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Emulating Deep-Sea Bioremediation: Oil Plume Degradation by Undisturbed Deep-Sea Microbial Communities Using a High-Pressure Sampling and Experimentation System

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Abstract: Hydrocarbon biodegradation rates in the deep-sea have been largely determined under atmospheric pressure, which may lead to non-representative results. In this work, we aim to study the response of deep-sea microbial communities of the Eastern Mediterranean Sea (EMS) to oil contamination at in situ environmental conditions and provide representative biodegradation rates. Seawater from a 600 to 1000 m depth was collected using a high-pressure (HP) sampling device equipped with a unidirectional check-valve, without depressurization upon retrieval. The sample was then passed into a HP-reactor via a piston pump without pressure disruption and used for a time-series oil biodegradation experiment at plume concentrations, with and without dispersant application, at 10 MPa and 14 °C. The experimental results demonstrated a high capacity of indigenous microbial communities in the deep EMS for alkane degradation regardless of dispersant application (>70%), while PAHs were highly degraded when oil was dispersed (>90%) and presented very low half-lives (19.4 to 2.2 days), compared to published data. To our knowledge, this is the first emulation study of deep-sea bioremediation using undisturbed deep-sea microbial communities.

Keywords: oil plume degradation; dispersant application; marine microbes; deep-sea; bioremediation; high-pressure sampler; high-pressure reactor

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1. Introduction

The risk of an oil spill accident in the deep-sea has increased in the past few years with the expansion of offshore drilling activity in ultra-deep waters [1]. To this day, different response strategies have evolved to treat oil spills on the sea surface, but little has been done for hydrocarbon releases in the deep-sea. The Deepwater Horizon (DWH) accident in the Gulf of Mexico in 2010 revealed the knowledge gap in subsea response strategies, and although more than 10 years have passed since then, it still provides insight for deep-sea accidents. The jetting of live crude oil that was released from the blowout preventer together with an unprecedented subsea dispersant injection led to the formation of small, neutrally buoyant oil droplets; more than 80% of the subsurface particles had diameters <70 µm [2]. These droplets were confined at depth within subsurface plumes [3–5], the largest and deepest of which was located at ~1000–1200 m depth and formed the deep plume [6].

It is estimated that about 41% of the hydrocarbons that were discharged during the DWH remained below the sea surface and were degraded [7]. Subsea dispersant application was associated with a decrease in the accumulation of surfaced oil above the

wellhead [8] and significantly enhanced the entrapment of oil in the water column. Since common response strategies could not be employed at these depths, hopes were pinned on microorganisms to combat the oil contamination. Microbial response to hydrocarbons was rapid after the spill [9,10] and indigenous microbial populations vigorously removed a major fraction of the deep plume of hydrocarbons [9]. Previous exposure of these microorganisms to oil contamination from natural seeps is believed to be responsible for their rapid response [11].

Following the DWH accident, numerous studies focused on oil bioremediation in the deep-sea to decode microbial metabolisms and provide biodegradation rates that could be applied in simulation models to predict the fate of oil in the case of a subsea oil spill. However, the collection of deep-sea microbial communities typically involved depressurization of the sample during retrieval and in most cases, subsequent incubation at atmospheric pressure [12–21]. In situ studies during DWH, using fluorometry, mass spectrometry, dissolved oxygen monitoring and next generation sequencing techniques have provided most of the documented data on microbial activity, accurately depicted the shifts in microbial communities, and pointed out the key microbial taxa that are involved in hydrocarbon degradation [9,22–29], without providing any information on biodegradation rates. Ever since, the interest for more accurate measurement of the metabolic activities of deep-sea microbial communities regarding oil degradation has led to studies where high-pressure is applied. In most of these studies, deep-sea samples are depressurized upon retrieval and re-pressurized in the lab [30–33].

In order to provide accurate and representative data on microbial processes in the deep-sea, pressure retaining samplers have been developed and used over the years for the collection and study of deep-sea microbial communities under in situ conditions [34–41]. These samplers are usually impractical, difficult to use and often quite expensive. In this study, we present a practical, low-cost apparatus for the deep-sea sampling of indigenous microbial populations without causing any pressure disruption upon retrieval and subsequent experimentation, in an effort to provide representative biodegradation rates. The sampling unit that is presented in this study, in association with a high-pressure experimental setup, has been successfully employed for the retrieval of deep seawater from the Eastern Mediterranean Sea (EMS) and the study of crude oil biodegradation rates. EMS faces new challenges, as existing oil and gas reserves in the area, along with the intensification of offshore oil and gas exploration activities in deep and ultra-deep waters, pose a threat for an accidental oil release in the deep-sea environment. Our current knowledge on microbial response in the deep EMS is still very limited [42–44] and the so-far available biodegradation rates derive from our previous work on enriched deep-water consortia that are grown at atmospheric pressure [44]. EMS consortia that were collected from surface and deep waters in our previous work showed that the latter resulted in a higher efficiency for hydrocarbon degradation within the first week following oil contamination [44]. In this study, we aim to investigate the microbial degradation of crude oil in the deep EMS under in situ conditions. For this purpose, seawater samples that were retrieved from a 600 to 1000 m depth were used for the emulation of a deep-sea oil release in the lab, in a time-series experiment where both crude oil and dispersed crude oil were introduced at plume concentrations, without decompression at any stage. To the best of our knowledge, this is the first attempt to provide a complete emulation study of deep-sea bioremediation using undisturbed indigenous microbial populations.

2. Materials and Methods

2.1. High-Pressure Sampling Apparatus

An engineered apparatus (HP-sampler) was designed for the collection of deep seawater without depressurization upon ascent (Figure 1). The HP-sampler is comprised of a 1 L stainless-steel tube (dimensions: 500 mm × Φ60.3 mm × 4 mm). The upper end of the tube carries a slot which closes with a screwed stainless-steel cap and seals tight with a rubber O-ring. Both the screwed cap and the closed end of the tube have integrated $\frac{1}{4}$″

valves (needle valve 1/4" NPT male, Parker Hannifin, Mayfield Heights, OH, USA) which are sealed tightly with liquid Teflon (Loxeal 55-14, LOXEAL Srl, Cesano Maderno, Italy). A unidirectional check valve (high-pressure relief valve, Parker Hannifin) is attached to the bottom-end needle valve to allow a flow of seawater inside the sampler. The check valve was set to open when ΔP was 0.2 MPa using a high-pressure piston pump (Model Isco 500D, Teledyne Isco Inc., Lincoln, NE, USA).

