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# Assessment of Crude Oil Bioremediation Potential of Seawater and Sediments from the Shore of Lebanon in Laboratory Microcosms

Khaled Sakaya<sup>a</sup>, Darine A. Salam<sup>a\*</sup>, Pablo Campo<sup>b</sup>

<sup>a</sup>Department of Civil and Environmental Engineering, Maroun Semaan Faculty of Engineering and Architecture, American University of Beirut, P.O.Box: 11-0236, Riad El Solh 1107 2020, Beirut, Lebanon

<sup>b</sup>Cranfield Water Science Institute, Vincent Building, Cranfield University, Cranfield, Beds, MK43 0AL, UK

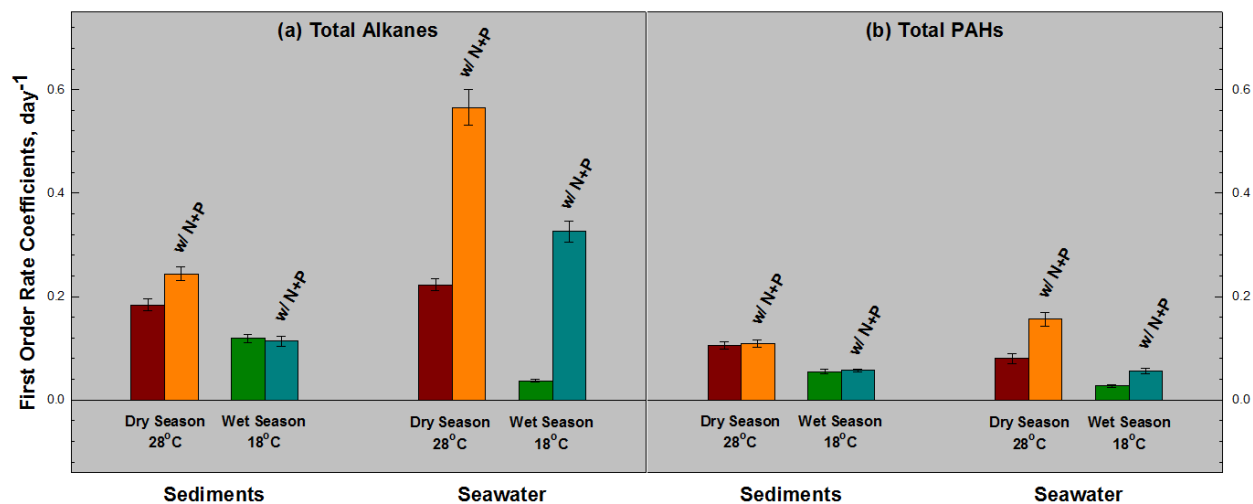
\*Corresponding Author: Darine A. Salam. American University of Beirut, Maroun Semaan Faculty of Engineering and Architecture, Munib and Angela Masri Bldg, M418. P.O.Box: 11-0236, Riad El Solh 1107 2020. Beirut, Lebanon. Email: ds40@aub.edu.lb, Phone: +961-1-350000-Ext: 3609, Fax: +961-1-744462

## ABSTRACT

With the planned oil and gas exploration activities off the coast of Lebanon, the risk of shoreline contamination with crude oil spills has become a major concern. This study aimed at assessing the crude oil bioremediation potential of the chronically polluted Lebanese shores in light of the continuous discharge of nutrient-rich sewage into the Mediterranean Sea and the long-lasting absence of proper sewage treatment systems. It was anticipated that, with the high pollution levels of the coastline, background concentrations of nutrients would be sufficient to sustain high intrinsic biodegradation rates without human intervention. Biodegradation experiments were conducted using crude oil-spiked beach sediments and seawater under natural attenuation and biostimulation conditions. The experiments were conducted at 18 and 28°C to account for seasonal variation in temperature, background nutrient levels, and microbial communities. The biodegradability of oil constituents – namely alkanes and polycyclic aromatic hydrocarbons (PAHs), was monitored over a 42-day period using gas chromatography-mass spectrometry (GC-MS). Under biostimulation conditions, significant enhancement in the overall biodegradation rates of alkanes and PAHs was observed in seawater at 18 and 28°C, while little to no improvement was measured at both temperatures in sediments where background nutrient levels were sufficient to induce near maximum intrinsic biodegradation rates. Under both natural attenuation and biostimulation treatments, the increase in temperature increased the oil biodegradation rates in sediment and seawater microcosms. In both instances, the overall trend in the biodegradation of individual alkanes and PAHs suggested a typical decrease in biodegradation rates with the increase in carbon number/rings and alkyl groups.

*Keywords:**Crude oil spills, bioremediation, background nutrient levels, marine sediments, seawater.*

# Graphical Abstract



## 1. INTRODUCTION

In 2010, the United States Geological Survey department (USGS) conducted a geological study on the Levant Basin Province, during which 1.7 billion barrels of recoverable crude oil and 122 trillion ft<sup>3</sup> of recoverable natural gas were estimated contained within the area, which includes Lebanon's 17,901 km<sup>2</sup> Exclusive Economic Zone (EEZ) (Schenk et al., 2010). In the outcome of this study, massive oil and gas exploration activities were planned off the eastern coasts of the Mediterranean Sea, including the shores of Lebanon, raising paramount concerns on the safety of its marine environment and highlighting the need to develop response options for the cleanup of potential oil spills.

Since the 1970's, bioremediation has proven environmental applicability, versatility, and efficacy in mitigating oil spills in marine environments as compared to the toxic, expensive, and partially successful physical and chemical remediation techniques (Nikolopoulou and Kalogerakis, 2010; Xu and Lu, 2010; Atlas and Bragg, 2009; Venosa and Holder, 2007; Röling et al., 2002; Atlas, 1995). Optimal rates of oil biodegradation depend on the presence of microorganisms capable of degrading petroleum hydrocarbons, as well as adequate supply of rate limiting nutrients (N and P) needed when significant amounts of hydrocarbons are released into the marine environment (Ron and Rosenberg, 2014; Tyagi et al., 2011; Röling et al., 2002). Therefore, developing suitable bioremediation strategies for the Lebanese shoreline should start by characterizing the biodegradation capacity of its water and beach substrates. While several studies addressed the microbiology of marine sites worldwide, a recent survey revealed a limited number of studies on the Southern coasts of the Mediterranean Sea, and a more restrictive number addressing its Eastern shores, including Lebanon (Daffonchio et al., 2013). To address this limitation, an EU-funded project (ULIXES) was established with the main objective of

increasing the knowledge of the bioremediation potential of contaminated sites from the Southern Mediterranean countries (Daffonchio et al., 2012). This leaves the Eastern shores of the Mediterranean, including Lebanon, ill-characterized.

The aim of this study was to assess the bioremediation potential of the Lebanese shores, in light of the major planned oil and gas extraction activities. Until today, little has been achieved regarding improvements in wastewater treatment and the continuous discharge of nutrient-rich raw sewage into the Mediterranean Sea along the shoreline of Lebanon. Therefore, it was anticipated that, in the short-run, the concentrations of nitrogen and phosphorus already present in the polluted areas along the shoreline are high enough to sustain high intrinsic oil biodegradation rates without human intervention. M. Rust and Linden O. (2008) conducted a field study on the distribution of petroleum hydrocarbons on the Lebanese shoreline after the major oil spill from the Jyeh power plant in July 2006. Three months after the spill, they measured petroleum hydrocarbon levels equivalent to background concentrations often found in coastal sediments due to anthropogenic sources (Rust and Linden, 2008). This suggests a high intrinsic oil biodegradation activity that could be attributed to high levels of nitrogen and phosphorous in the contaminated area.

