International Biodeterioration & Biodegradation 132 (2018) 216–225

Contents lists available at ScienceDirect

International Biodeterioration & Biodegradation

journal homepage: www.elsevier.com/locate/ibiod

Biosurfactant enhanced soil bioremediation of petroleum hydrocarbons: Design of experiments (DOE) based system optimization and phospholipid fatty acid (PLFA) based microbial community analysis

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ARTICLE INFO

Keywords:
Enhanced biostimulation
Rhamnolipids
Hydrocarbon degradation
PLFA analysis
Microbial community

ABSTRACT

The microbial mechanisms in nutrient and biosurfactant enhanced soil bioremediation of petroleum hydrocarbons (PHCs) were investigated. A systematic factorial design was conducted and a response surface reduced quadratic model was developed to determine the effects of nutrients and two surfactants (i.e., rhamnolipids and Tween 80) in the 36-day PHC degradation. A significant effect was observed from nutrient addition and a 92.3% removal of PHCs was achieved by applying rhamnolipids at a concentration of 150 mg/kg and nutrient solution at 1000 μL. Rhamnolipids promoted higher metabolic activities of indigenous soil microorganisms to assimilate hydrocarbons than Tween 80 during the kinetic investigation. Phospholipid fatty acid (PLFA) analysis indicated three different microbial transformation patterns were observed when the soils were treated by natural attenuation, enhanced by rhamnolipids and Tween 80, respectively. As indicated by PLFA biomarkers of the Gram-negative bacterial populations (cy17:0, cy19:0, 16:1ω7c and 18:1ω7c), Gram-negative bacteria are closely correlated with the amount of total soil biomass and are the sources of hydrocarbon degraders. PLFA compositional characteristics were also used to evaluate the physiological status of the indigenous microorganisms and elucidate biodegradation mechanisms linked to different soil treatments.

1. Introduction

Petroleum hydrocarbons (PHCs) are widespread environmental pollutants in soil habitats and have raised global concerns due to their negative impacts on all forms of life (Li et al., 2007; Obbard et al., 2004). There are many ways of PHC contamination caused by various anthropogenic activities, which include inappropriate transportation processes, oil wells leakages, improper disposal of petroleum wastes and accidental oil spills (Moldes et al., 2011). Although numerous remediation technologies for treating PHC contaminated soils have been proposed, bioremediation is an attractive and promising approach because of its advantages in simple maintenance, low cost and environmental impact, as well as applicability over large areas and a wide variety of organic contaminants (Philp et al., 2005).

During bioremediation, PHCs will possibly serve as organic carbon sources in most environments, which leads to an enrichment of oil-degrading microbial populations (Margesin et al., 2000). The stimulation of indigenous oil-degrading microorganisms is often beneficial and the appropriate addition of nitrogen (N) and phosphorous (P) based-nutrients will readily improve degradation competence of the native microbial consortia. A concern of biodegradation to be stressed out is the bioavailability issue when PHCs exist the deep pores of soils and are poorly accessible for hydrocarbon degraders. During such circumstances, microbial populations can produce oil dispersive compounds like biosurfactants (BS) that could accelerate the degradation of toxic compounds (Pacwa-Płociniczak et al., 2014; Ron and Rosenberg, 2002). In this regard, bioremediation enhanced by direct supplementation of BS exhibits great potential in improving the mobility and bioavailability of PHCs and subsequent biodegradation.

BS are functional amphiphilic compounds produced by a variety of microorganisms, either on the cell surface or secreted extra-cellularly, that reduce surface and interfacial tensions (Cai et al., 2014). BS may enhance PHC bioremediation either by increasing the nutrient availability for microorganisms or by active interaction with the cell surface to increase the hydrophobicity of the surface, allowing hydrophobic substrates to bind more easily to bacterial cells (Dias et al., 2012;

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https://doi.org/10.1016/j.ibiod.2018.04.009
Received 18 January 2018; Received in revised form 25 March 2018; Accepted 14 April 2018
Available online 30 April 2018
0964-8305/

Rahman et al., 2003). Although many studies have investigated the effects of nutrient and BS addition on PHC degradation (Cameotra and Singh, 2008; Nikolopoulou et al., 2013; Szulc et al., 2014), the factors were usually investigated through a simple one-factor-at-a-time (OFAT) approach. The approach typically exhibits less accuracy in estimating the optimal conditions and provides limited information on factor interactions when compared with designed experiments (Vasilev et al., 2014). In addition, precise knowledge was still needed to study the long-term and short-term effects of chemical surfactants and BS in enhancing soil remediation (Makkar and Rockne, 2003). Thus, a systematical design with factors including the type and dose of surfactant coupled with nutrient in PHC bioremediation will provide interesting information to elucidate the interaction of the factors and the fate of contaminants.

Soil microbial communities play an important role in the biodegradation of PHCs. The contaminants together with the addition of nutrients and BS will significantly influence the composition and activities of indigenous microorganisms in soil, thus selecting for microbes that survive and function under those conditions (Mrozik and Piotrowska-Seget, 2010). Natural soil microorganisms are very sensitive to any ecosystem perturbation (Nikolopoulou et al., 2013), and their rapid alteration in structure and biomass is considered as best indicators of soil pollution (Wang et al., 2016). Knowledge of microbial communities in overall biodegradation process is important for determining the potential for bioremediation, transformation, or persistence of pollutants (Pratt et al., 2012; Wang et al., 2013). However, there is a lack of message of microbial community and the associated biological mechanisms during the BS enhanced bioremediation.

Phospholipid fatty acid (PLFA) analysis, a rapid, inexpensive, sensitive, and reproducible tool for assessing soil structure (Frostegård et al., 2011; García-Orenes et al., 2013; Ławniczak et al., 2013), was used to evaluate the performance of microorganisms during PHC bioremediation. Specific PLFA patterns revealed robust information on microbial community structures, physiological and nutritional status, and the viable biomass of the microbial population in soil (Frostegård et al., 2011). PLFA analysis has been widely used to determine differences in microbial community structure on soil over various environmental factors, such as soil pore size (Ruamps et al., 2011), soil water availability (Ruamps et al., 2011), spatial patterns in marine sediments (Fischer et al., 2010) and spatial covariation in polluted soil (Torneman et al., 2008). It was also used in associated community composition studies on PAH contaminated riverbank sediment (Pratt et al., 2012), nutrient-stimulated (Margesin et al., 2007) and chemical surfactant-enhanced bioremediation of hydrocarbon-contaminated soil (Lai et al., 2009; Mair et al., 2013). Therefore, the PLFA approach is promising to be applied for the microbial community analysis and microbial dynamic investigation over biodegradation process, which are extremely limited in the literature.

The objective of this work was to investigate the microbial communities in PHC-contaminated soil supplemented with nutrients and a BS to track biodegradation mechanisms and provide potential biodegradable internal information through PLFA analysis. A chemical surfactant was also used for comparison purpose. Design of experiments (DOE) was applied to conduct the biostimulation under various conditions.

