

Characterization of Polycyclic Aromatic Hydrocarbons and Bioaugmentation Potential of Locally Isolated Beneficial Microorganisms Consortium for Treatment of Tar-Balls

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ABSTRACT

Oil spills are one of the environmental pollutions that commonly occur along coastal areas. Tar-balls are one of the products that come from the oil spill pollution. In this study, tar-ball pollution was monitored at 10 points along the coastline of Marintaman Beach in Sipitang, Sabah, Malaysia. This research determined the physical characteristics, composition, and concentration of Polycyclic Aromatic Hydrocarbons (PAHs) in tar-balls. The total number of tar-balls collected was 227 (n=227). The tar-balls were observed in various shapes and the sizes were recorded in the range of 0.1 cm to 6.9 cm. The composition and concentration of Polycyclic Aromatic Hydrocarbons (PAHs) in the outer and inner layer of tar-balls were determined. The results showed that the main Polycyclic Aromatic Hydrocarbons (PAHs) compounds were found in inner layers of the tar-balls with benzo (g,h,i) perylene (72.26 mg/kg), flourene (59.87 mg/kg), dibenzo (a,h) anthracene (44.48 mg/kg), indeno (1,2,3-c,d) pyrene (78.18 mg/kg), and benzo (e) fluoranthene (45.70 mg/kg), respectively. Further research was done with the bioaugmentation study of locally isolated beneficial microorganisms (LIBeM) consortium for treatment of tar-balls in an Aerated Static Pile (ASP) bioreactor system. The results showed that, after 84 days of treatment, this consortium, consisting of *C. tropicalis*-RETL-Cr1, *C. violaceum*-MAB-Cr1, and *P. aeruginosa*-BAS-Cr1, was able to degrade total petroleum hydrocarbon (TPH) by 84% as compared to natural attenuation (19%). The microbial population of this consortium during the biodegradation study is also discussed in this paper.

1. INTRODUCTION

Oil pollution is considered to be one of the major contributors to marine environment pollution. This organic contaminant comes from uncontrolled oil spillage during transportation, manufacturing, and run-off from terrestrial sources. As a result of oil spilled in the marine environment, many implications occur. One of them is the formation of tar-balls. Tar-balls are the product of weathered marine oils, regardless of source. Tar-balls come from many sources of petroleum and they are mostly derived from different activities such as tanker washing and routine shipping operations (Chandru et al., 2008). In Malaysia, a few studies on tar-balls have been

conducted, notably by Zakaria et al. (2002), Yong (2003), Johnson (2004), and Chandru (2005). Despite the ever-growing cases of accidental and non-accidental oil pollution in the sea, limited study has been done to understand the effects inflicted on the marine environment. This study contributes to providing information regarding the effect of oil pollution on the onshore environment, specifically in terms of the PAH levels. PAH is chosen among other compounds due to the carcinogenic and toxic properties. PAH negatively impacts the marine habitat, mainly affecting the filter and bottom-feeders as they lack PAH-metabolizing enzymes (Adzigbli and Yuewen, 2018). Analyzing the chemical makeup

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of tar-balls gives a further understanding on beach oil pollution. The Sipitang beach is also of concern as it serves as a developing recreational spot. Hence, this study describes the distribution and abundance of stranded tar-balls at the coastline of Marintaman Beach, Sepitang, Sabah. The analysis allows a better understanding on the precautions to the oil spill in the marine environment and the cause of variability in tar-ball accumulation nearby the coastal water. In addition, we introduce one practical and environmentally friendly method to treat tar-balls by using locally isolated microorganisms (LIBeM) which has the potential to degrade hydrocarbons as well as phenol (Piakong, 2006; Nurulhuda and Piakong, 2007).

2. METHODOLOGY

2.1 Study area

The Marintaman Beach ($5^{\circ}04'45.30''N$, $115^{\circ}33'02.95''N$), a beach in Sipitang, is located in the southwestern town of Sabah. The beach is a part of an extended bay that stretches for about 92.79 km. The beach consists of unique rock formations and boulders strewn on the right flank of its beach.

