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Effect of Triton X-100 on Bioremediation of PAHs of Medium Crude Oil in Soil

Saeed Minoui[1] • Dariush Minai-Tehrani[2] *

[1] Department of Environmental Pollutants, Environmental Sciences Research Institute, Shahid Beheshti University, G.C, Tehran, Iran
[2] BioResearch Lab, Faculty of Biological Sciences, Shahid Beheshti University, G.C, Tehran, Iran

Corresponding author: * **D_MTehrani@sbu.ac.ir**

ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are a group of chemicals that occur naturally in coal, crude oil, and gasoline. PAHs can also be released into the air during the burning of fossil fuels, garbage, or other organic substances. PAHs are found throughout the environment in the air, water, and soil, and can persist in the environment for months or years. Some PAHs are harmful to organisms. In this study, during a four-month period the effect of different concentrations of Triton X-100 (0-0.25%) on the bioremediation of medium crude oil obtained from Tehran refinery and its PAH components was studied in the soil obtained from a cultivation site near the Tehran refinery. Triton X-100 at low concentrations (0.01-0.025%) had a greater effect on the reduction of total PAHs than at a high concentration (0.05-0.25%). The highest crude oil and total PAH reduction was observed at 0.025% followed by 0.01% Triton X-100 samples, while the lowest was observed at 0.25% Triton X-100. The HPLC pattern of samples showed that naphthalene and acenaphthylene were reduced in all the samples as well as in the dry control. The highest reduction of three- and four-ringed PAHs such as phenanthrene, anthracene, fluoranthene, pyrene and chrysene was observed at 0.025% followed by 0.01% Triton X-100; least reduction occurred with 0.25% followed by 0.1% Triton X-100.

Keywords: bioavailability, biodegradation, detergent, HPLC, microorganisms, oil-contamination
Abbreviations: PAH, polycyclic aromatic hydrocarbon

INTRODUCTION

Oil pollution in soil arises from various sources including leakage from pipelines, refining process and transportation. The spillage of crude oil in soil can damage the environment and ecosystems. Crude oil contains aliphatic and aromatic fractions some of which are toxic to living organisms (Hammond *et al.* 1976; Gibbs 1997; Armstrong *et al.* 2004). Crude oil can be biodegraded by some bacteria such as *Rhodococcus*, *Nocardia, Acinetobacter and Pseudomonas* (Hamamura *et al.* 2006; Gouda *et al.* 2007). Polycyclic aromatic hydrocarbons (PAHs) are an oil component whose range of biological effects has been demonstrated, including toxicity, carcinogenicity and mutagenicity (Albert 1995). Crude oil contains different PAH compounds. Some PAHs are biodegraded by complex natural communities of fungi and bacteria (Cerniglia 1984, 1997). Various factors can enhance the biodegradation of crude oil and its components such as fertilizers, pH, salinity and some surfactants (Leahy and Colwell 1990; Mille *et al.* 1991; Kästner *et al.* 1998). Some reports indicated that the presence of surfactants inhibit the biodegradation of hydrophobic organic compounds and PAHs (Laha and Luthy 1991; Tiehm 1994). Surfactants may reduce the adhesion of bacteria to hydrophobic surfaces (Stelmack *et al.* 1999). This mechanism may be important for the biodegradation of virtually insoluble contaminants, and therefore the use of surfactants may not be beneficial. Other reports have suggested that the preferential utilization of surfactants by PAH degraders was responsible for the inhibition observed in the biodegradation of hydrocarbons (Deschenes *et al.* 1996). Other reports indicated that the presence of surfactants enhances biodegradation (Bury and Miller 1993; Doong *et al.* 1996; Wong *et al.* 2004; Hickey *et al.* 2007). The most important effect of surfactants on the interactions among soil and pollutants is stimulation of mass transport of the pollutant from the soil to the aqueous phase (Volkering *et al.* 1997).

