

THE EFFECT OF APATITE II™ ON THE BIODEGRADATION OF TNT AND PERCHLORATE IN CONTAMINATED SOIL SAMPLES

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ABSTRACT

Soil found on military gunnery ranges, old battle field sites, or ammunition repositories poses a hazardous threat to the environment and humans because it may be contaminated with 2,4,6-Trinitroluene (TNT) and perchlorate. The purpose of this research was to investigate the use of microorganisms to degrade TNT and perchlorate, and to examine the biodegradative enhancement with a fishbone-based nutrient source known as Apatite II. Tests consisted of column flow-through experiments in which influent and effluent samples were tested for TNT and perchlorate concentrations. The columns were amended with a combination of Apatite II, and a previously isolated TNT-degrading fungal strain which had the same ribosomal DNA sequence as *Penicillium spinulosum* (based on 561 nucleotide bases). When 10 ppm TNT was amended over a period of 16 months, HPLC analyses showed that during column flows with a 45-minute retention time, soil-only columns, soil + Apatite columns and soil + Apatite + fungal inoculum resulted in 47%, 69% and 95% of the TNT degraded. When 1 ppm perchlorate was amended to the columns, TNT degradation was unaffected, and perchlorate degradation followed the same trends of TNT degradation: that is, perchlorate degradation was stimulated synergistically by the presence of Apatite and the fungal inoculum. Experimental results clearly indicated that the Apatite II stimulated biodegradation of TNT and perchlorate, especially when combined with the TNT-degrading fungus, and that the Apatite II material maintains biodegradation for lengthy periods of time.

INTRODUCTION

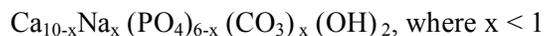
According to a recently released Environmental Protection Agency (EPA) report¹ as many as 294,000 contaminated sites must be cleaned within the next thirty years. Of these sites, 6,400 have been identified as Department of Defense (DOD) sites that contain soil contaminated with TNT, unexploded ordnance and perchlorate. Rosser et al. concluded that TNT remains a hazardous carcinogen and toxic substance long after an explosion has occurred². It is imperative, therefore, that contaminated sites be cleaned up and that scientists continue to research effective and inexpensive biological treatments that may be used in the remediation of explosives-contaminated soils.

Research has shown that the breakdown of TNT may be effectively accomplished by microorganisms such as bacteria and fungi³. Fungi, which play an important role in TNT biodegradation by virtue of their aggressive growth capability and their ability to degrade TNT, have been used successfully in the remediation process⁴. This experiment consequently examines biodegradation of TNT and perchlorate by investigating the use of a known fungal strain and a fishbone-based nutrient identified as Apatite II.

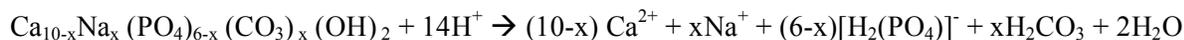
Apatite II is a natural phosphate material from fish bones that has been used to remediate lead (Pb)-contaminated range soil at the Solid Waste Management Unit (SWMU) B-20 at Camp Stanley Storage Activity, Texas, a former open burn-open detonation site⁵. The Apatite II concept was derived from research illustrating that fossil biogenic apatite retains a signature of paleochemical variations in seawater from the Cambrian period to the present. These studies showed that fossil apatite hard parts of marine organisms had incorporated trace metals post-mortally that were enhanced by six or seven orders of magnitude above the levels found in the seawater. Moreover, these trace metals were retained within the biogenic apatite over geologic time and subsequent burial and diagenesis. Apatite II works by four general non-mutually-exclusive processes, depending upon the metal, the concentration of the metal and the aqueous chemistry of the system⁶:

- Heterogeneous nucleation--supplying a small amount of PO₄ to solution to exceed the solubility limits of most metal apatites causing precipitation of new minerals
- At low pH, acts as a buffer--neutralizes acidity to pH 6.5-7 causing precipitation of many metal apatites
- Chemi-adsorption--uncompensated PO₄ and OH⁻ groups on the surface induce metal sorption, particularly transition metals
- Biological stimulation-- P and bioavailable organics can stimulate microbial community activity in many chemical systems, e.g., high SO₄ or NO₃

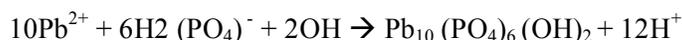
The premise for Apatite II applications in metal-contaminated soils has been that this particular product produced a phosphate-induced metal stabilization (PIMS) effect on metals such as lead (Pb), cadmium (Cd), zinc (Zn), copper (Cu), uranium (U) and plutonium (Pu). PIMS has been shown to effectively adsorb metals and transform them into a precipitate. Apatite II, which was developed from fish bones (U.S. Patent #6,217,775), has been defined chemically as⁷:



The reaction between the apatite and metals, e.g., Pb, consists of two parts: a dissolution reaction followed by a precipitation reaction:



and



The ability of Apatite II to stabilize metals by chemically binding them as stable phosphate minerals makes it ideal for remediation, especially when biodegradation of organic pollutants, such as TNT, is enhanced because the toxicity of these metals to microorganisms is reduced by Apatite II sequestration of the metals. Metals can have an adverse effect on the biodegradation process by influencing the makeup and ecology of degrading microorganisms. A study by Roberts et al.⁸ concluded that lead (total concentrations > 1000 mg/kg) delayed degradation of TNT by as many as nine days; Zinc (1,500 mg total zinc/kg) delayed degradation by eight days; and Copper (4,000 and 8,000 mg total copper/kg) completely inhibited the process. Metal toxicity, therefore, is an important and relevant consideration in the biodegradation of TNT because forty percent of hazardous waste sites are co-contaminated with metal pollutants.

