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Development of biological process for Kuwait crude oil contaminated soil

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ABSTRACT

Seven hundred ninety-eight oil wells were set fire, damaged, and gushed oil and resulted on a crude oil contaminated area over 300 km^2 include dry oil lakes at the end of Iraqi war. The United Nation Compensation Commission (UNCC) recommended remediation of crude oil contaminated soil as physical chemical and thermal processes due to the oil sludge and high concentration of TPH. In this study, indigenous oil-emulsifying bacteria in Kuwait crude oil contaminated soil was cultured via enrichment culture method. Bacterial composition of enrichment culture investigated by phylogenetic analysis of 16S rRNA gene sequences obtained through high-throughput sequencing. The majority of enriched bacteria belonged to the order of *Flavobacteriales* (56.38%), *Burkholderiales* (16.13%), and *Pseudomonadales* (12.22%). Using those indigenous microorganisms, biological process consists of bio-washing, biocatalytic, and biopile was operated for 20 days in lab-scale. Initial TPH concentrations measured by hexane extraction method (HEM) and GC-FID method were 46,096 mg/kg and 34,153 mg/kg, respectively. Removal rates of TPH after 20days operation were 68% (HEM) and 86% (GC-FID). Therefore, Kuwait crude oil contaminated soil can be remediated by combine biological process such as bio-washing and biopile process.

INTRODUCTION

Kuwait's oil wells were damaged and set fire during the Iraqi aggression in August 1990 and February 1991. It was the worst environmental pollution and ecological disaster recorded in the world as a result of oil spills and fire. In the Gulf War, over 700 wells were damaged and the amount of oil released to inland as well as coastline was about 25 million barrels, which is 40

times that of the Exxon Valdez oil spill in Alaska (Tawfiq and Olsen, 1993; Aisha-Al-Barood, 2015).

The Kuwait desert was affected by crude oil accumulations in shallow depressions forming over 300 oil lakes with an overall area of about 49 km^2 in the oil forming dry oil lakes. Crude oil from the oil lakes penetrated into soil 2 m below the oil lakes (Al-Awadhi et. al., 1995). After the Gulf War, United Nation Compensation Commission (UNCC), Kuwait National Focal Point (KNEP), Kuwait University (KU), and Kuwait Oil Company (KOC) carried out joint research for contaminated soil remediation of Phase I and Phase II (Al-Awadhi et. al., 1995). In the dry oil lakes, the crude oil components were transformed by weathering due to exposure to sunlight with high temperatures over 25 years. Crude oil had penetrated into the soil down to 1 m on average of which the top 30cm soil was found to be oily sludge like oil ball with over 40% Total Petroleum Hydrocarbon (TPH). Below the oily sludge layer, the TPH concentration was 20% or less.

They recommended physical/chemical remediation technologies such as solvent extraction and thermal processes for soils with over 40% TPH and bioremediation technologies such as land farming, windrow composting, and bioventing process for soil with less than 5% TPH. The efficient cleaning technologies for the crude oil contaminated soil are bioremediation approaches because microorganisms that can degrade a variety of petroleum hydrocarbon compounds, including aliphatic, aromatic alkanes, and PAHs, exist widely. Balba et. al (1998) found that up to 82.5% reduction in the TPH after 12 months under the optimum C:N:P ratio with compost and wood chips as carbons sources and at water holding capacity in the landfarming, windrow composting pile, and bioventing pile processes. Thus, bioremediation of crude oil contaminated soil could be applied to the contaminated fields with suitable nutrients, moisture, and oxygen.

Recently, oxidation of PAHs in the PAH contaminated soil using the catalytic reaction of hemoglobin and hydrogen peroxide has rapidly degraded 60% of PAHs within 1 day and 98% after 42 days. Oxidation of iron in heme protein with hydrogen peroxide has been reported for contaminated soil with hazardous organic compounds such as PAHs, PCP, and TPH (Kang, et. al., 2015; Jho et. al., 2016). They suggested that residual hemoglobin may be used as a primary carbon source and enhanced biodegradation of PAHs like co-oxidation reaction (Kang, et. al., 2015; Jho et. al., 2016).

The objective of this research was to evaluate the capability of biowashing, catalytic reaction between hemoglobin and hydrogen peroxide and biopile process for remediation of Kuwait crude oil contaminated soil.

MATERIAL AND METHODS:

Soil and chemicals

Crude oil contaminated soil with 4 to 7% TPH and <1% moisture contents from the Burgan oil contaminated field was obtained by Kuwait Oil Company (KOC), and placed in 4^{o}C refrigerator until experimental set up. The soil sample was loamy sand with 81% sand, <1% clay, and 18% silt, which was determined after crude oil removal by hexane since the soil was highly contaminated with crude oil.