2. Materials and methods

2.1. Soil characterization

PHC impacted soil used throughout the experiments was collected from a farm that was a part of a petroleum supply storage system in the province of Newfoundland and Labrador, Canada. The average daily temperatures in the farm range from −18.1 °C (January) to 15.5 °C (July) and the average precipitation is 949 mm. The soil was characterized by the material of peat, fine-to-medium grained sand and discontinuous silt layers.

The moisture content of the soil was measured by a gravimetric method from 5 g sample, in which the crucible with samples was dried at 105 °C in the oven overnight until a constant weight was achieved. The dried samples in crucible were further placed into muffle-furnace at 550 °C for 4 h and the weight of the ignited sample was used to calculate soil organic matter content. The C/N ratio was obtained from measuring total carbon and nitrogen contents by the LECO TruSpec CN Determinator (LECO Corporation, St. Joseph, MI) after a drying process in the oven (105 °C for 24 h). A bench top pH meter (EL20, Mettler Toledo) was used to measure the soil pH in a mixture with a soil: water ratio of 1:2 (weight/volume). The electrical conductivity (EC) was measured by bench top EC meter (Orion Star A222 and A322, Thermo Scientific).

2.2. Experimental design and kinetic settings of PHC degradation

A general factorial design was used to systematically investigate the factors of surfactant type, surfactant concentration and nutrient concentration on biostimulation. Rhamnolipids and Tween 80 were used to represent BS and synthetic surfactant, respectively, and the concentrations (0, 50 and 150 mg/kg) applied to the soil were referred from previous studies (Nikolopoulou et al., 2013; Szulc et al., 2014). The nutrient solution containing $NaNO_3$ and K_2HPO_4 at 10 g/l and 2 g/L, respectively, was applied to the soil at three levels (0, 400 and 1000 μL for each run) to support the growth of hydrocarbon metabolizing bacteria (Leys et al., 2005; Moldes et al., 2011). PHCs of each run were analyzed after 0, 3, 8, 17, 26 and 36 days of incubation. Apart from the 108 general factorial runs ($2 \times 3 \times 3 \times 6$), an extra of 36 duplicates was set to test experimental stability and yield pure errors in the analysis of variance (ANOVA) test. For each run, a 30 g of soil was independently added to a semi-open beaker and was stirred weekly to maintain aerobic conditions at ambient temperature. The sterilized and non-sterilized control were set to investigate the degradation kinetic of PHCs. Experiments with increased amounts of rhamnolipids and nutrients were conducted to further investigate the degradation potential of the microorganisms. Other detailed experimental settings of PHC degradation can be found in Appendix B.

2.3. PHC analysis

Quantification of the PHCs in soils was conducted by gas chromatography/mass spectrometry (GC-MS). Each individual set in the experimental design with 30 g soil was employed as an independent sample to ensure the accuracy of the PHC determination. Soil samples were dried with anhydrous Na_2SO_4 and extracted three times with a 30 mL solvent of dichloromethane (DCM) and hexane (1:1) through intermittent ultra-sonication (15s on/15s off pulses). All the extraction solvents were filtrated, collected and then concentrated to 10 mL via rotary evaporator in 35 °C water bath. The surrogate hexadecane-d34 was used to examine the methodological recovery while the internal standard nonadecane-d40 was added in the final solution before GC-MS analysis.

The PHC solution was analyzed on a GC-MS system (Agilent 7890A GC system coupled with a 5975C MSD) interfaced with an Agilent 7693 auto-sampler. Data acquisition, processing and evaluation from the full scan mode (range 50–500 m/z) were carried out using Agilent Chem Station software Version 2.01. PHCs were separated on a 30 m × 250 μm (internal diameter, i.d.) × 0.25 μm DB-5MS UI fused silica capillary column. An electronic pressure control (9.07 psi) was utilized to maintain a constant carrier gas (Helium of ultrahigh purity) flow of 1.2 mL/min throughout the oven program. Sample injections (2 μL) were conducted using a split/splitless injector (single tapered inlet liner, pulsed-splitless mode) at 200 °C under a pulse pressure of 25 psi. The initial oven temperature was set to 55 °C, followed by a temperature ramp of 7 °C/min up to 265 °C, a ramp of 15 °C/min to 295 °C,

and finally to 300 °C with a running time of 40 min.

2.4. Microbial PLFA identification

Soil samples for PLFA analysis were collected on Day 0, 17 and 54 to indicate the initial, thriving and decaying status of microbial activities. Samples from non-sterilized control as well as those amended by Tween 80 and rhamnolipids with 400 μL nutrient solution added were subjected to PLFA profiling. PLFA analysis was conducted following a modified Bligh and Dyer extraction method (Fan et al., 2017). Phospholipid standard C19:0 PC (1,2-dinonadecanoyl-sn-glycero-3-phosphocholine; Avanti Polar Lipids) was spiked in the beginning to determine phospholipid recovery and internal standards 14:1ω5c and 21:0 were spiked before GC-MS quantitative analysis (Ziegler et al., 2013). Quantification and identification of fatty acid methyl esters (FAMEs) were referred from several standards: Bacterial Acid Methyl Esters CP Mixture, FIM-FAME-7 Mixture, 10Me16:0, and 16:1ω7t were from Matreya LLC (Pleasant Gap, Pennsylvania, USA); 18:1ω7t was from Sigma-Aldrich (Oakville, Ontario, Canada).

Certain PLFAs were used as biomarkers to track relative differences in the activities of broadly separated functional groups relevant to PHC degradation (Li et al., 2007; Zelles, 1999). The fatty acids i15:0, a15:0, 15:0, i16:0, 16:1ω7c, 17:0, i17:0, cy17:0, 18:1ω7c and cy19:0 were used to represent bacterial biomass (bacterial PLFAs), and 18:2ω6,9c was chosen to indicate fungal biomass (fungal PLFA). The PLFAs of bacterial origin were further classified as Gram-positive bacterial PLFAs (i-C15:0, a-C15:0, i-C16:0, and i-C17:0) and Gram-negative bacterial PLFAs (cy17:0, cy19:0, 16:1ω7c and 18:1ω7c). PLFAs not assigned as biomarkers were included in total PLFA yields (total biomass). The ratios of saturated (SAT) and monounsaturated (MONO) PLFAs (Gómez-Brandón et al., 2011; Moore-Kucera and Dick, 2008) in conjunction with (cy17:0 + cy19:0)/(16:1ω7c + 18:1ω7c) were used as indicators of physiological or nutritional stress in bacterial communities.

2.5. Statistical analysis

Triplicate samples (in PLFA analysis, PHC experimental controls and enhanced nutrient & rhamnolipid study) were prepared and analyzed to ensure the reproducibility of results, and the error bars in the plotted data stand for the standard deviations of the mean values of triplicate samples. Principal component analysis (PCA; SPSS 18.0) was conducted to analyze patterns of intercorrelations among variables of PLFA species measured. The PLFA data were orthogonally transformed into a new coordinate system and principal components (PCs) with the greatest variances (80.4% and 10.0%, respectively) were used as coordinates. Correlations between the measured parameters (surfactant type, surfactant concentration, nutrient concentration and time) were analyzed by Design-Expert 8.0. The abundance of PHCs was transformed to a natural logarithmic scale to reduce the effect of outlying observations. An ANOVA of the response surface reduced quadratic model was conducted to analyze significant differences among the means of each PHC abundance group. Differences were considered statistically significant at $p < 0.05$.

