THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Microbial communities in biological electrochemical systems

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Cover:
Left: Illustration of a BES system
Right: Photo of the microbial biofilm on the electrode surface in a MEC

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ABSTRACT

Biological electrochemical systems (BES) can be used as biosensors and for recovery of resources from waste streams. BES utilizes microbial communities that grow on the surface of electrodes in the form of biofilms. Electrogenic bacteria residing in the anode biofilm initiate oxidation reactions, resulting in the release of electrons and subsequent electrical current generation. The electrons flow to the cathode where reduction reactions take place. Microbial biofilms may also be involved in the catalysis of cathode reactions. Many factors are involved in shaping the composition and performance of the microbial communities in BES, most of which remain poorly understood.

In this thesis, the impact of electrode material and biotic interactions on performance and microbial community assembly was investigated in microbial electrolysis cells (MECs) oxidizing volatile fatty acids at the anode. MECs are a type of BES that require an applied electric potential to generate products such as H_2, CH_4, and acetate at the cathode. MECs with mixed-culture biofilms on both the anode and the cathode were studied. Two experiments were conducted. The first was a comparison of MECs with three different cathode materials: carbon nanoparticles, titanium, and steel. The second was a comparison of MECs with three different anode materials: carbon cloth, graphene, and nickel. Furthermore, the effect of dispersal limitation as well as the presence of viruses and their associations with microorganisms was investigated. MECs with carbon cloth anodes had the highest current density and shortest lag time during startup. In contrast, no significant impact of cathode material on MEC performance was seen. The anode communities were dominated by electrogens from the *Desulfobacterota* phylum, while the cathodes were dominated by methanogens from the *Methanobacteriaceae* family. Stochastic initial attachment by competing electrogens on the anode explained variations in the startup time between replicate MECs. In each experiment at least two different *Desulfobacterota* species competed for dominance on the anode. MECs that enabled dispersal between the system tended to have the same dominating taxa. Biotic interactions also affected the microbial communities in the system. Network analysis showed that the anode communities had a greater number of negative interactions between taxa compared to the cathode. Due to the need for direct contact by electrogens to transfer electrons to the anode, there is a higher competitive element to the colonization of the anode biofilm. Viral infection is another type of biotic interaction. Analysis of the prokaryotic and viral communities resulted in the identification of CRISPR-based and prophage virus-host associations, indicating previous infections and prophage inductions of electrochemically active microorganisms. These findings suggest that while there is selective pressure for electrogenic bacteria on the anode, stochastic factors, and biotic interactions play a larger role compared to electrode material in shaping the anode community.

Keywords: bioanode, biocathode, bioelectrochemical system, microbial community assembly, microbial ecology. microbial electrolysis cells, viruses

In memory of my beloved grandmother, Zahra, whose unwavering belief in me and steadfast support has been my guiding force.

LIST OF PUBLICATIONS

This thesis is based on the following appended manuscripts:

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B. **Abadikhah, M.**, Liu, M., Persson , F., Wilén , B.-M., Farewell, A., Sun, J. and Modin, O. (2023). "Effect of anode material and dispersal limitation on the performance and biofilm community in microbial electrolysis cells." Biofilm **6**: 100161.

C. **Abadikhah, M.**, Persson F., Farewell A., Wilén, B.-M., and Modin, O. “Viral and prokaryotic diversity and interactions in microbial electrolysis cells.”, *Manuscript*

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Paper A: Conceptualization of the research goals and aims; Performance of laboratory work; Result analysis and visualization; Writing the original draft.

Paper B: Conceptualization of the research goals and aims; Performance of laboratory work; Result analysis and visualization; Writing the original draft.

Paper C: Conceptualization of the research goals and aims; Performance of laboratory work; Writing the original draft.

Paper D: Conceptualization of the research goals and aims; Performance of laboratory work; Result analysis and visualization; Writing the original draft.

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LIST OF ACRONYMS AND ABBREVIATIONS

ASV: amplicon sequence variant

BES: bioelectrochemical system

CNP: carbon nanoparticles

CV: cyclic voltammetry

HPLC: high performance liquid chromatography

MAG: metagenome-assembled genome

MEC: microbial electrolysis cell

MET: microbial electrochemical technology

MFC: microbial fuel cell

NM: nutrient medium

Contents

1 Preface

This doctoral thesis explores the microbial communities residing on the electrode surfaces of biological electrochemical systems (BES), specifically microbial electrolysis cells.

1.1 Research motivation and scope of thesis

Biological electrochemical systems (BES) utilize microorganisms to drive the reactions necessary for the systems to function. The use of BES can be implemented in many different areas. For instance, in resource recovery and as biosensors. Although these systems have been studied extensively to better understand how they function, there is still some uncertainties with regards to the microbial community assembly and dynamics. The focus up until recently has mainly been on deterministic factors, such as operational settings and electrode material, without considering the impact stochastic factors, such as dispersal and drift, may have on the microbial community development and system performance. Biotic interactions between prokaryotes as well as with predators within the system also play important roles in the development of the microbial community and system function. Given the pivotal role of the microbial community, it is of importance to study the impact that both deterministic and stochastic factors have on the microbial community assembly, performance, and stability in BES.

The goal of this thesis consisted of two aspects. The first was to investigate the impact of different aspects of the system on the microbial community and performance. Specifically, the impact electrode material, biotic interactions, drift, and dispersal had on the performance and microbial community. Secondly, we wanted to investigate the effect of different DNA sequencing and analysis approaches for diversity analysis and taxonomic classification of microorganisms in the systems. Thereby, we contribute more insight into how the different aspects of the MECs impact the function optimization and microbial community dynamics.

The findings of this research have been presented in four manuscripts (Paper A-D) included in this thesis, which address the following key questions:

How can we optimize the performance of microbial electrolysis cells?

In **Paper A** the effect of different cathode materials, carbon nanoparticle covered carbon paper, titanium, and steel, on the function of MECs was investigated. **Paper B** focuses on the performance of MECs with differing anode materials. Here the conventional material carbon cloth was compared against graphene, a carbon-based material, and nickel.

How does deterministic and stochastic factors impact the performance and development of the microbial communities residing on the electrode surfaces?

Paper A and **Paper B** also investigate the impact the electrode material has on the microbial community composition between replicate MECs as well as those with different materials, allowing for investigation of the influence of both deterministic and stochastic factors. The biotic interactions were investigated in both **Paper A** and **Paper C.** In **Paper A** the interactions between the microorganisms in the electrode communities were investigated using network analysis. **Paper C** explores the relationship between the bacteria and viruses present in the microbial community.

How does sequencing method affect the taxonomic classification and diversity analysis in bioelectrochemical systems?

In **Paper D** the choice of sequencing methods is evaluated for microbial communities in MECs. The extracted DNA from the microbial community investigated in **Paper A** was sequenced using, amplicon sequencing, short-read sequencing, and long-read sequencing, followed by a comparative analysis to evaluate the taxonomic classification and diversity analysis across these methods.

1.2 Scientific approach and limitations

Two different designs of microbial electrolysis cells (MECs) were constructed to investigate the deterministic and stochastic factors involved in the performance and microbial community assembly and development. The investigation of cathode material was done in single-chamber MECs batch fed with a nutrient medium containing acetate, propionate, and butyrate. To ensure that any effect seen was due to the differing cathode material, all MECs had carbon cloth anodes. The nutrient medium was replenished every 2-3 days. At the beginning of each batch cycle a high concentration of nutrient was available which became depleted over time. This affected the ability of the electrogenic bacteria responsible for the current generation to produce current at an equal level throughout the cycle. The anode material was investigated in single-chamber MECs hydraulically connected in a loop, allowing dispersal to occur between those MECs connected, and preventing dispersal between those from differing hydraulic loops. Since three MECs were connected to the same hydraulic loop with the circulating nutrient medium, the coulombic efficiency of each individual MEC could not be established. Replicate MECs were constructed for each electrode material to evaluate the reproducibility and statistical significance of the different parameters measured.

To study the impact of electrode material, dispersal, drift, and biotic interactions on the assembly and development of the microbial communities in the MECs, the biofilms of the electrodes as well as the biomass of the suspended liquid in the systems were sampled for taxonomic classification. Amplicon and metagenomic sequencing were utilized to identify the species present in the microbial communities. Downstream analysis consisted of relative abundance and diversity analysis as well as network analysis. Although the species present in the microbial community can be identified, it is difficult to determine whether they are active in the community based solely on the sequencing performed. Biotic interactions of the microbial community with predators, specifically viruses, were also investigated in the system. To establish the presence of viruses and their interaction with the prokaryotic community, the suspended liquid was sampled. The viral DNA was extracted and sequenced.

Three sequencing approaches were also evaluated for their ability to identify the microbial community composition of the MECs. Amplicon sequencing, Illumina short-read sequencing, and Nanopore long-read sequencing. The choice in sequencing- and analysis method may impact the results of the taxonomic classification and subsequent analysis of diversity and community composition. Amplicon sequencing amplifies conserved regions, which may result in over estimation of the relative abundances due to gene copy number. While short-read sequencing has the highest accuracy, there is a need to outsource the sequencing. Nanopore long-read sequencing can be done in lab using a portable sequencing machine, although the use of the method is limited by the quantity of DNA needed, since the lab scale MECs are

small resulting in lower DNA concentrations. Additionally, the high error rate associated with the sequencing results may also impact the taxonomic classification.

2 Introduction

Water is an important resource in society. As a result, the treatment of used water, known as wastewater, is of importance. Typically, wastewater refers to all effluent water from households, industry, municipalities, and agriculture (Nishat et al. 2023). The role of wastewater treatment plants is to gather and treat the water to remove pollutants before being released into the environment (Rashid et al. 2021). The treatment processes used typically require the use of both energy and chemicals. Beyond its role as a product that needs to be cleaned, wastewater is also a resource that is not fully utilized (Kehrein et al. 2020). There is a large quantity of organics, nutrients and metals that can be found in our wastewater. In addition to this, wastewater is also a source of energy (Modin et al. 2017, Kehrein et al. 2020). As a society we need to strive for a more sustainable future where we make use of all available resources to the fullest. Therefore, finding strategies that allows us to utilize and recycle the resources in wastewater is of importance. To attain this objective, the research and development of new technologies that can be implement in the wastewater treatment process is essential. Biological electrochemical systems (BES), specifically microbial electrochemical technologies (METs), e.g. microbial fuel cells and microbial electrolysis cells, are one such set of technologies that could contribute to the resource recovery in various ways.

In 1911, Potter et al. (1911) were able to demonstrate how certain microorganisms could initiate the generation of electric current through their microbial metabolism. These findings highlighted the potential for harnessing microorganism for their use in electrochemical processes. The first instance of what is today considered a microbial fuel cell was constructed in 1931, demonstrating how stacking of fuel cells containing bacterial biofilms on the electrode surfaces resulted in current production (Cohen 1931). Although these findings were of interest, the research did not progress further until the 1960s. At that time the research focused on the improvement of the generated current through the use of electron transport mediators as well as potential nutrient sources were studied (Davis et al. 1962, Bennetto et al. 1983, Allen et al. 1993). Metabolic pathways involved in the transfer of energy from the anode and cathode were discovered, specifically the importance of microbial electron transfer for the system function in bioelectrochemical system (BES) (Bennetto et al. 1983). These findings were followed by a decline in research into BES until the end of the 20th century when a revival of interest emerged (Schröder 2007, Arends et al. 2012). A multitude of studies were conducted to obtain better understanding of what materials and under what conditions the performance of BES could be optimized (Mier et al. 2021). However, there are still factors impacting the microbial community assembly and performance that are still not fully understood.

2.1 Bioelectrochemical systems and their applications

Bioelectrochemical systems (BES) refers to any system where the present microorganisms on the electrode surface are involved in the catalysis of redox reactions using the available organic materials (Costa et al. 2018, Das et al. 2022). Typically, these systems have distinct anodic and cathodic communities involved in oxidation or reduction reactions. The application of BES for use for different purposes, such as resource recovery and biosensors, is referred to as microbial electrochemical technologies (METs). These can be anything from technologies to recover and harvest resources, such as electric power generation in microbial fuel cells (MFCs) and production of energy carriers in microbial electrolysis cells (MECs), to uses as biosensors for

organic biodegradable organic compounds or toxicity (Modin et al. 2017, Kehrein et al. 2020). The general principle behind the function of BES is dependent on the electrogenic bacteria dominating the anode community (Mateo et al. 2018). The electrogenic bacteria initiate the oxidation of the organic materials resulting in the release of electrons (Fig 1) (Logan et al. 2015, Shin et al. 2017). The newly released electrons are then transferred to the anode surface by the electrogenic bacteria, culminating in current production (Logan et al. 2015, Hassan et al. 2021). Once the transported electrons reach the cathode, the microbial community of the cathode initiate reduction reactions (Contreras et al. 2022). Since the microorganisms active at the anode in BES consume organic substrates to facilitate their electroactive capabilities, they are good candidates to aide in wastewater treatment. The use of these types of technologies in the wastewater treatment process could reduce the required energy input. Not only would they be suitable for the reduction of the external input of energy, but they could also be used to recover other forms of energy such as hydrogen and methane gas, as well as other nutrients and important resources including metals (Kehrein et al. 2020). Furthermore, BES can also be implemented in the desalination of saltwater (microbial desalination cells) as well as in the removal of pollutants from the environment through microbial remediation systems (Bala et al. 2022). Additionally, the use of BES in the form of biosensors has also been implemented, to measure the changes in toxicity or biochemical oxygen demand (Kim et al. 2003, Patil et al. 2010, Adekunle et al. 2019).

Microbial fuel cells

Microbial fuel cells (MFC) are a type of BES which can convert chemical energy to electrical energy. The anodic community of MFC initiate the oxidation reactions necessary for the release of electrons. These electrons are then transported using the electrogenic bacteria residing on the anode. As the electrons are transported the electrical current is generated by the external circuit connected to the system (Ucar et al. 2017). The catalysis of the oxidation reaction occurring on the anode by the microbial community occur in the absence of an applied potential. The electrons then travel to the cathode where reduction reactions occur. Depending on the design of the cathode there are different types of reduction reactions that may occur. The cathodic reduction reactions can occur in both an aerobic and anaerobic environment. Aerobic cathodes use oxygen as an electron acceptor for the reduction reaction, typically leading to the production of either water or hydrogen peroxide (Rismani-Yazdi et al. 2008). Anaerobic cathodes on the other hand typically rely on the microbial communities present on the cathode as electron acceptors initiating reduction reactions, such as nitrate reduction (Park et al. 2005, Clauwaert et al. 2007).

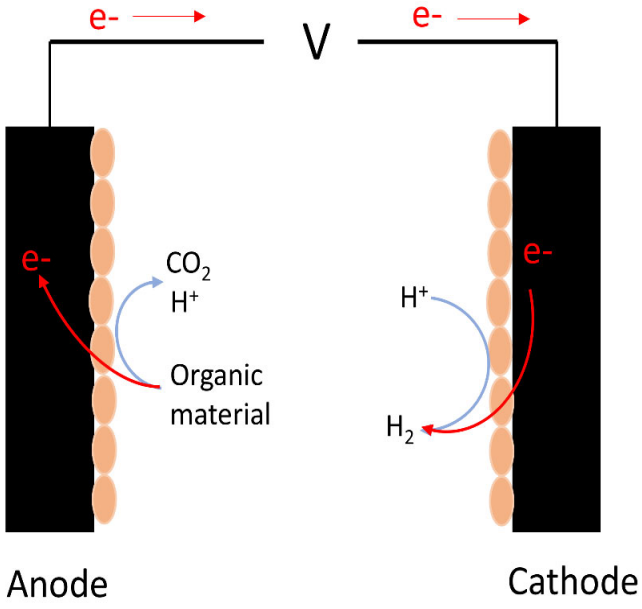

Figure 1. Example of the redox reactions that occur on the electrodes in MEC.

Microbial electrolysis cells

Microbial electrolysis cells (MECs) represent a type of BES which can both convert chemical energy into electrical energy as well as the reverse back into chemical energy. In contrast to MFC, an applied potential on the cell drives the electrochemical reactions occurring within the system (Rozendal et al. 2006, Nam et al. 2011, Hari et al. 2016). The anode community in MECs generate current in the presence of an applied potential, whilst the cathode community uses the transported electrons in the generation of hydrogen and methane gas (Li et al. 2019, Logan et al. 2019).

MECs can be constructed to either be a single-chamber system or a dual- chamber system, depending on the purpose and usage. In single-chamber MECs, the anode and cathode microbial communities reside in the same chamber. This results in the sharing of resources and nutrients between the two electrode communities (Call et al. 2008). A major benefit of using single-chamber MECs is the reduction in voltage loss (Kadier et al. 2016) . Since there is no separation of the compartments using an ion exchange membrane, the subsequent pH imbalance that typically occurs due to limited transfer of hydrogen and hydroxide ions though an ion exchange membrane, is not present (Call et al. 2008). Although there are some benefits with regards to minimizing voltage loss in single-chamber MECs, the main use of them is when the aim of the system is methane gas production and not hydrogen production. This is mainly due to the methanogens consuming a large portion of the hydrogen generated at the cathode, resulting in methane gas production (Li et al. 2019). Dual-chamber MECs separate the anode and cathode compartments by an ion conductive spacer, typically an ion exchange membrane is used. This separation leads to a higher overpotential since there is a larger space between the electrodes (Call et al. 2008, Krieg et al. 2019, Fathima et al. 2024). Even so, there are certain advantages to the dual-chamber MECs, such as reduced crossover of bacteria and organic material between the anode and cathode compartments due to the membrane separating the two compartments (Wang et al. 2021, Koul et al. 2022).

2.2 Electrode materials

There are certain aspects of importance when considering the performance of BES. These aspects include factors such as design, operational setting, and nutrient composition (Liu et al. 2020, Mier et al. 2021). An essential component to take into consideration when designing an optimal BES, is the electrode material. When choosing an appropriate material for the anode and cathode certain aspects need to be considered. Since most of BES function in aqueous environments the material used must be able to withstand the effects of water. Typically, materials that are carbon-based are chosen since they do not corrode in aqueous solutions (Zhang et al. 2020). Additionally, it is of importance that the material used for the anode is a good conductor of electric charge, to optimize the transfer of electrons from the bacteria (Mier et al. 2021). Furthermore, the cost of the material also must also be taken into consideration. Since excessive costs in the construction of the system could impede the widespread implementation of the developed system by appropriate actors. Conventionally, carbon-based materials such as carbon felt, carbon paper, and carbon cloth have been used as electrode materials, due to their high conductivity and low cost (Kaur et al. 2020). In recent years, a new carbon-based material, graphene, has become a good candidate as an electrode material. Graphene has a two-dimensional structure which has been reported to have a large surface area and high conductivity (Geim 2009, Bhatt et al. 2023). Based on studies carried out in BES, the implementation of graphene could result in improved performances. A study investigating the impact of graphene electrodes in MFCs noted an increase in the obtained power density compared to carbon cloth (Liu et al. 2012). Similarly, it has been noted that MECs using graphene based electrodes had an increase in hydrogen production (Dai et al. 2016). There are, however, some discrepancies in the reported research regarding the use graphene and its biocompatibility. There have been reports of the antimicrobial properties and its use in biomedical applications, specifically for drug delivery and substrate preparation for tissue engineering (Kumar et al. 2016, Xia et al. 2019). Studies have also shown the reduction of biofouling when using graphene coated forward osmosis membranes during water treatment (Wang et al. 2019, Firouzjaei et al. 2020). Some potential aspects which may impact whether graphene is biocompatible or not can be correlated to the manufacturing process (Yu et al. 2016). There are multiple methods that can be utilized during the manufacturing process, such as chemical vapor deposition and oxidation and reduction methods. Some of these methods result in toxic residues remaining on the surface of the material (Yu et al. 2016). Other potential causes for the observed antimicrobial properties could be the graphene structure. Certain form of graphene consist of sharp vertical flakes (Pandit et al. 2021). When the microbial organisms attach onto the surface of the graphene, they are most likely pierced resulting in cell death.

2.3 Microbial communities within BES

Anode microbial community

The oxidation of organic material resulting in the release of electrons is performed by the microbial community of the anode. Once the oxidation of organic material has occurred, the electrogenic bacteria within the anode biofilm, facilitate extracellular transport of the newly released electrons to the anode surface leading to current generation (Schröder 2007). The electrogenic bacteria prevalent in BES typically belong to the *Desulfobacterota* phylum, formerly referred to as *Deltaproteobacteria* (Waite et al. 2020). Those belonging to the

Geobacter genus have been extensively studied for their presence in BES (Logan et al. 2019, Kondaveeti et al. 2020). *Shewanella* sp. represent another category of electrogenic bacteria that have been well documented with regards to their function and abundance in METs (Silva et al. 2020).