To achieve the study objectives, biodegradation experiments were conducted under natural attenuation and nutrient-amended conditions (N and P) in oil-contaminated beach sediments and seawater under aerobic conditions. The experiments were carried out during the wet and dry seasons to account for the seasonal variation in temperature and nutrient levels. The biodegradation of oil constituents, namely alkanes and PAHs, was monitored in sacrificed microcosms throughout the experiments using gas chromatography – mass spectrometry (GC-MS), and the biodegradation rates of petroleum hydrocarbons were determined under both

natural and nutrients-enhanced conditions.

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## 2. MATERIALS AND METHODS

### 2.1 Samples Collection and Characterization

Eleven sites along the coast of Lebanon were selected to cover its beach sediments and seawater characterization during both the wet and the dry seasons (Figure S1). The designated sites consisted of sandy beaches and were distant from nutrient-rich effluent sources for a more representative characterization. At each sampling location, triplicate beach sediments and seawater samples were collected at water depths ranging from 30 to 50 cm, representative of the intertidal zone that is subjected to aerobic conditions.

Sediments and seawater samples were assessed for their nitrates-N, nitrites-N, ammonia-N, total Kjeldahl nitrogen, total nitrogen, phosphate-P, and total phosphorous contents by spectrophotometry using standard Hach methods. In the case of the sediments, the samples were first sieved using 2 mm pore size sieve, and aliquots of 30 g were then mixed with 100 mL distilled water at 300 rpm for 1 hour to extract the chemical compounds.

For the bacteriological analysis, sediment and seawater samples were processed for most probable number (MPN) analyses to determine the concentration of the hydrocarbon-degrading bacteria. For seawater, the samples underwent serial 10-fold dilutions in 96-well microtiter MPN plates, with 175  $\mu$ L of *Bushnell Haas* as a growth medium per well, and 2  $\mu$ L of crude oil per well as a carbon source for the bacteria. With respect to the sediments, 10 g wet weight aliquots were mixed in 90 mL of sterile detachment solution (1 g/L disodium pyrophosphate;  $\text{Na}_2\text{H}_2\text{P}_2\text{O}_7$ , and 20 g/L sodium chloride; NaCl) at 300 rpm for 1 hour, and the analysis was performed on the extract. The plates were incubated at 20°C for 14 days. Oil emulsion indicated positive results (Gómez-Ullate et al., 2008; Venosa et al., 1996). The purpose of the conducted bacteriological



analysis was to emphasize the existence of variations in microbial community concentrations that occur between the dry and the wet seasons, as well as between the different matrices used. It is however worth mentioning that bacteria are not the sole biodegraders of crude oil and that other microbial communities, namely fungal communities, play an important role in the oil bioremediation (Bovio et al., 2017). Identification of microbial communities potentially participating in crude oil biodegradation was beyond the scope of this study. The results of the chemical and bacteriological analyses of the beach sediments and seawater are presented in the supporting information (Tables S1-S6).

## **2.2 Crude Oil Characterization.**

Light Arabian crude oil supplied by Jordan Petroleum Refinery was used in the biodegradation experiments. Oil characterization was conducted to assess its physical and chemical properties and the results are presented in the supporting information (Table S7).

## **2.3 Biodegradation Experiments**

### ***2.3.1 Microcosms Preparation***

Two sets of batch biodegradation experiments were conducted. In the first set of experiments, seawater was used as the matrix in the biodegradation microcosms, while beach sediments were used in the second set. In each case, used seawater and sediment samples were collected from the shoreline of Beirut, being representative of the average characteristics of the Lebanese shoreline (Tables S1 and S6). To account for the seasonal variation in temperature and nutrient levels, biodegradation experiments were performed at 18 and 28°C, representative of shoreline temperature conditions attained during the wet and the dry seasons of the year, respectively. In each case, seawater and sediment samples from the shore of Beirut used for the

biodegradation experiments were collected directly after the shoreline characterization during the corresponding season to maintain similar characteristics to the ones from the same site previously characterized. The biodegradation microcosms consisted of 250 mL silanized shake flasks filled with 100 mL seawater for seawater microcosms, and 100 g (wet weight) of sieved beach sediments for sediments microcosms. In the latter case, 10 mL of seawater were added for ease of shaking and were replenished throughout the experiments as needed. Crude oil was spiked in all microcosms to achieve concentrations of 0.7 g/L of seawater and 0.7 g/kg of sediments, equivalent to 80  $\mu$ L of crude oil per flask. The oil loading was selected based on reported amounts of crude oil spills in aquatic environments (Sammarco et al., 2013), and previous conducted studies where similar spiked amounts showed to be fully degradable within the incubation period adopted in this study (Campo et al., 2013). The microcosms were covered with loose cotton plugs to allow oxygen flow through the cotton threads. The cotton plugs were removed for a short period of time on a regular basis throughout the experiments to ensure adequate aeration of the systems. The microcosms were continuously shaken at 200 rpm.

### **2.3.2 Treatments**

For each set of biodegradation experiments conducted at 18 and 28°C, two treatments were prepared: natural attenuation to quantify the effectiveness of the intrinsic biodegradation rates, and biostimulation via the addition of nutrients, to determine the effect of nutrients enhancement on the oil biodegradation rates. For the biostimulation treatment, nitrogen and phosphorous were added in the form of potassium nitrate ( $\text{KNO}_3$ ) and sodium triphosphate pentabasic ( $\text{Na}_5\text{P}_3\text{O}_{10}$ ), respectively. The final nitrogen and phosphorous concentrations in the biostimulated microcosms approached a C:N:P stoichiometric ratio of 100:5:1 for optimal oil biodegradation rates (Venosa et al., 2010; Venosa et al., 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 (UCSB ScienceLife, 2017; Nikolopoulou and Kalogerakis, 2009). No additional nutrients were introduced in the microcosms under natural attenuation conditions. For both treatments, oil biodegradation was carried out by the indigenous hydrocarbon-degrading microbial communities present in the seawater and beach sediments, with no added exogenous cultures.

### **2.3.3 Procedure**

Microcosm sampling events (11) were carried out throughout the biodegradation experiments which lasted 42 days. The sampling events took place at days 0, 2, 4, 8, 11, 14, 17, 21, 28, 35, and 42, during which triplicate seawater and sediments microcosms were sacrificed per each treatment. Therefore, a total of 132 ( $11 \times 3 \times 2 \times 2$ ) samples were prepared per each set of experiments. At each sampling event, residual oil in the sacrificed microcosms was extracted using dichloromethane (DCM) according to the methods described by Campo et al. (2013) and Venosa et al. (2010). Details of the residual oil extraction procedure are available in the Supporting Information.

