BENCH SCALE TESTING OF NEOZYMES® - NONTOX™ FOR DEGRADING PETROLEUM HYDROCARBONS IN SOIL

Experimental Program Study

Information in this report was provided by a Californian Environmental Engineering Company. It is the follow study to the first USAF base case study and was performed in collaboration with the manufacturer and it's representatives. The first study revealed that biocatalytic soil additives can preferentially degrade soluble fractions of total petroleum hydrocarbons (S-TPH)(Gaudette et al, 1996).

Soluble fractions of TPH are the most mobile in the environment and as such, tend to be of highest concern to regulatory agencies. The experimental program described herein focuses on confirming NONTOX's tendency to preferentially degrade S-TPH so that soluble fractions are degraded at highly accelerated rates. Oxygen, temperature and soil moisture will be held constant for the purposes of this work.

The experiment consists of monitoring TPH and S-TPH degradation over time as a result of NONTOX treatment as compared to controls. The work was carried out in a controlled laboratory environment with constant temperature and humidity.

The data in this study summarises the first four weeks of an ongoing study which was anticipated to last for up to twelve weeks. This document also provides the testing procedures an d quality control measures to be followed during implementation of the experimental program.

Experimental Procedure Description

Start-up procedure

- Latex gloves and laboratory gloves were during all procedures
- Twenty one 5 gal buckets of JP-7 contaminated soil were obtained from the USAF base from AOC-34. These buckets were labelled A-U, and stored in the California State University, Chico laboratory. One bucket of soil from the JP-7 fuel site was obtained that has no evidence of contamination. This bucket was labelled "V".
- Six litres of neat JP-7 fuel were obtained from the USAF base and stored in CSUC laboratory
- Five 37.85 litre glass test reactors (61cm x 25.4 cm x 25.4 cm) were used as testing containers, and eighteen 1 litre capacity aluminium scoops will be used to mix the soil during the course of the experiment.
- Testing containers and scoops were washed with a mild detergent and rinsed thoroughly, followed by a final rinse with distilled water.
- Testing containers and scoops were labelled 1-5. Label assignments will be summarised in the key below:

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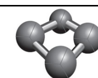

Labelling Key		
Scoop/Test Cell ID	Surfactant Concentration Ratio (Water: Surfactant)	Biocatalyst Concentration Ratio (Water: Biocatalyst)
1	None	None
2	None	None
3	NONTOX standard	NONTOX standard
4	None	NONTOX Biocatalyst only
5	NONTOX Surfactant only	None
(a)	Test cell no 2 was autoclaved to remove microbes prior to initiating testing	

- In order to ensure that the testing soil is relatively homogenous in both texture and JP-7 contamination, an equal amount of soil was taken from each of the 21 field sample buckets, labelled A-U, and placed in the labelled testing containers (glass aquariums). One scoop of soil was taken from each bucket consecutively (A-U). The total amount of soil to be placed in each aquarium was approximately 1 litre x 21 scoops = 21 litres = 5.5 gallons = 0.74cu.ft x 100lbs/cu.ft = 74 lbs = 33.6kgs. Each scoop was used with the corresponding testing container throughout the experiment to avoid any cross contamination. Soil from bucket V was used for test cell No. 2.

- Test cell No. 2 was sterilised to remove existing natural micro-organisms. Soil for test cell No. 2 was autoclaved at 15psi and 121^{0}C for a period of 30 minutes. A sample of the soil was then tested for microbes in accordance with procedure outlined below. Autoclaving was repeated until all existing micro-organisms has been eliminated.

- A sample of JP-7 fuel was sent to a California state certified laboratory and a gas chromatograph was run to determine the JP-7 fingerprint.

- Each of the testing containers was spiked with 200 ml of JP-7 fuel to ensure an initial soil concentration of at least 5,000 mg/kg JP-7 fuel, as demonstrated by the following calculation:
    - @ 1 gm/ml, 200 ml JP-7 weighs 200 gms = 200,000 mg;
    - we have 33.6 kg of soil
    - therefore concentration is 200,000/33.6 = 5950mg/kg

- Each sample was mixed thoroughly to achieve a uniform composition for each of the initial soil samples. Mixing was performed under a fume hood provided by CSUC. The samples were then stored in the environator and turned once per day for a period of one week. This provided "set up" time for the added JP-7 fuel. The air quality in the environator was checked daily with a Photo Ionisation Detector (PID) to monitor potential build up of volatile organic compounds. If the monitoring revealed air concentrations in excess of 100 ppm the equipment was to be delayed until special ventilation equipment could be installed.

