Results of Laboratory Microbial Testing for Enhancing Bioremediation of Petroleum-Contaminated Soils at the Hanford Site's 100-N Area – 14592

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ABSTRACT

Microbial studies were conducted to evaluate the ability of indigenous microorganisms to biodegrade weathered diesel number 2 and Bunker C (diesel number 6) as a part of the ongoing in situ bioremediation of petroleum contaminated soil at the Hanford Site's N Reactor. Aerobic, anaerobic, and fungi were identified in soil samples collected during drilling of bioventing wells and from soil remediation excavations. The predominant strains were further evaluated to identify species and determine if they have the ability to degrade petroleum hydrocarbons. Six of the petroleum degrading strains were then selected for hydrocarbon degradation studies and kinetic nutrient assays to evaluate the rate of microbial degradation of petroleum, to compare the performance of these indigenous bacterial species, and to determine if the addition of nutrients could enhance bioremediation. Bunker C collected from drained pipelines at the waste site and diesel # 2 provided by the laboratory were used as the carbon sources for the study.

INTRODUCTION

Traditionally, petroleum hydrocarbon contaminated soils have been dealt with by excavation and disposal to a landfill. Other treatments such as incineration, immobilization, chemical oxidation, and thermal desorption are generally expensive, may not be sustainable with respect to environmental impacts, or do not eliminate the problem.

Bacteria and fungi have a remarkable ability to metabolize hydrocarbon contaminants resulting in cell biomass and stable innocuous end products (carbon dioxide and water). Encouraging the growth and reproduction of indigenous microorganisms to enhance biodegradation of petroleum in the deep vadose zone may be necessary in order to meet cleanup timeframes at the Hanford Site's 100-N Operable Unit.

BACKGROUND

The 105-N Reactor operated between 1963 and 1987. Throughout the operational history of the 105-N Reactor, spills, releases, and discharges were documented in unplanned release reports. A number of these releases resulted in petroleum hydrocarbon contamination in the vadose zone and groundwater at the 100-N Area. These releases occurred through mechanisms inclusive of corrosion failure of piping systems used to transport diesel and fuel oil, overfilling of storage facilities, and spills during fuel transfers. Bulk quantities of Bunker C fuel oil (Grade No. 6) (5,469,903 L [1,445,000 gal]) and diesel oil (Grade No. 2) (1,786,709 L [472,000 gal]) were stored at the 100-N Area. Bunker C oil was used as fuel for the 184-N boilers. Diesel oil was used to fire the igniters in the boilers and to fuel the diesel-driven emergency low-lift, high-lift, and fog spray pumps.

The UPR-100-N-17 waste site is the largest documented petroleum release located within the Hanford Site 100-NR-1 Operable Unit, approximately 192 m (630 ft) northeast of the 105-N Reactor. The site is an unplanned release of diesel that occurred at the 166-N Tank Farm

sometime between August 1965 and September 1966. The release was from a 10-cm (4-in.) pipeline in the 166-N tank farm and was located approximately 140 m (460 ft) from the Columbia River (Fig. 1). The presence of light nonaqueous-phase liquid (LNAPL) was discovered beneath the facility in March 1967, when evidence of oil was observed at the bank of the Columbia River (100-N-65 waste site) approximately 100 m (328 ft) northwest of the 166-N Tank Farm. To alleviate diesel entering the river, a trench was excavated at the shoreline to intercept and accumulate the migrating diesel. Based upon reports estimated during the time of operation, the release may have resulted in up to 39,000 m^2 (1,377,272 ft^3) of soil contaminated with petroleum hydrocarbons.

