like this could be a good strategy for isolation of single species that can catalyze $H_2$ production in a cathode for further studies.

The two hydrogenase genes of known species that were detected in the AcnSL setup did not belong to an *Actinobacterium*. Possibly the detected hydrogenase genes were from species that were present but not detected in the clone library because at least one of the genes (Anae109_4306) was also present, although less abundant, in the AcL setup from which this setup was inoculated. The other AcnSL detected hydrogenase (azo3808) was also present in the AcL sample but just below the significant bright probe fraction detection limit. This similarity suggests those hydrogenase genes belong to species also present in the AcL setup. The hydrogenase genes from metagenomic data found in the AcnSL setup showed two FeFe-hydrogenases which did not have a match with any cultured species and might be derived from the dominant *Promicromonospora* sp. in this sample. To our knowledge nothing is known about $H_2$ production by *Promicromonospora*. This could be a possible novel characteristic of the *Promicromonospora* strain detected in our biocathode. A sequencing project for *Promicromonospora kroppenstedtii* which was isolated from garden soil is ongoing (Alonso-Vega *et al*., 2008). Hopefully this will provide insight in the possible $H_2$ metabolism of *Promicromonospora* spp.. A PROSITE scan (Gattiker *et al*., 2002; Marshall *et al*., 2012) of the draft genome, using the PROSITE motive as described by (Vignais & Billoud, 2007), and an IMG/GEBA search revealed three putative hydrogenases (IMG/GEBA identifiers: 2507527031, 2507526009, and 2507525797) which are, however, not related to any of the hydrogenases found in this study and also are not present on the Hydrogenase Chip.

### 3.5.2 Bacteria responsible for catalyzing $H_2$ evolution

In this study very distinct bacterial populations with members of different dominant phylogenetic groups (*Firmicutes*, *Proteobacteria* and *Actinobacteria*) were detected in setups that all catalyzed the production of $H_2$ at the biocathode. In the only other study on the microbial population of the biocathode for $H_2$ production a dominant group of *D. vulgaris* related species was found (and active) when the biocathode was enriched as anode fed with $H_2$ and acetate (Croese *et al*., 2011). This suggested that *Desulfovibrio* species might be the major players in $H_2$ production at the cathode. In the present study we only found *Desulfovibrio* in the large setups and only predominant in the BicL setup. Apparently, a wide range of phylogenetically different bacteria can grow in the biocathode which presumably catalyze, or are involved in $H_2$ production at the MEC biocathode.

Several mechanisms for electron transfer and $H_2$ production in cathode systems have been proposed (Rosenbaum *et al*., 2011). Hydrogenases are present in many bacteria from different phylogenetic groups (Vignais *et al*., 2001), and also in many of the bacteria that we identified in the biofilms on the biocathodes in the present study. It is not known if bacteria are able to conserve energy by formation of $H_2$ by reduction of protons with electrons derived from the electrode. It has been suggested that bacteria are able to grow in $H_2$ producing biocathode systems through the activity of energy converting hydrogenases (Ech) or via cytoplasmic hydrogenase activity resulting in a proton gradient that can be utilized by a membrane-integrated ATPase (Croese *et al*., 2011; Geelhoed *et al*., 2010).

Using a hydrogenase DNA microarray, we tried to identify the hydrogenase genes that are present in the biocathode samples. The hydrogenases from several species that were identified in the clone library were identified with the Hydrogenase Chip. First of all, *D. vulgaris* Miyazaki F which was identified in the BicL clone library and its NiFe hydrogenase genes were identified using the Hydrogenase Chip. The NiFe hydrogenases in *D. vulgaris* Miyazaki F have not been linked to $H_2$ production before but no other hydrogenase genes for $H_2$ production are known for this species, although it has been recorded to be able to produce $H_2$ (Tsuji & Yagi, 1980). In the AcL setup one clone of *Desulfovibrio* was found and also a FeFe hydrogenase gene was identified from *D. magneticus*. Furthermore, 4 *Leptothrix* clones were detected and one *Leptothrix* NiFe hydrogenase gene was identified using the chip. This hydrogenase belongs to the Hox-type or bidirectional $NAD(P)^+$ dependent hydrogenases which can be involved in cytoplasmic $H_2$ production and consumption (Tamagnini *et al*., 2007; Vignais & Colbeau, 2004; Worm *et al*., 2011). The other detected hydrogenases are from bacteria in the *Proteobacteria* phylum but did not match with any of the detected clones. Some of the dominant species that were detected in the clone library were not detected using the Hydrogenase Chip because either the abundance of their hydrogenase genes was too low to be able to detect them with the microarray or there are no hydrogenase genes known and/or sequenced for those species (e.g. *Promicromonospora*, *Hydrogenophaga, Azospira, Azonexus, Kaistella*).

In general, the detected hydrogenases seem to be mostly cytoplasmic, bidirectional, $NAD(P)^+$ dependent Hox-type hydrogenases. Interestingly, the Hox-type hydrogenases, although of the NiFe-type, have been associated with, (mostly fermentative) $H_2$ production before (Burow *et al*., 2012; Tamagnini *et al*., 2007).

Concerning the mechanism of biocathodic $H_2$ production, if the Hox-type hydrogenases are mostly involved, this would suggest a predominant role of cytoplasmic hydrogenases rather than of membrane integrated energy converting

hydrogenases. In that case electrons need to be shuttled from the outside to the cytoplasm by, thus far unknown, electron mediators. Although a microbial biofilm developed on the cathodes that catalyzed $H_2$ production, it cannot be excluded that part of the microbial community consumed $H_2$ and hydrogenases originate from those species. Furthermore, from our study we cannot fully exclude the possibility that free hydrogenases (derived from lysed cells) play a role in biocathodic $H_2$ formation. Immobilized hydrogenases were successfully applied in fuel cells (Armstrong *et al.*, 2009; Lojou *et al.*, 2002) but were shown to be unstable and needed regeneration. Active microbial cells can facilitate continuous regeneration of hydrogenase activity in an MEC. Likewise, other enzymes than hydrogenases could also be involved in biocathodic $H_2$ production. For example nitrogenases catalyze $H_2$ production under photoheterotrophic conditions (Kim & Kim, 2011), which might be functional in a similar manner when the cathode in an MEC is the external energy source.

In conclusion, the setup design had a major effect on the development and composition of the biofilm on the biocathode of the MEC. This effect was larger than the effect of differences in carbon source (autotrophic or heterotrophic) and therefore setup design needs to be carefully considered when designing experiments. We have revealed that a large diversity of bacteria is likely electrochemically active and involved in the production of $H_2$ in the cathode of the MEC. From an applied perspective this is very beneficial because mixed and diverse microbial communities rather than specific pure cultures can be used at the biocathode. To unravel the mechanisms underlying the production of $H_2$ in an MEC biocathode, more in depth research is essential. The Hydrogenase Chip offers excellent perspectives for further MEC biocathode studies.

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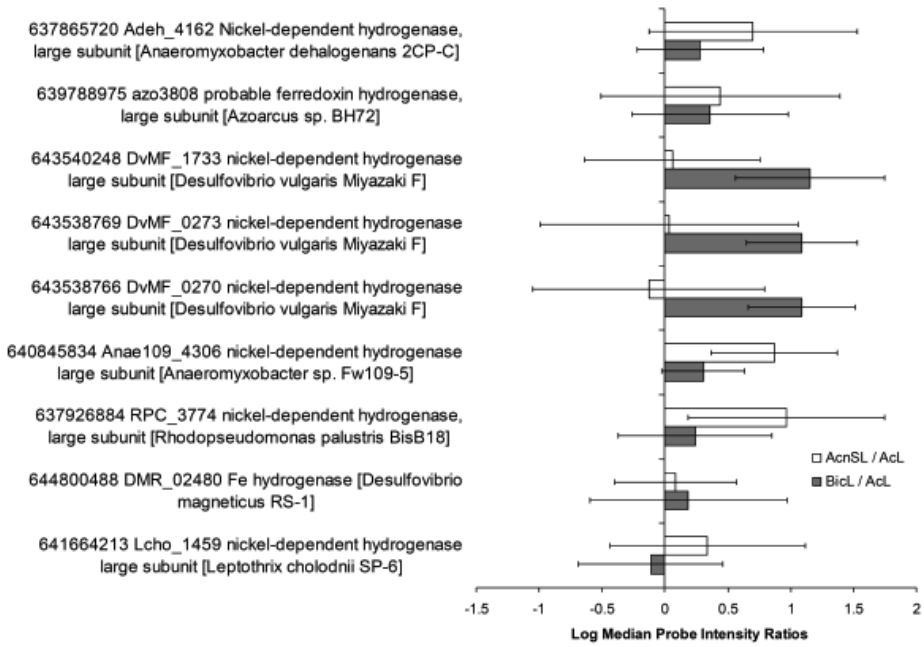
**Appendix A**: Phylogenetic tree of all clones from the biocathodes including closest related cultured species. Numbers indicate amount of clones within that cluster. *Sulfolobus tokodaii* was used as out-group.

**Appendix B** Hydrogenase microarray data. Digitally available from the authors upon request.




**Appendix C** Log median probe intensity ratios of the Loess-normalized data of the genes from bacterial isolates that showed above-threshold BPF values for samples BicL and AcnSL to sample AcL using the tileplot.double() function for quantitative comparisons.

# Chapter 4

## Startup of a microbial electrolysis cell (MEC) biocathode with a *Citrobacter* strain isolated from an MEC

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### 4.1 Abstract

In the microbial electrolysis cell (MEC) microorganisms can be used as catalyst for cathodic $H_2$ production. The understanding of the molecular mechanisms of microbial-mediated biocathodic $H_2$ formation is limited. So far, no bacteria have been isolated from $H_2$ producing biocathodes. Here, we describe the first isolate from a $H_2$ producing biocathode, strain PS2 (99% identity with *Citrobacter amalonaticus*), and initial tests on its ability to use electricity to form $H_2$. Inoculation of strain PS2, together with a continuous pyruvate feed, resulted in an active biocathode that produced 2.2-2.3 A $m^{-2}$ with 2.2-2.8 $m^3$ $H_2$ $m^{-3}$ reactor per day as sole product during the first run and minor amounts of acetate and formate during the second run. Nigericin or monensin addition to the active biocathode did not influence current or $H_2$ production, suggesting that membrane associated proton gradient driven processes are not involved in $H_2$ production by the biocathode. We show for the first time that it is possible to start up a MEC biocathode directly using a pure bacterial culture isolated from an MEC.

## 4.2 Introduction

The microbial electrolysis cell (MEC) technology offers great perspective as sustainable technology for $H_2$ production opposed to direct water electrolysis. By using organic waste streams as feed and microorganisms as catalysts at cheap carbon anodes and cathodes, the energy input and costs of the system can be very economical (Jeremiasse *et al.*, 2009; Liu *et al.*, 2005; Rozendal *et al.*, 2006; Rozendal *et al.*, 2008). In addition, the MEC is an interesting tool to study exocellular electron transfer from a solid surface to microorganisms that are able to form $H_2$ driven by electricity. It has been shown that microbial growth can occur close to the limit of what is thermodynamically possible (Dolfing *et al.*, 2008; Kim *et al.*, 2010). In an MEC those energetic limitations can be regulated by fixing the potential of the electron donor (cathode) or electron acceptor (anode) at a specific potential. $H_2$ formation from protons under standard biological conditions is endergonic and needs input of energy. In an MEC, $H_2$ production is driven by electricity, and the energy input can be regulated by the cathode potential. It has been shown that it is possible to catalyze $H_2$ production at a cathode using mixed microbial communities as catalyst (Jeremiasse *et al.*, 2009; Rozendal *et al.*, 2008). Thus far, no pure cultures have been obtained from MEC biocathodes but it has been suggested that energy conservation and growth is possible by producing $H_2$ with electrons derived from the cathode involving membrane associated energy converting hydrogenases (Ech) or by cytoplasmic hydrogenases that reduce protons and thus create a proton motive force that can drive ATP formation by a membrane-bound ATPase (Geelhoed *et al.*, 2010; Geelhoed & Stams, 2010). So far, many different species are known that are able to donate electrons to an anode (Logan, 2009; Sharma & Kundu, 2010) including members of the genus *Geobacter, Shewanella* and *Pseudomonas* (which produces electron shuttling components to do so). The uptake of electrons from a cathode for the reduction of a diversity of products has been described as well for several species like *Geobacter* and *Pseudomonas* species (Rosenbaum *et al.*, 2011). But to date *Geobacter sulfurreducens* PCA and *Desulfovibrio* G11 are the only two pure cultures that have been described to produce $H_2$ using electrons from a cathode (Croese *et al.*, 2011; Geelhoed & Stams, 2010). No pure cultures have been isolated so far from $H_2$ producing biocathodes. In this study we describe a new isolate which was isolated from an MEC biocathode and describe initial tests to show its bioelectrochemical activity in $H_2$ production.

## 4.3 Materials and methods

### 4.3.1 Cultivation and isolation procedures

A mix of catholyte and cathode biomass (1 mL) from an active biocathode of an MEC with acetate as substrate (Jeremiasse *et al.*, 2011) was used to inoculate anaerobic agar (1.6 % agar) tubes containing basic phosphate buffered medium as described before (Croese *et al.*, 2011) with 10 mM pyruvate and 10 mM sulfate. Separate agar tubes were inoculated with dilutions (10 and 100 fold) from the original biomass. After 2 weeks of incubation at 30°C, colonies were picked from the 100x dilution tubes under $N_2$ flow and transferred to anaerobic liquid medium (Croese *et al.*, 2011). These cultures were transferred in dilution series of $10^{-1}$ till $10^{-12}$ in the same media. Growth (increasing turbidity) was observed till the $10^{10th}$ dilution. A sample from this $10^{10th}$ dilution was then transferred to fresh basic phosphate buffered medium amended with only 10 mM pyruvate as substrate. Samples from this culture, which was microscopically pure, were used for further characterization. The new strain was named strain PS2.

### 4.3.2 Determination of growth parameters

PS2 was routinely grown in basic phosphate buffered medium with pyruvate. To determine growth parameters of PS2, it was transferred to medium with different carbon sources and electron acceptors. As carbon and energy source 10 mM of glucose, lactate, citrate, formate, acetate, $H_2$ (100 % in the gas phase) + 10 mM formate, $H_2$ + 10 mM acetate or $H_2$ + 10 mM bicarbonate, and as electron acceptor sulfate (10 mM), nitrate (10 mM) or oxygen (open to air) were used in combination with all mentioned carbon sources. To test actual consumption of oxygen, PS2 was grown on citrate with closed headspace containing sterile air. PS2 was also transferred to rich meat extract medium and Lysogeny broth (LB) under aerobic and anaerobic conditions. 5 mL of 20 mM pyruvate-grown culture was transferred to an API E20 strip (bioMérieux, La Balme les Grottes, France) and analyzed after 24 hours and again after 7 days of incubation at 37°C. PS2 cells were stained for Gram type using 2% (w/v) crystal violet and 2% (w/v) safranin S as counterstain. Light and scanning electron microscopy (see below) were used to observe microbial morphology and motility.

### 4.3.3 Molecular analysis and phylogeny

Genomic DNA was extracted from 2 mL of culture or 1 $cm^2$ electrode samples using the Fast DNA spin kit for soil (Bio101, Vista, CA, USA). Bacterial 16S rRNA genes were amplified with the primers Bact 27F-Univ1492R (Lane, 1991). PCR settings were initial denaturation for 2 min at 95°C, followed by 25 cycles of 30 s denaturation at 95°C, 40 s annealing at 52°C and 1.5 min elongation at 72°C. Post-elongation was 5 min at 72°C. The PCR samples were tested on a 1% agarose gel for amount and size of product. PCR fragments were sent to Baseclear (Leiden, The Netherlands) for sequencing using the 27F primer. The obtained partial bacterial 16S rRNA gene sequence (514 bp) was compared to the GenBank database using the NCBI blastn tool to identify the most closely related sequences. The sequence was deposited in the European molecular biology laboratory (EMBL) nucleotide sequence database (accession nr.: HE794999). The 16S rRNA gene sequences of the closest related species were selected and aligned with the PS2 sequence using the online Silva alignment tool (Pruesse *et al.*, 2007) and merged with the ARB database using ARB software package version 5.1 (Ludwig *et al.*, 2004). A phylogenetic tree was constructed using the ARB Neighbour Joining Algorithm and Jukes Cantor correction.

### 4.3.4 Denaturing Gradient Gel Electrophoresis (DGGE)

Partial bacterial 16S rRNA gene amplicons to be used for DGGE analysis were obtained using primers Bact968F (including GC clamp) and 1401R (Nübel *et al.*, 1996). PCR conditions were as above, except that 35 cycles were applied and an annealing temperature of 56°C was used with 1 min elongation steps. DGGE of the culture, catholyte and cathode samples was performed as described before (Croese *et al.*, 2011). DGGE profiles were compared to previously generated profiles from mixed culture MEC biocathodes (Chapter 3). For comparison, pictures of the DGGE profiles were digitally processed in BioNumerics version 4 (Applied Maths NV, Sint-Martens-Latem, Belgium) according to the manufacturer's instructions, checked manually and aligned using the 'Different bands' similarity with Ward alignment. Band classes were exported to view similarities in band classes. Band classes with < 0.5% difference were considered one band class. The band classes were numbered according to the height of the bands in the profiles.