- Appropriate treatment dilutions were made to each container according to the procedure described below and each test cell was mixed thoroughly.

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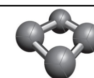

NONTOX Addition for Test Cell No. 3

Seven ml of neat NONTOX product was mixed into 28ml of distilled water creating a total volume of 35ml. This volume shall be added to the test cell No.3 and mixed into the soil.

- The moisture content of the soil in each container was tested by time domain reflectometery testing using an Environmental Sensors moisture probe as follows:
  - Insert probe 10cm into soil
  - Percent moisture was read from digital display
  - Instrument was calibrated weekly using soil with known water content; or if soil is not at 50% saturation, distilled water was added in 100ml increments until this level was obtained. The sample was mixed thoroughly after each water application. Fifty percent soil saturation was maintained throughout the experiment. Fifty percent soil saturation was approximately 10-15 percent on a mass basis (i.e. 10-15 grams water per 100 grams dry soil)

- Containers were placed in the temperature controlled environment chamber. Chamber temperature was maintained at 20^0C throughout the experiment.

- Start up soil pH readings were obtained using the following procedure:
  - A soil distilled water slurry was made using 1.0 gallons of soil vortexed in 9.0 ml deionised water for 10 seconds.
  - This was measured using either Cole Palmer hand held pH tester or a Beckman Zeromatic SS-3 pH meter. The meters were standardised using standards of pH 4, 7 and 10 daily before use.

- Obtain start up TPH samples for total TPH and S-TPH analysis using the following procedures:
  - Latex gloves and laboratory gloves were worn during all sampling procedures
  - Eight oz. glass jars with Teflon lid were provided by a California state certified laboratory.
  - Each jar was packed tightly with soil. There were no air spaces within the jar.
  - Each jar was labelled with the date and time of sampling; sample ID number (testing container number), and the requested tests. Samples were immediately packed on ice to maintain temperature at or below 4^0C.
  - Chain of custody forms were completed and samples were shipped to a California state certified laboratory for analysis. Samples were analysed for total petroleum hydrocarbons in the diesel range (TPH-D) using EPA Method 8015, total petroleum hydrocarbons in the gasoline range (TPH-G) using EPA Method GCFID/5030, and BTEX compounds using EPA Method 8020. Soluble TPH (S-TPH) was measured by performing a waste extraction test with deionised water (DI-WET) and analysed for the same compounds as for total TPH.

- Start up measurements of petroleum hydrocarbon degrading bacteria were obtained using the following procedures:
  - Two soil samples from each sample cell were taken on day 0,1,3,7 and every 7 days thereafter.

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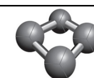

- 1.0 gallon of soil was vortexed in 9.0 ml of deionised water for 10 seconds.
- Appropriate dilutions were made to yield 30-300 CFU using the spread plate technique.
- 1:10 serial dilutions were prepared out 10^{-6}.
- 0.1ml of each dilution were plated onto media for enumeration of total heterotrophs, *psudomonads*, and petroleum degraders (see media below).
- Samples were incubated for 7 days at 28^0C.
- Plates were made with thick, 22ml media/disposable plate.

- Media preparation techniques for identification of petroleum hydrocarbon degrading bacteria:

Total Heterotrophies

- Basal mineral salts media (M-9)
- Na_2HPO_4(6.0g), KH_2PO_4(3.0g),NaCL (0.5g), NH_4CL (1.0g), Bacto-Agar (20.0g)
- Add deionised water to 979ml
- Autoclave and cool to 50^0C.
- Add 1 M $MgSO_47H_2$) (1.0ml) and 0.01 M $CaCL_2$ (10ml) to above media.
- Autoclave separately and cool to 50^0C.
- Add sterile 20 percent glucose (10ml).
- Add Amphotericin B (fungicide) to 10ug/ml and pour plates.

Pseudomonades

- Pseudomonades Isolation Agar (PIA)(Difco).
- Suspend 45g dehydrated PIA in 980 ml deionised water.
- Add glycerol (20ml) and boil to dissolve completely.
- Autoclave and cool to 50^0C.
- Add Amphotericin B to 10ug/ml and pour plates.

Petroleum Degraders

- Bacto Bushnell-Haas Broth (Difco).
- Dissolve 3.27g dehydrated broth in 990ml deionised water.
- Add Bacto-Agar (20g)
- Autoclave and cool to 50^0C.
- JP-7 was added to the media (10g/l). the JP-7 was prepared by adding silica gel (3.35g) to filter sterilised JP-7 (6.35g)
- Add Amphotericin B to 10ug/ml and pour plates.