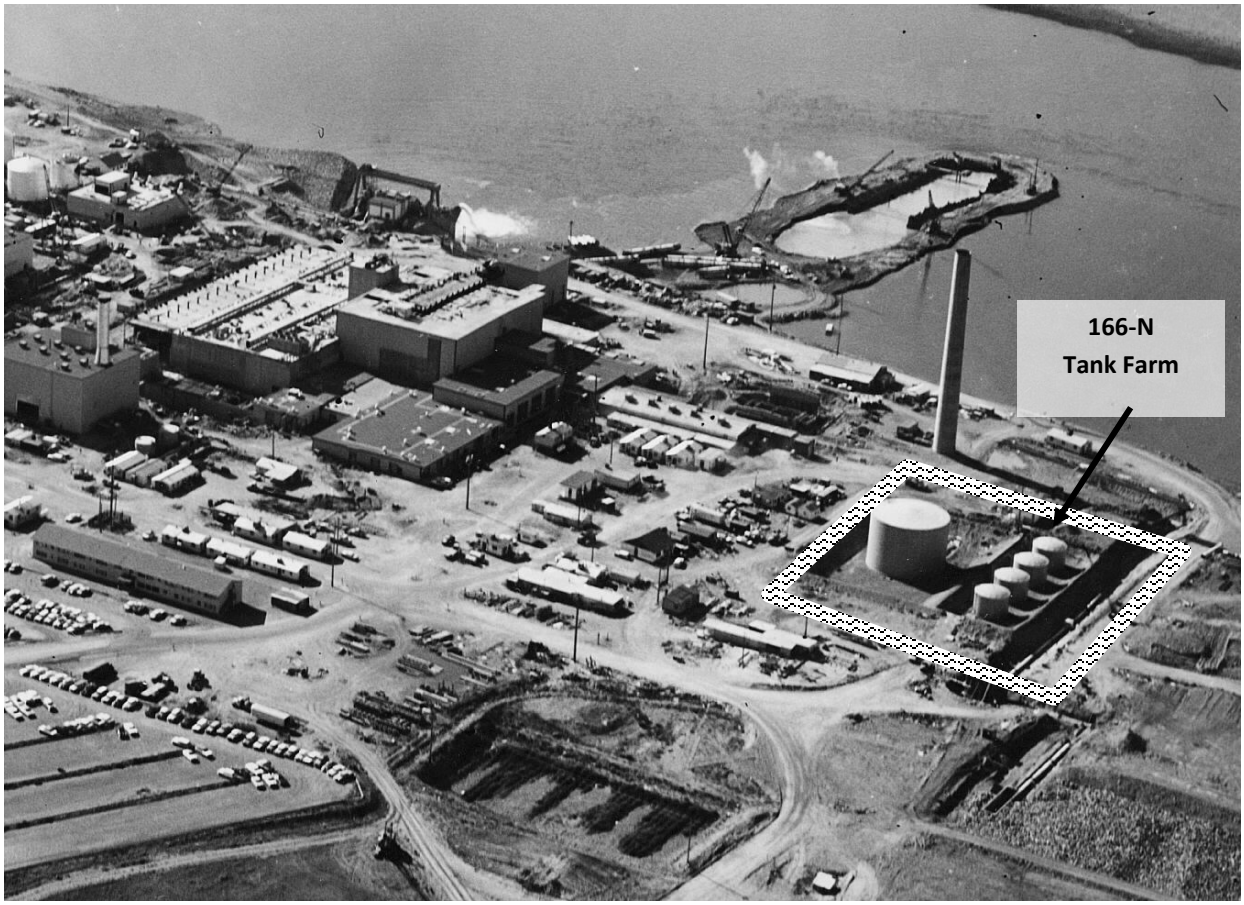

Fig. 1. 166-N Tank Farm Facility (Early 1960s).

Hydrocarbon products released at the 166-N Tank Farm facility have contaminated soil and groundwater near the facility, and petroleum contamination has reached near-shore sediments and the Columbia River via groundwater seepage (Fig. 2). Because of these releases, petroleum has been found in soil and groundwater as free product.

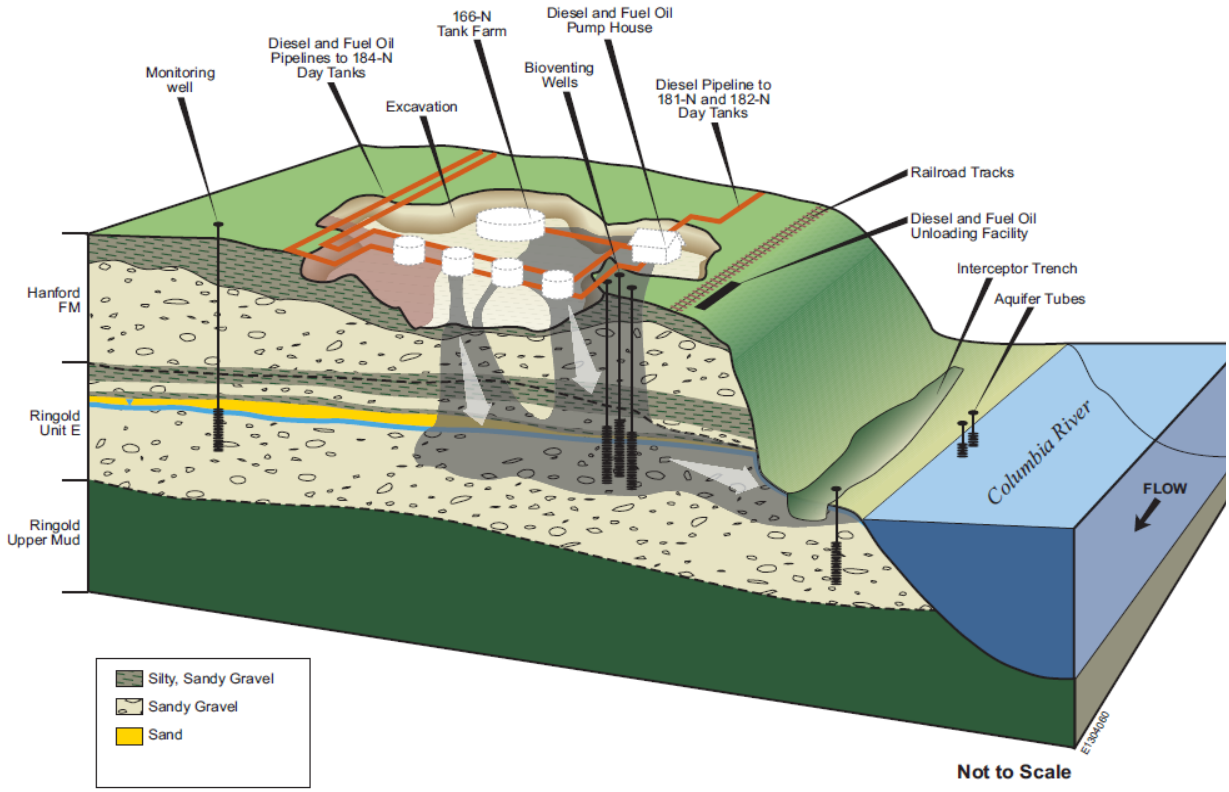

Fig. 2. Site Conceptual Model for Petroleum Contamination at 100-N.

