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Biodegradation of Soil Contaminated with Polycyclic Aromatic Hydrocarbons PAHS through Nitrogen Biostimulation

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Abstract

This study evaluated the effect of nitrogen on the biostimulation of microbial load in soil mesocosms contaminated with bunker oil. Urea was used as a nitrogen source, with C/N ratios of 60:1 and 100:10 established to analyze its impact on the biodegradation of polycyclic aromatic hydrocarbons (PAHs). Experiments were conducted in plastic trays containing 2 kg of soil contaminated with 8% bunker oil. Samples were taken on days 15, 30, 60, and 90, and chemical analysis was performed using gas chromatography-mass spectrometry (GC-MS).Results indicated that the treatment with a C/N ratio of 100:10 achieved the highest PAH reduction percentage (99.27%), demonstrating the efficacy of urea biostimulation. This study highlights the importance of optimizing nutrient proportions to enhance biodegradation efficiency in hydrocarbon-contaminated soils. The addition of urea is confirmed as a viable and economical solution for soil remediation, aligning with previous studies emphasizing the crucial role of nitrogen in microbial activity and the degradation of recalcitrant contaminants.In conclusion, incorporating urea as a nitrogen source proved to be an effective strategy for biostimulation and PAH biodegradation in soil mesocosms, offering a sustainable solution to mitigate hydrocarbon contamination

Keywords: *Bioestimulation, PAHs, Biodegradation.*

Introduction

Hydrocarbon contamination over several years has become a major global concern, especially in the environmental field (Gennadiev & Pikovskii, 2007; Varjani & Upasani, 2019). The exploitation of crude oil, leaks, fuel spills, and other incidents have caused significant environmental impacts. One of the groups of hydrocarbons present in crude oil are PAHs (polycyclic aromatic hydrocarbons), which due to their molecular structure are highly polluting and persistent in the environment (Sakshi, Singh, & Haritash, 2019; Torri, Cabrera, & Alberti, 2018). The recalcitrant nature of PAHs makes their degradation difficult, representing a significant challenge for remediation.

In this regard, fuel oil 6, known as bunker, is a product of fractional distillation of oil that has high viscosity, low volatility, and mobility, characteristics that make it difficult to treat (Lien, Yang, Chang, Tu & Kao, 2016; Tran *et al.,* 2018). Soil contamination by bunker generates significant environmental problems, leading to a considerable reduction in soil production capacity and quality (Gennadiev & Pikovskii, 2007). The presence of PAHs in soils contaminated with hydrocarbons poses a potential risk to human health and ecosystems due to their toxicity, mutagenicity, and carcinogenicity (Peng *et al*., 2020).

Given all the above, alternatives to address this environmental problem are being sought. In this area, biodegradation techniques are of great interest within the field of environmental biotechnology. The use of

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biological agents such as fungi, bacteria, and plants present minimal energy consumption, low operating costs, and does not generate secondary sources of contamination (Chen *et al.*, 2019; Lamichhane, Krishna & Sarukkalige, 2017; Lien *et al.*, 2016; Ramadan, Sari, Rosmalina, Effendi & Hadrah, 2018; Redfern *et al.*, 2019). Bioremediation is a natural process in which microorganisms transform or mineralize contaminants into less toxic products, such as carbon dioxide and water (Haritash & Kaushik, 2009). Recent research has highlighted the importance of proper nutrient balance to optimize PAH biodegradation (Wu et al., 2019; Martínez *et al.*, 2015). Nitrogen is an essential nutrient for microbial growth, and its availability can significantly influence the biodegradation of hydrocarbons. The addition of nitrogen sources, such as urea, has been shown to improve microbial activity and, consequently, the hydrocarbon degradation rate (Morales, Ehrmann & Pernía, 2014; Infante *et al.*, 2010). Urea hydrolyzes in the soil, releasing ammonia and increasing the content of available nitrogen, which can stimulate the growth of hydrocarbon-degrading microorganisms (Xu *et al.*, 2020).