Hemoglobin powder donated from the Shenzhen Taier Biotechnology (P. R. China) was dissolved in 50 mM phosphate buffer (pH 7.0). Hydrogen peroxide was purchased (Samchun Pure Chemicals, Korea) and prepared by diluting to 34% H_2O_2.

Phosphate buffer solution was prepared by combining potassium monobasic phosphate and potassium dibasic phosphate (Samchun Pure Chemicals, Korea). Potassium hydroxide (Samchun Pure Chemicals, Korea) was used for CO_2 trap solution in the reactor system.

Analytical methods

Total hexane extractable matter (HEM) was determined by extracting the soil, using hexane solvent and soxhlet apparatus. The extracted solvent was then evaporated by a N concentrator (Turbo Vap II, Biotage, USA) and the weight was measured (US EPA, 1986) at the beginning of treatment and every 4 days for 20 days. After measuring HEM, extracted matter was dissolved in hexane, cleaned up on Isolute EPH column (25mL, Biotage, USA), and analyzed by GC/FID (Agilent 6890N, USA) for TPH

Phylogenetic structure of *Bacteria* in enrichment culture

The V5-8 region of the 16S rRNA gene was amplified using 787F-1392R primers, which liked with a Roche 454 pyrosequencing adapter and barcode sequences. PCR reaction was performed in reaction mixture (24 μl) containing 20ng of template DNA, 0.5 μl of dNTP, 1μl (0.4 μM) of each fused primer, 2.5 μl of FastStart 10 buffer, 0.25 μl (5 unit/μl) of FastStart HiFi polymerase, and molecular biology grade water. PCR condition was as follows: initial denaturation (94^{o}C) for 3min, 35 cycle of denaturation (94^{o}C) for 15sec, primer annealing (55^{o}C) for 45sec, and final extension (72^{o}C) for 1min. Amplicon library was prepared following to standard protocol (Roche). Amplicons were purified by AMPure beads clean up method and quantification of amplicons was performed by fluorimeter using Quant-iT Pico-Green (Life Technologies). Emulsion PCR was performed using sequencing beads and recovered beads were put into a 454 Pico Titer Plate and sequences were analyzed with a Roche 454 GS FLX plus (Roche diagnostics, Basel, Switzerland) following the manufacturer's instructions. Sequences, of which quality score was lower than 20 (window size 35) with its length of > 200 bp, were removed

using Pipeline Initial Process provided on the Ribosomal Database Project website (http://rdp.cme.msu.edu/). Chimeric sequences were also sorted out using USEARCH (ver. 6.0) program in FunGene Pipeline website (http://fungene.cme.msu.edu). Taxonomic classifications of sequences were obtained from RDP classifier and data was visualized by using MEGAN (MetaGenome ANalyzer) program.

Abundance of *Bacteria*

Community DNAs were extracted from the samples by using a PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer's protocol. Abundance of *Bacteria* was estimated by using a real-time PCR assay with bacterial universal primer set (EUB338F, and BAC515R). SYBR Premix Ex Taq reagent (Takara, Shiga, Japan) was used for the real-time PCR. SYBR Green I and ROX were used as reporter and passive reference dyes, respectively. Reactions were carried out in MicroAmp optical eight-tube strips (Applied Biosystems, Foster City, CA, USA) by using a ABI Prism 7300 sequence detection system (Applied Biosystems). Each reaction contained 2 μl of template DNA, 25 μl of SYBR Premix Ex Taq, 1 μl of ROX dye, 1 μl (20 pmol) of each primer, and sterile water to bring the total reaction volume to 50 μl. Thermal cycling parameters were as follows: initial denaturation (95 °C) for 5 min, followed by 35 cycles of denaturation (95 °C) for 30 s, primer annealing (55 °C) for 30 s, and final extension (72 °C) for 30 s. Real-time PCR amplifications were performed in triplicate. The collected fluorescence signals were analyzed by using ABI Sequence Detection Software version 1.4 (Applied Biosystems). The fluorescence signal intensity of the reporter dye was normalized by using the signal intensity of the passive reference dye to correct for fluctuations in the fluorescence signal due to the changes in the concentration and volume of the reaction mixture. The threshold cycle (C_T) was taken to be the PCR cycle at which a significant increase in the normalized fluorescence signal was first detected. The threshold level was determined automatically by the detection system, using the default settings. Standard curve constructed in our preliminary study (Hong and Cho, 2014) was used for determining the copy numbers of 16S rRNA gene sequences of *Bacteria*.