Currently, a *Comprehensive Environmental Response, Compensation and Liability Act* Interim Action Record of Decision (ROD) for the 100-N Area identifies in situ bioremediation of the petroleum-contaminated soils in the deep vadose zone (4.6 m [15 ft] below ground surface extending to the groundwater interface) as the preferred remedy, with bioventing used as the selected technology [1]. In 2009, Washington Closure Hanford (WCH) conducted a pilot study to evaluate the effectiveness of bioventing for remediation of vadose zone petroleum contamination by supplying oxygen to the unsaturated zone to stimulate microbial activity and biodegradation of the contamination [2]. Five wells were constructed and screened within the deep vadose zone from 16.8 to 22.9 m (55 to 75 ft), and two were constructed and screened from 4.6 to 9.1 m (15 to 30 ft). During the well installation, soil samples were collected to quantify contamination levels and collect information to support the design and construction of the bioventing system. Respirometry testing performed during the pilot study indicated oxygen to be rate limiting for biodegradation at depths between 18.3 and 21.3 m (60 and 70 ft) below surface grade; however, oxygen was present at ambient levels in the shallow vadose zone soil from surface to 9.1 m (30 ft) depth and not found to be rate limiting. It is possible that addition of nutrients (nitrogen, phosphorous) could be used to enhance bioremediation of the shallow vadose zone soil. Additionally, radius of influence tests indicated sufficient air permeability to treat the area of vadose zone contamination using the installed bioventing system wells.

A full-scale bioventing system installed in 2012 is currently operating; however, the estimated timeframe for remediation calculated from respirometry tests have been variable, with some test results indicating long remediation timeframes extending beyond cleanup milestones. These estimates raise questions concerning whether biodegradation rates can be enhanced through the addition of nutrients and/or bacteria as provided in the interim ROD [1].

In July 2013, a laboratory study was initiated to determine if indigenous petroleum-degrading microbes are present in the contaminated soil at the site and, if present, determine the rate of microbial degradation including an evaluation of whether the addition of nutrients is needed to enhance biodegradation.

DESCRIPTION

Eight archived soil samples collected during drilling of the bioventing wells at the UPR-100-N-17 waste site at depths between 18.3 and 21.3 m (60 and 70 ft) below surface grade and two soil samples collected from the base of a shallow zone remedial excavation at a depth of 4.9 m (16 ft) were submitted for laboratory testing. The eight archived samples are from the smear zone associated with a diesel groundwater plume and the samples from the 4.9 m (16 ft) depth are where Bunker C contamination is present from historical releases. Bacterial and fungal counts were conducted on all samples. Isolation and incubation of selected samples was performed to identify bacterial strains and evaluate the ability of the strain to use petroleum as its only carbon source. Samples of Bunker C collected from drained pipelines at the waste site and diesel #2 provided by the laboratory were used as the carbon sources for the study. Six strains identified as excellent hydrocarbon degraders were further tested to determine petroleum degradation rates and to perform kinetic nutrient assays.

Bacterial Count

An aliquot of each soil sample was checked for weight and serially diluted. The dilutions were aseptically transferred in a laminar flow biological cabinet and plated for enumerating bacteria and fungi. Sample dilutions for fungi count were plated onto prepared and dried Sabouraud Dextrose Agar (SAB) medium in petri plates and then incubated at 24.5 °C for 3 and 5 days, with the results reported as total colony forming units per 1 gram of solid (CFU/g). Sabouraud Dextrose Agar is a selective medium that is formulated to allow growth of fungi and inhibit growth of bacteria. Sample dilutions for aerobic and anaerobic count were plated onto prepared and dried Trypticase Soy (Broth) Agar (TSA) medium in petri plates. Observations for colony-forming units per gram sample (CFU/g) were made after 24 and 48 hours of incubation at 30 °C for aerobic bacteria; anaerobic bacteria were counted after 48 and 72 hour of incubation at 37 °C under anaerobic conditions. The results of these enumerations are reported in Tables I and II.

TABLE I. Results of Bacterial and Fungal Colony Counts.