It is important to mention that several studies have shown promising results in the biodegradation of PAHs with the addition of urea. For example, a study by Zhang *et al.* (2021) showed that the addition of urea to soils contaminated with PAHs significantly increased microbial activity and the biodegradation rate. Another study by Liu *et al.* (2022) demonstrated that combining urea with other nutrients, such as phosphorus, can further enhance biodegradation efficiency.

Furthermore, the use of advanced technologies, such as biostimulation and bioaugmentation, in combination with the addition of nutrients, has shown great potential to improve the remediation of hydrocarbon-contaminated soils (Gong *et al.*, 2023). These technologies leverage the natural capacity of microorganisms to degrade contaminants and can be optimized by adding specific nutrients that promote their growth and activity. Biostimulation involves the addition of nutrients or conditioning agents to stimulate indigenous microbial populations, while bioaugmentation refers to the introduction of specific microbial strains with high contaminant-degrading capacity (Tyagi *et al.,* 2011).

Research has also explored the use of microbial consortia and plants in phytoremediation, combining the ability of plants to stabilize and extract contaminants with the degradative activity of microorganisms in the rhizosphere (Susarla, Medina & McCutcheon, 2002). These integrated approaches can offer more effective and sustainable solutions for the remediation of contaminated soils.

This study aims to evaluate the influence of nitrogen in the biodegradation process of soils contaminated with fuel oil 6, using urea as a nitrogen source. The results are expected to provide valuable information for the development of more effective and sustainable remediation strategies for hydrocarbon-contaminated soils.

Materials and Methods

This study was conducted in the Life Sciences laboratories at the Universidad Politécnica Salesiana. The type of soil used in the bioremediation tests was loam-clay texture, previously sieved through a 2 mm mesh. For the physicochemical analysis of the soil, the following methodology was considered (Table 1).

Table 1. Methods to Quantify Some Study Variables in The Soil.

Parameter	Method	Reference
pH	Potentiometric method	Bates, (1983)
Electrical conductivity	Electrometric method	Fernández et al., (2006)
Field capacity	Gravimetric method	Shukla et al., (2014)
Organic carbon	Walkley-Black method	García Galvis et al., (2005).
Total nitrogen	Internal method	

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To form the mesocosms, the soil was mixed with leaf litter at a proportion of 3% by weight to improve porosity and aeration. Bunker fuel was added as a contaminant at a concentration equivalent to 8% by weight. The biostimulation of the process was carried out using urea as a nitrogen source, considering the carbon-nitrogen ratio, in this case study 60:1 and another of 100:10, following the references of (Morales, Ehrmann, & Pernía, 2014).

The weight of each mesocosm was 2 kg and they were placed in plastic trays of 40 x 25 x 15 cm (Table 2). The evaluation of the effect of nitrogen and organic matter on the process was based on the different concentrations by weight.

The study was conducted over a period of 90 days, with field capacity maintained at 50% through the addition of water and aeration by turning three times a week. The average temperature in the study area was 18°C (Wilke, 2005). The experimental design was a completely randomized design (CRD) with 5 treatments, a control, and 3 replicates. The control mesocosm was coded as (TR) (Soil plus bunker fuel and no fertilization), while the treatments (T1) consisted of contaminated soil with bunker fuel, plant organic matter, and no nitrogen fertilization, (T2) contaminated soil with bunker fuel, plant organic matter, and urea as a nitrogen source, (T3) contaminated soil with bunker fuel, organic matter, and urea as a nitrogen source, (T4) contaminated soil with bunker fuel, without plant organic matter, and urea as a nitrogen source, and finally (T5) contaminated soil with bunker fuel, without plant organic matter, and with urea as a nitrogen source. The details are in Table 2.