Micro reactor and biopile reactor

A micro reactor was fabricated from a 250 mL Erlenmeyer flask with a side arm joined to a tube for trapping CO_2 as shown in Fig. 1. An ascarite (Sigma-Aldrich, Korea) trap was set up on the top of the Erlenmeyer flask. The ascarite trap removed CO_2 in the air when air was supplied to the reactor. The side arm tube for trapping CO_2 was placed with 12 mL of 0.01M KOH solution every day. A schematic of the biopile bioreactor is shown in Fig. 2.

Experimental procedures

Ten grams of Kuwait crude oil contaminated soil was placed in the Micro reactor with 4 mm glass balls for enhanced mixing efficiency. The harvested cultures, 30 mL, were placed in the reactor and four replicates micro reactors were set up. These micro reactors were shaken on the shaker table at 120 rpm at 30^{o}C (VS-8480SF, Vision Scientific Co., Korea) after adding 12mL 0.01M KOH solution in the side arm tube during the 4 day-biowashing process.

After biowashing, soil was removed from the micro reactor and the supernatant was removed. All of soils were homogeneously mixed, 10 g soil sample for analysis of TPH and PAHs. After mixing 0.3 g hemoglobin and 0.9 g of hydrogen peroxide were added immediately to the biopile reactor to evaluate the catalytic and biological processes.

The combined biological process was operated for 20 days with the following sequence; 4 days biowashing, 4 days biocatalytic, and 8 days biopile process. At the end of each process, a 10 g soil sample was collected for analysis of TPH and microorganisms.

RESULTS AND DISCUSSION:

The microbial consortium was enriched in a 3 L bioreactor with hemoglobin (0.1 g) as a specific nutrient for bio-surfactant producing bacteria. The effect of the hemoglobin in stimulation of bio-surfactant producers was already demonstrated in our preliminary study. The bioreactor was equipped with an impeller and an air diffuser for supplying air to microbial community. After 3 months, bacterial 16S rRNA gene amplicons in the enrichment culture were obtained by high-throughput sequencing. A total of 8,144 sequences of bacterial 16S rRNA gene were analyzed and 3,455 sequences were removed after quality filter and chimera check process. The quality filtered sequences (3,257) were subjected to the RDP classifier to determine phylogenetic affiliation. The majority (66.6%) of 16S rRNA gene sequences was phylogenetically assigned to genus *Chryseobacterium*, which belongs to family *Flavobacteriaceae* and the remaining sequences were affiliated to 12 different genera (Fig. 3). Species belonging to the genus *Alcaligenes*, *Acinetobacter*, *Pseudomonas*, and *Stenotrophomonas* were reported as bio-surfactant and bio-emulsifier producers. The bacterial diversity of the enrichment culture indicated that the surface activity derived from various types of bio-surfactants and bio-emulsifiers. Rhamnolipids, the most studied bio-surfactant, is low molecular weight glycolipid produced by *Pseudomonas aeruginosa*, and have been applied to several bioremediation technologies (Rahman et al, 2002). Production of polymeric bio-surfactants (i.e., high molecular weight bio-emulsifiers) by *Pseudomonas nautica* in marine habitats was also observed (Husain, 1997). Species belonging to the genus *Alcaligenes* and *Stenotrophomonas* are also known as glycolipid type bio-surfactant producers and species of the genus *Acinetobacter* was reported as bio-emulsifier producers in previous studies (Maneerat, 2005; Ron and Rosenberg, 2001; Kaplan et al, 1987; Navon-venezia et al, 1995; Walzer et al, 2006). Recently, a novel biosurfactant was identified using metagenomics approaches to the

microbial community in the slaughterhouse biofilm, which was dominated by the genus *Chryseobacterium* and *Flavobacterium* (Thies et al, 2016). In addition, the production of lipopeptide type bio-surfactant by *Lysinibacillus chungkukjangi* isolated from a rice bran oil sludge was reported. Although identification of bio-surfactant like molecules present in the supernatant of the culture of the *Brevundimonas diminuta* was incomplete, successive bioremediation of marine oil pollution was achieved using *Brevundimonas diminuta* culture (Wang et al, 2015). To determine the surface activity of the enrichment culture and the species responsible for the surface activity, isolation and species-level identification of each microorganism in the enrichment culture will be required in the future study.

