Research Article

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Removal Of Petroleum Hydrocarbon From Water By Combined Cultivation Of Azolla And Bacteria.

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ABSTRACT

Physico-chemical methods for removing low concentration dispersed Total Petroleum Hydrocarbons (TPH) from water are not cost-effective. The aim of this study was to remove TPH from water by combined cultivation of Azolla and bacteria. Concentrations of petroleum hydrocarbon in culture medium were 100,500 and 1000 mgL^{-1}. Study was conducted over 14 days in three modes by cultivation of Azolla, bacteria and combined Azolla-bacteria culture. Concentration of Petroleum hydrocarbon (TPH) in the water samples was measured using gas chromatography. For cultivation of Azolla, the TPH removal from water after 14 days of contact was 76%, 70%, and 62% for initial TPH concentrations of 100, 500, and 1000 mgL^{-1} respectively. For bacterial cultivation, for an initial TPH concentration of 1000 mgL^{-1}, *Pseudomonas aeruginosa* exhibited the lowest removal efficiency of 35%, while *Alcaligenes faecalis* showed the highest removal efficiency of 60%. In the combined cultivation of Azolla and bacteria, for an initial TPH concentration of 1000 mgL^{-1}, *Pseudomonas aeruginosa* and Azolla exhibited the lowest removal efficiency of 80%, while *Alcaligenes faecalis* and Azolla showed the highest removal efficiency of 100%. Combined cultivation Azolla and bacteria was effective to improve removal of petroleum hydrocarbon from water.

Keywords : *petroleum hydrocarbon, water, Azolla, bacteria.*

INTRODUCTION

Water pollution caused by petroleum hydrocarbons can occur through various ways, such as the produced water in petroleum extraction, fuel spills during the transport of petroleum products, leakage from storage tanks and pipes, and fuel spill in gas stations, as well as from cars, vehicles, and other sources. Petroleum derivatives such as petroleum hydrocarbon, gasoline, and kerosene, are a complex combination of alkanes, alkene, cyclic hydrocarbon, aromatics and molecules containing sulfur and nitrogen [1]. Water pollution with petroleum hydrocarbons endangers the health of all living organisms. According to the guidelines of the World Health Organization (WHO), the maximum concentration level (MCL) of petroleum compounds in drinking water for benzene, benzopyrene, toluene, xylene and ethylbenzene are 10, 0.7, 700, 500 and 300 micrograms per liter respectively and for aliphatic compounds up to C8 is 15 mg/L and for C9-C16 is 0.3 mg/L[2].The maximum allowable concentration for discharge of produced water in open bodies of sea water depending the country is typically 5 -30 mg/L, and for refinery wastewater containing fuel and grease is 10 mg/liter [3].

Petroleum hydrocarbon contains about 64% aliphatic hydrocarbons, 33% aromatics, 1% olefins and 0.5% BTEX [4, 5]. When diesel enters water, it quickly disperses and can remain in the water for an extended period of time. The heavier molecular fractions tend to sink deeper into the water. Water pollution caused by diesel is considered more harmful than pollution caused by crude fuel. This is because diesel undergoes physicochemical changes when mixed with

water [6]. The presence of petroleum hydrocarbon in polluted water in the form of dispersed micro and nano droplets was confirmed[7].

