Enhanced Hydrocarbons Biodegradation at Deep-Sea Hydrostatic Pressure with Microbial Electrochemical Snorkels

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Abstract: In anaerobic sediments, microbial degradation of petroleum hydrocarbons is limited by the rapid depletion of electron acceptors (e.g., ferric oxide, sulfate) and accumulation of toxic metabolites (e.g., sulfide, following sulfate reduction). Deep-sea sediments are increasingly impacted by oil contamination, and the elevated hydrostatic pressure (HP) they are subjected to represents an additional limitation for microbial metabolism. While the use of electrodes to support electrobioremediation in oil-contaminated sediments has been described, there is no evidence on their applicability for deep-sea sediments. Here, we tested a passive bioelectrochemical system named “oil-spill snorkel” with two crude oils carrying different alkane contents (4 vs. 15%), at increased or ambient HP (10 vs. 0.1 MPa). Snorkels enhanced alkanes biodegradation at both 10 and 0.1 MPa within only seven weeks, as compared to nonconductive glass controls. Microprofiles in anaerobic, contaminated sediments indicated that snorkels kept sulfide concentration to low titers. Bulk-sediment analysis confirmed that sulfide oxidation by snorkels largely regenerated sulfate. Hence, the sole application of snorkels could eliminate a toxicity factor and replenish a spent electron acceptor at increased HP. Both aspects are crucial for petroleum decontamination of the deep sea, a remote environment featured by low metabolic activity.

Keywords: crude oil; hydrostatic pressure; deep sea; alkanes; sulfide; sulfate

1. Introduction

Crude oil accidentally released into marine ecosystems results in persisting contamination, particularly in anaerobic sediments. Under anaerobic conditions, some microorganisms can still oxidize petroleum hydrocarbons, provided suitable terminal electron acceptors such as ferric oxide (Fe[III]) or sulfate (SO$_4^{2-}$) are present [1]. Electron acceptor availability often represents the limiting factor for microbial metabolism [2]. High carbon loads typical of oil-spill events may cause the rapid depletion of electron acceptors supporting biodegradation processes, resulting in the long-term persistence of the spilled hydrocarbons in sediments. Besides, the accumulation of sulfide (H$_2$S), originating from SO$_4^{2-}$ reduction, can have toxic effects and immobilize essential trace elements [3,4].

Electrodes applied in contaminated sediments can act as a virtually nonexhaustible electron acceptor, thereby overcoming this limitation in bioremediation processes. In such a bioelectrochemical system (BES), microorganisms can oxidize organic pollutants (and the resulting metabolic intermediates) while transferring electrons to an anode [5], as demonstrated for single aromatic hydrocarbons, e.g., benzene [6–8], phenols [9–11], toluene [12] and naphthalene [6,13], but also crude oil [14–16].
Petroleum contamination in deep-sea environments is an emerging threat. Crude oil spills occurring on surface waters may reach seafloors exposed to increased hydrostatic pressure (HP) in numerous ways, e.g., dissolution, emulsification and diffusion in the water column; through injection of chemical dispersants, which also enhances the risk of direct contact of underwater oil plumes with continental slopes; formation and sinking of heavier particles (tar) or marine snow; and in situ oil burning [17]. The spill may originate at depth, as in deep exploration and production platforms [18], accidents at deep-sea wells (e.g., the Deepwater Horizon disaster [19]) or oil seepage from pipelines [18] and sunken shipwrecks [20,21]. Microbial-induced corrosion in these shipwrecks can be enhanced by exposure to later oil spills [22], representing an additional risk for the deep sea. The study of deep seafloor decontamination is hampered by their remote location. The use of laboratory-scale high pressure reactors simulating deep-sea environments is an emerging tool in bioremediation studies, which can facilitate testing and upscaling of future technologies.

