

Evaluation of Methods for Bioremediation of Petroleum-Contaminated Soil

STATEMENT OF THE PROBLEM

If soil exposed to a petroleum concentration of 8% (w/w) is amended with cow manure for bio-stimulation, potato peelings for biosorption, and *Bacillus subtilis* for bioaugmentation as well as combination treatments, which treatment provides the most effective method for combating petroleum contaminated soil?

Bio-remediation Method						
Time (days)	Control (g)	Bioaugmentation: <i>Bacillus subtilis</i> (g)	Biosorption: Potato Peelings (g)	Bio-stimulation: Cow Manure (g)	Combination Treatment: Cow Manure and <i>Bacillus subtilis</i> (g)	Combination Treatment: Potato Peelings and <i>Bacillus subtilis</i> (g)
2	7.839	7.676	7.759	7.667	7.711	7.710
4	7.756	7.467	7.630	7.493	7.426	7.471
6	7.728	7.246	7.401	7.273	6.998	7.308
8	7.688	6.983	7.221	6.995	6.540	6.888
10	7.610	6.737	7.084	6.792	6.123	6.585
12	7.543	6.455	6.954	6.489	5.865	6.300
14	7.480	6.220	6.758	6.313	5.619	6.011
16	7.446	5.870	6.610	6.124	5.264	5.701
18	7.409	5.546	6.457	5.914	4.963	5.428
20	7.352	5.161	6.256	5.696	4.735	5.123
22	7.323	4.833	6.013	5.489	4.390	4.669
24	7.286	4.420	5.715	5.323	3.910	4.342
26	7.227	3.986	5.452	4.961	3.690	3.904
28	7.198	3.784	5.209	4.675	3.323	3.632
30	7.082	3.659	4.904	4.376	2.932	3.325

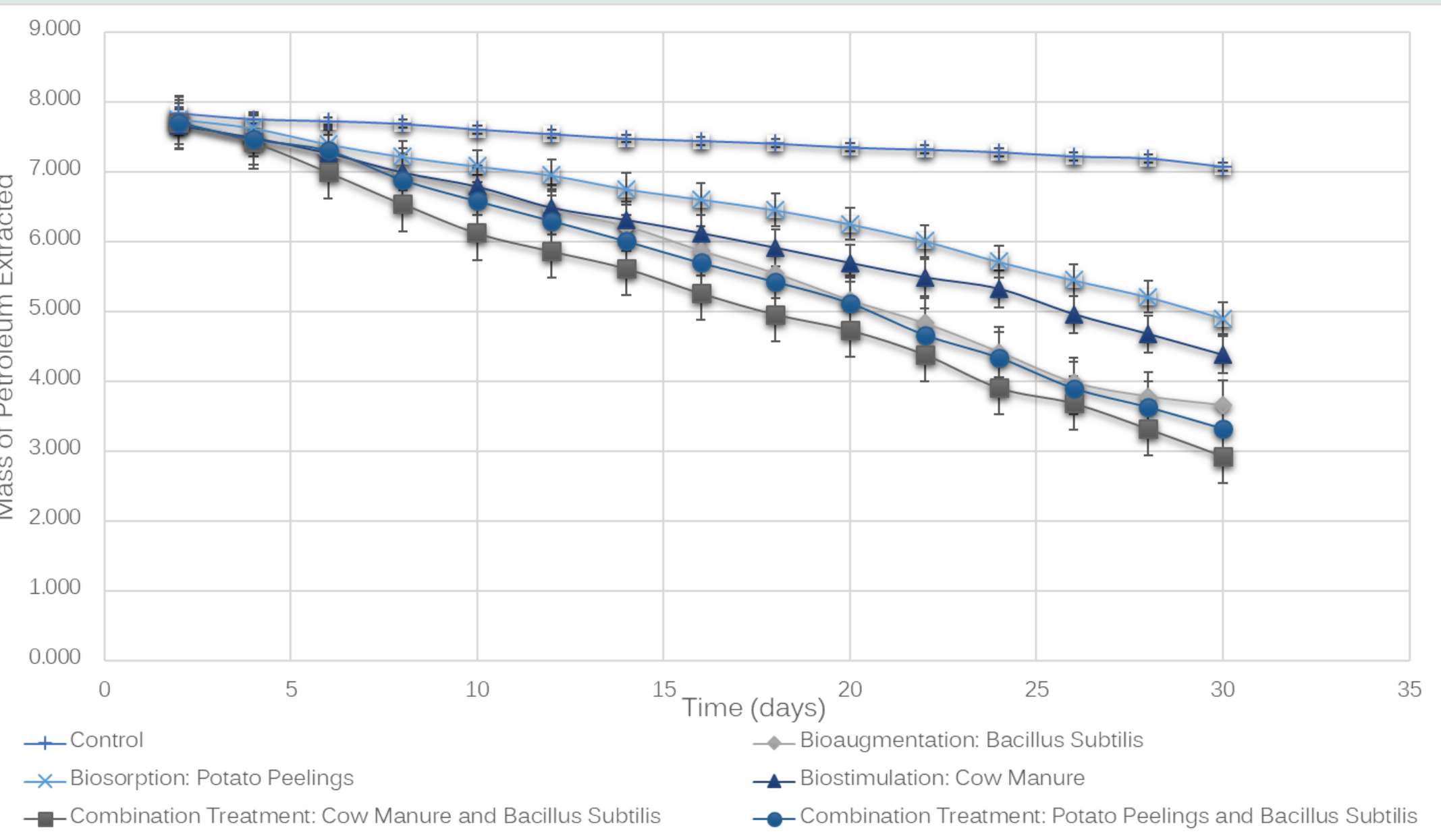
- Table 2** Average Mass of Petroleum Extracted over time from Soil Samples
- These are the values for the average mass of petroleum extracted from the samples over time for the five trials
 - The petroleum was extracted using the n-hexane method (n-hexane was the extraction solvent)
 - Mass of petroleum should decrease the most in the sample where degradation is occurring the most which in this case is combination treatment (bioaugmentation and bio-stimulation)
 - Time period for indigenous bacteria to acclimate to pollutants in soil sample amended with potato peels is longer than that for *Bacillus subtilis* since it took longer to degrade at least 1 gram petroleum
 - Cow manure was able to stimulate indigenous bacteria to reduce lag period to start biodegradation which means it's comparable to *B. subtilis* in this regards

