



Substrate Enhanced Biodegradation of Light Crude Oil Sludge by the Resident Methanogens of an Oil Storage Tank Sediment

Okoro Chuma Conlette*

Department of Biology, Microbiology and Biotechnology, Federal University, Ndufu-Alike, Ikwo, Ebonyi State, Nigeria

*Corresponding Author: Okoro Chuma Conlette, Professor, Department of Biology, Microbiology and Biotechnology, Federal University, Ndufu-Alike, Ikwo, Ebonyi State, Nigeria.

Received: February 21, 2018; Published: April 20, 2018

DOI: 10.31080/ASMI.2018.01.0052

Abstract

Introduction: Biodegradation of hydrocarbons under methanogenic conditions has been widely investigated for a variety of crude oil components but the influence of various substrates during methanogenic biodegradation is scanty in literature.

Objective: The main objective is to evaluate the role of metabolic substrates in methanogenic biodegradation

Materials and Methods: Methanogenic biodegradation of crude oil sludge was investigated using chemical and molecular approaches.

Results: 16S rRNA gene sequences recovered from the samples revealed significant presence of *Marinobacterium* (63%), *Pseudomonas* (3%) alongside with acetotrophic *Methanosaeta* (16%) and hydrogenotrophic *Methanobacterium* (5%). The resident microbial community was able to reduce the gravimetric weight of residual oil by 65.5% (with complete degradation of C₅-C₁₇ n-Alkane fractions) in non-amended samples and 94.13% (with complete degradation of C₅-C₂₅ n-Alkane fractions) in substrate amended samples during the 60-day incubation period. As biodegradation progressed, acetotrophs consume acetate at the rate of 0.41 Mm/day⁻¹ while hydrogenotrophs consume hydrogen at the rate of 0.59 Mm/day⁻¹.

Conclusion: Our results showed that the resident methanogenic archaea that dominated the anaerobic microbial community were largely responsible for the anaerobic degradation of hydrocarbons in crude oil sludge and degradation rates were enhanced with substrate amendment. Considering the relatively high number of facultatively anaerobic *Marinobacterium* and significant presence of *Pseudomonas* in the sequenced data, we speculate that the bacteria were at least partially responsible for biodegradation of crude oil components potentially acting as syntrophic organisms with methanogens to convert crude oil to methane.

Keywords: Methanogenic Biodegradation; Acetotrophs; Hydrogenotrophs; Crude Oil Sludge; Syntrophy; Anaerobic Biodegradation

Introduction

Crude oil is a mixture of hundreds of hydrocarbon compounds comprising of the n-alkanes, isoprenoids and the cyclics. These are the saturated fractions and they are the most studied because they constitute the greater percentage in composition [1,2]. Other fractions which are unsaturated include aromatic hydrocarbons, nitrogen, sulfur and oxygen (NSO) containing compounds and the very large highly branched asphaltene molecules. These unsaturated fractions are regarded as the most toxic and persistent. Biodegradation sequence proceeds from the n-alkanes, followed by the isoprenoids and then the steranes, hopanes and higher molecular weight aromatics [2,3]. Whereas degradation of hydrocarbons by oxygen respiring microorganisms has been known for more than a century, utilization of hydrocarbons under anoxic conditions has been investigated properly only during the past 20 years [4-8].

In hydrocarbon bearing and impacted subsurface environments, oil components can be anaerobically biodegraded via a number of anaerobic electron accepting processes including nitrate, iron and sulfate reduction, however when available electrons are depleted or not available, hydrocarbon biodegradation may proceed via methanogenesis [9]. Methanogenic hydrocarbon metabolism involves

the interaction between syntrophic bacteria and methanogens. During the interaction, syntrophic bacteria degrade hydrocarbon substrates to products such as acetate and or hydrogen and carbon dioxide which are then used by methanogens to produce methane [10,11]. Biodegradation of hydrocarbons under methanogenic conditions has been widely investigated for a variety of crude oil components such as n-alkanes [12-14], iso-alkanes [15], benzene [16], toluene [17,18] and Polycyclic aromatic hydrocarbons [19,20]. Recently reports have emerged demonstrating the susceptibility of whole crude oil components to methanogenic biodegradation [10,21-23]. Though the recent studies demonstrated that methanogenic hydrocarbon metabolism are usually dominated by the acetotrophic and hydrogenotrophic methanogenic species, none has clearly demonstrated how enrichments with individual growth substrates can influence methanogenic hydrocarbon degradation and methanogenesis. Considering the accumulation of huge amounts of growth substrates in oil storage tank sediments, wide varieties of hydrocarbon utilizing microorganisms and the anoxic environment, availability of required substrates may play some active roles in the biodegradation of petroleum hydrocarbons by the resident microbial community. This speculation provided strong incentives and motivation to investigate the roles of

substrate availability in the anaerobic biodegradation of crude oil components by the resident anaerobic microbial flora

In this paper, we investigated how substrate enrichments can enhance biodegradation of petroleum hydrocarbons in crude oil storage tanks by the resident anaerobic microbial flora dominated by methanogens.

Materials and Methods

Sample collection

Oil sludge samples were collected from oil storage tanks due for cleaning in 1 Liter sterile Nalgene sample bottles. They were all filled to the brim to exclude air. Samples were transported to the laboratory within 48 hrs of collection in iced bags for analysis. Samples for DNA analysis were shipped to the University of Calgary under low temperature and upon arrival to the laboratory were kept in a CO₂ anaerobic hood with an atmosphere of 90% N₂ and 10% CO₂ (v/v) for further analysis.

