Kill•Spill

Integrated solutions for combating marine oil spills

Deliverable D5.1

Report on factors affecting hydrocarbon biodegradation under sulphate reducing conditions

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1 About this deliverable

This deliverable forms part of Task 5.3: Enhancing anaerobic degradation and bioavailability, with partners UNEW, UNIBO, IRSA-CNR and UTex. The deliverable relates to Subtask 5.3.1: Identification of factors which limit rates of spilled oil biodegradation under sulphate-reducing conditions (UNEW). The subtask investigates the response of the trace metals, Nickel (Ni) and Cobalt (Co), on the biodegradation of crude oil in anoxic sediments. Ni and Co are required by key enzymes involved in anaerobic hydrocarbon degradation (e.g. carbon monoxide (CO)-dehydrogenase and methylmalonyl-CoA carboxytransferase). This was placed in the context of the effect of macronutrient (N and P) availability on crude oil biodegradation under sulfate-reducing conditions, a factor that is well known to influence crude oil degradation under oxic conditions. This subtask was performed using a series of sulphate-reducing microcosm experiments dosed with different levels of N and P or Ni and Co and amended with either North Sea crude oil or fuel oil (IFO180). Sediments from the Mediterranean (Messina & Gela, Italy), River Tyne (Newcastle, UK) or Amble Harbour (Amble, UK) were used as bacterial inoculum in microcosms. Sulphate-reduction rates were determined as a measure of hydrocarbon biodegradation in response to Ni and Co dosing in oil-amended microcosms. It is clear from comparisons of oil treated microcosms with killed controls and no oil controls that sulfate-reduction in oil treated sediments above that seen in no oil control incubations was driven by oil biodegradation. Thus sulfate reduction is a reliable proxy for oil degradation in these experiments.

2 Introduction

Natural hydrocarbon seeps are quantitatively the largest source of petroleum in marine systems, nevertheless, anthropogenic activities involved in the production transport and use of crude oil and oil products remain important sources of oil pollution (National Research Council, 2003). As a result of the localized release of relatively large quantities of oil, anthropogenic emissions may have effects on local ecosystems that are disproportionate to their contribution to global budgets of hydrocarbons in the sea. The incidence of major oil spills has decreased by 76% from 787 to 190 during the four decades from 1970 to 2010. In terms of volume this corresponds to a 93% decrease and, excluding the Deepwater Horizon blowout, the total quantity of oil spilt during 2010–2011 (13,000 tonnes from 13 recorded spills) was the lowest so far recorded (ITOPF, 2011). Although such statistics indicate that oil spills are generally declining, major accidents like the Deepwater Horizon blowout on 20th April 2010 in the Gulf of Mexico are a stark reminder that accidental oil spills remain an important environmental hazard.

Many bioremediation strategies for oil spills have focussed on aerobic systems due to rapid rates of degradation and an emphasis on treatment of surface sediments (Atlas and Bragg., 2009; Atlas and Hazen., 2011). Degradation of crude oil in oxic sediments by indigenous microorganisms may be limited due to a lack of inorganic nutrients, such as nitrogen and phosphorus, in the oil and/or sediments, which are a key requirement for microbial growth (Atlas & Bartha, 1972; Head et al., 2006). A useful bioremediation strategy for spilled oil, is the use of fertilisers to increase inorganic nutrient levels and enhance rates of oil biodegradation. This was used successfully in response to the Exxon Valdez oil spill, where large scale addition of fertilisers containing N & P nutrients indeed enhanced oil biodegradation in oil contaminated sediments in Prince William Sound, Alaska (ASM, 2011; Atlas and Hazen, 2011). Recently a systematic study of the effect of N and P on the biodegradation of crude oil in intertidal marine sediments determined fundamental kinetic parameters (K_s and q_{max}) for nitrate- and phosphate-stimulated crude oil biodegradation (Singh *et al*., 2014), such information is essential to model the fate of crude oil in bioremediation programmes that use inorganic nutrient addition to stimulate oil biodegradation.

However, in many cases stimulation of aerobic oil degradation may not be appropriate (e.g. in sensitive salt marsh environments) or may be logistically problematic (e.g. oil which has reached sediments underlying deep water either as partially-degraded residual heavy oil or oil which has been deliberately sunk using adsorbents). In marine systems, the most important inorganic electron acceptor is sulphate, however the factors which determine rates of spilled oil biodegradation under anaerobic SO_4-reducing conditions are still largely unknown (Musat & Widdel., 2008). There is little information on the effect of inorganic nutrients (N and P) or trace metal micronutrients (e.g. nickel, cobalt) on sulphate-driven oil-biodegradation. Indeed, key enzymes in sulphate-reducing bacteria require nickel (e.g. carbon monoxide (CO)-dehydrogenase and key enzymes for anaerobic alkane degradation (e.g. methylmalonyl-CoA carboxytransferase) require cobalt. Both elements are often present at suboptimal concentrations in contaminated sediments (Xu & Obbard., 2003) and Ni and Co concentration in crude oil, determined from a survey of more than 130 oils from around the world showed that the Ni and Co content of oils is highly variable (range 0.4 – 55 µg/g Ni; 3.1 – 900 ng/g Co) (Al-Shahristani & Al-Atyia., 1972, Ellrich *et al*., 1985, Frankenberger *et al*., 1994, Olsen *et al*., 1995, Duyck *et al*., 2008).

