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# Bioaugmentation and Biostimulation-Enhanced Bioremediation of Hydrocarbon-Contaminated Soil Evaluation of the Effect of Soil Washing Pre-Treatment and Bacterial Communities

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## ABSTRACT

Fuel, a petroleum derivative, contributes to soil pollution because of its hydrocarbons, which are difficult to decompose. Bioremediation can assist by introducing microbes that are capable of degrading hydrocarbons and enhancing this process by adding nutrients. This study validated previous research by adding the most optimum nutrients, bacteria with the right ratio, and pre-treatment of soil washing on the degradation value of hydrocarbons in crude oil-contaminated soil. Pre-treatment involved washing the soil with surfactant tween-80, determining the type of mixed bacterial culture variables, adding varying concentrations of inoculum and inorganic nutrients, and determining the optimal total petroleum hydrocarbon (TPH) reduction. The study found that the provision of nutrients, bacteria, and pre-treatment in soil washing had no significant effect on the original soil TPH.

**Keywords:** bioremediation, bioaugmentation, biostimulation, hydrocarbons, pollution control

## INTRODUCTION

One of the most common causes of soil contamination is the release of hydrocarbons from chemicals used and produced by humans onto the land surface. In the mining sector, fuel oil is a hydrocarbon contaminant with the potential to become an environmental pollutant (Putra, 2016). Mining activities, as well as processing and distribution, can cause pollution through leaks, accidents, and overfilling of tanks (Nurmalasari, 2018). Currently, Indonesia has 10 oil fields. The oil refinery is owned by PT Pertamina (Persero) and other private companies, with a total oil refinery processing capacity of 1.156 million barrels per day.

The oil refineries owned by PT Pertamina (Persero) are Pangkalan Brandan with a processing capacity of 4,500 barrels per day which has not been operating since 2007, Dumai (127,000 barrels per day), Pakning River (50,000 barrels per day), Musi (127,300 barrels per day), Cilacap (348,000 barrels per day), Balikpapan (260,000 barrels per day), Balongan (125,000 barrels per day), and Kasim

(10,000 barrels per day). Based on the information from the Ministry of Energy and Mineral Resources, the average consumption is 345,140,000 barrels per year, and the average consumption of fuel is 1.76% per year. This large development has created a deficit, thus requiring the construction of a new refinery with a capacity of 1,000,0000 barrels per day (Sa'adah et al., 2017). The exploration of petroleum found in Indonesia has yielded low contents of aromatic compounds and sulfur. The percentages of the main components of petroleum are 80%–89% carbon, 12%–14% hydrogen, 2%–3% oxygen, and 0%–3% sulfur, 0.3%–1% nitrogen. Impurities such as Cl, Ni, Mo, Fe, Na, and other elements are also part of petroleum manufacturing (Nugroho, 2010). Notably, the combination of hydrocarbon complexes is also the main ingredient in the manufacture of petroleum (Thalib, 1972).

Hydrocarbons are the main ingredients of this petroleum, causing the formation of soil pollution in several mines in Indonesia. One example of this pollution occurs near PT CPI Riau's Minas oil mine. This type of pollution is triggered by crude oil spills during manufacturing, transportation, and drilling (Charlena et al., 2009). Excessive hydrocarbon content was also observed in the oil mining of the Wonocolo, Bojonegoro, with the TPH value reaching 6.05%. This large percentage of pollution has exceeded the quality standard according to the Minister of Environment Decree No. 128 of 2003 by 1% (Barakwan, 2017).

The next problem of soil pollution caused by hydrocarbons also occurs at PT. Unilever Jakarta, which covers an area of 2.2 ha, while the PT. Caltex area is 8 ha. This pollution was triggered by a leak in the petroleum pipeline at PT. The length of Conoco Phillips is 300 m (Munawar, 2012). Several strategies have been used to reduce the effect of soil pollution, but the techniques used remain ineffective in overcoming these problems. The large and expensive functional cost is also a limitation. One of the current strategies for managing oil waste is to use bioremediation techniques. The bioremediation strategy is a waste management technique that is determined to return the general habitat to its original condition by using microorganisms (I Ketut Irianto, 2017). Bioremediation completed in Indonesia has passed the soil testing standard by utilizing total petroleum hydrocarbon (TPH). TPH plans to determine the level of protection for the climate by determining the level of crude oil content in the open ground (Handrianto, 2018). The achievement of bioremediation can be controlled by several elements. The element of nature can be considered the main factor. These ecological elements combine humidity, pH, and temperature (Cookson, 1995). The following variables have known effects: the increase in the actions of indigenous microorganisms, including degrading hydrocarbon organisms. This process is completed by adding a supplement containing two drugs (biostimulation and bioaugmentation-biostimulation mixture). The aim is for a slump to occur in hydrocarbons around the first three weeks of the incubation cycle (Aleer et al., 2011). The last factor is the treatment with air circulation media, which will increase the percent of TPH biodegradation when looking at media without air circulation to improve biodegradation. The final TPH was equal to 100% and the media without aeration was 79.6% (Wayoi, 2018).

The present research aims to enhance the bioremediation of hydrocarbon-contaminated soil by using bioaugmentation and biostimulation methods. This study used the optimum conditions from the results obtained by previous studies (Nurmalasari, 2018; Vyatrawan, 2015), namely the type of bacteria *P. fluorescens-P. putida*, the composition of the addition of 5% (*w*/*w*) inoculants, and the addition of nutrients with a composition of C:N:P=100:5:1. The surfactant composition used as pre-treatment had a soil-washing solution ratio of 50 g/L and a surfactant concentration of 0.02% (*v*/*v*). This study aimed to validate the results of previous studies with the addition of optimum nutrients, the addition of a bacterial consortium, and pre-treatment soil washing on land originally from Wonocolo Village, Kedewan District, Bojonegoro Regency.

## MATERIALS AND METHODS

### Collection of total petroleum hydrocarbon (TPH) contaminated soil

Soil sampling in petroleum mining in Wonocolo, Bojonegoro, was carried out on the refinery area land. The three sample points in this refining area have the following coordinates:

- Sample 1 = $7^0$ 02'44.5"S $111^0$ 39'34.4"E
- Sample 2 = $7^0$ 02'44.2"S $111^0$ 39'34.6"E
- Sample 3 = $7^0$ 02'44.3"S 111 $^0$39'34.1"E

### Pre-treatment soil washing

Pre-treatment soil washing was done on soil samples with petroleum contamination and

mixed with Tween-80. Tween-80 is a biodegradable, nonionic surfactant. According to Charlena et al. (2009), non-ionic surfactants are non-toxic essential ingredients with a threshold concentration of more than 100 g/kg.

### Preparation of bacterial cultures and suspensions

A 250 mL Erlenmeyer flask containing 100 mL of Nutrient Broth (Merck, USA) was prepared, and then three loops of each bacterium were transferred into each Nutrient Broth medium (Merck, USA) aseptically and incubated in a shaker incubator (150 rpm) at room temperature for 18 h (Imron et al., 2019; Purwanti et al., 2019). Cell cultures were prepared from harvested cell cultures in the mid-log phase with OD of 1.0 at $\lambda = 600$ nm. The bacterial cells from the media were separated using a centrifuge at 3,000 rpm for 15 min. The pellets formed were rinsed twice with physiological water (0.85% sterile NaCl). Next, 150 mL of sterile physiological water was added to the bacterial pellets in an Erlenmeyer flask. The dry weight of the pellets or bacterial biomass formed was measured to determine the number per gram of bacteria that entered the reactor. The dry weight measurement of the pellets was carried out by drying the pellets in an oven at 80 °C for 24 hours to remove the water content so that the measurement would only consist of the per-gram pellet formed.

### Preparation of inorganic fertilizer for treatment

Variation in the addition of nutrients to the soil at a C:N:P ratio of 100:5:1 was carried out based on the research of previous research (I Zam and mustafa, 2012; Nurmalasari, 2018), which showed the optimum degradation of hydrocarbonoclastic bacteria was added to the C:N:P ratio of 100:5:1. Elemental N was obtained from 46% of the urea of PT. Petrokimia Gresik, and elemental P was obtained from potassium dihydrogen phosphate. Urea and superphosphate fertilizers for treatment were prepared by smoothing them first by using a mortar and then sieving them through a 40-mesh sieve.

### Bioremediation of hydrocarbon-contaminated soil

The bioremediation test was carried out for 14 days in a round glass reactor (inoculum size). Approximately 50 g of the original soil was used, and a 5% bacterial consortium was added to each reactor. The nutrients were added with a ratio of C:N:P = 100:5:1 to the determined reactor.

## RESULTS AND DISCUSSIONS

### Soil washing process

The compilation of results obtained during the soil washing process is depicted in Figure 1.

