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# Enzymes for Degradation of Energetic Materials and Demilitarization of Explosives Stockpiles

**SERDP Final Report, 9/00**

S. C. Goheen
J. A. Campbell
Y. Shi
S. Aust

September 2000

Prepared for the U.S. Department of Energy
under contract DE-AC06-76RLO 1830

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## Summary

When this project started, the stockpile of energetic materials requiring disposal contained about half a million tons. Through 2001, over 2.1 million tons are expected to pass through the stockpile for disposal. Safe and environmentally acceptable methods for disposing of these materials are still needed.

The focus of this project was to develop safe, economical, and environmentally sound processes using biocatalysts (enzymes) to degrade energetic materials and to convert them into economically valuable products. Some alternative methods for destroying these materials are hazardous, environmentally unacceptable, and expensive. These methods include burning, detonation, land and sea burial, treatment at high temperature and pressure, and treatment with harsh chemicals. Enzyme treatments can operate at room temperature and atmospheric pressure in an aqueous solution.

A series of experiments demonstrated the ability of redox enzymes from spinach (ferredoxin NADP oxidoreductase, and glutathione reductase), buttermilk (xanthine oxidase), and *E. coli* (Oxyrase) to transform 2,4,6-trinitrotolune (TNT). All of these enzymes transformed TNT, some into 4-hydroxylamino-2,6-dinitrotoluene (4HADNT). The intermediate, 4HADNT was subsequently transformed to either 2- or 4-amino dinitrotoluene (ADNT) by some of the enzymes, but the Oxyrase enzyme complex did not form the 4-HADNT intermediate under all conditions. When the 4-HADNT intermediate was not observed, 2- and 4- aminodinitrotoluene (ADNT) were produced.

The relevant process engineering parameters for scale-up were addressed using mathematical modelling. Mathematical simulations were conducted for a proposed processing tank methodology for the enzymatic destruction of TNT. Two coupled rate equations contained terms for aqueous-phase transformation, solid-phase transformation, and solid TNT dissolution. Simulations showed that aqueous TNT concentrations rapidly became and remained low, limited by the relatively slow dissolution rate of solid TNT. They also showed that concentration versus time profiles contained a substantial initial period of linear decline. Sensitivity studies conducted for the operating parameters of the engineered tank showed that the time for 100% disappearance of the added TNT should be a linear function of the total enzyme concentration and the initial solid TNT concentration for the particular values of the other input parameters describing the expected processing tank system. We have confirmed a portion of this model by demonstrating a linear relationship between enzyme concentration and conversion rate in our previous work (1). Based on work conducted thus far, the simulated disappearance times were short enough to indicate the feasibility of this methodology for TNT destruction. A news release on the potential enzymatic process for the degradation of explosives provided evidence for public acceptance of the technology. It received attention and interest from end users, defense contractors, media, scientific magazines and regulatory agencies. The total circulation number for the coverage was 2.6 million.

Potential commercial applications of TNT derivatives were examined. There were two types of products that could be considered. One is a series of new products for which we can speculate some specific use. The other is products already on the market. Our initial effort was to explore all possible products, including those for which we could visualize a synthetic pathway. In our final several months of this project, we attempted to narrow this list to products already on the

market. These products have a clear monetary value and they can be sold much more readily than products for which applications and markets would need to be developed. The initial long list of products included aromatic hydroxylamines, aminophenols, and mixtures of those products. The most promising products, in order, appeared to be 1) antioxidants, 2) curing agents, 3) biocides/disinfectants, 4) monomers, and 5) pharmaceutical and agricultural chemicals. Flame retardants and azo dyes were also considered. All of these would have required further chemical processing of the main products produced in the discovered enzyme processes. We were unable to generate any of these value-added products under the limitations of this project. Instead, we identified other conceivable products, which are currently on the market. Only one product was identified that could be sold to an existing market that could be conceivably produced from TNT and enzymatic processing. That is nitrobenzoic acid. Nitrobenzoic acid is relatively inexpensive. Therefore the concept of generating high value currently marketable products from the enzymatic digestion of TNT was no longer pursued.

Sodium chlorite and potassium superoxide, a powerful oxidizer, was also shown to be an effective, safe treatment for TNT, hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) conversion. The small molecules can potentially penetrate the microstructure of bulk explosives, allowing the destruction of explosive devices that cannot be safely disassembled for treatment. Nitrate and nitrite were primary products of RDX and HMX degradation. Products of TNT degradation remain to be identified.

Finally, we explored the ability of fungi to digest TNT and DNT. We found that both of these explosives could be mineralized when certain fungi were used. This is in contrast to currently popular composting methods which appear to generate similar products to those we observed from our much simpler enzyme processes.

This project was not completed. Funds stopped at the end of the second year for what was planned to be a three-year project. We were left with some answers and many questions. In summary, we determined that enzyme processing of explosives may have limited value. Its greatest potential appears to be in the processing of waste streams. Yet, the effluent may remain as toxic as the products from composting. Sodium chloride may have its greatest potential in treating scrap. Yet we need better information on the products formed from this process. Degrading TNT and DNT using fungi appeared promising, but the data we collected was limited. Although mineralization took place, all the TNT was not mineralized. Therefore, each process had promise, but the results remain inconclusive.

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# 1.0 Project Summary

This project was initiated by Dr. Manish Shah in 1998. Dr. Shah's intent was to examine whether enzymes could convert explosives to high-value commercial products. Enzymes are often highly specific with regard to the substrate they act upon and the product they form. They operate under safe conditions, in an aqueous environment, and can tolerate a greater diversity of conditions than bacteria. Dr. Shah examined the ability of several different engineers to generate various products from TNT, RDX, and HMX. Typically, TNT was converted by several enzymes, but the same was not true of RDX or HMX. Another complication was that the products formed from TNT were similar or identical to those generated by various reducing bacteria (under reducing conditions). These products, HADNT and ADNT and presumably TAT were produced exclusively from TNT by some of the enzymes we used. These products are commonly observed by the popular composting process used commercially. Therefore, the initial premises that we would be able to generate high valued products from TNT using enzymes was no longer an expected outcome.

In the process of pursuing different enyzmatic pathways, we examined the abilities of hydroxyl radical, sodium chloride, and sodium bypochlorite to degrade TNT, RDX, and HMX. The outcome of these experiments was valuable. We observed good degradation of TNT, RDX, and HMX by one or more of these non-enzymatic processes. However, we were not able to reproduce these reactions with enzymes. And, the degradation of explosives by non-enzymatic means was outside the scope of this project. Therefore, we left several questions unanswered as we focused on enzyme processes rather than chemical processes. The finding that enzymes were not likely to produce high-valued products from TNT, RDX, or HMX dampened much of the enthusiasm for this project. The work continued for two rather than the planned three years. A third year of effort. would have probably revealed more evidence to support the claim, but may not have been as fruitful as other pursuits.

Some of the benefits that have evolved from this project include the following:

We are now confident that commercially available enzymes that will degrade RDX or HMX are hard to find.

- Most commercially available enzymes which convert TNT to another product are typically reducing enzymes probably with little or no specificity for the substrate.
- Sodium chlorite and/or hypochlorite degrade RDX, HMX, and TNT. This reaction needs further evaluation, by may be useful for cleaning explosives from scrap materials.
- Finally, we found that fungi mineralize TNT and DNT albeit slowly. This arena was examined to help discover new enzymes which could more completely degrade nitroaromatics.

These findings are described in more detail throughout this report.

## 2.0 Objective

The objective of the current project was to develop a safe, economical and environmentally sound process using biocatalysts (enzymes) to degrade energetic materials, with an option of converting them into value added products. The proposed process can operate at room temperature and atmospheric pressure in the aqueous phase. The proposed technology is much different from microbial processes as it can tolerate high concentrations of explosives or solvents and will use catalysts with the highest activity per unit weight of the catalyst. The process does not employ open burning or open detonation to destroy energetic materials. It can stand by itself or augment and support other technologies for treating energetic materials. An enzyme based process will also provide the alternative of converting explosives into commercially valuable chemicals because the transformation process can be controlled to accumulate desired intermediates. The proposed technology will not release air pollutants; the proposed enzyme technology could have met the regulations of Clean Air Act amendments, Resource Conservation and Recovery Act (RCRA) and Federal Facilities Compliance Act (FFCA). The development of this approach is now possible because of recent developments in enzyme technology. Robust and active enzymes can now be developed that will withstand harsh reaction environments. Alternatively, inexpensive enzymes can be used as disposable catalysts in the same manner as their use by the detergent and food industries.

In our early work, we discovered that enzymes from spinach leaves could degrade the explosive tetryl (2). More detailed research and development efforts lead to the further discovery that enzymes from spinach leaves could also degrade TNT (3) but not nitramine explosives such as RDX and HMX. The nitroreductase enzyme from spinach reacted with 2,4,6-trinitrotoluene (TNT) and synthesized 4-hydroxylamino 2,6-dinitrotoluene, which has potential uses in the production of antioxidants. This TNT conversion process could provide a zero-cost alternative for the disposal of unusable TNT stockpiles around the world. The rate of degradation of TNT by enzymes increased with increases in enzyme and TNT concentration. TNT was also degraded by enzymes above TNT's solubility limit of 100 ppm. Mathematical simulation studies suggested that the kinetics of the process are limited by the rate of dissolution of TNT. Mathematical simulation studies also suggested that insoluble TNT particles with diameters of 1 mm or less give the best kinetics of degradation (1). Our study also suggested that TNT degrading enzymes are probably ubiquitous in natural sources such as plants, dairy products, and microbial membranes. This is simply because these enzymes reduce biological molecules of higher redox potential and size in their natural function. This also may explain why TNT is toxic to living organisms: high concentrations of TNT could interfere with the natural functions of these enzymes.

To find additional enzymes that would degrade nitramine explosives, we decided to develop biomimetic processes. We discovered that the nitramine class of explosives could be treated more effectively using potassium superoxide. The mechanism used was similar to the natural defense system of humans, which produces superoxides to defend against environmental toxins or microorganism invasions. In addition to superoxide, we discovered that bleaching agents, such as sodium chlorite, could destroy TNT, RDX and HMX.

These results were very encouraging in our efforts to develop an environmentally benign digestion process for the destruction or conversion (in some situations) of explosives. We anticipated both enzymatic and biomimetic processes to achieve our initial objectives. Based on this knowledge, we

looked for enzymes to degrade RDX and HMX. We found one enzyme that could degrade HMX, but not RDX. We also investigated the degradation of metabolites of TNT formed by enzymatic processes. These metabolites appeared to be only slightly biodegradable by microorganisms. We evaluated industrial options for 4-HADNT-dinitrotoluene, as it is relative easy to synthesize from TNT. Our preliminary market assessment suggests 4-HADNT has very limited market value.

There are however, additional reasons for exploring the environmentally benign and safe methods for the synthesis and destruction of nitrocompounds. One is that these technologies could potentially provide an environmentally benign, safe, and cost-effective method for destroying explosives and other nitrocompounds. In addition, these enzymatic processes are the basis of bioremediation. Composting is the most recent technology used for treating explosive-contaminated soil. As we learn more about the enzymatic processes, we develop a deeper understanding of the bioremediation process. From the literature, it appears that bioremediation by composting may produce highly toxic products which bond strongly to soil (personal communication H. Hruel Lenke, September 9, 1999) (4). With the wide spread use of this technique, continued research to better understand the chemistry is warranted.

## 3.0 Technical Approach And Risks

The project was a joint effort between Pacific Northwest National Laboratory, Genencore International, Utah State University, and the Demil Technology Office (Defense Ammunition Center) at Savanna, Illinois. The overall technical approach for the proposed research involved developing enzymatic or equivalent biomimetic transformation of munitions such as TNT, RDX, and HMX in different forms (composition A, B, C, D, H-6, Tritonal) to intermediate products. The intermediate products, in some cases, were expected to have reduced or no toxicity and were evaluated for their toxicity by standard methods. In other cases, the intermediates could be imagined as a feed stock in the chemical industry or mineralized using microbial and chemical processes. We evaluated the toxicity of the intermediate products and final products.

We tested several biocatalysts that operate at room temperature and atmospheric conditions in an aqueous phase to degrade munitions. The first need for the project was an understanding of the kinetics and mass transport issues involved in explosive degradation by enzymes under heterogeneous conditions. The conversion of explosives (for example, TNT) by an enzyme-catalyzed heterogeneous solid-liquid system is different from a normal heterogeneous catalytic system since the catalyst (enzyme) is in the aqueous phase. The change in concentration of TNT will therefore require a TNT material balance. TNT is potentially degraded both in solution and on the surface of explosive particles. The size and mixing of TNT particles could have dramatic effects on the kinetics of the process. If the reaction occurs at a surface, the rate of reaction will be one of the boundary conditions for the kinetics. Additionally, the kinetics will have to account for the change in the surface area with the passage of time. The basic equation is a transient equation suitable for the moving boundary problem. Assuming a single TNT particle to be spherical with angular symmetry, the change in radius can be related to the velocity of the moving boundary. This equation, once solved for a single particle, can be integrated for the entire reactor volume to get the change in TNT concentration with time. Hence, the first task was to characterize the kinetics of the enzymatic process under heterogeneous conditions. We determined the kinetics for dissolved TNT. The rate limiting component for the heterogeneous catalysis was not identified due to time and resource constraints.

Ferredoxin NADP reductase enzyme (FNR) from spinach can reduce 2,4,6-trinitrophenylmethylnitramine (tetryl) and (TNT). A number of other redox enzymes (glutathione reductase, xanthine oxidase) could also exhibit explosive degradation activity. These enzymes are ubiquitous in nature and thus can be obtained in large quantity. The rate of transformation is estimated to be about 7 μmoles/min/mg of the enzyme. For TNT in solution, the estimated treatment time for 1 liter of waste stream containing 440 μM (100 ppm) of explosive TNT can be as low as 4 seconds for one gm of enzyme. This is a significant improvement over the traditional microbial processes, which would take anywhere from days to weeks or months for the degradation of explosives. However, the products in each case may be different. A complete mineralization of the metabolites is more likely after removal or reduction of the nitro group present in energetic materials. However, we have not yet demonstrated mineralization with enzymes. Nor has mineralization been demonstrated in the composting process, or degradation by bacteria (4,5,6,7). Enzymatic processes also provide an alternative, the accumulation of intermediates of TNT metabolism, which can potentially be of commercial value. We tried to identify commercial products that could be formed from TNT metabolites. It is unlikely that such a goal can be easily attained.

However, the basis for exploring enzyme degradation of explosives includes the manner by which the enzyme can act on explosives. Explosives can be attacked by enzymes and enzyme mediators under either oxidizing or reducing conditions. Another important point is that the size (less than one micron) of the enzyme molecule or its mediator (less than 100 angstroms) will be small, and they may be able to penetrate some porous plastic explosive matrices. Reactions of redox enzymes with nitroaromatic and nitramine explosives are summarized below.

I. Reductive Attack

**A**. Single- or two-electron reduction step process for the reduction of nitroaromatic explosives:

$$RNO_2 \rightarrow RNO \rightarrow RNHO \rightarrow RNH_2$$

**B**. Newly discovered single-electron reduction step process for nitramine explosives:

$$RN\text{-}NO_2 \rightarrow [RN\text{-}NO_2]^- \rightarrow RNH + NO_2^-$$

or: Hydride-based reduction of nitroaromatic chemicals:

$$RNO_2 + H^- \rightarrow RH + NO_2$$

II. Oxidoreductive Attack

This promised to be a very innovative and unique approach with tremendous potential. Under oxidoreductive attack, enzymes would be used to generate reactive cation radicals, anion radicals, or oxygen radicals of widely differing redox potentials. As a result, the process could be applicable for the treatment of a number of structurally diverse energetic materials. In addition to nitroreductase enzymes, we investigated the transformation of explosives by oxidative enzymes such as peroxidases. Peroxidase enzymes can catalyze both oxidative and reductive reactions. Peroxidases are available commercially in large quantity at a price of 250 billion units of activity for $10 (Enzymol, International, Inc.). Peroxidases can produce highly powerful oxidant radicals, such as aromatic cation radicals and hydroxyl radicals, or highly reducing carboxylate anion radicals and superoxide anion radicals. They can oxidize (iodide, cyanide, and methoxyphenols) or reduce (carbon tetrachloride, cytochrome c, ferric iron, and nitrobluetetrazolium) molecules under different reaction conditions. Genencore International, our collaborator, had several potential proprietary enzymes, which were available to us. One of them, laccase, an oxidative enzyme, uses molecular oxygen (air) for catalyzing oxidative reactions. Overall, our strategy of enzyme selection was cost and availability driven.

We believed that the proposed line of research had a high probability of success. The proposed enzyme technology can be viewed as a free-radical based process where enzymes are the free-radical generators. By selecting different combinations of enzymes, mediators, and reaction environments, one may change the redox potentials of free radicals. Such flexibility was never envisaged by any earlier biological or chemical process in the aqueous phase. Years ago, researchers at SRI International found that RDX and HMX can be mineralized with light. The light based degradation is mediated by free radicals. The proposed technology will work similarly but will use enzymes as the catalyst instead of light. White rot fungi have been shown to mineralize RDX and HMX; fungi use a free-radical based process to degrade recalcitrant chemicals. Unlike fungal or light-based systems, the proposed process will have the ability to control the free radical chemistry and process products, and could have significantly higher reaction rates.

The proposed technology appeared promising. Enzymes have the highest reactivity per unit weight of catalyst. The enzyme process operates under mild and safe conditions. The process can operate at room temperature, atmospheric pressure, and in the aqueous phase. The process does not require any special equipment, hardware, or software and, thus, has very low capital costs. A mobile system can be designed without any major technical or cost hurdles. Enzymes can be produced at low cost and in large scale by enzyme manufacturers; enzyme production is a very attractive business opportunity as it creates a new market opportunities for oxidoreductive enzymes.

Special features of the proposed enzyme technology are:

- excellent kinetics,
- no special equipment hardware and thus low capital cost,
- stability under harsh conditions (solvents, high concentration of explosives etc.),
- highest reactivity per unit weight of any catalyst,
- simplicity of operation,
- mobile systems can be designed,
- can be sprayed in remote locations,
- low operating cost,
- can operate at room temperature and atmospheric pressure,
- can be produced at low cost for large scale applications.

# 4.0 Summary of Progress

## 4.1. Digestion of TNT and Other Explosives by Oxyrase

A series of experiments were conducted on the digestion of 2,4,6-trinitrotoluene (TNT) with Oxyrase, a relatively low cost membrane-bound enzyme aggregate from *E. coli.* The experiments demonstrated the ability of Oxyrase to digest TNT rapidly at room temperature under aqueous conditions. The kinetics of the digestion process were characterized by high performance liquid chromatography with ultraviolet detection (HPLC-UV). The metabolites of the digestion were analyzed by high performance liquid chromatography with ultraviolet, and electrospray mass spectrometry detection (HPLC-UV-ESI-MS). The metabolites were identified as 4-amino-2,6-dinitrotoluene, and 2-amino-4,6-dinitrotoluene based on the spectral and chromatographic data. Detection limits of TNT and metabolites by HPLC-UV were also determined.
Similar experiments have also been carried out on the digestion of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX). However, the experiments have not demonstrated the ability of oxyrase to digest RDX at room temperature using aqueous conditions. In one experiment, we observed a significant loss of HMX in the presence of Oxyrase, but we could not find or identify any products by the most commonly used extraction, separation, and analysis methods. Due to the discontinuation of this project, we were unable to resolve this question. That is, was HMX really digested, and the products were not extracted correctly, or lost during analysis? Or, was HMX mineralized? Details of the digestion of TNT by Oxyrase are presented in Appendix A. The material from this appendix was presented in Seattle at HPLC 2000, and the text was submitted for publication to J. Chrom.

## 4.2 Digestion of Explosives by Enzymes Other than Oxyrase

Various other naturally occurring enzymes besides Oxyrase have been examined for the digestion of TNT, RDX, and HMX. Ferredoxin NADP oxidoreductase (FNR) and glutathione reductase from spinach demonstrated the ability to digest TNT. One metabolite was formed from the digestion of TNT by FNR. The metabolite was identified by HPLC-ESI-MS and HPLC-particle beam-MS as 4-hydroxylamino-2,6-dinitrotoluene (4-HADNT). Glutathione reductase, xanthine oxidase and xanthine were also able to digest TNT into 4HADNT. Most of these results have been reported previously (1). However new information from work performed in 1999 included efforts to digest RDX, HMX, and TNT using other enzymes. All our enzyme digestion results for these three explosives are summarized in Table 1.

The conditions used these enzymes are described in Table 2. Each characteristic used in the selection process from Table 1 was examined and used to help identify appropriate enzymes. A + indicates the enzyme was tested and the explosive reacted. A – indicates no reaction was observed in the presence of the enzyme. Blank spaces suggest the enzyme reaction was not attempted.

Some properties of these enzymes and conditions used are described in Table 2. Each characteristic used in the selection process from Table 3 was examined and used to help identify appropriate enzymes.

## 4.3 Marketable Products from Enzymatic Digestion of Explosives

Our initial hope for this project was to be able to generate a marketable product from surplus explosives. The process was to be environmentally benign, using enzymes in an aqueous suspension. Generating marketable products from waste can be a challenge in general, but when enzymes are used, there is the possibility that the enzymes could be specific enough to act on one waste component at a time and generate one product at a time. If the product is higher value than the waste material, the effluent could be sold to help recover processing costs.

