Beyond N and P: the impact of Ni on crude oil biodegradation

Obioma K. Mejeha [1,2*], Ian M. Head [1], Angela Sherry [1], Clare M. McCann [1], Peter Leary[1], D. Martin Jones [1] and Neil D. Gray [1]

[1] School of Civil Engineering and Geosciences, Newcastle University, Newcastle upon Tyne, NE1 7RU, United Kingdom

[2]Federal University of Technology, P. M. B. 1526, Owerri, Nigeria

*correspondence: obioma.mejeha@futo.edu.ng

Running Title: Nickel impacts on hydrocarbon degradation

Keywords: Biodegradation, Biostimulation, Bioremediation, Crude oil, Nickel

Current addresses of corresponding author:

Obioma K. Mejeha: Department of Microbiology, Federal University of Technology, P. M. B. 1526, Owerri, Nigeria; Phone: +2349035621829; email: obioma.mejeha@futo.edu.ng

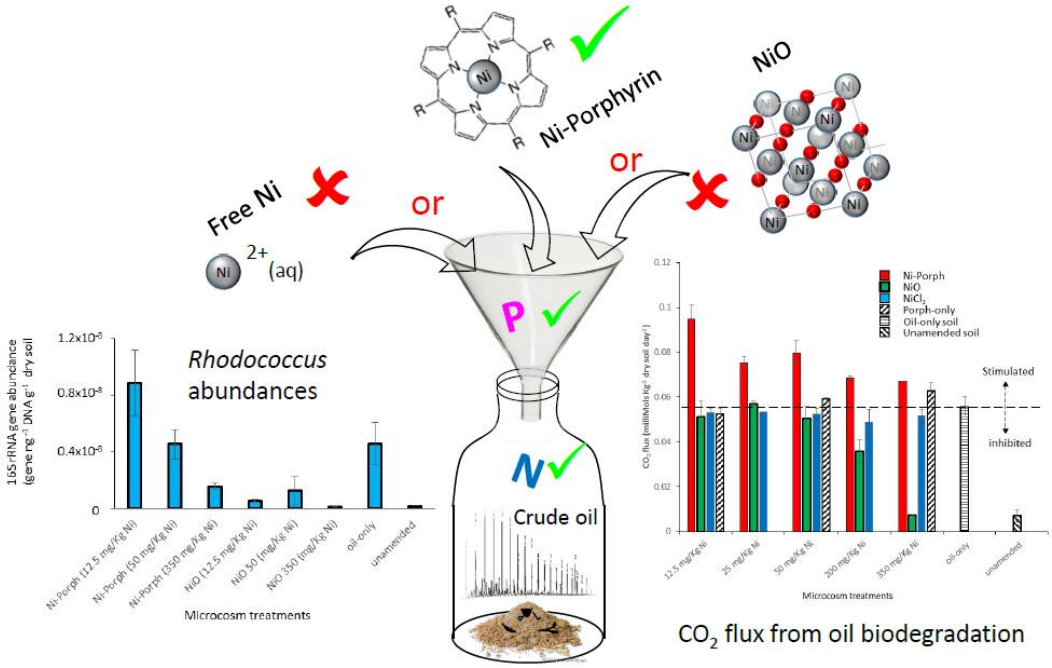
Abstract

N and P are the key limiting nutrients considered most important for the stimulation of crude oil degradation but other trace nutrients may also be important. Experimental soil

microcosms were setup to investigate crude oil degradation in the context of Ni amendments. Amended Nickel as NiO, $NiCl_2$, or, a porphyrin complex either inhibited, had no effect, or, enhanced aerobic hydrocarbon degradation in an oil-contaminated soil. Biodegradation was significantly (95% confidence) enhanced (70%) with low levels of Ni-Porph (12 mg/Kg) relative to an oil-only control; whereas, NiO (200 and 350 mg/Kg) significantly inhibited (36 and 87%) biodegradation consistent with oxide particle induced reactive oxygen stress. Microbial community compositions were also significantly affected by Ni. In 16S rRNA sequence libraries, the enriched hydrocarbon degrading genus, *Rhodococcus,* was partially replaced by a *Nocardia* sp. in the presence of low levels of NiO (12 and 50 mg/Kg). In contrast, the highest relative and absolute *Rhodococcus* abundances were coincident with the maximal rates of oil degradation observed in the Ni-Porph-amended soils. Growth dependent constitutive requirements for Ni-dependent urease or perhaps Ni-dependent superoxide dismutase enzymes (found in *Rhodococcus* genomes) provided a mechanistic explanation for stimulation. These results suggest biostimulation technologies, in addition to N and P, should also consider trace nutrients such as Ni tacitly considered adequately supplied and available in a typical soil.

Highlights

- Ni concentration and form added to soil dictated impacts on oil biodegradation
- Biodegradation was enhanced with low concentrations of porphyrin-complexed Ni (12 mg/Kg)
- At higher concentrations, Ni oxide but not porphyrin-complexed Ni was inhibitory
- Highest *Rhodococcus* abundances were coincident with porphyrin-complexed Ni stimulation


Ni-Porphyrin
NiO
Free Ni
Ni 2+ (aq)
or
or
P
N
Crude oil
Rhodococcus abundances
16S rRNA gene abundance (gene ng-1 DNA g-1 dry soil
1.2x10-5
0.8x10-5
0.4x10-5
0
Ni-Porph (12.5 mg/Kg Ni)
Ni-Porph (50 mg/Kg Ni)
Ni-Porph (350 mg/Kg Ni)
NiO (12.5 mg/Kg Ni)
NiO 50 (mg/Kg Ni)
NiO 350 (mg/Kg Ni)
oil only
unamended
Microcosm treatments
Ni-Porph
NiO
NiCl2
Porph-only
Oil-only soil
Unamended soil
Stimulated
inhibited
0.12
0.1
0.08
0.06
0.04
0.02
0
12.5 mg/Kg Ni
25 mg/Kg Ni
50 mg/Kg Ni
200 mg/Kg Ni
350 mg/Kg Ni
oil only
unamended
Microcosm treatments
CO2 flux from oil biodegradation

49

1. Introduction

Despite efforts to find alternative energy sources, oil consumption will continue to increase at least up to 2040 (International Energy Agency, 2015), peaking at over 100 million barrels per day, which is 9–10% higher than the present. With oil spills still occurring in upstream and downstream production activities, there is a continuing need to develop and refine remediation methods for oil contaminated sites. With respect to remediation, the international community now prioritizes the development of methods to protect ecosystems, for instance, the EUs sustainable approach for the remediation of crude oil spills (Kalogerakis et al., 2014). Bioremediation, a process of utilising biological systems to clean up contaminants is considered an efficient, cost effective and eco-friendly approach (Tyagi, et al., 2011) and has been employed for the remediation of major crude oil spills (Atlas and Hazen, 2011; Ron and Rosenberg, 2014).

Generally, microbial oil bioremediation is slow relative to more physical and chemical approaches (Macaulay and Rees, 2014). Hence, research studies prioritize improving rates (Macaulay and Rees, 2014) either by manipulating the environment to facilitate rapid proliferation of indigenous hydrocarbon degraders (biostimulation) or by augmentation with non-indigenous hydrocarbon degraders (bioaugumentation) (Tyagi et al., 2011; Nikolopoulou et al., 2013; Suja et al., 2014).