### 4.3.5 Operation in setup

The MEC design was as previously described (Jeremiasse *et al.*, 2010) with the exception that both electrodes consisted of 2.5 mm thick graphite felt and the membrane was a Ralex cation exchange membrane (Ralex CMH-PES, Mega A.S., Prague, Czech Republic). The phosphate buffered medium was as described before (Croese *et al.*, 2011), further referred to as catholyte buffer. Temperature was controlled at 303K and pH was controlled at 7 by dosing 1M HCl. The cell voltage was applied such that the cathode potential was -0.7 V vs SHE and manually adjusted if needed. The current, pH, anode potential and cathode potential were logged every 5 minutes (Memograph M, Endress + Hauser, Naarden, The Netherlands) and averaged per day for plotting of the current development. The anolyte (Fe(II)-cyanide) and catholyte buffer recycle speed was 60 mL $min^{-1}$. Catholyte buffer and carbon source were pumped from individual influent tanks and mixed in the tubing before entering the setup. When no carbon influent was applied, the carbon influent tank was replaced by demi-water to maintain a constant buffer concentration. Before inoculation the setups were cleaned with water and soap and fresh membranes and electrodes were used. Additionally, the PS2MEC2 cathode compartment was cleaned with 70% ethanol before inoculation. One liter of an anaerobic PS2 culture grown on 20 mM pyruvate (first run, PS2MEC1) or 20 mM citrate (second run, PS2MEC2) to the end log phase was centrifuged (8000 rpm, 15 minutes), and the pellet was resuspended in 10 mL anoxic phosphate buffered saline (PBS, pH 7.4) and used to inoculate the cathode. PS2MEC1 (inoculated with pyruvate grown cells) was first operated without influent for 2 days before starting the addition of catholyte buffer with 5 mM pyruvate at a rate of 2.6 mL $min^{-1}$. After 17 days the influent medium was changed to buffer + 2 mM bicarbonate. 24 days after inoculation the influent was changed back to buffer + 5 mM pyruvate. $H_2$ production ($H_2$ yield) tests were done on day 41 and 48. Prior to these tests the MEC was left without carbon source addition during two days. A polarization test was performed two days after the first $H_2$ yield test. The second setup, PS2MEC2 (inoculated with citrate grown cells), was first left 2 days without influent pumping after which the pumping was started. The influent buffer for PS2MEC2 was first supplemented with 1 mM of acetate and 2 mM of bicarbonate as carbon source for 7 days. After this, the setup was amended with pyruvate (10 mM) and left for two days for batch growth. After that, acetate and bicarbonate were again added to the influent buffer, and after two days these were changed for 5 mM citrate. Day 15 after inoculation, the influent was changed to buffer + 1 mM pyruvate. At day 31 the carbon source was omitted from the influent. At day 47 of the PS2MEC2 run, a polarization curve and at day 51 a $H_2$ production test was

performed. At the end of the run of PS2MEC2 the open circuit potential (OCP) of the cathode was determined. The OCP is the potential which the cathode reaches when no cell voltage is applied and thus the cathode is in equilibrium with the electron acceptor.

### 4.3.6 $H_2$ production test

When stable current was reached, a 24 h $H_2$ production test of the biocathode was performed (day 41 and 48 for PS2MEC1 and day 51 for PS2MEC2). The biocathode was operated in batch mode during the $H_2$ production tests. The liquid and gas outlet of the catholyte recycle was connected via a 1 L flask to a gas flow meter (Milligascounter ®, Ritter, Bochum, Germany). One milliliter gas samples were taken at 0h and 24h and two or three additional time points in between (PS2MEC1 first test: t = 3h and 7h and second test t = 3h and 20h and for PS2MEC2 t = 16h, 19h and 22h), via a septum in the 1 L flask. The $H_2$ production was calculated using the mass balance equation and corrections for water vapor as described by Jeremiasse *et al.* (2011).

### 4.3.7 Polarization

The catalytic activity of the biocathode was analyzed by making a polarization curve. To prevent disruption of the biocathodes during development, polarization curves were only recorded after their development. The polarization curves were made by measuring the current and cathode pH at cathode potentials of -0.50, -0.55, -0.60, -0.65, -0.70, -0.75, -0.80 V (recorded every 5 minutes). Each cathode potential was applied for 1 h for PS2MEC1 and 30 minutes for PS2MEC2. For each cathode potential, the last 5 measurements were averaged.

### 4.3.8 Nigericin and monensin test

Setup PS2MEC2 was used to test if a proton gradient over the microbial cell membrane is needed for $H_2$ formation on the biocathode. To test this, nigericin (Sigma-Aldrich, Saint-Louis, USA) was dissolved in pure ethanol to a concentration of 5 mM, and diluted in water for addition in the active biocathode. First, 2 µM nigericin (final 0.04% ethanol) and after 1h 50µM (final 1% ethanol) was added to the active biocathode. After 24h without influent pumping, the influent pump was started again with only influent buffer. After 4 days, monensin was added (Sigma-Aldrich, Saint-Louis, USA) (final concentration again 2µM and after 1h

50µM). Effluent samples were collected before and after each of the tests and fatty acids and total carbon (inorganic and organic) were determined using TOC and UHPLC. At the end of the MEC runs the microbial population on the cathode was inspected under the light microscope, and electrode scrape of the biocathode was transferred to a batch tube containing anaerobic phosphate buffered medium supplemented with 20 mM pyruvate. After 7 days incubation at 30°C a 2 mL sample of this culture was collected for DGGE.

### 4.3.9 Analytical methods

Substrate and product concentrations from the batch cultures and the setup influent and effluent were measured using gas chromatography for the gasses $H_2$, $CO_2$, $H_2S$ and $CH_4$ (Varian CP-4900 microGC, TCD detector, MS5 and PPU columns in parallel), total organic carbon analyzer (TOC) for bicarbonate concentrations (Shimadzu TOC-VCPH), ultra high performance liquid chromatography (UHPLC) for citric, pyruvic, malic, succinic, fumaric, lactic, formic, acetic, propionic and butyric acid (Dionex Ultimate 3000 system, UV detector and Phenomenex Rezex Organic Acid $H^+$, 300x7.8 mm column), and ion chromatography (IC) for sulfate, nitrate and nitrite concentrations (Metrohm 761 Compact IC, conductivity detector and a Metrosep A Supp 5 6.1006.520 column).

### 4.3.10 Scanning electron microscopy (SEM)

Electrode and liquid culture samples were fixed in 2.5 % glutaraldehyde for 2 hours and washed twice with 10 mM PBS buffer (pH 7.4). Subsequently, the samples were dehydrated in a graded series of ethanol (10%, 25%, 50%, 75%, 90% and twice in 100% during 20 minutes for each step) and dried in a desiccator. The samples were coated with gold and viewed using a JEOL JSM-6480LV scanning electron microscope (acceleration voltage 6 kV, HV-mode, SEI detector).

## 4.4 Results

### 4.4.1 Enrichment and isolation of strain PS2

PS2 was isolated from an MEC biocathode fed with acetate as sole carbon source. PS2 formed a white colony, selected from colonies growing on anaerobic pyruvate + sulfate agar medium. After transfer to liquid pyruvate + sulfate and dilution

series, the highest dilution ($10^{-10}$) in which growth occurred was transferred to medium with only pyruvate. Analytical methods showed that sulfate was not used (concentrations did not change) and therefore PS2 was further routinely grown on pyruvate medium. DGGE (Figure 4.1, lane 2), sequencing of the partial 16S rRNA gene and light microscopy confirmed the purity of the culture. The 16S rRNA gene sequence showed 99% identity with *Citrobacter amalonaticus* strain C5 TYA (Figure 4.2). Microscopic observation and Gram staining showed that PS2 is a Gram-negative, rod shaped (Figure 4.3a) motile bacterium. PS2 is able to grow by fermentation of glucose, pyruvate and citrate while producing $H_2$, among other products. When grown on glucose it produces mainly lactic acid and acetic acid and traces (< 1 mM) of formic acid, pyruvic acid, malic acid and succinic acid. When grown on pyruvate it produces mainly acetic acid and formic acid and traces of propionic acid, butyric acid and lactic acid. When grown on citrate it produces mainly acetic acid and traces of formic acid and malic acid. PS2 did not grow with lactate, $H_2$ + formate, $H_2$ + acetate or $H_2$ + bicarbonate, but was able to grow on acetate with $O_2$ as electron donor. PS2 is able to grow on rich meat extract medium and on LB broth. PS2 is able to grow aerobically and anaerobically. Nitrate is completely reduced to nitrite (not further reduced) and sulfate is not reduced. Furthermore, API strip analysis showed that PS2 is ONPG, ADH, ODC, CIT, GLU, MAN, SOR, RHA, SAC, AMY and ARA positive and LDC, $H_2S$ ,URE, GEL, IND, MEL negative (see Table 4.1 for explanation of the abbreviations).

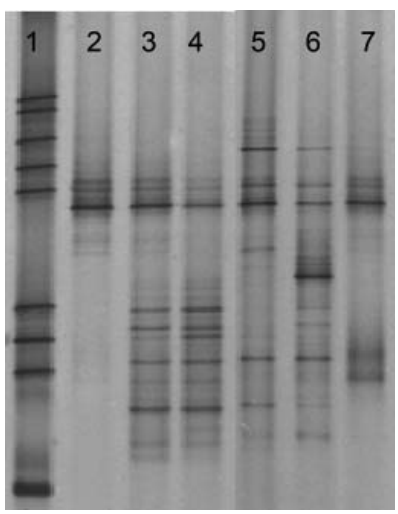


**Figure 4.1** DGGE fingerprinting profiles of PS2 pure culture and of PS2MECs. Lane 1: marker, Lane 2: PS2 isolate, Lane 3: PS2MEC1 cathode, Lane 4: PS2MEC1 catholyte, Lane 5: PS2MEC2 cathode, Lane 6: PS2MEC2 catholyte and Lane 7: PS2MEC2 cathode biomass grown in pyruvate batch tube.

**Table 4.1** Selected growth substrates and API strip analysis of PS2 compared to other *Citrobacter* species

| Compound or process | Strain PS2 | Other *Citrobacter amalonaticus* in %, n=16, and API strip results[1] | General to most known *Citrobacter*[1] |
|---|---|---|---|
| Citrate | + | 100 (+) | + |
| Glucose | + | 100 | + |
| Formate | - | | |
| Lactate | - | 100 | - |
| Pyruvate | + | + | + |
| LB | + | | |
| Meat extract | + | | |
| Acetate | + (only aerobic, not anaerobic) | 94 | - |
| $CO_2$ (with $H_2$ as energy source) | - | | |
| aerobic growth | + | 100 (+) | + |
| Motile | + | 100 | + |
| $H_2S$ production | - | 13 (-) | - |
| $NO_3 \rightarrow NO_2$ | + | 94 | + |
| $H_2$ oxidation | - | | |
| $H_2$ production | + | + | + |
| Arginine dihydrolase production (ADH)[2] | + | 100 (+) | - |
| O.-Nitrophenyl-p-D-galactosidase (ONPG)[2] | + | + | + |
| Lysine decarboxylase production (LCD)[2] | - | - | - |
| Ornithine decarboxylase production (ODC)[2] | + | 100 (+) | - |
| Urease production (URE)[2] | - | 94 ( -) | - |
| Indole production (IND)[2] | - | + | - |
| Gelatinase (GEL)[2] | - | - | - |
| Manitol fermentation (MAN)[2] | + | + | + |
| Sorbitol fermentation (SOR)[2] | + | + | + |
| Rhamnose fermentation (RHA)[2] | + | + | + |
| Melibiose fermentation (MEL)[2] | - | 6 (-) | - |
| Amygdalin fermentation (AMY)[2] | + | + | + |

+, growth; -, no growth detected

[1]Data from (Brenner *et al.*, 1999; Oh *et al.*, 2008). [2]API strip results

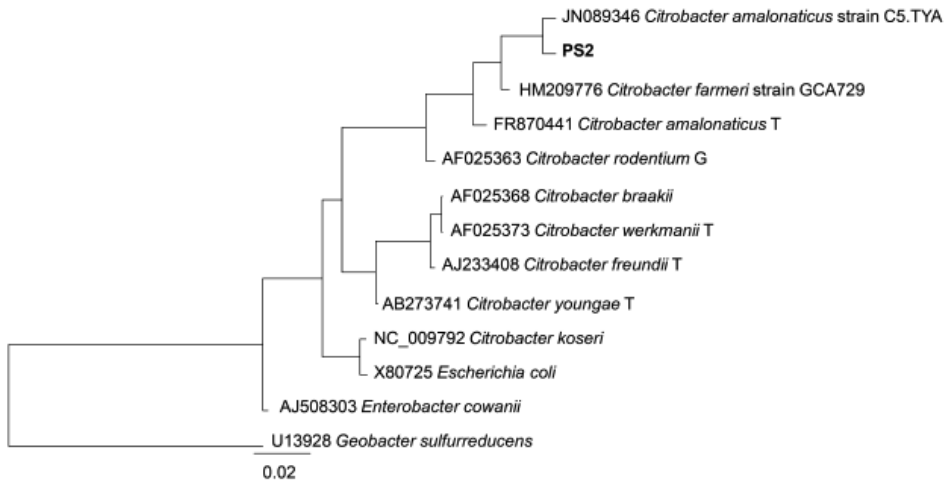


**Figure 4.2** Dendrogram of the 16SrRNA gene relatedness of strain PS2 with several other Citrobacter strains. Geobacter sulfurreducens was used as outgroup. The scale bar indicates the distance of 0.02 (or 2%) sequence identity.

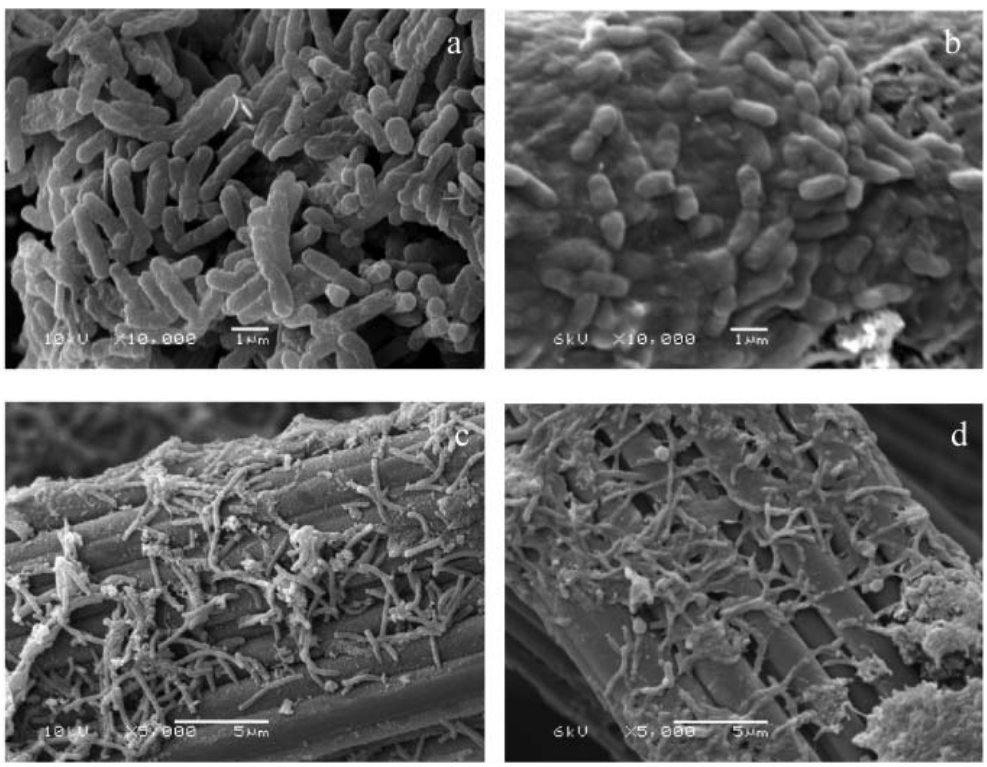


**Figure 4.3** SEM images of strain PS2 grown in batch on glucose (a) and PS2MEC cathode samples after operation as biocathode. (b) PS2MEC2 at a densely populated site, (c) PS2MEC1 and (d) PS2MEC2 at a less dense populated site. Scale bars indicate actual size in the picture.

### 4.4.2 Performance of PS2 in MEC cathode

Two separate MEC cathodes were inoculated with PS2. The background current of both MECs was between 0.1-0.5 A $m^{-2}$. Directly after inoculation no changes in current were observed. Batch operation of the setups to allow growth without washout of PS2 did not result in current increase. Neither did continuous feed of acetate and bicarbonate or citrate (data not shown). Continuous feed of pyruvate (either 1 mM or 5 mM) did result in increasing current up to average of 2.3 A $m^{-2}$ (PS2MEC1) and 2.2 A $m^{-2}$ (PS2MEC2) (Figure 4.4). The $H_2$ yields of 2.8 (PS2MEC1) and 2.2 (PS2MEC2) $m^3$ $H_2$ per $m^3$ reactor liquid volume per day, corresponded with a cathodic $H_2$ recovery (current to $H_2$) of 47% (PS2MEC1) and 46% (PS2MEC2) (Table 4.2). These values are calculated with the assumption that all $H_2$ was derived from electrons from the cathode, which is most likely since no pyruvate or other carbon source was fed into the system before and during the $H_2$ production tests. No other products than $H_2$ were detected in the effluent liquid or gas, just before or after the $H_2$ production test. For comparison, previous experiments (Jeremiasse *et al.*, 2011) showed that an uninoculated control setup produced 0.8 A $m^{-2}$ with $H_2$ yield of 0.32 $m^3$ $H_2$ $m^{-3}$ reactor per day and 14% cathodic $H_2$ recovery. The catalytic activity of the biocathodes was illustrated with a polarization curve that showed similar catalytic activity of PS2MEC1 compared to PS2MEC2, and was much higher than a control setup (control setup data from (Jeremiasse *et al.*, 2011) (Figure 4.5). In addition, the open circuit potential (OCP) of PS2MEC2 was -0.313 V (vs SHE). A control setup inoculated with PS2 but in which no current was produced had an OCP of 0.055 V (vs SHE).

In the effluent of PS2MEC1, traces (4 µM) of pyruvic acid were detected, and no other organic acids. In the effluent of PS2MEC2, formic acid (0.43 mM) and acetic acid (0.67 mM) were detected. SEM imaging of the electrode fibers showed that PS2-shaped cells were present at the electrode after operation and that the two biocathodes both had morphological similar biofilms attached to the electrode fibers (Figure 4.3).

Addition of the ionophores nigericin or monensin up to 50 µM to the active biocathode did not have any effect on current production or on the $H_2$ production. After the tests with nigericin and monensin, the PS2MEC2 was stopped, and the microbes inside were identified using DGGE, SEM and light microscopy.