Surfactants can help solubilize non-polar material in the liquid phase and increase its biodegradation. Triton X-100 is a non-ionic detergent widely used in extraction of membrane proteins. This compound may be toxic to microorganisms at high concentrations. The effect of this detergent on biodegradation of naphthalene and phenanthrene by *Pseudomonas* and microbial consortium has been studied (Allen *et al.* 1999; Kim *et al.* 2001).

In this report the effect of different concentrations of Triton X-100 on biodegradation of PAHs of medium crude oil in soil by microbial communities (fungi and bacteria) was studied for four months. The results of this work could help understand the role of non-ionic surfactants in bioremediation of contaminated soil in environment.

MATERIALS AND METHODS

Soil preparation

Non-polluted soil was obtained from a cultivation area near the Tehran refinery. The soil of this site was chosen because some of these areas (around Tehran refinery) are the most polluted sites in Tehran, and the non-polluted areas have a risk of becoming contaminated. The soil was analyzed for its texture (silty clay loam) by a hydrometer method (Bouyoucos 1962; Gee and Bauder 1986; Robertson *et al.* 1999). The soil characteristics are shown in **Table 1**. The soil was dried at 50°C for 48 h and well crushed to homogeneity. Medium crude oil (American Petroleum Institute, API gravity = 30) was also obtained from Tehran refinery (**Table 1**) and added to the soil to a final concentration of 2% (w/w). To mix the soil with oil, the soil was transferred into a pail and the oil was added to the soil. The lid of the pail was fastened tightly and the soil in the pail was shaken firmly by hand. Shaking was continued

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Table 1 The soil and crude oil characteristics used in this experiment.

Soil parameters	
Clay	33%
Silt	55%
Sand	12%
Organic matter	3.1%
Calcium carbonate	28%
pH	7.3
Total N	0.02%
Total P	5 ppm
Crude oil parameters	
API gravity	30
Sulfur (wt%)	3.16
Pour point (°C)	-18
Asphaltenes (wt%)	2.6

until the soil was homogeneously mixed (Minai-Tehrani 2008). To confirm the homogeneous contamination of the soil, two samples were taken from different parts of the soil and determined for the oil contamination using the method that has been explained in the crude oil extraction. The soil was divided into equal portions, each of which contained 500 g of soil and transferred to a 2-L pail. The dry oil-contaminated soil remained for a week to let the highly volatilized fractions of oil release from the soil. After a week, Triton X-100 (Merck analytical grade) at final concentrations of 0.01 to 0.25% (v/w soil) was solubilized in 200 ml of distilled water and solubilized Triton X-100 was added to the soils. The soil was mixed by hand, so Triton X-100 could be evenly and homogeneously distributed.

After determining the water holding capacity (field capacity) of the soil, the moisture of the soil was maintained to about 30% using distilled water in all pails, except for the dry control which had no water. The soil water content was measured by gravimetric method throughout the experiment (Robertson *et al.* 1999).

The soil was mixed every other day thereby inducing aeration in the pails. Aeration was done once by mixing the soil and by turning the whole soil within the pails upside down for about 10 min. The dry control sample did not contain Triton X-100 or moisture but it was aerated during the experiment. Each sample was prepared in triplicate.

For further study and to compare the amount of oil and PAHs reduction in treated samples after 4 months and the samples at start time, 20 g of dry contaminated soils from all samples were removed and kept at 4°C as time zero.

The pH of soil was determined to be 7.4 for soil-distilled water slurry (1:5, w/v). The amount of nitrate and phosphate of the soil are important factors for increasing the efficiency of oil biodegradation, as a result for each 1000 mg of crude oil about 150 mg of nitrate (NH_4NO_3) and 30 mg of phosphate (KH_2PO_4) were added to all pails (Rosenberg and Ron 1996).

All the pails were placed at room temperature (25°C ± 5) throughout the duration of the experiment.

Total colony count

Determination of the number of colonies in the soil was completed using the pure-plate method (Cappuccino and Sherman 1996) every two months and compared with time zero. From each sample, 1 g of soil was dissolved in 9 ml of autoclaved NaCl solution (9 g/L) and serial dilutions were prepared for each sample. Diluted samples were transferred to nutrient agar (Merck) plates. The plates were incubated at 30°C for 48 h and the number of colonies was counted.