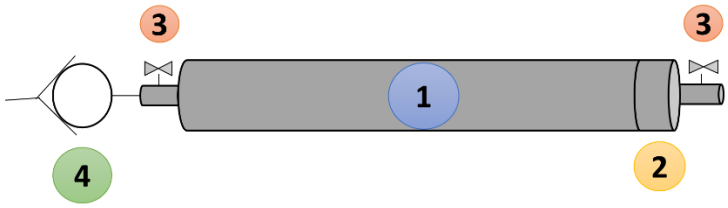

Figure 1. Schematic of the high-pressure sampler. (1) 1 L stainless-steel tube, (2) screwed stainless-steel cap, (3) integrated $\frac{1}{4}$" NPT male needle valves, (4) unidirectional check valve.

Two HP-samplers were pressurized on board with nitrogen gas at 60 MPa and were secured on the CTD rosette (Figure 2), an oceanography instrument that is used to measure the electrical conductivity, temperature, and pressure of seawater (CTD stands for conductivity, temperature, depth), which descended at a rate of 1 m/s. Pressurization was essential prior to sampling so that seawater would not be collected from the upper parts of the water column. When reaching a pressure of 60 MPa (600 m depth), the check valves opened and closed for every ΔP of 0.2 MPa, viz about every 20 m, until 1000 m depth, without reaching the bottom of the sampling station at 1185 m to avoid contamination of the seawater sample with sediments. Approximately 400 mL per sampler were collected within this depth range.

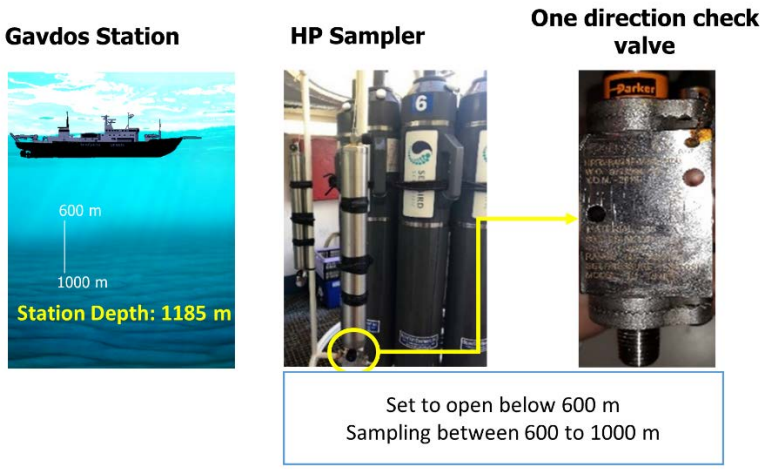

Figure 2. Schematic of the high-pressure deep-sea sampling. HP-sampler in the middle as attached to the CTD rosette on board. Close up of the one direction check valve on the right.

2.2. Sample Collection

Seawater samples were collected on board the R/V Aegaeo (Hellenic Centre for Marine Research) on 29 February 2020. The sampling station was located off South-east Crete, Greece (Gavdos station: 24.123194 E, 34.70177 N). The sampled depth range (600–1000 m) corresponded to an intermediate water mass (Transition Mediterranean Water; Theocharis et al. [45]) with an average temperature and salinity of 13.83 °C and 38.81 psu, respectively. The composition of microbial communities within the Transition Mediterranean Water were highly similar based on a PCoA analysis using the Bray–Curtis and Weighed UniFrac distances (data are provided in the Supplementary Materials, Figure S1). The samples were stored at 14 °C for 4 days until the oil biodegradation experiment started.

2.3. Time-Series Biodegradation Experiment

The Biochemical Engineering and Environmental Biotechnology lab at the Technical University of Crete is equipped with a High-pressure Reactor (HP-Reactor) (Parr Instrument Company, Moline, IL, USA) [46] with a maximum capacity of 1 L and able to achieve pressures up to 200 bar (20 MPa). The temperature is regulated with a cooling jacket and wave energy is emulated with an integrated stirrer. An electrode is used to monitor temperature.

Each of the HP-samplers described above were able to retrieve ~400 mL of seawater which was used for experimentation. For the present work, 300 mL of the collected sample was transferred via a high-pressure piston pump, without pressure disruption, into the HP-Reactor at 10 MPa pressure (Figure 3) and was incubated with crude oil at plume concentration (~1–10 ppm) for 77 days at in situ temperature (14 °C). The HP-Reactor was prefilled with light Iranian crude oil (density ~0.7821 g mL^{-1}) and 300 mL of sterile-filtered seawater that was collected from the sampling location using Niskin bottles that were pressurized at 10 MPa and acclimated at 14 °C prior to the sample transfer. The HP-Reactor operated at 60% capacity (600 mL total volume) and the stirrer was set at 100 rpm. For the first part of the experiment ("OIL" treatment), bioremediation was monitored for 35 days and the oil was replenished regularly to maintain the plume concentrations (~1 ppm). On day 35 (end of OIL treatment), ~450 mL of the volume in the HP-Reactor was collected for a metagenomic analysis of the microbial community, which will not be discussed in this work. The remaining volume was left intact in the HP-Reactor and acted as inoculum for the second part of the experiment, ("OIL + D" treatment). To regenerate the microbial community in the HP-Reactor, 400 mL of sterile-filtered deep seawater from the sampling station and light Iranian crude oil were added under pressure to replenish the volume inside the reactor, then left for a week before the start of the second part of the experiment. On day 42, oil and dispersant (1:25 v/v COREXIT™ EC9500A, COREXIT Environmental Solutions LLC, Sugar Land, TX, USA) were added under pressure (final concentration ~10 ppm) and replenished weekly. Bioremediation was monitored until day 77 to examine the effect of dispersant application. Samples were collected regularly from the bioreactor for a gas chromatography–mass spectrometry (GC-MS) analysis of the hydrocarbon concentrations. The microbial population was monitored via flow cytometry analysis.