Table 2. - Description of Treatments

Treatments	Soil (Kg)	Plant Organic Matter (Kg)	Bunker (ml)	Urea (g)
TR	1.84	……	160	…….
T 1	1.78	0.06	160	…….
T 2	1.78	0.06	160	3.60
T 3	1.78	0.06	160	23.43
T 4	1.84	……	160	3.60
T 5	1.84	……	160	23.43

Treatments 1, 2, and 3 contain 0.06 kg of plant organic matter, whereas treatments 2, 3, 4, and 5 have urea at varying concentrations as a nitrogen source, according to the C/N ratio proposed for this study.

Method for Quantification of PAHs

It was conducted using GC-Mass Spectrometry (EPA, 1986), Method 8100, on a Thermo Trace GC Ultra chromatograph coupled to a MS ISQ 7000 mass spectrometer with a commercial standard (PAH-mix9, Dr-Ehrenstorfer). Analysis was performed at 15, 30, 60, and 90 days after the start of the process (Barrios, Robayo, Prieto, & Cardona, 2017; Bento et al., 2005; Izquierdo, 2013). The GC-MS ISQ 7000 system was equipped with a non-polar TG-SQC column (15m x 0.25mm diameter x 0.25µm film thickness). For analysis, 1 µL of the standard was injected at an initial temperature of 80°C for 1 minute with ramps of 40°C min-1 until reaching 320°C and 5°C min-1 until 325°C; total runtime was 35.95 minutes. The equipment's detection limit was 0.0005-0.005 mg/kg (Balsamo, Riccardino, & Cojocariu, 2019). It is important to note that this study considered the quantification of 9 PAHs. According to current Ecuadorian environmental regulations, the quantification of these hydrocarbons is based on their toxicity. Benzo[a]pyrene, naphthalene, and fluoranthene are compounds of major concern due to their high carcinogenicity and persistence in the environment. For this reason, they are the most studied as they pose an imminent risk to public health and the environment. Monitoring 9 PAHs provides an adequate representation for assessing environmental risk (Yang et al., 2021; EPA, 2017; Wick et al., 2011; López et al., 2018; Ministerio del Ambiente de Ecuador, 2021).

Statistical Methods

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In this study, two separate analyses of variance (ANOVA) were conducted to evaluate the effects of treatments and days on the observed data. The first ANOVA was performed to assess the variability of the response variable across different days within each treatment group. This analysis aimed to determine if there were significant differences in the response variable on different days for each treatment. By examining the day-to-day variability within treatments, we aimed to capture the temporal dynamics and any potential day-specific effects that might influence the outcomes.

The second ANOVA focused on comparing the treatments across all days. This analysis aimed to identify whether the different treatments resulted in significant differences in the response variable when considering the data pooled across all days. This approach allowed us to assess the overall effect of each treatment while accounting for daily variations.

Box-Cox Transformation

To ensure the validity of the ANOVA results, Box-Cox transformations were applied to the data. The Box-Cox procedure is a powerful technique used to identify the appropriate power transformation needed to stabilize variance and make the data more normally distributed. The analysis suggested a logarithmic transformation as the most suitable normalization method for our dataset. This transformation was necessary to meet the assumptions required for ANOVA, including normality and homogeneity of variance.

Assumptions and Residual Analysis

After applying the logarithmic transformation, the assumptions underlying ANOVA were re-evaluated. The residuals were checked for normality, which is crucial for the validity of ANOVA results. Normality was assessed using visual inspections of Q-Q plots and statistical tests, confirming that the residuals followed a normal distribution.

The assumption of homogeneity of variance, which requires that the variance within each treatment group be approximately equal, was also tested. This was verified using plots of residuals versus fitted values and formal tests for equal variances. The analysis indicated that this assumption was largely satisfied, thereby supporting the robustness of the ANOVA results.

Handling Outliers, Leverage, and Influence Points

Regarding outliers and influential points, their identification and handling were carefully considered. Outliers can significantly impact the results of statistical analyses, especially in small sample sizes. Leverage points, which have the potential to influence the parameter estimates, and influence points, which can affect the overall fit of the model, were identified using standard diagnostic measures.