Chemical Analysis

The pH was analyzed with an Orion pH meter (model 370). The concentration of dissolved sulfide was analyzed by the diamine method [24]. Sulfate was assayed by ion chromatography using a conductivity detector (Waters 423) and an IC-PAK anion column with borate/gluconate buffer at a flow rate of 2 ml/min (4.6 x 150 mm, Waters). Ammonium was assayed with the indophenol method. Key organic acids (lactate, acetate, propionate and butyrate) were determined using an HPLC equipped with a UV detector (Waters, 2487 Detector) set at 220 nm and an organic column (Alltech, 250 x 4.6 mm) swept with 25 mM KH₂PO₄ (pH 2.5) as described in [25]. Total petroleum hydrocarbons were measured as described in standard methods of [26].

Biodegradation studies

Minimal salt media was prepared as described in Mills, *et. al* (1978) [27]. The medium was anaerobically dispensed in 150 ml aliquots into 250 ml serum bottles with a gas phase of 90% nitrogen and 10% carbon dioxide and closed with sterile butyl rubber stoppers. The experiment was carried out in three sets and each set was in triplicates to allow periodic measurements of oil content and methane production in individual bottles. Only one set was amended with substrates (10 mM acetate + 15 mM hydrogen) while 50 ml of sample was added in all the three sets. Methanogenic activity was inhibited in the control tube (minimal salt broth+ sample only) by addition of 2 mM of 2-bromoethane sulfonate as described in [28]. The media were anaerobically incubated for 60 days and at every 30 day interval, samples were withdrawn for estimation of residual hydrocarbon content and gas chromatographic analysis.

Estimation of residual hydrocarbon content

The estimation of the oil content of the sample was by partition gravimetric method as described in standard methods of [26], with little modifications in our laboratory. Fifty (50) ml of the sample was extracted with 100 ml of the solvent (Freon) and the solvent was allowed to evaporate in the oven after the extraction. If the organic solvent is free of residues, the gain in weight of the tarred

flask is mainly due to the oil content and is calculated thus;

$$\text{Oil content (ppm)} = \frac{(A-B) \times 1000}{\text{ml sample}}$$

A= Weight of tarred flask+ residue; B= Weight of residue

Gas chromatographic analysis of oil samples

1 µl of the extracted oil was injected by an auto injector (7683 B series, Agilent technologies, Santa Clara, CA) into a gas chromatograph (7890 N series, Agilent) that was connected to a mass selective detector (5975 C inert XL MSD series, Agilent). The gas chromatograph was equipped with an HP-1 fused silica capillary column (length 50m, inner diameter 0.32 mm, film thickness 0.52µm, J&W Scientific) with helium as a carrier gas. The GCMS system was operated as described in [29].

Determination of nC17/Pristane and nC18/Phytane ratios

Pristane and Phytane are low molecular weight isoprenoids used as biomarkers in the experiment to prove that biodegradation by microbial action actually took place. They have a retention time of 30 - 40 minutes are resistant to biodegradation. Hence measurement of the decreasing trends in the ratio of n-alkane peaks closest to pristane (n17) and that of phytane (n18) respectively is an indication of biodegradation and the rate of decrease indicates the severity of biodegradation. Measurement of nC17/Pristane and nC18/Phytane ratios were carried out as described in [30].

Measurement of Methane production

Maximum methane concentration at the head space was measured monthly by GC-FID using a Carlo ERBA HRGC 5160 fitted with Chrompak plot fused silica capillary column (30 m x 0.32 mm) using helium as a carrier gas. Methane was quantified on the basis of the peak area and calibrated using CH₄ standards (Scientific and Technical Gases Ltd. New Castle, UK) as described in [30].

DNA extraction, amplification, sequencing and bioinformatic analysis

Genomic DNA was extracted from 40 ml of sample using the FastDNA Spin kit (MP Biomedical, Santa Ana, CA). Extracted DNA (2 ng µL⁻¹) was then amplified through 25 PCR cycles [31]. The 16S rRNA genes were amplified by PCR (95°C, 3 minutes; 25 cycles of 95°C 30s, 55°C 45s, 72°C 90s; 72°C 10 minutes; final hold at 4°C) using the FLX Titanium amplicon primers 454T-RA and 454T-FB (20 pmol µL⁻¹) that have the sequences for 16S primers 926f (aaa ctY aaa Kga att gac gg) and 1392r (acg ggc ggt gtg tRc) as their 3'-ends. Primer 454T-RA had a 25 nucleotide A-adaptor sequence of CGTATCGCCTCCCTCGCGCCATCAG, whereas primer 454T-FB had a 25 nucleotide B-adaptor sequence of CTATGCGCCTTGCCAGCCC-GCTCAG. PCR product quality was verified on an 0.7% agarose gel and PCR products were purified with a QIAQuick PCR Purification Kit (Qiagen) following which their concentrations were determined on a Qubit Fluorometer (Invitrogen), using a Quant-iT dsDNA HS Assay Kit (Invitrogen) as described elsewhere [32]. PCR products (typically 100 ng) were sent to the Genome Quebec and McGill University Innovation Centre for pyrosequencing with an

FLX Instrument, using a GS FLX Titanium Series Kit XLR70 (Roche Diagnostics Corporation). Data analysis was conducted with Phoenix 2, a 16S rRNA data analysis pipeline, developed in house [32,33]. High quality sequences that remained following quality control and chimeric sequence removal were clustered into operational taxonomic units at 3% distance by using the average linkage algorithm [34]. A taxonomic consensus of all representative sequences from each of these was derived from the recurring species within 5% of the best bitscore from a BLAST search against the SSU reference data set SILVA102 [35]. Amplicon libraries were clustered into a Newick-formatted tree using the UPGMA algorithm with the distance between libraries calculated with the thetaYC coefficient [36] as a measurement of their similarity in the Mothur software package [37]. The Newick format of the sample relation tree was visualized using Dendroscope [38]. The entire set of the raw reads is available from the Sequence Read Archive at the National Center for Biotechnology Information (NCBI) under accession number SRR1508445.

