Analysis of Exoelectrogenic Bacterial Communities Present in Different Brine Pools of the Red Sea

Thesis by
Juan Fausto Ortiz Medina

In Partial Fulfillment of the Requirements
For the Degree of
Master of Science

King Abdullah University of Science and Technology, Thuwal, Kingdom of Saudi Arabia

(May, 2014)
The thesis of Juan Fausto Ortiz Medina is approved by the examination committee

Committee Chairperson: Pascal Elias Saikaly
Committee Member: Gary Lee Amy
Committee Member: Ulrich Stingl
Analysis of Exoelectrogenic Bacterial Communities Present in Different Brine Pools of the Red Sea

Juan Fausto Ortiz Medina

One contemporary issue experienced worldwide is the climate change due to the combustion of fossil fuels. Microbial Electrochemical Systems pose as an alternative for energy generation. In this technology, microorganisms are primarily responsible for electricity production. To improve the performance it is reasonable to think that bacteria from diverse environments, such as the brine pools of the Red Sea, can be utilized in these systems. Samples from three brine pools: Atlantis II, Valdivia, and Kebrit Deeps, were analyzed using Microbial Electrochemical Cells, with a poised potential at +0.2 V (vs. Ag/AgCl) and acetate as electron donor, to evaluate the exoelectrogenic activity by the present microorganisms. Only samples from Valdivia Deep were able to produce a noticeable current of 6 A/m². This result, along with acetate consumption and changes on the redox activity measured with cyclic voltammetry, provides arguments to confirm the presence of exoelectrogenic bacteria in this environment. Further characterization using microscopy and molecular biology techniques is required, to obtain the most amount of information about these microorganisms and their potential use in bioelectrochemical technologies.
ACKNOWLEDGEMENTS

I would like to thank all the people that helped me in the development of this project and the completion of this study. To Dr. Krishna Katuri, for all his knowledge about Bioelectrochemical Systems, the experience gained by working alongside him, and the support that he always provided, making me to be passionate in continuing on the research and development of these technologies. To Dr. Pascal Saikaly, for providing me the opportunity to work in his research group and guiding me on the development of this project, enhancing my knowledge about molecular biology and microbial community analysis. To Noura Shehab, which is also part of this project to apply it for her PhD studies. To my colleagues and friends on the MFC group and Red Sea Center: Rodrigo Jiménez, Craig Werner, Sirisha Kamireddy, Hari Anandarao, Manal Al-Qahtani and Yue Guan, for assisting me with knowledge and labwork whenever I needed. To the WDRC personnel and Lab Staff, especially Faisal Wali, Ahmed Kasmi, Ali Imran, and Riaan van der Merwe, for their support in the use of equipment and the following of laboratory practices. To all my friends, remarkably Flavio Siller, Laura Félix, Eriko Padrón, Daniel García, Adel Sarmiento, Malvina Vogler, Stephanie Saade, Ibrahim Gawish, Yasmeen Najm, Krystle Pertsch, Rodrigo Valladares, Liliana Pérez, Alejandro Durán, Octavio Salazar and Eduardo Gorrón, for all the encouragement and good friendship given to me during my term as Master Student. And finally, to my parents Fausto Ortiz and Ysabel Medina, and my sister Mónica Ortiz, for their infinite encouragement and love no matter where I am. I am highly confident that the job that is being done offers a really promising reward.
TABLE OF CONTENTS

Examination Committee Approval .................................................. 2
Copyright ....................................................................................... 3
Abstract .......................................................................................... 4
Acknowledgements .......................................................................... 5
List of Figures. .................................................................................. 8
List of Tables .................................................................................... 10
List of Abbreviations ........................................................................ 11
1 INTRODUCTION ........................................................................... 13
1.1 The Importance of Renewable Energies ..................................... 13
1.2 Environmental Biotechnology .................................................. 15
2 LITERATURE REVIEW ................................................................ 18
2.1 Microbial Electrochemical Systems ......................................... 18
  2.1.1 Microbial Fuel Cells ......................................................... 18
  2.1.2 Microbial Electrochemical Cells ................................. 20
  2.1.3 Microbial Desalination Cells ......................................... 21
2.2 Exoelectrogenic Bacteria .......................................................... 22
  2.2.1 Respiration process of living organisms .......................... 22
  2.2.2 Definition of exoelectrogenic bacteria ......................... 22
  2.2.3 Methods of electron transfer ........................................ 24
  2.2.4 Identification of exoelectrogenic microorganisms and commu-
      nities in the environment ...................................................... 26
2.3 The Red Sea .............................................................................. 29
2.4 The Brine Pools in the Red Sea ................................................ 30
  2.4.1 Identification of Brine Pools along the Red Sea ............. 30
  2.4.2 Physical and Chemical Characteristics .......................... 31
  2.4.3 Analysis on the microorganisms present in the Brine Pools .. 33
3 MATERIALS AND METHODS .................................................. 36
  3.1 Sample Collection .................................................................... 36
3.2 Determination of brine pools components ........................................ 36
  3.2.1 Metal content .............................................................. 36
  3.2.2 Anions content ............................................................. 37
  3.2.3 Organic carbon content .................................................. 37
  3.2.4 Bacterial count ........................................................... 38
3.3 Experimental Setup .............................................................. 38
  3.3.1 Reactor design and construction ........................................ 38
  3.3.2 Experimental arrangement and operation ............................... 39
3.4 Experimental measurements .................................................... 41
  3.4.1 Acetate concentration ..................................................... 41
  3.4.2 Sulfate concentration .................................................... 41
  3.4.3 Cyclic voltammetry ....................................................... 41
  3.4.4 Other sources of carbon ................................................ 42
4 RESULTS AND DISCUSSION .................................................. 43
  4.1 Characterization of Brine Pool samples .................................... 43
  4.2 Exoelectrogenic bacteria in Atlantis II Deep ............................. 45
  4.3 Exoelectrogenic bacteria in Kebrit Deep ................................ 48
  4.4 Exoelectrogenic bacteria in Valdivia Deep ............................... 51
  4.5 Factors influencing the growth of exoelectrogenic bacteria .......... 56
  4.6 Future work ..................................................................... 57
5 CONCLUSION ..................................................................... 59
References ............................................................................. 61
Appendices ............................................................................ 65
LIST OF FIGURES

1.1 Global carbon dioxide (CO$_2$) emissions from fossil fuel burning. 14
1.2 Proportion of the energy sources used in the global energy demand, as of 2008 14
2.1 Basic model of a Microbial Fuel Cell (MFC). 19
2.2 Basic model of a Microbial Electrolysis Cell (MEC). 20
2.3 Basic model of a Microbial Desalination Cell (MDC). 21
2.4 Potential losses occurring in the transfer of electrons from the electron donor to the anode. 24
2.5 Main recognized mechanisms of electron transfer. 25
2.6 Location of the Red Sea and its limits. 29
2.7 Geographic location of the different brine pools on the Red Sea 31
3.1 Graphical representation of the Microbial Electrolysis Cell (MEC) used for the experiment. 39
3.2 Reactors spatial setup for the experiment. 40
4.1 Cyclic voltammogram of Atlantis II reactors at initial time. 45
4.2 Chronoamperometry results for the samples for Atlantis II Deep. 46
4.3 Acetate concentration over time on Atlantis II reactors. 47
4.4 Sulfate concentration of Atlantis II reactors after two months of operation, compared with the obtained initial value. 47
4.5 Cyclic voltammogram of the reactors with Atlantis II Deep samples after 30 days of operation. 48
4.6 Cyclic voltammogram of Kebrit reactors at initial time. 48
4.7 Chronoamperometry results for the samples for Kebrit Deep. 49
4.8 Acetate concentration over time on Kebrit reactors. 50
4.9 Sulfate concentration of Kebrit reactors after two months of operation, compared with the obtained initial value. ........................................ 50
4.10 Cyclic voltammogram of the reactors with Kebrit Deep after 30 days of operation. ................................................................. 50
4.11 Cyclic voltammogram of Valdivia reactors at initial time. .............. 51
4.12 Chronoamperometry results for the samples for Valdivia Deep. ....... 52
4.13 Acetate concentration over time on Valdivia reactors. .................... 53
4.14 Sulfate concentration of Valdivia reactors after two months of operation, compared with the obtained initial value. ....................... 53
4.15 Cyclic voltammogram of the reactors with Valdivia Deep sample after 30 days of operation. ......................................................... 54
4.16 TOC measurements of Valdivia reactors after two months of operation, compared with the obtained initial value. ....................... 55
4.17 HPLC chromatogram with the detected volatile fatty acids in Valdivia brine pool samples. .......................................................... 55
LIST OF TABLES

1.1 Advantages and Limitations of Microbial-based Bioenergies . . . . . . . 16

2.1 Common redox half reactions occurring in Microbial Fuel Cells (MFCs) 23

2.2 Reported physical parameters and chemical composition of the studied
  Brine Pools. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 33