However, due to the relatively low number of observations in the dataset, a decision was made not to remove these points. Removing data points in small datasets can lead to a substantial loss of information and potentially bias the results. Instead, the analysis proceeded with all observations included, acknowledging the presence of these points but ensuring that the overall conclusions were not disproportionately affected by them.

Results

Physicochemical Analysis of Soil

The analysis considered the variables electrical conductivity, pH, moisture, organic carbon, and total nitrogen (Table 2). According to the results, the pH was slightly acidic, while the concentrations of organic carbon and total nitrogen were moderate.

Table 3. Physical-Chemical Analysis Results of Soil

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PARAMETERS	RESULTS
pH	6.69
Electrical conductivity	240 (µS)
Moisture	37.6%
Organic carbon	1.28%
Total nitrogen	0.23%

Initial Concentration of PAHs

Naphthalene, phenanthrene, and chrysene exhibited higher concentrations of 6.576 mg/kg, 4.661 mg/kg, and 4.560 mg/kg, respectively, while fluorene, anthracene, benzo(a)anthracene, benzo(k)fluoranthene, benzo(a)pyrene, dibenzo(a,h)anthracene, and benzo(g,h,i)perylene were found below the detection limit of the equipment according to the proposed analytical method (Balsamo et al., 2019).

Table 4. PAHs Concentration

	COMPOUND	CONCENTRATION mg/Kg
1	Naphthalene	6.576
2	Acenaphthylene	1.291
3	Acenaphthene	2.924
4	Fluorene	<0.005
5	Phenanthrene	4.661
6	Anthracene	<0.005
7	Fluoranthene	3.944
8	Pyrene	2.928
9	Benzo(a)anthracene	<0.005
10	Chrysene	4.560
11	Benzo(b)fluoranthene	1.721
12	Benzo(k)fluoranthene	<0.005
13	Benzo(a)pyrene	<0.005
14	Indeno[1,2,3-c,d]pyrene	1.404
15	**Dibenzo(a,h)anthracene**	**<0.005**
16	**Benzo (g,h,i) perylene**	**<0.005**

Fluorene, anthracene, benzo(a)anthracene, benzo(k)fluoranthene, benzo(a)pyrene, dibenzo(a,h)anthracene, and benzo(g,h,i)perylene are below the detection limit of the equipment

PAHs Concentration (Point 1)

After 15 days, naphthalene in T1 and T2 exhibited values below the detection limit, likely due to losses from abiotic processes such as volatilization (Table 5).

Table 5. Pahs Concentrations After 15 Days

Compound	Concentration (mg/kg)						
	TR	T1	T2	T3	T4	T5	T6
1.- Naphthalene	6.576	<0.005	<0.005	1.986	2.107	2.053	1.587
2.- Acenaphthylene	1.291	1.054	0.956	0.744	0.950	0.810	0.622
3.- Acenaphthene	2.924	2.562	2.598	1.971	2.353	2.028	1.759
4.- Phenanthrene	4.661	3.493	2.902	2.822	2.975	2.806	2.843

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5.- Fluoranthene	3.944	3.188	2.523	1.632	1.339	1.167	0.951
6.- Pyrene	2.928	2.491	2.475	2.394	2.207	2.167	1.930
7.- Chrysene	4.560	2.867	2.776	2.256	2.114	2.231	1.904
8.- Benzo(b)fluoranthene	1.721	1.015	0.645	0.603	0.971	0.890	0.964
9.-Indeno[1,2,3-c,d]pyrene	1.404	1.034	0.774	0.881	0.708	0.808	0.701

In treatments 1 and 2, the concentration of naphthalene is less than 0.05 mg/kg. A similar case occurs with Indeno[1,2,3-c,d]pyrene.

PAHs Concentration (Point 2)

The results after 30 days are shown in Table 6.