Several mechanisms for initial activation of alkanes for anaerobic hydrocarbon degradation have been described including fumarate addition, anaerobic hydroxylation with subsequent carboxylation and hydroxylation using oxygen generated from a range of oxyanions under anoxic conditions. Nevertheless there is still considerable debate over alternative anaerobic alkane activation processes (reviewed in Callaghan, 2013 and Agrawal & Gieg, 2013). Alkane activation by addition to fumarate, yields alkylsuccinate metabolites that can be used to indicate *in situ* anaerobic alkane metabolism. The alkylsuccinate metabolites as well as the gene encoding for the alkylsuccinate synthase enzyme (*ass*A) (Callaghan et al., 2008) are now considered useful biomarkers to provide evidence of *in situ* anaerobic alkane metabolism. Indeed the alkylsuccinate synthase gene (*ass*A) has been detected in conjunction with the *in situ* detection of alkylsuccinate metabolites involved in the metabolism of crude oil hydrocarbons under sulphate-reducing conditions in hydrocarbon-impacted environments (Callaghan *et al*., 2010), shallow aquifers and deep subsurface environments (Agrawal & Gieg., 2013) and microcosm enrichment cultures (Aitken *et al*., 2013).

3 Aims and Objectives

The working hypothesis underlying this part of the Kill•Spill project is that micronutrient enhancement can be a feasible strategy to speed up hydrocarbon biodegradation under SO_4-reducing conditions. In the context of a broader assessment of the effects of inorganic nutrients on anaerobic crude oil biodegradation we also investigated the effects of macronutrients (NH_4^+, PO_4^{3-}). An anaerobic sediment microcosm approach was used to investigate the effects of micronutrients (Ni, Co) and macronutrients (NH_4^+, PO_4^{3-}) on the enhancement or limitation of rates of oil biodegradation under sulphate-reducing conditions.

4 Material and Methods

Microcosm incubations were prepared to assess the effects of trace element concentrations (Nickel (Ni) and Cobalt (Co)) on hydrocarbon degradation under sulphate-reducing conditions.

4.1 Microcosm assembly

Sediment microcosms were assembled in glass serum bottles (120 ml, Wheaton) inside an anaerobic cabinet (Coy laboratory products, York, U.K.) fitted with an oxygen sensor and with an atmosphere of nitrogen (99.5%) and hydrogen (0.5%) (BOC Special Gases Ltd.). Sediments (10 g, Section 4.1.1) and

weathered oils (200 mg, Section 4.1.2) were weighed directly into glass serum bottles. Nutrient medium (90 ml) to support the growth of mesophilic, sulphate-reducing bacteria (Bak and Widdell, 1992 – Appendix A) was prepared, and dosed with Ni and Co at 0, 100, 200 and 400% of cell requirement (Section 4.1.3, Table 1). Sulphate-reducing conditions were established by the addition of Na_2SO_4 (28 mM) to the medium. The salt concentration was matched to the *in-situ* salinity at the sampling location of the sediment inocula (Section 4.1.1). After assembling, microcosms were sealed with rubber stoppers and aluminium crimp seals, providing a final volume of 100 ml sediment slurry with 20 ml headspace per bottle. Triplicate microcosms were prepared for each treatment (Table 2). Microcosms were removed from the anaerobic cabinet and flushed with N_2 gas to displace 0.5% H_2 gas from the headspace and to prevent H_2 from being used as a substrate by the microbes which may affect hydrocarbon degradation rates. Controls consisted of microcosms which were autoclaved (121°C, 20 min) on three consecutive occasions, and unamended (no oil) microcosms which were prepared to assess background levels of sulphate-reduction in the absence of oil (Table 2). All microcosms were statically incubated in a temperature-controlled room at 25°C in the dark.

4.1.1 Sediment inocula

Homogenised sediments (10 g) from the following locations were used as a source of bacterial inoculum in anaerobic microcosms.

- River Tyne, Scotswood, UK (Brackish salinity 7 g L^{-1} NaCl).
- Amble marina, UK (Seawater salinity 20 g L^{-1} NaCl).
- Messina harbour, Sicily (Seawater salinity 20 g L^{-1} NaCl).
- Gela oil spill incident, Sicily (Seawater salinity 20 g L^{-1} NaCl).

4.1.2 Weathering of oil and oil additions

Weathering of North Sea crude oil and fuel oil (IFO180) was performed by incubating the non-weathered oils in a glass Petri dish in the air stream of a fume hood at room temperature (0.4 m/s;~21◦C) for 14 days to remove volatile hydrocarbons. Weathered oils were subsequently added to microcosms (200 mg).

4.1.3 Calculation of Ni and Co concentrations for dosing of microcosms

Levels of Ni and Co amendment were determined from the Ni and Co content of methanogen cells (according to Gonzalez-Gil *et al*, 1999) and estimated Ni and Co requirements for growth on crude oil, where a final concentration of 2.3 µg/l (39 nM) Ni and 1.0 µg/l (17 nM) Co in microcosms corresponds to 100% requirement (Table 1**Fehler! Verweisquelle konnte nicht gefunden werden.**), according to the following calculation:

The Ni and Co content of methanogen cells was previously measured as 0.135 mg Ni g $cells^{-1}$ (2.3 µmol) and 0.06 mg Co g $cells^{-1}$ (1.02 µmol).

The cell yield on 1 g of North Sea crude oil was estimated as follows:

Oil contains 10% of *n*-alkanes = 0.1 g

Of which 85% is carbon = 0.085 g

Of which 10 % will be converted to cells = 0.0085g cells produced per g oil

Therefore the Ni requirement for 1 g of oil is

0.135 mg Ni g $cells^{-1}$ * 0.0085 g cells g oil^{-1} = 0.00115 mg = 1.15 µg Ni g oil^{-1} (0.196 µmol)

And similarly, the Co requirement for 1 g oil is

0.06 mg Co g cells^{-1} * 0.0085 g cells g oil^{-1} = 0.00051 mg = 0.51 µg Co g Oil^{-1} (0.008 µmol)

In microcosms of liquid vol. 100 ml containing 1 g oil, the concentration of these elements becomes 11.5 µg Ni l^{-1} and 5.1 µg Co l^{-1}.

For oil amendments of 200 mg per microcosm:

For 0.2 g of oil, of which 0.85 % will be converted to cells = 0.0017g cells produced per 200 mg oil

Ni requirement =

0.135 mg Ni g cells^{-1} * 0.0017 g cells g oil^{-1} = 0.0002295 mg = 0.2295 µg Ni (0.00391 µmol = 3.91 nmol)

Co requirement =

0.06 mg Co g cells^{-1} * 0.0017 g cells g oil^{-1} = 0.000102 mg = 0.10 µg Co (0.00169 µmol = 1.69 nmol).