4.1 Analyzed parameters of the studied Brine Pools. . . . . . . . . . . . . 44
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AEM</td>
<td>Anion Exchange Membrane</td>
</tr>
<tr>
<td>ARB</td>
<td>anode-respiring bacteria</td>
</tr>
<tr>
<td>CCD</td>
<td>charge-coupled device</td>
</tr>
<tr>
<td>CEM</td>
<td>Cation Exchange Membrane</td>
</tr>
<tr>
<td>CH₄</td>
<td>methane</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>CTC</td>
<td>c-type cytochrome</td>
</tr>
<tr>
<td>CV</td>
<td>Cyclic voltammetry</td>
</tr>
<tr>
<td>DGGE</td>
<td>denaturing gradient gel electrophoresis</td>
</tr>
<tr>
<td>EPS</td>
<td>exopolymeric substance</td>
</tr>
<tr>
<td>Ewe</td>
<td>potential of the working electrode</td>
</tr>
<tr>
<td>Fe(CN)₆³⁻</td>
<td>ferricyanide</td>
</tr>
<tr>
<td>GHG</td>
<td>Greenhouse Gas</td>
</tr>
<tr>
<td>H₂</td>
<td>hydrogen gas</td>
</tr>
<tr>
<td>H₂S</td>
<td>Hydrogen Sulfide</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>ICP-OES</td>
<td>Inductively coupled plasma optical emission spectrometry</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>KAUST</td>
<td>King Abdullah University of Science and Technology</td>
</tr>
<tr>
<td>MDC</td>
<td>Microbial Desalination Cell</td>
</tr>
<tr>
<td>MEC</td>
<td>Microbial Electrolysis Cell</td>
</tr>
<tr>
<td>MFC</td>
<td>Microbial Fuel Cell</td>
</tr>
<tr>
<td>MRM</td>
<td>Microbial Resource Management</td>
</tr>
<tr>
<td>MXC</td>
<td>Microbial Electrochemical Cell</td>
</tr>
<tr>
<td>NHE</td>
<td>Normal Hydrogen Electrode</td>
</tr>
<tr>
<td>NO$_3$</td>
<td>nitrate</td>
</tr>
<tr>
<td>NPOC</td>
<td>Non-purgeable Organic Carbon</td>
</tr>
<tr>
<td>O$_2$</td>
<td>oxygen</td>
</tr>
<tr>
<td>OCV</td>
<td>Open Circuit Voltage</td>
</tr>
<tr>
<td>OLR</td>
<td>Organic Loading Rate</td>
</tr>
<tr>
<td>OMC</td>
<td>Outer Membrane Cytochrome</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>ppm</td>
<td>parts per million</td>
</tr>
<tr>
<td>RSRC</td>
<td>Red Sea Research Center</td>
</tr>
<tr>
<td>SEM</td>
<td>scanning electron microscopy</td>
</tr>
<tr>
<td>SHE</td>
<td>Standard Hydrogen Electrode</td>
</tr>
<tr>
<td>TOC</td>
<td>Total Organic Carbon</td>
</tr>
<tr>
<td>VFA</td>
<td>volatile fatty acids</td>
</tr>
<tr>
<td>WDRC</td>
<td>Water Desalination and Reuse Center</td>
</tr>
<tr>
<td>WHOI</td>
<td>Woods Hole Oceanographic Institution</td>
</tr>
</tbody>
</table>
1.1 The Importance of Renewable Energies

One contemporary issue that the world faces is climate change due to global warming. The increase of gases such as carbon dioxide (CO$_2$) and methane (CH$_4$) in the atmosphere, due to anthropogenic activities, has led to an apparent rise on Earth’s mean temperature. The possible effects that global warming is projected to have on the planet are, among others, the rise on the sea level, ocean acidification and more extreme weather [1].

The major cause of climate change is the emission of the aforementioned gases, known as Greenhouse Gases (GHGs), due to the energy required for daily human and industrial activities. This energy supply is mainly done through the combustion of fossil fuels, which are responsible for the increased amount of emissions of CO$_2$ (Figure 1.1). Currently, according to Moomaw et al. [2], 85% of current primary energy driving global economies comes from the combustion of fossil fuels, and consumption of fossil fuels account for 56.6% of all anthropogenic GHG emissions.
Because of this relationship between the utilization of fossil fuels and its effect on the experienced climate change, renewable means of energy generation have been developed. Direct solar energy, geothermal energy, hydropower, wind energy, and bioenergy are among them; however, their use only represents 12.9% of the global energy supply (Figure 1.2)[3]. For this reason, it is necessary to optimize the technology used for these sources of energy so they can pose as viable alternatives against the use of fossil fuels.
1.2 Environmental Biotechnology

Biotechnology, as defined by Frazier and Geiss [4] is the use of biological systems for the improvement of the health and welfare of the mankind. One of the mentioned alternatives for renewable energy generation is bioenergy, which is obtained using biological sources such as plants and microorganisms. Therefore, this technology is covered within the scopes of Biotechnology, more specifically, by Environmental Biotechnology.

According to Verstraete [5], Environmental Biotechnology includes the microbial resources present in the water, air, and soil. These microorganisms can be harnessed to be used in a variety of processes such as wastewater treatment, remediation of hazardous contaminants, and production of environmentally benign chemicals[6]. Also, problems such as climate change can be mitigated by developing technologies for CO$_2$ sequestration, prevention of CH$_4$ emissions and, as contemplated before, alternate energy produced by living organisms.

Diverse technologies for energy generation using microorganisms have been conceived. They are considered renewable energies because they utilize organic matter to produce energy. This matter is recycled when the CO$_2$ generated during combustion is fixed by plants. The most representative of these technologies are: the anaerobic digestion of organic waste to produce methane as a combustion gas, the cultivation of photosynthetic microorganisms to generate biodiesel, and the degradation of organic waste to produce electricity in MFCs. The latter technology will be further explored in consequent chapters. These technologies and the advantages and limitations currently experienced are shown in Table 1.1.
Table 1.1: Advantages and Limitations of currently developed Microbial-based Bioenergies. Adapted from Rittmann [7]

<table>
<thead>
<tr>
<th>Energy option</th>
<th>Advantages</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anaerobic digestion to produce methane</td>
<td>Mature technology; can use residues and wastes, turning a pollution problem into an energy resource; CH$_4$ technology is in place</td>
<td>Conversion efficiency is not yet high enough; unit cost is higher than from natural-gas deposits today</td>
</tr>
<tr>
<td>Biofuel from phototrophic microorganisms</td>
<td>Biodiesel is a high-density fuel that substitutes ideally for petroleum; possible to have high-yield per unit area; allowing TW output; does not compete with food crops</td>
<td>Technology is at an early stage, may require a significant capital investment</td>
</tr>
<tr>
<td>MFCs to generate electricity from organic waste</td>
<td>Electricity infrastructure is in place; and MFC is a combustionless, pollution-free fuel cell technology that uses renewable organic fuel directly</td>
<td>Technology is nascent; conversion efficiency is not established</td>
</tr>
</tbody>
</table>

The importance of the microbial communities in the development of these technologies is evident. Therefore, it is necessary to understand the dynamics within these communities, and to look at the same time for new sources that can provide useful microorganisms. These objectives are defined by Verstraete [5] within the term Microbial Resource Management (MRM). Since microbial communities are likely to follow a Pareto distribution, where 20% of the total microbial species perform 80% of the functions of the community [5], rare microorganisms that can perform desirable duties in energy production could be found in most of the habitats.
Therefore, focusing this work on the technology known as Microbial Fuel Cells (MFCs), it is to be proven that environmental habitats that are largely unstudied, such as the brine pools of the Red Sea, can harbor microbial organisms that can perform efficient electricity generation. The scope of this document is to analyze samples from different brine pools, detecting the presence of exoelectrogenic bacterial communities with diverse analytical techniques. They can be further characterized and utilized in subsequent studies and applications.
CHAPTER 2
LITERATURE REVIEW

2.1 Microbial Electrochemical Systems

2.1.1 Microbial Fuel Cells

Microbial Fuel Cells (MFCs) are one of the promising technologies for renewable energy generation using biological sources. According to Rabaey and Verstraete [8], an MFC converts energy, available in a bio-convertible substrate, directly into electricity. This can be achieved when bacteria switch from the natural electron acceptor, such as oxygen (O$_2$) or nitrate (NO$_3$), to an insoluble component such as the MFC anode. The bacteria will use it as a way of respiration in order to obtain energy for their basic metabolic functions. Meanwhile, the electrons deposited in the anode travel through a resistor in order to reach the cathode, completing the circuit. In a basic configuration, the cathode can be either be exposed to the air or submerged into aerobic water. When the protons generated in the respiration process also travel to the cathode chamber, water is formed [9]. Electric current is generated in the end because of this flow of electrons. A basic scheme of the usual components of an MFC is shown on Figure 2.1.
Figure 2.1: Basic model of a Microbial Fuel Cell (MFC).