#### *Analysis of pH*

The data for measuring the pH of the original soil and the soil that underwent soil washing are shown in Figure 1a. The original soil pH values at points 1, 2, and 3 were obtained at 6.9, 7.1, and 7.2, respectively. The pH at points 1 and 2 increased after soil washing to 7.4 and 7.3, respectively. The pH increased because of the formation of nitrogen gas, or ammonia, from nitrate reduction (Cookson, 1995). At point 3, shrinkage occurred from pH 7.2 to 7.1. This phenomenon occurred because organic acids were formed (Cookson, 1995). The pH value can change if microbial development occurs. To control nitrogen shrinkage caused by ammonia evaporation at pH 7.5 (Mari et al., 2005). The results of the pH analysis on the original soil and the soil after soil washing were still in the range of 6–9. The pH values of the washing water at points 1, 2, and 3 were 6.0, 5.3, and 6.3, respectively.

An important factor for the degradation activity of introduced bacteria is soil pH, because it remarkably influences the maximum situation of carbon-degrading microorganisms. In microorganisms, pH participates in carrying out cell functions, transporting cell membranes, and balancing reactions. The growth of microorganisms increased at pH 6–9. The pH value of the washing water at the three points shown in Table 1 was lower than that of the original soil and the soil after soil washing. This finding was obtained because of the metabolic activity of cells resulting from the metabolites of organic acids and carbon dioxide. When carbon dioxide is dissolved in water, it produces acid, thus decreasing the pH (Vyatrawan, 2015).

#### *Analysis of soil washing water levels*

Results in the water content of the original soil and the soil that has been soil washed are shown

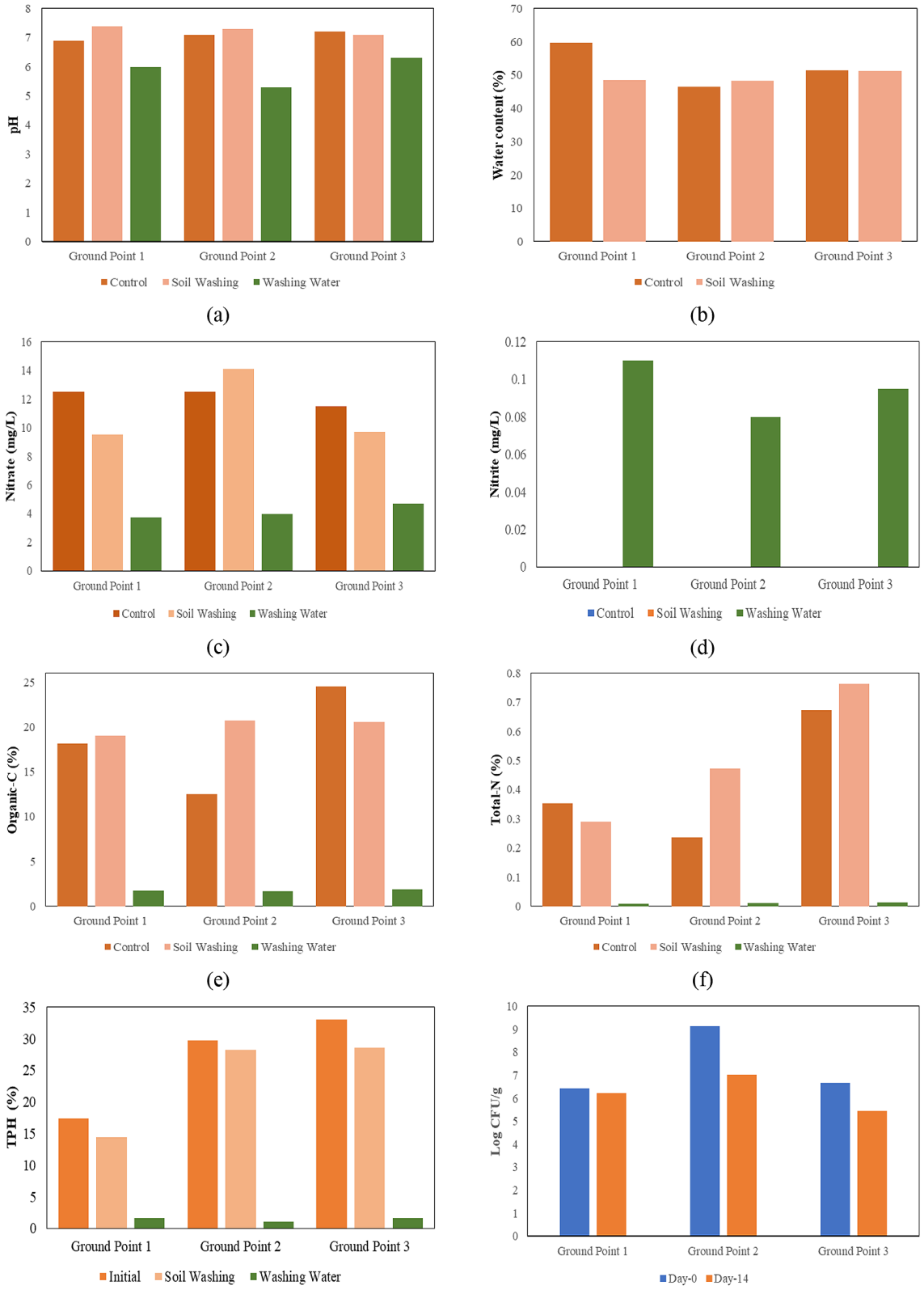


**Figure 1**. Results of (a) pH, (b) water content, (c) nitrate, (d) nitrite, (e) C-organic, (f) N-total, (g) TPH, and (h) TPC after soil washing

in Figure 1b. As shown in Figure 1b, the original soil of point 1 had a water content of 59.82%, and after soil washing, the value decreased to 48.55%. The original point 2 soil contained 46.42% water, and after soil washing, the value increased to 48.55%. The original soil water content at point

3 was 51.51%, and after soil washing, the value decreased to 51.19%. The soil in its natural state generally contains 15%–100% water content (Wesley, 1977). Gas transfer, the level of contaminant toxicity, and the distribution of microorganisms affect the water content of soil (Cookson, 1995). The limitation of hydrocarbon biodegradation in the soil environment causes a limited water supply for metabolism and microbial growth; therefore, the water content should not exceed 60% to optimize the hydrocarbon degradation process (Cookson, 1995). At points 1 and 3, according to Figure 1b, the percentage of the water content decreased because the microbes found in the original soil contaminated with oil utilized the oil as a carbon source to carry out metabolism, thus releasing the oil molecules attached to the soil pores and filling them with water.

### *Nitrate level analysis*

The nitrate content at 3 soil sampling points remained relatively small, but without an effort to overcome it, the accumulation of nitrate will be prolonged. As shown in Figure 1c, the nitrate content in point 1 soil was 12.50 mg/L, which decreased to 9.50 mg/L after soil washing. The nitrate content also decreased at point 3 in the soil from 11.50 mg/L to 9.70 mg/L. The nitrate in the soil decreased because the surfactant absorbed the nitrate anion. The nitrate levels decreased because of the evaporation of nitrogen in the form of ammonia into the air (Barakwan, 2017). At point 2, the nitrate content after soil washing increased from 12.50 mg/L to 14.10 mg/L.

The presence of nitrate is indicated by the result of chemical contamination used in petroleum mining management activities in Wonocolo Village. Nitrates contained in soil can contaminate groundwater. Groundwater containing nitrates and entering the human body can cause various risks of disease. Nitrates play a role in spontaneous miscarriage, thyroid disorders, birth defects, and the development of certain types of cancer in adults (Dewi et al., 2016). The nitrate content at three soil sampling points in Wonocolo village remains relatively small, but if no effort is made to overcome it, the accumulation of nitrate will be prolonged.

The nitrate content may increase because the bacteria contained in the soil experience a lot of death. Bacterial death causes an increase in nitrate because, when the bacteria die, the nitrate absorbed by the bacteria as cell-forming agents remains in the bacteria's body and is then released (Barakwan, 2017). The nitrate content in the washing water after soil washing treatment was 3.71 mg/L in point 1, 3.94 mg/L in point 2, and 4.70 mg/L in point 3. In the soil washing process, the surfactant washing solution adsorbed the nitrate anion so that the nitrate content could be found in the washing water. Microorganisms that die and dissolve during the soil washing process also contribute to the nitrate content in the washing water, because in this process, bacteria absorb nitrates contained in the soil or those formed through the nitrification process, and these nitrates are released when the bacteria die (Barakwan, 2017).

