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Microbial community evolution during the aerobic biodegradation of petroleum hydrocarbons in marine sediment microcosms: Effect of biostimulation and seasonal variations☆

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ABSTRACT

Evolution of the microbial community structure in crude oil contaminated marine sediments was assessed under aerobic biodegradation during wet (18 °C) and dry (28 °C) seasons experiments, to account for seasonal variations in nutrients and temperature, under biostimulation and natural attenuation conditions. NMDS showed significant variation in the microbial communities between the wet and the dry season experiments, and between the biostimulation and the natural attenuation treatments in the dry season microcosms. No significant variation in the microbial community and oil biodegradation was observed during the wet season experiments due to high background nitrogen levels eliminating the effect of biostimulation. Larger variations were observed in the dry season experiments and were correlated to enhanced alkanes removal in the biostimulated microcosms, where *Alphaproteobacteria* dominated the total microbial community by the end of biodegradation (54%). Many hydrocarbonoclastic bacterial genera showed successive dominance during the operation affecting the ultimate performance of the microcosms.

1. Introduction

Petroleum hydrocarbon contamination in aquatic environments is of a rising concern due to toxic effects on living organisms. Sediments in aquatic environments are especially important when addressing petroleum hydrocarbon contamination due to acting as a major sink for organic pollutants (Abbas et al., 2017; Adeniji et al., 2017). Petroleum hydrocarbons have a tendency to accumulate in the sediment layer in high concentrations over time (Adams et al., 2018; Kanzari et al., 2014). The characteristics of petroleum contamination in sediments has thus been of a recent interest, not only to prevent new pollution from entering the environment, but also to treat existing contamination (Meng et al., 2019; Oyo-Ita et al., 2016). The natural processes involved in the consumption of the pollutants is often hindered by a multitude of parameters such as the continuous input of contamination, the limited availability of nutrients (nitrogen and phosphorus) needed for sustained microbial metabolism, the high concentration of certain pollutants suppressing the growth of the microbial consortium, and the actual composition of the microbial ecosystem that might lack the presence of efficient hydrocarbon degraders capable of metabolizing existing pollution or properly adapting to the degradation of new pollution (Alegbeleye et al., 2017; Ghosal et al., 2016; Röling et al., 2002; Viñas et al., 2005). Bioremediation is one major technique that takes advantage of the biological ability of the sediments for degrading petroleum hydrocarbons, thus stimulating enhanced rates of attenuation of target pollutants. Such an approach is deemed important given that it provides a sustainable, less aggressive, less costly, and less energy demanding approach compared to common sediment remediation techniques such as dredging (Azubuike et al., 2016; Li and Yu, 2015; Perelo, 2010). Hence, research on optimizing existing bioremediation techniques is being performed (Abbas et al., 2017; Kurniati et al., 2016; Sakaya et al., 2019).

Bioremediation processes are characterized by successive dominance of various microbial populations during the different

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stages of the biodegradation. For example, when petroleum hydrocarbon contamination is introduced into the sediments, the microbial community usually develops generalist hydrocarbon degraders in response to easily biodegradable hydrocarbons, which is followed by successive restructuring favoring specialized groups capable of degradation of the more persistent hydrocarbons (Neethu et al., 2019; Sun et al., 2018). *Hyphomonas*, *Pseudomonas*, *Marinobacter*, *Methylophaga*, *Sulfurimonas* and *Helicobacter* are all examples of bacteria being involved in the natural removal of oil hydrocarbons contamination in marine sediments (Neethu et al., 2019).

Also, seasonal changes in the microbial structure of sediments in aquatic environments are driven by multiple factors including temperature variation, which highly affect potential bioremediation enhancement of specific pollutants (Bucci et al., 2014; Wilhelm et al., 2014; Zhang et al., 2019). Temperature affects the adaptation of certain microbes, favoring dominance of certain groups at different times (Bárcenas-Moreno et al., 2009; Gutiérrez et al., 2018). This highlights the importance of temperature as a factor affecting natural bioremediation processes such as the hydrocarbon removal, the microbial activity and the microbial community structure (Siles and Margesin, 2018).

Furthermore, nutrients concentrations could highly affect the microbial community structure. Evans et al. (2004) reported heavy changes in the microbial structure under biostimulation conditions for oil degradation in natural systems, compared to natural attenuation. In another study, Coulon et al. (2007) indicated that the supply of nutrients did not seem to have a large influence on the oil-degrading microbial community in biostimulated contaminated estuarine waters. The authors found that the presence of a pre-adapted oil-degrading microbial community, in the presence of sufficient initial levels of nutrients, can allow for high rates of oil removal with no further nutrients amendment. These studies stress the complex nature of bioremediation systems, showing that site specific characteristics usually dictate the bioremediation performance of oil contamination.

