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MICROBIAL DEGRADATION OF HIGH MOLECULAR WEIGHT
PAHS AND WEATHERED CRUDE OIL USING MARINE SMFCS

by
HAMDAN ZUHEIR HAMDAN

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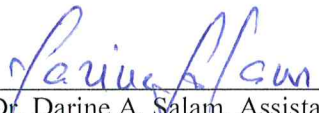
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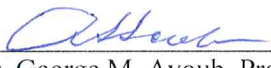
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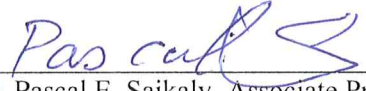
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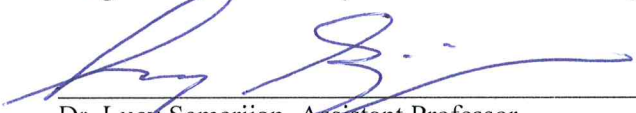
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AN ABSTRACT OF THE DISSERTATION OF

Hamdan Zuheir Hamdan for Doctor of Philosophy
Major: Environmental and Water Resources Engineering

Title: Microbial Degradation of High Molecular Weight PAHs And Weathered Crude Oil Using Marine SMFCS

Sediment Microbial Fuel Cells (SMFCs) are a type of bioelectrochemical systems, based on the Microbial Fuel Cell (MFC) technology, which can be used for bioremediation of various contaminants in aquatic sediments. Recently, there has been a rise in studies targeting applicability of SMFCs for biodegradation of lingering pollutants in sediments, such as petroleum hydrocarbons. The interest in the SMFC technology can be attributed to the overall simplicity of the system when applied as a bioremediation approach. SMFCs can thus provide a passive and a sustainable bioremediation system that does not heavily interfere with the existing environment. However, research targeting SMFCs is still limited, hindering the potential of applying SMFCs for treatment of contaminated sites. For example, existing literature is confined to few specific compounds rather than simulating actual field conditions with a multitude of coexisting contaminants. Additionally, given the importance of ferric iron reducing microbes in the electrochemical performance of the SMFCs, and given that ferric iron reducers are generally lacking in marine sediments, most of the research is focused on freshwater SMFCs.

To better understand the technology for bioremediation of marine sediments, we investigated the use of marine SMFCs for the bioremediation of petroleum hydrocarbon contamination. Thus, 2 sets of experiments were conducted utilizing ferric iron stimulated SMFCs for the removal of a mixture of Polycyclic Aromatic Hydrocarbons (PAHs) as well as for the removal of weathered crude oil from the sediments.

Volatilization was the major removal mechanism of low molecular weight PAHs (2 and 3 rings). In the case of high molecular weight PAHs, highest removal was observed under iron stimulation when sulfate reducing bacteria (SRB) were inhibited, irrespective of the involvement of anode reduction, compared to the controls. Additionally, significant variation in the microbial community composition was observed, with several electrochemically active microbes and hydrocarbonoclastic bacteria being identified in SRB inhibited and iron stimulated SMFCs, such as *Arthrobacter*, *Geoalkalibacter* and *Marinobacter*. Results also indicated that iron stimulation was a major contributor to the change in the microbial composition of the SMFCs.

For weathered crude oil contamination, limited biodegradation rates of total petroleum hydrocarbons were observed under all operating conditions, with no significant

difference being observed in the iron stimulated SMFCs compared to the controls. High levels of hydrocarbon degrading bacteria were identified in these SMFCs, such as *Marinobacter* and *Arthrobacter* in the case of the open circuit control SMFCs and *Gordonia* in the case of iron stimulated SMFCs, with limited contribution of electrochemically active bacteria. Iron stimulation was the major contributor to the variation in the most dominant microbial genera involved in the petroleum hydrocarbon degradation.

Results indicate that SMFCs might not be a sustainable nor a feasible approach given the observed low overall biodegradation efficiency. Therefore, findings suggest that a better understanding of the performance of the system is required when targeting complex sediment contamination in marine environments, in order to develop an improved and a more efficient SMFCs bioremediation approach for the treatment of polluted marine areas.

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ABBREVIATIONS

BAP: Benzo(a)Pyrene

CC: Closed Circuit

COD: Chemical Oxygen Demand

EPA: Environmental protection Agency

Fe: Ferric Iron

FeRB: Ferric Iron Reducing Bacteria

MFC: Microbial Fuel Cell

Mo: Molybdate

MW: Molecular Weight

NA: Natural Attenuation

NMDS: Non-metric Multidimensional Scaling

OC: Open Circuit

OTU: Operational Taxonomic Unit

PAHs: Polycyclic Aromatic Hydrocarbons

SMFC: Sediment Microbial Fuel Cell

SRB: Sulfate Reducing Bacteria

TEA: Terminal Electron Acceptor

TPH: Total Petroleum Hydrocarbons

USEPA: United States Environmental protection Agency

CHAPTER 1

LITERATURE REVIEW

1. Introduction

Recently, pollution of aquatic ecosystems has been of a rising concern due to the toxic effects on living organisms (Ayangbenro et al., 2017; Blumer et al., 1970). Water contamination is considered a major issue given that it acts as the medium transferring the pollution to aquatic ecosystems. Aquatic sediments around the world are considered as a major sink for a variety of environmental pollutants entering the ecosystem through the overlying water (Adeniji et al., 2017; Kaonga et al., 2016; Mortimer et al., 1999; Nel et al., 2018). This is especially important for persistent organic pollutants that tend to be adsorbed and accumulate in the sediments, reaching concentrations that are much higher than their corresponding counterparts in the overlying water column (Fairbairn et al., 2015; Philipp Mayer et al., 2000; Zhang et al., 2003). Sediment can thus contribute heavily to water pollution; the sediment layer can act as a long-term active source of contamination, releasing it back into the overlying water column (Björklund et al., 2000; Eggleton and Thomas, 2004; Mustajärvi et al., 2017). This key-link between sediment and water thus largely increases the challenge of dealing with contamination of aquatic environments worldwide, impeding the sustainability of current remediation regimens.

Currently, there are many practiced approaches for treatment of contaminated aquatic sediments. These treatments are mainly physicochemical practices that are highly aggressive in nature, requiring a heavy use of energy and demanding high costs for sustaining a reasonable effectiveness (Mulligan et al., 2001; Perelo, 2010). This hinders the widespread application of common remediation approaches such as dredging, electrochemical degradation and ozonation (Azubuike et al., 2016; Perelo, 2010).

On the other hand, bioremediation is a process which takes advantage of the natural biota present in various environmental sources, to convert the toxic compounds to less harmful byproducts (Adams et al., 2015). Bioremediation thus provides a much less complex approach to treatment of contaminated soil, sediment and water resources. This provides a method that is more feasible than the common expensive and invasive physicochemical techniques for removal of contaminants (Refugio RV, 2016). Bioremediation is therefore exploited heavily for a variety of in-situ as well as ex-situ treatment schemes. Few examples of the commonly practiced bioremediation techniques include phytoremediation, composting, bioventing and bioleaching (Vineet Kumar et al., 2018). Given that bioremediation is a natural process by itself, the indigenous microbes can be externally stimulated (biostimulation), such as in the case of external supply of an essential nutrient(s), or can be seeded by the external addition of an organism(s) (bioaugmentation) that is(are) capable of transforming the target pollutant(s) (Benyahia et al., 2016; Wu et al., 2016). Hence, a lot of research is being pooled into optimization of the current bioremediation processes and discovering new techniques that are more efficient and sustainable for the attenuation of target contaminants.

Bioremediation of natural resources, such as aquatic sediments, mainly target exploiting the metabolic capabilities of the naturally occurring sediment microbiological organisms for decomposition and detoxification of adsorbed pollutants. When microorganisms metabolize organic matter, they need a terminal electron acceptor (TEA) to combine with the electrons generated as part of their energy production process. Various electron acceptors are commonly utilized by microbes (Ladino-Orjuela et al., 2016). Microbial populations close to the oxygen rich water, namely in the top layer of the sediment, can generate more energy due to the high redox potential of oxygen. This is translated into a faster growth compared to utilizing other TEAs such as sulfate and

nitrates, and subsequently, faster utilization of organic compounds as electron donors, including organic pollutants (Carlton et al., 1989; Hazen, 2018; Lovley, 2011). Under aerobic respiration, microbes typically donate the generated electrons to oxygen in the form of hydrogen atoms (through dehydrogenation of the electron donors) to produce water (H₂O). In the deeper sediment layers, the scarcity of oxygen as a TEA is overcome through several mechanisms. Bacteria can simply use other available TEAs such as sulfates, nitrates, and iron; however, certain populations take advantage of the natural electron shuttling capability of specific compounds, such as iron oxides, to transfer the electrons from the deeper layers of the sediments to the oxygen near the surface of the sediments (Lovley, 2011). Yet, such a process is usually limited by the low concentration of electron shuttles within the sediments (Kappler et al., 2004).

The large diversity in the microbial populations inhabiting aquatic sediments allow for a range of microbial metabolic processes to take place, permitting thus the oxidation of simple organic compounds as well as complex organic pollutants such as petroleum hydrocarbons (Kurtböke et al., 2018). This renders the in-situ bioremediation of contaminants a possible treatment approach. However, the limitation in the natural attenuation process due to the scarcity of available TEAs hinders the continuous oxidation of organic compounds, slowing down the bioremediation process due to a limited microbial activity (Holmer and Storkholm, 2001). Typical microbial activity enhancement practices include the external addition of chemicals such as ferric iron salts, sulfates, nitrates and methanol, which adds to the cost of the treatment in addition to risking further pollution. Another approach targets stimulating aerobic degradation of contaminants by sediment aeration; however, air bubbling might risk an increased pollutant release rate into the water column caused by sediment perturbation. Such bioremediation limitations raised the interest among the bioremediation community for

having a sustainable bioremediation approach involving minimal chemical amendments (Li and Yu, 2015). This led to the exploitation of microbial electrochemical properties to provide an inexhaustible TEA, which provides a long-term stimulant to enhanced natural attenuation rates of contaminants trapped within the sediments.

One of the potential tools being tested for optimized bioremediation performance of aquatic sediment contamination is the Sediment Microbial Fuel Cell (SMFC) technology. SMFC technology is a subcategory of the Microbial Fuel Cell (MFC) technology, used as a power source and a method for bioremediation (Hamdan et al., 2017; Logan, 2008; Sherafatmand and Ng, 2015; Yan et al., 2012). In a SMFC, an electrical connection is established between the anaerobic sediment and the aerobic overlying water column. This can enhance the metabolism rates of microbial consortia within the sediments, which are usually slow, allowing thus for a sustained breakdown of complex compounds that are usually persistent within the sediment column (Logan, 2008). Figure 1 presents a simplified schematic of a SMFC.

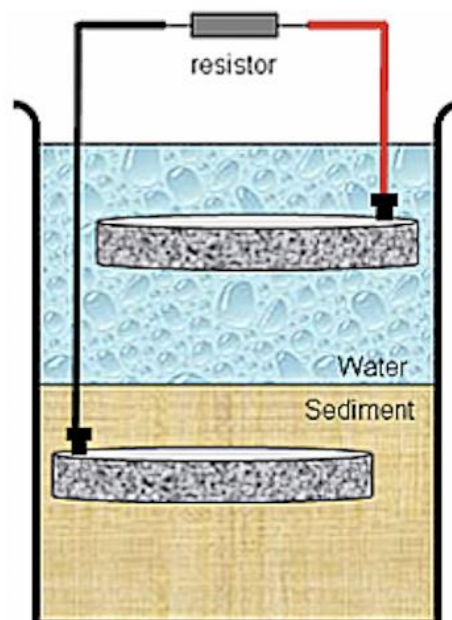


Figure 1. SMFC schematic

Recently, there has been an increased interest in using SMFCs for bioremediation of a multitude of persistent organic pollutants, which are trapped within aquatic sediments, including alkanes and PAHs, in both marine and freshwater environments (An et al., 2013; De Schampelaire et al., 2008; Hamdan et al., 2017; Sherafatmand and Ng, 2015; Viggi et al., 2015; Yan et al., 2012; H. Zhang et al., 2015). An electrode (anode) is embedded within the anaerobic sediment layer and can be utilized by microbes as a mediator for electron transfer to another connected electrode (cathode) placed in the overlying oxygen rich water layer. Electrons can thus be ultimately transferred to oxygen as a TEA. Utilizing a passive inexhaustible TEA, a SMFC setup thus does not consume large amounts of chemicals and energy that hinders the feasibility and the sustainability of the remediation technique. Microorganisms can oxidize the sediment contaminants near the surface of the electrode, and transfer the generated electrons to the anode either directly by using internally produced electron shuttles, or indirectly through the use of externally added electron mediators (Vivek Kumar et al., 2018). The bio-catalysis properties of a bioelectrical system, like the SMFC technology, has been reported as an effective method for removal of persistent petroleum compounds residing in sediments after an oil spill, especially given the thermodynamic favorability of oxidation of these highly concentrated compounds using oxygen compared to natural attenuation, with the added benefit of energy recovery through the resulting current from the flow of electrons across the electrodes (Chan and Li, 2014). Despite the significant enhancement of the natural attenuation rates of contaminants using SMFCs, traditional bioremediation approaches which rely on biostimulation of the contaminated sediments exhibit higher bioremediation efficiencies. However, due to the low cost, low maintenance, and the sustainability of SMFCs, this approach is considered as a better alternative to chemical amendment techniques. SMFC technology, in addition to simultaneous bioremediation

and energy production capabilities, have several advantages compared to common bioremediation practices. It is a flexible, a less aggressive and a simple mechanism for bioconversion of organic contaminants (Li and Yu, 2015), and results in minimal disruptions to natural aquatic habitats, avoiding further deterioration of the native ecosystem. SMFCs electrochemical properties can be also easily monitored during the bioremediation process for easier control of the system (Das, 2018).

2. SMFCs principles

2.1. Natural sediment redox characteristics

Natural redox gradients are usually spontaneously established in the sediment layer below the sediment-water interface in aquatic environments, which is due to the separation of the microbial redox reactions throughout the sediment layer (Aharon and Fu, 2003; Rosenberg et al., 2001; Vink, 2002). Oxygen is present in the water above the sediments. Diffusion of oxygen into the sediment layer allows for the top sediment layer to undergo aerobic processes. However, as the depth of the sediment layer increases, oxygen gets depleted, forming an anoxic environment where organic compounds are either oxidized using several TEAs such as nitrate, sulfate and iron oxides, or degraded through fermentative processes or methanogenesis (Asaoka et al., 2018; Chan and Li, 2014; Hansen et al., 1998; Lovley and Phillips, 1986). These processes are much slower than oxygen reduction due to the lower oxidation power offered by these TEAs and their limited availability and replenishment in the aquatic environment. This can sometimes halt the natural degradation process of organic compounds. However, a multitude of naturally occurring sediment microbial populations developed mechanisms to overcome this limitation to sustain continuous growth through extracellular electron transfer (Kumar et al., 2017). The presence of electron shuttles within the sediments allows for

several microbial populations to export the internally generated electrons to the extracellular medium, where the shuttles can carry them to the oxygen rich layer, practically allowing access for oxygen reduction even for microbes placed deeper within the sediments (Doyle and Marsili, 2015). In theory, the mediated electron transfer process can significantly accelerate the natural degradation process of organic compounds adsorbed onto the sediments; however, the process is extremely hindered by the low concentration of electron mediators. SMFCs thus turned out to be an appealing solution to the lack of proper electron transfer from the deeper sediments to the overlying oxygen.

2.2. SMFCs as an alternative reduction pathway for bioremediation

Deployment of SMFCs is usually performed at the bottom of an aquatic environment, where the sediment electrode is placed several centimeters below the sediment-water interface where oxygen is lacking (Logan, 2008). Organic compounds can thus be oxidized by the naturally occurring microbial community that takes advantage of the presence of the electrode onto which they dump their electrons, utilizing oxygen in the water as a TEA through the connection made across the 2 distant electrodes (De Schampelaire et al., 2008; Donovan et al., 2013; Jung et al., 2014; Sherfatmand and Ng, 2015). Thus, using this bridged artificial connection, the electrode can be used by nearby microorganisms as a strong TEA for oxidation of contaminants. The presence of the electrode can positively affect the rate of contaminant oxidation driven by the microbial redox reactions. Previous studies reported that the natural redox potential between the oxygen rich water and the anoxic sediment layer is usually below 800 mV (Lowy et al., 2006; Schulz, 2006). However, this potential is sometimes not sufficient for driving the oxidation of many persistent pollutants, especially due to overpotential being a major parameter in operating SMFCs (Noori et al., 2018). This sometimes limits the

practical application of SMFCs for bioremediation, especially that an external voltage is needed to provide the required redox potential for oxidation of such compounds (Erable et al., 2010).

2.2.1. Exoelectrogens

An electrochemically active microorganism, also known as an exoelectrogen or an electricigen, is a microorganism that has a natural ability to transfer the electrons generated during the cellular energy production process (oxidation of electron donors) to the extracellular medium (Chan and Li, 2014). Electrons can thus combine with extracellular electron acceptors, which can be insoluble, thus extending the capability of the involved microorganisms beyond the limited availability of soluble TEAs available for intracellular reduction (Logan, 2008). Commonly utilized external TEAs are strong oxidizers such ferric iron oxides and manganese oxides (LOVLEY, 2008; Mohan et al., 2019; Thamdrup, 2000).

To better understand the mechanisms involved in the SMFCs operation, microbial electrochemical properties must be also understood. Many microorganisms with electrochemical properties were identified in literature, of which numerous are observed as part of the microbial community inhabiting the electrodes in MFCs in general and SMFCs in specific. Multiple bacterial groups belonging to *Firmicutes*, *Acidobacteria* and *Proteobacteria* as well as yeast, fungi and microalgae are reported to be involved in current generation in SMFCs, with species such as *Shewanella spp.*, *Aeromonas hydrophila*, *Clostridium butyricum*, *Geobacter spp.*, *Rhodospirillum rubrum*, and *Enterococcus gallinarum* being commonly noted (Jothinathan et al., 2018b; Logan, 2008; Yu et al., 2017). During the extracellular reduction of ferric oxides, these microbes actively express the genes involved in the extracellular transport of electrons. For

example, *Geobacter* extracellular respiration involves active expression of conductive pili that are able to dump the electrons onto insoluble ferric iron (LOVLEY, 2008). *Geobacter* is a model bacterium among the MFC community. *Geobacter* is a motile bacterium that is capable of searching for new insoluble natural ferric oxides as electron acceptors (Childers et al., 2002). However, the placement of the anode within the anaerobic sediment, which can be used by *Geobacter* as a dump for electrons, permits the growth of this exoelectrogenic bacterium as a biofilm on the electrode, facilitating the optimized use of the anode as a TEA (Childers et al., 2002). Many known exoelectrogenic bacterial species can easily transfer electrons to an external electrode. *Rhodopseudomonas palustris*, *Geobacter sulfurreducens*, *Pseudomonas aeruginosa*, *S. spp.*, *Escherichia coli*, and even some pathogenic bacterial strains, such as *Ochrobactrum anthropi* and *Klebsiella pneumonia*, are all previously reported for their efficient use of an external electrode as a TEA (Bond and Lovley, 2003; Feng et al., 2018; Jothinathan et al., 2018a; Semenc et al., 2018; Zou et al., 2019).

2.2.2. Microbial communities in SMFCs

In a typical SMFC, the application of the anode as a TEA allows for a consortium of naturally occurring microbial communities to evolve and inhabit the electrode (De Schampelaire et al., 2008; Hamdan et al., 2017; Li and Yu, 2015). The evolution of the microbial community at the anode level is driven by the combination of environmental parameters, the indigenous microbial population and the presence and concentration of contaminants within the sediments (Song and Jiang, 2018). To ensure the stability of a field operated SMFC, the robustness of the system must be well studied and ensured to maintain a proper performance, which is mainly dependent on the microbiological aspect of the system. Knowing that exoelectrogens being involved in direct electron transfer to

the anode are not the only microbes involved in contaminants removal using SMFCs, identifying the different involved microbial communities and understanding the synergy among them during operation of SMFCs, remains essential to optimize and apply this technology for enhanced bioremediation in aquatic contaminants (Sajana et al., 2017).

Interaction between the microbes and the anode in SMFCs is divided into two stages. First, exoelectrogens are involved in the direct breakdown of target contaminants by metabolizing them and utilizing the anode as a TEA. Second, non-exoelectrogenic microbes can stimulate the electrochemically active microbes by breaking down complex organic matter and other sediment components into forms that are utilizable by the exoelectrogens inhabiting the anode, which in turn further stimulates the exoelectrogenic bioremediation capability of the anodic microbial community (Lovley, 2008).

In general, microbial populations involved in the operation of SMFCs show different characteristics based on the source of the sediments. The majority of the exoelectrogenic bacteria belong to two major classes belonging to the phylum *Proteobacteria*, which are *Deltaproteobacteria* and *Gammaproteobacteria* (De Schamphelaire et al., 2010; DENG et al., 2014; Wang et al., 2015). Members of *Deltaproteobacteria* are ubiquitous in sediments of marine environments, and many of them were identified as direct utilizers of the anode as a TEA in SMFCs, using organic matter or sulfur as electron donors, such as in the case of *Desulfobulbus* and *Desulfocapsa* (Daghio et al., 2018; Finster et al., 1998; Lam et al., 2018). *Geobacter* represents another bacterial group pertaining to the class *Deltaproteobacteria* capable of direct utilization of the anode as a TEA and is dominant in freshwater SMFCs. *Shewanella*, a widely distributed marine bacterium belonging to *Gammaproteobacteria*, is also known for its capability of direct anode utilization as a TEA (Chan and Li, 2014; De Schamphelaire et al., 2008; Jung et al., 2014). Studies also indicated that even in a single operated SMFC,

the utilized anode shows variation in terms of microbial composition along its length, with deeper sections showing a higher microbial diversity (De Schamphelaire et al., 2008). Such observations show that understanding the interactions among the various involved microbial populations in SMFCS is essential in order to maintain a dominant exoelectrogenic community capable of enhancing the SMFCS. Efforts to assess the sediment microbial ecology and its electrochemical potential, as well as microbial interactions in SMFC systems, are still lacking in the literature.

With the extreme importance of the anode microbial community as an essential component of successful implementation of SMFCs for bioremediation, studies are overlooking the importance of the cathode as an optimizable parameter. Cathodes can be a limiting factor in many SMFCs design given that the rate of oxygen reduction at the level of the cathode can inhibit proper performance of the system (Feng Zhao et al., 2006). Oxygen availability at the cathode level can thus dictate the success or the failure of the SMFC. Various designs are incorporating high surface area cathodes to avoid this limitation. Some researchers are incorporating photosynthetic biocathodes as part of their SMFC design to enhance the oxygen reduction rate, which can directly affect the oxygen availability at the cathode level for enhanced SMFCs performance due to in situ generation of oxygen at the cathode level, which is much higher than what is achievable under physical aeration of the cathodic compartment (Commault et al., 2014; Jiang and Zeng, 2019; Yang et al., 2018). De Schamphelaire et al. (2008), for example, was able to use a biocathode to significantly and sustainably increase the cathodic potential compared to control SMFCs. In addition, certain microbes can even directly accept the electrons at the cathode level to catalyze the oxygen related process (Dubé and Guiot, 2015). In addition, the aerobic environment at the level of the cathode allows for various microbes to inhabit the electrode. Only few studies have raised concerns about the ability of the

cathode to serve as a medium for biofilm growth, affecting the overall performance of the system (Commault et al., 2014; Wang et al., 2012). Information about the cathodic microbial characteristics and composition in SMFCs is still very limited.