For biostimulation, limiting factors such as the concentrations of the main nutrients (nitrogen (N) and phosphorous (P)), are often manipulated to improve biodegradation rates via stimulated enrichment of indigenous aerobic petroleum hydrocarbon degraders (e.g. Singh et al., 2014). Biostimulation has been implemented successfully at number of crude oil contaminated sites. For instance, Inipol EAP22 (an oleophilic fertilizer comprising 7.4% N and 0.7% P by weight) and Customblen (a slow nutrient-releasing encapsulated product consisting of 28% N and 3.5% P), enhanced the biodegradation rate of petroleum spilled from the Exxon

75 Valdez in Prince Williams Sound, Alaska, by 5 fold (Tyagi et al., 2011). On a sandy beach at
76 Haifa, Israel, 88% of oil pollutants were removed within 4 months using a nitrogen-enriched
77 culture derived from a polymerized formaldehyde-urea resin mixed with the polymerized
78 nitrogen fertilizer (Ron and Rosenberg, 2014).

79 Additionally, it is well known that trace heavy metals (Co, Cu, Fe, Mn, Mo, Ni, V, and Zn) are
80 required for microbial growth and can stimulate biodegradation activities. For instance,
81 although, excessive amounts of heavy metals become harmful, an absence or low concentration
82 of trace metal is often considered the main factor which limits the anaerobic digestion of
83 organic wastes to biogas (Demirel and Scherer, 2011). As further evidence, the addition of Cu
84 and Fe to a culture of *Rhodococcus jostii* RHA1 enhanced the degradation rate of
85 Tetrabromobisphenol A (Xu et al., 2018) and sub-lethal concentrations of metals (Cu and Cd)
86 have even been shown to stimulate biodegradation of 2-chlorophenol and 3-chlorobenzoate
87 (Kuo and Genthner, 1996).

88 There are indications to suggest that crude oil may contain substantial quantities of metals. For
89 instance, it has been reported that Ni and V can be present in crude oil up to 350 and 1580 ppm,
90 respectively, partly as metalloporphyrins (Filby, 1994). Porphyrins are thought to be derived
91 from phytoplankton or higher plant chlorophylls formed during diagenesis whereby this
92 original complexed magnesium is replaced under specific depositional conditions. Although
93 usually present in trace concentrations (see table S2), oil Ni content in relation to V has led to
94 an oil classification approach based on ratios and absolute concentrations (Barwise, 1990).

95 The aim of this present study was to determine, as an example of a potentially limiting trace
96 metal, the role of Ni as a biostimulant or, potential inhibitor, during petroleum hydrocarbon
97 biodegradation. The effects of different Ni chemical forms, including a porphyrin complex, at
98 different concentrations in soil were assessed. Such studies, for trace metals (akin to those for
99 biostimulation by N and P) when applied to real soils or sediments have not been carried out.

Given the typical chemical and physical complexity of such natural systems it is not yet known whether metal additions such as those made to anaerobic digesters or pollutant degrading laboratory cultures are necessary or feasible. For instance, trace nutrients may be adequately supplied by the mineral composition of soils regardless of increased requirements after a spill. Alternatively, the complexity and reactivity of organic matter and mineral phases may rapidly sequester amendments rendering them ineffective.

2. Materials and methods

2.1. Soil source, physicochemical analysis and microcosm set up

Soil was sourced from a fallow plot under organic management at Newcastle University's Nafferton farm (54°59'08.6"N 1°53'56.2"W) located 24 miles west of Newcastle upon Tyne, UK. This loamy clay soil was classified as a Cambic Stagnogley by the Agrifood Institute (Avery, 1973; Orr et al., 2012) and was considered pristine and suitable for experimental microcosms.

Soil pH was determined according to the ISO 10390-2005 Soil quality -- Determination of pH method. The total organic carbon and heavy metal contents of the soils was measured by Derwentside Environmental Testing Services (DETS) Durham, UK according to the Standing Committee of Analysts methods (UK Environment Agency). Soil water holding capacity was determined according to ISO 11274:1998 Soil quality — Determination of the water-retention characteristic.

Soil microcosms were set up using sterile 100 ml amber coloured serum bottles closed with rubber septa. Microcosms comprised 10g of air dried and sieved soil (2mm) amended, except for the unamended control, with 100 mg of an undegraded medium gravity North Sea crude oil. Nitrogen (as sodium nitrate) and phosphorous (as potassium dihydrogen phosphate) was added to give a C:N:P ratio of 100:10:1 which was considered sufficient to sustain aerobic

124 microbial growth. Soil moisture contents were adjusted to 60% of the soil water holding
125 capacity to facilitate diffusion of air for aerobic microbial growth in the soil. To understand the
126 effect of Ni on oil biodegradation, triplicate soil microcosm sets were amended with Ni at
127 varying concentrations (12.5, 25, 50, 200 and, 350 mg Ni per kg of dry soil (mg/Kg)) supplied
128 in varying chemical forms (Sigma Aldrich, UK), namely NiO, $NiCl_2$ and, 5,10,15,20-
129 tetraphenyl-21H,23H-porphine nickel(II) (Ni-porphyrin). Three sets of replicated control
130 microcosms were also prepared which: were not amended with oil or Ni (unamended controls);
131 were amended with oil but not Ni (oil-only controls); amended with oil and Ni-free porphyrin
132 (5,10,15,20-tetraphenyl-21H,23H-porphine) at levels equivalent to that added to the Ni-
133 porphyrin experiments (Ni-free porphyrin controls). All microcosms were incubated at 20^{o}C
134 for 15 days

135 *2.2. Microcosm CO_2 flux rates linked to hydrocarbon degradation*

136 As an oil biodegradation proxy, evolved CO_2 was measured (at 2-day intervals) in headspace
137 gas samples (100 µl) removed using a gas-tight syringe (SGE Analytical, Australia). Analysis
138 was performed using a Fisons MD800 quadrupole mass spectrometer (at 70 eV, filament
139 current 4 A, source current 800 µA, source temperature 200 °C, multiplier voltage 500 V,
140 interface temperature 150 °C) linked to a Fisons 8060 gas chromatograph fitted with an HP-
141 PLOT Q capillary column (30 m X 0.32 m) with 20-µm Q phase and helium as the carrier gas
142 (1ml/min, 65 kPa, split at 100m/min, 35^{o}C). Peaks (m/z = 44) were calibrated using CO_2 gas
143 standards (Scientific & Technical Gases Ltd, UK). O_2 concentrations (m/z = 32) were
144 simultaneously monitored in microcosm headspaces, which were flushed with air when O_2
145 dropped below 75% of atmospheric levels.

146 *2.3. Residual crude oil (n-alkanes) extraction and analysis*

147 Prior to extraction, 500µg (250µl of a 2000mg / L solution) of a squalane extraction standard

representing 0.5% of the added crude oil, was added to soils. Aliquots (10ml) of methanol, toluene and dichloromethane (DCM) were sequentially added to the samples in the microcosm flasks, mixed and vacuum filtered followed by two rounds of rinsing of the residue with the same sequence of solvents. The filtrates were then transferred to a separating funnel with DCM and methanol (30ml of each); deionized water (50 ml) was then added followed by gentle agitation. After settling (1hr), the organic solvent/organic extract phase of the solvent/water mixture was removed. Two additional liquid extraction steps were carried out each by addition of DCM (30 ml) with further agitation. The combined solvent extract was then reduced to ~5ml by rotary evaporation. All microcosm solvent extracts along with aliquots (x3) of the original oil as an undegraded control were then analyzed for total petroleum hydrocarbons. An aliquot of each organic extract or oil was re-suspended in DCM, solvent exchanged into hexane (200µl) and added to a 500mg/3ml capacity Isolute®C-18 Solid Phase Extraction (SPE) column (Biotage, UK) and eluted with hexane (5ml) (Bennett et al., 2007). This eluate was reduced in volume, transferred to an autosampler vial made up to 1ml with hexane, together with a known quantity of heptadecylcyclohexane internal standard. The hydrocarbon fraction samples were then injected via a split-splitless injector (held at 300°C) into an HP5890 Series II gas chromatograph (GC) fitted with a flame-ionization detector (FID). The GC was fitted with a 30 × 0.25 mm fused silica capillary column coated with HP-5 phase (0.25µm). Hydrogen was used as the carrier gas at a flow rate of 2ml/min. The oven temperature of 50°C was held for 2 minutes and then ramped to 300°C at 5°C/min., where it was held for 20min. Data were acquired and processed using Thermo Lab Systems Atlas software to identify n-alkane peaks and quantify their total mass by comparison to the internal standard (n-heptadecylcyclohexane). n-alkane extraction efficiencies were determined from the measured recovery of the added extraction standard (squalane) quantified by reference to the internal standard. Microcosm extraction efficiencies were 88.2±5.7% (mean±SE) and were used to

normalize alkane mass values from individual microcosm extracts.

