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Potentiality of *Azolla pinnata* R. Br. for Phytoremediation of Polluted Freshwater with Crude Petroleum Oil

Aya A. Mostafa [1], Ahmad K. Hegazy [2,*], Nermen H. Mohamed [3], Rehab M. Hafez [2], Ehab Azab [4,*], Adil A. Gobouri [5], Hosam A. Saad [5,*], Azza M. Abd-El Fattah [6] and Yasser M. Mustafa [3]

1 Biotechnology/Bimolecular Chemistry Program, Chemistry Department, Faculty of Science, Cairo University, Giza 12613, Egypt; aamoustafa@sci.cu.edu.eg
2 Botany and Microbiology Department, Faculty of Science, Cairo University, Giza 12613, Egypt; rehabhafez@sci.cu.edu.eg
3 Egyptian Petroleum Research Institute, Nasr City, Cairo 11727, Egypt; nermenhefiny@yahoo.com (N.H.M.); ymoustafa12@yahoo.com (Y.M.M.)
4 Department of Nutrition and Food Science, College of Science, Taif University, P.O. Box 11099, Taif 21944, Saudi Arabia
5 Department of Chemistry, College of Science, Taif University, P.O. Box 11099, Taif 21944, Saudi Arabia; a.gobouri@tu.edu.sa
6 Chemistry Department, Faculty of Science, Cairo University, Giza 12613, Egypt; azza682000@yahoo.com
* Correspondence: hegazy@sci.cu.edu.eg (A.K.H.); e.azab@tu.edu.sa (E.A.); h.saad@tu.edu.sa (H.A.S.)

Abstract: The pollution of freshwater resources with crude petroleum oil is a major environmental issue in oil-producing countries. As a result, the remediation of polluted aquatic ecosystems using eco-friendly and cost-effective technology is receiving increased global attention. In this study, the ability of *Azolla pinnata* R. Br. to remediate petroleum-polluted freshwater was assessed. The remediation potentiality was determined by evaluating the total petroleum hydrocarbon degradation percentage (TPH%) and changes in the molecular type composition of saturated and aromatic hydrocarbon fractions. TPH% was estimated gravimetrically, and changes in the molecular type composition of saturated and aromatic fractions were measured using gas chromatography and high-performance liquid chromatography, respectively. The results reveal that *A. pinnata* has the potential to phytoremediate freshwater polluted with low levels (up to 0.5 g/L) of petroleum hydrocarbons (PHs). After seven days of phytoremediation, the degradation rate of total PHs was 92% in the planted treatment compared with 38% in the unplanted positive control. The highest breakdown of PHs for the normal paraffinic saturated hydrocarbon fraction occurred in the presence of *A. pinnata* combined with *Anabena azollaea* (A-A), which showed a moderate degradation capacity toward total aromatic hydrocarbons (TAHs) and total polycyclic aromatic hydrocarbons (PAHs). The results indicate that *A. pinnata* effectively removed C18, a saturated PH, and acenaphthene (Ace), an aromatic PH. Therefore, this study suggests that *A. pinnata* is a useful tool for the remediation of freshwaters contaminated with low pollution levels of crude oil.

Keywords: total petroleum hydrocarbons (TPHs); n-paraffins; isoparaffins; polycyclic aromatic hydrocarbons (PAHs); crude oil degradation

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1. Introduction

The currently increasing demand for energy is accompanied by high global production and consumption levels of crude petroleum oil (hereafter, crude oil) and its refined products. As a consequence, environmental pollution with crude oil and petrochemical products has become a major environmental concern [1], especially when aquatic ecosystems become sinks for pollutants that result from oil spills, exploration, extraction, transportation and anthropogenic activities [2–4].

The deposition of these persistent pollutants in aquatic ecosystems threatens biodiversity [5–7]. The disruptive effects of oil-polluted water not only cause an ecological

imbalance but also lead to catastrophic consequences for human beings, ranging from infertility to cancers such as leukemia [8,9]. The severe toxic effects of crude oil are due to its petroleum hydrocarbon constituents. Crude petroleum oil generally consists of a wide range of inorganic and hydrocarbon-based components [10]. The inorganic fraction of crude oil includes nitrogen, sulfur, oxygen compounds and trace amounts of heavy metals, such as copper, iron and nickel [10]. The hydrocarbon fraction consists of saturated hydrocarbons such as cycloalkanes, normal alkanes, isoalkanes and aromatic hydrocarbons, including polycyclic aromatic hydrocarbons (PAHs) [11]. The U.S. Environmental Protection Agency (EPA) has classified 16 PAHs as code red environmental pollutants whose removal is considered essential for environmental remediation and human health and safety [12]. As these environmental problems are caused by oil pollution of water bodies, many established methods exist to remediate aquatic ecosystems that have been exposed to oil spills. These methods are categorized into conventional techniques, such as chemical, physical and thermal methods, and eco-friendly remediation methods that depend on biological principles [13]. Chemical remediation methods are based on treating the oil-polluted water with chemicals such as dispersants [14,15] or solidifiers [14,16], which cause physical or chemical changes in the spilled oil characteristics [17]. In physical remediation methods, suitable barrier structures such as booms and skimmers constructed in the oil-polluted water area to prevent the oil spill from spreading to other locations without changing the physical or chemical characteristics of the spilled oil [14,17,18]. The thermal remediation method is based on the in situ burning of oil spills [14]. Although conventional methods are commonly used because their remediation rates are faster than natural attenuation processes, they have operational disadvantages, such as high operation costs and secondary environmental pollution [14,19,20]. Therefore, the application of eco-friendly green technology for the remediation of crude oil-polluted water resources is increasing around the world [12,21]. In eco-friendly remediation technology, living organisms are used to accelerate the natural attenuation of low levels of oil pollutants [14,22]. For instance, a biological remediation process that applies microorganisms such as algae, fungi or bacteria to the polluted area is known as bioremediation [23]. Furthermore, a biological remediation process that uses plants is known as phytoremediation [24].