### *Nitrite levels analysis*

The data on the nitrite content obtained by soil washing can be analyzed, as shown in Figure 1d. Figure 1d shows that the starting soil at points 1, 2, and 3 did not contain nitrite. After soil washing was carried out on the three soils, the results showed no change in the nitrate content in the soil samples. The nitrite contents detected in the soil washing water after the soil washing process were 0.110, 0.080, and 0.095 mg/L at points 1, 2, and 3, respectively. The nitrite parameter is correlated with pH. The higher the pH value, the higher the formation of nitrite ($NO_2$-N). A negative correlation was observed between nitrate and pH, and an increase in pH encourages the formation of $NH_3$ and $NO_2$ gases and decreases the concentration of nitrate ($NO_3$) (Wantasen, 2015). Nitrite is formed in the middle of the nitrification and denitrification processes, causing nitrite to become an unstable compound (Nolan et al., 2011). Nitrification occurs in an aerobic situation where ammonium is converted to nitrite and forms nitrate. This change occurs because of the presence of *Nitrosomonas* bacteria (Ilma, 2021). The nitrite content was detected in the washing water after the soil washing process. The presence of bacteria, the content of ammonium, and nitrate will form a cycle in the nitrification and denitrification processes, allowing the process to proceed and nitrite to be formed. Nitrification can occur with assisted oxidation (Barakwan, 2017).

### *C-Organic levels analysis*

Data on C-organic levels were obtained by soil washing, as shown in Figure 1e. The C-organic values in the original soil for points 1, 2,

and 3 soils were 18.12%, 12.51%, and 24.54%, respectively. The C-organic values in the soil after soil washing obtained at points 1, 2, and 3 soils were 19.01%, 20.73%, and 20.55%, respectively. The values at soil points 1 and 2 increased, whereas those at point 3 decreased. C-organic is a source of energy used to perform metabolism in microorganisms (Imron et al., 2019). Organic C levels decrease faster if the number of microorganisms that consume it is large. The C-organic value increased because of the soil contact with surfactant organic matter containing high C content. Meanwhile, the decrease in C-organic can be caused by bacteria that use carbon (C) as an energy source continuously. This phenomenon causes soil contamination due to the presence of carbon in the soil. The decreased C/N ratio is caused by the reduced amount of carbon in the soil (Hanafi and Ocatvia, 2014). Ground washing water at points 1, 2, and 3 contained a little C of 1.77%, 1.66%, and 1.87%, respectively. This finding was obtained because the soil washing process uses a surfactant that also contains C.

### *N-Total soil washing levels analysis*

The N-total data of original soil and soil that has been soil washed are shown in Figure 1f. Figure 1f shows that the original soil point 1 has a total N-content of 0.354%, which decreased to 0.290% after washing the soil. The original soil points 2 contained an N-total of 0.236%, which increased to 0.472% after washing the soil. The original soil water content at point 3 was 0.673%, which increased by 0.763% after washing the soil. Ground washing water at points 1, 2, and 3 contained N-Total with small concentrations of 0.008%, 0.012%, and 0.014%, respectively. N-total is nitrogen in two forms, namely, organic and inorganic. The organic compounds are formed by enzymes, amino acids, and proteins, while the inorganic compounds are formed by ammonia, nitrate, and nitrite. The nitrogen levels at points 2 and 3 can increase because of the conversion of ammonium to nitrate in microorganisms, thus increasing nitrogen elements (Al-Ajalin et al., 2022; Buhari et al., 2022; Fitriani et al., 2023). In addition, the surfactants caused the decomposition of soil organic matter, binding by microorganisms, and an increase in organic matter with high nitrogen. Nitrogen levels undergo several changes caused by the loss of nitrogen in the form of ammonia, which evaporates into the air so that the nitrogen content temporarily decreases. The discovery of total N content in washing water can be caused by microorganisms that die and dissolve during the soil washing process so that the nitrate absorbed by bacteria as cell-forming is released and contributes to the N content in the washing water (Barakwan, 2017). N content can be found in proteins, enzymes, and nucleic acids from microbes (Alfia et al., 2013).

### *TPH in soil washing*

Figure 1g shows the results of the analysis of the TPH value for soil leaching. A feasible estimate of TPH by using the gravimetric technique was changed to US EPA-821-R-98-002 Teknik 1664 in 1999. After completing the investigation with the gravimetric technique, the introductory TPH fixation on the sample was 17.45% for point 1, 29.72% for point 2, and 33.06% for point 3.

The maximum biological value of TPH allowed to manage polluted soil with bioremediation was 15%. Considering that the concentration of petroleum hydrocarbons at the three points of the soil sample exceeds the regulation of KEPMEN LH NO 128, initial processing was carried out with the soil washing method to minimize hydrocarbon contamination in the soil up to 15%. The surfactant solution cannot completely remove the contaminants present in the soil, but the surfactant solution can minimize the contaminants bound in the soil (Tumpahan et al., 2015; Wilén et al., 2006).

In the process of binding contaminants with a surfactant solution in petroleum-contaminated soil, the surfactant solution will be formed as micelles, monomers, hemimicelles, and admis cells, depending on the surfactant concentration. Transfer of oil or hydrocarbon compounds into the micelles as surfactants or monomers can increase the solubility, thus improving the process of separation (Tumpahan et al., 2015). Based on the balance of TPH concentrations in the three soils after soil washing, washing water experienced an imbalance in the initial soil TPH. This imbalance is caused by the soil washing process, in which the TPH in the initial soil evaporates into carbon dioxide.

To test the mixture that is degraded through the dirt washing process, we carried out gas chromatography-mass spectrometry (GC-MS). Biodegradation of hydrocarbon compounds was determined from the value of the decline rate at the peak combining increasing hydrocarbons with the fixation value of TPH (mg/kg). The hydrocarbon

concentrations (mg/kg) in Tables 1, 2, and 3 show the follow-up effect of doubling the area (%) of each compound with TPH grouping at each starting point and after wasting soil.

Table 2 shows that methyl cyclopentane compounds were no longer detected in point 1 soil, compound 3 methyl pentane and methyl cyclopentane in point 2 soil, and n-hexane compounds were no longer detected in point 3 soil. Points 1 and 3 soils after the soil washing process still contained types of contaminants. Compound 2 ethyl-oxetane was observed at point 1 soil with a final hydrocarbon of 11,204.99 mg/kg and removal of 32.08%. At point 3, the compound was methyl cyclopentane with a final hydrocarbon of 11,014.88 mg/kg and a removal rate of 91.01%. The addition of surfactants not only removes hydrocarbon compounds but can also give rise to new compounds. Based on the chromatograms of point 2 and point 3 soils, 2-pentanone-4-hydroxy-4-methyl compounds appeared at 20 min (soil point 2 and point 3) and octane compounds, 2,4,6-trimethyl, at 30 min (ground point 3).

Table 3 shows the results of compounds bound to the soil washing water. Point 1 washing water contained compound 2 ethyl-oxetane with a concentration of 4.5 mg/kg. Referring to the initial point 1 soil with a hydrocarbon concentration of compound 2 ethyl-oxetane of 16,498.18 mg/kg, the concentration of the compound was 11,204.99 mg/kg after soil washing. The difference between two ethyl-oxetane compounds before and after the soil washing process was 5,293.19 mg/kg. Therefore, the content of compound 2 ethyl-oxetane in point 1 washing water is 5,293.19 mg/kg, but based on the results of the analysis, the content of compound 2 ethyl-oxetane is only 4.5 mg/kg. The peaks of hydrocarbon compounds extracted gravimetrically can be determined using the chromatogram as shown in the retention time of the compounds (as listed in Figures 2, 3, and 4).

GC-MS measurements were conducted after the soil washing process to determine the residual compounds and the effect of the addition of surfactants to the soil. The working principle of GC was the forwarding of gas flow through the stationary phase to separate organic compounds. The GC mooring time shows the identity of the compound, which can provide qualitative information, namely the presence or absence of certain

**Table 1.** Hydrocarbon components of GC-MS results in initial soil

| Point | TPH (mg/kg) | Compound name | Formula | Area (%) | Hydrocarbons (mg/kg) |
|---|---|---|---|---|---|
| Ground point 1 | 174,495.49 | 2 Ethyl-Oxetane | $C_5H_{10}O$ | 9.45 | 16,498.18 |
| | 174,495.49 | Methyl Cyclopentane | $C_6H_{12}$ | 2.81 | 4,895.46 |
| Ground point 2 | 297,171.04 | 3 Methyl Pentane | $C_6H_{14}$ | 6.90 | 20,519.33 |
| | 297171.04 | Methyl Cyclopentane | $C_6H_{12}$ | 1.08 | 3,221.10 |
| Ground point 3 | 330,559.05 | N-Hexane | $C_6H_{14}$ | 5.17 | 17,105.03 |
| | 330,559.05 | Methyl Cyclopentane | $C_6H_{12}$ | 0.37 | 122,549.32 |

**Table 2.** Hydrocarbon components of GC-MS results in soil washing

| Point | TPH (mg/kg) | Compound name | Formula | Area (%) | Hydrocarbons (mg/kg) |
|---|---|---|---|---|---|
| Ground point 1 | 144,031.74 | 2 Ethyl-Oxetane | $C_5H_{10}O$ | 7.78 | 11,204.99 |
| Ground point 2 | 282,776.35 | N-Hexane | $C_6H_{14}$ | 10.41 | 29,450.97 |
| Ground point 3 | 286,491.39 | Methyl Cyclopentane | $C_6H_{12}$ | 3.85 | 11,014.88 |