Electrogenic bacteria can utilize multiple strategies for extracellular transportation of electrons. These strategies are divided into two main categories: direct electron transfer and mediated electron transfer (Lovley 2011, Deutzmann et al. 2015). In mediated electron transfer the bacteria transfers the newly released electrons with the help of mediators (Patil et al. 2012). Typically, redox mediators are used by the electrogenic bacteria, such as riboflavin (Huang et al. 2018, Montoya-Vallejo et al. 2023). Artificial redox mediators can also be added to the system to aid the electron transfer, some commonly used artificial redox mediators include methylene-blue and neutral red (Gemünde et al. 2022). Redox mediators' function like a bridge between the bacteria and the anode surface. When the bacteria oxidize the organic substrate, thereby releasing the electron, the mediator receives it and transports it to the anode surface (Patil et al. 2012). Since the mediators can undergo reversible oxidation, this cycle of receiving and releasing the electrons can be done multiple times, allowing for continuous transfer of the electrons. Research has shown that the use of mediators in BES have improved the rate in which the electron transfer occurs in compared to direct electron transfer (Yi et al. 2021).

Direct electron transfer on the other hand, does not require the presence of a mediator for the transfer of the electrons from the bacteria to the anode surface. Instead, the bacteria directly attach onto the electrode surface and facilitate the transfer of electrons through membrane-bound or extracellular proteins (Kim et al. 2003, Liu et al. 2012). There are two types of direct electron transfer that the bacteria can utilize: redox proteins and nanowires. Redox proteins can be membrane-bound or found on the outer membrane of the bacteria. Membrane-bound cytochromes are one type of redox protein which uses heme groups that can accept and donate electrons (Costa et al. 2018, Thapa et al. 2022). Outer-membrane proteins such as c-type cytochromes directly interact with the anode surface allowing for the transfer of the electrons (Ueki 2021). Nanowires are a type of conductive pili, that can be used for the direct transfer of electrons between bacteria as well as to conductive surfaces (Reguera et al. 2006). The nanowires are made of conductive proteins, that allow for the construction of an electrically conductive pathway in which the electrons can travel (Subramanian et al. 2018).

Cathode microbial community

The cathode microbial community uses the electron transported to the cathode from the anode to initiate reduction reactions. Depending on the setup and design of the system, the substrate produced may vary (Ragab et al. 2020, Gatidou et al. 2022). Typically, the dominating species found in the cathode community consists of methanogens (Siegert et al. 2015, Li et al. 2019). Those from the *Methanobacteriaceae* family are typically found to dominate the cathode community. The methanogens may either directly take up electrons from the cathode and reduce CO_2 to methane or use abiotically produced H_2 as an intermediate (Cheng et al. 2009). Additionally, acetogens are also found on the cathode. Species belonging to the *Acetobacterium* genera are involved in the reduction of CO_2 to acetate, by using the cathode itself or hydrogen generated at the cathode as an electron donor (Balch et al. 1977, Nevin et al. 2011, Wang et al. 2018). Similarly, *Sporomusa ovata* is also a well-known electroactive

acetogen able to utilize the electrons in combination with CO_2 to produce acetate (Nevin et al. 2010).

Eukaryotes and viruses

In addition to the bacterial communities of the anode and cathode, METs also include other forms of microorganisms. Typically, eukaryotic microorganisms that are identified in these systems consist of fungi and protozoa (Logan et al. 2019). Research performed on fungi such as *Saccharomyces cerevisiae* have found they have electroactive capabilities under proper conditions and operational settings (Verma et al. 2023). There is however a lack of understanding how eukaryotic species found in diverse microbial communities within METs impact the development of the electrode microbial communities.

Viruses can also be found in BES. Viruses that target bacteria and archaea are referred to as bacteriophages. Typically, viruses can infect the bacteria through two distinct cycles, the lysogenic or lytic cycles. Those viruses that undergo the lysogenic cycle can also switch to the lytic cycle (Lee et al. 2018). During the lysogenic cycle the infected host incorporates the viral DNA into its genome, creating prophages (Casjens et al. 2015). The incorporated viral genome replicates together with the host genome, resulting in the formation of new host cells with the incorporated viral genome. The prophages can remain in the host unless something results in their activation. When the host cell experiences different form of environmental stressors, such as nutrient depletion or UV-light, the dormant prophages can undergo induction, triggering initiation of the lytic cycle (Czyz et al. 2001, Makky et al. 2021). The lytic cycle in contrast to the lysogenic cycle does not result in the viruses existing dormant within their hosts. Upon infection, the host replicative machinery is hijacked by the infecting virus. The virion and necessary viral proteins are synthesised using the host replicative machinery, before assembly of new phages occur. Once the virus has completed the assembly of all new phages, the virus initiates the lysis of the host cell, spreading new phages that are capable of infecting new potential bacterial hosts (Zhang et al. 2022).

Although the bacterial and archaeal communities in BES have been studied extensively, there is almost no information about the impact of viruses and the viral community in BES. Despite the limited studies on the impact of the viral communities in BES, studies in other environments have shed light on this aspect. It has been noted that modulation of the microbial communities in the observed environments can be correlated to the viral communities present. Investigation of the microbial and viral interactions in coastal marine ecosystems underlined the importance of the viral community in preservation of diversity and richness (Zhang et al. 2007). They determined that the overgrowth of specific microbial groups was prevented due to the presence of the viruses, indicating the virus's modulating ability. Similarly, investigations into the associations between the viral and prokaryotic communities of acid mine drainage showed positive correlations between the observed viral diversity and microbial diversity (Liu et al. 2023). A "killing the winner" pattern was also observed for viruses found in bioreactors treating industrial wastewater (Shapiro et al. 2010, Shapiro et al. 2011). Knowles et al. (2016) have proposed a "Piggyback-the-Winner" model, where it is thought that in environments with a high microbial density there is a low viral density, due to temperate dynamics being favoured allowing for an increase in lysogeny.

2.4 Microbial community assembly in BESs

The microbial communities on the electrode surfaces play a large role in the performance of BESs. They are the main players that initiate the redox reactions resulting in the generation of the desired products (Yates et al. 2012). Numerous studies since the late 20th century have been able to identify the microorganisms most commonly found in these systems (Logan et al. 2019). The significance of a high abundance of electrogenic bacteria such *Geobacter spp.,* has been extensively reported (Reguera et al. 2019, Fernandes et al. 2021). Although much is known about the identity of the main functional groups of microorganisms, the biofilm assembly and continuous development over time is still poorly understood. There have been studies highlighting the differences in performance and microbial community composition in replicate systems under identical operational conditions (Zhou et al. 2013). Investigation of the microbial community in pilot-scale MECs, operated on domestic wastewater, noted the importance of stochastic factors involved in the community assembly based on neutral community model analysis (Cotterill et al. 2018). Although the study concluded that most of the effect seen on community assembly in the pilot-scale MECs were attributed to stochasticity, there were still indications of the influence of deterministic factors as well (Cotterill et al. 2018). However, other studies have emphasized the importance of deterministic, not stochastic factors, influencing the similarities observed in microbial communities in BES (Yates et al. 2012).

Insight into the ecological processes involved in the shaping of the microbial community in BES is crucial for a more comprehensive understanding of the microbial community assembly. There are four processes involved in the ecological dynamics of microbial community structures. These processes are referred to as dispersal, diversification, drift, and selection (Nemergut et al. 2013). Most research into BES have focused on selection. Selection is the ability of a species to thrive under a specific set of conditions, typically referred to as fitness. For the microbial organisms in BES, the selection process allows for those bacteria that can transfer electrons resulting in the generation of current to thrive. The way selection of these bacteria is achieved is through deterministic factors such as operational conditions, substrate composition, and choice of material (Koch et al. 2019, Saheb-Alam et al. 2019). The stochasticity of the system is typically explained by the remaining three processes. Drift represents the random death and replication of the microorganisms, while diversification describes any changes resulting in new abilities that improve the organisms' chances of survival. These changes can occur due to mutations as well as horizontal gene transfer (Wiedenbeck et al. 2011, Arnold et al. 2022). Dispersal is the random attachment, detachment and movement that occurs in the system over time (Nemergut et al. 2013).

The way the BES is designed may impact how and to what extent the ecological processes impact the microbial community development. In both single- and dual-chamber MECs, there are at least two distinct habitats with unique ecological niches, namely the anode and the cathode. Consequently, selection pressure based on these ecological niches are one of the driving forces behind the initial microbial assembly. Since the anode community needs the ability to transfer electrons to the anode, species of electrogenic bacteria have an advantage in colonization of the anode surface (Logan et al. 2019). Similarily, hydrogen oxidziers are favoured during colonization of the cathode surface because the cathode communities need to have the ability to accept electrons, as well as initiating the reduction reactions responsible for the production of hydrogen and methane gas (Li et al. 2019).

Other than the location specific functions, there are other determinstic factors that may impact the selection pressure in MECs. Determinstic factors such as choice of electrode material, substrate compositon, operation, and design may alter which microroganisms are favoured to thrive (Li et al. 2019). For instance, the choice in substrates used for the nutrient medium may impact the complexity of the microbial community. Nutrient medium containing only acetate will allow for all microorganisms that can utilize acetate as a carbon source to prosper. In contrast, supplementing the system with a more complex food source, such as a mixture of acetate, butyrate, and propionate, selects for a more diverse community. This allows for microorganisms that can use one or all of these substrates as an energy source to grow and colonize the electrodes. The movement of the microroganisms is explained by dispersal. The way in which dispersal impacts the microbial communities in single- and dual chamber MECs is different. The anode and cathode are located in different compartments, seperated by an ion exchange memebrane, in dual-chamber MECs. This prevents the interaction of the microbial communitites of the two communities. Due to this, there is no competition between the microorganisms from the different habitats for the same space and resources. Conversely, single-chamber MECs consists of one compartment that the anode and cathode communities share. For single-chamber MECs with continous circulation, all microorganinsm have equal oppurtunity to access all locations and resources within the system. In addition to the movement of the microroganisms, dispersal also refers to the random attachment and detachment to the electrode surfaces. In single-chamber MECs this allows for dispersal of the microorganims between locations to occur. This, however is not possible in dual-chamber MECs due to the separation of the electrode compartments.

2.5 Sequencing approaches of the microbial community

Amplicon sequencing and shotgun metagenomic sequencing

The identification of the species present in the microbial communities of METs is an important aspect in understanding the dynamics involved in the microbial community assembly. DNA sequencing is a strategy generally implemented for taxonomic classification and information about the relative abundance of interesting species. There are multiple approaches that can be used to accomplish this. Which strategy is chosen depends on what type of information about the microbial community is needed. Two main categories exist for the sequencing of the microbial community: Amplicon and metagenomic sequencing.

In amplicon sequencing, targeted regions or genes of interest are sequenced (Rausch et al. 2019). All organisms contain conserved regions, typically involving genes that are essential for cell function. These conserved genes, tend to be unique for each species making it a good candidate for targeted sequencing. The 16S rRNA region in bacteria and the 18S rRNA region in eukaryotes are two examples of conserved gene regions that can be utilized for taxonomic classification (Woo et al. 2008). The region of interest is amplified using polymerase chain reaction (PCR) before purification, library preparation, and sequencing of the target region occurs using NGS sequencing methods (Ambardar et al. 2016). The sequencing data is then error-corrected and amplicon sequence variants (ASVs) are determined. The ASVs can be identified taxonomically by comparison to databases with known taxa.

Shotgun metagenomic sequencing targets the entire genome of all species in the sample (Hu et al. 2021). Metagenomic sequencing can be performed using either short-read or long-read sequencing, both of which belong to the class of DNA sequencing classified as next-generation sequencing (NGS). In short-read sequencing, the DNA undergoes fragmentation resulting in short DNA segments. Illumina next generation sequencing (NGS) uses adaptors to attach fragments of DNA onto the flow cell, where they undergo clonal amplification with bridge PCR (Ambardar et al. 2016). The sequencing is a paired-end, meaning that the reaction occurs on both ends of the DNA fragment, thereby allowing for more accuracy and higher coverage (Ambardar et al. 2016). These short fragments are then simultaneously sequenced in parallel before bioinformatical tools are utilized to assemble the reads (Hu et al. 2021). Long-read sequencing is also called third generation sequencing. In contrast to short-read sequencing, there is no fragmentation of the DNA into short sequences of nucleotides, instead the fragments are kept intact, usually ranging between 10 000 -100 000 bp (Adewale 2020). There are two main types of long-read sequencing: PacBIO by Pacific Biosciences and Nanopore by Oxford Nanopore Technologies. PacBIO uses a Single Molecule real-time sequencing (SMRT) (Rhoads et al. 2015). The DNA fragments are attached onto a chip containing multiple zero-mode waveguides (ZMWs). Each individual strand of DNA as well as the DNA polymerase is immobilized in a ZMW. As the nucleotides attach the emitting light is recorded, allowing for the identification of the complementary DNA sequence (Rhoads et al. 2015). In contrast, Nanopore sequencing uses a protein pore to read the bases of the DNA fragment, usually groups ranging from 2-6 bases are read together. This process leads to the generation of an electrical current (Hu et al. 2021). The base-calling software then uses the generated electrical current profiles to identify the order of the sequence (McCombie et al. 2019). For all types of NGS technologies, once the raw sequencing reads have been obtained, bioinformatical tools are used to for *de novo* assembly and subsequent analysis to sort the reads and identify the taxonomic classification and relative abundance of the species.

Both amplicon sequencing and shotgun metagenomic sequencing have their advantages and disadvantages. Even though amplicon sequencing is considered more cost-efficient in comparison to shotgun metagenomic sequencing, there are some drawbacks as well (Liu et al. 2021). Since amplicon sequencing targets a specific region of choice, information about the species function (indicated by other genes on the chromosome) cannot be obtained. Furthermore, the amplification of a specific gene, may result in overestimation of the abundances due to the difference in gene copy number between species (Rausch et al. 2019). Additionally, since the targeted region needs to be amplified using a PCR step, inter-kingdom samples cannot be classified simultaneously. Instead, separate sequencing needs to be done for the bacterial and eukaryotic species (Jo et al. 2016). Nevertheless, due to the low quantity of DNA needed as well as the shorter analysis time, amplicon sequencing is still considered a good method when the main interest is in taxonomic classification (Rausch et al. 2019).

Shotgun metagenomic sequencing is more expensive to perform compared to amplicon sequencing. The analysis of the obtained sequencing reads is also considered to be more time-consuming and complicated (Liu et al. 2021). Even so, since there is no need for the amplification of specific regions, both prokaryotic and eukaryotic DNA can be sequenced simultaneously (Jo et al. 2016). The lack of amplification before sequencing also eliminates the biases for genes with multiple copy numbers in the final abundance of each identified species. Furthermore, the sequencing of the genome in its entirety allows for determination of

functional genes and potential species function as well as resulting in a higher taxonomic resolution compared to amplicon sequencing.

There are also some advantages and disadvantages between different types of shotgun sequencing. Although short-read sequencing is considered quite accurate, there are some limitations due to the length of the reads. The genome may consist of large areas with repetitive sequences. These repetitive regions make it difficult to construct unique short reads, resulting in gaps or low coverage over certain areas during assembly (Adewale 2020). One of the reasons for the development of long-read sequencing was to address this issue. Long-read sequencing on the other hand, tend to have higher error rates, lower coverage of the genome, and a lower raw sequencing read yield (Amarasinghe et al. 2020). For Nanopore sequencing the reduction in accuracy and low sequencing yield can be attributed to factors pertaining to the protein pore. The chances of errors increase because of the length of the fragment travelling through the pore, as well as the fact that the pore measures the electrical current for a group of nucleotide bases (McCombie et al. 2019). Additionally, the pore is unable to control the speed in which the DNA fragment travels through which in turn can result in section of fragments not being sequenced (MacKenzie et al. 2023). Even so, Nanopore sequencing is a good choice for experimental designs where rapid analysis of the microbial community is needed, due to their portable DNA sequencers which can be used in lab or on site for DNA sequencing.

3 Materials and Methods

The doctoral thesis contains research from two experiments, presented in four papers (Fig 2). Both experiments investigate the impact electrode material has on the performance and microbial community in MECs. The first experiment (**Paper A**) examines the differences in cathode material. This experiment will be referred to as the cathode experiment henceforth. The microbial community from this experiment was further analysed for bacterial and viral associations (**Paper C**). Furthermore, different sequencing approaches were also investigated using the microbial community samples from this experiment (**Paper D**). The second experiment studies the impact the anode material and dispersal limitation has on the performance and microbial community assembly (**Paper B**) and will be referred to as the anode experiment.

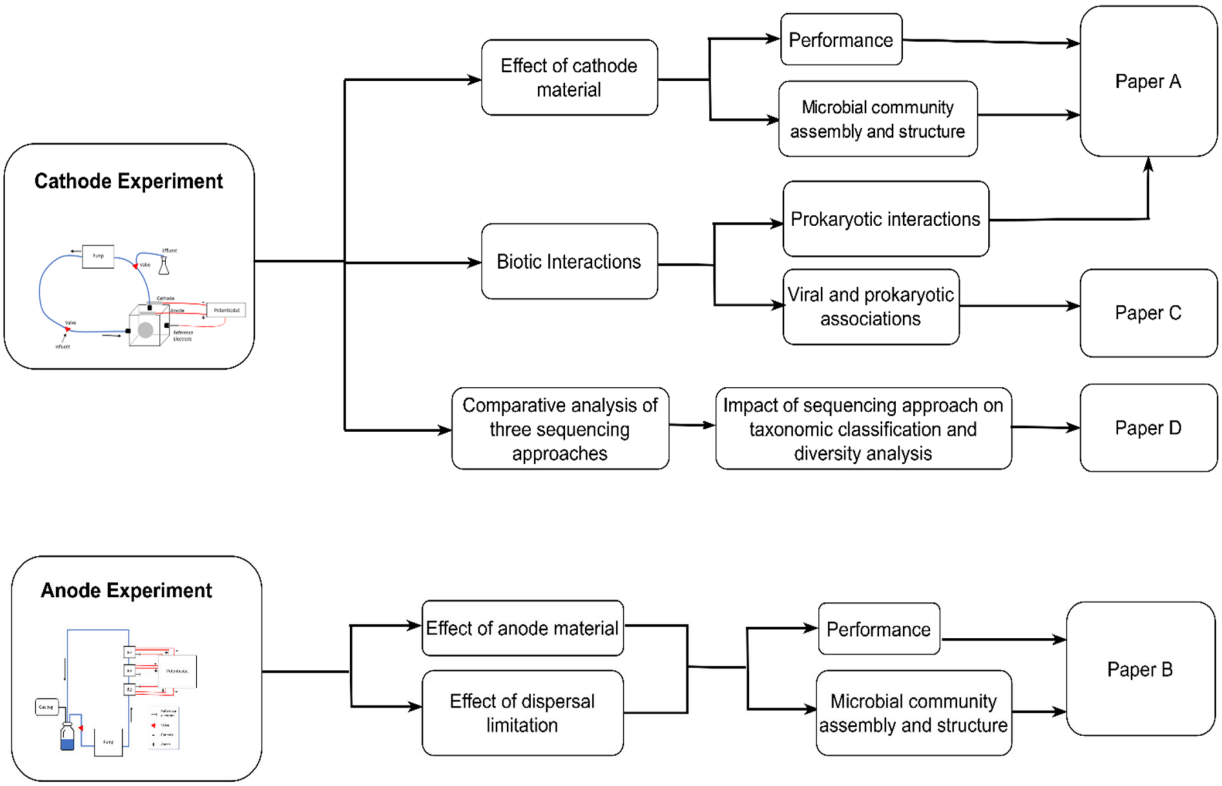

Figure 2. Schematic illustration of the two experiments and the corresponding studies.

3.1 Experimental design and operation

Single-chamber reactors were constructed to study the effects of different electrode materials on the performance of MECs. A nutrient medium (NM) containing acetate, propionate and butyrate was given to the systems with regular intervals (**Paper A**; **Paper B**). Samples were taken from the effluent after each feeding for further analysis. 5 mL of anaerobic mesophilic digester sludge was used to inoculate the experiments investigating different electrode materials. 3-4 replicate reactors for each material were constructed and operated to determine the consistency in the obtained results.

Reactor design for the cathode experiment

Three cathode materials were investigated: steel, titanium and carbon nanoparticle covered carbon paper (CNP) (Fig 3). A total of nine reactors were constructed and operated for 106 days, allowing for triplicates of each material. Carbon cloth was used as anode material for all reactors, to isolate the effects seen on performance and microbial community assembly to the cathode material. The total system volume for each reactor was 70 mL, and the nutrient medium was replenished every 2-3 days. An applied potential of 1 V was placed between the anode and cathode. The MECs with carbon nanoparticle cathodes are referred to as C1-C3, while the MECs with titanium cathodes are called T1-T3 and those with steel cathodes are referred to as S1-S3.