Here, the electrobioremediation capacity of a passive BES named “oil-spill snorkel” was tested with two crude oils at either ambient or increased HP (i.e., 10 MPa, equivalent to 1000 m below seawater level (bswl)). The BES consisted of nonpolarized, graphite electrodes (hereby referred to as “snorkels”), electrically bridging anaerobic crude-oil-contaminated sediments with oxygenated, overlying seawaters. The side of the snorkel buried in sediment (i.e., the anode) collects electrons deriving from the (bio)oxidation of organic and inorganic compounds; owing to the existing redox gradient, electrons flow to the upper side of the snorkel to combine with oxygen ($O_2$) and protons to form water [14]. Originally, the concept of such a microbial electrochemical snorkel, a simplified design of a “short-circuited” microbial fuel cell (MFC), was proposed as a means to accelerate the anaerobic treatment of wastewater relative to a traditional MFC. More recently, the potential of this technology to sustain redox processes over long periods, with no need for continued maintenance, has attracted the interest for broader applications, including environmental remediation [15,16,23] and also metal recovery [24]. However, application of BES to environments exposed to enhanced HP (i.e., 5 MPa) has been explored marginally: Reimers et al. operated benthic microbial fuel cells at a cold seep (~10 MPa [25,26]), and Kobayashi et al. conducted electromethanogenesis tests in laboratory-scale systems (5 MPa [27]).

Here, we report on the first use of a BES at increased HP for bioremediation purposes. Increased HP negatively impacts microbial oil degradation under aerobic conditions. This is apparently due to a reduction of tricarboxylic acid (TCA) cycle rates, which act as a negative feedback on sustained hydrocarbon oxidation [28]. Microbial oil degradation in anaerobic sediments subjected to increased HP is thus expected to be further slowed. However, little to no investigation has been carried out so far on how to relieve this metabolic pressure and sustain oil bioremediation at deep-sea conditions. The aim of the present study was to test whether the inhibitory effects of increased HP on oil biodegradation rates could be alleviated at least in part by allowing microorganisms in sediments a preferential access to $O_2$ via snorkels. Our hypothesis was that the oxidation of readily degradable petroleum constituents (e.g., alkanes) may rapidly deplete electron acceptors in the sediment, and potentially prevent the degradation of more recalcitrant components (e.g., polyaromatic hydrocarbons, PAHs) owing to the accumulation of toxic metabolites (e.g., $H_2S$ following $SO_4^{2-}$ reduction). Results therefore describe snorkel impact on petroleum hydrocarbons removal (alkanes and total petroleum hydrocarbons (TPH)) and on the cycling of $SO_4^{2-}$ and $H_2S$ as compared to nonconductive glass rods used as controls.

2. Results and Discussion

2.1. Bioelectrochemical Snorkels Enhance Alkanes Biodegradation at Deep-Sea and Ambient HP

Oil biodegradation was investigated in the HP range 0.1 to 10 MPa (surface to 1000 m bswl), which coincides with the epi- and mesopelagic zone. Here, seawater is commonly subjected to high mixing (e.g., during winter, or due to convection from surface...
to deep waters [29]) and high rate of sinking particles from the surface (up to $10^{10}$ bacteria per gram, which can sink up to ~4700 m bswl [30]). These deep seawaters and superficial seafloors are thus populated by a mixture of autochthonous (belonging in this environment) and allochthonous (originally from another environment, e.g., surface waters) prokaryotes. A fast, deep-sea bioremediation should theoretically rely on piezophilic (HP-requiring) oil-degrading prokaryotes; however, they do not appear to possess a competitive advantage at these mild depths. Seawaters [28,31–33] and sediments [34] collected from 1000 to 1500 bswl and cultivated at in situ HP have lower oil degradation capacity than depressurized subsamples at 0.1 MPa. Estimates on the decrease of microbial oil degradation for every 1 MPa increase indicate that cell division was reduced 5% in enriched seawater communities from 1100 m bswl using Macondo oil [33]; cell division and CO$_2$ production were reduced 8% and 9%, respectively, in synthetic communities originally collected 1000 m bswl using $n$-C$_{20}$ [28]; and alkanes biodegradation was reduced 4% in sediments from 1100 m bswl using sweet Louisiana crude [34]. The fact that depressurization-enhanced microbial activity suggests that (1) piezophilic microorganisms did not predominate at these deep sampling sites, rather allochthonous from surface waters not adapted to increased HP constituted a large part of the microbial community; and (2) HP can severely impact oil degradation already at 10 MPa. Stimulation of microbial activity in environments exposed to these mild HPs appears crucial to support bioremediation.