Ni and Co additions were added to the nutrient medium (Appendix A, Section 10.1.7), so that the medium could be prepared and dispensed in bulk.

Table 1 Final concentration of Ni and Co in sulphate-reducing, oil-degrading microcosms

% Dose	µg l^{-1}		nM	
	Ni	Co	Ni	Co
0	0	0	0	0
100	2.3	1.0	39.1	16.9
200	4.6	2.0	78.2	33.8
400	9.2	4.0	156.4	67.6

Table 2 Anaerobic microcosms prepared to assess the effect of trace metals, Ni and Co, on sulphate-driven oil biodegradation.

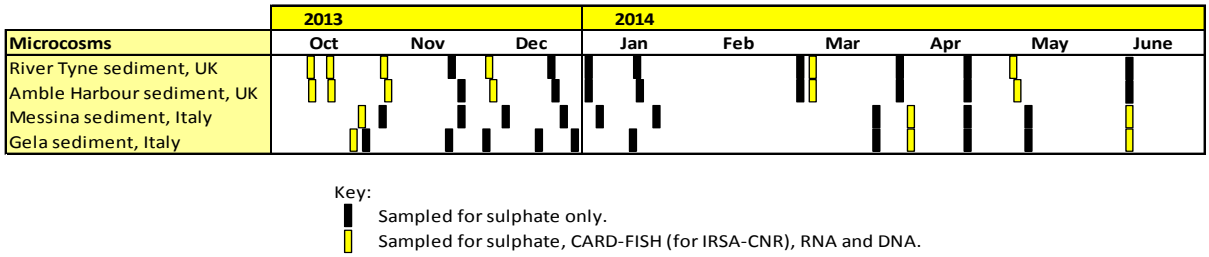
	North sea crude oil					Fuel oil IFO180			
	0% NiCo	100% NiCo	200% NiCo	400% NiCo	400% NiCo KILLED	0% NiCo	400% NiCo	0% NiCo No Oil	400% NiCo No Oil
River Tyne sediment, UK (UNEW estuarine reference sediment)	3	3	3	3	3	3	3	3	3
Amble Harbour sediment, UK (UNEW marine reference sediment)	3	3	3	3	3	3	3	3	3
Messina sediment, IT, Kill•Spill reference sediment)	3	3	3	3	3	3	3	3	3

	Oil contaminated sediment				Uncontaminated sediment			
	0% NiCo	100% NiCo	400% NiCo	400% NiCo KILLED	0% NiCo	100% NiCo	400% NiCo	400% NiCo KILLED
Gela sediment, IT (contaminated and clean sediments following an oil spill)	3	3	3	3	3	3	3	3

4.2 Routine sampling and sample preservation

Routine sampling of microcosms was performed for sulphate analysis, microbial community analysis (RNA and DNA) and CARD-FISH analysis (in collaboration with IRSA-CNR) (Table 3). In addition microcosms were assembled and were sacrificed by immediately freezing (-20°C) (10 g sediment + 90 ml media) to act as time 0 samples for oil geochemistry, microbial community analysis and trace metal analysis.

Table 3 Sampling schedule of anaerobic oil-degrading, sulphate-reducing microcosms.



Incubation start dates were staggered to accommodate regular sampling. The large number of microcosms involved meant that it would not have been practical to sample all microcosms in a single day. Start dates for each incubation were; River Tyne (09/10/2013), Amble Harbour (11/10/2013), Messina (28/10/2013) and Gela (22/10/2013).

4.2.1 Chemical analysis

Sediment slurries (1 ml) were collected from microcosms, and immediately frozen (-20°C) for chemical analyses (anions (sulphate) and VFA, if required).

4.2.2 Microbial community analysis

For DNA extraction and cell counts, sediment slurries (1 ml) were collected from microcosms and transferred into filter-sterilized (0.2 µm pore size) absolute ethanol (1 ml). Samples were mixed and stored at -20°C.

For RNA preservation, sediment slurries (1 ml) were collected from microcosms into filter-sterilized RNAlater RNA stabilization reagent (500 µl, Qiagen). Samples were mixed and incubated at 4°C for 3 hours before long term storage at -20°C.

4.2.3 CARD-FISH analysis

Sediment slurries (1 ml) were collected from microcosms and transferred into filter-sterilized 37% formaldehyde (130 µl). Samples were mixed and incubated at 4°C for 3 hours, followed by the addition of filter-sterilized 96% ethanol (1 ml). Tubes were stored horizontally at -20^{o}C.

4.3 Microplate assay to monitor sulphate-reduction

4.3.1 Barium-gelatin-HCl reagent preparation

The assay for the determination of sulphate was based on the barium-gelatin method of Tabatabai, 1974 as described in Laskov et al., 2007. Gelatin (0.75 g) was dissolved in 250 ml of boiling water, and cooled in an ice bath. Barium chloride (10 g) was added to the gelatin, and the barium-gelatin reagent was diluted 1:1 with hydrochloric acid (0.5N). The reagent (50 µl per well) was added to a 96-well microtitre plate before adding the samples (250 µl per well). The microtitre plate was agitated continuously at 150 rpm for 60 m, followed by measurement of turbidity at 450 nm (Infinite F200 Pro microplate reader, Tecan, Reading, UK). The pre-mixed barium-gelatin-HCl is stable only for 3 h and was freshly prepared for each assay.

4.3.2 Sample and standards preparation for sulphate analysis

Microcosm samples for chemical analyses were collected (Section 4.2.1) and were diluted 1:100 to a final volume of 250 µl. Sulphate standards in the range 0 - 280 µM and 0 – 28 mM sulphate were prepared in either deionised water or growth medium used for the preparation of microcosms (Appendix A). Triplicate absorbance measurements at 450 nm were performed on sulphate standards (Infinite F200 Pro microplate reader, Tecan, Reading, UK) and calibration curves were determined. A comparison of sulphate measurements from the microplate assay were compared to values from the same set of standards and samples performed using ion chromatography (IC) (Dionex ICS-1000 with AS40 autosampler).