2.4. Microbial community analysis

Soil genomic DNA (~0.25g) was extracted using the PowerSoil ® DNA Isolation Kit (MOBIO Laboratories, USA) in triplicate according to the manufacturer's instructions. The primer set F515/R926 (Quince et al., 2011) was used to polymerase chain reaction (PCR) amplify the variable V4/V5 region of the archaeal and bacterial 16S rRNA gene. F515 was ligated to a PGM™ linker primer/ adapter, a Golay barcode (unique for each sample) and a barcode spacer. R926 was ligated to a truncated P1 (TrP1) adapter at the 5' end (Hamady et al., 2008). Reactions used Bioline's TaqMan DNA amplification kit (Bioline, UK) with conditions of 95 °C for 3 minutes followed by 30 cycles (1 min at 95 °C, 45 seconds at 55 °C, 1 minute at 72 °C) and finally 10 minutes at 72°C. Amplicons were purified using an Agencourt Ampure XP purification Kit (Beckman Coulter Ltd, UK) and quantified using a Qubit® dsDNA HS Assay Kit (Life Technologies, USA). Amplicons were then pooled into an equimolar library of 500 pM DNA. Sequencing templates were generated by attaching the DNA samples to ion sphere particles using ion PGM™ Template OT2 400 kit (Life Technologies, USA) based on the manufacturer's instruction and using the Ion OneTouch™ 2 Instrument and the Ion OneTouch™ ES. Sequencing was performed using the PGM™ sequencing Platform with the Ion PGM™ sequencing 400 kit followed by filtering to remove low quality and polyclonal sequences. Data was analysed by the Qiime2 pipeline (https://qiime2.org/) (Caporaso et al., 2010) to trim and cluster sequences into amplicon sequence variants (ASVs), assign taxonomies and generate representative sequence and ASV frequency outputs. Principal component analysis (PCA) plots were generated from ASV frequencies in the STAMP v2 software package (Parks et al., 2014). These plots were generated to provide a two dimensional graphical (exploratory) representation of the variation in community composition. Representative sequences of selected, predominant, ASVs were compared (BLAST) to the

NCBI nucleotide database to identify cultured and environmental closely related sequences. Representative 16S rRNA sequence fragments and close relatives were aligned using MUSCLE in MEGA7 to construct a neighbour-joining phylogenetic tree supported by bootstrap analysis (Kumar et al., 2016).

2.5. Quantitative polymerase chain reaction (qPCR)

Bacterial abundances were determined by qPCR using previously developed primer pairs U1048F-U1371R and reaction conditions (Gray et al., 2011). An additional qPCR assay was developed using the PRIMROSE Software (Ashelford et al., 2002) to target the most enriched ASV (genus *Rhodococcus*) in the oil degrading microcosms using RhO1390F (5'-GGTACGGCTACCTTGTTACG-3') and RhO1454R (5'-CACAAGGGGTTAAGCCACCG-3'). The specificity of the qPCR assay was assessed using the RDP probe match analysis tool[40]. The U1048F/U1371R primer pair targeted 90% of all bacterial 16S rRNA sequences (>1200 bp), whereas, the RhO1390F/RhO1454R primer pair targeting a small number (128) of sequences classified in the RDP as genus *Rhodococcus* with no non-target matches. The amplicon size of the primer set was 82 bp and the optimum annealing temperature, determined using the thermal gradient facility of the CFX96 Real-Time system (BioRad, UK), was 62 ^{o}C. Melt curve analysis was used to determine amplification specificity. qPCR reactions included 5 µl of SsoAdvanced™ Universal SYBR® Green Supermix, 0.5 µl of reverse and forward primer (concentration of 10 picomole each), 1 µl of PCR-grade water and 3 µl of template. Reactions included an initial denaturation (7 min at 95°C), followed by 40 cycles of 30 s at 95°C, 30 s at 62°C, and 40 s at 72°C. Standards were prepared from a target 16S rRNA sequence cloned from an amplified pure culture of *Rhodococcus erythropolis* (T) DSM 43066). A one-point calibration (OPC) approach (Brankatschk et al., 2012), was used employing individual amplification reaction efficiencies.

2.6. Statistical analysis

To facilitate the robust statistical analysis of measured variables (i.e., CO_2 flux rates, n-alkanes, and 16s rRNA gene abundances), the experimental design involved replicated experimental soil microcosms randomly assigned into multiple sets of experimental Ni treatment groups (Ni-Porph, $NiCl_2$, NiO) comprised multiple replicated sub-treatments of different Ni concentrations which were then compared to various controls using one-way analysis of variance (ANOVA). These ANOVA tests were followed, post hoc, by Dunnett's multiple comparison of means which identified which individual sub-treatment mean was different in comparison to a specified single control group mean. For CO_2 flux data and 16S rRNA gene abundance data, comparisons were made both with oil-only and unammended controls. For residual total n-alkanes, comparisons were made with oil-only and undegraded oil controls. The outcome of comparisons are selectively reported in the text as p value thresholds indicative of varying levels of confidence but presented in full table S3. For p values of <0.05, <0.01, and <0.001, means are considered different with 95, 99, and 99.9% confidence, respectively. For p values <0.1, means were considered borderline significantly (i.e., 90% confidence), whereas, those >0.1 were not considered significant. In addition to these hypotheses tests, bivariate correlation analyses was performed on measured rates of hydrocarbon degradation (CO_2 flux rates) and qPCR derived 16S rRNA gene abundance data for the identified hydrocarbon degrading taxon (*Rhodococcus*). Correlation analysis was performed to examine the strength (Pearson correlation coefficient (R)) and significance of the linear relationship between these two variables. All ANOVA and correlation analyses were performed using IBM SPSS statistics version 24.

3. Results

3.1. Soil physicochemical characteristics

The soil's pH, total organic matter, water holding capacity and Ni concentrations were, respectively, 7.1 ±0.03, 3.1 ±0.1% by dry weight, 78.5±10.7%, and 9.1 ± 0.6 mg/kg (i.e., mg Ni per kg of dry soil). The concentrations (mg/kg) of other heavy metals were: Hg, 0.1±0.01; As, 11.2±4.4; Cd, 0.73±0.1; Cr, 17.3±1.5; Cu, 15±1; Ni, 9.1±0.6; Pb, 125.7±71.2; Se, <0.5; V, 27±3; Zn, 81.7±9.9. Pertinently, the average Ni concentration was below the UK Environment Agency soil guideline value of 130 mg/Kg (Environmental Agency, 2015). All other heavy metals were similarly at low levels.

