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A study on bio-treatment of petrogenic contamination in El-Lessan area of Damietta River Nile Branch, Egypt

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Abstract

Petrogenic pollution is the most important source for environmental contamination with polynuclear aromatic hydrocarbons (PAHs) and polyaromatic sulfur heterocycles (PASHs). These pollutants have acute or chronic toxicity, tumorigenic and carcinogenic activity to human health and other living organisms. El-Lessan area of Damietta River Nile Branch, Egypt was selected because of its crowdness with fishing boats, touristic activities and presence of fueling stations and maintenance workshops for boats which led to high petrogenic contamination. As a suggestive solution to minimize and treat petrogenic contamination in El-Lessan area; the capability of previously isolated *Corynebacterium variabilis* sp. Sh42 for bioremediation of a petrogenic polluted water sample collected from El-Lessan area individually and in consortium with a standard bacterium *Rodococcus erythropolis* IGTS8 was studied in batch flasks system for 28 d of incubation at 30°C. Follow up the effectiveness of biotreatment processes by different analytical techniques revealed that; bioaugmentation by *C. variabilis* sp. Sh42 and consortium (IGTS8-Sh42) are the most suitable biotreatments recording the highest decontamination effect on different petrogenic pollutants.

Keywords: Bioremediation, Petrogenic polluted water, *Corynebacterium variabilis* sp. Sh42, *Rodococcus erythropolis* IGTS8

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1. Introduction

Petrogenic pollutants have received the greatest attention internationally, politically, and scientifically [1-3]. There are three major categories: PAHs, heterocyclics (e.g. PASHs), and substituted aromatics co-exist as complex mixtures in petroleum refinery and oil polluted sites [4]. It is widely recognized that petrogenic contaminated aquatic environment is a potential threat to human health, and its continual discovery over recent years has led to international efforts to remedy many of these sites either as a response to the risk of adverse health or environmental effects caused by petrogenic contamination or to enable the site to be redeveloped for use [5, 6]. Over the last decades, there has been an increasing interest in biological methodologies, collectively indicated as bioremediation that may help to reduce the risk of petrogenic pollutants in soil and water and effectively restore polluted sites. Where, bioremediation is often considered as safe and cost effective means of restoring environmental quality [7-10]. In Egypt, most bioremediation efforts have been focused on the degradation of aliphatic and aromatic fractions rather than the heavy fractions, that is, resins and asphaltenes pollutants [11-15].

El-Lessan area of Damietta River Nile Branch in Egypt; the merge point between River Nile fresh water and Mediterranean seawater, is one of the water bodies that support fishing activities and tourism. Petrogenic pollution in that area previously studied throughout three representative sites. Extracted hydrocarbons from collected water samples revealed that; the studied sites are highly contaminated with petroleum hydrocarbons, PAHs and PASHs compounds (data not shown). Also, the hydrocarbon pollutants concentrations have been ranked in the following decreasing order: W1 > W2 > W3. Names and geographic locations of these sites are represented in Table (1) and Figure (1). Therefore, the present work aimed to minimize and treat petrogenic pollution in El-Lessan area by investigating the capability of the previously isolated *Corynebacterium variabilis* sp. Sh42 [16] for bioremediation and cleanup of polluted water sample collected from El-Lessan area individually and in consortium with a standard bacterium species *Rodococcus erythropolis* IGTS8 (ATCC 53968).

2. Materials and Methods

2.1. Sample Location

Petrogenic polluted water sample was collected from site W1 at El-Lessan area of Damietta River Nile Branch in Egypt.

2.2. Chemical reagents

Acetonitrile (Ace), Water (W) and 16 PAHs standard mixture used for HPLC analysis were of HPLC grade and all were purchased from Aldrich. All other chemical reagents employed in this study were of analytical grade and were purchased from Sigma Chemical Company, USA.

2.3. Microbial strains

Corynebacterium variabilis sp. Sh42, previously isolated from El-Lessan area for its capability to utilize phenolic, PAHs and PASHs compounds at high salinity ≈ 30,000 mg/L [16] and a standard bacterium species *Rodococcus erythropolis* IGTS8 (ATCC 53968) were used in this study**.**

2.4. Cultural Media

Tryptone glucose yeast extract medium (TGY) was prepared according to Benson [17] and was used for obtaining the biomass. TGY/agar plates were used for monitoring total viable count, TCFU (cells/mL).

2.5. Inocula preparation

C. varaiabilis sp. Sh42 and *R. erythropolis* IGTS8 were inoculated individually in TGY medium, with pH7, incubated at 30 ^{o}C for 24 hrs in a shaking incubator of 150 rpm. Cells were pelleted by centrifugation at 3000 rpm for 15 min and the supernatant is decanted and the cells pellets were washed twice with steriled saline (9 g NaCl / L distilled water) then re-suspended in fresh steriled saline.

2.6. Preparation of biotreatment batch flasks

The collected sample was divided into three groups; The 1st group (steriled); eight (250 mL) conical flasks each containing 100 mL of fresh polluted water sample supplemented with corn steep liquor CSL (1 g/L) as nutrients were autoclaved at 120^{o}C at 1.0 bar for 20 min. The 2nd group (non- steriled); the autoclaving step was omitted, ten (250 mL) conical flasks each containing 100 mL of polluted water sample supplemented with CSL (1 g/L). The 3rd group; four (250 mL) conical flasks containing 100 mL non- steriled polluted water sample without addition of CSL were used.

2.6.1. Study the effect of Sh42, IGTS8 and consortium of both on biotreatment of polluted water sample

Bacterial cultures were inoculated individually and in consortium into batch flasks of (1st and 2nd groups). The initial inocula were adjusted (TCFU ≈ 10^{6} cells/mL). Non inoculated steriled flasks were used under the same condition as negative control for each group.

2.6.2. Study the effect of biostimulation

The non-steriled flasks contain CSL (1 g/L) but without inoculation were used for studying the effect of the metabolic degradation activity of enriched native microorganisms naturally present in the studied area.

2*.6.3. Study the effect of natural weathering*

The non-steriled flasks without CSL or inoculation were used for evaluation of natural weathering effect on polluted water.

2.6.4. Incubation and biotreatment monitoring

All the flasks were incubated in a shaking incubator of 150 rpm at 30^{o}C for four wks. Monitoring of growth was done by TCFU (cells/mL) on TGY plates and pH changes were determined at time intervals of zero, two and four wks. At the prescribed time intervals of incubation, pH of the cultures was brought to pH 2 with 1M HCl to halt biological activity**.** Petrogenic pollutants were extracted, separated and subjected to different analytical techniques. Calculation of metabolic degradation activity for all compounds was done by comparing the difference between negative control and after different biotreatment processes.

Note; All the above steps were performed in duplicates and the listed data are average values of the obtained results.