3. Results and discussion

3.1. Soil characterization

The soil texture, as well as the content of organic matter, moisture, C/N ratio, pH, EC, and the PHC concentrations, were given in Table S1 (supplementary material). The standard deviations from triplicate tests were all below 10%. The soil investigated was a mixture of clay, sand and silt with low permeability. The relatively high moisture of 26.6% was due to the influence of offshore wet weather and onshore runoff distribution. From the crucible tests, the organic matter accounted for 31.2% and its enrichment was attributed to the presence of peat in soil. Most agricultural soils of Atlantic Canada are naturally acidic (Harmsen et al., 2007) due to the severe leaching of elements (e.g. Ca, Mg) from the surface soil caused by high precipitation. The soil pH detected was in the range of 5.4–5.8, and the EC was 653.6 mS/cm with a C/N ratio about 16.4. The results of PHC analysis indicated the contamination was mainly attributed to petroleum diesel. The above-mentioned parameters entail appropriate set-up design factors to allow the sufficient development of the microbial population with robust enzymatic activities.

3.2. System optimization

Enhanced bioremediation is directly associated with two factors that may limit biodegradation of petroleum pollutants on site. One is the catabolic potential of microorganisms used for biodegradation and the other is the bioavailability of the contaminants. The effects of surfactant type, surfactant concentration and nutrient concentration on the removal of PHCs by indigenous microorganisms were involved in the time-based general factorial design. The ANOVA results from for PHC microbial remediation are presented in Table S2 (supplementary material). The Model F-value is as high as 161.85, which implies the model is significant and there is only a 0.01% chance that a “Model F-Value" this large could occur due to noise. The experimental data were fitted to a response surface reduced quadratic model to describe the weights of each factor on the system. The final equation in terms of coded factors was obtained as:

$$\text{Ln (PHCs concentration)} = 6.69+0.032\times A-0.035\times B-0.24\times C-0.89\times D+0.032\times A\times C+0.054\times A\times D-0.25\times C\times D+0.053\times C^2+0.10\times D^2$$

where A is surfactant type, B is surfactant concentration, C is nutrient and D is time. Detailed description of the response surface reduced quadratic model was provided in Appendix C.

According to our model, the lowest PHC concentration was 201.88 mg/kg (92.3% removal of PHCs), and it was achieved by applying rhamnolipids at a concentration of 150 mg/kg and nutrient solution at 1000 μL. Therefore, we initially concluded that the application of rhamnolipids exhibited better performance than Tween 80 in PHC degradation under sufficient nutrient conditions.

The biostimulation of indigenous soil microorganisms is influenced by a series of environmental factors such as nutrient availability, oxygen content, water, pH, and temperature (Dias et al., 2012). The results indicated the nutrient addition, which is of great importance to microbial assimilation and dissimilation processes (Maki et al., 2003), contributed significantly to the biodegradation of PHCs. Nitrogen (N) is found in all amino acids, proteins, and enzymes, while phosphorus (P) is involved in energy trapping and transfer as adenosine triphosphate. Specially, the nutrient or fertilizer application can be essential in some environments with insufficient nutrient levels. Our results of significant nutrient bioremediation enhancement indirectly indicate the soils are infertile due to the leaching of nutrients by high precipitation in Atlantic area (Harmsen et al., 2007). Walworth et al. (1997) evaluated the relationship between soil water content and microbial response to soil nitrogen (N) in petroleum-contaminated soils and mentioned ineffective nutrient stimulation of degradation can be correlated with low water content in soil. Soil texture and organic matter are the key factors in soil to determine soil water holding capacity. Moreover, soil organic matter rich in nutrients such as nitrogen (N), phosphorus (P) can be an important sink and source of nutrients (McMurtrie et al., 2001).