In this study, the microbial structure evolution associated with the biodegradation of petroleum in oil contaminated marine sediments was assessed under Biostimulation (BS) and Natural Attenuation (NA) conditions in laboratory microcosms. The results were correlated to the achieved oil removal under the different treatments. The effect of temperature and nutrient concentration on the microbial community structure was analyzed with its subsequent implications on the bioremediation performance. The results from this study will help in better understanding the role of the microbiological ecosystem in oil contamination removal as it is influenced by different environmental factors. This would ultimately help in optimizing the bioremediation approach based on predictions of microbial communities' involvement and evolution.

2. Materials and methods

2.1. Biodegradation experiments

Marine sediments from the shore of Beirut, Lebanon, located on the eastern side of the Mediterranean Sea, were collected to prepare the laboratory microcosms. In each of the dry and wet seasons, surface sediment samples were collected at depths ranging from 30 to 50 cm below the water surface, which is representative of the intertidal zone that is subjected to aerobic conditions. Sediments were first sieved through a 2 mm sieve to remove large particles and then characterized in triplicate samples for chemical properties and exhibited respectively in the wet and the dry seasons; nitrate + nitrite 1.32 ± 0.05 and 0.48 ± 0.28 mg N/kg of sediment), total nitrogen (2.21 ± 1.14 mg N/kg of sediment and 0.7 ± 0.3 mg N/kg of sediment), reactive phosphorus (below detection and 0.19 ± 0.02 mg P/kg of sediment) and total phosphorus (0.15 ± 0.02 mg/kg of sediment and 0.19 ± 0.02 mg/kg of sediment).

The biodegradation experiments were conducted in 250 mL silanized Erlenmeyer flasks, filled with 100 g of wet sediments. Sediments were spiked with Light Arabian crude oil supplied by the Jordan Petroleum Refinery, at a concentration of 0.7 g/kg of wet sediments. Characteristics of the used petroleum oil are presented elsewhere (Sakaya et al., 2019). The amount of spiked oil was selected based on crude oil spills typical contamination levels previously reported in aquatic environments and based on quantities which were shown to be fully degradable within the incubation period adopted in this study (Campo et al., 2013; Sammarco et al., 2013).

Two sets of experiments were conducted, one set was performed during the dry summer season by incubating the microcosms at 28 °C, and another set during the wet winter season by incubating the microcosms at 18 °C, representing typical average water temperatures observed in each corresponding season in Beirut. In each case, sediments were collected just before the experiments during the corresponding season. In each set of experiments, two treatments were prepared. In one treatment, nutrients were added for BS of microbial activity, while no nutrient addition was performed under the NA treatment. Under biostimulation conditions, nitrogen (N) and phosphorus (P) were added in the form of potassium nitrate (KNO_3) and sodium triphosphate pentabasic ($Na_5P_3O_{10}$), respectively, to approach an optimal C:N:P of 100:5:1 for oil biodegradation, with a final concentration in the microcosms of 0.035 mg N/kg of sediment and 0.007 mg P/kg of sediment in the microcosms. The adopted C:N:P stoichiometric ratio of 100:5:1 is universally used by bioremediation personnel for attaining optimal oil biodegradation rates (Adriano et al., 1999; Dibble and Bartha, 1979; Martínez Álvarez et al., 2015; Venosa et al., 2010, 1996). The chosen water-soluble nutrient additives in this study were suitable for enclosed marine environments with low amplitude tides such as the Mediterranean Sea (Nikolopoulou and Kalogerakis, 2009; UCSB, 2017). For both treatments (BS and NA), oil biodegradation was carried out by the indigenous hydrocarbon-degrading bacteria present in the sediments, with no added exogenous cultures. Biodegradation experiments lasted for 42 days and were performed under continuous shaking at 200 rpm.

Eleven sampling events were performed during each of the biodegradation experiments corresponding to days 0, 2, 4, 8, 11, 14, 17, 21, 28, 35, and 42. At each sampling event, triplicate flasks from each treatment (BS and NA at 18 °C corresponding to the wet season experiment, and BS and NA at 28 °C corresponding to the dry season experiment) were sacrificed for analysis of residual oil. Residual oil was extracted from the sampled microcosms using dichloromethane and analyzed on a GCMS (Agilent Technologies 7890A GC-5975C MSD). The GC-MS method was based on the internal standard method described by Campo et al. (2013). Linear and branched aliphatic alkanes ranging from nC10 to nC35, in addition to pristane and phytane, were measured. For PAHs, the analysis included 2-, 3-, and 4-ring aromatic compounds and their alkylated derivatives (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).

2.2. Microbial community analysis

2.2.1. DNA extraction and analysis

Microbial community characterization was conducted by 16s

rRNA sequencing at days 0, 4, 8, 11, 21 and 42 of the experiments. PowerSoil® DNA Isolation Kit (QIAGEN) was used to extract the microbial DNA from the sediments in the microcosms. At each sampling event, 0.3 g of sediments from each of the sacrificed microcosms were used for DNA extraction according to the manufacturer's instructions. For each treatment, DNA extracts from triplicate sacrificed microcosms were pooled together and preserved at −50 °C. At the end of the experiments, all DNA extracts were shipped to MR DNA (Shallowater, TX, USA) for full characterization of the prokaryotic microbial communities by sequencing of the 16S rRNA gene V4 variable region (primers: 515/806) on a MiSeq (Illumina, USA).