The concentration of accumulated CO_2 is shown in Fig. 4 during the combined biowashing, biocatalytic, and biopile processes for the feasibility biological process. Biowashing was carried out for 4 days and the average amount of CO_2 generated was 140 mg/L. During the biocatalytic reaction with hemoglobin and H_2O_2, the CO_2 generated during Day 1 increased to the maximum value of 555 mg/L, and then decreased after Day 1 to reach 49 mg/L on Day 19. During this process, air was supplied at 50 mL/min for 5 min every day, as air seems to be a limiting factor for the biological degradation. Thus, air was supplied for 24 h on Day 19 and the CO_2 generated for 1 day was captured. The CO_2 concentration increased about 10 times to 488 mg/L. During the TPH degradation process, the Kuwait crude oil contaminated soil was mineralized to CO_2 which generated 4,460mg/L of accumulated CO_2 for 20 days.

The initial THP concentration of Kuwait crude oil contaminated soil was 46,096 mg/kg and 34,153±368 mg/kg by HEM and GC-FID analysis, respectively. HEM and GC-FID are shown in Fig. 5. Fig. 6 presents that individual TPH (C_8 to C_{40}) concentration at the end of biowashing, biocatalytic, and biopile processes. During the combined three processes, soil TPH concentration

decreased by 66% and 86% by HEM and GC-FID analysis, respectively. Most of TPH (75% removal) was removed during the biowashing process and the TPH concentration was 8,620 mg/kg. During the biocatalytic process, 21% TPH was removed. During the biopile process, 30% TPH was removed in 12 days. With the HEM analysis, the TPH removals were 62%, 10.2%, and 6.2% for the biowashing, biocatalytic, and biopile processes, respectively. Fig. 7 presents the observed individual THP removal for biowashing, biocatalytic, and biopile processes. The results show that most of the individual TPH concentrations were below 20 mg/kg after biowashing process. Overall, we suggest that hemoglobin can enhance the bioremediation for Kuwait crude oil contaminated soil because biosurfactant generation during the biowashing process with hemoglobin as a primary carbon source promotes release of crude oil from soil and enhance solubility of contaminants by emulsification. This showed that the highest TPH removal efficiency was observed in the biowashing process with hemoglobin.

Bacterial abundance was calculated from 16S rRNA gene copy numbers in the processes using real-time PCR analysis (Fig. 7). In Kuwait crude oil contaminated soil, bacterial 16S rRNA gene copy number was 3.9×10^5 copy number/g soil, which was slightly lower than that of soil environments reported previously (10^8 - 10^{11} 16S rRNA gene copy number/g soil). Bacterial abundance was increased during the processes due to elevation of water level and hemoglobin supplement. Bacterial 16S rRNA gene copy numbers of the soils from biowashing step, biocatalytic step, and biopile step were 5.1×10^6, 3.5×10^7, and 3.7×10^7, respectively. The increase of bacterial abundance in the soil sample after the process and the TPH removal rates indicated that biowashing process using biosurfactant producing microbial community and hemoglobin addition enhanced biological and catalytic degradation of crude oil in Kuwait soil.

CONCLUSION:

In this study, biological processes were studied to evaluate the effectiveness of (1) soil washing with hemoglobin, (2) biocatalytic process with hemoglobin and hydrogen peroxide, and (3) biopile process for TPH removal.

The indigenous oil-emulsifying bacteria in the Kuwait crude oil contaminated soil was cultured via the enrichment culture method with added hemoglobin as a primary carbon source. Three bacterial orders were classified by phylogenetic analysis of 16S rRNA gene sequences analyzed by a high-throughput sequencing technique. The majority of the enriched bacteria belonged to *Flavobacteriales* (56.38 %), *Burkholderiales* (16.13 %), and *Pseudomonadales* (12.22 %). Using those indigenous microorganisms, three biological processes were operated for 20 days, which has combined bio-washing, biocatalytic, and biopile processes in lab-scale experiments. The initial TPH concentrations were 46,096 mg/kg and 34,153 mg/kg, respectively, by hexane extraction method (HEM) and GC-FID method. After 20 days operation, 86% TPH was removed by GC-FID method and 68% by HEM. Therefore, the Kuwait crude oil contaminated soil can be remediated by combined biological processes such as bio-washing and biopile process.