**Table 3.** Hydrocarbon components of GC-MS results in soil washing water

| Washing water | TPH (mg/kg) | Compound name | Formula | Area (%) | Hydrocarbons (mg/kg) |
|---|---|---|---|---|---|
| Ground point 1 | 11.624 | 2 Ethyl-Oxetane | $C_5H_{10}O$ | 38.85 | 4.5 |
| Ground point 2 | 10.88 | 2 Ethyl-Oxetane | $C_5H_{10}O$ | 55.41 | 6.03 |
| | 10.88 | 3 Methyl Pentane | $C_6H_{14}$ | 30.94 | 3.37 |
| Ground point 3 | 16.17 | Allyl Acetate | $C_6H_{12}$ | 13.68 | 2.21 |
| | 16.17 | 2,3 Dimethyl Pentane | $C_7H_{16}$ | 65.63 | 10.61 |

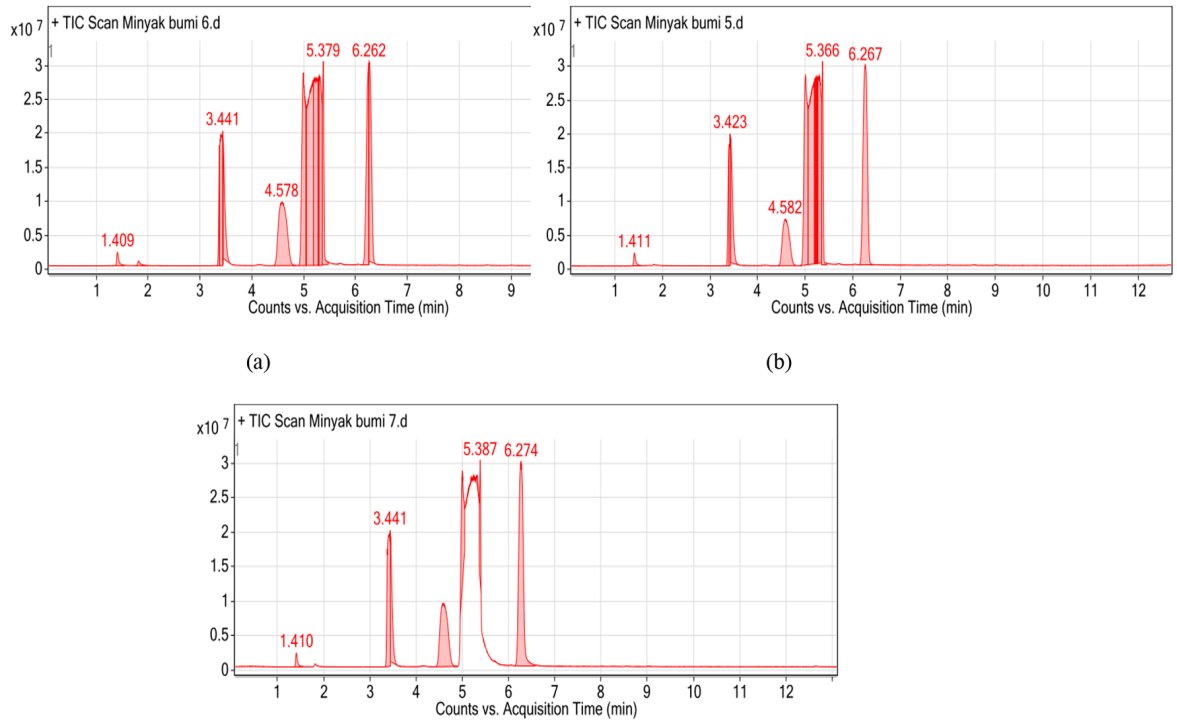


**Figure 2.** GC spectra of initial soil for (a) point 1, (b) point 2, and (c) point 3

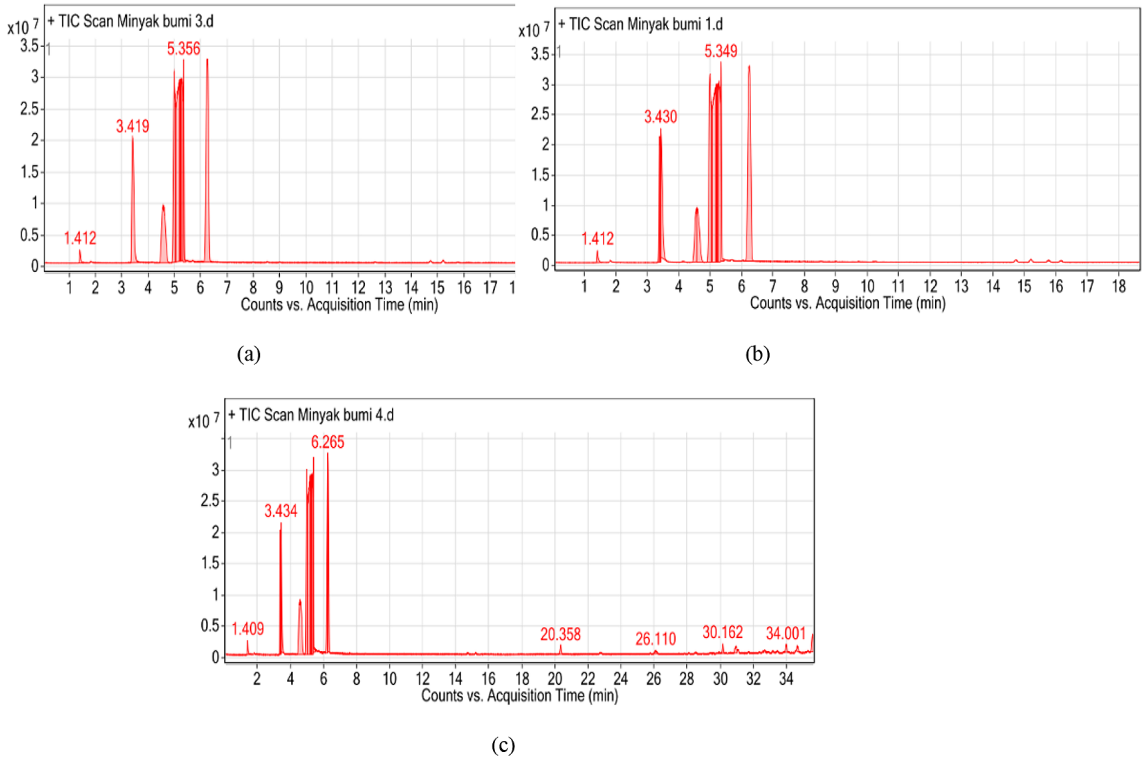


**Figure 3.** GC spectra after soil washing for (a) point 1, (b) point 2, and (c) point 3

compounds, and can indicate the amount of each compound in a mixture. The separation of compounds in the degradable hydrocarbon fraction can be detected using a GC device by observing the loss and growth of the identity of the hydrocarbon compounds (Hendayana, 2006). The