Substrate utilization tests

Serum bottles (125 ml Wheaton) containing 70 ml of sulfate free CSB-K medium (composition in g/L: NaCl, 1.5; CaCl₂·H₂O, 0.21; MgCl₂·H₂O, 0.54; NH₄Cl, 0.3; KCl, 0.1; KH₂PO₄, 0.05; resazurin (0.5 ml); trace elements solution, 1 ml and tungstate selenite (1 ml) were inoculated with 5 ml of oil sample under anaerobic conditions. The bottles were closed with butyl stoppers, crimp sealed and purged with oxygen free nitrogen. Triplicate microcosms were amended with methanogenic substrates (10 Mm of acetate and H₂). Control tubes do not have oil samples. Mild steel coupons (50 x 5 x 1 mm) were used as source of metallic iron. The coupons were pre-treated with HCl for 2 minutes to remove surface corrosion products and rinsed immediately with distilled water. Coupons were then washed with acetone, dried, carefully weighed and two were placed in each serum bottles. Methane concentration at the head space was measured weekly by GC-FID using a Carlo ERBA HRGC 5160 fitted with Chrompak plot fused silica capillary column (30 m x 0.32 mm) using helium as a carrier gas. Methane was quantified on the basis of the peak area and calibrated using CH₄ standards (Scientific and Technical Gases Ltd. New Castle, UK). Residual concentrations of acetate and hydrogen were also measured weekly and the consumption rate were as described in [28].

Results

Chemical characterization of oil sludge samples

Results of some relevant chemical parameters of oil sludge sample are shown in table 1. Sample pH was 8.25 with low salinity (2.76 mM) and zero sulfate but with significant values of accumulated volatile fatty acid substrates especially acetate (2.96 mM). Total petroleum hydrocarbon content of the oily sludge was 4890 ppm.

Parameter measured	Value obtained
pH	8.25
Sodium chloride	2.76
Conductivity	23.03
Sulfate	0
Sulfide	0.045
Ferrous iron	0
Ammonium	0.65
Acetate	2.96
Propionate	0.40
Butyrate	0.14
Total Petroleum Hydrocarbon (TPH)	4890

Table 1: Physicochemical analysis of oily sludge sample in (mM), conductivity in (mS/cm) and total petroleum hydrocarbon in (ppm).

Methanogenic biodegradation of crude oil

The indices used in this study to evaluate methanogenic biodegradation of crude oil are the gravimetric loss in oil weight as biodegradation progressed and also the volume of methane produced during biodegradation (Figure 1). Evidence of biodegradation were also revealed in the gas chromatographic analysis of residual oil and the decreases recorded in the values of nC17/Pristane and nC18/Phytane ratios as biodegradation progressed without substrate enhancement (Figure 2) and with substrate enhancement (Figure 3). Biodegradation studies showed 65.5% reduction in the gravimetric weight of oil and degradation of C₅-C₁₇ n-Alkanes in non-amended samples and 94.13% reduction in the gravimetric weight of oil and degradation of C₅-C₂₅ n-Alkanes in substrate amended samples. Control samples showed no observable reduction in the gravimetric weight of oil and none of the hydrocarbon fractions were degraded in the control samples. Gas chromatographic analysis also showed reduction in the values of nC17/Pristane from 1.47 at day 0 to 1.10 and 0 at day 30 and 60 respectively while nC18/Phytane ratios decreased from 3.30 at day 0 to 2.31 and 0.81 respectively in unamended samples. Substrate amended samples showed drastic decrease in the ratios of both the nC17/Pristane and nC18/Phytane as both biomarkers and the n-alkanes were degraded during the 30 day incubation period. Total methane produced in non-amended samples at the end of the 60 day incubation period was 0.68 mM while substrate amended samples produced about 1.60 mM of methane at the end of the incubation period.

