Assessment of the biodegradation capacity of *Azolla* on polycyclic aromatic hydrocarbons in crude oil

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Abstract

In this study, the potential use of *Azolla filiculoides* Lam. for the bioremediative solution to polycyclic aromatic hydrocarbon (PAH) pollution due to crude oil spills in freshwater was investigated. The plants were grown in nitrogen-free Hoagland nutrient solution media containing 0.05%, 0.1%, 0.2%, 0.3%, 0.4% and 0.5% crude oil by volume for 15 days under greenhouse conditions. Relative growth rates of *A. filiculoides* decreased in the presence of crude oil in a concentration-dependent manner. The probable influence of *A. filiculoides* on the biodegradation of polycyclic aromatic hydrocarbons was measured by using synchronous UV fluorescence spectroscopy. GC-MS analysis were also carried out to elucidate the behavior of the oil in experimental and control samples. Although 1-2 rings PAHs have not been encountered in control or plant samples, the measured intensity for 3-4 ring PAHs in plant samples was remarkably lower in comparison to the control. Furthermore, these results demonstrated that the predominant efficacy of the *A. filiculoides* was for 3-4 ring PAHs at the range 0.05 to 0.2% crude oil concentrations. It could be concluded that the bioremediative potential of *A. filiculoides* for the removal of polycyclic aromatic hydrocarbons strongly depends on the amount of oil in the contaminated water resource. In other words, *A. filiculoides* could be used more effectively after the removal of excess crude oil in the spilled freshwater areas.

Keywords: Relative growth rate; crude oil; *Azolla*; PAHs; biodegradation

1. Introduction

Crude oil has been a veritable source of economic growth to modern societies from the point of view of its energy and industrial importance (Okoh 2006). Moreover, increased energy demand places crude oil among the major issues for the next two decades (Aljuboury *et al*. 2017). Petroleum is a complex mixture of *n*-alkanes, aromatics, resins and asphaltenes. Many of these components are toxic, mutagenic and carcinogenic (Zappi *et al*. 1996; Propst *et al*. 1999). Natural crude oil contains significant amounts of polycyclic aromatic hydrocarbons (PAHs). PAHs are composed of two or more aromatic (benzene) rings. Because of their bioaccumulation potential and carcinogenic activity, they have gathered significant environmental concern. (Haritash and Kaushik, 2009). Therefore, their release to the environment is strictly controlled. Because of exponential increase in worldwide oil consumption, more frequent oil tanker accidents, and occasional leaks from oil pipelines, petroleum hydrocarbons have become one of the most abundant and harmful pollutants in soil and water resources (Vasudevan and Rajaram, 2001; Mrayyan and Battikhi 2005). In return, this fact has necessitated the development of new techniques for the removal of contaminants (Millioli *et al*. 2009). Phytoremediation is an emerging and the most promising technology, defined as the use of plants and associated microorganisms to remove, transform, stabilize, volatilize or assimilate toxic chemicals located in soils, water and air via various biochemical process (Rajkumar *et al*. 2013; Jagtap *et al*. 2014)

There is an increasing number of studies that evaluate the use of plants in the petroleum contaminated freshwater areas and in these researches different plant species such as *Landoltia punctata* (Ertekin *et al.* 2015), *Vetiveria zizanioides* (Effendi *et al.* 2017) and *Lemna minor* (Wang *et al.* 2017) have been used. *Azolla* has a special significance between the freshwater plants used in phytoremediation. It is a fast growing species and can double it's biomass in 5 to 10 days (Kollah *et al.* 2016) and for these reason *Azolla* is often used in phytoremediation studies (Kosesakal *et al.* 2016; De *et al.* 2017; Gomes *et al.* 2018).

The main purpose of this study was to elucidate the biodegradation potential of a freshwater fern species, *Azolla filiculoides,* on polycyclic aromatic hydrocarbons in crude oil.

