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# Hydrocarbon contamination affects deep-sea benthic oxygen uptake and microbial community composition

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ABSTRACT

Accidental oil well blowouts have the potential to introduce large quantities of hydrocarbons into the deep sea and disperse toxic contaminants to midwater and seafloor areas over ocean-basin scales. Our ability to assess the environmental impacts of these events is currently impaired by our limited understanding of how resident communities are affected. This study examined how two treatment levels of a water accommodated fraction of crude oil affected the oxygen consumption rate of a natural, deep-sea benthic community. We also investigated the resident microbial community's response to hydrocarbon contamination through quantification of phospholipid fatty acids (PLFAs) and their stable carbon isotope ($\delta^{13}C$) values. Sediment community oxygen consumption rates increased significantly in response to increasing levels of contamination in the overlying water of oil-treated microcosms, and bacterial biomass decreased significantly in the presence of oil. Multivariate ordination of PLFA compositional (mol%) data showed that the structure of the microbial community changed in response to hydrocarbon contamination. However, treatment effects on the $\delta^{13}C$ values of individual PLFAs were not statistically significant. Our data demonstrate that deep-sea benthic microbes respond to hydrocarbon exposure within 36 h.



## 1. Introduction

Modern societies remain largely dependent on crude oil as a material and energy resource. This has pushed the frontiers of oceanic oil drilling to exploit previously inaccessible reserves, for example those found on the continental slope. The 2010 Macondo oil well blowout was the largest accidental input of hydrocarbons into the deep sea from a single incident (DHSG, 2011), resulting in a prolonged (86 days) release of crude oil and gas into the deep (~1600 m) waters of the Gulf of Mexico. Consequently, the scale and complexity of this accident brought into focus how little was known about the impacts of oil spills on deep-sea organisms and ecosystem functioning. Therefore, as more drilling continues to take place in the deepwater regions beyond the continental shelf edge, we need to improve our understanding of the biotic processes that contribute to ecosystem function and how they may be affected by hydrocarbon contamination.

Following the Macondo oil well blowout and the subsequent sinking of the Deepwater Horizon drilling rig, leaking oil and gas entered a range of transport pathways (Ryerson et al., 2011). In addition to forming surface slicks and evaporating to the atmosphere, dissolved oil and small droplets formed multiple horizontal intrusions that were entrained into currents in the deep ocean (Reddy et al., 2011; Socolofsky et al., 2011). These hydrocarbon 'plumes' persisted for months, triggering blooms of bacteria (Hazen et al., 2010) that produced a local, persistent depletion in oxygen in the water column as oil respiration occurred (Kessler et al., 2011). Oil persisted at toxic concentrations for months after the initiation of the leak, both in the water column and in deepwater sediments on the continental slope (Paul et al., 2013).

Mechanisms through which Macondo oil arrived at the seabed have been discussed following the accident. Aggregates of oil with particulate organic matter were observed in the water column that could have been exported from the euphotic zone (Passow et al., 2012). Oil in subsurface intrusions could also be advected directly onto a slope according to the so-called 'toxic bathtub ring' hypothesis (Deepwater Horizon Oil Spill Principal Investigator Workshop Final Report, 2012). At the seabed, tidal pumping of pore waters and faunal ventilation of burrow structures may draw oil beneath the sediment surface. Hence, there are multiple pathways through which deep-sea benthos may come into contact with hydrocarbon contaminants following a spill. At the seabed,

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there was strong evidence that oil from the Macondo well caused stress to benthic fauna.

Impacts of oil industry activities and pollution have commonly been measured by assessing changes in species assemblages of benthic invertebrates in response to pollution (e.g. Gray et al., 1990). Other whole community measures, e.g. sediment community oxygen consumption (SCOC, the consumption of oxygen by all living biota within a sediment community) can also indicate status. Total oxygen uptake rate of sediments is indicative of whole community metabolism Glud, 2008). Oxygen demand also commonly correlates with indicators of stress in marine benthic ecology (Hyland et al., 2005).

Effects of hydrocarbons on benthic respiration rates have previously been studied experimentally, where oxygen demand of shallow (200–300 m) Arctic marine sediment communities increased in response to high concentrations of crude oil slurry (Olsen et al., 2007a). Sedimentation by drill cuttings also results in chronic increases in benthic respiration and changes to the structure and functioning of benthic communities (Schaanning et al., 2008; Trannum et al., 2011). Increases in respiration rates reflect the upregulation of compensatory mechanisms in response to hydrocarbon contaminants (Widdows et al., 1995; Olsen et al., 2007b).

Oil can induce stress in prokaryotes (Griffin and Calder, 1977), but also provides a metabolic substrate for hydrocarbon-degrading bacteria. Petroleum is isotopically distinct from contemporary marine organic matter (Peters et al., 2007) and has been traced as it is remineralised by bacteria in sediment slurry experiments. For example, the stable carbon isotope ratio of $CO_2$ respired by bacteria exposed to hydrocarbons shifted towards that of the oil and hence demonstrated the ability of those bacteria to perform bioremediation (Lapham et al., 1999). Bioremediation has also been demonstrated in the field, where natural $^{14}C$ analysis of bacterial phospholipid fatty acids (PLFAs) indicated in situ biodegradation of alkanes at a beach oil-spill site (Slater et al., 2006). We further hypothesized that incorporation of hydrocarbons into microbial biomass may be detected through the isotopic analysis of bacterial PLFA), which degrade quickly from non-living cells (White et al., 1979).

