ALKALINE HYDROLYSIS OF EXPLOSIVES

by

Catherine Elizabeth VanEngelen

A dissertation submitted in partial fulfillment
of the requirements for the degree

of

Doctor of Philosophy

in

Engineering

MONTANA STATE UNIVERSITY
Bozeman, Montana

January 2010

APPROVAL

of a dissertation submitted by

Catherine Elizabeth VanEngelen

This dissertation has been read by each member of the dissertation committee and has been found to be satisfactory regarding content, English usage, format, citation, bibliographic style, and consistency, and is ready for submission to the Division of Graduate Education.

Dr. Brent M. Peyton

Approved for the Department of Chemical and Biological Engineering

Dr. Ron W. Larsen

Approved for the Division of Graduate Education

Dr. Carl A. Fox

STATEMENT OF PERMISSION TO USE

In presenting this dissertation in partial fulfillment of the requirements for a doctoral degree at Montana State University, I agree that the Library shall make it available to borrowers under rules of the Library. I further agree that copying of this dissertation is allowable only for scholarly purposes, consistent with "fair use" as prescribed in the U.S. Copyright Law. Requests for extensive copying or reproduction of this dissertation should be referred to ProQuest Information and Learning, 300 North Zeeb Road, Ann Arbor, Michigan 48106, to whom I have granted "the exclusive right to reproduce and distribute my dissertation in and from microform along with the non-exclusive right to reproduce and distribute my abstract in any format in whole or in part."

Catherine Elizabeth VanEngelen

January 2010

DEDICATION

This work is dedicated to my parents, Patricia and Daniel Albaugh, and to my husband, Michael VanEngelen. Only their loving support and encouragement has made this possible.

ACKNOWLEDGEMENTS

Financial support for this research was provided by: the Thermal Biology Institute at MSU (TBI grant #NNG04GR46G), the Inland Northwest Research Alliance (INRA Student Fellowship), the Center for Biofilm Engineering at MSU, the College of Engineering at MSU (Benjamin Fellowship), and the Radford Army Ammunition Plant, Radford, VA.

I would also like to gratefully acknowledge the following people for their help and guidance throughout my time at Montana State: Shelley Thomas, Jill Newman, John Neuman, Ann Willis, John and Flower Aston, Melinda Clark, Angela and Dennis O'Brien, Chris and Angie Albaugh, Mary and Dean Lutz, Patrick Albaugh, John and Susan VanEngelen, Tamara and Rich Cullen, Jen and Tim Traynor, Kristen and Chip George, members of the Peyton Research Group, Ron Larsen, and my committee: Ross Carlson, Robin Gerlach, Anne Camper, and Brent Peyton.

TABLE OF CONTENTS

TABLE OF CONTENTS – CONTINUED

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES – CONTINUED

ABSTRACT

In the United States, ammunitions testing and manufacturing facilities must transform unused explosives into non-hazardous materials for disposal. 2,4,6-trinitrotoluene (TNT) is an explosive that has been found as a soil and groundwater contaminant at numerous ammunitions testing sites. Unused quantities of nitrocellulose (NC), another explosive, have also been accumulating at ammunitions manufacturing facilities. Transformation of both TNT and NC to non-explosive compounds has been studied using either chemical or biological approaches, each with limited success. With respect to TNT, the use of alkaline hydrolysis (degradation at high pH) as a chemical treatment had been tested at room temperature (20°C) under conditions where the hydroxide concentration exceeded that of TNT (pH > 10). These high hydroxide conditions were not directly amenable to biological treatment of the hydrolysis products. This study found that alkaline hydrolysis was effective for complete degradation of TNT at elevated temperatures (60°C and 80°C) when the concentration of TNT was less than the hydroxide concentration (pH 9 and 10). The resulting solution, or hydrolysate, contained no TNT. This hydrolysate was used as the carbon and nitrogen source for an aerobic bacterial enrichment from the Bozeman wastewater treatment plant. With respect to NC, the back-log of accumulated NC necessitates a degradation method that will process high NC concentrations (200g/L). Alkaline hydrolysis at 60°C was used with very high hydroxide concentrations to rapidly degrade high concentrations of NC, producing high nitrate and nitrite concentrations. The NC hydrolysate was neutralized and spiked into a denitrifying culture which was able to reduce both nitrate and nitrite. The goal of this work was to develop a dual component chemical-biological system for complete degradation of the explosives TNT and NC, which was achieved using alkaline hydrolysis as the chemical component and bacterial wastewater treatment enrichments as the biological component.

INTRODUCTION

Millions of pounds of explosives waste exist as contaminants in soil and groundwater or as excess munitions at manufacturing operations (Spain, 2000; Kim et al., 1998). These wastes are typically toxic to soil and groundwater microorganisms, require specialized storage, and are in need of safe disposal. Disposal methods reported to date have included chemical treatments as well as certain types of biological treatments. The efficiency and safety of these treatments is minimal and prompts further research for improved disposal methods. This dissertation seeks to characterize and improve a combined chemical and biological treatment method for the safe disposal of the explosive compounds 2,4,6-trinitrotoluene (TNT) and nitrocellulose (NC).