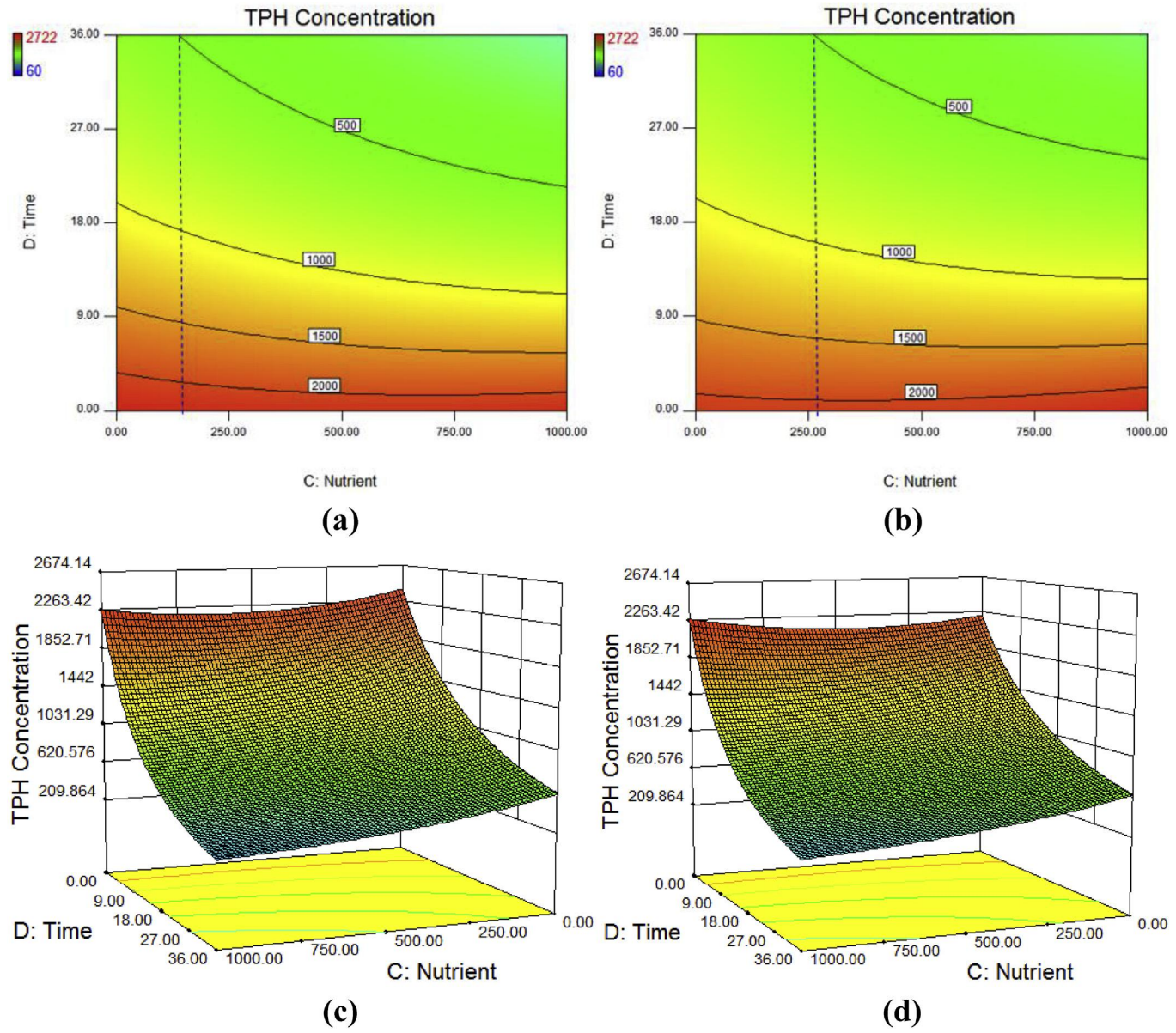

Fig. 1. Response surface plots depicting the interactions of independent variables (a) rhamnolipids at 75 mg/kg (b) Tween 80 at 75 mg/kg (c) rhamnolipids at 150 mg/kg (d) Tween 80 at at 150 mg/kg.

Consequently, adding appropriate organic (peat or muck) soils or other organic bulking agents (such as manure, vegetable wastes, etc) were recommended in practical large-scale remediation acts (Naseri et al., 2014) to change soil physical properties and increase soil organic matter.

The interactions of independent variables from the quadratic model were shown in Fig. 1. The results indicate that rhamnolipid treatment on contaminated soil facilitated the biodegradation more than the treatment of Tween 80 during the long-term investigation (Day 36) under same nutritional conditions (Fig. 1a and b). Less time or lower amount of nutrient is required to achieve the same degree of PHC degradation using the treatment of rhamnolipids than Tween 80, although the difference is not obvious during the initial 17 days. Response surface plots of Fig. 1c and d indicated that the concentrations of PHCs in soil decreased rapidly in the first stage (10 days), but the degradation rates of PHCs gradually slowed down after the period. Under the two circumstances, the nutrient concentrations were both found to be positively correlated with the PHC removal while the surfactant type only slightly affected the final PHC response. In general, a bit lower PHC level was obtained from the treatment of rhamnolipids than the identical treatment of Tween 80 on contaminated soil. The lowest predictions of PHC concentration from rhamnolipids and Tween 80 treatment on soil are 201.9 mg/kg (92.3% removal of PHCs) and 255.7 mg/kg (90.3% removal of PHCs), respectively. The lowest values are both obtained from a biodegradation of 36 days with 1000 μL nutrient solution and 150 mg/kg surfactant initially added. The biofriendly and biocompatible rhamnolipids better promoted metabolic activities of the population than Tween 80 in contaminant biodegradation.

3.3. Kinetics of enhanced biodegradation

Fig. 2 shows the kinetic behavior of PHC consumption during the bioremediation experiments after the supplementation of rhamnolipids (150 mg/kg) and nutrient (1000 μL solution) for 36 days. PHC concentration maintained in a relatively high value after the treatment of sterilization. However, a dramatic removal of PHCs was observed from all the different soil treatments in the initial 7 days. It's highly possible that soil PHCs volatilized quickly at an early stage (< 7 days), but the

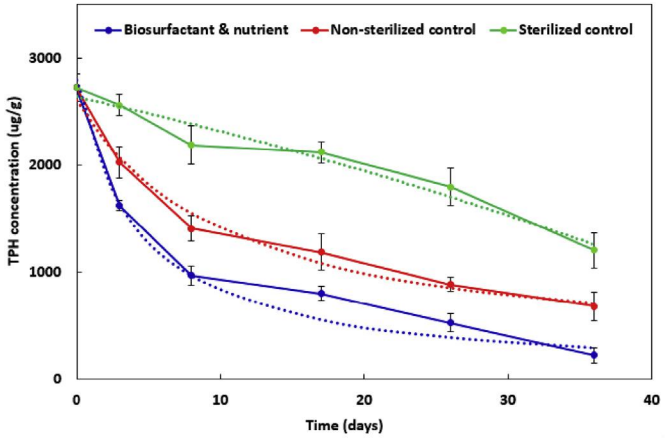

Fig. 2. Kinetics of the PHCs biodegradation in soil stimulated by rhamnolipids and nutrient. Data are presented by mean values of three replicates with standard deviations as errors. The equations of fitting curves for Sterilized control, Non-sterilized control and Biosurfactant & nutrient are $y = -0.2331x^2 - 29.891x + 2637.2$, $y = 2.2945x + \frac{29631.85}{x + 11.3884}$ and $y = \frac{11813.42}{x + 4.2284}$, respectively.

volatilization of PHCs was inhibited due to the appreciable PHC distribution below the surface and their deep binding to soil components (Clair et al., 2003). As revealed by the results, a different type of equation fitted the PHC concentration change under sterilized conditions. Once the hydrocarbons are present or absorbed in pores smaller than the size of microorganisms, the limited bioavailability restricts the PHC degradation (Clair et al., 2003). The results suggested that although a considerable 91.4% removal of PHCs (227.4 mg/kg at Day 36) was observed, certain hydrophobic contaminants can persist in the soil matrix for a long period of time (> 36 days). Apparently, during long-term residence in soil, organic contaminants can form stronger bonds with soil by adsorption and partitioning or can be incorporated in structural micropores (Moyo et al., 2014; Zhang et al., 2014).

In all the cases, the differences in biodegradation extent between natural/enhanced treatment and the sterilized control were always significant. The biodegradation occurred very fast once microorganisms adapted to the environment. Accordingly, the enhanced bioremediation by BS and nutrients will timely reduce the adverse environmental impact of pollutants, and possibly avoid any further movement or diffusion of pollutants to another matrix (Moldes et al., 2011). Similar results were achieved by Thavasi et al. (2011) during the bioremediation of contaminated sites with crude oil in laboratory scale microcosm experiment. During room temperature investigations, 75% of the crude oil was biodegraded in a period of 168 h and BS alone proved to be capable of promoting biodegradation to a large extent when soil moisture was below 100% (Thavasi et al., 2011).

In order to evaluate the microbial biodegradation potential, two batches of experiments were set while maintaining the highest levels of rhamnolipids or nutrient in general factorial design but doubling the amount of the other factor. The results in Fig. 3 showed the extra rhamnolipid addition beyond 150 mg/kg (300 mg/kg) accelerated the reducing of the final PHC concentration from 227.4 mg/kg (91.4% removal of PHCs) to 131.2 mg/kg (95.0% removal of PHCs). In contrast, no further increase in the extent of PHC biodegradation (238.4 mg/kg at Day 36) through increasing the nutrient concentration beyond 1000 μL (2000 μL) was observed. The results are in accordance with the conclusions proposed by Maki et al. (2003) and Singh and Lin (2009) that fertilization stimulated abilities of indigenous microorganisms mainly during the initial stages of degradation. In fact, excessive nutrient concentrations can also inhibit the biodegradation activity and the negative effects of high nutrient levels on the biodegradation of hydrocarbons were also reported (Chaillan et al., 2006; Chaîneau et al., 2005; Singh et al., 2014). An excess of nutrient amendment may have the potential to stimulate the activities of other microbes other than hydrocarbon-degrading microorganisms.