#	Sample Description	Petroleum Concentration (mg/kg)	Total Aerobic Plate Count (CFU/g)		Total Anaerobic Plate Count (CFU/g)		Fungi Count (CFU/g)	
			24 hr	48 hr	48 hr	72 hr	3 days	5 days
1	199-N-169 (60 ft)	3,800	**1.00×10^2**	**1.00×10^2**	**2.00×10^2**	**3.00×10^2**	$<1.00 \times 10^2$	$<1.00 \times 10^2$
2	199-N-169 (65 ft)	3,800	$<1.00 \times 10^2$	**1.50×10^4**	**1.10×10^3**	**1.90×10^3**	$<1.00 \times 10^2$	**5.10×10^3**
3	199-N-170 (60 ft)	2,300	$<1.00 \times 10^2$	**4.00×10^2**	**1.91×10^5**	**1.91×10^5**	$<1.00 \times 10^2$	$<1.00 \times 10^2$
4	199-N-170 (65 ft)	3,100	$<1.00 \times 10^2$	$<1.00 \times 10^2$	$<1.00 \times 10^2$	**1.00×10^2**	**1.00×10^2**	**1.00×10^2**
5	199-N-171 (65 ft)	3,400	$<1.00 \times 10^2$	**3.04×10^4**	**9.10×10^3**	**1.20×10^4**	**7.60×10^3**	**8.00×10^3**
6	199-N-172 (60 ft)	690	$<1.00 \times 10^2$	$<1.00 \times 10^2$	**1.00×10^2**	**5.00×10^2**	$<1.00 \times 10^2$	$<1.00 \times 10^2$
7	199-N-172 (65 ft)	2,200	$<1.00 \times 10^2$	**2.02×10^4**	**2.67×10^5**	**2.67×10^5**	**7.00×10^3**	**7.70×10^3**
8	199-N-172 (70 ft)	3,300	**1.00×10^2**	**9.73×10^4**	**2.10×10^5**	**3.20×10^5**	**4.90×10^3**	**7.20×10^3**
9	Excavation (16 ft)	14,000	**4.95×10^6**	**5.02×10^6**	**1.00×10^6**	**9.00×10^6**	**1.55×10^5**	**1.55×10^5**
10	Excavation (16 ft)	13,000	**5.12×10^4**	**3.95×10^4**	**1.13×10^6**	**1.18×10^6**	**2.94×10^4**	**4.47×10^4**

Microbial Identification

Table II shows the relative percentages of aerobic and anaerobic strains identified for each of the 10 soil samples. Fifteen of the highest occurring strains were selected for species identification using Vitek®, Biolog®, or 16S rRNA gene sequencing. Samples were gram stained for classification (i.e., gram-positive or gram-negative) prior to processing for Vitek® identification. An aliquot from the original agar plate was diluted in a saline solution to an optical density absorbance between 0.53 and 0.69. The diluted sample was then transferred to a Vitek® plate and incubated in the instrument until a positive identification against the database of species was obtained. Samples processed for Biolog® identification were streaked onto Biolog® universal growth agar and allowed to incubate at 30 °C for 24 hours. After 14 hours, the sample strain was suspended into an inoculating fluid and then the solution was loaded into the GEN III micro-plate. The plate was incubated at 30 °C and then, using an automated micro-plate reader, examined at 24 and 48 hours and compared against the Biolog®Gen III database to obtain the bacterial identification. Samples processed for 16S rRNA gene sequencing had genomic DNA extracted from the bacterial colony. The 16S rRNA gene was sequenced using dye terminator cycle sequencing chemistry. The sequence reactions were analyzed using automated DNA sequencers and software. Unknown bacterial samples were identified using the microbial identification software and the 16S rRNA gene sequence database as well as GenBank, a public database supported by the National Center of Biotechnology Information. Ten strains of bacteria were identified (Table III). Five of the strains could not be cultured again from the sample or were not identifiable.

TABLE II. Approximate Percentage of Bacterial Strains in Sample.

Strain	Sample Number									
	1	2	3	4	5	6	7	8	9	10
Aerobic Strains on TSA										
1	100	--	--	--	--	--	--	--	--	5
2	--	98	--	--	--	--	--	--	--	--
3	--	1	--	--	--	--	--	--	--	--
4	--	1	--	--	--	--	--	--	--	--
5	--	--	100	--	--	--	--	--	--	--
6	--	--	--	--	--	--	--	--	--	--
7	--	--	--	--	--	--	--	--	--	--
8	--	--	--	--	10	--	--	--	--	--
9	--	--	--	--	80	--	--	--	--	--
10	--	--	--	--	5	--	--	--	--	--
11	--	--	--	--	5	--	30	75	--	--
12	--	--	--	--	--	--	30	5	5	--
13	--	--	--	--	--	--	30	--	--	--
14	--	--	--	--	--	--	10	5	5	--
15	--	--	--	--	--	--	--	5	--	--
16	--	--	--	--	--	--	--	10	--	5
17	--	--	--	--	--	--	--	--	37	5
18	--	--	--	--	--	--	--	--	38	--
19	--	--	--	--	--	--	--	--	5	--
20	--	--	--	--	--	--	--	--	10	10
21	--	--	--	--	--	--	--	--	--	26
23	--	--	--	--	--	--	--	--	--	24
24	--	--	--	--	--	--	--	--	--	15
25	--	--	--	--	--	--	--	--	--	5
48	--	--	--	--	--	--	--	--	--	5
Anaerobic Strains on TSA										
1	100	--	--	--	--	--	--	--	--	--
2	--	--	--	--	--	--	5	--	--	--
8	--	--	--	--	44	--	--	--	--	--
9	--	--	--	--	48	--	--	--	--	--
11	--	--	--	--	--	--	15	--	--	--
12	--	--	--	--	--	--	--	--	--	5
13	--	--	--	--	--	--	--	--	--	70
16	--	--	--	--	--	50	--	--	--	--
18	--	--	--	--	--	--	--	90	--	--
20	--	23	--	--	--	--	--	--	--	--
23	--	7	--	--	2	--	--	--	--	--
24	--	23	--	--	1	--	--	--	--	--
27	--	1	--	--	--	--	--	--	--	--
28	--	23	--	--	--	--	--	--	--	--
29	--	23	--	--	--	--	--	--	--	--
30	--	--	100	--	--	--	80	--	--	25
31	--	--	--	100	--	--	--	--	--	--
32	--	--	--	--	1	--	--	--	--	--
33	--	--	--	--	1	--	--	--	--	--
34	--	--	--	--	3	--	--	--	--	--
35	--	--	--	--	--	50	--	--	--	--
36	--	--	--	--	--	--	--	--	10	--
37	--	--	--	--	--	--	--	--	45	--
38	--	--	--	--	--	--	--	--	45	--
49	--	--	--	--	--	--	--	10	--	--