2.2.2. DNA amplification and sequencing and data analysis

The V4 variable region of the 16S rRNA gene was amplified by PCR using the 515/806 primers, with barcode on the forward primer. 30 total cycles were performed using the HotStarTaq Plus Master Mix Kit (Qiagen, USA). The following conditions were applied during the amplification process: 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 53 °C for 40 s and 72 °C for 1 min. After that, a final elongation step was performed for 5 min at 72 °C. To ensure successful amplification, the PCR products were checked in a 2% agarose gel electrophoresis. Illumina DNA library was prepared by pooling together equal proportions of the amplified samples after purifying the pooled samples using calibrated Ampure XP beads.

Sequencing was performed on a MiSeq following the manufacturer's guidelines, and the resulting sequence data was processed using the MR DNA analysis pipeline (Shallowater, TX, USA) to produce the OTU tables. Sequence data were first joined, depleted of barcodes, then sequences <150 bp and sequences with ambiguous base calls were removed. Denoising was performed, OTUs were generated and chimeras were removed. OTUs were defined by clustering at 97% similarity (3% divergence). The final OTUs were classified using BLASTn against a curated database derived from RDPII and NCBI (www.ncbi.nlm.nih.gov, http://rdp.cme.msu.edu). Rstudio was then used to generate the Nonmetric multidimensional scaling plot (NMDS) using the ampvis2 package based on Bray-Curtis distance and using the Hellinger transformation, using the amp_ordinate command. Microbial evolution plots were then generated from the OTU table using Excel by plotting the variation of the most abundant taxa (classes and genera) as a function of time.

3. Results and discussion

3.1. Petroleum hydrocarbons degradation

Temporal variation of petroleum hydrocarbons in the sediments was monitored by determining the hopane-normalized concentrations of individual alkanes and Polycyclic Aromatic Hydrocarbons (PAHs), comprising the major components of the crude oil (n-C10 to n-C35 in addition to pristane, phytane, and hopane for the aliphatic portion, and 2-, 3-, and 4-ring aromatics and their alkylated derivatives for the aromatic portion of the oil). The temporal variation of the Total Petroleum Hydrocarbons (TPH) components in both the dry season and the wet season experiments are presented in Fig. 1.

3.1.1. Dry season (28 °C experiments)

In the case of the dry season experiments (28 °C), significant differences were observed in the biodegradation of total alkanes between the nutrient-enhanced treatment and the natural attenuation treatment during the first 2 weeks of operation, after which the concentration of the total alkanes overlapped under both treatments, and levelled thereafter with around 1.8% residual. First order decay rates showed an overall 33% improvement in the biostimulated microcosms (0.244 day^{-1}) as compared to the NA ones (0.184 day^{-1}). This observed enhancement in alkanes biodegradation under biostimulation conditions seemed to fall in the moderate range of improvement compared to previous similar studies. For example, Venosa et al. (1996) observed a 115% improvement in the alkanes biodegradation rates of in biostimulated sediments (Delaware Bay), while another study (Tate et al., 2012) showed no improvement in the alkane removal rates in salt marsh sediments. The slight improvement in the removal rates of alkanes in the dry season experiments of this study was found to be related to the background concentration of nutrients already present within the sediment, namely nitrogen (2.8 mg-N/L), which falls within the required range of nitrogen concentration (2–10 mg-N/L) to maintain near-maximum biodegradation rates of crude oil (Venosa et al., 2010). This explains the slight enhancement in the degradation rates of alkanes under biostimulation conditions as compared to the natural attenuation treatment where background nitrogen concentrations supported relatively high oil biodegradation rates. The relatively high background nutrients concentration in the sediments is due the continuous discharge of nutrient-rich sewage along the coast of Lebanon and the long-lasting absence of proper sewage treatment systems.

This was not the case for the more resistant PAHs, where similar biodegradation rates were reported under both biostimulation (0.109 day^{-1}) and NA (0.106 day^{-1}) treatments. In both treatments, the biodegradation levelled after 17 days of operation with about 13% residual PAHs (Sakaya et al., 2019) .

3.1.2. Wet season experiments (18 °C)

The biodegradation of both alkanes and PAHs in the wet season experiments showed no noteworthy improvement when biostimulation was applied. Indeed, in the wet season experiments, the sediments exhibited a high background nitrogen concentration compared to the dry season experiments, reaching a porewater concentration of 8.4 mg-N/L, thus allowing for maximal oil biodegradation rates to be achievable even under the natural attenuation process. The overall biodegradation rates were slower in the wet season experiments compared to the dry season, with biodegradation rates of 0.119 day^{-1} for natural attenuation and 0.114 day^{-1} for biostimulation in the case of alkanes, and 0.055 day^{-1} for natural attenuation and 0.057 day^{-1} for biostimulation in the case of PAHs (Sakaya et al., 2019).