The advantages of MFCs over other methods of energy production are diverse. Since bacteria are self-replicating, the degradation of organic matter is done without the need of replenishing catalysts. A wide range of temperature growth conditions and sources of biodegradable organic matter, such as wastewater, can be used in an MFC; examples include volatile acids, carbohydrates, proteins, alcohols, and recalcitrant materials like cellulose [10]. Also, the direct transformation of substrate energy into electricity enables high conversion efficiency, and their design enable them to be used in locations lacking electrical infrastructures [8].

Nevertheless, the application of MFCs is still limited to laboratory scale, and a lot of improvements must to be developed for the technology to be feasibly applied in larger demanding markets. The most important ones are: to develop cost-effective materials and new designs that can be used in large scale applications; to understand the conditions for the bacteria to grow favorably, such as pH, temperature and media; and to develop models that can calculate optimal Organic Loading Rates (OLRs) and mass transfer coefficients to increase the output production obtained from the organic substrates added to the reactors [11].
2.1.2 Microbial Electrochemical Cells

A Microbial Electrolysis Cell (MEC) is a different configuration of an MFC. While the basic components are mostly the same as every other electrochemical cell (i.e. a circuit with an anode and cathode), in the MEC the oxygen is completely removed and a small voltage (around 0.2 V) is applied between the electrodes. This allows to produce hydrogen gas in the cathode through the reduction of protons [12]. A basic design for an MEC is shown on Figure 2.2.

![Figure 2.2: Basic model of a Microbial Electrolysis Cell (MEC). An external power is applied to produce hydrogen gas in the cathode.](image)

The main advantage of using MECs is that the output is hydrogen gas (H\textsubscript{2}), a transportable fuel that can be used to produce energy without combustion and emission of GHGs. Also, using an MEC overcomes several problems associated with MFCs, the most prominent being the intrusion of O\textsubscript{2} in the anode chamber and the high potential loss for reducing this gas at the cathode [7].

However, the challenges experienced by MFCs are also applied to MECs in terms of reactor design and understanding the bacterial processes. In addition to that, the produced gas must have the least contact with the bacteria to avoid consumption of H\textsubscript{2}, and the capture and purification of the evolved hydrogen gas is a complicated and energy intensive process [10]. Consequently, MECs need to be further studied in
order to develop a truly sustainable way of producing this energy source.

2.1.3 Microbial Desalination Cells

Another variant of the MFCs are Microbial Desalination Cells (MDCs). These systems, as described by Cao et al. [13], consist of three chambers, with an Anion Exchange Membrane (AEM) next to the anode (forming the anodic chamber), a Cation Exchange Membrane (CEM) by the cathode (delimiting the cathode chamber), and a middle chamber between the membranes filled with water to be desalinated. Due of the degradation of organic matter by the bacteria present in the anode, the generated current leads to an accumulation of positive charges (protons) in the anode chamber, so the anions are diffused through the AEM. Simultaneously, the protons in the cathode chamber are consumed to produce water as in an MFC, so the chamber becomes negatively charged and the cations of the saline water are moved through the CEM. A basic design of an MDC is shown on Figure 2.3.

![Figure 2.3: Basic model of a Microbial Desalination Cell (MDC). While electric current is generated by the degradation of organic matter, the ions are moved to their respective chambers, desalinating the water in the middle chamber.](image)

While this technology is very promising to treat wastewater and produce desalinated water for consumption at the same time, there are still areas for improvement. Besides the inherent development that needs to be done as with the rest of Microbial Electrochemical Cells (MXCs), some specific problems that need to be addressed are
the substantial pH changes in the electrode chambers, the tradeoff between expecting 
high current density or high desalination rates, and the inhibition of bacterial activity 
at high salinities [14].

2.2 Exoelectrogenic Bacteria

2.2.1 Respiration process of living organisms

The respiration process of all living organisms is a transfer between an electron donor 
(usually a carbon source) and an electron acceptor that is diffused into the cells. This 
energy is calculated by the equation

\[
\Delta G^\circ = -nF(E_{\text{donor}}^\circ - E_{\text{acceptor}}^\circ)
\]

where \(\Delta G^\circ\) is the change of Gibbs free energy at pH = 7 and 25°C, \(F\) is Faraday 
constant (96,485 Cmol\(^{-1}\)e\(^{-}\)) and \(\Delta E^\circ\) is the biological standard potential of the 
electron donor and acceptor [15]. Therefore, if the difference between the donor and 
the acceptor is greater, the amount of available energy will be increased as well.

2.2.2 Definition of exoelectrogenic bacteria

The most important factors in the operation of MXCs are the bacteria that colonize 
the electrode, defined as exoelectrogenic bacteria or anode-respiring bacteria (ARB). They have the capability of extracellularly transfer the electrons gained by the respi-
ration process towards an exogenous electron acceptor.

Based on this concept, exoelectrogenic bacteria can transfer electrons to the anode, 
which will act as an insoluble electron acceptor to complete the oxidation of the 
electron donor. Therefore, the energy gained by the bacteria will be the difference of 
the potential between the electron donor and the electrode, \(E_{\text{donor}}^\circ - E_{\text{anode}}^\circ\). The 
electron acceptor present in the cathode chamber, which normally is either oxygen,
ferricyanide \((\text{Fe}(\text{CN})_6^{3-})\), or protons (in the case of MECs), will become reduced by accepting the electrons that moved through the circuit. The maximum energy that an MFC can generate is calculated in an analogous way to the energy gained by microorganisms, based on the difference of the potentials between the electron donor and acceptor’s redox reactions, i.e. \(E^\circ_{\text{anode}} - E^\circ_{\text{cathode}}\). Table 2.1 shows commonly used electron donors and acceptors occurring in the electrodes of an MFC and their given half-reaction potentials.

Table 2.1: Common redox half reactions occurring in MFCs. By IUPAC nomenclature, all reactions are written towards reduction of species. Adapted from Logan [10]

<table>
<thead>
<tr>
<th>Electrode</th>
<th>Reaction</th>
<th>(E^\circ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anode</td>
<td>(2\text{H}^+ + 2\text{e}^- \rightarrow \text{H}_2)</td>
<td>-0.414</td>
</tr>
<tr>
<td></td>
<td>(\text{CO}_2 + \text{HCO}_3^- + 8\text{H}^+ + 8\text{e}^- \rightarrow \text{CH}_3\text{COO}^- + 3\text{H}_2\text{O})</td>
<td>-0.284</td>
</tr>
<tr>
<td></td>
<td>(6\text{CO}_2 + 24\text{H}^+ + 24\text{e}^- \rightarrow \text{C}_6\text{H}_12\text{O}_6 + 6\text{H}_2\text{O})</td>
<td>-0.428</td>
</tr>
<tr>
<td>Cathode</td>
<td>(\text{O}_2 + 4\text{H}^+ + 4\text{e}^- \rightarrow 2\text{H}_2\text{O})</td>
<td>0.805</td>
</tr>
<tr>
<td></td>
<td>(\text{O}_2 + 2\text{H}_2 + 2\text{e}^- \rightarrow \text{H}_2\text{O}_2)</td>
<td>0.328</td>
</tr>
<tr>
<td></td>
<td>(\text{Fe}(\text{CN})_6^{3-} + \text{e}^- \rightarrow \text{Fe}(\text{CN})_6^{4-})</td>
<td>0.361</td>
</tr>
<tr>
<td></td>
<td>(\text{MnO}_2(s) + 4\text{H}^+ + 2\text{e}^- \rightarrow \text{Mn}^{2+} + 2\text{H}_2\text{O})</td>
<td>0.470</td>
</tr>
<tr>
<td></td>
<td>(\text{Fe}^{3+} + \text{e}^- \rightarrow \text{Fe}^{2+})</td>
<td>0.200</td>
</tr>
</tbody>
</table>

However, it is worth mentioning that there are losses that prevent the MFC to reach its maximum current generation capability, known as potential losses [16]. The most evident is the loss that occurs within the exoelectrogens, which is the difference between \(E^\circ_{\text{donor}}\) and the final potential reached in the electron transport chain in the outer membrane, defined as \(E^\circ_{OM}\). This potential loss represents the energy
gained by the bacteria to perform their metabolic functions. Also, there can be losses between the outer membrane and the interface before the anode is reached ($E_{\text{interface}}^o$), whose analogous energy is mostly dissipated as unusable energy such as heat. These definitions are illustrated in Figure 2.4. In summary, the anode will have a higher potential than $E_{\text{donor}}^o$ which is translated as less difference between $E_{\text{anode}}^o$ and $E_{\text{cathode}}^o$. It is necessary then to further understand the mechanisms occurring in the system in order to reach an optimum equilibrium between the energy available for bacterial growth and for electricity generation.