2.2.3. Electrodes

To ensure practicality and feasibility of SMFCs for bioremediation, electrodes, which are the major component, should be cost effective and durable (Mustakeem, 2015). Electrical conductivity is also a major contributor for maintaining stable operation of the cells. Copper, which is a highly conductive metal, is usually avoided due to its toxic impacts on the biofilm along with the corrosion of the metal. The use of stainless steel as electrodes was practiced in multiple studies due to the non-corrosive nature of the alloy. However, although the efficiency of microbial electron transfer to the stainless steel electrodes is well desired, the limitation of having a low surface area to volume ratio of these electrodes contributed to seeking a more effective alternative (De Schamphelaire et al., 2008; Wei et al., 2011). Carbon based electrodes thus came into competition by offering a versatile and durable material with corrosion and biofouling resistance, along with having a high surface area and being non-toxic to microorganism and being inexpensive (De Schamphelaire et al., 2008; Wei et al., 2011).

Carbon based materials that are commonly utilized as anodes in SMFCs are graphite rods/plates, carbon cloth, paper, fibers, sponges, etc., of which those with higher surface area per volume being more effective in SMFCs. Additional increase in the performance of carbon-based electrodes has been practiced by various chemical and physical modifications (Wei et al., 2011). Nonetheless, challenges, such as overcoming electrode passivation due to deposition of sulfur and other materials, are still to be

addressed in literature, especially that long term effect of biofilm growth on the anode in SMFCs is not properly researched (Peixoto et al., 2019; Prakash et al., 2018).

In many occasions, the low oxygen reduction rate at the cathode in SMFCs is a major limitation to the performance of the system. Carbon based cathodes are usually preferred due to the same advantages observed in the case of the anodes. There is a wide variety of cathode setups that were practiced in SMFCs to increase the oxygen availability for reduction, such as using rotating cathodes as well as coupling platinum as a catalyst (Wang et al., 2012; Zhou et al., 2018). This increases both the complexity of the system as well as the cost of operation. With the scaling-up endeavors of SMFCs for bioremediation of contaminated sites in mind, the current understanding and optimization of the cathode for enhanced operation of SMFCs should be further addressed in future studies, as the current knowledge is mainly focused on the anode performance.

Overall, the performance of SMFCs is strongly related to the limitations at the anode and the cathode levels, dictating successful or failed implementation of the system in various environmental settings. At the anode level, mass transfer of electron donors can heavily impact the oxidation of the pollutants in the sediments. Unlike regular MFCs (ex: wastewater MFCs), where mass transfer is not an issue due to the liquid nature of the medium, the solid sediment matrix in SMFCs hinders the microbial ability to efficiently utilize electron donors further away from the anode (Donovan et al., 2008). This is especially important when targeting trace organic pollutants as the effective area of the anode will not justify the implementation of the system for significant removal of the target pollutant(s).

2.2.4. Microbial electron transfer

Electrochemically active microbes can transfer the electrons to the extracellular medium, specifically the anode in the case of SMFCs, via different methods, which can be either direct or mediated. Four major mechanisms are responsible for the microbial electron transfer to the anode in SMFCs.

- Electron transfer via self-produced electron shuttles

Bacterial species such as *Pseudomonas aeruginosa*, *Geothrix fermentans*, *Shewanella oneidensis*, and *Lactococcus lactis*, have the ability to produce electron shuttles, enabling them to shuttle the internally generated electrons to an external insoluble TEA such as in the case of a SMFC (Glasser et al., 2017). *Geobacter fermentans*, for example, can produce riboflavin to mediate the electron transfer to the externally added ferric iron in the growth medium (Mehta-Kolte and Bond, 2012). *Shewanella* also has the ability to reduce amorphous ferric iron in the surrounding medium by producing flavin and riboflavin as soluble electron mediators (von Canstein et al., 2008). In one study, removing riboflavin from *Shewanella sp.* and *S. oneidensis* biofilm significantly reduced the electron to electrode transfer rate (Marsili et al., 2008).

- Redox-active proteins

Redox-active proteins are another mechanism utilized by bacteria for direct short-range electron transfer. For example, *G. sulfurreducens* expresses many types of cytochromes that protrude from the outer membrane, which are used to directly transfer the electrons from the intracellular medium to an external TEA such as elemental sulfur, metal ions, and anodes in SMFCs (Dantas et al., 2018; Hernandez-Eligio et al., 2018).

- Long-range electron transfer via conductive bacterial pili

This mode of microbial electron transfer was first observed in *G. sulfurreducens*, in which a dense network of pili was observed to have metallic properties, rendering the

overall biofilm conductive (Walker et al., 2018). The metallic characteristics of the pili extend beyond simple electrical conductivity. Conductive pili in *Geobacter* are actively expressed in the presence of insoluble electron acceptors, including manganese (IV) and ferric iron. However, in the case of the presence of soluble forms of these electron acceptors, the conductive pili are not expressed (Abbas et al., 2017). These observations, along with other related findings, suggested that *G. sulfurreducens* use their conductive pili as nanowires for long range dumping of electrons onto external insoluble TEAs (Filman et al., 2018). The properties of nanowires were ultimately correlated to the highest current densities when utilizing *Geobacter* as the exoelectrogenic microbial community in microbial fuel cells (Nevin et al., 2008). Other bacteria that can produce conductive pili are strains of *Synechocystis*, *Pelotomaculum thermopropionicum* and *Methanothermobacter thermautotrophicus* (Gorby et al., 2006; Sure et al., 2016).

- Direct electron transfer between different microbial species

Interspecies electron transfer was observed previously in a mixture of *G. sulfurreducens* and *Geobacter metallireducens*, in which the conductive pili contributed to the mutual growth of both species (Ueki et al., 2018). Non-metal reducing bacteria were also noted to be involved in direct inter-species electron transfer. For example, this was observed in co-cultures of *Pelobacter carbinolicus* and *G. sulfurreducens*, *G. metallireducens* and *Methanosarcina barkeri*, as well as a wide range of other bacterial species (Dubé and Guiot, 2015; Rotaru et al., 2014, 2012).

The various electron transport mechanisms utilized by bacteria thus highlight the importance of understanding the interactions between the anode biofilm and the anode, as well as inter-microbial interactions, which contribute to the overall performance of the SMFCs. Research targeting these microbial interactions is well established in the MFC community; however, such knowledge is still very limited in SMFCs, and most studies

only report the relative composition of the sediment and anode biofilm at the end of the experiments. To better understand the applicability and performance of SMFCs for bioremediation of specific pollutants, future research must be more focused on the microbial biofilm aspect.

3. SMFCs operating parameters

Studies targeting SMFCs for bioremediation tend to overlook major operating factors that contribute to the successful implementation of the system. While the focus is usually mainly related to the applicability of SMFCs to bioremediation of certain pollutants, anode and cathode modifications, microbial stimulation with external supplements, etc., little focus has been given so far for other important operational factors.

3.1. pH

The pH of a SMFC plays an important role in shaping the microbial biofilm for the removal of the target contamination. A change in the external pH could be due for instance to changes in the external ionic concentrations, or internal changes to microbial cytosolic pH, which might lead to the alteration of the proton and electron shuttling, as well as to a failure of proper formation of the biofilm (Abbas et al., 2017; He et al., 2008). Typically, SMFCs are preferred to operate under slightly acidic condition given the microbial acidophilic pathways. This is due to the high internal proton gradient formed during the microbial metabolism, which also aids in the electron transfer from inside of the bacterial cells to the external medium (Abbas et al., 2017).

SMFCs pH is normally influenced by the initial pH of the sediment and water, because the generation of protons as well as their consumption occur simultaneously as they diffuse from the anode to the cathode. Bacteria normally require a neutral medium

for maximal SMFCs performance, but since the local pH is affected by the anodic and cathodic processes, bacteria can adjust their intracellular pH by production of internal buffers that resist the external changes, maintaining thus a proper performance of the system (Kelly and He, 2014). However, in some cases, there is an imbalance between the generation of protons at the anode level and their consumption at the cathode level, requiring the addition of external buffers, such as glucose and phosphate, to maintain a neutral operating pH (Sajana et al., 2014). It was found that for optimal operation of SMFCs, the system is preferred to be maintained between a pH of 6 and 9 (An et al., 2013).

3.2. Temperature

Temperature also plays an important role for proper operation of SMFCs. A change in temperature could affect various aspects of a SMFC, such as mass transfer rates, kinetics and development of various bacterial species in the anode biofilm (Acosta-González and Marqués, 2016; Ye et al., 2016). Several studies reported an increased performance of SMFCs, both in terms of contaminant removal and power production, with an increased temperature, probably due to enhancement in the microbial metabolic pathways combined with increased conductivity of the sediments resulting in lower internal resistance (Sajana et al., 2014). Optimal operating temperatures of SMFCs were reported to be in the range of 30 to 45 °C, ensuring proper formation of the anode biofilm for a sustained and stable operation (Abbas et al., 2017). However, in many instances, given the variety of the microbial species growing within the anode biofilm, each having its own optimal temperature range, higher temperatures could result in denaturing of microbial enzymes that are required for anode reduction and contaminant oxidation. This could lead to biofilm inactivation. Therefore, the initial temperature of SMFCs has a huge

impact on the development and relative distribution of anode microbial cells, which can later tolerate temperature changes by adjusting their metabolic activities, maintaining better long term performance once the biofilm has been established (Domínguez-Garay et al., 2013).

3.3. Internal resistance

Internal resistance of SMFCs is an additional important factor necessary to understand for a better use of SMFCs for bioremediation purposes. Internal resistance is a function of several other design parameters of the SMFC, mainly the distance between the electrodes and the conductivity of the sediment and water (Sajana et al., 2014). A higher conductivity, such as in the case of marine SMFCs, will decrease the internal resistance, resulting in better electrochemical performance (Chen et al., 2016). The distance between the anode and the cathode affects the movement of the electrons and protons. For optimal results, the two electrode must be as close as possible to each other to lower the internal resistance; however, electron losses have been previously reported to be a limitation when the electrodes were very close to one another, resulting in lower overall performance (Abbas et al., 2017; Donovan et al., 2013). Hence, future studies should focus on optimization of anode/cathode distance in SMFCs as well as other factors affecting the internal resistance of SMFCs, to ensure maximal rate of electron acceptance throughout the system.

3.4. Target substrate

Finally, the selection of a target substrate(s) for biodegradation using SMFCs is an additional important parameter to be taken into consideration when designing for a bioremediation treatment. SMFCs might not be capable of significantly enhancing the

natural removal rates of certain persistent pollutants due to multiple factors such as the absence of specific microbes capable of degrading such compounds (Das and Chandran, 2011; Kim and Kwon, 2010). Additionally, the concentration of substrates in the sediment might also hinder the proper performance of the SMFC due to a low abundance of electron donors to enable a sustained growth of the anode biofilm.

4. Applications

4.1. Power generation

Due to the specific characteristics of SMFCs, their overall power generation is much lower than their wastewater MFC counterparts. Specifically, marine SMFCs were largely investigated for operating low power consuming aquatic instruments, such as remote environmental sensors, given that the higher conductivity of the marine SMFCs is translated into a better energy recovery compared to freshwater SMFCs. This application facilitates the operation of environmental monitoring program, as the powered equipment could help in recording and transmitting data such as pH and temperature in remote locations that are not easily accessible. Yet, in most cases, the low energy potential provided by the SMFCs is not sufficient and consistent to maintain continuous operation of the sensors, and thus, to overcome this limitation, a simple power control circuit could be used to store the power to supply continuous voltage to the operated sensors (Prasad and Tripathi, 2019). The advantage of SMFCs over traditional batteries is mainly due to being practically a maintenance-free system (Zhang et al., 2011). In one study, it was reported that operating multiple connected small SMFCs could provide significantly higher power than operating a single larger scaled-up SMFC, which is proposed to be a solution for equipment with higher energy demands (Ewing et al., 2014). In another study, a successful long-term operation of SMFCs was reported for effective use as a scaled-up

power supply tool for commercial electronic devices (Yang et al., 2015). SMFCs were also utilized in literature to successfully power a variety of devices, including a hydrophone, temperature sensors, environmental sensors, an underwater ultrasonic receiver and a wireless telecommunication sensor (Donovan et al., 2013, 2011; Thomas et al., 2013; Wotawa-Bergen et al., 2010; Zhang et al., 2011).

4.2. Water bioremediation

Overall, there are several limitations to using a SMFC as a power production tool, mostly due to the low power generated by the system in most environmental settings. This is especially important in areas where a sustained operation is not practical due to low sediment conductivity (especially in freshwater environments) or due to low sediment organic matter (Chen et al., 2016). As a result, research is being oriented more towards other environmental applications of the SMFC technology, mainly bioremediation of various aquatic pollutants. Many freshwater SMFCs studies focused on the remediation of water. For example, in one, study, significant wastewater COD removal was achieved by using a modified version of the SMFC technology, reducing it from 330 mg/L to around 125 mg/L within a day (Erable et al., 2011). Zhang and Angelidaki (2012) were able to apply SMFCs to remove nitrogen from a eutrophic lake, achieving 77% removal of total nitrogen contamination from the water. Sajana et al. (2013) demonstrated that SMFCs can be used for in situ reclamation of aquaculture water, with achieved COD and total nitrogen removal reaching 84.4% and 49%, respectively. Xu et al. (2018) showed promising phosphorus removal efficiencies in the water column in SMFCs.

4.3. Sediment bioremediation

The diversity in the indigenous microbial structure of the sediments allows for a wide range of sediment contaminants to be biodegraded as the SMFC biofilm evolves. For example, Williams et al. (2010) stimulated metal reducing microbial populations using SMFCs to attenuate uranium in contaminated groundwater sediment. SMFCs were also reported for enhanced bioremediation of several heavy metals and organic matter from contaminated sediment (Wu et al., 2017; Zhu et al., 2019). Furthermore, a lot of research is being pooled into understanding the use of SMFCs for the removal of organic contaminants from polluted sediments. This is especially important for petroleum hydrocarbon pollution in various aquatic ecosystems, due to the variety of polluting sources contributing to the daily increase in the contamination. Naturally occurring microorganisms in aquatic sediments can attenuate petroleum hydrocarbon deposited in the sediments (McGenity et al., 2012). There is a large diversity in the naturally occurring microbial population, which allows for a consortium of species to be involved in the natural attenuation process (Deng et al., 2014; Röling et al., 2002). Such a variation in the naturally occurring microbial ecosystem has a significant effect on the fate of individual hydrocarbons in affected sites and the success of applying SMFCs as a petroleum hydrocarbon bioremediation tool. For example, Yan et al. (2012) achieved successful enhancement of removal of selected Polycyclic Aromatic Hydrocarbons (PAHs) in freshwater SMFCs, which are phenanthrene and pyrene, achieving removal efficiencies of $96.14 \pm 1.28\%$ and $92.13 \pm 3.29\%$, respectively. Sherafatmand and Ng (2015) used lake sediments contaminated with PAHs to operate SMFCs, which were able to achieve high removal rates for naphthalene, acenaphthene and phenanthrene. In a recent study that we performed, high removal rate of phenanthrene was achieved in marine SMFCs contaminated with a mixture of low molecular weight PAHs (93.8% compared to 40.3%

in the control), while the other low molecular weight PAHs in the mixture (naphthalene and 1-methylnaphthalene), were mostly removed by volatilization rather than enhanced biodegradation (Hamdan et al., 2017). Xia et al. (2015) showed successful stimulation of bioremediation in heavily contaminated sediments using SMFCs, with the more polar compounds being more susceptible to the enhanced biodegradation, such as in the case of alkanolates and phthalates. Yan et al. (2015) also demonstrated the applicability of SMFCs for the bioremediation of a mixture of low molecular weight PAHs in contaminated sediments. Yang et al. (2015) showed that SMFCs is an efficient approach for bioremediation of sediment total organic carbon in heavily contaminated areas containing heavy metals, PAHs and polybrominated diphenyl ethers. Viggi et al. (2015) showed enhanced degradation rates of total petroleum hydrocarbons in crude oil spiked sediment using a variation of the SMFC technology termed an “Oil-Spill Snorkel”.

SMFCs bioremediation studies thus are mostly targeted at applicability to specific pollutants in small scale laboratory settings rather than field simulation studies. Even applicability studies mostly focus on easily biodegradable petroleum hydrocarbon compounds such as in the case of PAHs. For example, in a recent yet unpublished study that we have conducted, marine SMFCs, which were previously demonstrated to be applicable for remediation of low molecular weight PAHs, were found to be not very successful when applied to a mixture of PAHs containing high molecular weight ones. Additionally, the same was noted for SMFCs bioremediation of marine sediment spiked with a heavy contamination of crude oil, raising the concern of successful applicability of SMFCs to real world heavy aquatic contamination events rather than treatment of existing low concentration sediment pollution.

5. Conclusions

The natural anoxic environment provided by aquatic sediments, in combination with the capabilities of indigenous sediment microbial communities to adapt to enhanced biodegradation of pollutants, make SMFCs a strong candidate for extended future large-scale bioremediation studies. However, even with the increasing number of SMFC studies, targeting both bioremediation and energy production, the knowledge is still very limited compared to the wastewater MFCs. Knowledge from conventional MFCs cannot be simply transferred and applied to SMFCs for bioremediation attempts, due to the huge variation in the operation setup and conditions, in addition to other influential factors such as the complexity of the biological system combined with the mass transfer limitations.

SMFCs thus created their own line of research, which is oriented towards providing a controllable, economically feasible and sustainable technology for bioremediation of recalcitrant pollutants. While current research is mostly concentrated on laboratory scale remediation of simple contaminants in SMFCs, future research should better explore the capabilities of the system for bioremediation of complex contamination in natural sites to ensure its scalability. This will require addressing a variety of field related issues such as actual installation, electrode passivation, corrosion of connections and destruction by aquatic organisms. This will require knowledge beyond what is possible through laboratory operation. Yet, this does not belittle the importance of addressing the missing knowledge through laboratory experimentation for optimizing the system for pilot field studies. The continued pursue for understanding SMFCs for bioremediation will ultimately maintain the improvement of the technology. This will require the combined efforts of multiple disciplines, including electrochemistry, biotechnology, environmental sciences, material sciences, etc., and hence, the research

interest for successful implementation of SMFCs for bioremediation will continue to grow.

CHAPTER 2

FERRIC IRON STIMULATION OF SULFATE RICH MARINE SMFCs FOR THE REMOVAL OF LOW AND HIGH MOLECULAR WEIGHT PAHS

Abstract

Sediment Microbial Fuel Cells (SMFCs) take advantage of the natural ability of certain indigenous microbes to transfer their intracellularly generated electrons to external insoluble electron acceptors placed within the anaerobic sediment. This, coupled to the ability of these communities to adapt to and degrade organic contaminants, allows for using SMFCs for stimulating degradation of persistent organic pollutants such as polycyclic aromatic hydrocarbons (PAHs) by using an anode embedded in the anaerobic sediment as a favorable terminal electron acceptor (TEA). PAHs bioremediation studies in SMFCs are limited to a couple reporting low molecular weight PAHs (2 and 3 aromatic rings), which are mostly lost by volatilization rather than by biodegradation. High molecular weight PAHs (4 and more aromatic rings) are much harder to attenuate once settled in the environment. Additionally, SMFCs/PAHs studies are focused on freshwater sediments that are, unlike marine sediments, rich in ferric iron. Ferric iron is important for enrichment of the exoelectrogenic microbial community, which is the major direct utilizer of the anode as a TEA. However, no studies addressed ferric iron stimulation of exoelectrogens in marine SMFCs to aid competing with the dominant sulfate reducers, which interferes with the proper performance of anodes. Thus, assessment of marine SMFCs for the bioremediation of high molecular weight PAHs is essential to understand the applicability of the system as a treatment approach to real case scenarios, where contaminated sediments are usually affected by a variety of complex and persistent hydrocarbons.

In this study, we investigated the use of marine SMFCs for the simultaneous bioremediation of a complex mixture of PAHs highlighted in the Environmental protection Agency (EPA) priority pollutants which are the 2-ringed naphthalene, 3-ringed fluorene, 4-ringed pyrene and 5-ringed benzo(a)pyrene (BAP). Investigation was performed under anode reducing conditions in the presence and absence of sulfate reduction inhibition and ferric iron stimulation. Control SMFCs were operated under open circuit condition. Volatilization was the major removal mechanism of low molecular weight PAHs (2 and 3 rings) given the similar removal kinetics compared to the control SMFCs. In the case of the 4 ringed pyrene, highest removal was observed under iron stimulation when sulfate reducing bacteria were inhibited (37.69% for closed circuit and 37.92% for open circuit SMFCs), compared to the natural attenuation control which showed around 22.11% removal. In the case of the 5-ringed BAP, same observation was noticed with 37.89% and 41.07% removal in the case of iron stimulation and sulfate reduction inhibition, under closed and open circuit operation, respectively, compared to only 13.21% removal under natural attenuation. Additionally, significant variation in the microbial community composition was observed among the operating conditions, with several electrochemically active microbes and hydrocarbonoclastic bacteria being identified in SRB inhibited and iron stimulated SMFCs, such as *Arthrobacter*, *Geoalkalibacter* and *Marinobacter*. Additionally, similarity in the overall microbial

evolution profile in several of the conditions indicated that iron stimulation was a major contributor to the change in the microbial composition of the SMFCs.

Findings suggest that a better understanding of the performance of the system is needed when targeting complex combinations of PAHs, in order to develop an improved and a more efficient SMFCs bioremediation approach for the treatment of polluted marine areas.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous pollutants of the marine environment (Yang et al., 2019). Anthropogenic processes greatly impact the influx of PAHs into marine areas, largely affecting their concentration in coastal sediments nearby urban and industrial areas (de Almeida et al., 2018; Mirza et al., 2012). The United States Environmental protection Agency (USEPA) recognizes PAHs as primary contaminants, of which 16 are identified as priority pollutants (Ćirić et al., 2018). PAHs are further divided into two main groups based on their anthropogenic sources. Petrogenic sources of PAHs, such as crude oil and oil-refined products, contribute to marine contamination mainly through accidental oil spills and discharge of contaminated runoff. Pyrogenic sources further add to the marine pollution mainly through deposition of PAHs from incomplete combustion of organic matter (Barakat et al., 2011; Richter-Brockmann and Achten, 2018). As a result, those processes render the sediments in marine environments a major reservoir of PAHs pollution given the tendency of these hydrophobic contaminants to adsorb onto organic particulates, and also due to their slow natural biodegradation rates once settled (Barakat et al., 2011; Yang et al., 2019).