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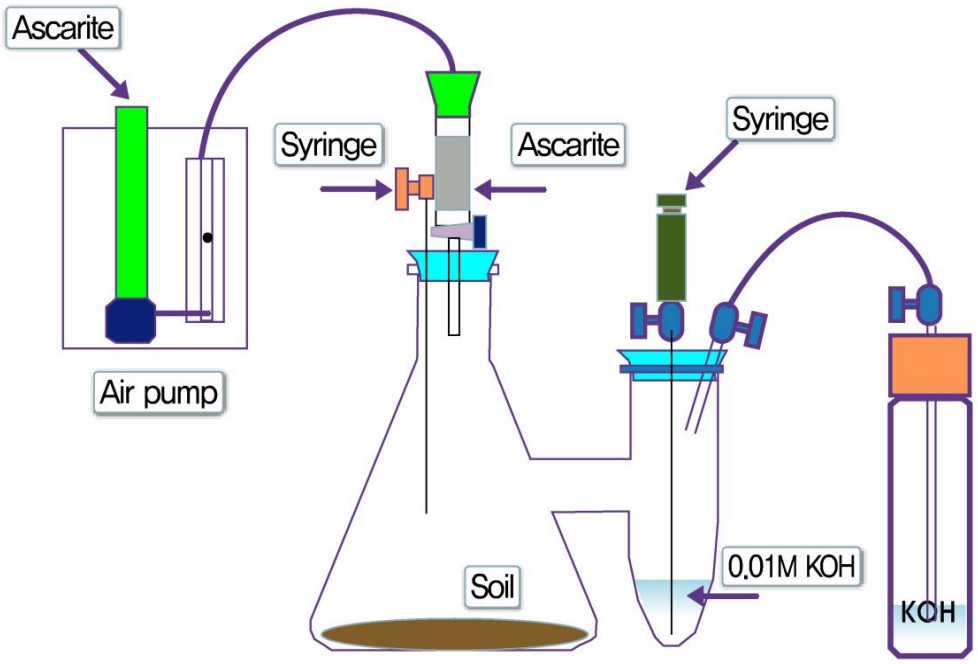

Fig. 1. Micro reactor diagram for trapping CO_2.

Fig. 2. Schematic diagram of the biopile process.

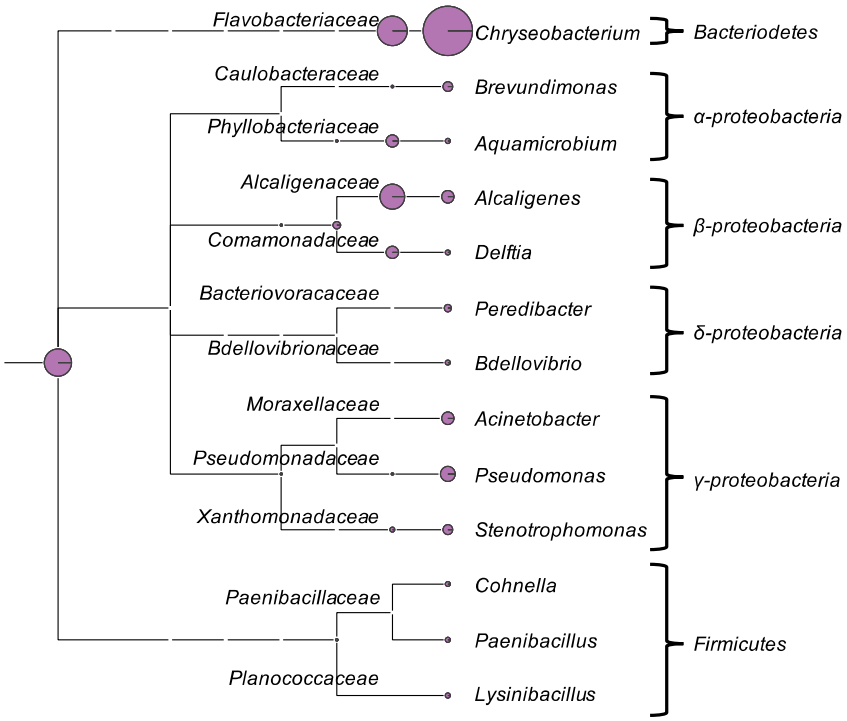

Fig. 3. Bacterial community structure in enrichment culture was investigated using high-throughput sequencing method. Relative abundances of bacterial 16S rRNA gene sequences affiliated phylogenetically are shown in size of the pie at the end and middle of the branch.