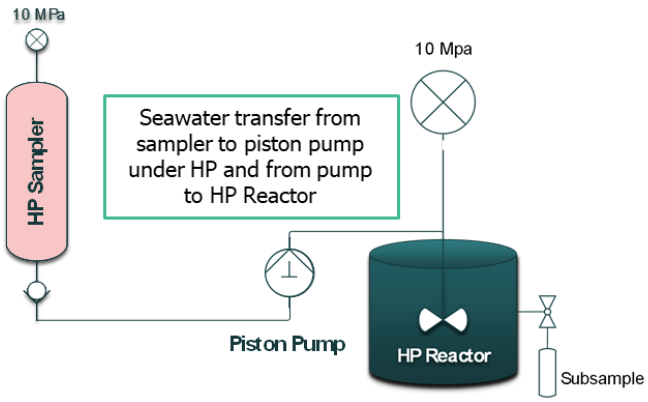

Figure 3. Schematic representation of the deep-sea water transfer from the HP-sampler to HP-Reactor in the lab.

2.4. Hydrocarbon Extraction and GC-MS Analysis

Liquid–liquid extraction was performed to obtain the microbial activity extract, free from the aqueous culture medium (deep seawater). For the extraction of the organic compounds, a small volume of dichloromethane (DCM Suprasolv®, Merck KGaA, Darmstadt, Germany) was used (3 × 5 mL for each extraction), in 100 mL Erlenmeyer flasks with a glass spout at the bottom. The flasks were shaken manually to assist the dissolution of the organic compounds in the solvent and the extract was collected in glass vials. Following solvent removal on a hot plate (~50 °C) with simultaneous nitrogen blow, the dried samples

were transferred to 4 mL vials with a small amount of DCM Suprasolv® and concentrated once again by evaporation at low heat on a hot plate (~50 °C) with simultaneous nitrogen blow. The solid extracts that occurred with weight >50 mg were then separated in saturated and aromatic hydrocarbon fractions by elution through SPE columns (Bond Elute TPH, Agilent Technologies, Inc., Santa Clara, CA, USA) with n-hexane Suprasolv® (Merck KGaA, Darmstadt, Germany) and DCM Suprasolv®, respectively. The rest were pre-concentrated to 100 μL glass inserts inside 2 mL GC-MS vials using a 1:1 mixture of DCM Suprasolv® and n-hexane Suprasolv® (Merck KGaA, Darmstadt, Germany). A GC-MS analysis was performed on an Agilent GC-MS HP 7890/5975C system, with an Agilent HP-5MS 5% phenyl methyl siloxane column (60 m × 250 μm × 0.25 μm). The hydrocarbon mixture consisted of an Oil Analysis Standard (Absolute Standards Inc.®, Hamden, CT, USA) containing 44 compounds, and a 17a(H),21b(H)-hopane (Chiron AS®, Trondheim, Norway). The standard composition of the hydrocarbon mixture was normal alkanes (C_{10}-C_{35}), pristane and phytane, and 16 polycyclic aromatic hydrocarbons (PAHs), fourteen of which classified as priority pollutants from the US EPA [47] (naphthalene, phenanthrene, anthracene, fluorene, dibenzothiophene, fluoranthene, pyrene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(e)pyrene, benzo(a)pyrene, perylene, indeno(g,h,i)pyrene, dibenzo(a,h)anthracene and benzo(1,2,3-cd)perylene).

2.5. Kinetic Evaluation

A kinetic analysis was used to estimate the degradation rates of the GC-MS detected hydrocarbon compounds. The biodegradation of crude oil is typically assumed to follow first-order kinetics, when hydrocarbons are consumed early in the process [48,49]. In order to compare the two treatments, we determined the degradation rate for each time frame. The bacterial cell concentrations differed between the two parts of the experiment, which should be taken into consideration when comparing biodegradation rates. We assume that the inorganic nutrients are not depleted because of the partial replenishment of water after each time frame so that first order kinetics apply. Hence, the concentration is given by Equation (1):

$$C(t) = C_0\, e^{-kt} \quad (1)$$

where C(t) denotes the concentration of hydrocarbons (ppm) at time t, t is the time expressed in days, C_0 is the initial concentration of hydrocarbons (ppm) and k is the apparent first-order degradation rate constant (day^{-1}), which depends on the average concentration of the hydrocarbon-degraders during the periods of interest.

The half-life ($t_{1/2}$), which represents the time that is needed for the C_0 to be reduced to half of its amount, was calculated by Equation (2):

$$t_{1/2} = \ln(2)/k \quad (2)$$

2.6. Flow Cytometry

For the flow cytometry analysis, the samples were fixated with 1% paraformaldehyde (4% stock) and 0.025% glutaraldehyde (25% stock) and appropriately diluted with 0.2 μM of filter-sterilized deionized water, in order to obtain a cell concentration of 10^6–10^7 cells mL^{-1}. Thereinafter, the microbial cells were stained with 1× thiazole green stain (1000× stock in DMSO, BIOTIUM, Fremont, CA, USA) and incubated for 15 min in the dark at room temperature. Microbial cell counts were measured on a CytoFLEX Flow Cytometer (Beckman Coulter Inc., Carlsbad, CA, USA) operating with CytoFLEX sheath fluid. CytoFLEX Daily QC Fluorospheres (Beckman Coulter Inc., Carlsbad, CA, USA) were used for the daily quality control of the instrument. The side-scatter (SSC) threshold was set to 10,000 (height) and a slow sample rate (10 μL min^{-1}) was selected for the analysis.