2.2 Sampling location

Tar-ball samples were collected from the coastline of the Marintaman Beach Sipitang based on the transect line area from the high tide to the low tide of the beach. Figure 1 shows the coastline area at

Marintaman Beach Sipitang that is located 39.93 km from the federal territory of Labuan. It was found that there were many oil production activities such as shipping, petroleum processing and excavation of crude oil carried out on and off the shore.

2.3 Sample collection

A total of 227 tar-balls along the Marintaman Beach was collected in February 2016. Samples were collected from a one-meter strip running across the beach from high tide line to the present water level. The samples were collected in 10 stations assigned along the 1.63 km of the coastline of Marintaman Beach. The tar-balls were wrapped with aluminum foil and put inside a glass jar and ziploc bag to avoid contamination. Collected samples were kept in a cold box at $4^{\circ}C$ prior to the bioaugmentation study.

2.4 Bioreactor design

Figure 2 shows a bioreactor designed with a size of $30\text{ cm} \times 20\text{ cm} \times 25\text{ cm}$. The bioreactor made of acrylic material was supplied with three tubes at the side of the reactor connected to the air pump (Model RESUN LP100 Low Noise Air Pump) and was used for bioaugmentation study (Piakong and Nur Zaida, 2018). There are three parts of the reactor which consists of gravel-sized (1-1.5 cm), sand, and soil on the surface. The soil that had been used in this study was mixed well meanwhile the effluent at the bottom of the reactor was collected in the universal bottle.



Figure 1. Sampling location of tar-balls in the coastal area of Sipitang, Sabah, Malaysia

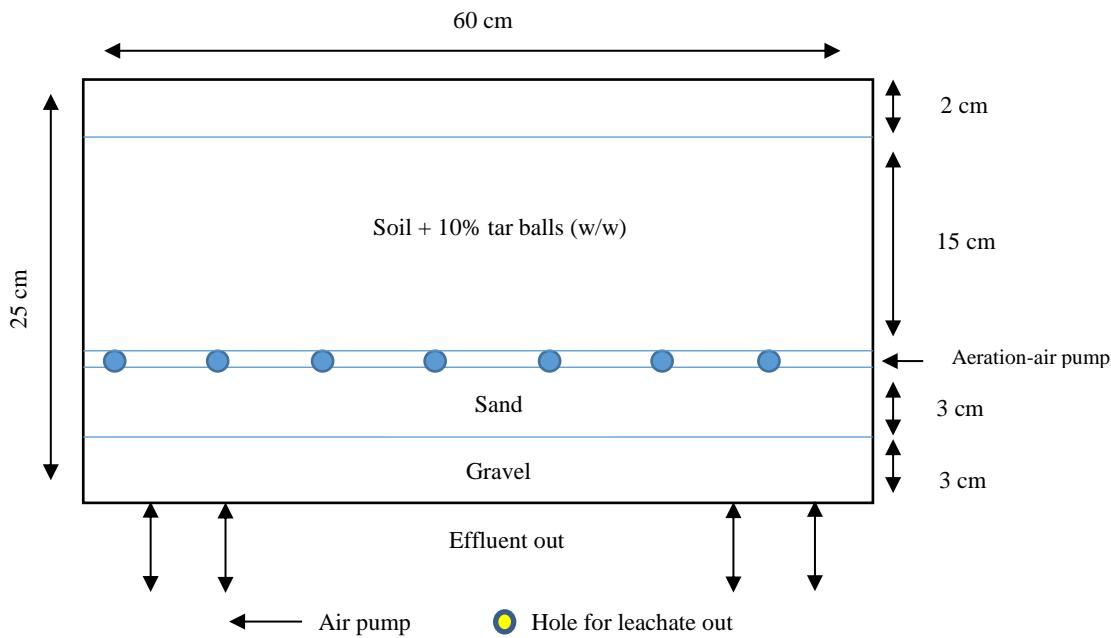


Figure 2. The layout of bioreactor used to treat tar-balls (Piakong and Nur Zaida, 2018)

2.5 Sources of microorganisms

The microorganisms were obtained from Environmental Microbiology Laboratory, Faculty of Science and Natural Resources, Universiti Malaysia Sabah. There are three species used, *Candida tropicalis*-RETL-Cr1, *Chromobacterium violaceum*-

MAB-Cr1, and *Pseudomonas aeruginosa*-BAS-Cr1. These cultures were proved to degrade oil sludge efficiently based on previous study by Piakong and Nur Zaida, (2018). The colony of these micro-organisms is shown in Figure 3.