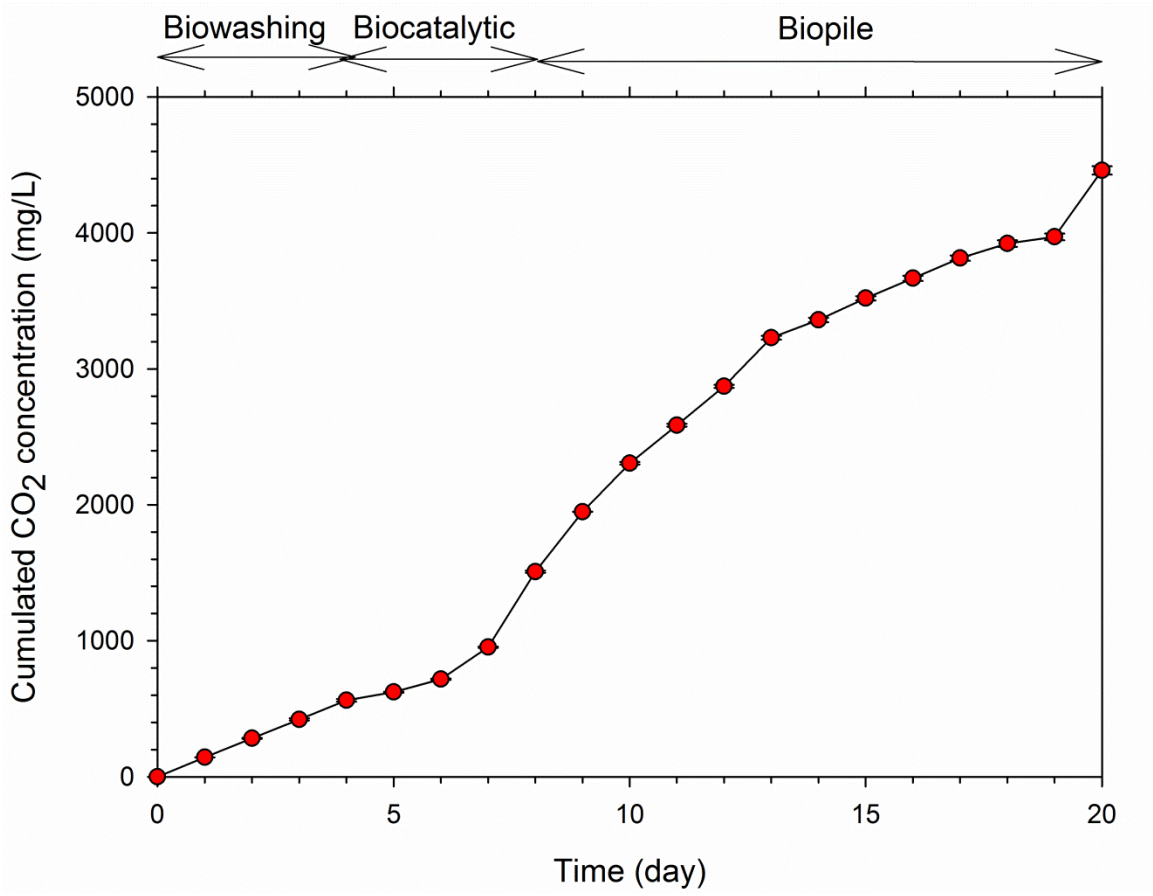

Fig. 4. Cumulated CO_2 concentrations vs. time.

