BIODEGRADATION OF MACONDO OIL BY AEROBIC HYDROCARBON–DEGRADING BACTERIA IN THE WATER COLUMN AND DEEPSEA SEDIMENTS OF THE NORTHERN GULF OF MEXICO

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BIODEGRADATION OF MACONDO OIL BY AEROBIC HYDROCARBON–DEGRADING BACTERIA IN THE WATER COLUMN AND DEEPSEA SEDIMENTS OF THE NORTHERN GULF OF MEXICO

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I wish to thank my advisor, Dr Joel Kostka. I cannot finish this without his tremendous help. Also, I would like to thank Dr Martial Taillefert and Dr. Yuanzhi Tang for being my committee members.

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LIST OF SYMBOLS AND ABBREVIATIONS

y_{growth}	Microbial Carbon Use Efficiency
μ	Carbon Assimilated into Biomass
R	Carbon Loss to Respiration
DWH	Deepwater Horizon
GOM	Gulf of Mexico
SI	Shannon Index

SUMMARY

Previous studies have come to contrasting conclusions regarding nutrient limitation of hydrocarbon biodegradation in the Gulf of Mexico, and rate measurements are needed to support oil plume modeling. Thus, this study investigates the rates and controls of biodegradation in seawater and sediments, largely in the deepsea. Sediment and seawater samples were collected on research cruises in the northern Gulf from 2012 to 2014, where the seafloor was impacted by the Deepwater Horizon (DWH) oil spill. Biodegradation was clearly limited by both nitrogen and phosphorus availability in surface waters with significant rates of CO_2 production (100 μmol CO_2 l^{-1} d^{-1}) only observed in treatments amended with ammonium and phosphate. In deepsea sediments, nutrient amendments resulted in an average of 6 fold higher degradation rates (0.49 μmol CO_2 g sed^{-1} d^{-1}) compared to unamended controls. Microbial communities responded to oil contamination rapidly in a series of enrichment cultures, and selection was observed for populations of native hydrocarbon–degrading bacteria. Temperature was shown to be a major factor in controlling microbial community composition in the enrichments. At room temperature, community diversity in the enrichments was significantly reduced in the presence of oil, while under 4 ℃, the community diversity and evenness remained relatively high upon oil amendment. From the same deepsea sediments, 30 strains of known oil–degrading bacteria (*Rhodococcus* and *Halomonas*) were enriched and isolated with hexadecane, phenanthrene, and Macondo oil as the sole carbon and energy source. Detection of these strains in sequence libraries indicates that they may have contributed to the degradation of oil deposited onto the sediments. *Rhodococccus* strain PC20

degraded approximately one–third of total petroleum hydrocarbons amended into cultures within 7 days. This work elucidates the controls of biodegradation and we provide model pure cultures to further elucidate the ecophysiology of hydrocarbon degradation, focusing on deepsea sediments of the northern Gulf of Mexico.

CHAPTER I

INTRODUCTION

The Deepwater Horizon (DWH) oil discharge represents the largest accidental marine oil spill in human history. Approximately 4.9 billion barrel equivalents of oil were released into the Gulf of Mexico (GOM) over a period of 84 days. One of the unique characteristics about the DWH spill is the depth at which it occured[1]. The oil was released from a depth of 1,500 m below the sea surface. According to the Oil Budget Calculator from the Federal response effort, only 25% of discharged oil was accounted for, and 75% remained in the environment[2]. Out of the unaccounted 75%, 10% contributed to the surface oil slick, and 35% was directly detected in the deepsea oil plume[3], which has been observed at a distance of up to 10 km from the wellhead[4,5]. A recent study estimates that a 3200 km^2 region was contaminated surrounding the Macondo well, and 4 – 31% of the discharged oil was sequestered in the deep ocean.[6] Therefore, an understanding of hydrocarbon degradation in the deepsea is necessary for assessing the environmental impacts of the spill.

Biodegradation of hydrocarbons by indigenous microorganisms is one of the major pathways by which oil contamination is eliminated from the oceans[7]. Hydrocarbon degrading bacteria are ubiquitously distributed in the marine environment, and their abundance increases significantly in the presence of hydrocarbon contamination[8,9,10]. Oxygen availability is an important factor controlling the oil biodegradation process in both the marine water column and in the sediments. Aerobic hydrocarbon oxidation provides biochemically the highest amount of energy in comparison with other electron

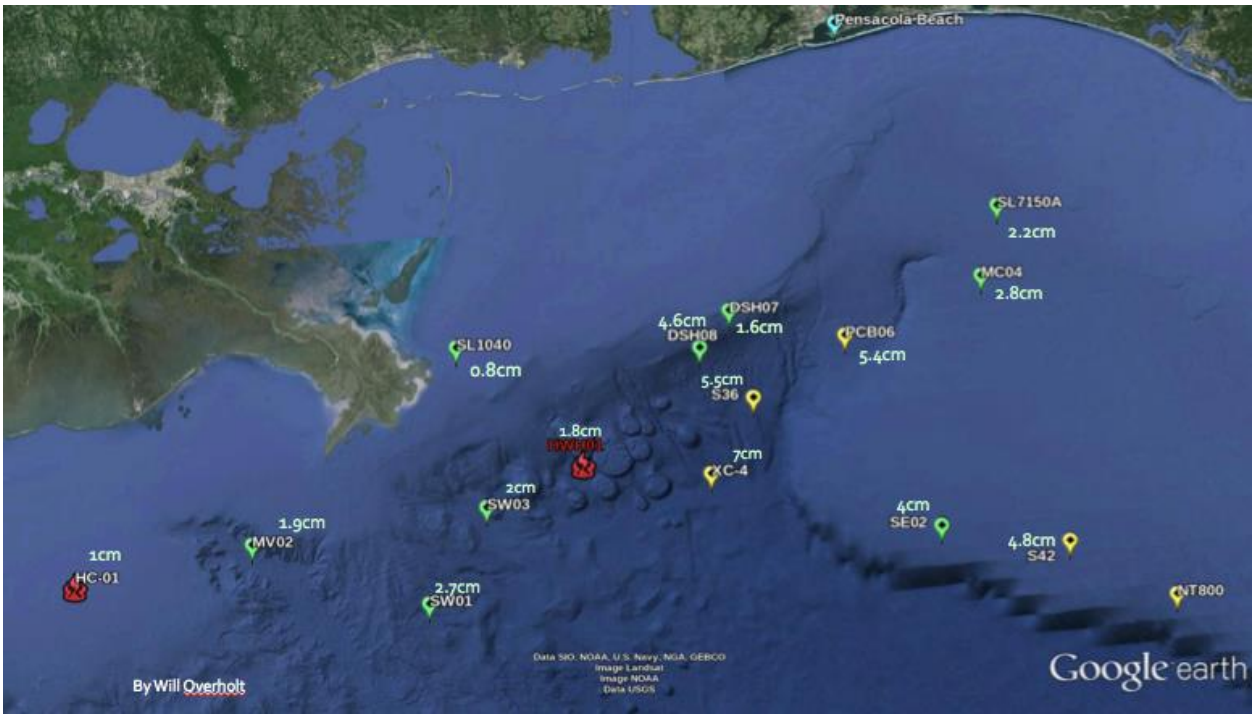


Figure 1. Map of average depth of oxygen penetration

accepting conditions and thus this process is favored for microorganisms. Oxygen saturation within the DWH oil plume averaged 59%, which was 8% depleted relative to outside the plume, suggesting oxygen was consumed by microbial activity within the plume. Also, a majority of the enriched microbial groups were known aerobic hydrocarbon–degraders, with the majority closely related to the *Gammaproteobacteria*[4,11].

Geochemical and microbiological evidence indicates that oil from the DWH spill was deposited under aerobic conditions at the seafloor of the GOM. A study of oxygen distribution at the sea floor in the northern GOM between 2012 and 2014 revealed that oxygen was not depleted in the top few centimeters around the wellhead and in the DeSoto Canyon area (unpublished results, Figure 1). Further, Mason et al. (2014) demonstrated that the most heavily oiled sediments in the top 1 cm depth were dominated by aerobic bacteria (uncultured *Gammaproteobacteria* and *Colwellia*) based on analysis

of 16S rRNA gene sequences[12]. Therefore, the aerobic degradation process likely dominated microbially mediated natural attenuation in both the water column and deepsea sediments in the northern GOM.