3.2. Effects of Ni on the biodegradation of crude oil in soil microcosms

Crude oil biodegradation in replicated soil microcosms was apparent from an approximately linear accumulation of headspace CO_2 over 15 days following oil amendment (see supplementary material, Figure S1). Maximal rates of CO_2 production in Ni and oil amended microcosms relative to oil-only and unamended controls (Figure 1) indicated that oil was readily degraded but the chemical form and concentration of added Ni influenced the rates of degradation. More specifically, amendment with Ni-Porphyrin significantly stimulated CO_2 production relative to the oil-only amended control ($p<0.001$, ANOVA see Table S3). Stimulation was greatest at 12.5 mg/Kg Ni (i.e., the lowest concentration) and progressively reduced with increasing Ni concentration. Maximal rates of CO_2 production (Figure 1) were significantly higher than the oil-only control (Dunnett's comparison of means) at 12.5 mg/Kg Ni ($p <0.001$) and to a lesser extent at 25 and 50 mg/Kg Ni ($p \leq 0.01$) but not at 200 and 350 mg/Kg ($p >0.1$). This pattern was also found for the final CO_2 yields (see table S4). Ni-free porphyrin amended controls did not show enhanced degradation since the mean CO_2 flux rates from the three different Ni-free porphyrin treatments were not significantly different to the mean rate of the oil-only control ($p >0.1$, Figure 1, Table S3). Contrary to the Ni-Porphyrin

experiments, rates (Figure 1, table S3) and yields (table S4), in NiO and $NiCl_2$ amended soils were not significantly different at 12-50 mg/Kg relative to the oil-only amended control ($p > 0.1$) and for NiO, there was actually significant inhibition of degradation ($p < 0.01$) at 200 and 350 mg/Kg Ni.

3.3. n-Alkane degradation in microcosms

Corroboratory analyses were conducted on residual petroleum hydrocarbons, specifically individual alkanes (n-C_{11} to n-C_{32}) recovered from treatment microcosms and controls. On the basis of CO_2 flux results, Ni-Porph and NiO at concentrations of 12.5, 50, and 350 mg/Kg Ni were selected for alkane analysis in comparison to the undegraded oil. The undegraded oil prior to degradation contained approximately 8.6% n-alkanes by total oil weight (i.e., 8675 ± 718μg/100 mg of oil (Figure 2)); whereas, the average total weight of n-alkanes recovered from the different Ni and oil amended soil microcosms ranged from just 828 ± 619 μg for 12.5 mg/Kg Ni as Ni- porphyrin to 5203.6 ± 1651.8 μg with 350 mg/Kg Ni as NiO. Total n-alkanes were significantly lower in all the soil microcosm treatments relative to the initial undegraded crude oil ($p<0.01$) but with less confidence for NiO at 350 mg/Kg ($p < 0.05$) consistent with the CO_2 flux patterns. At 12.5 and 50 mg/Kg Ni as Ni-porphyrin, residual alkanes were apparently lower than in the oil-only controls (Figure 2) although this was not found to be statistically significant (>0.1). Likewise, at 12.5 and 50 mg/Kg Ni as NiO, the total n-alkane recovered was found to be lower, albeit not significantly, relative to the oil-only amended control.

3.4. Microbial community structure and dynamics in Ni amended and control microcosms

Soil microcosms amended with Ni-Porph and NiO at 12.5, 50, and 350 mg/Kg (i.e., those that were stimulated and inhibited) were used to determine the structure and dynamics of soil microbial communities in relation to controls. The 16S rRNA PCR-DGGE analysis (see supplementary experimental procedures) identified variations in bacterial diversity between

different Ni chemical forms and critically, a higher degree of reproducibility between treatment replicates (see supplementary results and discussion and Figure S3). On this basis we reasoned that a 16S rRNA sequence library obtained from the DNA extract of an individual treatment replicate would be broadly representative of the other two replicates. Consequently, 8 barcoded 16S rRNA PCR amplicons each from a single but representative treatment replicate were sequenced. The average number of reads in individual binned libraries after filtering was 33806 sequences ranging from 23849 to 65672. However, individual libraries were rarefied to 15000 for comparative analysis. Sequences have been deposited in the NCBI's Sequence Read Archive (SRA) available under Bio Project PRJNA390800.

A principal component analysis (PCA) generated in the STAMP v2 software package (Parks et al., 2014) using ASV frequencies from individual libraries indicated clustering of communities based on Ni-form (Figure 3a). The most abundant ASV in the oil degrading soils was related to the genus *Rhodococcus*, irrespective of Ni form or absence (Figures 3b) and related *Rhodococcus* strains were identified in diverse environments including those that are hydrocarbon-contaminated (Figure 3c). However, the relative abundance of this *Rhodococcus* ASV represented 30, 32, and 28% in the Ni-porphyrin sequence libraries at 12.5, 50, and 350 mg/Kg Ni, respectively, whereas it was only 12 and 14% at 12.5 and 50 mg/Kg Ni as NiO. This taxon represented 16% of sequences in the oil-only control but was not enriched at all in the unamended control (0.1%) or with 350 mg/Kg Ni as NiO (0.09%). Thus, porphyrin complexed Ni promoted the proliferation of the *Rhodococcus* whereas the NiO was inhibitory to its growth. A very similar pattern of enrichment, albeit to a lesser extent, was observed for sequences related to the genera *Mycobacterium* and *Pseudomonas* (Figure 3b). In contrast, a *Nocardia* sequence type was most abundant in NiO amended soils at 12.5 and 50 mg/Kg implying the presence of this toxic mineral at moderate levels, selected disproportionately for *Nocardia* growth.

In contrast, to the obviously highly enriched and therefore clearly hydrocarbon degrading bacterial taxa such as *Rhodococcus*, the bacterial genera *Bacillus*, *Hyphomicrobium*, and *Bradyrhizobium*, as well as the archaeal family *Nitrososphaeraceae*, were the most abundant taxa in the unamended soil. These typical soil taxa (Figure 3c), were still observable, albeit at a reduced relative abundance, in all the Ni- and oil-amended microcosms. *Nitrososphaeraceae* was the most obviously reduced in relative abundance.

3.5. The influence of oil- and Ni-amendment on bacterial and Rhodococcal absolute abundances

At 12.5 mg/Kg Ni, bacterial 16S rRNA genes in the Ni-porphyrin soil were approximately 3-fold higher, albeit not significantly ($p > 0.1$, table S3), than in the oil-only soils and 9-fold higher ($p < 0.1$) than the unamended controls (Figure 4). At a Ni concentration of 50 mg/Kg, abundances were comparable to the oil-only microcosms; whereas, at 350 mg/Kg Ni abundances were broadly comparable to the unamended control. In all the NiO-amended soils, the mean bacterial 16S rRNA gene abundances were lower, albeit not significantly ($p > 0.1$), than the oil-only soils.

Oil-only amendment increased the abundance of *Rhodococcus* by more than an order of magnitude relative to that in the unamended control and this increase was borderline significant (Figure 4, table S3, $p < 0.1$). In the Ni-amended soils, however, abundances of *Rhodococcus* differed based on Ni concentration and Ni-form. In the 12.5 mg/Kg Ni-porphyrin amended oil degrading soil microcosms, the mean abundance of *Rhodococcus* was approximately a third higher, albeit borderline significantly, relative to the oil-only soil ($p < 0.1$) and approximately 2 orders of magnitude and very significantly higher than the unamended soil ($p < 0.001$). With Ni-porphyrin at 50 mg/Kg the mean *Rhodococcus* abundance was not significantly different relative to the oil-only amended control ($p > 0.1$) but was borderline significantly higher relative to the unamended soil control ($p < 0.1$). In

contrast, at a Ni-porphyrin concentration of 350 mg/Kg the *Rhodococcus* abundance was three-fold lower, albeit not significantly ($p > 0.1$), relative to the oil-only amended soil and was not found to be significantly different relative to the unamended soil ($p > 0.1$). In all the NiO-amended soils, *Rhodococcus* abundances were all not significantly different to the unamemded controls ($p > 0.1$).

