A TEST OF PLANT-AIDED PETROLEUM HYDROCARBON DEGRADATION

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ABSTRACT

One of the mandates of WTI's Assessment & Remediation Technical Area is to research and develop environmental restoration technologies, especially those applicable to contaminated industrial sites. One technology that may prove useful for restoring contaminated sites is *phytoremediation.* The applied use of plants for restoring anthropogenically-contaminated environments may prove to be a simple, cost-effective clean-up option for specific sites, under certain conditions.

A greenhouse-based phytoremediation experiment was performed to assess the potential impacts of three treatment factors on the degradation of petroleum hydrocarbons in contaminated soils. A 2^3 factorial experimental design - replicated twice for a total of 16 experimental units – was used. The three factors tested were: 1) turfgrass (a 50:50 mixture of tall fescue and perennial ryegrass); 2) BuRIZE™ (a commercial "vesicular-arbuscular mycorrhizal", or VAM, inoculant); and 3) fertilizer (a commercial liquid formulation). Controls consisted of untreated contaminated soil. The design allowed for testing of any significant differences in contaminant degradation rates resulting from use of the three treatments, individually and jointly. Four sampling events (i.e., an initial, two interim, and a final) occurred over a study period of 180 days. Statistical evaluation of the experimental results revealed a consistent, significant treatment effect for the "fertilizer" factor and a periodic treatment effect for the "grass" and "grass/VAM inoculant" interaction.

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Introduction

This publication describes the results of one part of a phytoremediation research program to assess the use of turfgrasses and other plants for enhancing the *in situ* degradation of petroleum hydrocarbons under conditions that would mimic potential large-scale phytoremediation operations. The overall research program involved two separate but related parts; Part I involved a multi-year field study, and Part II involved a greenhouse "potted plant" study. The field and greenhouse studies were designed to complement one another, such that results could be compared/corroborated. Only the greenhouse research study will be discussed here.

Phytoremediation[1] is the name given to a class of distinct technologies that use particular plant species to restore contaminated environmental matrices. Phytoremediation technologies exploit the unique abilities of the plant species to:

- degrade or facilitate degradation of predominantly organic contaminants alone or via microbial associations within the plant rhizosphere;
- hyperaccumulate or sequester predominantly inorganic contaminants within plant parts (e.g., roots, shoots, stems, leaves);
- bind / stabilize contaminants within plant parts or within the rhizosphere;
- uptake volatile organic contaminants from the rhizosphere and translocate and transpire them into the atmosphere;
- perform other metabolic functions that result in enhanced de-contamination of environmental matrices.

Methods to study, and enhance the biodegradation or bioremediation of organic contaminants such as petroleum hydrocarbons - or their constituent polycyclic aromatic hydrocarbon (PAH) compounds - have been researched by WTI scientists since the early 1980's (e.g., Bulman *et al.*, 1985, 1988; Hosler *et al.*, 1992; Hosler and Booth, 1996). This research revealed that biologically-aided contaminant degradation may often be enhanced by various treatments (e.g., adding nutrients in the form of inorganic fertilizers or manure; adding oxygen or other electron acceptors; modifying soil conditions, etc.). Recently, research has shown that contaminant degradation may also be enhanced in the rhizosphere or root-zone of various plant species (e.g., April and Sims, 1990; Banks *et al.*, 1997; Qui *et al.*, 1997). Remediation of particular contaminants may be achieved by exploiting the unique symbiotic associations between certain fungal species and plant roots, called "mycorrhiza" (literally, *fungus-root)*. Inoculation of soils with mycorrhizal fungi for enhancing agricultural production is a common practice supported by various commercial companies (e.g., Buckman Laboratories of Canada Ltd.). Plants have an inherent ability to aerate, bind and/or stabilize soils at contaminated sites (e.g., reducing or eliminating erosion, and regulating water infiltration), and thus their presence is undoubtedly advantageous. However, more research is needed to show that plants – in phytoremediation applications - provide a distinct and significant benefit above that of general "bioremediation" processes, which have proven effective in degrading various environmental contaminants, both *in situ* and *ex situ* (e.g., see AAEE/Wastech, 1995).