2. Materials and Methods

2.1. Cultivation of plants and application of crude oil

Azolla filiculoides plants were grown in nitrogen-free Hoagland nutrient solution 26-28 °C under greenhouse conditions. In this study, crude oil from Batman Refinery (Batman, Turkey) was used. The effect of different amounts of crude oil (0.05%, 0.1%, 0.2%, 0.3%, 0.4% and 0.5% by volume, for 15 day) on the growth of *Azolla filiculoides* was investigated. Oil applications were done as follows: The crude oil 0.005% 0.01%, 0.05%, 0.1%, 0.2%, 0.3%, 0.4% and 0.5% concentrations (v/v) were added into glass beakers (500 ml) containing N-free Hoagland nutrient solution. The beakers containing different oil concentrations containing N-free Hoagland nutrient solution used as negative control. 24 hours after the application of crude oil, 3 grams of plants were weighed from each experimental series and left to grow under greenhouse condition for another 15 days before the finalization of the experiment. All the experiments were performed with three replicates.

2.2. Analysis of plant growth

A. filiculoides relative growth rate (RGR) (g g^{-1} d^{-1}) was calculated by the formula;

RGR = (ln W2−ln W1/t, where W1 and W2 are the initial and final fresh weights, and t is the experimental time (Jampeetong and Brix, 2009).

2.3. Extraction and clean-up

To evaluate the degree of hydrocarbon degradation, liquid/liquid extraction was applied to the experimental growth media was employed with (DCM) dichloromethane (U.S. EPA, METHOD 3510C). After the termination of each crude-oil applications, the plant samples were taken from the experimental growth mediums, according to U.S. EPA METHOD 3541 automatic Soxhlet extraction (SER 6 VELP) was applied. 2-fluorobiphenyl (Dr. Ehrenstorfer, Germany) was added as internal standard. The extracts obtained from the samples according to U.S. EPA METHOD 3600C by column chromatography to be divided into 2 fractions as aliphatic and aromatic groups. Total extracts were fractionated by applying to Fluorosil columns. In brief, the extracts (0.5 to 1 ml) was added carefully and the column fractions were collected: (1) 10 ml of hexane (aliphatic hydrocarbons), and (2) 10 ml 1:1 dichloromethane: hexane (aromatic compounds). The extracts were concentrated to 100 microliters under the nitrogen gas flow.

2.4. Analysis of petroleum hydrocarbons

Total petroleum hydrocarbons (∑TPH) were analyzed by UV fluorescence (UVF) spectroscopy (RF-5301, Shimadzu). The samples were diluted to within the linear calibration range of the spectrofluorometer. Excitation and emission wavelengths were fixed to 310 and 360 nm for single measurement, respectively. The calibration curves were plotted in the range of 0.25-2 µg oil/ml hexane (Ehrhardt *et al*. 1992). The measurements were assured to be within the linear calibration range of the spectrofluorometer for Batman crude oil.

2.5. Spectrofluorometric analysis of PAHs

The samples were analyzed by synchronous UV fluorescence spectroscopy (SUVF, Jasco-6300, Shimadzu). For quantitative characterization, the excitation wavelengths scanned were at covered the range from 220 to 700 nm. The $\Delta\lambda$ interval between λ_{ex} and λ_{em} was constant and equal to 23 nm (Lloyd, 1971). Each sample was analyzed in triplicates in order to evaluate the reproducibility of the method. One cm length quartz cuvettes were used for the measurements.

2.6. GC/MS analysis

GC/MS analysis were performed with Perkin Elmer Thermo DSQ Turbo MSD system. The extracts were analyzed in splitless mode by using a fused silica capillary column HP-5 MS (30 m×0.25 mm ID×0.25 µm film thicknesses) and helium (1 ml/min) as carrier. The front inlet temperature was 280 °C and the injector was set for splitless injection. The GC oven temperature was programmed from 50 °C (held for 1 min) to 320 °C at a rate of 10 °C min^{-1} and maintained at 320 °C for 5 min. The MS temperature program for transfer line was 220 °C. The MS was operated in EI mode (70 eV) scanning from 50 to 600 amu. The library search was carried out using Wiley and NIST GC/MS library. The GC/MS was calibrated for aliphatic hydrocarbons (n-alkanes calibration mixture purchased from Dr. Ehrenstorfer, Germany) from nC_{10} to nC_{35} and the selected PAHs by using the internal standard calibration procedure described in US EPA Method 8000 and 8015 respectively. All of the solvents were of HPLC grade.