### **2.3.4 Residual Hydrocarbon Analysis**

Oil biodegradation was monitored through the analysis of residual petroleum hydrocarbons (alkanes and aromatics) in sacrificed microcosms. The analysis was performed by GC-MS (Agilent Technologies 7890A GC- 5975C MSD) using an internal standard method described elsewhere (Campo et al., 2013). Alkanes included normal and branched aliphatics ranging in carbon number from 10 to 35, plus pristane, phytane, and hopane. Aromatics included 2-, 3-, and 4-ring PAH compounds and their alkylated homologs (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). Analyte concentrations were normalized to that of hopane, which is assumed to be non-biodegradable throughout the 42-day experiments, to eliminate initial differences in the oil concentration which might have occurred through oil losses during its loading in the microcosms (analysis at time  $t=0$ ) and later during the microcosms sampling and extraction (Campo et al., 2013; Venosa et al., 1996).

## 2.4 Statistical Analysis

The biodegradation rate coefficients of individual alkanes and PAHs were examined by means of a nonlinear regression analysis using R-Studio (version 1.1.442 - © 2009-2018 RStudio, Inc). The data were fit to a simple first-order model:

$$C_1 = C_0 \times e^{-kt}$$

where  $C_0$  and  $C_1$  are the initial concentration (mg/mg hopane) and the concentration (mg/mg hopane) at any given time  $t$  (days), respectively, and  $k$  is the first-order biodegradation rate coefficient ( $\text{day}^{-1}$ ).

Statistically significant differences were tested by Student's  $t$ -test. The null hypotheses being tested were: (1) no difference in total analyte concentrations exists between natural attenuation and biostimulation, and (2) no difference in the first order biodegradation rate coefficients of total analytes exists between natural attenuation and biostimulation.

## 3. RESULTS AND DISCUSSION

### 3.1 Shoreline Characterization

On average, the shorelines presented measureable concentrations of total nitrogen in both beach sediments (0.69 mg-N/kg) and seawater (0.31 mg-N/L) during the dry season. These levels were significantly increased during the wet season (2.72 mg-N/kg in sediments and 0.48 mg-N/L

in seawater) (Table S1). The continuous discharge of untreated sewage along the shoreline partakes in maintaining such measurable nutrient levels. Moreover, the close proximity of farmlands to the shores, especially in the south of Lebanon (Saida and Tyre), as well as the seafront landfills and open dumps along the shoreline, explain the significant increase in background nutrient levels during the wet season, due to increased agricultural and surface runoffs.

The bacteriological analysis reported average shoreline hydrocarbon-degrading bacteria concentrations of 127.47 MPN/g (wet weight) in sediments and 21.87 MPN/mL in seawater during the dry season (Table S6). Those concentrations were higher during the wet season mainly due to the higher organic nitrogen content in sediments, as well as the overall higher background nutrient concentrations observed in both sediments and seawater (Table S4 and S5). Those concentrations attained on average 182.97 MPN/g and 30.59 MPN/mL in sediments and seawater, respectively (Table S5). The results of the bacteriological analysis demonstrate the shoreline's intrinsic potential ability to mitigate oil pollution through existing hydrocarbon-degrading microbial communities. Moreover, the bacterial concentrations in sediments were around 6-fold greater than those found in seawater during both seasons. This difference is due to the dilution and wash out of petroleum hydrocarbons, nutrients, and microorganisms in seawater by the action of waves, as opposed to their adsorption and fixation by the sediments.

Background nutrient and hydrocarbon-degrading bacteria concentrations measured on the shore of Beirut during the dry and the wet seasons were representative of the average shoreline values (Tables S1 and S6).

### 3.2 Dry Season Experiments (28°C)

#### 3.2.1 Total Alkanes and PAHs

The biodegradation of total alkanes and total PAHs at 28°C in sediments and seawater under biostimulation and natural attenuation conditions is presented in Figure 1(a-d).. Concentrations of total alkanes and total PAHs at each sampling event were calculated by summing the hopane-normalized concentrations of the individual alkane analytes ( $n$ -C<sub>10</sub> to  $n$ -C<sub>35</sub> plus pristane, phytane, and hopane) and the individual aromatic analytes (2-, 3-, and 4-ring aromatics with their alkylated homologues), respectively, with each data point being the arithmetic mean of three independent replicates.

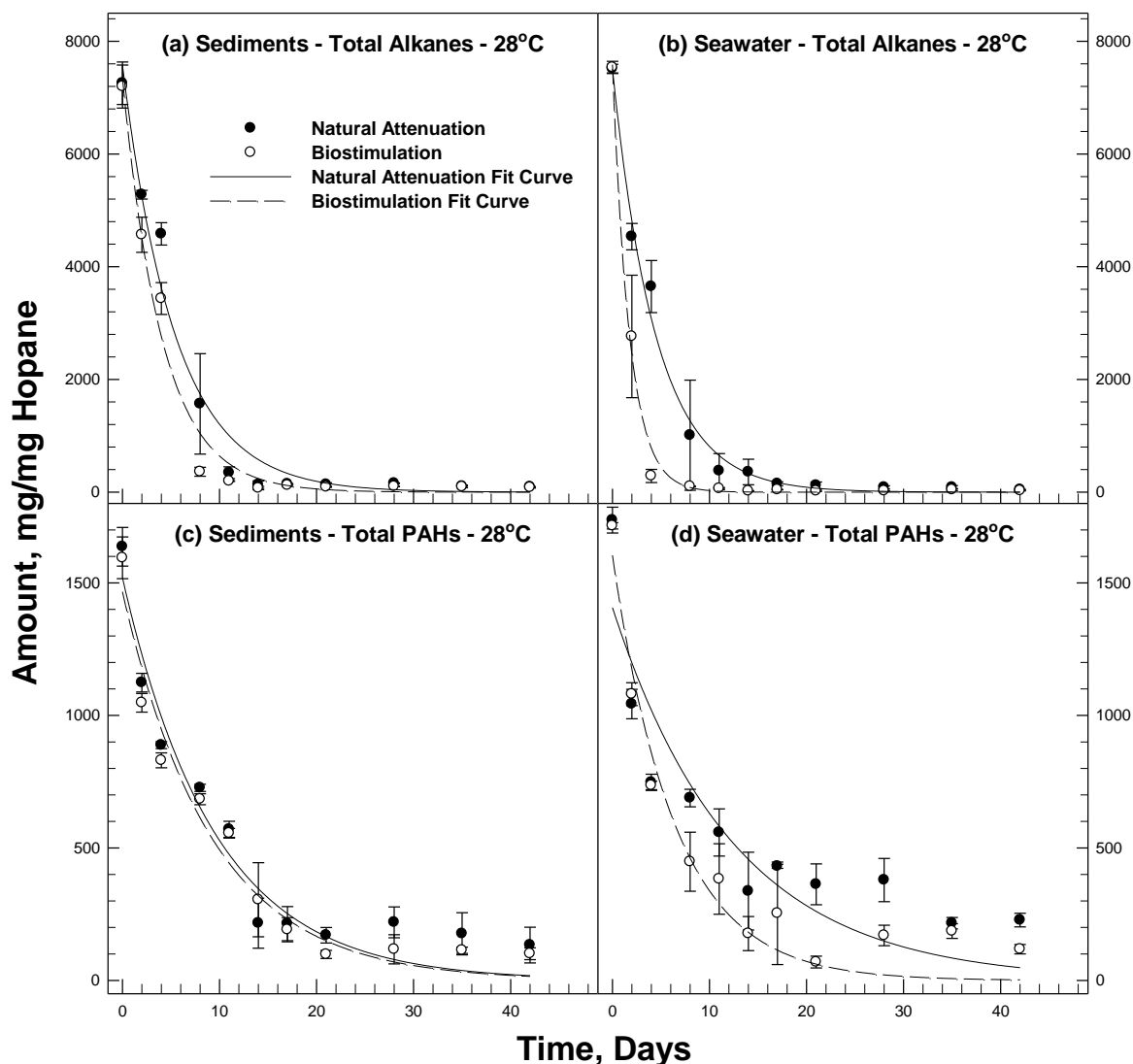


Figure 1. Biodegradation of total alkanes and total polycyclic aromatic hydrocarbons at 28°C, in marine sediments and seawater. Error bars represent  $\pm 1$  standard deviation unit.