More accurate determination of the rates and controls of hydrocarbon biodegradation in the GOM are necessary for improvement of models that estimate the fate of spilled oil. A variety of modeling approaches are currently used to predict oil transport and persistence. Many factors, besides oxygen, such as nutrients, temperature, or microbial community composition control biodegradation potential, and therefore at least some of these environmental parameters should be used to constrain the models[13]. For the DWH event and other major spills, many biological model inputs, such as degradation rate and carbon use efficiency, have not been adequately considered[14]. As a result, biological activity is often not incorporated into oil plume modeling[15,16,17]. Vilcaez et al. (2013) proposed a new model that incorporates biodegradation, but this model has not been applied extensively in the GOM[18].

A supply of major nutrients, nitrogen and phosphorus, is critical for the biodegradation process, especially in the oligotrophic offshore waters around the wellhead in the GOM. Previous studies provided contrasting evidences for in situ nutrient limitation between the surface water and subsurface plumes. In the subsurface, Baelum et al. (2012) reported that nutrients were not limiting for hydrocarbon degradation in oil plume waters (1100 to 1200 m water depth)[19], while in the surface oil slick, Edwards et al. (2011) reported that hydrocarbon degradation was limited by phosphorus in agreement with earlier studies of surface waters in the northern GOM[20, 21]. These contrasting results

make sense given current knowledge of oceanography in the GOM. However, the relationship between nutrient availability and microbial activity requires further study. Indigenous microbial communities responded rapidly to the oil discharge. Many groups of hydrocarbon degrading bacteria were enriched during the spill. By far, the changes of microbial community composition were mostly identified and reported in the water column[22,23] and in the shallow nearshore or coastal sediments[8,24]. Both Hazen et al. (2010) and Mason et al. (2012) reported that three major genera, *Colwellia, Cycloclasticus,* and a genus related to *Oleispira*, were enriched in the oil plume by using next generation sequencing approaches. In Rivers et al. (2013), metatranscriptomics revealed that *Colwellia*, *Cycloclasticus*, *Methylobacter*, and *Methylococcus* were active in the DWH oil plume. Kostka et al. (2011) found that groups affiliated with the *Gammaproteobacteria,* with representatives of genera *Alcanivorax, Marinobacter, Pseudomonas, and Acinetobacter,* were dominant in the microbial community in oiled beach sands. All of these groups were previously identified as hydrocarbon degrading bacteria, and these increases in relative abundance suggest that they were responsible for the *in situ* biodegradation process. Similar studies on the microbial community response to oil in deepsea sediments, however, are still rare. Mason et al. (2014) showed an uncultured *Gammaproteobacteria* and *Colwellia* increased in relative abundance in seafloor sediments near the Macondo well in response to oil deposition soon after the DWH discharge. Further investigation of the community responses will enhance the estimation of oil impacts in the deepsea environment.

In order to relate specific microbial taxa to the rates and pathways of hydrocarbon carbon degradation, further information is needed on the ecophysiology of hydrocarbon

degrading bacteria. Strains isolated from the GOM can be used as model systems for this purpose.

In previous research by the Kostka lab, 10 representative strains of hydrocarbon degrading bacteria were isolated and their genomes were sequenced[8,25]. As has been shown in previous studies, genome analysis indicated that metabolic and hydrocarbon degradation potentials are strain–specific[26]. Even for strains of the same species, the potential for hydrocarbon degradation may differ[25]. While hydrocarbon degrading bacteria communities have been well studied in the water column[19,22,27] , much less information is available from sedimentary communities, especially in the deepsea.

The objective of this study is to characterize the controls of aerobic hydrocarbon degradation in the water column and in deepsea sediments. Because of the paucity of information from the deepsea, hydrocarbon degrading bacteria were cultivated and representative strains were studied from that environment. While nutrient limitation affected hydrocarbon degradation in all environments studied, that limitation was most pronounced in surface waters[20,21].

CHAPTER II

METHOD

2.1 Site description and sample collection

Sediment and water samples were collected during multiple cruises (WB1306, WB1403, WB1411, and WB0814) on the R/V Weatherbird II from 2012 to 2014. Sites were sampled near the Macondo wellhead and in the DeSoto Canyon area, which is to the northeast of the wellhead in the northern GOM. The water depth of the sample sites varied from 1200 m to over 2000 m.

Water samples, both surface and plume water, were collected with Niskin bottles which were attached to a CTD sampling rosette (Sea–Bird Electronics INC., WA). Surface water samples were collected at 5 m below the sea surface. For the plume–depth samples, the samples were collected at 1000 m depth at site DWH01 and 10 m above the seafloor at site PCB06 (1200 m below sea surface). All water samples were stored at 4 ℃ prior to use.

Sediment cores were collected using a multi–coring device. During each deployment, a maximum of eight sediment cores were collected within 1 m diameter area of the seafloor. For microbial community characterization, cores were immediately sectioned into 2 mm intervals for the first 30 mm, 5 mm intervals for the 30 – 50 mm and 10 mm intervals for the 50 – 100 mm depth. Fractions were stored in sterile whirl–pak bags at –20 ℃ until transport to the lab, where they were stored at –80 ℃ before use. For cultivation work, cores were sectioned into three intervals, 0 – 3 cm, 3 – 5 cm, and 5 – 10 cm depth, with an extruder. Fractions were stored in sterilized plastic containers or Ziploc

bags at 4 ℃ before use. Sediment density and porosity were determined by drying a set volume of sediment to a constant mass at 60 ℃.

2.2 Oil chemistry

BP has established the Knox Storage Archive, managed by AECOM, for the storage and control of reference oil material following the DWH discharge. All oil samples used in this study were obtained from the Knox facility. MC252 source oil, collected from the DWH wellhead, was used in a subset of experiments, and surrogate oil, collected from the Marlin Platform of the Dorado field, was used for the remainder. The surrogate oil was shown by BP scientists to contain a nearly identical chemistry and toxicity to the source oil[28].

2.3 Seawater incubations

Seawater incubations were performed in triplicate in 165 ml sealed serum bottles with 10 ml of seawater amended with 0.5% v/v of BP surrogate oil. Treatments were conducted in triplicate and included the following three sets of amendments: 32 μM ammonium, 2 μM phosphate, and both 32 μM ammonium and 2 μM phosphate. Triplicate unamended controls were also included. For surface water samples, microcosms were maintained at room temperature in the dark; for plume water, microcosms were kept at 4 ℃ in the dark. Light was excluded to prevent confounding effects from photochemical reactions.

2.4 Sediment incubations

Incubations were conducted with PCB06 sediments, which were collected from 1200 m water depth. All the incubations were conducted at 4 ℃ in the dark to simulate the in situ condition. Sediment incubations were carried out in sealed 155 ml serum bottles in triplicate. In each bottle, approximately 8 g of wet sediment was mixed with 24 ml of minimal artificial seawater medium[29] or filtered bottom seawater from the same site, and amended with 0.5% v/v of BP surrogate oil as the sole carbon source. A large headspace was used to maintain oxic conditions and the headspace was monitored at regular intervals for CO_2.

2.5 CO_2 measurement

At each time point, 150 µl of headspace was removed with a gas tight syringe from the serum bottles. This volume was injected into a Shimadzu Gas Chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector. The packed column was maintained at 50 ℃, and the injector was set at 350 ℃. Dissolved inorganic carbon concentrations were calculated for the soluble and gas phase using Henry's law, with temperature and ionic strength effects taken into consideration.

2.6 Enrichment and isolation of aerobic hydrocarbon degrading bacteria

Enrichment cultures were established in 125 ml Erlenmeyer flasks with 2 g of sediment and 0.5% v/v MC252 oil in 20 ml of minimal artificial seawater medium, which was prepared according to Widdel (2010)[29]. The enrichments were successively

transferred when the cultures reached stationary phase. For microbial community composition analysis, 10 ml of well mixed culture was sacrificed for DNA extraction.

Subsamples were taken from successive transfers of the enrichment cultures, and used as inocula on agar plates for the isolation of pure cultures. Solid media consisted of Zobell marine agar (HiMedia laboratories Pvt. Ltd., India) or minimal artificial seawater medium[29] with crude oil (Macondo oil), hexadecane (Acros, New Jersey), or phenanthrene (Acros, New Jersey), dissolved in heptamethylnonane, added as sole carbon source. Temperature and substrates were systematically varied in order to obtain microorganisms with diverse metabolisms. All incubations were conducted in the dark to eliminate photochemical effects.