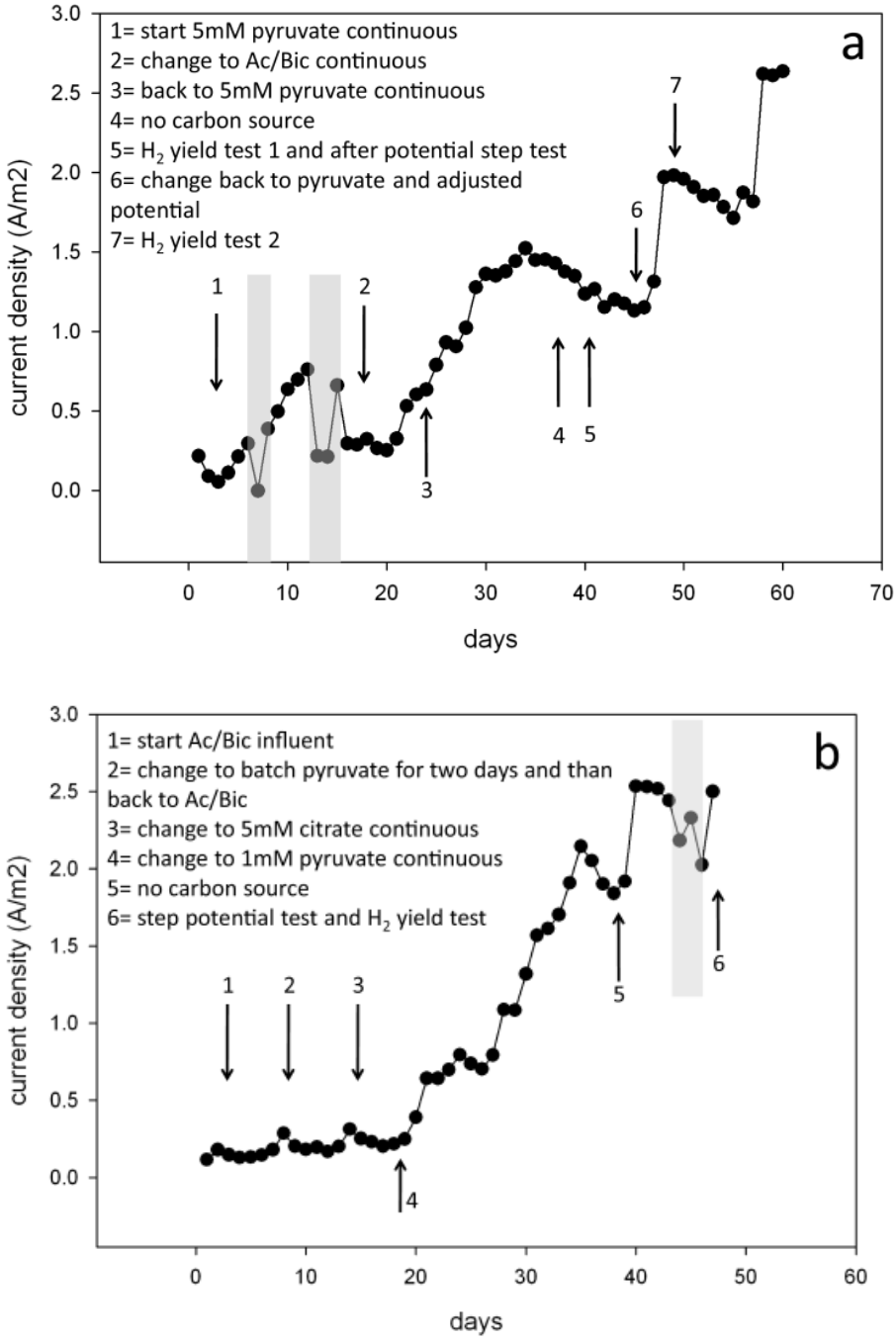


**Figure 4.4** Current development of PS2MEC1 (a) and PS2MEC2 (b). Arrows indicate events as described in the graph, gray areas indicate changes in current resulting from pH deviations from pH 7. Ac/Bic = 1mM acetate and 2mM bicarbonate.

**Table 4.2** Results of the $H_2$ production and current development of the PS2MECs.

| | $H_2$ yield ($m^3/m^3$reactor liquid/day) | Cathodic $H_2$ recovery (%) | Current density ($A/m^2$) |
|---|---|---|---|
| PS2MEC1 | 2.8 | 47 | 2.3 |
| PS2MEC2 | 2.2 | 46 | 2.2 |
| Control [a] | 0.32 | 14 | 0.8 |

$H_2$ yield and recovery are averages from 2 yield tests exept PS2MEC2. Current density is the measured average during the 24-hour yield test. [a]Data from (Jeremiasse *et al.*, 2011)

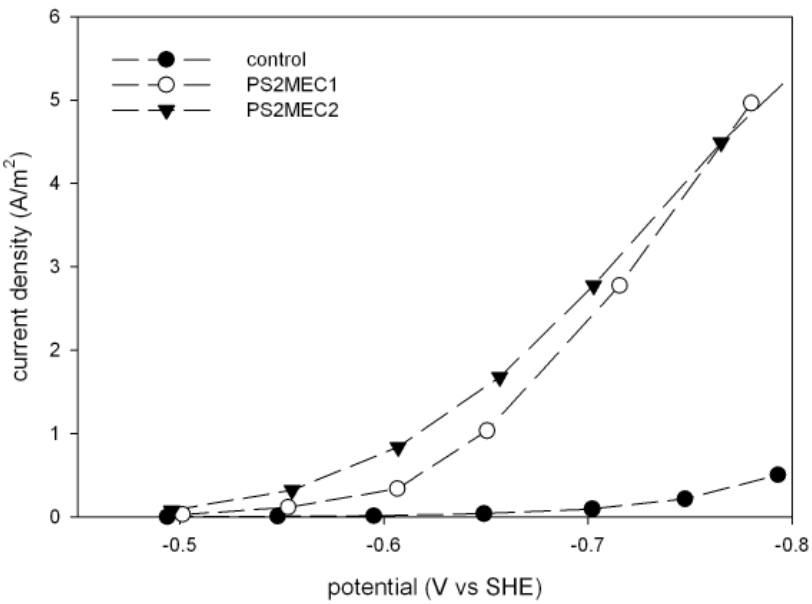


**Figure 4.5** Polarization curve of PS2MEC1 and PS2MEC2 compared to an uninoculated control setup.

### 4.4.3 DGGE

DGGE profiles of the biocathode samples compared to PS2 pure culture (Figure 4.1) showed that PS2 was dominantly present at the cathode of PS2MEC1 and PS2MEC2 and in the catholyte, but also some other bands were observed at the cathode after MEC operation. The DGGE profile of a batch culture of anaerobic phosphate buffered medium supplemented with 20 mM pyruvate, inoculated with electrode biomass suspended in effluent (1 mL), showed a single band at the height of PS2 and none of the other bands that were present at the PS2MEC cathodes (Figure 4.1 lane 7). Comparison of PS2MEC1 and PSMEC2 with mixed

culture biocathodes (described in chapter 3) showed that PS2 was not present in any of the other biocathodes, however, one band (Figure 4.6, band 52) was present in both PS2MECs and in AcL and BicL (see chapter 3 for conditions of those setups).

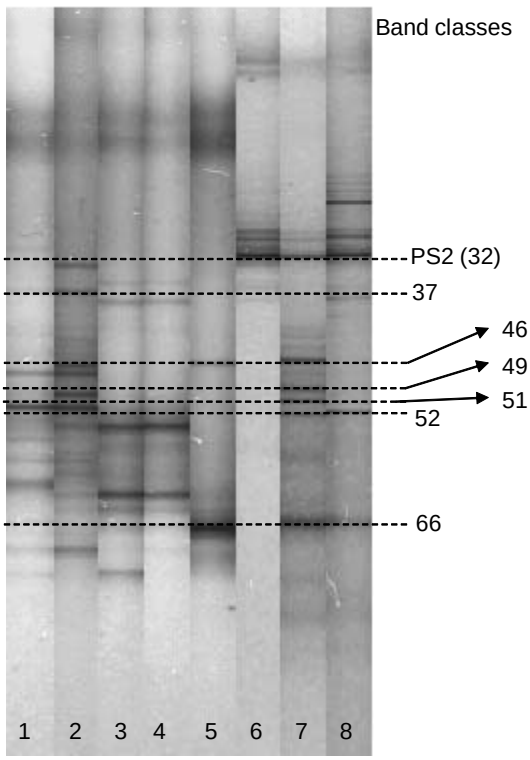


**Figure 4.6** Comparison of the DGGE profiles from PS2MEC1 and PSMEC2 cathodes with mixed culture setups from chapter 3. Lane 1: AcL, 2: BicL, 3: AcS, 4: BicS, 5: AcnSL, 6: PS2, 7: PS2MEC1 and 8: PS2MEC2. Dotted lines indicate band classes with numbers of the band classes. Band class 32 represents PS2. DGGE profiles were aligned in BioNumerics using a standard marker (not shown in figure).

## 4.5 Discussion

### 4.5.1 *Citrobacter* species

In this study *Citrobacter* strain PS2, was isolated from an MEC biocathode. *Citrobacter* is a well studied genus with many different species. Currently, 11 *Citrobacter* genomes are sequenced and 288 *Citrobacter* strains are known in the

NCBI database. *Citrobacter* belongs to the gamma-proteobacteria and the order of *Enterobacteriales*. Many *Citrobacter* species are capable of $H_2$ production from a large diversity of carbon sources including citrate (Borenshtein & Schauer, 2006). The isolated strain PS2 was similar to many other *Citrobacter* species and specifically to the most closely related *Citrobacter amalonaticus* strains (Table 4.1), such as fermentation and $H_2$ production from glucose, pyruvate and citrate. Strain PS2 and most other *Citrobacter* species are also able to reduce nitrate to nitrite and to grow aerobically.

### 4.5.2 *Citrobacter* in MEC biocathode

Inoculation of PS2 in a biocathode resulted in current generation and $H_2$ formation. Current generation in an MFC anode has been shown for other *Citrobacter* spp. (Gunasekaran *et al.*, 2011; Xu & Liu, 2011), when growing on various substrates. *Citrobacter* has to our knowledge so far not been tested in biocathode systems. PS2 was not dominant (not detected) in the clone library of the cathode from which PS2 was derived (sample AcL, chapter 3). Nevertheless, the electrochemical activity of other *Citrobacter* sp. in MFC systems and the presence (although not dominant) in the sample of a biocathode, makes it a possible candidate for the catalysis of $H_2$ production in an MEC biocathode. Dominant species that are detected in a mixed community using molecular techniques are not necessarily the most active species when several species from this community were tested in pure culture (Kiely *et al.*, 2010). *Citrobacter* is an interesting candidate for MEC biocathodes because it can ferment a large diversity of carbon sources with $H_2$ as one of the products. Fermentative $H_2$ production in combination with the ability to use a cathode as energy source is of specific interest for MEC biocathodes because it would theoretically allow complete conversion of the substrate with a maximum $H_2$ yield. In a first step $H_2$ would be released by fermentation and the fermentation products (e.g. acetate) could be converted to $H_2$ by the addition of electrical energy through the cathode. This would only be possible if the metabolic pathways are available to also convert the fermentation products. PS2 was able to metabolize acetate, but only under aerobic conditions. Metabolizing acetate could thus be possible with additional energy from the cathode. In PS2MEC1 no acetate was detected in the effluent and in PS2MEC2 only little amounts (0.67 mM) of acetate were detected. In this study two independent MEC setups were started which were inoculated with strain PS2 in the cathode compartment. Batch growth of PS2 in the cathode did not catalyze current and $H_2$ production, however for both setups continuous feed of pyruvate resulted in an increase of current. The current

and $H_2$ production rate (Table 4.2) were similar to the mixed community setups described in chapter 3 which ranged from 2.2 to 2.7 A $m^{-2}$ with a $H_2$ yield ranging between 2.2 and 2.7 $m^3$ $H_2$ $m^{-3}$ reactor liquid per day. Those setups were fed with acetate or bicarbonate instead of pyruvate, however, a few days before and during the $H_2$ production test, the carbon source feed was stopped (for all experiments) allowing only electrons to flow from the cathode to $H_2$, and not from the carbon source. The carbon source might however have had a large influence during the startup phase. In comparison, although operated with different carbon sources and in different setups, previous experiments with pure cultures of *Desulfovibrio* G11 (Croese *et al.*, 2011) or *Geobacter sulfurreducens* PCA (Geelhoed & Stams, 2010) resulted in current densities of 1.1 A $m^{-2}$ and 1.5 A $m^{-2}$, respectively, at a cathode potential of -0.7 V (vs SHE), which is much less than we observed in the present setups. The catalytic activity of the biocathode was confirmed with a polarization curve and by the OCP which was very different for PS2MEC (-0.315V) compared to a control in which no biocathode activity was detected (0.055V).

After opening the setup it appeared from DGGE (supported by SEM) that PS2 was dominant, but there were other ribotypes present on the electrode as well. Therefore, we have no full proof that PS2 alone can actively produce $H_2$ from electrons derived from the cathode. Additional experiments with a sterile setup are required to obtain conclusive evidence. However, previously MEC biocathodes were started with inocula from active MEC anode or cathode or MFC anode (Geelhoed & Stams, 2010; Jeremiasse *et al.*, 2009; Jeremiasse *et al.*, 2012). Only one other study (Croese *et al*. 2011) showed current and $H_2$ production after direct inoculation of *Desulfovibrio vulgaris* strain G11 in the cathode, however, this setup did not reach as high current densities as the mixed community biocathodes. In this experiment we showed that it is possible to start an MEC biocathode with PS2 directly inoculated in the cathode, also after disinfection with ethanol, preventing the presence of any species from earlier active biocathodes. Those biocathodes reached current densities comparable to biocathodes inoculated with anode enriched biomass. PS2 has a function in, at least, the startup phase of an MEC biocathode and most likely also in the $H_2$ production in the operational phase. The used procedure helps to get an active biocathode with high current and $H_2$ production. Except for the DGGE band indicative of strain PS2, also a few other bands were observed in the DGGE profiles of the PS2MEC cathodes. One of those bands (Figure 4.6, band 52) also appeared in the DGGE profiles of two of the previously operated mixed culture biocathodes. Possibly this species was also involved in current and $H_2$ production in the MEC biocathode. Although there may have been effects of contaminant species in the PS2MEC operated here,

inoculation of PS2 in combination with continuous pyruvate addition allowed development of an active biocathode for $H_2$ production.

### 4.5.3 Pyruvate essential for startup

The successful startup of the biocathode in these experiments might be explained by the addition of pyruvate to the system. The addition of strain PS2 only, was not sufficient to start up the biocathode, however, when continuous pyruvate was fed to the system, current and $H_2$ production increased and thus in our experiments pyruvate seemed essential for startup. When a sample of the active biocathode was incubated with pyruvate, DGGE analysis indicated that only PS2 was able to grow with pyruvate, while none of the other bacteria represented by the DGGE bands developed (Figure 4.1). This supports that PS2 is the only species growing on pyruvate. Pyruvate is a central metabolite for microbial growth which might allow activation of specific pathways or other metabolites that are necessary for performance of the biocathode. However, it could also mean that contaminants could only grow on pyruvate together with the cathode or on products of the breakdown of pyruvate such as acetate or formate together with the cathode. Although, growth on acetate is not very likely, because an increase in current was not observed when acetate was fed into the PS2MECs. Consumption of acetate with $H_2$ (only possible after PS2 produced $H_2$) would most likely produce methane, which was not detected. It is not well understood why the MEC biocathode only started to produce current after continuous feed of pyruvate. If pyruvate would serve as carbon source only, it should also be possible to start a biocathode with citrate feed because PS2 is also able to use citrate. Maybe specific intermediate products that are produced while growing on pyruvate are essential for biocathode performance of PS2. Exploring the variety of carbon sources that can be used for biocathode start-up would therefore be interesting future research.

### 4.5.4 Disruption of membrane potential

To investigate the involvement of a microbial cell proton gradient in $H_2$ production at the MEC biocathode we added nigericin and monensin to the active cathode. Both nigericin and monencin are ionophors that disrupt the proton, $K^+$ (nigericin) and $Na^+$ (monensin) gradient across the cytoplasmic membrane and in that way destroy proton motive force driven processes. The addition of nigericin or monensin to the MEC biocathode did not have any effect on the current. It has been suggested before that energy converting hydrogenases (Ech) might be involved in

$H_2$ production at a cathode (Geelhoed *et al.*, 2010). $H_2$ production by Ech is coupled to a membrane proton gradient (proton motive force) (Hedderich & Forzi, 2005; Vignais & Billoud, 2007). This suggests that active $H_2$ production that is driven by Ech is not the mechanism of $H_2$ production in the MEC biocathode. However, since we only applied this experiment with a stable, fully grown biocathode, this does not exclude that a proton gradient over the membrane is essential for growth at the biocathode.

### 4.5.5 Conclusion

A *Citrobacter* strain was isolated from an active biocathode. Current and $H_2$ production were observed when this strain was inoculated in a MEC biocathode compartment and a continuous pyruvate feeding was applied. In addition, disruption of the membrane potential has no influence on the current or $H_2$ production of the MEC. However, to draw clear conclusions on the role of PS2 in the MEC biocathode and the mechanism of MEC biocathode activity more research is essential. A control setup fed with only pyruvate without *Citrobacter* PS2, a setup with other *Citrobacter* species and a fully sterile setup would help to elucidate the role of PS2 in the MEC. In addition, studying PS2 using e.g. cyclic votametry would improve the understanding of the electrochemical properties of PS2.

## 4.6 Acknowledgements

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# Chapter 5

## Population dynamics in relation to the performance of microbial electrolysis cells: a statistical approach

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### 5.1 Abstract

The Microbial Electrolysis Cell (MEC) is a promising system for $H_2$ production, but little is known about the active microbial population in MEC systems. We analyzed the microbial community of 5 different MEC graphite felt anodes using denaturing gradient gel electrophoresis (DGGE). The results showed that the bacterial population was very diverse and there were substantial differences between anolyte and anode samples. The archaeal population in the anolyte and at the anodes was very similar. Also between the different MEC's the detected archaeal populations were similar. SEM and FISH imaging showed that Archaea were mainly present in the spaces between the electrode fibers and Bacteria were present at the fiber surface, which suggests that Bacteria are the main microorganisms involved in the electrochemical activity in the MEC. We used Redundancy analysis (RDA) and QR factorization based estimation (QRE) to link the composition of the bacterial community to electrochemical performance of the MEC. The effects of current and resistance on the populations are significant. Our results showed that the community composition is strongest correlated with current. Especially the

combination of RDA and QRE seems promising to get insight in the part of the microbial population that is actively involved in electrode interaction in the MEC.

