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Response of sediment microbial communities to crude oil contamination in marine sediment microbial fuel cells under ferric iron stimulation☆

Hamdan Z. Hamdan, Darine A. Salam*

Department of Civil and Environmental Engineering, Maroun Semaan Faculty of Engineering and Architecture, American University of Beirut, Beirut, Lebanon

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ABSTRACT

In this study, response of the microbial communities associated with the bioremediation of crude oil contaminated marine sediments was addressed using sediment microbial fuel cells (SMFCs). Crude oil was spiked into marine sediments at 1 g/kg of dry sediment to simulate a heavily contaminated marine environment. Conventional SMFCs were used with carbon fiber brushes as the electrode components and were enhanced with ferric iron to stimulate electrochemically active bacteria. Controls were operated under open circuit with and without ferric iron stimulation, with the latter condition simulating natural attenuation. Crude oil removal in the Fe enhanced SMFCs reached 22.0 ± 5.5% and was comparable to the measured removal in the control treatments (19.2 ± 7.4% in natural attenuation SMFCs and 15.2 ± 2.7% in Fe stimulated open circuit SMFCs), indicating no major enhancement to biodegradation under the applied experimental conditions. The low removal efficiency could be due to limitations in the mass transfer of the electron donor to the microbes and the anodes. The microbial community structure showed similarity between the iron stimulated SMFCs operated under the open and closed circuit. Natural attenuation SMFCs showed a unique profile. All SMFCs showed high relative abundances of hydrocarbon degrading bacteria rather than anode reducers, such as *Marinobacter* and *Arthrobacter* in the case of the natural attenuation SMFCs, and *Gordonia* in the case of iron stimulated SMFCs. This indicated that the microbial structure during the bioremediation process was mainly determined by the presence of petroleum contamination and to a lesser extent the presence of the ferric iron, with no major involvement of the anode as a terminal electron acceptor. Under the adopted experimental conditions, the absence of electrochemically active microbes throughout the biodegradation process indicates that the use of SMFCs in crude oil bioremediation is not a successful approach. Further studies are required to optimize SMFCs systems for this aim.

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 anoxic 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 in subsurface sediments, which are usually the most efficient pathway for oxidation of various contaminants, are usually limited to the top few

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* Corresponding author. American University of Beirut, Maroun Semaan Faculty of Engineering and Architecture, Munib and Angela Masri Bldg, M418. P.O.Box: 11-0236, Riad El Solh, 1107 2020, Beirut, Lebanon.
E-mail address: ds40@aub.edu.lb (D.A. Salam).

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millimeters of the sediment surface due to rapid depletion of oxygen with the increased depth into the sediments (Gerwing et al., 2015; Yazdani Foshtomi et al., 2018). Therefore, oxidation of organic pollutants in deeper sediments could be halted easily (Gerwing et al., 2015; Meysman, 2018; Morris and Jin, 2012; Nzila, 2018; Yazdani Foshtomi et al., 2018). 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). During the extracellular reduction of ferric oxides, these microbes actively express the genes involved in the extracellular transport of electrons, such as in the case of *Geobacter* involving active expression of conductive pili that are able to dump the electrons onto insoluble ferric iron (Lovley, 2008). Placement of an anode within the anaerobic sediment allows exoelectrogens to use it as a dump for electrons similar to ferric oxides, permitting the growth of the exoelectrogenic biofilm on the electrode and facilitating the optimized use of the anode as a Terminal Electron Acceptor (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., 2018; Semenec et al., 2018; Zou et al., 2019). 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). Therefore, identifying the different involved microbial communities and understanding the synergy among them during operation of Sediment Microbial Fuel Cells (SMFCs) is considered essential to optimize and apply this technology for enhanced bioremediation in aquatic contaminants (Sajana et al., 2017).