3. Results

3.1. Microbial Growth

Microbial growth was monitored throughout the experiment along with oil biodegradation. The microbial population grew ~20 times its original size in the first three days and by day 7 it had reached a maximum of 75 times its original size (Figure 4). Fluctuations during the OIL treatment may be attributed to the concomitant successional changes of the microbial community within the first time periods (0–3, 3–7 and 7–14). After day 14, the community seems to have reached a more stable phase. Even after removing a substantial amount of sample on day 35, a week of incubation was enough for the population to rehabilitate and return to its previous state. The addition of dispersant (OIL + D treatment) did not cause an increase in the population in the first three days, as observed in the beginning of the experiment, mainly because the community was already adapted to crude oil degradation. The stabilization of the cell densities after day 14 and throughout the second phase of the experiment coincided with a comparative stability in the community composition (data not shown).

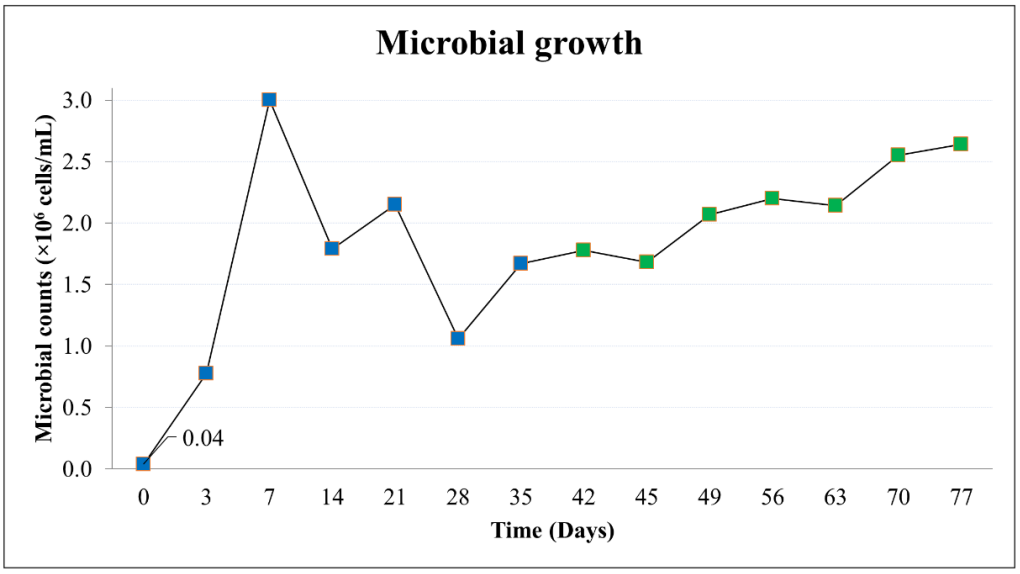

Figure 4. Microbial growth throughout the whole oil bioremediation experiment. Blue dots represent the microbial counts for the first phase of the experiment (OIL treatment), where only oil was added (0–35 days). Green dots represent the second phase of the experiment (OIL + D treatment), where oil and dispersant were added (42–77 days). Microbial counts on Day 0 denote the population of microorganisms in natural deep seawater.

3.2. Hydrocarbon Degradation

3.2.1. Concentration Diagrams

The results from the GC-MS analysis revealed an immediate degradation of crude oil at plume concentrations. The amount of dispersed crude oil that was recovered in the OIL + D treatment was higher due to limited losses from the adhesion of oil to the equipment parts, which translated in higher concentrations being measured by GC-MS. For the OIL treatment, the resulting total alkane concentrations that were determined by GC-MS were less than 1 ppm (Figure 5). For the OIL + D treatment, where oil was dispersed prior to addition, fewer losses were observed and the measured total alkane concentrations were almost 10 times higher (~10 ppm). Nevertheless, alkane degradation was rapid in both cases, as presented in Figure 5. Additional information about the total petroleum hydrocarbons (TPH) concentration are provided in the Supplementary Materials (Figure S2).

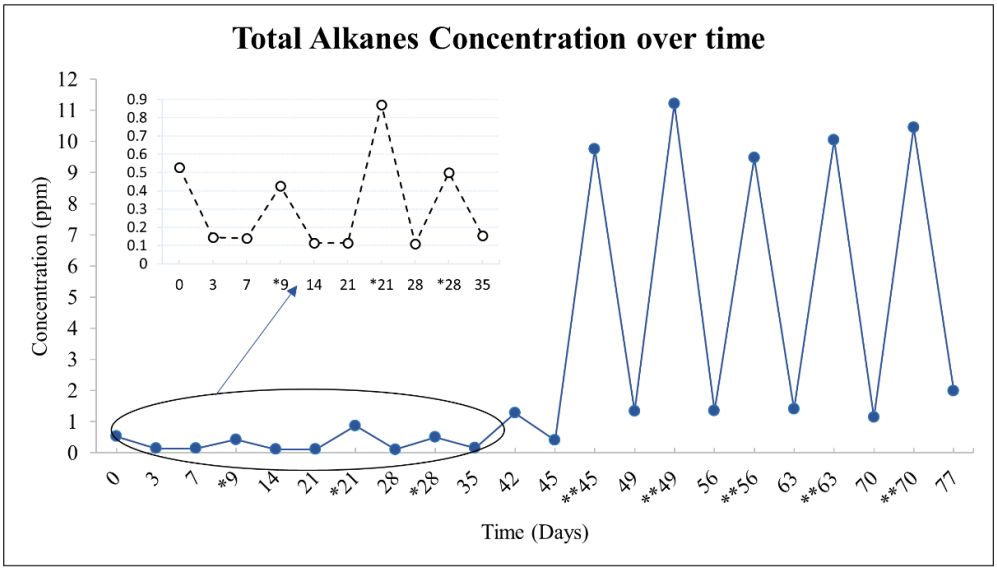

Figure 5. Concentration of the total GC-MS detected alkanes over the course of the experiment. * denotes the addition of oil and ** denotes the addition of oil and dispersant.

The measured polycyclic aromatic hydrocarbons (PAHs) concentrations were, as expected, lower than the alkanes. The concentrations of PAHs in the OIL treatment seem to stay constant at 61 ppb, without showing any significant decrease. For the OIL + D treatment (day 42 and thereafter), PAH concentrations after each replenishment were about 100 times higher than the corresponding ones in the OIL treatment (~1.2 ppm after the addition of the dispersed oil). The concentration of PAHs for the two treatments are shown in Figure 6.