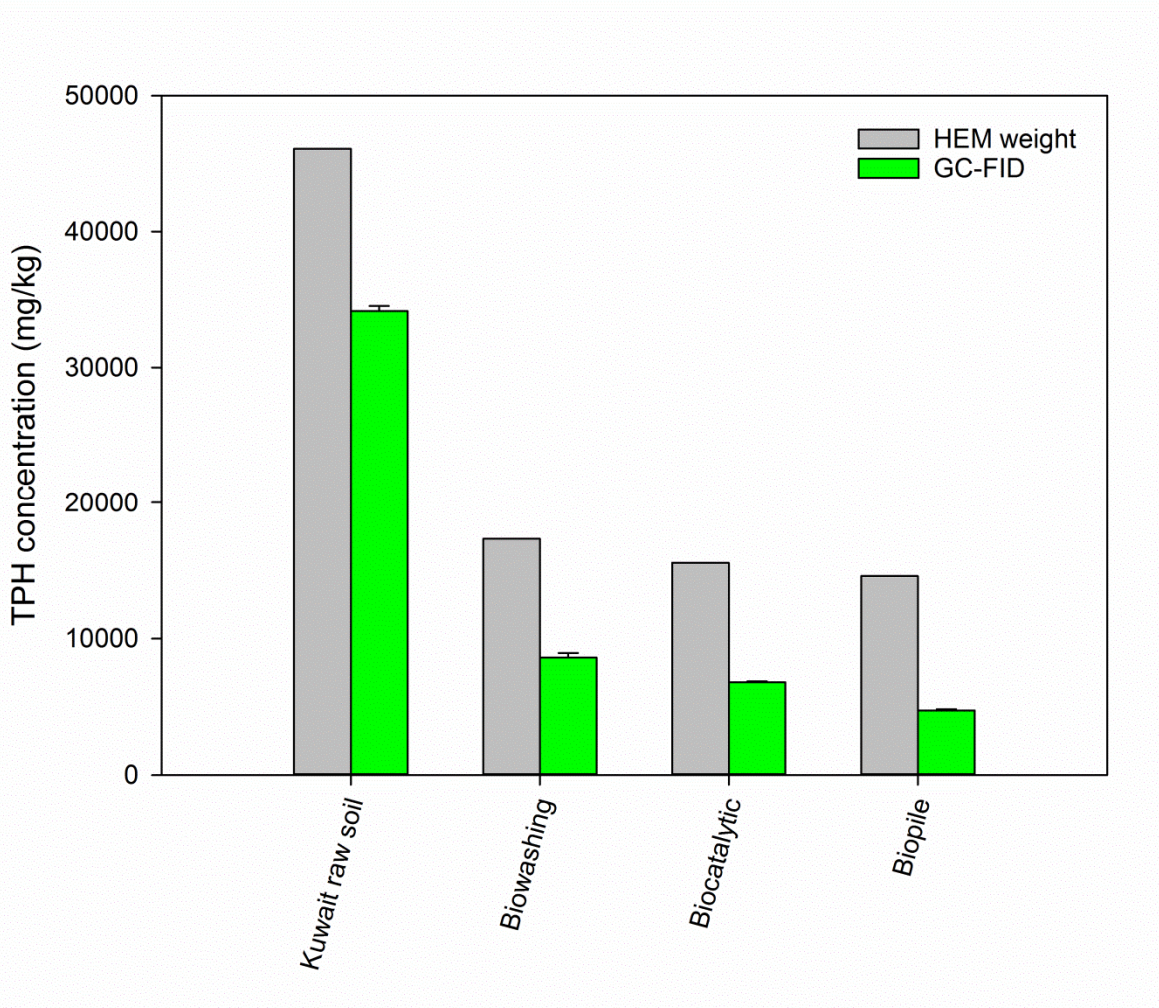

Fig. 5. Residual TPH concentrations consecutive three biological process such as biowashing, biocatalytic, and biopile process.

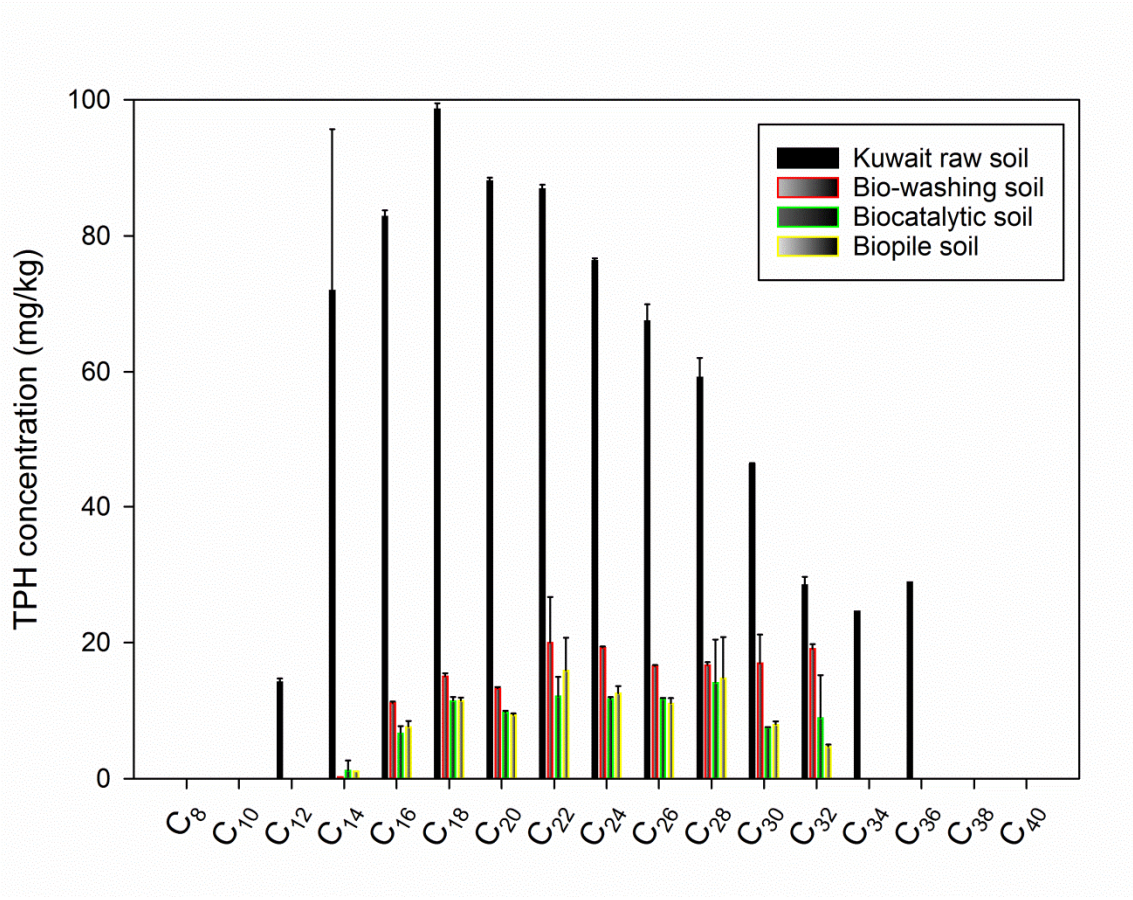

Fig. 6. Individual THP concentration as C8 to C40 at end of each process.