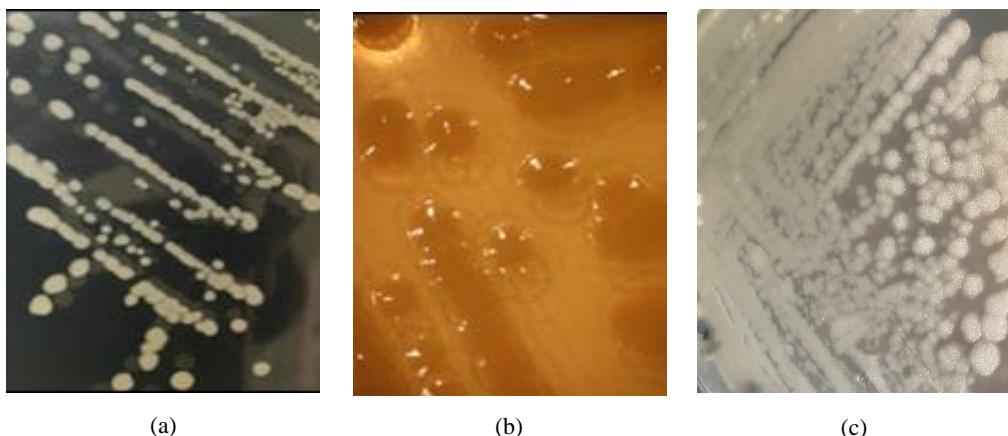


Figure 3. Colony morphology of LIBeM (a) *C. tropicalis*-RETL-Cr1, (b) *C. violaceum*-MAB-Cr1, and (c) *P. aeruginosa*-BAS-Cr1 used in this study.

2.6 Culture medium

The media used for inoculation of LIBeM consortium was Ramsay broth as mentioned by Frank et al. (2020). All of the ingredients were suspended in a Schott bottle with 1,000 mL of deionized distilled water. After that, the mixture was autoclaved at 121°C for 15 min. The Ramsay broth was cooled to room temperature and stored in a sanitized cabinet.

2.7 Inoculum preparation

The inoculation of LIBeM consortium into the tar-balls treatment reactor was conducted in liquid form. A single colony of bacteria isolate was inoculated into Ramsay broth at 30°C for 24 h in an orbital shaker at 200 rpm. A total of 10% of the cultured bacteria (1×10^7 CFU/mL) with OD 0.5 and above at 600 nm was used as inoculum. Then 10%

(v/v) inoculants of consortium cultures were added into the treatment reactor that contains 100 g of tar-balls that are crushed mixed with soil for bioaugmentation studies. The inoculation of LIBeM consortium into the treatment plot was done every 2 weeks.

2.8 Experimental set-up

A portion of 1,000 g of soil was sieved through a 0.20 mm sieve size to remove the remaining debris in the soil. A total of 100 g of tar-balls was mixed over the soil for further bioaugmentation studies. The following treatment in duplicate was performed for 84 days in ambient temperature as shown in [Table 1](#).

Table 1. Bioaugmentation of tar-balls by LIBeM consortium in liquid formulation

Treatment	Content
Treatment 1	Non inoculation of microorganism in the soil (Natural attenuation - NA)
Treatment 2	The tar-balls were treated with consortium (RETL-Cr1 + MAB-Cr1 + BAS-Cr1) (LIBeM-LIQ)

2.9 Determination of total petroleum hydrocarbon (TPH) in tar-balls

Total petroleum hydrocarbon was carried out based on the gravimetric method (Soxhlet extraction) (USEPA 3540C) ([Adeniji et al., 2017](#)). A 20 g soil sample was grained and placed in a thimble and extracted with dichloromethane (DCM). Then the thimble was placed in a Soxhlet extractor. 250 mL of dichloromethane was added into the round bottomed flask (RBF). The extraction process took place for 11 h. The total solvent was cleared completely with the vacuum evaporator at 40-50°C. The RBF together with the extract was cooled in a desiccator after being dried in an oven at 40°C. The percentage of total petroleum hydrocarbon (TPH) was calculated using the formula below:

$$\% \text{ TPH} = \frac{\text{Min extract weight in RBC}}{\text{Weight of sample}} \times 100$$