2.7 Phylogenetic characterization of pure cultures

For pure culture identification, distinct colonies were directly picked from agar plates and the 16S rRNA gene was amplified with primer set 27F and 907R by polymerase chain reaction (PCR). Amplicons were first tested with electrophoresis on an agarose gel for designated amplification. Successfully amplified samples were then purified with the QIAquick PCR cleanup kit (QIAGEN, CA) and sent to University of Illinois at Chicago Research Resources Center for Sanger sequencing.

For community composition analysis, total genomic DNA was extracted with the MoBio Powersoil DNA extraction kit (MoBio Laboratories, Carlsbad, CA) from enrichment cultures following the manufacturer's protocol. Extracts are then amplified for 16S rRNA genes with primer set 27F and 907R by PCR. An agarose gel was employed for confirming designated amplification products. The amplification products were then sent out for sequencing. Sequences were analyzed by the software package

QIIME[30]. The UCLUST algorithm was used to cluster sequences into operational taxonomic units (OTU) by 97% similarity[31]. OTUs were then classified using the RDP classification algorithm with a 50% confidence rating.

2.8 Total petroleum hydrocarbon degradation

Total Petroleum Hydrocarbons (TPH) were recovered from the culture medium for analysis. The entire culture volume was extracted using EPA method 3510C[32]. After extraction, a Turbovap (Biotage, Uppsala, Sweden) was used to accelerate the evaporation process. Extracts were then brought up to 1 ml with hexane (Sigma Aldrich, MO) and kept in 2 ml gas tight HPLC vials.

Shimadzu gas chromatography (Kyoto, Japan) with flame ionization detection and a capillary column was used for determining total petroleum hydrocarbon concentration. Helium was employed as the carrier gas at constant flow. During the separation of petroleum hydrocarbons, the initial oven temperature was set to 60 ℃ for 1 min, and then the temperature was gradually increased to 290 ℃ at the rate of 8 ℃/min, where it was maintained for 6.75 min.

2.9 Microbial carbon use efficiency

The growth yield is defined in this study as ratio of growth to assimilation according to the following equation[33]. The mathematical expression for this is

$$Y_{growth} = \frac{\mu}{\mu + R}$$

where μ is the amount of carbon assimilated into biomass, R is carbon loss to respiration.

CHAPTER III

RESULT

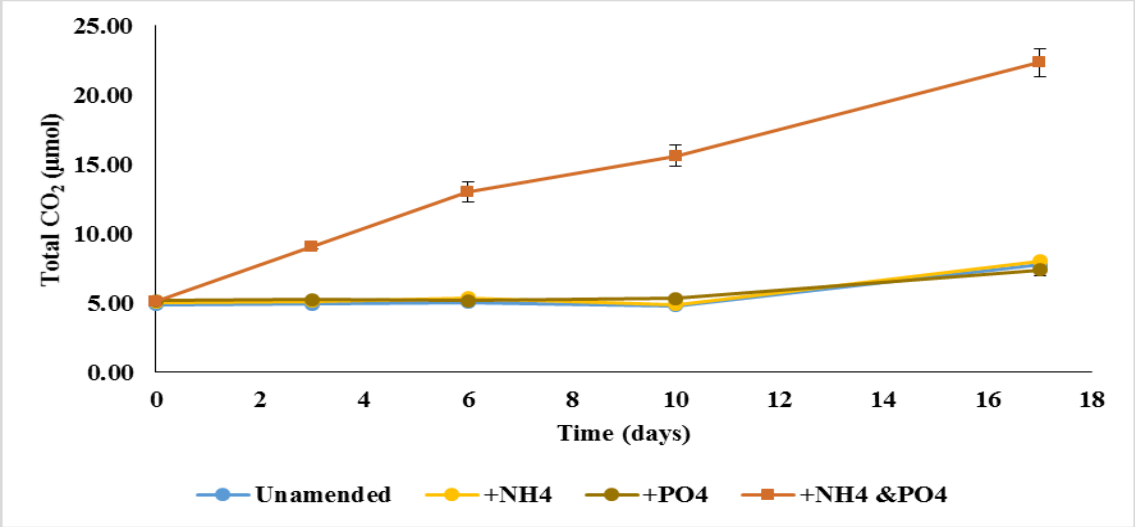
3.1 Nutrient limitation in the water column and deepsea sediment in the northern Gulf of Mexico

Hydrocarbon degradation rates were determined using the surface water from two sites (Figure 2. DWH01 and PCB06) with and without nutrient amendment. For both sites, little to no degradation was observed in unamended, $+NH_4^+$, and $+PO_4^{3-}$ treatments. Only the treatments with both NH_4^+ and PO_4^{3+} added showed a substantial degradation at averaged 100 μmol CO_2 L^{-1} day^{-1} (ranging from 80 to 127 μmol CO_2 L^{-1} day^{-1}).

Hydrocarbon degradation rates were measured in the deep water from the same sites. Hydrocarbon degradation was observed in all treatments beginning at 6 days of incubation. A higher rate and extent of hydrocarbon degradation was observed in treatments amended with both NH_4^+ and PO_4^{3-} (Figure 3a). In the bottom water from site PCB06 (1200 m below sea surface), there was a lag phase for 6 days, after that the hydrocarbon degradation gradually commenced at rates of 47.3 μmol CO_2 L^{-1} day^{-1} (ranging from 39.8 to 52.7 μmol CO_2 L^{-1} day^{-1}) in unamended, $+NH_4^+$, and $+PO_4^{3-}$ treatments until reaching a plateau on day 17. By comparison, treatments amended with both NH_4^+ and PO_4^{3-} revealed a 2X faster degradation rate averaging at 126 μmol CO_2 L^{-1} day^{-1} (ranging from 114 to 144 μmol CO_2 L^{-1} day^{-1}). Similar patterns were observed in DWH01 bottom water, which was collected at the depth of 1000 m below surface (Figure 3b). Biodegradation rates at DWH01 averaged 55 μmol CO_2 L^{-1} day^{-1} (ranging

from 39.7 to 61.2 µmol CO_2 L^{-1} day^{-1}) for unamended, $+NH_4^+$, and $+PO_4^{3-}$ treatments, while