PAHs can be attenuated from the environment through various physical and chemical mechanisms such as volatilization, chemical degradation, photolysis, and microbial degradation (Haritash and Kaushik, 2009; Zhao et al., 2016). PAHs, similar to other organic compounds, can be biologically degraded through their utilization as a carbon source by a variety of naturally occurring microbial populations (Haleyur et al., 2018). The natural biodegradation rates of PAHs; however, are usually limited due to suboptimal degradation conditions related to pH, temperature, dissolved oxygen, microbial adaptation, etc. (Ghosal et al., 2016; Yang et al., 2019). Yet, the major contributor to the persistence of PAHs is their high stability, which increases with the

increase of their molecular weight/number of rings (Abdel-Shafy and Mansour, 2016; Khuman et al., 2018). For example, Kanaly and Harayama (2000), reported that the 3-ringed phenanthrene half-life ranged from 16 to 126 days in various soil and sediment environments, compared to 229 to 1400 days for the 5-ringed benzo(a)pyrene (BAP).

A number of naturally occurring sediment microbial populations are hydrocarbon degraders capable of metabolizing PAHs contamination. These could include electrochemically active bacteria belonging to classes *Deltaproteobacteria* and *Gammaproteobacteria*, of which the former is ubiquitous in marine sediments (Li and Yu, 2015). Electrochemically active bacteria, also known as exoelectrogens, are capable of using external insoluble terminal electron acceptors (TEA) such as iron as part of their metabolism (Chen et al., 2019). Bioelectrochemical systems such as SMFCs take advantage of the natural ability of such microbial communities by providing an inexhaustible TEA within the anaerobic sediment to enhance the natural degradation rates of organic contaminants (X. Li et al., 2018; Y. Li et al., 2018). Iron reducing bacteria (FeRB) belonging to the class *Deltaproteobacteria*, such as *Geobacter*, and the class *Gammaproteobacteria*, such as *Shewanella*, are well known for their involvement as exoelectrogens in bioelectrochemical systems (Logan, 2008). This, coupled to the ability of these communities to adapt to and degrade organic contaminants, allows for using SMFCs for stimulating the degradation of persistent organic pollutants through using the anode as TEA at a rate that is higher than their natural attenuation rate (Lu et al., 2014). SMFCs then allow for the transfer of the electrons into a cathode, placed in the overlying oxygen rich water and connected to the anode, where the electrons can reduce oxygen; oxygen combining with the transferred electrons act as an ultimate TEA that is thermodynamically favorable over the use of other TEAs (X. Li et al., 2018; Logan, 2008)

Biodegradation of organic pollutants in SMFCs has been associated with the activity of a variety of microbial populations that utilize various anode oxidation mechanisms, which could be either direct or mediated (Logan, 2009). Anode biofilm is a critical factor in understanding and optimizing bioelectrochemical processes such as anode reduction in the case of SMFCs, and better enhancement of the performance of these systems needs careful analysis and understanding of the associated microbes (Jung et al., 2014). However, microbial transition from reduction of naturally occurring TEAs, such as the extremely abundant sulfate in marine sediments, to anode reduction in SMFCs, is not straightforward given that exoelectrogenic anode reducing microbes, namely FeRB, may not be able to outcompete the existing microbial populations. This is due to the limited abundance of FeRB compared to the highly established sulfate reducing bacteria (SRB). This was observed in a previous study where FeRB enrichment through amending the sediment with ferric iron failed to shift the sediment microbial community away from sulfate reduction, which was probably due to the low population size of FeRB (Coates et al., 1996). In another study, Yan et al. (2012) demonstrated successful enhancement of SMFCs performance for the removal of low molecular weight (MW) PAHs through addition of ferric iron to the sediments; however, this was performed in freshwater SMFCs in which FeRB are much more common compared to marine sediments. On the other hand, (Zhou et al., 2014) reported that amendment of freshwater SMFCs with ferric iron stably enhanced SMFCs voltage outputs with no apparent enhancement to the organic matter removal from the sediments.

Thus, the complexity of the sediment microbial structure often has a direct impact on the success or failure of the development of an anode biofilm capable of utilizing the anode as a TEA in a SMFC. The microbial structure will evolve upon the existing sediment characteristics (Logan, 2008). For instance, sulfate-rich marine sediments lack

a sufficient amount of iron that can support the growth of FeRB, which are typically outcompeted by the dominant SRB (Yang et al., 2019). This can restrict direct anode respiration and enhancement of biodegradation of targeted pollutants due to competition for space and resources (Zhou et al., 2014). The effect of the dominance of SRB on FeRB was observed in a previous study employing sulfate-rich marine SMFCs, in which anode respiring bacteria were detected at a relatively high relative abundance when SRB were inhibited (Hamdan et al., 2017). To further stress the importance of sediment characteristics on the anodic microbial structure, several studies report that freshwater and marine SMFCs show different microbial profiles, with the exoelectrogenic iron reducer *Geobacter* being enriched at the level of the anode in freshwater SMFCs while *Desulfuromonas* usually dominating in marine SMFCs (Chan and Li, 2014; De Schamphelaire et al., 2008; Jung et al., 2014). Iron reduction is an important biodegradation pathway in freshwater sediments, which is not the case in the sulfate rich marine environments where sulfate reduction predominates.

In this study, we assess FeRB enrichment in marine SMFCs operated for the purpose of bioremediation of a range of PAHs. This is especially important for systems that are deficient in FeRB such as in marine environments to shift the microbial metabolism towards having a higher abundance of FeRB, including exoelectrogens, in the presence of SRB competition. Additionally, information regarding application of SMFCs for the attenuation of high MW PAHs is very limited, especially in marine environments where such contamination is very common. Only few recent studies addressed PAHs attenuation in contaminated sediments using SMFCs (Hamdan et al., 2017; Sherafatmand and Ng, 2015; Yan et al., 2012). These studies focused on low MW PAHs which are usually 2-ringed or 3-ringed. Only one reported the use of a 4-ringed PAH which is pyrene (Yan et al., 2012). Low MW PAHs in SMFCs are usually lost by

volatilization rather than by biodegradation, namely 2-ringed PAHs such as naphthalene and its derivatives. Removal of semi-volatile 3-ringed PAHs, such as phenanthrene, is also enhanced due to volatilization. However, no studies had evaluated the use of SMFCs for the bioremediation of higher MW PAHs, which are more persistent and are much harder to remove once settled in the environment. Thus, in order to better understand the biodegradation process of PAHs using SMFCs, 2-, 3-, 4-, and 5-ringed PAHs, represented respectively with naphthalene, fluorene, pyrene and benzo(a)pyrene (BAP) were spiked into marine sediments to simulate a complex mixture of PAHs contamination. SMFCs were then operated under anode reducing conditions in the presence and absence of bacterial sulfate reduction inhibition and ferric iron stimulation. Furthermore, the evolution of microbial populations during operation of the SMFCs was monitored to provide a better understanding of the microbial communities involved in PAHs degradation in marine SMFCs. This would provide essential information for future optimization of the system for bioremediation approaches.

2. Materials and methods

2.1. Sediments and seawater

Marine sediments were collected near the Jiyeh powerplant in Lebanon, where a significant oil spill has occurred in 2006 releasing more than 15000 tons of heavy fuel oil on the shoreline (Maslo et al., 2014; Shaban et al., 2007). The site is also prone to regular oil contamination from daily powerplant activities such as shipping and loading/unloading of fuel oil. Sediment and seawater samples were collected from 5 points within 20 meters from each other, about 200 meters off the shore. Details about the sampling location and the sampling points are presented in APPENDIX A (Figure S1). Grab sediment samples were collected at a depth of around 5-6 meters below the

water surface and 30 cm below the sediment/water interface to ensure collection of anaerobic microbial communities. Five seawater samples were then collected from 1 meter above the sediment/water interface from the respective sediment collection points. Samples were transported in closed containers and stored at 4°C until further use.

Sediment samples were passed through a 2 mm sieve to remove any coarse materials that might interfere with the sediment analysis and the experimental setup. Sediments were then extracted with 100 mL of deionized water and analyzed for their physicochemical characteristics using standard methods (APHA, 2012). Sediments exhibited a pH of 8.23 ± 0.26 , sulfates 721 ± 83 mg/kg of dry sediment, nitrates 12.22 ± 2.68 mg/kg dry, total nitrogen 21.5 ± 1.23 mg/kg dry, total iron 0.168 ± 0.009 mg/kg dry, Fe^{3+} 0.107 ± 0.009 mg/kg dry, organic content $1.08\% \pm 0.2$, wet density 2.040 ± 0.007 g/mL, dry density 1.693 ± 0.004 g/mL, total phosphorous 0.32 ± 0.02 mg/kg dry, phosphates 0.41 ± 0.03 mg/kg dry. Seawater exhibited a pH of 8.18 ± 0.11 , sulfates 2980 ± 228 mg/L, sulfides 0.003 ± 0.001 mg/L, nitrates 0.66 ± 0.2 mg/L, total nitrogen 0.79 ± 0.02 mg/L, total phosphorous 0.02 ± 0.01 mg/L, and phosphates below minimum detection limit.

2.2. Experimental setup

2.2.1. SMFCs design

SMFCs were based on a cylindrical design to enhance the homogeneity of the system. Plexiglass reactors with a relatively large volume of 4.4 L (height=25cm; diameter=15 cm) were used. Detailed design of the reactors is presented in the Figure 1. The reactors were filled with a sediment column of 9 cm depth. Seawater was added to form a 12 cm layer above the sediments. The electrodes were made of cylindrical carbon fiber brushes twisted around titanium wiring (Mill-Rose Company; Ohio, USA). Anodes

(7 cm × 13 cm LxD) were placed vertically in the middle of the sediment column, while cathodes (13 cm × 9 cm LxD) were suspended horizontally in the middle of the water layer such that the electrodes were perpendicular to each other and 10.5 cm apart (center to center). The vertical placement of the anode in the middle of the sediment compartment permitted an equidistant distribution of the anode carbon fibers from the SMFC wall. There is no such essential requirement in the cathode compartment, and thus, the horizontal placement of the cathode in the seawater compartment was for ease of manipulation. An external load of 10 Ω (resistor) was provided in the closed circuit SMFCs.

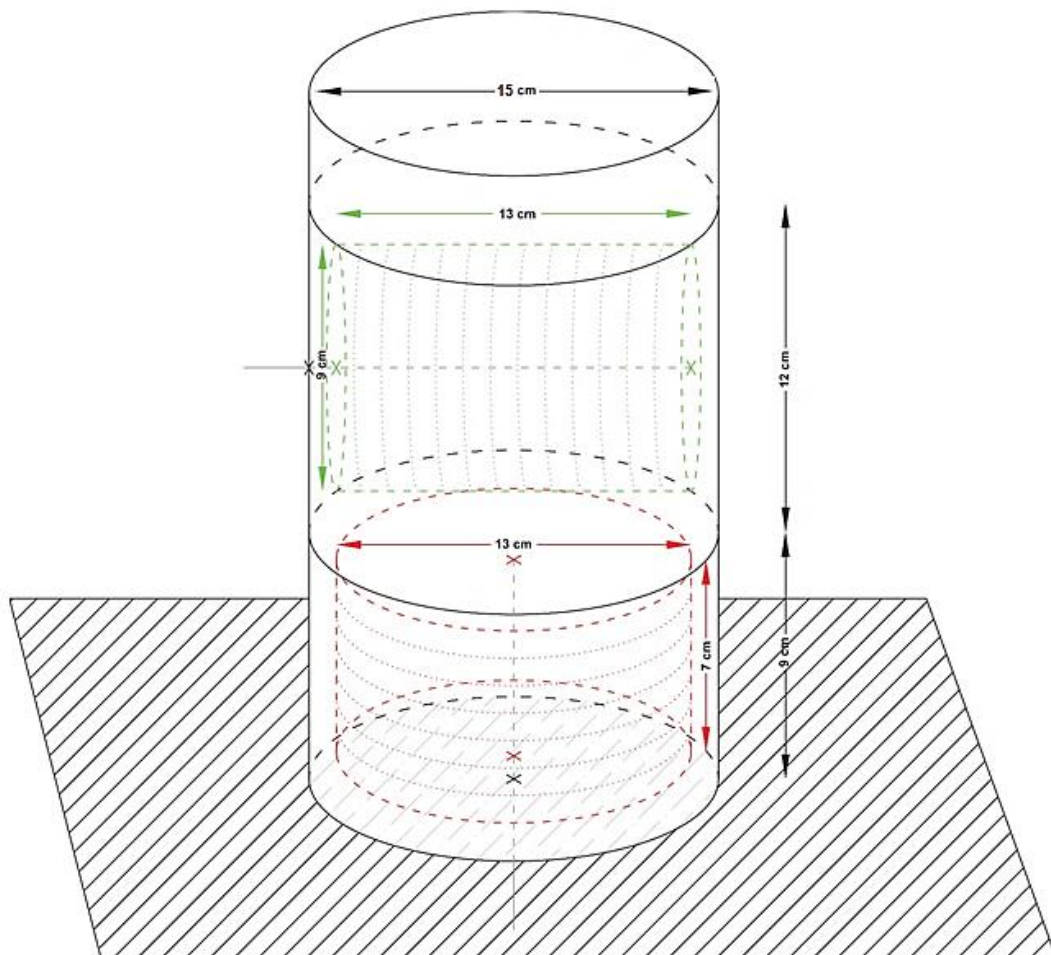


Figure 1. Schematic of the SMFC design

2.2.2. SMFCs operation:

A mixture of 2-ringed naphthalene, 3-ringed-fluorene, 4-ringed pyrene and 5-ringed BAP, listed by the EPA as priority pollutants, was prepared in dichloromethane and spiked into the collected marine sediments to achieve a concentration of 20 mg/kg of dry sediment of each of the studied PAHs, except for naphthalene, for which a higher concentration of 100 mg/kg dry sediment was initially targeted to compensate for expected losses during the spiking process due to its high volatility. This is representative of a high level of contamination in marine sediments (Botsou and Hatzianestis, 2012; Linden and Rust, 2008). Wet sediments were used during the spiking process by mechanically mixing the sediments at low speed for 45 minutes while slowly adding the PAHs solution to achieve a homogeneous distribution of the PAHs. The spiked sediments were then distributed into the different SMFCs to achieve a depth of 9 cm in each reactor. The SMFCs were then left to settle for few hours, after which the water layer settling on top of the sediments was discarded. After that, the cathode compartment was filled with seawater, and the SMFCs were left to equilibrate for one day before closing the circuit in the corresponding closed circuit SMFCs.

SMFCs were operated either under anode reducing condition (closed circuit) or under open circuit condition. Two main treatments were tested under each operating condition. The first treatment considered the conventional operation of the SMFCs under naturally occurring marine sediment chemical properties, while the second treatment considered the addition of ferric iron to assess potential enhancement of the SMFCs performance in the PAHs biodegradation process.

Iron amended SMFCs employed both soluble ferric iron (in the form of ammonium ferric citrate, $(\text{NH}_4)_5[\text{Fe}(\text{C}_6\text{H}_4\text{O}_7)_2]$) as well as amorphous ferric iron (in the form of amorphous ferric hydroxide, $\text{FeO}(\text{OH})$) to enhance the pore water ferric

availability in the sediments for FeRB enrichment. Soluble ammonium ferric citrate provided an easily accessible short-term source of iron in the pore water, compared to the amorphous ferric hydroxide which constituted a long-lasting source of ferric iron supply during the operation of the SMFCs. In a previous study utilizing freshwater sediments, Yan et al. (2012) amended SMFCs with 10g of amorphous ferric hydroxide per Kg sediments. In this study, a total concentration of 15 g/Kg of dry sediments of ferric iron was used, which were added as 10 g of amorphous ferric iron per Kg of dry sediment and an additional 5 g of soluble ferric iron per kg of dry sediment. Such a high amount of added ferric iron compared to literature was intended at significantly affecting the evolution of the microbial community towards FeRB, especially given their extremely low abundance in marine sediments. The combination of ferric iron addition and the use of anode as a TEA aimed at significantly shifting the microbial population on the anode toward FeRB, thus shifting the overall contaminant oxidation towards the thermodynamically faster and more efficient iron/anode reduction pathway.

Furthermore, control SMFCs with inhibited SRB activity were prepared to assess the role of FeRB populations in SMFCs in the absence of sulfate reduction. SRB inhibition was performed using a molybdate/sulfate molar ratio of 3 for complete inhibition of sulfate reduction (de Jesus et al., 2015; Predicala et al., 2008).

In total, 36 SMFCs were operated as quadruplicates representing 9 operating conditions. Five sets of SMFCs were operated under the anode reducing/closed circuit condition, while three sets were operated under the open circuit condition. A final set was operated under natural attenuation condition with no electrodes embedded in the reactors. Table 1 presents a detailed description of the different treatments and their respective controls, along with the rationale behind the selected operating conditions.

Table 1. Experimental setup

Operating condition	Closed/ Open circuit	Iron enrichment	SRB inhibition	Abbreviation	Description	Significance
1	Closed	-	-	CC SMFCs	SMFCs operated under closed circuit condition.	To evaluate the potential of SMFCs in enhancing PAHs biodegradation. This is expected to occur due to the activity of both SRB and exoelectrogenic bacteria associated with the anode.
2	Open	-	-	OC control SMFCs	Control SMFCs operated under open circuit conditions.	To assess the degradation of PAHs associated with the activity of indigenous SRB in the absence of the anode as a potential TEA. In this case the anode plays the role of a support medium allowing for assessment of the presence of the brush itself on the sediment biodegradation capacity
3	Closed	-	-	Abiotic control SMFCs	Autoclaved control SMFCs. Sediments were autoclaved twice for 30 minutes at 120°C and amended with sodium azide to kill all existing bacterial species. Closed circuit condition was then applied.	To monitor potential non-biological processes involved in the removal of PAHs from the sediments. These might include volatilization, photolysis, and chemical oxidation.
4	Closed	-	+	CC Mo SMFCs	SMFCs operated under closed circuit condition with molybdate addition to inhibit sulfate-reducing bacteria (SRB)	To assess the efficiency of the anode as the main terminal electron acceptor in the biodegradation of PAHs.
5	Closed	+	-	CC Fe SMFCs	SMFCs operated under closed circuit condition with iron enrichment	To assess the effect of iron addition in SMFCs performance. It was anticipated that iron addition will lead to a better utilization of the anode as a TEA. PAHs removal will result from the activity of competing SRB and FeRB.
6	Open	+	-	OC Fe SMFCs	Control SMFCs operated under open circuit conditions with iron enrichment.	To assess the effect of iron addition on the degradation of PAHs in an SRB dominated environment and in the absence of the anode as a potential TEA
7	Closed	+	+	CC Mo Fe SMFCs	SMFCs operated under closed circuit condition with molybdate addition to inhibit sulfate-reducing bacteria (SRB) and iron addition.	To assess the effect of iron addition on SMFCs performance in the absence of SRB. This allows to evaluate the extent of the involvement of FeRB and anode utilization as a TEA in PAHs degradation.
8	Open	+	+	OC Mo Fe SMFCs	Control SMFCs operated under open circuit conditions with iron enrichment and SRB inhibition.	To assess the biodegradation of high MW PAHs under the sole action of FeRB.
9	No electrodes reactors	-	-	Natural attenuation control; NA control SMFCs	Control reactors operated with no brushes.	To assess the sediments bioremediation potential of PAHs under natural conditions

2.2.3. SMFCs monitoring

PAHs biodegradation experiments using SMFCs were carried out over a period of 26 weeks with periodic sampling. Continuous monitoring of SMFCs voltages was performed by recording the voltage output across the electrodes once every 15 minutes using a data acquisition system (2700; Keithley Instruments Inc., United States) and the data were averaged daily.

Five SMFCs sampling events were performed at weeks 0, 1, 7, 18 and 26. During the first sampling event, a sediment sample from each operating condition was used for extraction and quantification of the starting PAHs concentrations. At each sampling event

thereafter, one of the quadruplicates of each of the operating conditions was sacrificed to characterize for the required parameters. Sacrificing the reactors required discarding the seawater column as well as the top sediment layer in contact with oxygen (top 1 cm). The remaining sediments were then disassembled from the anode brush, homogenized, and analyzed for PAHs, sulfates and iron. Sediments and anode microbial communities were also determined.

Soxhlet extraction was used to extract PAHs from the sediments. For each extraction, 100 g of wet sediments were mixed with anhydrous sodium sulfate and then placed in glass fiber thimbles. The mixture was then extracted over 18 hours using 250 mL of dichloromethane. Additionally, anodes used in the SMFCs were also extracted for PAHs determination using the same method and by placing the whole carbon brush in the Soxhlet apparatus with a glass wool plug at the bottom of the Soxhlet extraction compartment. The extracts were then collected and concentrated to a final volume of 25 mL using a rotary evaporator (Buchi R-205 Rotavapor System). PAHs were then determined following the method described by Yan et al. (2012) using High Performance Liquid Chromatography (HPLC) equipped with a Diode Array Detector DAD (Agilent 1100). Chromatographic analysis was performed using a C18 column (DISCOVERY HS C18, 25cm, 4.6 mm, 5 μ m). An isocratic elution was carried out using 90% methanol and 10% deionized water at a flow rate of 1 mL/min. The column temperature was maintained at 30°C. Naphthalene was best detected at 220 nm, fluorene at 264 nm, pyrene at 238 nm and BAP at 295 nm.

DNA was extracted from the sediments and the anodes of each SMFCs as triplicate samples, using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories) according to the manufacturer's instructions. Anode DNA was extracted from the carbon fibers after being randomly cut from each of the anodes of the different SMFCs prior to

the Soxhlet extraction. The triplicate DNA extracts were then combined and further processed for characterization using 16S rRNA gene pyrosequencing at MRDNA (MR DNA, Shallowater, TX).

3. Results and discussion

3.1. PAHs biodegradation

The starting concentrations of the PAHs varied compared to the spiked concentrations due to various losses before initializing the reactors. One major factor was the volatility of naphthalene bringing it down to an initial concentration of 18.87 ± 5.37 mg/kg of dry sediment at day 0 of the experiments (the spiked concentration was equal to 100 mg/Kg). Another factor affecting the initial PAHs concentrations was the discarding of the water settling on top of the sediments when setting the reactors up before filling the cathode compartment with seawater. The initial concentrations of fluorene, pyrene and BAP were 16.97 ± 0.99 , 18.95 ± 1.02 and 16.767 ± 1.16 mg/kg of dry sediment, respectively. Table 2 shows detailed starting concentrations of the used PAHs among the operating conditions. Figure 2 shows the PAHs degradation curves from the sediments during the 26 weeks of operation, and Figure 3 show the removal efficiency of the PAHs at the end of operation of the experiments. It is important to note that the spiked sediments were prepared as batches, with each batch corresponding to a single operating condition. The consistency maintained during the spiking process allowed for highly similar starting concentrations of the studied PAHs, except for naphthalene due to its highly volatile nature.