3. RESULTS AND DISCUSSION

3.1 Composition and concentration of PAH in tar-balls

The major PAH composition of outer and inner layered of tar-balls samples are presented in [Figure 4](#) and [5](#). The total cumulative concentrations of PAH levels are significantly higher in the inner layer (469.79 mg/kg), whereas the outer layer was lower

with 172.64 mg/kg. In the sample 2-outer, the PAH compound fluorene was the highest at 59.87 mg/kg, followed by benzo (b) fluoranthene at 45.70 mg/kg and benzo (g,h,i) perylene at 31.61 mg/kg. The lowest concentration for sample 2-outer was acenaphthylene, 5.22 mg/kg, benzo (a) pyrene, 9.75 mg/kg, and dibenzo (a,h) anthracene, 10.98 mg/kg, respectively. In sample 1-outer, the highest PAH concentration found was in benzo (e) pyrene, benzo (g,h,i) perylene, and benzo (b) fluoranthene with concentrations of 28.52 mg/kg, 24.58 mg/kg, and 23.65 mg/kg, respectively. In sample 2-outer, the distribution of PAH composition was spread out more evenly than sample 1-outer. Various concentrations of these elements may be caused by several weathering processes, such as evaporation, dissolution, photochemical oxidation, dispersion, emulsification, adsorption onto suspended particulate material, and sedimentation ([Leili et al., 2020](#)).

For the inner-layered samples, a different trend can be seen as in [Figure 5](#). The PAH concentrations for the inner layers of both samples were relatively higher than the outer-layered samples. The highest concentration was found in sample 2-inner and is followed by 1-inner. The highest PAH concentration in sample 2-inner was found in the compound indeno (1,2,3-c,d) pyrene, 78.18 mg/kg. Following that is benzo (g,h,i) perylene, 72.26 mg/kg, dibenzo (a,h) anthracene, 44.48 mg/kg, and 1-Methyl penanthrene, 33.59 mg/kg. The lowest PAH compounds found were benzo (a) pyrene (3.70 mg/kg), acenaphthene (3.71 mg/kg), and acenaphthylene (3.93 mg/kg). Outer-layered samples were seen to be dominated by PAH compounds of benzo (b) fluoranthene, benzo (k) fluoranthene, and benzo (g,h,i) perylene, respectively.

It shows that the concentration of PAH was higher in 2-inner than in 1-inner sample. The value of the total PAH concentration in inner layer was 61% more than the outer-layered samples. In the inner layers, benzo (g,h,i) perylene, dibenzo (a,h) anthracene, and indeno (1,2,3-c,d) pyrene had the highest concentration values with 72.26 mg/kg, 44.80 mg/kg, and 78.18 mg/kg, respectively. Naphthalene and its derivatives seem to come in second for high concentrations. As seen in both the inner and outer samples, naphthalene, acenaphthylene, and acenaphthene have relatively the same concentrations. Fluorene has nearly similar concentrations in the inner samples of 1-inner and 2-inner, 32.54 mg/kg and 25.79 mg/kg, respectively. In both the inner and outer layers, it can be seen that PAH compounds methyl-anthracene