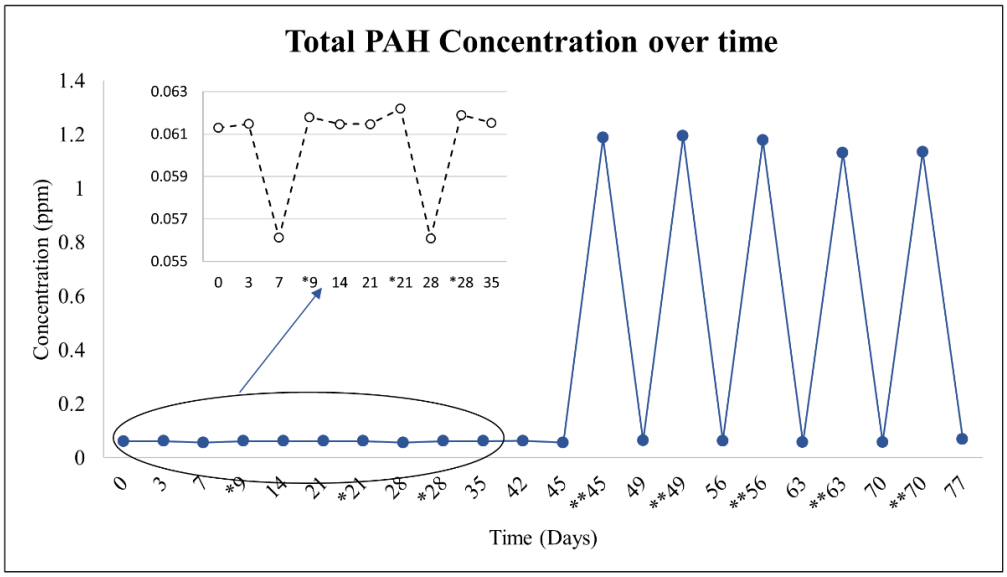

Figure 6. Concentration of the total GC-MS detected PAHs over the course of the experiment. * denotes the addition of oil and ** denotes the addition of oil and dispersant.

3.2.2. Degradation Efficiency

The degradation efficiency of the total alkanes and PAHs as detected by GC-MS is presented below (Figure 7A,B). Both crude oil and dispersed crude oil saturated compounds were rapidly consumed and resulted in significantly high degradation efficiencies. High degradation efficiencies (almost up to 90%) of the saturated compounds were achieved in both treatments. However, ~90% for the OIL treatment is achieved by the third week and

for the OIL + D treatment by the first week, which can be attributed to the acclimatization of the microbial community to oil degradation during the OIL treatment. Other than the microbial composition, dispersant application may have assisted achieving higher biodegradation efficiencies faster. PAH degradation was significantly enhanced with the dispersant addition (Figure 7B) and it reached more than 90% degradation efficiency after the 42–45 time period in the OIL + D treatment, while it was less than 10% for the OIL treatment. However, it should be noted that in the OIL treatment, PAH concentrations were extremely low to begin with (~61 ppb) and ~100 times lower than those of the second phase (~1.2 ppm). This means that biodegradation efficiency may have reached a limit and could not be properly assessed due to these initially low concentrations. Supporting data for the hydrocarbon degradation efficiency of each component can be found in the Supplementary Materials (Figures S3–S5).

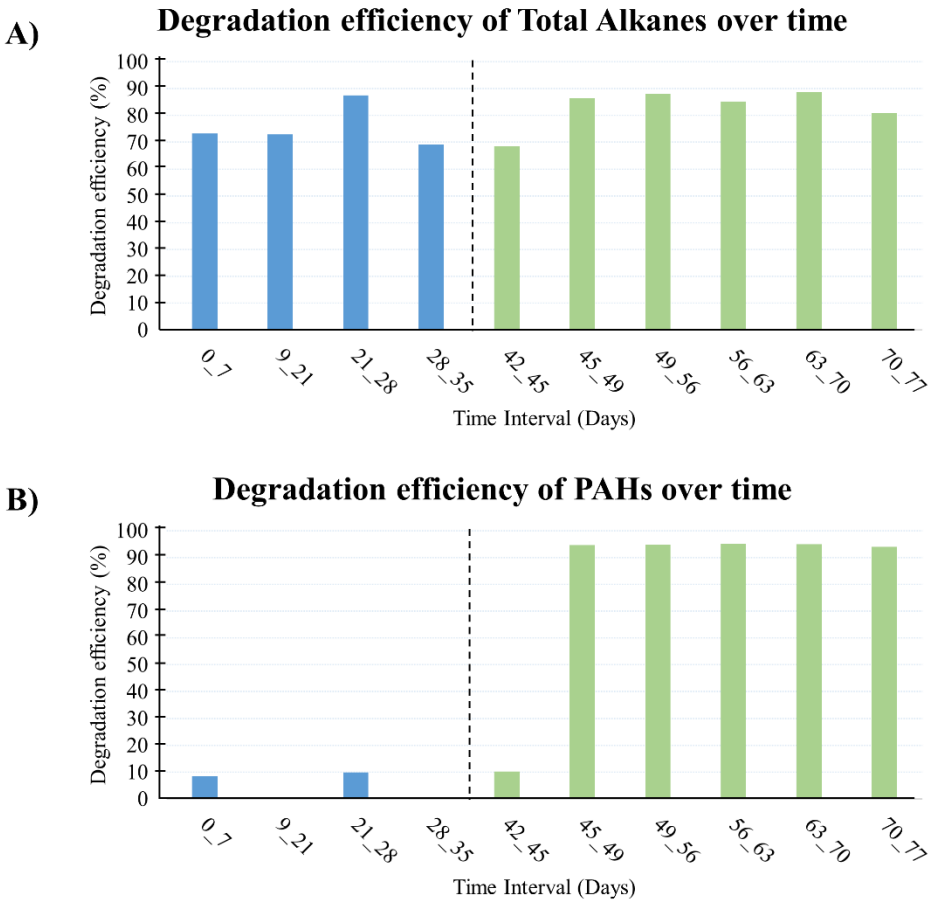

Figure 7. Total alkanes (**A**) and PAHs (**B**) degradation for every time interval. Blue bars represent the first part of the time-series experiment, where only oil was added (OIL treatment). Green bars represent the second part, where oil and dispersant were added (OIL + D treatment).