Table 2. PAHs starting concentrations

Operating condition	PAHs starting concentrations (mg/Kg of dry sediment)			
	Naphthalene	Fluorene	Pyrene	BAP
Abiotic control SMFCs	11.92	16.72	20.05	17.94
Natural Attenuation control SMFCs	16.74	15.96	17.72	15.21
CC SMFCs	14.35	17.70	19.98	17.70
OC control SMFCs	13.51	15.19	17.18	14.85
CC Mo SMFCs	17.78	16.40	18.17	16.82
CC Mo Fe SMFCs	23.44	17.10	18.97	16.19
OC Mo Fe SMFCs	24.72	17.84	19.36	17.43
CC Fe SMFCs	19.18	17.65	19.45	17.39
OC Fe SMFCs	27.41	18.20	19.64	17.33
<i>Average</i>	<i>18.78±5.37</i>	<i>16.97±0.99</i>	<i>18.95±1.02</i>	<i>16.76±1.16</i>

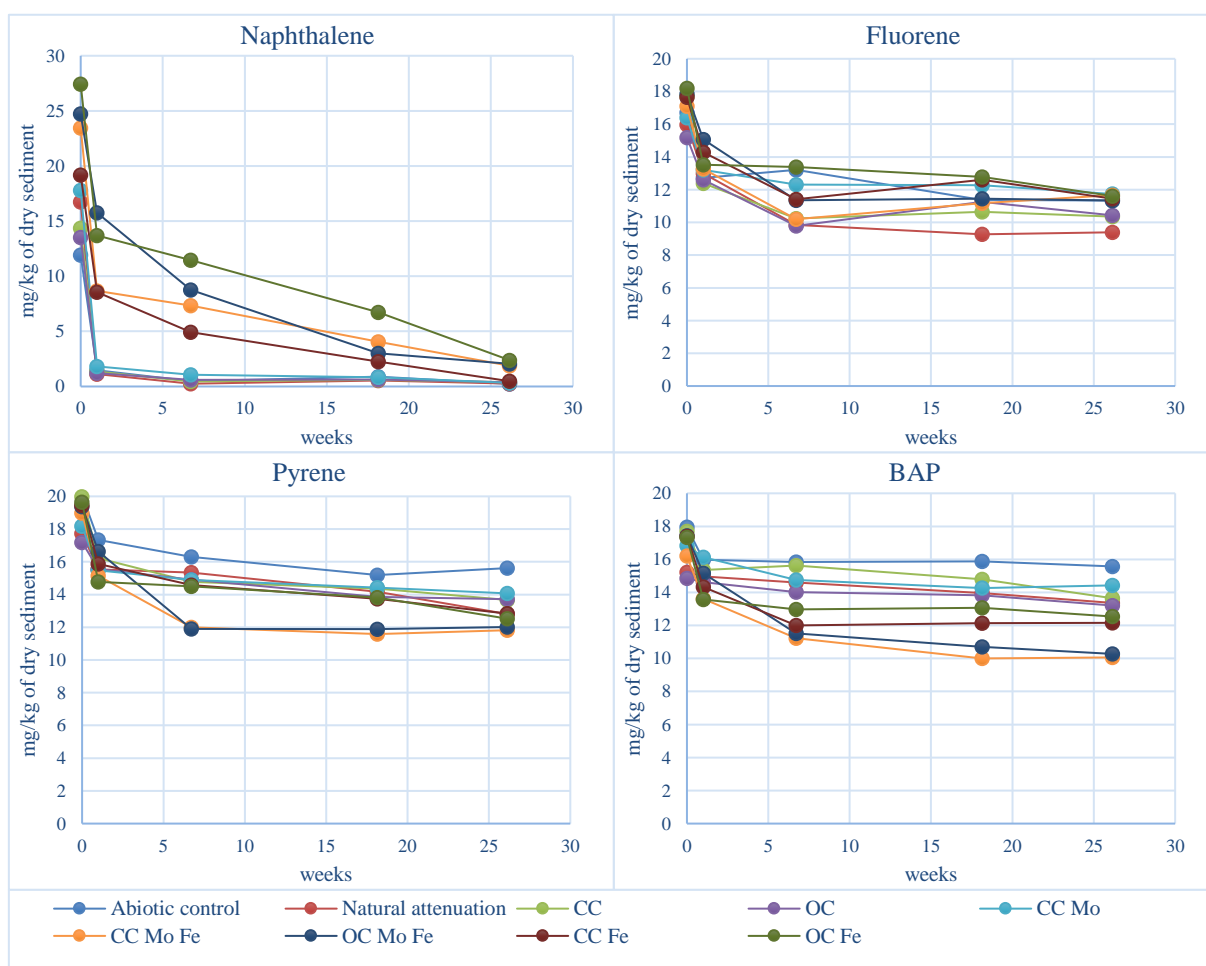


Figure 2. Sediment PAHs degradation

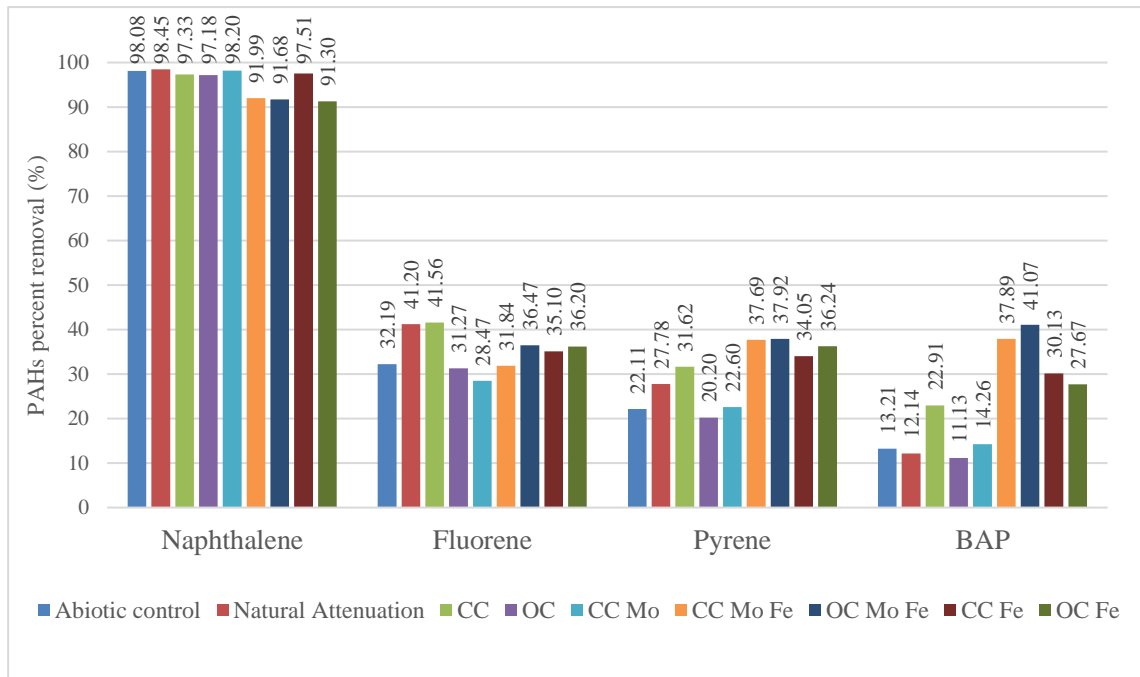


Figure 3. PAHs removal efficiency after 26 weeks of operation

More than 50% of naphthalene was lost during the first week of operation, after which it gradually disappeared to reach very low levels in all SMFCs after 26 weeks. SMFCs with the highest removal percent of naphthalene were CC Mo, NA SMFCs and abiotic control SMFCs at 98.20% (0.3 mg/kg of dry sediment remaining), 98.45% (0.2 mg/kg of dry sediment remaining) and 98.08% (0.2 mg/kg of dry sediment remaining), respectively, while all other SMFCs reached less than 2.3 mg/kg of dry sediment with more than 91% removal in all of them. This indicates that there was little contribution of actual biodegradation corresponding to the attenuation of naphthalene from the sediments. It is important to note that the variation in the concentrations of naphthalene at the end of the experiments is driven by the differences in the starting concentration of this highly volatile PAH. Although strict measures were taken to ensure that identical manipulation was performed during the spiking process, the unstable nature of naphthalene resulted in the starting concentrations to range between 11.92 mg/kg of dry sediment in the abiotic control SMFCs and 27.4 mg/kg of dry sediment in the OC Fe

SMFCs. This highlights the lower final concentrations observed in the abiotic control SMFCs in the case of naphthalene; however, the removal trend of naphthalene in all SMFCs was converging with time, showing that irrespective of the extent of involvement of biodegradation of naphthalene, the rate of volatilization dominates.

PAHs removal in this study was assumed to follow a first order decay. The degradation of PAHs was described by the first order kinetics equation $C=C_0e^{-kt}$ (Li et al., 2010) and are presented in table 3. C_0 represents the original PAHs concentration (time zero), C represents the PAHs concentration at time t , and k represents the first order decay constant. Naphthalene, although highly volatile in nature, was also assumed to follow the same process and its decay constants were also reported, albeit the correlation coefficients were very low in this case. Naphthalene volatility thus reflects on the large variability in the removal constants, which are all much higher than in the case of other PAHs and range from $5.1 \times 10^{-3} \text{ d}^{-1}$ in the CC SMFCs to $15.4 \times 10^{-3} \text{ d}^{-1}$ in the CC Fe SMFCs.

Table 3. PAHs decay constants

	naphthalene		fluorene		Pyrene		BAP	
	$k (\times 10^{-3} \text{ d}^{-1})$	r^2	$k (\times 10^{-3} \text{ d}^{-1})$	r^2	$k (\times 10^{-3} \text{ d}^{-1})$	r^2	$k (\times 10^{-3} \text{ d}^{-1})$	r^2
Abiotic Control	7.50	0.54	0.87	0.77	0.62	0.72	0.12	0.73
Natural attenuation control	5.28	0.35	1.59	0.62	1.09	0.94	0.63	0.99
CC SMFCs	5.10	0.51	1.06	0.97	0.85	0.87	0.69	0.82
OC SMFCs	5.29	0.78	1.04	0.94	0.74	0.94	0.50	0.91
CC Mo SMFCs	8.76	0.91	0.56	0.81	0.51	0.96	0.58	0.66
CC Mo Fe SMFCs	8.50	0.96	1.34	0.88	1.18	0.55	1.62	0.79
OC Mo Fe SMFCs	11.70	0.98	1.25	0.50	1.45	0.47	1.93	0.76
CC Fe	15.40	0.94	1.22	0.98	1.11	0.96	1.00	0.90
OC Fe	9.51	0.91	0.83	0.89	0.90	0.91	0.35	0.73

k , first order decay constant of PAH degradation; r^2 , correlation coefficient.

Abiotic removal of fluorene was significant as observed in the abiotic control SMFCs, with 32.19% of the original concentration being lost after 26 weeks of operation, reaching 11.3 mg/kg of dry sediment. Given the semi-volatile nature of 3-ringed PAHs, the removal of fluorene is highly driven by volatility, with no apparent trend affecting the

decay constants of this PAH. Highest removal was observed in the CC SMFCs (41.56% removal; reaching 10.35 mg/kg of dry sediment) and the natural attenuation control (41.20% removal; reaching 9.38 mg/kg of dry sediment). OC Mo Fe SMFCs showed a high removal, reaching 11.33 mg/kg of dry sediment at the end of incubation (36.47% removal). The removal of fluorene in the remaining conditions ranged between 28.47% for CC Mo SMFCs and 36.20% for CC Fe SMFCs, further indicating a significant involvement of abiotic losses in the case of this compound.

Abiotic losses were reached 22.11% in the case of pyrene and 13.21 in the case of BAP, with 15.61 mg/kg of dry sediment and 15.57 mg/kg of dry sediment remaining at the end of the experiment, respectively. Pyrene has been previously reported to show some volatility properties under wet settings (TOXNET, 2018). For both PAHs, the highest removal was observed in the OC Mo Fe SMFCs, reaching 37.92% and 41.07% removal, with final concentrations of 12.02 mg/kg of dry sediment and 10.27 mg/kg of dry sediment for pyrene and BAP, respectively. Relative significant removals of pyrene and BAP were also observed in the case of the CC Mo Fe and measured 37.69% and 37.89%, reaching final concentrations of 11.82 and 10.06 mg/kg of dry sediment, respectively, compared to the controls. A removal of 34.05% for pyrene and 30.13% for BAP were reported in the CC Fe SMFCs by the end of the incubation period, with final concentrations of 12.83 and 12.15 mg/kg of dry sediment, respectively, while 36.24% and 27.67% removals were measured in the OC Fe SMFCs, with final concentrations of 12.52 and 12.53 mg/kg of dry sediment, respectively. These results suggest that the major contributor to the enhanced biodegradation of the high MW PAHs is the presence of ferric iron, irrespective of the operation under anode reducing condition (i.e. closed circuit condition). The data also suggests that SRB inhibition combined with Fe addition further enhanced the removal of the high MW PAHs.

It is well known that PAHs can be readily biodegraded, especially low MW ones (2-ringed and 3-ringed PAHs), although sometimes a high concentration of these low MW PAHs could inhibit the degradation of high molecular weight PAHs (Haritash and Kaushik, 2009). The 4-ringed pyrene was described to be microbially degraded with previous reports of enhanced biodegradation in Fe amended sediments (Haritash and Kaushik, 2009; Yan et al., 2012). Some studies had reported several microbial groups that can utilize pyrene as the sole carbon source (Hu et al., 2011; Lease et al., 2011). However, 5 and 6 ringed PAHs are very hard to degrade, and although their biodegradation is inducible under certain settings (Haritash and Kaushik, 2009), the process is usually very slow. Wischmann and Steinhart (1997) measured substantial residues (54%) of BAP after 15 weeks of induced PAHs biodegradation in artificially contaminated soil. In one study, macrophyte enhanced freshwater SMFCs were able to induce pyrene and BAP removal from sediments up to 87.18% and 76.4%, respectively, after 367 days of operation (Yan et al., 2015). Such a variation in the reported sediment/soil capabilities of degrading complex mixtures of PAHs indicate that the potential for bioremediation is most probably correlated to the specific characteristics of the sediment/soil in question, namely with respect to the presence of an abundant and highly diverse microbial structure capable of flourishing when PAHs contamination is applied.

Additionally, it is previously reported that the addition of ferric iron into hydrocarbon contaminated sediments usually enhances the attenuation rates of target pollutants. For example, Zhou et al. (2014) reported in their biodegradation experiments an enhanced SMFCs voltage outputs under ferric iron amendment conditions. They also suggested that iron enrichment in SMFCs could stimulate organic matter oxidation. Kato et al. (2010) reported that the presence of ferrous iron, which could be added externally or produced internally from reduction of ferric iron, could act merely as an electron shuttle

between the bacteria and the anode. This could explain why the iron amendment could enhance voltage outputs more significantly than enhancing the biodegradation potential of the sediments. Finally, it is important to also note that Liu et al. (2011) reported that ferric amendment could hinder anode reduction in microbial fuel cells, which is because of the tendency of the microbial populations to simply use the available ferric iron as a TEA rather than the anode itself. Such reports further stress the observation that the biodegradation of high MW PAHs in this study was more noticeable in the presence of ferric iron even in the open circuit SMFCs where anode reducing condition was not enforced.

3.2. TEA utilization (ferric iron and sulfate)

Anaerobic microbial populations play an important role in pollutants decomposition using a variety of TEAs as compared to the single TEA (oxygen) utilized by the aerobic microbes in the top layer of the sediments (Li et al., 2009). Given that PAHs tend to accumulate more in deeper sediments, the presence of several anaerobic degradation pathways and the complex interactions among various microbial groups play an important role in the recycling of PAHs in contaminated sediments (Li et al., 2009). Figure 4 shows the temporal variation in the sediment ferric iron and sulfate concentrations, presented as remaining percentage compared to their original corresponding sediment concentrations.

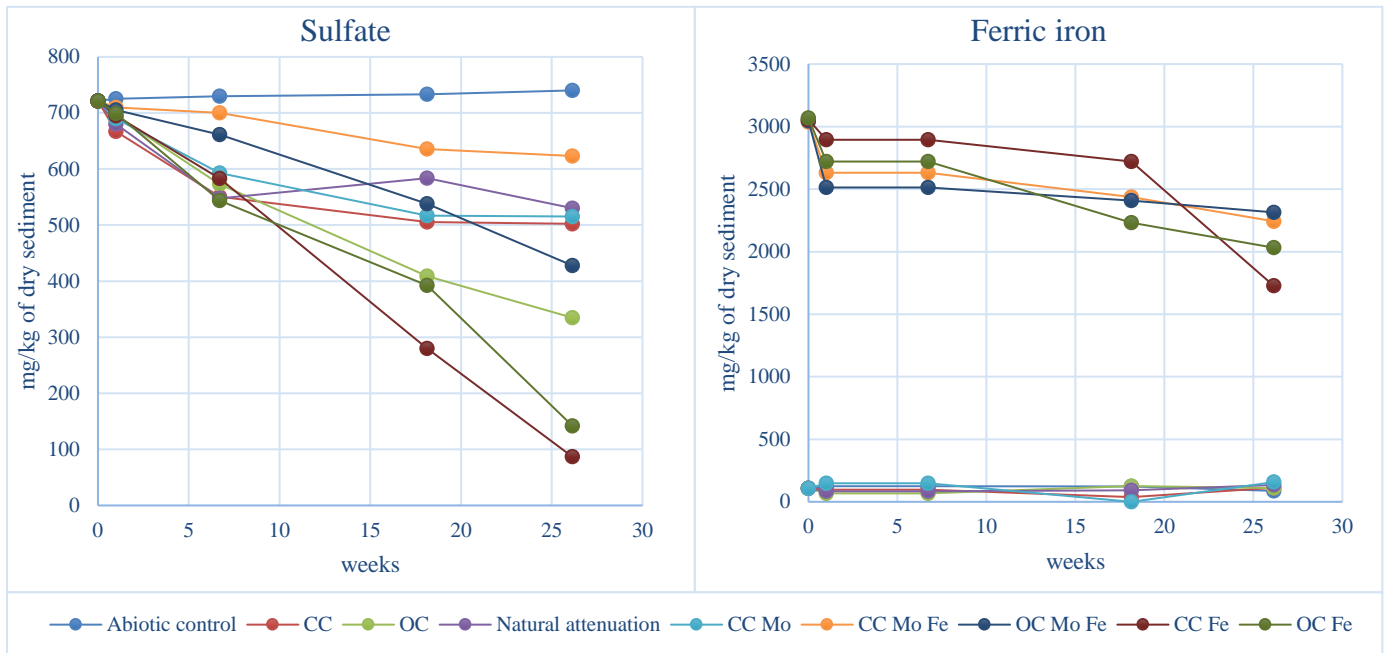


Figure 4. Sediment TEA utilization

Ferric iron consumption in Fe amended SMFCs showed a similar trend among the various conditions. Highest utilization of ferric iron was observed in the case of CC Fe SMFCs, reaching 1727 mg/kg of dry sediment at the end of the experiment (43.5% utilized). Lowest utilization was observed in SRB inhibited Fe amended SMFCs, reaching 2314 (24.01% utilization) and 2242 mg/kg of dry sediment (26.10% utilization) for CC Mo Fe SMFCs and OC Mo Fe SMFCs at the end of the experiment, respectively. Finally, OC Fe SMFCs showed moderate ferric iron utilization of 33.76%, with 2031 mg/kg of dry sediment remaining at the end of the experiment. A higher utilization of ferric iron could be mostly correlated to a higher activity of FeRB communities. The results suggest that the iron enrichment under anode reducing condition, favors the activity of FeRB in the corresponding SMFCs. SRB inhibition did not contribute to enhanced utilization of iron by FeRB in the absence of SRB competition. It is interesting to note that the overall utilization of ferric iron in the Fe-amended SMFCs was gradual over the duration of incubation of the SMFCs except for the CC Fe SMFCs, where ferric utilization was similar compared to the other SMFCs until week 18. After week 18, a dramatic increase

in the ferric utilization was observed until the end of the experiment. Under this condition (CC Fe), sulfate utilization was the highest compared the other operating conditions, reaching 86.6 mg/kg of dry sediment (88% utilization) after 26 weeks of operation, indicating the possibility that the decrease in the SRB activity, as the sulfate was consumed, has contributed to the spike in FeRB. Additionally, a rapid increase in the relative abundance of the iron reducing bacterium *Geoalkalibacter* was observed in the middle and late stages of operation of the CC Fe SMFCs (Section 3.5), which dominated the microbial community, reaching a relative abundance of 48.4% at the end of experiments compared to much lower relative abundances of other genera. This possibly contributed to the increased utilization of ferric iron during the later stages of operation.

Similar observation was noticed in the case of OC Fe SMFCs, showing slightly lower utilization of the available sulfate at 80.33% (141.8 mg/kg of dry sediment remaining at the end of the experiment), and significant ferric reduction (35.3%). This indicates that even in the presence of an active SRB population, FeRB were able to compete. This means that the presence of ferric iron itself at the used concentrations allowed FeRB to significantly compete. It was more noticeable under anode reducing condition (CC Fe SMFCs), allowing a better performance of the anode as TEA, and improved performance of the SMFCs. The lowest sulfate utilization rate was observed in SMFCs operated under SRB inhibition in the presence of iron and anode reducing conditions (CC Mo Fe SMFCs with 623 mg/kg of dry sediment remaining at the end of the experiment; 13.62% utilization). The utilization of sulfate that was observed even in the presence of molybdate as an inhibitor of SRB indicates that the effect of molybdate was not sustainable throughout the incubation period of the experiments. However, combined with the presence of the anode and the high concentration of ferric iron, SRB inhibition allowed for significant stimulation of non-SRB populations, allowing for

development of a non-SRB dominated microbial population. Significant consumption of sulfate was observed in the Mo Fe OC SMFCS (40.71%) where the highest enhancement of PAHs removal occurred, reaching a final concentration of 427.7 mg/kg of dry sediment after 26 weeks of operation. OC control SMFCs utilized 53.60% of the available sulfate over 26 weeks (334.7 mg/kg of dry sediment remaining at the end of operation), which was higher than all operating conditions except for Fe amended SMFCs (CC Fe SMFCs and OC Fe SMFCs). It was also higher than the natural attenuation control reactors which showed less sulfate utilization of 26.52% (530.1 mg/kg of dry sediment remaining at the end of operation). The high consumption of sulfates in this case was expected given that sulfate reduction is the dominant process involved in the attenuation of organic contamination from marine sediments. Additionally, compared to the natural attenuation control, the presence of the brush seems to have stimulated overall sediment biological activity by providing a high surface area for microbial biofilm attachment on the carbon fiber brush. However, Fe amendment, irrespective of enforcing the anode reducing condition, was related with higher utilization rates of sulfate, probably due to enhanced pore water availability of iron that can act as an electron mediator or shuttle for sulfate reduction within the sediment compartment of the SMFCs (H. Zhang et al., 2015; Zhou et al., 2014). Last but not least, compared to the other conditions, CC SMFCs, as well as CC Mo SMFCs, showed similar moderate consumption of sulfate, with 502.2 and 515.2 mg/kg of dry sediment remaining at the end of operation of the experiment, respectively (30.38% and 28.58% utilization, respectively), indicating that the presence of the anode itself can partially affect SRB through allowing other microbial communities to compete for electron donors, which is further enhanced by selectively inhibiting sulfate reduction (through molybdate addition) and stimulating other populations (through Fe and anode reducing conditions).

3.3. SMFCs voltage profile

SMFCs voltage profile was recorded throughout the biodegradation experiments and was used to monitor the microbial activity in the reactors. Figure 5 presents the SMFCs voltage profiles under different operating conditions.