TABLE III. Bacterial Strains Identified in Soil Samples.

Strain #	Sample #	Species	Identification Method	Biodegradation Potential	
				Bunker C	Diesel
38	9	*Comamonas testosteroni*	VITEK	Excellent	Inhibited
2	2,7	*Serpens flexibilis*	Biolog	Excellent	Excellent
11	5,7,8,9,10	*Microbacterium resistens*	Biolog	Good	Good
17	9,10	*Pseudomonas corrugate*	Biolog	Excellent	Excellent
18	8.9	*Serratia proteamaculans*	Biolog	Excellent	Excellent
20	2,8,9,10	*Pseudomonas chloroaphis ss aurantiaca*	Biolog	Inhibitied	Inhibited
24	2,5,8,9,10	*Bacilluls galactosidilyticus*	Biolog	Excellent	Inhibited
28	2	*Pseudomonas stuzeri*	Biolog	Excellent	Excellent
36	9	*Microbacterium maritypicum*	Biolog	Excellent	Excellent
9	5,7	*Streptomyces cellulosae*	16S rRNA	Inhibited	Inhibited

Biofeasibility Endpoint Assay

Six of the 10 bacterial strains were selected for biofeasibility endpoint assays. The tested bacterial strains were grown overnight on trypticase soy (broth) agar at 30 °C and then suspended in sterile saline to a turbidity of 40 to 50%. The strains were then aliquotted into a 96-well microtiter plate that contained growth medium of mineral salts, vitamins, and buffer without a major carbon source. The wells also contained a tetrazolium dye redox indicator system. Bacterial growth (metabolic respiration/oxidation of carbon sources) was monitored by tetrazolium reduction as measured at 590nm in a microplate reader. Samples of Bunker C (J1RPT1) collected from drained pipelines at the waste site as well as diesel #2 provided by the laboratory was added to selected wells to serve as the major carbon source. Trypticase soy broth served as the positive control for bacterial growth and water served as the negative control for bacterial growth in the assays. Total volume of each well was 150μL. Total growth was measured after 48 hours of incubation at 30 °C. Individual strain feasibility for biodegradation potential was reported as one of the following classifications: excellent degrader, good degrader, fair degrader, minimal degrader, no effect, or growth inhibited (Table II).

Biodegradation Study

The six bacterial strains were prepared as specified for the biofeasibility endpoint assays. Optical density readings measuring population growth were taken at 20-minute intervals over a time-period of 48 hours. At 48 hours, an endpoint reading was taken. The data was processed and given with background blank values subtracted. Figure 3 provides the test results for strain 17. Figure 4 shows the relative results for each of the six strain's ability to use Bunker C (J1RTP1) and diesel. Strains 2, 17, 18, and 28 were excellent degraders of both Bunker C and diesel. Strains 11 and 36 were good degraders.

Kinetic Nutrient Assays

The six bacterial strains were grown overnight on trypticase soy (broth) agar at 30 °C and then suspended in sterile saline to a turbidity of 40 to 50%. The strains were then aliquotted into a 96-well microtiter plate that contained growth medium of mineral salts, vitamins, and buffer without a major carbon source. The wells also contained a tetrazolium dye redox indicator

system. Bacterial growth (metabolic respiration/oxidation of carbon sources) was monitored by tetrazolium reduction as measured at 590nm in a microplate reader. Sample of Bunker C (J1RPT1) collected from drained pipelines at the waste site as well as diesel #2 provided by the laboratory was added to selected wells to serve as the major carbon source. A total of 50 μL of these inocula were dispensed into the test wells.