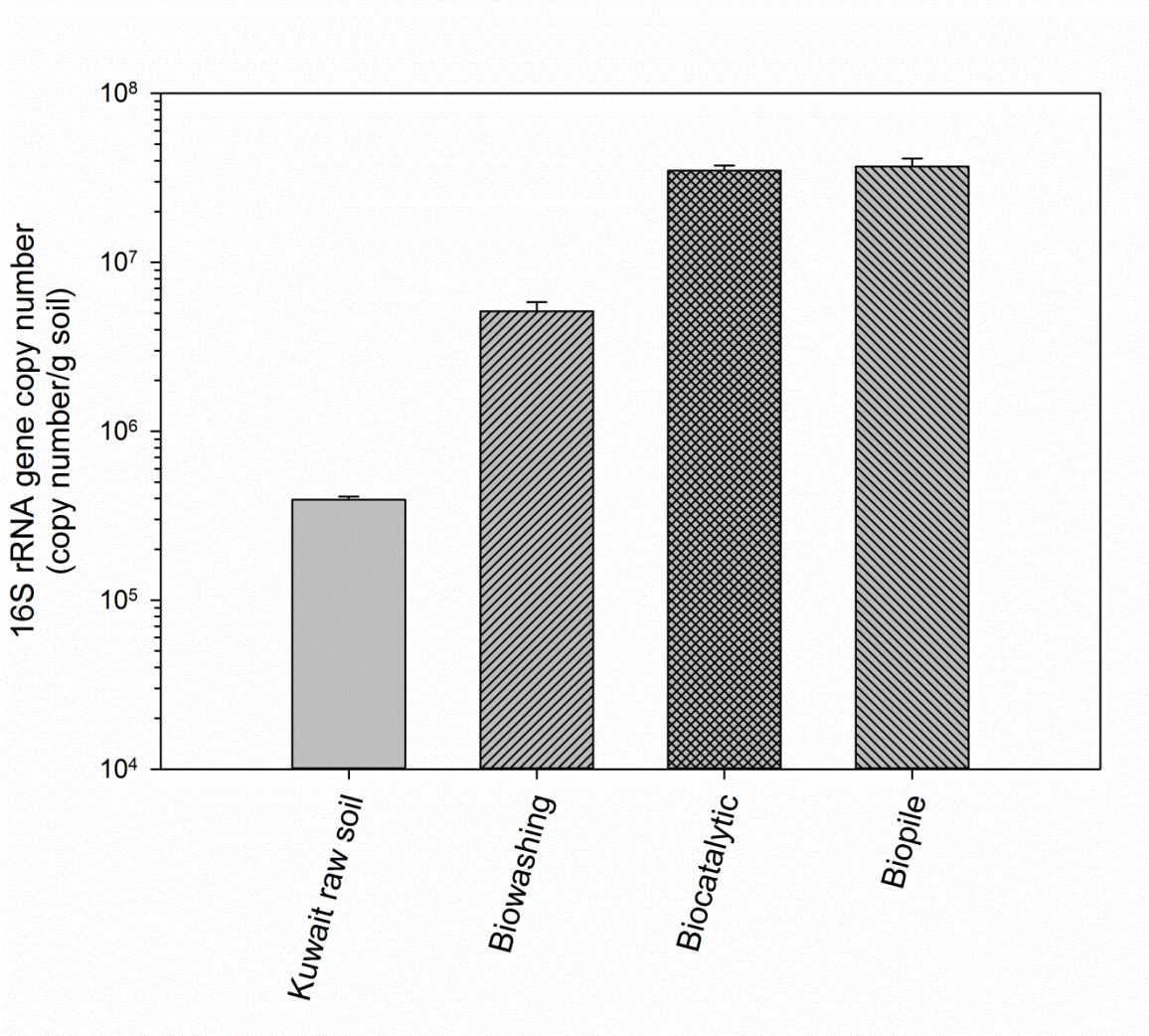

Fig. 7. Bacterial 16S rRNA gene copy numbers in different biological processes.