In the present investigation, we used an electrochemical snorkel to enhance microbial oil metabolism in marine sediments. TPH removal was generally higher in the alkanes-rich Statfjord than in the alkanes-poor Danish Underground Consortium (Duc) oil (the relative decrease with respect to initial concentrations ($C/C_0$) was $0.64 < C/C_0 < 1.03$ vs. $0.85 < C/C_0 < 1.04$; Figure 1A vs. Figure 1B). With Duc oil, no difference was observed in TPH or alkanes biodegradation when comparing snorkels to glass controls ($p > 0.05$, HPS vs. HPC, or APS vs. APC, Figure 1A,C). However, with the alkanes-rich Statfjord oil, both TPH and alkanes were biodegraded more when using snorkels at 0.1 MPa ($p < 0.001$, APS vs. APC, Figure 1B,D), a result also observed for alkanes at 10 MPa ($p = 0.004$, HPS vs. HPC, Figure 1D). Hence, bioelectrochemical snorkels could significantly enhance the removal of readily degradable hydrocarbons such as alkanes at deep-sea HPs within only seven weeks. The quality and quantity of the growth substrate can affect the microbial response to increased HP [35,36]. Here, the higher initial concentration of alkanes (315 vs. 139 µg g$_{DW}^{-1}$, Statfjord vs. Duc, Figure 1, Table S1) may have been a relevant factor for the sustained electrobioremediation at 10 MPa. Nonetheless, increased HP had a negative impact. For every 1 MPa increase, alkanes biodegradation was significantly reduced: 5% with Duc oil with snorkels (2% with glass controls, albeit not significantly), and 5% and 11% with Statfjord oil in snorkels and glass controls, respectively ($p < 0.05$, Figure 1). Despite sediments were collected at 30 m bswl, HP inhibition rates were thus comparable with those of samples from 1000 to 1500 m bswl [28,33,34]. This further confirms that microorganisms that are sensitive to increased HP (piezosensitive) and piezophiles are intermixed in the oceans [29]. Predominance of piezophiles underwater arises with long-term exposure to enhanced HP, as in deep, stratified waters [29]. Similarly, long-term exposure to petroleum may favor the evolutionary microbial adaptation to the fast, effective use of oil as a carbon and energy source. A better understanding of the metabolic requirements for sustained oil biodegradation at increased HP may be found at, e.g., deep hydrocarbon seeps [37] or chronically polluted deep-sea sites following anthropogenic spills. As the snorkel’s application for longer incubations (>1 year) efficiently reduced TPHs at ambient pressure [14,15], electrobioremediation of the most recalcitrant petroleum components should be tested with deep-sea samples from these long-term contaminated sites.
Figure 1. Final concentration (μg g⁻¹ dry weight sediment) of total petroleum hydrocarbons (A, B) and alkanes (between C₈ and C₃₃; C, D) at the end of 7 weeks of incubation in sediments contaminated with either Danish Underground Consortium (Duc) (A, C) or Statfjord (B, D) oil (n = 3; bars represent standard errors). Contamination levels at time zero are reported as horizontal dotted lines, with the grey areas representing the standard error (n = 3; data in Table S1). Degradation was assessed as the relative decrease with respect to the concentration at time zero (C/C₀), and is reported in orange for each condition. Abbreviations reported in the graph: HPS, high pressure with snorkels; HPC, high pressure with glass controls; APS, ambient pressure with snorkels; APC, ambient pressure with glass controls. Asterisks indicate statistically significant difference between the bars located at the two extremes of the parenthesis.