![Potential losses occurring in the transfer of electrons from the electron donor to the anode. The losses are the difference between certain potential and the next. From Torres et al. [16].](image)

2.2.3 Methods of electron transfer

There are three proposed methods in which exoelectrogenic bacteria transfer their electrons to the anode: through direct contact of the bacterial cells with the electrode, with the help of soluble electron shuttles, and by solid conductive components, such as nanowires, in a biofilm formation [16]. These mechanisms are illustrated in Figure 2.5 and discussed in the following paragraphs.
Direct contact of the exoelectrogenic bacteria with the electrode is possible because of the proteins that receive the electrons in the surface of the cells, such as Outer Membrane Cytochromes (OMCs). One family of these OMCs, called c-type cytochromes (CTCs), are heme-containing proteins that are located mostly in the outer cellular membrane and are part of the electron transport chain [17]. While the direct electron transfer through OMCs allows for few potential loses between the outer membrane and the anode, the current density is severely limited due to the very amount of electrochemically active bacteria on the anode surface[16], allowing only the bacteria that surround the electrode (a monolayer biofilm) to be able to produce electric current.

The second mechanism that exoelectrogenic bacteria can employ is using molecules known as 'electron shuttles'. They are small mediator compounds that receive the electrodes that are transported through respiration, and are secreted to reach the anode and transfer the electrons. According to Yang et al. [17], proper electron shuttles must be dissolvable, stable, reusable, environment-friendly, and to have a proper potential. Although these mediators can be added exogenously to facilitate
the transfer of electrons, electron shuttles that are generated inside the cells prove to be as effective and more sustainable in the operation of MFCs [18]. Examples of molecules endogenously produced to act as electron shuttles are flavins and phenazins [17]. While this method allows more bacteria to be able to transfer electrons, the produced current is limited because of the slow diffusion of reduced/oxidized electron shuttles [16], requiring high shuttle concentrations in order to overcome the mass transport limitations.

The third and most recently proposed method of electron transfer by exoelectrogens is the solid conductive matrix. The discovery of bacterial nanowires (electrically conductive pili [17]) and the capability of exopolymeric substances (EPSs) to act as semiconductors [16], allow bacteria forming a thick biofilm to transport the electrons through the formed matrix, the rate of transfer only being limited by its conductivity. Torres et al. [16] and Yang et al. [17] recognize this method as the most efficient to get high current densities and optimum bacterial growth; although the molecular composition of the nanowires and the matrix, and a clear understanding of the conditions that favor this mechanism, need to be further studied.

2.2.4 Identification of exoelectrogenic microorganisms and communities in the environment

One of the challenges in the operation of MFCs is to identify and understand the exoelectrogenic microorganisms that can operate in the reactors either as pure cultures or being part of a microbial community. Since the discovery, more than a hundred years ago, of the electrical potentials that bacteria and yeast can generate [19], the development of MXCs have encouraged the research on diverse sources of microorganisms that can be used in these systems. For this reason, a wide range of inocula from distinct environmental sources has been employed to assess their performance on the electrochemical systems.
One of the first approaches to understand the capability of bacteria to produce electricity was done by Roller et al. [20] using different pure cultures along with added mediators to improve the performance of the MFCs. The bacteria tested were *Alcaligenes eutrophus*, *Azotobacter chroococcum*, *Bacillus subtilis*, *Escherichia coli*, *Proteus vulgaris*, *Pseudomonas aeruginosa* and *Pseudomonas putida*; and the mediators added were dyes such as thionine, methylene blue, brilliant cresyl blue and benzyl viologen. Even though *E. coli* and *P. vulgaris* appeared to be the most efficient in the systems, the use of external mediators is discouraged because many of these mediators are toxic to the microorganisms, exerting adverse effects on the microbial population, and generating environmental issues related to their disposal [21].

It was later discovered by Chang et al. [21] that MFCs can operate without adding external mediators. By using cultures isolated from environments such as a rice paddy field, the bottom soil of a river, and an anaerobic septic tank of a sewage treatment work, the authors found effective current generation with bacteria that belonged to the iron-reducing species *Shewanella putrefaciens*. These bacteria are known to be incapable of absorbing electron acceptors into the cell, therefore the electrons are transferred to an OMC instead, reducing ferric ions [21].

In a similar strategy, Reimers et al. [22] discovered that an anode placed in marine sediments, and a cathode in the overlaying water, generate electrical current due to biological activity. Bond et al. [23] were subsequently able to identify that more than 70% of the bacteria colonizing the anode belonged to the class Deltaproteobacteria. This presence was mostly due to bacteria belonging to the family *Geobacteraceae*, which is known to anaerobically couple the oxidation of organic compounds with the reduction of insoluble ferric ions as well. It can be seen from the previous two studies that iron reduction may be a trait that exoelectrogenic bacteria possess in the environment, though not all the iron reducers can utilize a conductive anode
for respiration, suggesting that the mechanisms for extracellular electron transfer to ferric oxides and anodes are different [24].

Since then, a wide range of exoelectrogenic microbial communities have been found in the operation of MFCs. The performed analyses show that there is not a predominant organism responsible for electricity generation, mainly because of the diversity of microorganisms with this trait. Also the range of operating conditions, system architectures, electron donors and electron acceptors play a role in the diversity of results [25]. Among these organisms, electricity generation has been shown on four of five classes of Proteobacteria, the Firmicutes and Acidobacteria phyla, cyanobacteria such as _Synechocystis_ sp. PCC 6803, and even a species of yeast, _Pichia anomala_ [26].

It is interesting to note that many new pure cultures with electrode-reducing capabilities are obtained because of the selective pressure by the anode. Its surface might confer an energetic advantage even in fermentative habitats or in the presence of other electron acceptors [27]. Taking this role into consideration, some strategies have been applied into MXCs to enrich the presence of exoelectrogenic microorganisms in the reactors. One of the most commonly used ones is to poise the electric potential of the anode. This potential is analog to the concentration of a soluble electron acceptor in a conventional respiration process [16]. Therefore, regulating it with the help of a potentiostat and a reference electrode can create favorable conditions for certain type of bacteria capable of growth at that poised value.

Based on this approach, Miceli et al. [28] utilized single-chambered MECs with a poised potential of -0.3 V (vs Ag/AgCl) in order to enrich efficient communities of exoelectrogenic bacteria from diverse anaerobic environments, including mangrove swamps, anaerobic soils, a saline microbial mat, and an iron rich soil. Besides isolating commonly known exoelectrogens such as _Geobacter_, species belonging to other genus such as _Geoalkalibacter_ and _Desulfonispora_ were identified. This suggests that
there is the possibility of finding new communities of anode-respiring bacteria from environmental sources by running MXCs with a poised potential. Therefore, it is reasonable to think on a similar approach by utilizing MECs with poised potential to obtain exoelectrogenic communities in nearby environments such as the Red Sea and its brine pools.

2.3 The Red Sea

The Red Sea is a section of the Indian Ocean that is located between the African and Asian continents. Its approximate coordinates are in the parallels 27°N to 12°N and meridians 32°E to 44°E, extending approximately 1,900 km [29]. It is bordered on its west side by Egypt, Sudan, Eritrea and Djibouti; and on its east side by Saudi Arabia and Yemen (Figure 2.6). The sea has a relatively recent formation, originated by the separation of the African and Arabian tectonic plate systems approximately 32 million of years ago [30]. Because it is surrounded by desert and semi-desert areas, and does not receive any freshwater inlet, the mean temperature and salinity are rather high compared to other seawater bodies: 40°C and 39 PSU, respectively [29].

Figure 2.6: Location of the Red Sea and its limits. Obtained from Google Maps [31].
2.4 The Brine Pools in the Red Sea

2.4.1 Identification of Brine Pools along the Red Sea

One of the most intriguing characteristics of the Red Sea is the presence of high-salinity deposits of seawater in various deeps, being defined as deep-sea anoxic brines or brine pools. According to Antunes et al. [30], these brine pools are formed by a process of re-dissolution of evaporitic deposits buried at shallow depths, tectonic expulsion of interstitial brine associated with this evaporites and/or by hydrothermal phase separation. The salt-enriched waters migrate to the sea floor and accumulate in enclosed geographical depressions, which remain relatively stable as a result of their high density.