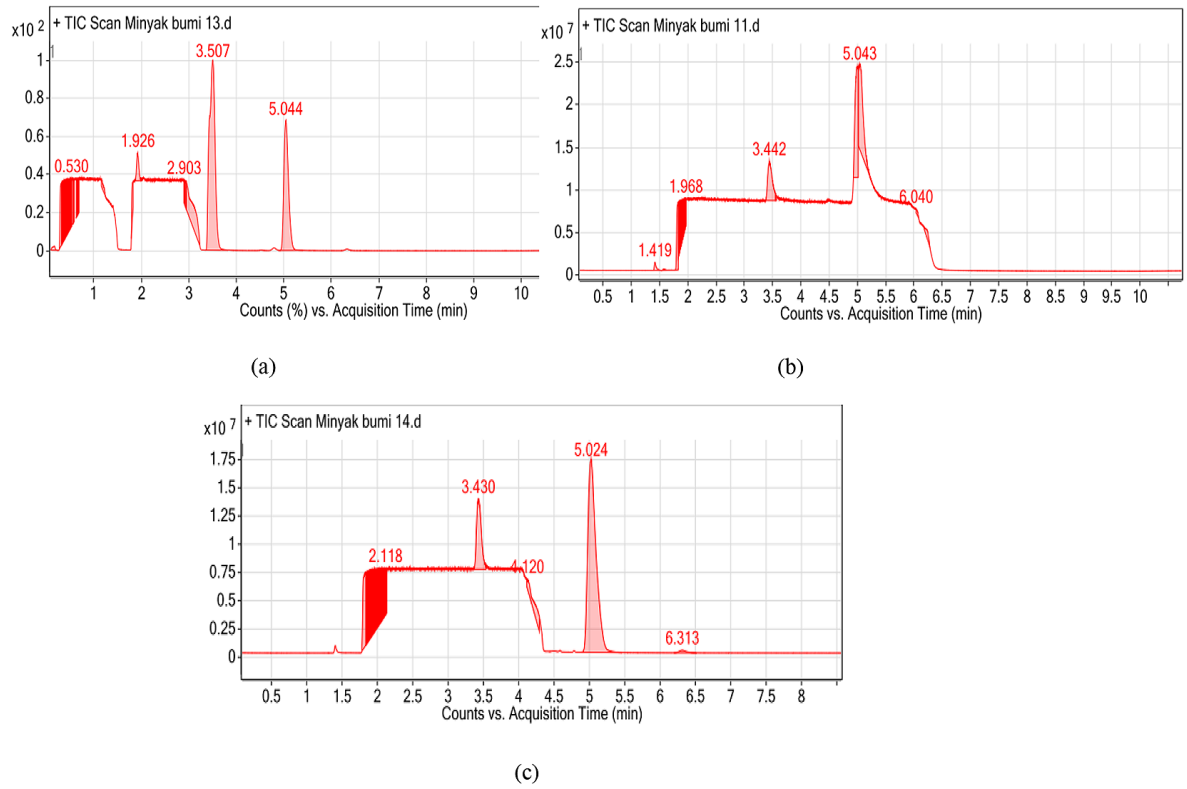


**Figure 4.** GC spectra of the washing water for (a) point 1, (b) point 2, and (c) point 3

increase in oil and grease content in the soil and the removal of polar contaminants can be done by adding surfactants to the soil. The GCMS results at the three initial soil points show that the hydrocarbon compounds contained in the soil were saturated aliphatic hydrocarbon compounds and cyclic hydrocarbon compounds with compounds containing 5 and 6 carbon chains. Surfactants in the soil can increase the oil and grease content of the soil and remove polar contaminants. The addition of surfactants not only removes hydrocarbon compounds but can also introduce new compounds. Based on the chromatograms of point 2 and 3 soils, 2-pentanone-4-hydroxy-4-methyl compounds appeared at 20 mins (soil point 2 and point 3) and octane compounds, 2,4,6-trimethyl, at 30 mins (ground point 3).

The emergence of this new compound is attributed to the interaction between surfactants, contaminants, and soil organics. Surfactants have a polar and a non-polar site, in which the non-polar side can bind hydrophobic organic contaminants such as paraffin (long-chain alkane compounds) to form new compounds (Tumpahan et al., 2015). The presence of new compounds indicates the exchange of positions in the surfactant with oil and soil organics.

### *Total plate count soil washing*

Total plate count *(*TPC) was used to count the number of bacterial colonies performed in this study. The first stage of this research process was to make growth media for microbes. The material used was sodium agar medium (NA). First, the NA media were transferred into the reaction tube and plugged with cotton. Before use, the tools and NA media were sterilized in an autoclave at 121 °C for 1 h. This sterilization process aims to eliminate the existing microorganisms in the material through heating (Al-Ajalin et al., 2022; Nimatuzahroh et al., 2022). After the sterilization process, 10 g of soil samples, from which the total microbial colonies will be determined, were placed in an Erlenmeyer flask with 150 ml of distilled water and then shaken for 60 min. Approximately 1 mL of sample was obtained using a clean measuring pipette, and then the NA medium was poured into a Petri cup. During this interaction, it is accomplished aseptically (near the fire) to limit the ascent of microorganisms from the outside climate into the medium. The petri dish containing the sample was allowed to stand for ± 15 minutes and then incubated at 37 °C for 24 hours to determine

the number of microbial colonies that were co-developed. The total microbial colonies before and after soil washing are shown in Figure 1h.

Total microbial colonies in soil samples were measured to count and observe oil-degrading microbial colonies in soil samples for each treatment change. Total microbial colonies were measured before and after soil washing. The results indicate a decrease in total bacterial colonies after soil washing. The decrease in total bacterial colonies was influenced by environmental factors that were not suitable for microbial growth. Indigenous bacteria are suspected to have died or are in the adaptation phase after the soil washing process because of environmental factors that are less than optimal or inappropriate, such as temperature, humidity, and oxygen ($O_2$) in the soil (Mrozik and Piotrowska-Seget, 2010).

If it relates to the addition of surfactant during the soil washing process, the number of bacteria decreases in the number of colonies, and it is suspected that the surfactant tween-80 solution can be toxic to microbes and destroy bacterial cell membranes (Shafiee et al., 2006). Microorganisms that degrade hydrocarbons can grow and adapt to temperature, oxygen, and the presence of contaminants. Optimal biodegradation in soil is influenced by environmental properties, such as soil type and parameters, that affect environmental factors (Purwanti et al., 2017b, 2018c).

## Bioremediation process

The results of the analysis of water content during the incubation period are shown in Figure 5. Figure 5 shows that the water content decreased significantly. The initial soil moisture content before the incubation process was in the range of 46%–60%. The most visible decrease in water content was found in the action of point 1 soil without soil washing with *P. putida-P. fluorescens* 5% (*w*/*w*) and the addition of nutrients C:N:P = 100:5:1. The success of a bioremediation process is highly influenced by environmental factors, including the water content of the soil. Water content is very influential on the process of oxygen exchange so that the activity of microorganisms can run well (Qi et al., 2018). Water content is very important for the metabolic activity of microbes in petroleum-contaminated soil because microbes remain active at the interface between oil and water (Aliyanta et al., 2012). The ideal humidity for microbial activity is 50%–80%, and the optimum water content required by bacteria is 50%–60% (Sari et al., 2014). The initial soil moisture content before the incubation process is 46%–60%. The decrease in water content in each treatment differed because of the addition of nutrients in some reactors.

The addition of nutrients makes microbes more active (Hanafi and Ocatvia, 2014). With the addition of these nutrients, microbes will produce oxygenase enzymes to oxidize hydrocarbons. The difference in water content reduction is also caused by the initial soil used for treatment. The soil used at this stage was treated with and without pre-treatment soil washing. Soil without pre-treatment soil washing experienced a greater decrease than pre-treatment soil washing because fewer microorganisms

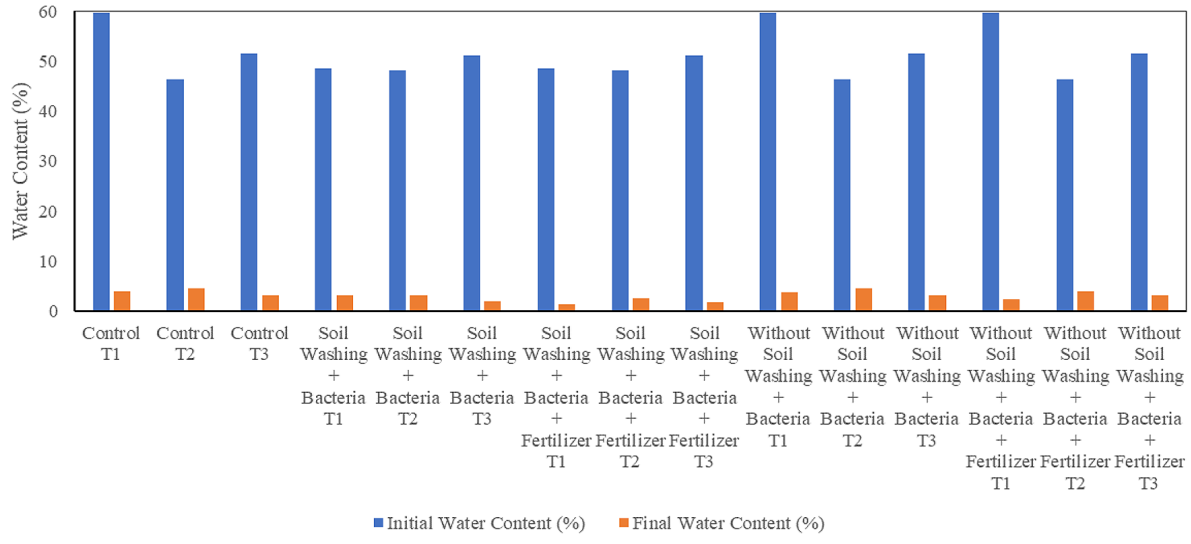


**Figure 5.** Water content of bioremediation process

were present in pre-treatment soil washing due to the large number of microorganisms that died during the soil washing process. In the present study, the pH parameter was tested, and the results obtained are shown in Figure 6.

Based on Figure 6, the pH values obtained from the beginning of the first day of the experiment to week 2 of the 14th day in each reactor fluctuated, ranging from 5.9 to 7.1. From day 0 to day 2, the pH value ranged from 6.8 to 7.1. From day 3 to day 4, the pH value slightly decreased to 6.8–7.1. The pH value on the 5th to 9th days decreased to 5.9–6.6. From days 10 to 14, the pH value increased to 6.5–7.0. The control reactor without bacteria and fertilizer and the non-soil washing+bacterial reactor showed pH values in the range of 6.0–7.0. The pH of soil washing + bacteria reactors, soil washing + bacteria + fertilizer reactors, and non-soil washing + bacteria + fertilizer reactors decreased to the range of 5.9–7.0.