2.2. Bioelectrochemical Snorkels Regenerate SO₄²⁻ in Sediments at Deep-Sea and Ambient HP

Aside growth substrate, microbial response to increased HP is affected by, e.g., temperature [38] and nutrient availability (quality and quantity) [39]. At low and middle latitudes, temperature in epi- and mesopelagic seawaters decreases along a permanent thermocline from as much as 30 to ~5 °C [40]. In this investigation, temperature was kept at 14 °C to test the sole impact of increased HP. Concerning nutrients, oxidation of petroleum hydrocarbons may use O₂ as terminal electron acceptors in seawater, or Fe[III] and SO₄²⁻ in anaerobic sediments. O₂ in epi- and mesopelagic seawaters decreases from as much as 350 μmol kg⁻¹ (or 11.2 mg L⁻¹) to a range 40–240 μmol kg⁻¹ (or 1.3–7.7 mg L⁻¹) according to the latitude [40]. In this investigation, values ranged between 8 and 0.03 mg L⁻¹ (beginning and end of the incubation, respectively). While O₂ in seawaters is continuously replenished by water circulation and vertical diffusion through the water column, Fe[III] and SO₄²⁻ in anaerobic sediments are limited reservoirs whose depletion can limit microbial metabolism [2]. In this investigation, application of the snorkel to oil-contaminated
seds regenared SO$_4^{2-}$, using O$_2$ in seawater as the ultimate electron acceptor. At the end of the incubation, all sediments were anoxic 2 mm below the sediment surface. Total sulfide (ΣH$_2$S, equal to [H$_2$S] + [HS$^-$] + [S$^{2-}$]) microprofiles were assessed in contaminated sediments with either crude oil (Figure 2A–D). The higher TPH biodegradation trend observed with Statfjord as compared to Duc oil (Figure 1) was mirrored by a higher ΣH$_2$S accumulation (Figure 2A–D). This suggests that electrons derived from the oxidation of hydrocarbons were primarily used to reduce SO$_4^{2-}$ thereby generating H$_2$S, with the alkanes-rich Statfjord oil accumulating more H$_2$S.

Notably, ΣH$_2$S was substantially lower in the presence of snorkels than in the respective glass controls at equivalent HP ($p < 0.001$, HPS vs. HPC, Figure 2A,C; or APS vs. APC, Figure 2B,D). This indicates that H$_2$S rapidly reacted with the anodic portion of snorkels buried in sediments. Anodic H$_2$S oxidation was suggested to be advantageous for microbial activity [41–43]: first, because H$_2$S scavenging reduces its toxicity to microorganisms [4]. This was most evident when using the alkane-rich Statfjord oil, where the higher abatement of ΣH$_2$S with snorkels ($p < 0.001$, Figure 2C,D) was consistent with the enhanced reduction of TPH (Figure 1B) and, particularly, alkanes (Figure 1D). Consistently lower ΣH$_2$S levels in the presence of snorkels as compared to glass controls across all conditions indicated that snorkels worked equally well at ambient and increased HP within a large range of ΣH$_2$S concentrations ($p < 0.001$, Figure 2A–D). Lower ΣH$_2$S levels at increased HP as compared to ambient pressure confirmed the decrease in SO$_4^{2-}$-reducing activity at 10 MPa, as mirrored in the lower alkanes degradation ($p < 0.05$ except for DUC HPC vs. APC, Figure 1C,D).

The second advantage of H$_2$S oxidation at the anode is that it regenerates oxidized sulfur species [16,44] (e.g., SO$_4^{2-}$), which may be used by SO$_4^{2-}$-or sulfur-reducing bacteria as electron acceptors [7,12,41]. In contaminated sediments, this would circumvent the risk for electron acceptor depletion and support prolonged periods of microbial, SO$_4^{2-}$-driven hydrocarbon degradation, a critical advantage for remote deep seafloor. Accumulation of H$_2$S in sediments implied that SO$_4^{2-}$ was used as an electron acceptor, as in fact observed in all incubations (Figure 2E,F). Nonetheless, application of conductive snorkels rather than nonconductive controls resulted in lower SO$_4^{2-}$ consumptions ($p < 0.05$ except for Statfjord HPS vs. HPC, Figure 2E,F). Together with ΣH$_2$S microprofiles (Figure 2A–D), this confirms that snorkels contributed to a sustained SO$_4^{2-}$ regeneration irrespective of the HP applied.