Apatite II can therefore be an important component in the overall TNT and perchlorate remediation process. It not only serves as a nutrient for TNT degrading microorganisms, but may also improve biodegradation by stabilizing metal concentrations at contaminated sites. By adding the Apatite II nutrient

in TNT contaminated soil, biodegradation of the TNT organic compound should significantly increase due to aggressive fungal growth.

MATERIALS AND METHODS

Biodegradation Column Set-up

Four glass columns (approximately 90cc volume) were loaded with an air-dried agricultural soil (a Brazito fine sandy loam), two with 50 cc of soil, and two others with 25 cc of soil plus 25 cc of Apatite II (crushed fish bones). The columns were supplied with ca. 10 ppm TNT in sterile water at a rate of 0.75 mL/ min (50 minute retention time). After 5 months of operation, two of the columns were inoculated with a TNT biodegrading fungus in July, 2004, and on June 10, 2005, all four columns were spiked with 1 ppm perchlorate in addition to the 10 ppm TNT.

Once a week, the column liquid was replaced with fresh 10 ppm TNT for 2 hours. After the first ten minutes a 1.5 ml sample of the four columns' effluent was taken. A second 1.5 ml sample was retrieved after the remaining hour and fifty minutes had passed. Influent and effluent samples were analyzed by HPLC for TNT, and ion chromatography for perchlorate.

TNT and Perchlorate Analyses

A Beckman System Gold HPLC was used for the analysis of TNT. A diode-array detector was used and the wavelength was 230nm with a 6 nm bandwidth. An isocratic solvent mixture of 70% MeOH and 30% H₂O was used to transport the TNT through the column at 1 ml/minute. The HPLC column used was a Supelco Discovery C18 with dimensions of 25cm x 4.6mm x 5µm. 20 µl of sample were injected giving an average TNT retention time of 4.84 minutes and a detection limit of 0.1 ppm. Aqueous samples were extracted with methylene chloride, which was dried, and the sample resuspended in methanol and injected into the HPLC. The extraction efficiency was approximately 80%. A typical TNT chromatogram is shown in Figure 1.

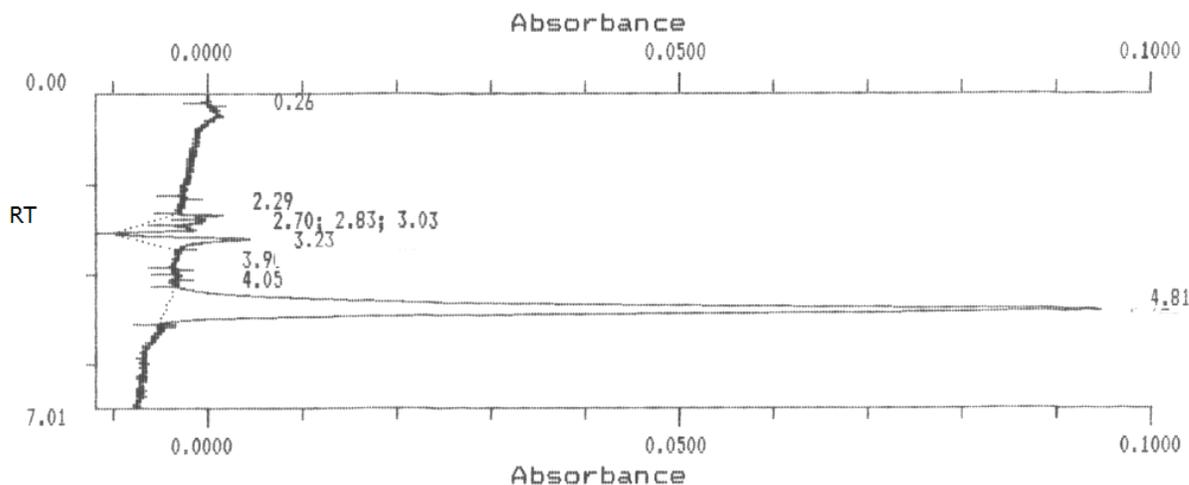


Figure 1 Chromatogram of 20ul injection of 10ppm TNT standard

Perchlorate detection was performed using a Dionex 2020i ion chromatograph (IC) and the data acquisition software was Dionex AI-450. A Dionex IonPac AS-16 analytical column was used with a Dionex ASRS ULTRA conductivity suppressor. The regenerant flowing through the suppressor was 30mM sulfuric acid and the eluent used was 40mM sodium hydroxide. Eluent flow was set to

1ml/minute. Argon gas was bubbled through the sodium hydroxide to prevent carbonate formation in the eluent from the sodium hydroxide reaction with air. The injection volume used was 1 ml of aqueous sample. An example of an IC chromatogram of perchlorate and its degradation products (chlorate, chlorite, hypochlorite, and chloride) are shown in Figure 2.