At present, phytoremediation is gaining global attention as a promising environmental restoration technology owing to its advantages over the established conventional cleanup techniques. These advantages include capital costs, reasonable remediation efficiency and lower environmental risks than conventional technologies [25–31]. The phytoremediation of environments containing organic pollutants has been studied by different methods using plants and microorganisms [32–35]. The mechanisms are grouped into intracellular and extracellular remediation. Intracellular phytoremediation depends on the plant's ability to take up the organic pollutant and enzymatically transform it to less toxic or harmless products within the plant tissues [33]. Extracellular phytoremediation depends on root exudate effects, including phytostimulation [36–38]. In phytostimulation, plants release mixtures of chemical signals in their root exudates to stimulaterhizospheric microbial communities that degrade hydrocarbon pollutants [39,40].

Many hydrophytes are considered to be convenient phytoremediators for polluted aquatic ecosystems, including *Azolla pinnata* [4,41,42]. Several potential characteristics, including its symbiotic relationship with *Anabena azollae*, may qualify *Azolla pinnata* as a green cleaning technology. The *Azolla pinnata*/*Anabena azollae* (A-A) combination is characterized by a fast growth rate, low operating cost, effective pollutant absorption and sustainable implementation [10,41–43]. To the authors' knowledge, previous studies have used this plant species with petroleum oil derivatives such as diesel [44] and produced water [44], but not crude petroleum oil. Therefore, this study investigated the potentiality of the *Azolla pinnata*/*Anabena* combination to phytoremediate oil-polluted waters by estimating the degradation of not only the crude petroleum oil but also the saturated and aromatic hydrocarbon fractions.

The objective of this study is the assessment of the phytoremediation potentiality of the *Azolla pinnata*/*Anabena azollae* combination grown in crude oil-polluted freshwater.

2. Materials and Methods

2.1. Plant Material and Its Propagation

The vegetative form of *Azolla pinnata* containing *Anabena azollaea* was provided by the Agricultural Research Center (ARC), Giza, Egypt, in July. The fronds were washed with freshly prepared growth medium. The *Azolla* growth medium was prepared according to previous studies [43,45]. The ferns were acclimated hydroponically using the growth medium inside a plastic basin for one week under the same climatic conditions as in a method in a previous study [43] in the greenhouse of the Faculty of Science, Cairo University. At the end of the acclimation time, about 150 g of biomass of acclimated *Azolla* was gathered for use in the experiment.

2.2. Assessment of Phytoremediation Potentiality

The experiment for the phytoremediation potentiality assessment of the *Azolla pinnata*/*Anabena azollaea* (A-A) combination was performed in the same location and under the same growth conditions used for *Azolla* acclimation.

There were three experimental groups: the negative controls, tested groups, and positive controls. The negative controls contained the growth medium that was used to plant the A-A combination. The results of this experimental group were used to ensure that the A-A combination grew healthily under the growth conditions over the experimental growth period. In the tested groups (hereafter, planted polluted water), the A-A combination was raised in growth media mixed with suitable amounts of crude oil (from Ras-Sidr, West Sinai, Egypt) to obtain different pollution levels (0.5, 1.0, 1.5, and 2.0 g/L). In the positive controls (hereafter, unplanted polluted water), the growth media were polluted with the same series of oil pollution levels as those in the tested groups but without the A-A combination.

The experimental period lasted for three weeks between July and August 2018. Three replications were conducted for all experimental groups using glass bowls, each of which was filled with one and half liters of the growth medium. To compensate for the lost growth medium in the bowls over the three-week growth period, freshly prepared growth medium was added up to the starting volume every day. For the negative controls and tested groups, four grams of the acclimated fronds was cultivated per bowl. To prevent anoxia in the oil-polluted waters, all experimental groups were aerated with an aeration system according to a previous study [43].

After each week of the three-week growth period, the phytoremediation capacity of the *Azolla pinnata*/*Anabena azollae* combination for the oil-polluted water was estimated from the total petroleum hydrocarbon (TPH) degradation percentage (%) and the residual amounts of total saturated hydrocarbons (TSHs) and total aromatic hydrocarbons (TAHs). In addition, subsequent fraction residues of TSHs, such as normal paraffins and isoparaffins, and of TAHs, such as polycyclic aromatic hydrocarbons (PAHs), were analyzed.

2.3. Determination of Petroleum Hydrocarbon Degradation

The residual TPH amounts were retrieved from the oil-polluted water growth medium using a liquid/liquid extraction method [46] at the end of each week of the experimental period. The extraction process was performed with 70 mL of dichloromethane. After that, the dichloromethane layers were pooled from the separating funnel into a labeled, clean, dry and pre-weighed 250-mL flask. The dichloromethane solvent was evaporated in a fuming cupboard for three days until the flask reached a constant weight. The extracted amounts of TPH were evaluated gravimetrically. The gravimetric analysis of the degraded amount of TPH was calculated as the difference in weight between the dried hydrocarbon-containing flask and the pre-weighed empty flask [47].

2.4. Fractionation of the Extracted TPH

TPH extracts were separated into insoluble and soluble fractions using n-hexane. The insoluble fraction (asphaltene) was precipitated by n-hexane, while the soluble fraction was further fractionated by column chromatography according to a previous study [48].

The used column was a glass column (1 cm in diameter and 60 cm in height) packed with 25 g of activated silica gel (mesh 60–200). The loaded fraction was eluted with organic solvents with different polarities. The eluted materials were categorized into two main groups, saturated and aromatic hydrocarbons. The saturated hydrocarbons (TSHs) were eluted with 100 mL of n-hexane, and the aromatic hydrocarbons (TAHs) were eluted with 100 mL of benzene.

2.5. Gas Chromatography for TSH Analysis

The saturated hydrocarbons were identified and quantified using PerkinElmer (Clarus 500) gas chromatography (GC) equipment equipped with a hydrogen flame ionization detector and a fused silica capillary column (0.32 mm in diameter and 60 m in length), filled with poly(dimethylsiloxane) HP-1 (nonpolar resin) with 0.5µm film thickness (Egyptian Petroleum Research Institute Cairo, Egypt). The injector was warmed to 350 °C. The carrier gas was nitrogen (oxygen-free) with a flow rate of 2 mL/min. The temperature program of the column was 100–300 °C at a constant rate of 3 °C/min. The detector was heated at 350 °C, and 0.1 µL of the melted sample was injected into the injector. The standard was a mixture of pure n-paraffins.