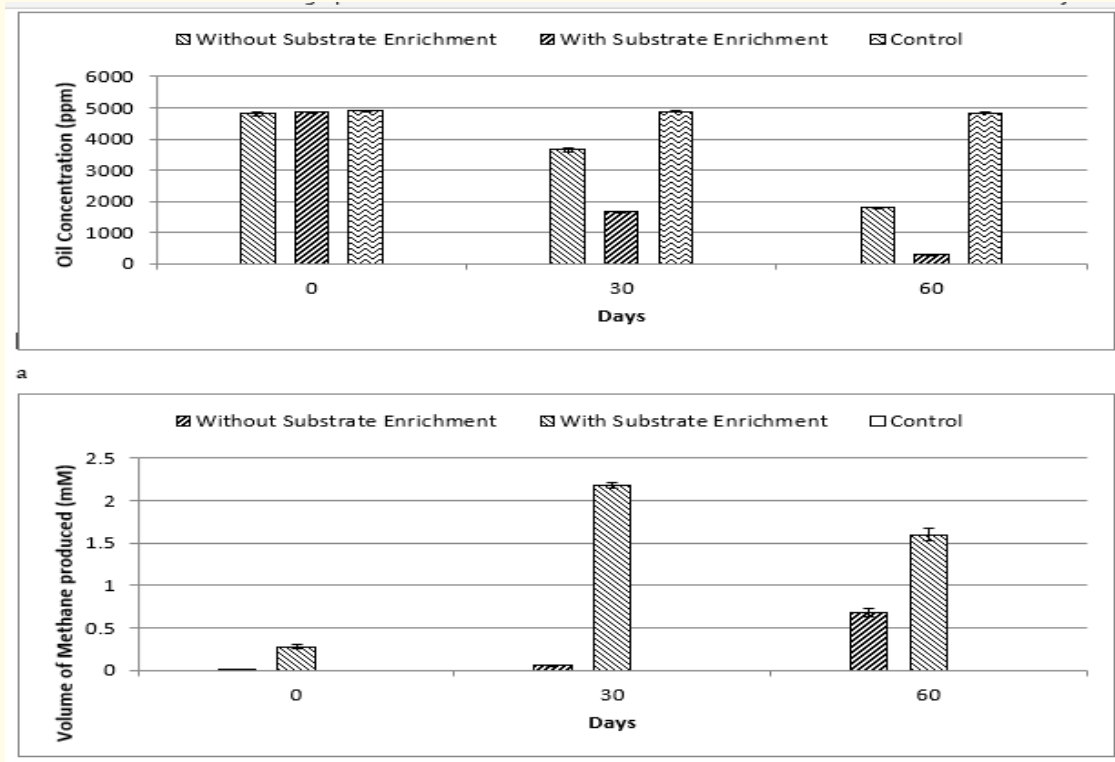
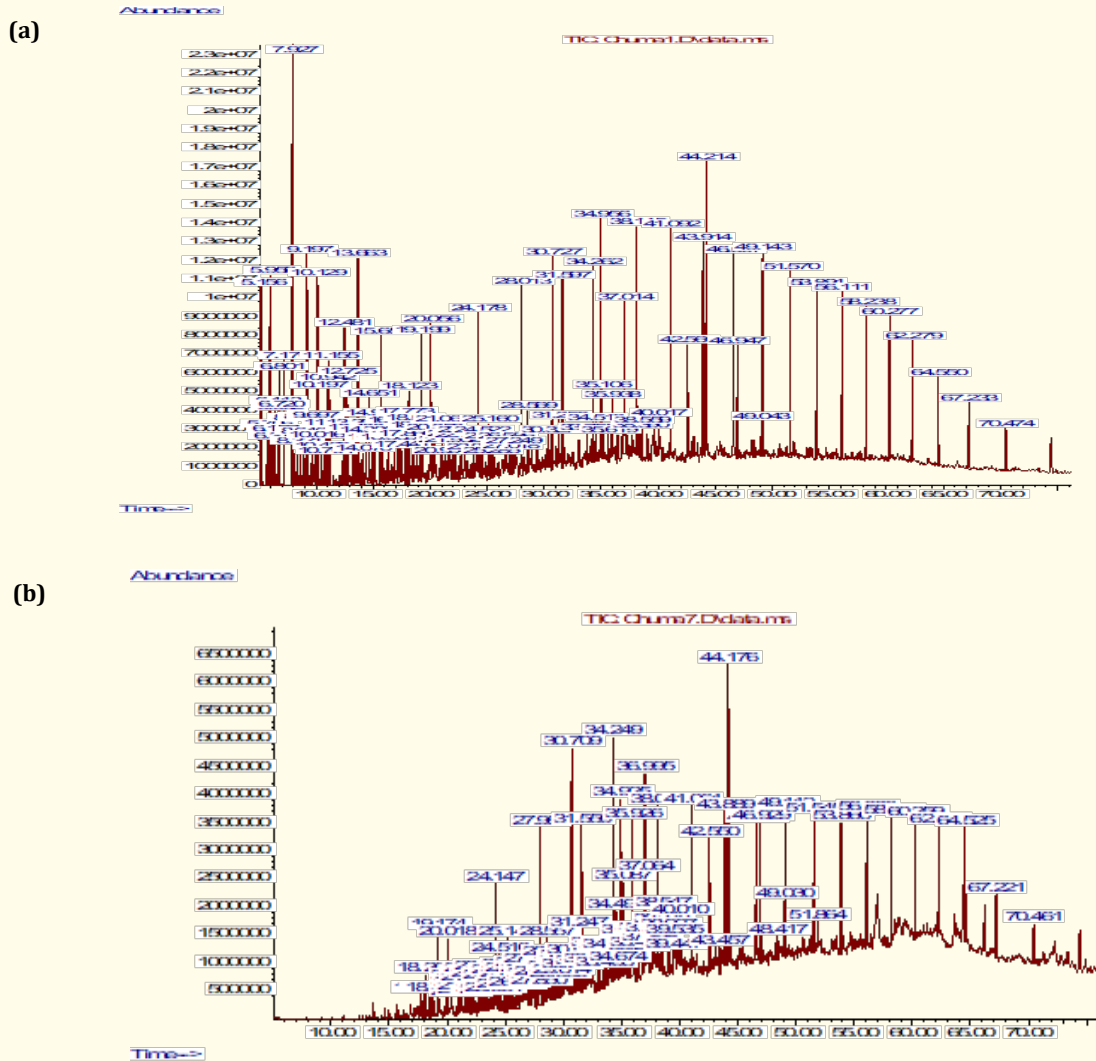


Figure 1: Methanogenic degradation of crude oil sludge showing a. Gravimetric loss in oil weight and b. Volume of methane produced with and without substrate enhancement during the 60 day incubation period.



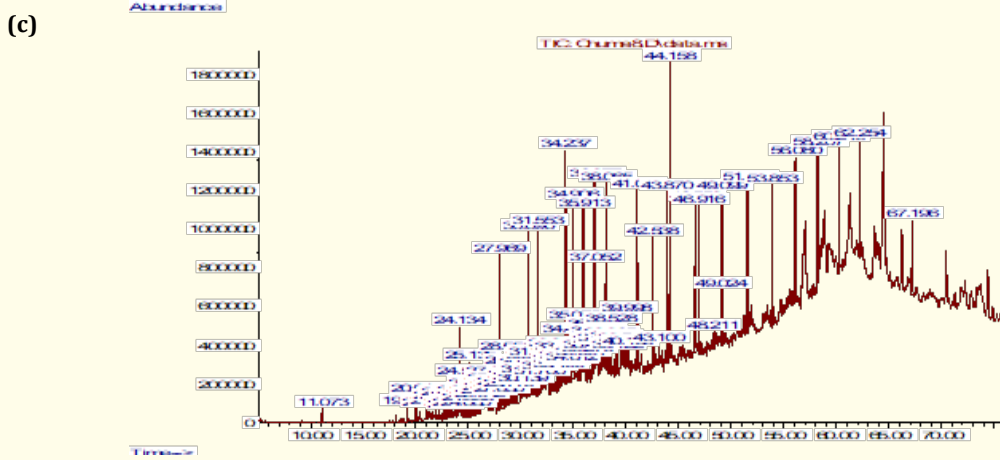


Figure 2: GC Chromatograms of oil sample subjected to anaerobic methanogenic degradation without substrate enhancement at day 0 (A; oil content = 4,800 ppm), day 30 (B; oil content = 3,600 ppm) and day 60 (C; oil content = 1,800 ppm).