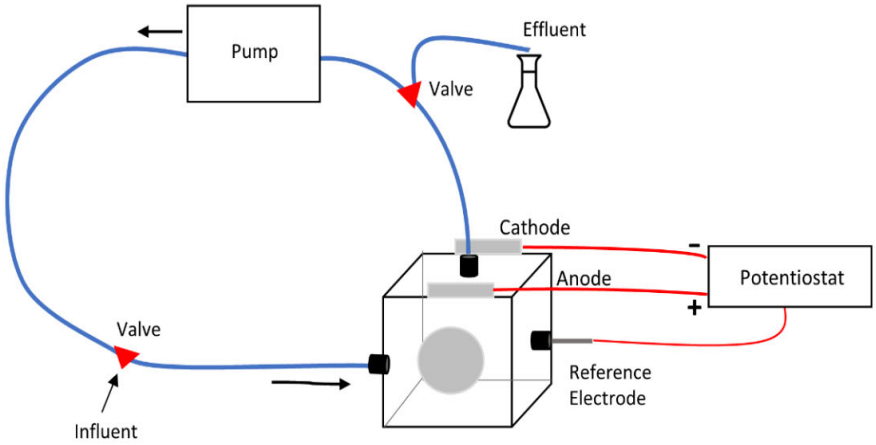

Figure 3. Schematic illustration of the setup of the cathode experiment.

Reactor design for the anode experiment

The anode experiment compared graphene and nickel with the more conventional anode material carbon cloth. A total of twelve reactors were constructed, four replicates for each material. The twelve reactors were placed in four replicate hydraulic loops, where each loop has three reactors with different anode materials (Fig 4). To eliminate the effect of the cathode, all reactors were constructed using steel as the cathode material. The total volume of the hydraulic loop was 225 mL. The reactors were operated for 56 days, under an applied potential of 1 V between the anode and cathode and fed at intervals of 4-5 days. The four hydraulic loops are labelled as hydraulic loop 1-4. The reactors are designated according to the initial letter of the material and the hydraulic loop number they are associated with. This means carbon cloth anodes are called C1-C4, graphene anodes are referred to as G1-G4, and nickel anodes as N1-N4.

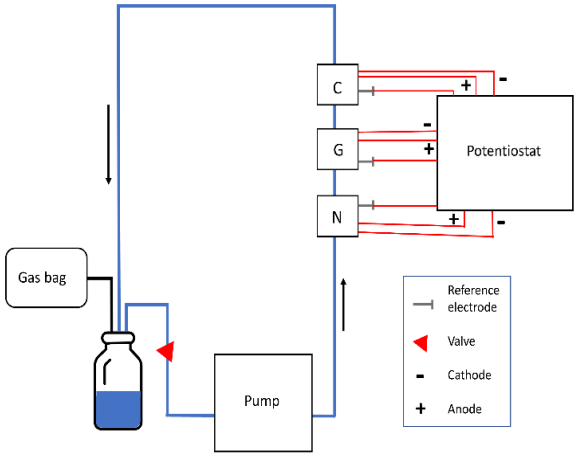

Figure 4. Schematic illustration of one of the hydraulic loops set up in the anode experiment.

3.2 Analytical methods for characterization of systems

The changes in carbon source concentrations for each system was measured throughout the experiment with a high-performance liquid chromatograph (HPLC) with a UV detector (Shimadzu) and an Aminex HPX-87H Ion exclusion column (BioRad). Sampling of the liquid in the systems were done before each feeding. To analyse consumption patterns the system were sampled at specific intervals, 0, 24 and 72 hours once a stable current production had been achieved.

The changes in bioelectrochemical activity between the start and end of the experiment was evaluated using cyclic voltammetry (CV) against a reference electrode (Ag/AgCl). Cyclic voltammetry characterizes the electrochemical reactions of the system, specifically the electrode of interest. This is achieved by applying a potential with a linear sweep to the working electrode, either the anode or the cathode, against a reference electrode with a known potential (Elgrishi et al. 2018). The bioelectrochemical activity is evaluated by measuring the generated current while the potential is applied to a working electrode. Thereby, we determine how well the electrode can catalyse reduction and oxidation reactions within the system. In addition to its use to evaluate the electrode ability to catalyse the redox reactions, cyclic voltammetry can also be used to detect the hydrogen generation that may occur in the system when the potential reaches negative values (Elgrishi et al. 2018). Since CV is used to evaluate the bioelectrochemical properties of the electrodes, it is suitable method for assess changes to the electrode surface due to microbial colonization in MECs. An increase in the ability of the electrode to catalyse redox reactions indicates the growth of biofilms on the electrode surface able to initiate these reactions. The current generation for all systems was measured using a potentiostat in accordance with methods described in **Paper A**.

3.3 Microbial community analysis

Sampling of the inoculum, biofilms residing on the electrode surfaces, the active viral community from the liquid as well as the biomass suspended in the liquid were done at the end of the experimental run, in-depth details can be found in **Paper A-D**. The FastDNA spin kit for Soil (MP Biomedicals) was used to perform the DNA extractions. A 0.2 µm polyethylene

sulfone filter was used to filter the viral samples to remove bacteria. A cellulose membrane filter with a 100kDa molecular weight cutoff (Amicon Ultra-15, Milipore) was then used to concentrate the samples. Treatment with DNase I (20 U, Invitrogen) was done to remove extracellular DNA before Norgen's Phage DNA Isolation Kit (Norgen Biotek) was used for viral DNA extraction.

Two sequencing strategies were used for the identification and classification of the taxa present in the microbial community composition of the MECs: amplicon and metagenomic sequencing (Fig 5). The anode experiment was analysed using shotgun metagenomic sequencing (**Paper B**), whilst the cathode experiment was analysed using both amplicon (**Paper A**) and metagenomic sequencing (**Paper C** and **Paper D**). A brief overview of the sequencing and downstream analysis is presented, and in-depth details can be found in **Papers A-D**.

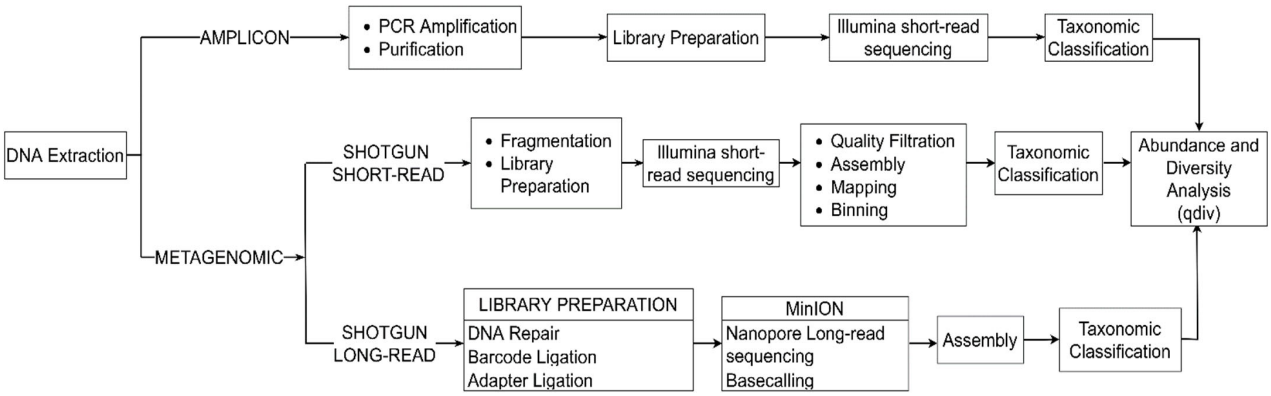

Figure 5. Schematic of the general workflow for the sequencing approaches.

Amplicon sequencing of the cathode experiment

The 16S V4 region of the 16S rRNA gene was amplified using the primer pair 515'F (GTGBCACMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) (Caporaso et al. 2011, Hugerth et al. 2014). Phusion master mix comprised of 0.4 µL Phusion hot start II polymerase, 8 µL 5x Phusion Buffer, 1.2 µL DMSO, and 23.6 µL ultrapure water was used (ThermoFischer r Scientific). 40 µL of the Phusion master mix was combined with 2 µL of the sample as well as the 2 µL of the forward and reverse primers respectively. The PCR was performed using a Bio-Rad Thermal Cycler. Activation was for 30 sec at 98ºC, followed by 24 cycles od denaturation for 10 sec at 98ºC, annealing for 30 sec at 55.8ºC and extension for 30 sec at 72ºC. The final elongation was for 10 min at 72ºC. The amplified sample was purified using MagJET NGS Cleanup and Size selection Kit (ThermoFischer Scientific). A Qubit Fluorometer (ThermoFischer Scientific) was used to determine the DNA concentration. An Illumina Miseq system with the Miseq reagent kit v3 and 2x300bp read length was used to sequence the pooled DNA samples. The raw sequencing data was processed using VSEARCH (Rognes et al. 2016) and DADA2 (Callahan et al. 2016) before being combined into a consensus table containing the ASVs identified by both methods. Taxonomical profile of the samples were identified was done using the Midas database (Nierychlo et al. 2020). Due to low read count, the cathode sample for T2 was excluded from downstream analysis of the amplicon sequencing data.

Metagenomic sequencing of microbial communities in MECs

Illumina short-read sequencing

In **Papers B**-**D** the microbial community was sequenced using short-read sequencing. Sequencing libraries were constructed from 10-50 ng DNA using SMARTer ThruPLEX DNA-seq Kit (cat#R400676, Takara) with HT dual indexes (cat#R400660, Takara). Fragmentation of the samples, targeting an insert size of 350-400bp was done using a Covaris E220 system. NovaSeq 6000 system (Illumina, Inc) was used to perform the paired-end sequencing.

In all three papers fastp v0.20.0 (Chen et al. 2018) was used for quality filtering of the raw sequencing reads. BBNorm (BBMap v38.61b, http://sourceforge.net/projects/bbmap/) was then used for normalization to a depth of 100. The data was processed both by co-assembly and individual assembly. The co-assembly was done using Megahit v1.2.9 (Li et al. 2015), mapping using Bowtie v2.3.51 (Langmead et al. 2012), and binning using MetaBat v2.12.1 (Kang et al. 2019). The individual assembly was done using Megahit, Minimap v2.24-r1122 (Li 2018) and then finally binning using Vamb v3.0.2 (Nissen et al. 2021). For the data in **Paper B** the bins obtained from the co-assembly and individual assembly were combined, whilst **Paper C** and **Paper D** selected consensus bins from the two assemblies using DAS Tool v1.1.4 (Sieber et al. 2018). CheckM v1.1.3 (Parks et al. 2015) was then used to control the completeness and contamination before the bins were dereplicated using dRep v3.4.0 with an average nucleotide identity (ANI) of 95% (Olm et al. 2017). GTDB toolkit (Chaumeil et al. 2022) was used to identify the taxonomic affiliation of the representative bins.
CoverM v0.6.1 (https://github.com/wwood/CoverM) was used to estimate the relative abundance of the identified representative species.

In **Paper** C, Metaviral SPAdes v3.15.3 (Antipov et al. 2020) was used for the assembly of the raw viral sequence reads into contigs. Both CheckV and VIBRANT was used to control the contigs. Contigs were classified as viral if they had at least one viral gene identified by CheckV, had a minimum length of 5 kb, and were classified as viral by VIBRANT. Virus species cluster were identified from the combined virus contigs and prophage sequences identified from the prokaryotic dataset using dRep. A species representative was chosen based on the calculated completeness using CheckV before relative abundance for each species was determined using CoverM. PhagCN (Shang et al. 2021) was used for taxonomic classification of the viral contigs.

Nanopore long-read sequencing

In **Paper D**, the prokaryotic samples were sequenced using long-read sequencing in addition to the short-read sequencing. The Native barcoding kit 24 V14 (Oxford Nanopore Technologies) was used in accordance with the manufacturer's instruction to prepare the sequence libraries and subsequent barcoding. Sequencing was done using the MinION Mk1c system (Oxford Nanopore Technologies). The wf-bacterial-genome workflow from EPI2ME (EPI2Me labs, https://github.com/epi2me-labs/wf-bacterial-genomes) was used to perform the assembly. The depth file was created from the bam files generated by Metabat v2.12.1. Kraken2 v2.1.1 (Wood et al. 2019, Lu et al. 2022) and Bracken v2.9 (Lu et al. 2017) was used for taxonomic classification and relative abundance estimation of the contigs. The abundance of each identified taxon was then calculated using the equation 1.

$$Relative\ Abundance = \frac{Average\ Coverage \times Fragment\ Length}{Total\ number\ of\ bases\ in\ sample} \quad \text{(Eq. 1)}$$

Virus-Host Associations

The sequenced prokaryotic and viral datasets were analysed for potential virus-host associations. One of two criteria had to be met to determine if a virus-host association existed. Firstly, existence of induced prophages was evaluated. If a prophage was identified from a sequence in one of the bins from the prokaryotic data, as well as found in the virus contigs, a virus-host association could be established (Kieft et al. 2020). Secondly, identification of CRISPR spacers found in both the prokaryotic and viral dataset could suggest the presence of a previous viral infection (Andersson et al. 2008).

Community diversity analysis

Alpha- and beta diversity was used to evaluate the microbial community diversity. The diversity within one sample is explained by the alpha diversity. A diverse community with a high number of species, result in a high alpha diversity (Whittaker 1960). Richness and evenness also further explain the diversity of the microbial community. Richness refers to the number of species found within the sample, whilst evenness describes the distribution of the abundances across the species found in the microbial community (Zhang et al. 2012). The differences of the microbial communities between samples are described by the beta diversity. A high beta diversity indicates that there is a high dissimilarity between the compared communities (Whittaker 1972). There are numerous frameworks that can be used to quantitatively evaluate the alpha- and beta diversity, such as, Shannon diversity index for alpha diversity as well as Bray-Curtis dissimilarity and UniFrac distance for beta diversity (Bray et al. 1957, Lozupone et al. 2005, Konopiński 2020). The framework used to obtain quantitative measures of the alpha- and beta diversity for all datasets in this thesis was a Hill-based framework using the python package qdiv (Modin et al. 2020). Hill numbers uses a diversity order to give weight to the relative abundance of different taxa in a community. For instance, a diversity order of 0 would place no importance on the relative abundance distribution of the species. Therefore, only absence or presence would be taken into consideration, while a diversity order of 1 weighs the taxa based on their relative abundance distribution. The higher the diversity order is, the more weight is placed on the relative abundance distribution (Modin et al. 2020).

A network analysis, described in Paper A, was performed to establish potential negative and positive interactions within the microbial community in a specific habitat of the analysis included 146, 113 and 229 unique ASVs on the anode, cathode, and suspension, respectively.

3.4 Assessment of MEC Performance

The MEC performance was evaluated for the systems in **Paper A** and **Paper B**. The performance parameters assessed were lag time, current density, and the electrical charge. Lag time is defined as the time it takes for 1 A/m^2 of current density to be generated, counted from the initial inoculation. The current density for the systems were calculated by dividing the generated current with the anode surface area. The coulombic efficiency of the MECs were also evaluated in **Paper A**. The proportion of the removed organic substrates resulting in the

generation of current is explained by the coulombic efficiency (Eq. 2). Finally, the electrical charge was calculated using equation 3.

$$Coulombic\ efficiency = \frac{\int_{t_1}^{t_2} I(t) \cdot dt}{F \cdot V \cdot (b_{ac} \cdot \Delta C_{ac} + b_{prop} \cdot \Delta C_{prop} + b_{but} \cdot \Delta C_{but})} \quad \text{(Eq. 2)}$$

$$Charge = \int_0^t I \cdot dt \quad \text{(Eq. 3)}$$

I is the current (A), t is time (s), F is Faraday's constant (96485.3 C/mol e^-), V is the liquid volume in the MEC (L), ΔC is the change in concentration of the substrate acetate, propionate, and butyrate (mol/L), and b is the number of electrons liberated when the substrates are oxidized to CO_2 (mol e^-/mol substrate).

3.5 Calculations and Statistical Analysis

The significance of the alpha diversity in **Paper A** was determined using a one-way single ANOVA followed by a post hoc (Tukey) test using Pinguoin (Vallat 2018). The beta diversity was visualized using a principal component analysis (PCoA) for all datasets and experimental setups. A Raup-Crick null model (Raup et al. 1979) was used to determine whether the observed differences could be attributed to random chance. Mantel test was performed to evaluate the correlation between differences in community composition and system performance (Mantel 1967). In addition to these, **Paper B** also evaluated the evenness based of the microbial communities based on Pielou's index (Pielou 1966) and permanova was employed to determine the impact the system and anode material on the microbial community composition. In **Paper D** regression analysis of the compared alpha and beta diversity was performed to determine if any significant correlations could be established.

In **Paper B**, two explanatory variables, namely hydraulic loop and anode material, as well as a series of responsible variables were assessed using a two-factor ANOVA with a paired sample t-test as a post hoc analysis. The responsible variables evaluated were the MEC performance and the microbial community composition, evenness, and a subset of the most abundant electrogens. Dominance analysis and multiple linear regression (Azen et al. 2003), as detailed in **Paper B,** was then performed to establish the percentage of contribution of the explanatory variables on the responsible variables.

4 Results and Discussion

4.1 Performance of MECs

Correlation between start-up time and the electrode material

The time it takes for the system to reach a sufficient level of current generation is defined as the lag time or startup time. In the systems investigated in this thesis, a current density threshold of 1 A/m^2 was determined as the minimum level for the system to be classified as actively generating current. There was no difference in lag time between the different cathode materials investigated in **Paper A** (Fig 6a). There were, however, variations observed in startup time between the replicates of each cathode materials, ranging from 5-17 days, although the titanium MECs showed the least amount of variation.

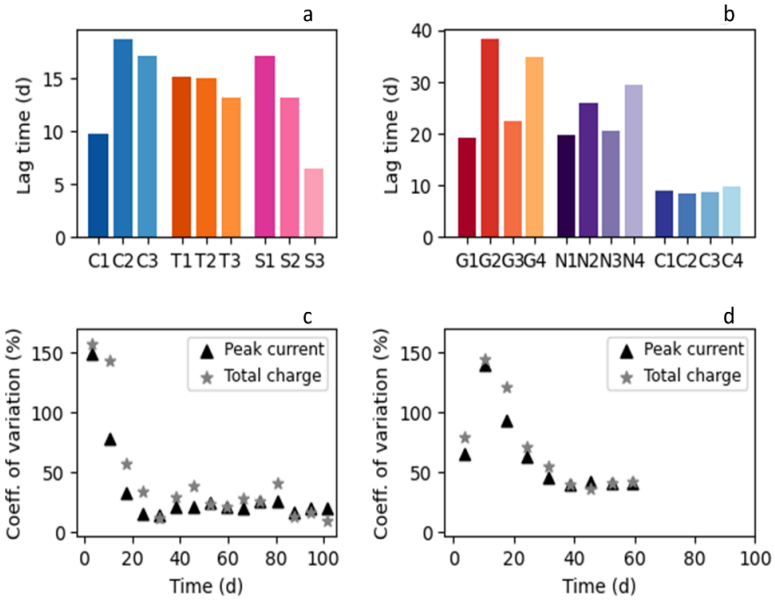

Figure 6. a) Bar graph of the lag time of the MECs for the cathode experiment. C1-C3 refer to MECs with CNP, T1-T3 refers to MECs with titanium and S1-S3 refers to MECs with steel cathodes. b) Bar graph of the lag time of the MECs for the cathode experiment. C1-C4 refer to MECs with carbon cloth, G1-G4 refers to MECs with graphene and N1-N4 refers to MECs with nickel anodes. c) Variation in peak current density and total charge per week for all MECs in the cathode experiment. d) Variation in the peak current density and total charge per week for all MECs in the anode experiment.

In contrast, the anode experiment (**Paper B**) showed clear variations in startup time between the different anode materials (Fig 6b). The carbon cloth anodes (C1-C4) had the shortest lag time and least variation between replicates, ranging from 8-10 days. The observed lag time for C1-C4 was similar to the lag time observed in the cathode experiment which also had MECs with carbon cloth anodes (Fig 6a), while there was a much higher variation for the replicates of both the graphene (G1-G4) and nickel (N1-N4) anodes. The observed lag time for these two materials ranged between 18-38 days. From the statistical analysis it was concluded that the effect of the anode material on lag time was significant ($p<0.05$, ANOVA). Subsequent post hoc test emphasized the difference in anode material effect between carbon cloth and the other

two materials ($p<0.05$, t-test). The total charge and average peak current for the first three weeks of the experimental run also reflected this variation in performance between different reactors for both experiment (Fig 6c-d).

Figure 7. Drops of water on the anode materials show the difference in hydrophobicity: graphene (a), nickel (b), and carbon cloth (c).