2.6. High-Performance Liquid Chromatography for TAH Analysis

The analysis of aromatic hydrocarbons was carried out using high-performance liquid chromatography (HPLC 600E) equipped with a Waters 2487 dual UV absorbance detector and Waters 717 Plus autosampler. The apparatus was attached to a computerized system with Millennium 3.2 software (Egyptian Petroleum Research Institute Cairo, Egypt). The PAH standard was supplied by Supelco. The separation conditions were established according to another study [49]. The column was 4.6 mm in diameter and 15 cm in length. The mobile phase was a gradient of 60–100% (v/v) acetonitrile/water, and the procedure was carried out for 45 min. The flow rate was 0.2 mL/min for 0–2 min and 1.0 mL/min for 2–45 min. The detector was adjusted to 254 nm.

2.7. Statistical Analysis

The experimental data are from three replications, and the results are expressed as the mean ± SE. The statistical analysis was performed with the Statistical Package for Social Sciences (SPSS 18.0 for Windows). The normality assumption was tested with the Shapiro–Wilk test. Two-way analysis of variance (ANOVA) was used for the comparison among different treatments within different periods using Duncan's 200 multiple range test ($p < 0.05$).

3. Results

3.1. Degradation of Total Petroleum Hydrocarbons

Figure 1 shows the degradation percentages (%) of total petroleum hydrocarbons (TPHs) in freshwater polluted with different crude oil concentrations and planted with the *Azolla*/*Anabena* combination (A-A) compared with unplanted water. The results suggest that A-A significantly degraded TPHs within three weeks compared with unplanted waters. The ranges of TPH degradation percentages for the planted water treatments were 92.90% ± 0.30%to 30.50% ± 0.10% after 7 days, 76.10% ± 0.10% to 16.30% ± 1.30% after 14 days, and 84.0% ± 1.20% to 25.90% ± 0.20% after 21 days. The ranges for the results in the unplanted water treatment were 38.20% ± 0.30% to 13.90% ± 0.40%, 40.60% ± 1.80% to 9.70% ± 0.10% and 61.0% ± 1.70% to 15.0% ± 0.30% after seven, fourteen, and twenty-one days, respectively.

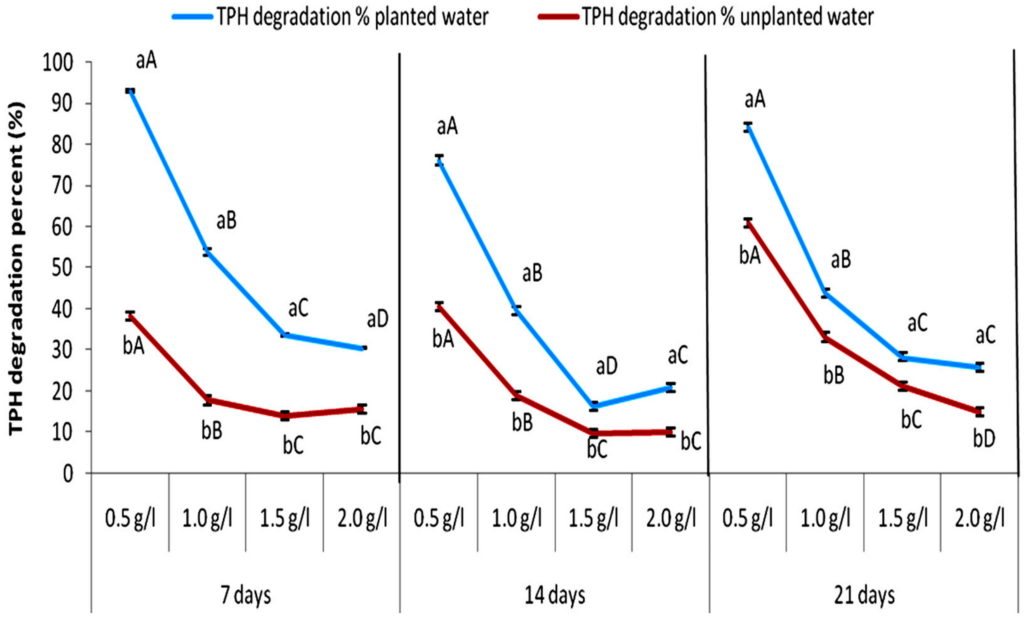

Figure 1. Time-dependent degradation percentages (%) of total petroleum hydrocarbons (TPHs) in freshwater treated with different concentrations of crude oil and planted with the *Azollapinnata*/*Anabenaazollaea* (A-A) combination for seven days, fourteen days and twenty-one days compared with unplanted water. Values are expressed as the mean ± SE. Different lowercase letters indicate significant differences between the planted and unplanted treatments at each oil concentration, and different capital letters indicate significant differences at each oil pollution level at $p \leq 0.05$ according to Duncan's multiple range test.

Although A-A significantly reduced the oil pollution level compared with the unplanted water treatments, its efficiency remarkably decreased as the oil pollution level increased between 7and 21 days. At 0.5 and 2 g/L oil pollution, the degradation was notably reduced from 92.9%, 76.1% and 84.0% to 30.5%, 16.3% and 25.9% after 7, 14 and 21 days, respectively. Moreover, the longer the growth period in oil-polluted water, the lower the efficiency of A-A phytoremediation. However, the extent of TPH degradation by A-A from days 7 to 21, in descending order, was 7 > 21 > 14 days.

3.2. The Molecular Type Composition of PHs

Further analyses were performed on the petroleum hydrocarbons in water polluted with 0.5 g/L oil after a 7-daygrowth period. The results show that the highest percentage of phytoremediation by the A-A combination reached more than 50%. One of these analyses was based on the determination of the oil molecular type composition before and after cultivation of the A-A combination compared with crude oil and the polluted unplanted control (Table 1).

Table 1. The molecular type composition of different petroleum hydrocarbon fractions (wt.%) extracted from oil-polluted freshwater after a 7-daygrowth period of the A-A combination following exposure to 0.5 g/L crude oil pollution. The results are compared with their initial and residual weight percentages in crude oil and the polluted unplanted control, respectively. Values are the mean ± SE, n = 3. The different lowercase letters from (a) to (c) indicate significant differences between crude oil and oil-polluted water without and with the A-A combination at $p \leq 0.05$ according to Duncan's multiple range test.