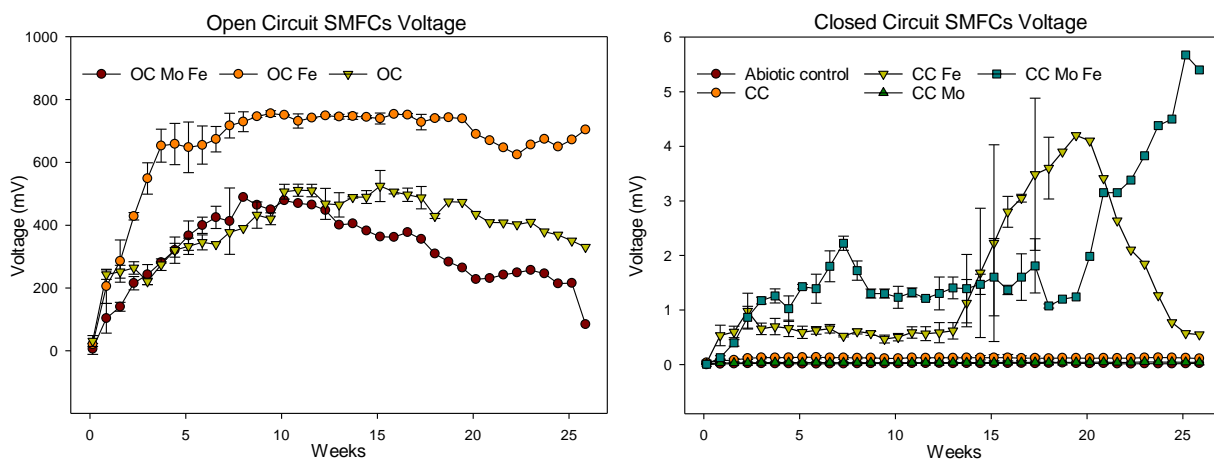


Figure 5. SMFCs voltage profile

No voltage was observed across the terminals of the abiotic control SMFCs, indicating successful inhibition of microbial activities in the reactors. All open circuit SMFCs showed a high potential difference across the electrodes. The Fe amended SMFCs (OC Fe SMFCs) showed the highest open circuit potential compared to the control open circuit SMFCs (OC control SMFCs). In both cases, the recorded voltage increased gradually from near 0 mV to reach a maximum after 5 weeks, which was around 700 mV in the Fe amended SMFCs compared to 450 mV in the case of the OC control. The voltage fluctuated around the maximum until week 18, after which the voltage dropped slightly to reach 650 mV and 320 mV at the end of the experiments for the OC Fe SMFCs and the OC control SMFCs, respectively. SRB inhibited SMFCs, which were operated under Fe amendment (OC Mo Fe SMFCs), showed a similar profile compared to the OC control, increasing gradually from zero to reach a maximum of around 450 mV at week 9 of operation; however, it significantly dropped after that compared to the control to reach

around 200 mV after 26 weeks of operation. The observed gradual increase in the SMFCs open circuit potential is mostly related to the changes to the properties of the reactors induced by the establishment of the microbial populations. The slight drop at the end of the operation of the SMFCs could be also due to additional changes in the properties of the SMFCs induced by the gradually diminishing microbial activity upon consumption of the available nutrients and substrates required for microbial metabolism.

Closed circuit SMFCs operated with no Fe amendment (both CC SMFCs and CC Mo SMFCs) showed a slight increase in the voltage from 0 to 0.04 mV and to 0.12 mV, within 2 weeks of operation, respectively, after which the voltage remained almost constant until the termination of the experiments. These observations are not consistent with the results obtained from previous experiments, where SRB inhibited SMFCs showed significantly higher voltages throughout the experiments indicating a higher microbial activity of the enriched FeRB in the absence of competing SRB (Hamdan et al., 2017). In the current study, the lower voltages recorded under SRB inhibition, are probably due to specific characteristics of the sediments used in the SMFCs preparation that could have affected the evolution pathway of the sediment microbial community, which, even under inhibition of SRB community, did not evolve towards anode reducers.

Fe amended SMFCs showed significant enhancement of the voltage output of the corresponding SMFCs compared to those operated with no ferric iron stimulation. CC Fe SMFCs showed an increase from 0 to 1 mV after 2 weeks of operation, after which it dropped slightly to fluctuate around 0.7 mV after week 3. After 13 weeks of operation, the voltage in these SMFCs started to rise again to reach a maximum of 4.2 mV at week 20, dropping afterwards to reach 0.5 mV after 26 weeks of operation. CC Mo Fe SMFCs had a similar trend compared to non-SRB inhibited iron amended C.C. SMFCs, with overall higher recorded voltages. The voltage in these SMFCs reached a peak of 2.2 mV

after 7.1 weeks of operation, after which it dropped to fluctuate around 1.2 mV from week 9 to week 18, when the voltage started to rise again to a maximum of 5.7 mV after 25 weeks of operation. The voltage started dropping after that; however, further data acquisition was not possible given that the experiments were terminated a week later. These observations indicate that the presence of Fe significantly enhanced the voltage outputs of the operated SMFCs, similar to what was reported previously by Zhou et al. (2014) in freshwater SMFCs. Additionally, the multiple peaks in the recorded voltages in these SMFCs could be due to oxidation of easily biodegradable materials followed by the gradual adaptation of the microbial community toward oxidation of other complex compounds as also reported by Zhou et al. (2014). Such activity is directly correlated to the involved microbial biofilm inhabiting the anode and the sediments in the SMFCs and its evolution throughout the biodegradation experiments. However, the enhanced voltages observed in Fe amended SMFCs compared to the controls were not translated into better removal of the contaminants. This stresses that the presence of iron enhanced the electrical properties of the SMFCs rather than their biodegradation capacity.

3.4. Microbial community analysis

3.4.1. Variation in the overall microbial community

Non-metric Multidimensional Scaling (NMDS) was used to show the similarity among the sediment and anode microbial structure of the operated SMFCs (Figure 6). Rstudio was used to generate the NMDS (beta diversity) plot using the ampvis2 package. The plot was generated using the amp_ordinate command based on Bray-Curtis distance and using the Hellinger transformation.

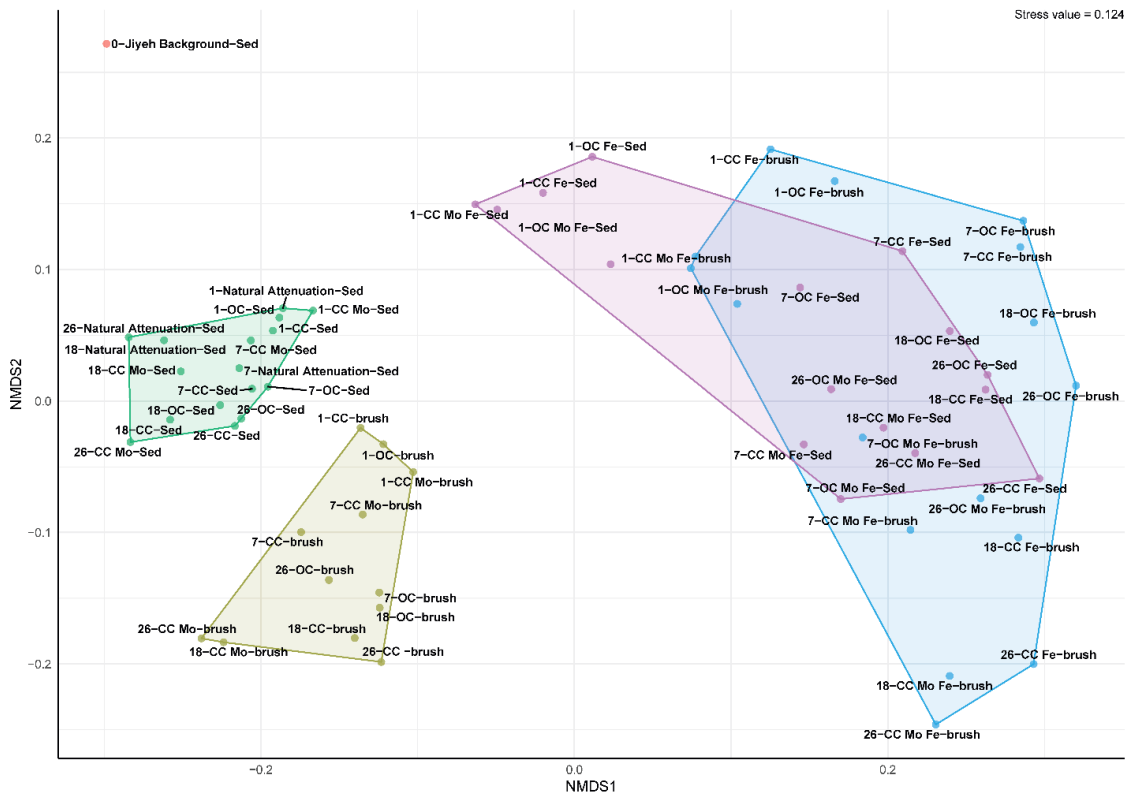


Figure 6. Nonmetric multidimensional scaling (NMDS) plot based on Bray-Curtis distance showing the relatedness of the microbial community structure. The numbers represent the time (weeks) of the experiments at which the microbial analysis was performed. Green and yellow groups represent sediment and anodic microbial communities of SMFCs operated without ferric iron amendment, respectively. Blue and pink groups represent sediment and anodic microbial communities of SMFCs operated with ferric iron amendment, respectively

NMDS revealed a clear separation between microbial communities of Fe-stimulated and non-Fe stimulated SMFCs. Microbial populations in the non-Fe stimulated SMFCs were grouped more closely together, with further clustering of the corresponding anode and sediment populations. On the other hand, Fe-stimulated microbial communities were more spaced out on the NMDS plot, with no clear distinction between anode and sediment microbial structures. These observations indicate that iron amendment had a significant effect on the observed deviation of the microbial communities in two distinct pathways as they evolved compared to the background community. The separate clustering of the anode and sediment microbial populations in non-Fe stimulated SMFCs indicates that specialization took place, with certain microbes being more anode-oriented due to the specific characteristics of the anode compared to the sediments. In Fe-

stimulated SMFCs, the anode and sediment microbial communities in the various applied conditions are overlapping on the NMDS plot, showing no significant effect of anode reducing conditions and SRB inhibition on the overall evolution of the microbial communities, which was mainly driven by the addition of Fe. This was in line with the observed similar performance of SRB-inhibited Fe-stimulated SMFCs (CC Mo Fe SMFCs and OC Mo Fe SMFCs), where no major differences in PAHs removal and ferric iron reduction rates were observed, irrespective of the involvement of the anode as a TEA. Similar performance was also noted in Fe-stimulated SMFCs operated in the absence of SRB inhibition (CC Fe SMFCs and OC Fe SMFCs), further indicating a major impact of ferric iron stimulation on the change of microbial composition of the anodes and the sediments, compared to no Fe stimulation and anode reducing condition. These Results indicate that although observable enhancement to the bioremediation potential of SMFCs was observed under Fe stimulation, the major impact of ferric iron amendment was enhanced voltage outputs, an observation that is not noted previously in marine SMFCs (Zhou et al., 2014).

3.5. Anode and sediment microbial structure and evolution

Figure 7 represents the SMFCs microbial community evolution plots throughout the experiments. Genera comprising the majority of the microbial community were presented to evaluate the microbial evolution under the different SMFCs operating conditions. The evolution plots data were extracted from the heatmaps that show the sediment and anode genera relative abundance at each sampling event. The data in the evolution plots shows the total SMFC microbial community in each operating condition by taking both the sediment as well as the anode microbial structure into consideration while generating the heatmaps from Operational Taxonomic Unit (OTU) table. Heatmaps

were generated using Rstudio through the ampvis2 package, using the amp_heatmap command. Detailed heatmaps are presented in APPENDIX B (Figure S2).

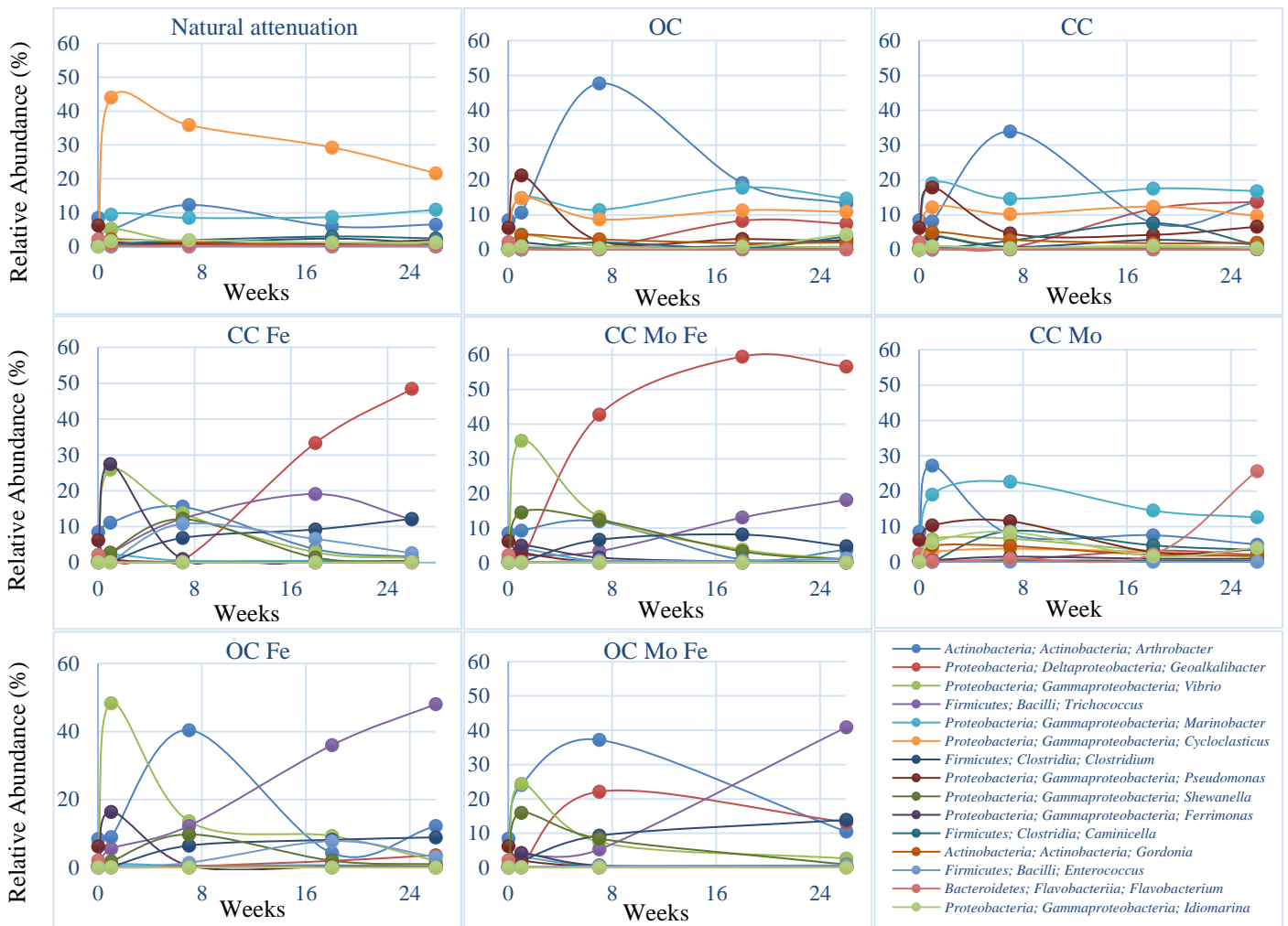


Figure 7. Microbial community evolution plots representing the variation in relative abundance (%) of the most abundant genera

The natural attenuation control SMFCs showed a rapid increase from less than 1% in the population of *Cycloclasticus*, to 44.0% during the first week of operation, after which it gradually decreased to reach 21.6% at the end of operation of the SMFCs. *Cycloclasticus* is a marine microbe specialized in the anaerobic oxidation of a range of PAHs in marine settings, including naphthalenes and pyrenes, which were used in this study (Cui et al., 2014; Staley, 2010). This explains the higher removal rates of PAHs observed in the natural attenuation control corresponding to the higher abundance of this genus, namely for fluorene. Additionally, this control showed better removal rates of

pyrene and BAP, compared to several other operating conditions which were originally proposed to enhance the natural decay rates of the introduced PAHs, such as in OC control SMFCs and CC SMFCs in the case of pyrene and BAP, and OC Fe SMFCs and CC Mo SMFCs in the case of pyrene. *Marinobacter* and *Arthrobacter* constituted the second and third most abundant microbes in the natural attenuation control, however at much lower relative abundances compared to *Cycloclasticus*, with both fluctuating around 8% during the operation of the SMFCs. *Marinobacter* is commonly and widely spread in marine environments, and is known for hosting species that are efficient degraders of aromatic and aliphatic hydrocarbons (Duran, 2010; Handley and Lloyd, 2013). Similarly, *Arthrobacter* is known to host efficient hydrocarbon degraders, of which many are able to grow using PAHs as the sole carbon source (Efroymsen and Alexander, 1991; Haleyur et al., 2018; Stevenson, 1967). The presence of *Marinobacter* and *Arthrobacter*, although at lower abundances compared to *Cycloclasticus*, further elucidates the high biodegradation performance of the natural attenuation control observed in this study.

Further inspection of the evolution of the microbial community in the applied SMFCs treatments revealed similar trends among various operating conditions. For example, both OC control SMFCs and CC SMFCs (with no iron amendment nor SRB inhibition) showed a similar profile of an increasing abundance of *Arthrobacter*, reaching a maximum after 7 weeks of operation (47.7% and 33.9%, respectively), after which it decreased to reach 10.4% and 13.7%, respectively. Other less abundant genera fluctuated in both conditions, ranging between 5 and 20%, namely, *Cycloclasticus* and *Marinobacter*. Furthermore, *Pseudomonas* in both conditions increased significantly from negligible levels to reach a maximum of 21.3% and 17.8%, respectively, after 1 week of operation, after which it dropped gradually to reach less than 2.2% and 6.5%,

respectively after 26 weeks of operation. Such a similarity between these 2 conditions explains the similar PAHs biodegradation performance of the SMFCs.

Furthermore, Fe stimulated SMFCs showed a similar microbial evolution pathway. OC Fe SMFCs and OC Mo Fe SMFCs showed continuous significant increase of *Trichococcus* reaching 48.0% and 40.8%, respectively, at the end of the experiments. *Trichococcus* has been previously reported to be involved in external electron reduction processes in fuel cell anode biofilms (Takahashi et al., 2016). However, its significant increase in the OC Fe SMFCs indicates that ferric iron stimulation as an external TEA was the main factor behind the enrichment of *Trichococcus*, rather than the use of an anode as an external inexhaustible TEA. Additionally, both conditions showed a sharp increase in the abundance of *Vibrio* during the first week of operation (up to 48.3% and 24.3 %, respectively), after which it decreased to reach less than 3% in both at the end of the experiment. Similarly, *Ferrimonas* increased in both treatments to reach a maximum after the first week of operation (16.3% and 16.9% respectively), dropping thereafter to less than 1% ultimately. *Ferrimonas* is a facultative ferric reducing bacterium previously identified in Mediterranean sediments and reported to be abundant in sediments exposed to PAHs (Rosselló-Mora et al., 1995; Zhou et al., 2009). *Ferrimonas* was also reported to be possibly related to *Shewanella* and is identified as a possible candidate for direct external electron transfer via nanowires in bioelectrochemical processes (Li and Nealson, 2015). Sequences of *Vibrio spp.* were identified previously in microbial fuels cells and were reported to be PAH-degrading marine bacteria (Gezginci and Uysal, 2015; Hedlund and Staley, 2001). The most noticeable difference between OC Fe SMFCs and OC Mo Fe SMFCs is that the exoelectrogen *Geoalkalibacter* showed a rapid increase to 22.14% after 7 weeks of operation in OC Mo Fe SMFCs, decreasing then slightly to reach 13.2% at the end of the experiments, compared to negligible levels in the OC Fe SMFCs. This

suggests that the higher PAHs removal rates observed in the case of OC Fe SMFCs are due to the evolution of the microbial community toward high abundances of PAH degraders that are also able to utilize iron as a TEA, without the need for involvement of the anode as a TEA. Additionally, the presence of *Geoalkalibacter* is probably the reason behind the significantly higher open circuit potential observed in the OC Mo Fe SMFCs.

Fe stimulated SMFCs operated under anode reducing condition, in the presence and absence of SRB inhibition (CC Fe SMFCs and CC Mo Fe SMFCs) presented similar microbial evolution profile. *Geoalkalibacter*, a known exoelectrogen, was successfully stimulated, increasing drastically from negligible levels to reach 48.4% and 56.6% in CC Fe and CC Mo Fe SMFCs at the end of the experiments, respectively. This is the highest abundance of a single genus observed, indicating successful specific stimulation of a direct anode reducer in marine SMFCs. Interestingly, *Geoalkalibacter* comprised almost all of the identified microbial community at the anode level in CC Mo Fe SMFCs (92.5%) and in CC Fe SMFCs (77.1%) at the end of the experiments (APPENDIX B; Figure S2), indicating significant involvement of the anode in driving the evolution of the total microbial community towards anode reducers in the presence of ferric iron stimulation, which was further enhanced by inhibiting SRB. *Geoalkalibacter* is known for producing high current densities through direct electron transfer in microbial fuel cells; however, it is not yet clearly correlated to degradation of aromatic hydrocarbons (Badalamenti et al., 2013). *Trichococcus* was also enriched in these two conditions, increasing gradually to 12.0% and 18.1%, respectively, at the end of the experiments. *Vibrio* was also present in both treatments, where it increased to 25.8% and 35.2%, respectively, at week 1 of operation, after which it decreased to about 1% at the end of the incubation period. In these 2 conditions, *Ferrimonas* was only noticeable in the non SRB inhibited Fe amended closed circuit SMFCs (CC Fe SMFCs), where it reached 27.4% at week 1 of operation,

then rapidly decreasing to less than 1% a week after, remaining negligible until the end of the experiments. Furthermore, *Clostridium* was significantly increased in the CC Mo Fe SMFCs, increasing gradually to reach 12.17% at the end of the experiments. *Clostridium* members were previously reported as hydrocarbon degraders including certain PAHs and for their involvement in current production in SMFCs, (Berdugo-Clavijo et al., 2012; Gieg et al., 2014; Hamdan et al., 2017; Logan, 2009). The slight difference in the microbial profile of the ferric iron stimulated closed circuit SMFCs, under both SRB inhibition and no SRB inhibition, explains the variation in their bioremediation performance and the voltage outputs. The CC Mo Fe SMFCs presented higher removal rates of PAHs compared to their non SRB inhibited counterparts (CC Fe Fe), aided by the higher abundances of potential PAHs degraders throughout the experiments. On the other hand, the better performance of the CC Mo Fe SMFCs in terms of the generated voltage is directly correlated to the higher abundance of direct anode reducers, which resulted in significantly higher sustained voltages throughout the experiments. This also explains the much higher voltages recorded in the Fe stimulated SMFCs compared to non-Fe stimulated SMFCs operated under anode reducing condition (closed circuit), in both SRB inhibition and non SRB inhibition conditions. This is due first to enhancement by the presence of iron that could act as an electron shuttle, and second by the presence of an actual significant population of FeRB capable of anode reduction.