Various methods have been proposed to remove fuel pollution from water, which include gravity separation, chemical coagulation, flotation, surface adsorption, chemical oxidation, membrane methods, bioremediation, and phytoremediation [8] . Studies have been conducted in the field of removing dispersed petroleum hydrocarbon from water by physico-chemical methods, such as activated carbon and bentonite[9] , hydrophobic nanosponge [10], graphene oxide [3], Rice bran ash [11] and ion exchange resin [12]. There are several disadvantages associated with physico-chemical processes, including the production of chemical fuel sludge and high costs.[13]. In the biological processes, microorganisms are used to remove fuel pollution. The efficiency of bioremediation is significantly improved by using methods such as improving environmental conditions to stimulate microbial growth (Biostimulation) and adding microorganisms with special abilities to the polluted environment (Bioaugmentation) [13, 14] . Research has been conducted for removal of petroleum hydrocarbons from water using bacteria, such as the removal of crude fuel from saline water by three species of Acinetobacter [15], Proteus vulgaris isolated from fuel-contaminated water [16], Proteus isolated from fuel sludge [17], Acinetobacter and fungi [18], Alcaligenes [19] and biodegradation of petroleum hydrocarbon using Enterobacter and Acinetobacter [20]. There are some studies on removal of petroleum hydrocarbon with the combined system of plants and bacteria, such as using bacterial flocs formed around Azolla roots[21], adding bacterial culture to the floating aquatic plant system [22], by bacterial flocs developed around the dead Azolla pinnata fronds [23] and by phytoremediation combined with bacterial inoculation [24]. Studies have also been conducted using Azolla for remediation of petroleum hydrocarbons from water, such as biodegradation of crude fuel in water by Azolla pinnata [25] and removal of crude fuel from water by Azolla filiculoides [26]. Azolla is a unique plant with distinct characteristics. It is an aquatic fern that grows and reproduces on the surface of water and has significant nutritional value [27]. The coexistence of nitrogen-fixing bacteria (cyanobacteria) with Azolla enables the plant to absorb and fix nitrogen from the air [28]. Azolla is a promising candidate for phytoremediation [29]. The removal of low concentrations of dispersed diesel in water is of great environmental importance. However, there have been limited studies investigating the removal of diesel from dilute diesel-water emulsions, and to the best of our knowledge, no study has yet been conducted using Azolla for this purpose. The innovation and different feature of this study compared to previous researches is the removal of low concentration dispersed petroleum hydrocarbon from water using Azolla, bacteria and combined cultivation of Azolla and bacteria. The aim of this study was to investigate the removal of petroleum hydrocarbon from water using Azolla filiculoides and in combination with different types of isolated bacteria. The study also determined the effect of bacterial type, total petroleum hydrocarbon (TPH) concentration, amount of Azolla, and contact time.

MATERIALS AND METHODS

Azolla filiculoides was collected from a paddy field in Sari-Iran, and cultivated under artificial light with an intensity of 10.0 k lux, using Hoagland medium (1.0 M, mL: 5.0 Ca(NO3)2 , 5.0 KNO3, 2.0 MgSO4.7H2O, 1.0 KH2PO4 and 1.0mL micronutrient solution). Bacteria were isolated from soil contaminated with diesel fuel. Bacterial strains were identified based on Bergey's manual of systematic bacteriology [30]. The identified bacteria were: Alcaligenes faecalis, Enterobacter aerogenes, Acinetobacter, Proteus vulgaris and Pseudomonas aerogenes. Minimum culture media (gL-1:1.0 KH2PO4, 1.0 K2HPO4, 1.0 (NH4)2SO4, 0.2 MgSO4, 0.05 FeCl3, and 0.02 CaCl2, pH 7) enriched by 1.0 gL-1 of glucose was used for bacterial culture. Commercial diesel fuel was sterilized and predetermined amounts of it were mixed with culture medium and then sonicated for 30 minutes to prepare 100, 500, and 1000 mgL-1 [3]. In the experiments involving bacteria, a bacterial inoculum of 100 mL consisted of a suspension of the each isolated bacteria in water (with an optical density of 0.6 at 600 nm) was added separately to the culture medium containing TPH. In the experiments involving Azolla, 3.0 grams of healthy fresh Azolla fronds were put into the flasks containing glucose enriched BH medium polluted by TPH in two different modes: with bacterial inoculum and without it. Experiments were conducted over a period of 14 days in three different modes: Azolla cultivation, bacterial cultivation, and combined cultivation (bacteria and Azolla). The cultivation was carried in an incubator with a light/dark cycle of 16/8 hours, day and night cycle, and temperature of 30°C, light intensity of 10000 lux. Positive controls, without plants and bacteria, were used to determine the amount of evaporated TPH, while a negative control, without TPH, was used to confirm the growth of Azolla. In all flasks the evaporated water was replenished by adding distilled water. Sampling were done on days 5, 10, and 14 after the start of cultivation. Analysis of TPH was performed by measuring TPH by GC-FID method [31].