Molecular Type Composition (wt.%)	0.5 g Crude Oil	Unplanted Polluted Water	Planted Polluted Water
Total saturated hydrocarbons	77.92 ± 0.04 [c]	79.80 ± 0.12 [b]	81.67 ± 0.18 [a]
Normal paraffins	77.03	58.39	47.88
Isoparaffins	0.89	21.41	33.79
Total aromatic hydrocarbons	22.10 ± 0.03 [a]	20.27 ± 0.18 [b]	18.67 ± 0.18 [c]

The total saturated hydrocarbon fraction (TSHs) remarkably accumulated in the planted polluted water; it reached 81.67 ± 0.18%, which is greater than that of the positive control (79.80 ± 0.12%) and crude oil before use (77.92 ± 0.04%). The normal paraffins (n-paraffins) and isoparaffins (Isoparaffins), subfractions of saturated hydrocarbons, were estimated as well (Table 1). Notably, the abundance percentages of n-paraffin decreased from 77.03% in crude oil before use to 58.39% and 47.88% in the positive control and planted polluted treatment, respectively. Conversely, the abundance percentages of isoparaffins were notably increased, from 0.89% in crude oil before use to 21.41% and 33.79% in the positive control and planted polluted treatment, respectively. This indicates that the A-A combination significantly reduced the total n-paraffin fraction by 10.51 units and increased isoparaffin by 12.31 units compared with the positive control. For the total aromatic hydrocarbon fraction (TAHs), the A-A combination exhibited a moderate phytoremediation effect at 0.5 g/L crude oil pollution after 7 days. The abundance percentage of TAHs was significantly reduced from 22.10 ± 0.03% in 0.5 g crude oil before use to 20.27 ± 0.18% in the unplanted control and 18.67 ± 0.18% in the planted polluted water. This means that the A-A combination actually reduced the TAH fraction by 1.63 units more than the control.

3.3. The Analysis of Normal Paraffins of TSHs

The differential changes in the abundance of total saturated hydrocarbons and then-paraffinic saturated compounds were analyzed (Figures 2 and 3 and Table 2). In Figure 2, the gas chromatograms represent the abundance changes in the total saturated hydrocarbon fraction in different samples. Figure 3 and Table 2 represent the abundance changes in normal paraffins only. The peaks of each chromatogram in Figure 2 represent the initial and residual amounts of paraffinic hydrocarbons in crude oil before use (Figure 2a), the unplanted polluted control (Figure 2b) and the oil-polluted planted water (Figure 2c).

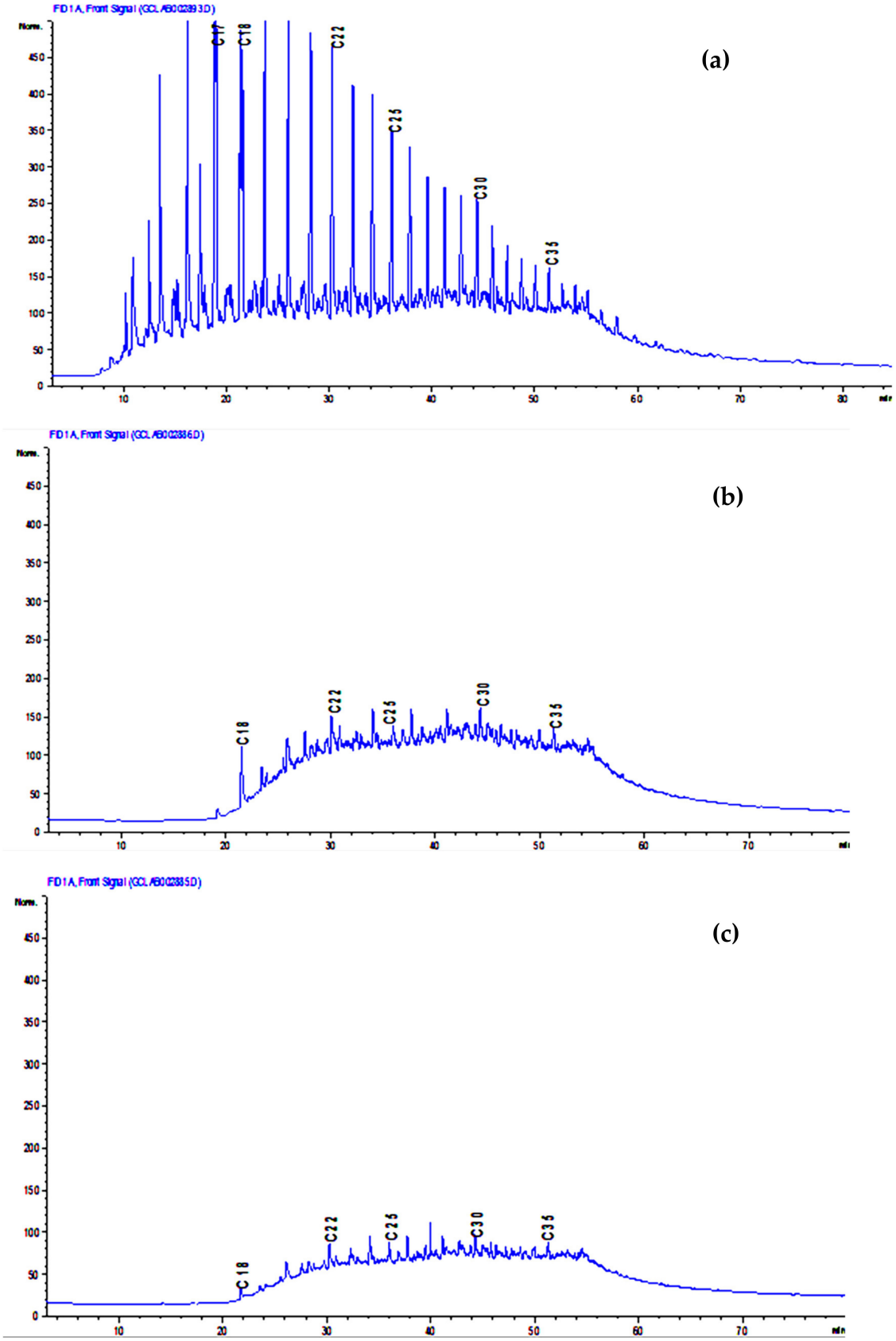

Figure 2. Gas chromatograms of saturated hydrocarbon fractions in 0.5 g crude oil before use (**a**), after a 7-daygrowth period in 0.5 g/L polluted unplanted water (**b**) and planted polluted water with the *A-A* combination (**c**).