### 3.2.1.1 Total Alkanes

It is evident from Figure 1(a) that the natural attenuation of total alkanes in sediments was slower than the nutrient-enhanced biodegradation at first (significant differences in concentrations ( $p < 0.05$ ) at Days 2, 4, and 8), but both treatments plateaued later on starting Day 14 at around 1.8% residual. The overall first-order rate coefficients ( $0.184 \text{ day}^{-1}$  for natural attenuation, and  $0.244 \text{ day}^{-1}$  for biostimulation) obtained through the nonlinear regression

analysis indicate a significant 33% increase in the biodegradation rate of total alkanes in sediments due to biostimulation as compared to the natural attenuation rate. In contrast with other studies, in the experimental oil spill field study conducted on the shoreline of Delaware Bay, the biodegradation of Bonny Light crude oil on the sandy beach was examined under natural attenuation conditions (background concentration of 0.82 mg of  $\text{NO}_3\text{-N/L}$  in interstitial pore water) and under nutrient-enhanced biodegradation (through the use of sodium nitrate  $\text{NaNO}_3$  and sodium triphosphate pentabasic  $\text{Na}_5\text{P}_3\text{O}_{10}$ ). The experiments, which were conducted in a temperate climate over the course of 98 days, resulted in a 115% enhancement in the biodegradation rate of total alkanes ( $\text{n-C}_{10-35}$  plus pristane and phytane) under biostimulation ( $0.056 \text{ day}^{-1}$ ) compared natural attenuation ( $0.026 \text{ day}^{-1}$ ) (Venosa et al., 1996). On the other hand, in the 180-day experimental oil spill field study conducted in a coastal Louisiana salt marsh, the biodegradation of Sweet Louisiana Crude Oil was investigated in salt marsh soils in control plots under natural attenuation conditions (nitrogen background concentrations were around 8.75 mg of  $\text{NO}_3\text{-N/L}$  and 4.3 mg of  $\text{NH}_4^+\text{-N/L}$  in porewater) and in plots fertilized with ammonium nitrate while others fertilized with a time-release urea. The study exhibited no improvement in the biodegradation rate of total alkanes ( $\text{n-C}_{10-36}$  plus pristane and phytane), which scored  $0.0059 \text{ day}^{-1}$  and  $0.0058 \text{ day}^{-1}$  under the application of ammonium nitrate and the time-release urea, respectively, compared to a rate of  $0.0054 \text{ day}^{-1}$  under natural attenuation conditions (Tate et al., 2012).

Similar observations can be made in Figure 1(b) with respect to the biodegradation of total alkanes in seawater, but with a more substantial gap between the two treatments ( $p < 0.05$  at Days 2 and 4). Both treatments reached a near-complete biodegradation extent, but at different instances (Day 8 for biostimulation vs. Day 17 for natural attenuation). The overall first-order



rate coefficients ( $0.223 \text{ day}^{-1}$  for natural attenuation, and  $0.566 \text{ day}^{-1}$  for biostimulation), show a significant ( $p < 0.05$ ) 1.5-fold increase in the biodegradation rate of total alkanes in seawater under the effect of biostimulation. Table 1 summarizes the obtained overall first-order rate coefficients from the biodegradation experiments conducted at  $28^{\circ}\text{C}$ .

Table 1. Summary of the biodegradation rate coefficients, standard errors of the mean (SEM), and curve-fitness data ( $R^2$ ) of the experiments conducted at  $28^{\circ}\text{C}$ .

		Alkanes			PAHs		
		Rate Coefficient	SEM	$R^2$	Rate Coefficient	SEM	$R^2$
<b>Sediments</b>	Natural Attenuation	0.184	0.012	0.965	0.106	0.007	0.941
	Biostimulation	0.244	0.013	0.979	0.109	0.007	0.955
<b>Seawater</b>	Natural Attenuation	0.223	0.012	0.977	0.080	0.009	0.815
	Biostimulation	0.566	0.035	0.979	0.156	0.013	0.930

The increase in the overall biodegradation rate of total alkanes in both sediments and seawater under biostimulation conditions can be attributed to the increase in nitrogen and phosphorous levels available to the hydrocarbon degraders, which has presumably improved their metabolic activity (Das and Chandran, 2011). The more pronounced effect of biostimulation in the case of seawater is associated with a relatively lower background nitrogen level ( $0.40 \text{ mg-N/L}$ ) in this matrix, allowing for a substantial biodegradation rate improvement with the addition of nutrients. This was not the case in sediments where the background nitrogen level of  $2.8 \text{ mg-N/L}$  of interstitial pore-water, was relatively high enough to maintain near-maximum biodegradation rates as compared to required nitrogen concentrations reported in the literature ( $2\text{-}10 \text{ mg-N/L}$ ) for optimum biodegradation (Venosa et al., 2010), which left minimal room for rate-improvement via the addition of exogenous nutrients.

It is interesting to note that, under biostimulation conditions, the overall biodegradation rate of total alkanes was significantly higher ( $p < 0.05$ ) in seawater as compared to their biodegradation rate in sediments. Dissolved nutrients are more readily available to the microorganisms in seawater than in sediments where the dissolved state and the attached state of the nutrients are exchangeable via the adsorption/desorption processes, making them less bioavailable to the active microbial communities at all times (Liang et al., 2013). This explains the slower biodegradation rates of total alkanes in sediments. Moreover, oil dispersion into fine droplets in seawater microcosms under the effect of mixing increased the oil surface area for the attachment of microorganisms, enhancing the biodegradation rates (Daffonchio, 2012). While the partitioning of oil on sediments also increases the surface area available for microbial activity, the limited aqueous phase in the sediment microcosms (as opposed to seawater microcosms) hindered the diffusion of the degradable hydrocarbons to the oil-water interface where their uptake by the hydrocarbon degraders occurs, leading to slower biodegradation rates (Tyagi et al., 2011; Atlas and Bragg, 2009).

### 3.2.1.2 Total PAHs

Regarding total PAHs in sediments, Figure 1(c) exhibits both treatments almost overlapping, and plateauing starting Day 17 at relatively considerable residual amounts (12-13% residual). The first-order rate coefficients (Table 1), which were quite similar in magnitude ( $0.106 \text{ day}^{-1}$  for natural attenuation and  $0.109 \text{ day}^{-1}$  for biostimulation), showed a statistically insignificant ( $p > 0.05$ ) 3% increase in rate under the impact of biostimulation. These rates were higher than the total PAH ( $\text{C}_{0-4}$ -naphthalene,  $\text{C}_{0-3}$ -fluorene,  $\text{C}_{0-3}$ -dibenzothiophene,  $\text{C}_{0-3}$ -phenanthrene,  $\text{C}_{0-3}$ -naphthobenzothiophene,  $\text{C}_{0-2}$ -pyrene, and  $\text{C}_{0-2}$ -chrysene) biodegradation rates obtained in the Delaware study ( $0.021 \text{ day}^{-1}$  under natural attenuation vs.  $0.031 \text{ day}^{-1}$  under

biostimulation), which demonstrated however a higher degree in biodegradation rate enhancement through nutrient addition (50% increase vs. 3% increase) (Venosa et al., 1996).

