The Biodegradation of Trinitrotoluene by Individual and Mixed Alcaligenes Strains

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ABSTRACT

A three-phase experiment assessed the trinitrotoluene (TNT) degradation by *Alcaligenes* strains isolated from a TNT contaminated soil sample. The purpose of the experiment was to show that *Alcaligenes* bacteria can degrade TNT and that a mixed population of *Alcaligenes* strains would degrade TNT to a greater extent than individual strains. The individual strains performed considerable reduction of soluble TNT, including strain I15 which decreased the TNT concentration from 70 mg/L to 1.4 mg/L. To identify a strain that would enhance TNT degradation when combined with I15, each remaining strain was fed the supernatant from a 48-hour degradation experiment using I15. I19 was chosen from this sequential feeding based on its performance on TNT and formation of metabolites. The mixed population of strains I15 and I19 was expected to perform better than the individual strains because of cometabolism. The mixtures, however, did not show increased TNT reduction.

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INTRODUCTION

The compound 2,4,6-trinitrotoluene (TNT) is the most widely used conventional explosive. At many military sites, TNT wastes from munitions production have led to soil and water contamination (4). This contamination is of concern because of the known harmful effects of TNT. Human exposure to TNT may produce aplastic anemia and toxic hepatitis. It is estimated that ingestion of 1 to 2 grams of TNT is lethal to humans (5). In an aquatic state, TNT concentrations of 1-2 mg/L is lethal to fish and invertebrates (3). In addition, TNT has tested positive in the Ames screening for mutagenicity (14). The chemical structure of TNT is shown in Figure 1.

Nitroaromatic compounds such as TNT have a high degree of chemical stability if protected from radiation and sunlight (13). Biodegradation has evolved as one method to break down these nitroaromatic wastes. An important consideration in biodegradation evaluation is that the degradation of TNT can only be considered complete if removal of all nitro groups occurs (7). Figure 2 demonstrates the reductive pathway of TNT to the diamino- and azoxytoluene compounds. The azoxytoluene compounds are not desirable degradation products because they exhibit toxic properties and accumulate without further break down.