a) DWH01



b) PCB06

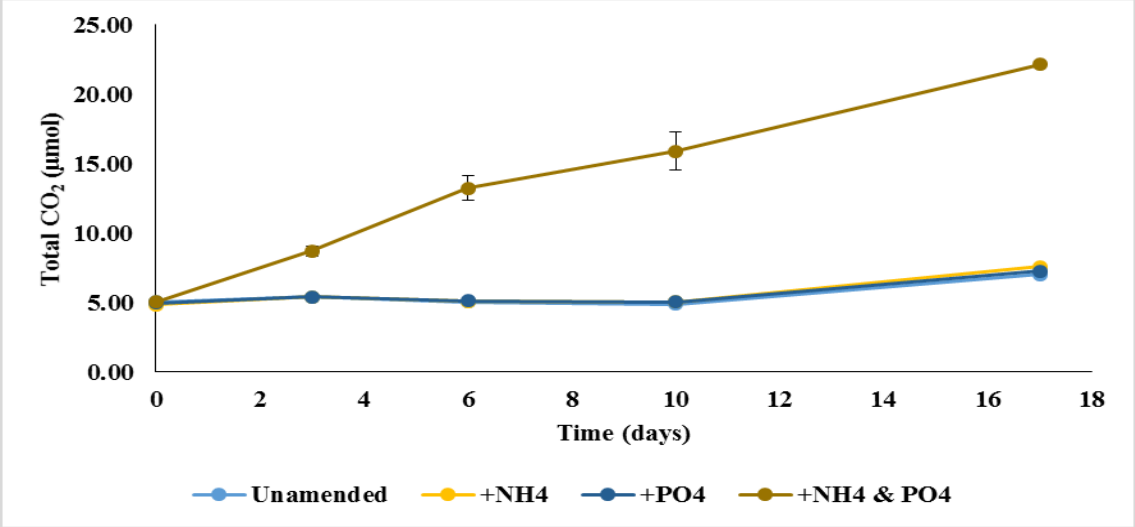
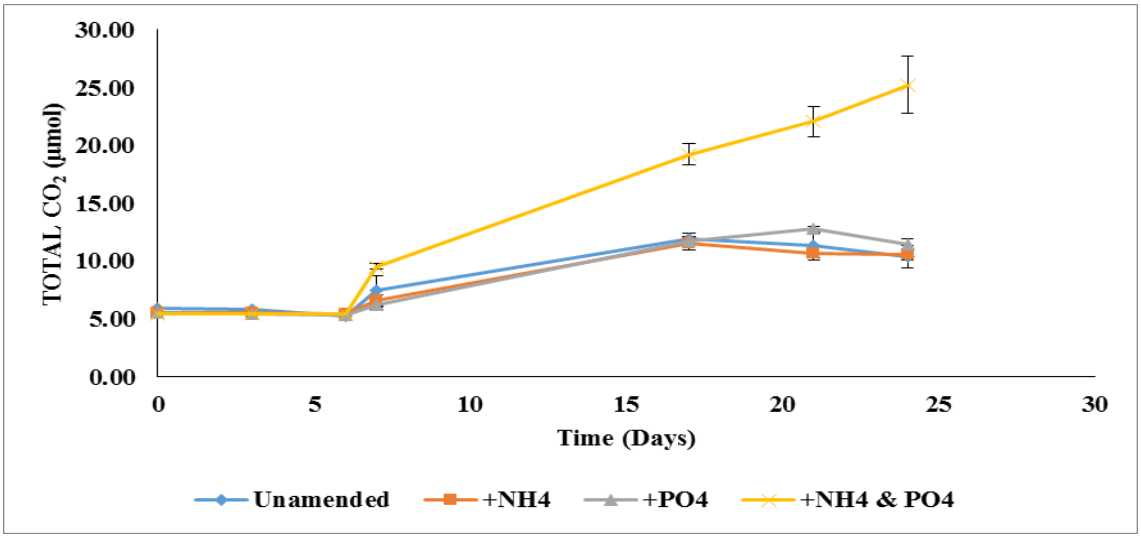


Figure 2. Accumulation of CO_2 in incubations of surface seawater collected from the northern GOM with 0.5% surrogate oil added as the sole carbon substrate. a) DWH01, adjacent to the Macondo Well., b) PCB06, 100 km to the northeast of the Macondo Well

a) DWH01



b) PCB06

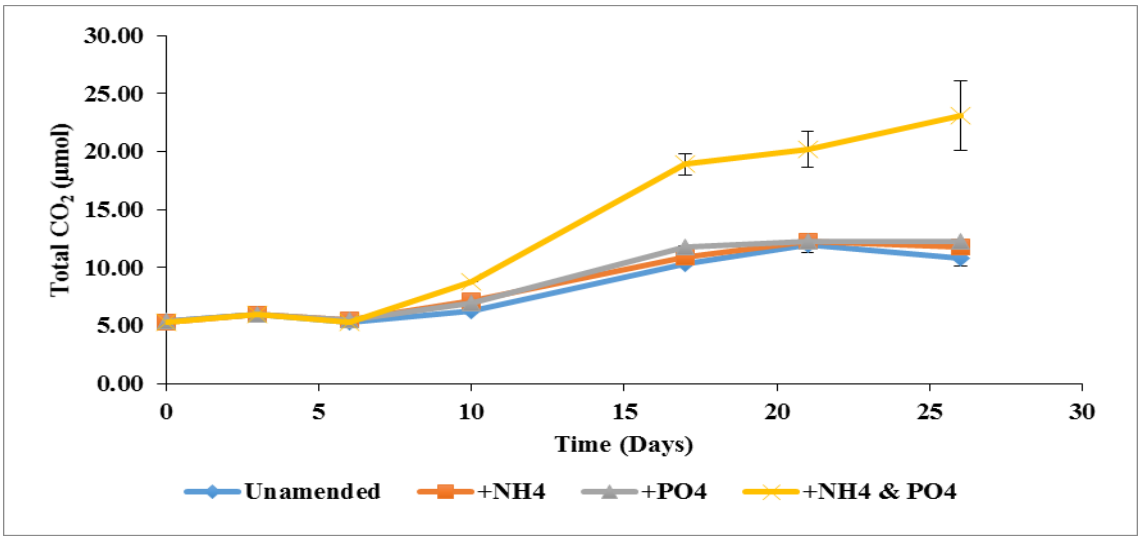
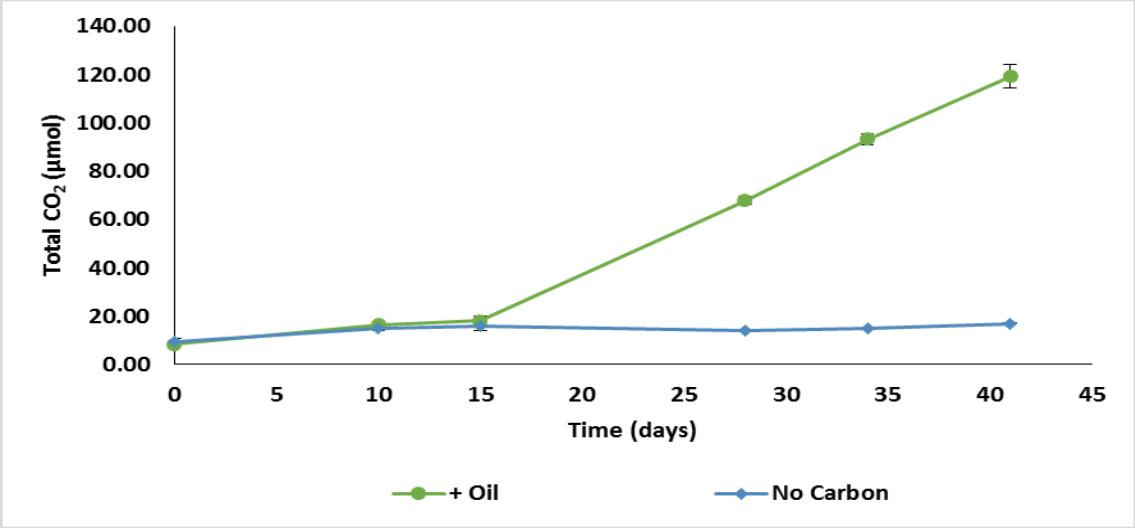


Figure 3. Figure 2. Accumulation of CO_2 in incubations of deep seawater collected from the northern GOM with 0.5% surrogate oil added as the sole carbon substrate. a) DWH01, adjacent to the Macondo Well., b) PCB06, 100 km to the northeast of the Macondo Well

a) Nutrient replete minimal medium



b) Filtered Seawater

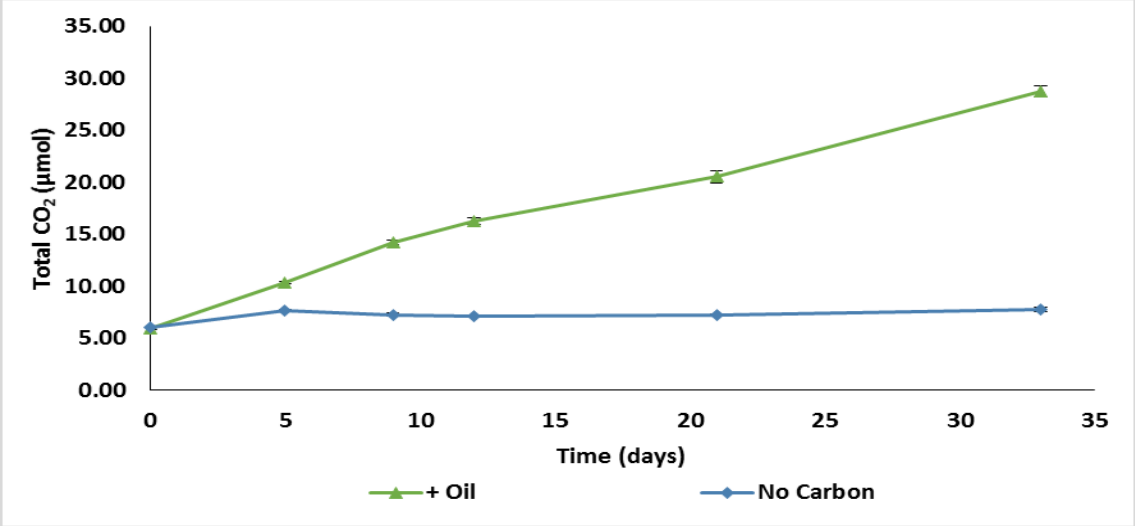


Figure 4. Accumulation of CO_2 in incubations of sediment collected from site PCB06 (1200 m water depth) in the northern GOM with and without Macondo oil added as the sole carbon substrate. a) Incubations conducted in minimal medium replete with major nutrients. b) Incubations conducted in filtered seawater without added nutrients.

degradation rates averaged 102 μmol CO2 L^{-1} day^{-1} (ranging from 95 to 122 μmol CO2 L^{-1} day^{-1}) in the treatments with both NH_4^+ and $PO4^{3-}$ added.