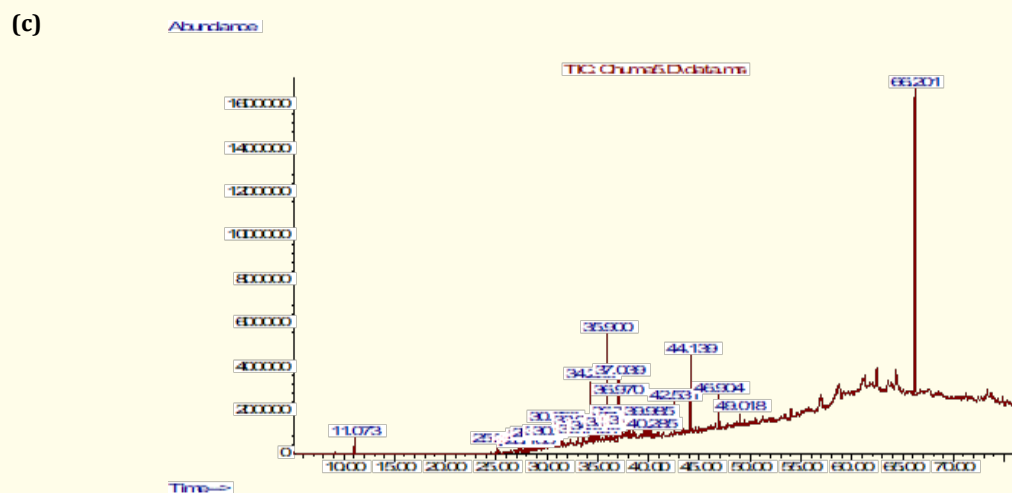
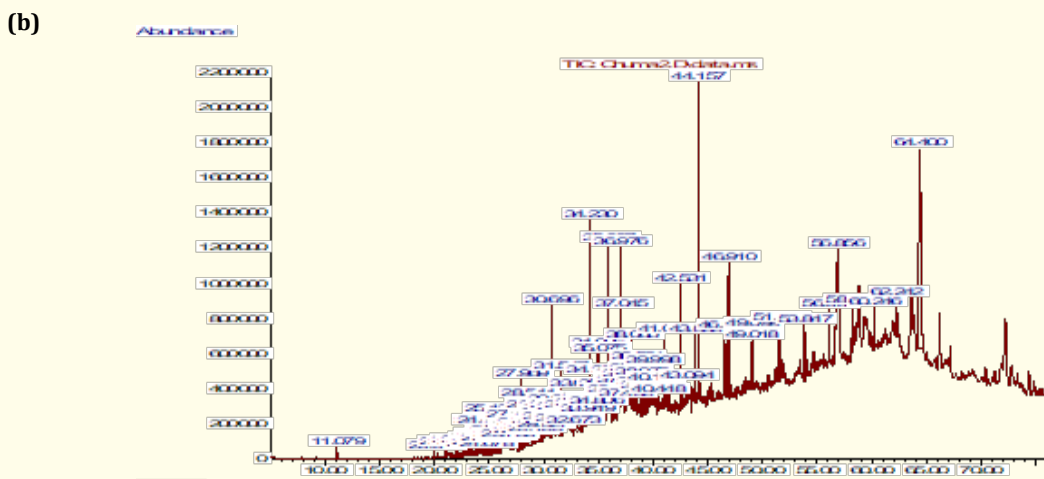
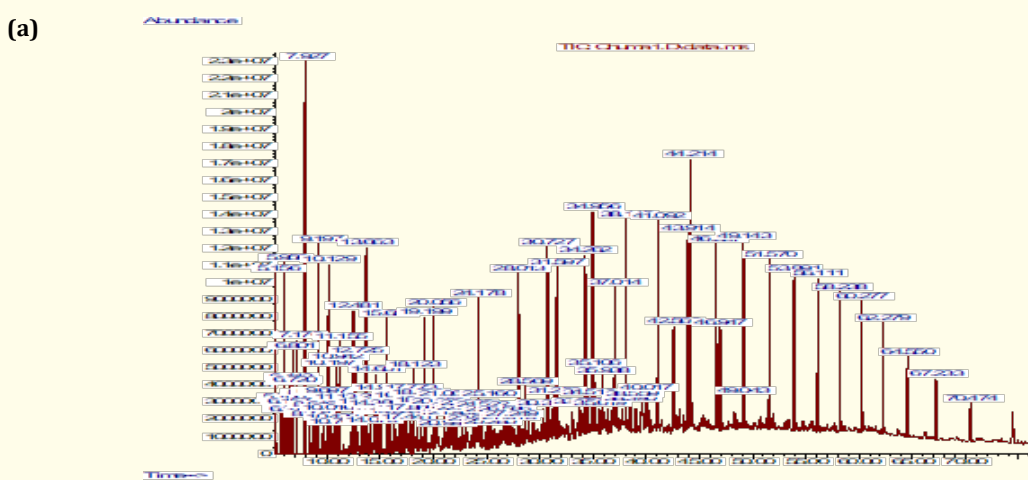


Figure 2: GC Chromatograms of oil sample subjected to anaerobic methanogenic degradation with substrate enrichment (acetate + H₂) at day 0 (A; oil content = 4,800 ppm), day 30 (B; oil content = 1,670 ppm) and day 60 (C; oil content = 285 ppm).