In regards to seawater, the overall trend in Figure 1(d) depicts an enhanced biodegradation rate of total PAHs in seawater under biostimulation. Both treatments however did not achieve a near-complete biodegradation extent within the 42-day period. The statistically different ( $p < 0.05$ ) first-order rate coefficients ( $0.080 \text{ day}^{-1}$  for natural attenuation and  $0.156 \text{ day}^{-1}$  for biostimulation) show around a one-fold increase in biodegradation rate of total PAHs in seawater due to nutrients addition (Table 1).

The same interpretation to the disparate increases in rates due to biostimulation can be applied to total PAHs as to total alkanes. The overall biodegradation rates of total PAHs however are lower than those of total alkanes. This is mainly due to the more complex and more biodegradation-resistant ring-structures of PAHs as compared to the easily biodegradable straight-chain n-alkanes (Adeniji et al., 2017; Das and Chandran, 2011; Tyagi et al., 2011; Atlas, 1995).

### **3.2.2 Individual Alkanes and PAHs**

Figure 2(a-d) summarizes the first-order rate coefficients with respect to individual alkanes (a and b) and PAHs (c and d) at  $28^{\circ}\text{C}$ , in sediments and seawater.

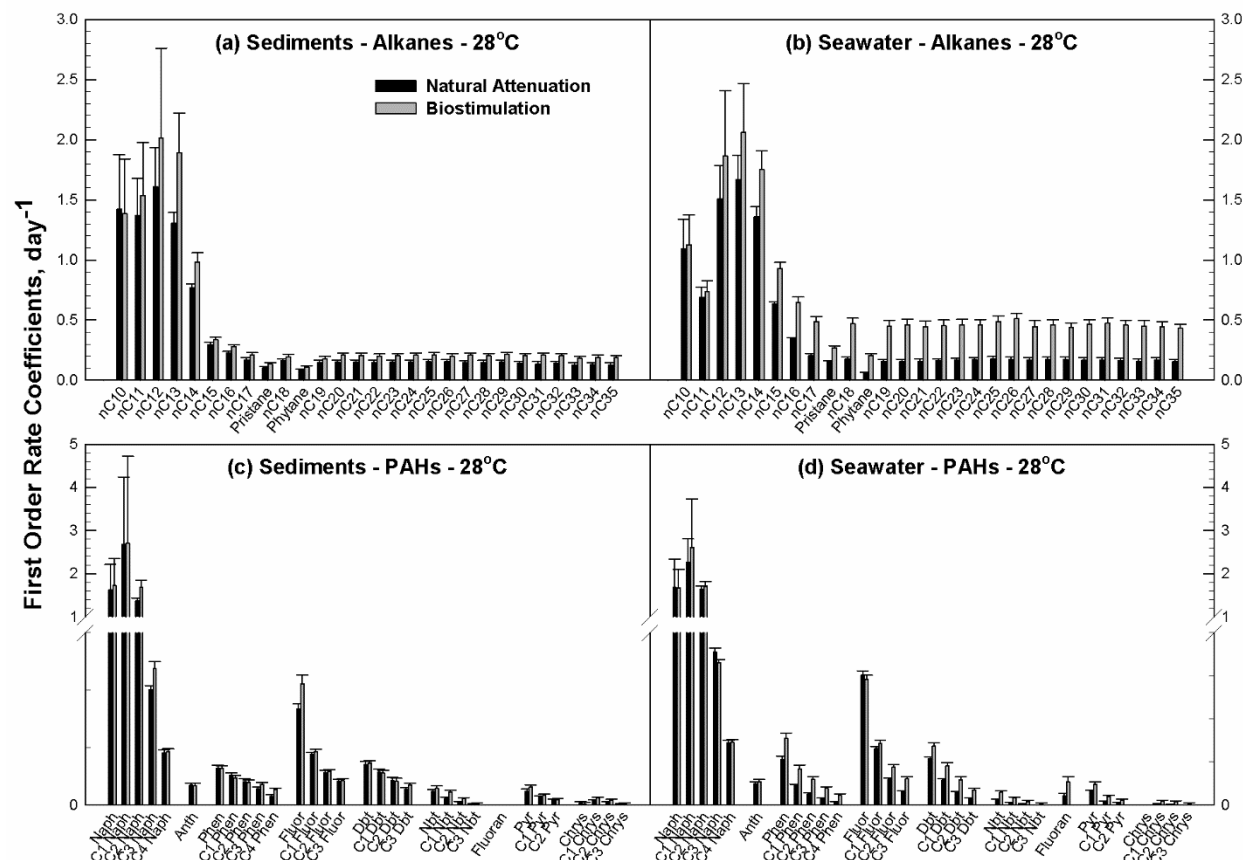


Figure 2. First-order biodegradation rate coefficients of individual alkanes and polycyclic aromatic hydrocarbons at 28°C, in marine sediments and in seawater. Error bars represent  $\pm 1$  standard error of the mean unit.

### 3.2.2.1 Individual Alkanes

In both sediments and seawater microcosms, the overall trend in the biodegradation of alkanes suggests a typical decrease in rate with the increase in carbon number, with biostimulation rates being slightly higher than the intrinsic rates in sediments [Figure 2(a)], and significantly higher in seawater (Figure 2(b)]. This trend is consistent with biodegradation behavior, as smaller hydrocarbon chains are more susceptible to biodegradation than longer chains (Venosa and Holder, 2007). Isoprenoid hydrocarbons (pristane and phytane) had relatively lower biodegradation rates under both treatments as opposed to the straight-chain alkanes. Isoprenoids are branched alkanes, which are more complex in structure than straight-

chain alkanes, and therefore more resistant to biodegradation (Campo et al., 2013; Venosa et al., 1996; Atlas, 1995). Deviation from the general trend was observed in the case of the lower carbon number alkanes  $n\text{-C}_{10}$ ,  $n\text{-C}_{11}$ , and  $n\text{-C}_{12}$  for which relatively lower biodegradation rate coefficients were reported. In general,  $n$ -alkanes with carbon number less than 15 can be lost by volatilization (Adeniji et al., 2017). This is expected to have reduced the initial amounts of the lower carbon chain alkanes ( $n\text{-C}_{10}$ ;  $n\text{-C}_{11}$ ;  $n\text{-C}_{12}$ ) at early stages of the experiments setup. Hence, the obtained rates for these particular alkanes cannot be attributed fully to biodegradation, which rather constituted the major pathway for the removal of higher chain alkanes.