Based on the findings in the cathode experiment, where all systems had the same anode material, the lag time observed indicate that there is some randomness involved in the initial colonization, highlighting the stochasticity of the initial microbial assembly. The length of the lag time may be impacted by which organism first colonizes the electrode surface. Previous research on the development of biofilms in BES have shown the importance of stochastic factors during colonization. A study investigating the initial colonization of two different *Shewanella* species were able to determine that the fittest, better performing species doesn't necessarily dominate the electrode surface. Instead, whichever of the two that first attaches becomes the dominating species (Kees et al. 2021). Similarly studies on the development of anammox biofilms as well as those investigating floating and settled granules have noted the importance of stochastic factors during assembly and development (Niederdorfer et al. 2021, Trego et al. 2021). The anode experiment on the other hand indicated differences in the lag time between materials (Fig 6b). This indicates that although cathode material may not have a direct influence on the lag time, the choice in anode material determines the length of the lag time. There are multiple factors that may impact the colonization and subsequent current generation on the anode. The hydrophobicity of the material is one aspect that may impact the ability of the microorganism to attach and colonize the surface (Muhammad et al. 2020). Typically, bacteria have a higher affinity for attachment onto hydrophobic surfaces (De-la-Pinta et al. 2019). The carbon cloth anodes were the most hydrophobic of the three materials tested (Fig 7), potentially explaining the short lag time and low variation seen. Although both carbon cloth and graphene are carbon-based material, they have a large difference in lag time (Fig 6b). Considering graphene's high conductivity and large surface area (Geim et al. 2007, McAllister et al. 2007), other aspects may be causing the delayed lag time. Studies have highlighted the antimicrobial properties that the material possesses (Bhatt et al. 2023). Some of these antimicrobial properties could be correlated with the manufacturing process. There have been reports regarding the presence of toxic residues from the manufacturing process resulting in antimicrobial outcomes (Yu et al. 2016). If so, a potential explanation could be the reduction of the toxic residues over time allowing for the delayed attachment of bacteria onto the anode surface. However, the manufacturing process used to produce the graphene in **Paper B**, does not typically result in toxic residues remaining on the surface (Yu et al. 2016). A more likely explanation for the observed lag time in the anode experiment is caused by the surface

structure of the graphene. The graphene consists of sharp flakes covering a nickel surface (Pandit et al. 2018). As the bacteria attach onto the surface, the sharp flakes pierce them causing cell death. The bacteria that subsequently attach onto the surface, have a layer of organic matter obstructing the sharp flakes and preventing cell death.

Comparison of current generation of the different electrode materials

In both experiments, a gradual increase in current could be seen until a stable level of current generation was observed (Fig 8). For the cathode experiment, no difference in generated current could be observed between materials once the system had stabilized (Fig 8a). All MECs reached a peak current density around day 30. In contrast, there were differences in current generation between the different materials in the anode experiment (Fig 8b). The carbon cloth anodes had a much higher current generation in comparison to both the graphene and nickel MECs. The cumulative total charge during the experimental run was much higher for carbon cloth MECs in comparison to both graphene and nickel MECs (Paper B: Fig 2c). Comparisons of the graphene and nickel MECs also highlighted some small differences in current generation, where graphene seemed to produce more current (Fig 8b). The peak current density for the carbon cloth anodes were reached around day 20, whilst the graphene and nickel MECs reached a peak current density between days 25-45. Further analysis highlighted the significant effect of anode material on the peak current density and total generated charge in the anode experiment ($p<0.05$, ANOVA). It was concluded based of the post hoc test that there was a significant difference between the peak current density and total generated charge of the carbon cloth anodes and the other materials ($p<0.05$, t-test). When the systems were assessed on a weekly basis, a significant effect of the hydraulic loops on the peak current density was established for the third week, which corresponds with day 7 to day 20 ($p<0.05$). The post hoc test showed there was a significant difference between hydraulic loop 1 and 4 ($p<0.05$, t-test), potentially indicating the impact the microbial community structure of the two different hydraulic loops may have on the performance.

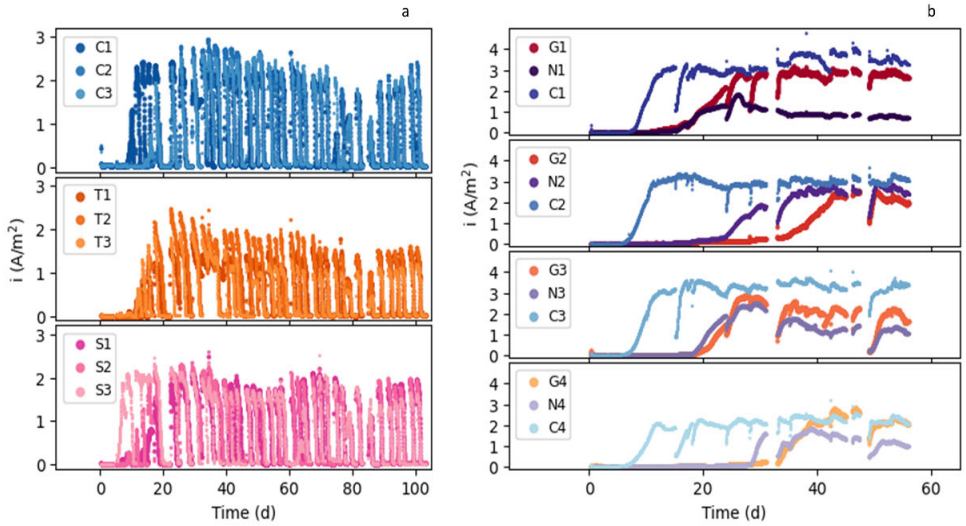

Figure 8. a) Chronoamperometry measurements of the current generation for the 9 MECs with different cathode materials. C1-C3 refer to MECs with CNP, T1-T3 refers to MECs with titanium and S1-S3 refers to MECs with steel cathodes. b) Chronoamperometry measurements of the current generation for the 12 MECs with different anode materials C1-C4 refer to carbon cloth, G1-G4 refers to graphene and N1-N4 refers to nickel anodes.

The current generation in the cathode experiment has continuous peaks in current followed by dips (Fig 8a). The cause of these dips is due to the access to carbon sources during the experiment. Since the concentration of the nutrients decreases between batch cycles as the bacteria consume them. As the bacteria consume the carbon the current generation increases. Once the carbon is depleted, there is a reduction in the produced current. This trend is not seen in the anode experiment since there is no depletion of the organic material in the system between batch cycles due to a larger volume of nutrient medium circulating in the hydraulic loop.

Once the peak current density had been reached in all systems, a slight reduction in the produced current could be observed (Fig 10). After this, the MECs in the cathode experiment had a stable current generation slightly lower than the peak current density observed (Fig 10a). A similar trend could be seen in the anode experiment; it was however much less prominent (Fig 10b). The duration of the experimental run might be a possible cause for the differences observed in the two experiments. The cathode experiment was operated for 106 days, while the anode experiment was run for only 56 days. The total charge per week and peak current density for the anode experiment highlight a downward trend (Fig 10b; Fig 11b). This indicates that the continuous operation of the anode experiment would have potentially resulted in a similar outcome as that of the cathode experiment. Once the initial colonization of the anode has occurred, the newly formed biofilm with electrogenic bacteria will have equal opportunity and conditions to initiate the oxidation of organic substrate and the following electron transfer and current generation. In the initial stages of biofilm formation and operation the biofilm consists of a thin layer, allowing all microorganisms in the biofilm equal access to the electrode surface and the nutrients circulating (Fig 9). This allows the electrogenic bacteria on the anode to achieve maximum electron transfer, thereby achieving maximum current generation. As the

biofilm continuous to grow, the access to both the electrode surface and the nutrients become more limited. This results in a reduction in the ability of the electrogenic bacteria to transfer the electrons, since some of the cells closer to the surface of the biofilm have unlimited access to the nutrient but limited access to the electrode surface to facilitate electron transfer. However, those closest to the electrode surface have a limited access to the nutrients.

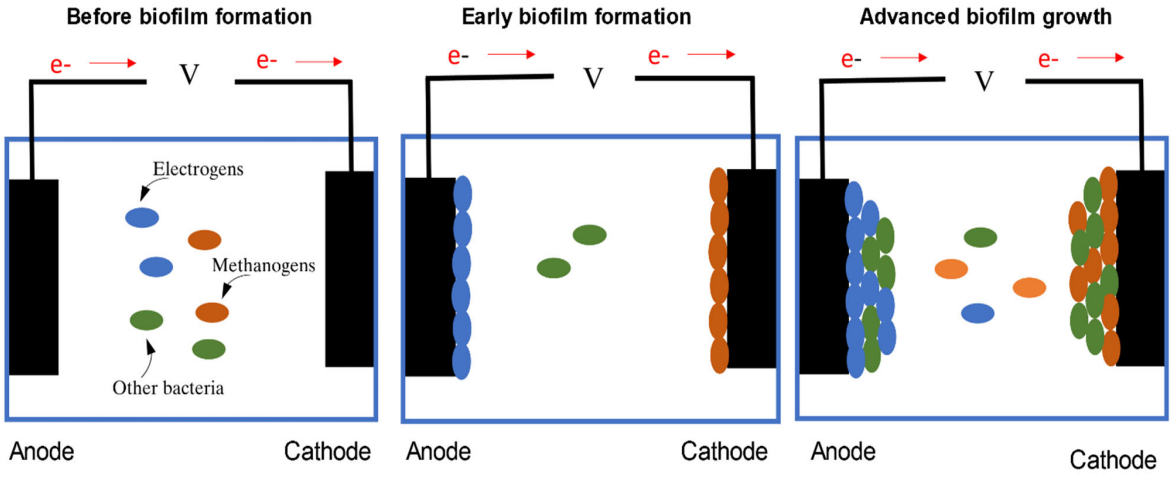

Figure 9. Illustration depicting the different stages of biofilm formation and growth in MECs.

Electrogenic bacteria have some mechanisms to overcome these limitations in access to both the nutrient and electrode surface. The use of nanowires for instance could help overcome this issue in the biofilm community and help improve electron transfer (Gorby et al. 2006, Reguera et al. 2006, Wang et al. 2023). The presence of nanowires has been noted in some prominent electrogenic bacteria such as those belonging to the *Geobacter* and *Shewanella* genera (Reguera et al. 2005, Subramanian et al. 2018). Nanowires are conductive pilis which can transport electrons between bacteria as well as to conductive surfaces (Reguera et al. 2005). The use of nanowires could potentially aid the electrogenic bacteria further from the electrode surface, by transportation of the electrons to other electrogenic species closer to the surface. Thereby, allowing for electrogenic bacteria that are further from the anode to contribute to the current generation. In addition to the electrogenic bacteria found in the biofilm community of the anode, there are also many species of fermenters. For instance, species belonging to the *Anaerolineaceae* family, *Clostridales* order and *Spirochaetaceae* family have been identified in the anode biofilm community. The species use a fermentative metabolism, where they produce hydrogen and acetate by utilizing sugars such as glucose (Menes et al. 2002, Maune et al. 2012, McIlroy et al. 2017). The presence of fermenters could potentially indicate a synergistic relationship, where the electrogenic bacteria and fermenter help each other though the transfer of electrons or nutrients. Additionally, the concentration of carbon sources present in the system is of importance for the generation of current. It is very likely that the higher the concentration of the nutrients in the system, the higher the rate of diffusion of these substrates is through the biofilm. This was seen when comparing the anode and cathode experiments, one had a limited access to the carbon sources whereas the other did not. Since the carbon sources are present in higher concentrations, the higher diffusion of these substrates through the biofilm is also a likely reason as to why the reduction in current is only observed to a small degree in the anode experiment.

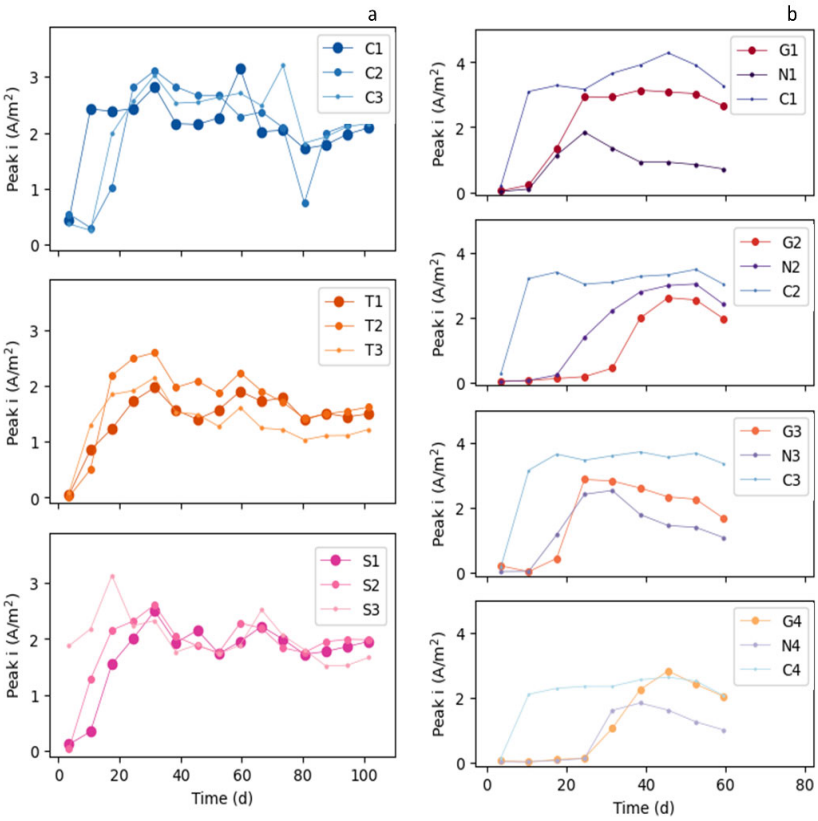

Figure 10. Peak current density (i) during the cathode and anode experiment. Weekly values are shown. a) The peak current density for the cathode experiment. C1-C3 corresponds with CNP, T1-T3 with titanium and S1-S3 with steel cathodes. b) The peak current density for the anode experiment. C1-C4 corresponds with carbon cloth, G1-G4 with graphene and N1-N4 with nickel anodes.

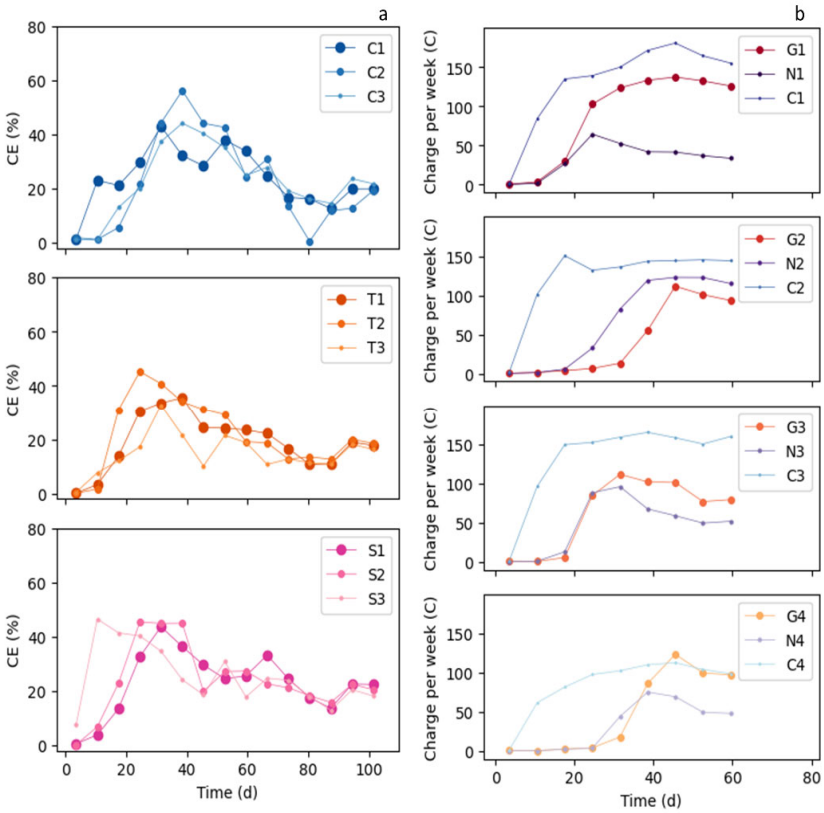

Figure 11. Coulombic efficiency (CE) and charge per week (C) during the cathode and anode experiments respectively. Weekly values are shown. a) The coulombic efficiency for the cathode experiment. C1-C3 corresponds with CNP, T1-T3 with titanium and S1-S3 with steel cathodes. b) The charge per week for the anode experiment. C1-C4 corresponds with carbon cloth, G1-G4 with graphene and N1-N4 with nickel anodes.

The ratio between the number of electrons transferred by the electrogenic bacteria to the anode from the consumption of the available carbon substrates and the theoretical maximum electrons available for transfer based on substrate concentration is referred to as the coulombic efficiency (Escapa et al. 2009). The observed coulombic efficiency for all MECs with differing cathode materials had a similar trend to the peak current density (Fig 11a). On day 40 a peak in the coulombic efficiency could be seen, ranging from 33 % to 56 %. Similar to the reduction in peak current density, the coulombic efficiency also showed a drastic reduction once the maximum levels had been reached. The reduced coulombic efficiency was approximately 20 %. Since the anode experiment consisted of four hydraulic loops, where each loop connected three MECs the coulombic efficiency for each MEC could not be determined. Instead, the charge generated per week was calculated. The carbon cloth MECs reached a peak in charge generated per week between day 20 and 30, except for C1 which reached a peak between day 40 and day 50 (Fig 11b). The graphene and nickel MECs both reached a peak in charge per week around day 30 and day 40 as well. Similar to the peak current density, once this peak in charge per week had been observed a slight reduction could be observed between day 40 and day 60. At this point the system stabilized. To investigate the patterns in carbon consumption for the cathode experiment in the absence of electrogenic activity, the potential was turned off for a batch cycle and sampled at the end for analysis of substrate concentrations (Fig 12). There were no major differences in the carbon consumption observed, indicating that the non-

electrogenic bacteria found in the system consume the resources rapidly. The degree of consumption seemed to vary slightly for acetate compared to propionate and butyrate. Acetate was consumed to a slightly lesser extent. This may be due to some fermenters favouring the more complex carbon sources such as propionate and butyrate while electrogenic bacteria favour acetate.

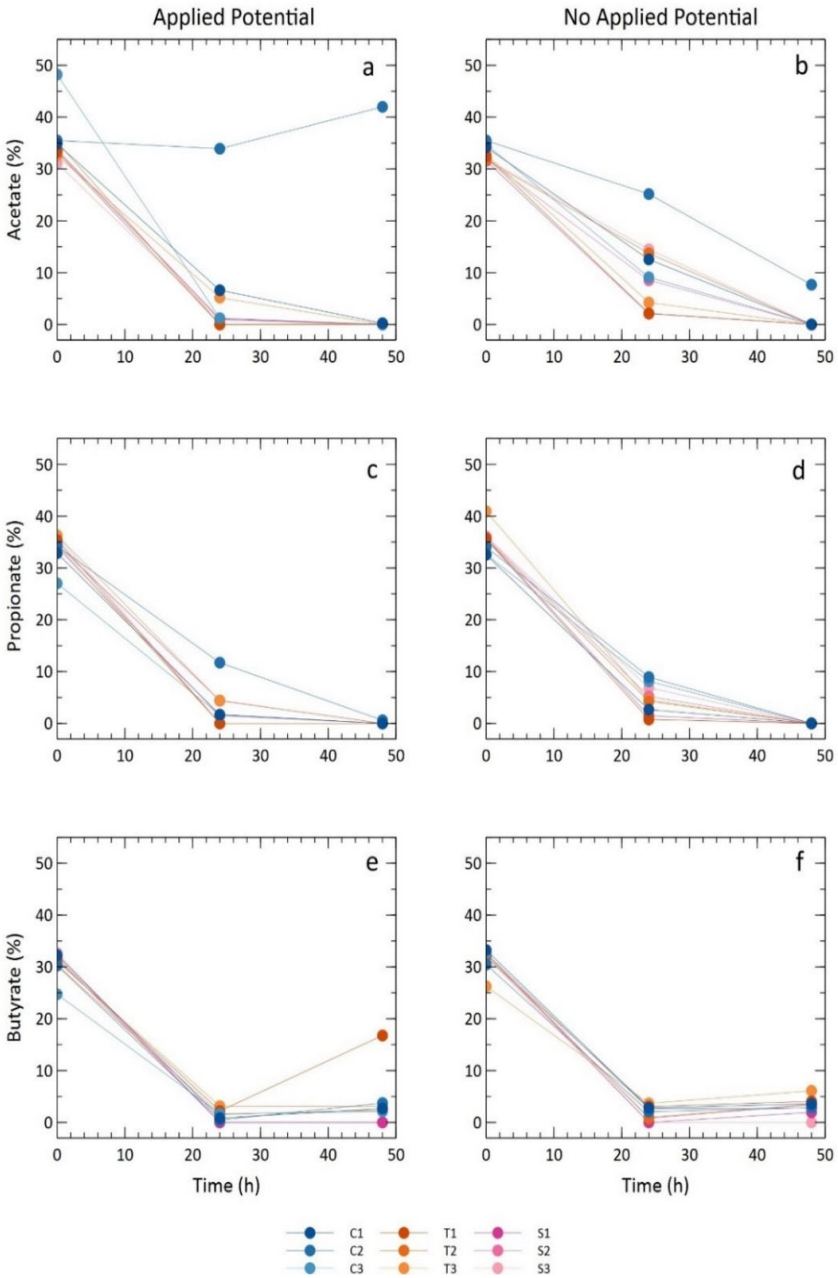

Figure 12. Concentration of acetate, propionate, and butyrate over time in the presence and absence of an applied potential on the system for the MECs with different cathode materials. The y-axis shows the reducing equivalent associated with the carbon source as a percentage of the total reducing equivalent of all carbon sources. The columns show presence **(a, c, e)** and absence **(b, d, f)** of an applied potential. The rows show the concentrations of the carbon sources acetate **(a, b)**, propionate **(c, d)**, and butyrate **(e, f)**. P1-P3 are MECs with carbon nanoparticle cathodes, T1-T3 have titanium cathodes, and S1-S3 have steel cathodes.