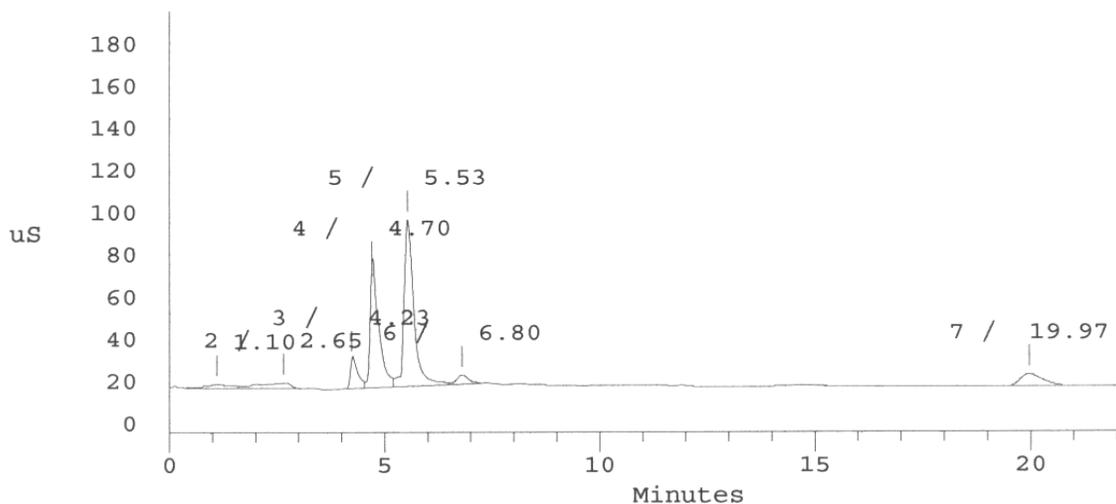


Figure 2 Ion Chromatograph of perchlorate (RT=19.97) and the degradation peaks, chlorite (RT=4.23) and chlorate (RT=5.53)

Organic Analysis of Apatite II

The organics were extracted from apatite using a soxlet extraction method and samples were extracted for 24 hours. Three different solvents were used: Acetonitrile was used to extract both the nonpolar and polar organics; methanol extraction followed to extract the polar organics; and hexane was used last to extract any nonpolar remaining organics.

Both HPLC/MS and GC/MS were used to analyze the extracted samples. A Benchmark MS in connection with a Rainin HPLC was used for initial analyses while Sun system software was used for data acquisition. The mass spectrometer was electron impact so fragmentation patterns were obtained, and these fragmentation patterns were compared to the NIST library. A Saturn GC/MS was also used for analysis of the extracted samples. Split/Splitless injection was used to concentrate the sample onto the capillary column. Ionization of the compounds occurred through electron impact in an ion-trap.

TNT-degrading fungus and PCR Amplification and Sequencing of fungal rDNA.

The TNT-degrading fungus was isolated previously⁹ from Sphagnum peat moss, and extracted DNA was amplified by the Polymerase Chain Reaction (PCR). Specifically, ribosomal RNA sequences for the 18-23S genes, separated by intergenic spacer (ITS) regions, were amplified. Subsequent profiles matched pattern rDNA fragments of *Penicillium spinulosum*⁹.

RESULTS

A. TNT Degradation

The data reported here was after the columns had been degrading TNT for the previous 6 months, and the fungus had been inoculated into 2 of the columns 1 month before the current data was taken. The

columns were being supplied once a week with 10 ppm TNT (for 2 hours, i.e., 2.4 pore volumes). The data shown in Table 1 represent effluent samples taken after 10 minutes of flow, and so represent the levels of TNT after 7 days of incubation. As noted in Table 1, results showed that Column 6, which contained soil, Apatite II and fungi, had no TNT traces demonstrating that all the TNT was eliminated from the sample. On the average, Column 8 (soil sample and fungi, but without the Apatite nutrient) did not degrade 3.03 ppm of TNT. Results were similar for Column 7, which consisted solely of soil sample. The TNT biodegradation percent amounts for Columns 7 and 8 have only 62 and 67 percent biodegradation, respectively. This means that without the Apatite nutrient, biodegradation of TNT does not immediately occur even if the fungi are present.

Results thus far indicate that biodegradation of TNT increases when Apatite II is added to samples of soil and samples of soil plus fungi. For example, Column 5 which contained soil and Apatite II had degraded 86 percent of the influent TNT, while Column 6 which contained soil, Apatite II and fungi had degraded 100 percent of the TNT.

TABLE 1: Initial 10-minute sampling--TNT remaining in columns after 7-day incubation.