### 3.2.2.2 Individual PAHs

The biodegradation of PAHs at 28°C exhibited similar trends in both sediments and seawater, suggesting a decrease in biodegradation rate with the increase in carbon rings and with the increase in alkyl groups on the ring structure, which is a typical biodegradation behavior [Figure 2(c-d)]. Some exceptions pertaining to naphthalene occurred in both sediments and seawater, where it had lower biodegradation rates than its alkylated homologues under both treatments. This could be mainly due to its high volatility, which has mostly affected the obtained removal rates of naphthalene rather than biological processes. The biodegradability of some of the 4-ring aromatics (fluoranthene, and  $\text{C}_0$ -chrysene) couldn't be properly evaluated due to their extremely low initial concentrations in oil. Overall, the biodegradation rates of PAHs in sediments were similar in magnitude under both treatments, while in seawater, rates were enhanced due to nutrients addition.

### 3.3 Wet Season Experiments (18°C)

#### 3.3.1 Total Alkanes and PAHs

The biodegradation of total alkanes and total PAHs at 18°C in sediments and seawater under biostimulation and natural attenuation conditions is summarized in Figure 3(a-d).

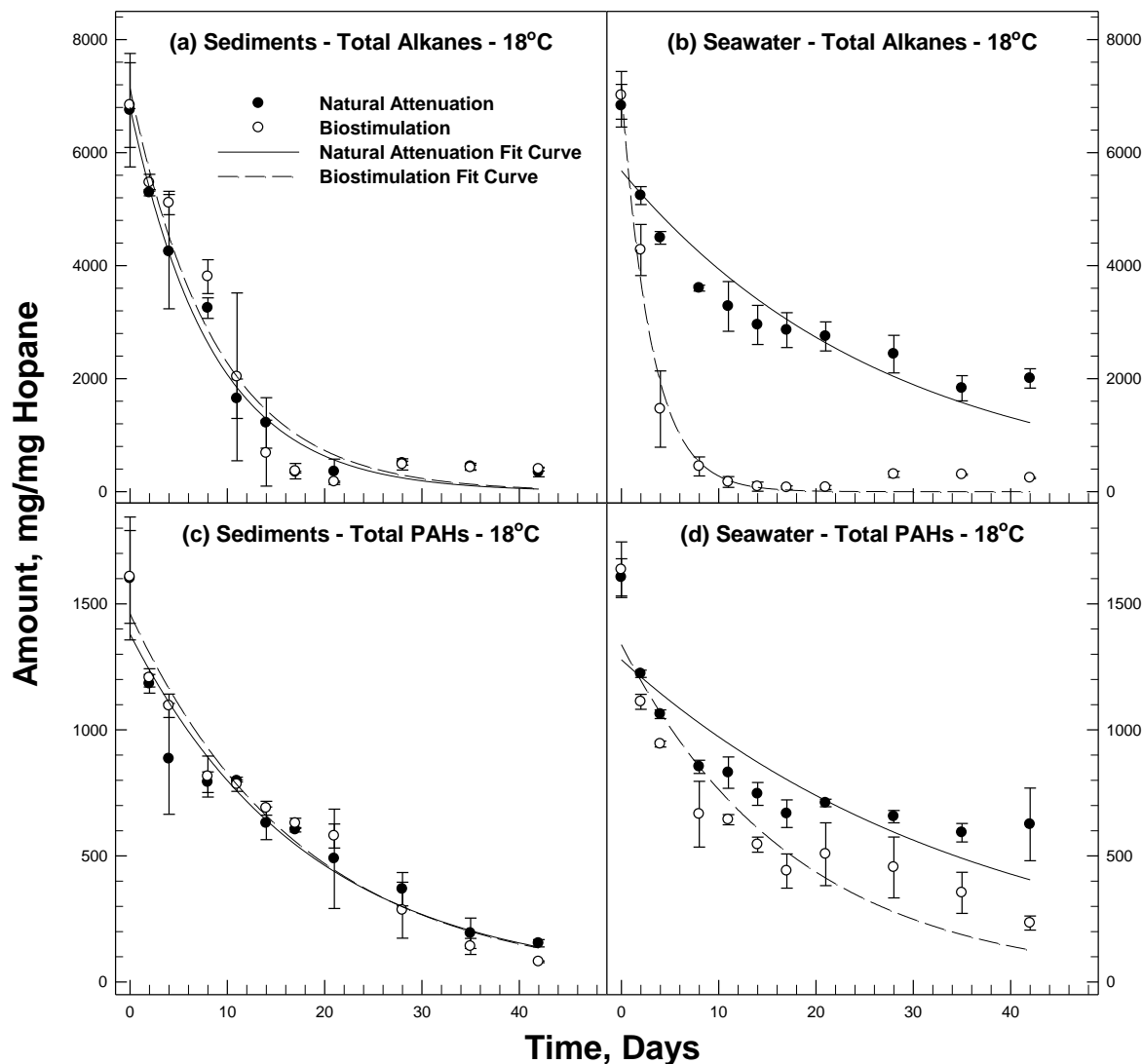


Figure 3. Biodegradation of total alkanes and total polycyclic aromatic hydrocarbons at 18°C, in marine sediments and seawater. Error bars represent  $\pm 1$  standard deviation unit.

### 3.3.1.1 Total Alkanes

In sediments, no differences in concentrations of total alkanes between the two treatments was observed at all instances ( $p > 0.05$ ) [Figure 3(a)], which illustrates the non-effectiveness of biostimulation. Also, both treatments reached near-complete total alkanes biodegradation at Day 21 (7 days behind the similar dry season experiments), and plateaued onwards. The almost equal ( $p > 0.05$ ) first-order rate coefficients ( $0.119 \text{ day}^{-1}$  for natural attenuation and  $0.114 \text{ day}^{-1}$  for biostimulation) confirm these observations. Those rates were lower in magnitude than the overall biodegradation rates of total alkanes in sediments at  $28^\circ\text{C}$ .

On the other hand, Figure 3(b) clearly depicts the substantial effect of biostimulation in enhancing the biodegradation of total alkanes in seawater at  $18^\circ\text{C}$ . Biostimulation plateaued at Day 11 (3 days behind the dry season experiments) at a near-complete biodegradation extent (3% residual alkanes), while 29% of the initially added alkanes amount was still available after 42 days of the biodegradation experiments under natural attenuation. The first-order rate coefficient measured under nutrient enhanced conditions was equal to  $0.326 \text{ day}^{-1}$  representing an almost 8-fold increase in alkanes biodegradation rates as compared to the intrinsic value  $0.037 \text{ day}^{-1}$ . Measured biodegradation rates under both treatments were lower than their respective values reported during the dry season experiments. Table 2 summarizes the obtained overall first-order rate coefficients from the biodegradation experiments conducted at  $18^\circ\text{C}$ .

Table 2. Summary of the biodegradation rate coefficients, standard errors of the mean (SEM), and curve-fitness data ( $R^2$ ) of the experiments conducted at  $18^\circ\text{C}$ .

		Alkanes			PAHs		
		Rate Coefficient	SEM	$R^2$	Rate Coefficient	SEM	$R^2$
<b>Sediments</b>	Natural Attenuation	0.119	0.008	0.951	0.055	0.005	0.879

	Biostimulation	0.114	0.010	0.922	0.057	0.003	0.945
<b>Seawater</b>	Natural Attenuation	0.037	0.003	0.835	0.027	0.003	0.716
	Biostimulation	0.326	0.020	0.973	0.056	0.006	0.827

The comparable biodegradation rates in the polluted sediments at 18°C under both natural attenuation and biostimulation treatments are associated with the high background nitrogen levels in the interstitial pore water (8.4 mg-N/L) measured during the wet season, which fall within the higher range of nitrogen concentrations necessary for maximum biodegradation rates (2-10 mg-N/L) (Venosa et al., 2010), and consequently left no room for biodegradation rates improvement under biostimulation. These background nitrogen levels were substantially higher than the pore water nutrient concentrations measured during the dry season (2.8 mg-N/L) and where the application of biostimulation resulted in 33% rate enhancement in the biodegradation of total alkanes.