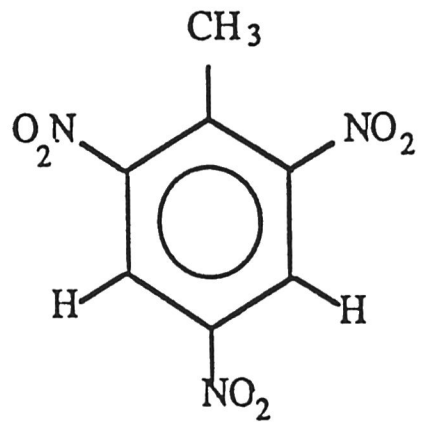

Figure 1. Chemical Structure of 2,4,6-trinitrotoluene

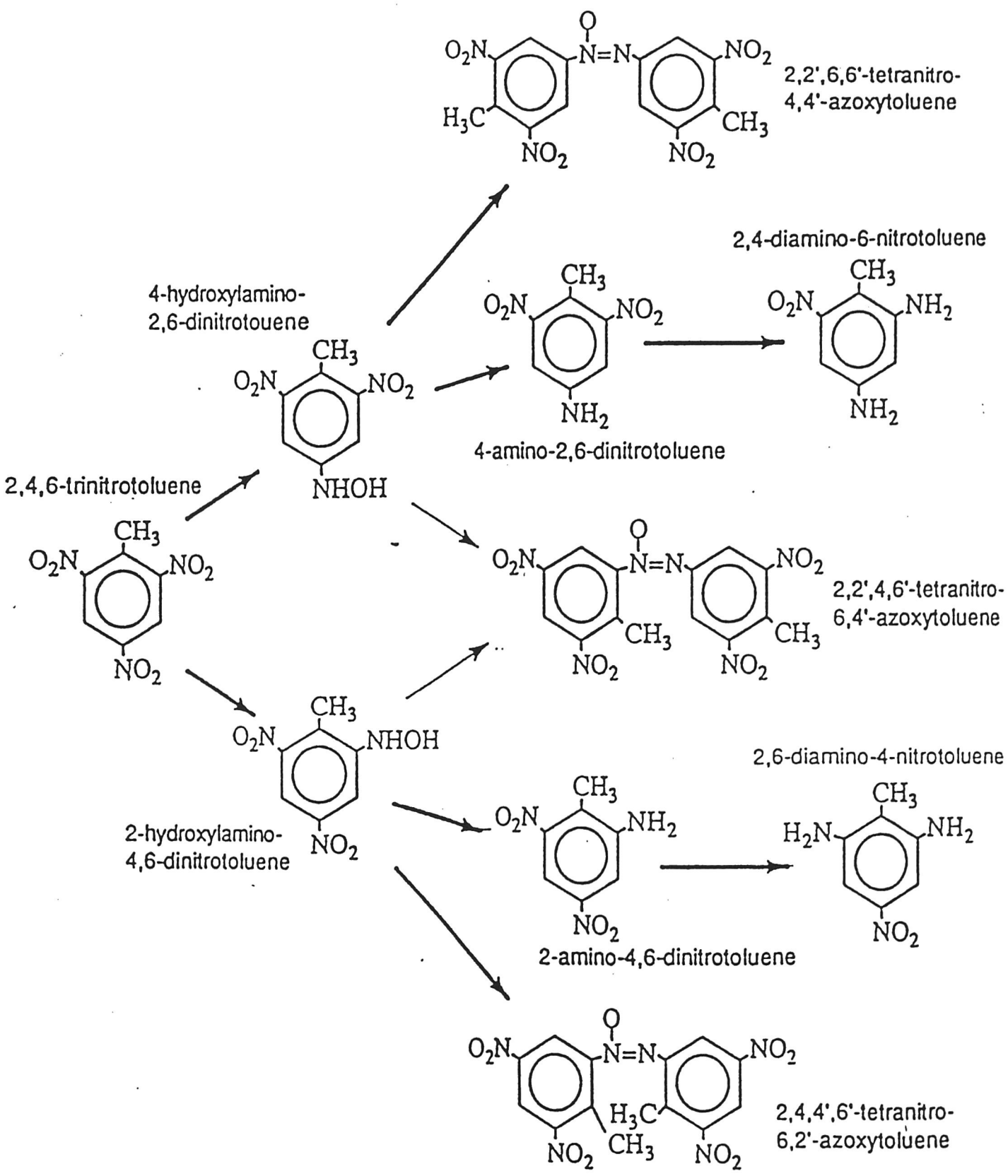

Figure 2. TNT Reductive Pathway in Aerobic Environments

(from Kaplan, 1993)

Biodegradation can take place in aerobic or anaerobic environments. Aerobic microorganisms use molecular oxygen as the final electron acceptor from a complex electron donor, such as TNT. Anaerobes use some other molecule as the acceptor. The environmental condition has been shown to influence which metabolites are formed from TNT, with diamino derivatives predominating under aerobic conditions (8). The experiments described herein used an aerobic environment.

The goal of bioremediation of TNT is to achieve mineralization, the complete breakdown of the ring structure into its basic component elements. To achieve mineralization of TNT, mixed microbial populations may be required. One of the reasons for this is cometabolism, the sharing of biotransformation duties. In this situation, one organism possesses the capability to transform the original substrate, but cannot metabolize the compound further. The initial biotransformation products are transported out of the first microorganism. These degraded forms can then be utilized by other organisms which do not posses the genetic coding necessary for the original transformation (8). In this way, the entire compound may be broken down, even if there is not any single organism in the consortia that can accomplish the goal. At the present, bacterial mediated aerobic mineralization of TNT has not been reported in the literature.

The purpose of this investigation was two-fold: (1) to determine whether *Alcaligenes* bacterium could biodegrade TNT and (2) to show that a selected mixed population of bacteria could degrade TNT to a further degree than any of the single organisms. Commonly used species for nitroaromatic biodegradation include *Arthrobacter*, *Pseudomonas*, and *Bacillus* because of their abundance (8). This biodegradation experiment, however, focused on six *Alcaligenes* strains that were isolated from soil contaminated with TNT.