## 5.2 Introduction

The microbial electrolysis cell (MEC) has shown potential for sustainable $H_2$ production (Liu *et al.*, 2005; Rozendal *et al.*, 2006). Insight in the microbial populations in those systems is essential to understand and optimize MECs. Microbial communities of electrochemical systems have mainly been studied in microbial fuel cells (MFCs) (Logan, 2009; Sharma & Kundu, 2010) in which electrical power is produced as the main product instead of $H_2$. So far, only a few studies describe the microbial community in MEC anode systems. Previous comparative studies showed that MEC anode bacterial populations are different from MFC anode populations (Chae *et al.*, 2008; Kiely *et al.*, 2011).

An important challenge in MEC research is the identification of those species, in mixed communities, that play a dominant role in the electrochemical activity and performance of MECs. Here we define performance as the quality of functioning. This can be e.g. $H_2$ production in the MEC, current generation in an MFC or delivery of electrons to an anode by bacteria. Several species have been identified to be able to exchange electrons with an electrode or identified as the dominant species present in mixed communities (Sharma & Kundu, 2010). Experiments with pure cultures are important to understand the molecular mechanisms that microorganisms employ for electron transfer. However, the large diversity of bacteria that are electrochemically active makes it difficult to specifically determine the species responsible for high performance in mixed communities (Logan, 2009; Nevin *et al.*, 2011; Phung *et al.*, 2004; Sharma & Kundu, 2010). So far, by molecular analysis of mixed bioelectrode communities no specific dominant species has been identified. It has been suggested that *Geobacteraceae* play an important role in electrochemical performance, but also other dominant species are detected and therefore *Geobacteraceae* may not be essential for good performance of the MEC (Chae *et al.*, 2008; Kiely *et al.*, 2011; Torres *et al.*, 2009). In general, the microbial populations that interact with the electrode seem to be able to adapt to a large variety of conditions. For comparison of microbial populations and the relation to MEC anode performance, we need to understand which parameters mostly influence the microbial population and overall performance of the MEC anode. Two important parameters that determine performance of the MEC anodes are i) the energy efficiency of substrate conversion and ii) the rate at which

this substrate is being oxidized. The energy efficiency is influenced by the anode potential which is the potential of the electron acceptor for the microorganisms. To gain as much energy for the microorganisms as possible, the redox potential of the terminal electron mediator inside the microbial cell or of the redox proteins at the surface of the microorganisms, should be as close to the anode potential as possible. The catalytic properties of the redox proteins influence the energy losses, which is referred to as overpotential. The overpotential is the difference between the thermodynamic potential of any electron donating reaction with the accepting reaction, and the actual measured potential. The second important parameter is the rate at which the organisms oxidize the substrate. When the anode is used as the sole electron acceptor, as we assume for the electrochemically active microorganisms which are responsible for the performance of the MEC anode, the substrate oxidation rate is directly related to the current density. The relationship between overpotential and current density is expressed as the anode resistance and is, at a wide range of applied voltages, calculated by Ohms law (V=I*R) (Logan *et al.*, 2008). For an optimal performance of the MEC system the overpotential, anode potential and resistance should be low and the current density high. As described above, the anode potential, overpotential, current and resistance are all correlated and influence each other. However, here we evaluate them separately as measures of anode performance because in that way it can tell us which parameter (e.g. energy efficiency or conversion rate) is most important for the overall performance of the microorganisms in the MEC anode.

For analysis of the total microbial community several methods are available (Rittmann *et al.*, 2008). We analyzed bacterial and archaeal communities of MEC anodes using denaturing gradient gel electrophoresis (DGGE) (Muyzer, 1999). DGGE gives a visual overview of the diversity within a sample and the dynamics of a population in time or when conditions change. It is assumed that dominant bands in 16S rDNA-based DGGE profiles represent dominant species in the community. Optical interpretation is the most straightforward method for interpreting DGGE profiles, but it is difficult to determine the dominant bands by eye when the diversity is high due to the high number of bands in the DGGE. In addition, the abundance of a band does not necessarily indicate an involvement of specific species with the performance of the bio-electrochemical system.

To understand the relationship between community composition and environmental parameters (e.g. performance of bioreactors) several methods have been used, such as estimates of species richness, dynamics and functionality of communities (Marzorati *et al.*, 2008; Read *et al.*, 2011). Moreover, statistical methods have been used to specifically link DGGE patterns to environmental data

such as applied conditions, environment or sampling time (Chen *et al.*, 2010; Joossens *et al.*, 2010; Liu *et al.*, 2011; Nakatani *et al.*, 2011). For analysis of MEC systems such methods have, to our knowledge, not been used before.

The objective of this study was to relate MEC anode performance to the composition of the anodic microbial community using the statistical methods redundancy analysis (RDA) and a QR factorization with column pivoting (QRE). RDA (Rao, 1964) is a multivariate linear ordination analysis widely used in microbial ecology to determine correlations between environmental conditions and microbial community structure (Ramette, 2007). Therefore, RDA was used to link the DGGE profiles to the experimental parameters: anode potential, overpotential, resistance and current. In addition to RDA, a numerically justified method was used. This method was based on the factorization of the data matrix in matrix Q and matrix R or so called QR factorization based estimation (Golub & Van Loan, 1996), with column pivoting, which is a ranking of the diagonal elements of the upper triangular matrix R. With these statistical approaches we identified the most important bands for electrochemical performance of MECs. We evaluate the difference in both approaches and evaluate the usefulness of these tools for MEC studies.

## 5.3 Materials and Methods

### 5.3.1 MEC operational conditions

All samples were collected from MEC anodes run in the period of end 2007 till end 2009. The inoculum originated from a full scale UASB reactor treating sulfate-rich paper mill wastewater (Industriewater Eerbeek, Eerbeek, The Netherlands), anodic effluent from a molasses-fed MFC and *Geobacter sulfurreducens* strain PCA (U. Michaelidou, personal communication) and was consequently enriched in several running MECs over a period of about four years previous to our experiments. Each time, 100 mL of effluent from a running MEC was transferred to the new system. The general operational conditions were as described previously (Sleutels *et al.*, 2009a; Sleutels *et al.*, 2009b; Sleutels *et al.*, 2010). The differences between the different runs are summarized in table 5.1, while the common operational conditions were as follows: The anode compartment contained a 6.5 mm thick graphite felt (National Electrical Carbon BV, Hoorn, The Netherlands). The cathode compartment contained a platinum coated (50 g $m^{-2}$) titanium mesh (thickness 1mm, specific surface area 1.7 $m^2$ $m^{-2}$ - Magneto Special Anodes BV,

Schiedam, The Netherlands). Anode and cathode compartment were separated by an ion exchange membrane. Both anode and cathode had a working volume of 280 mL and the anolyte and catholyte were circulated over the compartment at a flow rate of 340 mL $min^{-1}$. Anode, cathode and membrane all had a projected surface area of 250 $cm^2$. Potentials of anode and cathode were measured using Ag/AgCl reference electrodes (+0.200 V vs SHE: ProSense QiS, Oosterhout, The Netherlands) which were connected to the cell using capillaries filled with 3M KCl solution separated from the electrolyte by an agar salt bridge. The applied cell voltage (ES 03-5, Delta Electronica BV, Zierikzee, The Netherlands) was 1.0V for all experiments and the temperature was kept at 303 K. The anodes of all MECs were continuously fed with synthetic wastewater at a rate of 5 mL $min^{-1}$. The synthetic waste water contained (mM) 10 $NaCH_3COO.3H_2O$, 5 $KH_2PO_4$ , 5 $K_2HPO_4$, 10 KCl, 10 NaCl, 5.3 $NH_4Cl$, 0.4 $MgSO_4.7H_2O$, 0.7 $CaCl_2.2H_2O$ and 0.1 mL $L^{-1}$ of a trace element mixture (Zehnder *et al.*, 1980).

**Table 5.1** Differences in operational conditions for the analyzed MEC anodes

| Experiment | Anolyte flow[1] | Membrane | Reference |
|---|---|---|---|
| A | parallel | AEM<br>Fumasep FAA, FuMa-Tech GmbH, Germany | Sleutels *et al*. 2009a |
| B | perpendicular | CEM<br>Fumasep FKE, FuMa-Tech GmbH, Germany<br>AEM<br>AMX, Neosepta, Tokuyama Corp., Japan | Sleutels *et al*. 2010 |
| C | parallel | CEM<br>Fumasep FKE, FuMa-Tech GmbH, Germany | Sleutels *et al*. 2009a |
| D | parallel | AEM<br>AMX, Neosepta, Tokuyama Corp., Japan | Sleutels *et al*. 2009b |
| E | perpendicular | AEM<br>AMX, Neosepta, Tokuyama Corp., Japan | Sleutels *et al*. 2009b |

[1]the anolyte was either forced to flow parallel to the felt or perpendicular through the felt

Current and anode potential were constantly monitored and the average pH, current and potential, after current stabilized, were used for further analysis. Anode resistance per $m^2$ electrode surface and overpotential were calculated as described before (Sleutels *et al.*, 2009a) (see Table 5.2). Samples from the anode felt or surface of the felt and the anolyte were collected at the end of each experimental run except for samples 51 and 52 which were collected using a sterile

swab to collect biomass from the side of the anode felt after which the MEC setup was reassembled. When multiple samples were collected from the anodes, they were taken at 3 different locations. Location (1) is the anode close to the anolyte entrance, location (2) is at the middle of the flow path of the anolyte and location (3) is at the anode at the exit of the anolyte from the anode compartment. All samples were stored at -20°C until further analysis or directly treated further for SEM imaging as described in chapter 3.

**Table 5.2** Overview of performance of 5 MEC's

| Key | Experiment (location sample) | Current (A $m^{-2}$) | Anode potential (V) | pH anolyte | Standard potential (V) | Over-potential (V) | Anode resistance (m$\Omega$ $m^{-2}$) |
|---|---|---|---|---|---|---|---|
| 1 | B (1) | 3.6 | -0.42 | 7.0 | -0.51 | 0.09 | 24 |
| 15 | B (2) | 3.6 | -0.42 | 7.0 | -0.51 | 0.09 | 24 |
| 31 | B | 3.6 | -0.42 | 7.0 | -0.51 | 0.09 | 24 |
| 32 | B | 3.6 | -0.42 | 7.0 | -0.51 | 0.09 | 24 |
| 33 | D | 5.1 | -0.11 | 6.7 | -0.49 | 0.38 | 74 |
| 35 | E2 (2) | 13.6 | -0.33 | 6.5 | -0.47 | 0.15 | 11 |
| 37 | E2 (3) | 13.6 | -0.33 | 6.5 | -0.47 | 0.15 | 11 |
| 38 | E2 (2b) | 13.6 | -0.33 | 6.5 | -0.47 | 0.15 | 11 |
| 39 | E2 | 13.6 | -0.33 | 6.5 | -0.47 | 0.15 | 11 |
| 41 | E2 | 13.6 | -0.33 | 6.5 | -0.47 | 0.15 | 11 |
| 46 | E2 | 13.6 | -0.33 | 6.5 | -0.47 | 0.15 | 11 |
| 51 | E1 (1) | 4.4 | -0.01 | 6.5 | -0.47 | 0.46 | 104 |
| 52 | E1 | 4.4 | -0.01 | 6.5 | -0.47 | 0.46 | 104 |
| 55 | C | 2.3 | -0.13 | 6.1 | -0.44 | 0.32 | 139 |
| 65 | C (1) | 2.3 | -0.13 | 6.1 | -0.44 | 0.32 | 139 |
| 66 | C (2) | 2.3 | -0.13 | 6.1 | -0.44 | 0.32 | 139 |
| 67 | C (3) | 2.3 | -0.13 | 6.1 | -0.44 | 0.32 | 139 |
| 7 | A (1) | 5.3 | -0.43 | 6.7 | -0.49 | 0.06 | 11 |
| 71 | A (2) | 5.3 | -0.43 | 6.7 | -0.49 | 0.06 | 11 |
| 8 | A (3) | 5.3 | -0.43 | 6.7 | -0.49 | 0.06 | 11 |

### 5.3.2 Fluorescent in situ hybridization (FISH)

Electrode samples were fixed in 3% formaldehyde in 10 mM PBS (pH 7.4) for 1h at room temperature. After fixation the samples were washed twice with PBS and stored in 50% (v/v) ethanol in PBS at -20°C. Before hybridization, the samples were dehydrated using graded series of ethanol (50%, 80% and twice in 100% during 15 minutes for each step) and dried in an oven at 46°C. Subsequently, the samples were immersed in hybridization buffer (900 mM NaCl, 20 mM Tris/HCl pH8, 35% (v/v) formamide, and 0.01% (w/v) SDS in sterile MQ water). The hybridization buffer contained fluorescein labeled eubacterial probe mix 1:1:1 of EUB338I, -II and –III (Daims *et al.*, 1999) 0.5 ng $\mu L^{-1}$ final concentration and Cy3 labeled archaeal Arch915 probe (Stahl & Amann, 1991) 0.3 ng $\mu L^{-1}$ final concentration. All probes were synthesized by Eurofins MWG operon (Ebersberg, Germany). After 1.5 h of hybridization at 46°C the samples were washed in 48°C preheated washing buffer (20mM Tris/HCl pH8, 875 mM NaCl, 5 mM EDTA, 0.01% SDS in sterile MQ water) with 0.002% 4',6-diamidino-2-phenylindole (DAPI) at 48°C for 15 minutes and rinsed with 4°C MQ water. The samples were dried in a ventilated oven. Before imaging, the samples were immersed in a drop of Vectashield (Vector Labs, Burlingame, USA) on top of a cover slide and viewed using a Leica DMI6000B fluorescent microscope with a Leica DFC350FX digital camera (Leica Microsystems, Wetzlar, Germany) using the Leica Application Suite AF software package. The images were colored digitally using green for the fluoresceine detection, red for Cy3 detection and blue for DAPI detection. Overlapping Cy3 and DAPI was colored pink.

### 5.3.3 DNA extraction and amplification

Genomic DNA was extracted from the graphite felt (1 $cm^2$) and the anolyte liquid samples (4 mL) using the Fast DNA spin kit for soil (Bio101, Vista, CA, USA). Partial 16S rRNA genes were amplified with the primers Bact968F (including GC clamp) and 1401R (Nübel *et al.*, 1996) for bacteria, and Arch109(T)F and GC515R for archaea (Grosskopf *et al.*, 1998; Lane, 1991). PCR settings were initial denaturation for 2 min at 95°C, followed by 35 cycles of 30 s denaturation at 95°C, 40 s annealing at 56°C and 1 min elongation at 72°C. Post-elongation was 5 min at 72°C. The PCR samples were checked on a 1% agarose gel for quantity and size of the product.

### 5.3.4 Denaturing gradient gel electrophoresis (DGGE)

The bacterial and archaeal communities populating the electrode and effluent of 5 MEC setups were analyzed with DGGE. Amplicons were run on an 8% polyacrylamide gel containing a formamide and urea denaturant gradient of 30-60%, as described before (Martín *et al.*, 2007). Gels were run for 16 hours at 60°C and stained with silver nitrate (Sanguinetti *et al.*, 1994).

### 5.3.5 RDA analysis of DGGE profiles

Pictures of the DGGE profiles were digitally processed in BioNumerics version 4 (Applied Maths NV, Sint-Martens-Latem, Belgium) according to the manufacturer's instructions and checked manually. For analysis of the bands, the band classes of all electrode samples (20 samples from 5 MECs) were determined using the band matching tool and manually adjusted if necessary. The band classes and experimental data (current, anode potential, overpotential and resistance) belonging to the samples were imported in Canoco for Windows (version 4.52, Biometris-Plant Research International, Wageningen, The Netherlands). Direct linear gradient analysis (RDA) was performed in Canoco using interspecies correlation (Van Katwijk & Ter Braak, 2003). The band class scores were post-transformed by dividing them by the standard deviation. This analysis gave the eigenvalues for the different axes of which the first axis was constrained (or canonical axis) by the MEC experimental parameter. The analysis included a Monte Carlo permutation test with the null hypothesis that the DGGE band classes are randomly related to the MEC experimental parameters. This test was performed to test the significance of the ordination of the first axis. Bi-plots, or linear constrained ordination plots, of the DGGE band classes and MEC experimental parameters were created in CanoDraw (implemented in the Canoco program). RDA bi-plots describe the covariance of all the band classes with the MEC experimental parameter. The selection of the bands classes shown in the RDA bi-plots, was based on the percentage of covariance that could be explained by the first axis, setting the 'lower axis minimum fit' at the value of this percentage. From this selection the most important bands were selected based on the length and direction of the band class arrow in relation to the experimental (horizontal) axis.

### 5.3.6 QR factorization methods

To determine which DGGE bands correlated most to the specific experimental parameters, we also used QR factorization based estimation with column pivoting

(QRE). QR factorization is a commonly used method to solve linear least square problems (Golub & Van Loan, 1996). However, we included a method to overcome the problem of higher numbers of explanatory variables (band classes) compared to response variables (electrochemical measurements)

The relationship between band classes ($a_{ij}$) with $i = 1, ..., m$ and $j = 1, ..., n$ and response variable ($b_i$) can be given by the linear regression

$$b_i = \mathbf{a}_i^T \mathbf{x} \tag{1}$$

with $\mathrm{a}_i \in \Re^n$, the vector with intensities of the band classes, and $\mathrm{x} \in \Re^n$, the vector with regression coefficients. In matrix form

$$\mathbf{A}\mathbf{x} = \mathbf{b} \tag{2}$$

with $\mathrm{A} = \begin{bmatrix} \mathrm{a}_1^{\,r} \\ . \\ . \\ . \\ \mathrm{a}_m^{\,r} \end{bmatrix} \in \Re^{m \times n}$ $\mathrm{A} = \begin{bmatrix} \mathrm{a}_1^{\,r} \\ . \\ . \\ . \\ \mathrm{a}_m^{\,r} \end{bmatrix} \in \Re^{m \times n}$ , a rectangular matrix, and $\mathrm{b} \in \Re^m$ an

*m*-dimensional vector.