4.4 Statistical analysis

Sulphate-reduction rates (SRR) in oil-degrading sediment microcosms and controls were analysed by One Way analysis of variance (ANOVA) followed by pairwise comparisons of treatments (Tukey HSD), IBM SPSS statistics 19.

5 Results

5.1 Optimisation of a microplate assay to monitor sulphate-reduction

A microplate assay for the measurement of sulphate in microcosms was optimised by preparing sulphate standards in the ranges 0 - 280 µM and 0 – 28 mM sulphate in either deionised water or growth medium used for the preparation of microcosms. At low sulphate concentrations a linear response was elicited (Figure 1A), however at concentrations greater than approximately 3 mM sulphate the response was no longer linear (Figure 1B). This corresponded to absorbance measurements greater than about 1.5 as expected for photometric assays based on Beer-Lambert Law. The diluent used for sample dilution did not appear to influence the absorbance measurements, sulphate standards prepared with either deionised water or in growth medium used for the preparation of microcosms gave similar absorbance readings at the 0 - 280 µM sulphate range (correlation = 0.999) and the 0 – 28 mM sulphate range (correlation = 0.977). A comparison of sulphate measurements from the microplate assay were compared to measurements of the same set of standards and samples performed using an ion chromatography (IC) system. A strong positive

correlation between microplate assay and IC sulphate measurements was determined for sulphate standards (0.997, n=12) and microcosm samples (0.827, n=24).

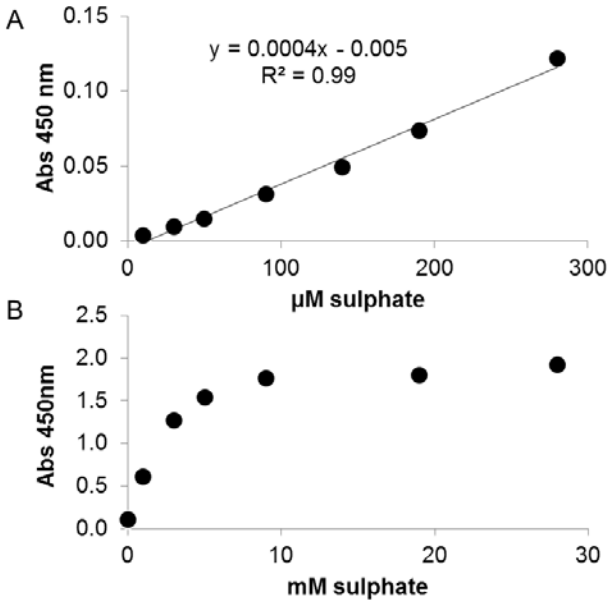


Figure 1 **Optimisation of colorimetric microplate assay for sulphate measurements.**
Triplicate absorbance measurements (450 nm) of sulphate standards prepared in deionised water over the range 0 - 280 µM sulphate (A) and 0 – 28 mM sulphate (B). Error bars show 1 x S.E. and were smaller than the symbols for all data points.

5.2 Effect of macronutrients (NH_4^+ and PO_4^{3-}) on sulphate-driven oil biodegradation

Unpublished findings (courtesy of Dr Luiza L Andrade, Newcastle University, UK) from a similar microcosm study investigating the effect of different concentrations of macronutrients, nitrogen (NH_4^+) and phosphate (PO_4^{3-}), on sulphate-driven oil biodegradation determined that low, medium and high concentrations of nutrients had a minimal effect on sulphate-reduction (Figure 2). In this study a non-weathered North Sea crude oil was used. Oil-amended microcosms displayed similar rates of sulphate-reduction when they were treated with ammonium and phosphate at high (100 mM NH_4^+, 10 mM PO_4^{3-}), intermediate (56 mM, 5.6 mM) or low (4.7 mM, 1.5 mM) levels. Sulphate reduction rates were 4.2 ±0.5, 5.2 ±0.5 and 5.3 ±0.9 µmol SO_4^{2-} day^{-1} g^{-1} sediment, with high, medium or low nutrient levels respectively. These values were not statistically significantly different ($p = 0.5$).

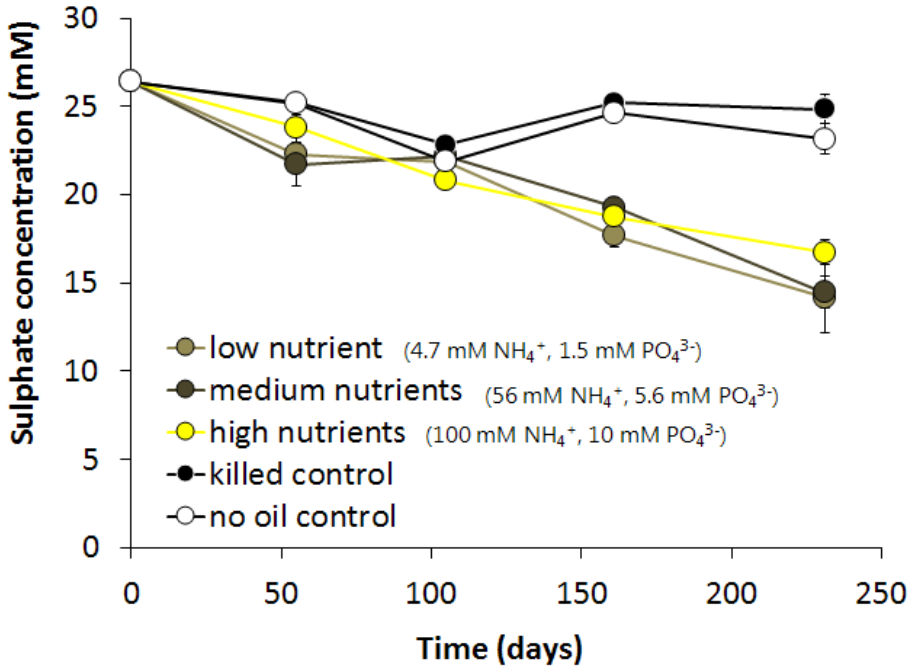


Figure 2 **Sulphate-reduction in oil-degrading sediment microcosms and controls amended with different concentrations of macronutrients, NH_4^+ and PO_4^{3-}.**
Error bars show 1 x S.E.