**Table 1. Enzymes Tested in Degradation of Energetic Materials**

| Enzyme | TNT | RDX | HMX |
|---|---|---|---|
| Glucose oxidase | | - | |
| Pepsin | | - | |
| Glutathione reductase | + | - | |
| Xylanase | | - | |
| Cellulase | | - | |
| Xanthine oxidase | + | | |
| Ferredoxin $NADP^+$ reductase | + | | |
| Oxyrase | + | - | + |
| Bio Systems enzyme solution | - | | |
| Horseradish peroxidase | | - | |
| Soybean peroxidase | | - | |
| Lignin peroxidase | | - | |

**Table 2. Key Factors Involving the Enzymatic Eegradation of TNT**

| Enzyme | pH | Temp. (°F) | Cofactors | Inhibitors | Avail. | Cost per unit enzyme |
|---|---|---|---|---|---|---|
| Glutathione reductase (GR) | 7.6 | 77 | NADPH | oxidizers/ base | Sigma | $0.584 [1] |
| Xanthine Oxidase (XO) | 7.5 | 77 | Xanthine | | Sigma | $2.89 [2] |
| Ferradoxin NADP+ Reductase (FNR) | 7.5 | 77 | NADPH | cofactor depletion | Sigma | $18.17 [3] |
| Oxyrase (OXY) | 8.3 | 66 - 99 | $NaHCO_2$ | Temperature above 131° F | Oxyrase | $10.00 [4] |
| Bio Systems Enzyme Solution | 7.5 | 40 - 110 | | | Bio Systems | |

[1] **GR Unit Definition:** One unit will reduce 1.0 micromoles oxidized glutathione/minute at 25° C and pH 7.6

[2] **XO Unit Definition:** One unit will oxidize 1.0 micromoles of Xanthine to uric acid/minute at 25° C and pH 7.5

[3] **FNR Unit Definition:** One unit will reduce 1.0 micromoles of NADP/minute at 25° C and pH 7.6

[4] **OXY Unit Definition:** One unit will reduce dissolved oxygen (in 1 mL of air saturated with 40 mM phosphate buffer pH 8.4 at 37° C) at the rate of 1%/second. The manufacturer has indicated costs can be diminished to $0.01 per unit for bulk processing.

In the first phase of this project, we examined the ability of various enzymes to degrade three explosives; TNT, RDX, and HMX. Our first success was our ability to convert TNT to another product as is described above. Our second success was to be able to identify potential products

that could be generated from the reduced species of TNT. All of the potential products we had identified in the 1998 Annual Report were not currently available commercially. Their commercial value and potential applications were speculative.

**Table 3. Key Factors Influencing the Choice of Enzyme.**

| Factor | Consideration |
|---|---|
| Specificity | Determined during screening*. |
| pH | Limits at/around neutral for safety and general applicability |
| Temperature | Near average ambient temperatures (cost reduction, safety, general applicability) |
| Activators/ inhibitors | Non-toxic and cost effective |
| Analysis methods | Cost effective |
| Availability | Readily available, safe handling |
| Cost | Per unit enzyme<br>Per unit of conversion<br>Percentage of processing cost |

* The specific action of our enzymes were determined during the screening process. The effectiveness of enzymatic degradation was measured against acceptable and undesired side activities of the enzyme in the reaction media. Enzymes are characterized on the basis of toxicity and potential commercial usage of the products formed. After meeting safety and economic criteria, candidate enzymes are placed in an order of precedence based upon the ease and cost efficiency of an industrial application. How the enzyme is introduced to the reaction matrix within the bio-reactor influences both procedural and enzyme/product recovery costs (i.e. Is the enzyme introduced on a support or in a slurry?). Enzyme stability and cost are factors which we considered in this phase of the selection process. Furthermore, we evaluated our enzymes on whether or not we may incorporate them into safe and cost efficient technologies. Our underlining criteria for enzyme selection was a safe and environmentally sound reaction pathway which can be conducted with ease in a cost efficient manner in either field or industrial applications.

The enzymes examined from May 13, 1998 to December 31 1999 are shown in Table 2.

Generating products which are currently not available is an inappropriate focus. Therefore, subsequent work was aimed at generating products with existing commercial value. The initial potential enzymatic pathways of TNT degradation were examined to help identify possible product targets. Our experiments indicated that TNT could be transformed easily to HADNT, 4-amino-2,6-dinitrotoluene (4-ADNT) and 2-amino-4,6-dinitrotoluene (2-ADNT). 4-ADNT and 2-ADNT might be further transformed to 2,4-diamino-6-nitrotoluene, which might be degraded further to 2-nitrobenzoic acid and cyclohexanone. The potential commercial applications of the TNT degradation products along the pathways were investigated. 2-nitrobenzoic acid is used as manufacture intermediates and a reagent for alkaloids. Cyclohexanone has been used as a solvent for cellulose acetate, nitrocellulose, vinyl resins, crude rubber, waxes, fats, and etc. These

products however, have a relatively low market value. Since these were the only commercially available products we could envision from the reduction products of TNT and this project was being discontinued, we did not pursue this avenue further.

## 4.4 Enzyme Ruggedness and Behavior in Organic Solvents

Some enzymes perform more effectively in dilute organic solvents than in pure aqueous or organic media. Similarly, antibody (another protein) coupling to a solid support is often carried out in the presence of an organic modifier to enhance the coupling process. Enzyme activity is also typically inhibited in the presence of excessive organic modifier. Furthermore, the solubility of all three explosives, TNT, RDX, and HMX is strongly dependent on the presence of organic modifiers. TNT is sparingly soluble in water, but HMX and RDX are essentially insoluble.

Earlier in this program, it was suggested that some enzymes may function in a manner which is not diffusion dependent. That is, that the enzyme acting on explosive components may take place at the liquid/explosive interface, as well as in solution. If the explosive/enzyme reaction is not diffusion dependent, then certain estimates can be made on reaction rates. We made the limiting assumption that if it did, or if there was very rapid mixing, then reaction rates would be maximal, and from that point, determined how quickly TNT could be converted to various metabolites.

Therefore, we were still faced with the issue of determining how effective at least one enzyme would have been in the presence of an organic solvent. Acetone is an effective solubilizing agent of RDX and HMX, is readily soluble in water, and has been shown to have minimal impact on some enzyme activities. We verified the solubility of TNT in the presence of acetone and buffer, and also examined the ability of the Oxyrase enzyme complex to convert TNT in the presence of various concentrations of acetone. The results are shown in more detail in Appendix 3. In summary, the Oxyrase enzyme complex activity was inhibited in the presence of acetone at nearly all concentrations. Activity was more severely inhibited at higher concentrations of acetone, as the solubility of TNT was enhanced. Therefore, there may be an ideal concentration of acetone, or other organic modifier in which the conversion of TNT is maximized. We were not able to determine that ideal concentration, or examine alternate organic solvents due to the early termination of this program.

## 4.5 Toxicity of TNT and Related Metabolites

Toxicity of the enzymatic metabolites of TNT were characterized using standard methods. The major metabolite of TNT from reduction by xanthine oxidase was identified as 4-hydroxylamino-2,6-dinitrotoluene (4HADNT). The subsequent metabolites of 4HADNT are polar and they elute with enzyme, buffer and other components. The separation was carried out using either of two approaches. The first was to use solid phase extraction cartridges. These removed nearly all of the interference from enzyme components, but were expensive. The second method was using a chloroform extraction step. The TNT metabolites appeared to extract into the chloroform phase, however we were unable to quantify the efficiency of this extraction process. Also, it is clear that some metabolites may have been lost when either of these methods are used. More extensive work is needed to better understand the efficiency of extraction for each metabolite for more efficient analysis of the products.

The toxicity of metabolites of TNT produced by enzymes to different microbial systems was evaluated. In the evaluation process, it was found that TNT pre-treatment with enzymes led to

reduction in toxicity of the TNT metabolite mixture when exposed to standard microorganisms as evidenced by increased microbial growth and conversion of TNT to the various metabolites.

TNT reacted with xanthine oxidase until all TNT had been reduced. The products were exposed to a *Ceridaphnia dubia* assay in which the toxicity of the metabolites to the organism was determined (see Appendix B). In parallel, toxicity was measured by the Microtox Assay. In both assays it was found that TNT conversion with xanthine oxidase led to a reduction in toxicity to microoorganisms.

In this experiment, all of the TNT was gone after being incubated with xanthine oxidase. In sample 2 there was some residual HADNT. DNT degradation was also monitored. Degradation of DNT seemed slower than for TNT. Some original material remained along with more of the HADNT. This is as expected as DNT is more difficult to reduce than TNT. This could account for some toxicity in the experiments with DNT. However, we also observed significant toxicity in the control. There are two possible explanations for this. First, uric acid, the oxidation product of xanthine, may be toxic to this organism. Secondly, the action of the enzyme may result in a product which is toxic. In the absence of another electron accepter, the enzyme will reduce molecular oxygen to superoxide which will dismutate to hydrogen peroxide. In these experiments, the enzymatic digestion was carried out under anaerobic conditions, but the toxicity test was performed under aerobic conditions, as is required by this standardized test.

## 4.6 TNT, RDX, and HMX Conversion by Sodium Chlorite and Hypochlorite

Sodium chlorite reacted with TNT, HMX, and RDX under aqueous conditions. We submitted a proposal to SERDP (as requested) to examine the products formed and the reaction conditions we should use to maximize the conversion of these explosives. The proposal was not funded. The products formed from reacting either sodium chlorite or sodium hypochlorite were not easily identified by liquid chromatography coupled with mass spectrometry. Further work should be carried out to determine the extent of the reaction and the products formed for each of these reactants.

## 4.7 Products of Enzyme (FNR) Processing of TNT

We had previously performed several experiments on the enzymatic conversion of TNT. However, the products formed are dependent on the enzyme that is used. One enzyme produced 4-HADNT, whereas another enzyme produced ADNT. A more careful examination of the products formed from the reduction of TNT by FNR revealed a single product, 4-HADNT, as reported previously. When two enzymes were present, FNR and glucose-6-phosphate dehydrogenase, two products were formed. These were 4-HADNT and ADNT. The quantities of product (HADNT and/or ADNT) were typically about 50% of the starting TNT, yet all TNT reacted. This suggests our analytical techniques were inadequate to identify all products. Yet, even NMR studies have shown low recoveries of metabolites (4). We suspect further reduction products such as TAT were also formed and related reduction products constitute the remaining unaccounted for material. The tri-amines are known to be strongly bound to humic acids. They may also bind strongly to HPLC and solid phase extraction materials. Further work would confirm or reject this suspicion.

## 4.8 Justification/logic for Selection of Enzymes in TNT conversion Process

We had previously summarized our justification for selecting the various enzymes for the conversion of TNT in the 1998 annual report. In it we indicated there are two types of enzymes which can be used for conversion of nitroaromatics. These are reductive and oxidoreductive. The first are those use a single or two-electron step process for the reduction of nitroaromatic explosives:

$RNO_2 \rightarrow RNO \rightarrow RNHO \rightarrow RNH_2$

The second is a newly discovered single-electron reduction step process for nitroamine explosives:

$RN\text{-}NO_2 \rightarrow [RN\text{-}NO_2]^{\cdot} \rightarrow RNH + NO_2^{\cdot}$

Or hydride-based reduction of nitroaromatic chemicals:

$RNO_2 + H^{\cdot} \rightarrow RH + NO_2.$

Therefore, the type of reaction we were attempting was one key consideration in selecting candidate enzymes. Several of the other key factors influencing our choice of processing enzymes are included in Table 3.

These factors together were used for enzyme selection. The overall cost of conversion could in the future be calculated once the costs of bulk materials can be determined. For example, oxyrase currently costs 1000 times more than the manufacturer has indicated it would if purchased in bulk. Thus significantly more effort would need to be placed on the cost issue if the goal was to accurately reflect processing costs.

## 4.9 Scale-up

We had previously calculated the processing rates for nitroaromatic conversion by enzymes. These calculations were entered into our website so that anyone could incorporate various assumptions into the equation and determine what the ideal reaction rate would be. The reaction rate assumed ideal conditions. For example, eliminating diffusion from the reaction simplifies the kinetics, and provides maximum reaction rates. Also, diffusion effects can often be minimized by vigorous stirring. We have expanded the website calculations further to include various techniques for converting the nitroaromatics and preserving enzyme activity while minimizing cost.

# 5.0 Other Accomplishments

Other accomplishments completed in this program include meeting specified milestones, and the presentation of data at various conferences, submission of manuscripts for publication, and submission of patent applications. In addition, there was a high volume of media coverage for this program in its early stages of development. These accomplishments are summarized in this section.

## 5.1. Milestones

**Milestone 1.0:** **Examine the effluent for toxicity (e.g., biodegradability) of TNT metabolites.**
**Due Date:** 2/28/99
**Complete Date:** 4/1/99

**Comment:** Toxicity and metabolites of TNT were characterized. The most common metabolite of TNT from enzyme degradation was identified as 4HADNT. Additional metabolites were later identified as ADNT. However, these two products do not appear to represent all the products of TNT metabolism by digestion with various enzymes. Some enzymes appeared to produce 4HADNT, and others formed ADNT. Additional products have not yet been identified within this project. However, we suspect DANT (diamino nitrotoluene) and triamino toluene (TAT) are likely additional products of TNT reduction by the enzymes we studied.

The toxicity of metabolites of TNT was determined, and the method for determining toxicity and the toxicity results are described in Appendix B. The metabolites were less toxic than controls generally, but even the controls had some toxicity. Therefore, it was difficult to fully determine whether the products could be dispersed to the environment without further treatment. Further studies would need to be carried out to evaluate this more completely if enzyme processing of nitroaromatics becomes a viable treatment process.

**Milestone 2.0:** **Improve the mathematical model by estimating necessary critical parameters. experimentally**
**Due Date:** 4/30/99
**Complete Date** 4/1/99

**Comment:** A mathematical model was developed and distributed in the 1998 annual report. Parameters needed to improve this model require a better understanding of the process, such as a well-defined final product. The products 4HADNT, ADNT, DANT, and/or TAT are of no particular commercial value. Therefore, defining processing parameters to reach either of these products may not be as important as identifying a viable process. In the refined model, some assumptions were made, such as the

reaction would not be diffusion limited. This can be approached with vigerous stirring. However, the materials being investigated are at best slightly soluble in water so that rapid stirring may be insufficient to overcome both the dissolution and diffusion barriers.

**Milestone 3.0** **Identify enzymes for transformation of RDX and nitrate ester**
**Due Date:** 7/30/99
**Complete Date:** 12/31/99

**Comment:** We investigated the conversion of RDX (and a much more limited number of tests for HMX digestion) using several enzymes. None of the tested enzymes digested RDX.

**Milestone 4.0** **Identify metabolites of RDX and nitrate ester effluent**
**Due Date:** 7/30/99
**Complete Date:** 12/31/99

**Comment:** Since we were unable to convert RDX (or HMX) using commercial enzymes, there were no metabolites to identify.

**Milestone 5.0** **Determine toxicity of effluent (RDX, nitrate ester)**
**Due Date:** 9/30/99
**Complete Date:** 12/31/99

**Comment:** See comments under milestones 3.0 and 4.0. Since we could not degrade RDX and/or HMX with any of our commercial enzymes, we were unable to measure the toxicity of the products.

**Milestone 6.0** **Market Evaluation of TNT conversion product**
**Due Date:** 9/30/99
**Complete Date:**

**Comment:** The product which is initially formed from FNR reduction of TNT is 4-hydroxylamino-2,6-dinitrotoluene (4-HADNT). However, using negative ion electrospray mass spectrometry we have confirmed the production of two additional products, 4-amino-2,6-dinitrotoluene (4-ADNT), and 2-amino-4,6-dinitrotoluene (2-ADNT) from the reduction of TNT by oxyrase. These three products are not easily obtained commercially but have little market value. Marketable products we could envision subsequent metabolism were also of low value. We estimate that the cost of generating a marketable product from waste TNT through enzyme processes would likely exceed destruction costs. However, we were unable to investigate processing costs since we were asked to terminate the program before we fully developed the process. Theoretically, one could produce nitrobenzoic acid from TNT. Nitrobenzoic acid has a retail value of approximately $100 per Kg when purchased in small quantities. In contrast, we could assume that waste TNT has a negative dollar value due to remediation costs.

**Milestone 7.0** **Process Economics**
**Due Date:** 10/30/99
**Completion Date:** Incomplete

**Status:** We were unable to complete this milestone because we had not yet determined a pathway for the process. Process economics should be determined after not only the products are determined, but the steps have been identified and verified in the laboratory. We identified a process, but the project was terminated before it could be verified.

**Milestone 8.0** **Interim to SERDP**
**Due Date:** 12/30/99
**Completion Date:** 10/1/00

**Status:** The present report is the final report. The delivery of this report was delayed because final data from our subcontractor arrived in early June, 2000. Since this report is the final for the project, an earlier submission would have been complete.

## 5.2 Patents and Patent Applications

- Patent on "Combined Enzymatic and Microbial Method for Destruction of Explosives". (submitted for internal review).

- Patent on "Combined Enzymatic and Microbial method for Destruction of Explosives". (In preparation).

- Patent was submitted on "Method for Transformation of Nitroaromatics by Redox Enzyme". This patent is on transformation of TNT using inexpensive reductant such as lactic acid, glucose, formic acid. The significance of the patent is that it allows the degradation of TNT without the use of expensive NADPH.

## 5.3 Manuscripts

- Shah, M. M., K. A. Bennett, J. D. Stahl and S.D. Aust. "Mineralization of TNT by Redox Enzymes in Combination with Microbial Treatment Systems". Submitted to Biotech. Bioengineering.

- Shah M. M. and S. K. Roach. 'Degradation of Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) by Sodium Chlorite under Mild Reaction Conditions". Submitted to ES&T, 7/15/99.

- Shi, Y, S. C. Goheen, and J. A. Campbell "Characterization of Biotransformation of TNT by Oxyrase Using HPLC-UV-EIS-MS". Submitted to J. Chrom., 6/00.

## 5.4 Presentations

1. SC Goheen, JA Campbell, SK Roach, Y Shi and MM Shah "Degradation Products after Digestion of TNT using Ferredoxin $NADP^+$ Reductase" to be presented at the 2nd International Symposium on Biodegradation of Nitroaromatic Compounds & Explosives, September 8,9, 1999, Leesburg, VA.

2. Y Shi, SC Goheen and JA Campbell "Characterization of Biotransformation of TNT by Oxyrase Using HPLC-UV-ESI-MS" to be presented at the HPLC 2000 Conference, June 24-30, 2000, Seattle, WA.

# 6.0 Acknowledgments

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# 7.0. References


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# Appendix A

## Characterization of Biotransformation of 2,4,6-Trinitrotoluene by Using High Performance Liquid Chromatography-Ultra Violet-Electrospray Interface-Mass Spectrometry

# Characterization of Biotransformation of 2,4,6-Trinitrotoluene by Using High Performance Liquid Chromatography-Ultra Violet-Electrospray Interface-Mass Spectrometry

Ying Shi[1], Steven C. Goheen, and James A. Campbell*
Battelle, Pacific Northwest National Laboratories
Richland, WA 99352

2,4,6-trinitrotoluene (TNT) is a major component of many explosives. During the manufacturing process, explosives have been inadvertently released and contaminated soil and ground water in areas throughout the United States and Europe. Biotransformation of TNT using microbes, as in composting, is a viable approach for the remediation of contaminated soil and water as it is safer, more economical, and more environmentally acceptable than other alternatives (e.g. detonation, harsh chemical treatment). Technologies using enzymes to transform TNT are still in the process of development. In the present study, a process to transform TNT under aqueous conditions employing Oxyrase, a complex mixture of enzymes from *Eschericia coli*, was characterized. TNT was incubated at room temperature in the presence of Oxyrase. Solid phase extraction was performed on the transformation mixture of TNT and Oxyrase. The extract was analyzed using High Performance Liquid Chromatography-Ultra Violet- Electrospray Interface-Mass Spectrometry. Two degradation products were separated by HPLC and identified as 4-amino-2, 6-dinitrotoluene and 2-amino-4, 6-dintrotoluene. Kinetics of the transformation process were studied by HPLC-UV. Detection limits of TNT and its transformation products were also determined. These characterization methods were used to help determine that treating TNT with Oxyrase gave similar results to the popular composting process. The advantage of enzyme processing is that it can be used to process contaminated streams.

## Introduction

2,4,6-trinitrotoluene (TNT) is commonly used in explosives and is often the major contaminant of soil and water at the sites of its production, processing, and disposal in the United States and Europe [1]. A recent survey showed that there were more than 50,000 contaminated sites, and concentrations of TNT as high as 100 µg/L in water and 12,000 mg/kg in soil, which could have a significant impact on the ecosystem [2-4]. TNT is toxic and mutagenic [5-8]. Therefore, remediation of TNT contaminated soil and water and safe disposal of explosives have become significant environmental and public health concerns.

Traditional treatment of TNT waste includes chemical and physical processes such as open detonation, open burning, chemical reduction, and burial. These treatments are expensive, hazardous and often generate toxic byproducts that will cause additional environmental problems [9,10]. The drawbacks of traditional treatment have resulted in the research for alternative technologies such as bioremediation [4,11]. Recently, composting of TNT contaminated soil has become widely used and has been evaluated by researchers [8]. However, the end products from those processes have not been well characterized and the amount of waste material might be increased [12]. In addition, a recent concern in bioremediation is that the finished compost product has been found to be highly genotoxic [8].

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1 Present address: Merial Limited, Iselin, NJ 08830-3077

Transformation of TNT using enzymes has not yet been accepted. Enzymatic transformation of TNT is a viable approach because it is safer, more economical, and more environmentally acceptable than detonation or burning. Enzymes are ubiquitous; they are the biocatalysts of plants, bacteria, and other living organisms. Advances in biotechnology, microbiology, and biochemistry have allowed enzymes to be mass-produced and purified at low cost. Enzymatic transformation can be carried out under atmospheric pressure and aqueous conditions. Several natural occurring enzymes such as ferredoxin NADP oxidoreductase (FNR) and glutathione reductase from spinach and xanthine oxidase from buttermilk have been studied for their ability to transform TNT. These enzymes were able to convert TNT to a major intermediate, 4-hydroxylamino-2,6-dinitrotoluene (4-HADNT). The enzyme-digested TNT can be treated further with either white rot fungi or microbial cultures. Mineralization of TNT metabolites has been observed using this approach [13]. Enzymatic and microbial studies have shown some potential for the development of a bioremediation process for TNT remediation of soil and water. However, transformation of TNT by these enzymes requires expensive cofactors, and the complete transformation of 4-HADNT has not been well characterized. We have identified a new, more cost-effective enzyme-cofactor system for TNT processing. To characterize this process, the kinetics, the metabolites, and the fate of the metabolites need to be analyzed thoroughly using appropriate analytical methods [14]. High performance liquid chromatography with ultraviolet detection (HPLC-UV) and gas chromatography (GC) with either electron capture or mass spectrometry detection are commonly used analytical methods for TNT and related compounds. As an example, tetryl and its transformation products in soil have been identified using GC/MS [15]. However, reproducibility suffers in GC analysis, especially for thermally labile compounds. Although HPLC-UV is the Environmental Protection Agency (EPA) standard method for explosive analysis, UV detection provides limited structural information for the determination of unknown explosive transformation products [16-18]. Until recently, the selectivity and specificity of the mass spectrometer as a chromatographic detector has been restricted to gas chromatography due to the difficulties associated with developing a suitable LC-MS interface. Introduction and improvements of ionization techniques such as electrospray (ESI) have allowed the mass spectrometer to become more widely used in liquid chromatography. The evolution of the LC-ESI-MS interface has enabled the development of methods for a variety of polar and thermally labile compounds including explosive and explosive transformation products [19-21]. Particle-beam LC/MS has been used to identify the major component resulting from enzymatic degradation of TNT with FNR [22]. In this research, Oxyrase, a low cost enzyme complex from *E. coli* that does not require high cost cofactors, was examined for the transformation of TNT. The objective of this research was to gain a better understanding of the enzymatic process of TNT reduction by Oxyrase. In the TNT reduction process, Oxyrase was able to transform TNT to two metabolites under aqueous conditions. The amount of product formed was approximately 35% of the starting material. The loss of 65% of the TNT without identification of metabolites has also been observed when composting is used [8]. The metabolites were isolated by solid phase extraction and identified using HPLC-UV-MS in negative ion mode. The HPLC-UV-ESI-MS method was developed and optimized. The kinetics of TNT transformation by Oxyrase was characterized using HPLC-UV. The characterization of the kinetics and metabolites of TNT transformation by Oxyrase will assist in the development of new bioremediation technologies and biosensors so that either value-added products or a mineralization pathway can be identified.