7 Day test		TNT ppm				
			Column 5	Column 6	Column 7	Column 8
Date	Sample Set	Influent (ppm)	25 cc of soil & 25 cc of Apatite II	25 cc of soil & 25 cc of Apatite II & fungi	50 cc of soil	50 cc of soil & fungi
8/26/04	1	6.8	0.3	0.0	5.7	3.3
9/13/04	2	10.2	0.8	0.0	3.3	2.9
9/23/04	3	11.4	1.4	0.0	2.2	1.9
9/30/04	4	10.6	1.5	0.0	2.4	3.1
10/7/04	5	9.2	1.6	0.0	2.9	4.1
10/14/04	6	8.6	2.6	0.0	3.4	5.2
10/28/04	7	10.3	1.5	0.0	4.6	3.9
11/8/04	8	8.1	0.0	0.0	3.5	1.8
11/30/04	9	9.5	0.0	0.0	3.0	2.3
12/14/04	10	10.31	0.0	0.0	3.62	2.43
1/27/05	11	7.9	0.0	0.0	2.96	2.37
	AVERAGE	9.3	.88	0.0	3.42	3.03

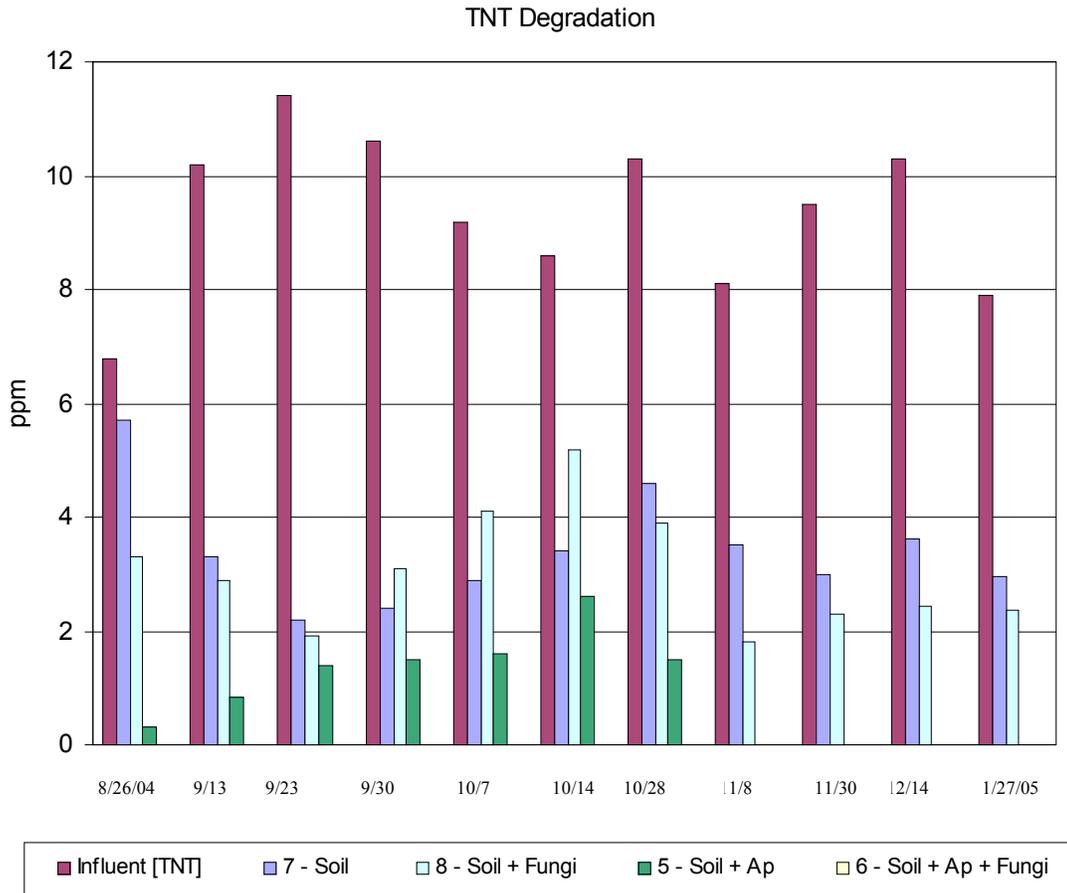


Figure 3. Results of 10 minute sampling.

Following the 10-minute sample, a 2-hour sample was taken which represents the amount of TNT degraded within the 50 minute column retention time. Samples were then taken and results tabulated in Table 2, and Figure 4. These results were a good indication of how the Apatite II stimulated degradation of TNT. For example, biodegradation of TNT was highest in Column 6, consisting of soil, Apatite II and fungi, where 90 percent of the influent TNT was degraded. Results also showed that when fungus was added to a simple soil sample, approximately 18 percent more TNT biodegradation occurs. This is evident in Column 7 (soil sample) which showed 42 percent degradation while Column 8 (soil sample plus fungi) degraded 60 percent of the TNT.

TABLE 2. Results of 2-Hour sampling, representing degradation after 50-minute retention time.