Alcaligenes are cocci to rod shaped chemoorganotrophic (use complex organic molecules as electron donors) gram-negative bacteria 0.5-1.2 by 0.5-2.6 μm in size.

They are motile with 1 to 8 peritrichous flagella (11). There are four *Alcaligenes* species listed in *Bergey's Manual* (11). *Alcaligenes* have not previously been used in TNT biodegradation, however, they have been utilized to convert acetophenone and halogenated analogs to esters (6), degrade 4-chlorophenol (1) and mixtures of monochlorophenols and phenol (9), and use 1,4-dichlorobenzene as a sole carbon source (6,12). It is reported that gram-negative bacteria, such as *Alcaligenes*, play a key role in biotransformation of TNT in natural environments (13).

MATERIALS AND METHODS

Organism Isolation. The organisms were isolated from a fed-batch reactor receiving TNT wastes that had been seeded with a soil sample from a contaminated site in Montana. The six organisms tested in this experiment were tentatively identified as *Alcaligenes* species by fatty-acid analysis. They were named strains I6, I13, I15, I18, I19 and I22 for internal laboratory investigation.

Enrichment Culture Conditions. The bacteria were grown in 250 mL flasks on a shaker table at room temperature (23^{o}C) in 100 mL of Oxoid Nutrient Broth #2 during the enrichment phase. No TNT was present in the broth.

Preparation of Washed Suspension of Bacteria. After seven days of growth in the broth, the solution was transferred to centrifuge tubes and centrifuged. The supernatant was extracted. The remaining biomass was washed with phosphate buffer and shaken for 30 minutes to resuspend the organisms. The solution was then recentrifuged and the supernatant was again extracted. The organisms were then resuspended in phosphate buffer. This suspension was added directly to the TNT reactors.

Experiment Growth Conditions. The first individual specie cultures were grown in 500 mL flasks with 250 mL of solution that were continuously stirred. The mixed cultures were grown on a shaker table in 250 mL flasks with 100 mL of solution. All flasks were covered with aluminum foil to prevent exposure of the TNT to light. TNT was provided as the main nitrogen source at 70 ppm, with ammonium nitrate as an alternate nitrogen source at 8.5 ppm. The alternate nitrogen source was provided in order to encourage microbial growth, which does not occur when only TNT is provided. Glucose was provided as the carbon source (15 g/L) and other necessary growth nutrients were provided as listed in Table 1. These quantities were determined by Emily McCreary at Texas A&M University.

Table 1. Growth Nutrients

Chemical Nutrient	Solution Concentration (mg/L)
$NaHCO_3$	226
$FeCl_36H_2O$	0.316
$MgSO_47H_2O$	36.97
$CaCl_2$	27.75
Na_2EDTA	4.26
H_3BO_3	1.0
Trace Metals:	
$CuSO_4$	0.0064
$ZnSO_47H_2O$	0.023
$CoCl_26H_2O$	0.0119
$Na_2MnO_42H_2O$	0.0066
Tri-Vitamin Solution:	
Biotin	0.005
B-12	0.0005
Thiamine	0.10
Adjust pH to 7.0 using Phosphate Buffer Solution	

Supernatant Collection. Supernatant from the degradation process was used in the second experimental phase. An initial biodegradation experiment using I15 was run with two 1000 mL reactors with 70 mg/L TNT for 48 hours. The reactor liquids were then centrifuged for 20 minutes. The supernatant was transferred to a main container. The supernatant (100 mL) was added to individual reactors to test the five remaining strains.

Chemicals. TNT with a purity of 99.9 percent was purchased from ChemService. The stock concentration was determined by HPLC measurement to be 16,800 ppm.

Analytical Methods. To determine TNT and its metabolites, high-performance liquid chromatography (HPLC) analyses were performed on a Supeleosid C-8 Reversible Phase column with methanol and water as the mobile phase at a flow rate of 1.4 mL/min. All compounds were detected at 230 nm with a Dionex detector by BumHan Bae of the Texas A&M University Civil Engineering Department. The compounds measured were TNT, 2-amino-4,6-dinitrotoluene (2amDNT), and 4-amino-2,6-dinitrotoluene (4amDNT).