Contrarily to the sediments, nutrients addition exceptionally enhanced the biodegradation rate of total alkanes in seawater mainly due to the relatively low background nitrogen levels in seawater observed during the wet season on the shore of Beirut (0.47 mg-N/L). While measured alkanes biodegradation rate was higher in the experiments conducted at 28°C, the effect of biostimulation was much more pronounced in seawater microcosms incubated at 18°C (1.5 vs. 8 folds increase in alkanes biodegradation rates at 28 and 18°C, respectively) despite the similar background nitrogen levels in seawater measured during both dry (0.40 mg-N/L) and wet seasons (0.47 mg-N/L). In general terms, biostimulation accelerated a relatively slow biodegradation process at 18°C, while it has improved an already well-operating biodegradation at 28°C, which gives more room for rate improvements during the wet season. The lower measured biodegradation rates of total alkanes in both sediments and seawater at 18°C as



compared to those measured at 28°C are attributed to a slower microbial activity at the lowest temperature. Temperature plays a crucial role in the biodegradation of petroleum contaminants. It directly affects the physiology and diversity of the microbial communities, as well as the chemistry of the pollutants (Tyagi et al., 2011; Das and Chandran, 2011; Nikolopoulou and Kalogerakis, 2009; Zkri and Chaalal, 2005).

A temperature sensitivity analysis was performed to estimate the differences in rates in the biodegradation of the hydrocarbon groups between similar treatments at the two temperatures to assess the effect of the 10°C temperature difference between the two sets of experiments. The temperature coefficient  $Q_{10} = \left(\frac{k_2}{k_1}\right)^{\frac{10^\circ\text{C}}{(T_2-T_1)}}$  where  $k_1$  and  $k_2$  represent the rate coefficients at the lower and the higher temperature, respectively, and  $T_1$  and  $T_2$  represent the lower and the higher temperature, respectively, was calculated to measure the temperature sensitivity of the biodegradation rates of total alkanes and total PAHs due to a 10°C difference in temperature. Table 3 summarizes the  $Q_{10}$  coefficients, which in this case reduce to  $\frac{k_{28}}{k_{18}}$  ratios, with respect to the biodegradation rates of total alkanes. The results of the sensitivity analysis indicate ratios revolving around 2 and 3, implying a typical biodegradation behavior as most biological reactions proceed at temperature coefficients  $Q_{10}$  between 2 and 3, meaning that reaction rates double or triple with every 10°C increase in temperature (Reyes et al., 2008). An exception pertaining to the biodegradation of total alkanes under natural attenuation conditions in seawater occurred and showed a temperature coefficient value of 6.027. Explanation to this occurrence could go back to the reasons previously discussed.

Table 3. Summary of the different rate coefficients ( $k$ ) and temperature coefficients ( $k_{28}/k_{18}$ ) for the biodegradation of total alkanes.

		Total Alkanes		
		k at 28°C	k at 18°C	k <sub>28</sub> /k <sub>18</sub> ratio
<b>Sediments</b>	Natural Attenuation	0.184	0.119	1.546
	Biostimulation	0.244	0.114	2.140
<b>Seawater</b>	Natural Attenuation	0.223	0.037	6.027
	Biostimulation	0.566	0.326	1.736

### 3.3.1.2 Total PAHs

In sediments, the overall biodegradation trend suggests no significant differences between the two treatments at any sampling event [Figure 3(c)]. Moreover, the relatively slow biodegradation of total PAHs under both treatments did not reach its full extent within the studied 42-day incubation period. The almost equal overall first-order rate coefficients (0.055 day<sup>-1</sup> for natural attenuation and 0.057 day<sup>-1</sup> for biostimulation) further confirm these observations (Table 2). These biodegradation rates were lower in magnitude than their corresponding rates in the dry season experiments.

On the other hand, Figure 3(d) depicts the positive effect of biostimulation on the biodegradation of total PAHs in seawater at 18°C, where significant differences ( $p < 0.05$ ) in PAH concentrations occurred starting Day 2, with biostimulation inducing around 1-fold increase in biodegradation rates (0.027 day<sup>-1</sup> for natural attenuation and 0.056 day<sup>-1</sup> for biostimulation) as shown in Table 2. Both treatments were not efficient enough to ensure near-maximum degradation extent within the 42 days of the experiments.

Similar interpretations of the obtained results can be applied to total PAHs as to total alkanes. The more complex structure of the PAHs however further decreased their biodegradation rates, and further hindered biodegradation rate improvements due to nutrients addition. This explains the non-effectiveness of biostimulation in enhancing the biodegradation of total PAHs in sediments, and the less significant enhancement in the biodegradation rate of

total PAHs in seawater as compared to total alkanes. The temperature sensitivity analysis (Table 4) demonstrates temperature coefficients indicative of typical biodegradation behavior against a 10°C increase in temperature (Reyes et al., 2008).

Table 4. Summary of the different rate coefficients (k) and temperature coefficients ( $k_{28}/k_{18}$ ) for the biodegradation of total PAHs.

		Total PAHs		
		k at 28°C	k at 18°C	$k_{28}/k_{18}$ ratio
<b>Sediments</b>	Natural Attenuation	0.106	0.055	1.927
	Biostimulation	0.109	0.057	1.912
<b>Seawater</b>	Natural Attenuation	0.080	0.027	2.963
	Biostimulation	0.156	0.056	2.786

### 3.3.2 Individual Alkanes and PAHs

Figure 4(a-d) summarizes the first-order rate coefficients with respect to individual alkanes (a and b) and PAHs (c and d) at 18°C, in sediments and seawater.