2 hr. Test		Ppm				
			Column 5	Column 6	Column 7	Column 8
Date	Sample Set	Influent(ppm)	25 cc of soil & 25 cc of Apatite II	25 cc of soil & 25 cc of Apatite II & fungi	50 cc of soil	50 cc of soil & fungi
8/26/04	1	6.8	4.9	1.6	6.2	4.7
9/13/04	2	10.2	1.5	0.0	5.1	2.0
9/23/04	3	11.4	2.0	1.3	5.3	3.2
9/30/04	4	10.6	2.4	0.8	5.4	4.2
10/7/04	5	9.2	1.8	0.6	4.6	2.7
10/14/04	6	8.6	3.6	0.9	5.3	4.8
10/28/04	7	10.3	3.8	1.4	6.6	6.2
11/8/04	8	8.1	1.5	1.0	4.6	4.4
11/30/04	9	9.5	2.94	0.49	5.06	5.15
12/14/04	10	10.31	1.55	0.0	5.42	4.98
1/27/05	11	7.9	2.07	0.0	4.3	4.0
	AVERAGE	9.3	2.55	0.73	5.26	4.21

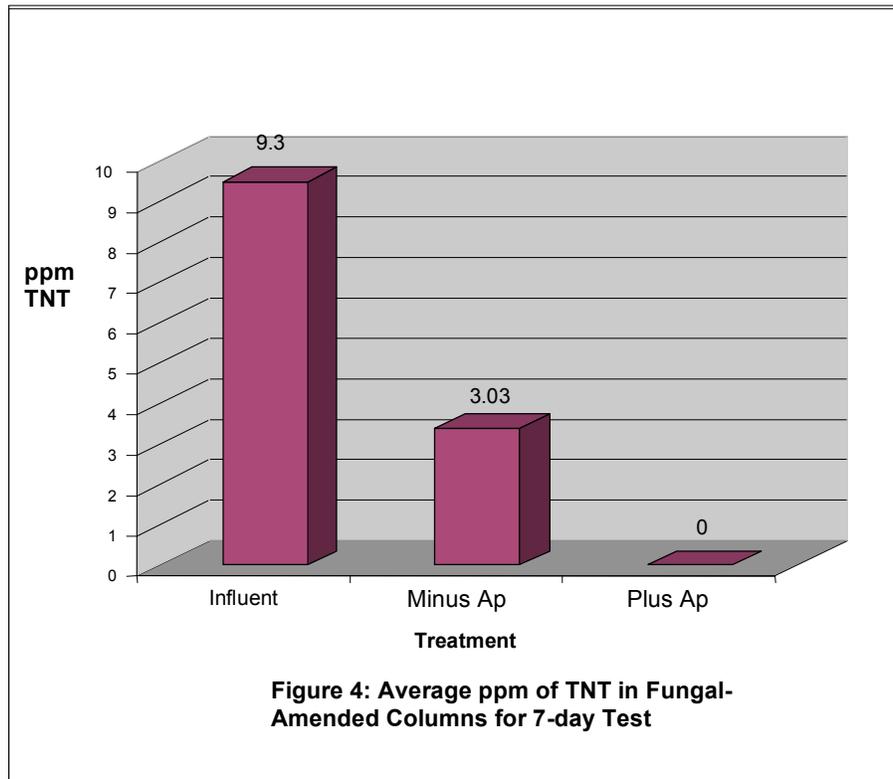


Figure 4 shows the average TNT (ppm) samples with and without Apatite II in fungal-amended columns sampled after 10 minutes (indicating results after 7 days of incubation).

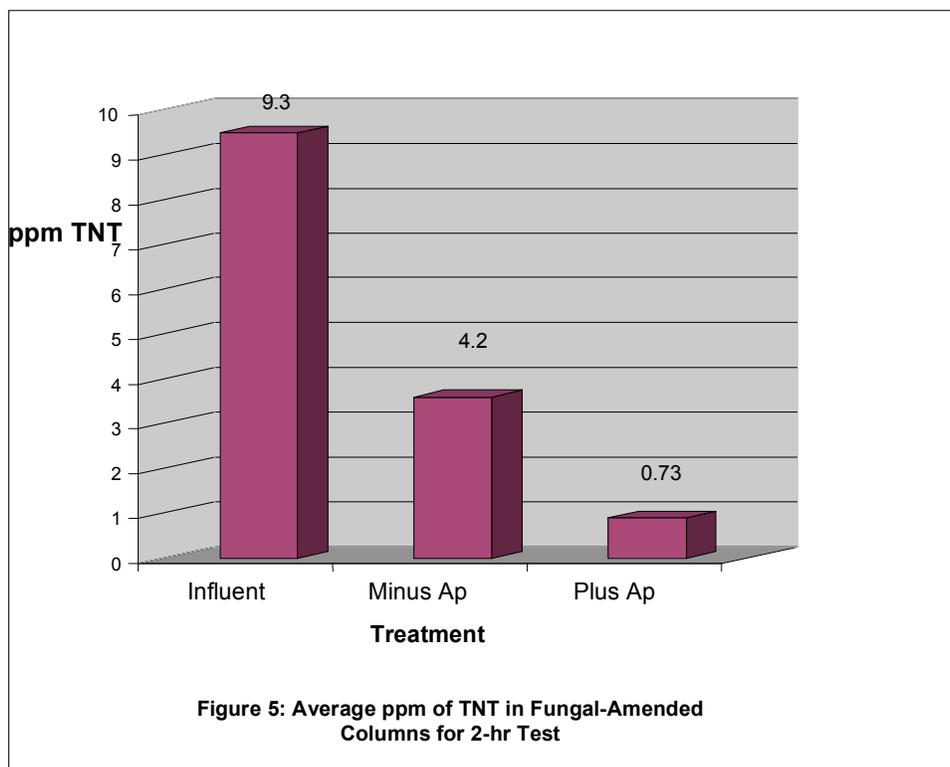


Figure 5 shows samples taken after a 2-hr period (indicating levels after the 50-minute retention time).