In addition, optical density readings were taken to approximate growth during the TNT degradation. The optical densities were obtained on a Milton Roy Spectronic 1201 spectrophotometer using a sample diluted 1:4 in phosphate buffer solution. Phosphate buffer solution was used as the zero reference reading.

RESULTS

Individual Strain Evaluation. The individual strains were evaluated first in two sequential experiments. Each strain was tested individually to determine the amount of TNT degraded during a 48 hour time period. The initial concentration of organisms was approximated by using optical density readings. The amount of bacterial suspension added to each reactor resulted in an optical density at 550 nm of approximately 0.4. The TNT concentrations were measured at 0, 12, 24, and 48 hour intervals. All of these results are summarized in Table 2. The first experiment tested two individual *Alcaligenes* strains, I13 and I15. The TNT concentration results are depicted in Figure 3. The second experiment involved I6, I18, I19, and I22 (see Figure 4). All of the *Alcaligenes* strains reduced the TNT concentration by degrading it to other compounds. The degradation rates of all strains was highest during the first 12 hours: I16, 2.1 ppm/hour; I13, 0.33 ppm/hour; I15, 3.85 ppm/hour; I18, 1.63 ppm/hour; I19, 0.83 ppm/hour; and I22, 3.38 ppm/hour. The rate of TNT reduction per hour decreases at each measured time interval. The degradation rate did not show a first-order trend, but resembled a natural logarithmic curve. At the 48 hour mark, all of the strains except I13 were still decreasing the TNT concentration. This indicates that a longer experiment time would have yielded even lower final TNT values.

Of the six strains, both I15 and I22 performed exceptionally well over the 48 hours, with a final TNT concentration of 1.4 and 5.1 ppm respectively. Because of strain I15's performance, it was identified as the best TNT performer.

Table 2. Individual Strain Experiment TNT Concentrations

Strain	TNT Concentration (mg/L)			
	0 Hour	12 Hour	24 Hour	48 Hour
I6	65.2	40.4	35.9	25.5
I13	67.7	63.8	49.1	48.3
I15	72.7	26.5	5.5	1.4
I18	65.5	45.9	42.2	30.2
I19	61.5	51.6	43.4	38.4
I22	68.0	27.5	19.3	5.1