Since the confirmed discovery in 1964 of the first brine pool, Atlantis II, by Miller [32] and Charnock [33], there have been numerous discovered brine pools that are mostly distributed along the rift axis of the Red Sea. They possess different physical and chemical characteristics, varying within close distances. The brine pools discovered as of 2011 are shown in Figure 2.7.
2.4.2 Physical and Chemical Characteristics

The most distinguishable feature of the brine pools is the hypersaline water, reaching values as high as 250 PSU [30]. This, along with the fact that most deeps are anoxic, dense, and possess a higher temperature than the normal seawater (up to 46°C) [35], makes in conjunction one of the most extreme environments for living organisms to thrive. In addition to these factors, all brine pools form characteristically sharp brine-seawater interfaces with typically steep gradients of salinity, temperature, density, O$_2$ and pH [30]. Therefore, a single deep can have as much micro-environments as the number of interfaces the column of water is comprised of, with differing physical and chemical characteristics within. This document will emphasize the characteristics of Atlantis II, Valdivia and Kebrith deeps, the brine pools where the samples for this work were obtained from. The information about their physical parameters and the concentration of their main chemical components are shown on Table 2.2.
The Atlantis II Deep is the largest deep-sea brine pool of the Red Sea [30]. It is located in the middle of the axial rift of the Red Sea (Figure 2.7) at a depth of 2194 m. Its main characteristic is that it is hydrothermically active, showing continuously an increase in temperature from the earliest record of 44.8°C to the last reported temperature of 68.2°C because of the influx of hot brine supplied by a geyser spring at the bottom [36]. According to Antunes et al. [30], besides presenting a high salinity of 25.7% (wt.vol) NaCl, Atlantis II has a high concentration of metalliferous sediments, highly enriched in iron, copper, zinc, and other heavy metals. It is also a highly acidic brine pool with a pH value of 5.3.

In the case of the Kebrit Deep, it is one of the smallest brine pools of the Red Sea, located in its northern section (Figure 2.7) and having a maximum depth of 1549 m. Compared with Atlantis II, its temperature is noticeably smaller (up to 23.3°C), but with comparable pH and salinity (5.5 and 26%, respectively) [30]. It is characterized for having substantial amounts of Hydrogen Sulfide (H₂S), up to 14 mg S (Sulfur) per liter [37]. Unlike Atlantis II, its temperature and physical and chemical structures has remained almost constant over time, suggesting that there is no tectonic or volcanic/hydrothermal activity [38]. Also, as it can be seen in Table 2.2, there is not sufficient reported data about many ions and metals present in comparison with the other deeps.

Regarding Valdivia Deep, it is positioned within the same basin as Atlantis II, but encased in a differently located depression that reaches 1,673 m in depth [34]. While it presents a similar salinity of 24.2%, its temperature at the bottom reaches 33.68°C [39]. Its pH is higher than the latter two deeps, reaching a value of 6.21. The most distinguishable feature is the high concentrations of sulfate and magnesium [30]. Also, according to Anschutz et al. [39], the temperature and salinity of Valdivia has increased over the years, suggesting that there is hydrothermal activity similar to Atlantis II.
Table 2.2: Physical parameters and chemical composition of the studied Brine Pools. NR = not reported in the literature. Information obtained from Antunes et al. [30], Anschutz et al. [39], and Backer and Schoell [34].

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Atlantis</th>
<th>Valdivia</th>
<th>Kebrit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na (M)</td>
<td>5.1</td>
<td>4.1</td>
<td>NR</td>
</tr>
<tr>
<td>Cl (M)</td>
<td>5.5</td>
<td>4.6</td>
<td>5.1</td>
</tr>
<tr>
<td>Ca (mM)</td>
<td>152.4</td>
<td>25.0</td>
<td>42.4</td>
</tr>
<tr>
<td>Mg (mM)</td>
<td>36.9</td>
<td>95.3</td>
<td>98.8</td>
</tr>
<tr>
<td>K (mM)</td>
<td>76.9</td>
<td>52.0</td>
<td>NR</td>
</tr>
<tr>
<td>SO$_2^-$ (mM)</td>
<td>10.8</td>
<td>72.0</td>
<td>22.9</td>
</tr>
<tr>
<td>B (mM)</td>
<td>1.2</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Sr (mM)</td>
<td>0.7</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Fe (µM)</td>
<td>1.6</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Mn (µM)</td>
<td>1.8</td>
<td>0.1</td>
<td>165.0</td>
</tr>
<tr>
<td>Zn (µM)</td>
<td>165.7</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Cu (µM)</td>
<td>6.3</td>
<td>traces</td>
<td>NR</td>
</tr>
<tr>
<td>Li (µM)</td>
<td>563.4</td>
<td>98.1</td>
<td>NR</td>
</tr>
<tr>
<td>Ba (µM)</td>
<td>10.9</td>
<td>0.5</td>
<td>NR</td>
</tr>
<tr>
<td>Rb (µM)</td>
<td>25.3</td>
<td>3.1</td>
<td>NR</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>68.2</td>
<td>33.7</td>
<td>23.3</td>
</tr>
<tr>
<td>pH</td>
<td>5.21</td>
<td>6.21</td>
<td>5.5</td>
</tr>
</tbody>
</table>

2.4.3 Analysis on the microorganisms present in the Brine Pools

Due to the extreme conditions present in the brine pools, early studies failed to find microorganisms using cultivation methods. This led to report the environment as sterile due to the synergy between high temperature, salinity and metal concentrations [40]. Nevertheless, with the advance in molecular biology tools to characterize microbial ecology, many microbial communities have been phylogenetically characterized and even some microbes have been isolated from the sediments, the overlaying water and the brine-seawater interface [30].
Most of the microbiological studies done so far have focused on Atlantis II Deep and the closely located Discovery Deep. Employing anaerobic culture enrichment of an Atlantis II sample on artificial media, Fiala et al. [41] achieved the isolation of *Flexistipes sinussarabici*. These bacteria were found to belong to a new phylum *Deferribacteres*, becoming the first fully described microbe from deep-sea anoxic brine pools [30].

Differences in the microbial components along the column of water in Atlantis II were addressed by Siam et al. [42] using 16S rDNA pyrosequencing. In the deepest layers, the abundance of pyrotags related to archaeal and bacterial sulfate-reducing genera (such as *Archaeoglobus* and *Desulfovermiculus* sp.) and anaerobic methane-oxidizing archaeal clusters (*Methanosarcinales* and *Methanomicrobiales*) made it possible to hypothesize on a community capable of sulfate reduction coupled with anaerobic oxidation of methane [42]. Also, Alphaproteobacteria was defined as the dominant bacterial class. Using similar methods, Bougouffa et al. [36] found instead a dominance of the class Gammaproteobacteria, mainly from the orders *Alteromonadales* and *Oceanospirillales*. Furthermore, Wang et al. [43] also performed the same strategy, in order to find the most probable metabolic pathways along the community, with their results establishing Betaproteobacteria as the dominant class of Atlantis II. Along with that, they concluded that the microbes present in this deep were generally heterotrophic bacteria that depended heavily on extracellular organic carbon like aromatic compounds, non-aromatic heterocyclic compounds, amino acids, storage sugars and structural sugars on the surrounding water.

The microbiota of Kebrit Deep was one of the first analyzed, though with the same scarcity as the rest of the brine pools. By cloning and sequencing Polymerase Chain Reaction (PCR) products of 16S rRNA genes, Eder et al. [44] were able to find a novel phylogenetic lineage in the sediments of this deep, denominated KB1 group, which is situated closer to the orders *Aquificales* and *Thermotogales*. Also, from the brine-
seawater interface, two halophilic isolates (KT-2/3-3 and KT-8-13) were isolated and cultivated, and later shown to belong to the family *Halanaerobiaceae* [37]. In contrast with the other brine pools, there is not a microbial community analysis on Valdivia Deep published so far.

These studies suggest that, in spite of the scarcity of analyses on the microbial communities of the brine pools of the Red Sea, some novel microorganisms have been found and there are some dynamic changes in the present microbiota, opening further the possibility of finding species capable of being exoelectrogenic. Therefore, by utilizing an approach similar to Miceli et al. [28], microbial electrochemical systems will be run to enrich the presence of exoelectrogenic bacteria from the Atlantis II, Valdivia and Kebrit brine pools of the Red Sea.
CHAPTER 3
MATERIALS AND METHODS

3.1 Sample Collection

The environmental samples for the project were obtained from the Red Sea Research Center (RSRC) of King Abdullah University of Science and Technology (KAUST). They were acquired during the second joint expedition of the Woods Hole Oceanographic Institution (WHOI) and KAUST, sailing on the R/V Aegeo in October 2011. Samples from different brine pools located in the Red Sea were collected, anaerobically, in 50 L containers. Afterwards, they were stored at 4°C inside the RSRC main laboratory. From these containers, 500 mL of sample from Atlantis II, Valdivia and Kebrit brine pools were obtained using anaerobic bottles, then stored at 4°C in the Water Desalination and Reuse Center (WDRC) facilities.

3.2 Determination of brine pools components

3.2.1 Metal content

In order to validate the chemical components of the brine pools and to address any possible change of composition due to hydrothermal activity [39], the samples were subjected to different analyses. To calculate the concentration of the most significant metals, Inductively coupled plasma optical emission spectrometry (ICP-OES) was done utilizing an Optima 8300 ICP-OES Spectrometer (Perkin Elmer), equipped with a custom designed solid-state charge-coupled device (CCD) array detector. The
samples were analyzed by the WDRC Laboratory Staff, using direct inductively coupled plasma analysis on axial view to detect elements present in parts per million (ppm), and in pre-concentration mode for trace elements.