Perchlorate Degradation.

When perchlorate was added to the columns in June of 2005, TNT degradation continued unabated in apatite-amended columns. Interestingly, perchlorate degradation followed the same trends already observed for TNT. That is, the apatite-amended columns degraded more perchlorate than the soil-only columns, and the presence of Apatite II stimulated further degradation in the fungus-amended column (Figures 6 and 7). The addition of perchlorate did not influence the degradation of TNT in the columns; indeed both contaminants were degraded simultaneously (Table 3).

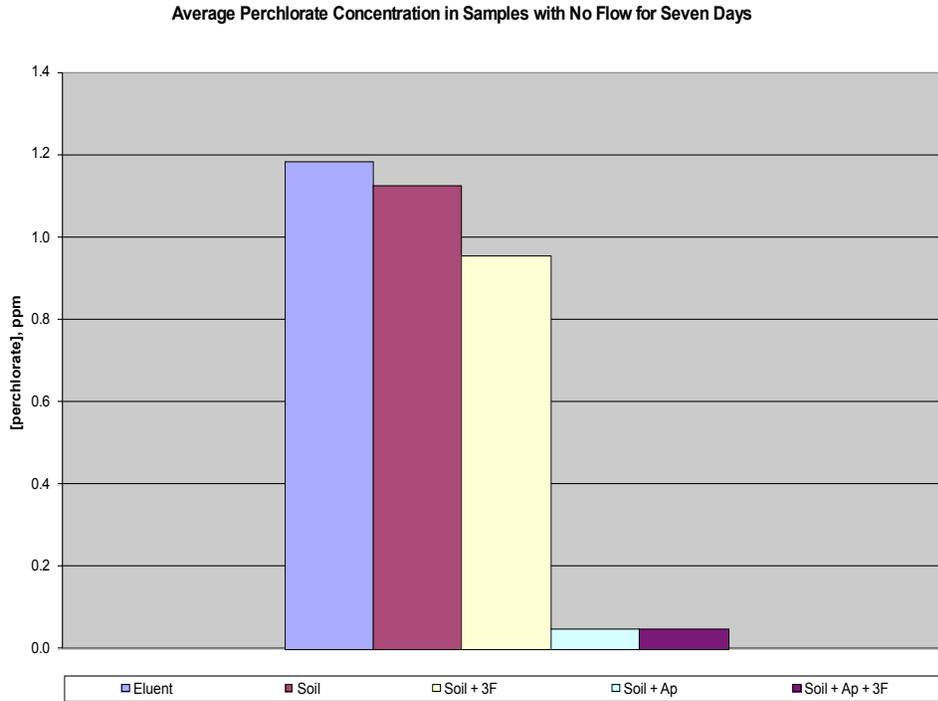


Figure 6. 10-minute samples. Perchlorate concentrations before (influent) and after treatment in the columns. Samples represent levels of perchlorate after 7 days of exposure to column materials.