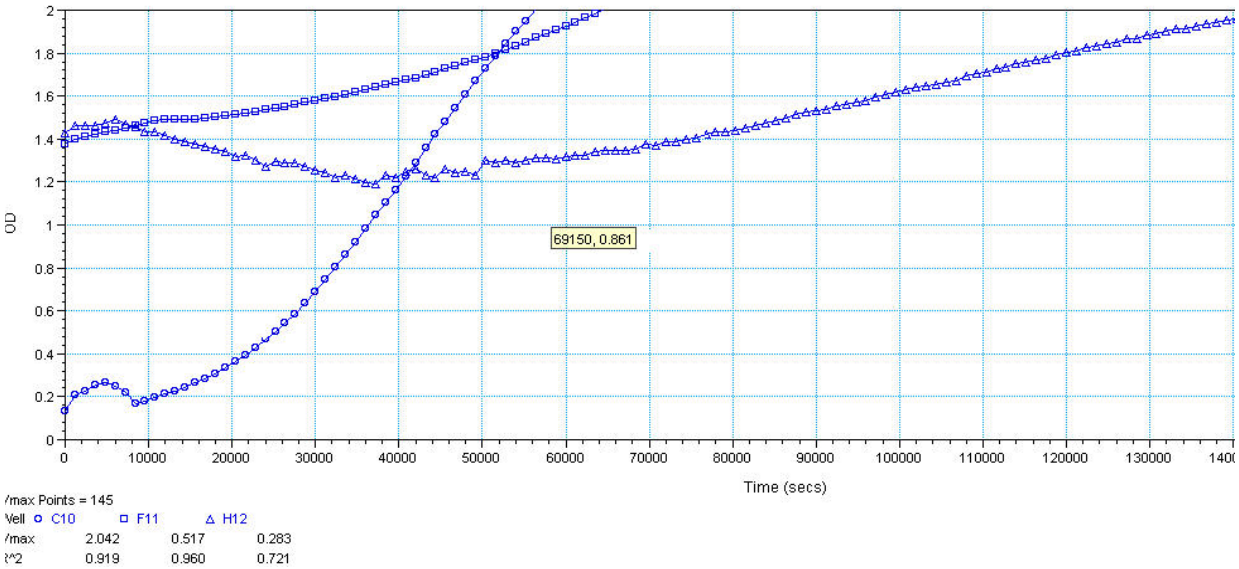

Fig. 3. Bacterial Growth Measured in Optical Density Measured over time for Strain 17.

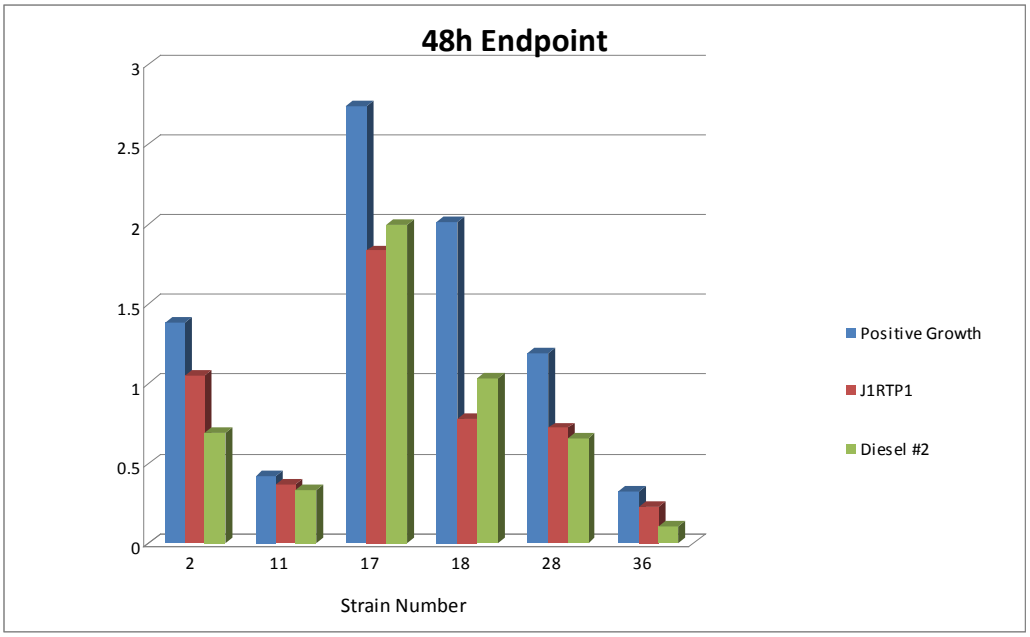

Fig. 4. Results of Biofeasibilty Endpoint Assays for the Six Bacterial Strains.

Trypticase soy broth served as the positive control for bacterial growth and water served as the negative control for bacterial growth in the assays. Davis Minimal Broth also served as a positive control for bacterial growth. Additionally, oxygenated water was used as a bacterial growth supplement with the two agars. Total volume of each well was 150 μL. Readings were

taken at 20 minute intervals over a time period of 48 hours. Total growth was measured after 48 hours of incubation at 30 °C. The data was processed with background blank values subtracted. Bar chart interpretation of the data is provided in Figure 5. The tested bacterial strains were then run with addition of nutrients only; no carbon source was added. The results of these assays are shown in Figure 6.