3.2. Microbial community analysis

3.2.1. Beta diversity

The overall similarity in the microbial communities observed throughout the biodegradation experiments under the different treatments was highlighted through Nonmetric multidimensional scaling (NMDS) based on Bray-Curtis distance (Fig. 2). Domain Bacteria was the main component of the background sediment microbial communities in both the wet (95.8%) and the dry season (99.3%) sediments. Bacteria were also the major component of the microbial communities during the biodegradation experiments (>99%).

Dissimilarity in the overall microbial community structure in the dry and wet season experiments was observed, indicated by the separate clustering of the corresponding points on the NMDS plot. This was expected due to seasonal variation in the sediment and seawater characteristics, which affects the microbial composition. In both the wet and the dry season experiments, the microbial structure gradually shifted from the background community. This is due to changes in the microbial structure upon adapting to and

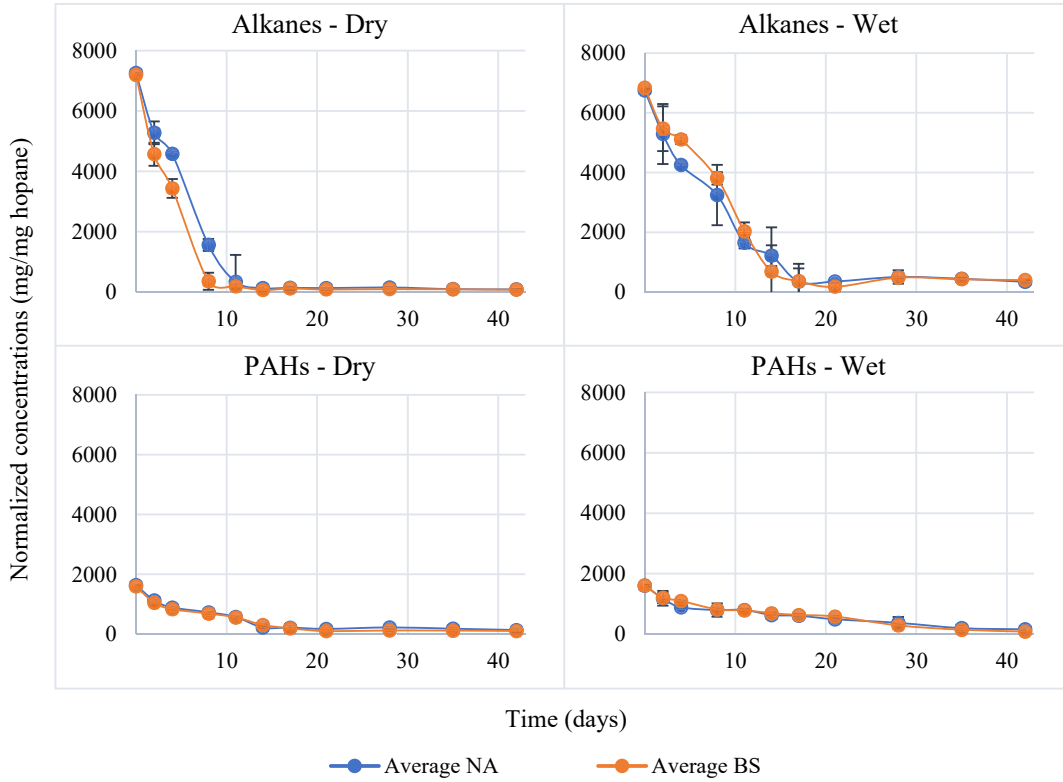

Fig. 1. Biodegradation profiles of crude oil in the wet (18 °C) and dry (28 °C) season experiments. Results are reported in terms of TPHs. NA indicates the Natural Attenuation treatment and BS indicates the Biostimulation treatment.