Due to the scarcity of sulfate as a TEA and the availability of iron oxides in freshwater settings, freshwater sediments are usually characterized by a higher abundance of iron reducing microbial populations (Hansel et al., 2015). Due to these conditions, freshwater SMFCs show significantly different overall microbial profile compared to marine SMFCs. Freshwater SMFCs are characterized by a higher abundance of iron reducers, such as *Geobacter*, which are capable of directly utilizing the anode as a TEA (Chan and Li, 2014; De Schamphelaire 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. For example, Yan et al. (2012) showed that dissimilatory iron reducing bacteria, such as *Geobacter*, were specifically identified in ferric iron stimulated freshwater SMFCs operated for the removal of phenanthrene and pyrene. In another study, Hamdan et al. (2017) successfully enriched for the dissimilatory iron reducing genus *Geoalkalibacter* in marine SMFCs operated for the removal of PAHs, namely when the dominant sulfate reducing bacterial community was inhibited, indicating that even in marine settings, anode reducing microorganisms that are important for successful operation of SMFCs can be stimulated under certain conditions. It was also indicated that the presence of these microbial taxa is sometimes correlated with better removal of target contamination (Hamdan et al., 2017; Yan et al., 2012). However, the transition of microbial communities from reduction of naturally occurring TEAs, such as the abundant sulfate in marine environments, is not only related to the enrichment for the target microbes or inhibition of the prevailing groups. Indeed, anode reduction enhancement in SMFCs is associated to different parameters, namely the background microbial consortium that can change in response to various environmental factors, even within the same study area (Hamdan et al., 2019). In this context, scarce FeRB background population may not be able to outcompete the well-established sulfate reducing microbial population in marine environments. For example, in the aforementioned study performed by Hamdan et al. (2017), the enrichment of FeRB was only observed when microbial sulfate reduction was inhibited. Coates et al. (1996) observed that FeRB enrichment through amending the sediment with ferric iron failed to shift the sediment microbial community in marine sediments away from sulfate reduction, which was probably due to the original low population size of FeRB (Coates et al., 1996). It is therefore important to study the different factors affecting the microbial community structure in an effort to optimize the bioremediation response.

While the use of SMFCs in the bioremediation of polluted water environments is well established in the literature, study of the evolution of the microbial structure associated with the biodegradation process is still lacking (Abbas et al., 2017; Kronenberg et al., 2017; Sherafatmand and Ng, 2015). Mostly, studies report the microbial communities inhabiting the SMFCs at the end of the system operation, or they even completely fail to describe the microbial aspect of the system, (Morris and Jin, 2012; Sherafatmand and Ng, 2015; Viggi et al., 2015; Yan et al., 2012; Zhou et al., 2014). Additionally, most studies on the use of SMFCs for pollutants remediation had targeted specific single or few combined pure compounds which do not represent the complex mixture of the different petroleum oil constituents, which in turn determines the oil physical characteristics and bioavailability for degradation (Sherafatmand and Ng, 2015; Xia et al., 2015; Yan et al., 2015, 2012).

In this study, the microbial community response to ferric iron amendment in marine SMFCs was assessed during the biodegradation of Total Petroleum Hydrocarbons (TPHs). Light Arabian crude oil was used to spike marine sediments to simulate a heavily contaminated marine environment. Iron amendment was applied using amorphous ferric hydroxide and was aimed at shifting the microbial community structure towards a higher abundance of FeRB, including exoelectrogens. Microbial community structure of the operated SMFCs was periodically monitored to allow for continuous assessment of the changes in the relative abundance of the most abundant taxa. The results from this study will allow a better comprehension of the temporal variation of the microbial community and will permit the assessment of the use of SMFCs for oil biodegradation in contaminated marine sediments.

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 15,000 tons of heavy fuel oil along the shoreline (Maslo et al., 2014; Shaban et al., 2007). The proximity of the powerplant to the sampling location allows for collection of sediment microbial communities that are acclimated to the degradation of petroleum hydrocarbons, which would help in avoiding a major biodegradation lag period when initializing the SMFCs. Additionally, the daily activities related to the operation of the power plant further contribute to the pollution of this area with petroleum hydrocarbons. Detailed description of the sampling site is presented in the supplementary information (Fig. S1). Grab sampling was used to collect anaerobic sediments at a depth of 5–6 m 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 m 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 ± 0.001% dry weight, wet density 1.84 ± 0.03 g/mL, dry density 1.57 ± 0.01 g/mL, sulfates (SO4-2) 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, and 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, and 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 = 25 cm; diameter = 15 cm) were used. Details about the design of the reactors are presented in Fig. 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 × 13 cm L × D) were placed vertically in the middle of the sediment half-cell, while the cathodes (13 cm × 9 cm L × 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. SMFCs were operated at room temperature. Given that oxygen availability at the cathode level is one of the major limiting factors for proper operation of SMFCs (Feng Zhao et al., 2006), the oxygen levels in the cathodic compartment were maintained at saturation by continuous aeration using and air diffuser system, and were monitored daily by measuring the dissolved oxygen in the seawater.