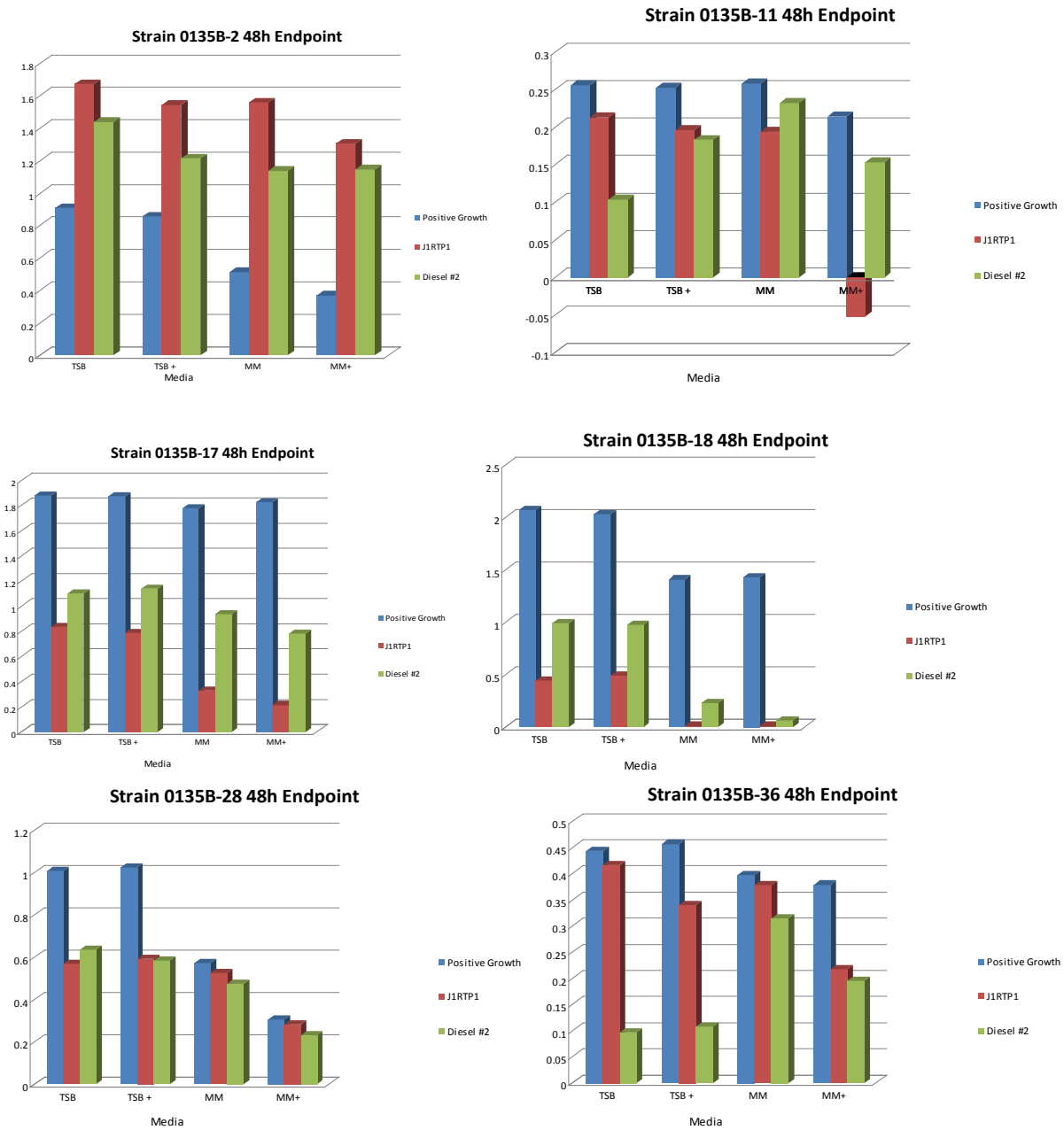

Fig. 5. Results of Kinetic Nutrient Assays for the Six Bacterial Strains with Carbon Source.