The reduction observed in both coulombic efficiency and generated electric charge after the initial peak can most likely be attributed to changes in the carbon source utilization pathways that occur as the microbial community of the anode develop over time. It is very common that methanogens dominate the community of the cathode biofilm in MECs (Siegert et al. 2015). In single-chamber MECs where the anode and cathode compartments are not separated, there is also a higher degree of competition between the methanogens and the electrogenic bacteria for the shared carbon substrates (Kadier et al. 2016). The changes and growth of the cathode community as well as the development of the anode biofilm over time could potentially explain the shift to a lower coulombic efficiency. Especially, as the methanogenesis pathway increases over time, as the methanogens increase.

Changes in bioelectrochemical activity of the electrodes during experimental run

Cyclic voltammetry was used to measure the bioelectrochemical activity of the electrodes during the experimental run. The bioelectrochemical activity of both the anode and cathode was measured against an Ag/AgCl reference electrode at the beginning of the experiment to characterize the initial properties of the material in the absence of a biofilm. Once a stable and consistent current generation had been obtained, the bioelectrochemical activity of the system was measured again to characterize any differences and changes (Fig 13; Fig 14). An improvement in the anode bioelectrochemical reactions could be seen across all materials, indicating that the bacterial growth on the anode surface resulted in improved performance across all MECs. The carbon cloth anodes had a greater ability to catalyse the redox reactions compared to the graphene and nickel MECs (Fig 14), which was also reflected in the current generation observed. The ability of the electrodes to generate hydrogen on the cathode can also be evaluated using a cyclic voltammetry. The hydrogen evolution can be observed at the negative potentials of a cyclic voltammogram where there is an exponential increase in negative current (Fernández-Valverde et al. 2010, Elgrishi et al. 2018). An improvement of the hydrogen generation was observed for the MECs with different cathode materials (Fig 13c-d). Comparison of the bioelectrochemical activity of the cathode at the start of the experiment and once the system had stabilized highlighted the improvement in hydrogen generation for both the steel and titanium cathodes. Although the CNP had a higher catalytic activity for hydrogen evolution reactions compared to steel and titanium cathodes, there was no changes observed when comparing the results from the start of the experiment and towards the end (Fig 13c-d). This lack of change in hydrogen generation is most likely due to the large surface area of the material itself. Since the carbon nanoparticles have a large surface area, the attachment of microorganisms onto the surface does not result in a sufficient increase in the surface area which may explain the lack of change observed in the hydrogen generation. The voltammograms for the cathode also indicated a reduction peak around -0.5 V (Fig 13c-d)., which may be associated with redox active compound in the biofilm residing on the cathode surface.

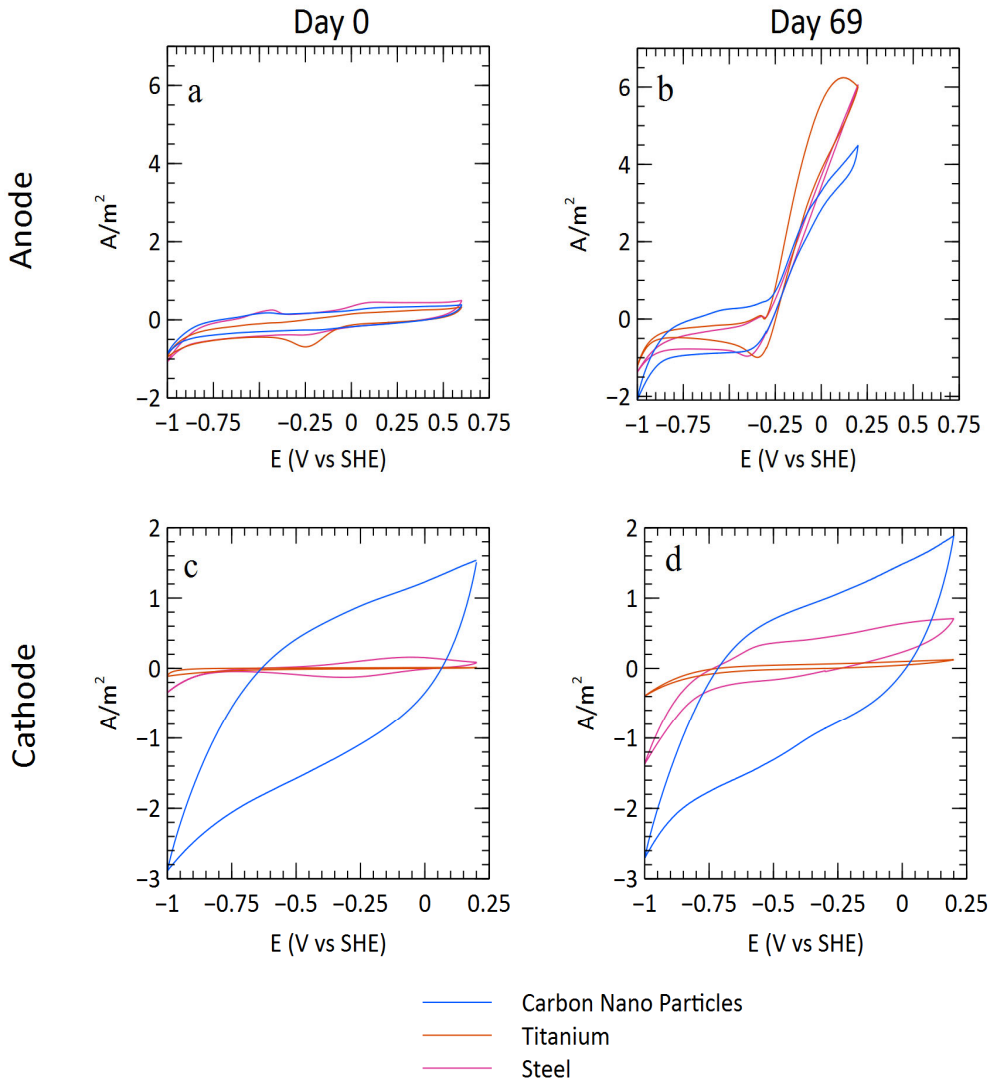

Figure 13. Cyclic voltammetry measurements from the start and end of the cathode experiment. the graph depicts a representative MEC for each material a) Anode vs reference electrode at start of the experimental run. b) Anode vs reference electrode at end of the experimental run. c) Cathode vs reference electrode at start of the experimental run. d) Cathode vs reference electrode at end of the experimental run.

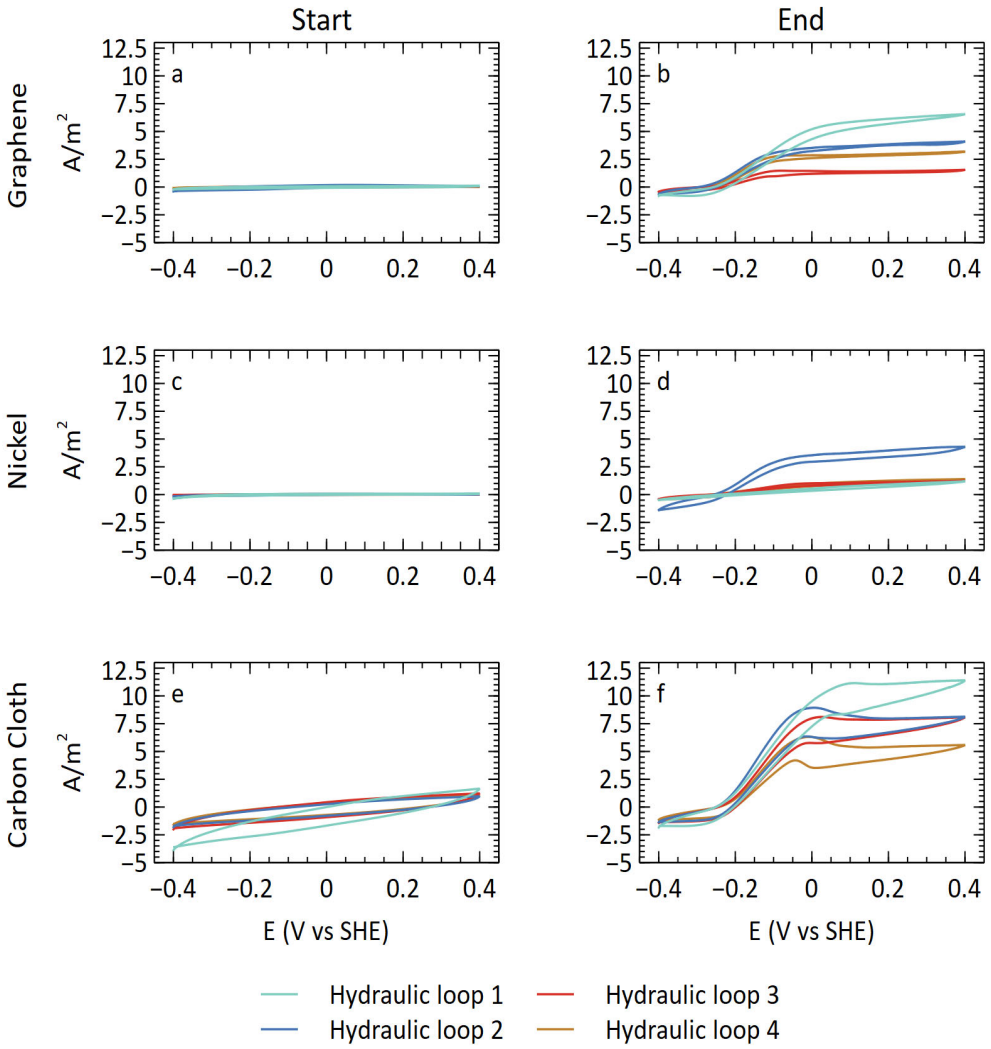

Figure 14. Cyclic voltammetry measurements of the anode from the start and end of the anode experiment. Each plot depicts the replicates from all four systems for each material. a) Graphene anodes from the four hydraulic loops at start of the experimental run. b) Graphene anodes at end of the experimental run. c) Nickel anodes at the start of the experimental run. d) Nickel anodes at the end of the experimental run. e) Carbon cloth anodes at the start of the experimental run. f) Carbon cloth anodes at the end of the experimental run.

4.2 Comparison of sequencing strategies in MECs

The sequencing strategy chosen to identify the species present in the microbial communities and their abundances is an important factor to consider when investigating the microbial communities in BES. Depending on the research question, a different sequencing approach may be the most appropriate. The microbial communities from the electrodes in the cathode experiment was sequenced using three different sequencing methods: amplicon sequencing, metagenomic short-read sequencing and metagenomic nanopore long-read sequencing.

In this section, the metagenomic short-read and long-read sequencing approaches will be referred to as shotgun short-read and shotgun long-read respectively. Although the amplicon sequencing also utilized short-read sequencing, it will be referred to as amplicon to differentiate it from the metagenomic sequencing. Some general statistics from the raw sequencing data can be found in Table 1. A total of 2279 amplicon sequence variants (ASVs) were identified based of the amplicon sequencing, whilst the shotgun short-read sequencing resulted in 278 bins after dereplication and identification of species representatives, and shotgun long-read sequencing downstream analysis resulted in identification of 462 species.

Table 1. Summary of the general information from the sequencing results after filtering and taxonomic classification.

	Amplicon	Shotgun Short-read	Shotgun Long-read
Total number of samples	18	19	19
Total number of ASV/Bins/Species	2279	278	462
Total raw reads	1627914	8.21×10^8	1844760
Minimum number of raw reads in a sample	42562	15260836	2806

Differences in microbial community composition and relative abundances were visualized using heatmaps (Fig 15; Fig 16; Fig 21). All three methods identified species belonging to the *Desulfobacterota* phylum to be dominating the anode community, whilst the cathode community was dominated by methanogens belonging mainly to the *Methanobacteriaceae* family. There were, however, differences in the specific species identified. Both the amplicon and shotgun short-read dataset identified two dominating species that were present on the anode (Fig 15; Fig 21). A species of *Geobacter* (ASV7+ASV8 and S46 bin 7) were found to be in high abundance on the carbon nanoparticle anodes as well as that of S3. Additionally, a species belonging to the *Desulfobacterota* phylum (ASV1) was identified by the amplicon dataset to be dominating most anodes, based on the shotgun short-read dataset this species corresponds with a species from the *Trichloromonas* genera (S53 bin 9) identified by the shotgun short-read dataset. Correlations of the calculated abundances of the dominating species on the anode highlighted the similarities in the abundances for these species in both the amplicon and shotgun short-read datasets (Fig 17a). In contrast, there were a larger number of electrogenic species identified based on the shotgun long-read dataset (Fig 16). A species of *Desulfuromonas* was found in high abundance in almost all MECs, additionally the *Geobacter* species *Geobacter sulfurreducens* and *Geobacter metallireducens* were also found in some of the MECs.

The cathode community composition determined by the amplicon and shotgun short-read dataset seemed to resemble each other (Fig 15; Fig 21). The amplicon sequencing identified the presence of two species from the *Methanobacteriacae* family (ASV3 and ASV10) as well as a *Acetobacterium* species (ASV5) in high abundance. For the shotgun short-read dataset the species found in highest abundance on the cathode consisted of several species belonging to the *Methanobacteriacaeae* family (S60 bin 16, S54 bin 14, S53 bin 25, S60 bin 14, and S53 bin 3)

as well as a species belonging to the *Acetobacterium* genera (S60 bin 5). Correlation of the relative abundances of the most abundant of these species between the amplicon and shotgun short-read dataset showed there were discrepancies in their abundances across the different MECs (Fig 17b-c).

Although the shotgun long-read dataset also identified the presence of methanogens from the *Methanobacteriaceae* family to be dominating the cathode community, there were differences in identification on species level (Fig 16). The species *Methanobacterium alkalithermotolens* was found to be dominating almost all cathode communities. Additionally, the presence of *Acetobacterim* and *Escherichia coli*, and DNA belonging to *Homo sapiens* were found in some of the MECs. The finding of the *Homo sapiens* sample may be due to the K-mer approach to the taxonomic classification in the shotgun long-read pipeline, as well as potentially caused by incomplete sequencing of the reads during sequencing resulting in similarity to *Homo sapiens* DNA. Another plausible explanation may be due to contamination during either the operation of the systems, since the environment and equipment used was not sterile, or during the DNA extractions.

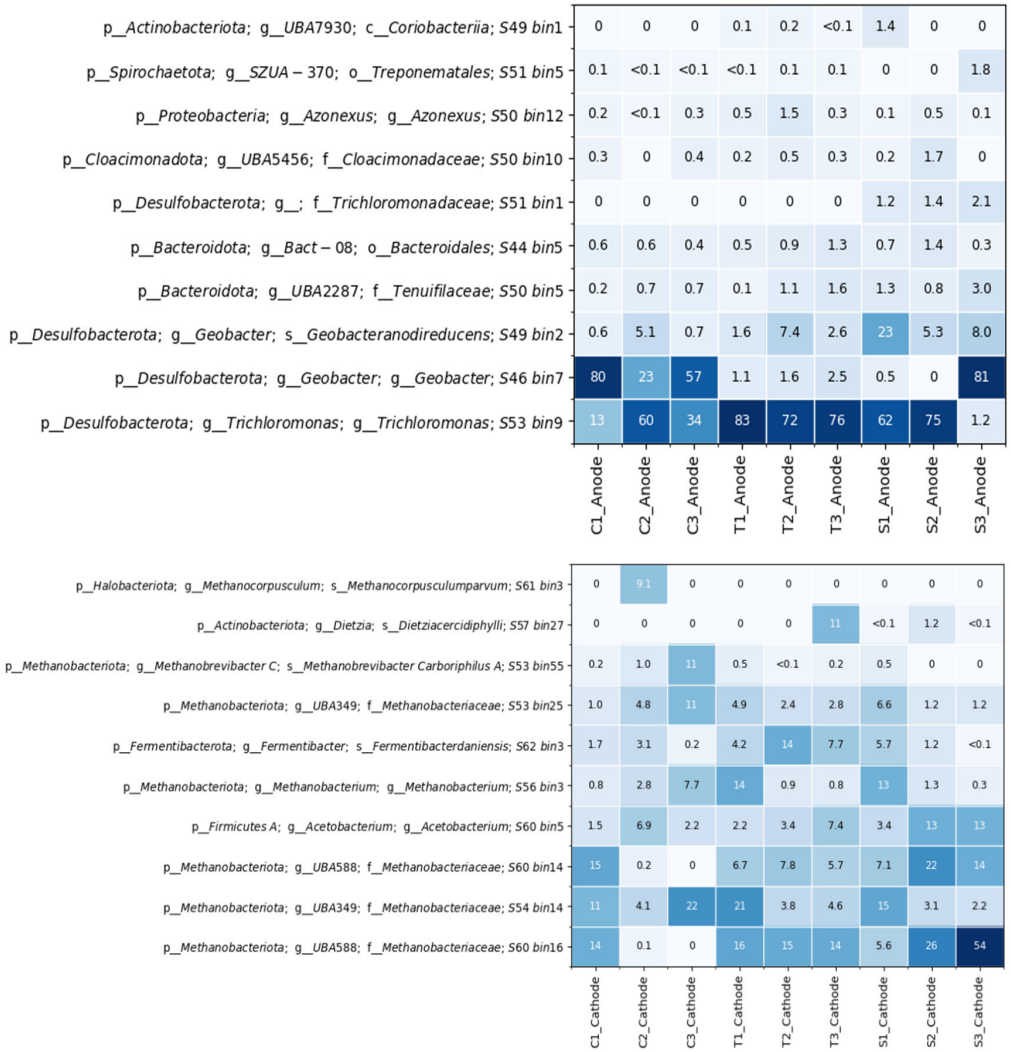

Figure 15. Heatmap showing the relative abundance of the 10 most abundant taxa in the reactors and the inoculum based on the Shotgun short-read sequencing data.

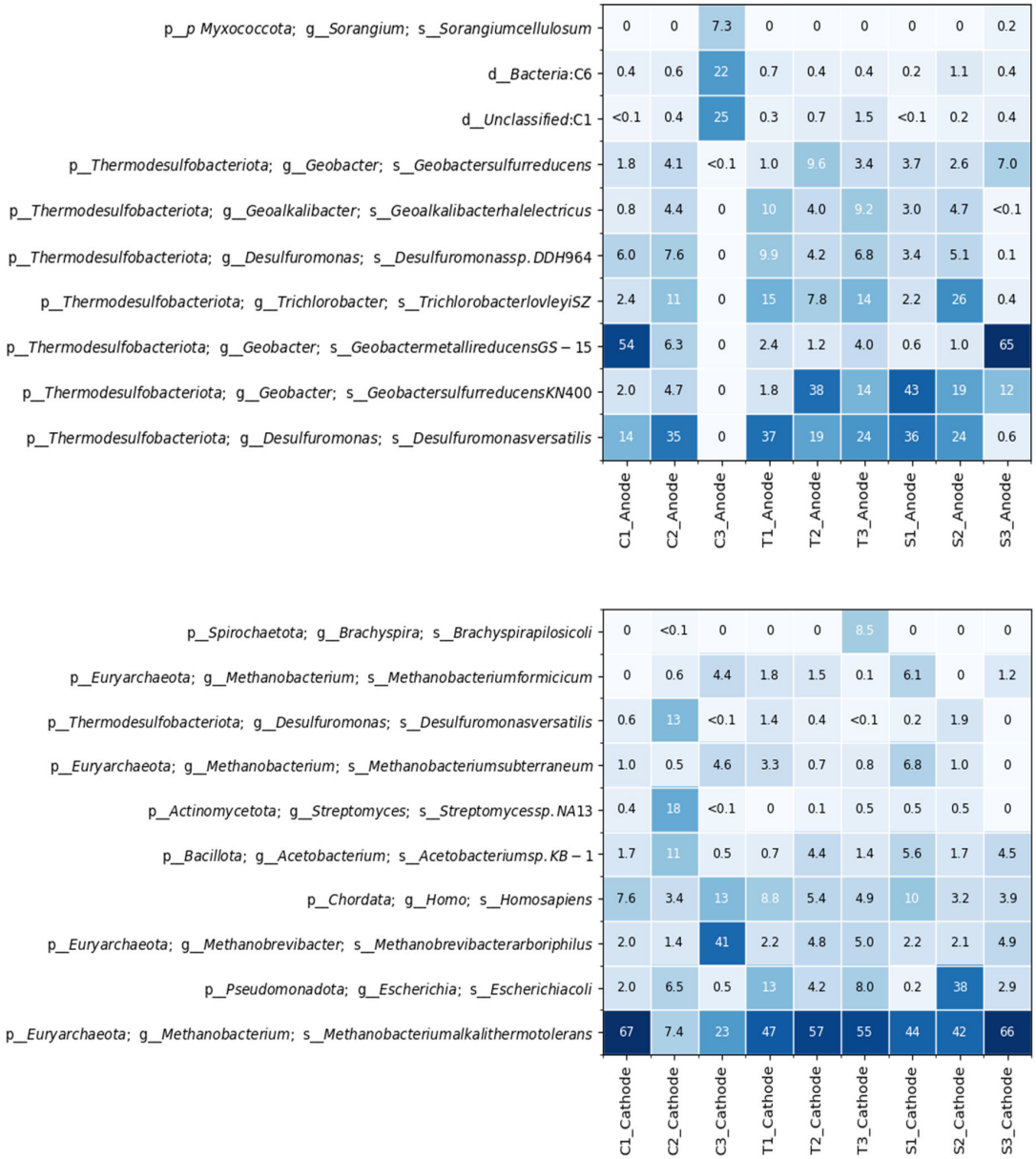

Figure 16. Heatmap showing the relative abundance of the 10 most abundant taxa in the reactors and the inoculum based on the Shotgun long-read sequencing data.