Our understanding remains incomplete with respect to the effects of oil on the structure of natural, deep-sea benthic microbial communities, their functioning with respect to respiration rates and their capacity to metabolise hydrocarbon contaminants. In order to predict the implications of future oil well blowouts to the functioning of benthic environments, we need to better understand the response of benthic remineralisation rates and microbial communities to oil-induced disturbance. We studied this by performing an ex situ microcosm experiment on sediments collected directly from an uncontaminated continental slope at ~1000 m water depth. We measured the effects of two treatment levels of hydrocarbons from a water accommodated fraction of crude oil (WAF; a medium prepared by low energy mixing of water and crude oil, Singer et al., 2000) on sediment community oxygen consumption (SCOC) rates, benthic bacterial biomass and community composition. Isotopic signatures of PLFAs were examined to investigate whether WAF exposure resulted in uptake of oil by benthic bacteria. The null hypotheses were that addition of hydrocarbons would not influence benthic respiration rates, bacterial biomass or community structure, or the isotopic composition of individual PLFAs.

## 2. Methods

### 2.1. Study location

Our study location, an area of the North Atlantic continental slope known as the Goban Spur (49° 35.5′ N, 011° 50.9′ W), is a non-drilled site at 995 m water depth, assumed to be free of anthropogenically spilled oil because of its distance ( > 100 km) from oil-industry activity. Near-bottom currents at the Goban Spur range between 10 and 35 cm $s^{-1}$ (Flach et al., 1998), and sediments have a relatively low organic carbon content (approx 0.5% by weight, Lohse et al., 1998); in contrast to other North Atlantic slope sites. At this location, SCOC is approximately equally attributable to bacteria and deposit feeding macrofauna (Heip et al., 2001). Sediment cores were collected from the continental slope of the Goban Spur during July 2012 (Table S1). The ambient bottom water temperature was 9.2 °C.

### 2.2. Preparation of water accommodated fraction of crude oil

Seawater was collected from immediately above the seabed at the study location prior to coring operations. This seawater was used to prepare a WAF using crude oil from the Wytch Farm oilfield (Dorset, UK, obtained fresh in June 2012). The stable carbon isotope ratio value of the whole crude oil was determined using a Flash EA 1112 Series Elemental Analyser connected via a Conflo III to a Delta$^{Plus}$ XP isotope ratio mass spectrometer (all Thermo, Bremen, Germany). The isotope ratios were calculated using $CO_2$ reference gas injected with every sample. The isotopic values of this gas was directly referenced using IAEA reference materials USGS40 and USGS41 (both L-glutamic acid); certified for $\delta^{13}C$ (‰$_{VPDB}$). Long term precision, over several months, for a quality control standard (milled flour) was: $\delta^{13}C$ $-25.5 \pm 0.29$‰ (mean ± sd, $n=200$). A standard low-speed mixing procedure was used to prepare WAF (Hokstad et al., 1999) at the in situ temperature (9.2 °C). In brief, 20 L of seawater and 613 mL fresh crude oil (the density of Wytch Farm crude oil[1] is 0.81 kg $L^{-1}$) were combined in a glass aspirator to produce an oil loading rate of 25 g $L^{-1}$ (Faksness et al., 2008). Low energy stirring of this solution, with no vortex present in the surface slick, was carried out for approximately 24 h. Samples of WAF (450 mL) were preserved with 50 mL hydrochloric acid (specific gravity 1.18, 12.2 M) for later laboratory analysis of WAF composition (ERT Fugro, UK). The resulting WAF was composed mainly of the monoaromatic hydrocarbons: benzene; toluene; ethyl benzene and isomers of xylene (Table S2).

### 2.3. Sediment core collection

A total of 24 sediment cores (10 cm diameter and between 20 and 40 cm sediment height) with overlying water were collected using a Bowers and Connelly megacorer and immediately transferred to an incubator set at the ambient bottom water temperature (9.2 °C). Cores were acclimated to experimental conditions for approximately 24 h in the dark.

### 2.4. Treating sediment cores with WAF

Sediment core tubes were contaminated with WAF at two treatment levels (eight replicates per treatment) and eight cores formed controls. Treatments of WAF were applied at low (25% overlying water volume) and high (50% overlying water volume) levels. In the controls, 25% of the overlying water was exchanged with uncontaminated bottom seawater. Samples of WAF (450 mL) were preserved with 50 mL hydrochloric acid (specific gravity 1.18, 12.2 M) for later laboratory analysis of WAF composition (ERT Fugro, UK). This was done at intervals spanning the cores treatment period in order to monitor stability of the mixture and provide an estimate of hydrocarbon concentrations in treatments (Table S2). All experimental apparatus, and the glass aspirator bottle used for WAF preparation on the ship, was cleaned

[1] http://www.bp.com/liveassets/bp_internet/bp_crudes/bp_crudes_global/STAGING/local_assets/downloads_pdfs/Wytch_Farm_Mar10.xls.

thoroughly and rinsed with hydrochloric acid followed by deionised water before use in the experiment. All glassware used for subsequent analytical work was combusted at 450 °C before use.

### 2.5. Incubation of microcosms

Following addition of WAF/seawater, the sediment cores were immediately sealed without a headspace and incubated at the ambient seabed water temperature. Oxygen concentration in the overlying water was measured at intervals of approx 6 h using a non-invasive PreSens Fibox oxygen sensor. The overlying water in each core was stirred manually for approximately 10 s every 2 h and for 10 s preceding measurement of oxygen. Incubations were ceased when (or before) core overlying water oxygen concentration reached ~80% of starting levels.

### 2.6. Slicing and preserving sediment horizons

All cores were immediately sectioned at depth horizons (0–1 and 1–2 cm) at the end of the incubation period. Four replicates from each treatment were frozen at −80 °C for subsequent microbial analyses, with the remaining four replicates from each treatment level (and controls) preserved in formalin for later macrofaunal analyses (details in Table S1).

### 2.7. Macrofaunal biomass

Macrofauna were removed from the sediment sections by washing on a 250 μm sieve. The animals were stained with Rose Bengal and were sorted into phyla. Wet weights were summed for the 0–1 cm and 1–2 cm sediment layers and an estimate of total macrofauna biomass (mmol C $m^{-2}$) was calculated (Table 2), assuming 12.4% carbon content for nematodes (Jensen, 1984) and 4.3% carbon content for all other animals (Rowe, 1983) except ophiuroids (1.9% carbon content).