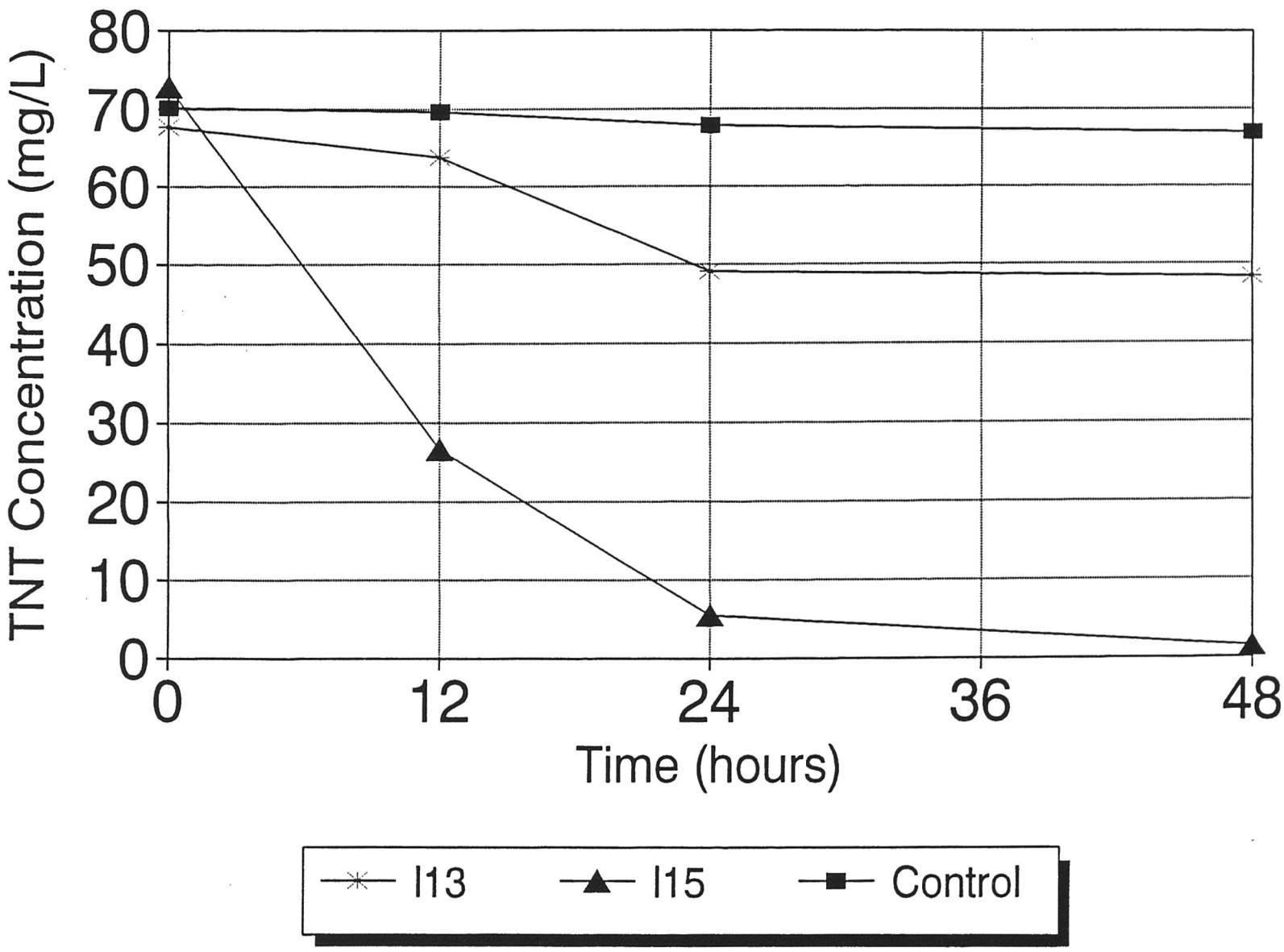

Figure 3. TNT Concentrations for Individual Strains I13 and I15

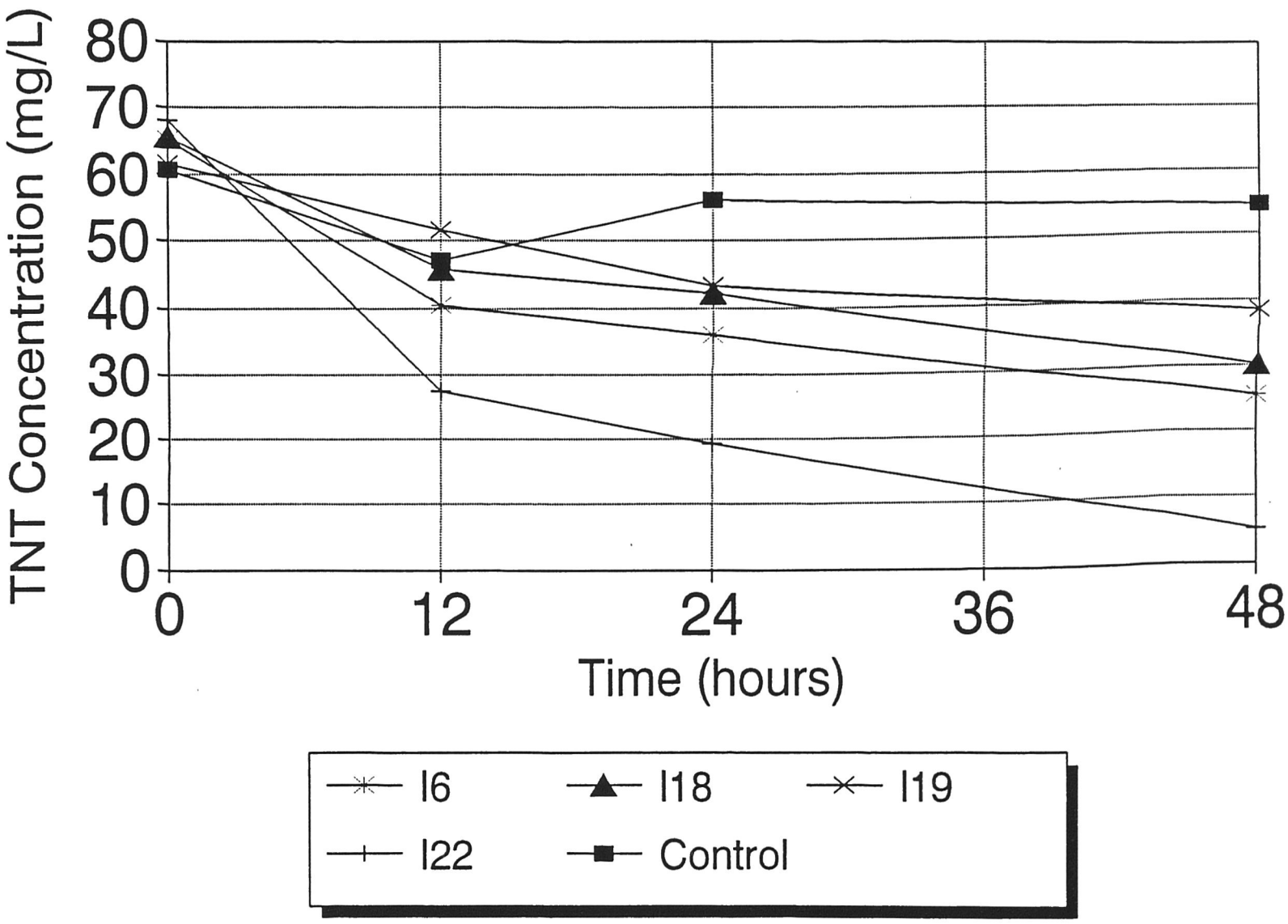

Figure 4. TNT Concentrations for Individual Strains I6, I18, I19 and I22

Additive Strain Evaluation. The next task was to identify an organism that would perform well in conjunction with I15, identified as the best performer in the initial experiment phase. I15 began with 70 ppm TNT and was allowed to degrade the TNT for 48 hours. The suspension was then centrifuged and the supernatant was used as a stock solution for each of the remaining five strains. The centrifuged solution contained approximately 50 ppm TNT, 1 ppm 2amDNT and 1.3 ppm 4amDNT. Each of five strains was grown in 100 mL of this solution for 48 hours. The 48-hour biodegradation products and corresponding concentrations for each strain are listed in Table 3. A mass balance was not performed to account for all of the chemical mass. I19 degraded more TNT and produced more 4amDNT than the other species. A high concentration of 4amDNT or 2amDNT indicates that the organism is converting the initial TNT and intermediate metabolites into simpler compounds. Because of this metabolite production, I19 was identified as the best additive microorganism.

Table 3. Additive Strain TNT and Metabolite Concentrations at 48 Hours

Strain	Chemical (mL/L)					
	TNT	% Change	4amDNT	% Change	2amDNT	% Change
I6	37.1	-25.8%	2.0	+53.8%	1.6	+60.0%
I13	42.6	-14.8%	1.9	+46.2%	0.7	-30.0%
I18	35.8	-28.4%	2.8	+115.4%	0.8	-20.0%
I19	9.8	-80.4%	6.8	+423.1%	1.4	+40.0%
I22	35.9	-28.2%	2.8	+115.4%	0.9	-10.0%

Mixed Population Evaluation. Strains I15 and I19 were mixed in three proportions of approximately 1:1, 4:1, and 1:4. The three different values were used to examine the microbial population interactions at the different concentrations of each strain. The proportions were determined from optical density readings of the washed suspension. Because optical density can be dramatically affected by bacterium size and shape, these proportions are only approximate. In order to better approximate the proportions, a correlation curve should be developed for optical density versus total suspended solids for each strain. Anand Sivaraju in the Texas A&M Department of Civil Engineering is currently developing these curves and the results are expected in the summer of 1994. At that time, a more accurate estimate of the microorganism proportions could be developed.