However, detailed oil geochemistry performed on the microcosm samples indicated that some effects of NH_4^+ and PO_4^{3-} on oil biodegradation were apparent (**Fehler! Verweisquelle konnte nicht gefunden werden.**). Following 231 days of incubation, sulphate-driven oil biodegradation was evident in the volatile alkanes (*n*C_5-*n*C_{10}), where a significant decrease in alkanes was observed in oil-degrading microcosms amended with low, medium and high nutrients compared to the initial conditions (0 days) and heat-killed controls. Furthermore, the degradation of volatile alkanes was significantly different between microcosms amended with low and high concentrations of macronutrients (**Fehler! Verweisquelle konnte nicht gefunden werden.**). Aromatic hydrocarbons (benzene and toluene) were also monitored. Toluene was completely removed by day 231 in microcosms amended with low, medium and high nutrient concentrations (**Fehler! Verweisquelle konnte nicht gefunden werden.**). In the case of the more persistent volatile aromatic, benzene, degradation was significantly greater in microcosms amended with medium and high nutrient concentrations, compared to low nutrient treatment and heat-killed controls (**Fehler! Verweisquelle konnte nicht gefunden werden.**).

To confirm selection of anaerobic alkane-degrading organisms that used the fumarate addition pathway in the sulphate-driven, oil-degrading microcosms treated with different concentrations of macronutrients, alkylsuccinate synthase (*ass*A) genes were analysed. Using primers designed and tested in Aitken *et al.*, 2013, *ass*A genes were detected in all nutrient amended microcosms from day 55 of the incubation (Figure 4). Moreover, *ass*A was absent in microcosms prepared without oil (Figure 4).

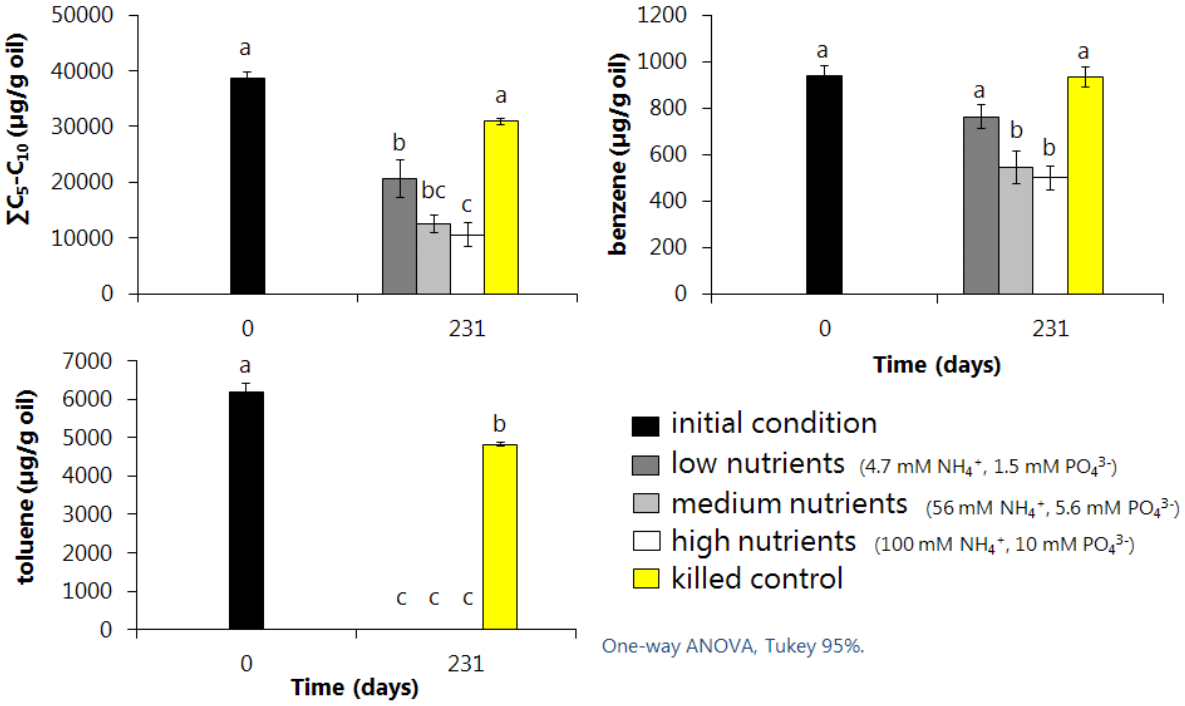


Figure 3 **Effect of macronutrient concentration (NH_4^+ and PO_4^{3-}) on biodegradation of alkanes (nC5-nC10), benzene and toluene in sulphate-reducing, oil degrading microcosms relative to controls.** Bars labelled with the same letter are not significantly different, bars with different letters are significantly different as assessed by One-Way ANOVA with Tukey HSD post-hoc testing. Error bars show 1 x S.E.