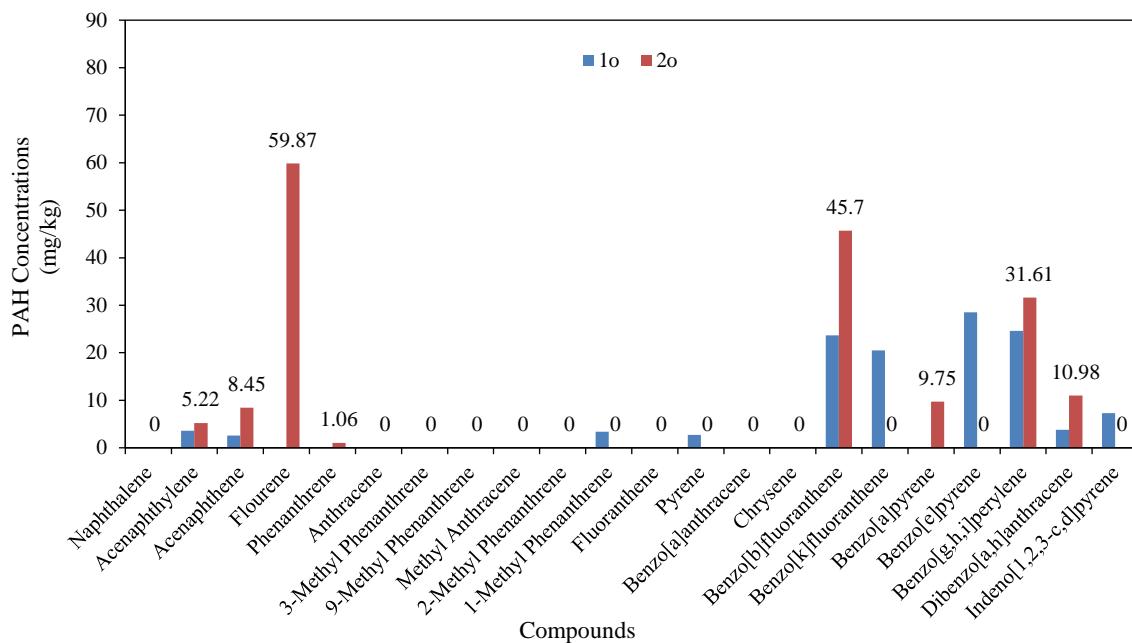


Figure 4. Characterization of PAHs compound in outer-layered samples of 1-outer (1o) and 2-outer (2o) (in mg/kg)

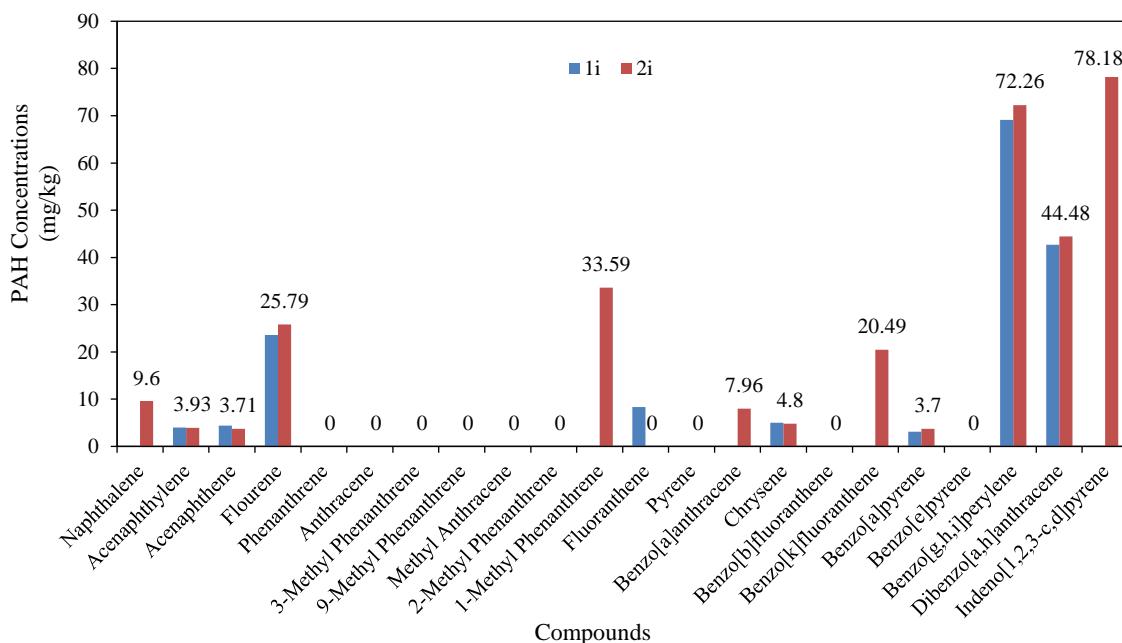


Figure 5. Characterization of PAHs compound in inner-layered samples of 1-inner (1i) and 2-inner (2i) (in mg/kg)

and the ones belonging to methyl-phenanthrene family were exceptionally absent in most of the samples. The compounds were either undetected by the GC/MS, the SIM method or had a negative value after the sample recovery.