Experimental Design

A 2^3 factorial experimental design was used in a greenhouse study to assess the degradation of "total petroleum hydrocarbons" (TPH) – the nomenclature that we will use to represent a "weathered crude oil" - in contaminated soil. Factorial treatment designs allow a researcher to evaluate the effects of one or more "factors", taking into account the simultaneous effects of other treatment factors, thus producing efficient experiments (Kuehl, 1994). Our design evaluated the effects of three factors:

(1) a 50:50 mixture by seed quantity - or 3:2 ratio by seed weight - of tall fescue [*Festuca arundinacea* var. "Bonsai"] and perennial ryegrass [*Lolium perenne* var. "Affinity"], applied at a rate of 5 g/pot;
(2) BuRIZE™ (a commercial vesicular-arbuscular mycorrhizal inoculant; Buckman Laboratories of Canada Ltd.; Vaudreuil, PQ), applied at a rate of 1 propagule VAM/cm^3; and,
(3) a commercial liquid fertilizer formulation ("Schultz-Instant®" water; Schultz Co., St. Louis, MO; guaranteed analysis of 10% total N, 15% available P_2O_5, and 10% soluble K_2O; mix formulation of 7 mL fertilizer/1L water), applied at the rate of ~150 mL mix per pot every three days.

[1] *Phyto* - combining form of the Greek word *phyton*, plant (Gage Canadian Dictionary, 1980)

The factors were tested at two levels each (i.e., either present or absent), and can be summarized as follows:

Treatment #1 Contaminated soil.....(no factors)
Treatment #2 Contaminated soil + grass.....(factor "A")
Treatment #3 Contaminated soil + fertilizer.....(factor "B")
Treatment #4 Contaminated soil + BuRIZE™ .....(factor "C")
Treatment #5 Contaminated soil + grass + fertilizer.....(factors "AB")
Treatment #6 Contaminated soil + grass + BuRIZE™ .....(factors "AC")
Treatment #7 Contaminated soil + fertilizer + BuRIZE™ .....(factors "BC")
Treatment #8 Contaminated soil + grass + fertilizer + BuRIZE™ .....(factors "ABC")

Treatment #1 represented the "control", as referred to in subsequent tables and Figure 1. There were two (2) replicates of the experimental units (i.e., ceramic plant pots, 205 cm in diameter and 153 cm in height; containing 1833 g of mixed soil) for each treatment, for a total of sixteen (16) pots. For a subjective evaluation of the quality and quantity of grass growth in uncontaminated versus contaminated soil, another two (2) pots were set-up with clean (uncontaminated soil) and all treatment factors were applied (i.e., the "subjective" control). This assessment of grass growth helped to determine if the contaminated soil had any toxic effect on the plants. This treatment was not sampled for TPH, as sufficient QA/QC samples had been included in the sampling program to assess "background" or uncontaminated soil TPH concentrations.

The plant pots were incubated on a table in a research greenhouse with relatively constant temperature (23°C+/-3°C), humidity, and a 12-hour photoperiod. Natural radiant energy was supplemented by two 400-watt metal halide lamps (HDK Luminaires; Philips Electronics Ltd.). A pot location grid was prepared on the table to facilitate the randomized pot placement; to avoid any confounding environmental variables (e.g., temperature, illuminance).

Experimental Set-Up

Contaminated soil - collected from the field site "hotspot" at a depth of approximately 25 cm - was sieved to <2 mm size (U.S.A. Standard Testing Sieve No. 10; Tyler Equivalent 9 Mesh, W.S. Tyler Inc.) and then stored at 4°C until ready for use. "Clean" soil from the A_h horizon (i.e., the dark-coloured horizon enriched in organic matter) of a non-cultivated rural agricultural field was similarly sieved and stored. Prior to and during their use in the study, both soils were characterized for basic physico-chemical properties previously shown to influence bioremediation processes (Sims *et al.*, 1989; Hosler & Booth, 1996). Also, three replicated samples of both contaminated and clean soils were characterized for total heterotrophic plate counts and quantity of TPH-degrading microorganisms using standard methods; diesel fuel was used as the organic substrate for isolating the TPH-degraders.