In our application $m < n$ and thus there are more unknowns ($n$) than equations ($m$). Consequently, if a solution exists, multiple solutions exist. QR factorization of **A** with column pivoting helps to find an appropriate solution. Standard QR factorization is defined as

$$\mathbf{A} = \mathbf{Q}\mathbf{R} \tag{3}$$

with $\mathrm{Q} \in \Re^{m \times m}$, an orthonormal matrix such that $\mathbf{Q}^T\mathbf{Q} = \mathbf{I}$ and thus $\mathbf{Q}^{-1} = \mathbf{Q}^T$ and $\mathbf{R} = \begin{bmatrix} \mathbf{R}_1 & \mathbf{R}_2 \end{bmatrix}$, an upper triangular matrix with $\mathrm{R}_1 \in \Re^{m \times m}$, $\mathrm{R}_2 \in \Re^{m \times (n-m)}$. If, however, the columns of **A** are interchanged a new QR factorization results. QR factorization with column pivoting, that is

$$\mathbf{A}\mathbf{E} = \mathbf{Q}\mathbf{R} \tag{4}$$

with $\mathrm{E} \in \Re^{n \times n}$ the column permutation matrix, allows a ranking of R such that $\left|\mathbf{R}_{11}\right| \geq \left|\mathbf{R}_{22}\right| \geq \ldots \geq \left|\mathbf{R}_{mm}\right|$, where $\mathbf{R}_{ii}$ is the $i^{\text{th}}$ diagonal element of the upper triangular matrix **R**.

As only *m* unknowns can be estimated from *m* measurements in **b**, a reasonable choice is to exactly estimate the coefficients from

$$\mathbf{x} = \mathbf{E}\begin{bmatrix} \mathbf{R}_1 & \mathbf{R}_2 \\ \mathbf{0} & \mathbf{I}_{n-m} \end{bmatrix}^{-1}\begin{bmatrix} \mathbf{Q}^T\mathbf{b} \\ \mathbf{0} \end{bmatrix} \tag{5}$$

which has at most *m* non-zero elements (see Appendix A for the proof). This estimate was calculated with Matlab's (MathWorks, Natick, Massachusetts, USA) backslash operation $\mathbf{x} = \mathbf{A} \setminus \mathbf{b}$.

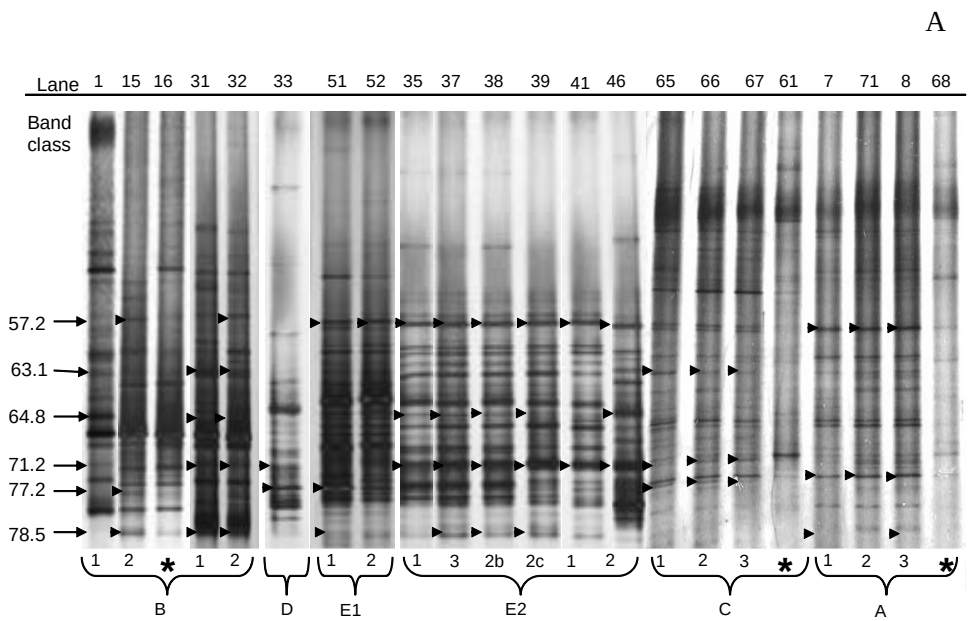
In our experiments we analyzed 20 electrode samples with related MEC experimental variables ($b_i$) and 54 DGGE band classes ($a_{ij}$). Therefore we made a pre-selection of a maximum of 20 (*m*) band classes based on QR factorization with column pivoting. In fact, the problem is to select the 'best' solution out of $\binom{54}{20}$ possibilities. The algorithm uses the ranking of R to provide a solution to find the band classes with the closest to the highest determinants. However, it also appears that this selection allows the determination of intense bands. The determinant of the selected data matrix represents the information content of the selected samples. After the pre-selection using the determinant, the coefficients x, that we will further refer to as 'weighing factors', can be determined for each band class with the specific experimental parameter. In this way, the weight of each band class on all specific experimental parameters (current, anode potential, overpotential or resistance) could be determined. Using all the selected band classes, the corresponding weighing factors determine the sensitivities (S) of the environmental data with respect to the band classes, that is $S_{ij} = \delta\, b_i/\delta\, a_{ij} = x_j$ (with $i$=1, ...20 samples and $j$=1, ...20 band classes). In other words, the selection method showed which of the specific values of the experimental data can be explained best by the presence and intensity of the bands.

The relative contribution of each experimental parameter was determined by setting the sum of all weighing factors (per experimental parameter) to 100%. Following, the relative value of each weighing factor for each band class was determined.

## 5.4 Results

### 5.4.1 DGGE profiling

A total of 20 anode samples and 5 anolyte samples from 5 different MEC setups were collected and analyzed using DGGE (Figure 5.1). Within MECs the location of sampling at the anode did not show a large variation in bacterial or archaeal community. However, anolyte samples were different in bacterial composition compared to the anodes (except for MEC B), whereas the archaeal population was similar in the anolyte and electrode samples. From 2 MECs (D and E) the anolyte samples did not give positive results for the PCR, most likely because there was not enough biomass present. Between MECs, the DGGE profiles showed a large bacterial diversity and a high similarity in Archaea. The Archaea consisted of 4 dominant bands that were present in all samples and up to 3 additional dominant bands that varied among MECs.



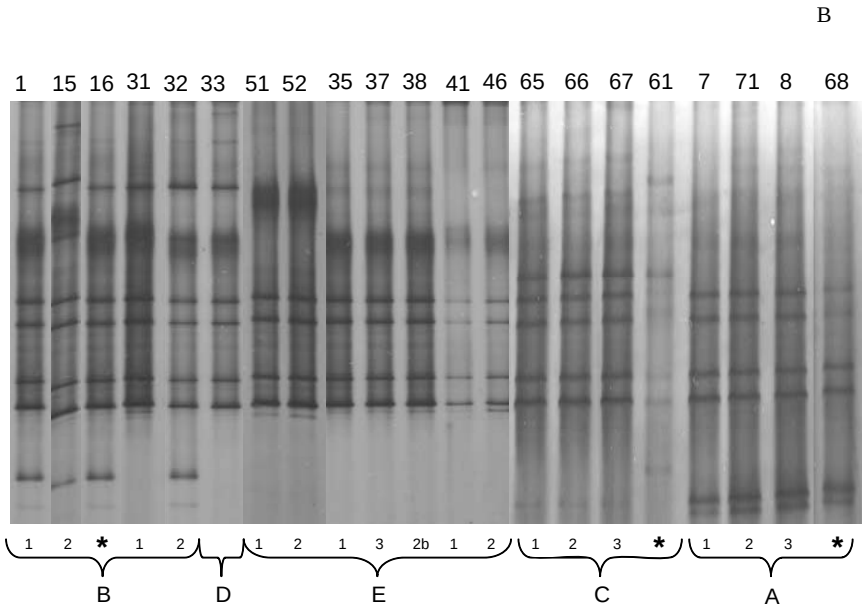



**Figure 5.1** DGGE profiles of bacteria (a) and archaea (b) in sample A, B, C , D and E (for explanation of MECs see table 5.1 and text) at different locations at the anode and effluent liquid (indicated with *). 1= influent site, 2= middle of anode, 3=effluent site. Number of the samples is indicated on top of the lanes. (a) Numbers at the left side refer to the most important band classes. Arrows indicate position of the band classes, * indicate location of bands belonging to each band class.

### 5.4.2 Imaging

SEM imaging showed that a high density of microorganisms with different morphologies were present on the anode felt. On the graphite fibers more rod-shaped microorganisms were observed (Figure 5.2a), where in the spaces between the fibers clusters of typical *Methanosarcina*-shaped cells (Figure 5.2b) were abundantly present in all the anode samples.

FISH imaging confirmed that rod-shaped Eubacteria were present at the electrode surface and typical *Methanosarcina*-shaped Archaea were present in the spaces between the electrode fibers. Archaea were not present on the fibers but more in the bulk between fibers (Figure 5.3).

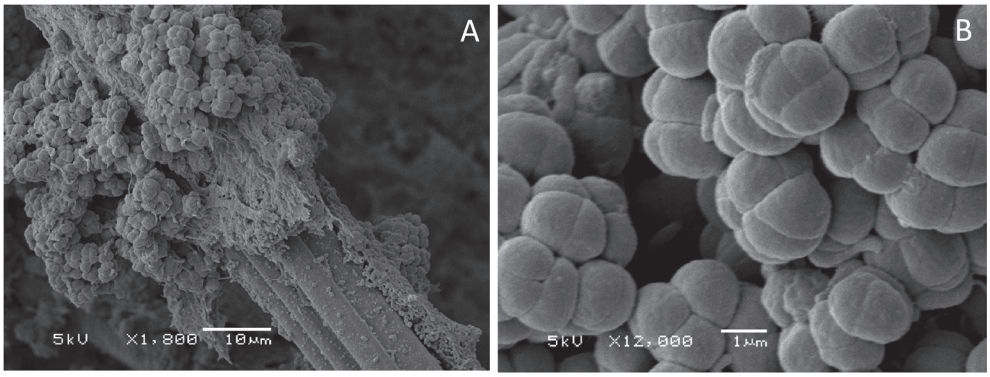



**Figure 5.2** SEM image of an MEC bioanode A) 1800x magnification of typical *Methanosarcina* cells located between fibers and other non-identified bacterial cells located at the fiber surface. B) 12000 x magnification of typical *Methanosarcina* cells. Bars indicate actual scale of the objects in the pictures.

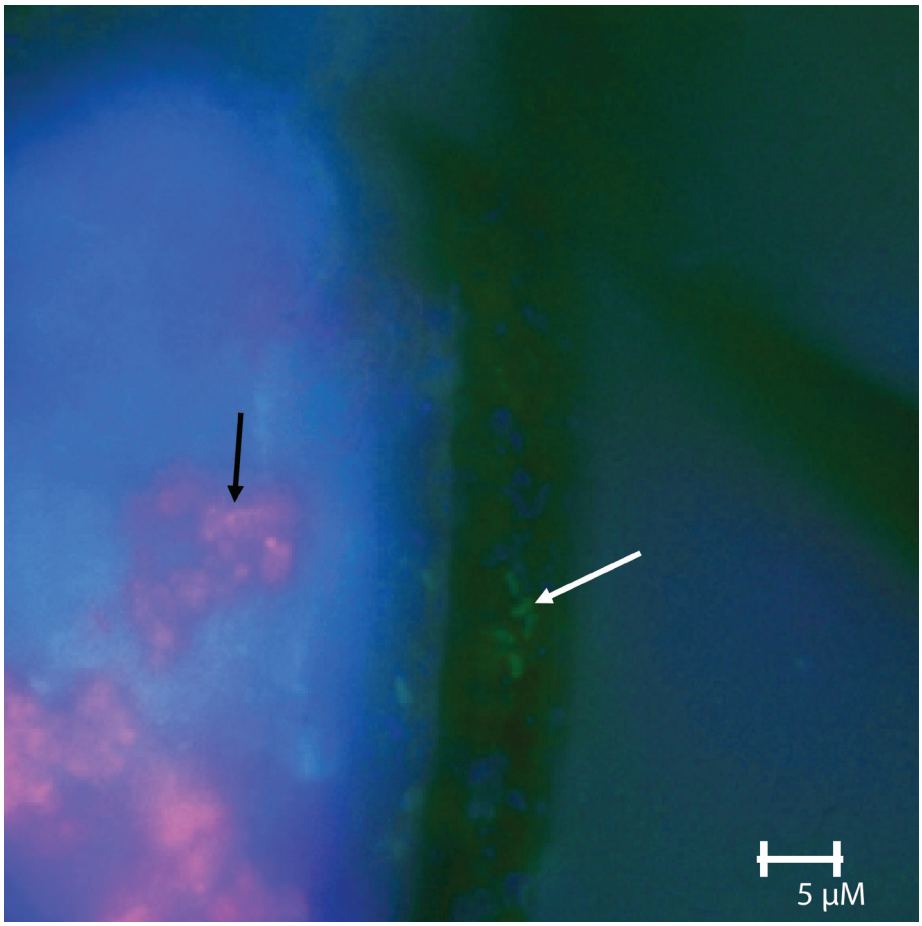



**Figure 5.3** FISH image of a MEC bioanode stained for eubacteria and archaea. Bacteria (white arrow) at the electrode fiber and archaea (black arrow) between the electrode fibers. Green: EUB; red/pink: Arch; blue: DAPI; black: electrode fibers.

### 5.4.3 RDA

RDA results are expressed by canonical eigenvalues (Table 5.3) and in bi-plots (Figure 5.4). The RDA bi-plots show vectors (arrows) for every dependent variable or band class (which can be interpreted as phylotype or species) including vectors for every environmental parameter (independent variable). The 4 experimental variables overpotential, anode potential, current and resistance were considered separately because it is recommended to use independent non-interacting measures as environmental variables (The ordination webpage, (Palmer)). Since current, resistance and (over)potential are, for a large part, related according to Ohms law, they should not be used together in one RDA plot. The direction of the vector points in the direction of maximum change of the experimental parameter, while the length of the vector gives the degree of change. When the species data are post-transformed (as was done in this assessment) the projection of the perpendicular of a species vector end-point on the experimental parameter vector assigns the dependence or covariance of that specific species on the experimental variable. The axes of the bi-plots of RDA do not have an intrinsic units, they merely express the degrees of change relative to the other parameters (Lepš & Šmilauer, 2003). Because we used one experimental variable per bi-plot, the horizontal axis is the constrained or canonical axis, expressing the direction in which the greatest species variability can be explained. The vertical axis is than the unconstrained axis which explains the variability among species. The canonical eigenvalue that results from RDA gives the percentage of variance that is explained by the experimental parameter on the horizontal axis (Table 5.3). Of the observed species variance, 12.8% can be explained by current, 10.3% by resistance, 8.7% by overpotential and 8.6% by the anode potential. The Monte Carlo permutation test showed, with a significance value of $P \leq 0.05$, that DGGE band classes are significally related to the MEC experimental parameters current and resistance, and show a trend ($0.05 \leq P \leq 0.1$) in relation to anode potential and overpotential (Table 5.3). The bi-plots showed that intensity and presence of bands 63.1, 64.8, 71.2 and 57.2 are most strongly related to current (Figure 5.4B). Bands 57.7, 77.2 and 57.2 are most strongly correlated to resistance (Figure 5.4D) and to overpotential (Figure 5.4C). Bands 57.7, 78.5 and 57.2 show the highest correlation with the anode potential (Figure 5.4A).

**Table 5.3** Tabular results from RDA and Monte Carlo permutation test.

| | Environmental variable | | | |
|---|---|---|---|---|
| | current | resistance | potential | overpotential |
| Eigenvalue axis 1 | 0.128 | 0.103 | 0.086 | 0.087 |
| Percentage of variance explained by axis 1 | 12.8 | 10.3 | 8.6 | 8.7 |
| F-value | 2.64 | 2.068 | 1.686 | 1.724 |
| P-value | 0.006 | 0.016 | 0.06 | 0.05 |

P<0.05 = significant axis 1

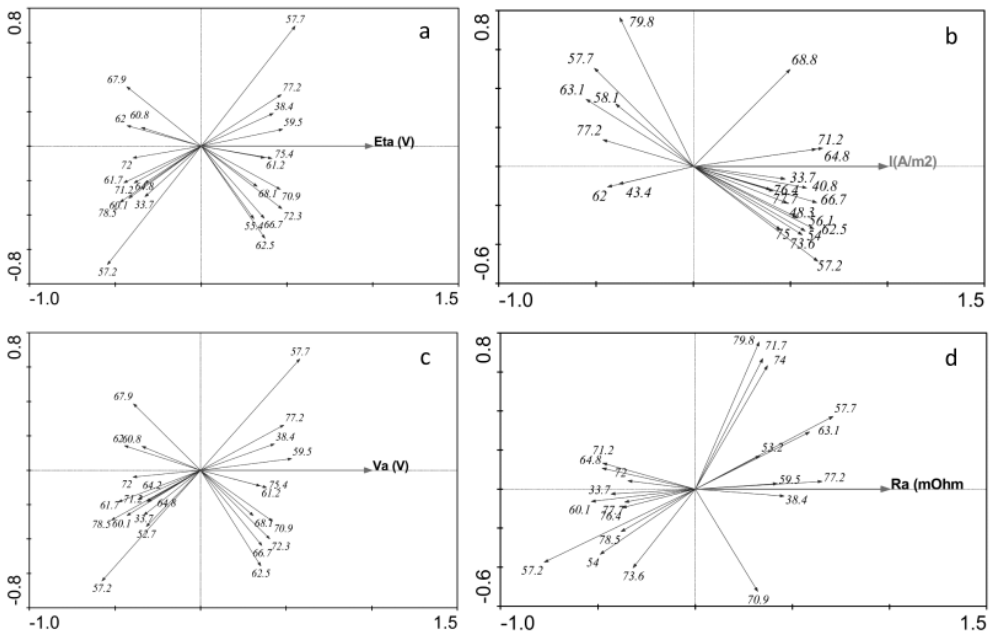



**Figure 5.4** RDA of the DGGE profiles for different environmental variables. A: overpotential (Eta); B: current (I); C: anode potential (V(a)); D: anode resistance (R(a)). The numbers of the species refer to a specific band class. The axes express the degree of change and do not have an intrinsic meaning.