Table 6. Pahs Concentration in Mg/Kg After 30 Days

			Concentration mg/kg				
COMPOUND	**TR**	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**
Naphthalene	6.576	<0.005	<0.005	<0.005	<0.005	0.234	<0.005
Acenaphthylene	1.291	0.475	0.229	0.050	0.086	0.585	0.199
Acenaphthene	2.924	2.219	2.074	2.276	2.038	2.220	1.905
Phenanthrene	4.661	2.396	2.162	2.285	2.427	2.130	1.485
Fluoranthene	3.944	2.128	1.560	1,369	1.163	1.109	0.874
Pyrene	2.928	1.847	1.807	1.883	1.809	2.047	1.613
Chrysene	4.560	1.505	2.029	1.806	1.491	1.518	1.232
Benzo(b)fluoranthene	1.721	0.503	0.506	<0.005	0.657	0.735	0.782
Indeno[1,2,3-c,d]pyrene	1.404	0.699	0.499	0.786	<0.005	0.255	0.691

Naphthalene in T1, T2, T3, T4, and T5 shows values below 0.005, which is the detection limit according to the analytical method. Meanwhile, Benzo(b)fluoranthene and Indeno present similar values in T3 and T4.

PAHs Concentration (Point 3)

The concentration of PAHs at 60 days showed a greater reduction in all treatments, with some values falling below the detection limit of the equipment according to the analytical method (Table 7).

Table 7. PAHs Concentration at 60 Days

			Concentration mg/kg				
COMPOUND	**TR**	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**
Naphthalene	2.570	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005
Acenaphthylene	1.291	<0.005	<0.005	<0.005	<0.005	0.052	<0.005
Acenaphthene	2.924	1.077	1.008	1.510	1.657	0.948	1.215
Phenanthrene	24.661	0.742	0.321	1.308	1.595	1.255	0.827
Fluoranthene	3.944	0.573	0.717	0.904	0.988	0.645	0.653

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Pyrene	2.928	1.363	1.437	1.374	1.285	1.300	1.060
Chrysene	4.560	0.477	0.943	0.666	0.515	<0.005	0.334
Benzo(b)fluoranthene	1.721	<0.005	<0.005	<0.005	0.333	<0.005	<0.005
Indeno[1,2,3-c,d]pyrene	1.404	0.247	<0.005	<0.005	<0.005	<0.005	<0.005

Naphthalene, except for TR, is below 0.005. A similar case occurs with acenaphthylene, except in T5, which has a value of 0.052. A similar situation occurs with Indeno[1,2,3-c,d]pyrene in T2, T3, T4, T5, and T6. For Benzo(b)fluoranthene in T1, T2, T3, T5, and T6, a similar pattern is observed.

PAHs Concentration (Point 4)

Table 8. PAHs Concentration after 90 Days of Experimentation

The degradation of PAHs is evident for the most part, reaching values below 0.005 mg/Kg wich is the detection limit according to the analytical method.

			Concentration mg/kg				
COMPOUND	**TR**	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**
Naphthalene	2.00	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005
Acenaphthylene	1.291	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005
Acenaphthene	**2.924**	**0.583**	**0.453**	**0.717**	**0.654**	**0.110**	**0.113**
Phenanthrene	4.661	0.162	<0.005	1.034	0.762	<0.005	<0.005
Fluoranthene	3.944	<0.005.	0.571	0.720	<0.005	<0.005	0.104
Pyrene	2.928	0.242	0.225	<0.005	<0.005	0.883	<0.005
Chrysene	4.560	0.333	<0.005	<0.005	<0.005	<0.005	<0.005
Benzo(b)fluoranthene	1.721	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005
Indeno[1,2,3-c,d]pyrene	1.404	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005

Except for acenaphthene, all compounds show a decrease in various treatments, reaching values below 0.005 mg/kg.

The statistical analysis of the means of data corresponding to 3 repetitions was conducted through inferential analyses to determine the possible difference between treatments with respect to degradation time.