3.2.3. Oil Biodegradation Rates and Half-Lives

The results of the kinetic evaluation for the two parts of the experiment are presented in Tables 1 and 2. We have chosen to present only the comparable time frames of the two experiments, on days 0 to 3, which represent the initial microbial response to oil contamination, and days 21–28 and 28–35, due to the microbial community reaching relative stability, in terms of cell numbers and composition, in these time frames. Alkane degradation in all cases was a matter of a few days, with the lighter components (C_{14}–C_{25}) presenting half-lives of 0.6 to 3.2 days for both treatments and the heavier alkanes (C_{26}–C_{35}) ranging between 2.1 and 7.7 days. An essential factor that should be taken into consideration for these comparisons is the microbial concentration for the two treatments, as the apparent

rate is strongly dependent upon it. The microbial counts for the time period 0–3 in the OIL treatment are considerably lower than the corresponding ones for the OIL + D. Nevertheless, similar alkane biodegradation rates were observed. Dispersant addition enhanced the heavier alkanes (HA) degradation and lowered the half-lives of both HA and total alkanes. The reduction was more obvious for the time frame 28–35 where the half-life of HA was reduced by about 5 days. As expected, the half-lives of PAHs were distinctly high in the OIL treatment, however as previously stated, these results are the outcome of very low initial concentrations which may also interfere with degradation rates and consequent half-lives. In spite of this, it is safe to say that dispersant addition enhanced the rate of PAH degradation, as witnessed by the distinctive reduction in the half-life of PAHs between days 0–3 (42–45, $t_{1/2}$ = 19.4 days), where the microbial community was introduced to the dispersed crude oil, and the last two time frames 21–28 and 28–35 (63–70, 70–77, $t_{1/2}$ = 2.2 and 2.9 days accordingly), where the community was accustomed to dispersant application (Table 2). The TPH biodegradation rates for the 28–35 time frame and the corresponding half-life that is reduced to half (from 5.1 to 2.8 days) also support the enhancement effect of dispersant application. Part of the enhancement however could also be due to higher microbial concentrations for the OIL + D treatment. Supporting data for the hydrocarbon degradation and the half-lives of each component can be found in the Supplementary Materials section (Tables S1 and S2).

Table 1. Initial rates and half-life times for total alkanes (C_{14}–C_{35}); light alkanes (C_{14}–C_{25}) (LA); heavy alkanes (C_{26}–C_{35}) (HA); polycyclic aromatic hydrocarbons (PAH); and total petroleum hydrocarbon (TPH). The data refer to the GC-MS detected components of the crude oil for the OIL treatment of the time-series experiment. Negative values due to detection errors from the GC-MS analysis have been removed.

	Time Frames					
	0–3		**21–28**		**28–35**	
Crude Oil Components	k_{app} (Days^{-1})	$t_{1/2}$ (Days)	k_{app} (Days^{-1})	$t_{1/2}$ (Days)	k_{app} (Days^{-1})	$t_{1/2}$ (Days)
LA	1.2067	0.6	0.7940	0.9	0.3638	1.9
HA	0.2027	3.4	0.1802	3.8	0.0901	7.7
TA	0.4326	1.6	0.2981	2.3	0.1687	4.1
PAH	-	-	0.0148	46.8	0.0009	770.2
TPH	0.3508	2.0	0.2479	2.8	0.1368	5.1

Table 2. Initial rates and half-life times for light alkanes (C_{14}–C_{25}) (LA); heavy alkanes (C_{26}–C_{35}) (HA); total alkanes (C_{14}–C_{25}); polycyclic aromatic hydrocarbons (PAH); and total petroleum hydrocarbon (TPH). The data refer to the GC-MS detected components of the crude oil for the OIL + D treatment of the time-series experiment.

	Time Frames					
	0–3 (42–45)		**21–28 (63–70)**		**28–35 (70–77)**	
Crude Oil Components	k_{app} (Days^{-1})	$t_{1/2}$ (Days)	k_{app} (Days^{-1})	$t_{1/2}$ (Days)	k_{app} (Days^{-1})	$t_{1/2}$ (Days)
LA	0.4983	1.4	0.2983	2.3	0.2148	3.2
HA	0.2491	2.8	0.3357	2.1	0.2970	2.3
TA	0.3848	1.8	0.4256	1.6	0.3992	1.7
PAH	0.0357	19.4	0.3108	2.2	0.2373	2.9
TPH	0.3571	1.9	0.3190	2.2	0.2471	2.8

4. Discussion

The aim of our study was to provide biodegradation rates under in situ conditions of pressure and temperature at environmentally relevant concentrations of oil, as there are no previous biodegradation studies in the deep EMS. For this purpose, we constructed a practical high-pressure sampling and experimentation system which could be used for deep-sea bioremediation studies, taking into account the high pressures of the deep-sea that indigenous microbial populations have adapted to. Having successfully developed such a high-pressure apparatus, we then proceeded in emulating a deep-sea oil plume after the hypothetical occurrence of a deep-sea oil spill and studied whether dispersant application enhances hydrocarbon degradation under these conditions. By studying the fate of a deep-sea oil plume, we were able to provide accurate biodegradation rates without causing any disruptions to the pressure continuum, which could apply to real-life scenarios of deep-sea oil spills. Dispersant application under high-pressure conditions was also imperative to study to examine whether dispersants under in situ conditions have a positive effect on biodegradation efficiencies.

In order to successfully emulate a deep-sea oil spill in the lab with dispersant application, and monitor the hydrocarbon biodegradation, it is essential to consider the rapid dilution of oil that occurs at sea. Most studies that focus on oil biodegradation do not take it into account and use unrealistically high concentrations of dispersed oil [2]. Taking this into consideration, the concentration of oil and dispersed oil in our study were adjusted to environmentally relevant levels. GC-MS detected concentrations for the first phase of the experiment, where only crude oil was introduced, were below 1 ppm, which is in accordance to reported field data following the DWH incident [9,50,51].