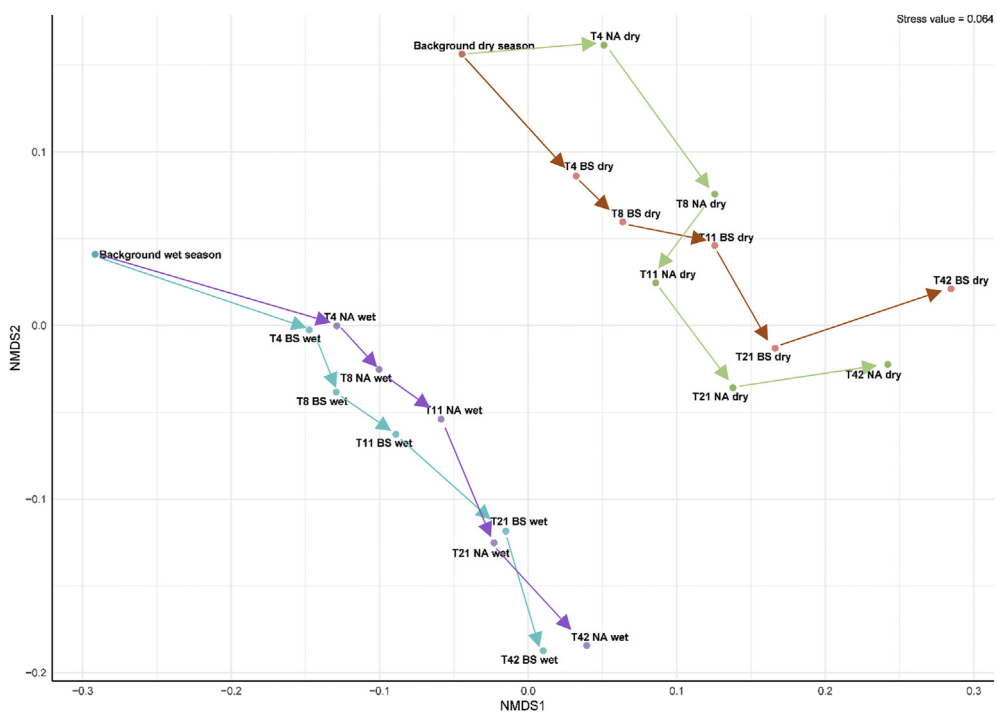

Fig. 2. NMDS plot. Background wet season and background dry season represent the original microbial community in the sediments. T0, T4, T8, T11, T21 and T42 represent the sampling events at days 4, 8, 11, 21, and 42, respectively, for microbial community characterization. Dry and wet correspond to the 28 °C and 18 °C experiments, respectively.

degrading various hydrocarbons as the experiments proceeded. It is interesting to note that although high degradation rates of TPH were observed in both the wet and the dry season experiments, the rates were determined by different microbial communities in each case. As shown in the genera heatmaps (SI), the background microbial composition in the dry season experiments showed that few genera dominated the community with *Arthrobacter* composing 25.7% of the total community, followed by *Glaciecola*, *Psychrosphaera*, *Alteromonas* and *Pseudomonas* at 14.8%, 7.1%, 5.7% and 4.6%, respectively. All, except for *Psychrosphaera* that has limited information regarding its function, are known to be capable of efficient degradation of petroleum hydrocarbons, with the latter 2 being known for their specific involvement in PAHs degradation (Dasgupta et al., 2013; Kumari et al., 2016; Lee et al., 2014; Math et al., 2012; Ren et al., 2018; Tremblay et al., 2017). On the other hand, the wet season sediments showed rather a similar distribution of few microbial genera, with the most abundant being *Pseudomonas*, *Achromatium*, *Oleiphilus* and *Alteromonas* at 8.3%, 7.6%, 6.1% and 5.1%, respectively, all of which are known to be hydrocarbon degraders except for *Achromatium* for which no such information is previously reported (Hamdan et al., 2019; Mangwani et al., 2015; Math et al., 2012). The presence of a large number of potential hydrocarbon degraders in the original wet and dry season sediments is correlated to lingering oil pollution along the shoreline of Lebanon, including the site from where sediments were collected for this study (Hamdan et al., 2019). In both experiments, however, a consortium of hydrocarbon degraders evolved to efficiently consume the available petroleum organics contaminating the sediments. This indicates that irrespective of the original composition of the microbial community, the overall microbial community evolution is highly affected by the presence of contamination, and many hydrocarbon degraders might dominate later during the bioremediation process even if they were undetected in the background community. Indeed, it is suggested that different microbial consortia under defined conditions will converge irrespective of the inoculum seeds (El-Chakhtoura et al., 2014; Yates et al., 2012).

In the wet season experiments, the microbial structure was very similar between the biostimulation and natural attenuation treatments throughout the biodegradation experiments, with only slight differences being observed at sampling days 8 and 11. This indicates that the adaptation of the microbial community and its evolution was not heavily driven by the studied variable, being the addition of nutrients to the microcosms. This conforms with the similar observed petroleum hydrocarbons biodegradation profiles in the wet season experiments under both BS and NA treatments (section 3.1.2). In both treatments, the microbial community converged towards a similar structure at day 42 of the biodegradation experiments. These observations indicate that the change in the microbial structure was more correlated to the actual petroleum contamination within the sediments rather than the added nutrients, probably due to the relatively high background nutrients measured in the sediments in the wet season experiments, sufficient for maintaining an optimal biodegradation rates as previously discussed.

In the dry season experiments, the microbial structure showed an immediate deviation from the original composition observed under both biostimulation and natural attenuation conditions. The evolution of the microbial communities in this case followed separate pathways in each treatment with higher dissimilarities being observed at early stages of the biodegradation experiments, namely at days 4 and 8. This is correlated with the observed variation in hydrocarbons removal rates in each of the treatments (Fig. 1). This indicates that the addition of the nutrients in the biostimulation treatment had a major effect on the change of the microbial structure in the sediments during the biodegradation process of the oil hydrocarbons. This is justified when considering the initial low nitrogen (0.70 ± 0.30 mg/kg of wet sediment) and phosphorus (0.19 ± 0.02 mg/kg of wet sediment) concentrations measured in the sediments during the dry season, and which could have affected the evolution of the microbial communities under natural attenuation conditions as compared to the biostimulated microcosms. Although addition of nutrients usually stimulates the petroleum degradation activity of microbial consortia in environmental samples such as soils and sediments; other factors, such as the sediments characteristics and the hydrocarbon pollution itself, can reduce the effectiveness of the nutrient stimulation, leading to insignificant enhancement in the oil removal compared to the controls, and can sometimes even hinder the natural bioremediation process (Dias et al., 2012; Jiang et al., 2016; Kim et al., 2018; Wu et al., 2016).