### 2.8. Extraction and quantification of PLFAs

Phospholipid fatty acids were extracted from freeze-dried sediment from the 0–1 and 1–2 cm horizons. All solvents used were HPLC grade. Following an extraction method modified from Bligh, 1959, lipids were extracted from 9.0 g freeze-dried sediment for each replicate sample using a single phase mixture of chloroform:methanol:citrate buffer 1:2:0.8 v-v:v) (Frostegård et al., 1991). The lipids were fractionated using 6 mL ISOLUTE SI SPE columns (solid phase extraction with 500 mg sorbent mass of silica, International Sorbent Technology Ltd, Hengoed, UK) preconditioned with 5 mL chloroform. Dry lipid material was taken up in 400 μL chloroform, vortex mixed twice then transferred onto the column. The vial was rinsed three times with 200 μL chloroform then added to the column on each occasion. The sample was then allowed to pass through the column. The columns were washed sequentially with 2 × 3 mL of chloroform then 2 × 3 mL acetone, these eluates were not retained. The column was then washed with a total of 10 mL methanol. All methanol eluates, containing the PLFAs, were collected in a glass vial, then slowly evaporated to dryness under nitrogen gas and stored at −20 °C until being processed further.

The PLFAs were subsequently derivitised with methanol to yield fatty acid methyl esters (FAMEs). Each sample was taken up in 1 mL of a 1:1 (v:v) mixture of methanol and toluene in a vial. Then, 1 mL of 0.2 M KOH in methanol was added with a known quantity of C19 internal standard (nonadecanoic acid, $CH_3(CH_2)_{17}COOH$), vortex mixed and incubated at 37 °C for 15 min. After cooling to room temperature 2 mL of isohexane:chloroform (4:1 v-v), 0.3 mL of 1 M acetic acid and 2 mL deionized water was added to each vial. The solution was vortex mixed, placed on an end over end mixer for 10 min, then centrifuged. The organic phase was transferred to a new vial. The aqueous phase was re-extracted with a further 2 mL of isohexane:chloroform (4:1) and centrifuged as before. Both resultant organic phases were combined, evaporated under nitrogen and stored at −20 °C. The residue was extracted with 3 × 100 μL isohexane:chloroform (4:1) and transferred to a glass vial. The solvent was evaporated with nitrogen and samples were stored at −20 °C. Samples were taken up in isohexane to perform analysis by gas chromatography–combustion–isotope ratio mass spectrometry (GC–C–IRMS).

The quantity and $\delta^{13}C$ value of individual FAMEs was determined using a GC Trace Ultra with combustion column attached via a GC Combustion III to a Delta V Advantage isotope ratio mass spectrometer (all Thermo Finnigan, Bremen, Germany). Samples (2 μL) were injected in splitless mode, via an inlet held at 250 °C, onto a J&W Scientific HP-5 column, 50 m length, id 0.2 mm with a film thickness of 0.33 μm (Agilent Technologies Inc, Santa Clara, USA). The He carrier gas was maintained at a constant flow rate of 1.5 ml $min^{-1}$. The GC oven was initially set at 100 °C, held for 1 min and then ramped at 20 °C $min^{-1}$ to 190 °C, then at 1.5 °C $min^{-1}$ to 235 °C and finally at 20 °C $min^{-1}$ to 295 °C; where the temperature was held for 15 min. The oxidation reactor on the interface was maintained at 950 °C and the reduction reactor at 650 °C.

The $\delta^{13}C_{VPDB}$ values (‰) of the FAMEs were calculated with respect to a $CO_2$ reference gas injected with every sample and traceable to International Atomic Energy Agency reference material NBS 19 TS-Limestone. Isodat 3.0 Gas Isotope Ratio MS Software (Ver 3.0) (ThermoFisher Scientific, Bremen, Germany) was used for data processing, and the final results were exported into Excel, and further processed using in house Visual Basic macros, which helped in selection of peaks of interest using relative retention time with respect to the internal standard. The macros also corrected $\delta^{13}C$ values of FAMEs for C added as a methyl group during derivatisation using a mass balance approach. Precision of the $\delta^{13}C$ FAME analysis was indicated by the $\delta^{13}C$ values determined for the C19 internal standard added to all samples; $\delta^{13}C = -32.44 \pm 0.67$‰ (mean ± sd, $n=23$). Measurement of the Indiana University reference material hexadecanoic acid methyl ester #1 (certified $\delta^{13}C_{VPDB}$ value $= -30.74 \pm 0.01$‰) gave a value of $-30.86 \pm 0.17$‰ (mean ± sd, $n=13$).

The combined area of all mass peaks ($m/z$ 44, 45 and 46) after background subtraction were collected for each individual FAME peak. These combined areas, relative to those of the internal standard, were used to quantify the 18 most ubiquitous FAMEs and subsequently the PLFAs from which they were derived, as described by Thornton et al. (2011).

Bacterial biomass was estimated from the concentration of the bacterial biomarker PLFAs: 15:0i, 15:0ai and 16:0i (Mayor et al., 2012; Moodley et al., 2005) assuming that these PLFAs make up 10% of total bacterial PLFAs and that there is 0.0056 g C PLFA/g C biomass. Estimating bacterial biomass on the basis of specific biomarker PLFAs has the potential to over- or under represent particular groups of bacteria (e.g. Gram-negative or Gram-positive), depending on the relative abundance of biomarkers in these organisms at the study location. Nevertheless, the PLFAs used to estimate bacteria biomass in our study,15:0i, 15:0ai and 16:0i, are widely used across a range of marine benthic ecosystems as generic bacterial biomarkers (e.g. Boschker and Middelburg, 2002; Moodley et al., 2005; Bouillon et al., 2006) and we feel that they provide a reasonable approximation for the bacterial biomass present.