Last but not least, CC Mo SMFCs showed a microbial evolution profile which differed from all other conditions. In this case, *Marinobacter* dominated during most of the operation of the experiments, increasing to a maximum of 22.6% after 7 weeks, then decreasing slightly to reach 12.6% at the end of the experiments. However, the first week of operation was dominated by *Arthrobacter*, reaching 27.0% after 1 week, then rapidly

decreasing to less than 8% after the 7th week of operation. *Flavobacterium* dominated the final 8 weeks of SMFCs operation, increasing from 2% to reach 25.6% at the end of the experiments. Similar to *Marinobacter* and *Arthrobacter*, *Flavobacterium* is known to be a degrader of PAHs in various environmental settings (Chaudhary and Kim, 2018; Hemalatha and VeeraManikandan, 2011). Thus, in these SMFCs, the slight enhancement of the bioremediation potential of the SMFCs, as well as the insignificant recorded voltages over the operation of the experiments, is explained by the moderate abundance of PAHs degraders combined with the lack of anode reducers. This means that in this treatment, the inhibition of SRB communities, under anode reducing condition, which was intended to stimulate anode reducers by eliminating competition, was rather hindered by the rapid multiplication of general PAH degraders in the presence of high concentrations of PAHs, which competed with potential anode reducers.

4. Conclusion

Ferric iron stimulation in marine SMFCs proved to be successful in terms of enrichment of electrochemically active microbial populations under anode reducing conditions, which was not possible under conventional SMFC setup employing only the anode as an alternative TEA. Such enrichment is heavily dependent on the sediment characteristics including the type of contamination and more importantly the original low abundance of iron reducers in marine sediments. SRB inhibition, which limited the dominance of sulfate reducers in the sediments, allowed the SMFCs microbial community to evolve towards heavy abundances of known exoelectrogens in the CC Mo Fe SMFCs, which is not previously reported in the literature, reaching 92.5% of the total anode microbial community for the deltaproteobacterial population *Geoalkalibacter*. However, the enhancement of the PAHs bioremediation potential of marine SMFCs appeared to be

better correlated to parameters that are different from simple employment of anode reducing conditions. This was most noticeable in the case of BAP, which is the heaviest and most persistent contaminant utilized in this study, where the highest removal was observed in the absence of anode as a TEA and in the presence of ferric iron stimulation when SRB communities were inhibited (41.07% removal). Although noticeable enhancement in the removal efficiency was observed in one of the closed circuit SMFC conditions (37.89% removal in the case of CC Mo Fe SMFCs), the slight enhancement in the removal is impractical given the long time needed to reach these results. Thus, the results indicate that Fe stimulation, especially when combined with the inhibition of sulfate reducing microbial populations, contribute to better overall removal of PAHs in contaminated marine sediments. The variation in the microbial community structure among the SMFCs was found to be heavily driven by the operating conditions, which is directly related to the observed variation in the performance of the SMFCs. In turn, removal of PAHs and the generated voltage were dependent on the composition of the microbial structure.

Results from this study suggest that implementation of SMFCs for the removal of persistent mixtures of PAHs could be more effective when stimulating the sediments with ferric iron rather than employment of an anode as a TEA within the sediments. Anode employment as a TEA may not be the best approach to enhance bioremediation in marine environments, especially in the presence of the well-established indigenous SRB population which is hard to compete with. An adequate method for ferric iron enrichment in open marine systems remains essential to harness its potential in enhancing removal of petroleum-related organic pollutants from sediments.

CHAPTER 3

BIOREMEDIATION OF CRUDE OIL CONTAMINATED MARINE SEDIMENTS USING SMFCs: EFFECT OF FERRIC IRON STIMULATION

Abstract

Contamination by petroleum hydrocarbons constitute a major problem in aquatic environments where large amounts of oil could be released. Even after many years of natural weathering of the spilled oil, a variety of organic contaminants can still be usually detected in high concentrations in the anaerobic sediments due to various limitations, including the stability of the organic compounds and the limited availability of oxygen and nutrients required for biodegradation. Although there is a variety of physical, chemical and biological methods used to handle an aquatic oil spill and the subsequent treatment of the resulting contaminated sediments, assessment of Sediment Microbial Fuel Cells (SMFCs) for the purpose of remediating the sediments in such cases is also important, given that the successful implementation of such a system will provide a passive method for the treatment of lingering oil with little external human intervention. It is thus important to assess the performance of SMFCs for oil hydrocarbons attenuation, rather than limiting the investigations to few target pollutants as is usually practiced. The results thus would be much representative of field conditions, demonstrating possible interactions among various contaminants that could be unnoticeable otherwise.

In this study, bioremediation of weathered crude oil in contaminated marine sediments was addressed using SMFCs. Weathered crude oil was spiked into marine sediments at a concentration of 1 g/Kg of dry sediment to simulate a heavily contaminated marine environment. Cylindrical SMFCs with a relatively large volume of 4.4 L were used for this aim. Conventional SMFCs were used with carbon fiber brushes as the anode and cathode components, which were simultaneously enhanced with ferric iron as a stimulant for electrochemically active bacteria that are an important parameter of operating a SMFC. A natural attenuation control was prepared and operated under open circuit condition with the anode and cathode placed in the bioreactors but left unconnected. A final ferric iron stimulation control was also used by operating open circuit SMFCs in the presence of ferric iron.

Limited biodegradation rates of total petroleum hydrocarbons were observed in all operating conditions, with no significant difference in the removal efficiency being observed among the different operating conditions, ranging between $16.2 \pm 1.0\%$ and $22.0 \pm 7.2\%$ in the iron stimulated SMFCs operated under open circuit and closed-circuit condition, respectively. The microbial community structure of the operated SMFCs showed similarity in the evolution pathways between the two iron stimulated SMFCs compared to the open circuit control SMFCs. All SMFCs showed high relative abundances of hydrocarbon degrading bacteria rather than anode reducing populations, such as *Marinobacter* and *Arthrobacter* in the case of the open circuit control SMFCs, and *Gordonia* in the case of iron stimulated SMFCs. This indicated that the performance of the system was highly driven by the presence of petroleum contamination rather than

the presence of the anode and ferric iron as a stimulant for enhanced biodegradation of the contamination. The results of this study indicate that using SMFCs as a bioremediation approach for petroleum-contaminated marine sediments may not be a sustainable nor a feasible approach given that the persistent pollutants will not readily degrade.

1. Introduction

Serious aquatic hydrocarbons contamination events are usually due to crude oil spills, which release large amounts of oil into the environment (Worthington et al., 2018). Even after many years of natural weathering of the spilled oil, a variety of organic contaminants can be usually detected in high concentrations in the anaerobic sediments due to various causes such as stability of the organic compounds (Ren et al., 2018; Venosa et al., 2010). This is mainly important for petroleum derived pollutants such as aliphatic and aromatic hydrocarbons that tend to be adsorbed onto sediment organic matter due to their hydrophobicity (Bach et al., 2005; Duan et al., 2018).

Natural attenuation of organic contaminants, which is mainly driven by microbial degradation in aquatic sediments, is often limited by the availability of efficient and sufficient electron accepting species required for oxidation of target contaminants (Fernando et al., 2018). Aerobic processes, which are usually the most efficient pathway, are usually limited to the top few millimeters of the sediment surface, and thus, oxidation of organic pollutants in deeper sediments, where the bulk of persistent organic pollutants occur, could be halted easily (Meysman, 2018; Morris and Jin, 2012; Nzila, 2018). Approaches that avoid the use of heavy chemical amendments for bioremediation schemes are usually preferred as ideal solutions given that they could provide a long lasting passive sinkhole for contamination on sediments (Morris and Jin, 2012). To overcome limitations in the terminal electron accepting process, certain microbial populations developed the ability to transfer the electrons generated during the oxidation process to an external terminal electron acceptor such as insoluble iron oxides, as in the case of *Geobacter* and *Shewanella* (Rojas et al., 2017). This, in addition to the natural capability of sediment microbes to degrade complex organic matter, allowed for the development of Sediment Microbial Fuel Cells (SMFCs) as a bioremediation approach

of contaminated aquatic sediments, by providing an inexhaustible insoluble sink for electrons for continuous oxidation of organic contaminants by utilizing oxygen in the overlying water as a terminal electron acceptor (TEA) (Namour and Jobin, 2018). SMFCs are bioelectrochemical systems that have been successfully used for stimulating biodegradation of various organic contaminants that are persistent in sediments of aquatic environments (Chan and Li, 2014; Hamdan et al., 2017). SMFCs employ an electrode (anode) embedded within the anaerobic sediments as a TEA (Sherafatmand and Ng, 2015; Yan et al., 2012). SMFCs then allow for the transfer of the electrons to another electrode (cathode), placed in the overlying oxygen rich water and connected to the anode, where the electrons can reduce oxygen; oxygen combining with the transferred electrons act as an ultimate TEA that is thermodynamically favorable over the use of other TEAs (Logan, 2008). Additionally, providing an unlimited source of electron acceptor presents a huge advantage in aquatic sediments to overcome the lack of sufficient naturally occurring TEAs, such as sulfate which could be rapidly exhausted in instances of significant contamination levels, hindering the natural attenuation process of petroleum hydrocarbons (Morris and Jin, 2012; Namour and Jobin, 2018).

Petroleum hydrocarbons constitute a major group of contaminants which can be biodegraded in SMFCs (Bach et al., 2005; Y. Zhang et al., 2015). Various studies reported successful employment of SMFCs for enhancing the natural degradation rates of petroleum derived pollutants using SMFCs; however, most of these studies targeted a small group of closely related contaminants and are usually more focused on freshwater SMFCs (Jobin and Namour, 2017; Kronenberg et al., 2017). Freshwater environments are known for the higher abundance of iron oxides and iron reducing microbial populations, which render freshwater SMFCs as a better candidate for evolution of the anode microbial community toward electrochemically active microbes, capable of

directly utilizing the electrode as a TEA (Hansel et al., 2015; H. Zhang et al., 2015). On the other hand, marine SMFCs performance is usually limited due to the low abundance of iron reducing microbial populations and an extremely dominant sulfate reducing bacterial community, driven by the high concentration of sulfates in the sediments and the seawater (Hansel et al., 2015). For example, Yan et al. (2012) reported high removal efficiencies of phenanthrene and pyrene from sediments in freshwater SMFCs in the presence of amorphous ferric hydroxide ($99.47 \pm 0.15\%$ and $94.79 \pm 0.63\%$, respectively). Sherafatmand and Ng (2015) reported the use of freshwater SMFCs for the removal of several low molecular weight PAHs, with removal efficiencies reaching 41.7%, 31.4% and 36.2% PAHs in aerobic SMFCs and 76.9%, 52.5% and 36.8% in anaerobic SMFCs for naphthalene, acenaphthene and phenanthrene, respectively, indicating that modifications to SMFC setups could highly affect the overall bioremediation performance of the system. Morris and Jin (2012) reported a 12 fold enhancement in the removal efficiency of total petroleum hydrocarbons (TPH) in conventional marine SMFCs compared to the control, reaching 24%. Viggi et al. (2015), measured up to 21% removal of total petroleum hydrocarbons in marine SMFCs compared to unnoticeable removal in control SMFCs. These studies; however, were more focused on the removal efficiency of the target compounds rather than the microbial community involved in their biodegradation.

Anodic microbial structure is well-studied in microbial Fuel Cells (MFCs); however, microbial evolution throughout the biodegradation process in SMFCs is still not emphasized. For example, among the aforementioned studies, only Yan et al. (2012) determined the microbial community structure under defined SMFCs conditions. Furthermore, it is established that SMFCs show significantly different overall anodic and sediment microbial profiles based on the origin of the utilized sediments. For example,

the exoelectrogenic iron reducer *Geobacter* is commonly enriched in freshwater SMFCs while *Desulfuromonas* usually dominates in marine SMFCs (Chan and Li, 2014; De Schampelaire et al., 2008; Jung et al., 2014). Given that iron reducers capable of direct anode reduction are usually the target organisms in SMFCs, iron enrichment was practiced in multiple SMFCs studies. Zhou et al. (2014), for example, reported that the amendment of sediments in freshwater SMFCs with soluble and colloidal ferric iron improved SMFCs performance, namely in terms of enhanced voltage outputs rather than sediment organic matter removal. Coates et al. (1996) showed that the addition of Fe(III) to petroleum-contaminated harbor sediments did not shift the terminal electron accepting process from sulfate reduction to Fe(III) reduction. The authors correlated this observation to the low population size of iron reducing bacteria (FeRB) in the sediments compared to sulfate reducing bacteria (SRB). On the other hand, Yan et al. (2012) provided evidence that ferric iron addition stimulated removal of target PAHs in freshwater SMFCs.

Previous marine SMFC studies often targeted bioremediation of few selected petroleum hydrocarbons rather than a simulation of an actual environmental contamination with high crude oil concentrations. This study provides a reference for future studies to address iron amendment in marine SMFCs, as such assessments were not noted in previous literature. In this study, ferric iron amended marine SMFCs were tested for enhancement of biodegradation potential of Total Petroleum Hydrocarbons (TPH) through stimulation of FeRB. Weathered light Arabian crude oil was used to spike marine sediments to simulate a heavily contaminated marine environment. This is proposed to shift the microbial metabolism towards having a higher abundance of FeRB, including exoelectrogens, in the presence of SRB competition, which is usually one of the major limitations when operating marine SMFCs. For this purpose, crude oil

contaminated marine sediments were amended with amorphous ferric hydroxide and employed in closed circuit SMFCs. Control SMFCs were operated as open circuit reactors with and without iron amendment to assess the effect of iron addition on TPH removal in the absence of anode reduction, and to assess the extent of TPH removal under natural attenuation conditions, respectively. Microbial community evolution was monitored in the three different treatments throughout the SMFCs operation.

2. Materials and methods:

2.1. Sediments and seawater

Sediment and seawater used in this study were collected from the shoreline of Jiyeh in Lebanon, previously affected by the 2006 petroleum oil spill from the Jiyeh power plant, which released more than 15000 tons of heavy fuel oil along the shoreline (Maslo et al., 2014; Shaban et al., 2007). Additionally, the daily activities related to the operation of the powerplant further contribute to the pollution of this area with petroleum hydrocarbons. Detailed description of the sampling site is presented in APPENDIX A (Figure S1). Grab sampling was used to collect anaerobic sediments at a depth of 5-6 meters below the water surface, and at a minimal depth of 30 cm below the sediment/water interface to ensure collection of anaerobic microbial communities. Seawater was collected at 1 meter above the sediment-water interface from above each sediment collection point. Collection containers were sealed under water directly after collection, and the collected samples were then promptly transported for storage at 4°C until further processing was conducted.

Collected sediments were sieved using a 2 mm sieve to exclude unwanted coarse materials. Deionized water was used to extract the sediments for their physico-chemical

characterization using standard methods (APHA, 2012). Sediments exhibited a pH of 7.3 ± 0.6 , organic content $1.059 \pm 0.001\%$ dry weight, wet density 1.84 ± 0.03 g/mL, dry density 1.57 ± 0.01 g/mL, sulfates (SO_4^{2-}) 665 ± 17 mg/kg of dry sediment, nitrates (NO_3^-) 16.20 ± 0.15 mg/kg of dry sediment, total nitrogen (N) 26.45 ± 0.92 mg/kg of dry sediment, iron (Fe) 0.10 ± 0.01 mg/kg of dry sediment, ferric iron (Fe^{3+}) 0.08 ± 0.02 mg/kg of dry sediment, total phosphorous (P) 24.61 ± 1.35 mg/kg of dry sediment, phosphates (PO_4^{3-}) 15.85 ± 4.41 mg/kg of dry sediment. Collected seawater samples were also analyzed for the physico-chemical parameters. Seawater exhibited a pH of 7.87 ± 0.1 , sulfates 1130 ± 70 mg/L, sulfides 0.002 ± 0.001 mg/L, nitrates 4 ± 1.4 mg/L, nitrites 0.51 ± 0.01 mg/L, total nitrogen 4.3 ± 0.4 mg/kg, phosphates 1.21 ± 0.28 mg/L, total phosphorous below minimum detection limit.

2.2. Experimental setup:

2.2.1. SMFCs design and operation:

A cylindrical design was adopted to be used for constructing the SMFCs reactors to ensure homogeneity of the reactors. Large volume Plexiglass reactors (height=25cm; diameter=15 cm) were used. Details about the design of the reactors are presented in Figure 1. Each of the SMFCs was filled with 9 cm of sediment and an overlying layer of 12 cm of seawater. The electrodes utilized in the reactors were cylindrical carbon fiber brushes combined with titanium wiring (Mill-Rose Company; Ohio, USA). The anodes (7 cm \times 13 cm L \times D) were placed vertically in the middle of the sediment half-cell, while the cathodes (13 cm \times 9 cm L \times D) were placed horizontally in the middle of the seawater half-cell. The electrodes were thus perpendicular to one another and positioned 10.5 cm apart from center to center. In each of the closed circuit SMFCs, a 10 Ω resistor was used to provide a small external load connecting the anode to the cathode.

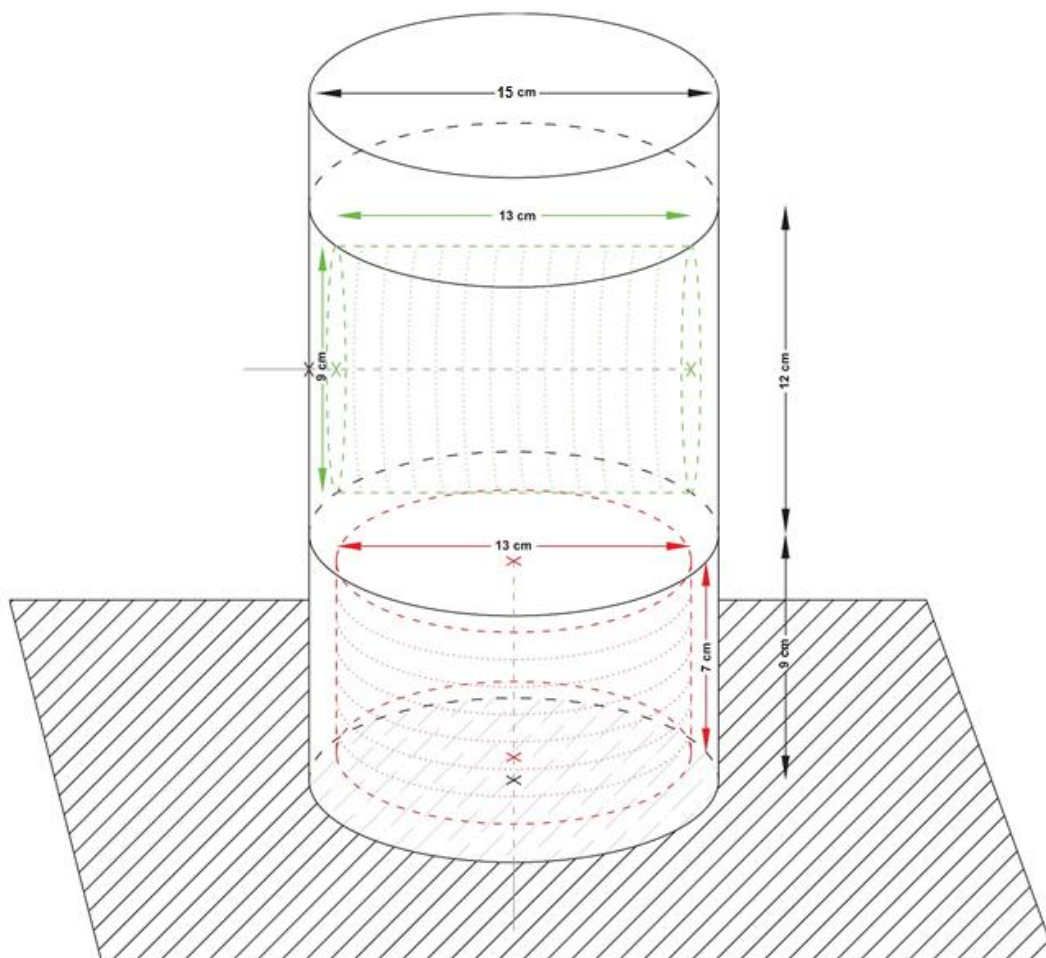


Figure 1. Schematic of the SMFC design

Three SMFCs treatments were prepared. In the first treatment (Fe C.C.), marine SMFCs were amended with ferric iron and operated under closed circuit condition to stimulate enrichment of exoelectrogenic populations for enhanced TPH removal. The two other treatments consisted of control SMFCs operated under open circuit condition. One set of controls was amended with iron (Fe O.C.) to assess the effect of iron on TPH removal in the absence of anode reduction. No iron amendment was applied to the second set of controls (O.C.) which was intended to evaluate the natural attenuation of TPHs.

Iron amended SMFCs were supplied with wet amorphous ferric hydroxide at a concentration of 20 g/kg of dry sediments. Sediments used in the SMFCs were spiked with light Arabian crude oil at 1 g/Kg of dry sediment to simulate a heavily contaminated marine environment. A single batch of sediment was used during the spiking process. To

ensure a homogeneous distribution of the crude oil, the required amount of the oil was added gradually to the sediments while being slowly tumbled inside a mechanical tumbler. The sediments were then mixed in the tumbler over a 30 minutes period to ensure complete homogenization of the oil in the sediments. Part of the crude oil spiked sediments were then used to prepare the control SMFCs with no iron amendment (O.C.). For the remaining portion of sediment which was used for iron amended SMFCs (Fe C.C. and Fe O.C.), the sediments were left in the tumbler for mixing with amorphous ferric hydroxide (FeOOH). Wet amorphous ferric hydroxide, which was prepared based on a method previously described by Lovley and Phillips (1986), was gradually added to the remaining sediments in the tumbler while mixing for homogenization. After about 15 minutes, a consistent reddish color stained the sediments, indicating complete homogenization of the amorphous ferric hydroxide within the sediment. After setting up the anode half-cells, all SMFCs were left to settle out for few hours. After that, the water layer that emerged on the top of the sediment was discarded from each SMFC before filling the cathode half-cells with seawater. SMFCs were then left to equilibrate for 1 day before connecting the anodes to the corresponding cathodes in closed circuit SMFCs.

2.2.2. SMFCs monitoring

SMFCs were run in triplicates over a period of 16 weeks (4 months), during which five sampling events were performed, corresponding to weeks 1, 2, 4, 8 and 16. At each sampling event, 3 SMFCs from each operating condition were sacrificed for chemical and microbial characterization. SMFCs were sacrificed by discarding the seawater layer as well as the top sediment layer in contact with the oxygen rich water, after which the anode half-cells were disassembled by separating the sediments from the anodes. Sediments were then homogenized before being extracted for TPH, sulfates and Ferric iron analysis.

DNA extraction from the sediments and anode brushes was also performed for microbial structure determination.

Sediment samples were extracted using an Accelerated Solvent Extractor (DIONEX ASE 350), following the method described by Richter (2000). Sediment samples were first sieved through a 2 mm sieve. Aliquots of 30 to 40 g (wet) of sieved sediment were mixed with a drying agent (Diatomaceous Earth; Thermo SCIENTIFIC) and loaded into stainless steel accelerated solvent extractor cells. Samples were then extracted with a 1:1 acetone:dichloromethane mixture at 175 °C, with 8 minutes heat-up time, 5 minutes static time, 70% flush, and 60 seconds nitrogen purge. Around 10-20 mL of dichloromethane was added to the extracts to facilitate the phase separation of water that was extracted along during the process. Extracts were then further dried using around 5 g of sodium sulfate. Dried extracts were then concentrated using a rotary evaporator and reconstituted to a final volume of 10 mL using DCM (Buchi R-205 Rotavapor System).