2.7. Petrogenic assessment

Extraction and gravimetric determination of total petrogenic hydrocarbons (TPH) from collected water samples before and after biotreatments were carried out according to the method described by Ali et al. [18].

2.7.1. Gas chromatographic (GC) analysis

This method was used to identify source of petrogenic pollution, degree of weathering and biodegradation efficiency of normal and iso-paraffins using (GC-FID). While GC-FPD was used to detect sulfur compounds in extracted petrogenic hydrocarbons and their BD efficiency. Analytical program of GC instruments are represents in Table (2).

2.7.2. High performance liquid chromatographic (HPLC) analysis

HPLC instrument model Waters 600E equipped with auto sampler Waters 717 plus and dual wavelength UV detector model Waters 2487 (set at 254 nm) was used for identification and quantification of 16 PAHs listed by US-EPA as priority pollutants in the extracted petrogenic HCs before and after biotreatments. Analytical program of HPLC instrument is represent in Table (3).

2.8. Statistical analysis

All statistical calculations were done using computer programs Microsoft excel version 10 and SPSS (statistical package for the social science version 20.00) statistical program at 0.05, 0.01 and 0.001 level of probability **[19].** Comparison of percentage was done using the one-way ANOVA and post hoc-LSD tests (the least significant difference). The Pearson correlation coefficient and discriminant analysis were estimated to show the relationship of all biotreatment flasks to each other **[20].**

3. Results and discussion

Visual observations of all biotreatment methods showed that; although of the absence of a chemical surfactant, metabolic degradation cultures are accompanied

by the formation of strong stable emulsions. These can be an indirect evidence for production of a biosurfactant or more likely a mixture of biosurfactants. The production of a large amount of biosurfactants by biocatalyst would enhance the bioavailability of hydrocarbons (HC) and poly aromatic compounds (PACs) and at the same time increase the metabolic reaction rate by improving and facilitate the hydrocarbon uptake through the hydrophilic outer membrane of microorganisms [3, 21]. Xu et al. [22]; Gesinde et al. [23] and Sobiecka et al. [24] reported that; *Corynebacterium* genera demonstrate potential as a petrogenic pollutants degrader and produces a range of biosurfactants e.g. phospholipids and lipopeptide biosurfactant. Also, *Rhodococcus erythropolis* IGTS8 genera are known to produce a range of biosurfactants to remove sulfur from DBT via a 4S route sulfur-specific pathway [25, 26]. Metabolic activity of strains under investigation for different petrogenic pollutants was evaluated by the traditional chemical analysis; GC-FID, GC-FPD and HPLC analyses to qualitatively and quantitatively evaluate effects of the individually augmented Sh42 or IGTS8 and their consortium on the hydrocarbons (HC), PASHs and PAHs contents of oil pollutant, respectively. Losses due to abiotic processes (Negative control, NC) are negligible. Gravimetric determination of initial HC content of collected polluted water sample, pH and indigenous microbial population recorded ≈ 195,214 mg/L, 7.06 and ≈ 5.2×10^3 cells/mL, respectively. It is obvious from the results that, the studied area has very high alarmingly petrogenic pollutants enough to cause lethal toxicity where the components of the HC especially PAHs and PASHs may interfere with cellular or sub-cellular processes in the living organisms [3, 27-28]. The chromatographic analysis (GC-FID) of the extracted HC (Figure 2) shows regularly spaced n-alkanes peaks nC_{11}- nC_{36} with a well-defined broad UCM and both pristane and phytane of Pr/Ph ratio ≈ 1.14. This indicates that the main origin of HC pollutants were of petrogenic origin. From the GC calculations, the sum of n- and iso-paraffin (TRP) and UCM represents ≈ 23.4% and 76.6%, respectively with TRP/UCM ratio ≈ 0.3 providing evidence that the sample was not affected by weathering and the water sample was freshly polluted with petroleum HCs which might be from the oil station and maintenance of fishing and touristic boats in the workshop located nearby the site under study. Results represented in Table (4) showed that; for all biotreatment flasks there were considerable decrease in pH values except for NW flasks. The largest decrease in pH was observed in Str and Nstr flasks inoculated with Sh42 and consortium. Decrease in pH might be due to production of organic acids as intermediate products from the metabolic degradation of the HCs. Follow up the effectiveness of biotreatment processes by chemical analysis alone may not be sufficient [29]. Thus monitoring of viable microbial count (cells/mL) of introduced microbial degraders and/or the indigenous microbial population throughout the incubation period for all the biotreatment flasks were also recorded. The obtained results revealed that; there was a significant sharp increase in viable cells count (cells/mL) after 14 d and extended to 28 d of incubation for all biotreatment flasks except Str flasks inoculated with IGTS8 which showed increase in cells count in the first 14 d and remained nearly sustained thereafter up to the 28 d recording ≈ 10^{10} cells/mL. The highest growth potential was observed in Nstr flasks inoculated with Sh42 and consortium that had nearly similar growth potential, recording ≈ 10^{12} and 10^{14} cells/mL after 14 d and 28 d, respectively. Flasks inoculated with consortium (IGTS8-Sh42); Sh42 is found to be the predominant one over IGTS8. This might indicate that Sh42 has a well-adapted metabolic genetic system capable to produce the required active enzymes needed to degrade many hazardous or persistent anthropogenic chemical compounds or various xenobiotic substances since; Sh42 was previously isolated from high oily polluted site from El-Lessan area for its ability to degrade different PACs [16]. It is obvious from data listed in Table (4) that, the amount of TPH consumed (BD %) was in parallel with the growth potential (cells/mL). Pearson Correlation (*r*), the most commonly bivariate correlation technique was used to evaluate the degree of relationship between the two quantitative variables; microbial growth (cells/ml) and removal of TPH (BD%) in different biotreatment flasks. It was found that there was a statistically strong positively bivariate correlation $r > 0.824$ at $P < 0.01$ in all biotreatment flasks i.e. as microbial growth increases the BD% increases. Where, there is no competition between Sh42 and indigenous microbial populations that explained the highest TPH BD% in Nstr flasks inoculated with Sh42. This can be due to the previous isolation of Sh42 from the polluted area under study. Results recorded in Table (4) revealed that, all biotreatment methods degraded more than 10 % of the oil, after 14 d except for Str flasks augmented by IGTS8 which showed no significant reduction in TPH recording ≈ 2.5% after 28 d. Also, there is no remarkable difference between the TPH BD% of Str flasks augmented by Sh42 alone or in consortium with IGTS8, which have nearly similar TPH BD% recording 31% and 33% after 28 d, respectively. Similar observation occurred in Nstr flasks augmented by Sh42 or consortium, TPH BD% recorded ≈ 42 % and 43% after 28 d, respectively. In BS flasks; TPH BD% recorded ≈ 25%, after 28 d. This may indicate that the indigenous microbial population is able to utilize petrogenic pollutants and have a considerable potential for remediation as they are self-adapted and are well adjusted to their own environment. According to the analysis of variance (ANOVA) at *P* value < 0.001 Table (4); it was obvious that there was statistical significant difference in BD% of TPH occurred in all biotreatment flasks and that of NC flasks. Where, there was low statistical significant difference in BD% occurred in Str flasks augmented with Sh42 or consortium; high statistical significant difference occurred in BS flasks and Str flasks augmented with Sh42 or consortium; very high statistical significant difference occurred in Str flasks augmented with IGTS8 and BS flasks, Str and Nstr flasks augmented with Sh42 or consortium and also very high statistical significant difference occurred in Nstr flasks augmented with IGTS8 and that augmented with Sh42 or consortium. But there was no statistical significant difference occurred in NW and Str flasks augmented by IGTS8; BS and Nstr flasks augmented by IGTS8; and Nstr flasks augmented by Sh42 or consortium. In addition, canonical discriminate was used for data analysis. The canonical discriminant analysis drives canonical discriminant functions (linear combinations of the quantitative variables) which facilitates differentiation of biotreatment groups. The groups to be differentiated are known as *priori*.