Hydrocarbon degradation rates were quantified in incubations of PCB06 sediment with and without added nutrients (Figure 4). In the nutrient replete medium, the average degradation rate was 2.21 μmol CO_2 g^{-1}dwt d^{-1} (ranging from 2.04 to 2.37 μmol CO_2 g^{-1}dwt d^{-1}) after a lag phase of 15 days. No lag phase was observed in the unamended treatments, and the average degradation rate was 0.37 μmol CO_2 g dwt^{-1} d^{-1} (ranging from 0.36 to 0.39 μmol CO_2 g dwt^{-1} d^{-1}).

3.2 Microbial Community changes in enrichment cultures

Microbial communities were characterized in a series of enrichment cultures, inoculated with PCB06 sediment and incubated at room temperature or 4 ℃ (Figure 5). Communities were also characterized in sediments used as a source inoculum for the enrichments. The in situ microbial community was determined to contain the highest microbial diversity and evenness with a Shannon Index (SI) of 2.5. The in situ community was evenly distributed between various groups, with the two dominant groups detected, *Xanthomondales* of the *Gammaproteobacteria* and the *Nitrosopumilus* of the *Crenarchaeota*, accounting for 5.7% and 5% of the total sequences, respectively. Primary enrichments demonstrated a lower microbial diversity when incubated both at room temperature and 4 ℃. At room temperature, the Shannon diversity significantly decreased in subsequent transfers, from a SI of 1 (primary enrichment) to 0.6 (4^{th} transfer) and finally to 0.05 (6^{th} transfer). A group of unclassified *Flavobacteriaceae* in the *Bacteroidetes* accounted for approximately 39% of sequences retrieved from the primary enrichment at room temperature, whereas *Microbulbifer* of the *Gammaproteobacteria*

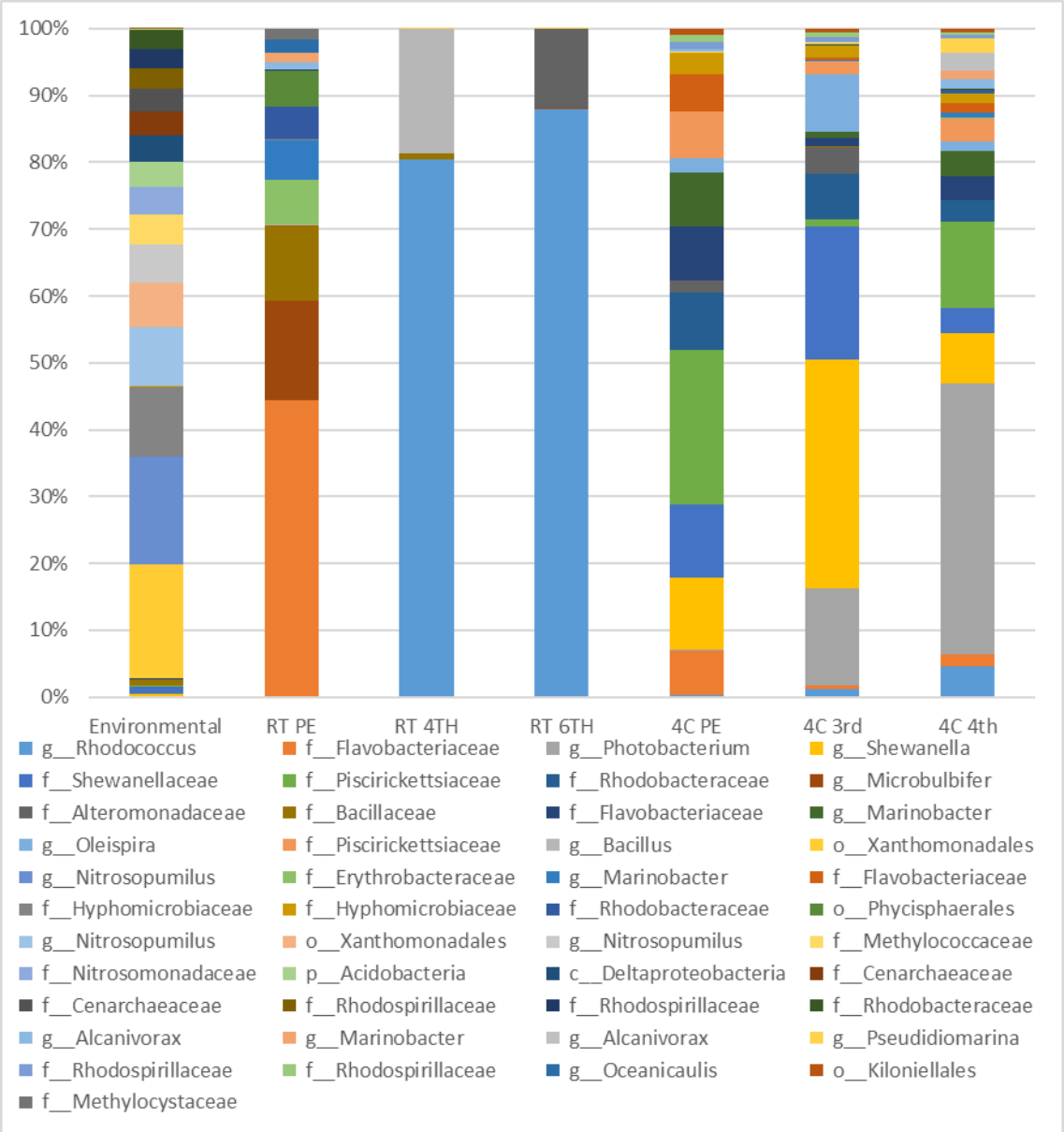


Figure 5. Characterization of microbial community composition in enrichment cultures by next generation sequencing of SSU rRNA genes. Histograms represent communities from the environment (1 from left) and the following enrichments: primary, fourth and sixth transfer incubated at room temperature (2–4 from left); primary, third, and fourth transfer incubated at 4 ℃ (5–7 from left).

was the second most abundant group, accounting for 13% of total sequences. In the later transfers, the community composition changed dramatically. In both the 4th and 6th transfers, the dominant microbial group was the genus *Rhodococcus* of the *Actinobacteria*. The abundance of this OTU reached 96% in the 6th transfer microcosms. In the 4 °C microcosms, the microbial community maintained a similar diversity among the primary and subsequent transfers, changing from a SI of 1.3 (primary enrichment) to 1 (3rd transfer) and rebounding to 1.2 (4th transfer). In the primary enrichment, the dominant group was an unclassified clade within the *Gammaproteobacteria*, accounting for 17% of the total sequences. The second most abundant group belonged to the family *Colwelliaceae* of the *Gammaproteobacteria*. In subsequent transfers, other OTUs were dominant. In the 3rd transfer, *Shewanella benthica* dominated, accounting for 31.8% of the total community. The *Colwelliaceae* family still remained at 18% of the total community. In the subsequent transfer, dominance shifted to the genus *Photobacterium* of the *Gammaproteobacteria*.

3.3 Isolates

Pure cultures were isolated from two different sites, PCB06 and DSH10. Twenty–six *Rhodococcus* strains (PC1–2, PC 5–28) and two *Halomonas* strains *(PC3, 4)* were isolated under various culture conditions from PCB06 sediment. Two strains of *Halomonas (PC29, 30)* were isolated on crude oil at 4 ℃ from DSH10 sediment. Sanger sequencing and RISA results indicated that all 26 *Rhodococcus* strains and the 4 *Halomonas* strains showed nearly 100% sequence identity of rRNA genes. The *Rhodococcus* strains, gram positive bacteria of the phylum *Actinobacteria,* share 99% sequence identity with *Rhodococcus erythropolis*. The *Halomonas* strains, a genus within

the *Gammaproteobacteria*, are nearly identical to *Halomonas campaniensis*[34]. Therefore, only representative isolates were chosen for phylogenetic and physiological characterization (Figure 6).