Bio-remediation Method												
Time (days)	Control (%)	Standard Deviation	Bioaugmentation: <i>Bacillus subtilis</i> (%)	Standard Deviation	Biosorption: Potato Peelings (%)	Standard Deviation	Bio-stimulation: Cow Manure (%)	Standard Deviation	Combination Treatment: Cow Manure and <i>Bacillus subtilis</i> (%)	Standard Deviation	Combination Treatment: Potato Peelings and <i>Bacillus subtilis</i> (%)	Standard Deviation
2	1.498	0.257	3.543	0.740	2.501	0.671	3.652	0.860	3.106	0.348	3.106	0.535
4	2.537	1.052	6.172	2.093	4.124	0.806	5.848	0.715	6.688	2.003	6.125	1.251
6	2.888	1.065	8.949	0.877	6.994	1.506	8.603	0.780	12.066	2.817	8.170	1.862
8	3.390	0.908	12.247	2.215	9.264	1.348	12.096	1.187	17.816	2.104	13.446	0.611
10	4.373	0.913	15.338	3.705	10.978	0.825	14.654	2.052	23.054	2.863	17.258	1.404
12	5.210	0.906	18.892	3.222	12.619	0.769	18.464	2.836	26.298	2.046	20.830	3.090
14	6.004	0.699	21.835	3.622	15.082	1.089	20.671	2.739	29.392	2.343	24.471	2.956
16	6.429	0.815	26.238	3.292	16.934	0.105	23.041	2.412	33.858	1.171	28.365	3.945
18	6.904	0.718	30.314	3.424	18.862	1.142	25.682	1.947	37.635	1.262	31.795	3.608
20	7.612	0.904	35.152	3.745	21.382	3.152	28.424	2.274	40.498	0.774	35.620	3.408
22	7.974	0.814	39.266	3.434	24.438	3.337	31.020	2.653	44.840	1.293	41.324	3.395
24	8.439	0.698	44.463	2.767	28.191	3.155	33.106	3.335	50.867	1.092	45.434	3.401
26	9.181	0.588	49.915	3.823	31.485	1.486	37.665	2.343	53.634	1.034	50.945	2.223
28	9.548	0.424	52.448	2.879	34.544	1.540	41.254	0.713	58.241	2.442	54.355	3.011
30	11.010	1.143	54.024	2.668	38.382	1.756	45.014	1.261	63.159	1.223	58.216	1.853