Crude oil extraction from soil

Extraction of crude oil was conducted according to the method previously established (Minai-Tehrani and Herfatmanesh 2007). For 48 h, 2 g of soil were dried at 50°C then crushed well. A total of 10 ml of CH_2Cl_2 (Aldrich) was added to the soil and shaken firmly. The sample was centrifuged (3000 × *g* for 10 min) to precipitate the soil, and the solvent phase was removed. This solvent extraction was repeated twice. The solvent was vaporized for 24 h and the amount of oil was measured gravimetrically and its reduction was compared with time zero.

HPLC analysis

After extraction of the crude oil by the above mentioned method, the residue was dissolved in 5 ml *n*-hexane (Merck) and filtered. The sample was loaded to a 1 × 25 cm column filled with 20 cm Silica Gel and 5 cm Na_2SO_4 (Merck). The column was pre-washed by *n*-hexane. A total of 30 ml of *n*-hexane was used as the mobile phase to release the aliphatic fractions.

To release PAHs fraction from the column, a total of 30 ml of *n*-hexane/dichlromethane (1:1, v/v) was used and the PAHs were collected and the solvent was evaporated. The residue was weighed to determine the amount of total PAHs of each sample. The residue was dissolved in 5 ml acetonitrile (Fluka, HPLC grade) and 20 µl were injected into the HPLC column (Shimadzu LC 10A HPLC system equipped with a C18 column), with water/acetonitrile (1:2, v/v) as the mobile phase and a flow rate of 1 ml/min equipped by a UV detector at 254 nm. Some PAHs such as naphthalene, acenaphtylene, phenanthrene, anthracene, fluoranthene, pyrene and chrysene were prepared as standards (Supelco mix PAHs standard) and injected into the HPLC column. The regions of their exit from the column were used to localize them in main graphs.

The total peak area of each compound in the graphs was used to determine the reduction of PAHs and compared with time zero.

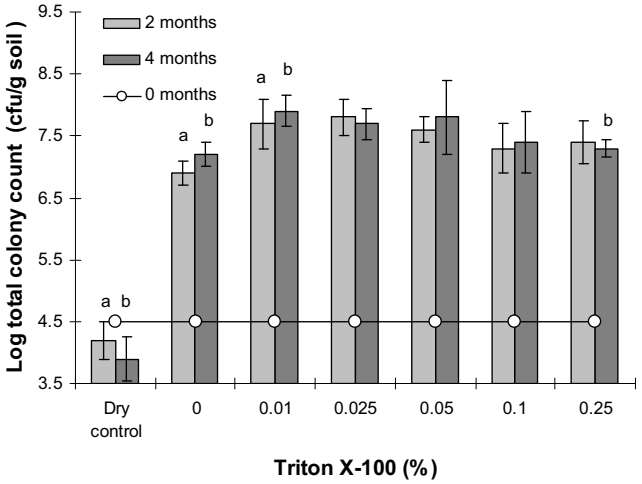

Fig. 1 Total bacterial count (CFU/g soil) in time zero and after months 2 and 4. In all treated samples the number of bacteria has increased. Average values given ± standard deviation ($n = 3$).

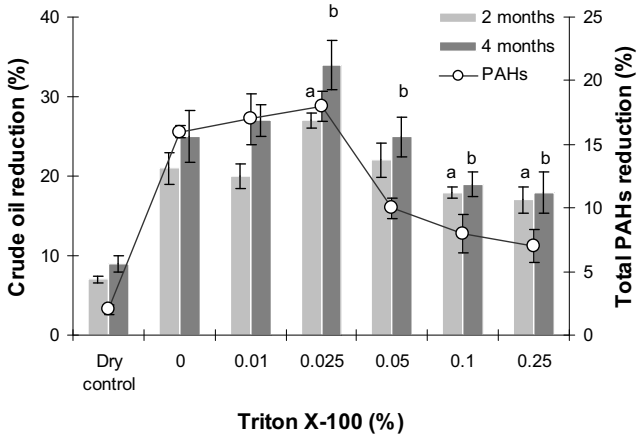

Fig. 2 Reduction of total crude oil reduction in month 2 and 4 and the reduction of total PAHs ($n = 3$).