3.4 Total petroleum hydrocarbon degradation in pure culture

The hydrocarbon degradation ability of *Rhodococcus sp.* strain *PC20* was tested (Figure 7). Strain PC20 appeared to produce its own dispersant as the oil layer was emulsified and formed aggregates after three days of incubation. At room temperature, after a 3 day lag phase, GC amenable compounds decreased by approximately 33% in inoculated treatments relative to uninoculated controls. Over the same period, an average of 1.3 mg C was assimilated into biomass in each culture, while 0.5 mg C was mineralized to DIC and CO_2, resulting in a carbon use efficiency on crude oil of approximately 72%

Similar results were obtained over a longer incubation period with *Rhodococcus* at 4 ℃ (Figure 8). After 21 days of incubation, an average of 33% of the amended oil was utilized. Meanwhile, 1.3 mg C was assimilated into biomass and 0.5 mg C was respired. The growth yield estimated by carbon use efficiency was 71%.

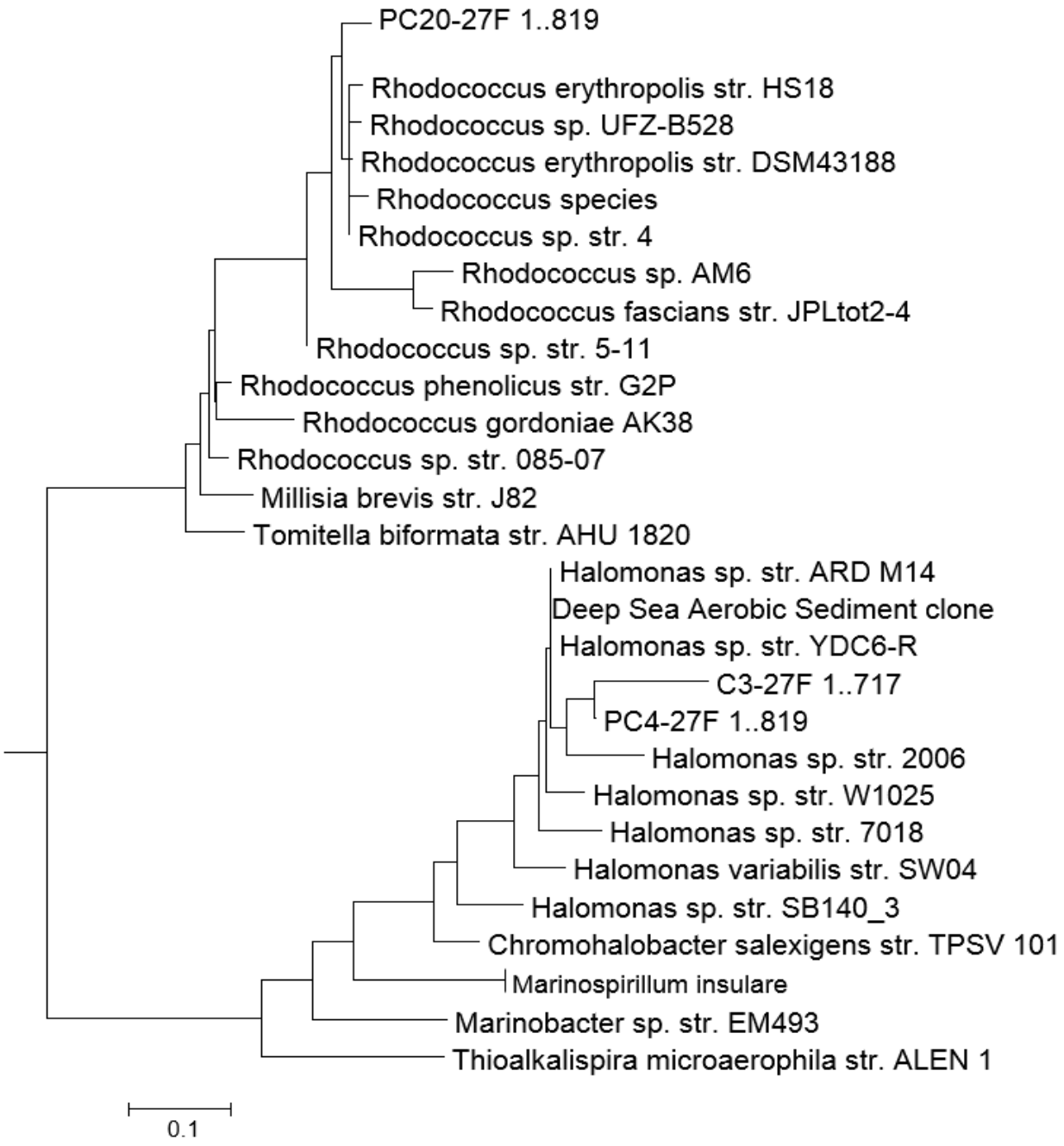
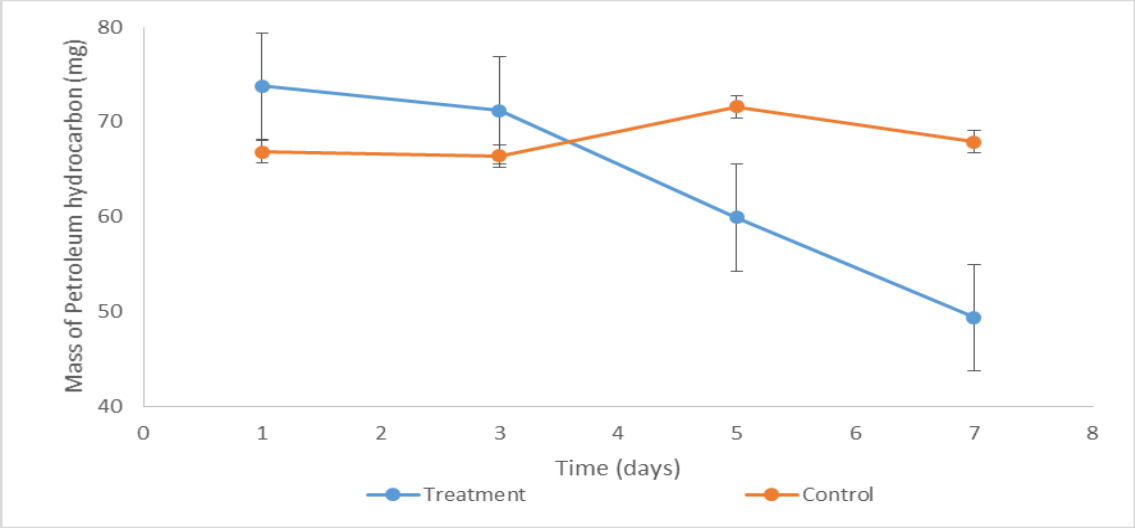


Figure 6. Phylogenetic analysis of SSU rRNA genes from pure cultures of hydrocarbon degrading bacteria isolated from deepsea sediments collected from the nothern GOM. Isolates show high sequence identity to known oil–degraders of the *Rhodococcus* and *Halomonas* genera.