For this study, an approximate 1:4 mix ratio of contaminated to clean soil was used, because preliminary testing had shown that the 3.5% - 5% range of contamination in the "raw" contaminated soil was severely detrimental to plant growth. The contaminated soil was mixed with the clean soil as follows. A pre-weighed amount of contaminated soil was mixed with a pre-weighed amount of clean soil within a Nalgene-brand HDPE container ~45 cm x 70 cm x 35 cm deep, by successively applying small aliquots of contaminated soil to a single layer of clean soil. This soil was then mixed thoroughly, and the process repeated until the entire mixture was visually homogenous - after approximately 20 minutes of hand mixing. The mixed soil was then distributed to individual experimental units (pots) in a randomized order. Soil sampling was then performed (described in detail below). After the sampling, all pots received equal amounts of sufficient water to initiate seed

germination, followed by additions of specific treatment amendments (i.e., grass, BuRIZE™ VAM inoculant, fertilizer)(see **"Treatment Application"**, below).

Soil Sampling

Soil sampling was performed in a uniform manner using a stainless steel scoopula. An approximate 50 g sample was taken from one of four quadrants or designated spots in each pot; a separate quadrant was used for each sampling event. Samples were thoroughly mixed prior to submission to an independent analytical laboratory. Appropriate and sufficient numbers of QA/QC samples (i.e., controls, spikes, duplicates, and method blanks), were included in the study. After grass growth had occurred in specific treatment pots, soil sampling involved "coring" the turf and removing it, removing all adhered soil from the plant roots, and adding it to the remainder of the soil sampled from deeper in the pot.

The sample schedule was originally designed to have three sampling events, but this was later expanded to four when the exhibited TPH degradation was lower than anticipated after three periods. Timing of the sampling events was based upon "typical" TPH degradation rates reported in the literature (e.g., Huesemann, 1994; Visser *et al.*, 1987).

Treatment Application

After initial soil sampling, an equivalent amount of moisture was added to all experimental units (pots). Hydrocarbon-contaminated soils are typically very hydrophobic and moisture addition to "air-dried" soils can often be problematic. Extensive mixing was thus required before the soils absorbed enough water to achieve the desired moisture content of 70% of maximum water holding capacity - as determined by standard methods.

Grass seeds were planted in designated treatment pots as follows. A suitable quantity of a 50:50 mixture of tall fescue and perennial ryegrass seeds (60:40 mixture by weight) was first prepared. The surface layer of soil in the respective treatment pots was then removed and held in a tinfoil weigh-boat. At this point, pots designated for inoculation with BuRIZE™ VAM (prepared as per the manufacturer's instructions) received the liquid inoculant; added to the exposed subsoil. An appropriate quantity of grass seed was then distributed onto the wetted soils and dry soil was sprinkled over-top to secure the seeds. More water - which may or may not have had liquid fertilizer added as per the designated treatments - was then applied.

Germination of the plants occurred after approximately seven days but was not uniform because a thin soil crust prevented "normal" germination in some pots. Plants attained a height of >8 cm in the uncontaminated controls whereas plants in the contaminated pots were noticeably less prolific. The soil crusts persisted in most pots after two weeks and were then manually "disrupted" to allow uniform grass development. After three weeks, grass growth was relatively uniform amongst all treatment pots, however clean soil controls continued to exhibit superior growth.

Results

The physico-chemical characterization of both "clean" and "contaminated" soils revealed that the critical environmental factors necessary for bioremediation of petroleum hydrocarbons (e.g., temperature, moisture content, pH, cation exchange capacity, etc.) were within acceptable levels (e.g., see Table 3., J.L. Sims *et al.*, 1989). Characterization of the heterotrophic and TPH-degrading microbial populations revealed that both of the soils individually possessed "reasonably-sized" heterotrophic and TPH-degrading microbial populations (i.e., > 1.0×10^5 cfu/g). It would thus be reasonable to expect some biodegradation of the hydrocarbon contamination during the course of the study.

Initial soil sampling results from the 16 experimental units revealed a relatively uniform mixing of contaminated hotspot soil with clean soil, displaying an average TPH concentration of 6,875 ppm with a standard deviation of 522 and a coefficient of variation (CV) of 7.6%[2]. Summary statistics for the overall TPH data are presented in **Table 1.** It would be expected that as the experiment progressed, the variation in the overall mean TPH value would increase because the differences between treatments and controls (i.e., treatment effects) should increase (i.e., a greater dispersion of the data around the overall mean), and this result did occur.

Table 1. Summary statistics for the TPH data; combined treatments and controls.