Increases in *Rhodococcal* abundances were broadly consistent with their relative enrichment in sequence libraries (Figure 3b), which corroborates the importance of this taxon in oil degradation. Furthermore, bacterial and *Rhodococcal* abundances were strongly correlated with each other ($R = 0.81$; $p < 0.05$) consistent with the finding that, regardless of overall stimulatory or inhibitory effects of Ni on the bacterial community, *Rhodococcus* was the most enriched by oil degradation. For instance, 22.5, 60.5, and 33.4% of the bacterial 16S rRNA gene abundances were accounted for by *Rhodococcus* in the Ni-porphyrin amended soils at 12.5, 50, and 350 mg/Kg, respectively. In NiO-amended soils, *Rhodococcal* abundances accounted for 8.8, 18.9, and 2.7% of bacterial abundances at 12.5, 50, and 350 mg/Kg. Furthermore, correlation analysis indicated a strong positive linear relationship between *Rhodococcus* gene abundances and maximal rates of hydrocarbon degradation ($R = 0.86$; $p < 0.05$).

4. Discussion

Agarry et al., (2013) have previously reported stimulatory effects of low levels of Ni on gasoline degradation in a gasoline-Ni co-contaminated soil. The results presented here, however, provide a broader perspective on this topic and indicate a hitherto overlooked control on hydrocarbon biodegradation dependent, not just on the amounts, but chemical form that metals such as Ni are present or provided in.

The organisms enriched in this study were unsurprising since close relatives have been previously identified and/or isolated from oil degrading systems (as evidenced in the phylogenetic tree in Figure 3c) (Pandey et al., 2016). The large genomes of the metabolically versatile *Rhodococcus* typically encode enzymes (monooxygenases, cytochrome P450, dioxygenases, peroxidases, dehydrogenases, acyl-CoA synthase, and fatty acid synthase) for catabolism of alkanes and biosurfactant production. This is certainly the case for the closely related (98% 16S rRNA sequence homology) *Rhodococcus* ruber IEGM 231 (Ivshina and Kuyukina, 2014). A critical question, however, is; why, in the context of Ni amendment, do we see this pattern of hydrocarbon degrader stimulation or inhibition?

4.1. The solubility, chemical fate, availability and toxicity of Ni amendments

Ni is an essential nutrient required for cell growth and maintenance (Gadd, 2010) but is not thought limiting in culture due to its trace presence in media reagents. Ni in natural environments is more problematic because Ni^{2+} solubility is low; 2 ppb in sea water or 0.3 ppb in fresh water (Eitinger and Mandrand-Berthelot, 2000). In a soil the availability of Ni was found principally dependent on the total Ni content, whereby, added amounts of $NiCl_2$ correlated positively with dissolved Ni concentrations (Zhang et al., 2015). However, at pHs similar to the current study, Ni was rapidly removed from solution via sorption to organic matter, Fe and Al oxides and clay (Zhang et al., 2015). In this current study, the fate of added Ni determined through sequential extraction (see supplementary material) indicated that Ni was not present in an easily exchangeable form.

Despite Ni insolubility, artificial (EDTA) or biological (siderophores) chelating agents are a mechanism for solubilising such heavy metals. For instance, the highly efficient assimilation of metalloporphyrins documented for indigenous microorganisms of the Kuperferschiefer black shale, Poland was followed by intracellular degradation of the porphyrin, freeing metal ions within cells (Matlakowska and Sklodowska, 2010). We speculate that porphyrin chelated

Ni was more available for uptake and hence more stimulatory to hydrocarbon degradation in our microcosms than similar levels of insoluble NiO or, initially, soluble $NiCl_2$. Of course, Ni availability, is also dependent on cell uptake requiring regulatory, uptake, chaperone and accessory trafficking proteins (Eitinger and Mandrand-Berthelot, 2000) many of which were ubiquitously observed in 173 *Rhodococcus* genomes searched using the Integrated Microbial Genomes & Microbiomes (IMG/M) system (Markowitz et al., 2012). The alkane oxidising *Rhodococcus* ruber IEGM 231 (Ivshina and Kuyukina, 2014) possessed nixA (a high-affinity nickel-transport protein) and Ni permeases.

At high concentrations Ni is toxic (Gadd, 2010); however, suppression levels are likely attributable to the nature and fate of amendments (Nie et al., 2015). For instance, transport via metalloporphyrins is a microbial strategy not only to facilitate metal uptake as discussed above, but also to mitigate intracellular toxic effects Lemire et al., 2013). In contrast, excessive intracellular free metal accumulated from high extracellular Ni^{2+} concentrations or attachment and internalisation of particles (e.g., NiO, Latvala et al., 2016) may result in cell damage. Referring to the previous discussion on the fate of the $NiCl_2$, its toxicity at higher levels is likely mitigated by the rapid sorption of Ni^{2+} to organic and mineral phases (Zhang et al., 2015) explaining the limited impact of this soluble but un-complexed form relative to the severe impacts of the insoluble NiO powder. With respect to NiO, nanoparticles induce superoxide production and hence reactive oxygen stress (Baek and An, 2011) and supported NiO surfaces promote abiotic superoxide formation during peroxide breakdown (Anpo et al., 1999). Furthermore, NiO cytotoxicity in human epithelial cell lines occurs by the uptake of particles rather than Ni^{2+} with internalisation inducing cytotoxic effects including oxidative stress and DNA damage (Karlsson et al., 2014).

4.2. Impact and putative mechanism of Ni stimulated biodegradation at low levels of amendment

417 At the other end of the Ni concentration range tested, stimulation of oil bioremediation,
418 especially with Ni-porphyrin, suggests Ni was limiting despite the presence of comparable
419 indigenous concentrations. This limitation is interesting because N and P are considered most
420 important during oil bioremediation (Atlas and Hazen, 2011; Coulon et al. 2005). However,
421 in comparable experiments, which investigated the effect of nitrate-N on oil bioremediation in
422 this same, organically managed soil, it is found that nitrogen was not limiting (Mitchell, 2014).

423 Mechanistically, Ni acts as co-factor for 9 enzyme systems (Diekert et al., 1981; Boer et al.,
424 2014). Seven of these are, however, unlikely to stimulate aerobic oil biodegradation and
425 include: glyoxalase 1, responsible for detoxification of methylglyoxal produced during
426 glycolysis; lactase racemase, which catalyses racemization of lactic acids; Ni-Fe hydrogenase,
427 involved in reverse activation of hydrogen in anaerobic (Diekert et al., 1981) and aerobic
428 hydrogen oxidizing systems (Grzeszik et al., 1997); and methyl-coenzyme M reductase
429 involved in anaerobic methane oxidation and methanogenesis (Boer et al., 2014). Ni dependent
430 enzyme are also involved in CO_2 reactions. Carbon-monoxide hydrogenase catalyses the
431 anaerobic oxidation of CO to CO_2 and Acetyl-CoA synthase/decarboxylase anaerobically
432 synthesises acetyl-CoA (Boer et al., 2014). The two remaining Ni associated enzyme systems
433 are more pertinent to aerobic oil biodegradation and are discussed.