Electrons generated by H$_2$S oxidation at the anode eventually reach the cathode (i.e., the snorkel side in contact with overlying waters) where they react with O$_2$ as the ultimate electron acceptor. The cumulative O$_2$ consumption in the water column above contaminated sediments was generally higher in the presence of snorkels than in the glass controls at equivalent HP (HPS vs. HPC, or APS vs. APC, Figure 2G,H). As cell densities in the water column were comparable in snorkel and glass controls ($p > 0.05$, Figure S1), the difference in O$_2$ consumption in overlying waters depended on the electrochemical connection with electron donors in sediments provided by snorkels. Cumulative respiration in overlying waters within ~50 days (14–15 mL O$_2$ per graphite rod; 14 °C, 0.1 MPa) was comparable with other oil-contaminated sediments (7–10 mL O$_2$ per graphite rod; 20 °C and 0.1 MPa [14]). Estimates on the O$_2$ consumption owing to electrochemical H$_2$S oxidation alone accounted for no more than 1 mg O$_2$ L$^{-1}$ day$^{-1}$, explaining ~10% to ~40% of the differential O$_2$ consumption between snorkels and glass rods, with either crude oil and HP (see Supplementary Information). This suggests that, along with mediating H$_2$S oxidation back to SO$_4^{2-}$, snorkels served as electron sinks for other reduced (organic and/or inorganic) substances in contaminated sediments, possibly including petroleum hydrocarbons or metabolic intermediates derived from hydrocarbon anaerobic degradation.
Figure 2. Microprofiles of total hydrogen sulfide ($\Sigma H_2S = [H_2S] + [HS^-] + [S^{2-}]$) concentrations (A–D), sulfate ($SO_4^{2-}$) in sediment pore water (E,F) and cumulative $O_2$ respiration in seawater overlying contaminated sediments at the end of 7 weeks of incubation in sediments contaminated with either Duc (A,B,E,G) or Statfjord (C,D,F,H) oil (n = 3; bars represent standard errors). Microprofiles of $\Sigma H_2S$ at time zero (A–D) are indicated as dotted lines. Initial $SO_4^{2-}$ concentrations (E,F) are indicated as horizontal dotted lines, with the grey areas representing the standard error (n = 3). The cumulative amount of $O_2$ in seawater provided through the 7 weeks of incubation was ~55 mg L$^{-1}$. Abbreviations reported in the graph: HPS, high pressure with snorkels; HPC, high pressure with glass controls; APS, ambient pressure with snorkels; APC, ambient pressure with glass controls. Asterisks indicate statistically significant difference between the bars located at the two extremes of the parenthesis.
3. Materials and Methods

3.1. Sediment Sampling

Sediment samples were collected from Aarhus Bay (Denmark) onboard the Aurora Research vessel (Aarhus University), station M5 (56°06′20″ N, 10°27′48″ E; 30 m bswl), using a box corer. Onboard, the upper 10–12 cm of sediment was discarded to exclude large burrowing animals. The sediment was sieved (mesh 0.5 mm) to remove solid residues that could interfere with microsensor measurements, homogenized and stored at 14 °C in airtight bags for three weeks. Exposure to air was minimized during handling procedures. The initial concentration of nitrite (NO$_2^{-}$) and nitrate (NO$_3^{-}$) in the pore water of incubated sediments was below detection limit (<10 µM), while that of Fe$^{2+}$ ranged between 6.3 and 16 µM.