Sample Period	Mean TPH (ppm)*	Standard Deviation (ppm)	%CV
#1 (0 days)	6,875	522	7.6%
#2 (66 days)	4,606	580	12.6%
#3 (88 days)	4,000	661	16.5%
#4 (188 days)	2,731	953	34.9%

* = Note that this is for all treatments and controls, averaged together.

The replicate average TPH concentrations, with standard deviation values, are presented for all treatments for the four time periods in **Table 2**.

Table 2. Replicate average TPH concentrations (ppm) versus time, with standard deviations.

Treatment	Time (d)	TPH Avg.	Std. Dev.	Treatment	Time (d)	TPH Avg.	Std. Dev.
Control	0	6350	919	Control	66	4800	141
A	0	7000	141	A	66	5200	283
B	0	7100	566	B	66	4850	354
C	0	6900	849	C	66	3900	0
AB	0	7200	141	AB	66	5400	141
AC	0	7150	495	AC	66	4200	849
BC	0	6800	707	BC	66	4150	71
ABC	0	6500	283	ABC	66	4350	212

[2] The *anticipated* or *target* TPH concentration had been 7,066 ppm TPH, based upon hotspot soil sample characterization.

Table 2. ***continued.***

Treatment	Time (d)	TPH Avg.	Std. Dev.	Treatment	Time (d)	TPH Avg.	Std. Dev.
Control	**88**	4800	283	Control	**180**	3600	283
A	**88**	4300	990	A	**180**	3750	495
B	**88**	4550	71	B	**180**	3250	495
C	**88**	4150	778	C	**180**	1900	707
AB	**88**	4650	71	AB	**180**	3700	141
AC	**88**	3100	283	AC	**180**	1900	283
BC	**88**	3450	71	BC	**180**	1500	424
ABC	**88**	3800	141	ABC	**180**	2250	71

The TPH "loss" data for all treatments for the four sampling periods is graphically presented in **Figure 1**, which displays a 3-D column chart of the "ranked" treatments (i.e., highest "loss" treatment [BC] shown in the foreground, and lowest "loss" treatment [Control] shown in the background).

The experimental hypothesis for this study was that the use of turfgrass should result in greater TPH degradation as compared to those treatments without turfgrass. The use of a factorial treatment design allows one to assess a number of factors to determine if there are significant effects on TPH degradation from using the treatments, or factors, alone (i.e., "simple effects"), or in combination (i.e., "interactions"). The analysis of the data was performed in a manner similar to methods outlined in Chapter 5 of Cochran & Cox (1992), however using a *STATISTICA®* software package (StatSoft®, Tulsa, OK). The data was analysed for all (four) time periods – individually, and in combination (i.e., periods #1, #2, #3, & #4, periods #1&#2, periods #2&#3, periods #3&#4, and periods #1&#4). All individual and combinations of factors were assessed to determine which effects were statistically significant for enhancing TPH "loss" (assumed to be "degradation"). The results may be summarized as follows:

- For time periods #1&#2, both "fertilizer" and "time" factors were statistically significant, and there was a significant "fertilizer/time" interaction;
- For time periods #2&#3, both "fertilizer" and "time" factors were statistically significant, and there was a significant "grass/time" interaction;
- For time periods #3&#4, both "fertilizer" and "time" factors were statistically significant, and there were significant "grass/VAM inoculant" and "fertilizer/time" interactions;
- For time periods #1&#4, both "fertilizer" and "time" factors were statistically significant, and there was a significant "fertilizer/time" interaction.

For brevity, only one of the ANOVA tables is presented below to illustrate the type of analysis performed (**Table 3**). This ANOVA table shows two significant factors and one interaction; ***all time periods were considered for the analysis.***

Table 3. ANOVA for the 2^3 factorial experiment; all time periods; significant effects are bolded.