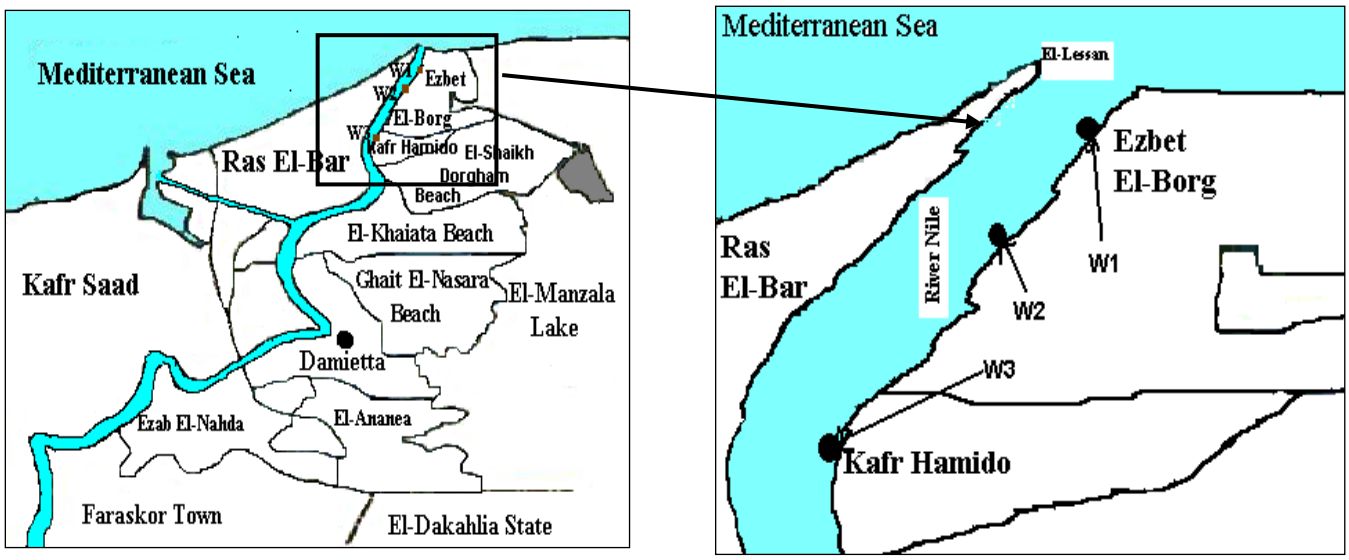


Fig.1. Damietta River Nile Branch in Egypt

Table 1: Sample sites and locations

Sample ID	Location
W1	Ezbet El-Borg Village, Damietta River Nile Branch in Egypt, 0.8 Km distance from El-Lessan (nearby private workshop for boat maintenance)
W2	Ezbet El-Borg Village, Damietta River Nile Branch in Egypt, 1.5 Km distance from El-Lessan (nearby fueling station)
W3	Kafer Hamido Village, Damietta River Nile Branch in Egypt, 4.5 Km distance from El-Lessan (nearby marina for fishing boats)

Table 2: Programmable setup of GC instruments

	GC-FID	GC-FPD
Instrumentation	Agilent 6890	Berkin Elmer Clarus 500
Column	Capillary HP-1 (30 m X 0.25 mm X 0.5 µm)	Capillary HP-1 (60 m X 530 µm X 5.0 µm)
Detector	FID (320^{o}C)	FPD (300^{o}C)
Oven Temp.	From 80^{o}C to 320^{o}C at fixed rate 3^{o}C/min.	From 50^{o}C to 300^{o}C at fixed rate 8^{o}C/min.
Injector Temp.	300^{o}C (Split 1:50)	300^{o}C (Split 1:100)
Sample Size	1 µL	0.5 µL
Carrier gas	Nitrogen gas (2 mL/min flow rate)	
Run Time	90 min	30 min
Standards	The identification of n-paraffin peaks was established by chromatographing a standard mixture of n-paraffins of known composition.	The identification of the individual PASHs peaks was established by chromatographing a standard mixture of Th, BT, DBT, 4-MDBT and 4,6-DMDBT.

Table 3: Programmable setup of HPLC instrument

Column	SUPELCOSIL LC-PAH (15 cm X 4.6 mm, 5µm)
Sample Size	2 µL
Mobile Phase	A: Acetonitril (Ace) B: Water (W) HPLC grade
Mobile Phase program	Gradient program; 40% Ace (v/v) for 5 min then gradually increased to 100% Ace at 30 min for 15 min, and decreased again to 40% Ace at 50 min for 2 min.
Flow Rate	1.0 mL/min
Run Time	52 min
Standards	The identification of PAHs peaks was established by chromatographic a standard mixture of 16 PAHs standards of known composition listed by EPA as priority pollutants.