On the other hand, the increased amendment of rhamnolipids increased the assimilation of PHCs, especially after 17 days of incubation (Fig. 3). The observed further decrease of PHCs was attributed to the increment of the bioavailability of hydrophobic contaminants to hydrocarbon-degrading microorganisms. As stated above, although the application of rhamnolipids at 50 mg/kg and 150 mg/kg did not show overall obvious variance in the PHC removal within the study period, the further elevated addition of nutrient accelerated the assimilation of PHCs in each level of rhamnolipid application. Further analysis on degradation behavior of rhamnolipids and Tween 80 can be found in Appendix D.

3.4. PLFA profiles of soil microbial community

From the results of long-term hydrocarbon biodegradation, most of the PHCs were consumed within 54 days. The observed PHC concentrations from long-term treatments by natural attenuation, biostimulation by rhamnolipids and Tween 80 were 188.1 mg/kg, 34.6 mg/kg, and 34.5 mg/kg, respectively, which corresponded with 92.9%, 98.7%, and 98.7% of PHCs removal. Day 54 was thus used to represent microbial activities in the final stage of PHC biodegradation. According to all the PHC degradation results, PLFA patterns on Day 0, Day 17 and the end of the experiment (Day 54) were determined for the assessment and interpretation of microbial viability and vitality. The obtained PLFA profiles from different experimental runs could represent soil microbial community structure at the initial stage, the most active stage, and the last stage of PHC degradation, respectively. A total of 22 different PLFA biomarkers were identified from the soils contaminated by PHCs although their patterns varied under different soil treatments. Major fatty acids identified in the soil samples include 14:0, 16:0, 18:1ω9c, the cis and trans isomers of 16:1ω7, 18:1ω7 and two cyclopropane fatty acids, cy17:0 and cy19:0. Fatty acids i15:0 and a15:0 were also found in appreciable amounts from the unstimulated soil structures, and these PLFAs accounted for 77.6%–88.9% of the total PLFAs present in each sample. Compared with other studies, the distinguishing feature in the PLFA profiles was the significant coexistence of cyclopropyl PLFAs cy17:0 and cy19:0 with their monoenoic precursors of 16:1ω7 and 18:1ω7 (Li et al., 2007; Main et al., 2015). High amounts of cy17:0 and cy19:0 may result from the microbial stress response to harsh environments in the North Atlantic region. Small amounts of fatty acids with carbon numbers of 12, 13, 17, 18, and 20 as well as were also detected. To achieve a consistent and reliable interpretation, only 11 of the PLFA indicators were selected to represent bacterial or fungal groups in the soil (Frostegård et al., 2011; Wixon and Balser, 2013). For instance, 10Me16:0 was widely accepted as *Actinomycetes* in soil samples (Kong et al., 2011; Moore-Kucera and Dick, 2008), but it was also used as sulfate-reducing biomarker (*Desulfobacter*) in anaerobic environments (Córdova-Kreylos et al., 2006; Pratt et al., 2012). Fatty acid 18:1ω9c may serve as ambiguous indicators for fungal biomarker (Barreiro et al., 2015; Kong et al., 2011; Lazcano et al., 2013) or bacterial biomarker (Covino et al., 2016; Helfrich et al., 2015).

Overall, the total microbial biomass, estimated as total PLFAs, ranged from 27.0 to 40.0 nmol/g during the initial stage of soil remediation. The amounts of PLFAs reached an appreciable abundance of 45.3–52.0 nmol/g at Day 17, but varied to 12.0–52.1 nmol/g at Day 54 for all the samples with different treatments. The PCA performed with the whole PLFA data set of the soil samples collected at different sampling times (0, 17, and 54 days after the treatments) revealed that the PC1 and PC2 factors contained 80.4% and 10.0% of the total variance, respectively (Fig. 4). PC1 revealed major differences (80.4%) in the microbial communities and differentiated all the samples, especially for those time-oriented samples taken from natural attenuation and

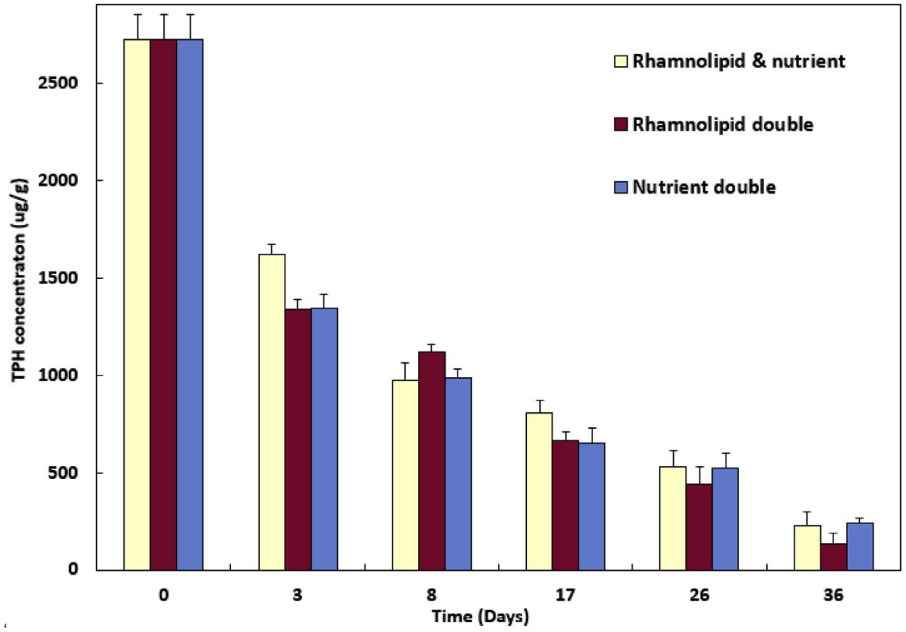

Fig. 3. The biodegradation behavior of PHC by the supplementation of increased amounts of rhamnolipids and nutrients to investigate the degradation potential of the microorganisms.

rhamnolipid enhanced soil remediation. It was observed that PLFA indicators 18:1ω7c, 16:1ω7, 16:1ω7t, cy17:0, and cy19:0 for Gram-negative bacteria, 18:2ω6,9c for as Fungi, as well as some general PLFAs 14:0, 16:0, 18:0 and 20:0 showed significant differences in PC1 loadings. These PLFAs are seen as major indicators to differentiate microbial structures among different PHC degradation stages. These PLFAs carrying positive loadings on Component 1 were present in greater abundance in all samples on Day 17 and in soil samples treated by Tween 80 on Day 54. Component 2 mainly separated samples taken from various periods of Tween 80-enhanced soil remediation. The major differences can be indicated by PLFAs, 18:1ω7c, cy17:0, i16:0, i15:0 and a15:0, which include both Gram-negative (18:1ω7c and cy17:0) and Gram-positive bacteria (i15:0, a15:0 and i16:0) indicators. Bacterial PLFA indicator 2OH 14:0 (Willers et al., 2015; Zaady et al.,