As the models initially did not meet the assumptions, BoxCox transformations were performed, suggesting a logarithmic change on the response variable.

Two analyses of variance were conducted. The first ANOVA considers the variation between treatments on the day they were observed. The second analysis corresponds to a repeated measures ANOVA to observe the evolution over the days.

The analysis at 15 days shows that no treatment is different from the reference. However, at 60 days, all treatments are effective. Analyzing the evolution of each treatment separately, methods 5 and 6 are observed to be the most effective at 15 days. Furthermore, treatment 5 is better as there are consecutive differences

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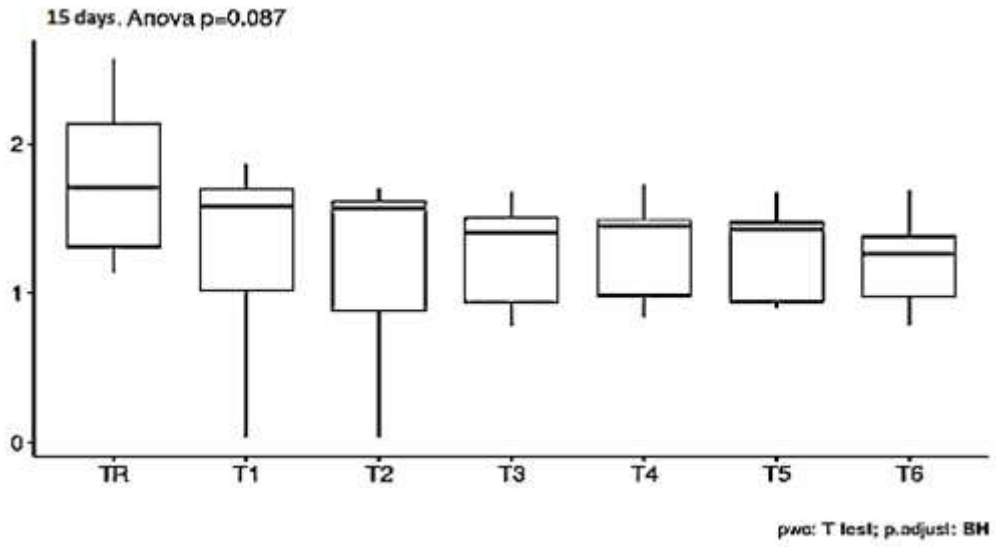
between all days, except from 30 to 60, unlike treatment 5, where there are no differences in the reduction of PAH concentration from day 15 to 30.

Indeed, at 15 days of observation, no treatment is effective, with a 95% confidence and a p-value of 0.087, as indicated in Figure 1.

Figure 1. Boxplot Across All Treatments Observed At 15 Days. There Are No Significant Differences in Means Between Them.



However, all treatments are effective after 60 days of observation, with a p-value in the analysis of variance equal to 0.005. However, none differ from each other. To affirm this, Tukey tests were conducted and p-values were adjusted according to multiple hypotheses using the Benjamini and Hochberg method, thus controlling the false positive rate.