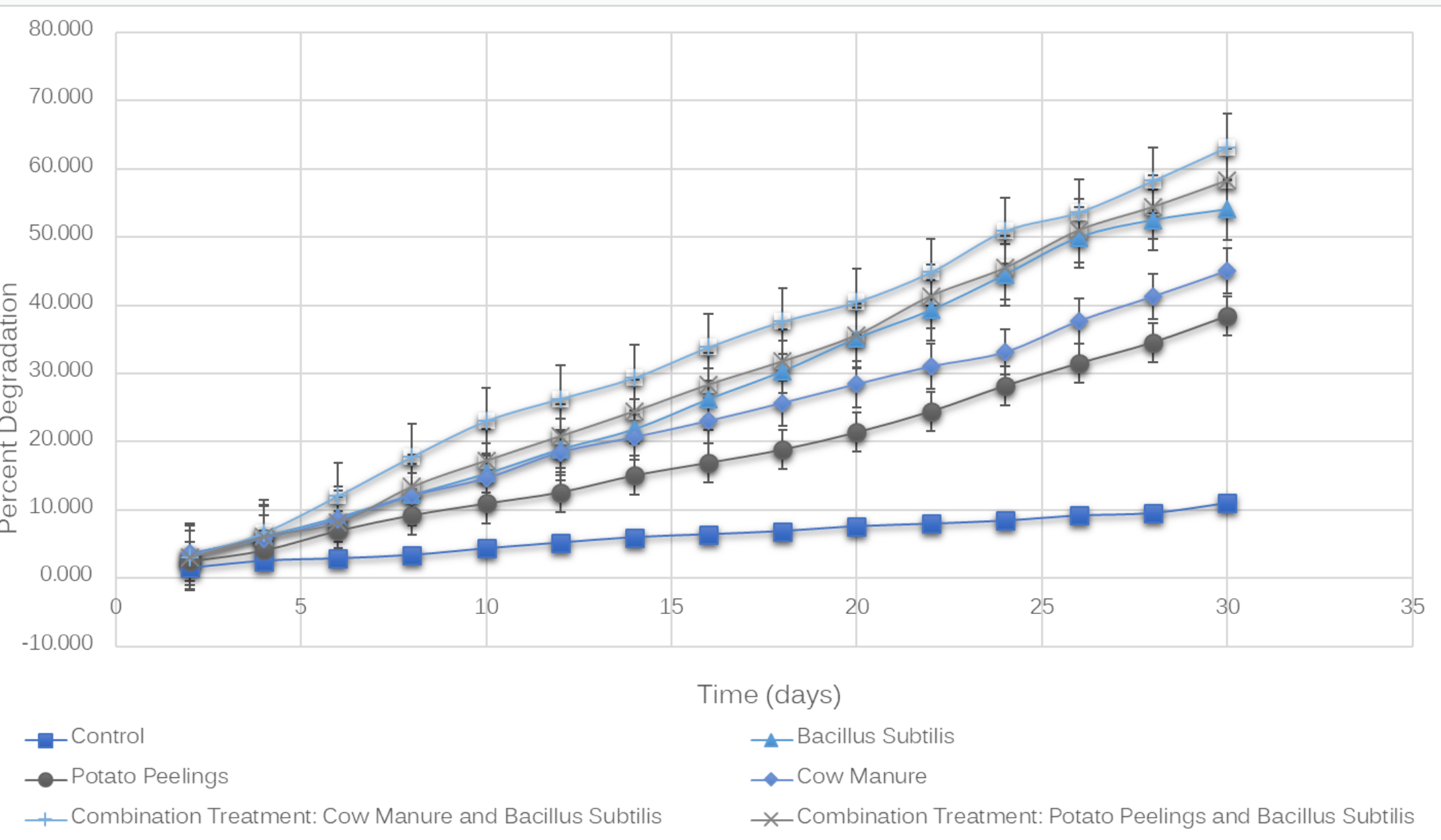
- Table 3** Average Percent Degradation of Petroleum over time Using Various Bio-remediation Methods
- These are average percentage degradations for each day for the five trials performed
 - Calculated from the mass of petroleum extracted over time
 - It can be noted that out of the individual treatments the *Bacillus subtilis* was the best
 - Natural attenuation or degradation by naturally occurring bacteria which occurs in the control was ineffective because it degraded around only 11%
 - Out of the combination treatments bioaugmentation and bio-stimulation were the best, bioaugmentation and biosorption followed it
 - Potato peelings did not contribute much to degradation

Table 4 Statistical Analysis of Percentage Degradation of Petroleum at the end of 30-day period			
	Mean (%)	Variance (%)	Standard Dev. (%)
Control	11.01	1.31	1.14
Bacillus subtilis	54.02	7.12	2.67
Potato Peelings	38.06	1.47	1.21
Cow Manure	45.01	1.59	1.26
Combination Treatment: Cow Manure and Bacillus subtilis	63.16	1.5	1.22
Combination Treatment: Potato Peels and Bacillus subtilis	58.22	3.43	1.85

- At the end of the 30-day treatment, the mean percentage degradation is clearly highest in the case of combination treatment of cow manure and *B. subtilis*
- The mean percentage degradation is the least in the control
- The data involving degradation of petroleum in *B. subtilis* differs more from the mean due to its variance being higher
- Standard deviation for combination treatment of cow manure and *B. subtilis* suggests the reliable nature of the method as it yields a data set that is not as spread out