3.2.2. Microbial dynamics

3.2.2.1. Enriched microbial taxa. Fig. 3 represents the variation of the microbial classes observed throughout the dry and wet season experiments. Detailed description of the microbial genera dynamics throughout the biodegradation experiments is presented in SI.

The class *Gammoaproteobacteria* comprised the majority of the microbial population identified in the wet season experiments and constituted 57.0% of the total background community. *Gammaproteobacteria* increased significantly after 4 days to reach 84.2% and 80.3%, gradually decreasing after that to reach 74.0% and 70.8% after 42 days in the BS and NA treatments, respectively. This class was significantly lower in relative abundance in the dry season experiments, with a larger variation observed between the BS and the NA treatments. *Gammaproteobacteria* in this case composed only 46.3% of the original background microbial community, increasing rapidly to a maximum of 84.2% for BS after 4 days, and to a maximum of 72.3% for NA after 11 days, decreasing gradually thereafter to reach 34.2% and 37.8% for BS and NA after 42 days, respectively. *Gammaproteobacteria* is a class of bacteria known for hosting most important obligate and generalist hydrocarbon degrading genera in marine environments (Romanenko et al., 2010; Gutierrez, 2017). Hence, such a high relative abundance of this class in the background microbial community indicates adaptation of the indigenous microbial ecosystem to the presence of hydrocarbons in the sediments, and permitted a rapid initiation of the oil biodegradation.

Alphaproteobacteria comprised a major portion of the microbial community in the dry season experiments, and was less prominent in the wet season experiments. According to Kim and Kwon (2010), there are contrasting reports on the impact of oil on the marine composition of *Alphaproteobacteria*, with certain studies indicating a decrease in the relative abundance of this class, and others indicating an increased abundance of specific hydrocarbon degraders. Kostka et al. (2011) indicated that following oil exposure, *Alphaproteobacteria* dominates marine sediments although to a lesser extent compared to *Gammaproteobacteria*. In this study, *Alphaproteobacteria* increased gradually with time in both the wet and the dry season experiments. In the case of the dry season experiments, *Alphaproteobacteria* increased from an original relative abundance of 10.5%–54% and 41% under BS and NA conditions after 42 days, respectively. The difference in both treatments in the dry season experiments was more noticeable compared to the difference observed in the case of the wet season experiments, in which *Alphaproteobacteria* increased slightly throughout the experiments from 9.3% to 15.8% and 16.3% after 42 days in BS and NA, respectively. These observations, coupled to the high bioremediation capacity of the sediments, namely in the dry season, indicate that

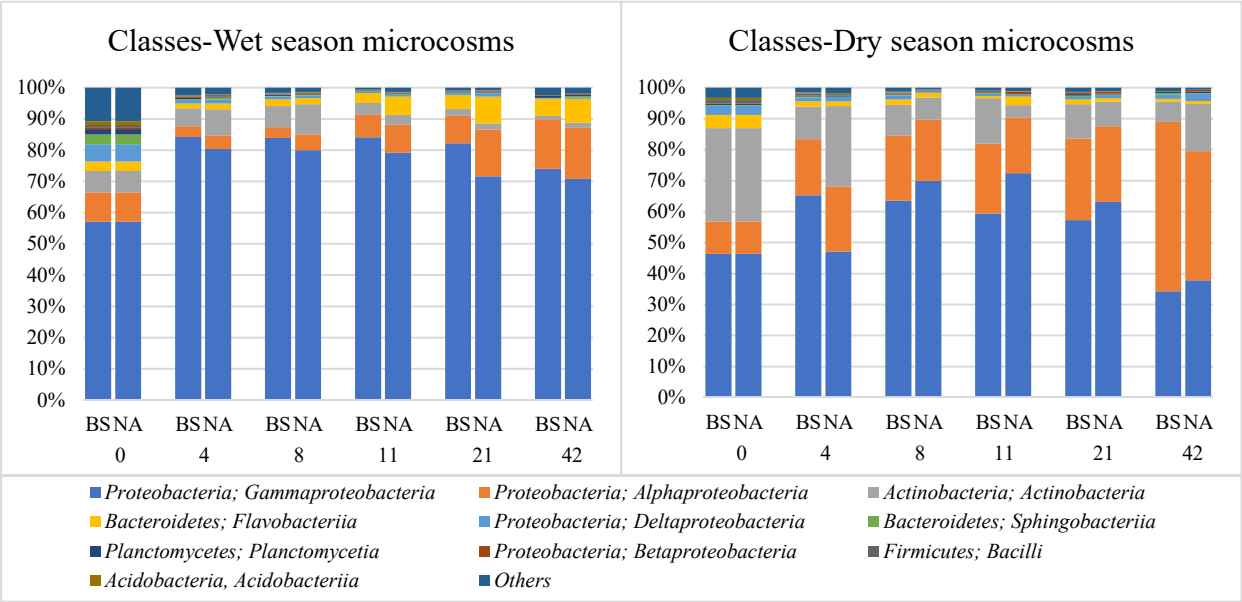

Fig. 3. Microbial classes evolution.

there is a major involvement of *Alphaproteobacteria* for hydrocarbons degradation. The increase of the class relative abundance in this case compared to the less prominent involvement in the wet season experiment could be due to specific characteristics of the background microbial community in the dry season experiments in relation to the existing nutrient levels in the sediments, allowing thus for a different microbial evolution pathway that facilitated proliferation of *Alphaproteobacteia*.