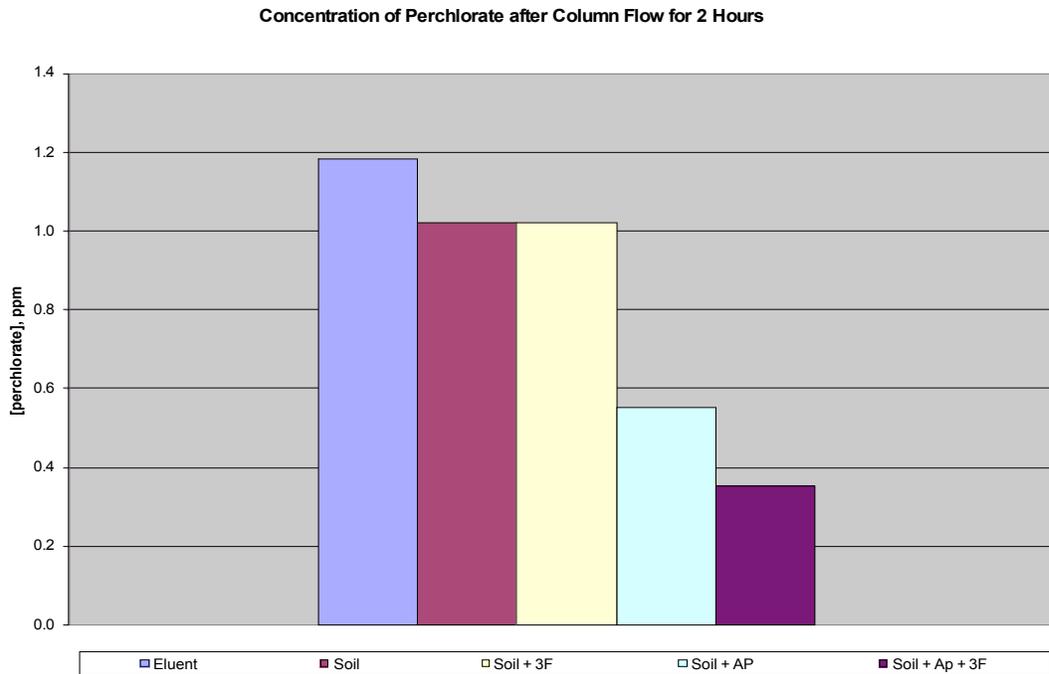


Figure 7. 2-hour samples. Perchlorate concentrations in the influent and effluents of columns during 50 minutes of column flow.

Table 3. Levels of TNT and Perchlorate in columns after 10 minute sampling—indicating levels remaining in columns after 7-day incubation.

		Column 5	Column 6	Column 7	Column 8
Date	Influent (ppm)	25 cc of soil & 25 cc of Apatite II	25 cc of soil & 25 cc of Apatite II & fungi	50 cc of soil	50 cc of soil & fungi
7/26/05	11 ppm TNT	0	0	4	2.2
7/26/05	1.1 ppm Perchlorate	0	0	1.1	0.9
8/2/05	11.1 ppm TNT	0	0	3.9	2
8/2/05	0.9 ppm Perchlorate	0	0	1.2	1.1

Chemical Analysis of Apatite II.

Like the HPLC/MS, the fragmentation patterns of the compounds were compared to the NIST library. The HPLC chromatogram and a mass spectrum of cholesterol are shown in Figures 8 and 9. Fig. 5 is an example of a GC chromatogram. Similarly, after the hexane extract, the final peak to elute (with an RT=1650 seconds) was a compound that closely matched cholesterol. The earlier eluting peaks were matched by MS analysis to long chain fatty acids, though their exact structure remains to be identified.

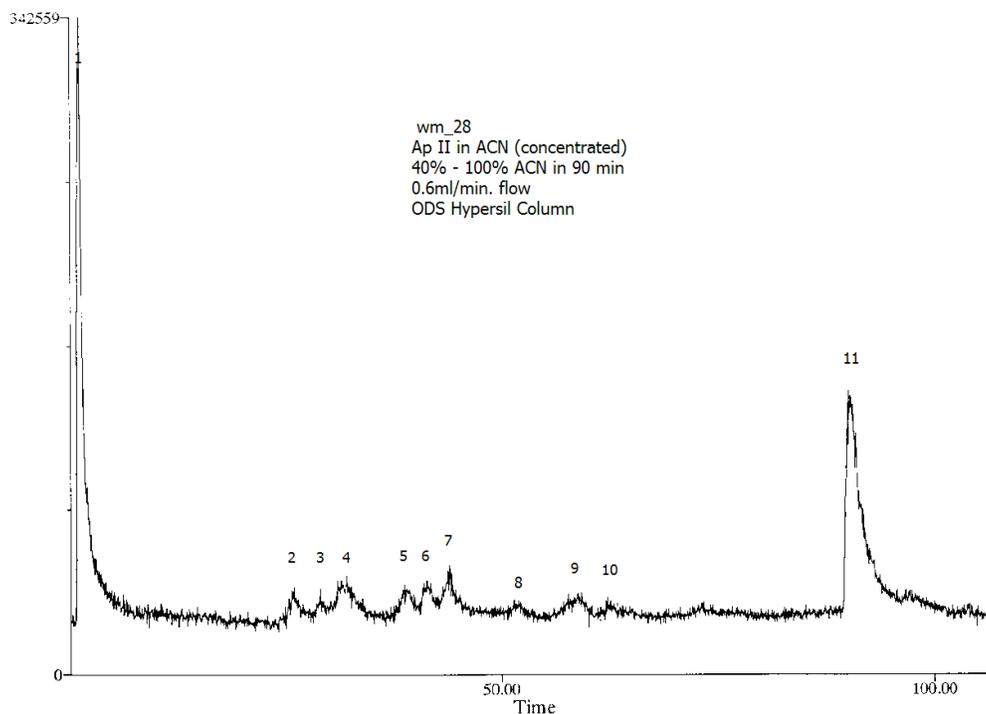


Figure 8 HPLC chromatogram of Apatite II after extraction by acetonitrile. MS analysis of Peak 11 indicated a close match with cholesterol, shown below in Figure 9.