- Fig. 1** Average Mass of Petroleum Extracted over time from Soil Samples
- Between two points sometimes there is a larger increase and sometimes a smaller
 - Indicates times when the bacteria are performing biological processes
 - Biosorption seems to be the least efficient individual treatment
 - Bioaugmentation and biosorption combination is the second most effective of the combination treatments. However, if performed on larger scale, combination treatment (bioaugmentation and bio-stimulation) will outperform the others due to cow manure's ability to provide essential nutrients for oxidative degradation



- Fig. 2** Average Percent Degradation of Petroleum over time Using Various Bio-remediation Methods
- Most degradation activity in combination treatment with cow manure and *Bacillus subtilis*
 - Points for *Bacillus subtilis* and combination treatment (*Bacillus subtilis* and potato peelings) seem to overlap on some days
 - Shows that adding potato peels does not enhance *Bacillus subtilis* performance as much

Table 5 ANOVA Two Factor With Replication Summary					
Source of Variation	df	MS	F	P-value	F crit
Sample	14	5397.872273	1137.283959	4.9387E-288	1.719322373
Columns	5	7102.818718	1496.501099	2.3223E-238	2.239060369
Interaction	70	185.1742531	39.01457776	4.5649E-131	1.332919998
Within	360	4.746283663			
Total	449				

- One factor is the type of treatments and second factor is interval (number of days)
- f-value was much larger than the f-crit value
- If the null hypothesis were true then F would have been closer to 1.0 but in the table above F is much larger than 1 showing that the null hypothesis is false
- The p-value was far lower than the chosen alpha value of 0.05 both of which validate my hypothesis and shows that the data is statistically significant. The p-value of 4.9387E-288 for the samples was extremely low suggesting that if the null hypothesis were true and there was no correlation between the method used and the percent biodegradation, then the chances of acquiring the data that was recorded through this experiment would be highly unlikely
- Column factor p-value is much lower than .05 which means that the differences seen between each column (type of treatment) in percent degradation over time is not due to chance

DISCUSSION

Although the combination treatment of bioaugmentation and bio-stimulation was the best, **among the individual treatments**, *Bacillus subtilis* seems to be the most effective as the amount of petroleum extracted decreased the greatest in the presence of this bacteria making it a sound replacement to other methods which are time-consuming. When choosing a remediation technology is it is important to consider the amount of time it takes to reach a significant level in the reduction of petroleum as the longer the petroleum stays in the soil the greater the chance of plant, human, and animal exposure. A greater percent degradation with *Bacillus subtilis* may also have been possible due to the presence of petroleum-degrading oxygenase and the bacteria's ability to produce surfactin. Surfactins are biosurfactants which are a group of surface-active chemical compounds that enhance the emulsification of the petroleum by increasing the oil surface area making the oil available for bacteria usage.

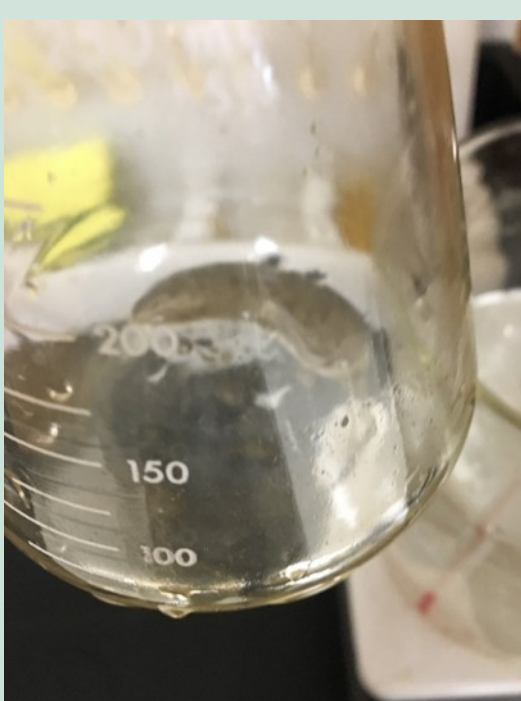
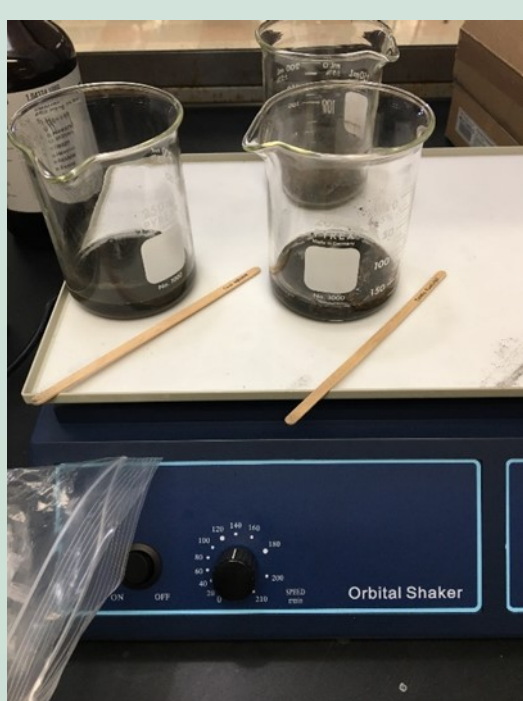
With bio-stimulation, the mass of petroleum decreased on average from 7.667 g to 4.376 g – a 45% reduction in total petroleum hydrocarbon. Providing the naturally occurring bacteria a source of nutrients was beneficial for its cell growth and division. As petroleum is an organic pollutant, microbes can perform oxidative biodegradation by intaking the hydrocarbons and transforming them into CO₂ and H₂O plus other waste products and energy for its biological processes. The nutrients present in the cow manure act as electron donors stimulating bioremediation by being the oxidized electron acceptor in this process.