Four 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 TPHs removal. Another set was operated as a closed-circuit abiotic control to monitor for abiotic losses of the petroleum hydrocarbons. These abiotic SMFCs utilized autoclaved sediments and seawater, which were amended only with sodium azide (50 mM) (Cabrol et al., 2017). Sodium azide addition ensured complete deactivation of all biotic processes inside these SMFCs by resisting possible microbial contamination during the operation and preventing any interference from biotic factors. The last two 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 TPHs removal in the absence of anode reduction. No iron amendment was applied to the

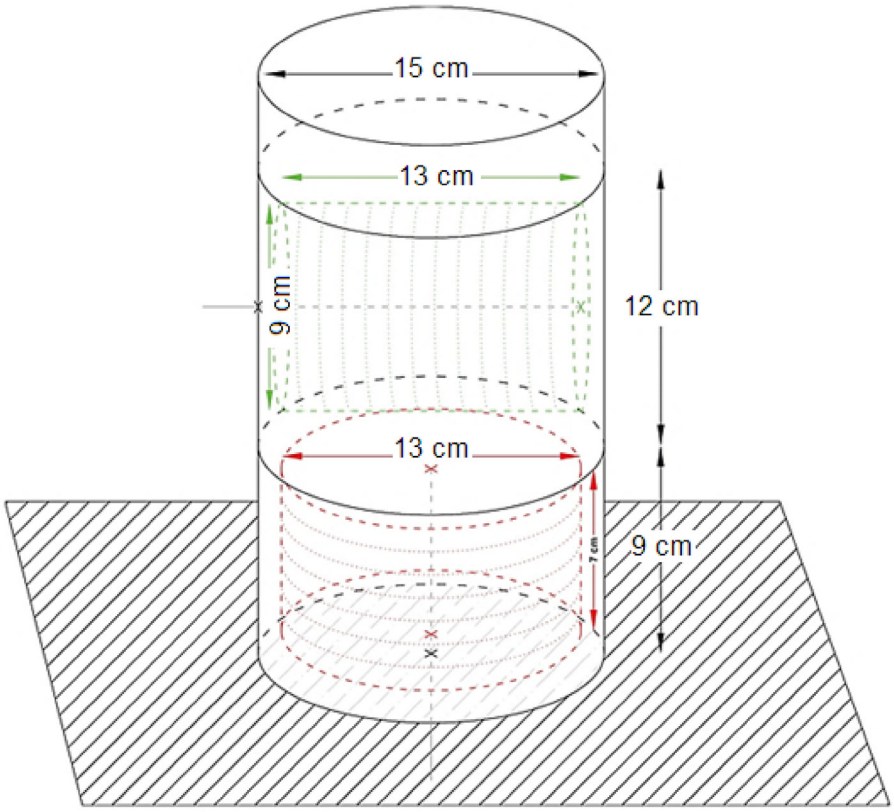

Fig. 1. Schematic of the SMFC design.

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 (FeOOH)at a concentration of 20 g/kg of dry sediments. The supplied amount of FeOOH resulted in a starting Fe^{3+} concentration of 633 ± 1.9 mg/kg of dry, which is within the range that is commonly applied to sediments in similar sediment Fe-stimulation studies (Yan et al., 2012). 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 min 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. 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 min, 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

For each of the four treatments, 15 replicate SMFCs were operated with a total of 60 SMFCs being performed. Continuous monitoring of SMFCs voltages was performed by recording the voltage output across the electrodes once every 15 min using a data acquisition system (2700; Keithley Instruments Inc., United States) and the data were averaged daily. Five sampling events were conducted over a period of 16 weeks and corresponded to weeks 1, 2, 4, 8 and 16 of operation. At each sampling event, triplicate SMFCs were sacrificed from each treatment. 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 TPHs, sulfates and Ferric iron analysis. DNA extraction from the sediments and anode brushes was also performed for microbial structure determination. The triplicate SMFCs that were sacrificed from each treatment were analyzed separately, and the results from each replicate were averaged for each data point.

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 30–40 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 min heat-up time, 5 min static time, 70% flush, and 60 s 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).

TPHs were calculated based on the summation of the major extracted petroleum components, which are alkanes and polycyclic aromatic hydrocarbons. 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).

2.3. Microbial characterization

The V4 variable region of the 16S rRNA gene was amplified by PCR using the 515/806 primers, utilizing a barcode on the forward primer. HotStarTaq Plus Master Mix Kit (Qiagen, USA) was used to perform a total of 30 cycles using the following programfor amplification: 94 °C for 3 min, which was followed by 30 cycles of 94 °C for 30 s, 53 °C for 40 s and 72 °C for 1 min. The final elongation step was performed after that at 72 °C for 5 min. PCR products were then checked on a 2% agarose gel electrophoresis to confirm successful amplification. The amplified samples were then purified using calibrated Ampure XP beads. The Illumina DNA library was then prepared by pooling the purified samples together in equal proportions. A MiSeq was used for sequencing following the manufacturer's instructions. Sequence data was then processed using the MR DNA analysis pipeline (Shallowater, TX, USA) to produce the OTU tables. Sequence data was first joined, after which it was depleted of the barcodes. Sequences less than 150 bp and sequences with ambiguous base calls were excluded. Denoising was then performed, and finally the OTUs were generated after removing chimeras. The OTUs were defined by clustering at 3% divergence (97% similarity), which were classified using BLASTn against a curated database that was derived from RDPII and NCBI (http://rdp.cme.msu.edu, www.ncbi.nlm.nih.gov). Nonmetric multidimensional scaling plot (NMDS) was generated through Rstudio by using the ampvis2 package, based on Bray-Curtis distance and using the Hellinger transformation. This was performed by running the amp_ordinate command. Microbial community response plots were then generated from the OTU tables using Excel by plotting the variation of the most abundant genera as a function of time. The complexity and community response 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