The pH in the soil sample for each treatment variation was measured to determine and control the pH value because it influences the life of microorganisms in the hydrocarbon removal process. The elimination of microorganisms mostly occurs at a neutral pH. In some soil types, extreme pH values will negatively affect the speed of the hydrocarbon removal process. The decrease in pH in the range of days 3 to 9 and the treatment reactor can be caused by the activity of the microbial consortium that is adapting to new environmental conditions to form acid metabolites (Permatasari et al., 2022; Purwanti et al., 2019). Another possibility occurs because the process of removing alkanes contained in crude oil can form alcohol and then become fatty acids. These fatty acids are oxidized to form acetic acid and propionic acid, which can reduce the pH value (Nugroho, 2010; Rosenberg et al., 1969). By the 14th day, the pH value increased again to 6.5–7.0. The increased activity of microorganisms indicates an increase in the pH value to form ammonia compounds (Buhari et al., 2023; Tangahu et al., 2018).

The increase in pH was caused by the ability of bacteria to respond to acid tolerance by exchanging $K^+$ from within the cell with $H^+$, which is abundant in their environment, so that environmental acidity can be reduced (Nugroho, 2010). Based on the results of the analysis of pH parameters, the pH values fluctuated, as influenced by the activity of microorganisms. Changes in pH occurred because the decomposition of organic material produced ammonium compounds, which cause the pH value to rise, and organic acids, which cause the pH value to decrease. Bioremediation temperature estimation in the soil test was carried out to determine its effect on the gasoline hydrocarbon biodegradation cycle in each treatment variation, especially in the metabolic cycle and the rate of bacterial development.

The temperature expansion was caused by the action of microbes using the carbon source of gasoline. Thus, the higher the action of microorganisms, the higher the natural temperature

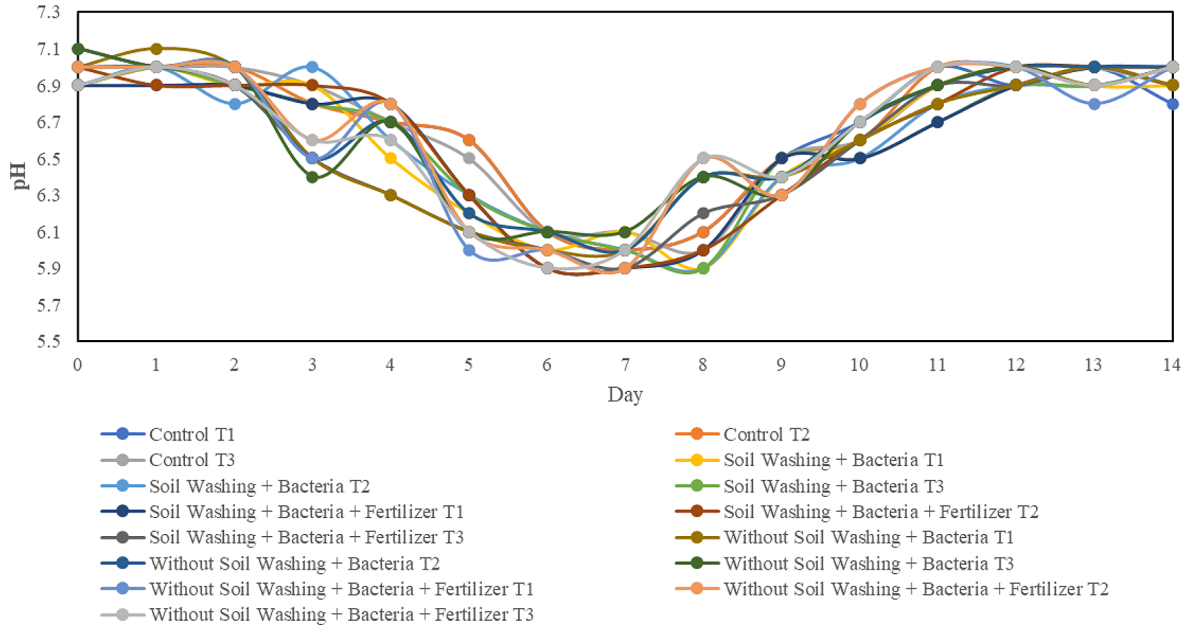


**Figure 6.** pH of bioremediation process

(Larasati and Mulyana, 2016). The estimated temperature range for all reactors with the expansion of test microbes and supplements generally remained stable in the mesophilic temperature range (20–40 °C) required for bacterial growth. This finding is based on the assertion of Vidali (2001) that the interaction of oil biodegradation occurs organically in mesophilic conditions. The temperature expansion also affects the water content, the higher the temperature, the lower the water content in the reactor. In the present study, temperature parameters were tested, and the results obtained are shown in Figure 7.

Temperature measurement was carried out simultaneously with pH from the first day to the 14th day. Based on the results, for 14 days, the recorded temperature was in the range of 28–31 °C. The temperature range of 10–40 °C was the maximum temperature for most microorganisms in soil (Larasati and Mulyana, 2016), and the optimum temperature for bacterial growth is 28–33 °C (Nurmalasari, 2018). Changes in temperature during the bioremediation process fluctuated in the range of 29–31 °C.

To determine the percentage of hydrocarbon chains in soil contaminated with petroleum after going through bioremediation, we carried out TPH measurements. We carried out earth bioremediation by using soil after pre-treatment soil washing and without pre-treatment soil washing and determined the optimum mixed culture of *P. putida* and *P. fluorescens* 5% (*w*/*w*) and the addition of nutrients with the optimum ratio of C:N:P = 100:5:1 for 14 days (Nurmalasari, 2018). TPH levels were measured at the beginning and end of the study. The gravimetric method with Soxhlet extraction was the TPH method used. The results of the analysis of the hydrocarbon content are shown in Figure 8.

Figure 8 shows a decrease in the concentration of hydrocarbons in each variation within 14 days. The decrease was not influenced by the addition of nutrients, the addition of bacteria, or pre-treatment with soil washing. This phenomenon can be seen in the control point 3 soils, which has a higher percentage of settlement compared to the treatment of points 1 and 2. The highest decrease in TPH levels occurred at point 3 soil, both in control, variation of soil washing, and bacteria. *P. putida-P. fluorescens*, variation of soil washing + bacteria *P. putida-P. fluorescens* + nutrients (C:N:P=100:5:1), variation of soil washing + bacteria *P. putida-P. fluorescens*, variation of soil washing + bacteria of *P. putida-P. fluorescens* + nutrients (C:N:P 100:5:1), variation without soil washing + bacteria of *P. putida-P. fluorescens*, and soil washing variation + bacteria of *P. putida-P. fluorescens* + nutrients (C:N:P = 100:5:1).

This phenomenon occurred because indigenous microbes in soil contaminated with hydrocarbons contaminated with petroleum can reduce TPH even without the addition of bacteria and nutrients. This finding is consistent with Cookson's (1995) finding, in which hydrocarbon compounds were used by microbes as sources of nutrients and energy for metabolism