### 2.9. Data analysis

Data exploration (Zuur et al., 2010) and analysis were conducted using the statistical programming language R (R Core Team, 2013) with the 'nlme' (Pinheiro et al., 2014) and 'vegan' (Oksanen

et al., 2013) packages. Oxygen concentration- and benthic biomass data were examined using linear regression techniques (Pinheiro and Bates, 2000; Zuur et al., 2009; Mayor et al., 2012). The effects of time, treatment (Control, 25% WAF and 50% WAF) and a time × treatment interaction on oxygen concentrations in core overlying water were assessed with a linear mixed effects (LME) statistical model. Core identity was included as a random effect (*L.* ratio = 337, df = 1, $p < 0.001$) to allow for the correlation of data collected within each core (Zuur et al., 2009). Treatment effects on the biomass of bacteria and macrofauna were examined using generalised least squares (GLS) models that included variance covariates to account for instances of heterogeneity. Preliminary analyses examining the interaction between treatment and sediment depth revealed strong heterogeneity in the residual plots. GLS models that allowed the residual spread to vary by treatment level were also used to examine treatment effects on the $\delta^{13}C$ values of individual PLFAs. Biomass data from the 0–1 and 1–2 cm sediment horizons were therefore analysed separately. The optimal structures of all linear models were determined using backwards selection based on the *L.* ratio test with maximum likelihood estimation.

Relative abundance (mol%) and stable carbon isotope ratios ($\delta^{13}C$) of the 18 identified PLFAs were examined using redundancy analysis (RDA). Both of these datasets differed significantly between the 0–1 cm and 1–2 cm depth horizons (Supplementary Figs. S1 and S2) and were therefore analysed separately. The significance of treatment effects were examined using a permuted Monte Carlo test ($n = 9999$; Zuur et al., 2007; Mayor et al., 2013).

## 3. Results

Oxygen concentrations were affected by a highly significant Time × Treatment interaction (Tables 1 and S3), indicating that the rate of SCOC was significantly affected by WAF treatment level (Fig. 1). Remineralisation rates, estimated using a respiratory quotient of 1, were calculated as 1.50 mmol C $m^{-2}$ $d^{-1}$ in control cores; 2.66 mmol C $m^{-2}$ $d^{-1}$ in 25% WAF treated cores and 3.08 mmol C $m^{-2}$ $d^{-1}$ in 50% WAF treated cores.

Bacterial biomass present in the microcosms was over an order of magnitude greater than that of metazoans (Table 2). There was a significant treatment effect on bacterial biomass in both the 0–1 and 1–2 cm sediment layers (Tables 1, 2, S4 and S5); increasing concentration of hydrocarbons typically resulted in a decrease in bacterial biomass. By contrast, macrofaunal biomass in the 0–1 and 1–2 cm sediment horizons remained unaffected by hydrocarbon contamination (Tables 1, 2, S6 and S7).

The full set of PLFA compositional (mol%) and isotopic data ($\delta^{13}C$ values) from the 0–1 and 1–2 cm sediment horizons are presented in Tables S8 and S9. There was a significant effect of treatment on the relative composition of PLFAs in the surficial layer (Table 1; Fig. 2a and b). Control cores were discriminated from those in the 25% WAF treatment along the first axis, which explained 24% of the variance in the data. This axis had positive loadings of 18:1($n-7$) and negative loadings of 14:0, 15:0i, 16:0, 16:0i, 17:0 and 17:0ai. The 50% WAF treatment was discriminated on the second axis, which explained 7.0% of variation. This axis had positive loadings of 16:1($n-5$), 19:1($n-5$) and negative loadings of 18:1($n-9$). Replicate observations of the composition of PLFAs in the 1–2 cm layer also grouped by treatment (Fig. 2c and d), although treatment effects were not statistically discernible (Table 1). The first axis, which explained 14% of the variation, had positive loadings of 19:0cy and negative loadings of 17:0, 18:0 and 12Me16:0. The second axis explained 9.0% of the variation and had positive loadings of 18:1($n-9$) and 16:1($n-5$), and negative loadings of 16:1($n-7$) and 19:1($n-6$).

The $\delta^{13}C$ values of individual PLFAs in the 0–1 and 1–2 cm depth layers were not significantly affected by treatment (Table 1). Replicate observations in these horizons were visibly discernible by group, but with high variance (Fig. 3). In the 0–1 cm layer (Fig. 3a and b) the first axis, which explained 15% of the variation, progressively discriminated the control cores from the WAF-treated cores. This axis had positive loadings of 17:0ai, 17:0, 16:1($n-5$) and 19:0cy and negative loadings of 15:0 and 16:0i. The second axis, which explained 3.0% of the variation, discriminated the replicate 25% WAF cores from the control cores. This axis had weak positive loadings of 16:0i, 18:0 and 19:1($n-6$) and negative loadings of 18:1($n-7$). In the 1–2 cm layer (Fig. 3c and d), only a low proportion of the variance was explained by the first (8.9%) and second (5.5%) axis. Axis 1 had positive loadings of 19:1($n-6$) and 18:0; axis 2 had a strong, positive loading of 19:0cy and negative loadings of 17:1($n-8$)c and 16:0i.

Two compounds (17:0 and 17:0ai) became progressively depleted in $^{13}C$ in the surficial sediment, shifting from values of approximately −24‰ in the controls to < −27‰ in the 50% WAF treatment (Table S9). However, when investigated further, there was appreciable variance in the estimates at the 50% WAF level and treatment effects were not regarded as significant (17:0ai: *L.* ratio = 5.79, df = 2, $p = 0.06$; 17:0: *L.* ratio = 5.10, df = 2, $p = 0.08$).