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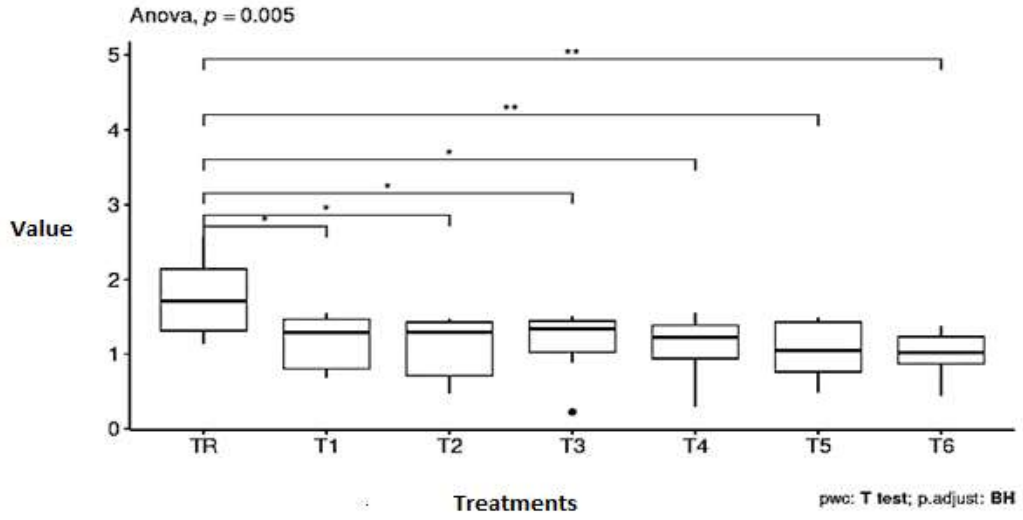
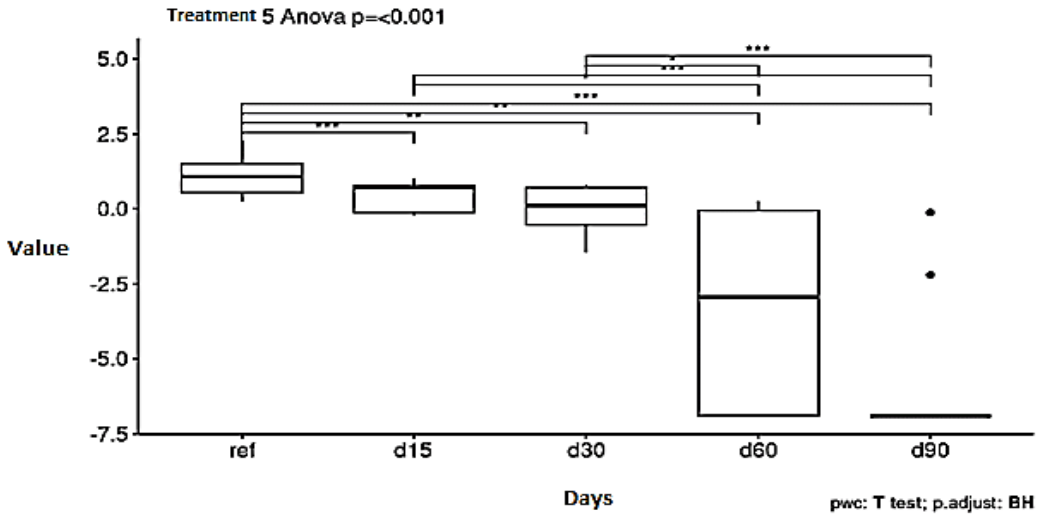
Figure 2. Boxplot Indicating Pairwise Comparisons from Post-Hoc Analysis Using Tukey's Test.



The analysis shows a significant difference among all treatments compared to the reference control. Treatments 4 and 6 tend to differ more from the control.

At the end of the experimentation period of 90 days, naphthalene, acenaphthene, Benzo(b)fluoranthene, and Indeno[1,2,3-c,d]pyrene were completely reduced to levels below the detection limit of the equipment according to the analytical method, thus preventing statistical analysis. The treatments demonstrating the highest efficacy were T5 and T6, despite no significant difference being found between treatments. As previously mentioned, no differences were found between treatments and the control at 15 days. However, in later days, a highly significant difference was observed among all treatments, with T5 and T6 being the most effective (Figures 1-2). Furthermore, when analyzing each treatment separately over time (Figures 3-4), differences between treatments 5 and 6 were observed at 15 days.

Figure 3. Analysis of Variance for T5



In the analysis of variance for T5, it can be observed that there is a difference starting from day 15; however, there is no significant difference between the subsequent 60 days.