Our results revealed a rapid degradation of the aliphatic compounds, regardless of dispersant application with the lighter alkanes (C_{14}–C_{25}), presenting half-lives of a few hours to a couple of days (between 0.6 and 3.2 days) in both treatments. On the other hand, dispersant application enhanced the degradation of the more recalcitrant compounds of oil, such as the heavier alkanes (C_{26}–C_{35}), where half-lives reduced to more than half for the 21–28 and 28–35 time periods (from 3.8 to 2.1 and from 7.7 to 2.3 days, respectively) after the dispersant application, and PAHs, where there was a distinct effect of the oil dispersion, especially in the 28–35 time period. PAH bioavailability in the OIL + D treatment resulted in a dramatic increase on the biodegradation rate by mature oil-degrading microbial communities (almost 265 times higher for the OIL + D treatment, compared to OIL for the time period 28–35). Our results are in accordance with Hazen et al. [10], who were the first to provide biodegradation rates from the deep plume that formed during the DWH accident. In their study, GC-MS detected alkanes underwent rapid biodegradation which translated in a half-life of days (between 1.2 and 6.1 days). Succeeding oil biodegradation studies have focused on reproducing the conditions of the DWH deep-sea plume in the lab, mainly at atmospheric pressure conditions. Here, we discuss only those conducted at oil concentrations within the range that was observed in the DWH deep plume, similarly to ours. Wang et al. [12] examined biodegradation rates of Gulf of Mexico seawater samples to 2 ppm initial oil concentration and used a dispersion generator and COREXIT 9500 to create 10 μm and 30 μm of oil droplets. Bacterial counts in this case peaked at 40 days of incubation, yet there was no association with higher degradation efficiencies by this point. Degradation also started after a considerable lag time, which for the more recalcitrant compounds (>C_{29} and some PAHs) reached up to 20 days. Excluding the lag phase, the lighter alkanes (C_{13}–C_{25}) presented half-lives between 0.6 and 9.5 days, the heavier alkanes (C_{26}–C_{35}) between 18 and 40 days and PAHs between 10 and 64 days. Another attempt to simulate the oil plume degradation was conducted by Hu et al. [16] with deep-sea water from Mississippi Canyon block MC-294, 2 ppm initial oil concentration and 1:100 dispersant to oil ratio. As in Wang et al. [12], biodegradation efficiencies were accompanied by a significant lag phase. The half-lives for the different oil components were 8.14 days for the lighter alkanes (C_{14}–C_{25}), 22.2 days for the heavier alkenes (>C_{25}) and ranged from 25.3 to more than 64 days with an increasing number of rings for the PAHs, notably higher than

the ones from the 0–3 time point of the OIL + D treatment in our study, where we detected the higher half-lives of PAHs; 1.4 days for the lighter alkanes, 2.8 for the heavier alkanes and 19.4 days for the examined PAHs.

Chemical dispersants have been widely used as a response strategy to oil spills. After the DWH accident, where a significant amount was injected in the subsea, numerous studies have attempted to clarify whether their use actually enhanced oil biodegradation, but without a definitive answer so far. Besides the previously mentioned studies, Kleindienst et al. [13], in deep-water microcosm experiments with seawater from the Gulf of Mexico, found that chemical dispersants did not stimulate oil biodegradation. Similarly, Rahsepar et al. [52] tested different hydrocarbon degrading bacterial cultures in artificial seawater and different dispersant to oil ratios and reported an inhibition of chemical dispersants on oil biodegradation. Tremblay et al. [53] and Sun et al. [54] on the other hand, found that oil biodegradation rates were enhanced by the addition of dispersants. It is thus obvious that there is no consensus of views on subsea dispersant application. Studies vary on the microbial community composition, type of dispersant used, dispersant to oil ratio, nutrient addition and the pressure at which the experiment is conducted. None of the above studies were conducted under high-pressure conditions with an undisturbed microbial community from the deep-sea. High-pressure can affect the efficiency of dispersants; in a recent study by Antoniou and Kalogerakis (2022) [46], it was found that increased pressure adversely affects dispersant efficiency, and depending on the type of dispersant, this can drop by up to 50% for pressures up to 10 MPa.

Many other studies have tried to provide biodegradation rates of crude oil and dispersed crude oil by utilizing native microbial populations from different seas and altering a number of factors, such as temperature, nutrient availability, the initial concentration of oil and hydrostatic pressure (reviewed in [55]). Prince et al. [55] also conducted their own research on crude oil and dispersed crude oil biodegradation under atmospheric pressure. Their results showed that dispersant addition (COREXIT 9500 to 1:15 ratio) significantly enhanced oil bioremediation and decreased the half-lives of both alkanes and PAHs from dozens of days to only a few days, which was in accordance with their previously reported data [56,57]. The effect of hydrostatic pressure on oil biodegradation has been studied only scarcely and, in all cases, concerned either surface seawater microbial communities that were subjected to high-pressure, or depressurized deep-water communities that were re-pressurized in the lab. Prince et al. [58] measured oil biodegradation at 5 °C and 15 MPa using surface seawater with its indigenous microbes, 3 ppm of crude oil, and dispersant COREXIT 9500 at a dispersant to oil ratio of 1:15. Even though alkanes were rapidly degraded (almost complete consumption within 7 days), there was an inhibitory effect on the biodegradation of the heavier alkanes (>C_{23}) and PAHs with increasing pressure. TPH half-lives were 13 and 16 days at atmospheric and 15 MPa pressure, respectively, significantly higher than the results that were obtained from our OIL + D treatment (1.9–2.8 days). Microbial response to crude oil (1% v/v) at in situ pressure and temperature conditions was also described by Marietou et al. [31] using deep water (~1000 m depth) from the Gulf of Mexico. They observed a lag in microbial activity and lower growth yields at high-pressures (15 and 30 MPa) which was associated with slower microbial response at depth to anthropogenic oil input. The inhibitory effect of high-pressure on biodegradation rates is more pronounced for compounds of a higher mass and structural complexity. The lighter alkanes in all the aforementioned studies are degraded within a few days regardless of pressure, dispersion of oil and type of seawater. A clear differentiation arises when comparing the heavier alkanes and PAHs, where pressure has a distinct effect on biodegradation rates. In a deep-sea study of Nguyen et al. [59], sediment samples were used at their in situ conditions (9.4, 11.1, and 15.3 MPa pressure at 4 °C) and amended with 0.1% v/v crude oil to monitor oil biodegradation for 18 days, without being able to provide conclusive results. The authors concluded that a non-disruptive experimentation was needed in order to give more definitive conclusions. Having used a non-disruptive experimentation system, our

study is expected to provide more reliable biodegradation rates in the deep sea, compared to previous studies.