2.7. Statistical analysis

All the experimental data were obtained in 3 replicates. The experimental results are expressed as mean ± standard deviation (SD) of triplicate measurements and analyzed. Statistical analysis was performed using GraphPad Prism version 5.2 for windows (GraphPad Software, San Diego, CA). Significant differences between the means were determined by the the Dunnett's Multiple Comparison Test and Tukey's Multiple Comparison test.

3. Results and Discussion

3.1. Plant growth

The relative growth rate of the control plants was 0,148 g $g^{-1}d^{-1}$ at the end of the 15-day experimental period (Table 1). While RGR slightly impeded at 0.05% and at 0.1% oil concentrations, the oil concentration above 0.2% noticeably reduced the growth rate (Table 1). In this study, plant growth was inhibited in a dose dependent manner. Furthermore, our results indicates crude oil concentrations > 0.2% had a negative effect on plant growth by reducing RGR values. This is consistent with previous reports of the effect of diesel and crude oil on the RGRs of *S. grossus* (Al-Baldawi *et al.* 2015) and *L. punctata* (Ertekin *et al*. 2015) respectively. The hindering effect of oil on plant growth could be as a result of the phytotoxicity of the increased number of aromatic rings (Baek *et al*. 2004). Thus it could be stated that crude oil increased in growth inhibition effect according to their concentration and number of aromatic rings.

3.2. Total petroleum hydrocarbons (ΣTPH)

To determine the relative change in the amount of total petroleum hydrocarbons as a result of the application of plants at different oil concentrations, ΣTPH was measured from negative control media, plant growth media, and plant material by UVF (Table 2). TPH amounts obtained from the plant-free growth medium (negative control) increased, as expected, depending on the applied concentrations of oil (0.05%, 0.1%, 0.2%, 0.3%, 0.4% and 0.5%). On the other hand, the amount of TPH in growth media with the plants was remarkably decreased relative to the control media at the end of 15-day application period (Table 2). As also expected, the amount of TPH in plants were also increased with the increase in the percentages of oil (Table 2). Huang *et al*. (2005) reported that the amount of removed TPH from

soil was different for different systems ranging from 90%, 55%, 40%, and 20% for multi-process phytoremediation, phytoremediation, bioremediation and land-farming respectively at the end of the 8 months. It could be suggested that degradation of TPHs is likely to be as a result of physical, chemical and biological actions all together. In the unplanted control areas, the degradation is often caused by volatilization and photolysis (Peng *et al*. 2009). In this study, TPH amounts obtained from the planted growth media were significantly ($p<0.05$) less with respect to the unplanted control media (Table 2).

3.3. Determination of polycylic aromatic structure

The fluorescence of crude oils derives from the presence of PAHs formed by combinations of one or more highly fluorescent benzene rings (Vo-Dinh, 1984; Abbas, 2006). In synchronous spectra, compounds with different numbers of fused aromatic rings exhibit their maximum emissions at particular wavelengths (Kister *et al*. 1996). According to the number of aromatic rings, the US EPA priority PAH compounds are divided into three groups, representing two-, and three-, four-, and five-, and more-ringed PAHs (Law, 1981). SUVF values obtained from Batman crude oil and the control and the experimental samples shown in Fig.1 and Fig.2 Petroleum hydrocarbons in crude oil and light refined products are dominated by the lowest molecular weight PAHs with only trace levels of the penta- and hexa- aromatics present. However, 1-2 rings PAHs have not been encountered in our control and plant samples. It appears likely that the more volatile PAHs are rapidly lost in growth media, through evaporation and photooxidation (Fig. 2). Similarly, at the 0.05% oil applications 3-4 rings PAHs have not been encountered in both the control and experimental samples (Fig. 2). For oil concentrations 0.1-0.5%, the intensity of 3-4 ring PAHs was lower than the control, especially at 0.1% and 0.2% oil applications (Fig. 2). Similar results were also found in our previous study (Kosesakal *et al.* 2015). Thus, it could be said that the bioremediation capacity of *A. filiculoides* on crude oil is much better especially at lower concentrations.