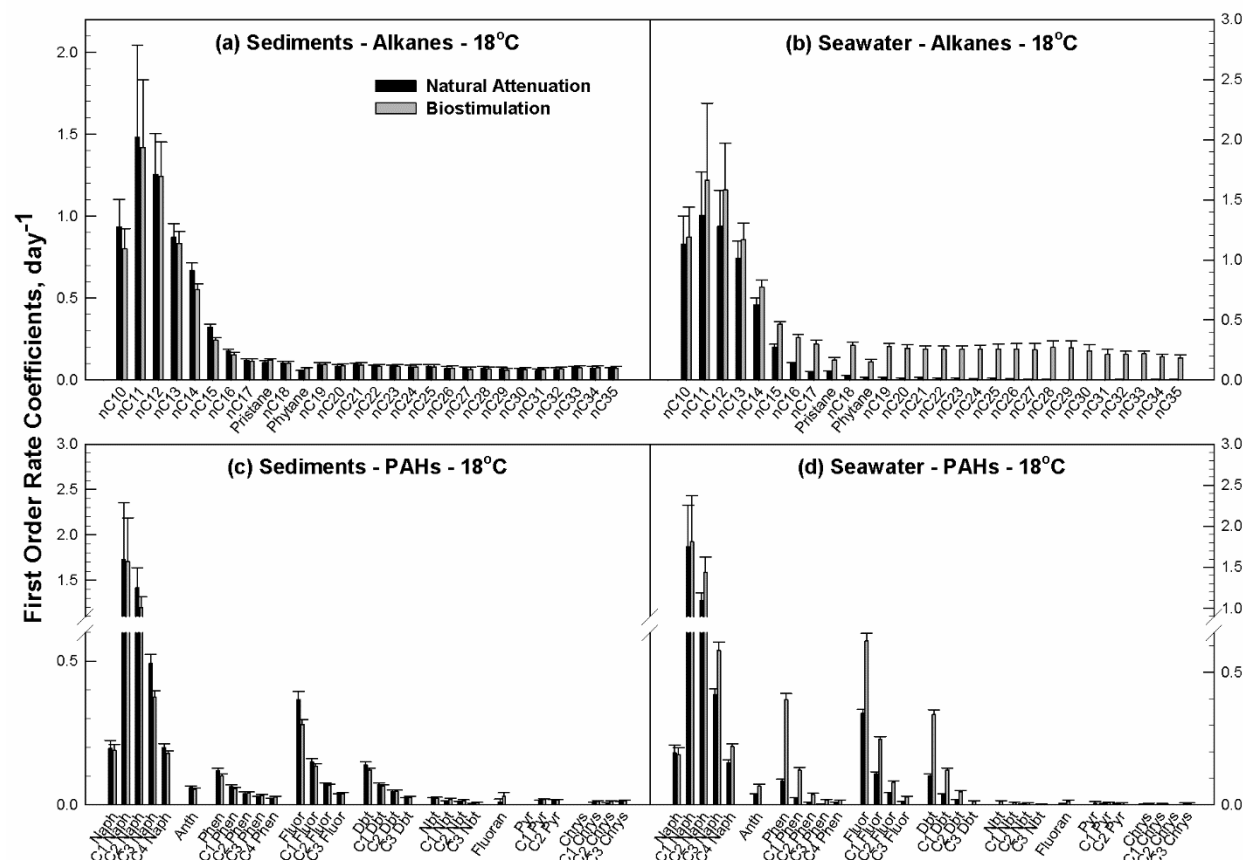


Figure 4. First-order biodegradation rate coefficients of individual alkanes and polycyclic aromatic hydrocarbons at 18°C, in marine sediments and in seawater. Error bars represent  $\pm 1$  standard error of the mean unit.

### 3.3.2.1 Individual Alkanes

Similar observations to the ones previously discussed concerning individual alkanes can be made from Figure 4(a and b), except that at 18°C, both treatments showed almost equal first-order rate coefficients in sediments, while in seawater, significant differences between the two treatments were expressed in the calculated biodegradation rates. Moreover, rates of both treatments in both matrices were relatively lower than the rates measured in the experiments conducted at 28°C. It is interesting to note that, the natural attenuation of the longer chain alkanes ( $n$ -C<sub>27-35</sub>) did not occur in seawater at 18°C.  $n$ -C<sub>20-40</sub> alkanes, usually called waxes, are hydrophobic solids at physiological temperatures which could explain their persistence.<sup>9</sup> Those

compounds were however fully degraded under the effect of biostimulation, justifying the significant improvement observed in the overall biodegradation rate of alkanes. Moreover, alkanes ranging between  $n\text{-C}_{22}$  and  $n\text{-C}_{32}$  exhibited an unusual small increase in concentrations in both sediments and seawater and under both treatments after Day 21, which was not observed at 28 °C. This issue was confronted by Venosa and Holder (2007) ( $n\text{-C}_{22-30}$ ) and by Pi et al. (2015) ( $n\text{-C}_{25-30}$ ), however no explanation to this occurrence was provided. An interesting explanation to this increase could relate to certain bacteria producing waxes while degrading crude oil (Tyagi et al., 2011). The faster microbial metabolic rate at 28°C could have countered the wax production rate which could explain why this occurrence was not observed at 28°C. On an individual basis, the temperature sensitivity analysis with respect to alkanes showed temperature coefficients around 2 and 3 particularly in the heavier alkanes ( $n\text{-C}_{17-35}$ ). Some exceptions in the lighter alkanes ( $n\text{-C}_{10-16}$ ) demonstrated insensitivity to the change in temperature ( $Q_{10} \sim 1$ ) which could imply that other processes (i.e. volatilization) or physical losses impacted the removal rates of those particular compounds at early stages of the experiments, as previously discussed.

### 3.3.2.2 Individual PAHs

Regarding the biodegradation of individual PAHs at 18°C, similar observations to the ones discussed at 28°C can be made from Figure 4(c and d), except that at 18°C, both treatments showed almost equal first-order rate coefficients in sediments, while an overall increase in rates was noticed in seawater under the effect of biostimulation. The measured rates were lower than the corresponding ones at 28°C. Moreover, the 3-ring PAHs ( $\text{C}_{0-4}$ -phenanthrene,  $\text{C}_{0-3}$ -fluorene,  $\text{C}_{0-3}$ -dibenzothiophene) demonstrated temperature coefficients typical of the biodegradation

behavior in face of 10°C change in temperature, unlike 2-ring PAHs (C<sub>0-4</sub>-naphthalene) and 4-ring PAHs (C<sub>0-3</sub>-naphthobenzothiophene, fluoranthene, C<sub>0-2</sub>-pyrene, C<sub>0-3</sub>-chrysene) which higher volatility and extremely low initial concentrations, respectively, led to their improper evaluation, as previously discussed.

## WORK SIGNIFICANCE

With the discovery of oil and gas and the planned exploration activities off the shore of Lebanon, the risk of oil spills should be seriously addressed. In this context, the bioremediation potential of the coast of Lebanon under different temperature conditions and nutrient concentrations was assessed. The results from this study would provide guidelines that can be used by policy makers and spill responders for an effective bioremediation of potential oil spills on the shoreline of Lebanon.

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# Assessment of Crude Oil Bioremediation Potential of Seawater and Sediments from the Shore of Lebanon in Laboratory Microcosms

Khaled Sakaya<sup>a</sup>, Darine A. Salam<sup>a\*</sup>, Pablo Campo<sup>b</sup>

<sup>a</sup>Department of Civil and Environmental Engineering, Maroun Semaan Faculty of Engineering and Architecture, American University of Beirut, P.O.Box: 11-0236, Riad El Solh 1107 2020, Beirut, Lebanon

<sup>b</sup>Cranfield Water Science Institute, Vincent Building, Cranfield University, Cranfield, Beds, MK43 0AL, UK

\*Corresponding Author: Darine A. Salam. 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. Email: ds40@aub.edu.lb, Phone: +961-1-350000-Ext: 3609, Fax: +961-1-744462

## Highlights

- Biostimulation significantly enhanced crude oil biodegradation rates in seawater
- Biostimulation did not enhance crude oil biodegradation rates in sediments
- High background nutrients ensured optimum biodegradation rates by natural attenuation.
- Biodegradation rates decreased with increasing carbon chains/rings and alkyl groups
- Biodegradation rates of alkanes and PAHs were higher at 28°C than at 18°C

# Assessment of crude oil bioremediation potential of seawater and sediments from the shore of Lebanon in laboratory microcosms

Sakaya, Khaled

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