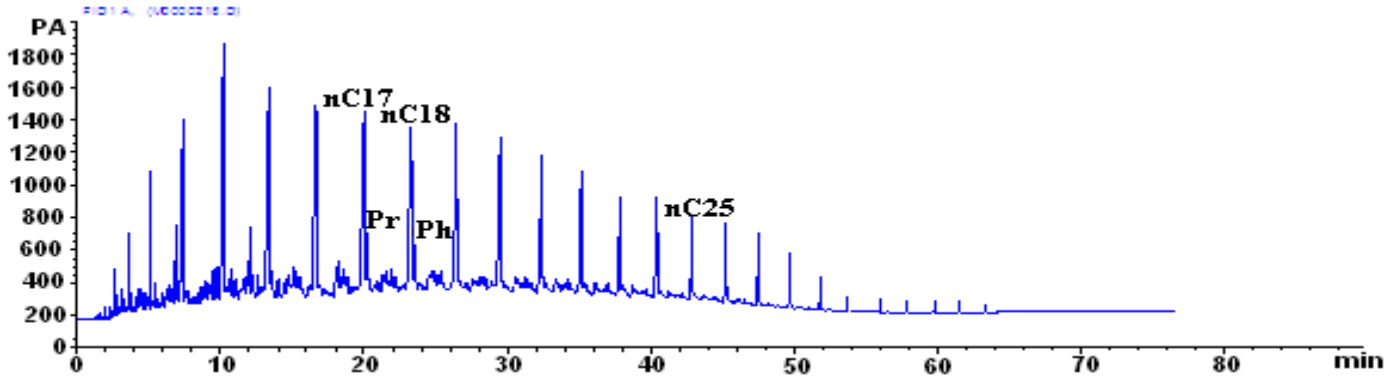


Fig. 2. GC-FID chromatogram of extracted petrogenic HCs before any biotreament methods.

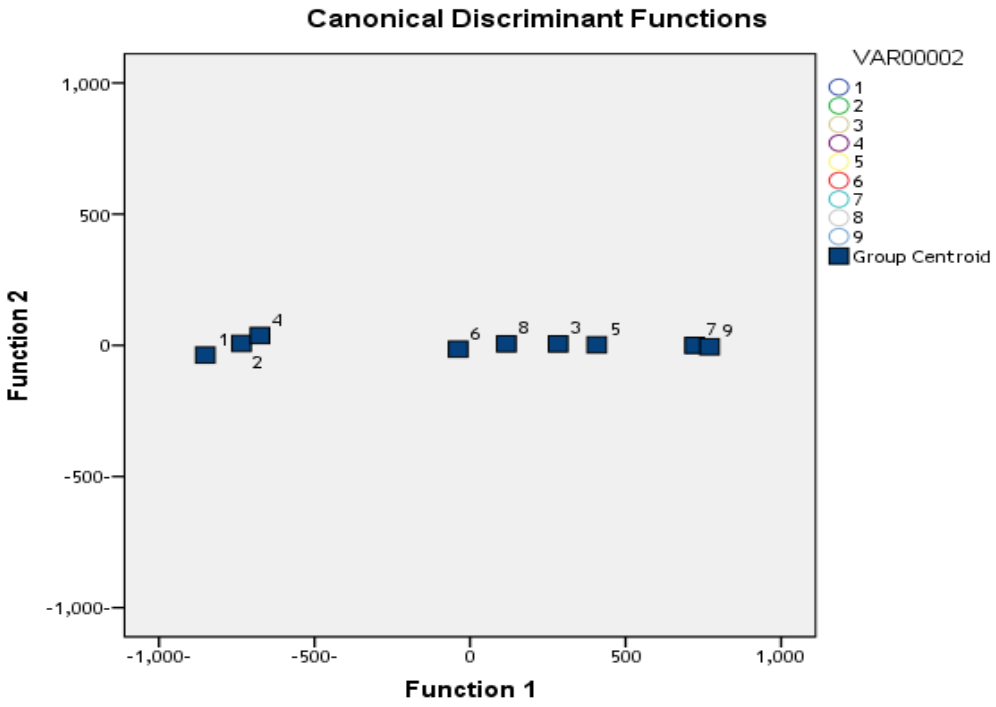


Fig.3. A discriminant function scatterplot. (1) NC; (2) NW; (3) Str and inoculated with Sh42; (4) Str and inoculated with IGTS8; (5) Str and inoculated with consortium; (6) BS; (7) Nstr and inoculated with Sh42; (8) Nstr and inoculated with IGTS8 and (9) Nstr and inoculated with consortium.

Table 4: Results of one way analysis of variance (ANOVA) and comparison between biotreatment methods, regarding the mean values of total viable count (cells/mL), TPH, pH and their statistical significance

Biotreatment flasks		cells/mL			TPH				pH	
Time		0 d	14 d	28 d	14 d		28 d		14 d	28 d
					mg/L	BD%	mg/L	BD%		
Negative control (NC)		0 **a**	0 **a**	0 **a**	194,98 **a**	0.12 **a**	194,59 **a**	0.32 **a**	7.02 **a**	7.02 **a**
Natural weathering (NW)		5.2×10^{3} **b**	3×10^{5} **b**	4.1×10^{6} **b**	193,07 **ab**	1.1 **a**	192,09 **b**	1.6 **ab**	6.89 **a**	6.82 **a**
Steriled (Str) and	Sh42	2.1×10^{6} **c**	1.4×10^{11} **cd**	9.6×10^{13} **ce**	171,01 **cd**	12.4 **b**	135,28 **d**	30.7 **d**	6.14 **a**	6.02 **a**
	IGTS8	3.0×10^{6} **c**	4.0×10^{10} **c**	8.4×10^{10} **d**	191,51 **b**	1.9 **a**	190,33 **b**	2.5 **b**	6.71 **a**	6.52 **a**
	Consortium	4.4×10^{6} **c**	6.1×10^{11} **cd**	1.2×10^{13} **c**	173,35 **d**	11.2 **b**	130.40 **e**	33.2 **e**	6.02 **a**	5.90 **a**
Biostimulation (BS)		5.2×10^{3} **b**	2.2×10^{7} **cb**	1.0×10^{11} **d**	169,84 **c**	13 **bf**	147,28 **c**	24.6 **c**	6.60 **a**	6.49 **a**
Non – sterilized (Nstr) and	Sh42	2.3×10^{9} **d**	4.6×10^{12} **d**	5.0×10^{14} **ce**	166,91 **e**	14.5 **be**	113,03 **f**	42.1 **f**	6.11 **a**	5.94 **a**
	IGTS8	6.4×10^{9} **d**	6.4×10^{10} **cd**	7.5×10^{12} **c**	175,30 **d**	10.2 **b**	144,85 **c**	25.8 **c**	6.58 **a**	6.45 **a**
	Consortium	9.0×10^{9} **d**	3.4×10^{12} **d**	9.0×10^{14} **e**	166,32 **e**	14.8 **ef**	110,69 **f**	43.3 **f**	6.01 **a**	5.82 **a**
F ratio		94.117	145.786	209.980	708.131	252.754	5925.692	1865.681	1.347	1.273
P value		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.283	0.317

Each value is mean of replicates. Means in a Column with similar letters are NS= not significantly different according to LSD. *** = significant at P value < 0.001.