Source	Sums of Squares	Df*	Mean squares (effect)	F-value	p-value
"A" (Turfgrass)	360000	1	360000	1.669	.205
"B" (BuRIZE VAM)	15625	1	15625	.072	.789
"C" (Fertilizer)	1314000	1	1314000	**60.942**	**.000**
"T" (Time)	142655700	3	47551900	**220.530**	**.000**
"AB"	250000	1	250000	1.159	.289
"AC"	122500	1	122500	.568	.456
"BC"	105625	1	105625	.489	.489
"AT"	1046250	3	348750	1.617	.204
"BT"	153126	3	51042	.236	.870
"CT"	5213124	3	1737708	**8.058**	**.000**
"ABC"	62500	1	62500	.289	.594
"ABT"	1328751	3	442917	2.054	.125
"ACT"	116250	3	38750	.179	.909
"BCT"	655626	3	218542	1.013	.399
"ABCT"	163749	3	54583	.253	.858

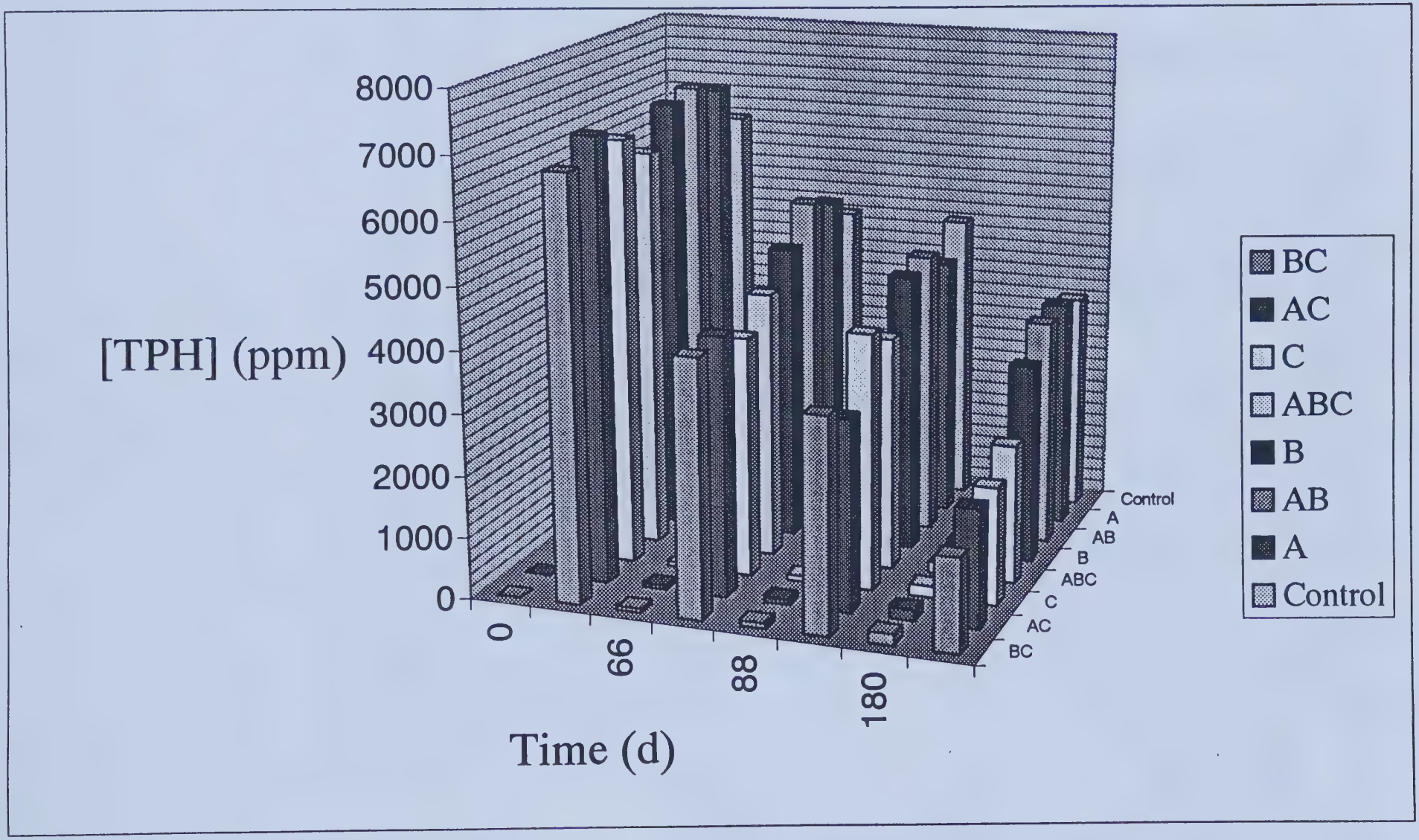
* = degrees of freedom for the effect.