434 Ni is a cofactor for urease catalysis of urea to ammonia and carbonic acid (Boer et al., 2014).
435 Interestingly, studies use urease assays to monitor hydrocarbon biodegradation because such
436 activities are highly correlated (Margesin et al., 2000) whereby ammonia release by urease
437 allows growth on organo-nitrogen compounds. However, urease is often 'constitutive': for
438 instance, nitrogen fixing Cyanobacteria regulate significant levels of urease even in the
439 presence of N_2 and NO_3^- (Singh, 1992) with urease conforming to a simple growth-rate
440 dependence expression model. Bacterial isolates have indicated that constitutive urease activity
441 is a common trait in soil (Burbank et al., 2012). Most compellingly, levels of soil urease activity

directly linked to diesel biodegradation occurred regardless of presence or absence of inorganic (NH_4^+, NO_3^-) or organic (urea) nitrogen forms (Margesin et al., 2000). It follows, therefore, that hydrocarbon degrader growth likely necessitates uptake of Ni to fulfill constitutive requirements for urease potentially explaining the stimulatory effect of Ni. To support this conclusion, genes coding for urease proteins were ubiquitously identified in *Rhodococcus* genomes.

Ni is also, however, a cofactor in superoxide dismutase (SOD) responsible for the detoxification of reactive oxygen species (ROSs) (Lemire et al., 2013). ROSs (i.e., superoxide, hydrogen peroxide, and hydroxyl radicals) are generated by redox active heavy metal contaminants such as Ni but are also by-products of aerobic respiration. Fe-, Mn-, Ni-, and Cu/Zn-containing SOD isoforms catalyse superoxide breakdown into H_2O_2 and O_2 (Fridovich, 1997). In combination with catalases (which convert H_2O_2 into H_2O and O_2) SODs, therefore, reduce oxidative stress. It follows that rapid rates of aerobic respiration (indicated in this study by an order of magnitude increase in the CO_2 production from oil) produces a demand for ROS detoxification via superoxide dismutase. Interestingly, in contrast to other SODs, Ni-SODs are primarily found in the bacteria (Fridovich, 1997), specifically, the Cyanobacteria and, intriguingly, the *Actinobacter* (Miller, 2012), which includes the *Rhodococcus* genus. Thus, rapid growth of this hydrocarbon degrader may have necessitated uptake of Ni for Ni-SOD production. However, while Ni-SODs are relatively common in the *Actinobacter* phylum, the *Rhodococccus* genomes survey identified only one Ni-SOD (*Rhodococcus sp. R04*) relative to large numbers of genes for Cu-Zn and Fe-Mn SOD families.

5. Conclusion

Empirically our results indicate a hitherto overlooked limit on hydrocarbon biodegradation dependent on the availability and toxicity of Ni. Explanations (i.e., Ni-dependent urease or

466 super oxide dismutase production) which explain the Ni-stimulated biodegradation patterns
467 observed which will require future work to resolve.

468 More generally the results indicate that, although, biostimulation can be considered an
469 efficient, cost effective and environmentally friendly approach to organic contaminant
470 removal, improvements can be made. Moving beyond provision of N and P, the technology
471 should consider the supply of trace nutrients (i.e. metals) tacitly assumed to be supplied by the
472 environment. Furthermore, it has been demonstrated that the chemical form and fate of the
473 metal and not just its quantity may be important.

474 **Acknowledgements**

475 This work was supported by Petroleum Development trust fund (PTDF) Nigeria and Newcastle
476 University, United Kingdom.

477 **References**

478 Avery, B.W., 1973. Soil classification in the soil survey of England and Wales. J. Soil Sci.
479 24, 324–338.

480 Agarry, S.E., Aremu, M.O., Aworanti, O.A., 2013. Biostimulation and phytoremediation
481 treatment strategies of gasoline-Nickel co-contaminated Soil. Soil Sediment Contam. 23,
482 227–244.

483 Anpo, M., Che, M., Fubini, B., Garrone, E., 1999. Generation of superoxide ions at oxide
484 surfaces. Top. Catal. 8, 189–198.

485 Ashelford, K.E., Weightman, A.J., Fry, J.C., 2002. PRIMROSE: a computer program for
486 generating and estimating the phylogenetic range of 16S rRNA oligonucleotide probes
487 and primers in conjunction with the RDP-II database. Nucleic Acids Res, 30, 3481–
488 3489.

489 Atlas, R.M., Hazen, T.C., 2011. Oil biodegradation and bioremediation: A tale of the two
490 worst spills in U.S. history. Environ. Sci. Technol. 45, 6709–6715.

491 Baek, Y.-W. and An, Y.-J., 2011. Microbial toxicity of metal oxide nanoparticles (CuO, NiO,
492 ZnO, and Sb_2O_3) to *Escherichia coli*, *Bacillus subtilis* and *Streptococcus aureus*. Sci.
493 Total Environ. 409, 1603–8.

494 Barwise, A.J.G., 1990. Role of nickel and vanadium in petroleum classification. Energy Fuels
495 4, 647–652.

496 Bennett, B., Noke, K.J., Bowler, B.F.J., Larter, S.R., 2007. The accurate determination of
497 C0–C3 alkylphenol concentrations in crude oils. Int. J. Environ. Anal. Chem. 87, 307–
498 320.

499 Boer, J. L., Mulrooney, S. B., Hausinger, R. P., 2014. Nickel-dependent metalloenzymes.
500 Arch. Biochem. Biophys. 544, 142–152. doi:10.1016/J.ABB.2013.09.002.

501 Brankatschk, R., Bodenhausen, N., Zeyer, J., Burgmann, H., 2012. Simple absolute
502 quantification method correcting for quantitative PCR efficiency variations for microbial
503 community samples. Appl. Environ. Microbiol. 78, 4481–4489.

504 Burbank, M.B., Weaver, T.J., Williams, B.C., Crawford, R.L., 2012. Urease activity of
505 ureolytic bacteria isolated from six soils in which calcite was precipitated by indigenous
506 bacteria. Geomicrobiol. *J* 29, 389–395.

507 Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K.,
508 Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T.,
509 Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D.,
510 Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J.,
511 Yatsunenko, T., Zaneveld, J., Knight, R., 2010. QIIME allows analysis of high-
512 throughput community sequencing data. Nat. Methods 7, 335–336.

513 Coulon, F., Pelletier, E., Gourhant, L., Delille, D,. 2005. Effects of nutrient and temperature
514 on degradation of petroleum hydrocarbons in contaminated sub-Antarctic soil.
515 Chemosphere 58, 1439–1448. doi:10.1016/j.chemosphere.2004.10.007.

516 Demirel, B., Scherer, P., 2011. Trace element requirements of agricultural biogas digesters
517 during biological conversion of renewable biomass to methane. Biomass Bioenergy 35,
518 992-998

519 Diekert, G., Konheiser, U., Piechulla, K., Thauer, R. K., 1981. Nickel requirement and factor
520 F430 content of methanogenic bacteria. J. Bacteriol. 148, 459–464.

521 Eitinger, T., Mandrand-Berthelot, M.A., 2000. Nickel transport systems in microorganisms.
522 Arch. Microbiol. 173, 1–9.

523 Environmental Agency, 2015. Environment Agency withdrawal of 2009 Nickel SGV 1. 2.
524 doi:10.2903/j.efsa.2015.4002.

525 Filby, R.H., 1994. Origin and nature of trace element species in crude oils, bitumens and
526 kerogens: implications for correlation and other geochemical studies. Geol. Soc.
527 London, Spec. Publ. 78, 203–219.

528 Fridovich, I., 1997. Superoxide anion radical (O_2), superoxide dismutases, and related
529 matters. J. Biol. Chem. 272, 18515–18517.