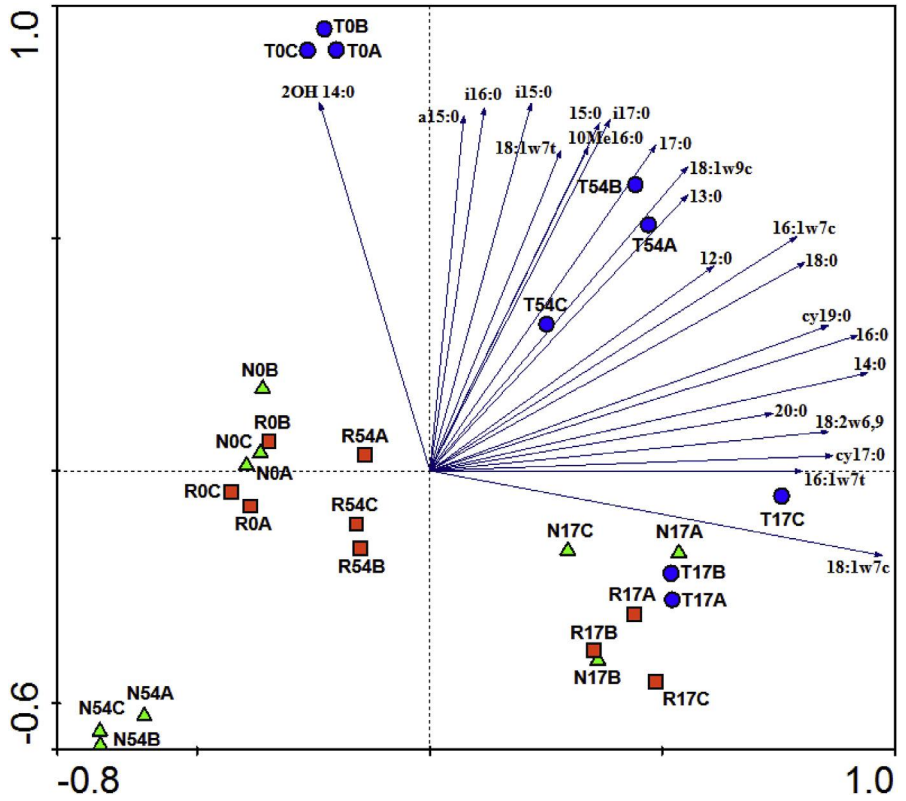

Fig. 4. Principal component analysis (PCA) plot of the soil microbial community structure with loadings of separate PLFAs along the sample distribution patterns. Samples are denoted by the treatments of soil (N for natural attenuation, R for rhamnolipids, and T for Tween 80) coupled with the sampling days.

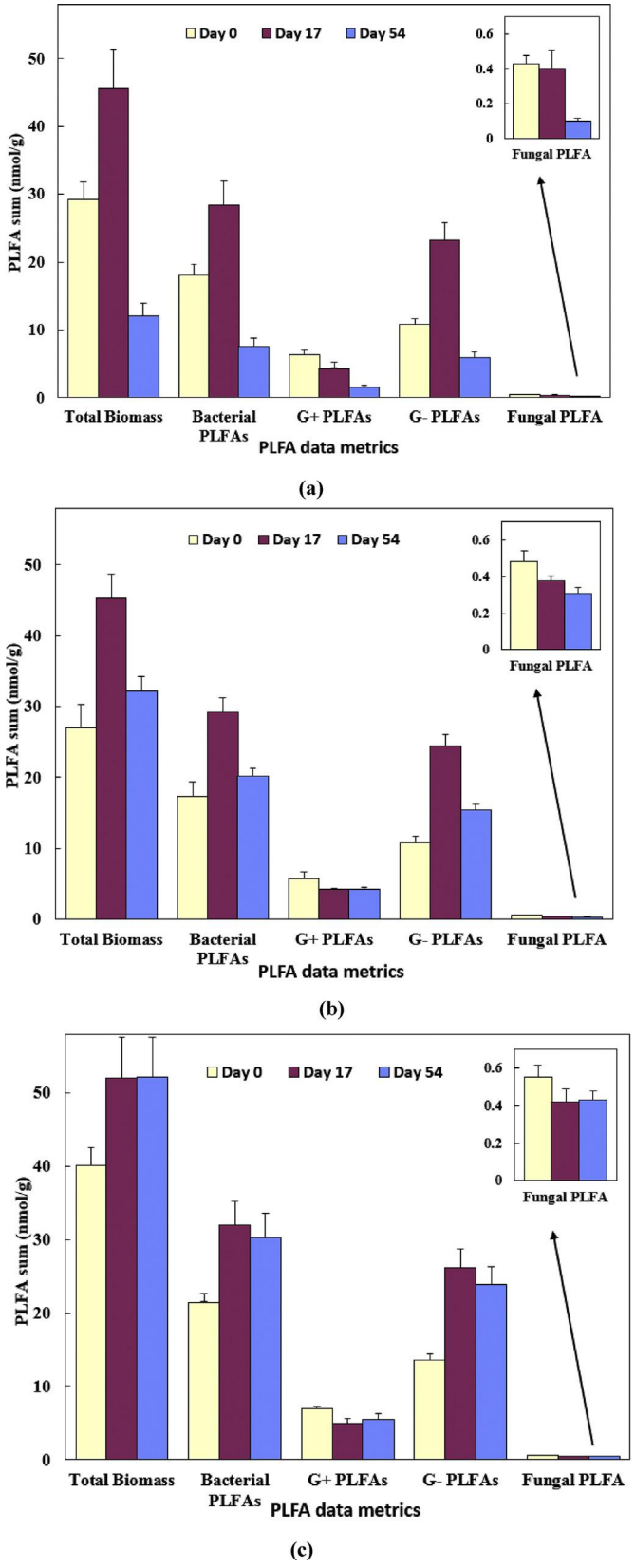

Fig. 5. Total PLFA biomass and sum of bacterial and fungal PLFA concentrations over the contaminated soil treated with natural attenuation (a) and biostimulation of rhamnolipids (b) and Tween 80 (c). Error bars are derived from standard deviations of samples (n = 3).

2010) and PLFAs characteristic of Gram-positive bacteria (i15:0, a15:0 and i16:0), which carried high positive loadings on Component 2, were enriched in soil samples treated by Tween 80 on Day 0.