3.2. PAHs and alkylated homologous PAHs

At the end of the experimental period, in order to elucidate the behavior of the oil at applied concentrations in experimental and control samples, GC-MS analysis were carried out. PAHs and alkylated homologous of PAHs in control and experimental samples were determined by library screening of the chromatograms (Table 3 and 4). PAH compounds are relatively stable and the distribution of alkylated PAH homologues have been used as environmental fate indicators. (Boehm *et al*. 1997). Generally, the degree of PAH degradation decreases with increasing ring size and within a homologous series and decreases with increasing alkylation (Barakat *et al*. 2001). In control samples, while the C0, C1 naphthalene compounds have not been encountered, C2 and C3 naphthalene compounds were determined at higher oil concentrations, 0.2%-0.5% (Table 3). On the other hand, for the experimental samples, while C0, C1, C2, naphthalene compounds have not been encountered only C3 naphthalene compounds were measured at the applied oil (0.05%-0.5%) concentrations (Table 4). Phenanthrene and alkylated homologues were determined in both control (Table 3) and experimental (Table 4) samples. Briefly, in this study, naphthalene and alkylated naphthalenes and phenanthrene and alkylated phenanthrenes were determined in both experimental and control samples. On the other hand, dibenzothiophenes, alkylated dibenzothiophenes, fluorenes and alkylated fluorenes have not been encountered in the experimental samples at 0.05% and 0.1% oil applications. Thus, it could be concluded that although the SUVF technique is a useful tool to determine polycyclic aromatic structure of a compound, GC/MS provides more precise data for the characterization of the present aromatic compounds.

4. Conclusion

In conclusion, the contribution of *A. filiculoides* to the biodegradation of PAHs was not significant at dense oil contaminations. Furthermore, the bioremediative capacity of *A. filiculoides* for PAHs strongly depends on the concentration of crude oil and its toxicity to the plants. Results from this study indicated that *Azolla filiculoides* is likely to be more effective at lower oil concentrations for the phytoremediation of crude oil-contaminated freshwater areas.

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Figure 1. Synchronous excitation/emission (nm) fluorescence spectra of aromatic hydrocarbon fractions of Batman crude oil.
Figure 2. Synchronous excitation/emission (nm) fluorescence spectra of aromatic hydrocarbon fractions of the control and plant samples at 0.05% - 0.5% crude oil concentrations.

Table 1. Relative growth rates of *A. filiculoides* treated with different crude oil concentration. "±" indicates standard deviation. Significant differences were determined by Tukey's multiple comparison test ($P<0.05$) and are indicated by different letters.

Table 2. The values of ΣTPH obtained from the negative control and the growth media with plants and the plant samples at different crude oil concentrations (0.05% - 0.5%) after 15 days of growth. "±" indicates standard deviation. Significant differences determined by the Tukey's Multiple Comparison test ($p<0.05$) are indicated by different letters. "*" indicates significant difference from negative control media. Significant differences determined by the Dunnett's Multiple Comparison Test ($p<0.05$).