530 Gadd, G.M., 2010. Metals, minerals and microbes: Geomicrobiology and bioremediation.
531 Microbiology 156, 609–643.

532 Gray, N.D., Sherry, A., Grant, R.J., Rowan, A.K., Hubert, C.R.J., Callbeck, C.M., Aitken,
533 C.M., Jones, D.M., Adams, J.J., Larter, S.R., Head, I.M., 2011. The quantitative
534 significance of Syntrophaceae and syntrophic partnerships in methanogenic degradation
535 of crude oil alkanes. Environ. Microbiol. 13, 2957–2975.

536 Grzeszik, C., Lubbers, M., Reh, M., Schlegel, H.G., 1997. Genes encoding the NAD-
537 reducing hydrogenase of *Rhodococcus opacus* MR11. Microbiology 143, 1271–1286.

538 Hamady, M., Walker, J.J., Harris, J.K., Gold, N.J., Knight, R., 2008. Error-correcting
539 barcoded primers for pyrosequencing hundreds of samples in multiplex. Nat. Methods 5,
540 235–237.

541 International Energy Agency, 2015. World Energy Outlook 2015 Factsheet.

542 Ivshina, I.B., Kuyukina, M.S., 2014. Draft Genome Sequence of Propane- and Butane-
543 Oxidizing Actinobacterium Rhodococcus ruber IEGM 231. ASM - Genome Announc. 2,
544 2–3.

545 Kalogerakis, N., Nikolopoulou, M., Coivini, P.F.X., Aulenta, F., 2014. Recent advances of
546 EU's FP7 project killspill - Integrated biotechnological solutions for combating marine
547 oil spills. in Proceedings of the 37th AMOP Technical Seminar on Environmental
548 Contamination and Response (Environment Canada), 455–467.

549 Karlsson, H.L., Gliga, A.R., Calleja, F.M.G.R., Goncalves, C.S., Wallinder, I.O., Vrieling,
550 H., Fadeel, B., Hendriks, G., 2014. Mechanism-based genotoxicit screening of metal
551 oxide nanoparticles using the ToxTracker panel of reported cell lines. Part. Fibre Toxic.
552 11, 41–54.

553 Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: Molecular Evolutionary Genetics
554 Analysis Version 7.0 for Bigger Datasets. Mol. Biol. Evol. 33, 1870–1874.

555 Kuo, C.W., Genthner, B.R.S., 1996. Effect of added heavy metal ions on biotransformation
556 and biodegradation of 2-chlorophenol and 3-chlorobenzoate in anaerobic bacterial
557 consortia. Appl. Environ. Microbiol. 62, 2317–2323

558 Latvala, S., Hedberg, J., Di Bucchianico, S., Moller, L., Odnevall Wallinder, I., Elihn, K.,
559 Karlsson, H.L., 2016. Nickel release, ROS generation and toxicity of Ni and NiO micro-
560 and nanoparticle. *PLoS One* 11, e0159684.

561 Lemire, J.A, Harrison, J.J., Turner, R.J., 2013. Antimicrobial activity of metals: mechanisms,
562 molecular targets and applications. Nat. Rev. Microbiol. 11, 371–84.

563 Margesin, R., Zimmerbauer, A., Schinner, F., 2000. Monitoring of bioremediation by soil
564 biological activities. Chemosphere 40, 339–346.

565 Markowitz, V.M., Chen, I.-M.A., Palaniappan, K., Chu, K., Szeto, E., Grechkin, Y., Ratner,
566 A., Jacob, B., Huang, J., Williams, P., Huntemann, M., Anderson, I., Mavromatis, K.,
567 Ivanova, N.N., Kyrpides, N.C., 2012. IMG: Integerated microbial genomes database and
568 comparative analysis system. Nucleic Acids Res. 40, D115–D122.

569 Matlakowska, R., Sklodowska, A., 2010. Uptake and degradation of copper and cobalt
570 porphyrins by indigenous microorganisms of Kupferschiefer (Fore-Sudetic Monocline,
571 Poland). Hydrometallurgy 104, 501-505.

572 Miller, A.-F., 2012. Superoxide dismutases: Ancient enzymes and new insights. FEBS Lett.
573 586, 585–595.

574 Macaulay, M.B., Rees, D., 2014. Bioremediation of oil spills: a review of challenges for
575 research advancement. Ann. Environ. Sci. 8, 9–37.

576 Mitchell, T.B., 2014. The effect of nutrient kinetics on bioremediation of hydrocarbon
577 contaminated agricultural soil. Newcastle University MSc dissertation thesis.

578 Nie, J., Pan, Y., Shi, J., Gua, Y., Yan, Z., Duan, X., Xu, M., 2015. A comparative study on
579 the uptake and toxicity ofnickel added in the form of differnt salts to maize seedlings.
580 Int. J. Environ. Res. Public Health 12, 15075–15087.

581 Nikolopoulou, M., Pasadakis, N., Kalogerakis, N., 2013. Evaluation of autochthonous
582 bioaugmentation and biostimulation during microcosm-simulated oil spills. Mar. Pollut.
583 Bull. 72, 165–173.

584 Orr, C.H., Leifert, C., Cummings, S.P., Cooper, J.M., 2012. Impacts of organic and
585 conventional crop management on diversity and activity of free-living nitrogen fixing

586 bacteria and total bacteria are subsidiary to temporal effects. PLoS One 7.
587 Pandey, P., Pathak, H., Dave, S., 2016. Microbial ecology of hydrocarbon degradation in the
588 soil: a review. Res. J. Env. Toxicol. 10, 1-15.
589 Parks, D.H., Tyson, G.W., Hugenholtz, P., Beiko, R.G., 2014. STAMP: statistical analysis of
590 taxonomic and functional profiles. Bioinformatics 30, 3123–3124.
591 Quince, C., Lanzen, A., Davenport, R. J.,Turnbaugh, P. J., 2011. Removing noise from
592 pyrosequenced amplicons. BMC Bioinformatics 12, 38.
593 Ron, E.Z., Rosenberg, E., 2014. Enhanced bioremediation of oil spills in the sea. Curr. Opin.
594 Biotechnol. 27, 191–194.
595 Singh, A.K., Sherry, A., Gray, N.D., Jones, D.M., Bowler, B.F.J., Head, I.M., 2014. Kinetic
596 parameters for nutrient enhanced crude oil biodegradation in intertidal marine
597 sediments. Front. Microbiol. 5, doi:10.3389/fmicb.2014.00160.
598 Singh, S., 1992. Regulation of urease cellular levels in the cyanobacteria Anacystis nidulans
599 and Nostoc muscorum. Biochem und Physiol der Pflanz 188, 33–38.
600 Suja, F., Rahim, F., Taha, M.R., Hambali, N., Rizal Razali, M., Khalid, A., Hamzah, A.,
601 2014. Effects of local microbial bioaugmentation and biostimulation on the
602 bioremediation of total petroleum hydrocarbons (TPH) in crude oil contaminated soil
603 based on laboratory and field observations. Int. Biodeterior. Biodegrad. 90, 115–122.
604 Tyagi, M., da Fonseca, M.M.R., de Carvalho, C.C.C.R., 2011. Bioaugmentation and
605 biostimulation strategies to improve the effectiveness of bioremediation processes.
606 Biodegradation 22, 231–241.
607 Xu, S., Wang, Y-F., Yang, L-Y., Ji, R., Miao, A.J., 2018. Transformation of
608 tetrabromobisphenol A by *Rhodococcus jostii* RHA1: Effects of heavy metals.
609 Chemosphere 196, 206-213.
610 Zhang, X., Li, J., Wei, D., Li, B., Ma, Y., 2015. Predicting Soluble Nickel in Soils using Soil
611 Properties and Total Nickel. PLoS One 10, e0133920.