a) Substrate



b) Product

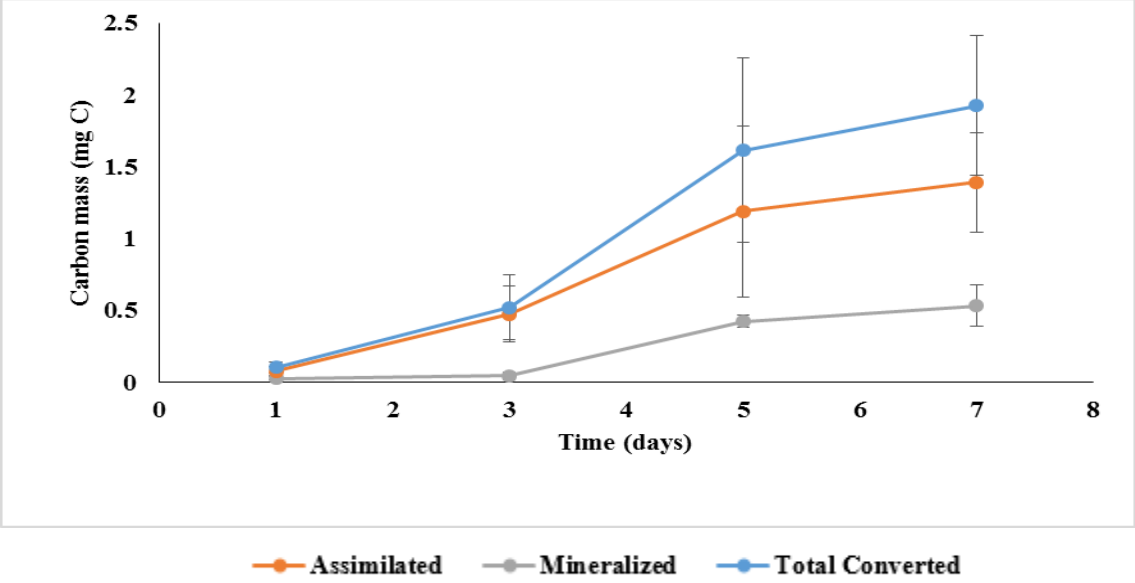
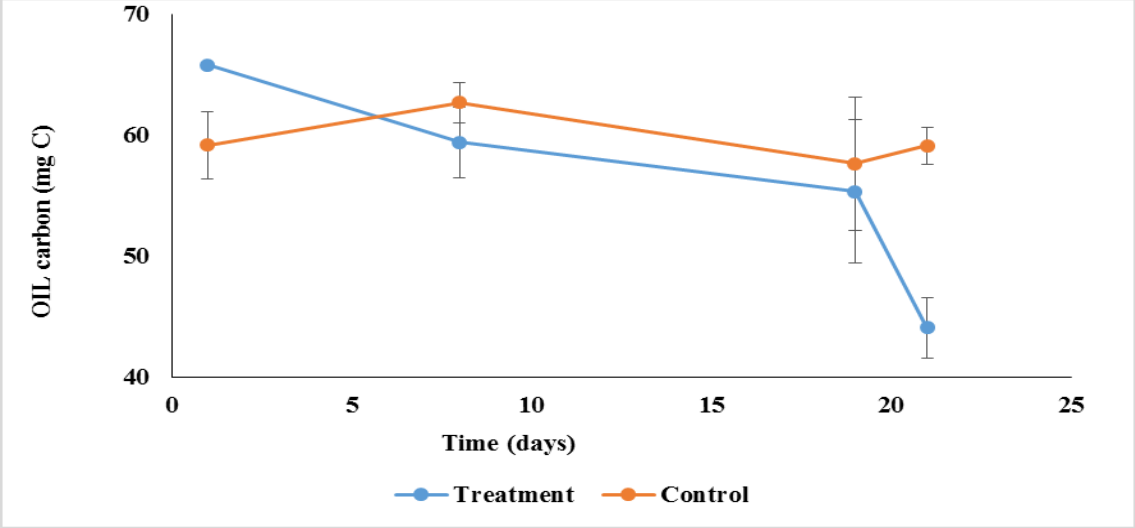


Figure 7. Degradation of crude oil by a pure culture of *Rhodococcus* strain PC20 at the room temperature as: a) mass loss of total petroleum hydrocarbons, and b) accumulation of CO2 or cell carbon.

a) Substrate



b) Product

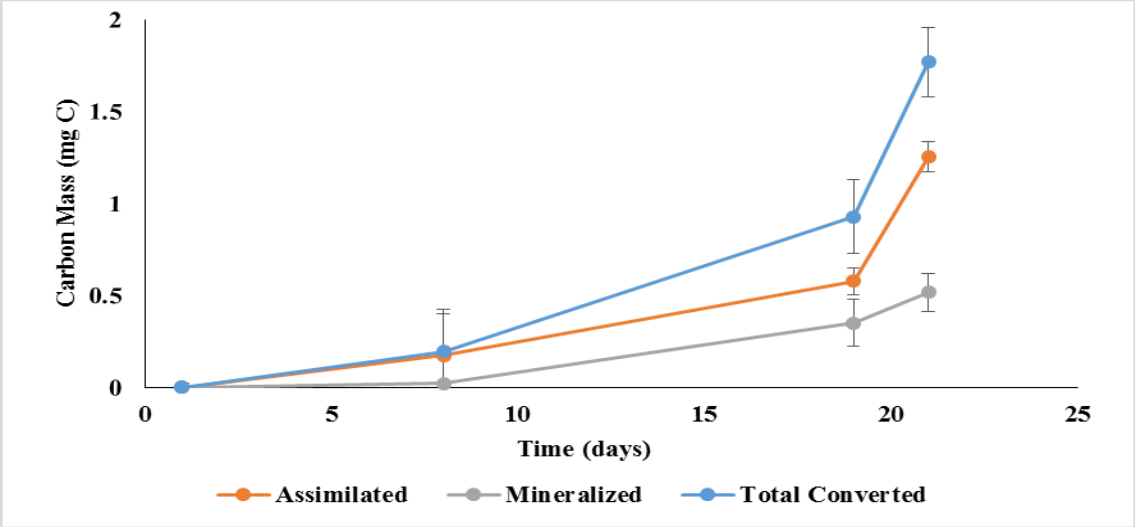


Figure 8. . Degradation of crude oil by a pure culture of *Rhodococcus* strain PC20 at the 4 ℃, as: a) mass loss of total petroleum hydrocarbons, and b) accumulation of CO2 or cell carbon.

CHAPTER IV

DISCUSSION

4.1 Hydrocarbon degradation and potential for nutrient limitation

Microbial communities responded rapidly to oil contamination from the DWH oil discharge, but this response was likely limited by many factors, including the availability of major nutrients[14]. Previous studies have come to contrasting conclusions with regard to nutrient limitation of the hydrocarbon degradation in the surface and deep waters of the northern GOM, where ambient nutrient concentrations differ. In offshore oligotrophic waters, nutrients, especially phosphate, are depleted in the top 200 m depth due to uptake by photosynthetic microorganisms, and nutrient concentrations gradually increase with depth below the photic zone due to organic matter remineralization. Therefore, in this study, we investigated and compared the degradation potentials between the surface and deep water in the northern GOM. We hypothesized that 1) nutrients will be limiting for hydrocarbon–degrading microbial communities in the surface water but not in bottom water, and 2) the different ambient temperatures at each depth, the degradation rate will be slower in the deep water.

The most pronounced nutrient limitation was indeed observed in incubations of surface water. Previous studies showed that offshore surface water of the GOM was oligotrophic, and phosphorus addition increased respiration rates and bacterial secondary production[21]. Edwards et al. (2011) reported that alkaline phosphatase activity was up regulated within oil slicks, which indicates enhanced phosphorus stress[20]. In the present study, the production of CO_2 from aerobic respiration was used as a proxy for

hydrocarbon degradation. Only surface seawater incubations amended with both NH_4^+ and PO_4^{3-} showed substantial hydrocarbon degradation, which indicates that nitrogen availability also limits activity at the surface. This observation agrees with the findings of Turner et al. (2013), which showed that microbial growth was limited by nitrogen or co–limited by both N and P in the northern GOM[35]. Respiration rates reported here are approximately three times higher than those reported by Edwards et al. (2011) for the Gulf surface water[20]. The difference in the rates could be caused by the low oil concentration (1.9 mg l^{-1} : 0.0002% v/v)[36] in the oil slicks studied by Edwards et al. (2011), which was at least three orders of magnitude lower than oil additions in this study.

As predicted from ambient nutrient levels, nutrient limitation had less of an effect in incubations of deep water. Hydrocarbon degradation did not appear to be nutrient limited in the early stages of the incubations. In the later stage of the incubations, however, the degradation terminated possibly due to the depletion of nutrients. In the unamended controls as well as treatments amended with NH_4^+ or PO_4^{3-} only, respiration rates (approximately 56.8 μmol CO_2 L^{-1} day^{-1}) were similar to those reported by Baelum et al. for the subsurface oil plume[19]. The treatments amended with NH_4^+ and PO_4^{3-} exhibited higher respiration rates, which were about twice those observed in all other treatments at both sites investigated in the present study.