Discussion

The statistical data analysis suggests the following conclusions regarding the important factors for enhancing TPH "degradation" during the course of the study:

- Two factors were consistently significant during the full study period (i.e., for the four time intervals tested). Significant effects were displayed by both the "fertilizer" and "time" factors. One would assume that the "time" factor would be significant since TPH degradation is known to occur abiotically in soils during time periods much less than the 180-days studied here. A significant effect for the "fertilizer" factor is reasonable given that bioremediation of petroleum hydrocarbons is commonly enhanced by the addition of inorganic fertilizers.
- A "grass / time" and a "grass / VAM inoculant" interaction each occurred in just one instance. The "grass / time" interaction occurred between periods #2 and #3 (66 and 88 days) and it was during this time that the rate of grass growth was quite high. The "grass / VAM inoculant" interaction might possibly be attributed to a mycorrhizal growth effect that enhanced TPH degradation during the last 92 days of the study (i.e., between periods #3 and #4).

Figure 1. **3-D column chart of TPH concentration versus time for the 8 "ranked" treatments; highest "loss" treatment appears in the foreground and lowest "loss" treatment appears in the background.**



As displayed in **Figure 1**, all treatments - including the control - showed decreasing TPH concentrations (replicate averages) during the course of the experiment, however the control showed the lowest decrease. Some of the reduction in TPH concentrations may be attributed to physico-chemical processes that were not experimentally controlled - such as volatilization, leaching, sorption, hydrolysis, and photodegradation - however these processes are assumed to be negligible in this study. Thus, decreasing TPH values may reasonably be labelled as "degradation" or more likely "biodegradation".

In conclusion, this experiment was designed to allow an assessment of several potentially important factors for enhancing the "loss" or "degradation" of TPH from contaminated soil. All of the treatments showed greater TPH "loss" – assumed to be caused by biological degradation - than the control. The detailed statistical analysis revealed a consistent, significant effect for the "time" factor, the "fertilizer" simple effect, and a "fertilizer/time" interaction. A "grass/time" interaction and a "grass/VAM inoculant" interaction significantly enhanced TPH degradation, but only for two time periods. The possible reasons for these results may be as follows:

1. For the "grass" treatment, the level of contamination used in this study (~6875 ppm) had an obvious deleterious effect on the growth of the grass plants – possibly due to the hydrophobic nature of the petroleum and its negative effect on soil moisture retention and soil compaction. The plants in the uncontaminated, fertilized, "subjective" controls displayed much more vigorous and healthy-looking growth than all other plants, including those in the contaminated soil treatment receiving all three factors (ABC).
2. For the BuRIZE™ VAM inoculation, fungal hyphae were observed in only one of the two "grass + inoculated" treatment pots, at the second sampling period (i.e., at 66 days). After this period however, hyphae were not readily observed in the pots. It is possible that VAM populations did not establish sufficient populations for aiding TPH degradation during the full course of the study.
3. The contaminated soil contained a large amount of "microbially-available carbon". It is well-documented in the bioremediation literature (e.g., Sims *et al.*, 1989) that a "carbon:nitrogen ratio" of approximately 12:1 (or thereabouts) is required for microbial growth and function via substrate metabolism. Since non-leguminous (i.e., non-Nitrogen-fixing) plants were used, it is possible that the amount of Nitrogen available to the microorganisms was the limiting factor for microbial growth, and the presence of plants and/or mycorrhizae could not improve upon this situation.

Although this experiment did not show a consistent, significant enhancement of TPH degradation resulting from turfgrass planting, it did reveal periodic enhancements resulting from the factors tested. There are several important benefits that plants may provide to contaminated site remediation that were not tested in this study. Plants have an inherent ability to aerate, bind and/or stabilize soils at contaminated sites (e.g., reducing or eliminating erosion, and regulating water infiltration). Recent research has shown that phenolic compounds released as root exudates from certain plant species (e.g., Mulberry, Osage Orange), may enhance the degradation of specific compounds such as polyaromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs)(Fletcher, 1998). If contaminant degradation and/or stabilization can be achieved *in situ* by plants, remediation costs for specific sites could be considerably reduced versus the use of more intensive bioremediation technologies such as biopiles, bioventing, or physico-chemical technologies such as soil washing or thermal destruction. Further testing of the specific benefits that may be provided by plants at contaminated sites is recommended in order to facilitate optimization of phytoremediation processes.

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