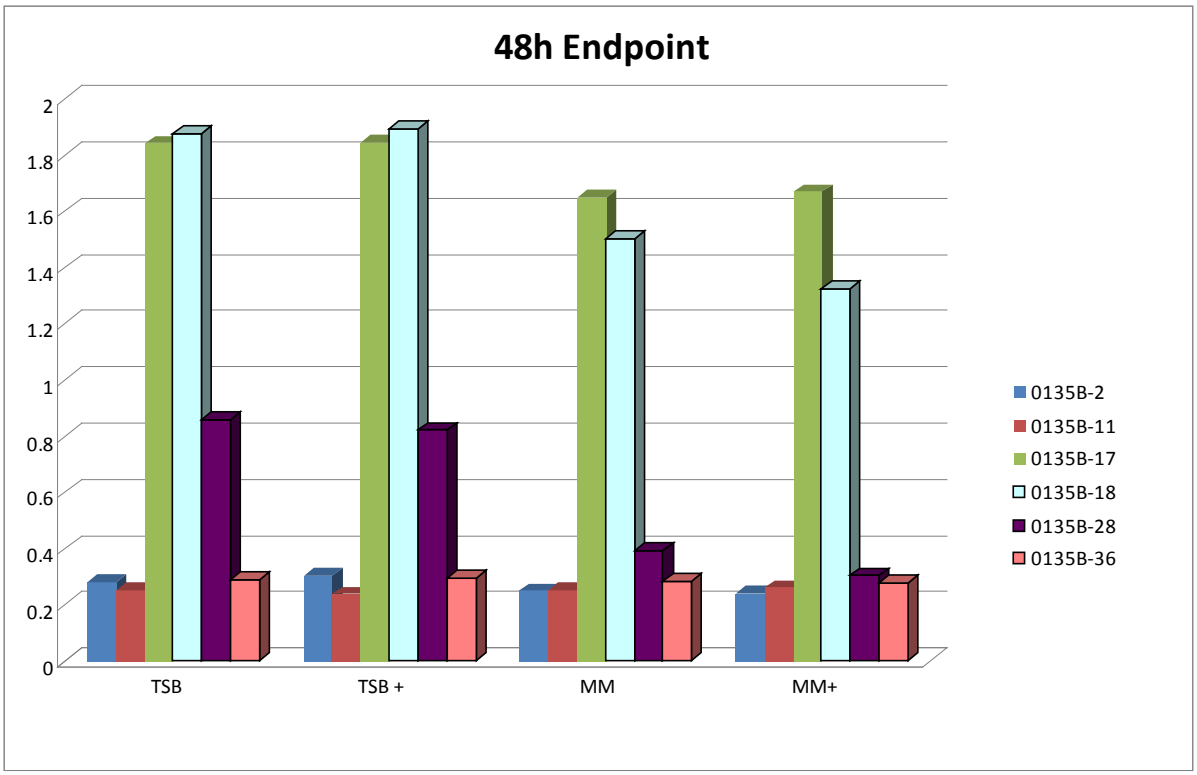

Fig. 6. Results of Kinetic Nutrient Assays for the Six Bacterial Strains Without Carbon Source.

DISCUSSION

The laboratory microbial testing was initiated to investigate the subsurface microbes in samples from the UPR-100-N-17 waste site and determine if indigenous bacteria are using petroleum contamination as part of in situ bioremediation, if the addition of nutrients will enhance biodegradation, and to estimate degradation timeframe for remediation. Aerobic, anaerobic, and fungal colonies were identified in soil samples collected from a depth of 16 ft at the base of a large Bunker C soil excavation and in archived soil samples collected from 18.3 to 21.3 m (60 to 70 ft) from drilling of bioventing wells for bioremediation of a deep vadose zone diesel plume. The highest colony counts were for samples collected at 4.9 m (16 ft) from the Bunker C contaminated soil. Samples collected from the diesel smear zone did not have measurable aerobic colonies until after 48 hours, likely because these samples were archived for 3 years; however, anaerobic colonies were present at 24 hours. Fungi were not measured in any of the 18.3 m (60 ft) depth samples. This depth is at the top of the historic high groundwater elevation during operations and the depth where free product would have been present on top of the water table.

Ten species of bacteria were identified with seven strains excellent degraders of Bunker C and five strains excellent degraders of diesel (Table III). These populations are present in the Bunker C contaminated soil at 4.9 m (16 ft) and in the diesel plume (18.3 to 21.3 m [60 to 70 ft]). Two of the strains were microbacterium and two were pseudomonas, known to be good petroleum degrading bacteria.

Biofeasibility testing on strains 2, 11, 17, 18, 28, and 36 showed that strains 2, 17, 18, and 28 excellent degraders of Bunker C and diesel and strains 11 and 36 are good degraders of Bunker C and diesel (Fig. 5). Kinetic nutrient testing indicated that nutrients are not necessary

to enhance biodegradation (Fig. 5 and 6).

Microbial diversity in subsurface soil at the Hanford Site has been reported with more than 700 operational taxonomic units detected in samples collected across geologic formations extending into the water table [4]. Heterogeneities in grain size distribution, porosity, nutrient availability, and redox status within the upper soil and transition to the smear zone and saturated soil may drive differences in microbial communities. This diversity is also reflected in the results of the microbial testing that indicate the microbial community has colonized the site and adjusted to their environment as influenced by the age of the contamination (over 45 years old), the chemical composition of the petroleum (Bunker C or diesel), and the diversity of the physical environment. Geologic logging indicates silty sandy gravel from 18.3 to 19.8 m (60 to 65 ft), sand from 19.8 to 21.3 m (65 to 70 ft), and sandy gravel below 12.3 m (70 ft). Bunker C is present in shallow vadose soils and diesel is present in the deep vadose soil associated with fluctuating groundwater, known as the smear zone (Fig. 2). The microbial results support previous studies that indicate diverse microbial habitats presumably driven by small-scale physical and chemical heterogeneities [4].

Minimum project length projections from these test results are currently being calculated to provide estimates of remediation timeframes.

CONCLUSIONS

Encouraging the growth and reproduction of indigenous microorganisms to enhance biodegradation of petroleum in the deep vadose zone may be necessary in order to meet cleanup timeframes at 100-N Area. The microbial testing results are expected to support a decision of whether indigenous petroleum microbes are sufficient to degrade petroleum within an acceptable timeframe and if not, whether the addition of nutrients and possibly laboratory bacterial strains as inoculum should be used to enhance biodegradation. If a decision is made to add nutrients and/or bacteria to the subsurface soil, engineering designs would need to be developed and finalized to ensure optimal addition without causing negative impact to the indigenous bacterial population or groundwater.

Subject to receiving the final laboratory report at the end of November, the microbial laboratory testing indicates that sufficient petroleum degrading microbes are present to degrade both Bunker C and diesel contamination present in the soil at 100-N Area and addition of bacterial strains is not required. Both aerobic and anaerobic petroleum degrading bacteria are present. Additionally, the testing indicates that nutrients are not required for enhancing bioremediation.

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ACKNOWLEDGEMENTS

The laboratory microbial work was performed by Microbe Inotech Laboratories, Inc. under subcontract to Washington Closure Hanford for the U.S. Department of Energy. Valuable assistance for sequencing the tests and interpretation of the test results was provided by Dr. Bruce Hemming.