RESULTS

Isolated bacteria from diesel contaminated soil were identified by biochemical test methods that the results is shown in **Table 1**.

Test: GR = Gram Reaction, XI= Oxidase, MOT= Motility, NI=

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Nitrate, LYS= Lysine, GLU= Glucose, XYS= Xylose, ORN= Ornithine, H2S, MAN= MANOSE, XL= XYLASE, IND= Indole, UR= Urease, VP= Voges-Proskauer, CIT= Citrate, MAL= Maltose, SUC= Sucrose, LAC= Lactose, STH= Starch Hydrolysis, CAT = Catalase, TSI= Triple Sugar Iron Agar, GEL = Gelatin Hydrolysis , A1= Alcaligenes.faecalis , A2= Enterobacter. Aerogenes , A3= Acinetobacter. Spp, A4= Proteus. Spp , A5= Pseudomonas aerogenosa

Table1. Result of biochemical tests of bacteria isolated from oil-contaminated soil.

Biochemical tests																							
GR	**XI**	**MOT**	**NI**	**LYS**	**GLU**	**XYS**	**ORN**	**H2S**	**MAN**	**XL**	**IND**	**UR**	**MR**	**VP**	**CIT**	**MAL**	**SUC**	**LAC**	**TSI**	**GEL**	**STH**	**CAT**	**separation**
+	+	+	_	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	N	-	-	+	A1
-	-	+	+	+	+	+	-	-	+	+	-	-	-	+	+	-	+	+	A/A	+	-	+	A2
-	-	-	-	-	+	+	-	-	+	N	-	-	-	-	-	-	-	-	K/K	-	-	+	A3
-	-	+	+	-	+	N	N	+	-	N	+	+	+	-	-	+	-	-	A/A	+	-	-	A4
-	-	+	+	+	+	-	+	-	-	+	-	+	+	-	+	-	-	-	K/K	+	-	+	A5

Figure 1. Flasks containing 3.0 grams Azolla and 1000 mgL-1 TPH : first day (left), after 14 days (right)