For biosorption, the average mass of petroleum decreased from 7.759 g to 4.904 g and the average percent reduction was 38%. Potato peels are made of porous material that allows the contaminants to adsorb to its surface. This removes the petroleum pollutants from the soil allowing the indigenous bacterial populations to recover from the initial shock of the pres-

This method is somewhat effective, but not as much as bioaugmentation. This may be because the number of microorganisms that are naturally present in the soil may not be there in a sufficient amount to commence the remedial action right away while the addition of *Bacillus subtilis* can reduce the lag period that it takes for the microorganisms to acclimate to the addition of the pollutants and multiply in a short period of time.

The control seems to offer the least amount of bioremediation. Over a period of 30 days, the mass of petroleum reduced on average from 7.839 g to 7.082 g showing that it failed to even degrade 1 gram of petroleum. Though the control does show some type of biodegradative activity, the indigenous bacteria are not capable as *Bacillus subtilis*. *Bacillus subtilis* which is one of the naturally occurring soil bacteria is not present in sufficient quantities in the topsoil already which is why its addition would be ideal rather than trying to just stimulate native bacteria populations.

The combination treatment of bioaugmentation and bio-stimulation offered the **highest** percent at 63% of petroleum degraded on average of **all** the soil samples. The *Bacillus subtilis* was able to stabilize itself quickly in the presence of the pollutants and utilize the cow manure as well. Nutrients such as nitrogen, phosphorus, and occasionally iron are vital to successful degradation of hydrocarbons. Cow manure seems to offer the necessary nutrients for *Bacillus subtilis* growth more than potato peels as it was able to degrade more petroleum in the presence of cow manure. Aerobic degradation of hydrocarbons is an oxidative process that can be enhanced by the addition of an organic fertilizer such as cow manure.



ABSTRACT

The world runs on oil. While notable oil spills involving tankers catch the eye of the public, oil spillage caused by other anthropogenic activities such as consumer use often goes unnoticed and is the major cause of petroleum contamination. Petroleum components include hazardous organic chemicals that pose a threat to environmental and human health. In this experiment, a potential solution involving the microbial bioremediation for degrading petroleum-contaminated soil was examined. Bioremediation stands as an internationally accepted, cost-effective, eco-friendly technology that can enhance the removal of petroleum pollutants. The gravimetric method was utilized to monitor the reduction of total petroleum hydrocarbon over a period of 30 days in six soil samples exposed to a petroleum concentration of 8% (w/w). The soil samples were amended using *Bacillus subtilis* for bioaugmentation (introduction of hydrocarbon utilizing microorganisms), cow manure for bio-stimulation (addition of nutrients to stimulate microbial growth), and potato peels for bio-sorption (allows for adsorption of pollutant) as well as combination treatments. It was hypothesized that the combination treatment of bioaugmentation and bio-stimulation would offer the highest percent of petroleum degradation due to the unique partnership between highly productive petroleum degrader *B. subtilis* and nutrient-rich bio-stimulant cow manure which is essential to microorganism activity. Overall, the average percent of petroleum degraded was the highest at 63% in the combination treatment of bioaugmentation and bio-stimulation which validates the hypothesis. Among the individual treatments, bioaugmentation showed the highest average percent degradation at 54% conveying that the application of microbes to contaminated soil is effective. The control showed around 11% average degradation signaling that petroleum degradation in untreated soil is inefficient.

OBJECTIVE

The purpose of this experiment is to utilize various methods of bioremediation to treat petroleum contaminated soil and reduce the presence of petroleum pollutants such as hydrocarbons in soil. Bioremediation refers to the method of applying microorganisms, plants or microbial processes to remove or degrade the pollutants/contaminants in the soil. The adverse effects of petroleum pollutants extend beyond the environment and can impact human health as well. While many methods for controlling the effect of oil contamination have been investigated, bioremediation provides the most cost-effective and environmental friendly process of remediation. This experiment will test different methods of bioremediation including the use of cow manure, potato peels, and the microorganism- *Bacillus subtilis* to evaluate the extent to which petroleum is degraded within the soil. The microbe, bio-stimulant, and bio-sorbent material chosen are representative of the bioremediation techniques.