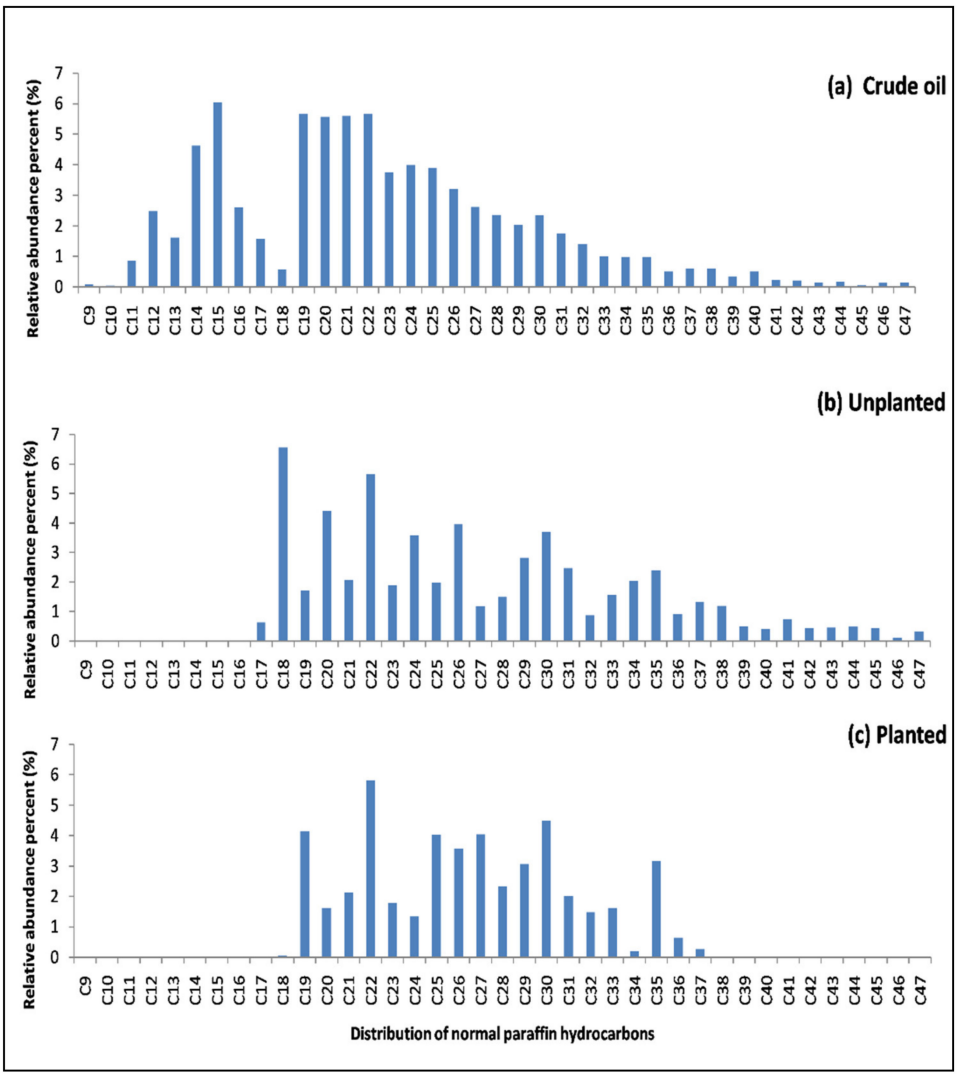

Figure 3. Histograms abundance of different normal paraffins of the saturated hydrocarbon fractions analyzed at 0.5 g crude oil before use (**a**), after a 7-daygrowth period in 0.5 g/L polluted unplanted water (**b**) and planted polluted water with the *A-A* combination (**c**).

Table 2. Relative quantitative GC analysis of the n-paraffinic saturated compounds (% of TSHs) extracted from oil-polluted freshwater after 7 days of growth of the A-A combination following exposure to 0.5 g/L crude oil pollution. The results are compared with their initial and residual amounts in crude oil and the polluted unplanted control, respectively.

Category	Carbon Number	0.5 g Crude Oil	0.5 g/L Planted Water	0.5 g/L Unplanted Water
First Class	C9	0.096	0	0
	C10	0.048	0	0
	C11	0.856	0	0
	C12	2.48	0	0
	C13	1.619	0	0
	C14	4.622	0	0
	C15	6.036	0	0
	C16	2.6	0	0

Table 2. *Cont.*

Category	Carbon Number	0.5 g Crude Oil	0.5 g/L Planted Water	0.5 g/L Unplanted Water
Second Class	C18	0.568	0.069	6.553
	C34	0.992	0.201	2.042
	C37	0.602	0.274	1.325
	C38	0.608	0	1.193
	C39	0.346	0	0.505
	C41	0.233	0	0.753
	C42	0.223	0	0.452
	C43	0.15	0	0.468
	C44	0.172	0	0.49
	C45	0.058	0	0.441
	C47	0.158	0	0.331
Third Class	C17	1.578	0	0.636
	C20	5.562	1.626	4.395
	C23	3.753	1.799	1.89
	C24	3.987	1.344	3.585
	C40	0.502	0	0.411
	C46	0.141	0	0.105
Fourth Class	C19	5.652	4.133	1.722
	C21	5.599	2.139	2.078
	C22	5.654	5.808	5.642
	C25	3.906	4.028	1.98
	C27	2.614	4.046	1.173
	C28	2.364	2.342	1.503
	C32	1.4	1.485	0.882
Fifth Class	C26	3.21	3.574	3.962
	C29	2.041	3.062	2.819
	C30	2.345	4.491	3.693
	C31	1.764	2.018	2.469
	C33	1.006	1.634	1.568
	C35	0.981	3.155	2.399
	C36	0.508	0.655	0.927

The hump feature in the chromatogram represents a complicated mixture of higher molecular weight hydrocarbons. The peak area of each n-paraffinic compound reflects the amount of this compound in the TSH fraction. Therefore, the relative abundance of each n-paraffinic compound was calculated as a percentage of its peak area relative to the total peak areas of TSHs (Figure 3 and Table 2). Therefore, the lower peak area percentage of a compound reflects its lower amount relative to the TSH fraction in a sample. Based on the GC chromatographic conditions, the crude oil before use contained 39 n-paraffinic compounds with carbon numbers ranging from 9 atoms (C9) to 47 atoms (C47). According to the relative abundance percentages (Figure 3 and Table 2), the n-paraffins could be categorized into five classes.