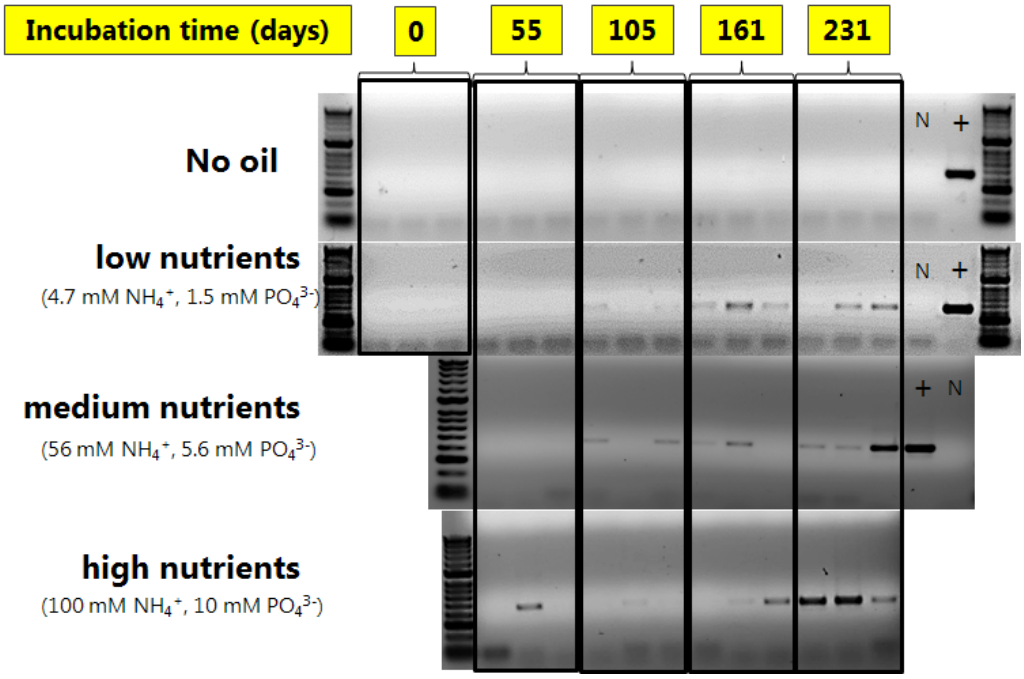


Figure 4 **Presence of the alkylsuccinate synthase gene (*ass*A) was determined with PCR in sulphate-reducing, oil-degrading microcosms amended with different concentrations of macronutrients.** +, PCR positive control; N, PCR negative control.

5.3 Effect of micronutrients (Ni and Co) on sulphate-driven oil biodegradation

Sulphate reduction rates (SRR) were highest in River Tyne sediment microcosms amended with North Sea crude oil and 100% Ni and Co (16.2 ±0.9 µmol $SO4^{2-}$ day^{-1} g^{-1} sediment) or 0% Ni and Co (13.1 ±2.5 µmol SO_4^{2-} day^{-1} g^{-1} sediment) (Figure 5A). SRR were significantly higher than in Tyne microcosms amended with 200% Ni and Co (6.9 ±2.6 µmol SO_4^{2-} day^{-1} g^{-1} sediment, $p = 0.026$), 400% Ni and Co (4.2 ±1.2 µmol SO_4^{2-} day^{-1} g^{-1} sediment, $p \leq 0.033$), and 400% Ni and Co killed controls (4.8 ±0.2 µmol SO_4^{2-} day^{-1} g^{-1} sediment, $p \leq 0.050$) (Figure 5A). Overall, SRR were higher in response to North Sea crude oil amendment compared to IFO180 fuel oil amendment (cf. Figure 5A and Figure 5B). In River Tyne microcosms amended with IFO180 and containing no Ni and Co amendment (10.2 ±0.8 µmol SO_4^{2-} day^{-1} g^{-1} sediment) significant differences in SR rates were observed in comparison to microcosms without oil and amended with 400% Ni and Co (3.9 ±0.7 µmol SO_4^{2-} day^{-1} g^{-1} sediment) ($p = 0.040$) (Figure 5B).