Table (5): GC-FID parameters of extracted petrogenic pollutants before and after 28 d of incubation**.**

Parameter	Zero Time	Natural Weathering	Sterilized flasks			Biostimulation	Non-Sterilized flasks		
			Sh42	IGTS8	Consortium		Sh42	IGTS8	Consortium
TRP (BD%)	--	5.8	83.0	7.3	85.3	66.3	99.5	64.5	99.9
UCM (BD%)	--	0.9	63.4	4.1	57.2	72.1	85.7	69.8	99.6
n-C_n (BD%)	--	8.9	83.6	11.6	92.1	75.4	99.9	73.0	99.7
Iso-C_n (BD%)	--	4.5	82.7	4.2	75.4	53.3	98.9	52.6	99.4
Pr (BD%)	--	3.4	80.4	6.1	71.7	69.9	99.6	67.0	99.3
Ph (BD%)	--	2.7	75.6	3.2	62.7	63.0	99.4	61.2	95.5
nC_{17} (BD%)	--	3.6	94.1	10.7	91.7	80.9	96.7	77.7	99.2
nC_{18} (BD%)	--	4.5	94.3	6.8	91.1	67.4	94.9	64.5	99.8
TRP/UCM	0.30	0.29	0.14	0.29	0.10	0.37	0.01	0.36	0.06
n-Cn/Iso-Cn	1.56	1.49	1.47	1.44	0.50	0.82	0.19	0.89	0.51
nC_{17}/Pr	4.82	4.81	1.44	4.59	1.41	3.05	1.53	3.26	0.08
nC_{18}/Ph	5.16	5.06	1.21	4.97	1.23	4.54	0.97	4.72	0.02
Pr/Ph	1.14	1.13	0.92	1.11	0.87	0.93	0.25	0.97	0.83

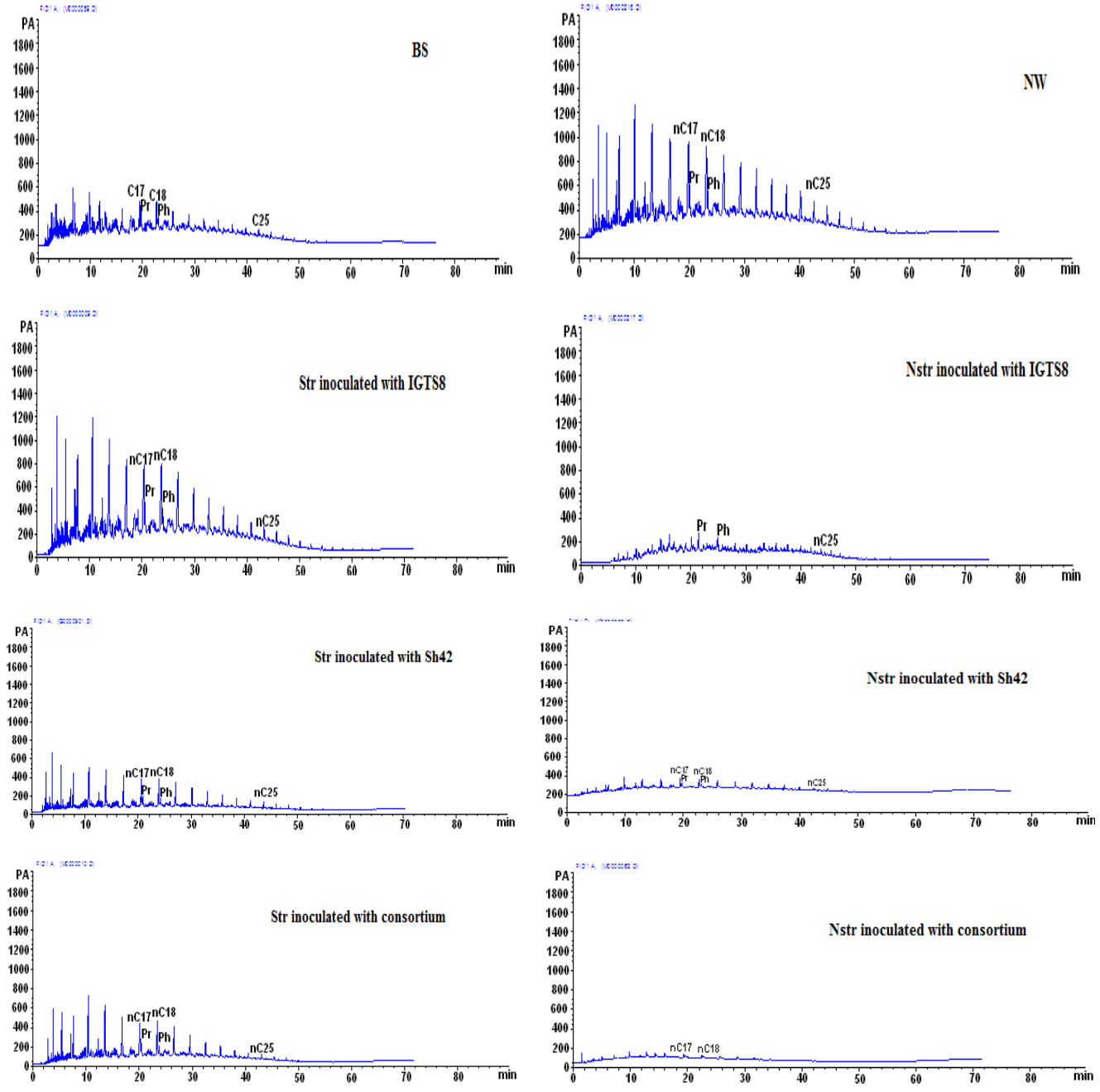


Fig.4. GC-FID chromatogram of extracted petrogenic hydrocarbons after 28 d of all biotreatment methods