# Experimental

## Chemicals

2,4,6-trinitrotoluene (TNT) was obtained from Chem Service (Westchester, PA, USA). 4-amino-2, 6-dinitrotoluene (4-ADNT) and 2-amino-4, 6-dinitrotoluene (2-ADNT) were purchased from Radian International (Austin, TX, USA). Sodium formate and HPLC grade acetonitrile were obtained from J.T. Baker (Phillipburg, PA, USA). TRIZMA Base (tris[hydroxymethyl]aminomethane) was acquired from Sigma (St. Louis, MO, USA). The enzyme Oxyrase was obtained from Oxyrase, Inc. (Mansfield, OH, USA). HPLC grade methanol was from Aldrich (Milwaukee, WI, USA). The Sep-Pak cartridges were purchased from Waters (MA, USA). Ultra pure deionized water was obtained from a water filtering system manufactured by Millipore (Molsheim, France). A standard small molecule negative ion mode tuning mixture was purchased from Hewlett Packard (Palo Alto, CA, USA).

## Sample Preparation

**Standards.** A range of concentrations of TNT, 4-A-2,6-DNT, and 2-A-4,6-DNT standards were prepared. TNT, 4-A-2,6-DNT, and 2-A-4,6-DNT standards were prepared by diluting 1000 µg/mL standards with acetonitrile. The stock solutions were prepared by dissolving the solid standards in acetonitrile.

**Buffer.** Tris/sodium formate buffer (50/60 mM) was prepared by dissolving 102.3 mg of sodium formate and 150.2 mg of tris[hydroxymethyl]aminomethane in deionized water. The pH was adjusted to 8.4 with hydrochloric acid. The final volume was 25 mL.

**Transformation.** A mixture containing TNT, tris/sodium formate buffer, water and Oxyrase was prepared in an amber vial and incubated at room temperature for various periods of time. The total volume of the mixture was 2 mL. Final concentrations of the components were 100 µg/mL (TNT), 17.5/21 mM (tris/sodium formate buffer), and 9 unit/mL (Oxyrase). Controls that contained the same concentrations of TNT and buffer but without Oxyrase, as well as blanks, were also prepared along with the TNT and Oxyrase reaction mixture.

**Extraction and Filtration.** Two sample cleanup methods have been used for TNT and metabolite analysis. One was solid phase extraction and the other was filtration. The TNT and Oxyrase reaction mixture was filtered with a 0.02 µ syringe filter. The filtrates were then analyzed by HPLC-UV-ESI-MS. Metabolites of the TNT transformation were extracted by solid phase extraction. A Sep-Pak cartridge for the extraction was conditioned immediately prior to sample extraction with 15 mL of acetonitrile followed by 30 mL of deionized water. The sample mixture was loaded onto the Sep-Pak cartridge and passed through by gravity. Metabolites were then extracted with 5 mL of acetonitrile. The extract was concentrated under a gentle stream of nitrogen to a final volume of approximately 2 mL and transferred to an amber vial. Solid phase extractions were also performed on blanks and TNT controls in the same manner as for the TNT and Oxyrase mixture.

## Instrumentation

All analyses were performed using high performance liquid chromatography with ultraviolet and electrospray mass spectrometry detection (HPLC-UV-MS). The HPLC was a HP1090 model with a built-in auto-sampler and a photodiode array detector (Hewlett Packard, Palo Alto, CA, USA).

The sample was injected onto HPLC in a volume of 20 μL. Metabolites were separated on a reversed-phase platinum $C_{18}$ column (150 mm X 4.6 mm ID) with an on-line filter kit (Alltech, Deerfield, IL, USA) using an isocratic mobile phase of 50% methanol and 50% water at a flow rate of 0.5 ml/min and ambient temperature. The UV signal was monitored at 254 and 230 nm. The electrospray MS was a HP 59987A/5989B electrospray ionization mass spectrometer (Hewlett Packard, Palo Alto, CA, USA). Negative ion mode was used and a voltage of 3.5 kV was applied to the cylinder. Pure air was used as the nebulizing and drying gas. The drying gas was heated to 200 °C and the nebulizing gas pressure was regulated at 80 psi. The quad temperature was set at 100 °C. Mass signals were scanned in the range of 50-400. HPLC-UV-ESI-MS was controlled by two personal computers with ChemStation software.

## Results and Discussion

### Identification of Metabolites

Previous work has shown that nitroaromatics have a great tendency to form negative ions due to the charge stabilizing electronegative nitro groups. For this reason, negative ion mode ESI-MS was used for detection throughout this study. ESI-MS is a very gentle ionization technique, which generally converts low molecular mass species to singly-charged deprotonated molecular ions in the negative ion mode [23]. The ESI-MS was tuned and calibrated in the negative ion mode with the standard tuning mix before any analysis. This was done to optimize instrumental parameters and calibrate the mass assignment. The filtrates and extracts of the TNT control and TNT transformation mixtures were analyzed by HPLC-UV-ESI-MS along with the TNT standard. The components were separated by HPLC and chromatograms were acquired by UV and ESI-MS detection. TNT in the control sample eluted at about 9.3 minutes, which is consistent with the TNT standard. Figure 1 shows the mass 196 chromatogram of TNT and Oxyrase transformation mixture incubated for 379 hours. Two metabolites were observed and eluted at approximately 11.0 and 11.9 minutes, while TNT was not observed in the chromatogram, indicating that TNT degraded completely in the transformation reaction. According to the mass spectra of the metabolites shown in Figure 2, molecular masses of two metabolites were determined. Metabolite 1 and metabolite 2 have the same molecular mass of 197; under negative ion conditions, $(M-H)^-$ is typically observed for nitroaromatics. The nitrogen rule of mass spectral interpretation suggested that the metabolites must have an odd number of nitrogen atoms in each molecule [24].
Oxyrase is an enzyme system derived from the cytoplasmic membrane of the microorganism, *E. coli*. Oxyrase is a biocatalytic oxygen reducing agent that provides anaerobic conditions for chemical and biological process. In anaerobic conditions, biotransformation pathways of nitroaromatics often follow reductive, oxidative or hydrolytic sequences as illustrated in Figure 3 [12]. By considering molecular masses, the nitrogen rule, and possible biodegradation pathway of TNT, standard 4-ADNT and 2-ADNT were selected and analyzed by HPLC-UV-ESI-MS in the same way as the digested TNT. The ESI mass spectra for the amino standards are shown in Figure 4. Metabolite 1 and 2 were identified as 4-ADNT and 2-ADNT, respectively by the comparisons of corresponding retention times, molecular masses, UV spectra, and mass spectra (see Figure 3 and Figure 5). No metabolites were detected in the controls and blanks. In comparison, the major metabolite identified in the degradation of TNT by FNR was 4-hydroxylamine-2,6-dintrotoluene (HADNT) with minor components identified as the amines. The FNR system represents a different enzyme and buffer system [13,22]. However, it is not clear why the metabolites formed from FNR and Oxyrase differ. ADNT is produced from reducing TNT one step further than HADNT (Fig. 3). FNR is only capable of catalyzing the first two reduction steps. Oxyrase has additional reduction capabilities for TNT than does FNR. Furthermore, since

we cannot account for all of the products of the reduction of TNT by oxyrase, it may be able to reduce TNT even further than ADNT.

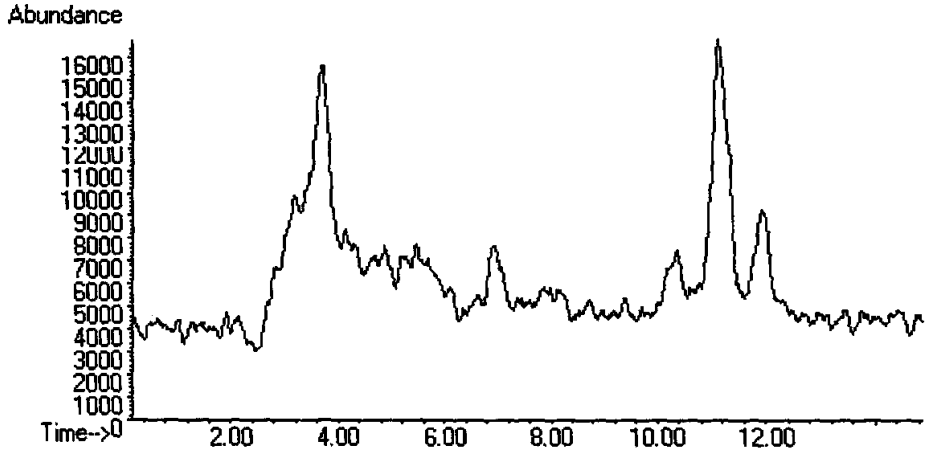


**Figure 1**. Total chromatogram ion of the organic extract of TNT transformation mixture. Components marked with * indicate metabolites.

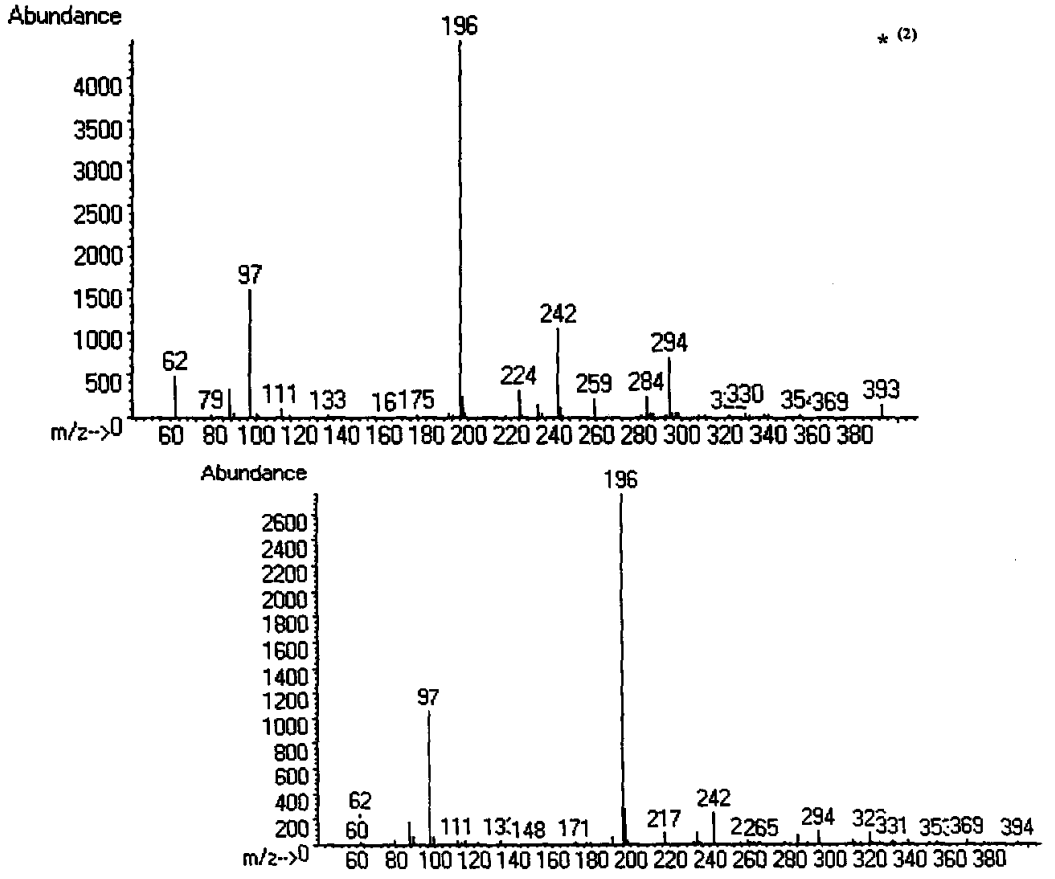


**Figure 2**. Mass spectra of (a) metabolite 1 and (b) metabolite 2

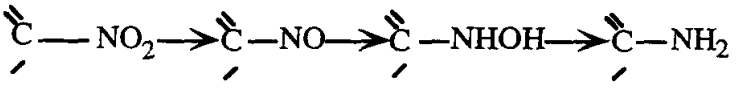


**Figure 3.** Pathway for Nitroaromatic Degradation.

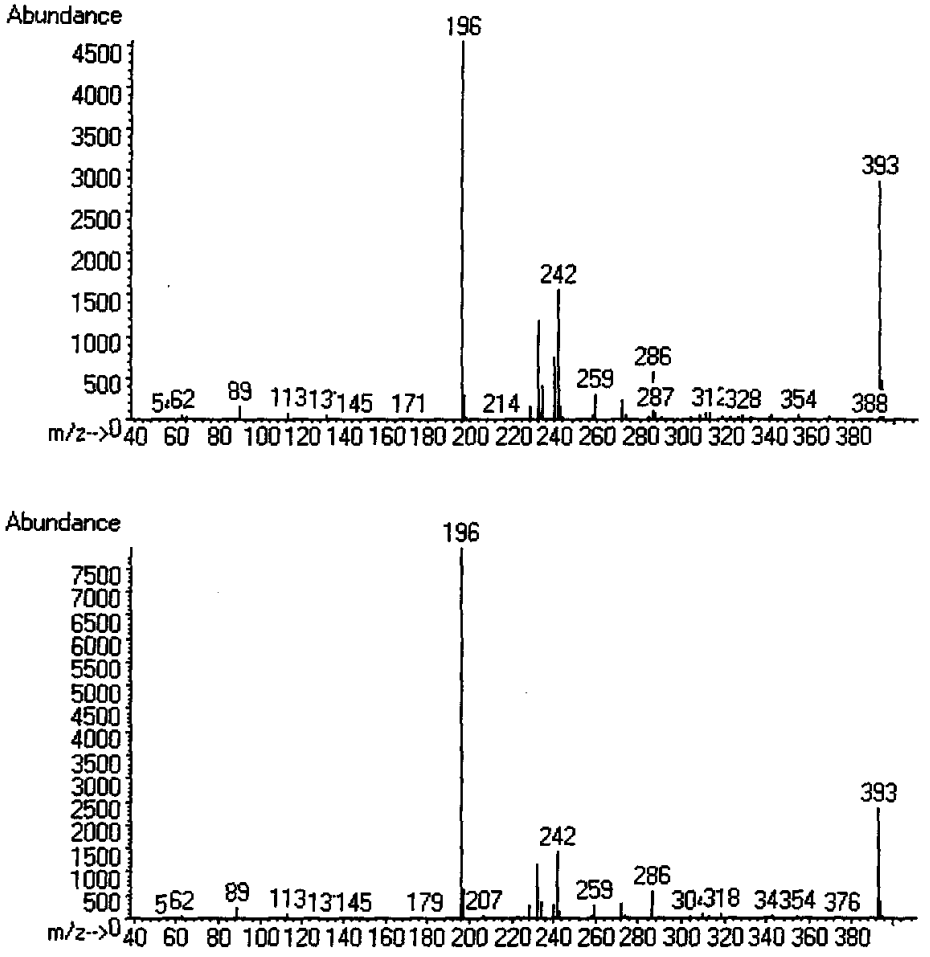


**Figure 4.** Mass Spectrum of (a) top, 4-amino-2,6-dinitrotoluene and (b) 2-amino-4,6-dinitrotoluene standards

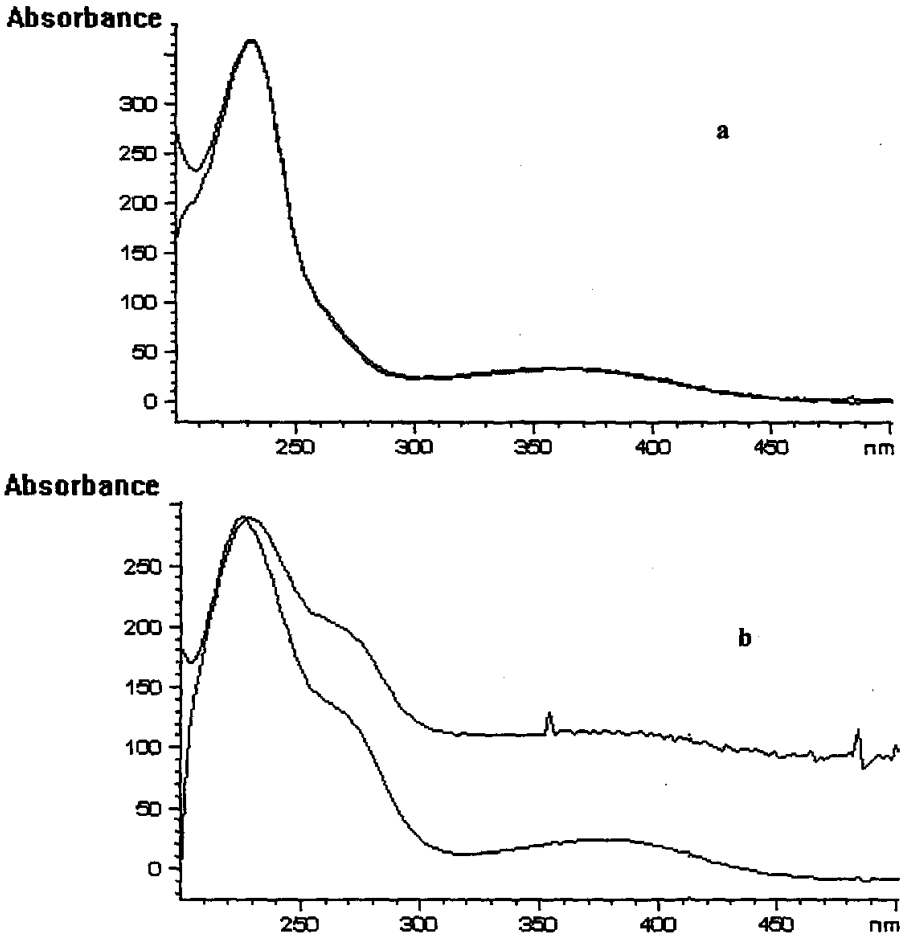


**Figure 5**. UV spectra of (a) metabolite 1 and standard 4-amino-2, 6-dinitrotoluene, (b) metabolite 2 and standard 2-amino-4, 6-dinitrotoluene.

## Kinetics of TNT transformation

The kinetic experiments of TNT transformation by Oxyrase were carried out by incubating the mixture of TNT, Oxyrase and tris/formate buffer, and the control containing only TNT and buffer at room temperature in amber vials. Aliquots were taken from the reaction mixture and the control at intervals from time 0 to 2, 4, 8, 16, 24, 48, and 379 hours. The aliquots were filtered with 0.02 μm syringe filters. The filtrates were analyzed by HPLC-UV. The kinetics of TNT transformation by Oxyrase is shown in Figure 6. TNT was transformed to a mixture of metabolites including 4-ADNT and 2-ADNT. The transformation was rapid during the beginning 24 hours, slowing down afterwards. About 1 mole of 4-ADNT and 0.5 moles of 2-ADNT was formed from every 4 moles of TNT.

## Calibration and Limits of Detection

The response of HPLC-UV-ESI-MS was calibrated with standard solutions of TNT, 4-ADNT, and 2-ADNT. Concentrations of the standards ranged from 0.5 μg/mL to 100 μg/mL corresponding to a range of material injected from 10 ng/injection to 2000 ng/injection. Calibration curves were obtained by linear regression curve fitting. The correlation coefficients of the linear regression were approximately 0.9989 to 0.9999. The limits of detection (LOD) were estimated

from the analysis of the standard solutions at various concentrations by HPLC-UV-ESI-MS. The criteria for estimating instrument detection limits was the smallest amount injected that yielded a signal-to-noise ratio greater than 3 for the chromatogram of HPLC-UV-ESI-MS. Limits of detection were estimated to be approximately 10 ng/injection for full scan. The LOD would be less using selected ion monitoring (SIM).