3.5. Microbial community structure shifts and dynamic change

Amounts of PLFAs specific for total soil biomass, total bacterial biomass, Gram-positive and Gram-negative bacteria as well as soil fungi over the three sampling periods were shown in Fig. 5. Three different total biomass transformation patterns over the study period were observed when the soils were treated by natural attenuation, enhanced by rhamnolipids and Tween 80, respectively. The total soil biomass raised from the initial level to a higher level after 17 days and then it dramatically dropped to a much lower amount at the end in the process of natural attenuation. In contrast, the total soil biomass went through the similar trend to a high point after 17 days, but it eventually decreased to a level higher than the initial amount during the rhamnolipid-enhanced soil treatment. Interestingly, although the total PLFA amount from Tween 80-enhanced soil treatment exhibited the raising period like the other two, the microbial biomass maintained high yields as much as it was on Day 17. Consequently, it can be speculated that the soil employed in this study has microbial biomass not inhibited by the presence of PHCs and some indigenous heterotrophic microorganisms were stimulated by nutrient and surfactant injection to degrade PHCs.

Notably, bacterial PLFA biomass and PLFA amounts of Gram-negative bacteria were closely correlated with the amount of total soil biomass and experienced the same transformation patterns as the total soil biomass over the three periods. The significant correlation between PLFAs specific for the Gram-negative bacterial populations and total microbial biomass clearly revealed that they were the hydrocarbon-degrading populations during all soil treatments. On the other hand, no raised PLFA yields from Gram-positive bacteria and Fungi were observed across the study period from all the soil treatments. The two specific microbial groups are assumed not actively involved in the PHC biodegradation activities and PHCs incurred environmental stress on them. Margesin et al. (2007) monitored the changes in microbial community composition and activity during biostimulation treatments of diesel oil contaminated soil and found Gram-negative bacterial community significantly increased. Similarly, Gram-positive population was not significantly affected by PHC content or biostimulation treatment (Margesin et al., 2007). Saturated PLFAs 15:0 and 17:0 were reported to increase in a consortium of oil-degrading marine bacteria cultivated by light petroleum (Aries et al., 2001), however, no significant increase of their concentrations was observed in all of the current soil treatments. The increase of even-numbered MONO PLFAs 16:1ω7, 18:1ω9c and 18:1ω7 in rhamnolipid treated soil was accorded with the understanding that the removal of hydrocarbon contaminants are accompanied by the increase of MONO PLFAs (Main et al., 2015).

It was noticed from close clustering in Fig. 4 (N0 and R0 series) that although the soils were applied with different treatments in bioremediation practice, all the time zero points were supposed to have similar PLFA patterns. Soils initially treated with Tween 80 resulted in relatively different PLFA profiles, especially in the differences of 2OH 14:0. PC 2 (10.0% of the variance) tended to separate soil samples collected at time 0. Fig. 6 showed the whole picture on distribution of PLFA biomarkers of original soil and soils treated with rhamnolipids, Tween 80 on Day 0. The results indicated that although generally consistent results were obtained from all the PLFA compositions, the biggest differences of PLFA concentrations between the three soils lied in the variances in three PLFAs 2OH 14:0, 16:1ω7c and 18:1ω9c. Soil samples with the addition of Tween 80 exhibited relatively higher values of PLFAs with regard to the other supplement treatments, especially for the three fatty acids listed above. This is attributed to the inherent analytical bias using PLFA profiling, which involves the

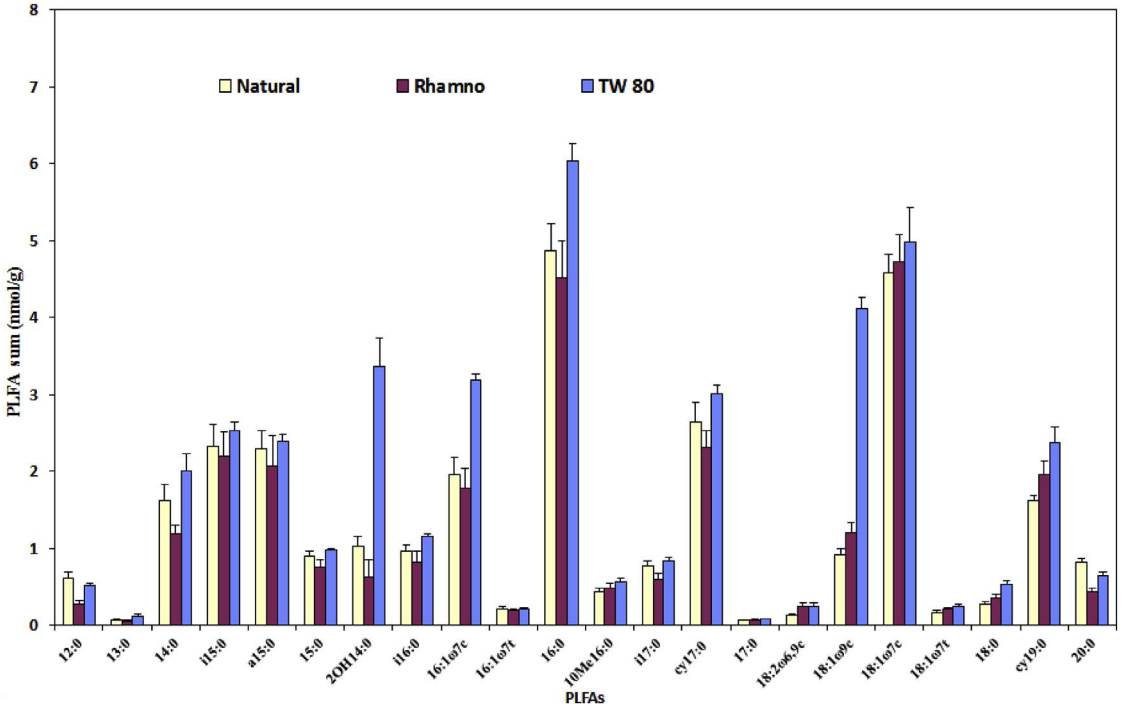

Fig. 6. Distribution of PLFA biomarkers of original soil and soils treated with rhamnolipids, Tween 80 on Day 0 from the hydrocarbon-contaminated site.

transesterification of phospholipids into FAMEs. The methylation reaction (transesterification) is applicable to various types of lipids, which involves the cleaving of the ester bond by an alcohol and form new esters (Anastopoulos et al., 2009). Tween 80 containing polyoxyethylene groups and oleate groups could be extracted and further involved in the methylation reaction. The hydroxyl groups and oleate groups of Tween 80 are believed to contribute the extra increase of PLFA abundance.