3.1. Biodegradation of TPHs

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 (17α(H),21β(H)-Hopane), which is considered to be non-biodegradable throughout the experiments duration (Venosa et al., 1997). The starting concentration of TPHs, total alkanes and total PAHs at the beginning of the experiments after equilibrating the SMFCs were 10,586 ± 447, 9211 ± 397 and 1374 ± 50 mg/mg hopane. Fig. 2a shows the degradation profile of TPHs as well as total alkanes and PAHs. Fig. 2a shows the degradation profile of TPHs while Fig. 2(b) and (c) show respectively the concentration profiles of the petroleum components, alkanes and PAHs, under different operating conditions.

Abiotic losses in the SMFCs were minimal as indicated by the slight decrease in the TPHs concentration over the SMFCs incubation period. A slight drop in the TPHs concentration was observed by the fourth week of operation, dropping to 10,120 mg/mg hopane, after which the concentration remained relatively constant, reaching 10,030 mg/mg hopane after 16 weeks of operation. The corresponding drops in alkanes and PAHs concentrations in the abiotic control SMFCs also occurred during the first few weeks of operation, reaching 8750 mg/mg hopane and 1280 mg/mg hopane, respectively, after 16 weeks of operation. The observed abiotic losses were statistically significantly different from those measured in the other operating conditions, despite the relatively low removal rates of TPHs, alkanes and PAHS in the latter ones, indicating significant contribution of microbial degradation of petroleum hydrocarbons. The degradation of TPHs showed no significant difference in their removal throughout the operation of the experiment (Fig. 2a). A relatively fast drop in TPHs was observed during the first 2 weeks of operation, followed by a slower removal rate until the last week of operation. TPHs dropped from 10,586 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 TPHs 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 TPHs.