Enzymes and compost

Mass balance experiments conducted in composted TNT using NMR and labeled compounds compared favorably with our data (Table 1). The enzyme method for degrading TNT can be compared with a composting method used on TNT-contaminated soil. The recoveries from Oxyrase degradation of TNT were calculated from peak areas of known compounds. It is likely that the unidentified products (70% of the starting material) were so numerous and diverse that they could not be easily identified without greatly increasing the sample size. Table 1 shows a

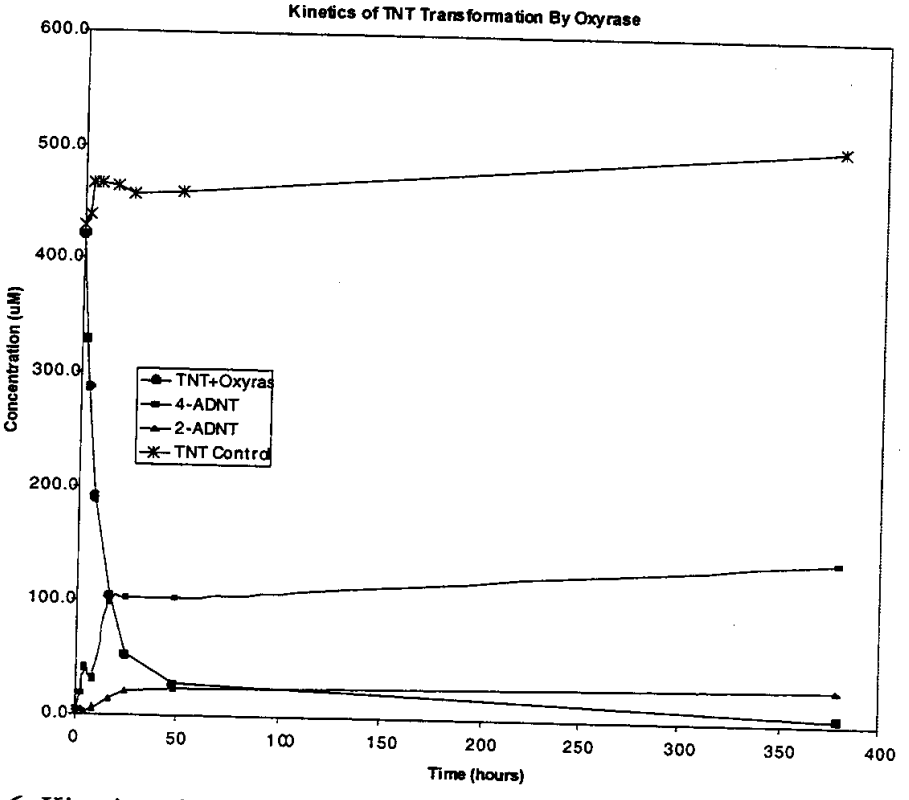


**Figure 6.** Kinetics of TNT transformation by Oxyrase at room temperature.

comparison where TNT was digested by the Oxyrase system and by composting in different experiments. Results are similar for the two processes. Both the loss of TNT and the products generated are similar, but the compost method requires the presence of other nutrients. These nutrients (fecal material and cellulose) may not be compatible with the treatment of contaminated streams even though the method is useful for treating soils. However, others have used anaerobic bioremediation techniques with living microorganisms to treat TNT contaminated water [25]. In addition, the kinetics of TNT conversion by Oxyrase is rapid (Figure 6), which supports further exploration of the enzyme method for the treatment of contaminated streams. However, lab and field conditions differ considerably and such an application should be studied in more detail.

**Table 1.** Concentrations of TNT and Its Transformation Products in Compost[1] (μg per gram compost) and Oxyrase Metabolite

| | $T_o$ TNT | $T_{20}$ TNT | % left | TNB | 4 - ADNT | 2 - ADNT | 2,6 - DANT | 2,4 - DANT | 4,4' - AZOXY | Total |
|---|---|---|---|---|---|---|---|---|---|---|
| **Oxyrase** | 500 | 10 | 2.0 | ND | 150 | 30 | ND | ND | ND | 36% |
| Compost | 26,000 | 2,190 | 8.4 | 6.2 | 936 | 343 | 12.6 | 261 | 91.6 | 13.4 % |

[1] Data from Pennington et al (8) Abbreviations include trinitrobenzene (TNB), diaminanitrotoluent (DANT) and 2,2',6,6'-tetranitro-4,4'-agoxytoluene (4,4'AZOXY).

## Conclusion

HPLC-UV-ESI-MS was utilized in the present study to characterize the transformation of TNT by Oxyrase (an enzyme from *E. coli*). Two metabolites of TNT were observed, metabolite 1 as 4-amino-2, 6-dinitrotoluene, and metabolite 2 as 2-amino-4, 6-dinitrotoluene. Both metabolites were identified by the comparisons of mass spectra, UV spectra, and retention times of the metabolites and standard compounds. The present study demonstrates that naturally occurring enzymes such as Oxyrase could potentially be applied to the remediation of TNT contamination.

## ACKNOWLEDGEMENTS

This research was funded by the Strategic Environmental Research and Development Program (SERDP). Pacific Northwest National Laboratory is a multiple program laboratory of U.S. Department of Energy operated by Battelle Memorial Institute under contract DE-AC06-76RLO 1830. Authors would like to thank Manish Shah for initiating this work, Eissa Hanna and Shannon K. Roach for their technical assistance. The authors would like to thank Kari Gaither for her assistance in editing and preparing this manuscript.

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# Appendix B

# Toxicity of TNT Degradation Components

# Appendix B
# Toxicity of TNT Degradation Components

## July 8 Report from USU: Preliminary Ecotoxicity Data for TNT Degredation

The folowing tests were performed and results are described in this letter report.

### *Ceriodaphnia dubia* Tests

*Ceriodaphnia* toxicity tests were performed in accordance with Coastal Bioanalysts' protocols. Protocols comply with methods described in EPA/600/4-90/027. The general test designs and methods are described below; test-specific details can be found in subsequent portions of the report and/or accompanying copies of laboratory bench sheets.

Dilution water consists of moderately-hard synthetic freshwater made up with ASTM Type I de-ionized water and ACS reagent-grade chemicals. Test organisms are obtained from in-house cultures and are cultured in the same type water used as test dilution water. Cladocerans are <24-h old at the start of tests.

The tests consist of randomized block designs in which four replicates of five organisms each are exposed to sample serially diluted (e.g., 100%, 50%, 25%, 12.5%, 6.3% and 0%) with dilution water. Solutions are prepared in a single flask for all replicates and tests to decrease within – and between-test variability. Test chambers of borosilicate glass contain 15 mL each of test solution. Temperature, pH, conductivity and dissolved oxygen concentration are measured daily or at the beginning and end of test in one replicate of each treatment. Survival is recorded for all chambers daily. Tests are maintained at 20±1°C.

LC50s are calculated for tests exhibiting sufficient toxicity using the Trimmed Spearman-Karber (Montana State University program), Probit (EPA program) or Linear Interpolation (graphical) methods are described in EPA/600/4-90/027. Printouts of program results are attached as appendices as applicable.

Acute toxicity tests using potassium chloride as the reference toxicant are performed monthly on each species of animal cultured in-house to insure animal vigor and validity of test materials and methods. In addition, each test is monitored by a QA/QC officer to insure adherence to test protocols.

### *Microtox* Tests

Microtox tests were performed in strict accordance with the specific protocols published by AZUR Environmental (Carlsbad, CA). Tests were performed on a model 2055 analyzer. All samples were osmotically adjusted by addition of 200 mg NaCl ACS reagent grade) per 10 mL of solution. Bacteria (Lot ACV017-2R, exp. date 11/2000) were stored frozen until reconstituted immediately prior to testing. Reference toxicant tests using $ZnSO_4$ $2H_2O$ were run periodically throughout the sample runs and compared well with ZAUR acceptance criteria for the lot.

Utah State
UNIVERSITY

Steven D. Aust, Professor
Chemistry and Biochemistry
Biotechnology Center
Logan UT 84322-4705
Telephone: (435) 797-2730
FAX: (435) 797-2755
Email: sdaust@cc.usu.edu

July 8, 1999

Dr. Steve Goheen
Pacific Northwest Laboratory, Battelle
PO Box 999, MS P7-41
Richland, WA 99352

Dear Dr. Goheen,

Herewith is a report on the status of our project entitled "Enzymatic Degradation of Nitroenergetic Compounds".

Task 1. Characterize the products of TNT reduction by xanthine oxidase.

Samples resulting from the reduction of TNT with xanthine and xanthine oxidase have been submitted to the mass spectroscopy laboratory. No results have been reported as the thermospray unit has become clogged recently and the samples have not been analyzed. A further complication is the low concentration of the metabolites and their lack of stability. New samples will therefore need to be prepared once the thermospray has been repaired. Results should be forthcoming within the next couple of weeks.

Task 2. Evaluate the ecotoxicology properties of TNT, xanthine oxidase-generated TNT products and metabolites formed during biodegradation of xanthine oxidase-generated products by *P. chrysosporium*, a mixed bacterial culture and soil microbes.

The results for ecotoxicity of TNT and xanthine oxidase-generated TNT products were reported by Coastal Bioanalyst Incorporated. The results are summarized in Table 1.

Table 1. Ecotoxicity of TNT and xanthine oxidase-generated TNT products.

| | | Toxicity (units)[a] | | | |
|---|---|---|---|---|---|
| | | *Ceridaphnia dubia* Assay | | Microtox Assay | |
| Sample[b] | Replicate | Toxicity | Average | Toxicity | Average |
| TNT | 1 | 14.6 | 14 | 64.1 | 42 |
| | 2 | 12.7 | | 19.6 | |
| TNT, xanthine, buffer | 1 | 13.7 | 13 | 13.2 | 18 |
| | 2 | 12.3 | | 22.1 | |
| xanthine, buffer, | 1 | 21.6 | | 1.7 | 1.7 |
| xanthine oxidase | 2 | --- | | --- | |
| TNT, xanthine,buffer, | 1 | 22.6 | 21 ± 2 | 7.3 | 12 ± 6 |
| xanthine oxidase | 2 | 18.7 | | 18.2 | |
| | 3 | 22.2 | | 12.1 | |

[a] One unit is equal to 100/LC50.

[b]Complete reactions contained TNT (111 mg/L), sodium phosphate buffer (22 mM, pH 7.8), xanthine (2.8 mM) and xanthine oxidase (0.09 U/ml). Reactions were made anaerobic by purging with argon prior to athe ddition of xanthine oxidase.

One of the control sample bottles was broken during shipment. Also, there is high variability in the toxicity of TNT by Microtox assay. New samples will be sent with the next batch of samples to confirm the toxicity of these two samples. In general, the reduction of TNT by xanthine oxidase did not seem to significantly reduce toxicity to *Ceriodaphnia dubia.* Comparing the toxicity of TNT to *C. dubia* to the toxicity of TNT in the presence of xanthine and the reaction buffer demonstrated that xanthine and the phosphate buffer (22 mM, pH 7.8) contributed to the observed toxicity. The toxicity resulting upon the addition of TNT was about the same as that found with just xanthine and xanthine oxidase. The results suggested that any reduction in toxicity to the *C. dubia* associated with the reduction of TNT was masked by the toxicity associated with oxidation of xanthine.

The results of the Microtox assay are difficult to analyze due to the large variation in the toxicity of TNT alone. However, if this anomoly (replicate) is removed, which seems reasonable as the toxicity in the second control (TNT and xanthine but not xanthine oxidase) was about the same (18) as for TNT alone in replicate 2 (19.6). The enzymatic reaction without TNT did not seem to affect the Microtox assay (1.7) although this must be repeated as one sample was lost. The toxicity resulting from the complete reaction as somewhat less (12 ±6) than for the controls, suggesting that the action of xanthine oxidase decreased toxicity.

The xanthine oxidase reduced TNT products are currently being incubated with wastewater bacteria from the Logan Waste Treatment Facility and with the white-rot fungus, *Phanerochaete chrysosporium*. Ecotoxicity of the fluid after 30 day incubations will be determined Coastal Bioanalyst Inc. in August.

The concentrations of a chemical that inhibits 50% of the activity in the *C. dabia* or Microtox assays are often reported. This value can be easily determined by dividing the initial TNT concentration (111 mg/L) by the toxicity units. This conversion is reported in Table 2.

Table 2. Ecotoxicity of TNT and xanthine oxidase-generated TNT products.

| | | $EC_{50}$[a] | | | |
|---|---|---|---|---|---|
| | | *Ceridaphnia dubia* Assay | | Microtox Assay | |
| Sample | Replicate | Conc (μM) | Average | Conc. (μM) | Average |
| TNT | 1 | 33.5 | 36 | 7.6 | 16 |
| | 2 | 38.5 | | 24.9 | |
| TNT, xanthine, buffer | 1 | 35.7 | 38 | 37.0 | 30 |
| | 2 | 39.7 | | 22.1 | |
| TNT, xanthine, buffer[b], xanthine oxidase | 1 | 21.6 | 23 ± 2 | 67.0 | 45 ± 20 |
| | 2 | 26.2 | | 26.9 | |
| | 3 | 22.0 | | 40.4 | |

[a]EC50 is concentration of TNT or accumulative TNT metabolites (μM) that inhibited 50% of activity.

[b]Complete reactions contained TNT (111 mg/L), sodium phosphate buffer (22 mM, pH 7.8), xanthine (2.8 mM) and xanthine oxidase (0.09 U/ml). Reactions were made anaerobic by purging with argon prior to the addition of xanthine oxidase.

Note: An EC50 concentration for the xanthine/xanthine oxidase control should not be calculated because no TNT or TNT metabolites were present in the sample.

This conversion allows comparison of the present results to those reported by Drzyzga *et.al.* (1995, Arch. Environ. Toxicol., 28, 229-235). They reported the toxicity of TNT and its major metabolites, DNT, and other explosives including RDX, HMX and PETN by the Microtox assay. The present result for TNT (~23 μM) was similar to that previously reported (16 μM). More importantly, Drzyzga *et.al.* (1995) reported that amino- (>380 and 108 μM), diamino- (287 and >598 μM) and triamino-metabolites (738 μM) from TNT were significantly less toxicity than TNT as measured by the Microtox assay. We observed the toxicity was somewhat decreased upon reduction but the decrease was not as dramatic as would have been expected based upon the Drzyzga *et.al.* (1995) results. This could be due to the fact that the enzyme alone with xanthine resulted in some toxicity.

The ecotoxicity of DNT and xanthine oxidase-generated DNT products is also being investigated. This was done since we are contracted to investigate the mineralization of DNT and the product of DNT reduction by xanthine oxidase by bacteria and a white-rot fungus (Task 3). The ecotoxicity of DNT and reduced DNT are reported in Table 3.

Table 3. Ecotoxicity of DNT and xanthine oxidase-generated DNT products.

| | | Toxicity (units)[a] | | | |
|---|---|---|---|---|---|
| | | *Ceridaphnia dubia* Assay | | Microtox Assay | |
| Sample | Replicate | Toxicity | Average | Toxicity | Average |
| DNT | 1 | 4.1 | 5 | 1.4 | 1.4 |
| | 2 | 5.6 | | 1.4 | |
| DNT, xanthine, buffer | 1 | 6.9 | 7 | 2.0 | 1.7 |
| | 2 | 6.8 | | 1.4 | |
| xanthine,buffer, | 1 | 21.6 | 22 | 1.7 | 1.7 |
| xanthine oxidase | 2 | --- | | --- | |
| DNT, xanthine, buffer, | 1 | 33.6 | $46 \pm 27$ | 3.2 | $2.6 \pm 0.6$ |
| xanthine oxidase [b] | 2 | 76.9 | | 2.2 | |
| | 3 | 27.9 | | 2.3 | |

[a] One unit is equal to 100/LC50.

[b]Complete reactions contained DNT (111 mg/L), sodium phosphate buffer (22 mM, pH 7.8), xanthine (2.8 mM) and xanthine oxidase (0.09 U/ml). Reactions were made anaerobic by purging with argon prior to addition of xanthine oxidase.

DNT was much less toxic than TNT in both assays. Also, the presence of xanthine and the phosphate buffer did not significant increase the toxicity of DNT. However, reduction of DNT by xanthine oxidase dramatically increased the toxicity to *C. dubia* and mildly increased the toxicity as measured by the Microtox assay. The DNT or total DNT metabolite concentration necessary to inhibit 50% of the activity in the two assays are reported in Table 4.

Table 4. Ecotoxicity of DNT and xanthine oxidase-generated DNT products.

| | | $EC_{50}$[a] | | | |
|---|---|---|---|---|---|
| | | *Ceridaphnia dubia* Assay | | Microtox Assay | |
| Sample | Replicate | Conc (μM) | Average | Conc. (μM) | Average |
| DNT | 1 | 149 | 129 | 436 | 436 |
| | 2 | 109 | | 436 | |
| DNT, xanthine, buffer | 1 | 88.4 | 89 | 305 | 370 |
| | 2 | 89.7 | | 436 | |
| DNT, xanthine, buffer, xanthine oxidase[b] | 1 | 18.1 | $16 \pm 7$ | 278 | $244 \pm 47$ |
| | 2 | 7.9 | | 265 | |
| | 3 | 21.9 | | 359 | |

[a]EC50 is concentration of DNT or accumulative DNT metabolites (μM) that inhibited 50% of activity.

[b]Reaction contained DNT (111 mg/L), sodium phosphate buffer (22 mM, pH 7.8), xanthine (2.8 mM) and xanthine oxidase (0.09 U/ml). Reactions were made anaerobic by purging with argon prior to addition of xanthine oxidase.

Note: An EC50 concentration for the xanthine/xanthine oxidase control should not be calculated because no DNT or DNT metabolites were present in the sample.

Drzyzga *et.al* (1995) reported that 248 μM DNT was required to inhibit 50% of the activity in the Microtox assay while we have found that almost double this concentration was required.

Task 3. Quantitate the mineralization of DNT and the products of xanthine oxidase reduced DNT by *Phanerochaete chrysosporium*, a mixed culture of bacteria from a local waste treatment facility and soil microbes.

The reduction of DNT by xanthine oxidase has been performed as part of Task 2. This was required to produce the reduced DNT product for ecotoxicity evaluation. However, mineralization of DNT and the products of xanthine oxidase reduced DNT is indefinitely delayed as the radiolabelled DNT is currently backordered at Chemsyn Laboratories. Once the $^{14}$C-DNT arrives this task should require approximately 6 weeks to complete.

Task 4. Develop a method for reduction of high concentrations of TNT by oxyrase.

Research on the reduction of high concentrations of TNT by oxyrase has only just begun. The optimal concentration of organic solvent for the reduction of TNT by oxyrase is being investigated first. Oxyrase, Inc. has not performed research on the stability of oxyrase in organic solvents over extended periods of time (personal

communications with Dr. Copeland, President of Oxyrase, Inc.). Low solvent concentrations (1-5%), should increase the reduction of TNT. However, higher solvent concentrations (15-20%) may denature the oxyrase enzyme. Several experiments have been designed and will be performed within the next 6 weeks to answer some of these questions.

Task 5. Investigate pentaerythritol (PETN) and trinitroglycerin (GTN) degradation by hydrolases and oxidoreductases.

A Beckman System Gold HPLC system with a Beckman autosampler and a Supelco LC-18 analytical column (4.6 mm x 25 cm, 5 μm pore size) is being used to analyze PETN. The mobile phase initially consisted of methanol:water (55:45) at a flow rate of 1.0 ml/minute. The methanol fraction was increased at a rate of 2%/minute to a maximum of 70%. The PETN peak were monitored at 215 nm and quantitated by comparison with standards of known concentrations.

The only enzyme found so far to degrade PETN is xanthine oxidase (Figure 1). PETN was completely degraded within 16 hours in the presence of the enzyme while no PETN was degraded in its absence. The concentration of one metabolite was observed to initial increase then decrease. This may be the trinitrate but this has not been confirmed. No PETN degradation with the extracellular enzymes from *P. chrysosporium* (lignin peroxidase, manganese-dependent peroxidase, cellobiose dehydrogenase) has yet been observed. However, none of the reaction conditions are optimized as yet so all findings should be considered very preliminary.

Enzymes chosen for this study include the major enzymes involved in the oxidation and reductive reactions white-rot fungi use to degrade pollutants (CDH, peroxidases, laccase), two cellulases, two esterases, xanthine oxidase and one nitrate reductase. The fungal enzymes were chosen since they have been implicated in the degradation of many other pollutants. PETN degradation by cellulases will be evaluated since this is a major class of hydrolases. The nitroester bond of PETN should be susceptible to hydrolytic reactions. PETN degradation by esterases will be evaluated as the nitroester bond is very similar in size and bond angles to carbon-ester bonds. This similarity may be sufficient to allow the nitroester to access the enzyme pocket of the esterases and become cleaved. PETN degradation by reductases has been reported. The reduction of the nitroester bond followed by hydrolysis to liberate nitrite was suggested by Binks *et.al.* (1996, Appl. Environ. Microbiol., 62, 1214-1219). A similar mechanism for GTN degradation was reported by Servent *et.al.* (1991, Biochim. Biophys. Acta, 1074, 320-325). Degradation of GTN by these same enzymes will be performed upon completion of the PETN degradation research.

It would seem that there are two reasons to investigate enzyme-catalyzed degradations. One is in case a very effective and relatively inexpensive enzyme might be found. Secondly, the results may suggest organisms and/or conditions for effective biodegradation.

In summary, the project is going very well, although we are hampered by the late delivery of $^{14}$C-DNT. Hopefully it will be here shortly.

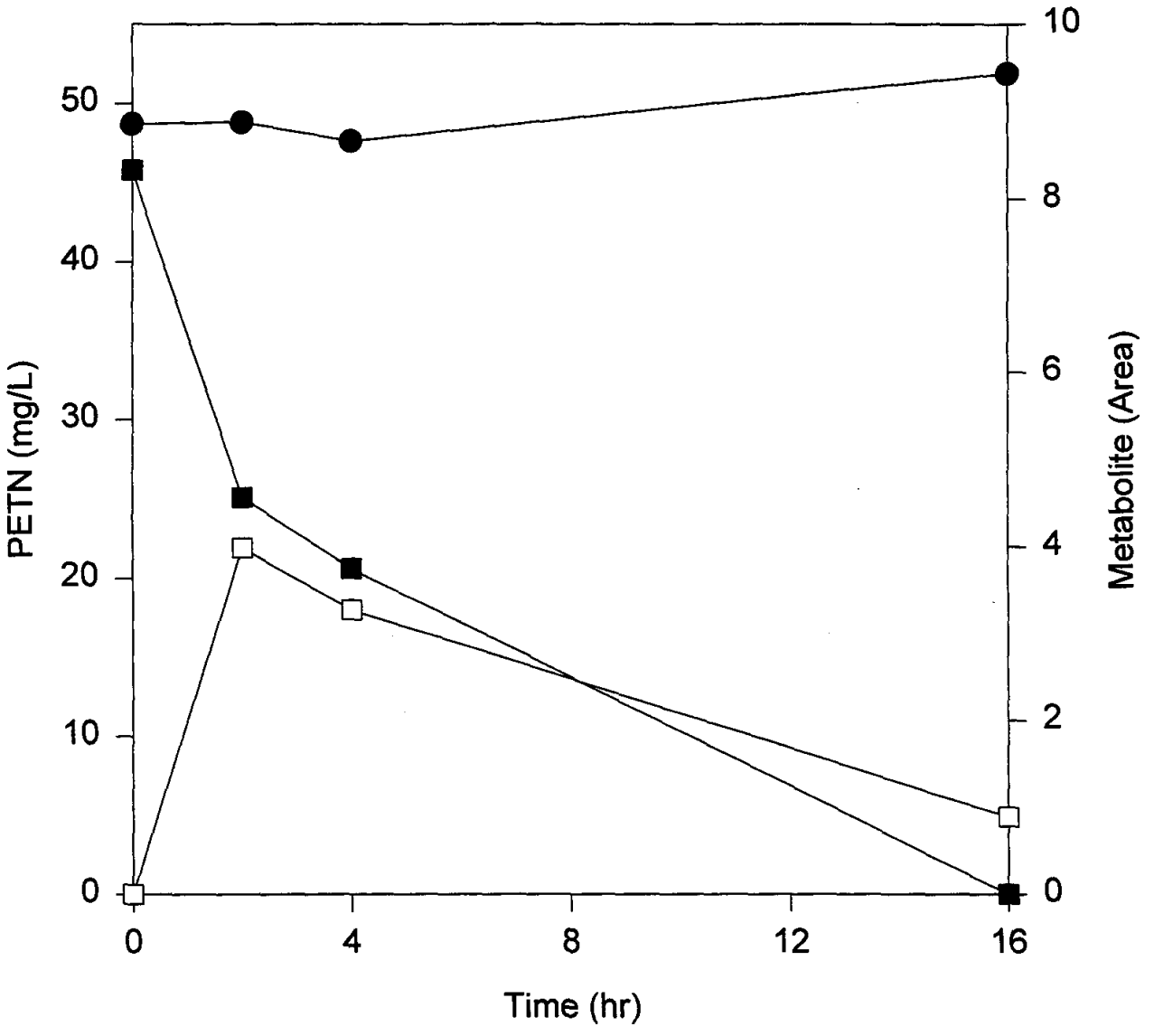
We hope this interim report will provide up-to-the-minute results. If you have any questions please contact me. We don't need to be listed as an author in your report but would appreciate an acknowledgement such that our contribution can be determined.