Table 3. PAHs and alkylated homologous PAHs determined in control samples at (% 0.05 - % 0.5) crude oil concentrations. (- : not determined)

Table 4. PAHs and alkylated homologous PAHs determined in plant samples at (% 0.05 - % 0.5) crude oil concentrations. (- : not determined)

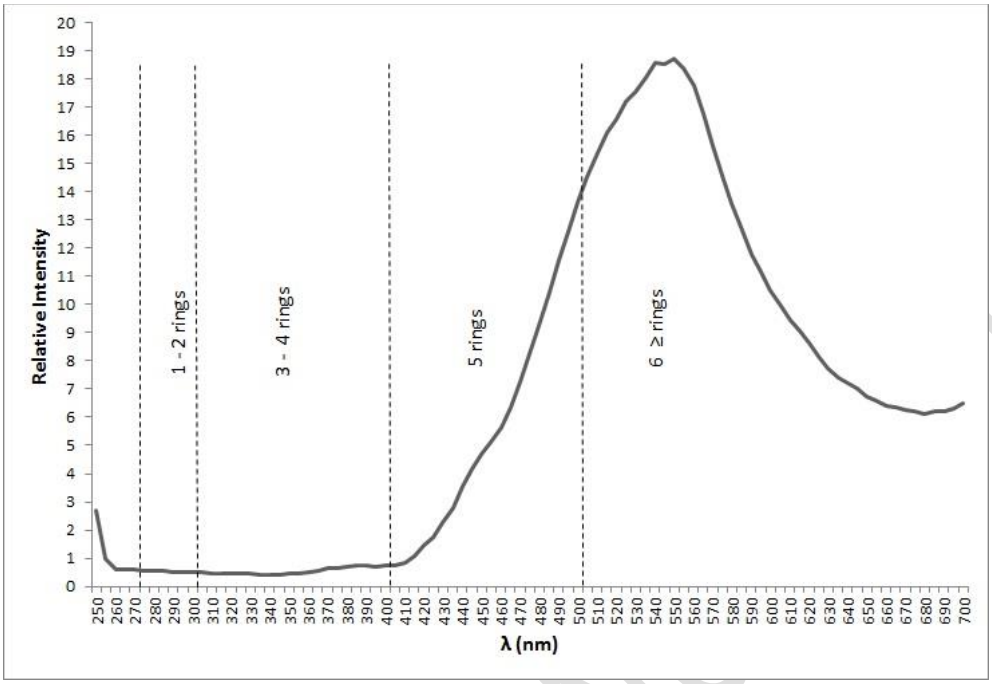

Figure 1. Synchronous excitation/emission (nm) fluorescence spectra of aromatic hydrocarbon fractions of Batman crude oil.