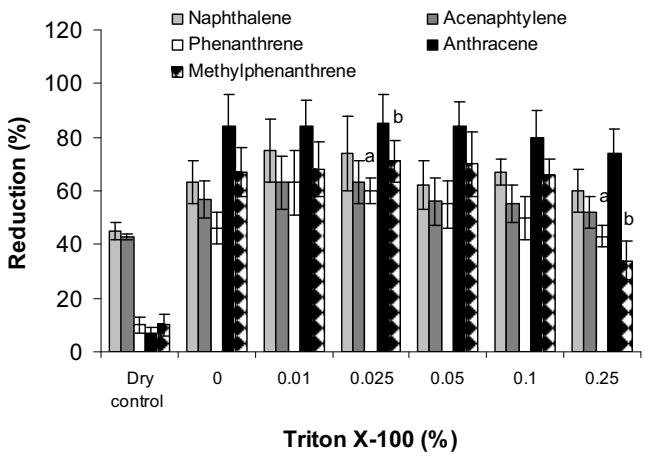

Fig. 4 Reduction of two and three rings PAHs (naphthalene, acenaphtylene, phenanthrene, methylphenanthrene and anthracene) in the samples (*n*=3).

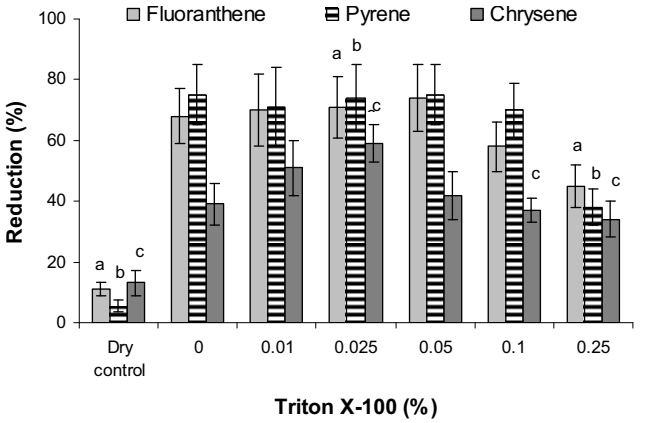

Fig. 5 The reduction of four rings PAHs (fluorenthene, pyrene and chrysene) after 4 months (*n* = 3).

Statistical analysis

Results were expressed as mean ± standard deviation (±SD) and the analysis of variance and statistical significant difference ($p<0.05$) was performed by one-way ANOVA with mean separation using Tukey's test. The statistical results were analyzed by Graphpad Prism 5 program.

RESULTS

Total colony count

Total colony count showed that in all the samples the population of bacteria has been increased in comparison with time zero (**Fig. 1**). There was no significant difference on colony count in all samples between the second and fourth months. However, the difference between the samples with 0.01 and 0.025% of Triton X-100 with 0% (wet control) was significant.

Total crude oil and PAHs reduction

Fig. 2 shows the effect of Triton X-100 on biodegradation of total crude oil and PAHs after 4 months. The lowest reduction was seen in 0.25% followed by 0.1 and 0.05% of Triton X-100. The highest reduction was observed in 0.025% followed by 0.01 and 0% of Triton X-100. The value of reduction of total crude oil and PAHs was significant between 0.025% sample and the samples with concentrations higher than 0.05% of Triton X-100 (0.05-0.25%), while it was not significant between 0.025% and the samples with lower concentrations of Triton X-100 (0-0.01%).