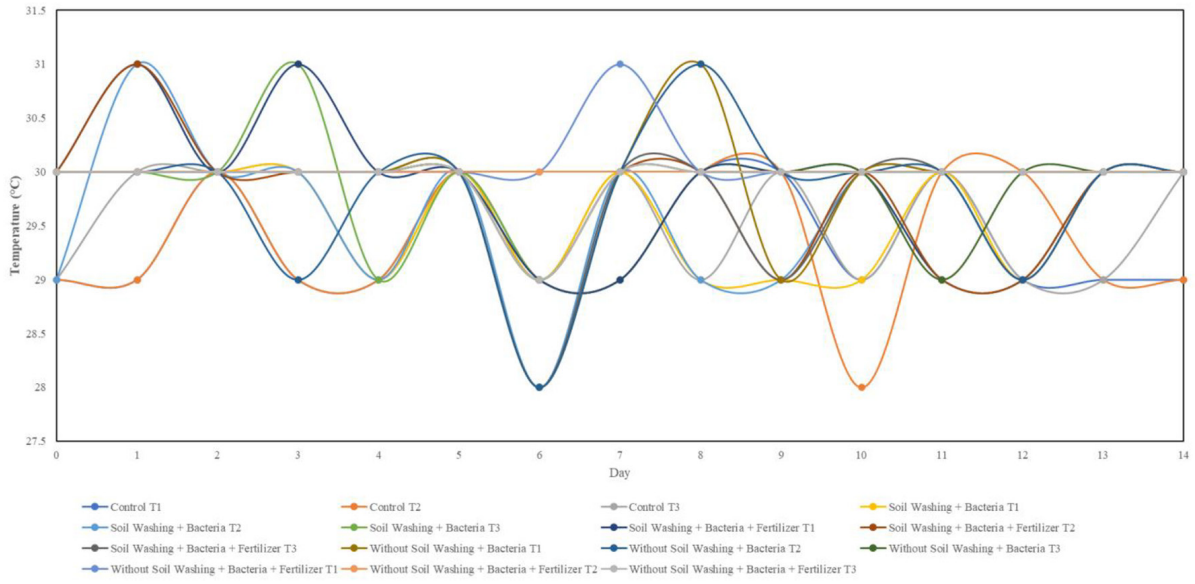


**Figure 7.** Temperature of bioremediation process

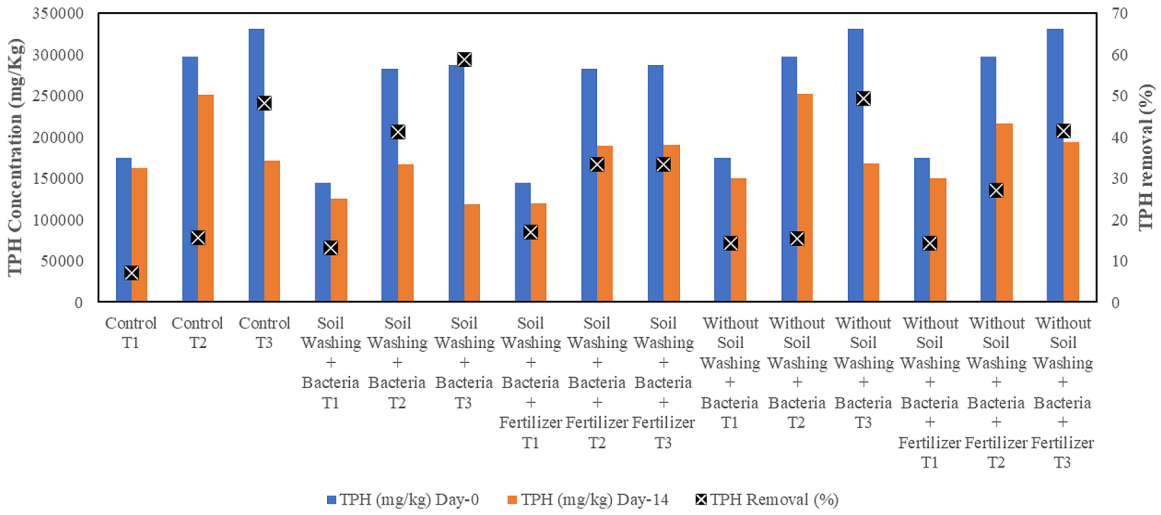


**Figure 8.** TPH concentration of bioremediation process

and reproduction, and an increase in microbial populations occurs when an environment is rich in hydrocarbons (petroleum). This condition happens because carbon clastic microbes can stick to hydrocarbons, can produce emulsifiers, and have a mechanism for desorption from hydrocarbons through the synthesis of hydrocarbon oxidizing enzymes encoded by microbial chromosomes and mutated plasmids.

Chromosomal and plasmid mutations affect the process of breaking down hydrocarbon molecules because hydrocarbon compounds are natural organic compounds; hence, many types of microbes have evolved to use hydrocarbon compounds (Imron et al., 2020; Othman et al., 2022; Rahim et al., 2022). This finding was confirmed in the present study, where point 3 soil that had been pre-treated with soil washing and without pre-treatment soil washing had a high TPH content with a higher percentage of settlement than points 1 and 2 soil, which had a lower TPH content. The decrease in TPH at point 3 soil with pre-treatment soil washing + *P. putida* + *P. fluorescens* and without pre-treatment soil washing + *P. putida* + *P. fluorescens* was higher than the control. This finding was obtained because the consortium formed in mixed culture accelerates the biodegradation process completely. This phenomenon occurs because the mixed culture of bacterial isolates degrading petroleum contaminating the soil has high biodegradability, which is better than that of pure culture (Alkatiri, 2017; Purwanti et al., 2018b; Tangahu et al., 2019). In general, bacteria only carry out one or two stages of a metabolic pathway, and if the bacteria are a mixed culture, the usual stages of the metabolic pathway will be longer and more profitable if a synergistic consortium occurs. The TPH value obtained in the present study was above the quality standard threshold required by the Ministry of Environment and Forestry, which is below 1%.

Figure 9 shows that from the first day to day 14, the bioremediation process showed a very sharp increase in the number of bacterial cells. The selective dominance of microbes caused an increase in bacterial cells. This finding was obtained because these microbes can convert hydrocarbon compounds into energy and carbon sources. The bacterial count was analyzed based on the variation of point 3 soil washing and *P. putida* and *P. fluorescens* by using the TPC method. This analysis aimed to determine the total number of bacterial colonies after bioremediation. By knowing the addition or reduction of the number of degrading microbes, microbiologically, the occurrence of hydrocarbon degradation can be predicted. The TPC method can count bacterial colonies on agar media (Imron et al., 2021, 2019; Nimatuzahroh et al., 2022). The principle of this TPC method is direct research with breeding and colony formation by growing living microbial cells.

Microbial activity in the reactor involves two groups of microbes, namely groups of indigenous microbes that already exist in the soil and groups of microbes resulting from seeding (inoculation). The involvement of the two microbial groups has an important role in the hydrocarbon degradation process. The oil content in soil can be absorbed by soil particles and other plant matter

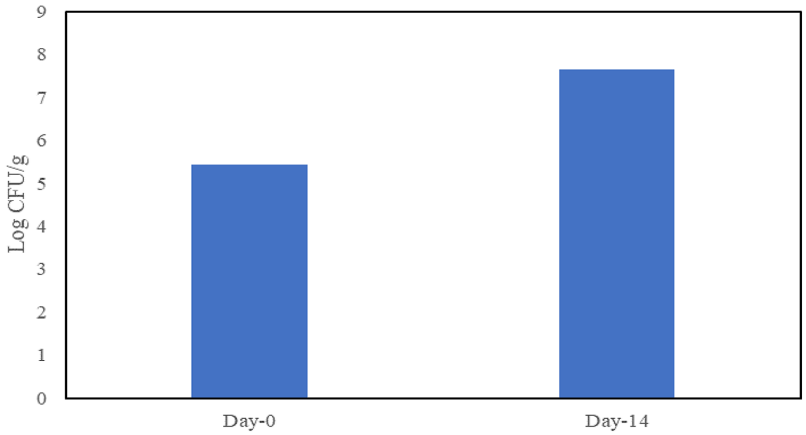


**Figure 9.** Total plate count (TPC) bioremediation

(Almansoory et al., 2019; Haritash and Kaushik, 2009). This condition affects the distribution of the number of hydrocarbon-degrading microbial colonies, in which the greater the area of the environment containing petroleum, the more colonies that grow and the faster the biodegradation process. This finding supports the results of TPH on point 3 soil washing and bacteria, which showed the highest level of TPH degradation and had the highest initial TPH content of the other variations.

The results of bacterial identification by using NGS from the three soil samples, namely the initial soil, after soil washing, and treated soil, show different types of species. The results of the initial bacterial identification at sampling point 3 are shown in Figure 10. Figure 10 shows the number of identified soil bacterial species: 1,113. The bacterial species with the highest number of individuals are *Immundisolibacter cernigliae, Pseudoxanthomonas spadix BD-a59, Calditerrivibrio nitroreducens DSM 19672, Mahella australiensis 50-1 BON, Hydrogenophilus thermoluteolus, Thermodesulfovibrio yellowstonii DSM 11347, Aerosticca soli, Syntrophus aciditrophicus SB,* and *Deferribacter desulfuricans SSM1. I. cernigliae* showed a promising result as a PAH-degrading bacteria. The shape of the coccobacilli cells is approximately 0.56 µm ×0.35 µm. Colonies on solid medium are small, convex, and circular with a yellow-orange color. Growth occurs aerobically between 20 °C and 36 °C (optimally between 28 and 30 °C), pH 6.5–8.0 (optimal 7.0), and at 1% salinity (optimal 0%). Growth was not observed under anaerobic conditions. Cells can metabolize ketobutyric acid, methyl pyruvate, mono-methyl-succinic acid, and ketovaleric acid and can grow on polycyclic aromatic compounds as sole carbon sources and nitrates and ammonia as nitrogen sources. Cells were negative for gelatin hydrolysis, cellulase, skim milk protease, starch hydrolysis, nitrate and urease dissimilation reduction, and positive for lipase activity