2,4,6-Trinitrotoluene

Because of its energetic properties, the nitroaromatic compound 2,4,6-trinitrotoluene (TNT, Figure 1) is a chief component of several types of explosives. Classified as a Class A explosive (US DOT), TNT poses a detonation hazard at high concentrations if subjected to sufficient friction or impact. It is stable

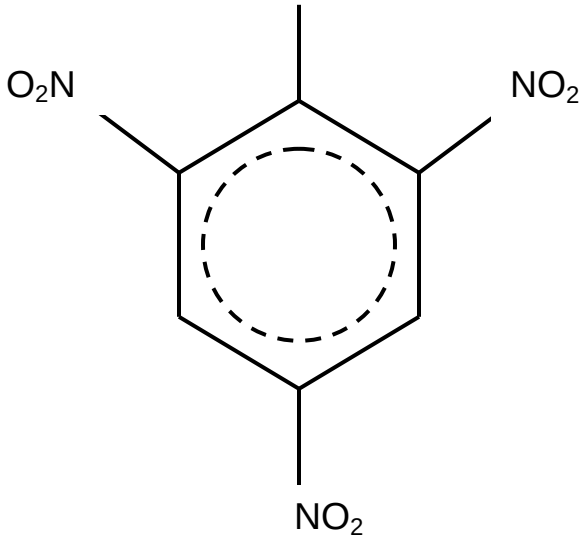


Figure 1: Chemical structure of TNT. Molecular mass = 227g/mole.

at low concentrations for temperatures below ~240°C (auto-ignition temperature). TNT is also very stable in water, for which the solubility is approximately 88mg/L at 20°C (Ro et al., 1996).

There are 40 sites within the United States with TNT soil and groundwater contamination, all of which are munitions manufacturing or testing facilities (Spain et al., 2000). In Europe, some TNT-contaminated sites are used for commercial and residential purposes (Spain et al., 2000). The need for clean-up of these sites is critical based on the toxic nature of TNT, not only to growth of plants and microorganisms, but also to humans. TNT is a suspected carcinogen, and prolonged TNT exposure has been shown to cause infertility, liver damage, and anemia (Yinon, 1990). To address these risks, treatment of soil and groundwater tainted with TNT has been the focus of many experimental studies, as described below.

Abiotic Treatments

The most widely employed method for destruction of TNT is incineration (Spain et al., 2000; Zupko et al., 1999). This method has been discouraged in recent decades due to environmental issues with air quality, as well as safety issues related to TNT's Class A explosive characteristics. Also, soil contaminated with TNT is consistently co-contaminated with metals and asbestos-containing materials; therefore incineration of these soils draws concern for more than just nitroaromatics in burn exhausts. Incineration trials of TNT-contaminated soil were performed to monitor both carcinogenic and non-carcinogenic health risk quotients (the ratio of the potential exposure to effect) of the burn exhaust (Zupko et al., 1999). For 72,000 tons of soil incinerated by Zupko et al.

(2000), the resulting emissions for TNT and related nitroaromatic compounds were measured to have a carcinogenic health risk quotient of 5.098×10^{-10} (accepted limit 1×10^{-6}) and a non-carcinogenic health risk quotient of 1.36×10^{-5} (accepted limit 1.0). The current acceptable limit for TNT in post-incineration soil is 57ppm according to the U.S. E.P.A. (1994), which still presents a significant toxic risk for soil and marine biota (Won et al., 1976; Zupko et al., 2000). Additionally, public opposition continues to hinder consistent use of incineration (Admassu et al., 1998; Esteve-Nunez et al., 2001; Heilmann et al., 1996; Labidi et al., 2001; Li et al., 1997; Symons et al., 2006), especially for non-military contaminated areas outside of the United States.

Another tested method is the use of the Fenton reaction for oxidation of TNT, which has been shown to completely destroy TNT in contaminated water, soil-water slurries, and aqueous extracts of contaminated soil (Li et al., 1997; Hess and Schrader, 2002; Kroger and Fels, 2007; Schrader and Hess, 2004). The Fenton reaction involves the use of ferrous iron (Fe^{2+}) and hydrogen peroxide (H_2O_2) to generate hydroxyl radicals as an oxidizing agent in aqueous solution. Because of the hydroxyl radical's strong oxidizing nature, many nonspecific oxidation reactions occur with organic compounds, leading to a variety of low molecular weight products. Complete mineralization of TNT to carbon dioxide, water, and nitrate is possible with excess hydroxyl radicals in the reaction solution. For TNT specifically, the Fenton reaction was most effective under conditions of low pH (optimal at pH 3 of tested range pH 2.5-7.5), elevated temperature ($\geq 45^{\circ}C$), and high concentrations of both Fe^{2+} (320 mg/L) and H_2O_2 (5.0%). The nitrogen from the TNT molecule was released stoichiometrically as nitrate (NO_3^-), which is three