PAHs are ubiquitously found in the sea and their concentrations vary with season and location depending on the inputs from natural and anthropogenic activity. Due to their chemical stability and low water solubility, PAHs are very persistent in the environment [60]. The USEPA has listed 16 PAHs as priority pollutants in aquatic and terrestrial ecosystems [47], with some of them being life-threatening for humans. Background concentrations of PAHs in the Mediterranean Sea are very low. Lipiatou et al. [61] inspected 11 of the 16 PAHs that were examined in this study and estimated a total concentration of 780 $\times$ 10^{-6} ppb in the water column of the Western Mediterranean Sea (WMS), with phenanthrene, fluorene and pyrene presenting the highest values. Another study of Castro-Jiménez et al. [62] on 30 PAHs, based on different Mediterranean sub-basins, also reported very low concentrations of the measured PAHs (19–38 $\times$ 10^{-6} ppb) with dibenzothiophene presenting the highest values. In our study, initial PAH concentrations in the OIL treatment were very low (~61 ppb), while in the OIL + D treatment they were relatively high (~1.2 ppm). After the DWH oil spill, PAHs comprised approximately 1.4% of the total source oil that was released [63]. Prior to the DWH oil spill, there were no documented PAH concentrations. According to Boehm et al. [63], the total detectable PAHs presented slightly elevated concentrations (>1 ppb) in the deep plume in a radius of ~20 km from the wellhead but decreased significantly within weeks after the capping of the wellhead. They also stated that changes in PAH composition that are associated with the decrease in concentrations could be due to differential solubilization, photodegradation, evaporation, and/or biodegradation of individual PAH compounds. In our study, we can exclude all of these processes, except biodegradation. Therefore, even for the OIL treatment where PAH concentrations were very low (~61 ppb), we can link the slight decrease in concentrations to biodegradation. Since background concentrations of PAHs and reported concentrations after the end of the oil discharge from the DWH were even lower than the ones that are presented in our study, we can assume that given more time, these concentrations could drop even more. Future work could focus on examining the limits of biodegradation with the use of the presented HP apparatus. Our findings that >90% of PAHs were removed with dispersant application are of great importance, since dispersant application has been controversial for a long time, without any final conclusions regarding their implementation in deep-sea oil spills. Our study, which was conducted under high-pressure with undisturbed deep-sea microbial communities, does provide significant and representative data both for the biodegradation rates and the effect of dispersants.

5. Conclusions

To the best of our knowledge, this is the first attempt to provide insight into the biodegradation rates of oil in the deep sea, in a complete emulation study of a deep hydrocarbon plume. We developed and successfully employed a high-pressure apparatus for sampling and experimentation with seawater that was collected from depths down to 1000 m, without any pressure disruptions during sample retrieval and with an uninterrupted pressure flow between each step. Our previous work with enriched consortia from the deep waters of EMS [44] showed their capability for effective crude oil bioremediation in the case of an oil spill. Following this work and focusing on a real-life scenario of a deep-sea oil release where oil is entrapped in plumes, we successfully proved that indigenous microbial populations from EMS are readily available to combat subsea oil spills. Background nutrient levels in deep seawater have proven to be sufficient to sustain microbial degradation. The dispersant COREXIT™ EC9500A, which we used in this study, enhanced the degradation of PAHs, which is critical information for the application of dispersants for deep-sea oil-spill response strategies. The sampling and experimentation apparatus that are presented in this study could be employed in a plethora of experiments and even used to further examine the limits of biodegradation for alkanes and PAHs in the water column. The representative biodegradation rates that are produced in this study could subsequently

be used for better predictions of the fate of oil in simulation models, thus offering the chance for more accurate response strategies in the case of a deep-sea oil release.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/en15134525/s1, Figure S1: Principal Coordinate Analysis (PCoA) on Bray–Curtis distances of samples collected from various depths in the water column of the EMS (AW: Atlantic Water; LIW: Levantine Intermediate Water; EMDW: Eastern Mediterranean Deep Water). The ellipse includes the EMDW samples indicating the similarity in the composition of microbial communities within this water layer. Figure S2: TPH Concentration as measured from UV and GC-MS. * denotes the days when oil was added in the bioreactor, ** denotes the days when oil and dispersant were added in the bioreactor. Figure S3: Degradation efficiency of light alkanes. (A) Degradation efficiency of each component per time frame. Only oil is added. (B) Degradation efficiency of each component per time frame. Oil and dispersant are added. Figure S4: Degradation efficiency of heavy alkanes. (A) Degradation efficiency of each component per time frame. Only oil is added. (B) Degradation efficiency of each component per time frame. Oil and dispersant are added. Figure S4: Degradation efficiency of PAHs. (A) Degradation efficiency of each component per time frame. Only oil is added. (B) Degradation efficiency of each component per time frame. Oil and dispersant are added, Table S1: Total Petroleum Hydrocarbons (TPH) Concentration for the GC-MS measured components. * denotes the days when oil was added in the bioreactor, ** denotes the days when oil and dispersant were added in the bioreactor, Table S2: Initial rates and half-life times per time frame for each GC-MS detected component of alkanes and PAHs for the first part of the time-series experiment where only oil was added. Negative values due to detection errors from the GC-MS analysis have been removed, Table S3: Initial rates and half-life times per time frame for each GC-MS detected component of alkanes and PAHs for the second part of the time-series experiment where oil and dispersant were added. Negative values due to detection errors from the GC-MS analysis have been removed. Reference [64] is cited in the Supplementary Materials.

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