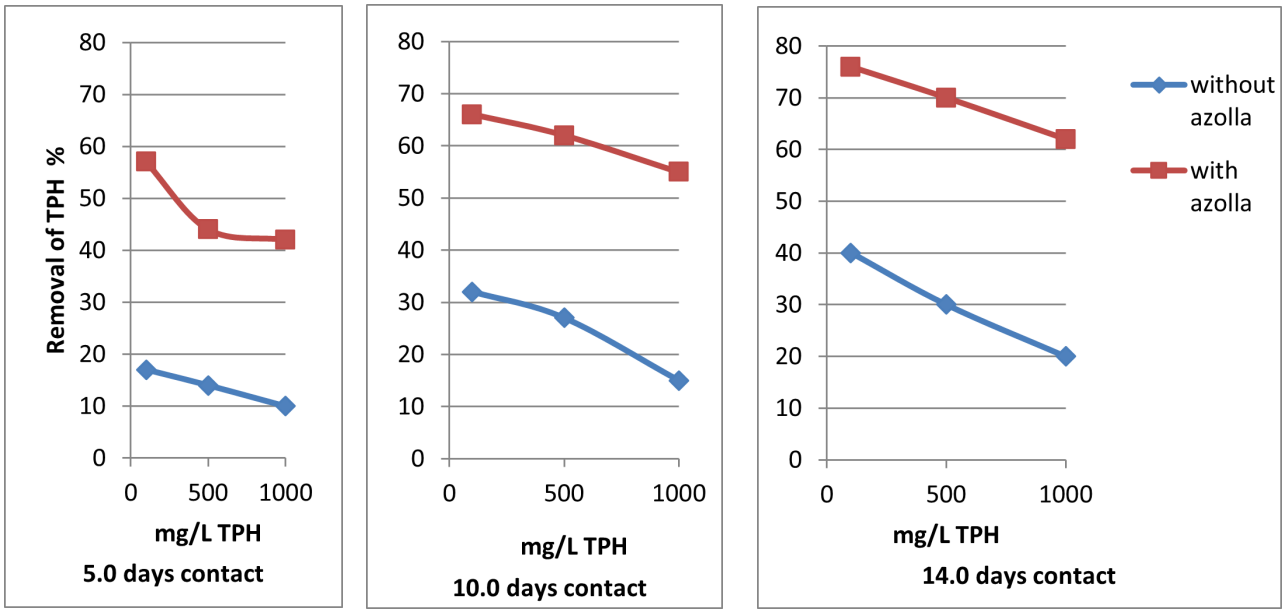
Removal of TPH by Azolla

Figure 2 shows the removal of 100, 500, and 1000 mgL^{-1} of TPH from water using 3.0 grams of Azolla at contact times of 5.0, 10.0, and 14.0 days.

The graphs in **Figure 2** illustrate that the flask containing Azolla achieved a TPH removal efficiency of 57% after 5.0 days of contact with a TPH concentration of 100 mg/L. However, when the TPH concentration was increased to 1000 mg/L, the removal efficiency decreased to 42%. Under the same conditions, in the flask without Azolla, the evaporation resulted in a reduction of TPH concentration by 28% and 18% for initial TPH concentrations of 100 mg/L and 1000 mg/L, respectively. Furthermore, Figure 2 illustrates that the TPH removal efficiency increased significantly for all three TPH concentrations as the contact time was extended to 10 and 14 days.

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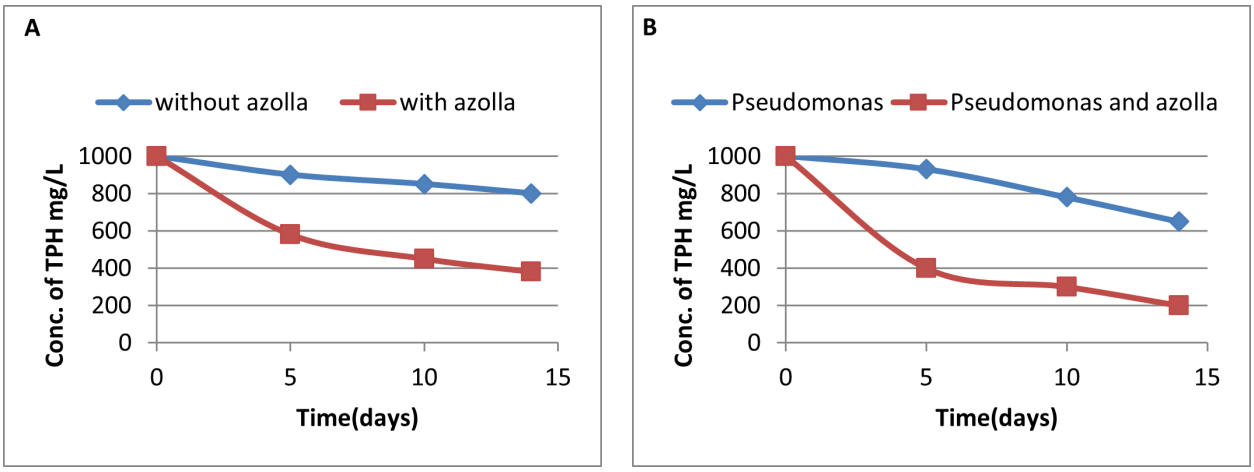
Figure 2. Reduction of TPH concentration in water using Azolla under varying TPH concentration and contact time



Removal of TPH by bacteria and combined Azolla-bacteria

Figure 3 illustrates the removal of TPH at a concentration of 1000 mg/L using bacteria and combined Azolla-bacteria culture. Graph A shows that in the control flasks, the concentration of TPH decreased by around 20% after 14 days of contact. Among the examined bacteria, only alcaligenes was able to remove approximately 60% of TPH (graph F), while the other bacteria showed a removal rate of around 40% (graphs B to E). For all bacteria, the removal of TPH was slow during the first 5 days, but then increased more rapidly. In the combined culture, the highest removal rate with 100% efficiency was achieved by Alcaligenes and Azolla, while the lowest removal rate with 80% efficiency was achieved by Pseudomonas and Azolla.