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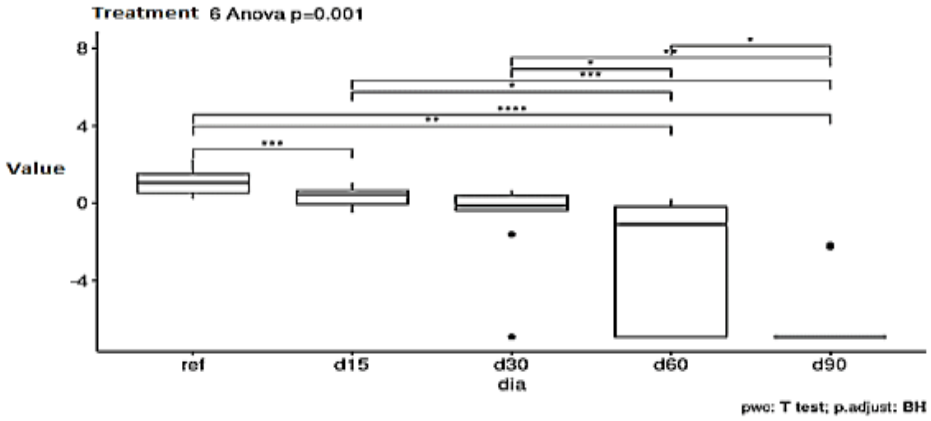
Treatment number 5 appears to be more effective in the early days compared to treatment 6.

Fig. 4 Analysis of Variance for T6



According to the analysis of variance in T6, significant differences between the means are observed, except from day 15 to 30.

As a conclusion from the statistical analysis, treatments 5 and 6 are effective after the 15-day period. All treatments differ from the control at 30 days.

Discussion

This study has demonstrated the efficiency of biostimulation using urea as a nitrogen source, resulting in a positive effect on PAH biodegradation. It has been proven that nitrogen is an essential nutrient in this process, promoting microbial activity and growth. Based on the results obtained, the C/N ratios used can be adjusted to maximize PAH biodegradation. Particularly, treatment T6 with a C/N ratio of 100:10 showed higher effectiveness in reduction, achieving a decrease in PAH concentration from 30.010 mg/kg to 0.218 mg/kg.

These findings are consistent with previous research (Otiniano, 2004), which highlights how nitrogen addition in the form of urea and ammonium salts accelerates hydrocarbon degradation in terrestrial and marine systems. Ammonium salts have been observed to achieve 92.67% efficiency, followed by urea at 70.66%.

In contrast, treatments T2, T3, and T4 showed lesser reduction in PAH concentration, possibly due to excessive nutrient presence that could have salinized the soil and inhibited microbial development. Despite this, all treatments achieved removal rates exceeding 90%, with greater degradation observed in the initial days of experimentation.

These results align with prior studies (Chemlal et al., 2013) that evaluated diesel-contaminated soil bioremediation through biostimulation, achieving removal rates of 85%. In some cases, the decrease in PAH concentration may be influenced by abiotic factors such as volatilization, but it is crucial to note that biological activity stimulated by increased carbon and nitrogen availability is the primary driver of degradation.

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In this context, it has been evidenced that bioaugmentation is effective in cleaning up sites contaminated with PAHs. However, various studies have pointed out that both abiotic and biotic factors significantly influence the efficacy of bioaugmentation processes (Cho et al., 2000; Bento et al., 2005; Wolski et al., 2006).

Conclusions

The biodegradation of PAHs was significantly improved due to the presence of nitrogen in any of its ratios, and based on this study, its use could be suggested to optimize PAH biodegradation in the field. The C/N ratio of 100:10 achieved the highest degradation rate, corresponding to T6, reaching results greater than 90% degradation of the contaminant. In T3, a lower degradation percentage was reported compared to the other treatments; however, all treatments showed a greater decrease compared to the control. Finally, it is important to mention that, according to current environmental regulations, the concentration of PAHs should present values <2 mg/kg for bioremediated soil and agricultural use. Our results in T1, T2, T4, T5, and T6 would be within the permissible limits, except for T3.

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