Ability of methanogens to utilize acetate and hydrogen as growth substrates and produce methane

Ability of methanogens in sample to utilize acetate and hydrogen as growth substrates are shown in figure 4. About 57% of acetate concentration present in the medium was utilized while 83.3% of hydrogen was utilized by methanogens present in the medium during the 14 day incubation period. Substrate consumption rate were 0.41 mM/day^{-1} for acetate and 0.59 mM/day^{-1} for hydrogen. Total volume of methane produced by methanogens during the 14 day incubation period was 1.25 mM.

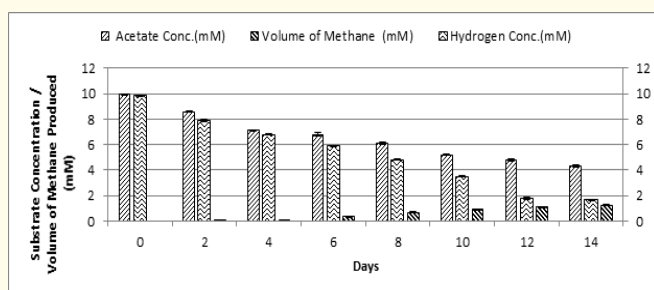


Figure 4: Utilization of acetate and hydrogen by methanogens present in the sample to produce methane.

Microbial communities of oil sludge sample

Phylogenetic classification of pyrosequencing reads for the oil sludge sample showed total reads of 4077, numbers of OTUs and taxa were 97 and 96 respectively. Bacterial taxa (75.82%) however dominated archaeal taxa (24.18%). Relative abundances of major bacterial and archaeal groups among sequences recovered from oil sludge samples as shown in figure 5 include; *Marinobacterium* (63%), *Methanosaeta* (16%), *Methanobacterium* (5%), *Petrobacter* (4%), *Kosmotoga* (4%), *Pseudomonas* (3%), *Methanomicrobiales* (3%) and *Desulfuromonadaceae* (2%).

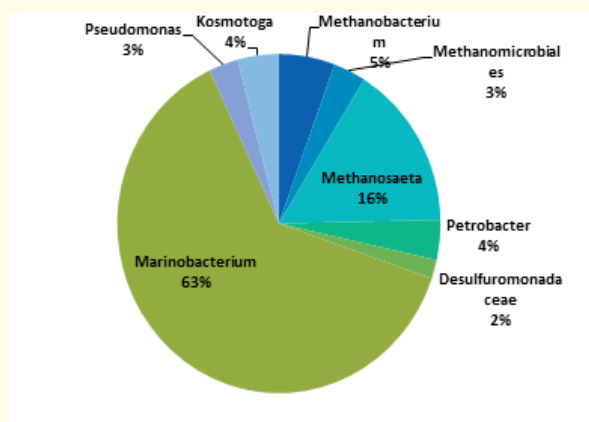


Figure 5: Relative abundances of major bacterial and archaeal groupings among sequences recovered in sample Oil Sludge sample.

Discussion

According to [6], under natural environmental conditions, biodegradation of oil reservoirs takes place over long geological time scales with the process taking millions of years to degrade up to 50% or more of light crude oil accumulation but in crude oil storage tanks, oil biodegradation is expected to be faster under anoxic conditions as a result of huge accumulation of organic nutrients and abundance of hydrocarbon degrading microbial communities. To date there is no clear consensus regarding the length of time oil can stay in storage tanks before it can undergo significant biodegradation but it is expected that when oil stays a reasonable length of time in the storage tank, it can undergo some degree of biodegradation which can lead to decrease in saturated and aromatic hydrocarbon fractions and API gravity (a measure that correlates with oil value). Sulfur content, acidity, viscosity and metal content of the oil also decreases and all these have negative impacts on the economic value of oil [14,39]. Some reports have also shown that severe light crude oil biodegradation can be fuelled by increasing organic matter availability [18,28].

In the current work, we monitored biodegradation of light crude from crude oil storage tank bottom sludge by resident microbial flora over a period of 60 days under anoxic conditions. We also experimented on how amendments with suitable substrates could influence the rate of biodegradation. Pyrosequencing surveys from our study indicated that the sample contained some fractions of both aerobic (*Marinobacterium*, *Pseudomonas*) and anaerobic (*Methanosaeta*, *Methanobacterium*, *Methanomicrobiales*, *Desulfuromonadaceae* and *Kosmotoga*) taxa. *Marinobacterium* and *Pseudomonas* though considered aerobic can also be facultative and they have always been associated with anaerobic oil environments [8,20,22,40]. Presence of methanogens alongside with syntrophic hydrocarbon degraders and biofilm forming organisms like *Marinobacterium* and *Pseudomonas* in oil storage tank is expected to facilitate degradation of petroleum hydrocarbon in the storage tank because it is suspected that while the syntrophic bacteria degrade the hydrocarbon substrates to products such as acetate, hydrogen or carbon dioxide, methanogens use these substrates to produce methane [10,21-23].

The resident oil sludge microbial community used in the present study which comprised of acetotrophic and hydrogenotrophic methanogens along with syntrophic *Marinobacterium* and *Pseudomonas* were capable of reducing the gravimetric weight the residual oil by 65.5% in non-amended samples and 94.13% in substrate amended samples during the 60-day incubation period. During this period, we also observed the complete removal of C_5 - C_{17} n-Alkanes in non-amended samples and C_5 - C_{25} n-Alkanes in substrate amended samples. This was similar to the observations made by [41] where significant populations of acetotrophic and hydrogenotrophic methanogens in oil sludge were able to degrade progressively the n-Alkane fractions of the hydrocarbon.