Figure 3. Removal of TPH in different days using pure bacterial culture and mixed culture of 3 grams of Azolla and bacteria A: Control and Azolla B: *Pseudomonas* and Azolla C: *Proteus* and Azolla D: *Acinetobacter* and Azolla E: Enterobacter and Azolla F: *Alcaligenes and Azolla*



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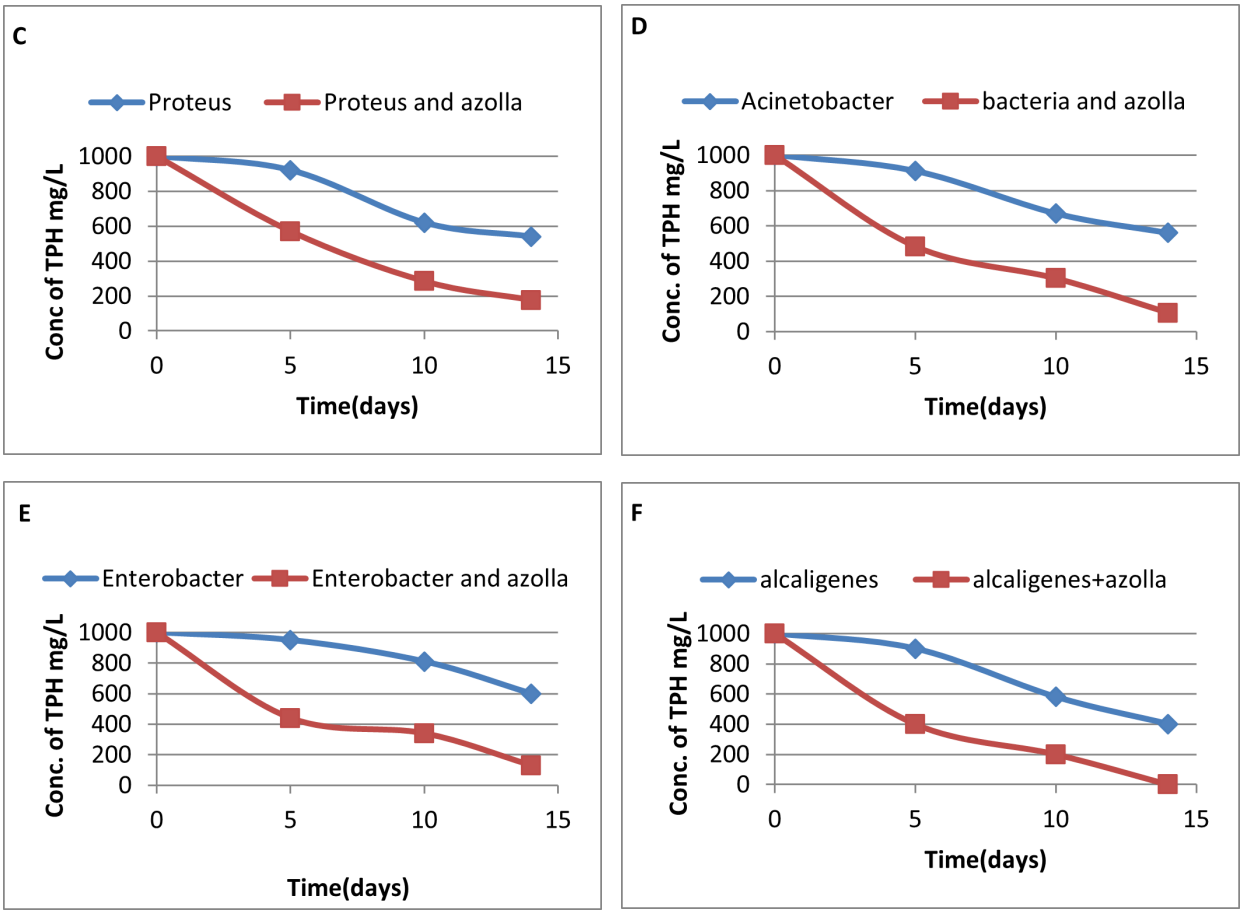
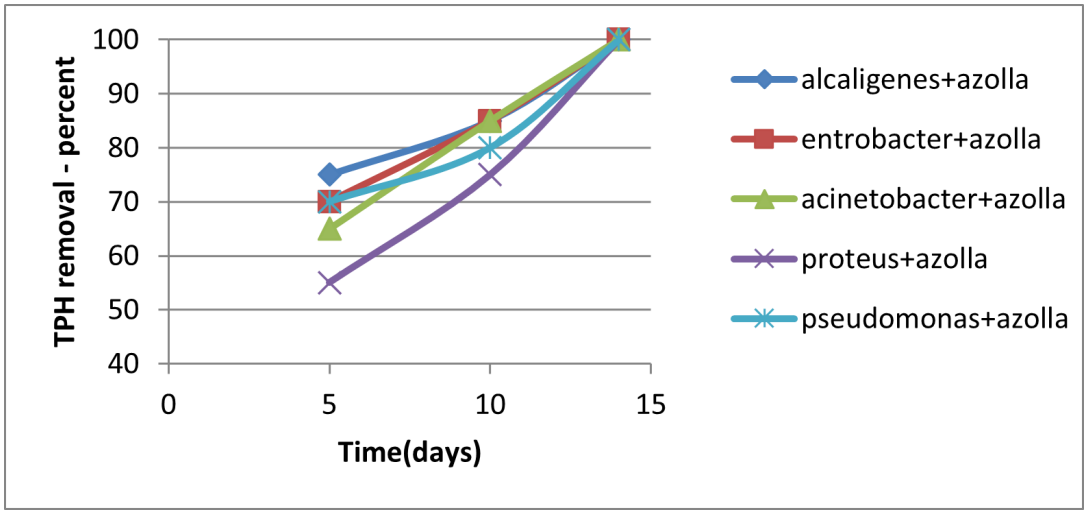