## 4. Discussion

This study demonstrated that SCOC rates and bacterial biomass, estimated using biomarker PLFAs, in a deep sea benthic community

**Table 1**
Summary of results from the statistical tests (LME, linear mixed-effects; GLS, generalized least squares; RDA, redundancy analysis).

| Response | Model | Model term | df | *L*-ratio | *F* | *p* Value |
|---|---|---|---|---|---|---|
| Oxygen concentration | LME | Treatment × time | 2 | 69.6 | | < 0.001 |
| Bacterial biomass (0–1 cm) | GLS | Treatment | 2 | 7.78 | | 0.02 |
| Bacterial biomass (1–2 cm) | GLS | Treatment | 2 | 9.27 | | 0.01 |
| Macrofaunal biomass (0–1 cm) | GLS | Treatment | 2 | 3.67 | | 0.16 |
| Macrofaunal biomass (1–2 cm) | GLS | Treatment | 2 | 0.16 | | 0.92 |
| PLFA composition (0–1 cm) | RDA | Treatment | 2 | | 2.05 | 0.03 |
| PLFA composition (1–2 cm) | RDA | Treatment | 2 | | 1.38 | 0.13 |
| PLFA $\delta^{13}C$ (0–1 cm) | RDA | Treatment | 2 | | 1.02 | 0.42 |
| PLFA $\delta^{13}C$ (1–2 cm) | RDA | Treatment | 2 | | 0.757 | 0.77 |

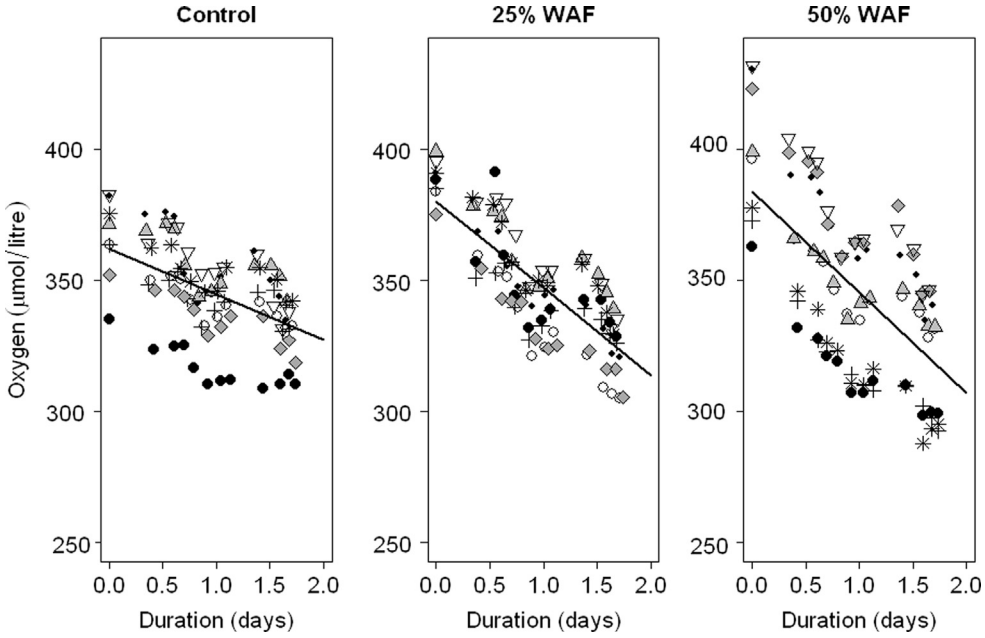


**Fig. 1.** Oxygen concentrations in water overlying the cores during the 36-h incubations. Cores are grouped by treatment in each panel, where symbols of same type show data from the same core. Lines show linear models fitted to the replicate data ($n=8$). Control cores had 25% of their water exchanged with uncontaminated bottom seawater at the start of the experiment.

**Table 2**
Bacterial and macrofaunal biomass in the sediment horizons investigated.

| | Mean bacterial biomass (mmol C $m^{-2} \pm$ SE) | | Mean macrofaunal biomass (mmol C $m^{-2} \pm$ SE) | |
|---|---|---|---|---|
| Depth horizon | 0–1 cm | 1–2 cm | 0–1 cm | 1–2 cm |
| Control | $68 \pm 14$ | $69 \pm 7.7$ | $2.9 \pm 0.7$ | $7.4 \pm 4.0$ |
| 25% WAF | $43 \pm 3.8$ | $37 \pm 4.1$ | $8.5 \pm 4.6$ | $6.1 \pm 2.6$ |
| 50% WAF | $28 \pm 5.7$ | $53 \pm 10$ | $7.7 \pm 3.2$ | $7.2 \pm 2.6$ |

were significantly affected by exposure to increasing levels of WAF. The consistent grouping of the PLFA compositional and isotopic data by treatment suggests that the bacterial community composition and their metabolic functioning were also affected by exposure to WAF. The estimated rate of carbon remineralisation in our control cores, 1.5 mmol C $m^{-2}$ $d^{-1}$, was very similar to the value of 1.8 mmol C $m^{-2}$ $d^{-1}$ reported previously at our experimental location (Heip et al., 2001) and the rate of carbon remineralisation in the upper WAF treatment level was approximately double that in controls. The significant increase in SCOC rate in response to WAF clearly demonstrates that exposure to these hydrocarbons affects deep-sea benthic organisms and hence the functioning of this environment, as has also been observed in shallower marine benthic habitats (Olsen et al., 2007a).