Sincerely,

Steven D. Aust, Ph.D.
Professor

tm

Figure 1. PETN degradation by xanthine oxidase.




Complete reactions contained phosphate buffer (22 mM, pH 7.5), xanthine (10 mM) and xanthine oxidase (0.13 U/ml). PETN concentration in the absence (closed circle) and presence (closed square) of xanthine oxidase and one metabolite formed in the presence of xanthine oxidase (open square) are shown.

# Appendix C

## The Influence of Acetone on Enzymes that Reduce TNT

# Appendix C

## The Influence of Acetone on Enzymes that Reduce TNT

Summary:

This appendix is a letter report from Utah State University from August 5. The main focus of this report is on acetone. Acetone solubilizes the explosives TNT< REX, and HMX effectively. Some enxymes can tolerate low concentrations of organic solvent. However, this report shows that acetone severely inhibits the TNT reduction process by Oxyrase.

Utah State
UNIVERSITY

Steven D. Aust, Professor
Chemistry and Biochemistry
Biotechnology Center
Logan UT 84322-4705
Telephone: (435) 797-2730
FAX: (435) 797-2755
Email: sdaust@cc.usu.edu

August 5, 1999

Steve Goheen
Pacific Northwest Laboratory, Battelle
PO Box 999, MS P7-41
Richland, WA 99352

Dear Dr. Goheen,

Herewith is a status report on our project entitled "Enzymatic Degradation of Nitroenergetic Compounds".

Task 1. Characterize the products of TNT reduction by xanthine oxidase.

Three more experiments have been conducted in attempts to produce sufficient quantities of the transcient TNT metabolites formed during the reduction of TNT by xanthine oxidase. The only detectable metabolites formed were the hydroxylamino-dinitrotoluenes and aminodinitrotoluenes. Further work needed to complete Task 1 has been postponed until Task 4 is completed.

Task 2. Evaluate the ecotoxicology properties of TNT, xanthine oxidase-generated TNT products and metabolites formed during biodegradation of xanthine oxidase-generated products by *P. chrysosporium* and a mixed bacterial culture.

Xanthine oxidase-generated TNT products are currently being incubated with *P. chrysosporium* under ligninolytic and nonligninolytic conditions as well as with a mixed bacterial culture. These incubations will be concluded on August 5, 1999. At that time the resulting product will be characterized by HPLC and sent to Coastal Bioanalysts for evaluation of the ecotoxicological properties. The relative ecotoxicity of the various mixtures previously tested by Coastal Bioanalysts (TNT, TNT & xanthine, xanthine & xanthine oxidase, and the complete reaction mixture) will be determined by comparing the germination of tomato seeds in soil wetted to its 100% water holding capacity (0.67 g solution/g soil) with the various solutions.

Task 3. Quantitate the mineralization of DNT and the products of xanthine oxidase reduced DNT by *Phanerochaete chrysosporium*, a mixed culture of bacteria from a local waste treatment facility and soil microbes.

Procurement of 14C-DNT from Chemsyn Laboratories has been a problem. The material has been repeatedly backordered. In the last phone call, the Vice President of Chemsyn indicated that it might be ready by the middle of this week. This will cause a significant delay. Once the 14C-DNT arrives this task should require approximately 6 weeks to complete.

Task 4. Develop a method for reduction of high concentrations of TNT by oxyrase.

The bulk of the research this month has been spent on characterizing the reductive reaction of oxyrase. The Km of oxyrase for lactate was found to be approximately 1 mM (Figure 1). Further, reduction rates by oxyrase were relatively unaffected when lactate was present in concentrations above 10 mM. The oxyrase (1% by volume) used in this experiment yielded a Vmax of approximately 55 nmole/min or 80 μmole/day. Initially, 24 hour incubations will be conducted with the proposed bioreactor so 100 mM lactate was chosen as the amount to be added. However, in a continuous flow system, the lactate concentration can be kept at approximately 10 mM.

The stability of oxyrase in various concentrations of acetone was investigated. Addition of an organic solvent was necessary as the carrier solvent of TNT. Acetone was chosen because approximately 5% solutions of TNT can be prepared in acetone. Thus, the amount of organic solvent in the reactor can be minimized. Further, it was proposed that addition of acetone to the bioreactor would increase the solubility of TNT and thus its reduction rate by oxyrase. However, oxyrase proved to be relatively unstable in acetone containing solutions (Figure 2). Oxyrase lost ~12% of it activity in the solution that contained as little as 2% acetone. Therefore, the concentration of acetone in the proposed bioreactor should be kept to a minimum. Acetone can not be eliminated because the TNT was added in an acetone solution. TNT is most soluble in acetone making it the best choice for the organic. A concentration of 2% was chosen as a maximum allowable limit for the proposed bioreactor.

The stability of oxyrase versus time in a 2% acetone solution was also investigated (Figure 3). The enzyme only lost 5% of its activity during the first 4 hours incubation but lost ~60% over a period of three days. This is a concern since the enzyme would need to be active for much longer periods of time in the proposed bioreactor. Oxyrase is a membrane bound enzyme complex and increased concentration of the membranes may decrease the effects of the acetone. However, this has yet to be evaluated.

The equilibrium concentration of TNT in aqueous solutions containing various concentrations of acetone is shown in Figure 4. It was much more efficient to study TNT reduction by oxyrase when TNT was not added in excess of its solubility limit. A TNT

concentration of ~120 mg/L was found in solutions containing 2% acetone. This was the amount of TNT added to initially characterize the TNT reduction rate by oxyrase.

TNT reduction was directly correlated with the amount of oxyrase present in the reaction (Figure 5). Approximately 35 mg/L/hr TNT was consumed in the presence of 2.0 U/ml oxyrase. In these reactions, TNT was first reduced to hydroxyamino-dinitrotoluenes and then to aminodinitrotoluenes, just as was observed with xanthine oxidase. No other TNT-metabolites were observed.

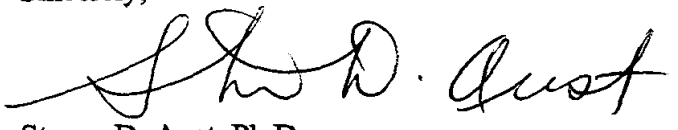
A target degradation rate of 41 mg/L/hr was initially proposed for the reactor. Thus the proposed bioreactor will contain 2.0 U/L oxyrase, 100 mM lactate, 1000 mg/L TNT, 1% acetone, in Tris buffer (10 mM, pH 8.4). Oxyrase will be separated from the reaction solution every 24 hours by filtration and resuspended in a fresh solution containing the mixture mentioned above. A small aliquot of the suspended mixture will be tested for oxyrase activity. This will be done by determining the ability of oxyrase to reduce dichloroindophenol. The TNT concentration in the reaction supernatent collected during the filtration will be determined. The next objective is to determine the sustained ability of oxyrase to degrade TNT in this system.

Task 5. Investigate pentaerythritol (PETN) and trinitroglycerin (GTN) degradation by hydrolases and oxidoreductases.

There is no new data to report for this task at present.

We hope this information is suitable. If you have any questions or need additional information please give me a call.

Sincerely,



Steven D. Aust, Ph.D.
Professor

tm

enclosures

Figure Legends

Figure 1. Determination of Km for lactate oxidation by oxyrase.
Oxyrase (0.06 U) was incubated with dichlorophenyl indophenol (0.2 mM) and sodium lactate (1.0, 2.0, 5.0, 10.0, 20.0, 30.0, 40.0 or 50.0 mM) in Tris buffer (50 mM, pH 8.4). Activity was based upon the DCPIP reduction rate measured spectrophotometrically at 522 nm. Data represents the average and standard deviation of triplicate analyses.

Figure 2. Effect of acetone concentration on oxyrase activity.
Oxyrase (0.12 U) was incubated with dichlorophenyl indophenol (0.2 mM), sodium lactate (100 mM) and acetone (0, 1, 2, 5, 7, 10, 12, 15, 17, 20%) in Tris buffer (50 mM, pH 8.4). Activity was based upon initial DCPIP reduction rate measured at 522 nm. Data represents the average and standard deviation of triplicate analyses.

Figure 3. Effect of acetone concentration on oxyrase stability over time.
Oxyrase (0.10 U/ml) was incubated with dichlorophenyl indophenol (0.2 mM), sodium lactate (100 mM) and acetone (2%) in Tris buffer (50 mM, pH 8.4). Aliquots (1.0 ml) were periodically collected and activity determined based upon initial DCPIP reduction rate measured at 522 nm. Data represents the average and standard deviation of triplicate analyses.

Figure 4. Effect of acetone concentration on TNT solubility in water.
TNT (1.0 mg) was incubated 24 hours in water containing acetone (1, 2, 4, 6, 8,10, 15 and 20%). Aliquots (0.1 ml) of each solution was decanted and analyzed for TNT concentration by HPLC. Data represents the average and standard deviation of triplicate analyses.

Figure 5. Effect of oxyrase concentration on TNT reduction.
Oxyrase (0.1, 0.5, 1.0, 1.5, 2.0 U/ml) was incubated with TNT (120 mg/L), sodium lactate (100 mM) and acetone (2%) in Tris buffer (50 mM, pH 8.4). Aliquots (0.05 ml) were periodically collected and analyzed for TNT concentration by HPLC. Data represents the average and standard deviation of triplicate analyses.

Figure 1

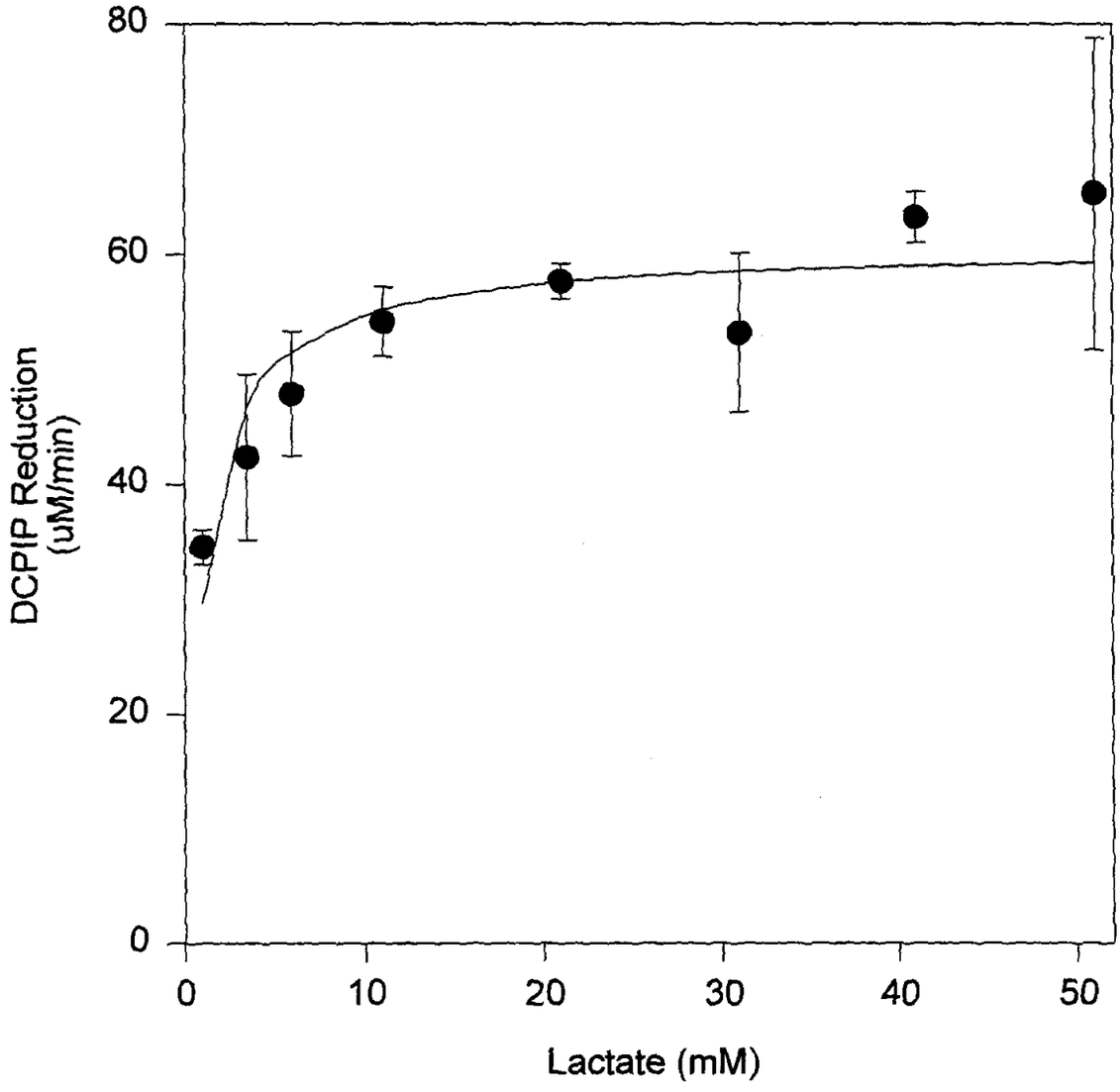


Figure 2

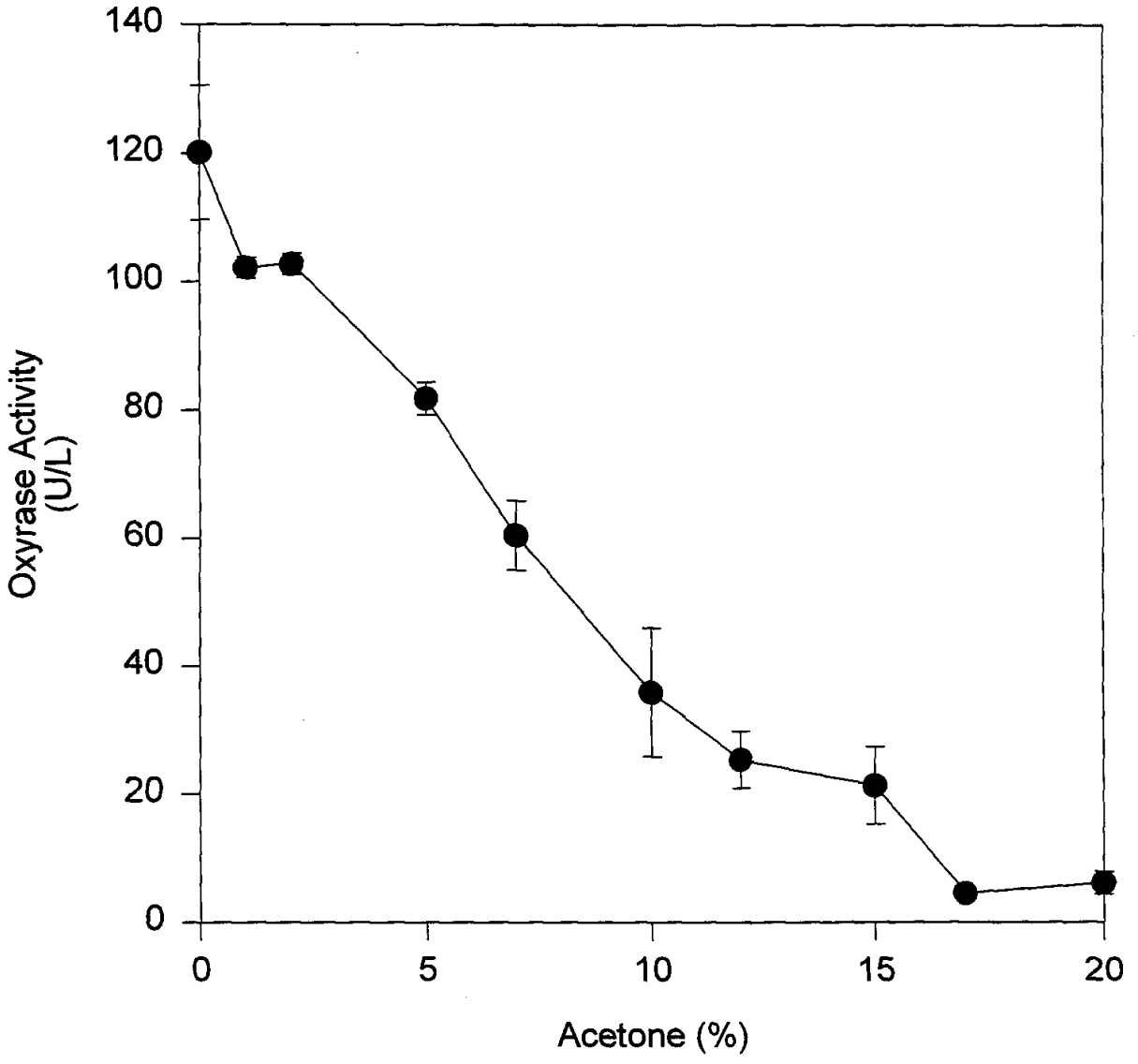

140
120
100
80
60
40
20
0
Oxyrase Activity (U/L)
0
5
10
15
20
Acetone (%)