Optical density and HPLC readings were taken at 0, 12, 24, and 48 hours. The optical density values show little growth for any of the mixtures (Figure 5). The small density change may be because the initial concentration of organisms was not adequate.

The solution used with the mixed populations initially contained 63 mg/L TNT with no metabolites present. The HPLC concentrations of TNT and measured metabolites at the 48 hour time are listed in Table 4. The amounts reveal an unexpected result. All of the reactors with a large proportion of I15 (I15, Mix 1:1, and Mix 4:1) produced the same percentage decrease in TNT and created the same amount of metabolites. The TNT in these three mixtures was decreased 20-22 percent; in addition, approximately 3.6 percent of the initial TNT was converted to 4amDNT and 0.6 to 1.0 percent changed to 2amDNT. The I15, Mix 1:1, and Mix 4:1 mixes contrast starkly to I19 and Mix 1:4 which showed 0 to 9 percent TNT reduction, respectively. These two mixtures also produced less 4amDNT and 2amDNT than the mixtures that contained a higher concentration of I15. The small TNT reduction in I19 and Mix 1:4 compared to the other mixes indicates that

strain I15 was performing the majority of the degradation when it was present in combination with I19.

Table 4. Mixed Population TNT and Metabolite Concentrations at 48 Hours

Mixture	Chemical (mL/L)		
	TNT	4amDNT	2amDNT
I15	49.5	2.4	0.4
I19	60.0	1.1	0.1
Mix 1:1	47.5	2.2	0.6
Mix 4:1	51.6	2.3	0.4
Mix 1:4	55.4	1.9	0.4