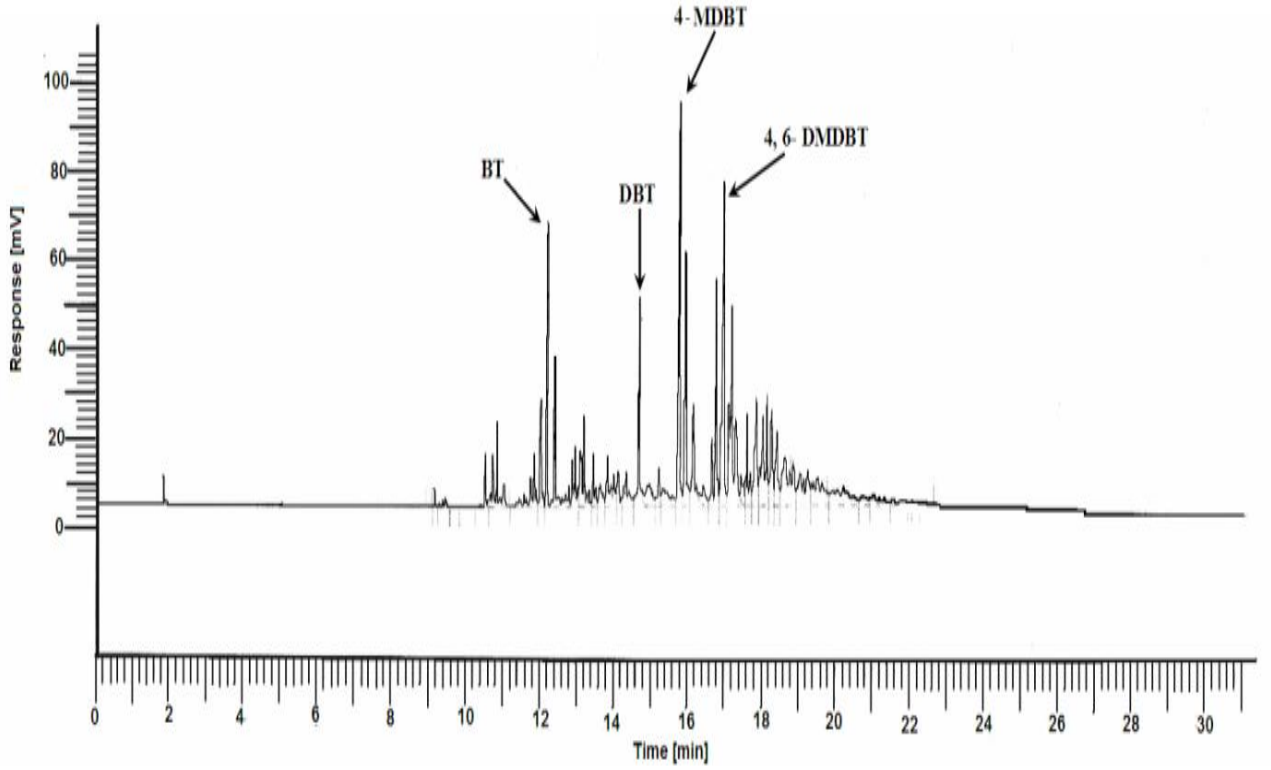


Fig. 5. Representative GC-FPD chromatogram of petrogenic HCs extracted from polluted water sample before any biotreatment

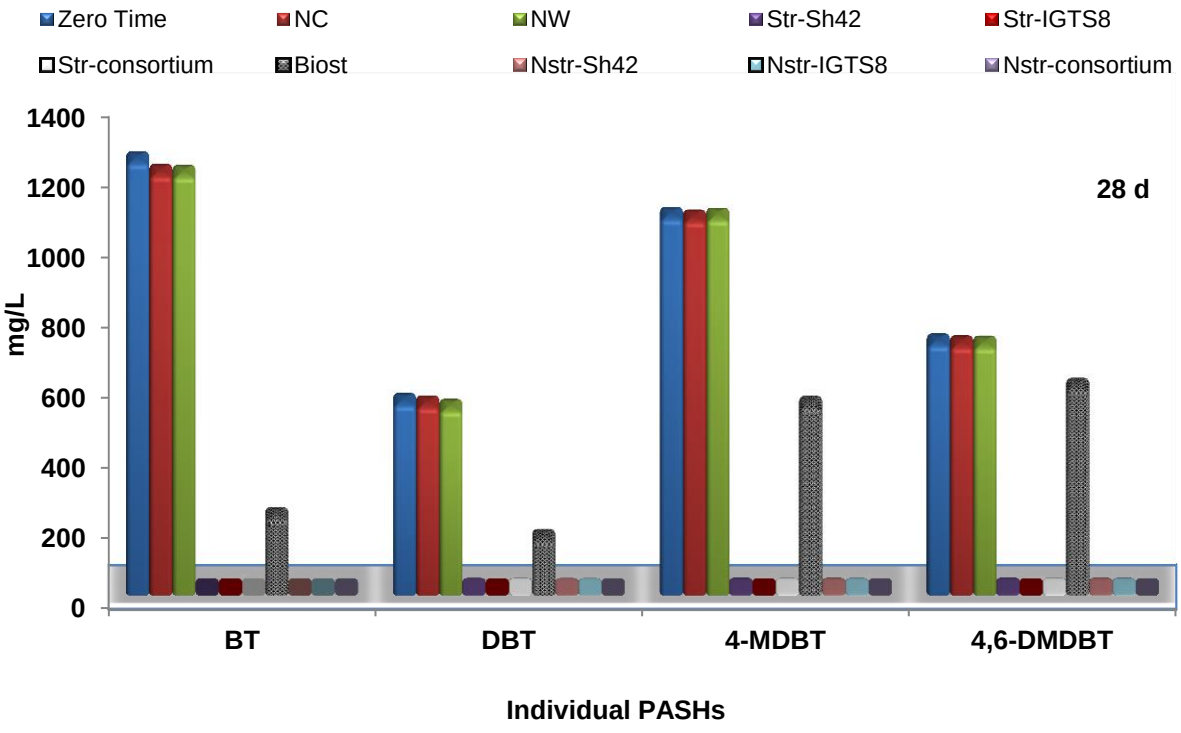


Fig. 6. Histograms of different PASHs removal using different biotreatment methods