We found no clear effects of treatment on macrofaunal biomass, but this is not surprising given the relatively short incubations and the inability of fauna to migrate out from the enclosed microcosms. In contrast, bacterial biomass in the WAF-exposed cores was consistently lower than that in the controls, suggesting that short-term exposure to hydrocarbons caused mortality and/or reduced growth rates (Griffin and Calder, 1977). The observed reductions in bacterial biomass in response to WAF-exposure are consistent with results of Syakti et al. (2006) who found that the same PLFAs used to estimate bacteria biomass in our study, 15:0i, 15:0ai and 16:0i, decreased in a coastal marine benthic microbial community exposed to artificially weathered crude oil. Lower bacterial biomass does not necessarily imply that the microbial contribution to SCOC in our experiment was lower. It is probable that exposure to WAF elicited stress responses in the fauna and the majority of active bacteria in the microcosms, resulting in increased respiration in both of these groups of organisms. Oil-induced stimulation of hydrocarbon degrading bacteria could also have contributed to the observed increase in oxygen consumption (Lee and Lin, 2013), even though these organisms potentially only represent a relatively minor component of the benthic community (Mazzella et al., 2005; Syakti et al., 2006). Little is known about how the physiology of deep-sea prokaryotic and eukaryotic organisms respond to oil exposure (Vevers et al., 2010). Hence, it is not possible to predict how the relative faunal and bacterial contributions of SCOC in our experiment changed from our biomass estimates alone. The relative contributions to increased oxygen consumption by either stress-induced respiration or remineralisation of the introduced hydrocarbons are also not discernible.

Changes in the composition of sediment PLFAs and their isotopic signatures have previously been used to investigate how natural microbial communities respond to external stressors (e.g. Pelz et al., 2001; Mayor et al., 2013), and $^{13}C$ labeling techniques have traced the incorporation of oil-derived carbon into the biomass of hydrocarbon-degrading bacteria (Rodgers et al., 2000; Pelz et al., 2001). The observed significant oil-driven shift in the composition of PLFAs in surficial sediments is consistent with the understanding that hydrocarbon exposure drives rapid changes in benthic microbial community structure (Hanson et al., 1999) and the composition of their PLFAs (Aries et al., 2001; Mazzella et al., 2005; Syakti et al., 2006). Nevertheless, interpreting PLFA data from natural sediment communities exposed to hydrocarbons is difficult, not least because only a proportion of the extant microbial community is likely to be capable of metabolising hydrocarbons. It is also possible for microbially-driven hydrocarbon degradation to occur without discernible increases in the concentrations of their biomarker PLFAs (Pelz et al., 2001). Our results therefore potentially reflect contrasting physiological responses in different components of the microbial community.

Some consistent patterns of interest emerged in our data. For example, the PLFAs that correlated positively with the WAF-treated sediments were typically saturated moieties, e.g. 14:0, 15:0, 16:0, 16:0i, 17:0, 17:0i and 18:0, many of which are frequently used as generic bacterial biomarkers. This observation agrees well

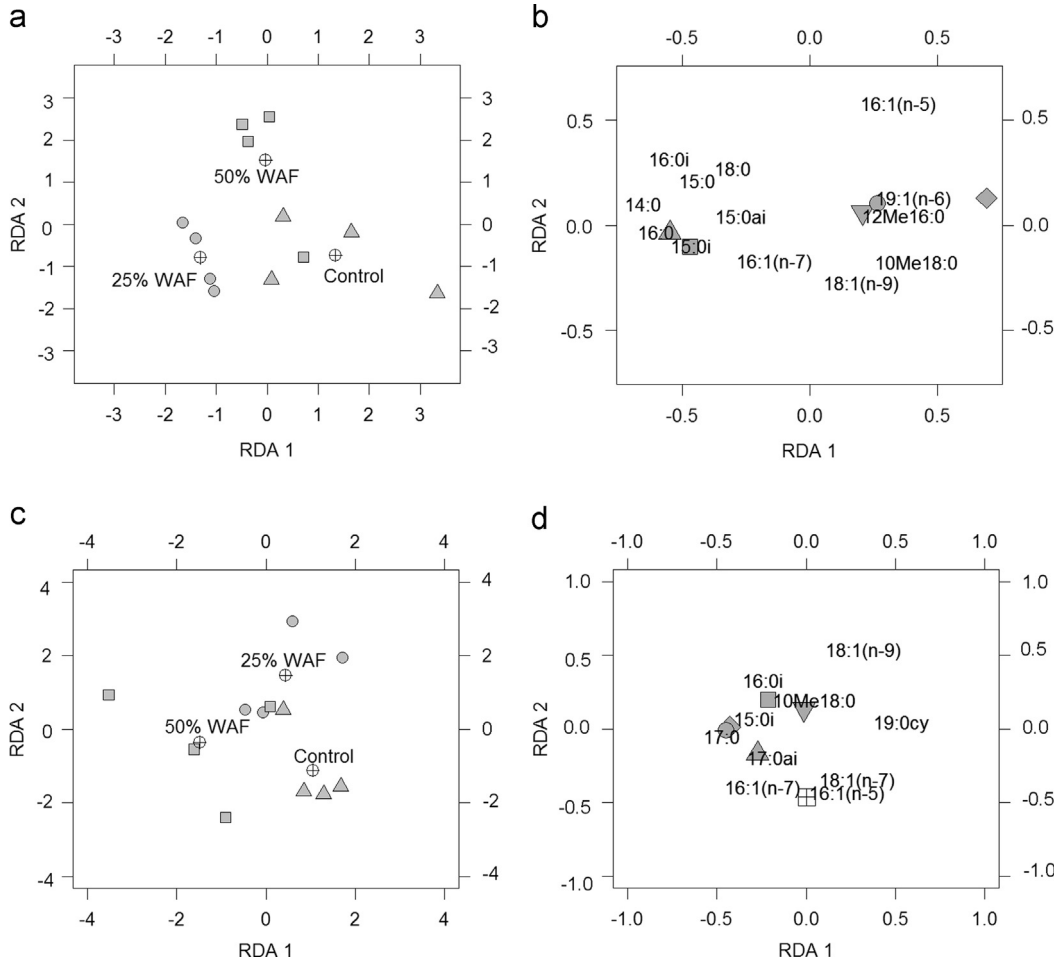


**Fig. 2.** Redundancy analysis plots showing the effect of treatment on microbial PLFA proportion data in the top 0–1 cm (panels a and b) and 1–2 cm (panels c and d) of sediment at the experiment end. Ordination of individual cores is shown by plotting different symbols for each treatment (panels a and c). Triangles = controls; circles = 25% WAF; squares = 50% WAF. First axis explains 24.2% of the variation. Second axis explains 7.0% of the variation. Effects of each treatment are shown by crossed circles. Ordination of individual PLFAs, indicated by their names (panels b and d). The following have been plotted with filled symbols for visual clarity: (b) square = 17:0ai; upward pointing triangle = 17:1($n-8$); downward pointing triangle = 17:0. (d) square = 14:0; upward pointing triangle = 15:0; downward pointing triangle = 16:0; diamond- = 12Me16:0; circle = 18:0; crossed square = 19:1($n-6$).