612

613 **Figure Legends**

614 Figure 1. A) Maximal rates of CO_2 production in: Ni- and oil-amended soils; Ni-free porphyrin
615 and oil amended soils; oil-only and; unamended-controls. The horizontal dotted line indicates,
616 for comparison, the maximal mean CO_2 production rate of the oil-only control. Treatments
617 with rates above or below the dotted line are considered stimulatory or inhibitory, respectively.
618 The x-axis indicates microcosm added Ni- or equivalent porphyrin concentration. Each bar
619 represents the mean maximal rate (n=3). Error bars represent 1 x standard error.

620 Figure 2: Mass of n-alkanes recovered after 15-day incubations of replicated soil microcosms
621 amended with oil (100 mg) and different quantities and types of Ni in comparison to the mass

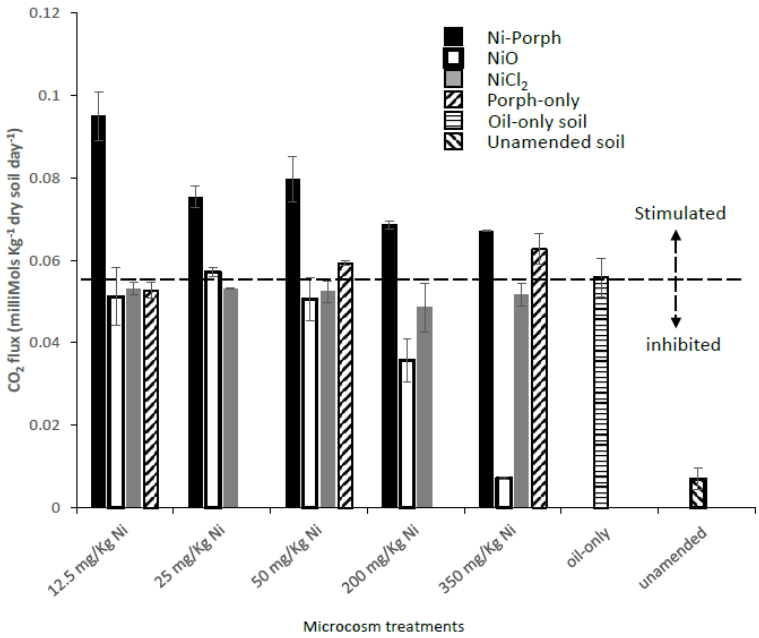
622 of alkanes recovered from the oil-only amended controls. For comparison the n-alkane content
623 of replicated 100 mg aliquots of fresh undegraded crude oil are also shown. Error bars represent
624 1 x the standard error (n=3).

625 Figure 3. (A) Principal Component Analysis (PCA) of 16S rRNA sequence libraries from
626 representative Ni-oil amended, oil-only, and unamended soil microcosms. (B) Fractional
627 abundances of selected dominant taxa in 16S rRNA sequence libraries representative of Ni-oil
628 amended, oil-only and unamended soil microcosms. (C) Phylogenetic distance tree
629 (Neighbour-Joining) of selected dominant bacterial and archaeal taxa identified in microcosm
630 sequence libraries and their close relatives. The tree is based on a comparative analysis of
631 selected partial 16S rRNA sequences and taxa identified in this study are indicated by
632 individual codes assigned during pipeline analysis. The percentage of replicate trees in which
633 the associated taxa clustered together in bootstrap analysis (1000 replicates) are shown next to
634 the branches.

635 Figure 4: Absolute abundances of bacterial and *Rhodococcus* 16S rRNA genes in soil
636 microcosms after 15 days incubation. Each bar represents the mean 16S rRNA gene abundance
637 (in genes ng^{-1} DNA g^{-1} soil) from triplicate samples. Error bars = 1 x the standard error.

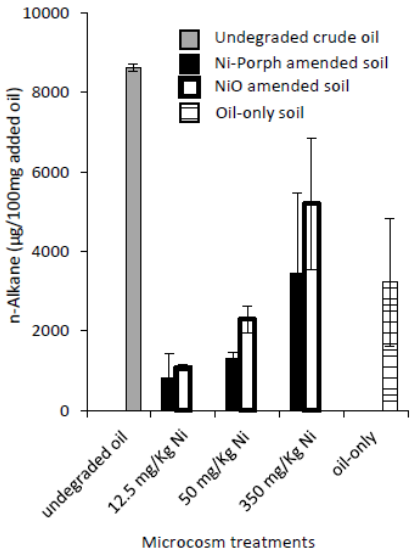
638

Mejeha et al. figure 1



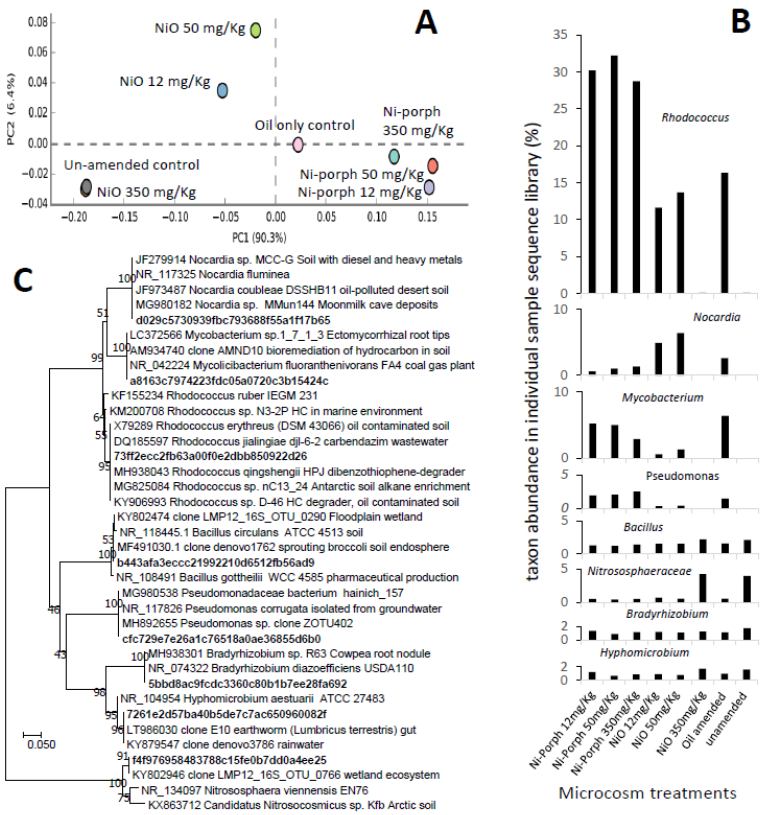
639

Mejeha et al. figure 2



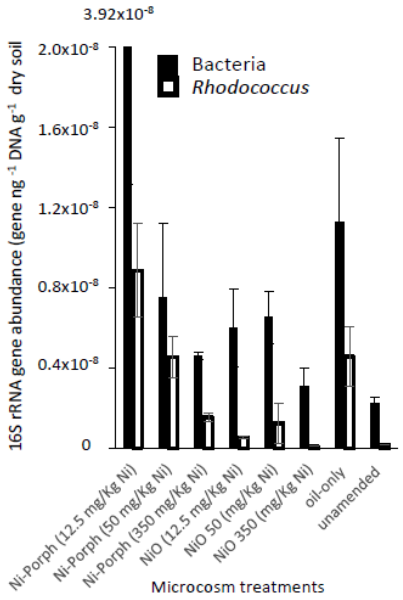
640

Mejeha et al. figure 3



641

MeJeha et al. figure 4



642