### 5.4.4 Results QR factorization

In addition to RDA, all DGGEs of the bacterial communities on the anode felt were analyzed using QRE. The column pivoting selection method showed that

the band classes 43.4, 53.2, 57.2, 60.1, 61.2, 63.7, 67.3, 70.3, 70.9, 71.2, 71.7, 72, 72.3, 73, 73.6, 75.0, 75.4, 77.2, 78.5 and 79.8 (20 selected) (Table 5.4) contained a maximum degree of information in determining the variation in band patterns. The bands with the highest weighing factors, when taking into account all samples, were band 77.2 for overpotential and resistance, band 57.2 for current and 78.5 for anode potential. To determine the influence of the different experimental parameters, we calculated the relative weighing factor per experimental parameter. The weighing factor of band class 57.2 for current had the highest contribution to all weighing factors (18.7%) followed by the resistance (17.7 %), overpotential (16.3%) and anode potential (12.3%) (Table 5.4).

## 5.5 Discussion

The aim of this study was to identify the ribotypes from a mixed microbial community that are most likely responsible for electrochemical performance of an MEC. In addition, we explored the usefulness of statistical methods for in-depth analysis of DGGE profiles to understand the relationship between the composition of microbial communities on the anode and MEC anode performance.

### 5.5.1 Bacteria are responsible for direct electrons transfer to the anode

Between different MECs there is a large difference in the DGGE pattern representing the bacterial communities on the anodes (Figure 5.1). By contrast, the archaeal populations on the anodes were similar in the different MECs. Stability of archaeal populations relative to bacterial populations has also been observed in other studies (Liu *et al.*, 2009; Nelson *et al.*, 2011). SEM and FISH images showed that the Bacteria are mainly present at the surface of the electrode fibers while the Archaea are present in the spaces between the electrode fibers. Because Archaea seem not physically attached to the anode they are most likely not involved in the electron donation to the anode. Several Archaea can grow by consuming acetate to produce methane and $CO_2$, therefore direct contact with the anode is not needed which explains the presence of the Archaea in the anolyte as much as on the anode (Figure 5.1b). Methane formation is a well-known problem leading to decreased efficiencies (substrate to $H_2$) in MECs (Chae *et al.*, 2010; Sleutels *et al.*, 2011a). The similarity in bacterial population profiles from different locations of the MEC anodes (Figure 5.1a) suggests that the location of sampling is not important for analysis of MEC systems. From our evaluation it is clear that analysis of anolyte

samples is not useful to identify the community responsible for current generation (Figure 5.1a). In this study the focus was on the microorganisms involved in direct interaction with the anode. Therefore, we studied the bacterial population on the anodes.

### 5.5.2 RDA analysis

In this study RDA was used to determine the interaction between composition of the bacterial community and electrochemical performance (electron transfer rate and energy efficiency) of the MEC anode. In addition we tried to pinpoint which ribotypes from DGGE profiles are likely responsible for electrochemical interaction with the electrode.

First, we analyzed which of the experimental variables was significantly affected by the species composition (Table 5.3). The results showed that the effect on potential and overpotential showed a trend while the effect on current and resistance was significant. Second, the canonical eigenvalues that resulted from RDA gave the percentage of variance that is explained by the parameter on that axis. These eigenvalues showed that 12.8% of the variance in species could be explained by current and 10.3% by resistance.

As described above, a good performance of an MEC means low overpotential, high current, low resistance and low anode potential. From a bacterial point of view a high anode potential and high overpotential would be more favourable, because this would theoretically allow more energy gain for the bacteria. However, in practice, when starting an MFC without applied voltage, the anode potential at the time of inoculation can be as high as the cathode potential but as soon as bacteria start to grow the anode potential drops (Torres *et al.*, 2009). This suggests that it is more important to remove the terminal electrons (to the electrode) than to gain more energy for growth. It has also been shown that initial low anode potentials select for species that can produce more current (Torres *et al.*, 2009). In addition, the growth of microorganisms on an anode serves as a catalyst which can lower the overpotential. Clearly, the requirements of the microorganisms are flexible and for a good performance of the microorganisms in the MEC it is more important to look at the effect of current and resistance (how well bacteria perform) rather than to anode potential or overpotential (what is needed for bacteria). Our RDA confirmed the significance of current and resistance relative to anode potential and overpotential.

In addition to the prediction of the importance of each experimental parameter, RDA was used to relate specific species (bands) to MEC anode performance. From

**Table 5.4** QRE selected band classes with weighing factors and relative weighing factors

| band class | 43.4 | 53.2 | 57.2 | 60.1 | 61.2 | 63.7 | 67.3 | 70.3 | 70.9 | 71.2 |
|---|---|---|---|---|---|---|---|---|---|---|
| **Weighing factor for** | | | | | | | | | | |
| Overpotential | 0.0039 | 0.0058 | -0.021 | 0.0128 | 0.049 | 0.0163 | -0.006 | -0.01 | -0.0035 | 0.0116 |
| Resistance | 5.9319 | 1.4265 | -16.1711 | 3.1834 | 12.8308 | 2.0792 | -2.1398 | -0.1498 | 1.613 | 5.2542 |
| Current | -0.29 | 0.2715 | 1.591 | -0.2812 | 0.2112 | 0.0346 | 0.103 | -0.3609 | -0.3742 | 0.3097 |
| Anode potential | 0.0098 | -0.01 | 0.0262 | -0.0159 | 0.0251 | -0.0265 | 0.0041 | -0.0398 | -0.0185 | -0.0382 |
| **Percentage for** | | | | | | | | | | |
| Overpotential | 1.22 | 1.81 | 6.55 | 3.99 | 15.28 | 5.08 | 1.87 | 3.12 | 1.09 | 3.62 |
| Resistance | 4.30 | 1.03 | 11.72 | 2.31 | 9.30 | 1.51 | 1.55 | 0.11 | 1.17 | 3.81 |
| Current | 3.40 | 3.19 | 18.68 | 3.30 | 2.48 | 0.41 | 1.21 | 4.24 | 4.39 | 3.64 |
| Anode potential | 2.34 | 2.39 | 6.26 | 3.80 | 6.00 | 6.33 | 0.98 | 9.51 | 4.42 | 9.13 |

**Table 5.4** continued

| band class | 71.7 | 72 | 72.3 | 73 | 73.6 | 75 | 75.4 | 77.2 | 78.5 | 79.8 |
|---|---|---|---|---|---|---|---|---|---|---|
| **Weighing factor for** | | | | | | | | | | |
| Overpotential | 0.0324 | 0.019 | 0.01 | -0.0074 | -0.0143 | 0.002 | 0.0072 | 0.0524 | -0.0138 | -0.0222 |
| Resistance | 16.155 | 10.4533 | -1.2401 | -3.7446 | -7.8694 | 5.4762 | 5.0413 | 24.3924 | -0.6265 | -12.2381 |
| Current | 0.0887 | -0.022 | 0.6983 | 0.5262 | 0.7052 | -0.1189 | -0.8738 | -0.7189 | -0.7491 | 0.1893 |
| Anode potential | -0.0069 | -0.0168 | 0.0124 | 0.0305 | 0.0232 | -0.0232 | -0.0086 | 0.0193 | -0.0517 | -0.0118 |
| **percentage** | | | | | | | | | | |
| Overpotential | 10.11 | 5.93 | 3.12 | 2.31 | 4.46 | 0.62 | 2.25 | 16.34 | 4.30 | 6.92 |
| Resistance | 11.71 | 7.57 | 0.90 | 2.71 | 5.70 | 3.97 | 3.65 | 17.67 | 0.45 | 8.87 |
| Current | 1.04 | 0.26 | 8.20 | 6.18 | 8.28 | 1.40 | 10.26 | 8.44 | 8.79 | 2.22 |
| Anode potential | 1.65 | 4.01 | 2.96 | 7.29 | 5.54 | 5.54 | 2.05 | 4.61 | 12.35 | 2.82 |

the RDA bi-plots it was possible to determine which bands had the most influence on current, resistance, anode potential or overpotential. As shown in Figure 5.4, band 57.2 is important for all tested experimental parameters. Furthermore, band 57.7 was important for anode potential, overpotential and resistance and 77.2 and 64.8 were specifically important for current and 78.5 for anode potential. It is important to keep in mind that this only shows that changes in the abundance of these species influenced the experimental variable. This does not necessarily mean that these species are responsible for, for example, higher current. But, in general terms, higher values for e.g. current do mean more relative effect of changes (Lepš & Šmilauer, 2003). Further, statistically, we can either explain the effect as an effect of species composition on the experimental variable or as an effect of the variable on the species.

### 5.5.3 Interpretation QRE

In addition to RDA, a numerically justified method, QRE, was used to analyze DGGE. QRE is based on linear regression. Linear regression is a numerical technique that is widely used to measure response variables against explanatory variables in a linear way. However, a problem that appeared in this study was that the number of measurements of each experimental parameter is smaller than the number of band classes. Usually a minimum length solution or regularization techniques are applied to solve these problems (Fletcher, 1980). Alternatively, constraints in the parameter space and prior knowledge can be added to reduce the parameter regression. In this study, we chose to apply QR factorization based estimation with column pivoting to solve the problem. Using this technique, most likely, the set of the selected band classes is chosen such that the set contains a maximum degree of information. However, this cannot be proven or guaranteed as in our case $\binom{54}{20}$ band classes can be selected.

In visual interpretation of the DGGE profiles one would select for the bands with the highest intensity (most dominant bands) to be most important, but after evaluation of the results it was found that the information content of the dominant bands is always smaller or comparable to the QRE results. Or, in other words, selection of the most intense bands could explain comparable or less of the variation in bands than the band classes with the highest determinant, calculated based on the column pivoting method. Calculation of this so called 'determinant' may help to understand the microbial communities better than when we only look at the dominant species present for any DGGE analysis, not only in our study, but also for future research. Further analysis of those band classes in combination

with the experimental data could tell us which bands of the profiles were mostly influencing the specific experimental parameter. Band 57.2 was most important in relation to current and band 77.2 was most important in relation to resistance (Table 5.4).

Comparison of the results of RDA and QRE shows that both methods give band classes 57.2 and 77.2 as most important bands and both analyses identified current as the most important experimental parameter. The difference between RDA and QRE lies in the amount of band-classes considered in the final judgement of the importance of band classes. In RDA, all band classes are taken into account and the best fit axes are determined after which a regression analysis is performed. RDA finds the best, but not an exact, way to relate all species with experimental parameters and might experience noise of bands that are not important (Figure 5.4). In QRE, first a selection is made of band classes that explain most of the variance, after which it is possible to exactly determine the weighing factors for each band class. A problem that can occur with QRE is the case when two band classes appear in a similar way (and thus have a similar importance), one will be selected and the other will be set to 0. For example, in the data set analysed with RDA, band classes 71.2 and 64.8 are both pointing in the same direction with the similar length for current (Figure 5.4B). In QRE band class 64.8 was selected to be set to 0 because it does not explain more of the variation than band class 71.2 already does. Physiologically however, both species 64.8 and 71.2 show the same correlation to current, which is not recognized in QRE.

Summarizing, RDA and QRE showed comparable results in bands classes that appear to be important. In this study band 77.2 and 57.2 are likely to be the most important species in MEC anode performance. Furthermore, both RDA and QRE gave statistical solid arguments that the species composition of the MEC anode is mostly correlated with current. The added value of QRE to RDA is the possibility to exactly select the most important bands not only based on dominance but based on the determinant which showed to have a larger explanatory power than only selecting the dominant bands. For future studies it is therefore suggested to use both methods to get most reliable information from the DGGE profiles.

### 5.5.4 Implications

It was shown for the first time that RDA and QRE are useful tools for bioelectrochemical system analysis. The results from RDA were confirmed with QRE. RDA with additional QRE can be used for future studies to pinpoint which experimental parameters and more specific which bands from DGGE profiles

(representing species) are most important for electrochemical performance. These methods can help to focus on specific species from complex microbial communities. Further studies with isolation and pure culture experiments of these species would be necessary to know what the exact function of these species is and to understand their physiological role in the microbial communities on the anodes.

## 5.6 Acknowledgements

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## Appendix A

Given

$$\mathbf{x} = \mathbf{E}\begin{bmatrix} \mathbf{R}_1 & \mathbf{R}_2 \\ \mathbf{0} & \mathbf{I}_{n-m} \end{bmatrix}^{-1}\begin{bmatrix} \mathbf{Q}^T\mathbf{b} \\ \mathbf{0} \end{bmatrix} \tag{A1}$$

then from (A1)

$$\begin{aligned} \mathbf{Ax} &= \mathbf{AE}\begin{bmatrix} \mathbf{R}_1^{-1} & * \\ \mathbf{0} & \mathbf{I}_{n-m} \end{bmatrix}\begin{bmatrix} \mathbf{Q}^T\mathbf{b} \\ \mathbf{0} \end{bmatrix} \\ &= \mathbf{AE}\begin{bmatrix} \mathbf{R}_1^{-1}\mathbf{Q}^T\mathbf{b} \\ \mathbf{0} \end{bmatrix} \\ &= \mathbf{Q}\begin{bmatrix} \mathbf{R}_1 & \mathbf{R}_2 \end{bmatrix}\begin{bmatrix} \mathbf{R}_1^{-1}\mathbf{Q}^T\mathbf{b} \\ \mathbf{0} \end{bmatrix} \\ &= \mathbf{QQ}^T\mathbf{b} \\ &= \mathbf{b} \end{aligned} \tag{A2}$$

Where $\mathbf{R}_1^{-1}\mathbf{Q}^T\mathbf{b}$ contains at most *m* non-zero elements.

# Chapter 6

## General discussion

The ability of microorganisms to transfer electrons out of or into the cell offers many possibilities for biotechnological applications, such as $H_2$ production by microbial electrolysis in microbial electrolysis cells (MECs), current generation in microbial fuel cells (MFCs), bioremediation, (prevention of) corrosion and biological leaching of metals. This study focused on the MEC in which $H_2$ production through electrolysis is catalyzed by microorganisms, which at the anode consume organic compounds (e.g. from waste or wastewater) and at the cathode use electricity to form $H_2$. The mechanism of the uptake of electrons from a solid surface by microorganisms for $H_2$ production at the MEC biocathode is not fully understood. Understanding the mechanism will help to get insight into the essential parameters for the development of MEC biocathode applications. In contrast, the mechanism by which electrons are transferred outside the microbial cell to a solid surface, such as to bioanodes, is relatively well studied (Bouhenni *et al.*, 2010; Gorby *et al.*, 2006; Gralnick & Newman, 2007; Logan, 2009; Lovley, 2008; Richter *et al.*, 2012; Strycharz *et al.*, 2011). An important aspect in MEC research is to get insight into which types of microorganisms develop in these systems. In this study we used molecular techniques to explore the biodiversity present in MEC biocathode compartments and postulated an initial hypothesis on the mechanisms of electron transfer from electrodes to microorganisms.

### 6.1 Phylogeny of microorganisms at the biocathode

Electrochemically active microorganisms have been detected mainly within the phylum *Proteobacteria*, but also members of several other taxonomic groups possess electrochemical activity. The microbial community of the MEC biocathode for $H_2$ production analyzed in this thesis, consists of several dominant species

belonging to the *Proteobacteria, Firmicutes, Bacteroidetes* or *Actinobacteria* phylum. The composition of the microbial consortium on the biocathode depends in part on the type of setups and operational conditions used (chapter 3). Phylogenetically there is no obvious common characteristic among the dominant phyla that we found in the MEC systems to explain their presence on the biocathode. *Actinobacteria* are Gram-positive and contain DNA with a high G-C content (>55%). They are morphologically and physiologically diverse (Embley & Stackebrandt, 1994; Servin *et al.*, 2008). *Firmicutes* are also mainly Gram-positive, but low in G-C content (<50%) Many are spore forming and able to survive in extreme environments. The *Proteobacteria* are Gram-negative bacteria with a phototrophic or chemoheterotrophic metabolism. The *Bacteroidetes* are also Gram-negative generally anaerobic, with a chemoheterotrophic metabolism. The large variety of dominant groups found in the MEC biocathodes, might reflect a general trait present in all those groups, associated with the catalytic activity at the biocathode. It might be that the presence of (possibly exocellular) hydrogenases is sufficient for bacteria to maintain themselves in the biocathode. Hydrogenases, the enzymes that catalyze both $H_2$ production and $H_2$ consumption, have been identified in species of different phyla of the order Bacteria, Archaea and Eukarya (Vignais & Billoud, 2007). Hydrogenases are categorized according to their (redox active) metal site. In Figure 6.1 the abundance of the different (FeFe and NiFe) hydrogenases are shown per phylogenetic group. The presented hydrogenase genes are from the analysis of Vignais & Billoud (2007) and a recent search in the NCBI webpage (http://www.ncbi.nlm.nih.gov/gene, search terms "NiFe hydrogenase" or "FeFe hydrogenase not Fe") are presented. Most studied and described hydrogenases belong to species within the *Proteobacteria* phylum of which the majority belong to the *Gammaproteobacteria*. Most described hydrogenases are of the NiFe type, but the (cytoplasmatic) FeFe hydrogenases are thought to be commonly involved in $H_2$ production. FeFe hydrogenases are mostly found in *Firmicutes*, but also in the delta-subclass of the *Proteobacteria* (Figure 6.1). The Fe hydrogenases are restricted to some Archaea (Vignais & Billoud, 2007) (not shown in Figure 6.1). Apart from the hydrogenase genes as used for Figure 6.1, many more species may contain putative hydrogenase genes in their genome not yet listed as such.