with a range of studies that have investigated how the presence of hydrocarbons affects the PLFA composition of soils and sediments. For example, the PLFAs 15:0, 16:0, 17:0 and 18:0 have been reported to increase in soil microbial communities where hydrocarbon degradation occurred via the activities of sulfate-reducing bacteria (Hanson et al., 1999; Pelz et al., 2001). Exposure to artificially weathered crude oil has also been reported to increase the prevalence of saturated PLFAs such as 15:0, 17:0 and 18:0 in a consortium of oil degrading marine bacteria (Aries et al., 2001), in a natural coastal benthic microbial community (Mazzella et al., 2005; Syakti et al., 2006) and in individual species extracted from therein (Mazzella et al., 2005). The monounsaturated PLFAs 16:1($n-5$), 18:1($n-7$) and 19:1($n-6$) also contributed to significant discrimination between the treatments in our experiments, with 16:1($n-5$) and 18:1($n-7$) correlating positively with the control cores. These observations agree with the understanding that even-numbered chain length, monounsaturated PLFAs typically increase when hydrocarbon contaminants are removed (Aries et al., 2001; Syakti et al., 2006).

It is important to note that the proportions of PLFAs in the surficial sediments did not respond to the WAF treatment level in a linear manner; many of the saturated moieties increased in the 25% WAF treatment, whereas in the 50% WAF treatment they remained similar to values in the 25% WAF treatment or decreased towards control levels (Table S8). This pattern of responses, and the association of these saturated compounds with hydrocarbon degrading bacteria (Hanson et al., 1999; Aries et al., 2001; Pelz et al., 2001) suggests that a component of the natural sediment microbial community was stimulated by the presence of 25% WAF but inhibited (Muñoz et al., 2007) or even killed in the highest, 50% WAF treatment.

A component of the microbial community may have been able to use the oil as a metabolic substrate (Lapham et al., 1999). Bacterial isotopic signatures change to reflect their nutrition source as they incorporate substrate-derived carbon into their lipids through the turnover of existing PLFAs and the production of new biomass (Boschker and Middelburg, 2002). Therefore, we would expect the isotope ratios of certain PLFAs (of oil-degraders) to eventually shift towards that of the WAF. The oil used to create WAF for our experiments had a bulk $\delta^{13}C$ value of $-29.2‰$. The $\delta^{13}C$ values of monoaromatic hydrocarbons have been found previously to be within 1.0‰ of bulk oil.

The non-significant effect of treatment found by multivariate ordination of PLFA $\delta^{13}C$ signatures indicates either that the majority of PLFAs investigated did not respond consistently, or were not strongly affected by WAF. Given the short incubation time of 36 h, it is perhaps not surprising that such effects could not be distinguished statistically. The microbial community in our experimental sediments