Observations on nutrient limitation generally supported our hypothesis that rates would track with the in situ availability of major nutrients[4,21,37]. Moreover, when major nutrients were not limiting, similar hydrocarbon degradation rates were observed in both surface and deep waters, despite the large difference in incubation temperatures (~18

℃)[38]. This suggests that for indigenous microbial communities in the deep water column of the northern GOM, temperature is not the primary limiting factor for hydrocarbon degradation potential. Rather, the availability of major nutrients and carbon appears to be more important for *in situ* degradation processes. Although biodegradation rates were quantified in surface oil slicks and subsurface plumes in the northern Gulf immediately after the DWH discharge, this study reports the first comparison of rates measured across nutrient gradients at different water depths and in sediments of the same site.

Nutrient addition had a less pronounced effect on hydrocarbon degradation in incubations of deepsea sediments. However, nutrient amendment still accelerated the degradation process. Similar observations were also made by Mortazavi et al (2013)[39] in incubations of sandy sediments from a beach at the Dauphin Island. Hydrocarbon degradation rates were two times higher in sediments amended with laurylcholine, a carbon and nitrogen source, in comparison to unamended controls. A maximum respiration rate of 55 μmol CO_2 g dwt^{-1} d^{-1} was observed in incubations of beach sands amended with oil, and with laurylcholine chloride addition, the rate increased by 67%. Singh et al. (2014) also reported that when excess amounts of nitrogen and phosphorus were added to beach sand microcosms, hydrocarbon degradation rates were increased by approximately 16 times. In the present experiment, nutrient addition increased degradation rates by approximately 6 times. And the observed lag phase could result from the time required for microorganisms to adapt to the elevated nutrient concentrations. Both Mortazavi et al. (2013) and Singh et al. (2014) demonstrated much higher degradation rates than in the present study. This was likely due to the origin of the sediments. In both previous studies, samples were collected from shallow sandy

sediments, which generally maintain much higher rates of biogeochemical processes compared to deepsea sediments (Jorgensen, 1992)[40].

4.2 Hydrocarbon degrading bacteria cultivated from deepsea sediments

Characterization of microbial community composition in the enrichment cultures identified the active hydrocarbon degrading microbial groups in deepsea sediments as well as providing further evidence for oil as a selective force impacting indigenous microbial communities. The *in situ* microbial community was dominated by members of the *Xanthomonadales* in the *Gammaproteobacteria*, which is commonly observed in the marine environment. Genera from this order have been previously reported to degrade hydrocarbons[41,42]. In the present study, however, the abundance of this group was significantly reduced in oil amended enrichments, indicating that the group may not mediate hydrocarbon degradation in the sediments studied here. The second most dominant group detected in situ was the genus *Nitrosopumilus* of the phylum *Crenarchaeota* which is also ubiquitously distributed in deepsea environments. Nitrosopumilus is a chemolithoautotrophic nitrifier that thrives under oligotrophic conditions.

Known hydrocarbon degrading microbial groups detected in this study was present as rare taxa in situ and then increased in relative abundance in cultures. At room temperature, the diversity of microbial communities rapidly decreased upon transfer in successive enrichments. Although a number of taxa were detected which have been linked to oil degradation (*Flavobacteraceae*, *Alteromonadaceae*, *Microbulbifer*), *Rhodococcus* appeared to outcompete nearly all other microbial groups by the fourth

transfer. The genus *Rhodococcus*, which belongs to the family *Nocardiaceae* of the Phylum *Actinobacteria*, is an aerobic, gram positive bacterial group. *Rhodococcus* isolates obtained in this study share 100% similarity with a *Rhodococcus erythropolis* strain, a well–studied hydrocarbon degrading species, which was first isolated from an oil–contaminated site in Antarctica[43]. The *Rhodococcaceae* were detected in multiple environments impacted by the DWH oil discharge. For example, within months of the DWH discharge, *Rhodococcus* was detected in deep subsurface oil plumes sampled near the Macondo well. The organism was linked to hydrocarbon degradation in the deep plumes based on the enrichment of alkane 1–monooxygenase genes detected using a functional gene array. Although the relative abundance of *Rhodococcus* was low in 16S rRNA gene sequences retrieved from deepsea sediments, this study provides evidence that the genus may have contributed to the degradation of Macondo oil deposited onto the deep seafloor from the DWH discharge. Because the organism has been cultivated from the deep GOM as well as from Antarctica, *R. erythropolis* is likely active and may act as a good model organism for understanding the ecophysiology of hydrocarbon degradation under cold conditions.

In the 4 ℃ enrichment cultures, microbial communities were more diverse and remained so upon successive transfer. Members of the *Gammaproteobacteria,* which is known to contain many aerobic hydrocarbon degrading taxa, were predominant in the transfers. For example, members of the *Piscirickettsiaceae* showed a high relative abundance, and this family contains the genus *Cycloclasticus*, which was detected in many other studies of the DWH spill[6,22,23]. Valentine et al. (2010) reported that this group was among the most important microbial groups capable of oil degradation the deepsea

subsurface plumes[44]. *Oleispria* of the *Gammaproteobacteria* was also abundant in our cold enrichments, and this group was shown to be active in hydrocarbon degradation in the deep subsurface plumes in previous work[4]. OTUs from many other groups with hydrocarbon–degrading members, were also abundant in the 4 ℃ enrichments, including *Ochrobactrum*[45], *Pseudomonas*[46], *Hyphomonas*[47], *Alcanivorax*[48], *and Marinobacter*[49]. Several of these genera were associated with the biodegradation process after the DWH discharge[4,8,23].

Although microbial community composition largely differed between enrichment cultures incubated at room temperature and 4 ℃, rates of hydrocarbon degradation were similar. Thus, the results implicate functional redundancy and a role for temperature in the selection of hydrocarbon degraders. Since many of the dominant hydrocarbon degrading bacterial groups were not shared between room temperature and 4 ℃ enrichments, this may mean that community composition is not as important as nutrient limitation of hydrocarbon degradation[50]. Interestingly, many of the taxa that increased in relative abundance at 4 ℃ were known piezophiles. It appears that temperature could be more important than pressure as a selective force in the structuring of hydrocarbon–degrading bacterial communities, as many piezophiles were abundant in the 4 ℃ enrichment, including as *Shewanella*[51], *Pseudomonas*[52], *Alcanivorax*[52], *Marinobacter*[52], *Rhodococcus*[52], *and Photobacteria*[53]. This information supports the findings of Schedler et al. (2014)[54], which demonstrated that for a piezophilic microorganism, pressure had only a minor effect on hydrocarbon degradation activity.

4.3 Total petroleum hydrocarbon degradation

The sequestration and mineralization of the spilled oil will potentially affect the carbon cycle in the northern GOM. Using model microorganism to monitor carbon balance comes to the conclusion that polarized hydrocarbon concentration increases significantly during the biodegradation process, therefore further studies are required to understanding the degradation pathways for the biodegradation processes.

The carbon balance between substrate and products was not balanced with a GC–FID measurement. During the hydrocarbon biodegradation, the complexity of hydrocarbon significantly increases. Ruddy et al. (2014) showed the oil collected from Pensacola Beach doubled its complexity than MC252 source oil and dominated by oxygenated compounds[55]. Major portion of these compounds were too polar to be measured by GC–FID[55]. Aeppli et al. (2012) also provided evidence of oxyhydrocarbons were enriched due to biodegradation[56].

The carbon use efficiency was high for strain PC20 when growing on crude oil. According to Widdel et al, for a perfect ideal aerobe, the carbon use efficiency is approximately 68% [57]. This high incorporation capability can be possibly explained by the selective incorporation of hydrophobic n–alkanes into cell membranes[58], and still requires further investigation.

In conclusion, our results revealed that: first, nutrient limitation affects the hydrocarbon degradation rates, and indigenous microbial communities are not limited by temperature, even in the deep water. Second, the characterization of sediment microbial

community responding to oil contamination reveals the functional redundancy and temperature has been identified as a major factor in selecting hydrocarbon degraders. And third, *Rhodococcus* and *Halomonas* strains have been isolated from the deepsea sediments. Further investigation on this topic will be focusing on the relationship between nutrient availability and microbial community composition, as well as identify more hydrocarbon-degrading bacteria.

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