3.2.2 Anions content

The anions present in the brine pools, mainly the concentration of chloride and sulfate, were analyzed utilizing ion chromatography. The samples were filtered through 0.45 \( \mu m \) syringe filters (Corning Incorporated), and then diluted by 1:2000 for the anions’ concentration to be below 200 mg/L, reaching a volume of 5 mL. They were placed in a Dionex ICS-1600 Ion Chromatography System (Thermo Scientific), equipped with a Dionex Reagent-Free Controller to prepare 30 mM of a KOH solution used as a mobile phase, and utilizing a high-performance conductivity detector. The employed column was an IonPac AS15 Capillary column (Thermo Scientific). The system operated for 15 minutes per sample at a flow rate of 0.3 mL/min. The resulting peak areas were converted to concentrations using a standard curve prepared with a Seven Anion Standard solution (Thermo Scientific).

3.2.3 Organic carbon content

To measure the amount of organic matter already present in the brine pool samples, Total Organic Carbon (TOC) measurements were performed. 100 \( \mu l \) were taken from each sample, filtered through 0.45 \( \mu m \) syringe filters and placed in TOC glass vials, adding water until a volume of 20 mL was reached (dilution of 1:200). The samples were analyzed using an On-line TOC-VCSH Total Organic Carbon Analyzer (Shimadzu), utilizing combustion catalytic oxidation at 680°C, nitrogen as carrier gas, and using the Non-purgeable Organic Carbon (NPOC) protocol by sparging 2 M hydrochloric acid (HCl). The detected peaks were converted to concentration units (mg/L) with the help of a standard curve already set in the system.
3.2.4 Bacterial count

The total number of bacteria in every brine pool sample was counted using flowcytometry. 700 µl of each brine pool sample were taken into 1.5 mL centrifuge tubes and incubated at 37°C for ten minutes. 7 µl of SYBR Green 100x solution were added to each tube, and again incubated for ten minutes at 37°C. 200 µl of each sample were taken in triplicate and put into a 96-well plate. The plate was loaded into an Accuri C6 Flow Cytometer (BD Biosciences), using a medium flow (35 µl/min) and the gates previously established to detect living bacteria.

3.3 Experimental Setup

3.3.1 Reactor design and construction

The enrichment of exoelectrogenic bacteria was done using single-chamber MECs. They were assembled using 100 mL glass reactor bottles (Adams & Chittenden Scientific Glass) with anode, cathode, and reference electrode placed in different openings of the reactor. The working electrodes were graphite rods of 7.5 cm length (7 cm inside the reactor) and 0.5 cm diameter. The counter electrodes consisted of titanium mesh squares, 5 cm by 5 cm, which were rolled into cylinders to avoid contact with the working electrode. The reference electrodes used were Ag/AgCl reference electrodes (Bioanalytical Systems, Inc.), with a potential of -0.2 V vs Normal Hydrogen Electrode (NHE). The space between all the electrodes was around 0.5 cm. A rubber cap was put in the membrane opening of each reactor to seal it, and septum caps were put in the unused openings to obtain liquid measurements when necessary. The bottles were sterilized and sparged with pure nitrogen before the electrodes were assembled, and every opening was sealed using epoxy glue to avoid leakage. The assembled design is shown in Figure 3.1.
3.3.2 Experimental arrangement and operation

Initially, a total of 10 reactors were assembled. Three contained brine pool water from Atlantis II Deep, three from Valdivia Deep and three from Kebrit Deep; the remaining one contained Atlantis II sample that was previously autoclaved twice to serve as control reactor. Around forty five days after the initial operation, two additional reactors were assembled with Valdivia brine pools, one of them autoclaved as well. Every reactor was filled with 100 mL of the respective sample, along with 10 mM of acetate as the electron donor. Everything was done inside an anaerobic glove box to avoid oxygen intrusion.

Using an enrichment method similar to Miceli et al. [28], the reactors were operated in MEC mode using a VMP3 multipotentiostat (BioLogic Science Instruments). In order to provide enough potential for the bacteria to deliver the electrons to the working electrode, the poised potential of all reactors was set to be +0.2 V vs Ag/AgCl (0.405 V vs NHE). This value was chosen to overcome the high concentration of electron acceptors such as iron and sulfate that can potentially decrease the produced
current [45]. Current measurement was done using the potentiostat’s chronoamperometry program, recording the measurements every two minutes.

In order to emulate the growth conditions inside the brine pools, the Atlantis II reactors were placed in an Isotemp Digital-Control water bath (Fisher Scientific) at the set temperature of 70°C. In the case of Valdivia and Kebrit brine pools, since preliminary experiments failed to detect any significant current at their ambient temperature (30°C and 23°C, respectively), two reactors of each set were placed in the water bath, with a third one being left operating at 30°C inside the temperature controlled room. The autoclaved reactors with Atlantis II and Valdivia Deep samples were also placed in the water bath, while the remaining reactors were not connected to the potentiostat, operating therefore at Open Circuit Voltage (OCV). The reactors operated for 60 days (with the exception of the ones later assembled) to address the current produced within this period. The experimental setup with the utilized reactors is shown on Figure 3.2.

Figure 3.2: Reactors spatial setup for the experiment. The reactors inside the water bath were operated at a temperature of 70°C, while the ones outside were at 30°C.
3.4 Experimental measurements

3.4.1 Acetate concentration

The concentration of acetate remaining in the reactors was monitored every week using High Performance Liquid Chromatography (HPLC). Using sterile syringes, 0.5 mL of each reactor was taken, and then filtered through a 0.45 \( \mu \text{m} \) syringe filter into 1.5 mL centrifuge tubes. 100 \( \mu \text{l} \) were taken from each tube and placed into HPLC vials, along with 900 \( \mu \text{l} \) of distilled water to have a dilution of 1:10. The samples were analyzed with an Accela HPLC System (Thermo Scientific) equipped with a photodiode array (PDA) detector, where the peaks were detected at 210 nm. The column utilized was an Aminex HPX-87H Ion Exclusion Column (Bio-Rad Laboratories) and the mobile phase was a 5 mM sulfuric acid solution, flowing at 0.55 mL/min. Since the acetate was calculated, from previous experiments, to elute at approximately 16 minutes from the injection, the area of the detected peak (calculated automatically by the analyzer) was written down and converted into concentration using the standard curve.

3.4.2 Sulfate concentration

Because the amount of sulfate present on the samples could act as a potential electron acceptor, its content in the reactors was analyzed. The samples were taken after two months of operation, and then analyzed using ion chromatography following the steps mentioned in section 3.2.2.

3.4.3 Cyclic voltammetry

Cyclic voltammetry (CV) was performed on all reactors, with the objective of knowing the oxidation and reduction processes that occur due to microbial activity in comparison with the other components of the media. This technique was performed
using the potentiostat, measuring the current obtained at certain potential of the working electrode (Ewe). The range employed at initial time was from -1.0 V to 1.0 V (vs Ag/AgCl), scanning at a rate of 1 mV/s. After 30 days of operation, the technique was repeated with a scanning range depending on the sample: for Atlantis II, the range was from -1.0 V to 0 V; while for Kebrit and Valdivia was from -0.8 V to 0.2 V.

3.4.4 Other sources of carbon

Due to the low rate of acetate consumption in the working reactors, it was necessary to analyze the influence of other carbon sources such as the originally present organic matter and the volatile fatty acids (VFAs) products of bacterial fermentation. TOC measurements were performed after two months of operation, following the same protocol as above. Also, HPLC was utilized to identify VFA other than acetate, using the same method as explained before, with a time of operation of 60 minutes to identify the volatile acids eluting at different retention times along this period.
CHAPTER 4
RESULTS AND DISCUSSION

4.1 Characterization of Brine Pool samples

The brine pools of the Red Sea are unique environments that were deemed sterile before, due to the extreme conditions of salinity and temperature. Nevertheless, some novel microorganisms have been isolated and some new lineages have been found to inhabit these environments [30]. Therefore, it is reasonable to think that they could harbor some microorganisms capable of electricity generation to be used in Microbial Electrochemical Cells (MXCs), especially considering the scarce information available.

Different analyses were performed on the brine pool samples to assess their physical and chemical properties compared to the values reported previously in review articles [30]. This study is one of the few times where components of these brine pools are measured, with the concentration of some metals reported for the first time, especially for the less studied Kebrit and Valdivia brines. Table 4.1 shows the measured values.

In the case of metals and anions, the majority of components have the same order of magnitude when compared with values reported in the literature (Table 2.2). It is worth noticing, though, the higher concentration of iron in Atlantis II by 3 orders of magnitude, and a slight increase in salinity (Na and K ions) in both Atlantis II and Valdivia brine pools. Also, the sulfate concentration was considerably higher in Atlantis II and Kebrit samples. These results indicate that the natural conditions
of the brine pools may be still changing, most likely due to hydrothermal activity as deduced before by Anschutz et al. [39]. Therefore, it is expected to find dynamic microbial communities that can adapt to these changes and grow in a wide range of environmental conditions.