Sediment extracts were analyzed for alkanes and polycyclic aromatic hydrocarbons (PAHs) by GC-MS (Agilent 7890A gas chromatography system coupled to an Agilent 5975C mass spectrometer), using an internal standard method described by Campo et al. (2013). Normal and branched aliphatic alkanes including hydrocarbon chains ranging from nC10 to nC35, along with pristane and phytane, were analyzed. PAHs included 2-, 3-, and 4-ring aromatic compounds and their alkylated homologues (i.e. C0-4-naphthalenes, C0-3-fluorenes, C0-3-dibenzothiophenes, C0-4-phenanthrenes, anthracene, fluoranthene, C0-3-naphthobenzothiophenes, C0-2-pyrenes, C0-3-chrysenes).

At each of the scheduled sampling events, sediments as well as anodes were characterized for the relative abundance of the microbial communities. PowerSoil® DNA

Isolation Kit (MO BIO Laboratories) were used to extract for DNA from the sediment samples and the anode samples from each SMFC. During each sampling event, triplicate sediment sample from each of the sacrificed SMFCs as well as triplicate samples from each anode of the corresponding SMFCs were extracted. All the extracted sediment DNA from replicate SMFCs at each sampling event were combined. The same was performed for the anode DNA extracts. This totaled 9 sediment DNA extractions and 9 anode DNA extractions per sampling event per operating condition. The combined DNA extracts were then processed for pyrosequencing at MRDNA (MR DNA, Shallowater, TX). The complexity and evolution of the sediment and anode microbial structure in SMFCs is a major indicator of the actual performance of the system, directly influencing the success or the failure of the setup to achieve the intended purpose. While most of the studies report the final microbial structure at the end of the SMFCs operation, the monitoring of the temporal changes in microbial structure was performed in this study for a better understanding of the degradation profiles and of the target contaminants.

3. Results and discussion

3.1. Biodegradation of TPH

To eliminate initial differences in the oil concentration in the SMFCs which might have occurred through oil losses during the loading of the reactors and later during their sampling and extraction, the concentrations of petroleum hydrocarbons (alkanes and PAHS analytes) were normalized to hopane, which is considered to be non-biodegradable throughout the experiments duration. The starting concentration of TPH, total alkanes and total PAHs at the beginning of the experiments after equilibrating the SMFCs were

10586±447, 9211±397 and 1374±50 mg/mg hopane. Figure 2 shows the degradation profile of TPH as well as total alkanes and PAHS.

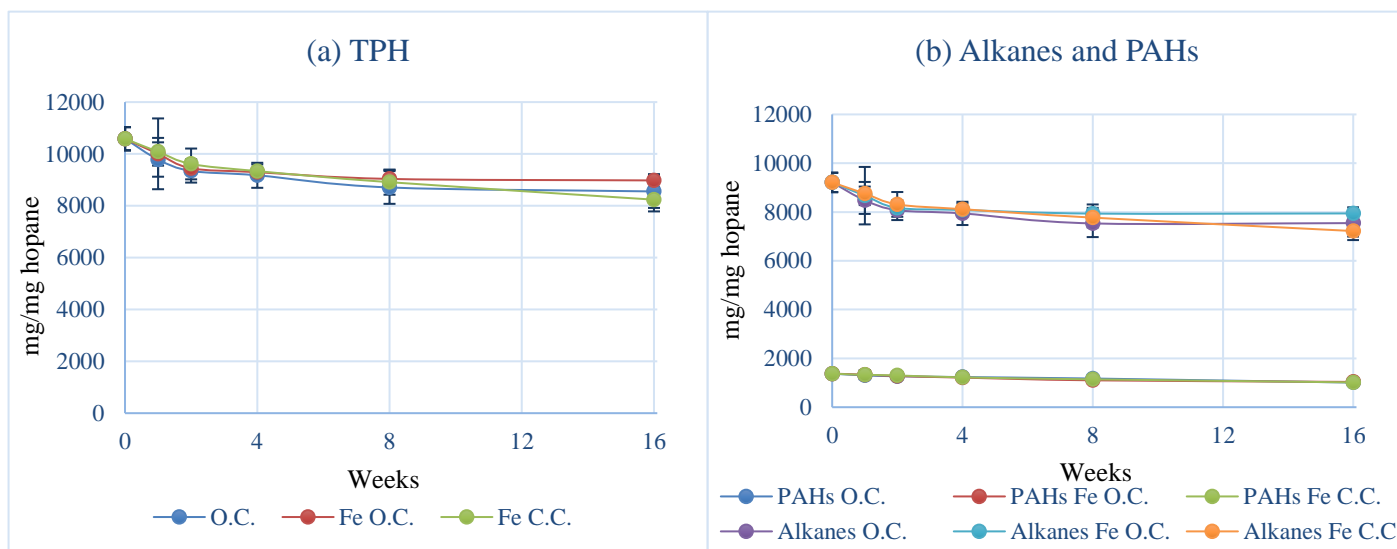


Figure 2. Concentration profile of petroleum hydrocarbons in mg/mg hopane in the SMFCs during the 16 weeks of operation. (a) TPH and (b) alkanes and PAHs

The degradation of TPH showed no significant difference in their removal throughout the operation of the experiment (Figure 2a). A relatively fast but limited drop in TPH was observed during the first 2 weeks of operation, followed by a slower removal rate thereafter until the last week of operation. TPH dropped from 10586 mg/mg hopane at the start to reach 9344±448, 9448±214 and 9614±593 mg/mg hopane after 2 weeks, dropping then to final concentrations of 8553±636, 8978±242 and 8236±453 mg/mg hopane at the end of the experiments in OC, Fe OC and Fe CC SMFCs, respectively. The similarity in the removal of TPH among applied treatments indicates that there was little impact of the ferric iron stimulation and the anode availability as a TEA on the biodegradation of TPH.

The two major constituent groups of TPH were also taken into consideration when comparing the degradation profiles of the hydrocarbons in this study (Figure 2b), which are alkanes and PAHs. As for PAHs, which composed only 12.9% of TPH, degradation

was extremely similar among the operating conditions, dropping from 1374 ± 50 mg/mg of hopane at the start of the experiment to 1036 ± 7 , 1016 ± 88 and 1016 ± 84 mg/mg of hopane in Fe O.C., Fe C.C. and O.C., respectively, which were not significantly different. Total alkanes, composing the majority of TPH (87.1%), presented a similar degradation trend to the TPH plot, with Fe C.C. dropping from 9211 ± 397 mg/mg of hopane to 7219 ± 368 mg/mg of hopane, Fe O.C. reactors reaching 7941 ± 250 mg/mg of hopane and natural attenuation control reaching 7545 ± 500 mg/mg of hopane after 16 weeks of operation.

Table 1 represents the fitted 1st order decay constants of TPH, total alkanes and total PAHs throughout the operation of the SMFCs.

Table 1. TPH, total alkanes and total PAHs decay constants

	TPH		Total alkanes		Total PAHs	
	Decay constant (d ⁻¹)	r ²	Decay constant (d ⁻¹)	r ²	Decay constant (d ⁻¹)	r ²
O.C.	0.0016	0.69	0.0014	0.60	0.0025	0.97
Fe O.C.	0.0012	0.59	0.0010	0.48	0.0025	0.91
Fe C.C.	0.0020	0.90	0.0019	0.88	0.0026	0.97

k, first order decay constant of PAH degradation; r², correlation coefficient.

The decay rates showed negligible enhancement in the biodegradation of petroleum hydrocarbons, namely alkanes, in the case of Fe C.C. treatment (0.002 d^{-1} for TPH and 0.0019 d^{-1} for alkanes) as compared to the control SMFCs (O.C. and Fe O.C. SMFCs with 0.0016 d^{-1} and 0.0012 d^{-1} for TPH, and 0.0014 d^{-1} and 0.0010 d^{-1} for alkanes, respectively). PAHs showed identical decay rates among the operated SMFCs (0.0025 d^{-1} for O.C. and Fe O.C. SMFCs and 0.0026 for Fe C.C. SMFCs). The slight observed enhancement in the TPH decay rate in the case of the Fe C.C. SMFCs however cannot be considered as an indication for successful stimulation of oil degradation, given that the degradation plots were not significantly different. The same can be said with respect to alkanes removal given that alkanes composed most of the spiked oil. The

similarity in the PAHs removal rates further stresses the little variability in the performance of the operated SMFCs.

Although some studies reported enhanced removal of certain petroleum hydrocarbons in freshwater sediments through amendment of SMFCs with ferric iron (Sherafatmand and Ng, 2015; Yan et al., 2012), the observations from this study suggest that there was no major involvement of the anode as a TEA in the oil biodegradation process. There was also no major impact of iron amendment on improvement of the petroleum biodegradation under the studied experimental conditions. Although the operated conditions can easily affect the microbial structure in the sediments, which constitutes a fundamental parameter in operating SMFCs, the similarity in the biodegradation performance also indicates possible overloading of the SMFCs biodegradation capacity with the high amount of the added oil, thus resulting in similar biodegradation performance irrespective of the applied treatment. Compared to marine sediments, high abundance of iron reducers in freshwater sediments allows for better utilization of electrochemically active microbes, which is different in the case of the sulfate rich marine sediment where sulfate reducing bacteria dominate and outcompete other existing microbial populations (Zhou et al., 2014). In both environments, however, iron could be used as a potential shuttle for electrons rather than as a stimulant for biodegradation, resulting in the redox pathways to shift only slightly. This affects the conductivity and electric properties of the sediments, resulting in enhanced voltage outputs in the case of iron stimulated SMFCs rather than consistent enhancement of the degradation rates of sediment organic material (Zhou et al., 2014). Additionally, in some studies, ferric iron amendment did not even increase the power outputs to MFCs due to the characteristics of the microbial community; bacteria could end up simply using the available ferric iron as a TEA rather than as a stimulant for biodegradation of target

contaminants or, more importantly, as a stimulant toward evolving anode reducing populations (Liu et al., 2011). Furthermore, Zhou et al. (2014) also reported that the effects of iron amendment in SMFCs are determined by the form of the ferric iron. For example, soluble and amorphous forms of ferric iron are more likely to be utilized by FeRB compared to crystalline ferric oxides, aided by the higher high surface area and the higher solubility; however, the presence of these ferric species could sometimes result in competition between iron reducing processes and anode reducing processes, resulting in lower power densities in some cases of MFC operation as reported by Liu et al. (2011).

3.2. TEA utilization

Figure 3 shows the temporal variation in the sediment sulfate and ferric iron concentrations. Significant portion of the available sulfate was consumed in all SMFCs, which presented a continuous but gradual drop in the concentration of the available sulfate over the operation duration of the experiments. However, there was no significant difference neither in the trend of the consumed sulfate nor in the concentrations of the sulfate during the operation of the different SMFCs conditions. Sulfate decreased from an original concentration of 665 ± 58 mg/kg of dry sediment in all of the SMFCs to reach a final concentration of 383 ± 38 , 410 ± 17 and 414 ± 67 mg/kg of dry sediment in O.C., Fe O.C. and Fe C.C. SMFCs, respectively, after 16 weeks of operation.

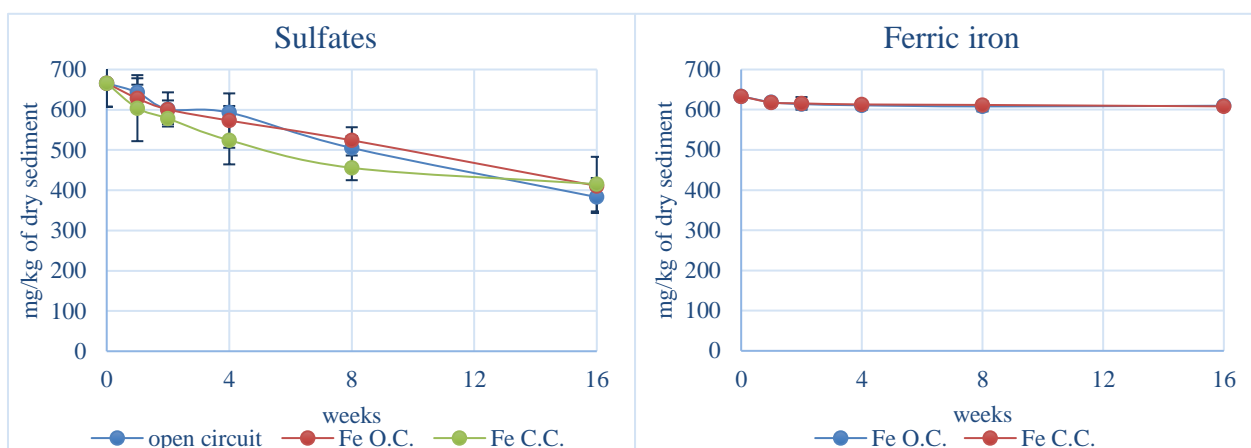


Figure 3. TEA utilization

Utilization of ferric iron was minimal in the iron amended SMFCs and showed also no significant difference in utilization of ferric iron. Both iron amended SMFCs (Fe O.C. and Fe C.C.) showed a very slight drop in the available ferric iron, which was observed mainly during the first 2 weeks of operation. Ferric iron dropped from 633 ± 1.9 mg/kg of dry sediment at the start of the experiment to 614 ± 2.9 and 615 ± 7.7 mg/kg of dry sediment after 2 weeks, leveling thereafter with minimal iron utilization until the end of the experiment after 16 weeks, when ferric iron concentration was 609 ± 1.7 and 608 ± 7.8 mg/kg of dry sediment in Fe O.C. and Fe C.C., respectively (3.9% and 3.7% utilization, respectively). The similarity in the ferric iron utilization shows that the presence of the anode reducing condition in the Fe C.C. SMFCs had minimal impact on the TEA utilization. This, in combination with the observed similarity in sulfate utilization in the natural attenuation control SMFCs, compared to both iron amended SMFCs, indicates that the overall microbial community did not significantly shift away from sulfate utilization toward anode reduction even in the presence of ferric iron. This is probably correlated to the original sediment microbial structure that is lacking iron and anode reducers, failing thus to shift the TEA utilization away from the dominant sulfate reduction pathway.

3.3. Recovered voltage

Figure 4 shows the average voltage profile generated by the SMFCs over 16 weeks of operation. Open circuit SMFCs showed a high open circuit potential, which is typical. Originally, open circuit potential was 600 mv in the O.C. control SMFCs and 400 mV in the Fe O.C SMFCs. It then increased to around 650 mV and 900 mV under these respective operating conditioning, fluctuating around these values during the first 6 weeks of operation, to converge later and stabilize at around 700 mv. The increase in the open circuit potential of in the O.C. and Fe O.C. SMFCs during the first few weeks of operation indicate an active microbial community driving such changes in the voltage across the electrodes. In the Fe C.C. treatment, an initial voltage of zero was measured upon closing the circuit. The voltage rapidly increased during the first two weeks of operation to reach a maximum of 0.05 ± 0.01 mV, dropping gradually thereafter to reach 0.011 ± 0.001 mV by the end of the experiments. Such a trend in the recorded voltage indicates that the anode was utilized as a TEA; however, taking into consideration that SRB were not outcompeted as indicated by the similar high sulfate utilization in all operating conditions, the results show that the electrochemically active microbial population contributed minimally to the biodegradation of TPH in the Fe C.C. SMFCs.

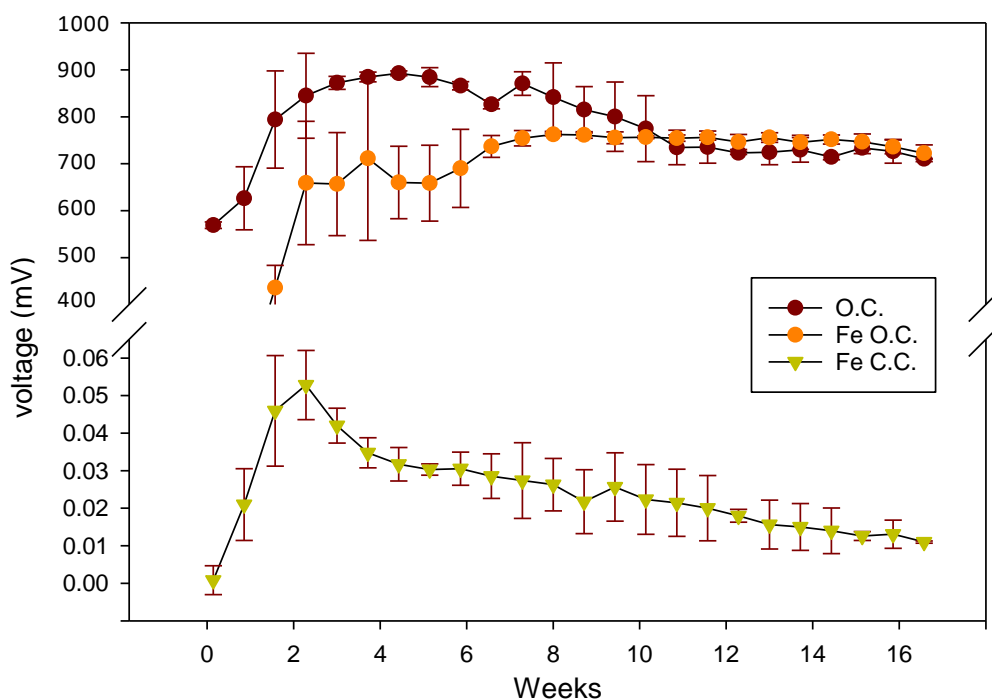


Figure 4. SMFCs voltage profile in the SMFCs over 16 weeks of operation

3.4. Microbial community analysis

3.4.1. Beta Diversity

Beta diversity of the anodic and sediment microbial biofilms were assessed through Non-metric Multidimensional Scaling (NMDS) (Figure 5). Rstudio was used to generate the NMDS plot using the ampvis2 package. The plot was generated using the amp_ordinate command based on Bray-Curtis distance and using the Hellinger transformation. The NMDS plot showed similar microbial structure among the Fe-stimulated SMFCs compared to the natural attenuation controls, with further sub-clustering of the anode and sediments microbial populations. The similarity between the microbial communities in the sediments and anodes of the O.C. and Fe C.C. SMFCs indicates that the presence of the anode did not affect the evolution of the microbial structure, with the major contributor being the presence of ferric iron in the sediments. This is further indicated by the separate clustering of the microbial population of the

natural attenuation control (O.C.) compared to the both Fe-stimulated SMFCs (Fe O.C. and Fe C.C.), which means that iron stimulation had a significant role in changing the evolution pathway of the microbial community in marine sediments in the presence of crude oil contamination. Furthermore, the sub-clustering of anode and sediment microbial communities on the NMDS plot indicates that the presence of the carbon brush itself, contributed to the specialization of the microbial structure given the specific characteristics of the carbon fiber.

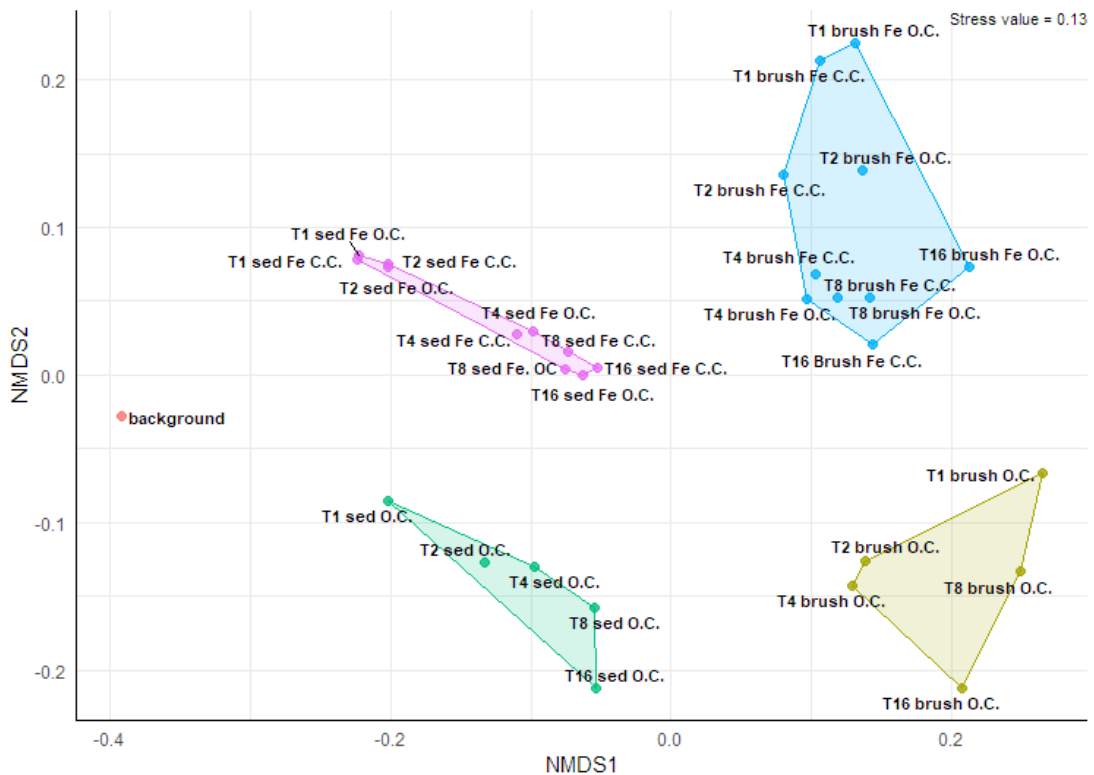


Figure 5. Nonmetric multidimensional scaling (NMDS) plot based on Bray-Curtis distance showing the relatedness of the microbial community structure. The numbers represent the time (weeks) of the SMFCs operation when the microbial structure was identified. Green and yellow groups represent sediment and anode microbial communities of the O.C. SMFCs, respectively. Blue and pink groups represent the anode and sediment microbial communities of the Fe-amended SMFCs, respectively. Brush represent the anodic microbial community and sed represent the sediment microbial community

3.4.2. Microbial community evolution

Figure 6 represents the SMFCs microbial community evolution plots that were monitored throughout the experiments. Data in the plots are extracted from heatmaps that were generated using Rstudio through the ampvis2 package using the amp_heatmap command. Detailed genera heatmaps are presented in APPENDIX B (Figure S3). The data points represent the average total SMFCs microbial community structure by taking into consideration the anode and sediment microbial communities while generating the heatmaps from the Operational Taxonomic Unit (OTU) table.