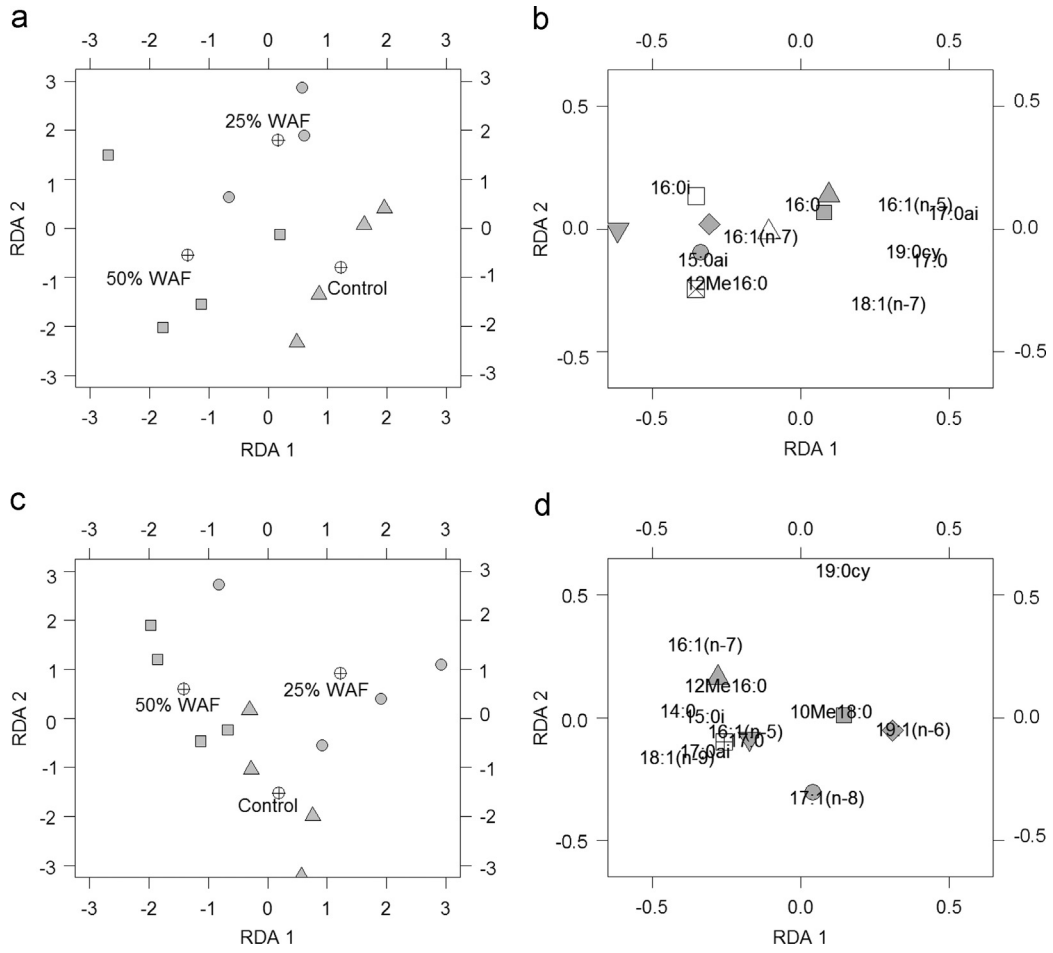


**Fig. 3.** Redundancy analysis plots showing the effect of treatment on $\delta^{13}C$ values of microbial PLFAs at the end of the experiment in the 0–1 cm (panels a and b) and 1–2 cm (panels c and d) layers. Ordination of individual cores is shown by plotting different symbols for each treatment (panels a and c). Triangles = controls; circles = 25% WAF; squares = 50% WAF. Effects of each treatment are shown by crossed circles. Ordination of PLFAs, indicated by their names (panels b and d). The following have been plotted with symbols for visual clarity: (b) square = 14:0; circle = 15:0; upward pointing triangle = 15:0i; downward pointing triangle = 17:1($n-8$). (d) square = 15:0; upward pointing triangle = 15:0ai; downward pointing triangle = 16:0; circle = 16:0i; diamond = 18:0; crossed square = 18:1($n-7$).

had no known previous exposure to hydrocarbons, and thus likely contained few, if any, metabolically active hydrocarbon degrading organisms. A metabolic shift towards hydrocarbon degrading organisms in our incubated sediment could require days, if not weeks, of acclimation. However, the visibly discernible groupings by treatment in the 0–1 and 1–2 cm horizons (Fig. 3) potentially suggest the beginning of a WAF-induced shift in the $\delta^{13}C$ values of the investigated PLFAs. The depletion of $^{13}C$ in the PLFAs 17:0 and 17:0ai in the surficial sediments, albeit only marginally significant, is interesting because this shift is consistent with the incorporation of the isotopically-depleted, oil-derived carbon used in our experiment. The observation that odd-numbered chain length PLFAs, including 17:0, in marine benthic sulfate-reducing bacteria become isotopically enriched when exposed to $^{13}C$-labelled toluene (Pelz et al., 2001) supports this suggestion.

An important caveat to this interpretation is the assumption that the isotopic signatures of compounds within the WAF were similar to the value for the whole crude oil, as has been found previously. We do not know the isotopic signatures of each compound within the WAF, and therefore our data do not conclusively demonstrate hydrocarbon degradation. A range of other processes could also have caused shifts in the isotopic signatures of individual PLFAs (Table S9). For example, anaerobic microbial communities strongly discriminate against $^{13}C$ during fatty acid biosynthesis relative to those growing under aerobic conditions (Teece et al., 1999). WAF-driven changes in the $\delta^{13}C$ values of 17:0 and 17:0ai may thus reflect a reduction in oxygen availability and a shift towards anaerobic metabolism, itself an effect of WAF-induced stress of the benthic community (Fig. 1). Equally, the degradation of faunal biomass as a result of stress-induced mortality could also result in significant isotopic shifts in sediment PLFAs (*sensu* Mayor et al., 2013); $\delta^{13}C$ values of different deep-sea benthic animals can vary by $>10‰$ (Gontikaki et al., 2011). We still lack a detailed understanding of processes that govern the turnover rates of individual PLFAs, the microorganisms that produce them or the metabolic pathways that result in isotopic fractionation in natural sediment systems (Lerch et al., 2011). Better knowledge of these processes is required if we are to meaningfully interpret isotopic shifts in natural bacterial communities in response to unwanted releases of hydrocarbons into the natural environment.

There is an emerging understanding that consortia of marine bacteria provide an intrinsic level of bioremediation in the event of hydrocarbons being released into the pelagic deep sea (Hazen et al., 2010; Bælum et al., 2012; Gutierrez et al., 2013). Benthic microbes also actively contribute to the removal of oil contaminants that reach sediments of the deep sea (Kimes et al., 2013; Mason et al., 2014), but observations are currently scarce. Our data indicate that that the contamination of deep-sea sediments with hydrocarbons will affect the structure and metabolic functioning of the resident microbial communities. However, we cannot conclusively attribute these changes to a shift towards a

hydrocarbon-degrading community of benthic bacteria owing to the paucity of information on the factors that cause the composition and isotopic signatures of PLFAs to change. There is a clear need for more detailed information on how the physiology of deep-sea benthic organisms responds to hydrocarbon contamination so that the mechanisms underpinning our results can be more clearly understood. Future studies should not be limited to understanding the effects on sediment-dwelling microbes; benthic fauna contribute directly to SCOC and other ecosystem functions such as the recycling of nutrients. They also influence ecosystem functioning by mediating changes in the microbial community structure through their physical activities (e.g. Laverock et al., 2010; Mayor et al., 2013). Hence, the net, community-scale response to hydrocarbon contamination will likely reflect a range of interactive processes that are currently not possible to predict. Understanding the disturbance-induced perturbations in benthic processes such as remineralisation give an indication of whole-community responses that will be a useful part of environmental assessment as our understanding of biogeochemical cycling continues to develop. As drilling operations continue to develop in the deep sea, a better understanding of how oil affects all benthic and pelagic processes, over both short and long time scales, is required to foresee the impacts of future blowouts.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.dsr.2014.12.008.

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