The first class of compounds (C9-C16) completely disappeared from the TSH fractions of oil-polluted waters with and without the A-A combination after 7 days at 0.5 g/L crude oil pollution. In the second class, the A-A combination was most effective in removing eleven n-paraffinic compounds, which had the lowest peak area percentages in the planted treatment compared with the crude oil and the unplanted polluted control. These compounds were C18, C34, C37-C39, C41-C45 and C47. Among these compounds, C18 was the most degraded by the A-A combination, as it was lower than that in the polluted unplanted control and its initial amount in crude oil by 6.5 units.

In the third class, n-paraffins were not as highly degraded by the A-A combination, as their peak area % were slightly lower than that of the control, which, in turn, were lower than that of the crude oil for C17, C20, C23, C24, C40 and C46. C23 and C46 were the least degraded by the A-A combination; their peak area %values in the planted water were

reduced by only 0.1 unit compared with the control. For the fourth class, n-paraffins were present at higher relative abundance percentages after plantation than in either the polluted control only such as (C19, C21 and C28) or the unused crude oil and the control (C22, C25, C27 and C32). Finally, in the fifth class, the peak area % values were significantly higher in the planted water and the control than in the unused oil. These compounds were C26, C29–C31, C33, C35 and C36, especially C30 and C35, the percentages of which increased from 2.3% and 0.9% in crude oil to 4.5% and 3.7% with the A-A combination and to 3.2% and 2.4% in the polluted control, respectively.

3.4. The Analysis of Polycyclic Aromatic Hydrocarbons of TAHs

Similar to TSHs, the differential changes in the presence of polycyclic aromatic hydrocarbons (PAHs) in the total aromatic hydrocarbon fraction (TAHs) were analyzed using HPLC (Table 3). The crude oil before use contained five classes of PAHs according to the number of constituent benzene rings, namely, two, three, four, five, and six rings. For each sample, the total PAHs and each PAH compound are represented in mg/L, while each PAH class is expressed as a percentage (%) of the total PAHs.

Table 3. Quantitative HPLC analysis of PAHs (mg/L) of 0.5 g crude oil before the experiment and those extracted from the 0.5 g/L crude oil–polluted control and planted treatment with the A-A combination after a 7-day growth period. Nap = naphthalene, A = acenaphthylene, Ace = acenaphthene, F = fluorene, Phe = phenanthrene, Ant = anthracene, Flu = fluoranthene, Pyr = Pyrene, BaA = benzo (a) anthracene, BaP = benzo (a) pyrene, BbF = benzo (b) fluoranthene, BkF = benzo (k) fluoranthene, Chr = chrysene, DahA = dibenzo (a,h) anthracene, and BP = benzo (g,h,i) perylene.

Number of Rings	PAHs	0.5 g/L Oil before Use	0.5 g/L Oil After	
			Planted Water	Unplanted Water
2 rings	Nap	0	0	0
% 2 rings/total PAHs		0	0	0
3 rings	A	0.24	0	0
	Ace	0	0	8.5
	F	0.32	0.36	0.41
	Phe	0	0	0
	Ant	0.12	0.34	0.21
% 3 rings/total PAHs		3.012	3.655	43.804
4 rings	Flu	0	0	0.012
	Pyr	0	0	0.093
	BaA	0.86	3.53	0
	Chr	0.14	0	0.52
% 4 ring/total PAHs		4.429	18.433	3.002
5 rings	BbF	0.21	12.86	9.74
	BkF	0.26	0.25	0.23
	BaP	0.71	0.31	0.09
	DahA	10.49	0	0.17
% 5 ring/total PAHs		51.683	70.078	49.135
6 rings	Bp	7.58	1.47	0.84
	IP	1.64	0.01	0
% 6 ring/total PAHs		40.833	7.728	4.035
Total PAHs		22.58	19.15	20.82

For PAH phytoremediation, the results show that the *Azolla*/*Anabenae* combination was not an effective phytoremediator for this petroleum hydrocarbon fraction. The remaining amounts of total PAHs decreased from 22.58 mg/L in crude oil to 20.82 mg/L in the positive control and to 19.15 mg/L in the planted polluted treatment. This indicates that the A-A combination only reduced total PAHs by 1.7 units compared with the positive control.

Generally, environmental (biotic and abiotic) factors in the polluted unplanted control reduced the abundance % of the higher molecular weight (HMW) PAH classes with six,

five, and four rings from the initial % in crude oil, especially those with six rings, as the six-ring PAH class decreased significantly from 40.8% in the unused crude oil to 4.0% in the polluted control. With the A-A combination, the abundance % of the same HMW classes increased compared with the unplanted control. These increases ranged from minute increases for the six-ring class to a significant accumulation of five- and four-ring classes, which were even higher than their initial % in the unused oil. The five-ring class increased from 49.1% in the control to 70.1% after plantation and 51.7% in the crude oil. Similarly, the four-ring class increased from 3.0% in the control to 18.4% after plantation and 4.4% in the crude oil.

The A-A combination efficiently decreased the abundance of the lower molecular weight (LMW) PAH class with three rings from 43.8% in the control to 3.7% after a 7-day growth period. This result means that the A-A combination actually removed about 40 units of three-ring PAHs. Among the three-ring PAH class, acenaphthene (Ace) was 8.5 mg/L in the control, but it was not detected in either the crude oil or the planted treatment. Therefore, the three-ring PAH compound that was most decreased by the A-A combination was Ace. The two-ring PAH class was represented by one compound, naphthalene (Nap), which was not detected in any sample.

4. Discussion

The phytoremediation capacity of the *Azolla*/*Anabena* (A-A) combination for freshwater polluted with different total petroleum hydrocarbon (TPH) concentrations was estimated in this study, and its bioremediation mechanism is represented in Figure 4. During the experimental period, the introduction of A-A to oil-polluted water for phytoremediation significantly enhanced TPH degradation. These results are consistent with previously reported findings that revealed that aquatic plants, such as *Lemna paucicostata* and *Lemna minor* [50,51], as well other species of *Anabena* spp. such as *Anabaena variabilis* [52], are notably efficient in TPH degradation.