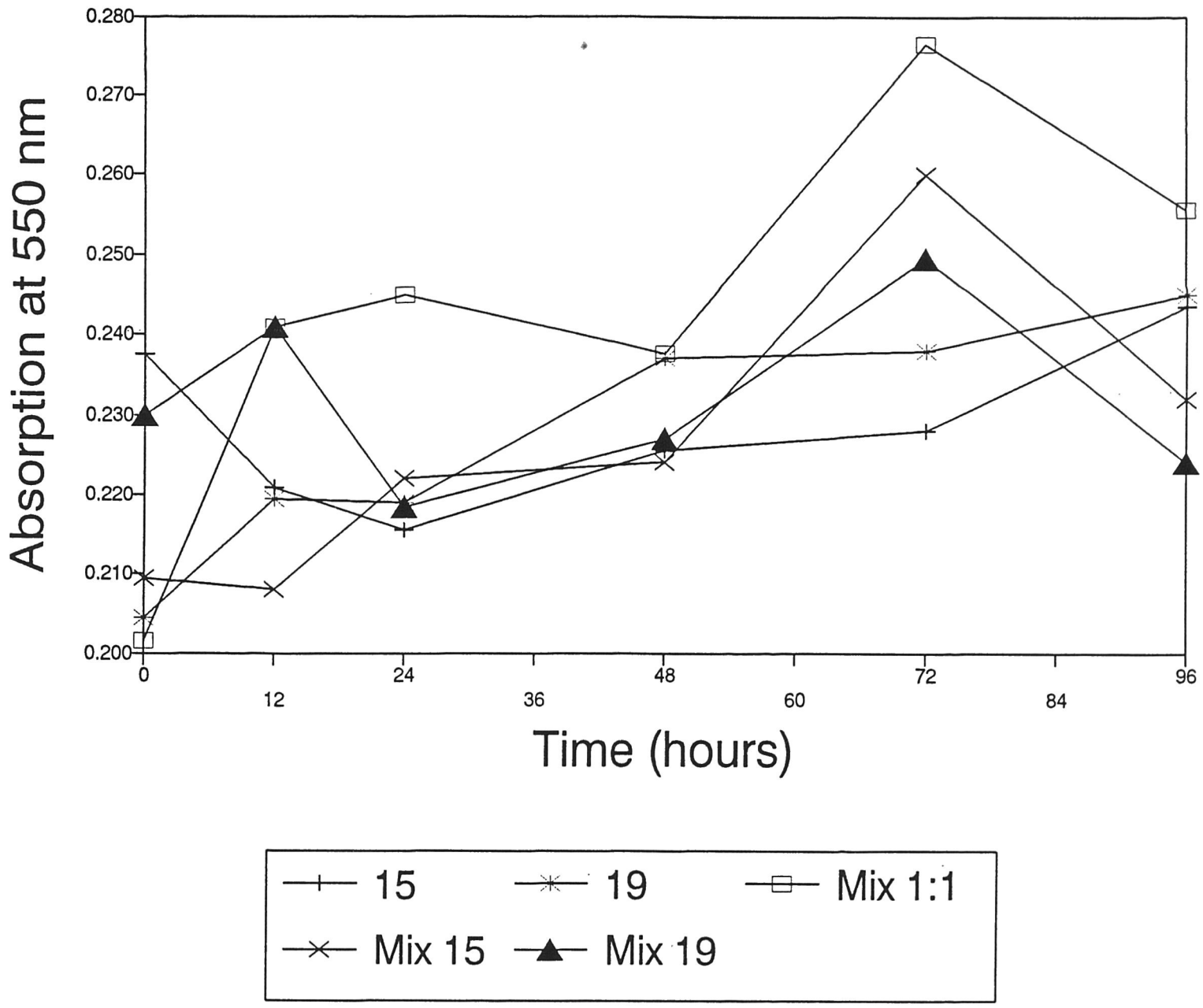

Figure 5. Optical Densities of Mixed Populations at 0, 12, 24, and 48 Hours

CONCLUSION

Waste TNT will continue to pose a threat to human health and the environment until a viable solution is found to break down TNT to its component elements. The findings from this experiment help define a new possibility for degrading explosive nitro-compounds. This work suggests that *Alcaligenes* do possess the capability to biodegrade TNT, which had not been tested until this experiment. The capability of *Alcaligenes* to degrade TNT, however, is strongly dependent on the initial microbial concentration. The initial experiment phase used high bacterial concentrations (0.4 optical density) and achieved almost full TNT break-down. In the last phase, however, the concentration was about half of the individual experiment concentration (0.20-0.23 optical density) and the results show much less degradation. For future experimentation and applications, high concentrations of the microorganisms may be required to achieve the desired TNT degradation.

The mixed microbial population experiment did not show that the mixtures performed better than the individual strain I15. All of the mixtures with a high I15 concentration performed comparably. This indicates that I15 was performing most of the TNT degradation in the mixtures. Strain I19 had been chosen for two trends: high TNT degradation and formation of 4amDNT from the I15 supernatant; however, a high 4amDNT concentration was not observed in the mixed populations. Metabolite degradation was not optimized by the combination of these two strains. This may indicate an adversarial relationship with I15 as the dominate strain.

Although the mixtures did not perform better than the individuals, the idea of cometabolism was not disproved; the experiment simply reveals that cometabolism is not used between these two strains. Other *Alcaligenes* strain combinations may show a marked difference in TNT biodegrading characteristics.

Research with many various combinations of *Alcaligenes* is under investigation by Anand Sivaraju at Texas A&M University.

Finally, when evaluating the results, the concepts of adhesion and absorption must be presented. TNT often adheres to the cell surface and is also absorbed into the cell without transformation. The degree of adhesion and absorption varies for each bacterium species and may vary be strain. Therefore, what appears as a TNT decrease with strain I15 may be a result of stronger TNT affinity to adhere/absorb to strain I15 than I19. Kathy Fredriksen is currently performing research on this topic at Texas A&M University. When her findings are complete, the conclusions from this experiment should be reevaluated.

Special thanks to Dr. Robin Autenrieth for her guidance and support as my advisor, Anand Sivaraju for his lab instruction and help , and BumHan Bae for assistance in experimental set-up and obtaining the HPLC results.

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