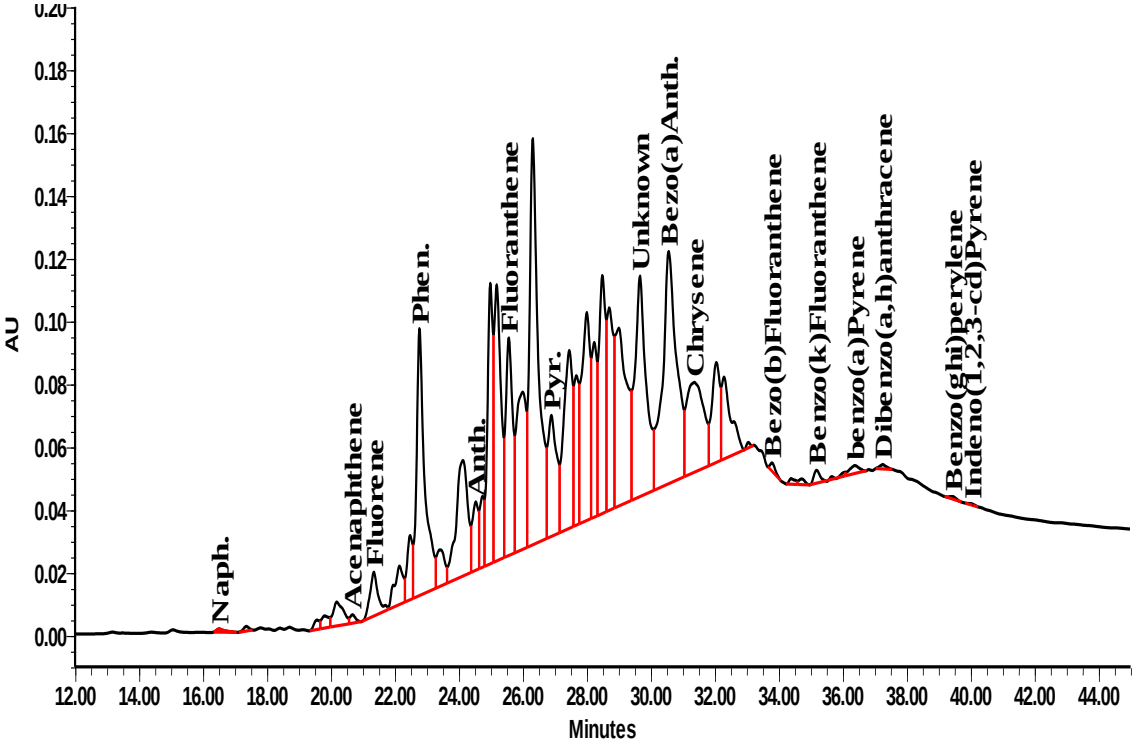
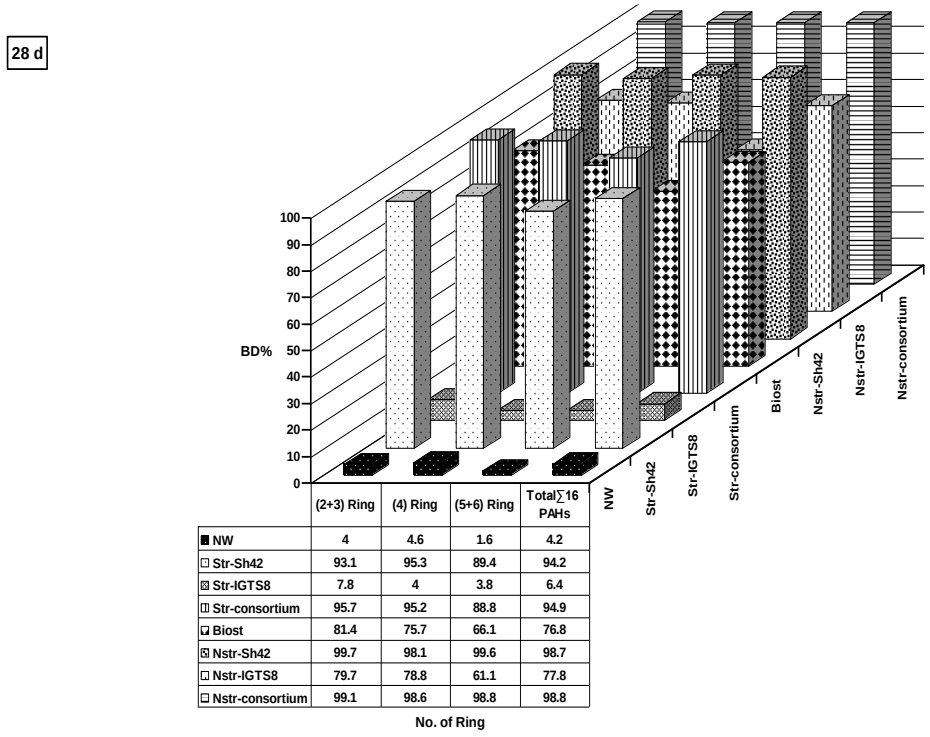
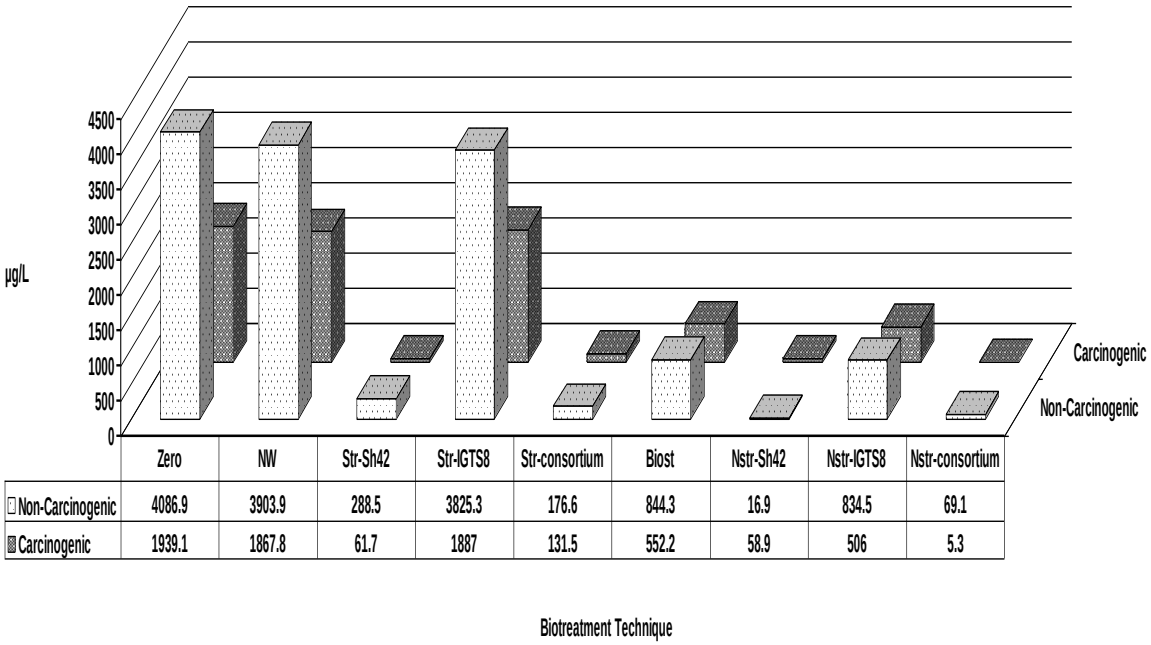


Fig.7. HPLC chromatogram of the 16PAHs listed by US EPA in oil extracted from collected water sample (site W1)



	(2+3) Ring	(4) Ring	(5+6) Ring	Total∑16 PAHs
NW	4	4.6	1.6	4.2
Str-Sh42	93.1	95.3	89.4	94.2
Str-IGTS8	7.8	4	3.8	6.4
Str-consortium	95.7	95.2	88.8	94.9
Biost	81.4	75.7	66.1	76.8
Nstr-Sh42	99.7	98.1	99.6	98.7
Nstr-IGTS8	79.7	78.8	61.1	77.8
Nstr-consortium	99.1	98.6	98.8	98.8