However, the results indicate that the A-A capacity to remove crude oil significantly decreases as exposure is prolonged from 7 to 14 days and as oil pollution levels increase. Preliminary tests in a previous study [53] estimated the phytotoxic effects of high petroleum hydrocarbon concentrations on *Azolla pinnata*. This marked decline in TPH degradation by A-A is due to the destructive effects of petroleum compounds on many living organisms [43,54,55]. However, the 21-day results show that the rates of TPH removal in the unplanted and planted waters were higher than that in the 14-daygrowth period, but they are lower than that the results obtained after 7days.

The increase in TPH degradation with a longer growth period in oil-polluted unplanted water may be due to longer exposure to environmental factors that induce oil decomposition via abiotic and biotic oil breakdown processes [4]. Among the abiotic oil weathering factors is photooxidation, in which solar radiation induces the oxidation of petroleum hydrocarbons such as polycyclic aromatic hydrocarbons (PAHs) [4]. As a result, the photooxidized hydrocarbons become more hydrophilic than their parent compounds, increasing their bioavailability [56]. The bioavailability not only increases oil toxicity in water bodies but also is considered a prerequisite for phytoremediation of environments contaminated with crude petroleum oil [2]. In the oil-polluted planted waters, there is an additional biological factor that may be responsible for the significant TPH degradation increase compared with the positive controls. Although plant health deteriorates at higher oil concentrations and with a longer growth period, *Anabena azollae* blooms in these conditions, forming a thick green layer on the polluted water surface [43]. Therefore, the main reason for the high rates of petroleum cracking in planted treatments with longer periods and at higher oil concentrations may be the over growth of *A. azollae*.

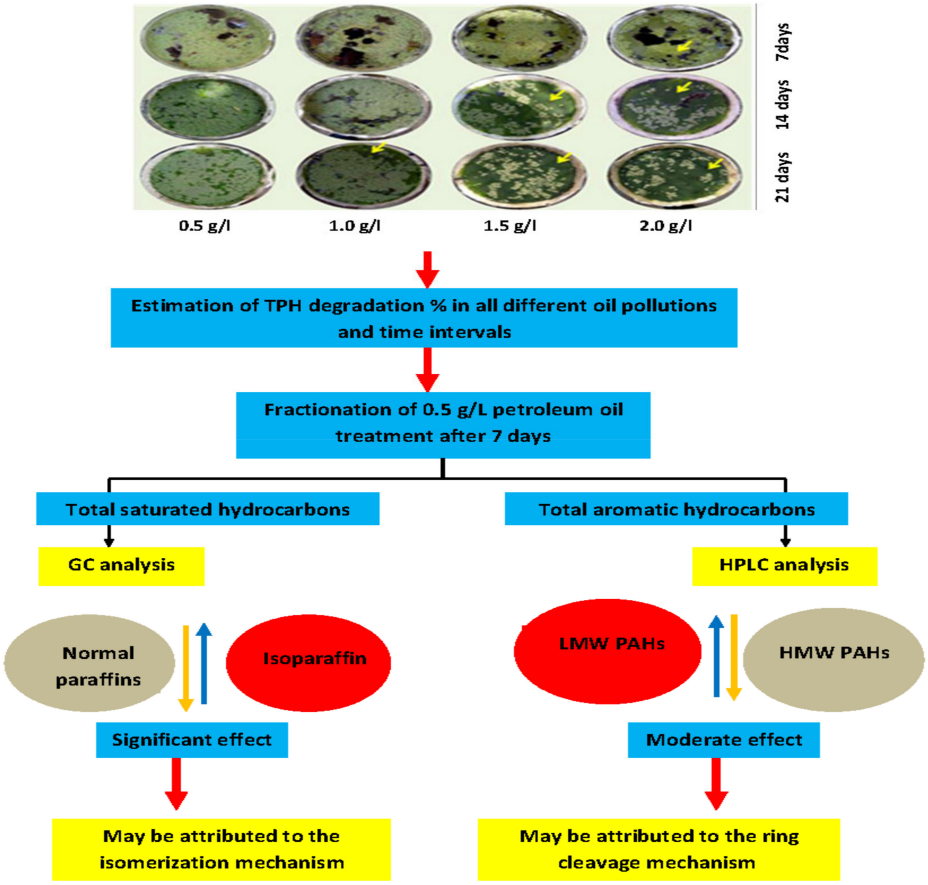

Figure 4. The effect of different pollution levels and exposure times on the bioremediation potentiality of the *Azolla*/*Anabena* combination and changes in the molecular type compositions of the remaining petroleum hydrocarbons at 0.5 g/L after a seven-day growth period. Abbreviations: TPH (total petroleum hydrocarbon), GC (gas chromatography), HPLC (high performance liquid chromatography), HMW PAH (high molecular weight polycyclic aromatic hydrocarbon) and LMW PAH (low molecular weight polycyclic aromatic hydrocarbon).

The results for the molecular type composition of the total saturated hydrocarbon fraction (TSHs) show that A-A had an effective phytoremediation effect on normal paraffinic saturated hydrocarbons (n-paraffins). This significant phytoremediation effect of A-A was due to the easily biodegradable chemical structures of these compounds, especially shorter n-paraffins such as C18 [34,57]. The n-paraffins with shorter carbon chains ranging between C9 and C16 were decreased due to environmental weathering factors, without the need for the A-A combination. These compounds were detected in the crude oil before use but not in either the planted polluted treatment or the polluted control. A wide range of n-paraffinic compounds with carbon chains lengths between C18 and C47 were among the most degraded by the effect of the A-A combination. Their relative abundance % values were significantly increased in the unplanted polluted control compared with their initial % in the unused oil. Furthermore, their percentages were significantly reduced in the planted polluted treatment and were lower than their initial percentages. The complete capture of these compounds, especially heavy ones ranging from C38 to C47, in the planted treatment may be attributed to the effects of indigenous microflora in the oil-polluted water and to *Azolla pinnata* cultivation. Many studies have demonstrated the effective microbial degradation potentialities for heavy normal paraffins such as C37–C40 via their oxidative enzymes [58,59]. Moreover, the presence of *Azolla pinnata* may further improve the microbial degradation capacity of these compounds compared with the unplanted case, and this may be due to the phytostimulation process caused by the plant [60]. In this process,

the plant releases exudates from its roots that trigger the microbial degradation of such pollutants [60]. The fate of the degraded n-paraffins may be their "isomerization", meaning the chemical conversion of an n-paraffin compound to an isoparaffin compound [61]. These results are similar to the effects of graminaceous plants on n-paraffins [61]. Evidence that supports this explanation includes the significant reduction in the total n-paraffin fraction with a significant increase in the isoparaffin fraction after A-A was grown for 7 days compared with the control and the crude oil. In addition, isoparaffins are more difficult to be biodegraded than n-paraffins [62]. Thus, isoparaffins were significantly higher in the control and in the polluted planted treatment than the in the crude oil.