The PAH found in outer-layered samples were lower than the inner-layered samples. The main reason for this is that the outer part is exposed, and hence easier

for degradation processes to occur. PAH compounds are exceptionally sensitive to ultraviolet light (Abdel-Shafy and Mansour, 2016) and degrade easily. Jennifer et al. (2019) have also stated the significant linear correlation between PAH half-lives and solar radiation intensity. Thus, it can be inferred that exposure of tar-balls to sunlight will affect the concentration in the outer-layered samples.

3.2 Biodegradation of polycyclic aromatic hydrocarbons (PAHs) compounds by LIBeM consortium

Figure 6 shows the percent degradation of PAHs compound in tar-balls treated with LIBeM consortium (*C. tropicalis*-RETL-Cr1, *C. violaceum*-MAB-Cr1, and *P. aeruginosa*-BAS-Cr1). The results explain that the LIBeM consortium are able to degrade PAHs compound as compared to natural attenuation (control). It was found that LIBeM consortium were efficient in degrading the naphthalene, acenaphthalene,

flourene, and benzo (b) fluoranthene at 84.62%, 80.37%, 83.87%, and 80.04%, respectively.

This finding agrees with [Ana et al. \(2020\)](#) who reported that most of hydrocarbon clastic bacterium were able to degrade n-alkanes and PAHs with four-rings PAH. It is interesting to note that in this present work, LIBeM consortium was found to degrade most of PAHs compound, especially pyrene. The result also showed that LIBeM consortium was able to degrade high molecular weight (pyrene) known to be recalcitrant to microbial attack ([Gabriela et al., 2018](#)).

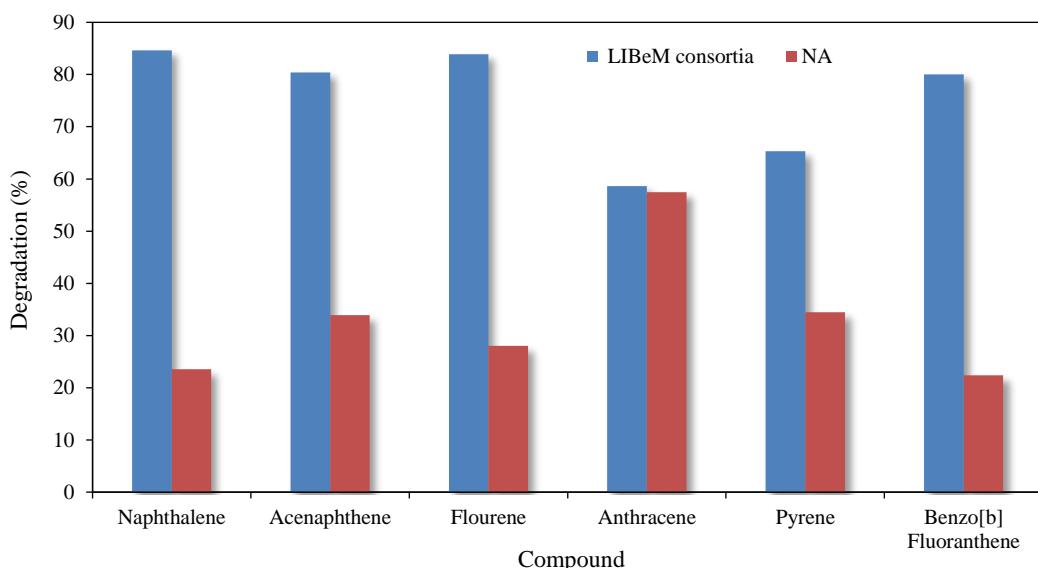


Figure 6. Biodegradation of polycyclic aromatic hydrocarbon (PAHs) of tar-balls by consortium LIBeM and Natural Attenuation (NA) after 84-days treatment.

Bioaugmentation with inoculation of LIBeM consortium appeared to be the most appropriate way to degrade PAHs compound while natural attenuation was capable to degrade a maximum of 57% of these elements. An increased in number of molecular weights will increase the hydrophobicity and decreased the solubility for indigenous microorganisms to degrade the compound ([Ana et al., 2020](#)). However, for LIBeM consortium the production of biosurfactant produced by *P. aeruginosa* promoted the high degradation of PAHs compound. Thus, it can be concluded that the performance of LIBeM consortium to treat tar-balls achieved more than 80% as compared to natural attenuation in the control plot. It is noteworthy that the physical and chemical properties of PAHs compounds as well as their molecular weight have a considerable effect on microbial assimilation and biodegradation rate of the soil study ([Sakshi and Haritash, 2020](#)).