Figure 4 illustrates the removal of petroleum hydrocarbon from water at a concentration of 500 mg/L using a combined culture of bacteria and Azolla. As shown in the figure, the combined culture was able to remove TPH with an efficiency of 85% and 100% after 10 and 14 days, respectively. The lowest removal rate, at 55%, was observed for Pseudomonas and Azolla after 5 days of contact time. The results of all experiments indicate that the removal efficiency of TPH increased as the contact time was increased.

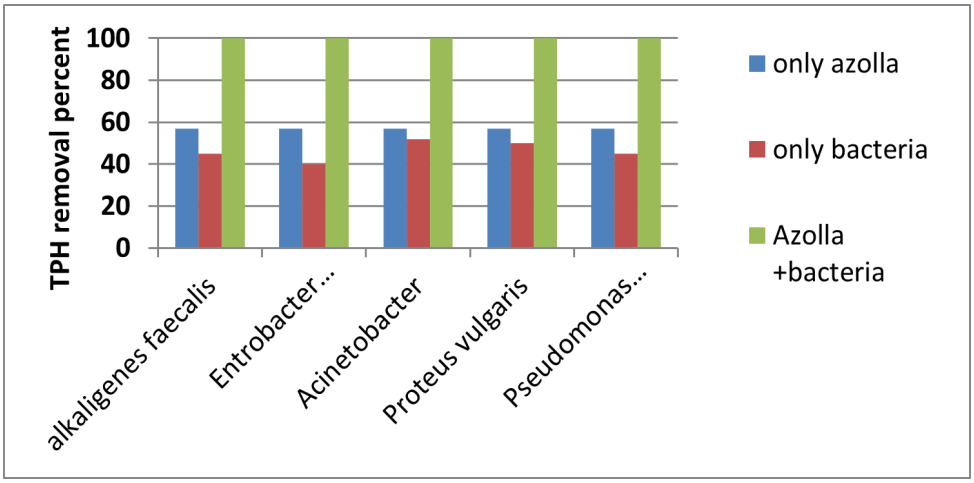
Figure 4. Removal of 500 mgL-1 of TPH by combined culture of Azolla-bacteria at different contact times.