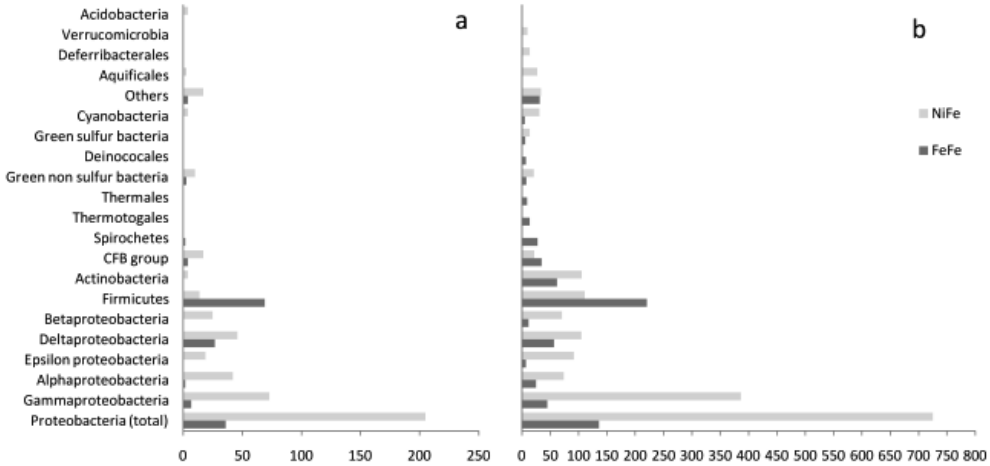


**Figure 6.1** Taxonomic groups and numbers of hydrogenase genes detected within the different taxonomic groups. a) Data obtained from Vignais & Billoud 2007. b) data obtained from NCBI gene webpage March 27th 2012 (http://www.ncbi.nlm.nih.gov/gene) with search terms "NiFe hydrogenase" or "FeFe hydrogenase not Fe". CFB group = *Cytophaga-Flavobacteria-Bacteroides group,* NiFe = Nickel Iron hydrogenases, FeFe = Iron Iron hydrogenases.

In our studies, the dominant species (ribotypes) that we detected were members of the phylogenetic groups *Firmicutes* and *Proteobacteria*, for which many different hydrogenase genes have been identified, but also many *Bacteroidetes* of which fewer hydrogenases have been identified. The abundance of *Firmicutes* in the analyzed biocathode samples suggests that FeFe hydrogenases are abundant in those MEC cathode species. However, analysis of the hydrogenase genes present in the MEC biocathode, showed the presence of one FeFe hydrogenase gene that is found in *Desulfovibrio* spp. (a *Deltaproteobacterium*), and further several NiFe hydrogenase genes (Chapter 3). The dominant species and hydrogenases that we detected in the MEC biocathode are assumed to be most important for catalysis of $H_2$ production at the MEC cathode. Yet, microbial species and hydrogenase genes that are not dominant could play an important role as well. Future research might disclose new hydrogenases or possible other traits that are specifically important for MEC application. Although hydrogenases are likely to be essential for $H_2$ production in a biocathode, other proteins that catalyze $H_2$ production such as nitrogenases (Hillmer & Gest, 1977) or proteins with other functions such as cytochromes which are involved in electron transfer, could be involved in $H_2$ production in MEC. Electron transfer, either directly or through shuttle compounds, from the electrode surface to the microbial cell, is essential for the

microorganisms to conserve the electrical energy. Cytochromes are present and abundant in bacteria with a respiratory metabolism. Cytochromes are involved in electron donation to an anode by *Geobacter* and *Shewanella* species (He *et al.*, 2007; Leang *et al.*, 2003; Lovley, 2008; Nevin *et al.*, 2009; Strycharz *et al.*, 2011). The presence of specific cytochromes could be essential for the uptake of electrons from the cathode and thus for biocathode functionality. Interestingly also Gram-positive bacteria (*Thermincola potens* within the *Firmicutes*) contain cytochromes, spanning the cell wall, that are involved in exocellular electron transfer (Carlson *et al*. 2012).

## 6.2 Physiology of biocathode microorganisms

Based on 16S rRNA gene cloning and sequencing, the dominant clones in our MEC biocathodes were identified as related to known species of the genera *Desulfovibrio*, *Promicromonospora*, *Kaistella*, *Azonexus* and *Hydrogenophaga*, but also clones with less than 97% identity to cultured species were detected such as clones with only 91% identity to *Desulfotomaculum* sp. Ox39 or *Clostridium cylindrosporum* as closest related species (Table 6.1). Assuming that the dominant species are responsible for the catalysis of the $H_2$ production in the biocathode, knowledge about those species can give information on the mechanism involved in electron transfer and $H_2$ production. For example *Desulfovibrio* spp. contain multiple hydrogenases (Pereira *et al.*, 2008; Tatsumi *et al.*, 1999). They also contain pilin like structures (Clark *et al.*, 2007) and several cytochromes (Lovley *et al.*, 1993) that might function in a similar but reverse way as in anode systems (Lovley *et al.*, 2011). Also members of the genus *Hydrogenophaga* are able to produce and consume $H_2$ (Willems *et al.*, 1989) but less is known about their hydrogenases. Thus far the ability of *Kaistella* spp. to produce $H_2$ was not reported. However, putative hydrogenases are present in the genome of *Chryseobacterium* spp. (Integrated Microbial Genomes (IMG) search on http://img.jgi.doe.gov/), which is a member of the same genus (synonym genus name) as *Kaistella*. Also the draft genome of *Promicromonospora kroppenstedtii* RS16 (DSM19349), which is the only sequenced *Promicromonospora* sp. so far, revealed putative hydrogenases both using IMG search (http://img.jgi.doe.gov/geba) and a PROSITE scan (Gattiker *et al.*, 2002) (Chapter 3). For *Azonexus* spp. no genome has been sequenced and it is not known if they contain hydrogenases. Genome sequence analysis might reveal the function of dominant species in mixed communities of biocathodes. Most detected species contain genes putatively coding for hydrogenases, but

not all coding for the same type of hydrogenase, e.g. coding for hydrogenases containing different metal sites. For example *Desulfovibrio* sp. have NiFe and FeFe hydrogenases and *Promicromonospora* sp. contain genes coding for putative NiFe $F_{420}$ reducing hydrogenases but no putative genes coding for FeFe hydrogenases. If these species are involved in $H_2$ production in the MEC it would mean that different hydrogenases are used by different microorganisms in the MEC biocathode.

**Table 6.1** Dominant ribotypes found in MEC biocathodes.

| Closest related species to dominant ribotype (identity) | % of total clones | Type MEC[1] | Chapter | Current density (A/m²) |
|---|---|---|---|---|
| *Desulfovibrio vulgaris* Hildenborough (98%) | 25 | 1 | 2 | 1.2 |
| *Clostridium cylindrosporum* (91%) Also present in BicS 19% | 40 | 2 (AcS) | 3 | 1 |
| *Desulfotomaculum* sp. Ox39 (91%) | 40 | 2 (AcS) | 3 | 1 |
| *Desulfotomaculum* sp. Ox39 (91%) | 59 | 2 (BicS) | 3 | 0.8 |
| *Kaistella koreensis* (99%) | 23 | 3 (AcL) | 3 | 2.7 |
| *Hydrogenophaga flava* strain 2 (97%) | 13 | 3 (BicL) | 3 | 2.3 |
| *Desulfovibrio vulgaris* Miyazaki F (99%) | 13 | 3 (BicL) | 3 | 2.3 |
| *Azonexus caeni* (97%) | 13 | 3 (BicL) | 3 | 2.3 |
| *Promicromonospora* sp. CPCC100077 (99%) | 96 | 3 (AcnSL) | 3 | 2.2 |
| *Citrobacter amalonaticus* (99%) | Inoculated as pure culture | 3 | 4 | 2.2/2.3 |
| *Desulfovibrio* G11 | Inoculated as pure culture | 3 | 2 | 0.8 |

[1] MEC setups types: type 1 as described by Rozendal *et al.* (2008), type 2 small setup as described by Ter Heijne *et al.* (2008) and type 3 large setup as described by Jeremiasse *et al.* (2010).

To study the function of the bacteria further, it is important to obtain isolates derived from MEC biocathode systems and try to get functional biocathodes with the isolated strains in separate experiments. We inoculated a clean cathode with *Desulfovibrio* G11 (DSM 7057) (Chapter 2) which showed current increase (up to an average of 0.8 A $m^{-2}$) and $H_2$ production at the cathode. Additionally, we isolated a strain PS2, with 99% rRNA gene identity to *Citrobacter amalonaticus*. Inoculation of strain PS2 in the cathode compartment resulted in current and $H_2$ production (up to 2.3 A $m^{-2}$ and 2.8 $m^3$ $H_2$ $m^{-3}$ reactor liquid per day). However,

after operation the biocathode appeared to contain a few contaminants. PS2 (and possible contaminants) produced about 3 times more current than a pure culture of *Desulfovibrio* G11. The differences in current generation of *Desulfovibrio* and strain PS2 might reflect physiological differences, but a role of the contaminants in the biocathodes together with strain PS2 cannot be excluded. Physiological differences such as the sensitivity for oxygen (*Desulfovibrio* G11 is a strictly anaerobe and strain PS2 is a facultative anaerobe) might be important in the ability to grow and perform well in an MEC. Although conditions at the cathode are reduced, the used system might have suffered from leakage of trace amounts of oxygen via e.g. the tubing or the influent into the cathode compartment. In addition, in one of the studied biocathodes, an almost pure culture of a *Promicromonospora* strain developed (AcnSL, chapter 3). *Promicromonospora* are generally described as aerobic bacteria. This setup performed similar to the PS2 setups and produced about 3 times more current than *Desulfovibrio* G11.

## 6.3 Mechanism of electron transfer at the biocathode

The mechanism of electron transfer in the biocathode is not understood. The electron transfer mechanism at the cathode may involve different mechanisms than at the anode (Holmes *et al.*, 2008). Most likely cytochromes for electron shuttling and hydrogenase for $H_2$ production are involved. It has been suggested that an energy conserving hydrogenase (Ech) could play an important role in $H_2$ production (Geelhoed *et al.*, 2010). However, both the results from chapter 3 (hydrogenase chip analysis) and chapter 4 (using the uncouplers nigericin and monensin), suggested that Ech are not involved in $H_2$ production at the biocathodes that we studied here. Although the diversity of detected species suggests that several hydrogenases can be involved, the most abundant hydrogenases genes that we detected in MEC biocathodes were bidirectional cytoplasmic $NAD(P)^+$-dependent (Hox-type) hydrogenases. Therefore, we hypothesize that $H_2$ is produced intracellularly by cytoplasmic hydrogenases (Figure 6.2). For $NAD(P)^+$-dependent hydrogenases, NAD(P)H is the (primary) electron donor for $H_2$ formation (Vignais & Billoud, 2007). Cytoplasmatic $NAD(P)^+$ can be reduced by electrons derived from the cathode through a sequence of cytochromes that deliver the electrons to the quinone pool (MQ). The reduced quinone ($MQH_2$) delivers the electrons to $NAD(P)^+$, producing NAD(P)H and $H^+$ through an inner membrane associated protein (e.g. NAD(P)H dehydrogenase) (Figure 6.2). The cytoplasmatic $H_2$ production creates a proton gradient that can drive a membrane-

associated ATPase to produce ATP (Figure 6.2). The difference between the thermodynamic potential of the $H^+/H_2$ couple (-0.41V) and the $NAD^+$/NADH couple (-0.32V) is small and the direction of reaction is therefore susceptible to changes in the concentrations of the reactants and products (e.g. $H_2$ partial pressure) (Lauterbach *et al*., 2011). However, $H_2$ production is possible if the $H_2$ partial pressure is kept low. Another possibility to drive the reaction towards $H_2$ formation is the presence and use of a (more reduced) co-electron donor such as ferrodoxin (Fd) (not shown in Figure 6.2). In that case, Fd could be reduced first by electrons derived from the cathode and partly drive the $H_2$ formation by a so-called confurcating hydrogenase in the cytoplasm. Confurcating hydrogenases simultaneously use NAD(P)H and reduced ferredoxin in a 1:2 ratio as electron donor. In *Thermotoga maritima*, a FeFe hydrogenase with confurcating activity was described (Schut and Adams, 2009). For putative NiFe hydrogenases and formate dehydrogenases from several different organisms confurcating activity has been suggested (Müller *et al*., 2010). Electron transfer from the outside of the microbial cell towards the inside would possibly involve a chain of several cytochromes (Figure 6.2). In the closest relatives of the species detected in biocathode samples (Table 6.1), multiple cytochromes are present (IMG search).

The involvement of cytochromes in the electron shuttling from the inside of the cell to the outside has been shown for species growing on anodes (Nevin *et al*., 2009). The reactive centres of specific cytochromes have different redox potentials. For example, multiheme cytochrome OmcZ has a large range of reduction midpoint potentials (from -0.42V till -0.06V) (Lovley *et al*. 2011). However, each cytochrome has an optimal potential dependent on the type of cytochrome and the reactive centre (Wei *et al*., 2010). The energy that can be harvested by the microorganisms in the MEC biocathode is the difference between the potential ($E^{o'}$) of an electron accepting outer membrane protein (e.g. cytochrome) that accepts the electrons from the cathode, and the terminal electron accepting reaction, which is $H^+$ to $H_2$. The potential of this outer membrane protein should be close to the cathode potential, specifically to allow the microorganisms to conserve the maximum amount of energy from the reaction. Since the potential of the cathode for $H_2$ production is different from the potential of an anode it is likely that other cytochromes are involved in the cathode reaction than in the anode reaction. In future studies it would be interesting to analyze in detail which cytochromes are involved in biocathodic $H_2$ production e.g using cyclic voltametry. For this, it is important to include a variation in applied potentials since the potential at which a biofilm is active might influence the expression of specific cytochromes. Analysis of the RNA (e.g by sequencing or use of microarrays) or protein profiles of a MEC

biocathode biofilm could clarify which specific cytochromes or other proteins are involved in biocathodic $H_2$ formation at different applied potentials. When a pure culture model organisms and a genetic system for a biocathode bacterium is established, possibly using strain PS2 or *Geobacter sulfurreducens*, more in-depth experiments on the mechanisms are possible, such as deletion of genes coding for cytochromes or hydrogenases or measurements on biofilm conductivity as described before for *Geobacter* species (Lovley *et al.*, 2011).

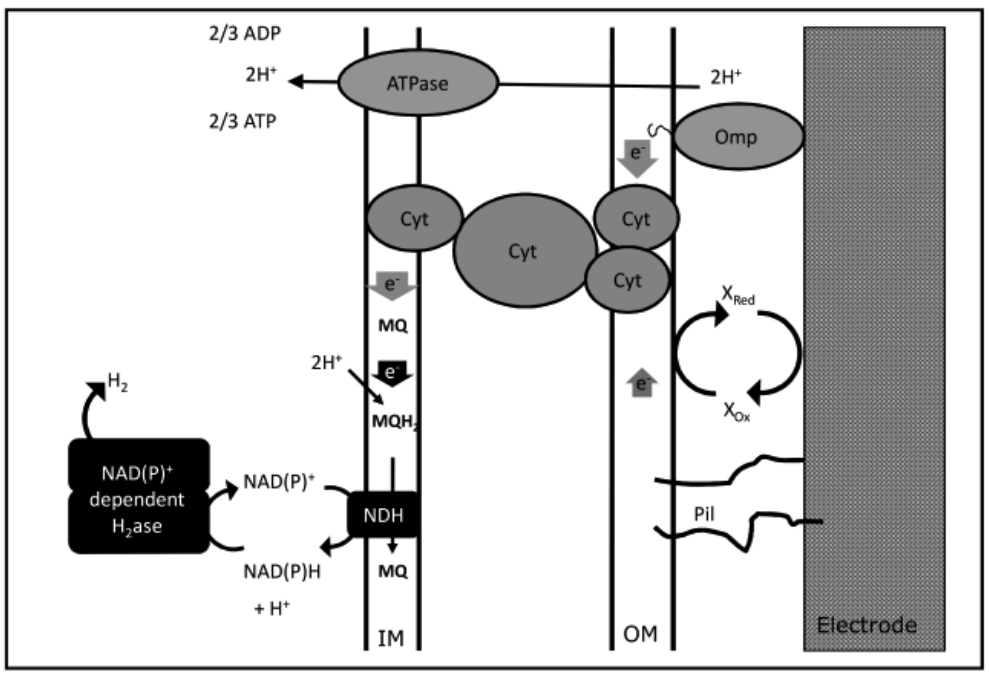


**Figure 6.2** Proposed mechanism for $H_2$ production with electrons ($e^-$) derived from a cathode and possible energy conservation. Electron uptake from the electrode may involve outer membrane proteins (omp) (possibly cytochromes), pillin-like structures (Pil) or shuttle components (X). The electrons may be transferred to the quinone pool (MQ) in the inner membrane by a sequence of cytochromes (Cyt). The quinone gets reduced (MQH2) by the electrons derived from the cathode and may subsequently deliver electrons to a membrane associated NAD(P)H dehydrogenase (NHD) resulting in the reduction of $NAD(P)^+$ to NAD(P)H and $H^+$. The NAD(P)H and protons are then used by a $NAD(P)^+$ dependent hydrogenase for production of $H_2$. Cytoplasmic $H_2$ production creates a proton gradient that can drive ATP formation by a membrane associated ATPase. IM = inner membrane, OM = outer membrane.

Direct exocellular electron transfer in anode systems is often compared to reduction of insoluble metal oxides in natural systems (e.g. manganese oxides

or iron oxides). Species able to reduce metal oxides are often also able to grow with an anode as electron acceptor such as for example *Geobacter* spp. (Lovley *et al*., 2011), and therefore many studies on the molecular mechanisms of direct exocellular electron transfer deal with metal oxides as electron acceptor. The uptake of electrons from a solid surface is often compared to microbial corrosion. Anaerobic corrosion is stimulated by microorganisms, however, it remains unclear if during corrosion electrons are taken up directly from a solid metal surfaces (e.g. Fe(0)) or only through intermediate compounds such as Fe(II) or $H_2$ (Hamilton, 2003; Mehanna *et al*., 2009). Interestingly, sulfate-reducing bacteria such as *Desulfovibrio* spp., which we found in several MEC systems, are often associated with corrosion (e.g. Iverson, 2001; Jan-Roblero *et al*., 2008). This suggests that indeed similar mechanisms are involved in corrosion and electron uptake from biocathodes. Natural systems in which electrons are taken up from a solid surface are poorly studied, which makes the biocathode systems a very challenging but also interesting system to study this mechanism under controlled conditions.