Figure 3

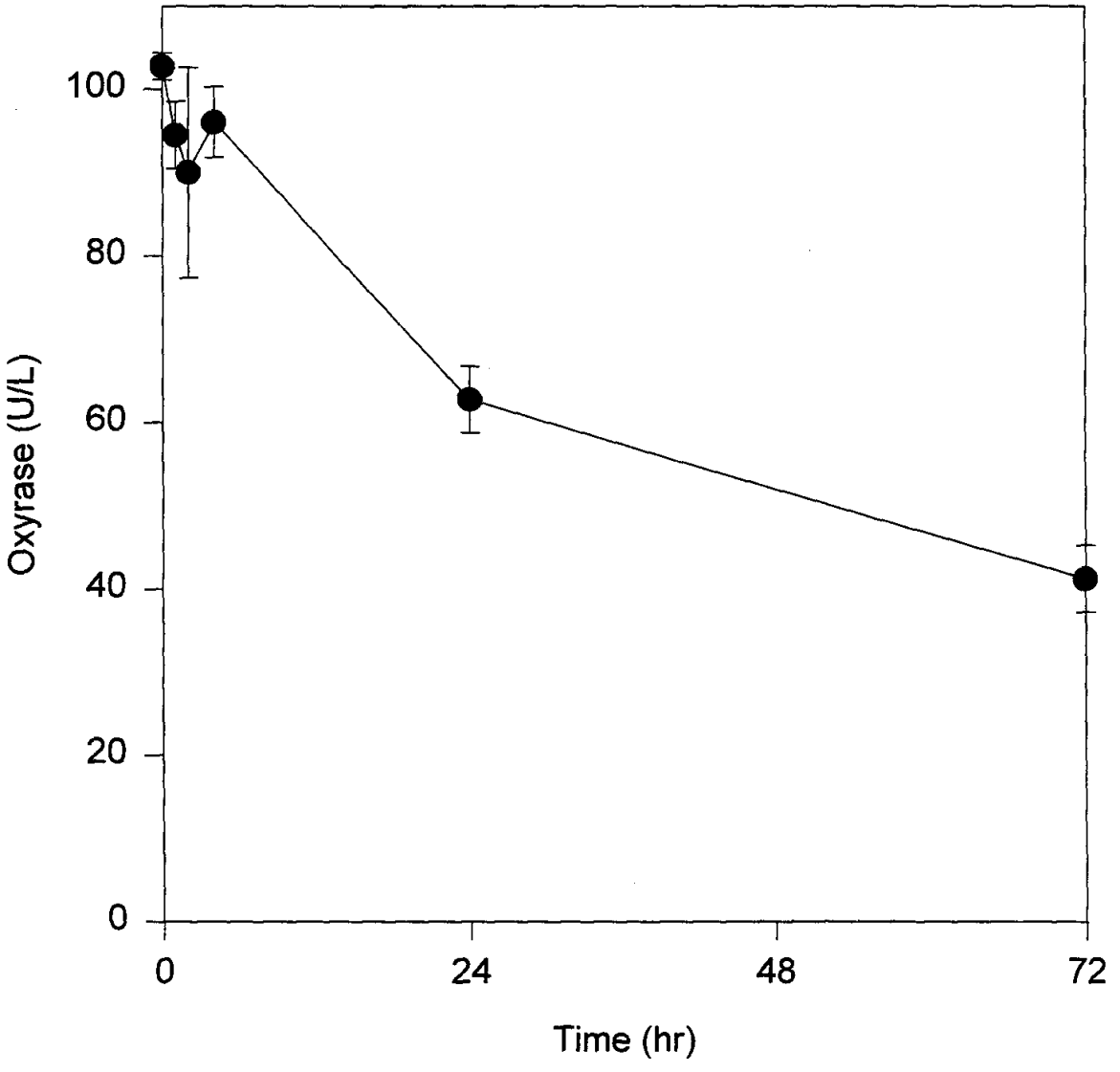


Figure 4

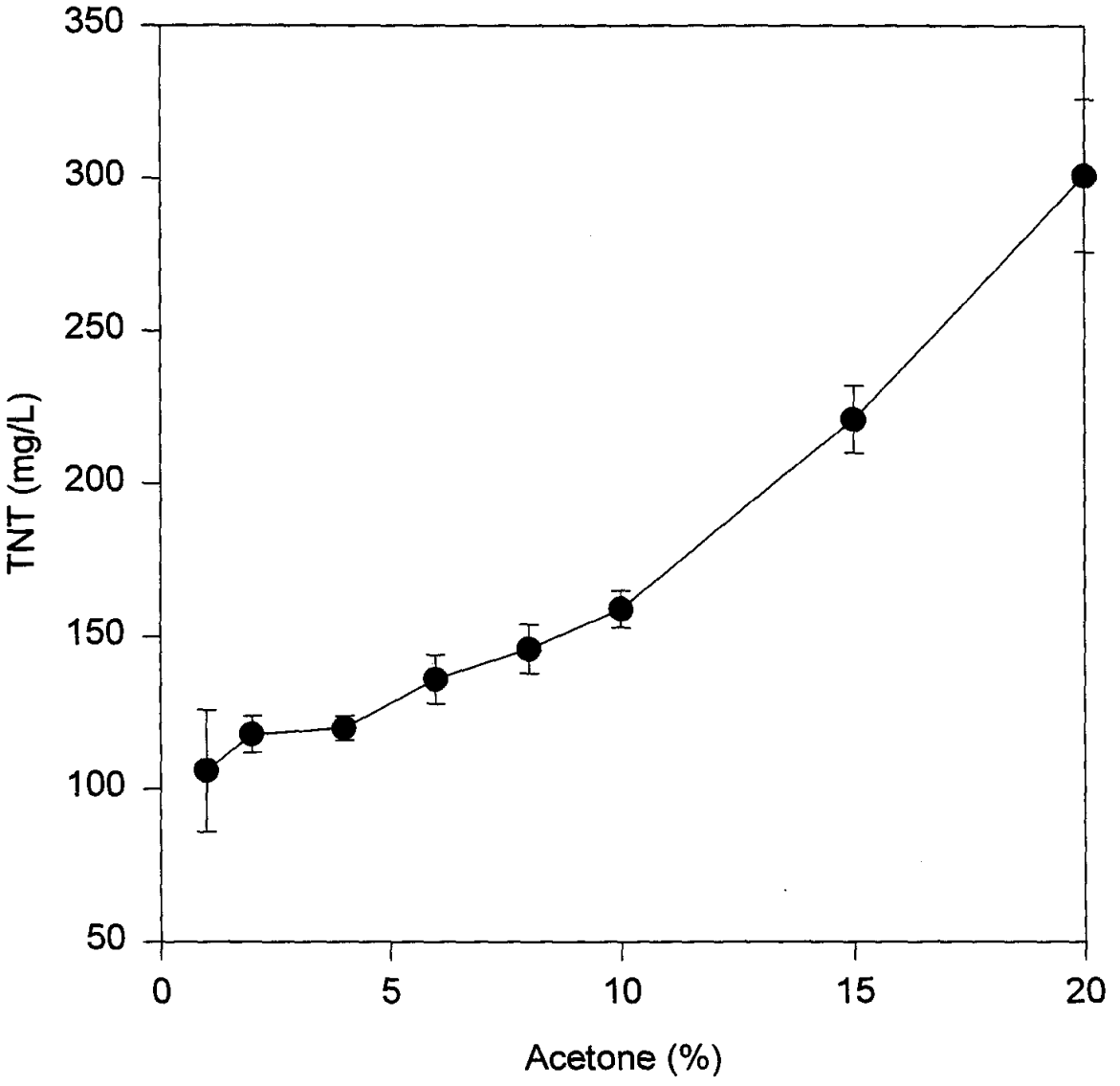
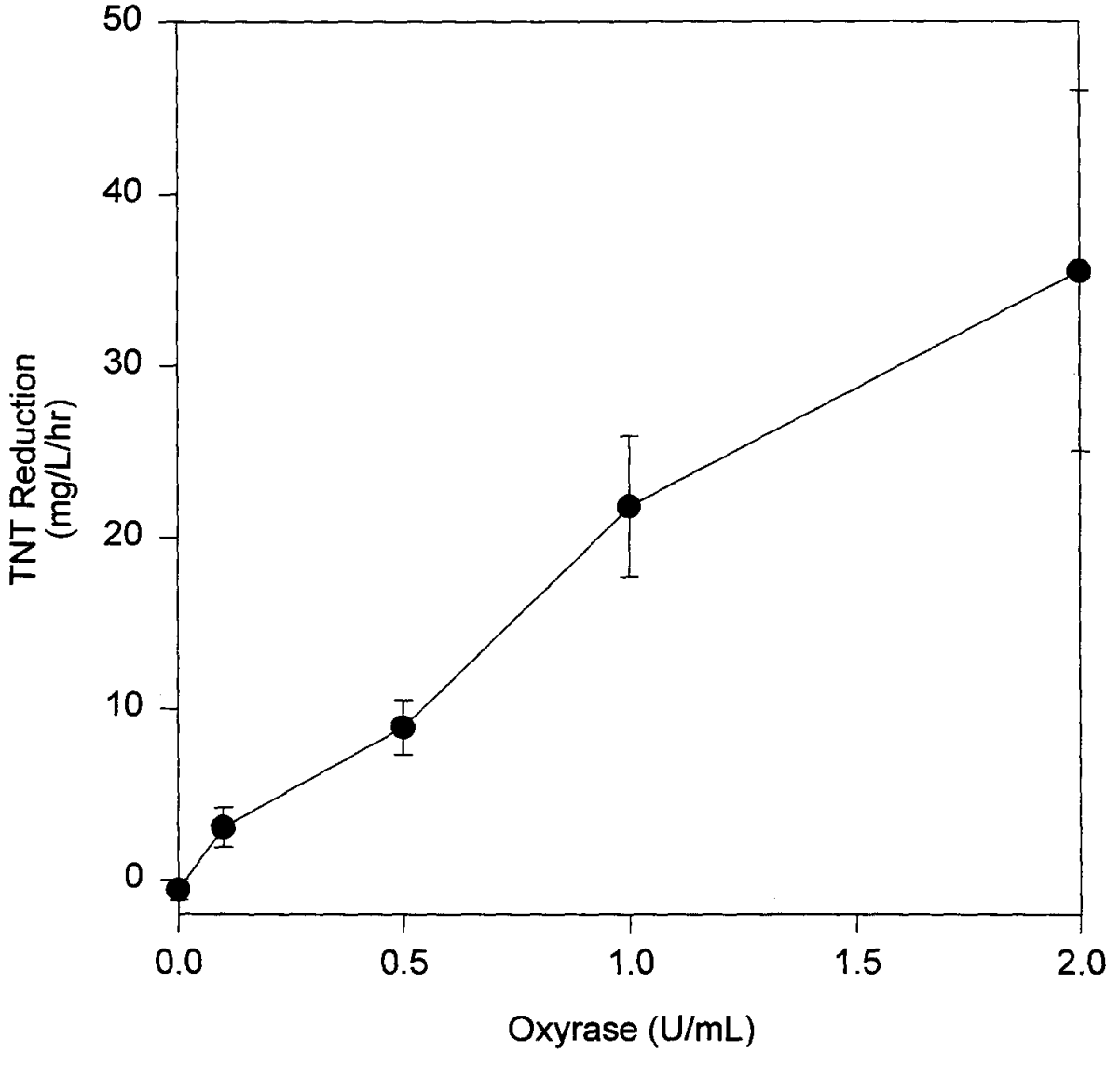


Figure 5



# Appendix D

## Additional Toxicity Testing of TNT Metabolites.

# Appendix D

## Additional Toxicity Testing of TNT Metabolites

Summary:
This appendix gives data subsequent to that of Appendix B. Here, digestion of TNT is first by the enzyme, xanthine oxidase, and second either by organisms from a waste water treatment plant, or white rot fungus. The results of this work suggest the following:

- Products from digesting TNT with xanthine oxidase are toxic.
- Both fungus and waste treatment plant organisms decreased the toxicity of the enzyme digest.
- When the fungus was grown under high nutrient conditions, toxicity was decreased the most.
- Further work was carried out to investigate the mineralization of TNT and DNT by microorganisms in wastewater treatment facilities and microorganisms in soil. For these studies, radiolabeled TNT and DNT were used. Mineralization occurred at a slow rate either by microorganisms from a wastewater treatment plant or soil. These fungal effects should be studied in more detail. Degradation of trinitroglycerine (GTN) was investigated using xanthine oxidase.

No enzymatic digestion was detected. The enzymatic activity of Oxyrase was studied as a function of methanol concentration. Like acetone, methanol inhibited enzyme activity, even at low concentrations.

Utah State
UNIVERSITY

Steven D. Aust, Professor
Chemistry and Biochemistry
Biotechnology Center
Logan UT 84322-4705
Telephone: (435) 797-2730
FAX: (435) 797-2755
Email: sdaust@cc.usu.edu

FAX - 509-373-0169

December 22, 1999

Steve Goheen
Pacific Northwest Laboratory, Battelle
PO Box 999, MS P7-41
Richland, WA 99352

Dear Dr. Goheen:

Herewith is a report on the additional work we were able to complete on the project entitled "Enzymatic Degradation of Nitroenergetic Compounds". Also included is the interim report submitted on July 8, 1999 which contained other results.

Task 1. Characterize the products of TNT reduction by xanthine oxidase.

No results have been obtained from the mass spectroscopy laboratory. The lack of results could be due to the fact that the reduced products are not sufficiently stable for mass spectroscopy. It is also possible that the instrument was not operating correctly.

Task 2. Evaluate the ecotoxicology properties of TNT, xanthine oxidase-generated TNT products and metabolites formed during biodegradation of xanthine oxidase-generated products by *P. chrysosporium*, a mixed bacterial culture and soil microbes.

Ecotoxicity results of TNT and xanthine oxidase reduced TNT were reported previously (attached).

In this period we incubated the xanthine oxidase generated material either with bacteria from the Logan, Utah wastewater treatment plant, or with the white-rot fungus *Phaneochaete chrysosporium*. The xanthine-xanthine oxidase reactor was 2.5 liters containing 270 mg of TNT and 5.4 mM xanthine oxidase in 0.05 M potassium phosphate buffer, pH 7.8. The reactor was operated anaerobically for 96 hours. Aliquots were then used for bacterial or fungal biodegradation studies. The fungus was grown under two nutrient nitrogen conditions, one (low nutrient nitrogen, 2.4 mM ammonia) in which the fungal peroxidases are excreted and one (high nutrient nutrogen, 24 mM) condition in which the peroxidases are not secreted (Bumpus et al.

Science 228, 1434-1436, 1985). Maximum rates of reduction but no oxidation of TNT metabolites should occur under high nutrient nitrogen conditions. The fungal cultures were in 2.8 l Fernbach flasks containing 110.5 ml of the xanthine-xanthine oxidase reaction mixture, 6.5 ml of fungal spore inoculum ($OD_{650}$ = 1.0), and 13 ml of 10X BIII medium (Bumpus and Aust, BioEssays 6, 166-170, 1986). In the case of the high nutrient nitrogen conditions, 12 mM diammonium tartrate was used instead of 1.2 mM. These cultures were grown aerobically at 37°C for 30 days. The fungal mats were removed before submitting the reaction for ecotoxicity studies.

Bacterial incubations were in 500 ml Wheaton bottles containing 212.5 ml of the xanthine and xanthine oxidase reaction product or an equal amount of water, 12.5 ml of wastewater treatment plant inoculum, and 25 ml of LB media (Difco Laboratories, Detroit, MI). The cultures were grown aerobically at 37°C for 30 days. Oxygen was provided by gassing the flasks with oxygen every 3 days. The cultures were centrifuged at 5000 xg for 30 minutes to remove the bacteria prior to submission for ecotoxicity analyses.

Ecotoxicity analyses were conducted by Coastal Bioanalysts, Inc., as described (attached).

The results of ecotoxicity analysis of samples submitted to Coastal Bioanalysts, Inc. are reported in Table 1. Data are included for another test of TNT toxicity as there was an anomoly reported previously. The new data agreed with one previous analysis and our previous conclusions as to the validity of the 19.6 toxicity units in the Microtox Assay. Therefore the value of 64.1 was discarded. Also included is a control for the fungus (no TNT) with both low and high nutrient nitrogen media, the xanthine and xanthine oxidase reaction above (as we were suspicious of toxicity of uric acid, reported previously), and a control for the bacterial medium.

The concentrations of chemical that inhibits 50% of the activity in the *C. dubia* and Microtox Assays were calculated by dividing the original concentration of TNT (108 mg/l) by the toxicity units. These are reported in Table 2.

It would appear that our previous suspicion (interim report) that the xanthine oxidase reaction produced something that was toxic in the ecotoxicity assays was correct. This control showed extreme toxicity (Table 2). Both the waste treatment plant bacteria and fungus were able to decrease this toxicity. The fungus grown under high nutrient nitrogen conditions reduced toxicity even more. This might indicate that the fungus was detoxifying some product of the xanthine oxidase reaction, perhaps uric acid, under better growth (high nitrogen) conditions.

Table 1. Ecotoxicity of xanthine oxidase reaction products of TNT and the subsequent incubation with bacteria or fungus reported as toxicity units.[a]

| | | Toxicity Units | | | |
|---|---|---|---|---|---|
| | | Ceridophnia Dubia Assay | | Microtox Assay | |
| | Replicate | Toxicity | Average | Toxicity | Average |
| TNT Control | 1 | 11.3 | 12.9[b] | 19.0 | 19.3[b] |
| Fungal Control | | | | | |
| Low nitrogen | 1 | 10.2 | - | 37.9 | - |
| High nitrogen | | 7.3 | - | 192.3 | - |
| Xanthine/Xanthine Oxidase | | | | | |
| Control | 1 | 14.5 | - | 0.97 | - |
| LB Media Control | 1 | 27.3 | 25.4 | 256 | 284 |
| | 2 | 23.5 | | 312 | |
| Xanthine/Xanthine | | | | | |
| Oxidase, TNT | 1 | 58.5 | | 233 | |
| Bacteria | 2 | 45.9 | 51.2 | 303 | 228 |
| | 3 | 49.3 | | 149 | |
| Xanthine, Xanthine | | | | | |
| Oxidase, TNT | | | | | |
| Fungus | | | | | |
| Low Nitrogen | 1 | 44.4 | | 35.2 | |
| | 2 | 36.1 | 39.3 | 31.6 | 32.9 |
| | 3 | 37.2 | | 32.0 | |
| High Nitrogen | 1 | 122 | | 50.3 | |
| | 2 | 89 | 24.1 | 40.8 | 44.1 |
| | 3 | 30.0 | | 41.2 | |

[a]One unit is equal to 100/LC50.
[b]The average includes data obtained originally with the exclusion of the value of 64.1 toxicity units for the Microtox Assay.

Table 2. Ecotoxicity of xanthine oxidase reaction products of TNT and the subsequent incubation with bacteria or fungus reported as $EC_{50}$.[a]

| | | Toxicity Units | | | |
|---|---|---|---|---|---|
| | | Ceridophnia Dubia Assay | | Microtox Assay | |
| | Replicate | Toxicity | Average | Toxicity | Average |
| TNT Control | 1 | 11.3 | 12.9[b] | 19.0 | 19.3[b] |
| Fungal Control | | | | | |
| Low nitrogen | | 10.2 | 8.75 | 37.9 | 115.1 |
| High nitrogen | | 7.3 | | 192.3 | |
| Xanthine/Xanthine Oxidase | | | | | |
| Control | | 14.5 | - | 0.97 | - |
| LB Media Control | 1 | 27.3 | 25.4 | 256.4 | 284.5 |
| | 2 | 23.5 | | 312.5 | |
| Xanthine/Xanthine | | | | | |
| Oxidase, TNT | 1 | 58.5 | | 232.6 | |
| Bacteria | 2 | 45.9 | 51.2 | 303.0 | 228.3 |
| | 3 | 49.3 | | 149.3 | |
| Xanthine, Xanthine | | | | | |
| Oxidase, TNT | | | | | |
| Fungus | | | | | |
| Low Nitrogen | 1 | 44.4 | | 35.2 | |
| | 2 | 36.1 | 39.3 | 31.6 | 32.9 |
| | 3 | 37.3 | | 32.0 | |
| High Nitrogen | 1 | 122.0 | | 50.3 | |
| | 2 | 89.3 | 80.5 | 40.8 | 44.1 |
| | 3 | 30.3 | | 41.2 | |

[a]$EC_{50}$ is the concentration of TNT or metabolites in μM that inhibited 50%.
[b]The average includes data obtained originally with the exclusion of the value of 64.1 toxicity units for the Microtox Assay.

Task 3. Quantitate the mineralization of DNT and the products of xanthine oxidase reduced DNT by *Phanerochaete chrysosporium*, a mixed culture of bacteria from a local waste treatment facility and soil microbes.

Studies on the mineralization of DNT were seriously delayed because the material was not received from the company responsible for its synthesis until very late in the project. The experiments are continuing. Mineralization of $^{14}$C-DNT in soil by *P. chrysosporium* was analyzed as described in Bumpus et al. Science 228, 1434-1436, 1985. The fungus was added to 10 g of soil (from the USU greenhouse) 9.4 g of sawdust inoculated with fungus 3 weeks previously, and 10 ml of water. Each incubation (in quadruplicate) contained 1.6 x $10^6$ dpm $^{14}$C-TNT and 260 ppm TNT in the soil. A very low but linear rate of mineralization is being observed (Figure 1). The study of mineralization of DNT by the fungus after reduction by xanthine oxidase was a failure due to failure of the inoculum. This experiment is being repeated.

In another experiment, the mineralization of $^{14}$C-DNT by soil and wastewater treatment plant bacteria both with and without treatment with xanthine and xanthine oxidase. Treatment of the $^{14}$C-TNT with xanthine oxidase was done as described above for the ecotoxicity testing. The untreated material was the same except that the enzyme was not included. The medium for the wastewater treatment bacteria was 1 ml of LB media in 10 ml total volume. Incubations were in quadruplicate. For mineralization by soil bacteria, 10 g of garden soil (from the USU greenhouse) was incubated with 8.5 ml of the TNT solutions (non-treated or treated with xanthine oxidase) and 1.5 ml of water, each in quadruplicate. The TNT concentration was 0.12 mg/ml containing 1.6 x $10^6$ dmp $^{14}$C-TNT total, in the liquid cultures (wastewater treatment plant bacteria) or 0.12 mg/g soil. The bacterial mineralization reactions (in quadruplicate) for the samples not treated with xanthine oxidase contained about 81,000 dpm, total, while those incubated with xanthine oxidase contained about 28,000 dpm of $^{14}$C-DNT. Essentially no mineralization by either source of bacteria has been observed (Figure 2).

Task 4. Develop a method for reduction of high concentrations of TNT by oxyrase.

An oxyrase reactor was designed to contain 100 mM lactate, 2 ml of oxyrase and 1000 ppm TNT in 100 mM Tris buffer, pH 8.4, 10 ml, final volume. The oxyrase reactor was run with dichlorophenolinopherol (DCPIP) as an assay for reduction. Samples were assayed before and after filtration to determine if the enzyme would stay in the reactor upon removal of solution by filtration to keep the particulate enzyme in the reactor. Enzyme activity was essentially all retained upon filtration and some activity remained after 200 hours of incubation but most was gone after 172 hours. Methanol and acetate were tested as solvents suitable for solubilizing high concentrations of TNT but not affecting enzyme activity up to 20% (volume/volume) of the solvents were tested (Figures 3 and 4).

Task 5. Investigate pentaerythritol (PETN) and trinitroglycerin (GTN) degradation by hydrolases and oxidoreductases.

PETN reduction by xanthine oxidase was reported in the interim report (attached). No degradation of trinitroglycerin by xanthine oxidase was observed. The tests with other enzyme are still being conducted.

If you have any questions please contact me.

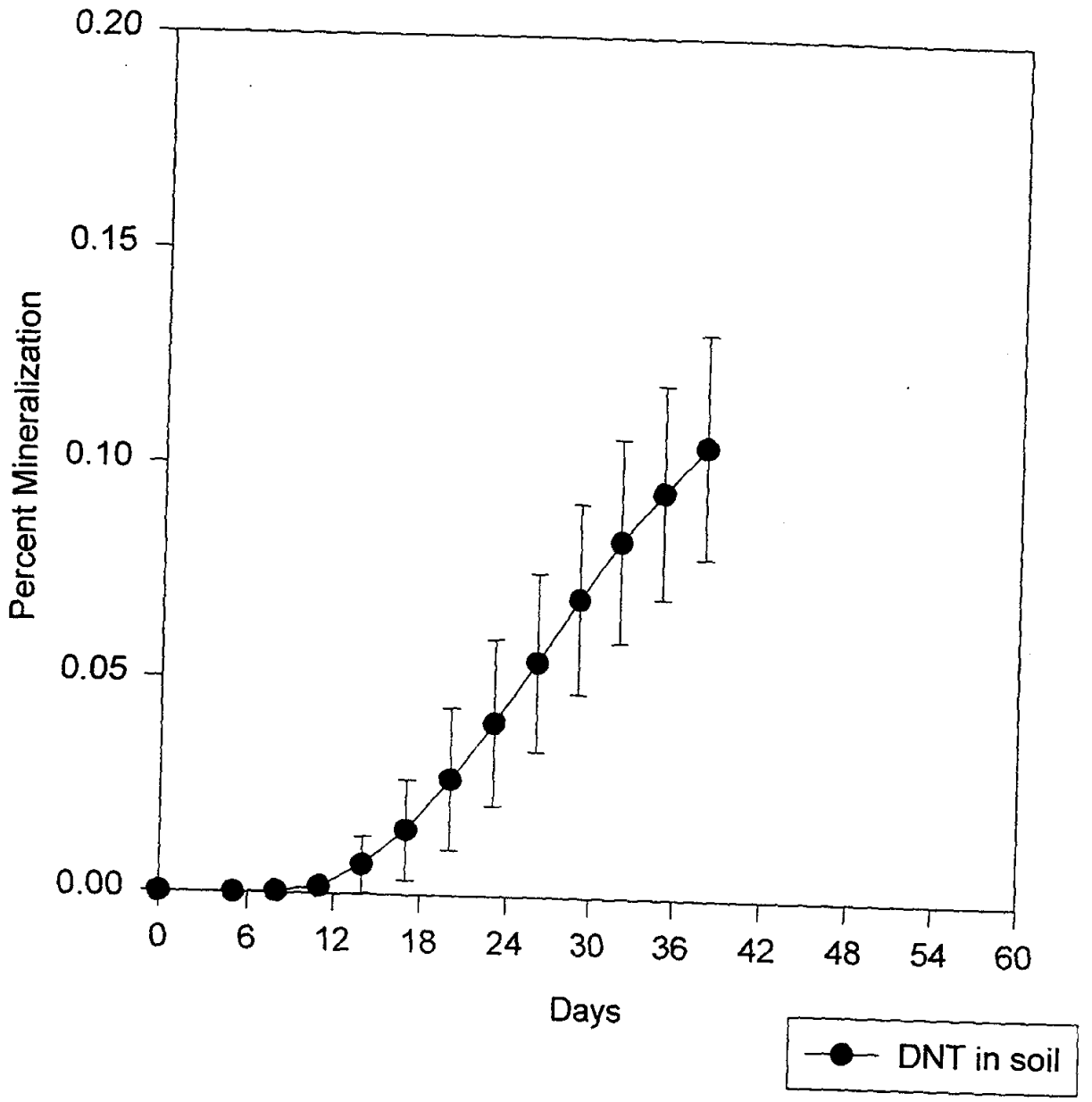
Sincerely,

Steven D. Aust, Ph.D.
Professor

tm

enclosures

Figure 1. Mineralization of TNT in soil by Phanerochaete chrysosporium.