Actinobacteria was similarly highly represented in the dry season experiments compared to the wet season experiments. In the dry season experiments, *Actinobacteria* represented 30.0% of the original microbial community, decreasing consistently until reaching a minimum of 6.5% and 15.2% after 42 days under BS and NA conditions, respectively, further highlighting the difference in the microbial composition of the two dry season treatments affecting the biodegradation performance. In the wet season experiment, *Actinobacteria* also decreased gradually throughout the experiments, but from a very low relative abundance of 6.8% at the start to less than 1.5% in both BS and NA at after 42 days. *Actinobacteria* include members capable of aerobically and microaerobically degrading hydrocarbons in soils (Björklöf et al., 2009).

Deltaproteobacteria and *Flavobacteria*, with an original relative abundance in the dry season experiments of 2.9% and 4.3%, respectively fluctuated around 1% in thereafter and until the end of the experiments. However, in the wet season experiments, *Deltaproteobacteia* were negligible throughout the biodegradation experiments, with an original relative abundance of 2.9%, while *Flavobacteria* decreased slightly from 3.1% at the start of the experiments to 1.5% and 1.0% at day 4, to increase thereafter reaching 5.2% and 7.2% after 42 days under BS and NA conditions, respectively. *Deltaproteobacteria* include several anaerobic hydrocarbonoclastic bacteria that are able to metabolize a variety of alkanes as well as PAHs (Davidova et al., 2018). *Flavobacteria* also hosts several hydrocarbonoclastic bacteria capable of consuming hydrocarbon contamination from polluted sediments (Berry and Gutierrez, 2017).

To identify the OTUs which were significantly different in abundance among the different treatments, STAMP software (Statistical Analysis Of Taxonomic And Functional Profiles) was utilized by applying the Welch's 2-sided *t*-test with a confidence interval of 0.95 (Parks et al., 2014). Since most of the degradation of petroleum hydrocarbons occurred mainly during the first 2 weeks of operation in both the wet and the dry season experiments, bioinformatics statistical analysis was carried out on the microbial communities identified in the sediments that were sampled at early stages of the experiments, and until sampling day 11. The analysis was performed at the genus level. Fig. 4 shows statistical differences in microbial genera between BS and NA treatments corresponding to the dry season and wet season experiments.

The results show that, during the initial active biodegradation period of the dry and wet season experiments (up to day 11), only a few genera were significantly different in abundance among the respective BS and NA treatments. In the dry season experiments, among these significantly different genera between BS and NA, only *Marinobacter* was highly abundant. All other genera were practically negligible in abundance, each composing less than 0.1% of the total sediment microbial community at all times during the biodegradation experiments. *Marinobacter* was more significant under BS conditions compared to NA. It increased from 2.6% to a maximum of 15.4% and 13.3% after 4 days, then decreased slightly to reach 14.5% and 9.1% after 11 days, ultimately reaching 10.4% and 10.3 after 42 days of operation in BS and NA treatments, respectively. *Marinobacter*, a common and widely spread marine bacterial genus, is known for hosting efficient degraders of aliphatic and aromatic hydrocarbons (Duran, 2010; Handley and Lloyd, 2013). This explains such enrichment of this genus in the dry season experiments. *Marinobacter* was also abundant in the wet season experiments; however, there was no statistical difference between BS and NA. *Marinobacter* fluctuated in both NA and BS treatments in the wet season experiments between 4.8 and 8.0% throughout the biodegradation experiments. The significant increase in the abundance of *Marinobacter* under BS in the dry season experiments, compared to no significant increase in its abundance in the wet season experiment under BS, indicates that this genus was more active at higher temperature. This enrichment in the abundance of this hydrocarbon degrader could also explain the observed slight enhancement in the degradation of alkanes, which was observed in the BS microcosms in the dry season experiments compared to the