3.2. Reactors Configuration

The sediment was artificially contaminated with crude oil and incubated for a total of seven weeks. Experiments used either Duc oil or Statfjord oil. The sediment contamination procedure [16,45], minimized the chemical and microbiological perturbation of the samples while allowing a homogeneous distribution of the oil in the whole sediment mass. Contaminated sediment was transferred into a glass cylinder (external diameter 4.2 cm, glass thickness 0.2 cm, height 33.5 cm). The final contamination (expressed as TPHs) for Duc and Statfjord oil was 3.2 ± 0.2 and 2.1 ± 0.2 mg TPH per gram of sediment dry weight (g$^{-1}$ DW), respectively (Table S1). These two crude oils were selected for their different content in alkanes, which was ~4.6 times lower in Duc (4.32% ± 0.08) as compared to Statfjord oil (14.92% ± 1.09; Table S1). The final volume of contaminated sediment was ~110 mL.

Bioelectrochemical petroleum degradation was assessed by preparing glass cylinders with either conductive graphite rods (snorkels) or nonconductive glass rods (controls), and applying either ambient (0.1 MPa) or increased (10 MPa) HP. Four types of cylinders were prepared: (1) glass controls at ambient pressure, (2) snorkels at ambient pressure, (3) glass controls at increased HP and (4) snorkels at increased HP. In snorkel treatments, two graphite rods (>99.9995% purity; Alfa Aesar, Milan, Italy; diameter 6.15 mm, length 150 mm) were embedded into the sediment for 8 cm, with the remaining 7 cm above the sediment surface. The two rods were connected at the top by a carbon felt disc (thickness 10 mm, carbon content >97%, specific resistance 0.18–0.22 Ω per cm; Hi-Tech Carbon Co., Limited-China) to extend the cathodic electrode surface. Control treatments had an identical set up, except for the replacement of conductive graphite rods with nonconductive glass rods of comparable dimensions (diameter 6.1 mm, height 150 mm).

Glass cylinders were filled with ~200 mL of sterile, artificial seawater (salinity 30%; Red Sea Salts, Red Sea Fish Pharm Ltd., Eilat, Israel). Seawater was oxygenated at the beginning of the experiment by flushing sterile air (98% O$_2$ saturation). O$_2$ concentration was monitored via fiber-optic sensors connected to a reader (PyroScience, Germany). Cylinders were sealed at each side with rubber stoppers. No gas phase was left inside the cylinders. Experiments were conducted at 14 ± 2 °C, and cylinders wrapped in aluminum foil to maintain darkness. At increased HP, cylinders were placed in a 5 L high-pressure reactor (capacity 0.1 to 60 MPa; Dustec Hochdrucktechnik GmbH, Germany) operated at 10 MPa. HP was changed manually via a HP pump (Enerpac, Netherlands) following compression and decompression rates, as described in [46]. As all experiments were conducted in three independent replicates, a total of 24 cylinders of ~310 mL each were tested for crude oil biodegradation, with six additional glass cylinders (three for each crude oil) entirely sampled before incubation to determine initial time points.

3.3. Sampling Procedure

Cylinders were sampled every seven days for O$_2$ concentration in seawater via fiber optics at 17, 9 and 1 cm above the sediment surface. Seawater was then reoxygenated to 98% of O$_2$ saturation, and the cylinders incubated for another seven days. After seven weeks of operation, following O$_2$ measurement, high resolution microprofiles of H$_2$S, pH
and O$_2$ were conducted in the sediments. Afterwards, bulk sediments were sampled to determine TPH and alkanes, and SO$_4^{2-}$ in the pore water.

### 3.4. Analytical Procedures

Cell numbers were assessed by flow cytometry as described in [46]. TPH and n-alkanes quantification in sediments was performed by gas chromatography combined with mass spectrometry (GC–MS) as reported elsewhere [16]. In brief, sediment samples (approximately 10 g) were air dried overnight and extracted with a Thermo Scientific ASE (DIONEX ASE 150) using a dichloromethane (DCM): hexane (1:9, v/v) mixture at 100 °C and a system pressure of 1500 psi. The extract was evaporated to a final volume of 5 mL under a gentle nitrogen stream. A sample of the extract (1 µL) was then injected (in pulsed splitless mode) into a GC–MS (PerkinElmer Clarus 680/600; column: HP-5 MS [Agilent] 30 m, ID 0.25 mm, 0.25 mm film thickness; carrier gas: helium at 1 mL/min; injector temperature: 280 °C; oven temperature program: initial temperature 40 °C, 18 °C/min to 250 °C, 10 °C/min to 280 °C, hold for 17 min; MS-scan 30–600, 2–32 min).