The two major constituent groups of TPHs, which are alkanes

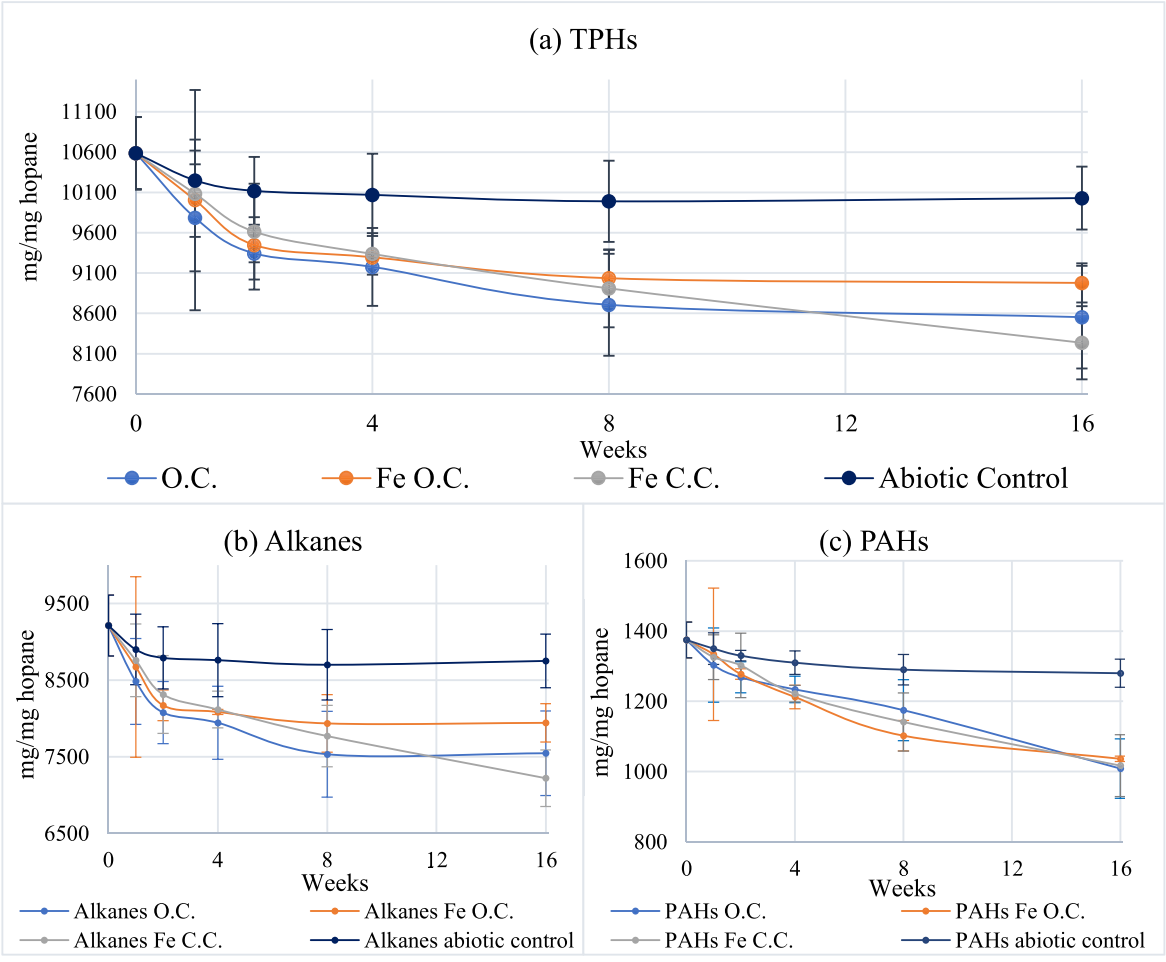

Fig. 2. Concentration profile of petroleum hydrocarbons in mg/mg hopane in the SMFCs during the 16 weeks of operation. (a) TPHs, (b) alkanes, and (c) PAHs.

and PAHs, were also taken into consideration when comparing the degradation profiles of the hydrocarbons in this study (Fig. 2b). As for PAHs, which composed only 12.9% of TPHs, 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 ($p > 0.05$). Total alkanes, composing the majority of TPHs (87.1%), presented a similar degradation trend to the TPHs plot, dropping from 9211 ± 397 to 7219 ± 368 mg/mg hopane in the Fe C.C., 7941 ± 250 mg/mg hopane in the Fe O.C., and reaching 7545 ± 500 mg/mg hopane in the natural attenuation controls, after 16 weeks of operation.

Analysis of variance (ANOVA) of the degradation of TPHs, alkanes and PAHs showed similar removal in all treatments except for alkanes removal which was significantly different ($p < 0.05$) between the Fe O.C. and Fe C.C. SMFCs. In this case, although only a slight difference in alkanes removal was measured, the statistical analysis suggests that simultaneous application of ferric iron and anode reduction could enhance SMFCs performance; Table 1 represents the fitted 1st order decay constants of TPHs, total alkanes and total PAHs.

3.2. TEA utilization

Fig. 3 shows the temporal variation in the sediment sulfate and ferric iron concentrations. Significant portion of the available sulfate was consumed in all SMFCs, with no significant differences ($p > 0.05$) being observed among treatments in the concentrations of the sulfate throughout the experiments' duration. This was different from the abiotic controls in which minimal drop of sulfate was observed, reaching 616 mg/kg of dry sediment after 16 weeks of operation, indicating significant sulfate reduction pathways taking place in the biotic SMFCs. 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.

Utilization of ferric iron was minimal and comparable in both iron amended treatments. Both iron amended SMFCs (Fe O.C. and Fe C.C.) showed a very slight drop in the available ferric iron, mainly observed 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, and reached 609 ± 1.7 and 608 ± 7.8 mg/kg of dry sediment after 16 weeks of operation in Fe O.C. and Fe C.C., respectively (3.9% and 3.7% utilization, respectively).

3.3. Recovered voltage

Fig. 4 shows the average voltage profile generated by the SMFCs over 16 weeks of operation. Abiotic control SMFCs showed no voltage production during the operation period of the experiment. 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 conditions, 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 in the O.C. and Fe O.C. SMFCs during the first few weeks of operation indicates and 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, since SRB population was 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 TPHs in the Fe C.C. SMFCs.