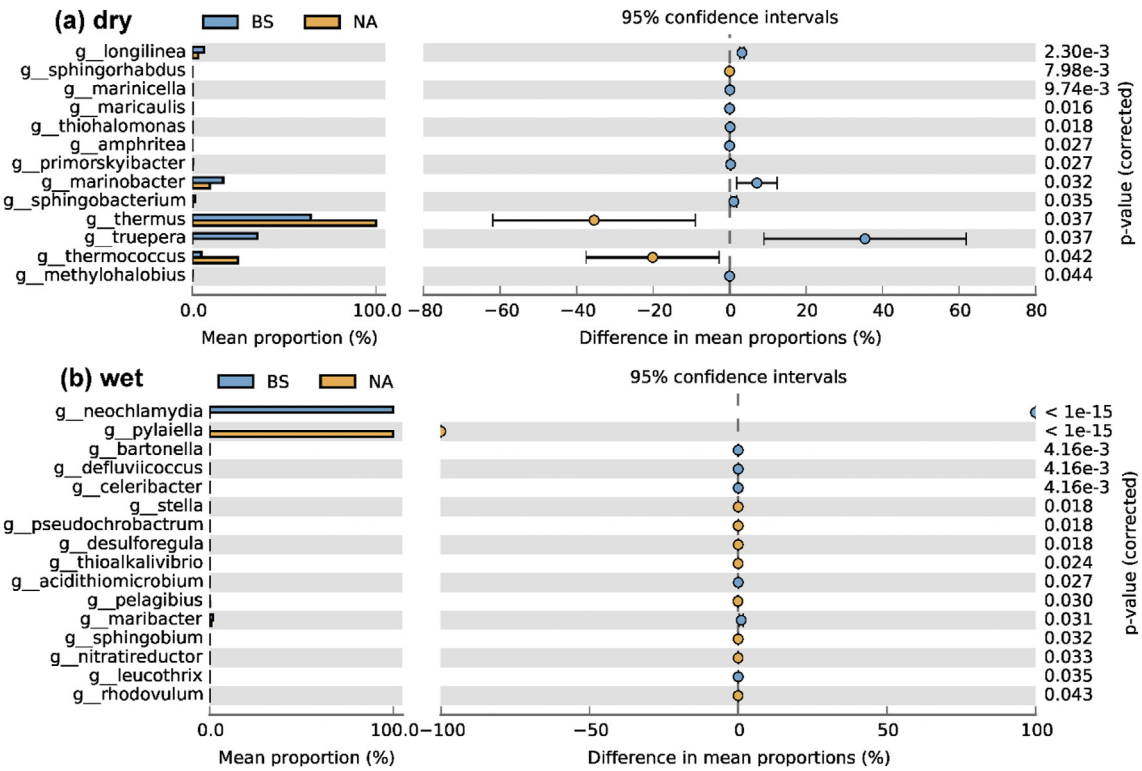

Fig. 4. Statistical differences in microbial genera between BS and NA in the (a) dry season and (b) wet season experiments.

NA microcosms.

For the wet season experiment, the genera which were found to be significantly different between BS and NA treatments Fig. 4b), were negligible in abundance in the overall composition of the sediment microbial community. This means that microbial communities under both NA and BS conditions were practically the same, with no observed variation among the most abundant genera. This also means that the biodegradation process in the wet season experiment was mainly driven by the contamination itself rather than by the addition of the nutrients. These results further confirm the observation that background nutrients concentrations in the sediments of the wet season experiments were sufficient to maintain high biodegradation rates, leaving no room for further enhancement under biostimulation conditions.

Detailed explanation about abundance and functions of the most abundant genera, which were active during the biodegradation process in both the wet and the dry season experiments, and which were found not to be significantly different between the respective NA and BS treatments, is included in SI. These genera mainly included *Glaciecola*, *Alteromonas*, *Pseudospirillum*, *Psychrosphaera*, *Pseudomonas*, *Kangiella*, *Idiomarina* and *Methylophaga* belonging to *Gammaproteobacteria*, *Litorimonas*, *Erythrobacter*, *Altererythrobacter*, *Algimonas*, *Maritimibacter*, *Primorskyibacter* and *Tropicibacter* belonging to *Alphaproteobacteria*, *Arthrobacter* and *Gordonia* belonging to *Actinobacteria*, and *Dokdonia* and *Winogradskyella* belonging to *Flavobateria*.

4. Conclusion

This study provided a detailed characterization of the microbial community structure and its evolution during crude oil biodegradation in contaminated marine sediments during the wet and dry seasons and under biostimulation and natural attenuation conditions. Significant variation in the microbial structure and evolution was observed between the wet and dry season experiments and was attributed to the initial difference in the original microbial structure in the collected sediments, affecting the evolution pathway of the microbial community in each case. A comparable microbial composition was observed under biostimulation and natural attenuation conditions in the wet season experiments and resulted in no significant variation in the biodegradation rates in both treatments. The diversion in the microbial structure between the biostimulation and natural attenuation treatments was more noticeable in the case of dry season experiments and was reflected by a moderate enhancement (33%) in the biodegradation rates of alkanes observed under biostimulation conditions. The complex combination and succession of the identified bacterial genera allowed for high removal rates of petroleum hydrocarbons that were comparable in both biostimulated and naturally attenuated microcosms during each of the dry and wet season experiments.

Main finding

Biodegradation of petroleum hydrocarbons under natural attenuation and biostimulation in marine sediments showed successive dominance of different hydrocarbonoclastic microbial genera.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2020.114858.

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