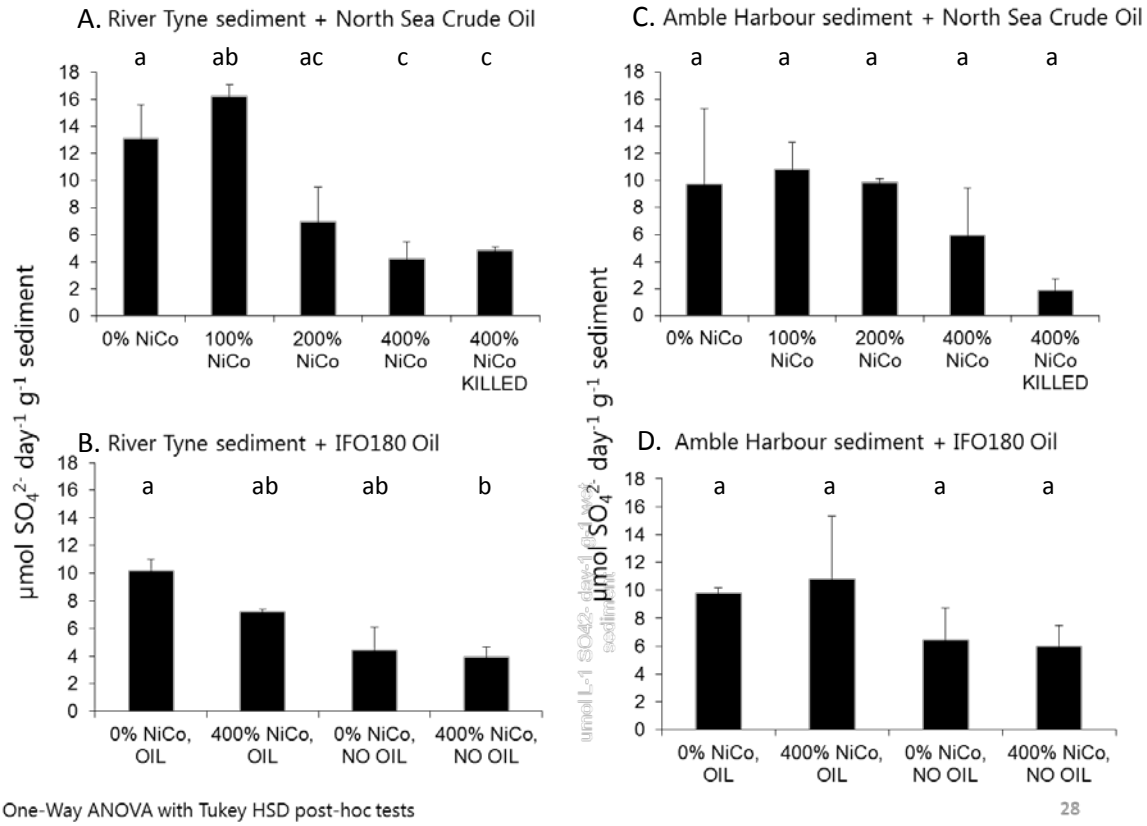


Figure 5 **Sulphate-reduction rates (SRR) in oil-degrading sediment microcosms and controls amended with different concentrations of trace metals, Ni and Co.**
SRR was monitored in River Tyne sediment microcosms (A & B) and Amble Harbour sediment microcosms (C & D) amended with either North Sea crude oil (A & C) or fuel oil IFO180 (B & D). Bars labelled with the same letter are not significantly different, bars with different letters are significantly different as assessed by One-Way ANOVA with Tukey HSD post-hoc testing. Error bars are 1 x SE of microcosm replicates.

In hydrocarbon-degrading microcosms prepared with sediments from Amble harbour, UK, generally SR rates were highest in oil-amended and Ni and Co-amended microcosms with both, North Sea crude oil (Figure 5C) or IFO180 oil (Figure 5D), however the rates were not significantly different from heat-killed or no oil controls. It is however interesting to note that in River Tyne sediments where oil-

driven sulphate reduction relative to killed and no oil controls was observed (Figure 5A), higher levels of Ni and Co appeared to be inhibitory (Figure 5A & 5B).

By contrast, Messina and Gela sediment microcosms showed low levels of sulphate reduction across the whole sample set (Messina: 0.26 ±0.41 µmol SO_4^{2-} day^{-1} g^{-1} sediment (n=27); Gela: 0.57 ±0.25 µmol SO_4^{2-} day^{-1} g^{-1} sediment (n=24)) (Figure 6) with no apparent significant differences between all of the treatments (Table 2).

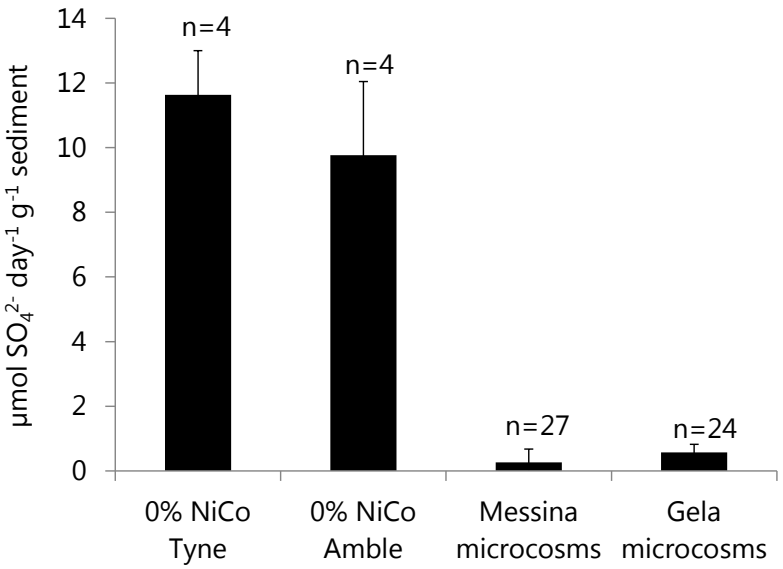


Figure 6 **Differences in sulphate-reduction rate in oil-degrading microcosms in response to sediment type** SR was monitored in River Tyne and Amble sediment microcosms containing North Sea crude oil or IFO180 fuel oil, without Ni and Co amendment (n=4), and compared to the full complement of microcosms prepared with Messina sediment (n=27) and Gela sediment (n=24). Error bars are 1 x SE.

6 Discussion

A microcosm study investigating the effect of different concentrations of macronutrients, nitrogen (NH_4^+) and phosphorus (PO_4^{3-}), on sulphate-driven oil biodegradation determined that sulphate reduction was driven by the presence of the oil as no sulphate-reduction occurred in heat-killed controls or controls without oil. Despite this, different concentrations of macronutrients had a minimal effect on the sulphate-reduction rates. Detailed oil geochemistry did however determine some effects of NH_4^+ and PO_4^{3-} on oil biodegradation, volatile alkanes were significantly more degraded in microcosms with a high concentration of nutrients compared to those with a low concentration of nutrients suggesting that higher nutrient concentrations may have had a stimulatory effect on the microbes involved in alkane metabolism. This was confirmed by the detection of *ass*A in all nutrient-amended microcosms shown to be degrading volatile alkanes. Similar findings were shown with the more persistent volatile aromatic hydrocarbon, benzene, suggesting that higher nutrient concentrations may also have a stimulatory effect on the microbes involved in aromatic hydrocarbon metabolism. Apart from this study, little is known about the effect of nutrient concentration on anaerobic benzene degradation, and the genes involved in the pathways of anaerobic metabolism of benzene have only recently been elucidated (Zhang *et al*., 2014).