Archaeal 16S rRNA gene sequences recovered from the oil samples revealed significant presence of acetotrophic *Methanosaeta* (16%) and hydrogenotrophic *Methanobacterium* (5%) and substrate amendments with acetate and hydrogen enhanced the volume of methane produced and the rate of biodegradation. Other investigations have implicated the dominant roles played by acetotrophic and hydrogenotrophic methanogens in crude oil biodegradation [18,22,41] but ours emphasized on how metabolism of required substrates by methanogens can enhance biodegradation. Our study showed the ability of methanogens to metabolize the required substrates and generate methane as biodegradation progressed. As biodegradation progressed, acetotrophs consume acetate at the rate of 0.41 mM/day⁻¹ while hydrogenotrophs consume hydrogen at the rate of 0.59 mM/day⁻¹ and total volume of methane generated during the 14 day incubation period was 1.25 mM [18] made a similar observation during methanogenic degradation of petroleum hydrocarbon where substrate consumption rates were 0.75 Mm/day⁻¹ for hydrogenotrophs and 0.46 mM/day-1 for acetotrophs. Both results confirm that the rate of utilization of hydrogen by hydrogenotrophs were higher than that of utilization of acetate by acetotroph [7] also confirmed in his studies that hydrogenotrophic methanogenesis were dominant in systematic biodegradation of oil in crude oil reservoirs. Nevertheless [42] demonstrated how acetate consumption can enhance methanogenic degradation of petroleum hydrocarbons. Our results however showed that addition of acetate and hydrogen enhanced methanogenic activity by both acetotrophs and hydrogenotrophs but did not indicate which of the activities dominated. Another observation that seem to agree with our findings is the postulation that volatile hydrocarbons (nC₅-nC₁₀) inhibits methanogenic alkane biodegradation by hydrogenotrophic and acetotrophic methanogens by [43]. We observed that from day 0 - 30 when some of these volatile hydrocarbons were present, biodegradation was slower but after day 30 when they must have been removed, biodegradation became faster indication that volatile hydrocarbons might have slowed down biodegradation rate.

Statistical data showed that there is a strong negative correlation between oil degradation and the volume of methane produced in both non-amended samples ($r = -0.949$; $p < 0.01$) and amended samples ($r = -0.819$; $p < 0.01$) indicating that as the gravimetric weight of oil is reduced, methane production increased. There is also a strong negative correlation between acetate concentration and volume of methane produced ($r = -0.920$ $p < 0.01$) and hydrogen concentration and volume of methane produced ($r = -0.960$; $p < 0.01$) indicating that as the substrates are being utilized and reduced, the volume of methane increased.

Conclusions

In conclusion, it is evident from the results obtained in the present study that the resident methanogenic archaea (*Methanosaeta* and *Methanobacterium*) were largely responsible for degradation of hydrocarbons in crude oil sludge and degradation rates were enhanced with amendments with suitable substrate but considering the relatively high number of facultatively anaerobic *marinobacterium* (63%) in the sequenced data, we speculate that the bacteria were at least partially responsible for the biodegradation of crude

oil components potentially acting as syntrophic organisms in conjunction with methanogens to convert crude oil to methane. Future research should focus on establishing the estimated shelf life of light crude stored in storage tanks considering the biodegradative potentials of the resident microbial community.

Acknowledgement

This work was supported by grants from the Nigerian Petroleum Technology Development Fund (PTDF). We acknowledge the earlier DNA analysis and pyrosequencing carried out with grants from Gerrit Voordouw, University of Calgary, Alberta Canada.

Bibliography

- Okoro CC. "Biodegradation of hydrocarbons in untreated produced water using pure fungal cultures". *African Journal of Microbiology Research* 2.8 (2008): 217-223.
- Wapes DW. "Geochemistry in petroleum explorations". IHRDC (Publishers). 137 New bury street, Boston MA 0216 (1985): 215.
- Andrade LL, et al. "Microbial diversity and anaerobic hydrocarbon degradation potential of an oil contaminated mangrove sediment". *BMC Microbiology* 12 (2012): 186.
- Cruz GF, et al. "Could petroleum biodegradation be a joint achievement of aerobic and anaerobic microorganisms in deep sea reservoirs?" *AMB Express* 1 (2011): 47.
- Gray ND, et al. "Methanogenic degradation of petroleum hydrocarbons in subsurface environments. Remediation, Heavy oil formation and Energy recovery". *Advances in Applied Microbiology* 72 (2010): 137-161.
- Head IM, et al. "Biological activity in the deep subsurface and the origin of heavy oil". *Nature* 426.6964 (2003): 344-352.
- Jones DM, et al. "Crude oil biodegradation via methanogenesis in subsurface petroleum reservoirs". *Nature* 451 (2007): 176-180.
- Li D, et al. "Microbial biodiversity of a Malaysian oil field and a systematic comparison with oil reservoir worldwide". *Archives of Microbiology* 194.6 (2012): 513-523.
- Widdel F, et al. "Anaerobic hydrocarbon degrading microorganisms. An overview". In: Handbook of hydrocarbon and lipid microbiology. Timmus KN (ed.). Springer-Verlag Berlin Heidelberg, Germany (2010).
- Berdugo-Clavijo C and LM Gieg. "Conversion of crude oil into methane by a microbial consortium enriched from oil reservoir production waters". *Frontiers in Microbiology* 5 (2014): 197.
- Cheng L, et al. "DNA-SIP reveals that syntrophaceae play an important role in methanogenic hexane degradation". *PLOS ONE* 8.7 (2013): e66784.
- Anderson RT and DR Lovely. "Hexadecane decay by methanogenesis". *Nature* 404.6779 (2000): 722-730.
- Bian XY, et al. "Insights into anaerobic biodegradation pathways of n-alkanes in oil reservoir by detection of signature metabolites". *Scientific Reports* 5 (2015): 9801.