HPLC analysis

Fig. 3 shows HPLC pattern of PAHs in samples after 4 months. The peaks of PAHs in HPLC graphs of treated samples were compared with the peaks of PAHs in HPLC graph at time zero. The reduction of all peaks was higher in 0.025% and it was lower in 0.25% and 0.1% Triton X-100 samples. Some PAHs such as naphthalene and acenaphthylene disappeared in nearly all samples as well as dry control (**Fig. 4**). The reduction of three rings PAHs such as phenanthrene, anthracene and methylphenanthrene was higher in 0.025% followed by 0.01% samples, while their reduction were lower in 0.25% sample (**Fig. 4**). However, there was no significant reduction between the 0.025% sample and other samples containing Triton X-100 except the 0.025% sample in which the reduction of phenanthrene and methylphenanthrene was significant. The reduction of the four-ring PAHs such as fluoranthene, pyrene and chrysene was also higher in 0.025% followed by 0.01% samples, while it was lower in 0.25% sample (**Fig. 5**). The reduction of chrysene was lower in all samples in comparison to other four rings PAHs. The reduction of fluoranthene, pyrene and chrysene was significant between 0.025% and 0.25% sample.

DISCUSSION

This study focused on bioremediation of some PAHs in the presence of Triton X-100 in soil. Our results showed that microbial population in the samples increased after month 2 and it remained nearly constant until month 4 (end of experiment) suggesting that the conditions in the soil were suitable enough for microbial growth during the experiment. The comparison of total crude oil and PAHs reductions in treated samples (**Fig. 2**) suggests that biodegradation of crude oil and its PAHs components has been done efficiently in lower concentrations of Triton X-100 (0-0.025%) while biodegradation was less efficient in higher concentrations of Triton X-100 (0.1-0.25%). Some reports indicated that addition of Triton X-100 at a concentration greater than its CMC (critical micelle concentration) inhibited adhesion of bacteria to solid surface, which in turn prevented degradation of both hexadecane and naphthalene (Efroymson and Alexander 1991; Ortega-Calvo and Alexander 1994; Hickey *et al.* 2007).

Since the main factor of reduction of PAHs in the dry control sample was volatilization, the high reduction of naphthalene and acenaphtylene in all the samples as well as the dry control, suggests that volatilization was the main factor for reduction of these PAHs. Biodegradation and volatilization are two factors that reduce the amount of crude oil and its fractions in soil (Atlas 1981; Leahy and Colwell 1990; Nicodem *et al.* 1997).

In contrast the three and four-ring PAHs were not reduced with high efficiency in the dry control sample, while their reduction in treated samples was almost high suggesting that biodegradation has played the main role rather than volatilization. The reduction of total PAHs was higher in 0.025 and 0.01% of Triton X-100 in comparison with 0% samples suggesting that Triton X-100 could increase the biodegradation of PAHs in 0.025 and 0.01% Triton X-100 samples. However, looking at the histograms in **Figs. 4** and **5**, the vast majority of the differences in individual PAH (except chrysene) reduction between the 0.025% Triton X-100 treatment and those receiving higher concentrations of the surfactant (0.1 and 0.25%) were statistically significant. Some studies have reported that the use of surfactants was not beneficial for hydrophobic contaminants biodegradation (Deschenes *et al.* 1996; Stelmack *et al.* 1999; Makkar and Rockne 2003). There were also significant differences between dry control and all the other treatments which are presumably due to biodegradation resulting from the addition of water to the soil plus aeration, indicating that the individual PAHs were reasonably bioavailable in the soil in