HYPOTHESIS

Of the five possibilities of treatment, the combination treatment of cow manure and *Bacillus subtilis* should offer the highest percent of reduction in petroleum. *Bacillus subtilis* is a type of oil-degrading microorganism capable of utilizing a range of hydrocarbons in petroleum for its biological processes. In addition to the presence of this bacteria, some types of indigenous bacteria that naturally dwell in the soil are can perform hydrocarbon degradation as well thus offering an additional outlet through which the contaminants can be removed. Supplying the microorganisms with a bio-stimulant such as cow manure maybe more useful than other amendments in providing an alternative source of Nitrogen and Phosphorous. Having appreciable quantities of N and P can result in stimulation of bacterial biodegradative activities.

Null Hypothesis

Regardless of which treatment is used, there is no difference in the percent degradation or mass of petroleum extracted of the contaminated soil sample (amended and unamended).

INTRODUCTION

- As the petroleum industry continues to be developed further, it is inevitable that spillages will occur. This issue has garnered more attention over the years as the effects of accidental spillages on the environment are vast and destructive to the flora and fauna in the area.
- Releasing petroleum hydrocarbons through spillages and leakage from underground tanks, steamers, unplugging of oil wells or abandoned oil refinery sites causes contamination of surface soil and bodies of water. If left untreated, the area affected by the accidental spillage can cause the petroleum to seep further underground and contaminate groundwater which can affect plants in the surrounding area, because layers of oil in the soil inhibit the metabolism of plants and suffocate them.
- Fat soluble components may accumulate in the organs of animals that ingest petroleum and may enter the food chain. These dilemmas are not limited to the environment and animals; humans are very much influenced by the poor soil quality.
- Petroleum hydrocarbons bind to soil particles and are difficult to be removed
- Components of petroleum include various aliphatic, branched, and aromatic hydrocarbons; among the most toxic and carcinogenic compounds found in petroleum include poly-aromatic hydrocarbons. These hydrocarbons have widespread effect on human body and prolonged exposure to these contaminants may cause liver and kidney diseases, bone marrow damage, or lead to the development of cancer.
- Another major consideration is the reduction in soil fertility caused by petroleum contamination. In developing countries such as Nigeria this could be particularly detrimental as the availability of arable land is already quite low and contamination prevents the country from achieving greater food security.
- A possible solution includes the application of hydrocarbon-utilizing microorganisms or utilizing those that are already indigenous to the soil at the contaminated site. Hydrocarbon-Utilizing Bacteria (HUB) are capable of releasing biosurfactants to impact the uptake of hydrocarbons.
- Various HUB and Fungi possess emulsification capabilities (breaking down fat globules for easier digestion) due to extra cellular or whole cell's surface-active compounds.
- Pectin, a compound found in potatoes, is known to have emulsification properties giving it the ability to break down petroleum as well
- The presence of nutrients in the right quantities is essential to the enhancement of TPH degradation since the rate of degradation depends on the concentration of the contaminant and availability of nutrients

Remedial Technologies	Cost per Cubic Meter (\$)
Bio-stimulation	40-120
Bioaugmentation	26-105
Biosorption	12
Bio-piling	140
Land Treatment	100
Bioreactor	260+
Bio-slurping	32
Conventional/Enhanced Bioventing	23-73

Table 1 Analysis of Cost-Effectiveness of Various Bioremediation Techniques

- Based on the table above, it can be observed, that bio-stimulation, bioaugmentation, and biosorption are among the cheapest methods present
- Phytoremediation takes months possibly years for the plants to fully grow and break down the petroleum components and other methods such as bio-piling are ex-situ methods which require the excavation of the contaminated soil making it not the most cost-effective.
- Bio-remediation offers the best of both worlds by being able to perform environmental-friendly remediation of soil in a shorter period and being less expensive compared to other methods.