## 6.4 Implications and future studies

In this thesis we showed that a large diversity of microorganisms can be found in the MEC biocathodes that produce $H_2$. A large diversity of species able to catalyze $H_2$ production at the cathode is advantageous for MEC application, because it will increase the chance that functional species are able to develop at the cathode. A big challenge is to understand which part of the microbial population catalyzes $H_2$ production and if that conversion is coupled to growth. One important issue would be to know if the microbial communities of duplicate experiments are the same. This can provide more information on specific effects of changes in conditions such as carbon source feed, type of electrode or applied potential on the active microbial population. In chapter 5 we showed how statistical techniques can be used to get insight which part of the microbial community is responsible for electrochemical performance (e.g. current production) of the MEC anodes. Those statistical techniques might be helpful in future studies to target the microorganisms for further isolation attempts. In our study we isolated one species from an active MEC biocathode. However, it is important to isolate more species from biocathode biofilms and investigate their performance in pure and mixed biocathodes to be able to understand if the mechanism of biocathode $H_2$ production is as general for microorganisms as we suggest here. Small scale systems, such as the ones described by (Call & Logan, 2011) are ideal to test

multiple species for electrochemical activity. Such a system could also be used for specific enrichments and isolation of microorganisms by making dilution series of inocula and applying selective conditions to produce $H_2$ in MEC. We also showed that restricted conditions (e.g. lack of sulfur source, chapter 3) can be helpful for enrichment and isolation of bacteria in the MEC biocathode. A metagenomic and proteomic approach could be used to study the enriched communities in the MEC systems. We showed that the hydrogenase chip (Marshall *et al.*, 2012) (chapter 3) can be applied to study the hydrogenases within the microbial population of the MEC biocathode. In addition to the detection of genes, this chip can also be applied to study which hydrogenase genes are expressed in active MEC biocathodes. Furthermore, using the GeoChip (He *et al.*, 2007), or other functional chips, would provide valuable information for understanding the microbial activities in the MEC biocathode. Functional analysis could also include protein analysis and enzyme activity tests. However, these types of analyses will give most information about the activity and possibly involved enzymes in $H_2$ production in MECs when pure cultures can be used.

## 6.5 Implications and challenges for MEC technology

During the last decade, research efforts have brought the MEC to a stage at which the main challenges are the up-scaling of the system and to find applications where this technology is cost effective. At present the most optimal MEC system seems, after up-scaling, to be cost-competitive with water electrolysis and distributed (not produced at the spot where it is used) $H_2$ obtained by biomass gasification (Jeremiasse, 2011). Reduction of the construction costs and improvement of the efficiencies will make the system even more attractive. Jeremiasse *et al.* (2010) showed that chemical cathodes such as nickel foam and nickel-cobalt or cobalt-molybdenum alloys on a non-noble metal electrode offer great perspectives as alternative for platinum, which was used in the first MEC systems (Liu *et al.*, 2005; Rozendal *et al.*, 2006). The biocathode would offer even cheaper possibilities because the cathode material consists of non-metal conductive materials that serve as a support for the biofilms. The efficiency of the biocathode is not as high as the chemical cathodes yet. However, the current densities of the latest biocathode (Jeremiasse *et al*. 2012) compared to the very first MEC biocathode (Rozendal *et al*. 2008) increased significantly, due to setup design, operation, decrease of losses (e.g. less to no methane production), and inoculation material. A large step forward would be the decrease of efficiency losses. Currently, cathodic efficiencies

are about 50%, which are thought to be due to $H_2$ leakage over the membrane and tubing (Rozendal *et al.*, 2006), but could also include microbial $H_2$ consumption. Improvement of the system in such a way that $H_2$ can be collected or flushed out, including the improvement of the system to increased current densities, will improve cathodic efficiencies and decrease microbial $H_2$ consumption.

MEC and MFC anodes have been studied in the last decades. The MEC biocathode is a new technology that needs further research to apply it as a technology for 'green' $H_2$ production. From our results it is clear that a large diversity of species is capable or even essential for $H_2$ production at a cathode. Secondly, our results show that specific conditions such as pyruvate addition in combination with inoculation of strain PS2 is useful to activate an MEC biocathode. Furthermore, the microbial community that develops in a biocathode might be largely influenced by the physical conditions of the setup such as e.g. the electrode material. In addition to $H_2$ production at the cathode, the MEC offers also perspectives for the production of other, economically interesting compounds. Examples are acetate production (Nevin *et al.*, 2011), ethanol production (Steinbusch *et al.*, 2010), methane production (Cheng *et al.*, 2009), or succinate production (Gregory *et al.*, 2004) from relatively oxidized carbon sources. The biocathode offers many possibilities in biotechnology, but further studies are essential to improve the system to make it a technological interesting alternative for MEC systems with chemical cathodes.

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# Summary

One of the main challenges for improvement of the microbial electrolysis cell (MEC) has been the reduction of the cost of the cathode catalyst. As catalyst at the cathode, microorganisms offer great possibilities. Previous research has shown the principle possibilities for the biocathode for $H_2$ production with mixed microbial communities. In this thesis we analyzed the microbial communities from several biocathodes for $H_2$ production. The microbial population of the very first MEC biocathode for $H_2$ production (Chapter 2) showed a dominant population of *Desulfovibrio* spp.. A member of these dominant species, *Desulfovibrio* strain G11 was reinoculated in a biocathode and produced current and $H_2$. On the basis of previous knowledge of known Desulfovibrio spp., the molecular mechanism of electron uptake from a cathode with $H_2$ production was proposed to have similarities to mechanisms that have been proposed for syntrophic growth.

In Chapter 3 the microbial population of 5 more MEC biocathodes was analyzed. The MECs were fed with either acetate or bicarbonate and two different designs were used. The results showed that the microbial communities from the same setup design are more similar than fed with the same carbon source. Furthermore, ribotypes from the phyla, *Firmicutes*, *Proteobacteria*, *Bacteroidetes* and *Actinobacteria* were found to be dominant. To understand the mechanisms of $H_2$ production in the MEC in more detail, a hydrogenase gene microarray was used to analyze the hydrogenase genes present in 3 of the cathode samples. The results showed that genes coding for bidirection $NAD(P)^+$ dependent hydrogenases were mostly present in the MEC biocathode. This suggests that a mechanism involving cytoplasmatic $NAD(P)^+$ dependent hydrogenases is present rather than energy converting hydrogenases as proposed before.

To understand the molecular mechanisms it is important to obtain pure cultures from the MEC biocathode and test them for biocathode activity. In chapter 4 we describe a *Citrobacter* species strain PS2 which was isolated from the MEC biocathode. PS2 was very similar to other *Citrobacter* spp. able to produce fermentative $H_2$ from a diversity of carbon sources. When inoculated in the MEC biocathode and fed with pyruvate, current increased and $H_2$ was produced with comparable efficiencies and production rates as the mixed cultures biocathodes. Addition of membrane potential uncouplers nigericin and monensin showed

no change in current and $H_2$ production rates, suggesting that the molecular mechanism does not involve membrane potential driven processes.

Finally, in chapter 5, we explored the usefulness of statistical methods to pinpoint which species are most important for MEC performance. We analyzed DGGE profiles from 5 different MEC anodes using two distinct statistical techniques, Redundancy analysis (RDA) and QR factorization (QRE), and tried to link the DGGE profiles to current, resistance, potential and overpotential. The results showed that current was mostly related to species composition. We were able to pinpoint a few bands from DGGE that were influencing changes in experimental parameters the most. The results showed that both RDA and QRE are useful methods, of which RDA takes all bands into account, but is therefore less precise; QRE is numerical precise but by eliminates bands that explain least of the variation and therefore using QRE might neglect an effect of these bands. Altogether, RDA combined with QRE is useful to give an indication of which species from a mixed community are important for MEC performance and can be used to find a focus in mixed community analysis.

From our results we conclude that a large diversity of bacteria is able to catalyze $H_2$ production at the cathode. The species that develop at a cathode might be largely influenced by the design of the used setup, which has to be considered when comparing different experiments. In addition, our results suggest that a general mechanism present in many different bacterial species is involved in $H_2$ production in MEC. We propose a molecular mechanism involving a series of cytochromes and cytoplasmatic $H_2$ production by $NAD(P)^+$ dependent bidirectional hydrogenases that use electrons derived from the cathode. The biocathode is a promising technology for application in the MEC, although to date the chemical cathodes still outcompete the biocathode. The biocathode offers great possibilities for future applications including production of other products such as ethanol, methane, succinate or acetate.

# Samenvatting

De microbiële elektrolyse cel (MEC) is een veelbelovend systeem voor duurzame waterstof gas ($H_2$) productie. De MEC bestaat uit twee elektroden in een buffer die verbonden zijn door een elektrisch circuit. Aan de anode zijn micro-organismen aanwezig die organisch materiaal consumeren en de elektronen die vrij komen bij metabolische processen afgeven aan de anode. Deze elektronen gaan door het elektrisch circuit naar de kathode waar een reducerende reactie plaatsvindt. Door toevoegen van een kleine hoeveelheid energie kan aan de kathode $H_2$ gevormd worden. Eén van de belangrijkste uitdagingen voor verbetering van de MEC is het reduceren van de kosten van de kathode door vervanging van het kostbare platina. Een van de mogelijkheden is om micro-organismen te gebruiken om de reactie aan de kathode te katalyseren. In eerder onderzoek is aangetoond dat gemengde microbiële gemeenschappen als kathode katalysator gebruikt kunnen worden waardoor de kosten van de kathode veel lager worden. In dit onderzoek zijn de microbiële gemeenschappen van verschillende $H_2$ producerende biokathodes geanalyseerd.

De eerste biokathode voor $H_2$ productie die voor deze studie onderzocht is (hoofdstuk 2) bevatte een dominante populatie van *Desulfovibrio* soorten. In een vergelijkbare opstelling is daarom een bekende *Desulfovibrio* stam, *Desulfovibrio* stam G11, geënt in een kathode wat resulteerde in de productie van stroom en $H_2$. Op basis van de kennis van bekende *Desulfovibrio* stammen, is een moleculair mechanisme opgesteld om stroomproductie en $H_2$-vorming aan de kathode te verklaren. Dit mechanisme is vergelijkbaar met het mechanisme dat voor syntrofe groei met formiaat is opgesteld.

In hoofdstuk 3, zijn de microbiële gemeenschappen van nog vijf andere MEC biokathodes geanalyseerd. Deze biokathodes waren gevoed met twee verschillende koolstofbronnen, acetaat of bicarbonaat, in combinatie met twee verschillende MEC ontwerpen. De resultaten laten zien dat de bacteriële gemeenschap van de biokathodes die zich in dezelfde type opstelling ontwikkelen, meer gelijk zijn aan elkaar dan de microbiële gemeenschappen die zich ontwikkelen in verschillende opstellingen maar gevoed zijn met dezelfde koolstofbron. De dominante ribotypes behoorden tot de phyla *Firmicutes*, *Proteobacteria*, *Bacteroidetes* en *Actinobacteria*. Om het mechanisme van $H_2$ productie in de MEC beter te kunnen begrijpen, is

een micro-array gebruikt waarmee bepaald kon worden welke typen hydrogenases aanwezig waren in de microbiële populatie op de biokathode. De resultaten laten zien dat genen coderend voor $NAD(P)^+$ afhankelijke (Hox-type) hydrogenases het meest voorkwamen in de drie geanalyseerde MEC biokathodes. Dit impliceert dat cytoplasmatische $NAD(P)^+$ afhankelijke hydrogenases betrokken lijken te zijn bij $H_2$ productie in de MEC en niet, zoals eerder voorgesteld, energie-conserverende hydrogenases (Ech).

Voor een goed begrip van het mechanisme van biokathodische $H_2$ productie is het belangrijk om bacteriën te isoleren uit de MEC biokathode en deze reincultuur apart te testen in de MEC biokathode. In hoofdstuk 4 is een *Citrobacter* stam, PS2, die geïsoleerd was uit de MEC biokathode beschreven. Stam PS2 was, net als andere *Citrobacter* stammen, in staat fermentatief $H_2$ te maken uit verschillende koolstofbronnen. Na enting van stam PS2 in een kathode gevoed met pyruvaat, nam de stroom en $H_2$ productie toe. De $H_2$ productie-efficiëntie en $H_2$ productiesnelheid waren vergelijkbaar met wat met gemengde cultuur biokathodes werd behaald. Membraanpotentiaal–ontkoppelaars, nigericine en monensine, hadden geen effect op de stroom of $H_2$ productie, wat impliceert dat membraanpotentiaal-gedreven processen geen rol spelen bij $H_2$ productie in de biokathode.

In hoofdstuk 5 is gekeken naar de bruikbaarheid van statistische methoden om aan te tonen welke soorten micro-organismen het belangrijkste zijn voor de activiteit van de MEC. Hiertoe zijn DGGE-profielen van vijf verschillende MEC bioanodes geanalyseerd met twee verschillenden statistische technieken, redundancy analyse (RDA) en QR factorisatie (QRE). Met behulp van deze technieken is geprobeerd een verband te leggen tussen de DGGE-profielen (waarmee de microbiële populatie kan worden gevisualiseerd) en stroomproduktie, anode potentiaal, overpotentiaal en weerstand. De resultaten laten zien dat stroom het meest gecorreleerd is met is voor de soortensamenstelling. Daarbij konden een aantal banden van de DGGE profielen aangewezen worden die het meest gecorreleerd waren met de verschillende experimentele parameters. De resultaten laten zien dat RDA en QRE beide bruikbare technieken zijn: RDA houdt rekening met alle banden maar is daardoor minder exact. QRE is numeriek exact maar elimineert banden die het minste van de variatie verklaren en kan daardoor het effect van de overblijvende banden onderschatten. Samengevat geeft RDA in combinatie met QRE de meeste informatie over welke microbiële soorten het meest waarschijnlijk betrokken zijn bij de activiteit van de MEC en deze methoden kunnen in de toekomst gebruikt worden als leidraad voor analyses van gemengde microbiële cultures.

Uit onze resultaten kunnen we concluderen dat veel verschillende micro-organismen in staat zijn om $H_2$ productie aan een kathode te katalyseren. De

bacteriën in de MEC worden mogelijk sterk beïnvloed door verschillende factoren in het ontwerp van de MEC. Bij een goede vergelijking van de resultaten van verschillende MEC experimenten moet dit aspect dan ook worden meegenomen. Daarbij geven de resultaten aanleiding tot de voorlopige conclusie dat er een algemeen moleculair mechanisme betrokken is bij $H_2$ productie in de MEC dat aanwezig is in veel verschillende soorten bacteriëen. Wij stellen hierbij een mechanisme voor waarbij een serie van cytochromen de elektronen die van de kathode komen, overdragen aan een cytoplasmatische $NAD(P)^+$ afhankelijke hydrogenase waarmee protonen worden gereduceerd tot $H_2$. De biokathode is een veelbelovende techniek voor toepassing in de MEC, hoewel op dit moment de kathodes met chemische katalysatoren (zoals platina of nikkel) beter werken maar over het algemeen wel duurder zijn. Dit onderzoek heeft laten zien dat een biokathode functioneel is en dat er nog voldoende mogelijkheden zijn om dit systeem efficiënter te laten werken. Een MEC biedt veel mogelijkheden voor toekomstige toepassingen, niet alleen voor $H_2$ productie maar ook voor de vorming van andere producten zoals ethanol, methaan, succinaat en acetaat.

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Groningen, 22 juli 2012

# About the author

Elsemiek Croese was born in Leidschendam on the 5th of June 1980. She went to the primary and secondary school of the Vrije School in Den Haag, after which she spent one year to get her VWO diploma at het Haags Montessori Lyceum. After finishing her VWO she went to Groningen to study biology. In the specialization phase she focused first on medical topics but later decided to focus more on the microbiology. Her graduation topics were on halophilic microorganisms and on re-growth of human cells on heart valves. After she finished her masters she worked a few months for a small, starting company DNA-art. Searching for more challenges, she applied for a job at Wetsus where she was hired to do a PhD within the Laboratory of Microbiology of Wageningen University. The product of this PhD project is presented to you here.

Netherlands Research School for the
Socio-Economic and Natural Sciences of the Environment

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The Netherlands Research School for the
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**SENSE PhD courses**

- Environmental Research in Context
- Research Context Activity: Co-organizing and communicating Biocatalyzed Electrolysis meeting (24 October 2007 at Wetsus, Leeuwarden)
- Advanced course on Environmental Biotechnology

**Other PhD and MSc courses**

- Electrochemistry
- Visualization cellular processes
- Scientific writing
- ARB course: Training in use of a software environment for sequence data analysis
- Real time polimerase chain reaction course
- Teaching microbial physiology and water microbiology practical courses

**Oral Presentations**

- Microbial communities in the microbial electrolysis cell, NVVM, 2 April 2008, Papendal, The Netherlands
- Microbial communities in the microbial electrolysis cell, Harvard University 15 April 2009, Boston, USA and Rutgers University, 20 April 2009, New Brunswick, USA
- Microorganisms in the microbial electrolysis cell, Sense symposium Innovative techniques for a sustainable environment, 20 February 2009, Wageningen, The Netherlands
- Hydrogen producing microbial community of the biocathode in the microbial electrolysis cell, 3th international MFC symposium, 6 – 8 June 2011, Leeuwarden, The Netherlands
- Microbial communities on the MEC biocathode, Wetsus Internal conference, 28 November 2009 and 24 November 2011, Leeuwarden, The Netherlands

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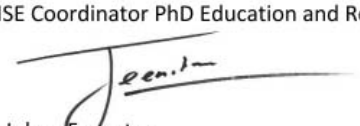

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