Optional Procedures

- Each testing container was aerated daily by mixing thoroughly with the designated scoop.

- The environmental chamber temperature was checked and recorded daily. Humidity was measured using a GECNO Sling Psychrometer using the following procedures:

  - The sock was wetted with deionised distilled water.
  - The psychrometer was swung rapidly at waist level for 60 seconds.
  - Temperatures on the wet and dry thermometers was read

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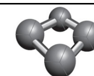

- Relative humidity was determined from a standard chart based on the dry temperature and the difference between the wet and dry temperatures.
- Soil moisture content was checked daily using the procedure specified in item 12 of the Start Up Procedures. Any adjustments that were made were recorded in the daily log book, including date of adjustment, initial moisture content, final moisture content, and required amount of distilled water to reach 50% saturation.
- Soil removal for sampling purposes (date, amount), changes in protocol, or deviation from the specified parameters (temperature, sampling procedures, etc) during the course of the experiment must be recorded in the daily log book
- Soil samples were taken for pH measurement as specified in item 16 of the Start Up Procedures. In addition to the pre-treatment testing, soil pH testing were performed on day 1,3,7 and weekly thereafter for the duration of the experiment.
- Soil samples were taken for total TPH and TPH-DI Wet analysis as specified in item 17 of the Start Up Procedures. In addition to the pre-treatment testing, TPH testing occurred on day 1,3,7 and weekly thereafter for the duration of the experiment.
- Soil samples were taken for bacterial analysis as specified in item 18 of the Start Up Procedures. In addition to the pre-treatment testing, bacteriological testing was done on day 1,3,7 and weekly thereafter for the duration of the experiment.

Results and Discussion

Reductions in total TPH in soil could not be evaluated in this study because rapid reductions in untreated control soil clearly indicated that volatilisation, rather than degradation, was responsible for most of the TPH lost in all treatments. Therefore, the role of NONTOX in the breakdown of TPH could not be discerned in these data. However, the data do suggest that the presence of NONTOX or the NONTOX surfactant actually reduced the rate of volatilisation.

The primary mechanism by which surfactants alone could reduce the rates of volatilisation of TPH from soil involves the formation of a hydrophobic environment around individual soil particles. Surfactant molecules are amphiphilic, having both hydrophobic and hydrophilic regions. As a surfactant monolayer is formed around individual particles, the hydrophobic region orients towards the TPH impacted particle and the hydrophilic region orients outwards towards the aqueous environment. The association of TPH with the hydrophobic region of surfactant molecules would result in a reduced tendency for loss by volatilisation.

The disappearance of soluble TPH is summarised in the table below. Degradative mechanisms appear to be responsible for the disappearance of soluble TPH. Soil treated with NONTOX showed greater reductions in soluble TPH than untreated controls or soil tested with surfactant alone or biocatalyst alone. Those results confirm that surfactants and biocatalysts combined behave as a bioorganic catalyst (BOC) which is responsible for soluble TPH reduction. It is likely that the tendency of NONTOX components to organise into clusters, aggregates, or gas filled bubbles provides a platform for reactions to occur. The resulting localised increase in reactant concentrations and reduction in transition energy for reactions significantly increase

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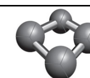

the rate of catalysis. These aggregates of BOC and partially degraded TPH may also facilitate the presentation of the TPH to micro-organisms for complete mineralisation.

Plate count data indicate that specific hydrocarbon degrading populations of bacteria emerge much more quickly in NONTOX treated soil and remained at least 10 fold higher during the study interval.

In conclusion, optimisation of treatment parameters resulted in a four fold decrease in the time required for treatment and a 50% reduction in the cost of NONTOX to meet clean up criteria as compared to the original study using NONTOX. The results of the current optimisation study confirms that the cost of NONTOX treatment is comparable to the cost of fertiliser in the removal of soluble TPH from oil. Based on these results, the environmental Engineer anticipates USAF and regulatory agency approval for the use of remediating impacted soil at the USAF base in the summer of 1997.

SOLUBLE TPH (JP-7 RANGE) DATA SUMMARY (mg/l)					
	CELL IDENTIFICATION NUMBER				
Days	1 Untreated	2 Sterile untreated	3 NONTOX	4 Biocatalyst alone	5 Surfactant alone
0	70	30	88	100	63
1	52	18	11	25	35
3	52	24	5.1	11	39
7	61	29	13	37	50
14	49	33	14	44	21
21	45	34	11	60	21
28	51	27	8.4	34	14.2
Total reduction	27%	10%	90%	66%	77%

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