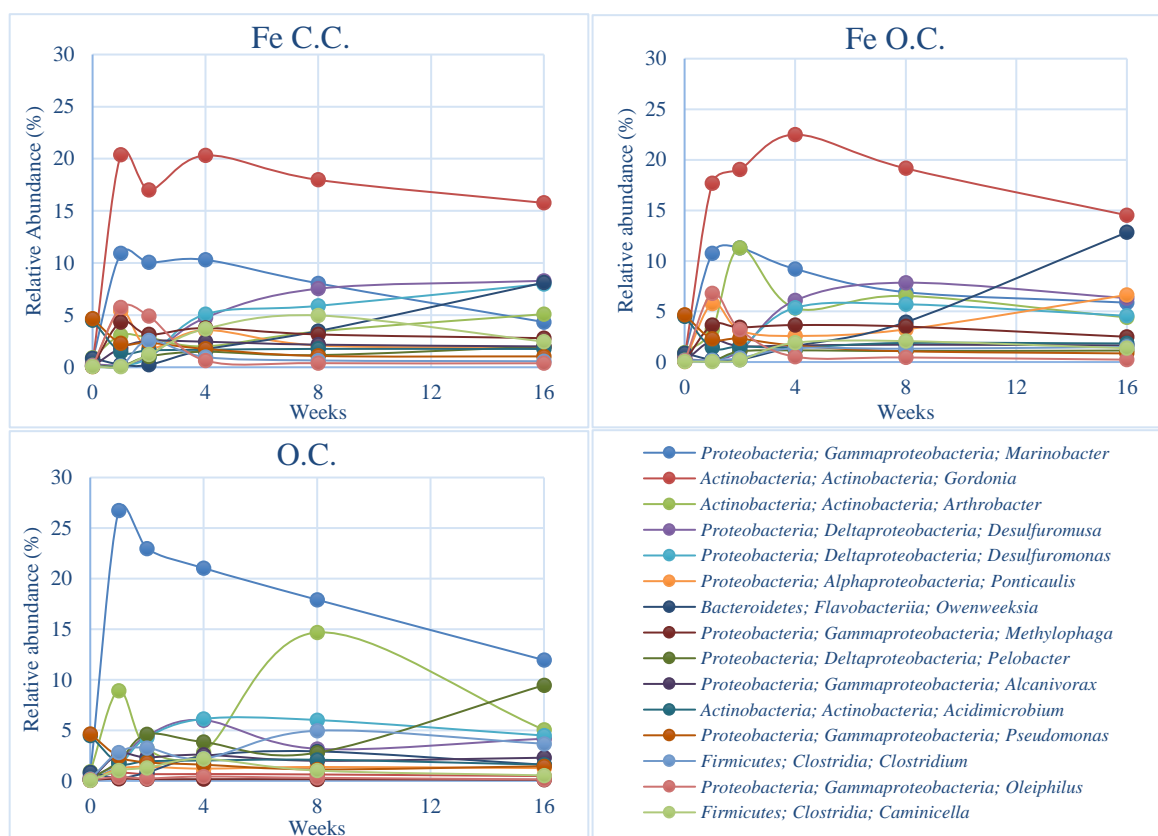


Figure 6. Microbial community evolution plots representing the variation in relative abundance (%) the most abundant genera

In the natural attenuation control, *Marinobacter* significantly dominated other microbial genera during the operation of the SMFCs. A rapid increase to a maximum of 26.7% was observed within a week of operation followed by a slow decrease to 11.9% after 16 weeks of operation. *Pelobacter*, rapidly increased after 8 weeks of incubation to

reach 9.4% after 16 weeks of operation. *Pelobacter* is known to be common in various natural environments such as marine sediments and is also commonly found in hydrocarbon-harboring environments (Sun et al., 2010). *Arthrobacter* peaked at weeks 1 (8.9%) and 8 (14.6%) of the experiments and then decreased to reach 5.0% after 16 weeks of operation. *Clostridium* represented a relatively major portion of the microbial community. It increased gradually to reach a maximum of 4.9% after 8 weeks, then decreased slightly to reach 3.6% after 16 weeks of operation. *Clostridium* was previously identified as a part of the microbial consortium involved in the biodegradation of crude oil, and thus its enrichment in the natural attenuation control is most probably due to the changes enforced on the sediments due to the addition of crude oil as a contaminant (Berdugo-Clavijo and Gieg, 2014).

Compared to the natural attenuation control, Fe-stimulated SMFCs (Fe C.C. and Fe O.C.) showed a similar trend in terms of the overall evolution of microbial communities. Most noticeably was the rapid increase in the n-alkane degrading actinomycete *Gordonia* (Kim et al., 2018; Quatrini et al., 2007), which increased to a maximum of 20.31% and 22.48 % at week 4 of operation, after which it slightly decreased to reach 15.8% and 14.5 % after 16 weeks in Fe C.C. and Fe O.C., respectively. *Gordonia* was reported to be capable of degrading PAHs in various environmental samples (Kurniati et al., 2016; Xue, 2003). The flavobacterium *Owenweeksia* also was significantly enriched in the Fe-stimulated SMFCs starting week 4 to gradually reach 8.1% and 12.8% at week 16 in Fe C.C. and Fe O.C. treatments, respectively. Few members of the class *Flavobacteria*, including *Owenweeksia*, are reported to increase in abundance during the middle phases of soil hydrocarbon bioremediation, and for being hydrocarbonoclastic microbes correlated to crude oil contamination in marine environments (Salam et al., 2018). *Marinobacter*, which is widely spread in marine

environments, and known for hosting species that are efficient degraders of aromatic and aliphatic hydrocarbons (Duran, 2010; Handley and Lloyd, 2013), also represented a significant portion of the microbial community in Fe stimulated SMFCs, increasing to a maximum of 10.0% and 11.2% in the first 2 weeks of operation, then gradually decreasing to reach 4.3% and 5.8% after 16 weeks, respectively. Finally, *Arthrobacter*, known for being efficient aliphatic and aromatic hydrocarbon degrader, was enriched gradually in the Fe stimulated SMFCs reaching 5.0% and 5.4%, respectively. The noticeable difference between the two iron treatments was the enrichment of *Ponticaulis* in Fe O.C. reaching 6.6%, compared to 1.9% in Fe C.C. SMFCs. No information is available about *Ponticaulis* correlation neither with aliphatic nor aromatic hydrocarbon contamination, and its enrichment could indicate a possible role in bioremediation of petroleum hydrocarbons in marine sediments.

Desulfuromusa and *Desulfuromonas* also increased significantly in the Fe enriched reactors to reach 8.3% and 7.9% in Fe C.C., and 6.3% and 4.5% in Fe. O.C. at the end of the experiments, respectively. These two genera were also noted in O.C. SMFCs contributing to 4.1% and 4.4% of the final microbial community, respectively. *Desulfuromonas*, a sulfur reducing bacterium capable of oxidizing some multi-carbon organic substances, was previously associated with current production in marine SMFCs, which could indicate a role in crude oil remediation in marine sediments (Hamdan et al., 2017). Also, *Desulfuromusa* was previously identified in biofilms associated with marine SMFCs (Jung et al., 2014), and species of *Desulfuromonas* and *Desulfuromusa* were reported to be FeRB (Vandieken, 2006).

Methylophaga, which was negligible in the O.C. SMFCs microbial community, represented a major portion of the microbial population in the Fe C.C. and Fe O.C. SMFCs, increasing to reach a maximum of 4.3% and 3.6% at week 1 of operation, then

slightly decreasing to reach 2.8% and 2.5% at the end of the experiments, in these respective treatments. *Methylophaga*, a methylotrophic bacterium, is involved in degradation of petroleum hydrocarbons and is reported to be metabolically active in oil-contaminated sites (Gutierrez and Aitken, 2014). *Oleiphilus*, described as novel marine bacterium which obligately utilizes hydrocarbons (Golyshin, 2002), increased noticeably in iron-stimulated SMFCs, both Fe C.C. and Fe O.C., to reach a maximum of 5.7% and 6.7%, respectively, after 1 week of operation, after which it rapidly decreased in to reach less than 1% at week 4 of operation in both treatments, remaining negligible until the end of the experiments.

The majority of the identified microbial communities, especially in the Fe C.C. treatment, were not previously reported to be involved in external electron transfer in bioelectrochemical systems. This explains the insignificant voltage recorded in these SMFCs. It also explains the lower voltage in the open circuit Fe stimulated SMFCs compared to the OC natural attenuation controls. In a previous experiment that we performed utilizing a similar experimental setup using PAHs instead of crude oil, recorded voltage in CC Fe enriched SMFCs reached about 5 mV, a 100-fold higher than its respective treatment in the current study (0.05 mV in Fe C.C. SMFCs), and was correlated to the enrichment of anode reducers. The presence of potential hydrocarbon degraders in both Fe-stimulated SMFCs and the OC controls dictates the similarity in the overall bioremediation performance observed in this study across all treatments. This also explains the similar consumption of the sulfate and ferric iron as TEAs and the insignificant differences observed in the sulfate and iron utilization. This is due to the low abundance of potential electrochemically active microbes, which were expected to be enriched under ferric iron stimulation combined with anode availability as a TEA.

These observations suggest that the evolution of the microbial community is strongly dependent on the heavy crude oil contamination within the sediments rather than the employment of an anode as an alternative TEA. Additionally, the similarity between the Fe C.C. and Fe O.C. microbial evolution indicates that the presence of ferric iron contributed to the deviation of the microbial evolution compared to the natural attenuation control, yet with no major impact of the anode as a TEA. This means that the microbial populations within the sediments mainly benefited from the available ferric iron as an alternative TEA rather than from the anode as an external one. The results thus demonstrate that application of SMFCs for treatment of heavy hydrocarbon contamination could be of a little impact on the bioremediation enhancement in marine sediments.

4. Conclusion

This study assessed the effect of Fe-stimulated SMFCs for enhanced bioremediation of heavy crude oil contamination in marine sediments. TPH removal in the Fe amended SMFCs (Fe O.C. and Fe C.C.) was similar to the non-amended natural attenuation control (O.C.), with insignificant enhancement due to ferric iron enrichment. Microbial community structure and evolution in the Fe C.C. and Fe O.C. SMFCs were also similar irrespective of the application of anode as an alternative external TEA, indicating that the presence of ferric iron rather than applying anode reducing condition was the main factor behind the shift in the microbial evolution compared to the natural attenuation iron free controls. The similar bioremediation performance of the different treatments indicates that TPH biodegradation is mostly driven by the total consortium of microbes within the sediments in relation to the heavy oil contamination, including

hydrocarbonoclastic microbial populations, rather than to specialized iron and anode reducers.

CHAPTER 4

FUTURE OUTLOOKS AND CHALLENGES

To ensure better understanding of SMFCs for future bioremediation endeavors, certain aspects of the system must be highlighted. This will ensure providing proper knowledge for possible large-scale future implementation of SMFCs for treatment of contaminated aquatic areas.

1. Modeling

SMFCs studies often follow no clear basis or criteria for the experimental setups being used. This includes the type of the tested pollutants for bioremediation, the sediment and water depth, the design and the position of the electrodes, etc., all of which can significantly affect performance. As a result, there should be distinguished criteria to be followed for designing a proper SMFCs bioremediation setup. One of the best methods that can be utilized is to create models based on previous research, which lays the road for systematic SMFCs design for future research. The SMFC technology is very complex when taking the interactions among the various components of the system with the environmental parameters and the composition of the anode biofilm into consideration. Thus, modeling would not be an easy effort; however, it would make it easier to better develop the technology for real world application (Ma et al., 2019).

2. Monitoring

Proper monitoring of SMFCs operation is needed for better understanding of the results being observed during a bioremediation study. Due to the many aspects involved in the operation of a SMFC, many studies target specific parameters, such as the concentration of the target pollutant, voltage and concentration of specific TEAs, with no

comprehensive understanding of the combined interactions among the parameters. Such a comprehensive approach could prove difficult for smaller studies due to the huge amount of effort and cost required to cover the analysis for most of the involved parameters. Even if performed, further research is needed to understand the nature of the interactions involved in the operation of the system (Abbas et al., 2017). A better controlled environment for operating the SMFCs, if created, could mean better comprehension of the system rather than description of few aspects, and will better highlight the microbial activity in terms of growth and structure, namely at the level of the anode.

3. Electromicrobiology

There is a need to identify optimal electrochemical active biofilms in SMFCs required for better implementation for purposes such as bioremediation and biosensing. Electrochemically active biofilms are often affected by the thickness of the biofilm itself, reducing the efficiency of the anode as a TEA and hampering the function of the SMFCs (Kondaveeti et al., 2018). Thus, a better focus on the formation and functioning of the biofilm is required. By developing an optimized biofilm, enhanced microbial/anode electron transfer is thus achieved, reducing the time required for transforming the target substrates into less harmful byproducts. Additionally, SMFCs rely on the sediment indigenous microbial community to develop the anode biofilm, and hence, the development of an efficient anode reducing population capable of degrading persistent pollutants is commonly hindered by the original microbial community being deficient in the needed microbial species (Kim and Kwon, 2010). On the other hand, pre-seeding the anode with a known biofilm to initiate anode reduction in contaminated sediment could also fail due to competition with the indigenous bacteria (Pan et al., 2017). Therefore,

careful considerations must be considered in future research to maintain an active anodic biofilm that is also simultaneously capable of consuming the target pollutants.

4. Sediment characteristics

Sediment conductivity is a major contributor to the performance of SMFCs. For example, marine sediment is much more highly conductive than freshwater sediment due to the increased salinity. The added conductivity can facilitate the electron and proton transfer processes, translating into better bioremediation performance (Song et al., 2012). On the other hand, marine sediment often lack the iron reducing microbial community required for proper operation of SMFCs, due to the low concentration of iron and due to the extremely dominant sulfate reducing microbial community aided by the high sulfate concentration in marine ecosystems (Hamdan et al., 2017). Additional local parameters can also affect the possible success or failure of the SMFCs in both freshwater and marine environments, such as the presence and concentration of organic matter, nutrients, or even certain pollutants, which all affect the existing sediment microbial composition and their evolution pathway (Mathuriya et al., 2018). As a result, it is important to create a future methodological approach for designing a successful SMFC setup based on the characteristics of the sediments rather than based on few parameters such as the target contaminant destined for bioremediation.

5. Overlying water

In addition to the importance of the sediment characteristics for the successful operation of a SMFCs, water characteristics should be also taken into account (Touch et al., 2017; Xu et al., 2019). Dissolved oxygen is the most important factor in the water for proper oxygen reduction rates at the level of the cathode. Other parameters are also

important to monitor such as pH, temperature, dissolved solids and even the microbial composition in the water, which can affect cathodic biofilm growth.

6. Scaling up

Manipulation of SMFCs for optimal operation, especially at a scale that is larger than laboratory-based microcosm studies, is difficult because of the complex nature of the sediment microbiology in aquatic environments. This hinders the scalability of laboratory-based findings to field studies (Seelam et al., 2018). In a recent yet unpublished work that we conducted, sediment and water characteristics, as well as the microbial composition of the sediments and anodes were found to vary greatly with time. Thus, a successful SMFC bioremediation laboratory study might fail when scaled up to field application, even in the same area where the sediments were originally collected, simply due to the temporal variation in the sediment and seawater characteristics. Furthermore, development of appropriate electrodes is a key factor given that at the larger scale, the electrodes have to withstand the harsh nature, while at the same time maintaining the feasibility, stability and durability for a long-term bioremediation capability (Das, 2018; Seelam et al., 2018).

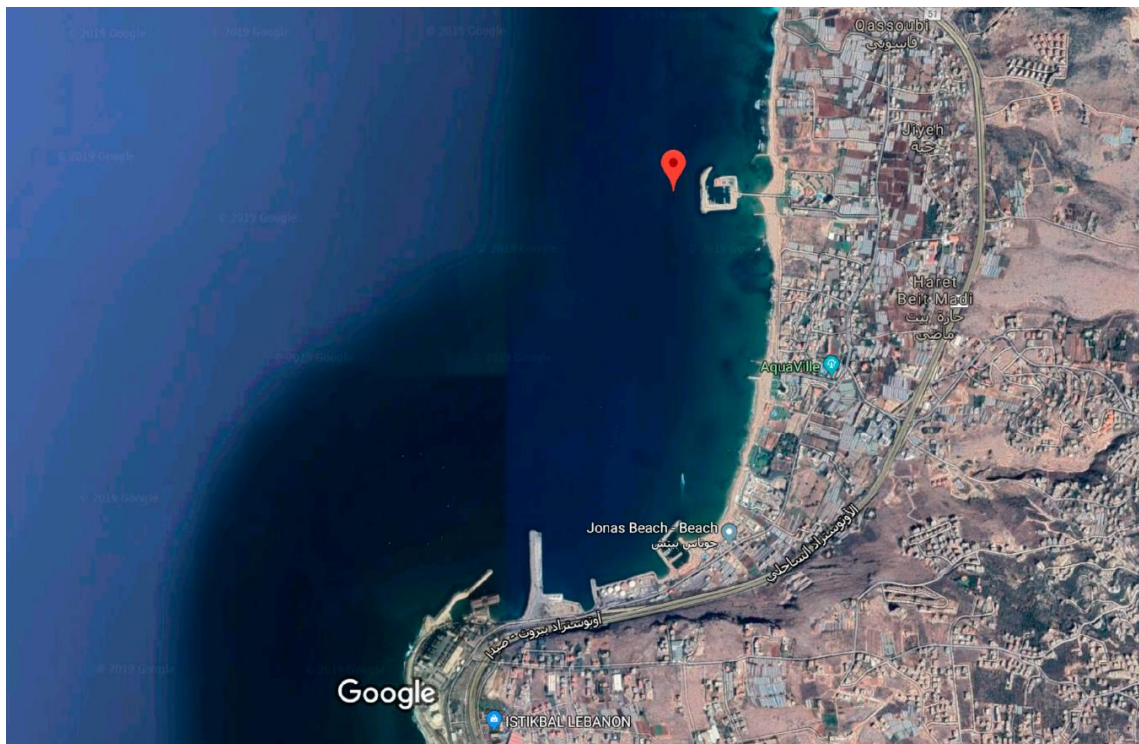
7. Conclusion

SMFCs could have a significant impact on the enhancement of bioremediation potential of marine sediments; however, this is dependent on many factors that could rather limit such enhancement. For example, a heavy contamination with petroleum hydrocarbons might force the sediment microbial community to evolve towards generalist hydrocarbon degraders before any observable effect of the anode and the iron reducing conditions takes place, thus preventing possible enrichment of electrochemically active

bacteria. The potential of marine SMFCs for the bioremediation of petroleum hydrocarbons could be thus limited to low-level contamination such as in the case of many lingering compounds, compared to serious contamination events such as in the case of a spill, when common practices such as biostimulation might be more practical and more effective.

APPENDIX A: COLLECTION OF SEDIMENTS AND SEAWATER

Anaerobic sediment samples were collected from about 2 km north of the Jiyeh power plant, a location with a long history of petroleum-hydrocarbon contamination (Figure S1). Five sediment samples were collected from the sampling point indicated in Figure S1 (33°40' 03.5" N; 35° 24' 44.0" E). The five samples were collected from 5 points within 20 meters of the indicated sampling location. Depth of the sampling points ranged between 5 and 6 meters. The sediment samples were collected at a depth of about 30 cm below the water-sediment interface to ensure collection of anaerobic bacterial communities. five seawater samples were collected 1 m above the respective sediment samples.



Imagery ©2019 CNES / Airbus, TerraMetrics, Data SIO, NOAA, U.S. Navy, NGA, GEBCO, Map data ©2019 Google, ORION-ME 500 m

Figure S1. Sediments and seawater sampling location

Figure S3 represents the genera heatmaps of the SMFCs operated during the second set of experiments (crude oil biodegradation experiments).

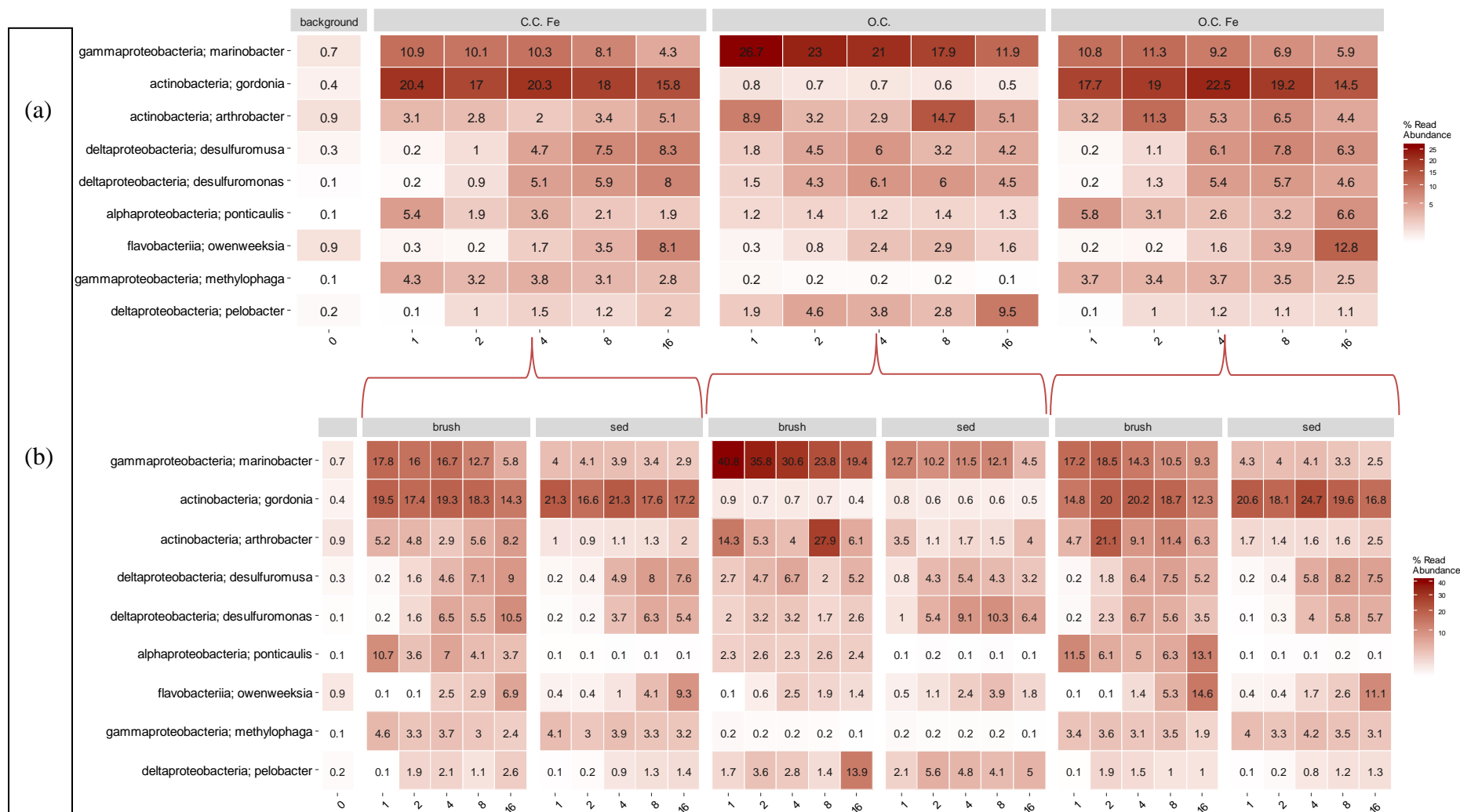


Figure S3. Genera heatmaps of the operated SMFCs showing the average microbial community (a) and the sediment and anodic microbial community (b). The numbers below the figures represent the week of operation when the microbial community structure was determined. Sed refers to the sediment microbial community and brush refers to the anodic microbial community. Fe refers to iron amended SMFCs, O.C. refers to the open circuit condition and C.C. refers to the closed circuit condition.

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