Bio-remediation: refers to the removal or transformation of contaminants to harmless or less harmful substances

- Bioaugmentation: the process where added microbes use organic petroleum contaminants as single most carbon and energy sources and, in the process, break down the hydrocarbon contaminants into other substances eventually producing simple compounds such as carbon dioxide and water
- Bio-stimulation: the modification of the environment to stimulate existing indigenous bacteria capable of bioremediation
 - This can be done by adding number of nutrients and electron acceptors such as phosphorus, nitrogen, oxygen, or carbon which are otherwise available in quantities low enough to constrain microbial activity
- Biosorption: Leads to adsorption of the contaminant by affinity of the surface to bind with the pollutant through the porous structure of the sorbent material (potato peels)

MATERIALS

- Lab apron
- Rubber gloves
- Safety goggles
- Breathing Mask
- 500 mL distilled water
- (5) 600g of topsoil batches
- 8mm sieve
- 10 Russet potatoes
- (30) 250 mL glass beakers or containers
- 250-watt electric blender
- 250 mL of petroleum or engine oil
- Sterilized Pipet or medicine dropper
- 1 L n-Hexane
- 250 mL Erlenmeyer flask
- Whatman filter paper (11 micron)
- Black Kow Organic Cow Manure
- Erlenmeyer flask clamp
- Corning Hot plate
- Digital thermometer (accurate to 1 degree Celsius)
- Analytical Balance (accurate to 0.001 g)
- 50mL graduated cylinder
- CO-Z Orbital shaker Adjustable Speed at 0-210 RPM (manufacturer: CO-Z ASIN B07FCY2S1P
- (2) *Bacillus subtilis*, MicroKwik Culture (strain ATCC 6633)
- (2) Nutrient Agar, Prepared Media Bottle, 125 mL
- 1000 mL Beaker
- Buchner funnel
- Parafilm
- HFS @ New Desktop Electric Centrifuge Lab (manufacturer: Hardware Factory Store ASIN B00MWKA8V2)
- 15mL Falcon tube

PROCEDURE

1. Take 900 grams of garden soil and pass it through an 8mm sieve to remove any lumps.
2. Dry the soil at ambient temperature (22-23 degrees Celsius) for 48 hours to remove moisture from the soil.
3. Peel 10 potatoes and dry the potato peels at 70°C for 3-4 hours to remove moisture.
4. Powder the dry potato peels in a 250-watt blender.
5. Add 100 grams of dried soil to 6 beakers – one for control, one for bioaugmentation, one for biosorption, one for bio-stimulation, one for bio-stimulation + bioaugmentation, and one for biosorption + bioaugmentation.
6. Add 60 mL of distilled water to each of the soil samples to raise the moisture content of the samples to 60% (w/w).
7. Add 10 mL of petroleum to each of the 6 beakers and mix well to ensure that it is evenly distributed throughout the soil.
8. Cover the tops of the beaker with parafilm to avoid contamination and keep water and petroleum from evaporating.
9. Leave the samples for 48 hours to let the petroleum permeate through the soil.
10. Since the *Bacillus subtilis* is freeze-dried, it must be reactivated. Using proper laboratory techniques, reactivate the *Bacillus subtilis* in a sterile environment under the fume hood using sterile pipets and allow to incubate for a period of 48 hours.
11. Then inoculate *Bacillus subtilis* into nutrient agar (liquid growth medium) following its reactivation in a fume hood.
12. Add 20 mL of active *Bacillus subtilis* suspension to the soil samples for bioaugmentation and the combination treatments.
13. Add 10 grams of dried potato skin powder (bio-adsorbent) to the beaker for biosorption and the beaker for combination treatment (biosorption + bioaugmentation).
14. Add 10 grams of cow manure (bio-stimulant) to the beaker for bio-stimulation.
15. Do not add any other substances (cow manure, potato peelings, or bacteria) to the beaker meant to serve as the control.
16. Add around 6 mL of distilled water every other day to maintain a moist environment.
17. Maintain the soil samples at room temperature and away from sunlight.
18. Oil extraction of samples should be done every other day by the gravimetric method for a total of 30 days.
19. Suspend 10 grams of each of the soil samples in 20 ml of n-Hexane in 100 mL beakers.
20. Place the beakers on an orbital shaker for 30 minutes at 140 rpm.
21. Centrifuge each of the mixtures at 4000 rpm for 10 minutes.
22. Perform vacuum filtration using a Whatman filter paper and Buchner funnel into an Erlenmeyer flask of known mass.
23. The solvent should be allowed to evaporate completely at 70°C which is the boiling point of n-hexane. To do so, prepare a hot water bath in a large 1000 mL beaker using a corning hot plate. Once the water temperature reaches 70°C, turn off the hot plate. Next, use a beaker clamp to hold the beaker steady in the hot water until n-hexane evaporates.
24. The beaker will now only contain residual petroleum and its new mass multiplied by 15 (to scale up the amount of petroleum present in 150 grams) can be recorded.