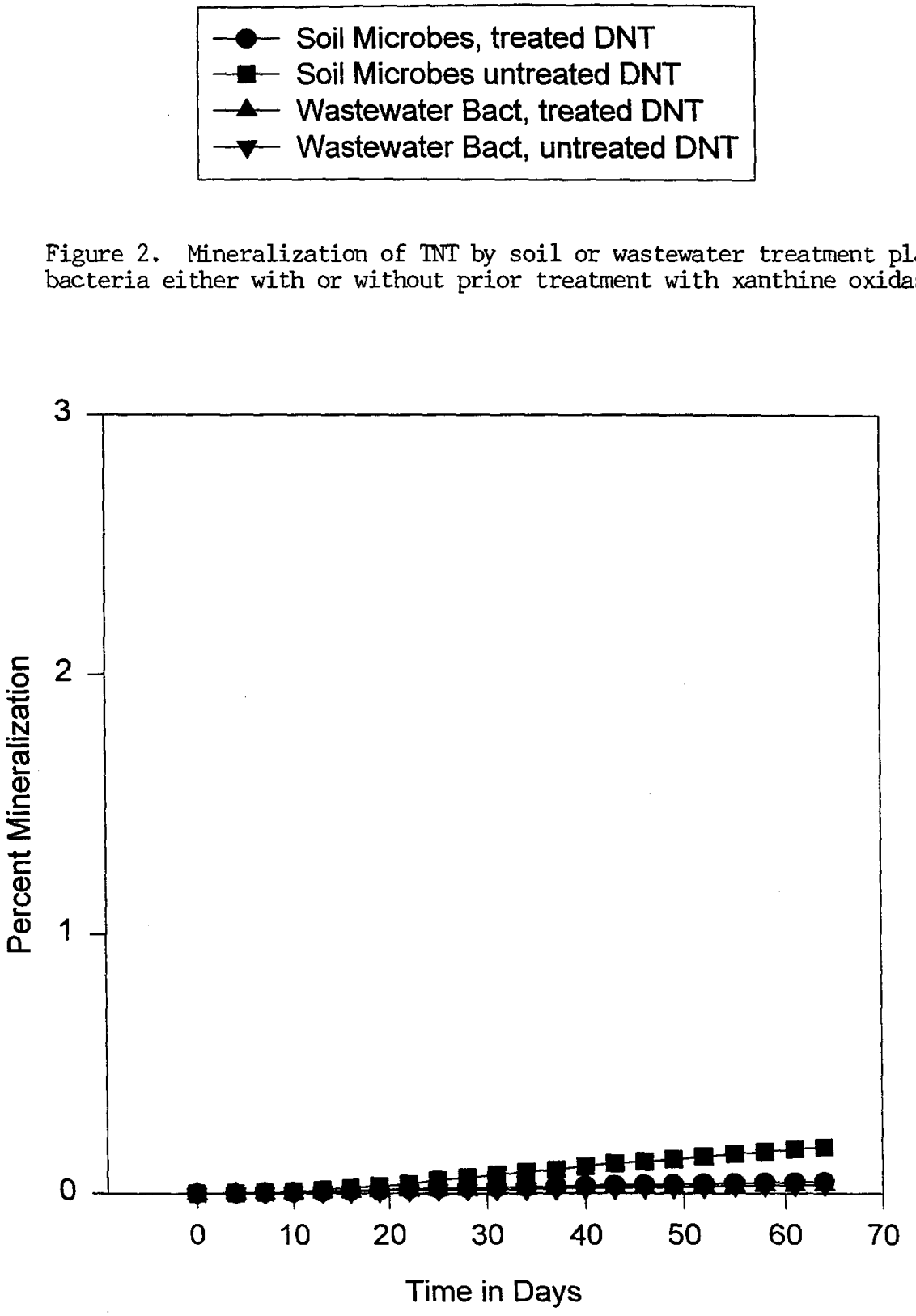



Figure 2. Mineralization of TNT by soil or wastewater treatment plant bacteria either with or without prior treatment with xanthine oxidase.

Figure 3. Effect of acetone concentration on initial oxyrase activity.

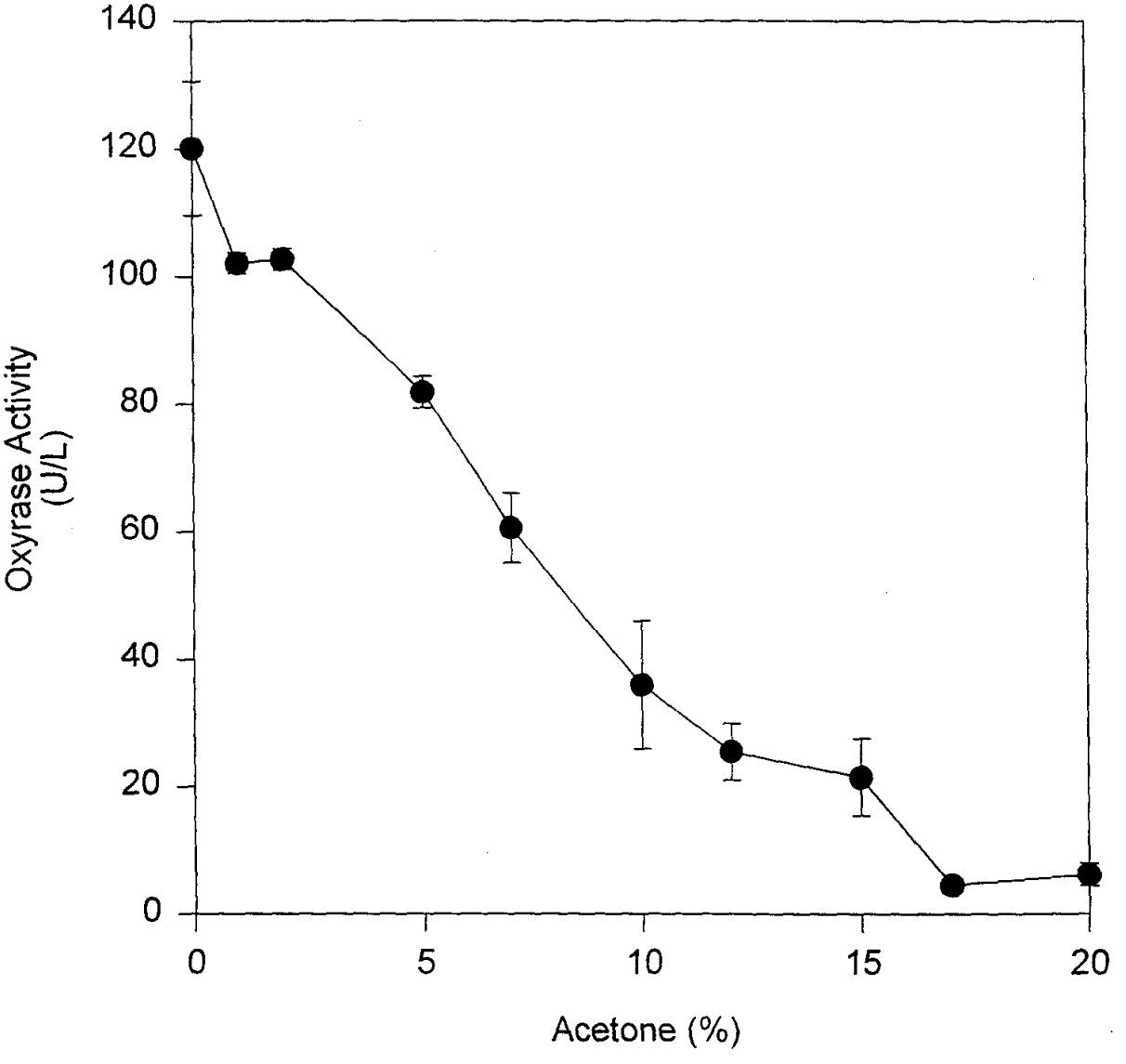
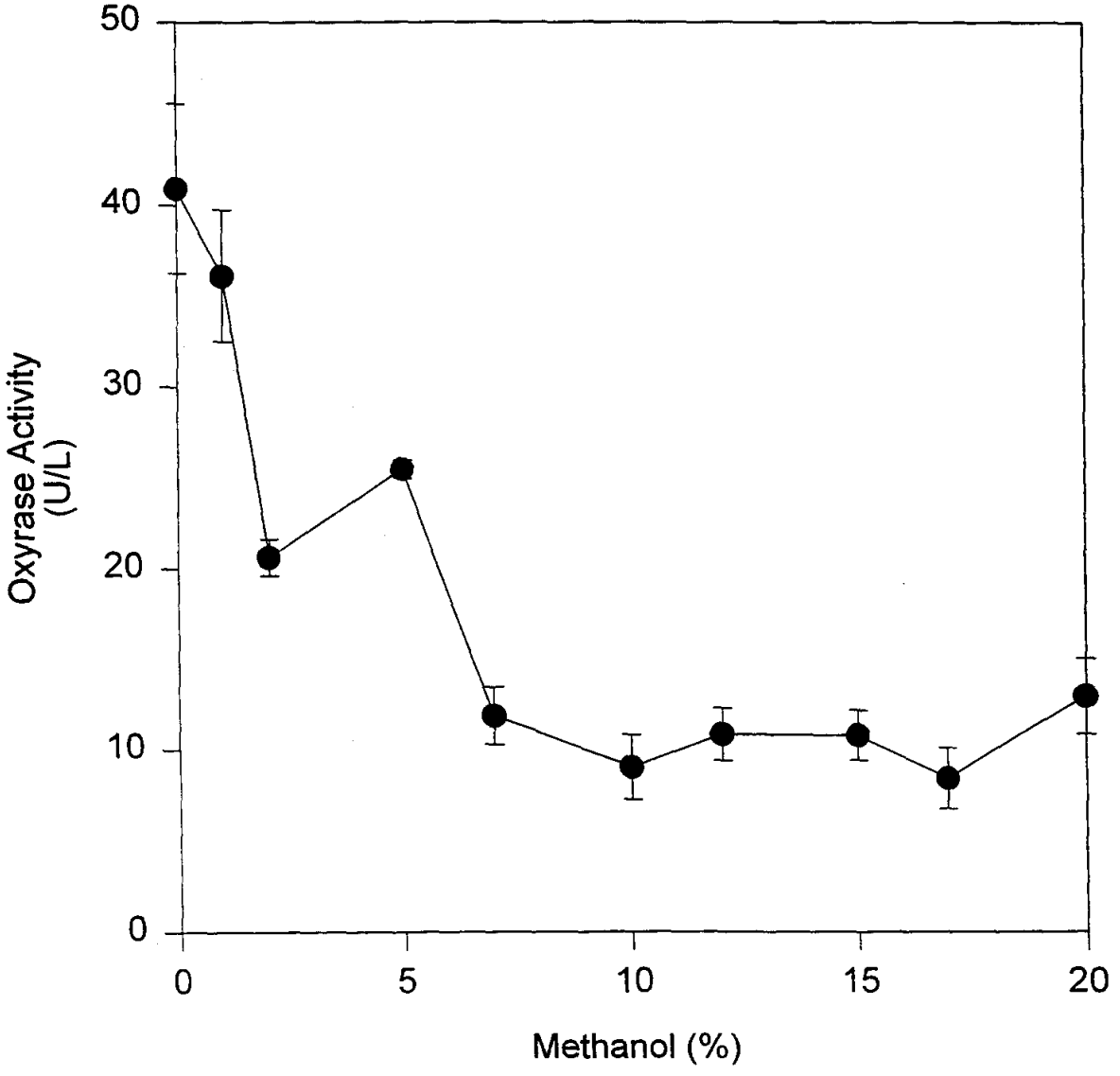


Figure 4. Effect of methanol concentration on initial oxyrase activity.



Coastal Bioanalysts, Inc.

## I. MATERIALS AND METHODS

*Ceriodaphnia dubia* Tests

*Ceriodaphnia* toxicity tests were performed in accordance with Coastal Bioanalysts' protocols. Protocols comply with methods described in EPA/600/4-90/027. The general test designs and methods are described below; test-specific details can be found in subsequent portions of the report and/or accompanying copies of laboratory bench sheets.

Dilution water consists of moderately-hard synthetic freshwater made up with ASTM Type I de-ionized water and ACS reagent-grade chemicals. Test organisms are obtained from in-house cultures and are cultured in the same type water used as test dilution water. Cladocerans are <24-h old at the start of tests.

The tests consist of randomized block designs in which four replicates of five organisms each are exposed to sample serially diluted (e.g. 100%, 50%, 25%, 12.5% , 6.3% and 0%) with dilution water. Solutions are prepared in a single flask for all replicates and tests to decrease within- and between-test variability. Test chambers of borosilicate glass contain 15 ml. each of test solution. Temperature, pH, conductivity and dissolved oxygen concentration are measured daily or at the beginning and end of test in one replicate of each treatment. Survival is recorded for all chambers daily. Tests are maintained at 20±1 $^{o}$C.

LC50s are calculated for tests exhibiting sufficient toxicity using the Trimmed Spearman-Karber (Montana State University program), Probit (EPA program) or Linear Interpolation (graphical) methods as described in EPA/600/4-90/027. Printouts of program results are attached as appendices as applicable.

Acute toxicity tests using potassium chloride as the reference toxicant are performed monthly on each species of animal cultured in-house to insure animal vigor and validity of test materials and methods. In addition, each test is monitored by a QA/QC officer to insure adherence to test protocols.

Microtox Tests

Microtox tests were performed in strict accordance with the specific protocols published by AZUR Environmental (Carlsbad, CA). Tests were performed on a model 2055 analyzer. All samples were osmotically adjusted by addition of 200 mg NaCl (ACS reagent grade) per 10 ml of solution. Bacteria (Lot ACV017-2R, exp. date 11/2000) were stored frozen until reconstituted immediately prior to testing. Reference toxicant tests using $ZnSO_4 \cdot 2H_2O$ were run periodically throughout the sample runs and compared well with AZUR acceptance criteria for the lot.

# Appendix E

## Enzymatic Degredation of PETN and GTN as well as Fungal Mineralization of DNT

# Appendix E

## Enzymatic Degredation of PETN and GTN as well as Fungal Mineralization of DNT

This is the final letter report from our subcontractor, Steve Aust from Utah State University. In this report, enzymatic degredation of explosives other than TNT, RDX, and HMX are explored. In addition, Dr. Aust had been examining the ability of fungi to mineralize TNT and DNT. These studies were undertaken initially to determine whether enzymatic mineralization was possible. Previously, we had only been able to generate reduced products of TNT by a single enzyme. These were seemingly dead end products. However, if fungi can mineralize TNT, then enzymes must exist to further degrade TNT beyond TAT. These results are meaninigful not only to suggest that other enzymes can be used to further process TNT beyond the limited products we observed,. But also that fungi alone may be useful in the bioremediation process. This is not a new finding, but fungi have been used less extensively than bacteria to degrade explosives.

Another interesting outcome of this set of experiments Is the relative rate of DNT and TNT degredation. Most TNT is contaminated with DNT due to the methods by which these explosives were produced. Yet, the relative rate of reactions between degredation enzymes and DNT vs. TNT is not well understood. These results may provide early insights into the relative environmental stability of these two energetic materials.

Utah State
UNIVERSITY

Steven D. Aust, Professor
Chemistry and Biochemistry
Biotechnology Center
Logan UT 84322-4705
Telephone: (435) 797-2730
FAX: (435) 797-2755
Email: sdaust@cc.usu.edu

FAX 509-376-2329

May 24, 2000

Steve Goheen
Pacific Northwest Laboratory, Battelle
PO Box 999, MS P7-41
Richland, WA 99352

Dear Steve:

Enclosed are additional results of our work on the Enzymatic Degradation of Nitroenergetic Compounds. These have resulted from work we continued because the $^{14}$C-DNT was delivered so late. I am really sorry for this delay but it was beyond our control.

We are still working on the mineralization of DNT. We had previously observed very low rates of DNT mineralization in soil. We are suspicious that something was wrong with the fungal cultures, either that we had a mutant, which could have resulted from the mutagenicity of DNT metabolites, or simply overgrowth by some fungus that might have been in the soil as the soil was not sterile. We have gone back to what one might consider the beginning, to obtain the rate of mineralization in a culture of the fungus in the standard liquid medium. Note that we are now observing a significant rate of mineralization of the DNT by *P. chrysosporium*, over 30% in about 30 days (Figure 1, attached). We have now gone on, since we have this "baseline" to obtain the rate of mineralization in soil. Unfortunately we are still just preparing things for this experiment.

We have also investigated the mineralization of DNT under various nutrient conditions designed to demonstrate that several different biochemical systems can degrade DNT. This is important in that it shows that conditions are not absolutely critical, although some conditions give faster rates of mineralization. In summary, mineralization of DNT by *Pycnoporus cinnabarinus* occurred under all conditions but was very low (~1.5% in about 30 days, Figure 2) under very rich nutrient conditions (high nitrogen with glucose as the carbon source). The highest (~15%, Figure 3) was obtained under conditions (nutrient nitrogen-limiting) when the peroxidases are present. When conditions were used to only have cellobiose dehydrogenase (nutrient nitrogen-sufficient and

cellulose as the carbon source), mineralization was still significant, almost 5% (Figure 4). Under conditions when both the peroxidases and cellobiose dehydrogenase should be present (limited nutrient nitrogen and cellulose), mineralization was about 10% (Figure 5).

Also enclosed are data on the enzymatic degradation of PETN. The only enzyme that seemed active for the degradation of PETN was xanthine oxidase. We had extreme problems with the insolubility of PETN in buffer and the loss of enzyme activities upon attempts to add sufficient solvents to have soluble PENT. Unfortunately we were unable to get any experiments done with GTN for very serious but perhaps unreasonable problems. In any case, we cannot continue so this aspect of the project must be considered complete. When we get additional data on the mineralization of DNT in soil I will forward it to you just in case it can be used for something.

Sincerely,

Steven D. Aust, Ph.D.
Professor

tm

enclosures

Enzymatic Degradation of PETN

| Enzyme (incubation time) | % degradation[1] | Rate (ppm/hr) |
|---|---|---|
| XO[2] | | |
| Control | 0 | 0 |
| +XO (2 hrs) | 45 | 10.3 |
| + XO (4 hrs) | 54 | 6.3 |
| + XO (16 hrs) | 100 | 2.9 |
| Oxyrase[3] | | |
| Control | 0 | 0 |
| + Oxyrase | 0 | 0 |
| CDH[4] | | |
| Control | 0 | 0 |
| +CDH (39 hrs) | 0 | 0 |
| MnP[5] | | |
| Control | 0 | 0 |
| +MnP (24 hrs) | 0 | 0 |
| Nitrate Reductase[6] | | |
| Control | 0 | 0 |
| Nitrate Reductase (24 hrs) | 0 | 0 |
| Laccase[7] | | |
| Control | 0 | 0 |
| Laccase (24 hrs) | 0 | 0 |

[1]PETN degradation was quantitated by disappearance, assayed by HPLC.
[2]Xanthine oxidase (XO, 0.64 units) was incubated anaerobically with 100 ppm PETN in 50 mM potassium phosphate buffer, pH 7.8, and 100 mM xanthine.
[3]Oxyrase (2.2 units) was incubated anaerobically with 100 ppm PETN in 100 mM Tris buffer, pH 8.4, containing 100 mM lactate.
[4]Cellobiose dehydrogenase (CDH, 2.5 units) was incubated anaerobically with 100 ppm PETN in 10 mM succinate buffer, pH 4.5, with 10 mM cellobiose.
[5]Manganese peroxidase (MnP, 0.133 μM) was incubated aerobically with 100 ppm PETN in 10 mM tartrate buffer, pH 4.5, 0.139 mM manganese and 0.1 mM hydrogen peroxide.
[6]Nitrate reductase (1 U from *Aspergillus sp.*) in 50 mM MOPS buffer, pH 7.0.
[7]Laccase (1 U from *Rhus vernificera*) in 10 mM Tris, pH 6.8.