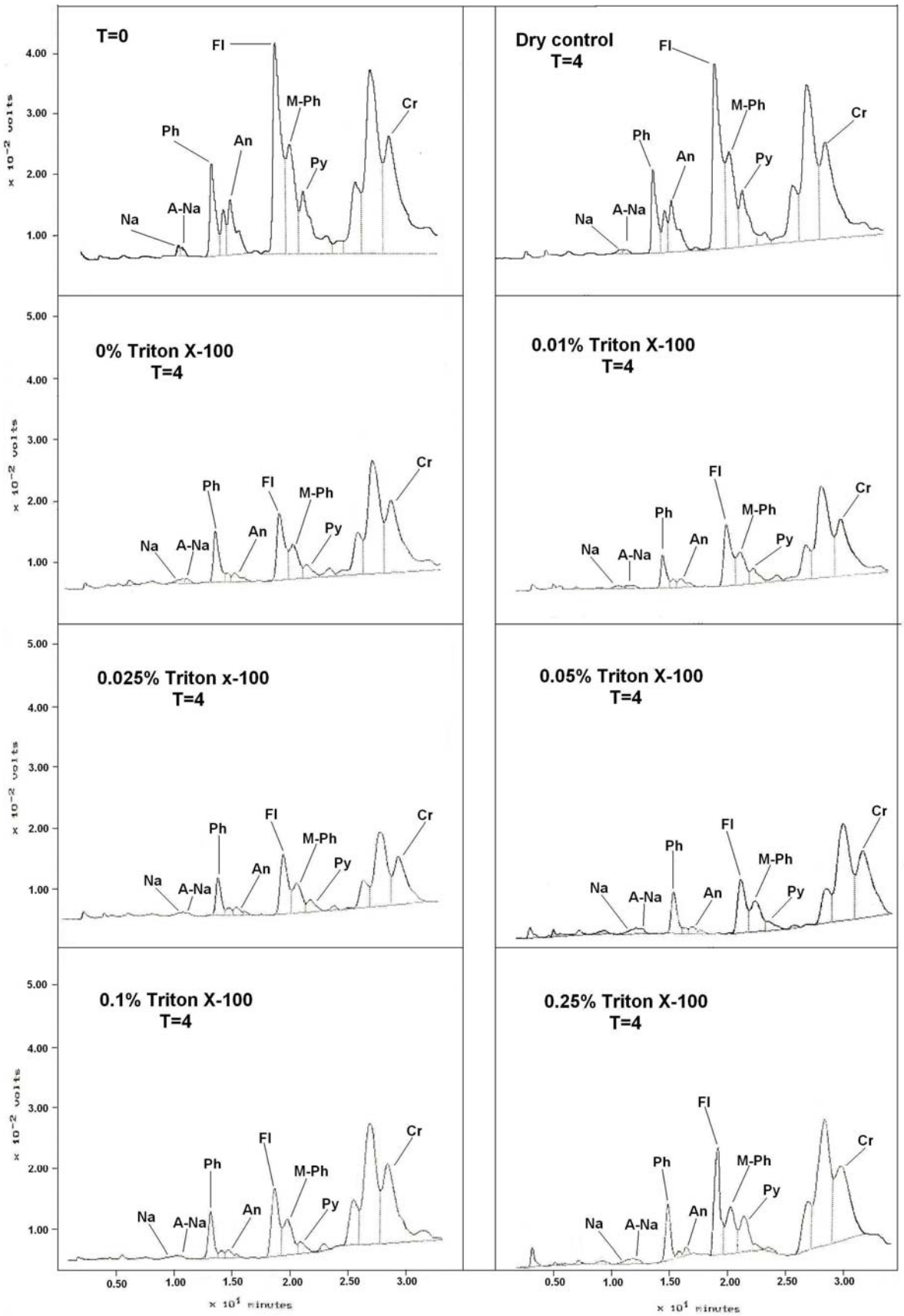

Fig. 3 HPLC pattern in treated samples after 4 months (T = 4). All the peaks of PAHs were compared with the peaks at time zero (T = 0). (Na = naphtalene, A-Na = acenaphtylene, Ph = phenanthrene, An = anthracene, Fl = fluoranthene, M-Ph = methylphenanthrene, Py = pyrene and Cr = chrysene).

the lower concentrations of Triton X-100.

CONCLUSION

Triton X-100 with low concentrations should have an apparent significant effect on the reduction of total PAHs. Our result also suggests that chrysene and the PAHs with more than four rings with lower solubility might be more bioavailable than the four-ring PAHs in the presence of low concentrations of Triton X-100.

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