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Figure 5 demonstrates that using the combined culture of Azolla and bacteria, 100 mg/L of TPH was removed with 100% efficiency within 5 days of contact time. The efficiency of Azolla alone in removing 100 mg/L of TPH was 57% within 5 days. The efficiency of each bacteria alone ranged from 40% to 50%, but increased to 100% when using the combined culture method.

Figure 5. Comparison of removal of 100 mgL-1 TPH in different test modes at 5 days contact time.



DISCUSSION

Removal of TPH by Azolla

In this study, findings indicate that longer contact times led to higher TPH removal, while higher concentrations of petroleum hydrocarbon led to decreased TPH removal. Observations of Azolla in culture flasks indicated that the withering of Azolla increased with higher concentrations of petroleum hydrocarbon and longer contact times. At a concentration of 1000 mg/liter of petroleum hydrocarbon, Azolla was found to be 50% dead. Al-Baldawi et al. studied the exposure of petroleum hydrocarbon on Azolla and found that Azolla was able to survive when exposed to concentrations of 500, 1000, and 3000 ppmv of petroleum hydrocarbon in water for 10 days. However, when exposed to a concentration of 7000 ppmv for 10 days, Azolla completely withered[32].

One study investigated the use of 4.0 grams of Azolla pinnata to remove crude fuel concentrations ranging from 0.5 to 2 g/L from water. The results indicated that Azolla has the capacity to remove petroleum hydrocarbons up to 0.5 g/L. After being in contact with 0.5 g/L of crude fuel for 7 days, the beaker containing Azolla showed a removal efficiency of 90% and under the same conditions the removal efficiency for a concentration of 1000 mg/L of crude fuel was approximately 55%.[25] . In our study for a concentration of 0.5 g/L of petroleum hydrocarbon, using 3.0 g of Azolla and contact time of 5 days resulted in a removal efficiency of 44%. In another study, crude fuel concentrations ranging from 0.005 to 0.5% vol. were introduced into a culture medium containing Azolla filiculoides and left in contact for 15 days. Concentrations of 0.005% and 0.01% vol. of crude fuel did not have negative effects on the growth of Azolla. After a 15-day contact period, the removal of petroleum compounds was found to be in the range of 60 to 90 percent, depending on the initial concentration of crude fuel [26].

A study on the biodegradation of petroleum hydrocarbon by Azolla revealed the formation of bacterial colonies around the dry mass of Azolla. The results indicated that the concentration of petroleum hydrocarbons around the dead Azolla biomass was 62% lower than the control flask without Azolla. The bacteria isolated from the sfuel around the dead Azolla biomass exhibited a dense growth in mineral culture medium with 4% petroleum hydrocarbon, leading to the conclusion that the release of nitrogen from Azolla causes the formation of bacterial colonies that aid in the biodegradation of hydrocarbons [33] .

Phytoremediation takes place through different mechanisms, including degradation, stabilization, volatilization, extraction and rhizofiltration. Furthermore the presence of suitable conditions for bacterial growth around the roots of Azolla suggests that there is a possibility of native bacterial growth and proliferation in the root zone of Azolla without bioaugmentation that can aid in the biodegradation of petroleum hydrocarbons.

Removal of TPH by bacterial culture

In this study for 1000mgL-1 of TPH, the alcaligenes bacteria was able to remove 60% of TPH after 14.0 days of contact (graph F of Fig.3). Under the same conditions, the removal of TPH by Proteus vulgaris and Acinetobacter was 45 percent (graph C &D). TPH removal by Enterobacter and Pseudomonas aeruginosa was 40 and 35 percent respectively (graph E &B). In a study halotolerant bacterial consortium containing 6 bacterial species including three strains of