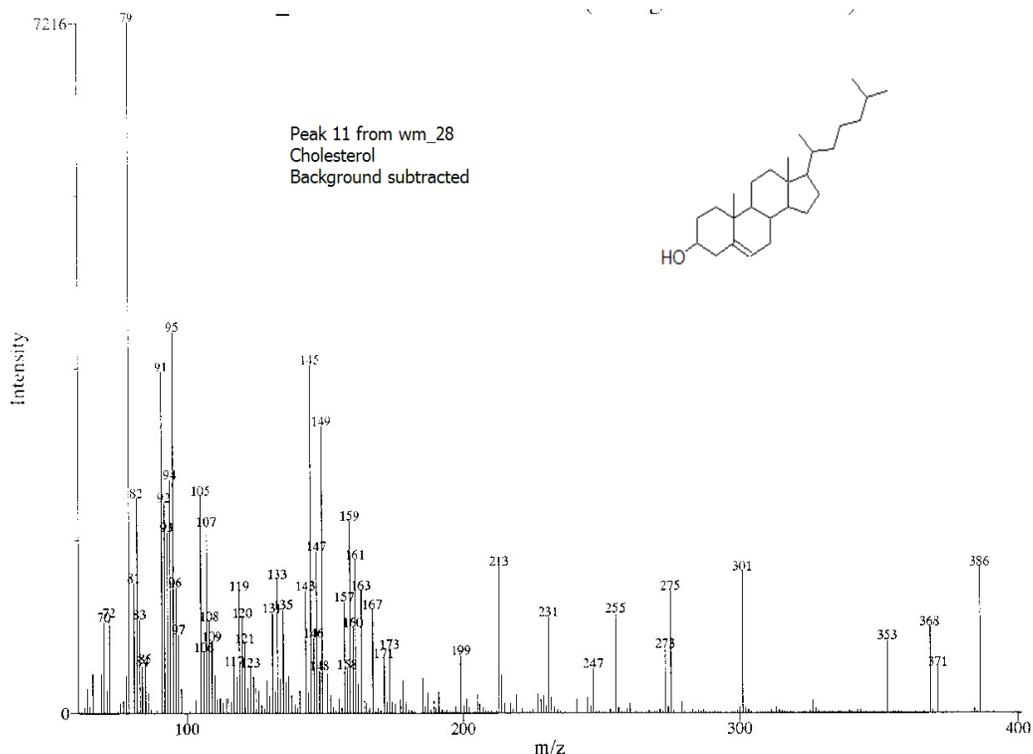


Figure 9 Mass spectrum of cholesterol

DISCUSSION

The reasons for this experiment are as follows: first, microbes such as fungi have been successfully used in the biodegradation of TNT, and second, Apatite II, a commercially produced reactive phosphate, has been effectively used as a microbe nutrient and to remediate zinc (Zn), lead (Pb), and cadmium (Cd) from metal-contaminated soil and acid mine drainage^{6,7}. Removal of metal toxics to microbes led to the assumption that this technique could be successfully applied to remediate soils contaminated with other compounds.

Aside from its capability for metal adsorption, in particular the transition metals, Apatite II may serve as a microbial nutrient. For this reason, Apatite II was introduced into this experiment in the hope that it would stimulate microbial growth to aid in bioremediation efforts. It is also noteworthy that Apatite II does not pose any adverse environmental impacts and can be easily mixed with contaminated soil. Application of Apatite II to the environment, therefore, was not an issue to be contended with.

As previously mentioned, fungus has provided a mechanism through which the biodegradation process can occur. In particular, research has shown that the white rot fungus, i.e., *Phanerochaete chrysosporium*,¹⁰ has been reported to degrade a wide variety of contaminants because of its wood-rotting enzymes. It has been reported that white rot fungus degradation of TNT has been successful in laboratory settings. It has also been determined that it works best in nitrogen-limited environments and that high TNT or PCP concentrations in soil can inhibit growth of white rot fungus. One study suggested that one particular species of white rot fungus was incapable of growing in soils contaminated with 20 ppm or more of TNT. On the basis of these studies, the application of microorganisms in the TNT degradation process was investigated. Research was performed so that a resilient fungal strain could be identified—one that did not have the problems of the white rot fungus—and that would lend itself to Apatite II applications.

Also of interest, was the finding of cholesterol, or a cholesterol-like lipid, as a dominant lipid present in Apatite II. This was somewhat surprising considering that Pollack, the fish that is used to generate the fishbone material, is a non-fatty fish. However, Pollack does have 80 mg of cholesterol per 3 oz. serving, a level higher than many oceanic fish (e.g., per 3 oz., Halibut has 30 mg, Mackerel has 60 mg and Orange Roughy has 20 mg)¹¹.

CONCLUSIONS

Overall, results indicate that the Apatite II nutrient can be used to promote the biodegradation of TNT and perchlorate. It stimulated native microflora to degrade the contaminants, and evidently stimulated the activity of a TNT-biodegrading fungus. Importantly, simultaneous TNT and perchlorate biodegradation was stimulated by the presence of Apatite II, since in its absence perchlorate was not degraded at all or TNT was only partially degraded. Additionally, the effect of apatite amendment was long-lived since the TNT has been continuously degraded over the past 18 months.

In conclusion, Apatite II shows promise as a natural means of enhancing TNT and perchlorate bioremediation processes. Future analysis may include the evaluation of samples containing a combination of TNT and metals such as lead and copper, and the effect that Apatite II has on the bioremediation process.

ACKNOWLEDGEMENTS

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