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

	TPHs		Total alkanes		Total PAHs	
	Decay constant (d^{-1})	r^2	Decay constant (d^{-1})	r^2	Decay constant (d^{-1})	r^2
OC	0.0018 ± 0.0006	0.69	0.0017 ± 0.0005	0.6	0.0026 ± 0.0004	0.97
Fe O.C.	0.0014 ± 0.0004	0.59	0.0011 ± 0.0005	0.48	0.0026 ± 0.0003	0.91
Fe C.C.	0.0019 ± 0.0004	0.9	0.0018 ± 0.0004	0.88	0.0025 ± 0.0004	0.97
Abiotic Control	0.0008 ± 0.0002	0.64	0.0008 ± 0.0001	0.60	0.0011 ± 0.0002	0.88

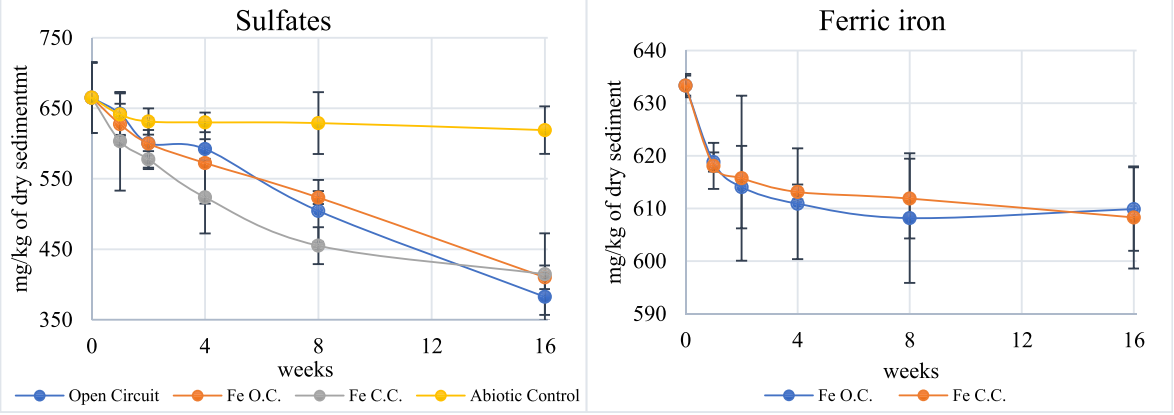

Fig. 3. TEA utilization.

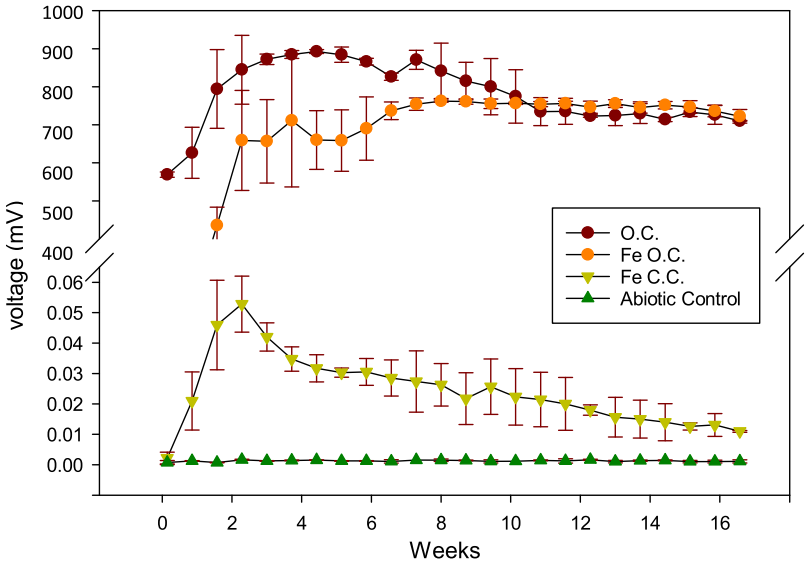

Fig. 4. SMFCs voltage profile in the SMFCs over 16 weeks of operation.

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) (Fig. 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 observed similar clustering of the Fe amended SMFCs indicates that ferric iron had a major impact on the overall microbial community in the SMFCs. The sub-clustering of the anode microbial communities in both Fe stimulated conditions (Fe O.C. and Fe C.C.) indicates that the presence of the anode itself had an impact on the microbial community response irrespective of it being connected to the cathode. This