3.3 Biodegradation ratio of n-C₁₇:pristane, n-C₁₈:phytane, and pristane:phytane

The results of isoprenoids n-C₁₇ and n-C₁₈ for consortium LIBeM from initial week and after biodegradation study are shown in [Table 2](#). These isoprenoids are highlighted due to the biodegradation and microbial activity that occur during the treatment of tar-balls. It is worth to note that the reduction of n-C₁₇ and n-C₁₈ in this study indicates the microbial degradation of tar-balls to less degradable compounds ([Ruben et al., 2020](#)). The results demonstrated that n-C₁₇:Pr and n-C₁₈:Ph ratios for LIBeM consortium sample compared to the control decrease with increasing the incubation time and reached its maximum after 12 weeks of incubation. The n-C₁₇:Pr and n-C₁₈:Ph ratio ranged from 0.1 to 3.8 and 0.5 to 3.2 while the Pr/Ph ratios range from 1.2 to 9.9 respectively. By comparing the results, it was found that n-C₁₈:Ph ratio was lower than n-C₁₇:Pr ratio.

Table 2. Comparison of biodegradation ratios of LIBeM consortium and natural attenuation.

Treatment	Week	C ₁₇ :pristane	C ₁₈ :phytane	pristane:phytane
LIBeM consortium	0	3.8	1.0	1.4
	12	0.4	0.8	1.2
Natural attenuation (Control)	0	1.2	3.2	9.9
	12	0.1	0.5	2.0

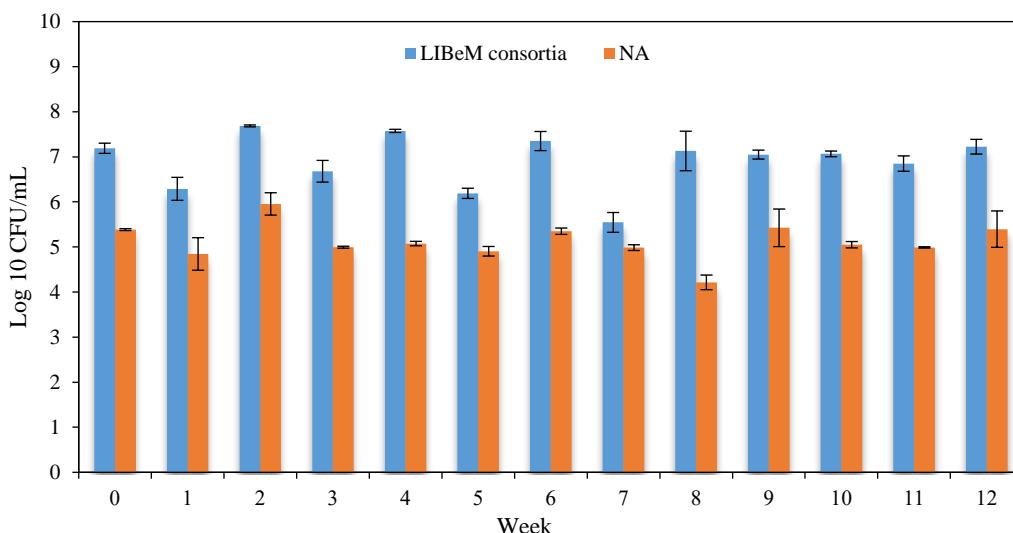
The results indicate that microbial degradation was actively involved during the treatment. This can be explained with the presence of biosurfactant by LIBeM consortium, thus make it easier to degrade both C₁₇ and C₁₈ as compared to pristine and phytane (Nasser et al., 2020).