Repeat this entire process of biodegradation (over 30 days) and the gravimetric method 4 more times to have a total of five trials

The percentage of biodegradation of petroleum per sample - per trial can be calculated by using the following formula:

$$\text{Percentage Biodegradation} = \frac{\text{Initial Mass of oil (~7.958 grams)} - \text{Mass of Extracted Oil}}{\text{Initial Mass of the oil}} \times 100$$

Variables	Constants	Controls
- Independent Variable: - Method of bioremediation (bioaugmentation, biosorption, bio-stimulation) - Dependent Variable: - Percent of the degradation of petroleum	Polluted soil with no added microorganism, bio-stimulant, or bio-sorbent material	- Indigenous bacteria naturally occurring in soil - Amount of soil - Amount of soil taken for n-hexane method - Amount of petroleum added to the soil - Soil samples maintained at room temperature (22 degrees Celsius) - Moisture content of the soil samples (60% (w/w))

FUTURE ENHANCEMENTS

- Use high pressure liquid chromatography to identify specific components of petroleum such as 1,3-Butadiene-a powerful carcinogen- and evaluate its depreciation over time
- Use a consortium of hydrocarbon degrading bacteria including but not limited to Pseudomonas putida, Acinetobacter spp., Flavobacterium spp., Capnocytophaga spp., Moraxella spp., Corynebacterium spp., Providencia spp
- Use hydrocarbon degrading fungi as well such as genera aspergillus, penicillium, fusarium, Trichoderma, and yeast
- Additional testing with different types of bio-stimulants such as compost made from wood chips and sewage sludge; brewery spent grains, banana, and spent mushroom compost; poultry droppings; and tea leaf and soy cakes
- Examining how effective the methods tested in this experiment are in other types of soil (clay, sand, etc.)

REAL WORLD APPLICATION

- Evaluating viable methods of degradation has led to the conclusion in this experiment that combination treatment may be the best method for contaminated topsoil. There may already be microorganisms capable of degrading hydrocarbons and stimulating these organisms serves to increase their population thereby increasing the rate of degradation, ultimately rendering the soil usable for vegetation.
- Furthering this process is the addition of *Bacillus subtilis* to supplement the indigenous population. In some cases, the indigenous bacteria population may not be capable of degrading petroleum hydrocarbons. Even if they are, perhaps they may not be able to degrade a wide range of potential hydrocarbons present or may be in a stressed state due to the introduction of a contaminant.
- *Bacillus subtilis*, native to most soil types is found to be a safe and effective bio-degrader and can even break down hydrocarbons with high molecular weights as well as reduce the lag period to start bioremediation. This is supported by the data which validates how quickly this method works as it displayed large decreases in the amount of petroleum present.
- It is estimated that the amount of natural crude oil seepage is about 600,000 metric tons per year. With such high rates of seepage, it is essential to utilize technology that will serve to decrease the impact of any oil spillages that may occur on the environment.
- Bio-remedial methods have already been employed in various EPA studies.
 - At the Scott Lumber Company Superfund site in Missouri, 16,000 tons of soils contaminated with polyaromatic hydrocarbons were treated through bioremediation processes and the PAH concentrations were reduced by 70%
 - Slurry-phase bioremediation was used to treat 300,000 tons of lagoon sediment and tar-like sludge contaminated with toxic compounds at the French Ltd. Superfund site in Texas.
- The decrease in TPH shows that hydrocarbons with higher molecular weights such as aromatic compounds and cyclic alkanes which will enhance the reduction of carcinogenic compounds and heavy metals thus improving environmental health

CONCLUSION

- The hypothesis was validated; the combination treatment with cow manure and *Bacillus subtilis* proved to be the most effective
- Cow manure has nutrients such as phosphorus and nitrogen that supplement the diet of the bacteria while it's using petroleum as its carbon source for biological processes
- The presence of hydrocarbon degradative enzyme systems and catabolic genes involved in the hydrocarbon degradation in the species contributed to its efficient degradation
- Cow manure in addition to stimulating growth of *Bacillus subtilis* also stimulates already present soil microorganisms
- Significant reduction in the mass of petroleum extracted from soil using n-hexane method demonstrates the reduction of total petroleum hydrocarbon and therefore poly-cyclic hydrocarbons
- **Individually**, most bio-remediation methods take long periods of time for percent degradation to reach a non-negligible point
- Within a span of 30 days which is a relatively short amount of time considering how long most bio-remediation methods take, the percentage of petroleum present was able to decrease over 50% in the **combination** treatments
- The addition of hydrocarbon utilizing bacteria and a bio-stimulant was found to be the ideal method to initiate bioremediation under the conditions tested in this experiment. In the long run, to ensure the complete degradation of substances with high molecular weight such as cyclic alkanes it is probably best to add another hydrocarbon utilizing microorganism as well. Although *Bacillus subtilis* is known to degrade a wide variety of hydrocarbons including the alkanes and aromatic hydrocarbons present in petroleum, no single microorganism is capable of fully degrading the petroleum. In addition, depending on the conditions of the environment, the concentration of pollutants, type of soil, and other biotic/abiotic factors a different combination of microorganisms and bio-stimulant may have to be used.

NOTABLE REFERENCES

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