It has been suggested that alteration of the concentration of micronutrients, particularly Nickel and Cobalt, may stimulate oil biodegradation, due to the requirement of these trace metals by key enzymes involved in anaerobic hydrocarbon degradation (e.g. carbon monoxide (CO)-dehydrogenase and methylmalonyl-CoA carboxytransferase). River Tyne sediment microcosms showed the highest rates of oil-driven sulphate reduction when amended with 100% of cell requirements of Ni and Co trace metals, suggesting that exogenous Ni and Co will have little effect on oil-driven sulphate reduction. Furthermore, at higher levels of Ni and Co dosing (200% and 400%) an inhibitory effect on oil-driven sulphate reduction was apparent. Oil-driven sulphate reduction was greater with light oil (North Sea light crude) than heavy oil (IFO180), presumably because the lighter crude contained a greater proportion of lower molecular weight, more readily biodegradable, less recalcitrant compound classes, compared to the heavier fuel oil. SR rates were highest in fine, anoxic sediments (River Tyne) compared to sandier sediments (Amble, Messina and Gela), suggesting that oil-driven sulphate reduction is likely to be minimal in sandy coarse grained sediments compared with fine grained, organic rich sediments.

7 Conclusions

- A microplate assay for monitoring sulphate was optimised and has proved useful for routine monitoring of sulphate in microcosm sub-samples.
- At a gross level macronutrients (NH_4^+ and PO_4^{3-}) had a minimal effect on oil-driven sulphate reduction
- Detailed oil chemistry indicated that volatile alkanes and benzene were significantly more degraded in microcosms amended with high levels of macronutrients compared to low levels
- SR rates in oil-degrading microcosms were highest when microcosms were dosed with 100% of cell requirements of Ni and Co indicating that exogenous Ni and Co have little effect on oil-driven sulphate reduction
- High levels of Ni and Co (200% and 400%) were shown to have an inhibitory effect on oil-driven sulphate reduction.
- Oil-driven sulphate reduction was greater with light oil (North Sea light crude) than heavy oil (IFO180).
- SR rates were highest in fine, anoxic sediments (River Tyne) compared to sandier sediments (Amble, Messina and Gela), suggesting that oil-driven sulphate reduction is likely to be minimal in sandy coarse grained sediments compared with fine grained, organic rich sediments.

8 Future work

- Measuring Ni and Co levels and assessing bioavailability in sediments
- Oil chemistry
- Microbial community analysis
- Functional gene analysis (*assA*)
- CARD-FISH and GENE-FISH analysis (in collaboration with IRSA-CNR)
- Quantitative PCR
- SYBR green cell counts
- Calculation of cell-specific sulphate reduction rates to further define the effects of Ni and Co on the microbial communities

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10 Appendix A

10.1 Nutrient medium preparation

A defined medium was prepared containing all the minerals, trace elements, and vitamins that may be required by sulphate-reducing bacteria (Widdel and Bak, 1992), according to the following:

All solutions were prepared in deionised water:

10.1.1 Basal Salts Medium

Prepared in 970 ml deionised water, the final 30 ml is added as $NaHCO_3$ solution. Autoclave at 121°C for 20 min.

	Brackish (g)	Seawater (g)
NaCl	7.0	20
$MgCl_2 .6H_2O$	1.2	3
$CaCl_2 .2 H_2O$	0.1	0.15
NH_4Cl	0.25	0.25
KH_2PO_4	0.2	0.2
KCl	0.5	0.5
Na_2SO_4	4.0	4.0

10.1.2 Non-chelated Trace Elements Solution.

Prepared to 1L, without the addition of Ni and Co.

Deionised water	975 ml
HCl (25% = 7.7M)	12.5 ml
$FeSO_4 . 7 H_2O$	2.1 g
H_3BO_4	30 mg
$MnCl_2 . 4 H_2O$	100 mg
$CuCl_2 . 2 H_2O$	2 mg
$ZnSO_4 . 7 H_2O$	144 mg
$Na_2MoO_4 . 7 H_2O$	36 mg

Add Fe last.

10.1.3 Selenite-Tungstate Solution.

Prepared to 1L in deionised water.

	mg	g
NaOH	400	0.4
Na_2SeO_3 . 5 H_2O	6	0.006
Na_2WO_4 . 2 H_2O	8	0.008

10.1.4 Resazurin Indicator Solution (0.5g/l)

Prepare by adding 0.5 g sodium resazurin salt to 1 L autoclaved water (which should be degassed during the autoclaving procedure).

10.1.5 $NaHCO_3$ solution (1.0 M)

Degas 30 ml sterile water in a 100 ml Wheaton serum bottle for 10 min using N_2. Add 2.5 g $NaHCO_3$ under N_2. Seal bottle with butyl rubber stopper and aluminium crimp seal. Autoclave for 15 min at 121°C.

10.1.6 Oxygen Scavenger Solution (Na_2S, 0.2M)

Degas 50 ml sterile water in a 100 ml Wheaton serum bottle for 10 min using N_2. Add 2.4g Na_2S under N_2. Seal bottle with butyl rubber stopper and aluminium crimp seal. Autoclave for 15 min at 121°C.

10.1.7 Ni and Co amendments

Final concentrations in Basal Salts Medium (Section 10.1.1) (i.e. final concentrations in microcosm liquid phase – except the oil addition):

% Dose	Conc. TE microcosm^{-1} (µg l^{-1})	
	Ni	Co
0	0	0
100	2.3	1.0
200	4.6	2.0
400	9.2	4.0

Ni and Co were prepared as concentrated stocks (1000 µg l^{-1}) and added to the media components in the anaerobic cabinet – make up to 1 L volumes using volumetric flask and individual media components.

To prepare 10 mg l^{-1} stock, using $NiCl_2.6H_2O$ and $CoCl_2.6H_2O$ 237.93

RMM $NiCl_2.6H_2O$ = 237.69, of which Ni = 58.69/237.69= 0.2469

10 mg of Ni = 10 / 0.2469 = 40.50 mg (0.04 g) in 1 L deionised water.

RMM $CoCl_2.6H_2O$ = 237.93, of which Co = 58.93/237.93= 0.2476

10 mg of Co = 10 / 0.2476 = 40.38 mg (0.04 g) in 1 L deionised water.

Dilutions into the Basal Salts Medium (1L vol.):

400 % dose: Ni soln. = 920 µl, Co soln. = 400 µl

200 % dose: = 460 µl, = 200 µl

100 % dose: = 230 µl = 100 µl