It is important to note that n-alkanes have been degraded faster than isoprenoids, thus lead to decrease in ratio values of n-C₁₇:Pr and n-C₁₈:Ph. Dashbozorg et al. (2019) stated that by considering the thermal maturity of the tar-balls, the biodegradation of n-alkanes is faster than isoprenoids. However, according to Lijmbach (1975), the value of Pr/Ph mainly comes from the different sources of the samples. He stated that different samples of tar-balls may come from different origin places, such as aquatic depositional environments (marine and brackish

water) with value Pr/Ph (<2.0). Meanwhile, for Pr/Ph (2-4) these samples may come from fluviomarine and coastal swamp environments while high values of Pr/Ph (>10) are related to oxidizing conditions such as peat swamp.

3.4 Microbial population during biodegradation of tar-balls

Figure 7 shows the microbial population of LIBeM consortium and indigenous microorganisms in the control plot. The results showed that the highest microbial population was found in week 2 (LIBeM consortium), 8.8×10^8 CFU/mL. The results indicated that the inoculation of LIBeM consortium in treatment plot increased the microbial population due to the carbon consumption in the tar-balls. This leads to the significant TPH reduction respectively.

**Figure 7.** Microbial population of LIBeM consortium and natural attenuation during 12 weeks of tar-balls treatment.

On the other hand, the control plot which consists of indigenous microorganisms in the soil recorded the lowest microbial population in range of 6.2×10^7 - 7.7×10^8 CFU/mL through the treatment period. The depletion of microbial population in the control plot might be due to the toxic effect of the tar-balls towards the indigenous population. This explained the inability of these microorganisms to

tolerate with tar-balls, and thus cause insignificant removal of TPH in the control plot. The biodegradation of tar-balls by LIBeM consortium was proven with scanning electron microscopy (SEM) image captured during the treatment study.

Figure 8 shows the SEM image of LIBeM consortium with 10^7 cells/g detected in the treated soil. The existence of microbial density of LIBeM

consortium is dependent on the availability of gas exchange, nutrients content, and the physiochemical properties of the tar-balls treatment. There are pores on the surface filled with a number of small grains and holding a lot of floc, forming irregular structure of the shape of tar-balls in soil. The distribution of LIBeM consortium shows a heterogenous morphology as

shown with enclosed dotted line area. The attachment of LIBeM consortium to the soil confirms that the formation of biofilm is an initial step in the biodegradation process of tar-balls. However due to homogeneously distributed and incubated period, the images were found non-uniform to cause the strain multiply.

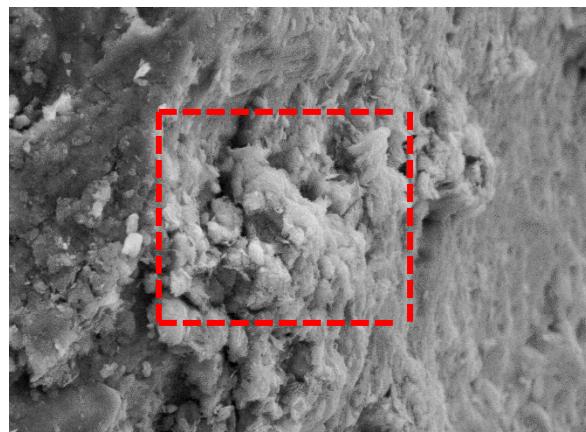


Figure 8. Scanning electron micrographs (SEM) images of LIBeM consortium with tar-balls contaminated soil under 1,000 x magnification.

4. CONCLUSION

This study concluded that the highest PAH concentration found in the tar-ball samples consisted of benzo (g,h,i) perylene, flourene, dibenzo (a,h) anthracene, and indeno (1,2,3-c,d) pyrene. Among these compounds, two of those are listed as Group B carcinogens. The top three compounds are benzo (g,h,i) perylene (72.26 mg/kg), flourene (59.87 mg/kg), dibenzo (a,h) anthracene (44.48 mg/kg), and indeno (1,2,3-c,d) pyrene (78.18 mg/kg). They are mostly found in the inner layers of the sample. Biodegradation of tar-balls by LIBeM consortium represents TPH biodegradation efficiency of 84% within 84-days period. The ASP-biodegradation has showed a great potential and integrated approach for treatment of tar-balls after oil-spill in marine ecosystem. Therefore, further research is recommended to carry out the ASP-enhanced bioaugmentation of tar-balls using other LIBeM products formulation, such as in powder and capsule form (LIBeM-POW and LIBeM-CAP).

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