Fig.8. Biodegradation percent of 16PAHs with different number of rings using different biotreatment methods



	Zero	NW	Str-Sh42	Str-IGTS8	Str-consortium	Biost	Nstr-Sh42	Nstr-IGTS8	Nstr-consortium
Non-Carcinogenic	4086.9	3903.9	288.5	3825.3	176.6	844.3	16.9	834.5	69.1
Carcinogenic	1939.1	1867.8	61.7	1887	131.5	552.2	58.9	506	5.3

Fig.9. Concentrations of the carcinogenic and non-carcinogenic PAHs compounds before and after 28 d of biotreatment

In such case, canonical discriminant analysis is a powerful tool in determining biotreatment methods with highest BD% of TPH and discrepancies among them. It can be concluded from the analysis based on the canonical discriminant function examination and the relative positions of the data cases and group centroids illustrated in Figure (3) that there was a great diversity in all biotreatment methods. Where, results showed that Nstr flasks augmented with Sh42 or consortium were strongly similar with highest BD% while NW flasks and Str flasks augmented with IGTS8 were strongly similar with no significant difference in BD% relative to NC flasks.

The effect of different biotreatments on the hydrocarbon skeleton of the extracted petrogenic pollutants was quantitatively studied using GC-FID analysis and all data are recorded in Table (5) and represented in Figure (4). Calculations from GC-FID chromatograms of extracted petrogenic HC before and after different biotreatment methods showed that; Sh42 exhibits a marked capability to metabolic degradation of TRP as well as UCM which are nearly consumed after 28 d in Str and Nstr flasks inoculated with Sh42 and consortium. This indicates that Sh42 has high capabilities to degrade a broad versatility of different hydrocarbon compounds; aliphatic and aromatic components. On the other hand, it was obvious that; there was no significant recordable change in NW or Str flasks inoculated with IGTS8. This confirmed that IGTS8 is unable to utilize the hydrocarbons since it is a bacterium used for biodesulfurization of PASHs only and lake the enzymatic system required for attack of hydrocarbon skeleton [30- 31]. Yu et al. [32] and Caro et al. [33] reported that, the Gram-positive bacterium *R. erythropolis* IGTS8 able to extract sulfur from a variety of organosulfur compounds in oil without altering its hydrocarbon skeleton and thus maintaining TPH content.

Several parameters are used depending on gas chromatographic data where the change of these indexes is generally used to monitor the progress of metabolic activity. Among these ratios are; nC_{17}/Pr, nC_{18}/Ph, Pr/Ph and TRP/UCM. All biotreatment methods showed decrease in these ratios up till the stage that the recalcitrant Pr and Ph were nearly completely metabolized except in NW and Str flasks inoculated with IGTS8 which showed no recordable change in these ratios.

From the GC-FPD chromatogram of the extracted petrogenic HCs (Figure 5); it is clear that the main sulfur compounds are; BTs and DBTs and its alkylated forms, which are represented as major peaks. GC-FPD histograms (Figure 6) illustrate the effect of different biotreatments on PASHs. There is approximately complete degradation of all studied sulfur compounds (BT, DBT, 4-MDBT and 4,6-DMDBT) in all bioaugmented Str and Nstr flasks after 28 d. Also, BA by consortium (Sh42-IGTS8) in Str and Nstr showed the highest BD% after 14 d recorded ≈ 100% for all studied sulfur compounds. While, BS flasks, recorded ≈ 83%, 72%, 50% and 18% for BT, DBT, 4-MDBT and 4,6-DMDBT after 28 d, respectively.

Measuring the success of bioremediation of petrogenic pollutants is based on several parameters, among these, the metabolic rate of individual 16PAHs listed by US-EPA as priority pollutants in the contaminated site. Qualitative and quantitative determinations of individual 16PAHs were done using HPLC analysis (Figure 7). HPLC chromatograms showed approximately complete metabolism of 16PAHs occurred after 28 d in Str and Nstr flasks inoculated with Sh42 individually or consortium with IGTS8. Quantitative determination of the 16PAHs at 28 d is summarized in Figure (8) and confirmed the above observation. NW flasks showed no significance effect on ∑16PAHs recording BD% of ≈ 4% after 28 d except for 2-

ring PAHs (Naphthalene) recording BD% of ≈ 36%. From results illustrated in Figure (8), it is well observed that there is no significant difference in BD% of ∑16PAHs between; (i) Nstr flasks inoculated with Sh42 or consortium, both recorded BD% of ≈ 99%, (ii) Str flasks inoculated with Sh42 or consortium, both recorded BD% of ≈ 95%, (iii) BS and Nstr flasks inoculated with IGTS8, both recorded BD% of ≈ 77 and (iv) Str flasks inoculated with IGTS8 after 14 d and 28 d, both recorded BD% of ≈ 5%. Generally in all biotreatment flasks, biodegradation percentages of different types of PAHs can be ranked in the following decreasing order: 2+3-ring > 4-ring > 5+ 6-ring, which coincided with the general idea that PAHs with less rings are more easily degraded. It can be noticed that PAHs degradation was in parallel with growth potential except for Str flasks inoculated with IGTS8. This confirms that IGTS8 is unable to utilize PAHs as carbon and energy source despite of their sufficient growth which might indicate its lack of enzyme system responsible for PAHs biodegradation.

It is well observed from Figure (9) that; the carcinogenic PAHs compounds were very high in the extracted petrogenic hydrocarbons (recording ≈ 1940 µg/L) while after 28 d these high values are completely removed in Nstr flasks inoculated with Sh42 and consortium recording BD% of ≈ 97% and 99%, respectively.

4. Conclusion

In conclusion, this study showed;

(i) The advantages of using consortium cultures instead of pure cultures in bioremediation processes. These advantages could be attributed to synergistic interactions among the members of the association, which can be complex and favor petrogenic HCs degrading mechanisms. This is possible when one species removes toxic metabolites (Sh42) of another species (IGTS8) that begins the biodegradation process, or when two species work in succession with the first partially degrading compounds and the second finishing the job. This phenomenon is completely discussed in further work (in press).

(ii) In addition, mixed populations (consortium) with broad enzymatic capacities and co-metabolic relationships are more favorable to attack and metabolize not only aliphatic or aromatic compounds but also nitrogen, sulfur and oxygen (NSO) compounds.

(iii) *C. variabilis* sp. Sh42, has high capabilities to bioremediate different hydrocarbons i.e. aromatic, hetero-aromatic and aliphatic compounds.

(iv) Finally, the results of this experiment show that bioaugmentation with *C. variabilis* sp. Sh42 may be considered as a valuable method to enhance the bioremediation process and remove petrogenic hydrocarbon pollutants in the studied polluted area (El-lessan area).

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