14. Evans CR, *et al.* "Evolution and alteration of petroleum in western Canada". *Chemical Geology* 8.3 (1971): 147-170.
15. Abulaban NA, *et al.* "Biodegradation of C7 and C8 isoalkanes under methanogenic conditions". *Environmental Microbiology* 17.12 (2015): 4898-4915.
16. Ulirich AC and EA Edwards. "Physiological and molecular characterization of anaerobic benzene degrading mixed cultures". *Environmental Microbiology* 5.2 (2003): 92-102.
17. Edwards EA and D Grbic-Galic. "Anaerobic degradation of toluene and o-xylene by a methanogenic consortium". *Applied and Environmental Microbiology* 60.1 (1994): 313-322.
18. Fowler SJ, *et al.* "Identification of toluene degraders in a methanogenic enrichment cultures". *FEMS Microbiology Ecology* 89.3 (2014): 625-636.
19. Cheng W, *et al.* "Polycyclic aromatic hydrocarbon (PAH) degradation coupled to methanogenesis". *Biotechnology Letters* 28.6 (2006): 425-430.
20. Zhang F, *et al.* "Microbial diversity in long term water flooded reservoir with different in-situ temperature in China". *Scientific Reports* 2 (2012): 760.
21. Fowler SJ, *et al.* "Community structure in the methanogenic enrichments provides insight into syntrophic interactions in hydrocarbon impacted environments". *Frontiers in Microbiology* 7 (2016): 562.
22. Gieg IM, *et al.* "Syntrophic biodegradation of hydrocarbon contaminants". *Current Opinion in Biotechnology* 27 (2014): 21-29.
23. Tan B, *et al.* "Comparative analysis of metagenomes from three methanogenic hydrocarbon degrading enrichment cultures with 41 environmental samples". *ISME Journal* 9.9 (2015): 2028-2045.
24. Trüper HG and HG Schlegel. "Sulfur metabolism in Thiorhodaceae. Quantitative measurements in growing cells of *Chromatium okechii*". *Antonie van Leeuwenhoek* 30 (1964): 225-238.
25. Okoro C, *et al.* "Comparison of microbial communities involved in souring and corrosion in offshore and onshore oil production facilities in Nigeria". *Journal of Industrial Microbiology and Biotechnology* 41.4 (2014): 665-678.
26. Eaton AD, *et al.* "Standard methods for the examination of water and waste water (19th edition)". United Books Press Inc. Baltimore Maryland USA (1995).
27. Mills AL, *et al.* "Enumeration of petroleum degrading marine and estuarine microorganisms by the most probable number method". *Canadian Journal of Microbiology* 24.5 (1978): 552-557.
28. Kleikemper J, *et al.* "Activity and diversity of methanogens in a petroleum hydrocarbon contaminated aquifer". *Applied and Environmental Microbiology* 71.1 (2005): 149-158.
29. Agrawal A, *et al.* "Toluene depletion in produced oil contributes to souring control in a field subject to nitrate injection". *Environmental Science and Technology* 46.2 (2012): 1285-1292.
30. Okoro CC. "Microbial Communities of Light Crude from Nigeria and Potential for In-situ Biodegradation, Souring and Corrosion". *Petroleum Science and Technology* 34.1 (2016): 71-77.
31. Ramos-Padrón E, *et al.* "Carbon and sulfur cycling by microbial communities in a gypsum-treated oil sands tailings pond". *Environmental Science and Technology* 45.2 (2011): 439-446.
32. Park HS, *et al.* "Effect of sodium bisulfite injection on the microbial community composition in a brackish-water-transferring pipeline". *Applied and Environmental Microbiology* 77 (2011): 6908-6917.
33. Soh J, *et al.* "Phonenix 2. A locally installable large scale 16S rRNA gene sequence analysis pipeline with web interface". *Journal of Biotechnology* 167.4 (2013): 393-403.
34. Schloss PD and SL Westcott. "Assessing and improving methods used in OTU-based approaches for 16S rRNA gene sequence analysis". *Applied and Environmental Microbiology* 77.10 (2011): 3219-3226.
35. Pruesse EC, *et al.* "SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB". *Nucleic Acids Research* 35.21 (2007): 7188-7196.
36. Yue JC and MK Clayton. "A similarity measure based on species proportions". *Communications in Statistics - Theory and Methods* 34.11 (2005): 2123-2131.
37. Schloss PD, *et al.* "Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities". *Applied and Environmental Microbiology* 75.23 (2009): 7537-7541.
38. Huson D, *et al.* "Dendroscope: an interactive viewer for large phylogenetic trees". *BMC Bioinformatics* 8 (2007): 460-463.
39. Meredith W, *et al.* "Influence of biodegradation on crude oil acidity and carboxylic acid composition". *Organic Geochemistry* 31.11 (2000): 1059-1073.
40. Huu NB, *et al.* "Marinobacter aquaeolei sp. nov. A halophilic bacterium isolated from a Vietnamese oil producing well". *International Journal of Systematic Bacteriology* 49.2 (1999): 367-375.
41. Cheng L, *et al.* "Progressive degradation of crude oil n-alkanes coupled to methane production under mesophilic and thermophilic conditions". *PLOS ONE* 9.11 (2014): e113253.
42. Chunshuay L, *et al.* "Effects of temperature, acetate, nitrate on methane generation from petroleum hydrocarbons". *Scientific Research* 16.4 (2014): 24-31.
43. Sherry A, *et al.* "Volatile hydrocarbons inhibit methanogenic crude oil degradation". *Frontiers in Microbiology* 5 (2014): 131.
44. Okoro CC, *et al.* "Methanogen Population of Oil Production Skimmer Pit and the Effects of Environmental factors on Methanogenesis". *Microbial Ecology* 72.1 (2016): 175-184.
45. Zengler K, *et al.* "Methane formation from long chain alkanes by anaerobic microorganisms". *Nature* 401.6750 (1999): 266-269.

Volume 1 Issue 5 May 2018

© All rights are reserved by Okoro Chuma Conlette.