TPHs were determined by summing up both the unresolved and resolved components eluting from the GC capillary column between the retention times of n-C$_8$ and of n-C$_{40}$, using solutions of diesel motor oil and diesel mineral oil in hexane as calibration standards. Degradation was assessed as the relative decrease with respect to initial concentrations (C/C$_0$). Alkanes calibration was performed with standards (C$_8$-C$_{40}$ Alkanes Calibration Standard, Sigma-Aldrich). Sediment not used for petroleum analysis was centrifuged at 8000 rpm for 15 min and ~1.5 mL of supernatant sampled to determine SO$_4^{2-}$ in the pore water by ion chromatography (Dionex IC-2500, Thermo Fisher Scientific).

### 3.5. Microsensor Measurements

High-resolution depth profiles of H$_2$S, pH and O$_2$ were recorded with microelectrodes built at Aarhus University [47–49], microprofiling and microsensor calibrations were conducted as described in [50]. Microprofiles were recorded at 100–400 µm vertical resolution and measured at ~2 cm from graphite or glass rods. A reference electrode (REF201 Red Rod electrode; Radiometer Analytical, Denmark) was used for pH measurements. Total hydrogen sulfide (ΣH$_2$S = [H$_2$S] + [HS$^-$] + [S$^{2-}$]) concentrations were calculated at each depth from the measured H$_2$S and pH values [49].

### 3.6. Statistical Analysis

Results are the mean value of experiments made in three independent replicates. Deviation was determined as standard error. Statistical significance was assessed with a t-test, two-tailed, 95% confidence interval.

### 4. Conclusions

Application of BESs has great potential for the biodegradation of hazardous compounds in anaerobic, electron-acceptor-limited sediments [51]. Here we show that in sediments exposed to 10 MPa, snorkels could accelerate alkanes biodegradation within only seven weeks (from 1.02 to 0.82, C/C$_0$), while reducing toxic H$_2$S accumulation (up to 85%, at the highest concentrations) and regenerating SO$_4^{2-}$ (up to the initial concentration). Both factors are crucial to degrade petroleum hydrocarbons over extended periods of time, particularly in remote deep seafloors.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/2073-4344/11/2/263/s1, Supplementary Information; Figure S1: Cell number in the water column at the end of 7 weeks of incubation in sediments contaminated with either Duc or Statfjord oil; Table S1: Duc and Statfjord crude oil alkanes profiles in contaminated sediments at Time zero; Table S2: Estimation of the total H$_2$S (nmoles) accumulated in sediments, which were incubated with either Duc or Statfjord crude oil, with snorkels or glass controls, at 10 or 0.1 MPa; Table S3: Estimates of the total O$_2$ respiration in seawater due to electrochemical oxidation of H$_2$S in sediments, and of the
relative contribution to the electrochemical O$_2$ respiration to the total, in marine sediments incubated with either Duc or Statfjord crude oil, with snorkels or glass controls, at 10 or 0.1 MPa.

**Author Contributions:** Experimental design, A.S., F.A. and U.M.; high pressure incubations, E.P. and A.S.; total H$_2$S microprofiles, E.P. and U.M.; petroleum hydrocarbons analysis, E.P. and C.C.V.; bulk sulfate measurements, E.P. and U.M.; O$_2$ measurements, E.P., A.S. and U.M.; data analysis, all authors; manuscript preparation, A.S. and F.A.; contribution to manuscript final version, all authors. All authors have read and agreed to the published version of the manuscript.

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