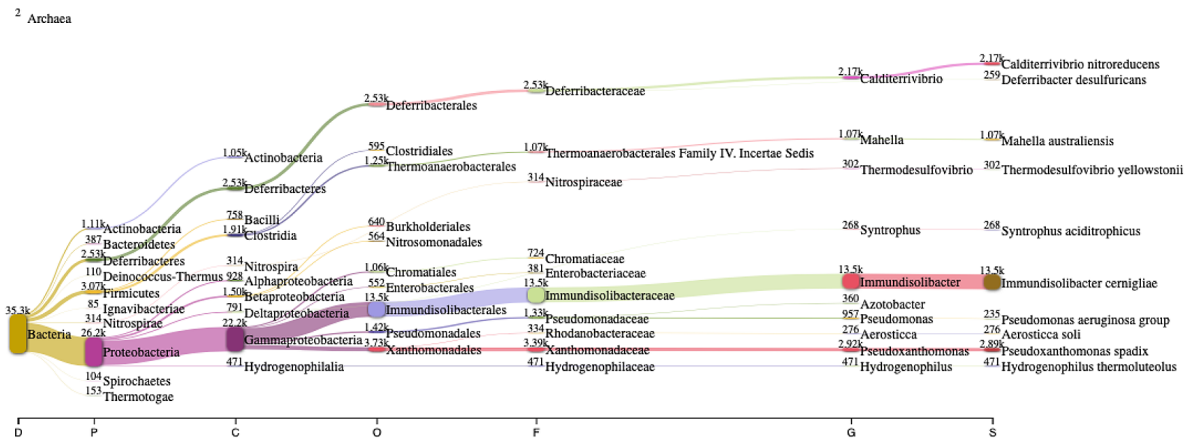


**Figure 10.** Result of bacterial identification in the initial sample

(Corteselli *et al.,* 2017). Remediation of PAH-contaminated soil that underwent soil washing produces bacteria, as shown in Figure 11.

Figure 11 shows the results of the bacterial identification of the initial soil samples after soil washing. The results of the initial bacterial identification after soil washing with the 10 most identified species were as follows: *I. cernigliae, Aquabacterium olei, P. spadix BD-a59, C. nitroreducens DSM 19672, Caulobacteraceae bacterium OTSz_A_272, Sphaerotilus natans subsp. Sulfidivorans, Roseateles depolymerans, Desulfoglaeba alkanexedens ALDC, Brevundimonas mediterranea, and Parvularcula bermudensis HTCC2503.* The bacteria *I. cernigliae* and *C. nitroreducens* DSM 19672 were still found in the soil samples after remediation with soil washing, which indicates that PAHs are still present in the soil. Soil samples that have been remedied with soil washing are then continued with bioremediation. The results of bacterial identification after bioremediation are shown in Figure 12.

The results of bacterial identification in Figure 12 show different species. The most identified bacterial species are *Pseudomonas aeruginosa, Pseudomonas oleovorans, Pseudomonas* sp. S150, *Escherichia coli, Pseudomonas aeruginosa* LES431, *Achromobacter xylosoxidans, Klebsiella pneumoniae, Pseudomonas citronellolis,* and *Pseudomonas taetrolens*. The bacterial species found in soil samples after the bioremediation process were different from those found in bacterial sepsis at the beginning of the soil samples and after soil washing. Naturally occurring bacteria in soil and plants generally include the genera Bacillus, Acetobacter,

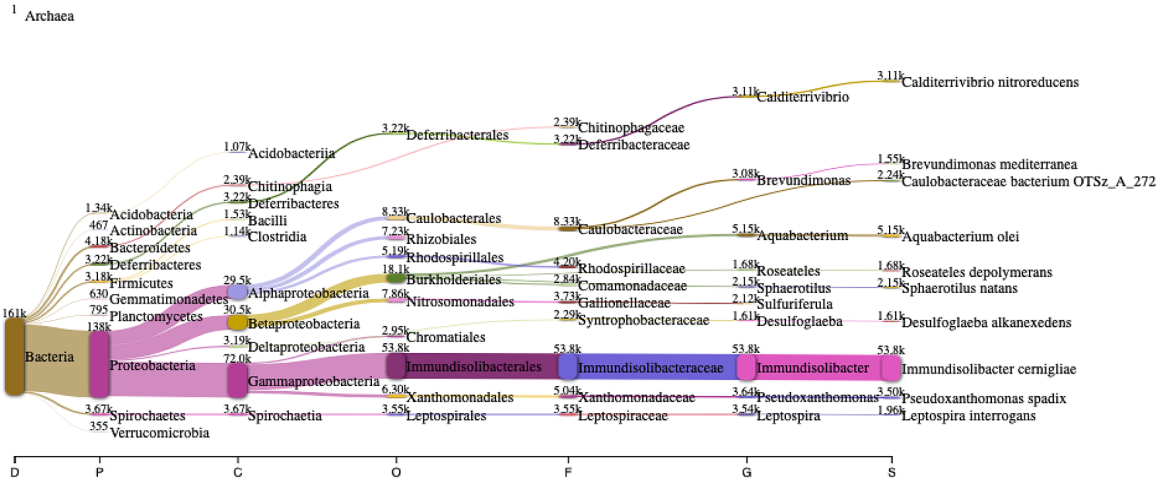


**Figure 11.** Results of bacterial identification after soil washing

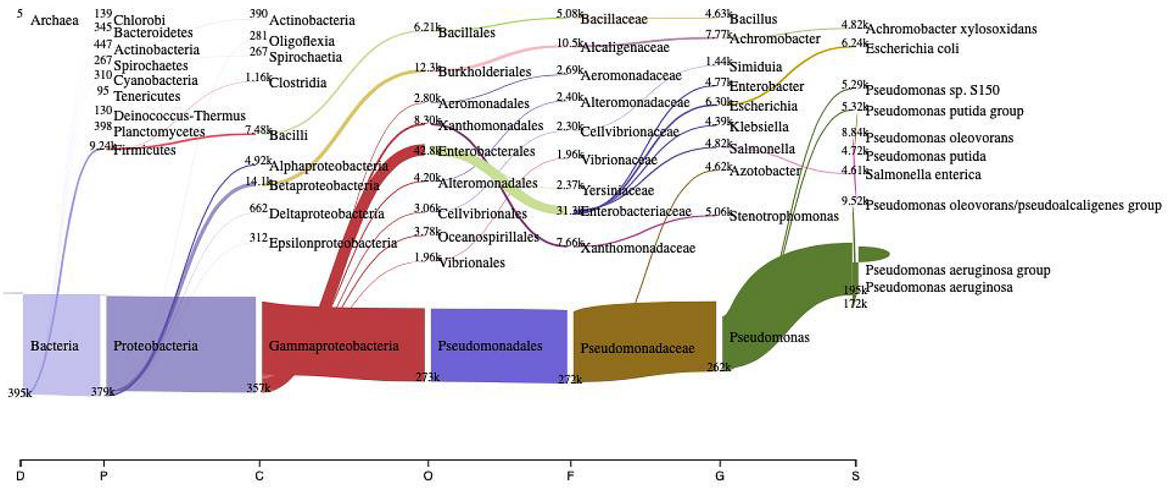


**Figure 12.** Result of bacterial identification after bioremediation

Actinobacteria, and Pseudomonas (Arliyani et al., 2023; Fauzul Imron et al., 2019; Grostern and Edwards, 2009; Mohan and Tippa, 2019; Purwanti et al., 2018a, 2017a).

## CONCLUSIONS

Pre-treatment soil washing can reduce the total content of petroleum hydrocarbons (TPH) in polluted soil by up to 4.41%. The highest decrease was observed at point 3, from 33.06% to 28.65%. The addition of a mixed culture of *P. fluorescens* and *P. putida* bacteria showed no significant effect on the level of degradation of petroleum hydrocarbons during the 14-day incubation period ($p > 0.05$). The highest decrease in TPH value was observed at point 3 of soil variation. Pre-treatment soil washing and mixed culture of *P. fluorescens* and *P. putida* bacteria resulted in a degradation rate of 57.47%. In addition, based on the addition of nutrients with a ratio of C:N:P = 100:5:1, no significant effect was observed on the level of degradation of petroleum hydrocarbons during the 14-day incubation period ($p > 0.05$). Furthermore, pre-treatment soil washing had no significant effect on the level of degradation of petroleum hydrocarbons during the incubation period of 14 days ($p > 0.05$). TPH levels decreased under the best bioremediation process conditions in point 3 soil, both in the control, soil washing variation + *P. putida-P. fluorescens*, soil washing variation + *P. putida-P. fluorescens* + nutrients (C:N:P=100:5:1), soil washing variation + *P. putida-P. fluorescens*, soil washing variation + *P. putida-P. fluorescens* + nutrients (C:N:P=100:5:1), no soil variation washing + *P. putida-P. fluorescens*, and variation of soil washing + bacteria *P. putida-P. fluorescens* + nutrients (C:N:P=100:5:1) groups. Finally, the addition of nutrients, pre-treatment of soil washing, and mixed culture of bacteria did not have a significant effect because of breeding, metabolism, and increasing the number of microbes by using hydrocarbon compounds.

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