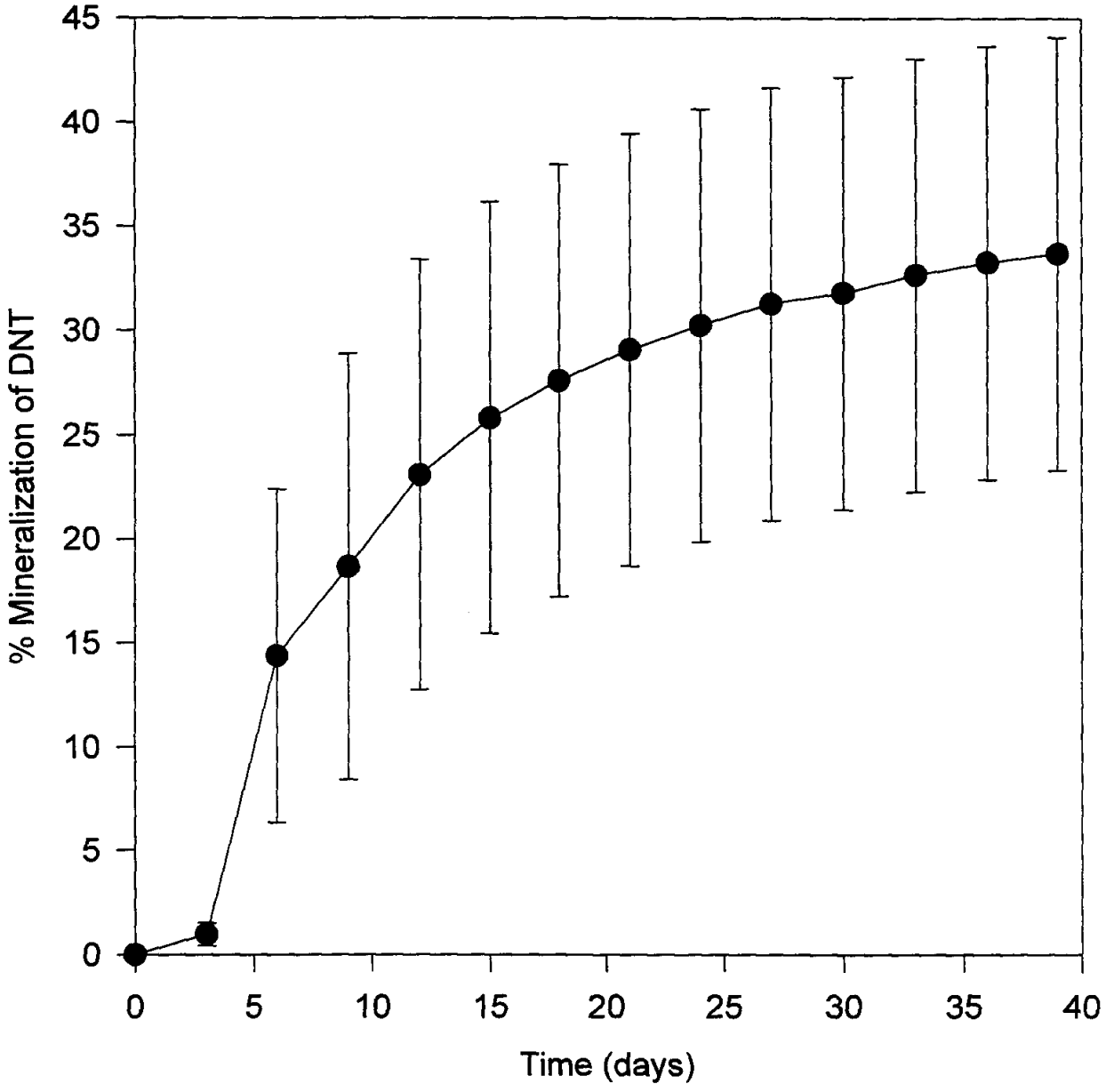



Figure 1. Mineralization of DNT by *Phanerochaete chrysosporium.* Liquid cultures (10.0 ml) of *P. chrysosporium* containing glucose (2%), ammonium tartrate (1.2 mM), sodium phosphate (2.0 g/L), dimethyl succinate buffer (10 mM, pH 4.5), thiamine (1 mg/L), mineral salts and 25 ppm $^{14}$C-labeled DNT (~500,000 dpms) were prepared in 250 mL Wheaton bottles. Cultures were incubated at 37°C and the gas in the headspace exchanged with oxygen every 3 days. Carbon dioxide in the headspace gas was collected every three days in a mixture of scintillation cocktail: methanol: ethanolamine (50:40:10) and its radioactivity determined by liquid scintillation spectroscopy. Data represent the average and standard deviation of quadruplicate cultures.

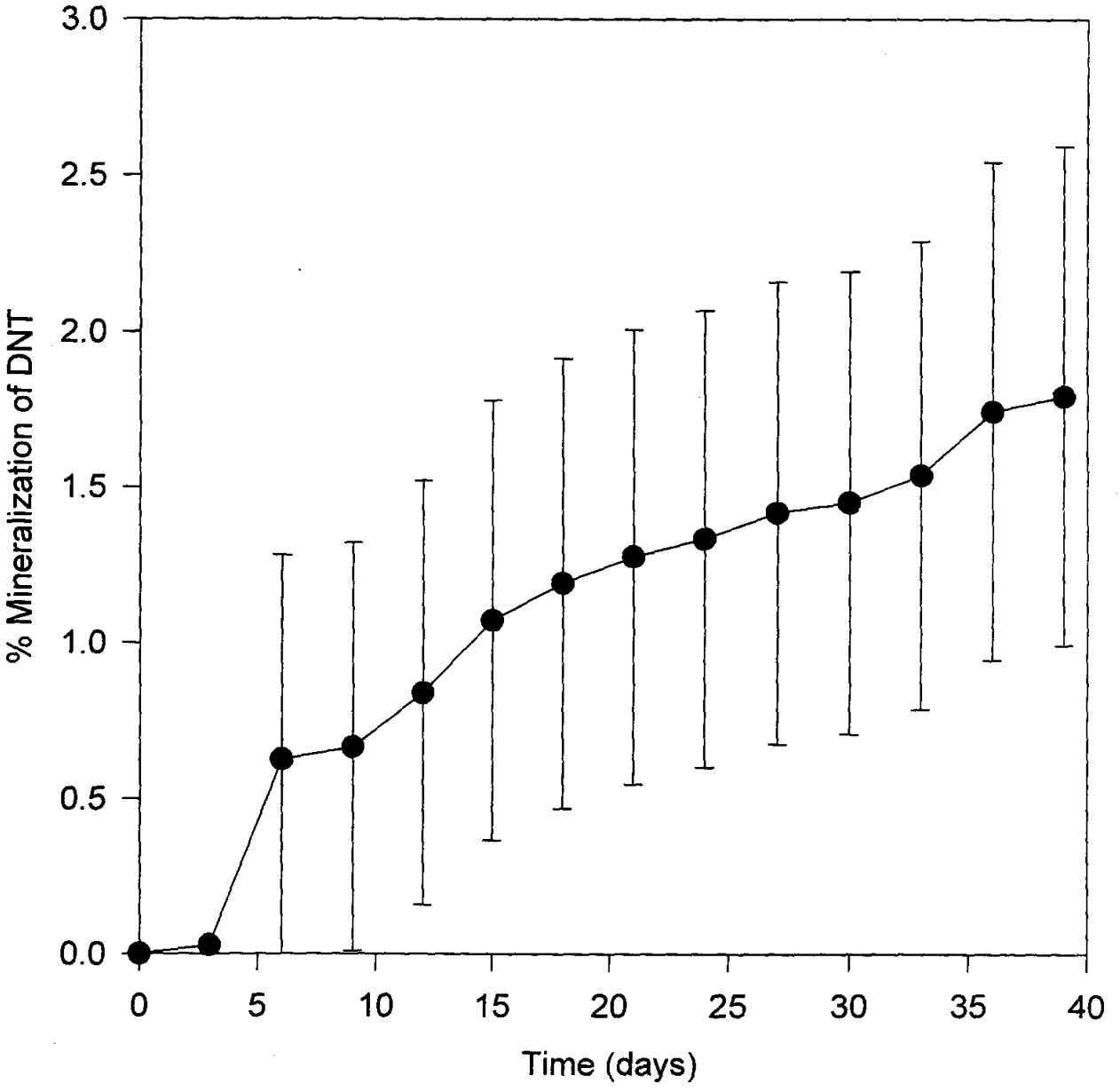



Figure 2. Mineralization of DNT by *Pycnoporus cinnabarinus* in nutrient nitrogen-sufficient liquid cultures with glucose as the carbon source. Liquid cultures (10.0 ml) of *P. cinnabarinus* containing glucose (2%), ammonium tartrate (12 mM), sodium phosphate (2.0 g/L), dimethyl succinate buffer (10 mM, pH 4.5), thiamine (1 mg/L), mineral salts and 25 ppm $^{14}$C-labeled DNT (~500,000 dpms) were prepared in 250 mL Wheaton bottles. Cultures were incubated at 37°C and the gas in the headspace exchanged with oxygen every 3 days. Carbon dioxide in the headspace gas was collected every three days in a mixture of scintillation cocktail:methanol: ethanolamine (50:40:10) and its radioactivity determined by liquid scintillation spectroscopy. Data represent the average and standard deviation of quadruplicate cultures.

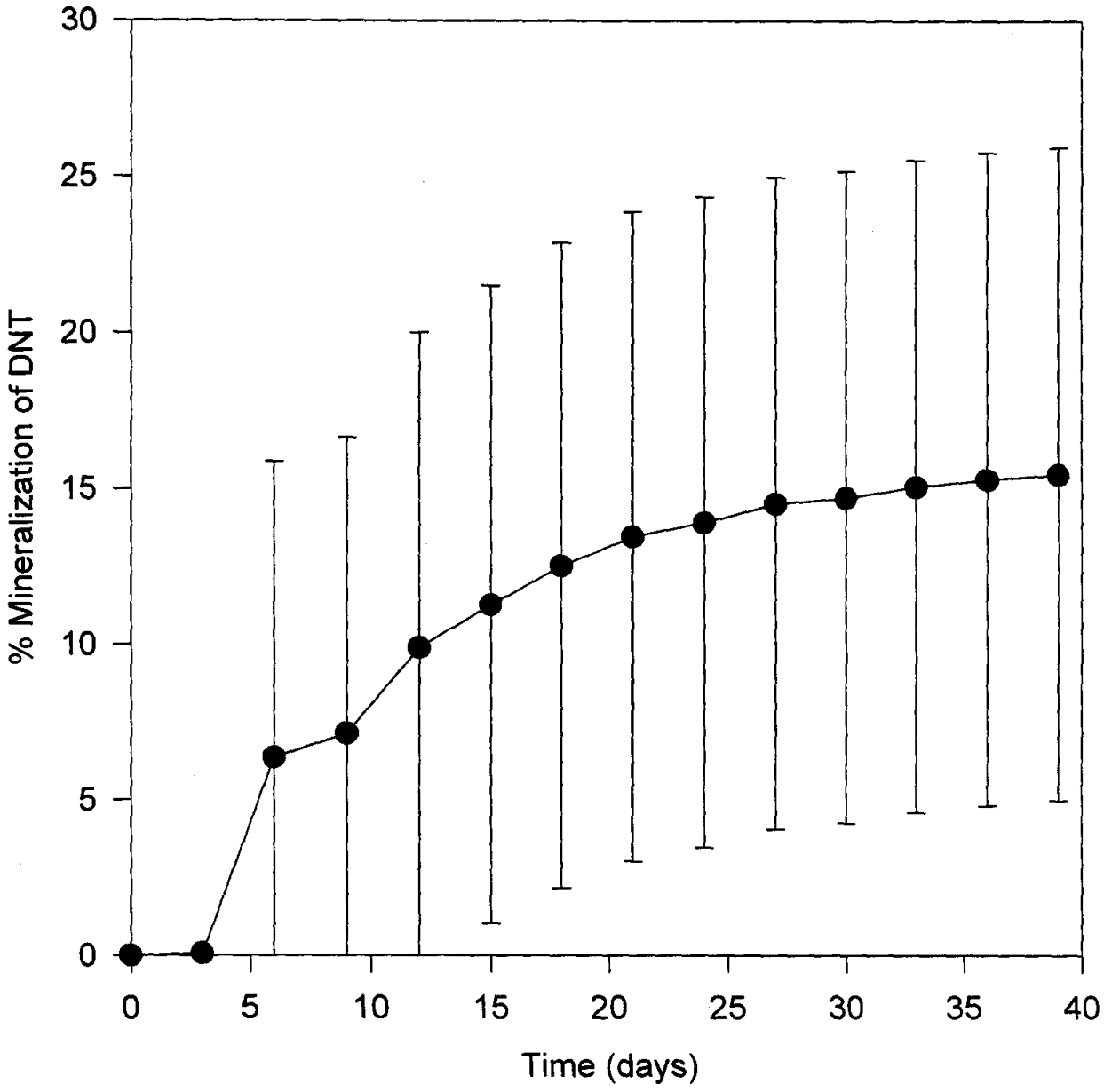



Figure 3. Mineralization of DNT by *Pycnoporus cinnabarinus* in nutrient nitrogen-limiting liquid cultures with glucose as the carbon source. Liquid cultures (10.0 ml) of *P. cinnabarinus* containing glucose (2%), ammonium tartrate (1.2 mM), sodium phosphate (2.0 g/L), dimethyl succinate buffer (10 mM, pH 4.5), thiamine (1 mg/L), mineral salts and 25 ppm $^{14}$C-labeled DNT (~500,000 dpms) were prepared in 250 mL Wheaton bottles. Cultures were incubated at 37°C and the gas in the headspace exchanged with oxygen every 3 days. Carbon dioxide in the headspace gas was collected every three days in a mixture of scintillation cocktail: methanol:ethanolamine (50:40:10) and its radioactivity determined by liquid scintillation spectroscopy. Data represent the average and standard deviation of quadruplicate cultures.

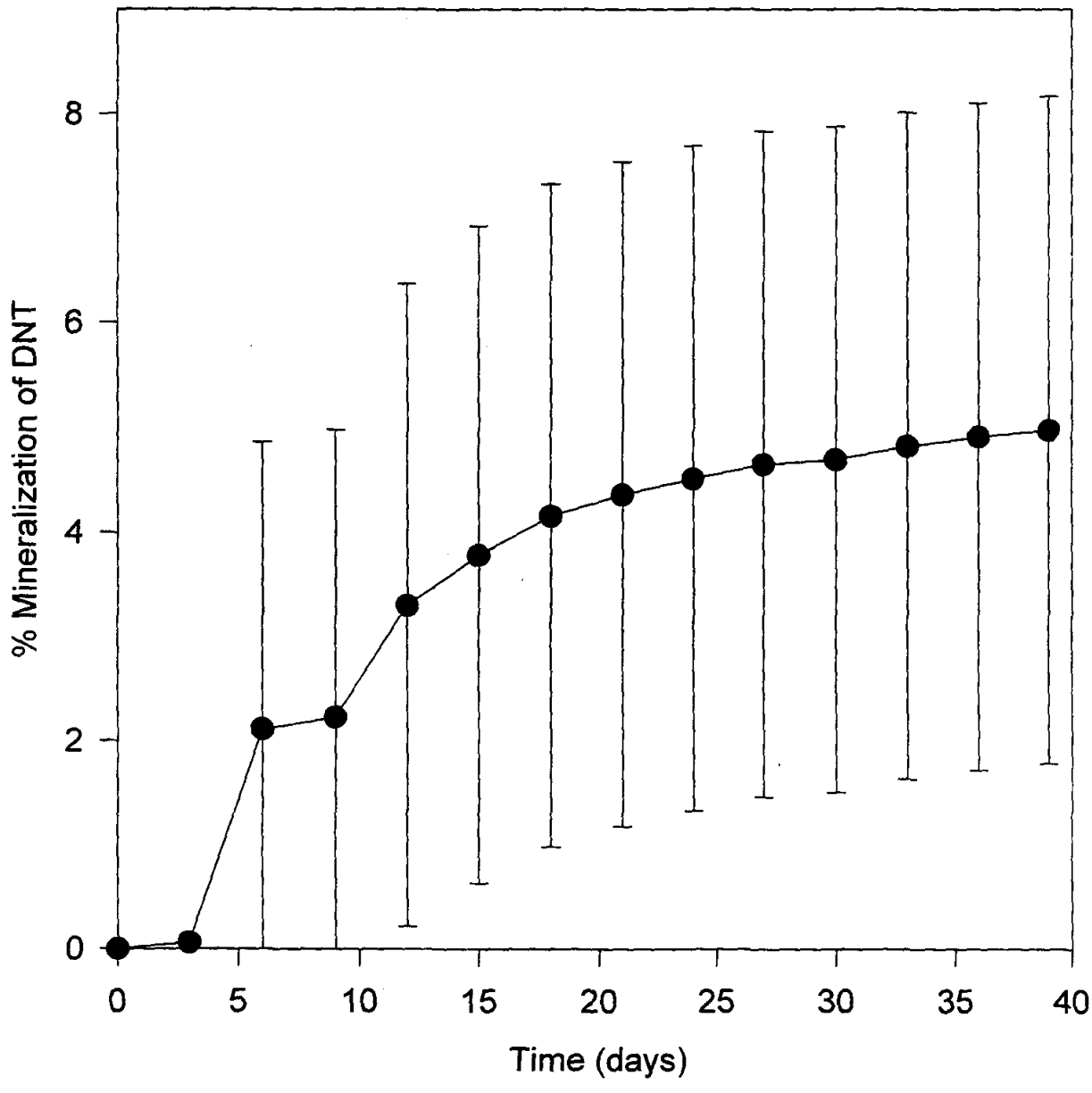



Figure 4. Mineralization of DNT by *Pycnoporus cinnabarinus* in nutrient nitrogen-sufficient liquid cultures with cellulose as the carbon source. Liquid cultures (10.0 ml) of *P. cinnabarinus* containing glucose (0.1%), 2% cellulose, ammonium tartrate (12 mM), sodium phosphate (2.0 g/L), dimethyl succinate buffer (10 mM, pH 4.5), thiamine (1 mg/L), mineral salts and 25 ppm $^{14}$C-labeled DNT (~500,000 dpms) were prepared in 250 mL Wheaton bottles. Cultures were incubated at 37°C and the gas in the headspace exchanged with oxygen every 3 days. Carbon dioxide in the headspace gas was collected every three days in a mixture of scintillation cocktail: methanol: ethanolamine (50:40:10) and its radioactivity determined by liquid scintillation spectroscopy. Data represent the average and standard deviation of quadruplicate cultures.

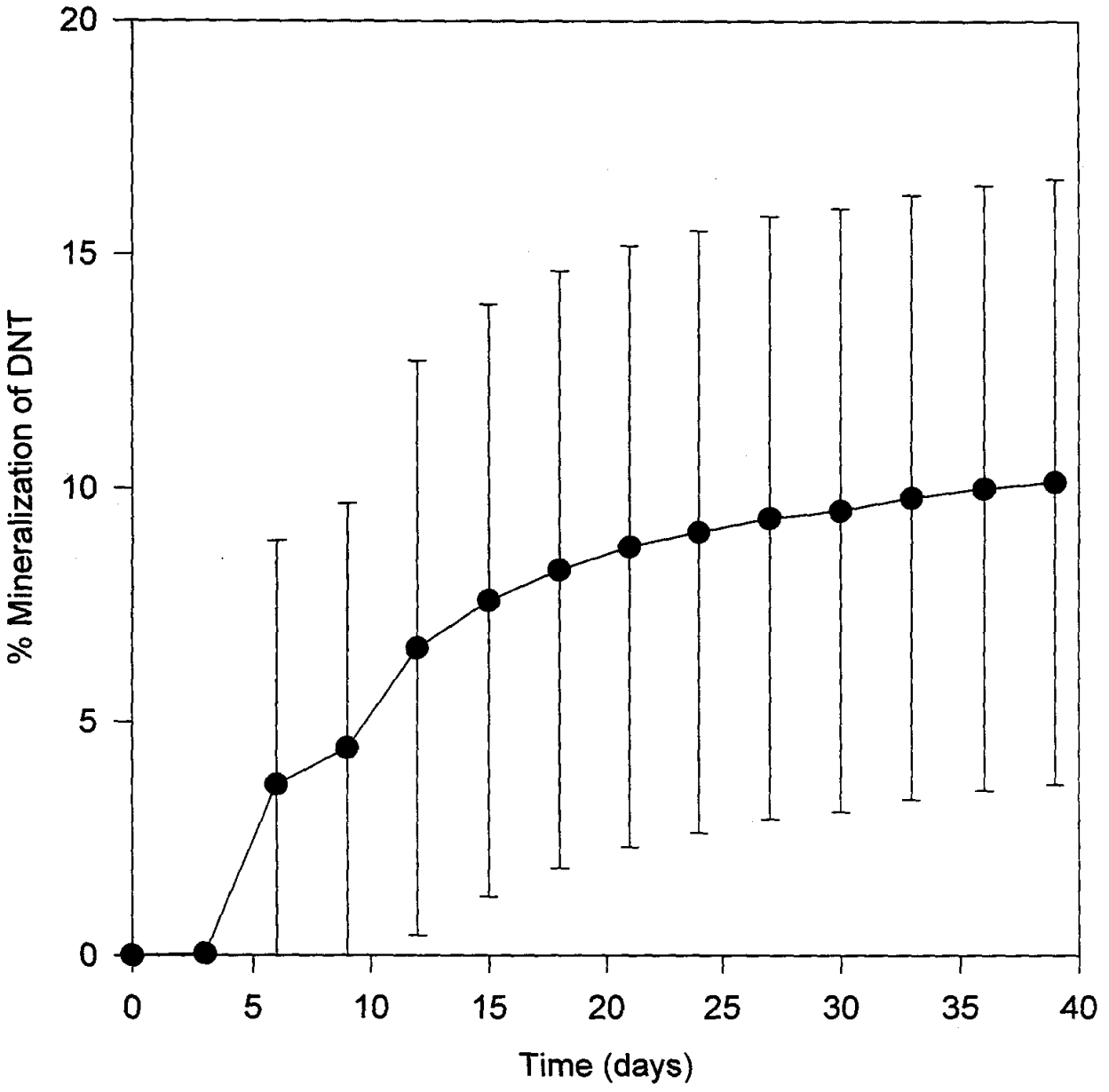



Figure 5. Mineralization of DNT by *Pycnoporus cinnabarinus* in nutrient nitrogen-limited liquid cultures with cellulose as the carbon source. Liquid cultures (10.0 ml) of *P. cinnabarinus* containing glucose (0.1%), 2% cellulose, ammonium tartrate (1.2 mM), sodium phosphate (2.0 g/L), dimethyl succinate buffer (10 mM, pH 4.5), thiamine (1 mg/L), mineral salts and 25 ppm $^{14}$C-labeled DNT (~500,000 dpms) were prepared in 250 mL Wheaton bottles. Cultures were incubated at 37°C and the gas in the headspace exchanged with oxygen every 3 days. Carbon dioxide in the headspace gas was collected every three days in a mixture of scintillation cocktail: methanol: ethanolamine (50:40:10) and its radioactivity determined by liquid scintillation spectroscopy. Data represent the average and standard deviation of quadruplicate cultures.

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