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Pseudomonas aeruginosa was removed by 83% of crude fuel [34]. In a research, the decomposition of 3% vol. of crude fuel in culture medium by Pseudomonas aeruginosa was 60% [35] . In a study, Proteus vulgaris was isolated from petroleum contaminated water samples from Delta of Niger. in the 1.0% concentration of petroleum compounds in water containing mineral medium, the removal rate of petroleum hydrocarbon, kerosene, benzene and naphthalene was 85, 90, 70 and 62% respectively [16] . In another study, Proteus mirabilis was isolated from fuel sludge and was grown in mineral medium containing crude fuel as the sole carbon source. The percentage of TPH decomposition reached to 70% after 60 days [17]. Degradation of crude fuel in water by Acinetobacter and Talaromyces fungus was studied. Within 14 days, normal alkane compounds were completely removed and removal of total petroleum hydrocarbon (TPH) was 80% [18]. Three strains of Acinetobacter were studied for removal of petroleum hydrocarbons in saline water. Saline water (3.5 and 35 g/L) and crude fuel (2 g/L) were examined. removal of TPH by Acinetobacter was 15 to 35% , while in deionized water was 7 to 12% [15]. The ability of bacteria or plants to biodegrade petroleum hydrocarbons is dependent on the concentration of these compounds. High concentrations of hydrocarbons can result in their accumulation, leading to uneven distribution in water bodies. Also, high concentration reduces the rate of biodegradation due to the effect of toxicity and conversely, while very low concentrations of hydrocarbons may not have toxic effects on microorganisms, they can still limit biodegradation by causing problems in the supply of substrate for microbial growth [36]. Increasing the contact time allows bacteria more time to break down TPH, resulting in a higher percentage of removal. Bacteria require time to adapt to new environmental conditions, but once they have acclimatized to a stable state, they can break down hydrocarbons at a relatively higher rate [37] .

Removal of TPH by combined culture of Azolla and bacteria

The data from the graphs in Figure 3 demonstrate that the amount of TPH removal in the Azolla-bacteria combined culture is 40-45% higher than in the pure bacterial culture. The bioremediation of petroleum hydrocarbons using plant-microbe systems has been studied, and it has been reported that the compounds identified around the Azolla roots are similar in molecular structure to the aromatic compounds found in petroleum hydrocarbon. Additionally, Azolla has been shown to aid in the formation of microbial flocs by providing nitrogen for the degradation of petroleum hydrocarbons [21]. In a research study, petroleum hydrocarbon (1.0% w/v) was removed from water using Phragmites australis and bioaugmentation with three bacterial strains including two strains of Acinetobacter and one strain of Bacillus megatrium. In the polyethyelen tank (microcosm) containing only bacteria, the TPH concentration decreased from the initial 10,000 mg/L to 9,000 mg/L after 15 days. In the microcosm containing only Phragmites , the TPH concentration decreased to 7,500 mgL-1 , and in the microcosm containing plant and bacteria, the TPH concentration decreased to 4,500 mgL-1 [22] . In the another study, treatment of wastewater from a bioethanol production plant was investigated using Azolla and Pseudomonas aeruginosa that the findings indicated that the combination of 0.2 kg of Azolla and a Pseudomonas was the most effective in reducing the biochemical oxygen demand (BOD) of the wastewater [38]. The relationship between plants and microbe is one of mutual support, as plants create suitable conditions such as oxygen supply and the release of nutritious organic compounds in the root area to support microorganisms [39]. Bacteria also benefit plants by synthesizing and secreting plant hormones such as Gibberellin and cytokinin, preventing ethylene production, fixing nitrogen, and mobilizing phosphorus and other minerals. In the root zone, plants secrete and release organic substances such as terpenes, flavonoids, and lignin compounds that have chemical formula structures similar to petroleum hydrocarbons. This similarity can lead to the creation of genes that enable root zone microorganisms to degradation of petroleum compounds [40].

CONCLUSION

Pollution of water by petroleum hydrocarbons can have various impacts that pose a threat to the environment and living organisms. Petroleum hydrocarbon in low concentrations is dispersed in water in the form of micro and nano droplets. Removing low concentrations of TPH from water by physico-chemical methods is not economical. The findings of this research indicate that phytoremediation using Azolla along with bacterial inoculation can effectively remove low concentrations of TPH dispersed in water.

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