The higher concentration of iron present in Atlantis II makes this environment more likely to harbor potential exoelectrogenic bacteria, because most of these bacteria are reported to possess the capability of iron reduction [23]. It is not to be discarded the presence of these bacteria in Kebrit and Valdivia brine pools, since a small amount of iron was found.

Table 4.1: Analyzed parameters of the studied Brine Pools. ND = not detected with the performed method.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Atlantis II</th>
<th>Valdivia</th>
<th>Kebrit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na (M)</td>
<td>5.68</td>
<td>5.15</td>
<td>5.72</td>
</tr>
<tr>
<td>Cl (M)</td>
<td>4.07</td>
<td>4.45</td>
<td>4.20</td>
</tr>
<tr>
<td>Ca (mM)</td>
<td>150.96</td>
<td>23.6</td>
<td>55.96</td>
</tr>
<tr>
<td>Mg (mM)</td>
<td>36.33</td>
<td>107.10</td>
<td>135.82</td>
</tr>
<tr>
<td>K (mM)</td>
<td>125.71</td>
<td>99.60</td>
<td>67.34</td>
</tr>
<tr>
<td>SO$_4^{2-}$ (mM)</td>
<td>49.04</td>
<td>76.50</td>
<td>58.37</td>
</tr>
<tr>
<td>F (mM)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>NO$_2^-$ (mM)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Br (mM)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>NO$_3^-$ (mM)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>PO$_4^{3-}$ (mM)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>B (mM)</td>
<td>0.90</td>
<td>0.96</td>
<td>2.40</td>
</tr>
<tr>
<td>Sr (mM)</td>
<td>0.44</td>
<td>0.17</td>
<td>0.52</td>
</tr>
<tr>
<td>Fe (µM)</td>
<td>786.10</td>
<td>0.267</td>
<td>0.304</td>
</tr>
<tr>
<td>TOC (mg/L)</td>
<td>159.6</td>
<td>170.8</td>
<td>219.2</td>
</tr>
<tr>
<td>Microbial cell density (10^4/ml)</td>
<td>0.157</td>
<td>28.0</td>
<td>6.36</td>
</tr>
</tbody>
</table>
4.2 Exoelectrogenic bacteria in Atlantis II Deep

Atlantis II Deep offered high expectations of exoelectrogen presence due to their capability of iron reduction [23]. The cyclic voltammogram of the brine pool sample (before the induction of biofilm growth under applied potential) shows one oxidation peak at -0.05 V vs Ag/AgCl, and a separate redox moiety at -0.55 V (oxidation peak at -0.6 V and reduction peak at -0.5 V, Figure 4.1). It is highly possible that the first mentioned oxidation potential corresponds to the oxidation of iron (Fe\(^{2+}\) to Fe\(^{3+}\)) in the sample, which indicates that the iron is mostly existing in its oxidized form.

![Cyclic voltammogram of Atlantis II reactors at initial time.](image)

After the sample background CV analysis, the reactors were subjected to chronoamperometry at a constant applied potential (+0.2 V vs. Ag/AgCl) for ≈60 days (Figure 4.2). Although there was an initial current response from the electrodes in both reactors, it is likely due to the abiotic oxidation of chemical compounds present in the sample by the electrode itself. After 20 days of operation, stable current was detected in all the reactors which can correspond to the acclimation of the microbial components to respire using the anode as electron acceptor. The current density generated by the reactors operating with Atlantis II samples produced a maximum value of 0.4 A/m\(^2\); in comparison, noticeable current was not observed in the control reactor that operated with autoclaved sample during the study period. This evidences that bacte-
ria present in the original sample are involved in the catalytic reactions that produce electricity from the provided organic matter. It is worth considering, though, that the bacterial community is not considered to be efficient according to Miceli et al. [28], which classified exoelectrogenic microbial communities as efficient when its current density was above 1.59 A/m².

Figure 4.2: Chronoamperometry results for the samples from Atlantis II Deep. All the reactors were operated at 70°C, the autoclaved sample being used as a control.

In agreement with the current density, the acetate consumption over the reactor operation time decreased very slowly (Figure 4.3), making more evident that the rate of substrate electrons turnover to anode though biocatalysis was slow. The perceived fluctuations can be attributed to the generation of acetate from the already present organic matter due to fermentation processes by native microorganisms, and the utilization of sulfate as an alternative electron acceptor by sulfate reducing bacteria, evidenced by the reduction on the concentration of this ion by almost 5% compared with the initial concentration and the autoclaved sample (Figure 4.4).
During amperometric analysis, after 30 days of operation, the catalytic activity of the bioanode was subjected for CV analysis (Figure 4.5). The voltammetric response of the biofilm grown on the anode shows a membrane bound redox couple expression around -0.55 V vs Ag/AgCl (oxidation at -0.4 V and reduction at -0.7 V) which can be attributed to the exoelectrogen transfer mechanism from bacteria to the anode. A similar response was not observed from the anode placed in the autoclaved sample. More studies must be performed to evaluate the influence of the already present electrochemical components and the organic matter on the growth of this microbial community, in order to improve the efficiency of the exoelectrogenic bacteria existing in this sample.
4.3 Exoelectrogenic bacteria in Kebrit Deep

The high amount of organic matter, a considerable cell density and the discovery of microorganisms belonging to a novel lineage such as the KB1 group [44], made Kebrit Deep an attractive environment where exoelectrogenic bacteria could be found. In comparison with Atlantis II, the initial cyclic voltammogram shows also an oxidation peak at -0.1 vs. Ag/AgCl but with lower current density, providing overall a similar possibility of finding an exoelectrogenic community if they respire through the iron present in the environment.
The current density reached a maximum value of 0.05 A/m² regardless of the temperature of operation (Figure 4.7); this current density was considerably lower than the threshold established by Miceli et al. [28]. There is not a noticeable acetate consumption (Figure 4.8) nor a decrease in other electron acceptors such as sulfate (Figure 4.9), indicating the absence of biological activity in the reactors. This is further confirmed by a similar shape of the cyclic voltammograms under imposed potential in both temperatures, without exhibiting additional redox peaks to provide evidence of bioelectrochemical activity (Figure 4.10). Therefore, it is likely that there is no presence of exoelectrogenic bacteria in this environment capable of growing with the tested conditions. In consequence, operational parameters such as the poised anode potential and the temperature can be changed to raise the possibility of finding exoelectrogens.

![Figure 4.7: Chronoamperometry results for the samples from Kebrit Deep. The reactors operated either at 70°C or at 30°C.](image-url)
Figure 4.8: Acetate concentration over time on Kebrit reactors.

Figure 4.9: Sulfate concentration of Kebrit reactors after two months of operation, compared with the obtained initial value.

Figure 4.10: Cyclic voltammogram of the reactors with Kebrit Deep after 30 days of operation. The voltammetry was performed on reactors operating at 30°C and 70°C.
4.4 Exoelectrogenic bacteria in Valdivia Deep

The combination of high concentration of bacterial counts, presence of organic matter and the steady increase in temperature due to reported hydrothermal activity, provided Valdivia Deep with the possibility of harboring many types of microorganisms, among them an exoelectron transferring community. The voltammetric response of this sample shows a reduction peak at -0.2 V vs Ag/AgCl; and an oxidation peak at -0.1 V vs Ag/AgCl, analogous to the peak found in Atlantis II and Kebrit Deeps which corresponds to the presence of iron. Consequently, iron-reducing bacteria that can potentially become exoelectrogens are expected to be found as in the other brine pool samples.

![Cyclic voltammogram of Valdivia reactors at initial time.](image)

Figure 4.11: Cyclic voltammogram of Valdivia reactors at initial time.

In contrast with the former anoxic deeps, Valdivia reactors at 70°C were able to generate noticeable current compared with the reactors operating at 30°C, reaching values around 1 mA during the potential induced growth and a current density normally ranging between 5 and 7 A/m² (Figure 4.12). Although the reactor with autoclaved sample was operated for a shorter time, its current density is as negligible as the reactor operating at 30°C, confirming that the generated current in Valdivia reactors is due to bioelectrochemical activity from exoelectrogenic bacteria.
In agreement with the produced current, the acetate concentration decreased over time in comparison with the other reactors (Figure 4.13). Correspondingly, negligible sulfate removal was observed at 70°C (Figure 4.14), which explains that the acetate oxidation is linked to the anode reduction, whereas in the case of the reactor operating at 30°C, electrons that are harvested from acetate oxidation by bacteria were preferably delivered to sulfate reduction whose concentration decreased by almost 5%. Therefore, these results provide more arguments to support the existence of exoelectrogenic bacteria that are preferably growing at 70°C using this sample. Although the natural temperature of Valdivia Deep is 33.7°C, the hydrothermal activity reported by Anschutz et al. [39] and these results offer the possibility of finding thermophilic microorganisms that are also capable of exoelectrogenesis in Valdivia Deep brine pool. In consequence, operational parameters closer to the natural conditions must be chosen in the reactors if microorganisms, among exoelectrogenic bacteria, are desired to be identified and cultivated.
Figure 4.13: Acetate concentration over time on Valdivia reactors. The autoclaved and OCV reactors started at a later time, so their monitoring time is shorter than the other reactors.