Nonetheless, the analytical bias for certain PLFAs caused by the presence of Tween 80 was not permanent during the study period. As revealed by Fig. 7, although significant difference of the three fatty acids was observed from the Time 0, the variations of 2OH 14:0 and 16:1ω7c were eliminated after 17 days. Referred from the total biomass and bacterial PLFA transformation patterns in this period (Fig. 7), the results were believed to represent bacterial compositions due to the elimination of determination interference caused by properties of Tween 80. The high abundance of 18:1ω9c on Day 17, however, still indicated that certain amounts of FAMEs were derived from Tween 80 itself but microbial sources. After 54 days, the amount of 18:1ω9c decreased while the total PLFA biomass maintained the same level as Day 17, an obvious sign that Tween 80 were degraded to some extent accompanied by long-term PHC degradation. This is supported by differential total biomass patterns on Day 54 and the fact that the 18:1ω9c concentration increased from the initial value when they are stimulated. In summary, although certain biases of PLFA species were involved in the profiling of microbial community structure due to the characteristic properties of Tween 80, the analytical error is a minor factor in evaluating the effectiveness of each microbial group. The

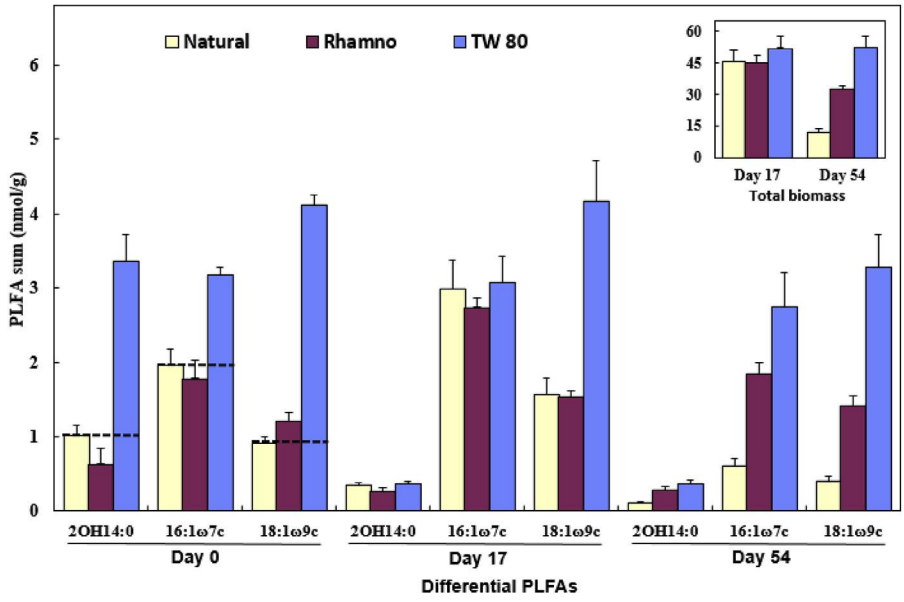

Fig. 7. The determination bias changes of three fatty acids yielded from PLFA analysis due to the addition of Tween 80 into the contaminated soil over time.

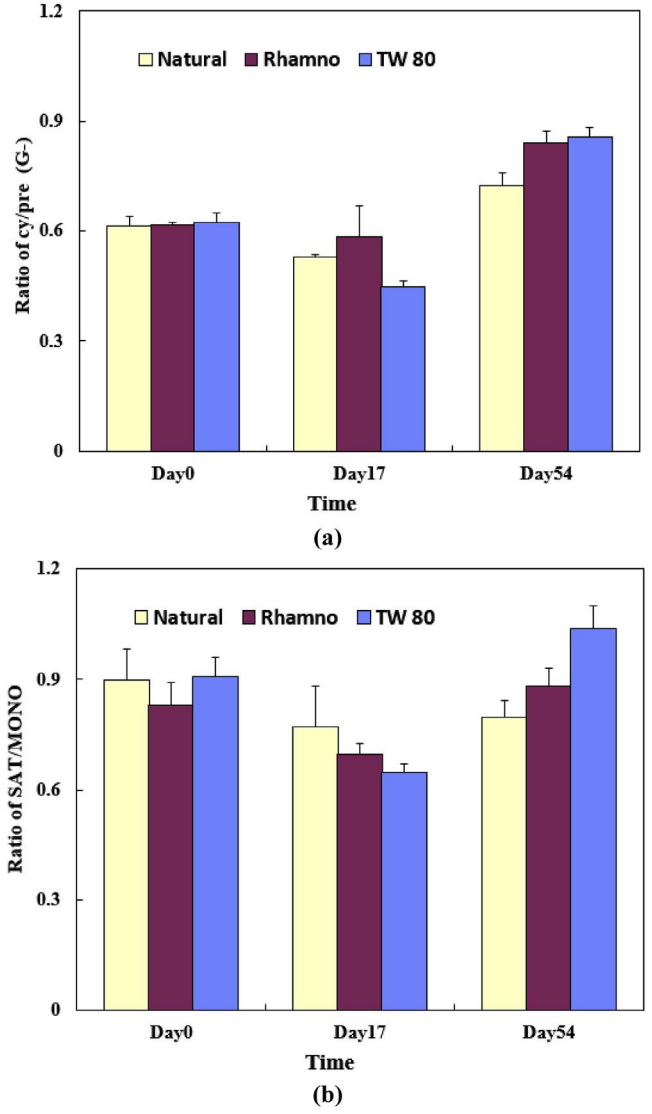

Fig. 8. Physiological stress status changes of the indigenous microorganisms in the environment indicated by cyclopropyl PLFAs to their precursors (a) and saturated to monounsaturated PLFAs (b) ratio over time.

results were in accordance with Nielsen and Petersen (2000) that the PLFA yields from non-microbial sources accounted for no more than 5–10% of the total amounts. Moreover, the concentration of Tween 80 in soil decreased over time to a final ignorable level so that its impact on PLFA analysis became limited.

The PLFA compositional characteristic is an important indicator of the change of membrane fatty acid composition to study microbial adaptive reaction against the environment alteration. Physiological status was determined using the ratios of cyclopropyl PLFAs to their monoenoic precursors (cy/pre) and the ratios of SAT to MONO (S/M) PLFAs (Fig. 8). The cy/pre ratios of all the soils treated by three incubation methods showed a decreasing tendency at Day 17, but the values were significantly higher at Day 54. Although a heating event with ample nutrition may induce the re-growth of bacteria and increase the cy/pre ratio under nonstressful conditions (Bárcenas-Moreno et al., 2011), this ratio would typically increase with other stresses caused by insufficient nutrients, low pH, pesticide use (Wixon and Balser, 2013), water (Moore-Kucera and Dick, 2008) and other factors. From our results, the microorganisms experienced the starvation during the last stage of biodegradation and the physiological status of the microbes was correspondingly reflected. The similar conclusions could be deduced from the results indicated by S/M ratios (Fig. 8b). The physiological change or stress responses from the indigenous microorganisms were closely correlated with the degradation stage of the contaminants and the availability of nutrients.

4. Conclusions

In this study, an enhanced PHC biodegradation system was investigated and optimized by DOE. PLFA analysis elucidated soil microbial community, shifts and dynamic change of the microbial community structure, and associated biodegradation mechanism during natural attenuation, and rhamnolipids or Tween 80 amended bioremediation. The outputs provide valuable guidance in understanding bacterial communities and further BS-enhanced bioremediation practices.

Acknowledgment

The authors would like to express their gratitude to Natural Science and Engineering Research Council of Canada (NSERC) (grant number #RGPIN 402371-2012) for the support.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibiod.2018.04.009.

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