Conversely, other n-paraffins, such as C19, C21, C22, C25, C27, C28 and C32, were significantly increased in the planted treatment compared with either the control or their initial % in the crude oil. There are two possible explanations for this phenomenon. One of them is attributed to the possible weak bioremediation capacity of the A-A combination toward these compounds. The other explanation is related to the effective degradation of higher molecular weight n-paraffins, such as C41–C45 and C47, by A-A and therelease of byproducts with shorter C-chains. It is worth mentioning that large n-paraffins such as C41–C45 and C47 were significantly increased in the control sample compared with their initial percentages in the oil. There are also two possible explanations for this phenomenon. One of them is the degradation of a complicated mixture of higher molecular weight hydrocarbons. Figure 2b shows that the hump feature is smaller than that of the crude oil. The other possibility is that extracellular hydrocarbons may be produced by *Azolla pinnata* itself, similar to other plants [63], or by freshwater microflora such as *Clostridium* spp. and *Pseudomonas fluorescens* [64,65]. The latter possibility may explain the increase in the total saturated hydrocarbons in the total petroleum hydrocarbons in the control and after *Azolla*/*Anabenae* plantation compared with its initial % in the oil before use.

Based on the results of the molecular type composition of the petroleum hydrocarbons, A-A is not an effective candidate for removing total aromatic hydrocarbons (TAHs), especially polyaromatic hydrocarbons (PAHs), due to their chemically stable structures [66]. However, A-A exhibited a preferential degradative capacity toward the three-ring PAH class over higher molecular weight classes with four, five, and six rings. This trend is consistent with the general principle of PAH biodegradation, in which the molecular weight of PAHs is inversely correlated with its biodegradability [67,68]. However, low concentrations of these large PAH classes can be degraded effectively by biotic and abiotic environmental conditions [2,4]. This was evident from the significant reductions in the % of remaining six-five- and four-ring PAH classes in the unplanted control polluted with 0.5 g/L crude oil compared with the initial % in the crude oil. Thus, the decrease in the abundance % of the six-ring PAH class in the planted treatment with A-A was due to environmental weathering factors. As a consequence, there were jumps in the concentration % of the smaller PAH classes with five and four rings in the planted treatment. This tradeoff between the concentrations of large and smaller PAH classes may be due to the fact that the lower molecular weight PAHs are byproducts of the degradation of larger PAHs [34,69]. Larger molecular weight PAHs are transformed to lower molecular weight PAHs via the oxidative ring cleavage mechanism [70]. In this mechanism, one aromatic ring of the compound is first oxidized, producing dihydrodiols, which are then cleaved to produce derivatives of lower PAHs [70]. For instance, the ring cleavage of fluoranthene, a four-ring PAH, may yield derivatives of acenaphthene, a three-ring PAH [71]. This chemical reaction is catalyzed by either light irradiation (photooxidation) [72] or the oxidative enzymes of microbes and plants [73]. The absence of Naphthalene (Nap), which represents the two-ring PAH class, from the polluted control and the planted treatment is attributed to its low molecular weight, which facilitates its degradation via photooxidation [74]. As pointed out by another study [75], besides photooxidation, oil constituents with lower molecular weights such as diaromatic hydrocarbons are easily converted from the liquid state on the water surface to gaseous particles, which are introduced to the atmosphere surrounding the polluted water body and contaminate the atmospheric ecosystem.

5. Conclusions

Based on our findings in this study, we conclude that the *Azolla pinnata*/*Anabena azollae* combination (A-A) is a good candidate as a phytoremediator of freshwater resources contaminated with low concentrations of total petroleum hydrocarbons (TPHs) of up to 0.5 g/L, and it can perform this function within a week. Due to the chemical structural variations of petroleum hydrocarbon compounds, A-A exhibited preferential degradation capacities toward crude oil constituents. This biological system was remarkably effective in the removal of normal paraffinic saturated hydrocarbons (n-paraffins), especially C18, and less effective in eliminating the isoparaffinic saturated fraction (isoparaffins). A-A showed a moderate capacity to remove total aromatic hydrocarbons (TAHs) and total polycyclic aromatic hydrocarbons (PAHs). However, A-A markedly affected the lower molecular weight class of PAHs (LMW PAHs), especially acenaphthene (Ace) at up to 8 mg/L. Therefore, further studies are required to elucidate the phytoremediation mechanisms of the A-A combination to understand how to improve its bioremediation efficiency for freshwater contaminated with TAHs. The development of this biological system, especially *Azolla pinnata*, to increase its phytoremediation efficiency is worthwhile due to its ability to rapidly reproduce, resulting in a high biomass within a week. Hence, this biomass can be used in polluted freshwaters with higher pollution levels of crude petroleum oil.

Future studies need to focus on testing the phytoremediation of plants and associated organisms that have the ability to consume crude petroleum oil and its derivatives (such as diesel) in polluted waters. Compared with the use of microbes only, vegetative-based remediation is characterized by additional simultaneous cleaning mechanisms over than microbial use only, such as the accumulation, immobilization, degradation of organic pollutants, and can also improve microbial bioremediation as well.

Among different hydrophytes, there is an urgent demand for studying and assessing the phytoremediation potentiality of *Azolla pinnata*/*Anabena* symbioses in oil-polluted waters. This biological system has the potential for rapid reproduction, doubling its biomass within one week, an important asset for its use in the remediation of freshwaters polluted with crude petroleum oil.

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