Figure 4.14: Sulfate concentration of Valdivia reactors after two months of operation, compared with the obtained initial value.

Overall, the approximate acetate consumption rate resulted to be very slow at 70°C, approximately 0.12 mM/d which influence the rate of electrogensis. Furthermore, the cyclic voltamogram on the reactors at 70°C show a noticeably different shape (Figure 4.15), where the initial redox peaks are absent even when compared with the reactors at 30°C, and no further redox peaks appear related to bioelectrocatalysis as happened with Atlantis II. It might be possible that the electron transfer happened through soluble mediators such as quinones and humic substances coming from the sediments, posing as an alternative method of current generation independent of membrane bound proteins.
Figure 4.15: Cyclic voltammogram of the reactors with Valdivia Deep sample after 30 days of operation. The voltammetry was performed on reactors operating at 30°C and 70°C.

It was hypothesized that additional sources of carbon could be influencing the development and performance of the possible exoelectrogenic bacterial community, making reasonable to measure the TOC concentration and the presence of other VFAs than acetate using HPLC. Compared with the initial amount of TOC when acetate was added, there was a significant decrease on the concentration on all the reactors, independently of the generated current (Figure 4.16). Based on the HPLC analysis of reactor samples (Figure 4.17), besides the acetate detection (peak eluting at 16 minutes retention time), other VFAs corresponding to n-valerate and propionate were detected which are not originally present in the original sample. This observation confirms that TOC was fermented to VFAs, and these intermediates contribute additionally for current generation.
Therefore, an exoelectrogenic microbial community is present in the reactors with this environmental sample due to the current generated and the acetate consumed. It can be proposed that this community can have microorganisms capable of utilizing additional carbon sources that are part of the total organic matter in processes such as fermentation, evidenced by the TOC consumption and the presence of fermentation products. Although the cyclic voltammograms do not show clear bioelectrocatalytic activity, there is the possibility of having soluble redox mediator molecules, such as humic substances and quinones, that are consumed in the electron transfer process. Further studies need to be performed to address the components of this commu-
nity and their electrochemical characteristics in order to obtain novel exoelectrogenic microorganisms that can be efficiently utilized in bi-electrochemical reactors.

4.5 Factors influencing the growth of exoelectrogenic bacteria

The fact that only Valdivia samples were able to generate a higher amount of current at 70°C, compared with the other brine pools, might be influenced by the operating conditions, namely the anode potential, the temperature and the intrinsic conditions of the brine itself. Regarding the anode potential, it is reasonable to think that a higher poised potential will increase the rate of bacterial growth, in agreement with the higher potential difference between anode and electron donor already explained. However, Torres et al. [46], using MECs showed that lower potentials are beneficial to microorganisms such as *Geobacter sulfurreducens* that have more efficient electron transfer mechanisms, allowing them to grow faster, dominate the community and produce more current more efficiently. Similarly, Bond [27] shows that a lower potential may select for only exoelectrogens that are capable of respiration at that given value, but in higher potentials many organisms with identical maximum rates of current flux to the electrode can be grown. In the end, due to this lack of consensus regarding the optimum potentials for either MFC or MEC operation [15], it is necessary to experiment with a range of potentials in order to find an optimal anode potential value for the enrichment of exoelectrogens.

The temperature of operation is another factor that could favor the growth of certain microorganisms present only in the Valdivia Deep. Even though the brine temperature is reported as 33.7°C [39], the generated current of the reactor at 30°C was not noticeable compared to the reactors operating at 70°C. It was already discussed that there could be a constant increase in temperature due to geothermal activity in this Brine Pool [39], making feasible for thermophylic microorganisms to be present. This temperature also provides faster microbial kinetics and higher rates
of complex organic matter hydrolysis [47], resulting in faster growth and electricity production for the bacteria resisting the conditions. Nevertheless, it is also needed to experiment with different temperature ranges in all the studied brine pools, in order to evaluate the community dynamics and the effect of temperature on exoelectrogens and other type of microorganisms.

4.6 Future work

The task of finding microbial communities from a novel extreme environment is a long and demanding process. Many experiments need to be performed in order to get the most amount of knowledge of the bacteria of interest, in this case, exoelectrogenic bacteria. The analysis done in this work supports the feasibility of finding these kind of bacteria in the brine pools, particularly in Valdivia Deep which showed a significant amount of current. It is not to be discarded, however, the existence of efficient exoelectrogenic bacteria in Atlantis II and Kebrit, so experimenting with the operational parameters is desired to obtain information about the environmental conditions where these kind of bacteria can grow if they are present.

Nevertheless, the difficulty of operating systems and components at extreme conditions of salinity and temperature poses a high challenge where a lot of undesirable outcomes can be possible. Of special attention is the occasional leakage of the functioning reactors due to the hydrogen production for being operated as MECs, and the damage of connections and reference electrodes after long periods of time. It is therefore necessary to utilize materials that can withstand the operational conditions, for the reactors and the bacterial behavior to be better understood and the results to be completely reproducible.

Until the completion of this document, only chemical and electrochemical analyses were performed to conclude that there are indeed exoelectrogenic bacterial communities in the studied brine pools. In order to completely confirm the presence of
these microorganisms and to know their identity, it is essential to utilize additional techniques. First of all, it is required to observe the surface of the electrode using microscopy techniques such as scanning electron microscopy (SEM). By doing this, the presence of microorganisms, their morphology and their spatial arrangement on the anode surface can be known, as it was performed on several studies involving the isolation of bacteria from MXCs [14, 48, 47, 49, 50].

Also, the encountered microbial community must be addressed by using molecular biology techniques, such as targeting, amplifying, and sequencing the 16S ribosomal RNA gene to get information about the taxonomy and classification of the present microorganisms. Methods such as cloning and sequencing of fragments [37, 44] and pyrosequencing [36, 42] have been used to know the composition of the microbial communities in brine pools. Therefore, the analysis of this gene is a required procedure to address exoelectrogenic bacteria growing inside MXCs, as it has been done with bacterial communities found in these systems with samples from the environment, either by employing cloning and sequencing [48], denaturing gradient gel electrophoresis (DGGE) [45], or pyrosequencing [28]. Finally, it is crucial to understand the underlying bacterial community in Valdivia Deep since there are no reports focusing on this brine pool yet. With the increasing interest in the study of these environments [30], more information about this Deep and others can be obtained, allowing more studies related to exoelectrogenic bacteria to be performed.
CHAPTER 5

CONCLUSION

The search of efficient bacteria capable of delivering electricity is one of the approaches to research in the development of Microbial Electrochemical Cells (MXCs), for this technology to pose as a viable alternative source of renewable energy. The brine pools of the Red Sea are extreme environments that have been scarcely studied, where these type of bacteria can thrive due to the unique physical and chemical conditions. With this objective in mind, samples from three distinct brine pools: Atlantis II, Kebrit, and Valdivia Deeps, were analyzed with Microbial Electrolysis Cells (MECs) to identify any possible exoelectrogenic bacterial community.

Using electrochemical analyses and monitoring of components in the media, it was found that reactors operating with Valdivia and Atlantis II Deep samples were able to produce current. The changes in oxidation and reduction behavior and the consumption of the added carbon source provide evidence that a exoelectrogenic bacterial community can be found. Therefore, further experimentation considering these brine pools is encouraged to characterize its microbial components responsible for the electricity generation. This will make possible to establish optimal growth conditions and to isolate individual components, understanding their properties with detail.

The search and isolation of microbial communities of interest is by no means an easy task, especially when the analyzed samples are from almost unknown environments such as the brine pools of the Red Sea. More specific technologies to study
these sources need to be developed in terms of equipment and laboratory techniques. This is required to reproduce the extreme conditions in the laboratory for bacteria to grow accordingly, and provide all the required information without possible interference of the components. The research on the biological components of microbial technologies for energy generation is only one of the challenges that needs to be looked upon. Nevertheless, it is valid to think of this study as a step towards the more frequent utilization of renewable technologies in the future, so the problems related to climate change can be mitigated and the inhabitants of this planet can live within the realm of sustainability.
REFERENCES


APPENDICES

A Utilized Standard Curves

Figure A.1: Standard curve for the calculation of acetate concentration based on peak area.

Figure A.2: Standard curve for the calculation of sulfate concentration based on peak area.