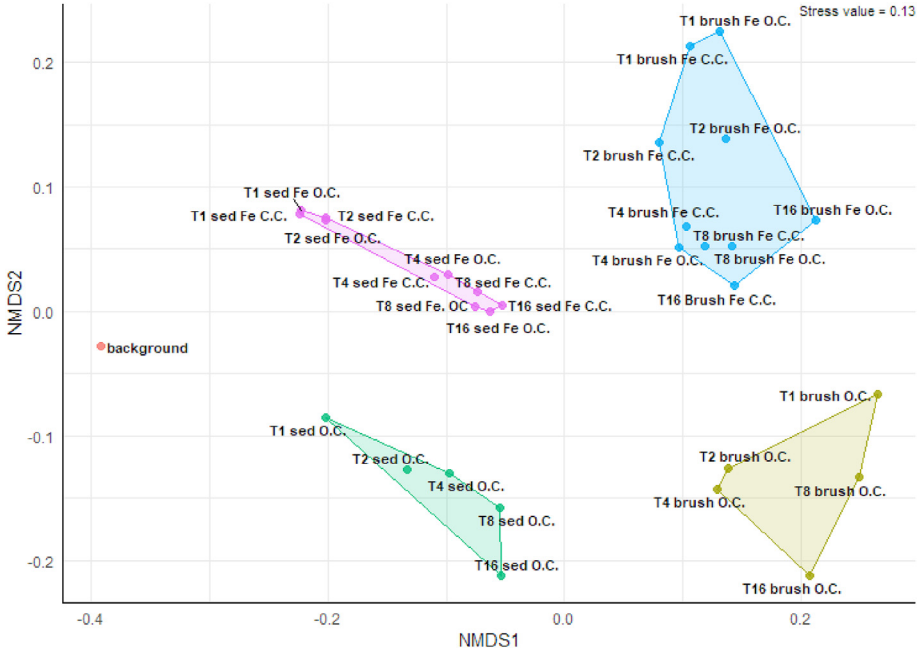

Fig. 5. Nonmetric multidimensional scaling (NMDS) plot based on Bray-Curtis distance showing the relatedness of the microbial community structure. The numbers represent the time (weeks) of the SMFCs operation when the microbial structure was identified. Green and yellow groups represent sediment and 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. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

further explains the minimal electrical activity observed in the Fe C.C. SMFCs in the presence the anode as a TEA.

3.4.2. Microbial community response

Fig. 6 represents the SMFCs microbial community response 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 supplementary information (Fig. S2). 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.

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 response 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). It is interesting to note that despite the aerobic nature of Gordonia (Arenskotter et al., 2004), it dominated the microbial communities in both of the iron-stimulated SMFCs in the anaerobic anode half-cell compartment. No information about the electrochemical capabilities of *Gordonia* is available in literature; however, several studies reported association between the growth of *Gordonia* and the presence of iron. For example, Liu et al. (2017) reported that enrichment of *Gordonia* was observed in the cathodes of single chamber microbial fuel cells enriched with ferrous iron compared to those with no enrichment. Ortega-Cabello et al. (2017) reported that the growth of *Gordonia* is highly impacted by the presence and type of iron salts added to the growth media used to culture Gordonia. Furthermore, Drzyzga (2012) mentioned that strains of *Gordonia* are capable of producing Mycobactin, a siderophore used to shuttle free extracellular iron ions into the cytoplasm of the bacterial cells. This might suggest an electrochemical potential in *Gordonia*, or might also suggest possible utilization of iron as a mediator to access the distant oxygen in the cathodic compartment of the SMFCs. The flavobacterium *Owenweeksia* also was significantly enriched in the Fe-