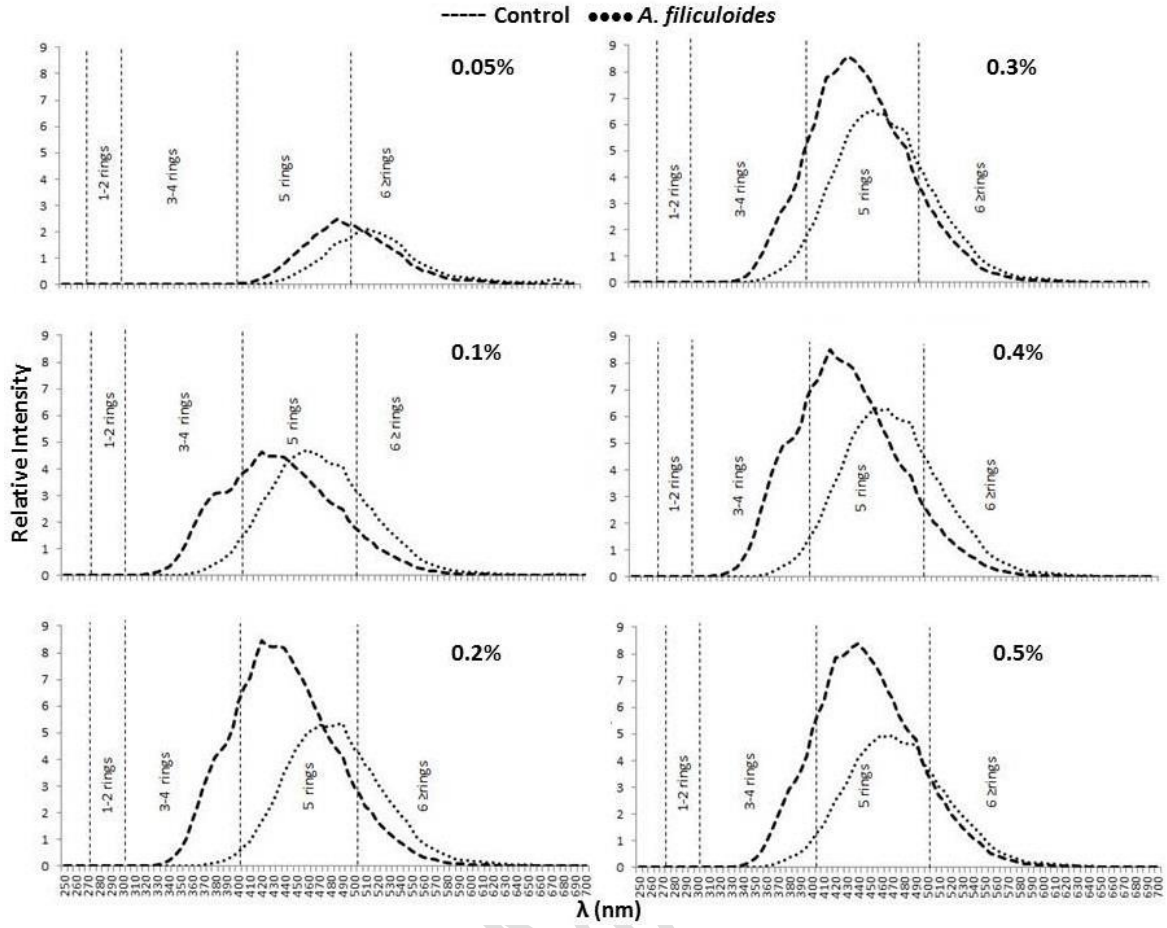

Figure 2. Synchronous excitation/emission (nm) fluorescence spectra of aromatic hydrocarbon fractions of the control and plant samples at 0.05% - 0.5% crude oil concentrations.

Table 1. Relative growth rates of *A. filiculoides* treated with different crude oil concentration. "±" indicates standard deviation. Significant differences were determined by Tukey's multiple comparison test (P<0.05) and are indicated by different letters.

(%) Crude oil	RGR ($g\ g^{-1}d^{-1}$)
Control	0.148 ± 0.005 a
0.05	0.138 ± 0.004 b
0.1	0.124 ± 0.001 c
0.2	0.104 ± 0.004 d
0.3	0.075 ± 0.006 e
0.4	0.067 ± 0.007 e
0.5	0.073 ± 0.001 e

Table 2. The values of ΣTPH obtained from the negative control and the growth media with plants and the plant samples at different crude oil concentrations (0.05% - 0.5%) after 15 days of growth. "±" indicates standard deviation. Significant differences determined by the Tukey's Multiple Comparison test ($p<0.05$) are indicated by different letters. "*" indicates significant difference from negative control media. Significant differences determined by the Dunnett's Multiple Comparison Test ($p<0.05$).

	Total Petroleum Hydrocarbons Amounts (∑TPH)		
(%) Crude Oil	**Control Media µg/ml)**	**Plant Media (µg/ml)**	**Plant (µg/gFW)**
0.05	1151.40 ± 86.76 e	6.24 ± 0.47 d *	448.86 ± 87.57 d *
0.1	1510.75 ± 113.83 e	12.68 ± 0.96 d *	1152.54 ± 139.86 cd *
0.2	9150.52 ± 689.45 d	14.30 ± 1.08 d *	2766.59 ± 390.62 cd *
0.3	11561.76 ± 871.13 c	34.18 ± 2.58 c *	4977.08 ± 452.75 c *
0.4	18372.47 ± 1384.30 b	57.86 ± 4.36 b *	15122.34 ± 3077.53 b
0.5	38997.32 ± 2938.29 a	258.23 ± 19.46 a*	38618.38 ± 7031.14 a