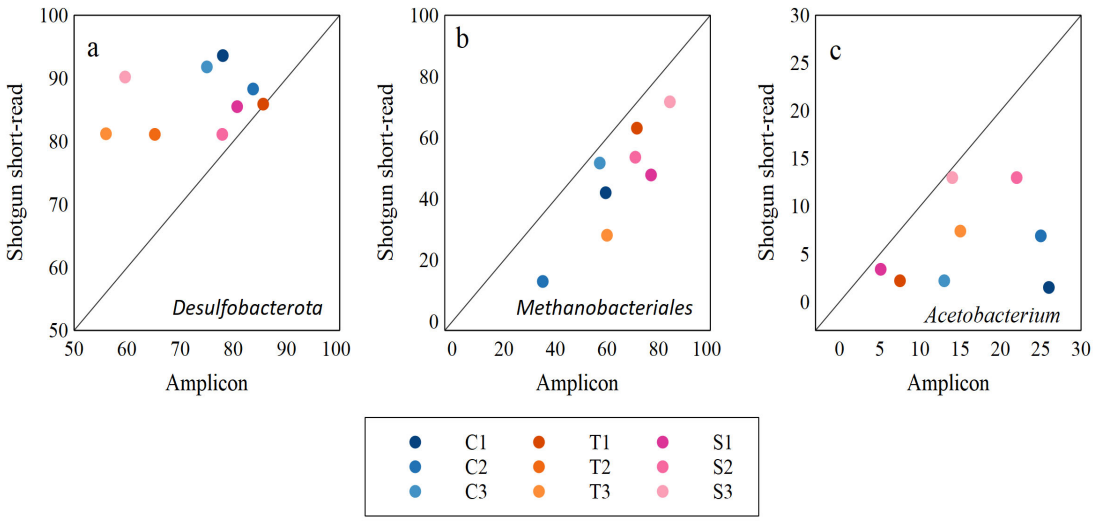

Figure 17. Abundance comparison between Amplicon and Shotgun short-read datasets for taxa found in highest relative abundance. a) *Desulfobacterota* b) *Methanobacteriuales*c) *Acetobacterium*

The differences in the taxonomic classification observed could be due to the sequencing methods and the subsequent analysis strategy (Kleikamp et al. 2023). The amplicon sequencing focuses on conserved regions of the 16S rRNA that can be used for taxonomic classification (Woo et al. 2008, Rausch et al. 2019). Due to this only the amplified short segment of DNA is sequenced. Resulting in less issue with sequencing errors compared to methods such as long-read sequencing. Additionally, there are numerous extensive databases that can be utilized for taxonomic classification of data obtained using amplicon sequencing (McCombie et al. 2019). Even though amplicon sequencing has a lower sequencing error rate, there may still be some issues during taxonomic identification, mainly due to the reads being too short and the consideration of only one specific gene. Additionally, there may be issues regarding the calculated abundances based on the sequencing data. Since the copy number for each taxon within the community is not known, there may be overestimation of the abundance of some species, which could explain the differences seen in abundance compared to the shotgun short-read dataset. Metagenomic sequencing using either short-read or long-read sequencing on the other hand does not focus on specific regions, instead it sequences everything in the sample (Hu et al. 2021). This may reduce overestimation caused by copy numbers in the calculated abundances. Additionally, more information of the function of the microorganism may be obtained through identification of their functional genes. Although, factors such as sequencing errors as well as errors during assembly may results in issues during taxonomic classification (Adewale 2020). For short-read sequencing it is very common to encounter issues during assembly of highly repetitive regions of the genomes (Whiteford et al. 2005). In contrast, in long-read sequencing, unique read patterns are more easily found for highly repetitive regions due to the length of the fragments. This results in better coverage for these areas (Amarasinghe et al. 2020). Based on previous research it has been noted that the main difficulty with Nanopore long-read sequencing can be attributed to low sensitivity and issues with the rate in which the DNA fragments move through the pores, resulting in sequencing errors (Ambardar et al. 2016, Adewale 2020). This may to some extent explain the differences observed in the taxonomic classification of the electrode communities in comparison to both the amplicon and shotgun short-read dataset. The downstream analysis of the sequencing results and the choice of database may also impact the taxonomic classification of the microbial communities. In this study three different databases were used: Midas, GTDB-tk and NCBI reference databases. The shotgun short-read sequencing undergoes binning after assembly and mapping has been performed. The taxonomic classification of each bin is then identified. The

shotgun long-read sequencing on the other hand utilizes k-mers for the taxonomic identification of the sequenced reads. These approaches may result in differences in the taxonomic identification obtained, which may in turn impact other aspects such as alpha diversity. Furthermore, studies on long-read sequencing have highlighted the lower yield obtained compared to other commonly used sequencing methods, which may in turn impact the calculated abundances for each classified taxon when taken into consideration with the higher sequencing error rate (Amarasinghe et al. 2020).

Comparison of the alpha and beta diversity

The alpha diversity describes the diversity within one sample. The diversity order (q) determines the weight placed on the relative abundance of the taxa. A diversity order of 0 places no weight onto the taxa, meaning only absence or presence is taken into consideration. A diversity order of 1 takes the relative abundance into consideration. While a diversity order that is higher than 1, places more weight onto those taxa found in higher abundance. The alpha diversity obtained for the same samples differed between the different sequencing approaches (Fig. 18). The amplicon and shotgun long-read dataset had similar alpha diversity (q=1) for the anode communities (Fig. 18b). The shotgun short-read dataset on the other hand tended to have lower values. Although the cathode samples had a higher alpha diversity compared to the anodes, the shotgun short-read dataset had a higher alpha diversity across all cathode samples. In contrast, when the alpha diversity with a diversity order of 0 was considered, the amplicon dataset had a higher alpha diversity across all samples (Fig. 18b). Regression analysis was performed to identify potential correlations between the alpha diversities of the different sequencing approaches (Paper D: Fig 7). Comparative analysis of the alpha diversity at different taxonomic levels showed a significant positive correlation between the three sequencing approaches for the taxonomic level: class for both diversity orders (ANOVA, $p<0.05$). Comparison of alpha diversities (q=0) from the amplicon and shotgun short-read indicated a significant positive correlation for all taxonomic levels (ANOVA, $p<0.05$). In contrast, comparisons between amplicon and shotgun long-read alpha diversities (q=0) only had a significant correlation for the taxonomic levels order and family in addition to class. Correlation analysis of the alpha diversities (q=1) for the three methods highlighted the significant correlation between shotgun short-read and shotgun long-read for the taxonomic levels order and family, while a significant correlation was observed for amplicon and shotgun long-read when ASV/Species was considered (ANOVA $p<0.05$).

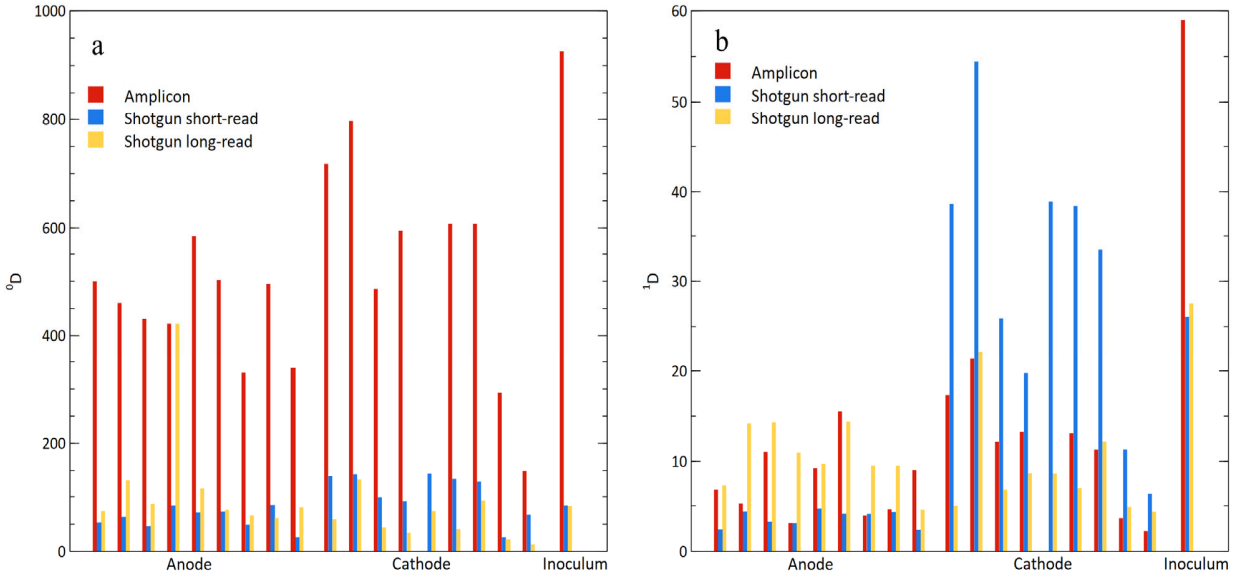

Figure 18. Bar graphs depicting the alpha diversity with a diversity order of 1 (a) and 0 (b). The graph illustrates the results for each location (Anode, Cathode add Inoculum) for all three sequencing methods. bar graph for each location (Anode, Cathode, Inoculum) for each sequencing method. Amplicon (Red), shotgun long-read (Yellow) and shotgun-short-read (Blue).

The PCoA visualising the dissimilarities between the samples, showed clear separation for all datasets based on location (Paper D: Fig 8). Similarly, when a diversity order of 0 was considered, the separation of the different habitats became less distinct for all datasets (Paper D: Fig 9). The pairwise dissimilarities of the beta diversity were done for all three datasets to determine if any correlations existed (Fig 19; Fig 20). The regression analysis indicated a significant positive correlation between the dissimilarity indices for all datasets ($p<0.05$, ANOVA).

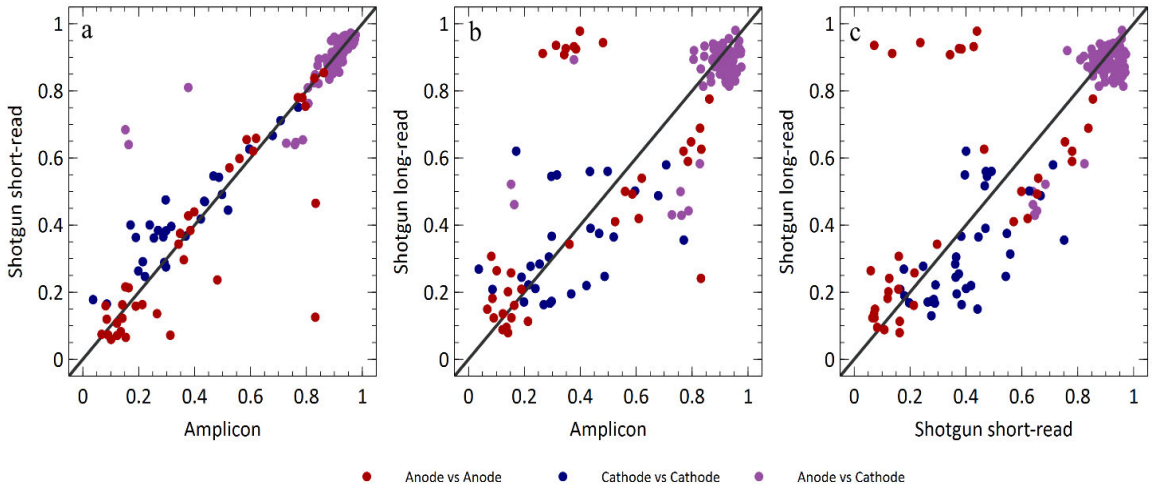

Figure 19. Correlation of the dissimilarity matrices (q=1) obtained with different sequencing methodology. a) Shotgun short-read vs Amplicon, b) Shotgun long-read vs Amplicon and c) Shotgun long-read vs Shotgun short-read.

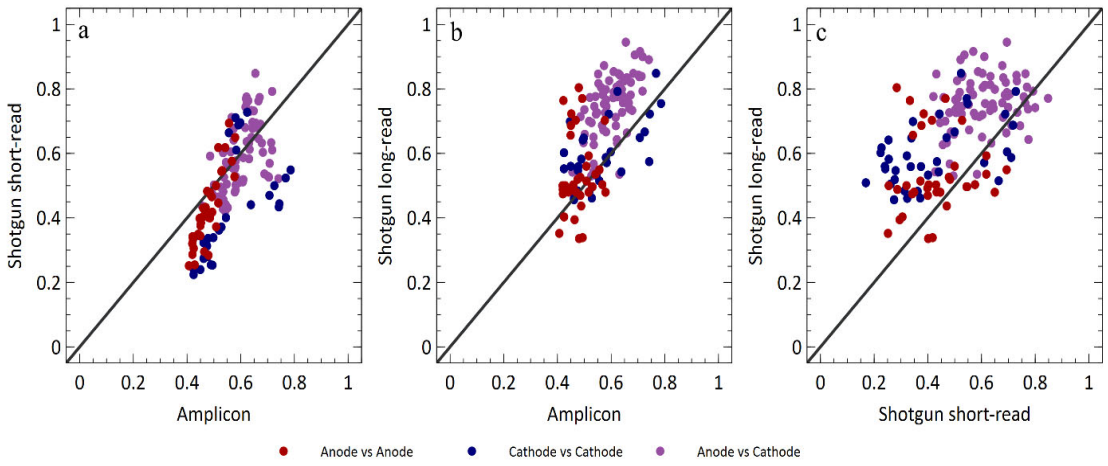

Figure 20. Correlation of the dissimilarity matrices (q=0) obtained with different sequencing methodology. a) Shotgun short-read vs Amplicon, b) Shotgun long-read vs Amplicon and c) Shotgun long-read vs Shotgun short-read.

The relative abundance of the microbial species identified, impacts the alpha diversity when a diversity order of 1 is considered. Amplicon sequencing has the potential to overestimate relative abundances, due to differences in copy number for the 16S rRNA gene in different species as well as PCR bias (Rausch et al. 2019). This could explain the differences seen between the two metagenomic sequencing datasets and the amplicon sequencing for the anode samples. Since both metagenomic methods sequence and assemble contigs based on the raw reads, copy number does not impact the relative abundance of the species. The shotgun short-read dataset had a higher alpha diversity for the cathode samples compared to both amplicon and shotgun long-read. Sequencing errors could be a potential explanation to taxa belonging to the same groups being classified as different by the shotgun short-read downstream analysis. This may shed some light on the differences observed in the alpha diversity of the shotgun short-read and long-read datasets.

Although the different sequencing approaches may vary slightly in the more specific levels, such as species level, of taxonomic classification, all three seem to be able to identify the species to the same degree in the higher orders such as phylum and in some cases genus. Therefore, depending on the degree of taxonomic information that is needed different methods may be of interest. Comparisons of the diversity analysis indicated similar diversity profiles regardless of methods for the beta diversity. The alpha diversity on the other hand differed between the datasets. Nonetheless the trends observed between the different habitats within the MECs were still reflected. Since amplicon sequencing relies on amplification of conserved regions, it is optimal to use in those instances where the quantity of DNA in the sample is quite small (Rausch et al. 2019). Furthermore, amplicon sequencing typically has a more beginner friendly downstream analysis compared to those involving metagenomic sequencing. This makes it optimal for those instances where the functional identity or the complexity of the microbial community may not be of the highest priority. In contrast, metagenomic sequencing, encompassing both short-read and long-read sequencing has a more difficult downstream analysis which require more experience, making it more difficult for beginners (Liu et al. 2021). These approaches are optimal in instances where the microbial community dynamics and microbial function are studied. In research focusing on the stochasticity of the microbial community assembly in BES these approaches may result in more in-depth information about these aspects. Additionally, there are long-read sequencers that can be used to sequence the microbial community in real-time as the experiment is underway. This could potentially allow for the potential adjustment in operational settings and

mapping of their impact on the microbial community over time. Nanopore long-read sequencing typically need approximately 400 ng of extracted DNA during the library preparation based on the manufacturer's instruction, which can become problematic in smaller BES. Since the electrode surfaces are quite small this may cause insufficient DNA concentration, making it difficult to sequence using Nanopore long-read sequencing.

4.3 Microbial community analysis

Microbial community composition of the suspension

The different habitats within the MECs had distinct microbial community compositions. The microbial community of the suspension had the most similar microbial composition profile to that of the inoculum (Fig 21), most likely due to sludge used for inoculation of the MECs remaining in the system over time. The microorganisms in the suspension could utilize carbon sources as well as hydrogen released from the cathode biofilm and cellular debris as nutrient sources. A high abundance of syntrophic bacteria, such as S*yntrophomonas* and *Syntrophorhabdus* were found in the suspension. Additionally, several species involved in hydrogen consumption was also identified, such as *Hydrogenophaga*. There were also a number of methanogens present in the microbial community of the suspension, such as *Methanothrix, Methanomasillicoccus, Ca Methanofastidiosum,* and *Methanolinnea*. Of these methanogens, *Methanothrix* was found in highest relative abundance. *Methanothrix* are known to use acetate in the production of methane (Patel et al. 1990). Based on these findings, the microbial community in the suspension primarily ferments butyrate and propionate via the syntrophic bacteria present and generates methane by the methanogens, either via hydrogen or acetate.

Microbial community composition of the cathode

The community composition for the 20 most abundant species for the cathode community in the cathode experiment can be seen in Figure 21. The cathode community is mainly composed of different methanogens. Some of the methanogens found in high relative abundance on the cathodes were species from the *Methanobacteriaceae* family. In addition to the methanogens, acetogens were also found in high abundance. Based on the microbial community profile of the cathode, it can be deduced that hydrogen generation is an important factor in the development of the microbial community. Based on the network analysis performed on the cathode communities from the cathode experiment (Paper A: Fig 6b; Paper A: Table 1) the cathode community had fewer negative interactions (7.6 %) compared to the anode microbial community (30 %). Since the hydrogen that is generated by the cathode surface can diffuse through the biofilm, there is less of a competition to obtain access to the hydrogen. Thereby allowing more species to colonize and grow on the cathode.