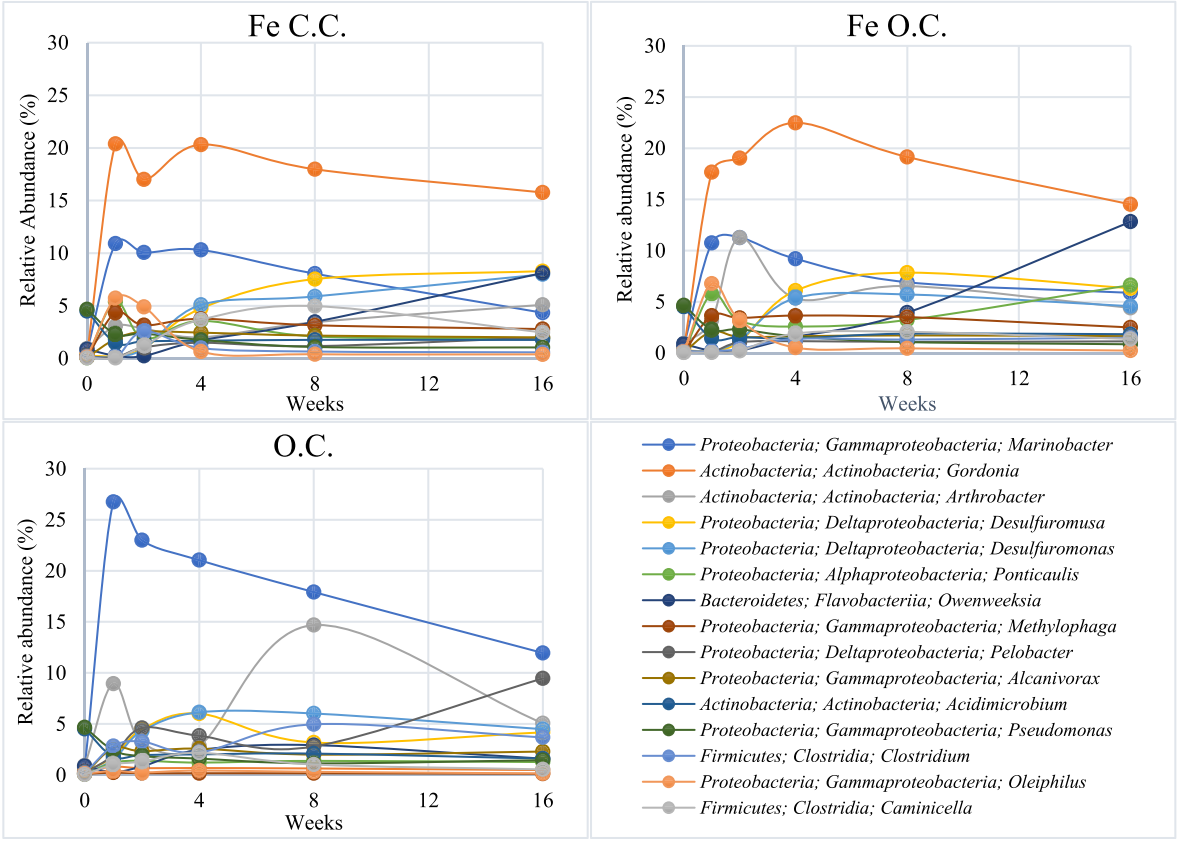

Fig. 6. Microbial community response plots representing the variation in relative abundance (%) of the most abundant genera.

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.

There are many studies that indicate the involvement of *Flavobacteria* in the degradation of petroleum hydrocarbons in marine environments, with many of them reporting its strong correlation with the presence of heavy petroleum contamination, such as the one following the tragedy of the Deep Water Horizon (Hu et al., 2017; Kimes et al., 2014; Liu and Liu, 2013). 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). *Owenweeksia* was also previously reported to predominate cultures involving crude oil degradation (Al-Mailem et al., 2015). *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.

4. Discussion

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 in improving petroleum oil biodegradation under the studied experimental conditions compared to the natural attenuation of the crude oil. High abundance of iron reducers in freshwater sediments allows the proliferation of electrochemically active microbes, which is not the case in 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 an 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 in 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. In all cases, the variation in the microbial structure involved in the degradation of oil under the different operating conditions would provide knowledge about the behavior of the sediment microbiota depending on the existing reducing condition and the type of contamination.

The similarity in the ferric iron utilization in the iron amended treatments shows that the presence of the anode reducing condition in the Fe C.C. SMFCs had minimal impact on the TEA utilization. This, besides the observed similarity in sulfate utilization in the natural attenuation control SMFCs as 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. This also explains the similarity in the open circuit potential profile in the O.C. SMFCs and the Fe O.C. SMFCs, indicating that the absence of potential anode reducers did not change the reduction potential of the anode embedded within the sediment.

The similarity between the microbial communities in the sediments and anodes of the O.C. and Fe C.C. SMFCs as shown by the NMDS plot, indicates that the presence of the anode did not affect the response 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 response 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.

In addition, 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 Fe enriched closed circuit 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 hydrocarbon degraders in both Fe-stimulated SMFCs and in 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, such as *Clostridium*, *Pseudomonas*, and *Desulfuromonas*, which were expected to be enriched under ferric iron stimulation combined with anode availability as a TEA (Chan and Li, 2014; De Schamphelaire et al., 2008; Jung et al., 2014; Semenec et al., 2018).

These observations suggest that the response 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 in the microbial community response between the Fe C.C. and Fe O.C. indicates that the presence of ferric iron contributed to the shaping of the microbial composition and its differentiation from that in 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 to sulfate, rather than as a stimulant for anode reduction. The results thus demonstrate that application of SMFCs for the treatment of heavy hydrocarbon contamination in marine sediments might not be a sound alternative.

5. Conclusion

This study assessed the effect of Fe-stimulated SMFCs for enhanced bioremediation of heavy crude oil contamination in marine sediments. TPHs 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 response 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 community structure compared to the natural attenuation iron free controls. The similar bioremediation performance of the different treatments indicates that TPHs 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.

Author statement

All authors discussed the results and contributed in multiple roles to the final manuscript.

Declaration of competing interest

The authors declare no competing interests.

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Appendix A. Supplementary data

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