Table 3. PAHs and alkylated homologous PAHs determined in control samples at (% 0.05 - % 0.5) crude oil concentrations. (- : not determined)

			Control					
Compound	Ring numbers	Target ions	0.05%	0.1%	0.2%	0.3%	0.4%	0.5%
Naphthalene								
C0-naphthalene	2	128	-	-	-	-	-	-
C1- naphthalenes	2	142	-	-	-	-	-	-
C2- naphthalenes	2	156	-	-	-	-	+	+
C3- naphthalenes	2	170	-	-	+	+	+	+
C4- naphthalenes	2	184	-	-	-	-	-	-
Phenanthrenes								
C0- phenanthrene	3	178	+	+	+	+	+	+
C1- phenanthrenes	3	192	+	+	+	+	+	+
C2- phenanthrenes	3	206	+	+	+	+	+	+
C3- phenanthrenes	3	220	+	+	+	+	+	+
C4- phenanthrenes	3	234	+	+	+	+	+	+
Dibenzothiophenes								
C0- dibenzothiophene	3	184	+	+	+	+	+	+
C1- dibenzothiophenes	3	198	+	+	+	+	+	+
C2- dibenzothiophenes	3	212	-	-	-	-	-	-
C3- dibenzothiophenes	3	226	-	-	-	-	-	-
Fluorenes								
C0- fluorene	3	166	-	-	-	-	-	+
C1- fluorenes	3	180	-	+	+	+	+	+
C2- fluorenes	3	194	+	+	+	+	+	+
C3- fluorenes	3	208	-	-	-	-	-	-
Chrysenes								
C0- chrysene	4	228	-	-	-	-	-	-
C1- chrysenes	4	242	-	-	-	-	-	-
C2- chrysenes	4	256	-	-	-	-	-	-
C3- chrysenes	4	270	-	-	-	-	-	-

Pyrene	4	202	-	-	-	-	+	+

Table 4. PAHs and alkylated homologous PAHs determined in plant samples at (% 0.05 - % 0.5) crude oil concentrations. (- : not determined)

			A. filiculoides					
Compound	Ring numbers	Target ions	0.05%	0.1%	0.2%	0.3%	0.4%	0.5%
Naphthalene								
C0-naphthalene	2	128	-	-	-	-	-	-
C1- naphthalenes	2	142	-	-	-	-	-	-
C2- naphthalenes	2	156	-	-	-	-	-	-
C3- naphthalenes	2	170	+	+	+	+	+	+
C4- naphthalenes	2	184	-	-	-	-	-	-
Phenanthrenes								
C0- phenanthrene	3	178	+	+	+	+	+	+
C1- phenanthrenes	3	192	+	+	+	+	+	+
C2- phenanthrenes	3	206	+	+	+	+	+	+
C3- phenanthrenes	3	220	+	+	+	+	+	+
C4- phenanthrenes	3	234	+	+	+	+	+	+
Dibenzothiophenes								
C0- dibenzothiophene	3	184	-	-	-	-	-	-
C1- dibenzothiophenes	3	198	-	-	-	+	+	+
C2- dibenzothiophenes	3	212	-	-	-	-	-	-
C3- dibenzothiophenes	3	226	-	-	-	-	-	-
Fluorenes								
C0- fluorene	3	166	-	-	-	-	-	+
C1- fluorenes	3	180	-	-	+	+	+	+
C2- fluorenes	3	194	-	-	-	-	+	+
C3- fluorenes	3	208	-	-	-	-	-	-
Chrysenes								
C0- chrysene	4	228	-	-	-	-	-	-
C1- chrysenes	4	242	-	-	-	-	-	-

C2- chrysenes	4	256	-	-	-	-	-	-
C3- chrysenes	4	270	-	-	-	-	-	-
Pyrene	4	202	-	-	-	-	-	-