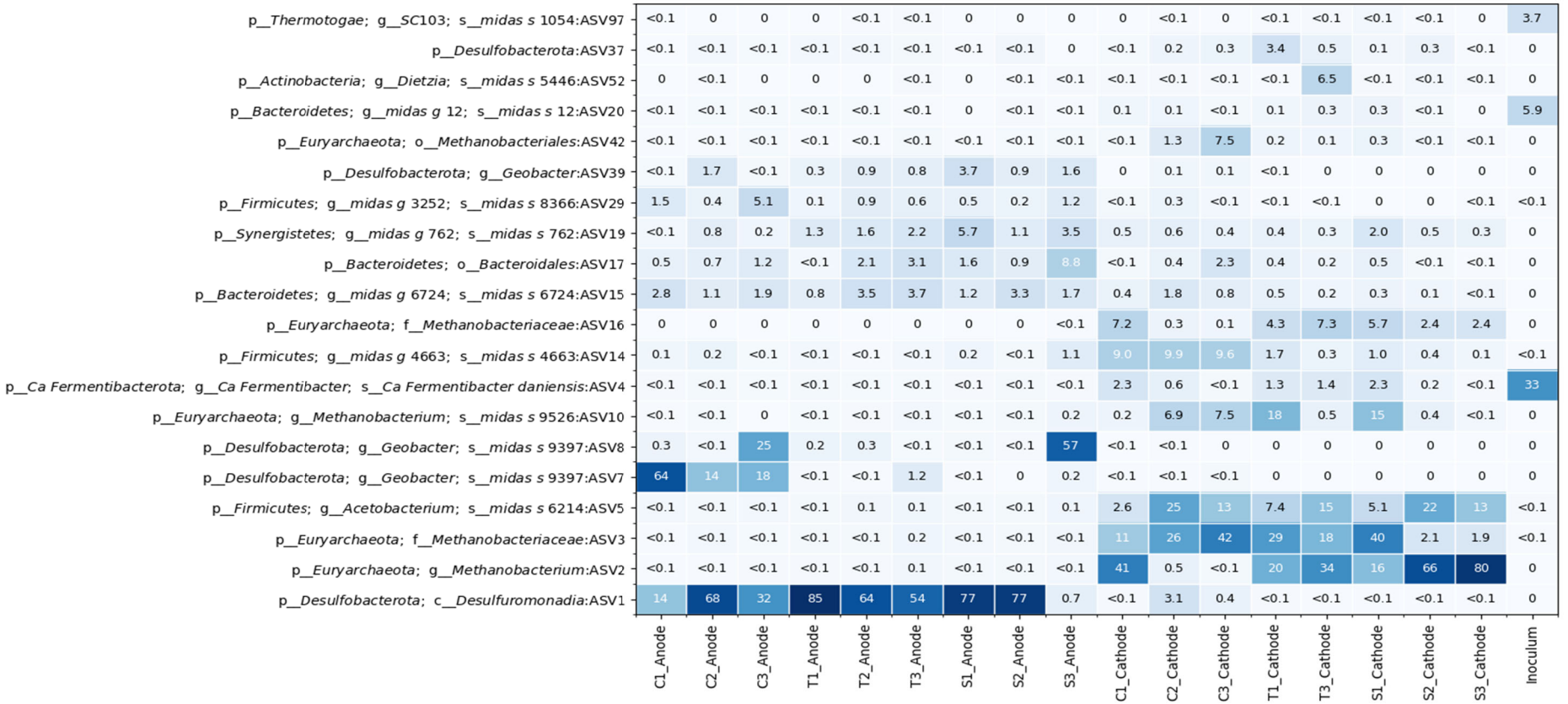

Figure 21. Heatmap depicting the relative abundance of the top 20 most abundant taxa present in the 9 MECs with different cathode materials, the inoculum as well as the foam samples from C2 and S3. R5 cathode was excluded due to low number of reads. The y-axis shows the phylum and the ASV tag for each taxon.

Microbial community composition of the anode

The anode community of both experiments were dominated by electrogenic bacteria capable of transferring electrons to the anode surface (Fig 21; Fig 23). The most commonly found species belonged to the *Desulfobacterota* phylum, formerly known as *Deltaproteobacteria*, a known group of electrogens commonly found in BES systems (Logan et al. 2019). In the anode experiment between 64-90 % of the communities were represented by MAGs belonging to the *Desulfobacterota* phylum. Of these MAGs two *Geobacter* species were found in high relative abundance in all MECs. Hydraulic loop 1 was dominated by *Geobacter* (S71_927), while the remaining loops were dominated by *Geobacter* (S78_1107). Both *Geobacter* species were found to be most similar to *Geobacter benzoatilyticus*. Additionally, it was found that *Geobacter* MAG S74_483 was similar to *Geobacter hydrogenophilus.* Moreover, three *Tricholoromonas* MAGs were determined to be most similar to *Desulfuromonas acetexigens*.

The negative interactions of the electrogenic bacteria could be established from the network analysis of the anode community from the cathode experiment (Paper A: Fig 6a), which highlights the competitive nature of the colonization of the anode surface. The electroactive microorganism needs to have direct contact with the anode surface to facilitate electron transfer (Thapa et al. 2022), since there is a limited space on the anode surface, the initial colonization becomes highly competitive. The competitive nature of the anode community is further illustrated by the correlation between ASV1 and ASV7+ASV8 (Fig 22). Based on the network analysis it was concluded that when ASV1 is found in high abundance, ASV7+ASV8 are found in low abundance, highlighting the negative interaction of these taxa. It should be noted that ASV7 and ASV8 only differed by one nucleotide and belong to the same species.

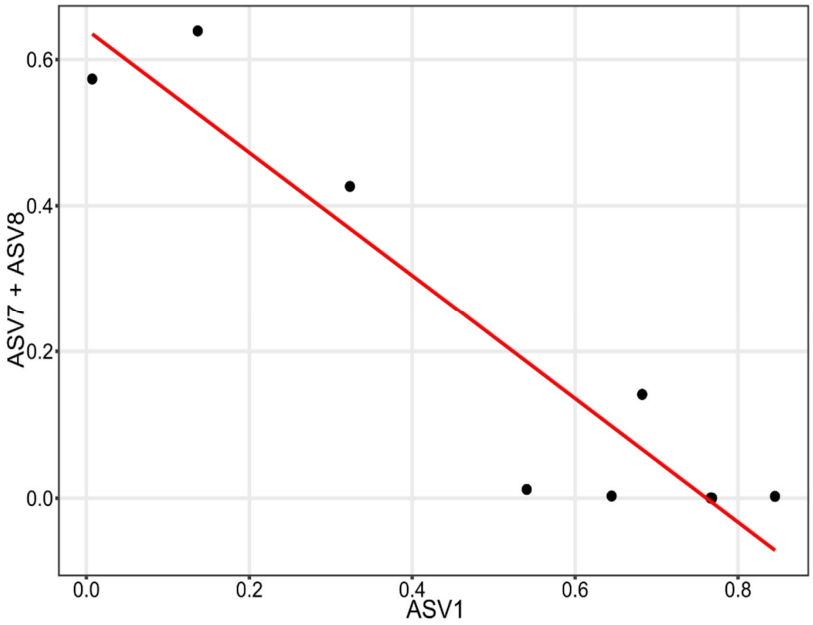

Figure 22. the correlation between the combined abundance of ASV7 and ASV8 with the abundance of ASV1 in the anode DNA samples for the 9 cathode reactors.

In addition to the electrogenic bacteria, some fermenters such as those from the *Synergistaceae, Anaerolineaceae, Spirochaetaceae,* and *Dysgonomonadaceae* families were found. These families are involved in processes resulting in the production of acetate, butyrate and hydrogen (Rees et al. 1997, Maune et al. 2012, Tomazetto et al. 2018).

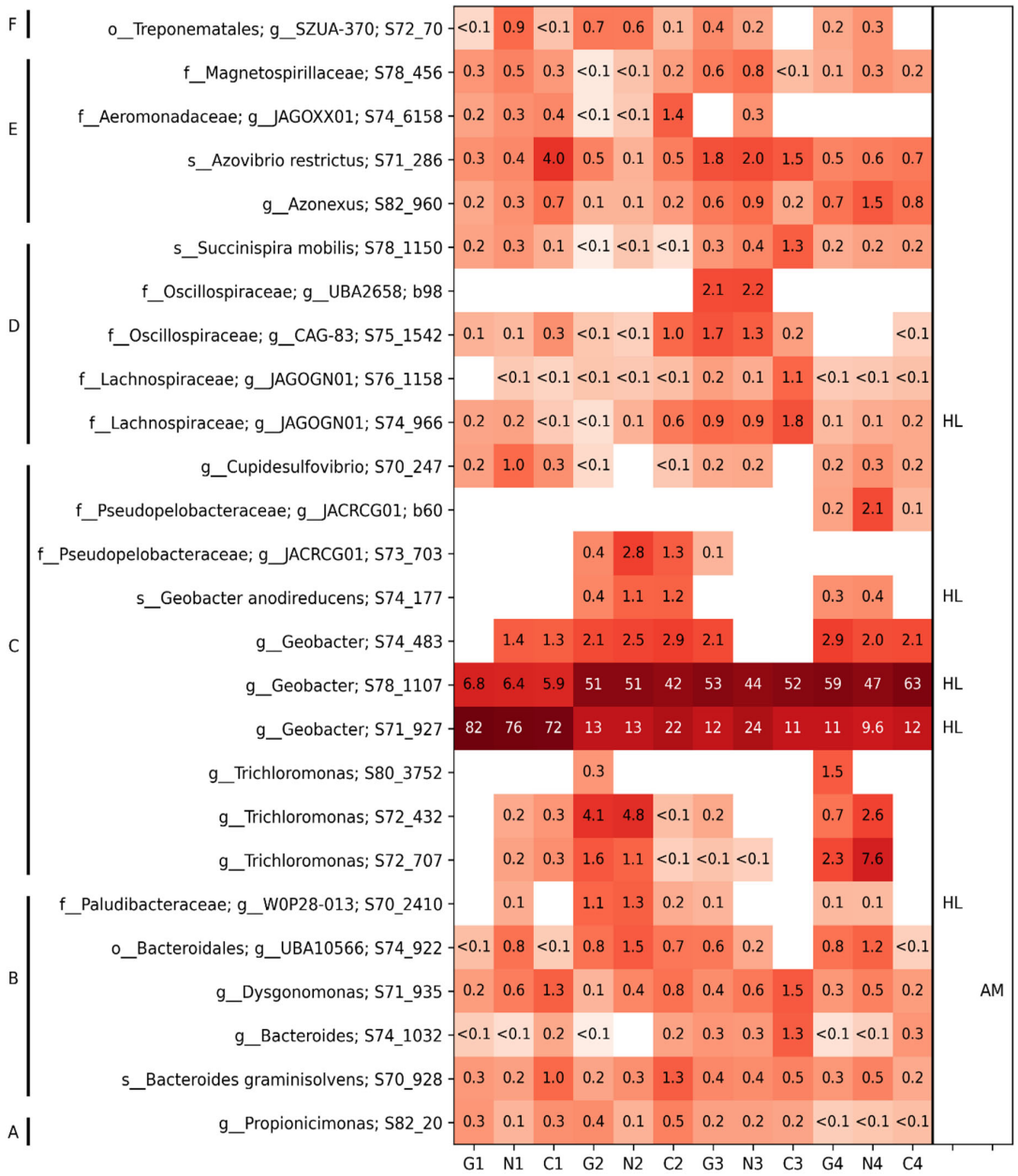

Figure. 23. Relative abundance (%) of the most abundant MAGs in the anode biofilms from MECs with different anode materials. The MAGs are grouped based on phylum: *Actinobacteriota* (A), *Bacteroidota* (B), *Desulfobacterota* (C), *Firmicutes* (D), *Proteobacteria* (E), and *Spirochaetota* (F). Statistically significant effect on the relative abundances of the MAGs by either hydraulic loop (HL) or anode material (AM) is shown in the right panel ($p<0.05$, ANOVA).

Microbial diversity of the different habitats within the MECs

When comparing the alpha diversity with a diversity order of 1, (Fig 24a), a significant difference can be observed in the diversity of the different habitats for the cathode experiment ($p<0.05$, ANOVA). The alpha diversity of the suspension was significantly higher than that of either electrode ($p<0.05$, ANOVA). However, there was no significant impact of the cathode material on the alpha diversity of the electrode microbial communities ($p>0.05$, ANOVA). The diversity of the anode communities from the anode experiment was done considering a diversity order of 0, 1, and 2 as well as evenness (Fig 25a-d). A diversity order of 0 allows for the evaluation of the richness of the sample, meaning the number of taxa present in the community. For the anode communities with different anode material the number of taxa present ranged from 40-107 species. A diversity order of 1 resulted in an alpha diversity ranging from 1.7-7.4, whilst a diversity order of 2 resulted in an alpha diversity between 1.3-3.2. The evenness obtained from the 12 MECs ranged from 0.17-0.44. Further analysis of the obtained diversity indices indicated the significant effect off the hydraulic loop on the alpha diversity with a diversity order of 1 as well as the evenness ($p<0.05$, ANOVA). Specifically hydraulic loop 1 had both the lowest diversity and evenness when compared to the other three hydraulic loops ($p<0.05$, t-test). The anode is mainly dominated by electrogenic bacteria (Thapa et al. 2022). Typically, there is a need for the electrogenic bacteria to have direct contact with the electrode surface to facilitate the proper circumstances for electron transfer to occur. Due to this, there is a limited amount of surface that the electrogenic bacteria compete for during the colonization of the anode surface. This may have an impact on the diversity of the anode community, since the limited surface could result in a limited number of species growing in the biofilm, when compared to less restrictive habitats such as the suspension. Similarly, there is also a lower community diversity on the cathode in comparison to the suspension. Conversely, the cathode still has a slightly higher degree of diversity when compared to the anode community. Because the hydrogen produced by the biofilm on the cathode can diffuse through the biofilm, it becomes more readily accessible to both the biofilm as well as the bacteria near the cathode surface This reduces the degree of competition present in the habitat, allowing for more species to coexist. In general, the electrode surfaces have a much lower alpha diversity in comparison to both the suspension and inoculum. An explanation for this observed difference in alpha diversity could be attributed to the selection of bacteria with desirable characteristics that are involved with the specific function of either the anode or cathode, e.g. electrogenic bacteria are selected on the anode, while there is a selection for methanogens or acetogens on the cathode.

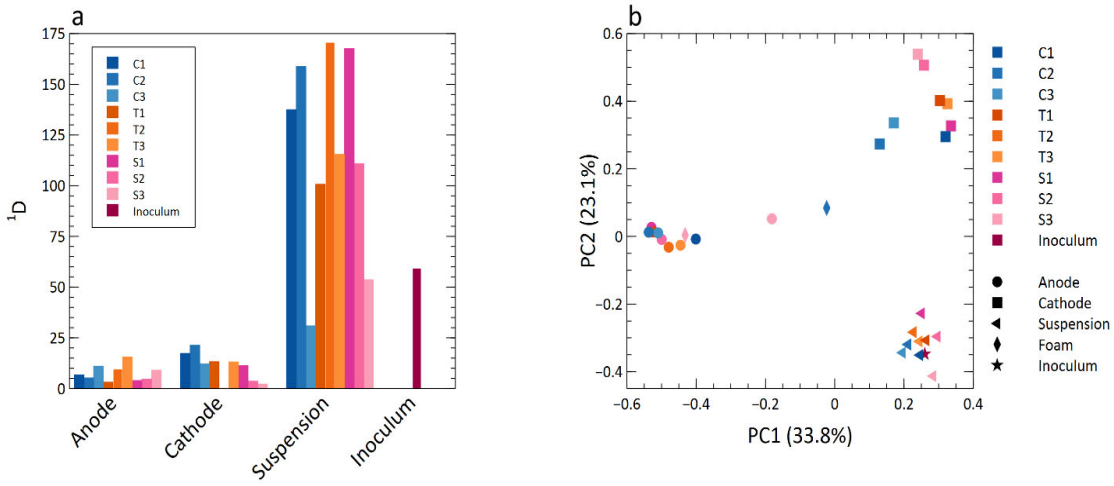

Figure 24. a) Bar graph of the alpha diversity for all samples with a Hill number order of 1, for the cathode experiment. b) Principal coordinate analysis from the dissimilarity matrix with a Hill number order of 1, for the cathode experiment.

The dissimilarity of the microbial communities between samples is described by the beta diversity. The beta diversity can be illustrated using a principal coordinate analysis (PCoA). The PCoA from the cathode experiment shows the clear separation of the microbial communities based on location (Fig 24b). This separation emphasizes the differences in microbial niches corresponding to each different habitat within the MECs. A null model analysis was performed to determine whether the dissimilarity observed between locations could be attributed to random chance (Paper A: Table S1, Supplementary material). The null model analysis randomly redistributes the ASVs present in the communities being compared while keeping the number of ASVs in a sample intact. Based on this new redistribution the dissimilarity for each pair of communities is recalculated and compared to the dissimilarity calculated from the actual communities. Thereby, determining whether any dissimilarity observed between communities can be attributed to random chance or not. In almost all instances, the null model analysis indicated that the dissimilarity observed was due to stochasticity and not the difference in material. The dissimilarity between the anode communities for the anode experiment was visualised for a diversity order of 0, 1, and 2 using a PCoA (Fig 25e-g). The anode communities visualised in the PCoA, separated mainly based on which hydraulic loop they belonged to and not anode material. Based on statistical analysis of the obtained dissimilarity it was concluded that the hydraulic loop had a significant effect on the dissimilarities observed for all three diversity orders ($p<0.05$, permanova).

Factors influencing the microbial community and performance in MECs

A dominance analysis was performed to determine the relative contribution of the two explanatory variables anode material and hydraulic loop on the response variables of interest (Fig 26). The response variables that were considered were, alpha diversity with a diversity order of 0, 1 and 2, evenness, a selection of species found in high abundance, as well as peak current density, lag time and total charge. The analysis of these factors, allow for the investigation of the impact selection, drift and dispersal have on the microbial community assembly and development. Based on the findings from the dominance analysis it could be concluded that the anode material explained most of the variances seen in the variables

describing the performance, whilst the hydraulic loop explained most of the variables related to the microbial diversity as well as the relative abundances of the species considered. These observations further highlight the stochasticity involved in the assembly and development of the anode microbial community over time. Considering these findings and the similarities between the anode communities within the same hydraulic loop it is very likely that the MECs within the same hydraulic loop undergo homogenizing dispersal resulting in similarities in the communities, which is not possible in the replicate MECs from different hydraulic loops. Furthermore, it can be noted that the dispersal limitation placed on the MECs, the larger differences in microbial community structure allowed for the observation of the impact of drift on the microbial community development.

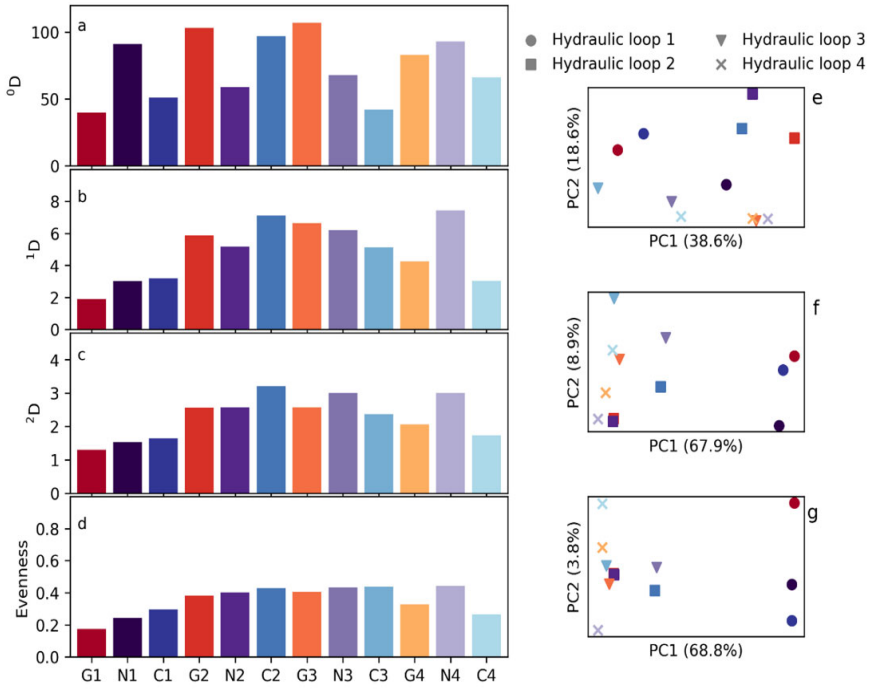

Figure 25. Diversity analysis of the anode communities from the MECs with different anode materials (Anode experiment). a-c) Alpha diversity calculated as Hill numbers with diversity order 0 (a), 1 (b), and 2 (c); d) evenness; and e-f) PCoA ordination based on dissimilarity matrices calculated with diversity order 0 (e), 1 (f), and 2 (g). The colours in a-d corresponds to the colours in e-g.

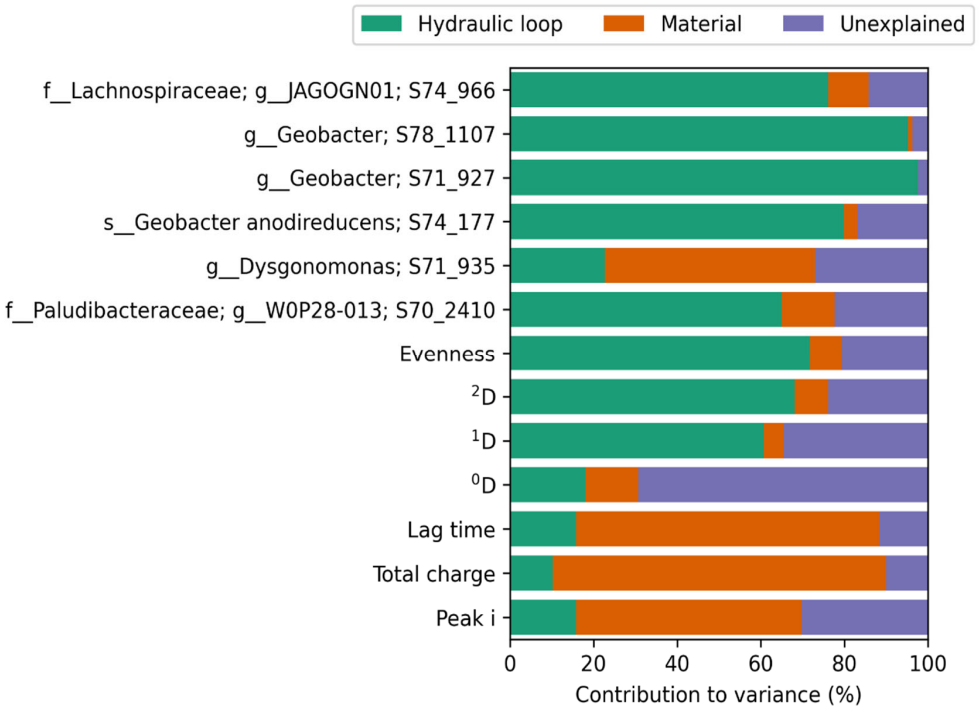

Figure 26. Dominance analysis showing the contribution of the explanatory variables hydraulic loop and anode material to the variance of several response variables for the anode experiment. The species of bacteria included are selected MAGs from the anode biofilms.

The viral communities in MECs

In addition to the bacterial communities residing in the different habitats, there are also other microbial organisms that may impact the bacterial growth and assembly. Viruses, also known as bacteriophages, are one such group of microbial organisms. Although not much is known about the viruses present in the viral communities in MECs. In **Paper C** the viral community of microbial electrolysis cells inoculated with anaerobic mesophilic activated sludge from the cathode experiment was investigated. Samples were taken from the prokaryotic community as well as samples from the suspended liquid to harvest potential phage DNA. From the prokaryotic samples, a total of 852 bins were identified after assembly and binning. From these 852 bins, a total of 278 species were identified, of which 17 were determined to be anode-affiliated and 40 cathode-affiliated, the remaining species had no electrode specific or suspension specific affiliation (Fig 27). Out of the 17 anode-affiliated prokaryotes, 8 had prophages. For the cathode-affiliated 27 out of 40 identified species had prophages. CRISPR are regions of prokaryotic DNA which consists of short repetitive sequences. These are used by the prokaryote as a defence mechanism against foreign genetic element, such as viruses (Barrangou et al. 2007). Identification of CRISPR regions was used to determine the presence of previous infection. For those prokaryotic species affiliated with the anode, 12 were found to have CRISPR regions, while the cathode affiliated species had 27 detected with CRISPR regions. For both identification of prophages and CRISPR regions the completeness of the bins had a large impact, the more complete the bins were the higher the likelihood of being able to identify a viral association (Paper C: Fig 3).

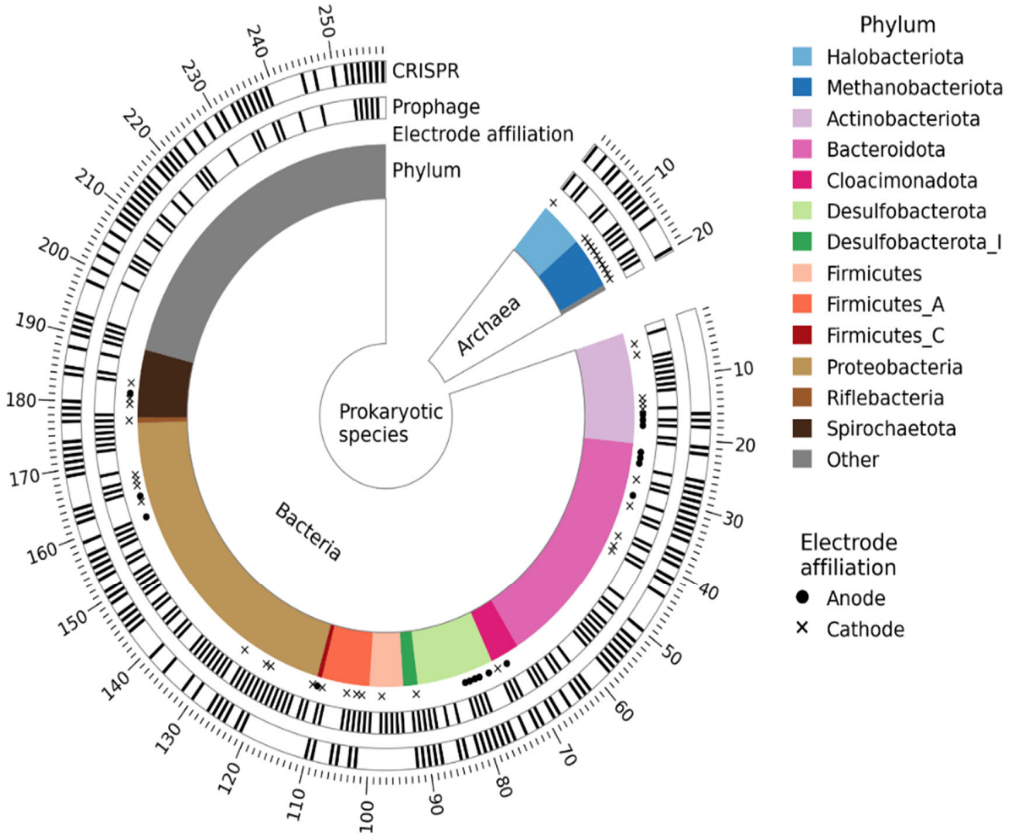

Figure 27. A diagram depicting the 278 prokaryotic species (MAGs) identified from the cathode experiment (**Paper C**). The phyla with electrode-affiliated species are coloured. The electrode-affiliation is indicated by • (anode) or x (cathode). Those phyla that have no electrode-species are grouped as "Other". Black lines indicate species that contained a prophage or a CRISPR region.

After taxonomic classification of the viral sequences, a total of 325 species were identified. Interestingly, many of the identified viruses were only found in some of the MECs with a relative high abundance (Fig 28). The families found in highest abundance all belonged to the *Caudoviricetes* class, formerly referred to as the *Caudovirales* order (Turner et al. 2023). Turner et al. (2023) have reported on the abolition of the viral families *Siphoviridae, Myoviridae* and *Podoviridae*. The method used for taxonomic classification of the viral species in **Paper C**, PhagCN, still uses the old family names. Due to this, the former family names will be used to discuss the viral species present in the system. The most found species belonged to the *Siphoviridae, Myoviridae* and *Podoviridae* family of viruses. Species belonging to the *Myoviridae* family are viruses with long contractile tails and linear dsDNA (Zinke et al. 2022). Previous studies have noted that they are temperate phages targeting mainly species belonging to the *Proteobacteria* phylum (Ackermann 1998). Both *Podoviridae* and *Siphoviridae* are viruses with non-contractile tails, in contrast to *Myoviridae* (Schouler et al. 1994, Goulet et al. 2020). *Podoviridae* has been reported to target species belonging to the *Enterobacter* and *Pseudomonas* genera (Ceyssens et al. 2008, Ceyssens et al. 2010). The viruses from the

Siphoviridae family were found in highest abundance across most MECs. These viruses typically target *Proteobacteria,* although previous research has also linked some phages belonging to this family with host associations to methanogens belonging to the *Methanobacterium* genus (Meile et al. 1989). More recent studies into the viral communities affecting methanogens have found association between siphovirus-like phages, named *Sperovirdae*, and species belonging to the *Methanobacteriales* order (Ngo et al. 2022).

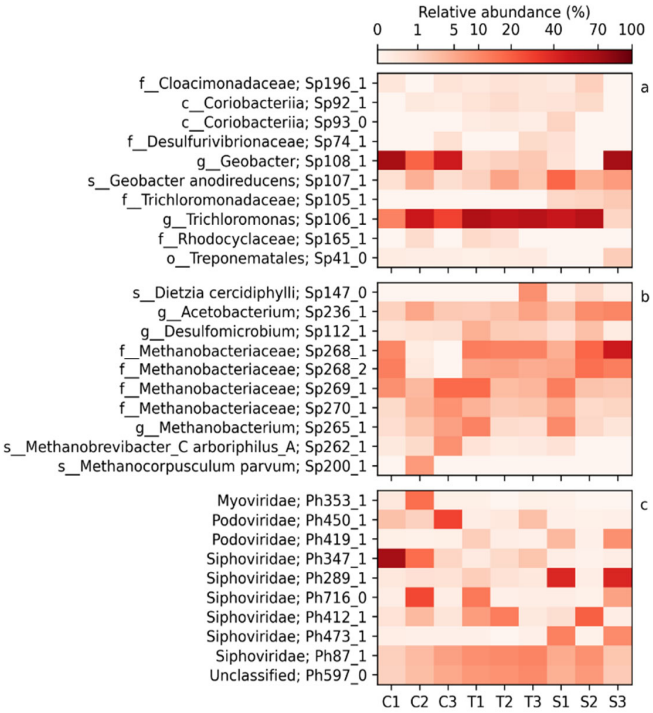

Fig. 28. Heatmap of the most abundant prokaryotic species on the anode (a) and the cathode (b), and the most abundant phage species suspended in the liquid of the MECs (c) from the cathode experiment (**Paper C**). Taxonomic classifications are shown together with our identification code for the prokaryote or phage species.

To identify potential infection at the time of sampling, the association between the prokaryotic samples and the identified phage samples suspended in the liquid were evaluated. If a CRISPR spaces could be identified in the phage sequences from the phages suspended in the liquid which matched with that from a prokaryote, an association could be established. Additionally, if a match between a prophage and the phages could be identified, this would indicate the potential induction of the prophage. A total of 50 CRISPR based matches could be identified and a total of 28 prophage matches for the phages suspended in the liquid (Fig 29). Most of these virus host association were between the viruses and the microbial community of the cathode, such as *Methanobrevibacter, Methanobacterium* and other genera within *Methanobacteriaceae.* The anode-affiliated species on the other hand only had induced prophages associated with a species from the *Coriobacteriia* class (Fig 29). Previous research has shown the modulating impact viruses can have on the prokaryotic communities (Liu et al. 2023). Specifically, in marine ecosystems, the presence of viruses have been shown to help modulate the communities by preventing overgrowth of specific groups (Enke et al. 2018).

Since the anode community relies on direct contact with the anode surface to facilitate electron transfer (Costa et al. 2018), there is more competition involved in the formation and growth of the electrogenic bacteria early in the anode biofilm formation. As other organisms, such as fermenters, attach and colonize the anode, the different layers of the biofilm may have different compositions. This could result in the inner layers having a larger quantity of electrogenic bacteria compared to the outer layers. Since the viruses may have a limited access to the inner layers of the biofilm, it is likely that induced prophages identified from the suspended liquid may not target the electrogenic bacteria to the same extent. In contrast, there is less of a competitive environment on the cathode since the generated hydrogen gas can diffuse through the biofilm. This allows all microorganism within the cathode biofilm as well as those close to its surroundings to utilize hydrogen gas, allowing for instance methanogens to take over the community (Siegert et al. 2015, Li et al. 2019), which would result in the identification of a higher number of induced prophages associated with the cathode in the suspended liquid. Like the virus-host associations identified for the induced prophages, most CRISPR-based associations were also cathode-affiliated. Of the two CRISPR-based associations connected to an anode-affiliated prokaryote, only one *Myoviridae* virus was associated with an electrogenic bacteria (*Geobacter anodireducens)* (Fig. 29). Like the prophage matches, a potential explanation of the greater number of cathode-affiliated matches may be caused by the less competitive nature of the cathode allowing for methanogens to colonize different layers of the cathode biofilm without it impacting their access to the diffusing hydrogen. Another important factor to consider is the operational design of the MECs and how that may impact the viral community and virus-host associations. The MECs were batch fed every 2-3 days. This resulted in the dilution of the microbial community suspended in the liquid every batch cycle. Since sampling of the viral community was done at the end of the experimental run, the changes that may have occurred in the viral community over time become difficult to establish. As the anode surface becomes colonized by the anode-affiliated prokaryotes, it is likely that the associate virus also increases in abundance. The changes that occur in the microbial communities as they develop over time, would also result in changes in the viral community.

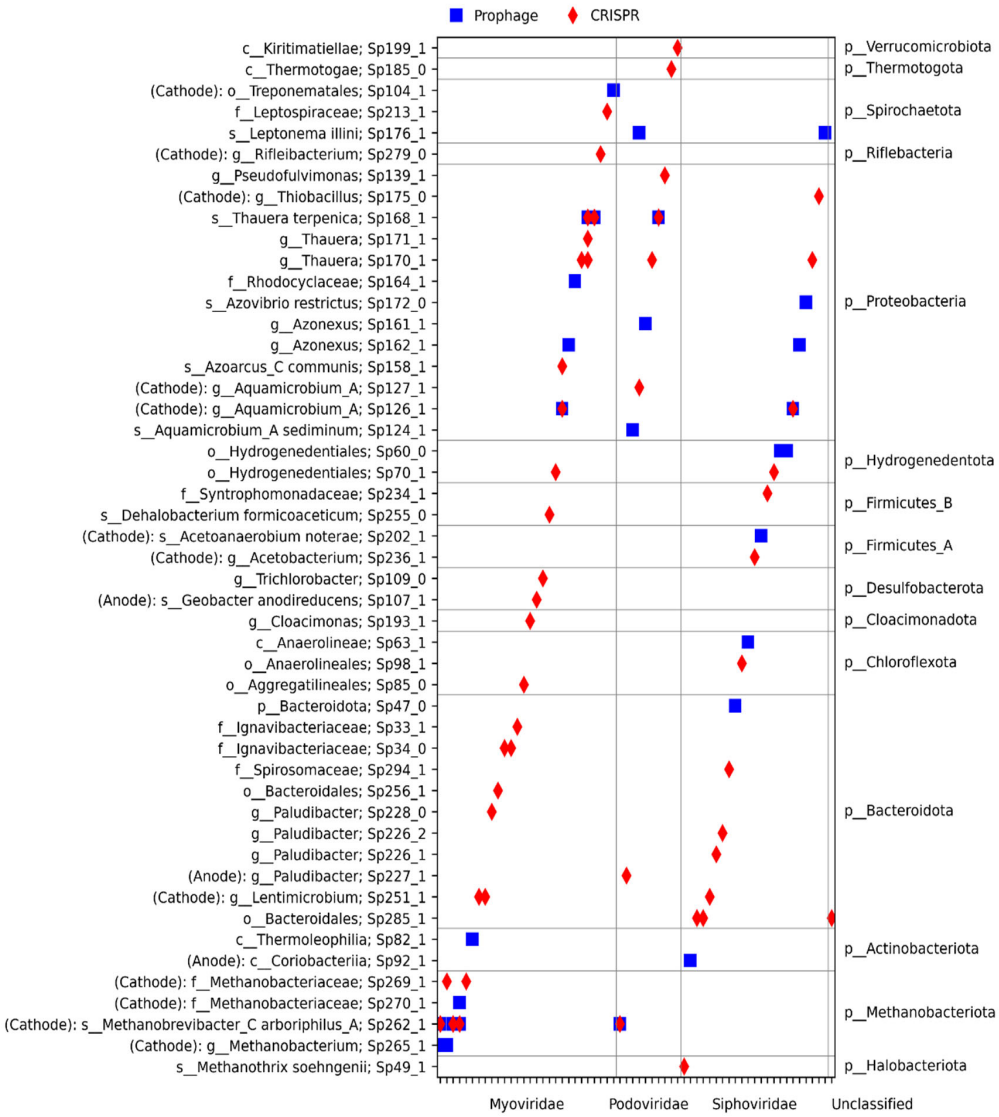

Fig. 29. Associations between 49 prokaryotic species and 62 phage species found in the cathode experiment (**Paper C**). Prokaryotic species classified as either anode- or cathode affiliated are indicated.

5 Conclusions

In this thesis the impact of electrode material, selection, dispersal, drift, biotic interactions, and phage predation, on the microbial communities in bioelectrochemical systems was investigated.

The choice of cathode material did not impact the performance of the MECs or their ability to generate current once the system had stabilized and reached a steady state. The hydrogen evolution observed on the cathode surface improved over time for the titanium and steel cathodes, whilst the carbon nanoparticles cathodes remained the same, most likely due to the already large surface area of the material. There was a variation in the lag time of 5-38 days, which then reached a peak in current production around day 40 at which point the systems current generation reached a steady state at a slightly lower level. The variation in lag time could be contributed to stochasticity influencing the initial colonization of the electrogens on the anode surface. Investigation of the effect of the anode material on performance and microbial community assembly highlighted the importance of anode material with regards to the performance of the system but not microbial community assembly and development. Indicating that although there is stochasticity involved in the initial colonization of the anode, the material has a direct impact on the ability of the electrogenic bacteria to attach and initiate electron transfer. The conventional anode material, carbon cloth, generated higher current compared to both that of graphene and nickel MECs. Although graphene has been reported to have a high conductivity and surface area, the structure consisting of sharp flakes, likely resulted in cell death of the initial colonizing bacteria. This in turn causing a reduced ability to transfer electrons by the bacteria attaching on the surface after initial colonization. There is a decrease in the peak current density observed over time. This is explained by the shift in selection from electroactive to non-electroactive bacteria due to the growth of the biofilm overtime leading to a changed environment.

The microbial community of the cathode were mainly dominated by methanogens, able to utilise hydrogen to produce methane gas. The anode community in turn was mainly dominated by electrogenic bacteria from the *Desulfobacterota* phylum. The microbial community of the different locations showed clear differences, indicating selection of function-specific species based on habitat. Differences seen in the profile of the dominating electrogenic bacteria of the anode biofilms could be explained by the stochasticity of the initial colonization between competing taxa. It was also noted based on the network analysis that the anode community showed a higher degree of competitive biotic interactions in comparison to the cathode. Dominance analysis, showcasing the effect of the hydraulic loop on the microbial community structure and diversity further highlights the impact of stochastic factor on microbial community development. It was noted that in the presence of dispersal limitations, larger differences could be observed between microbial communities indicating the impact of drift on the microbial community assembly. In addition, three families of viruses were identified in varying abundances in the MECs. Specifically, those belonging to *Siphoviridae, Myoviridae* and *Podoviridae* families. Virus-host associations were identified between both cathode and anode associated prokaryotic species, and the phages found suspended in liquid. These findings indicate biotic interactions between the phages and prokaryotes that may have a modulating effect on the prokaryotic communities within the MECs. Indicating for the first time, the active role of phages in shaping the prokaryotic community in bioelectrochemical systems.

Evaluation of the potential sequencing strategies used for taxonomic classification of the microbial communities highlighted the importance in research question when choosing methods. Depending on whether the identification or potential function is of importance different sequencing strategies could be more appropriate. Shotgun metagenomic sequencing using short-read sequencing gives the most information about both species' abundance and

their potential functional genes, although both time consuming and costly. While amplicon sequencing was a good strategy if only the identity of the microbial community and not necessarily the function is of importance. Metagenomic sequencing using long-read sequencing is an interesting tool which could be utilized to sequence samples more easily in lab or in the field. However, due to the quantity of DNA needed, it may be more difficult to implement in smaller systems, such as MECs where DNA concentrations are lower.

6 Implication and future research

Based on these findings, the importance of both deterministic and stochastic factors impacting the performance and microbial community of the MECs were highlighted. The choice in anode material directly impacted the ability of the system to maximize its capabilities to generate current. Whilst the performance could be correlated with the anode material (a deterministic factor), the findings summarized in this thesis highlight the important role stochastic factors play in the assembly and development of the microbial community on the electrodes in MECs. For better optimization of these types of systems, it is of importance to further study the impact of stochastic factors involved in the microbial assembly and development over time as well as the intra- and inter-community interactions.

Typically, sampling of the microbial communities in these types of systems are done at the end of the experimental run, although this is of interest many facets involved in the continuous development of the community is lost. Therefore, it would be of interest to further study the development of the microbial community focusing on the community composition over time during different stages of the biofilm assembly starting from the initial colonization until the end stages before complete shutdown of the system. Furthermore, although the species present in the microbial community can be identified, their activity status cannot. Therefore, it would also be of interest to use methods such as stable isotope probing to identify the active microorganisms in the biofilm and their potential functional role to further understand the microbial community dynamics.

The focus of the research done on the microbial communities in BES have been on the prokaryotic community. Although in lab conditions the impact of other forms of microorganism may not be of interest, to implement the systems in real life settings it is of importance to understand the dynamics behind the different biotic interactions in the system. It would therefore also be of interest to further study and understand the impact predators, such as viruses and eukaryotes, may have on the prokaryotic community. With regards to the eukaryotes, it would also be interesting to study how the competition for resources and space in the system impacts the development of the prokaryotic communities.

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