moles NO_3^- per mole TNT. The products of TNT oxidation from the Fenton reaction (CO_2, H_2O, NO_3^- and low molecular weight organic acids) are non-toxic and safe for disposal directly to wastewater treatment facilities. However, to prevent adverse effects on any subsequent biological treatment, neutralization and removal of excess Fe^{2+} as well as removal of hydroxyl radicals is necessary. No reports of TNT oxidation by Fenton reaction were found that addressed the possibility of nitrate conversion to nitrite, of which high concentrations would be detrimental to wastewater treatment microorganisms. While the Fenton reaction has been proven effective for complete transformation of TNT in solution, the need for multi-step treatment of the post-reaction solution to accommodate downstream applications is a clear disadvantage.

The use of alkaline hydrolysis as a chemical treatment process alternative to incineration and the Fenton reaction has been frequently reported (Bajpai et al., 2004; Bishop et al., 2000; Davis et al., 2006; Emmrich, 1999; Felt et al., 2002; Hwang et al., 2005; Karasch et al., 2002; Mills et al., 2003; Saupe et al., 1998; Thorn et al., 2004). The synthesis of TNT is done by sequentially nitrating toluene with nitric acid under acidic conditions, which makes the application of alkaline conditions a promising method for TNT degradation. The electron-withdrawing effect of the three nitro groups on TNT makes it susceptible to nucleophilic attack by OH^-. Reports of alkaline hydrolysis of TNT have focused on reaction conditions when the concentration of hydroxide exceeds that of TNT. Significant degradation of TNT by alkaline hydrolysis has been assumed to occur only when the reaction pH is 11 or greater (Emmrich, 1999), which corresponds to an excess of hydroxide relative to TNT. For example, at 20°C when the maximum solubility

of TNT in water is approximately 88 mg/L (388μM), an equal molar amount of hydroxide ion corresponds to pH 10.6. This has been demonstrated in the literature: alkaline hydrolysis studies involving TNT have only tested conditions of pH ≥ 11. Additionally, higher pH has been correlated to faster rates of TNT disappearance (Saupe et al., 1998; Mills et al., 2003; Bajpai et al., 2004). Elevated temperatures (>20°C) have also been shown to further increase alkaline hydrolysis rates (Saupe et al., 1998; Bajpai et al., 2004). However, the combination of elevated temperatures with pH values less than 11 has not been represented in the literature with respect to alkaline hydrolysis of TNT.

Previously reported products of TNT hydrolysis have included: nitrate, nitrite, ammonia, formate, acetate, oxalate, various nitroaromatics, TNT dimers, and other uncharacterized polymers (Bishop et al., 2000; Felt et al., 2002; Hwang et al., 2005; Mills et al., 2003; Saupe et al., 1998; Thorn et al., 2004). While these products are less toxic and less energetic than the parent TNT molecule, some may be generated at high enough concentrations to inhibit subsequent biological treatment at a wastewater treatment facility if left undiluted (Won et al., 1976). In addition, the high hydroxide concentrations require considerable dilution and/or neutralization before any biological treatment can be applied.

Biological Treatments

As an alternative to chemical methods, microbiological transformation of TNT under near-neutral conditions (pH 6-8) has also been reported. While TNT is generally toxic to a wide range of microorganisms, some mesophilic (20-40°C) cultures can transform the TNT molecule without significant adverse effects on cell growth or

viability (Admassu et al., 1998; Borch et al., 2005; Bruns-Nagel et al., 1996). The most commonly observed degradation pathways involve reduction or denitration of one or more nitro group substituents, (Claus et al., 2007; Conder et al., 2004). Residual nitroaromatic metabolites of TNT degradation have been found to be less toxic to microbial cultures than TNT itself (Popesku et al., 2006). The majority of biological treatment studies describe the transformation of TNT as a co-metabolic process, requiring a separate carbon and nitrogen source for microbial growth (Daun et al., 1999; French et al., 1998; Khachatryan et al., 2000). In most cases, aromatic ring cleavage is minimal, as is mineralization to CO_2. Figure 2, from Borch and Gerlach (2004), shows the nitroaromatic compounds and pathways that have been observed for biological TNT transformation in soil and water by *Pseudomonas*, *Clostridium*, and *Desulfovibrio* species.

Under aerobic conditions, complete co-metabolic transformation of TNT was observed for undefined soil cultures (both bacteria and fungi) (Bruns-Nagel et al., 1996; Khachatryan et al., 2000) as well as for isolated strains of *Escherichia coli, Raoultella terrigena, Pseudomonas putida, and Methylobacterium* species (Kurinenko et al., 2005; Claus et al., 2007; Park et al., 2003; Van Aken et al., 2004). Some TNT degraders were taken from cultures enriched on other organic substrates such as activated sludge, s-triazine, or petroleum-based crude oil (Kim et al., 2002; Oh and Kim, 1998; Popesku et al., 2006). The most frequently reported metabolites for aerobic TNT transformation were

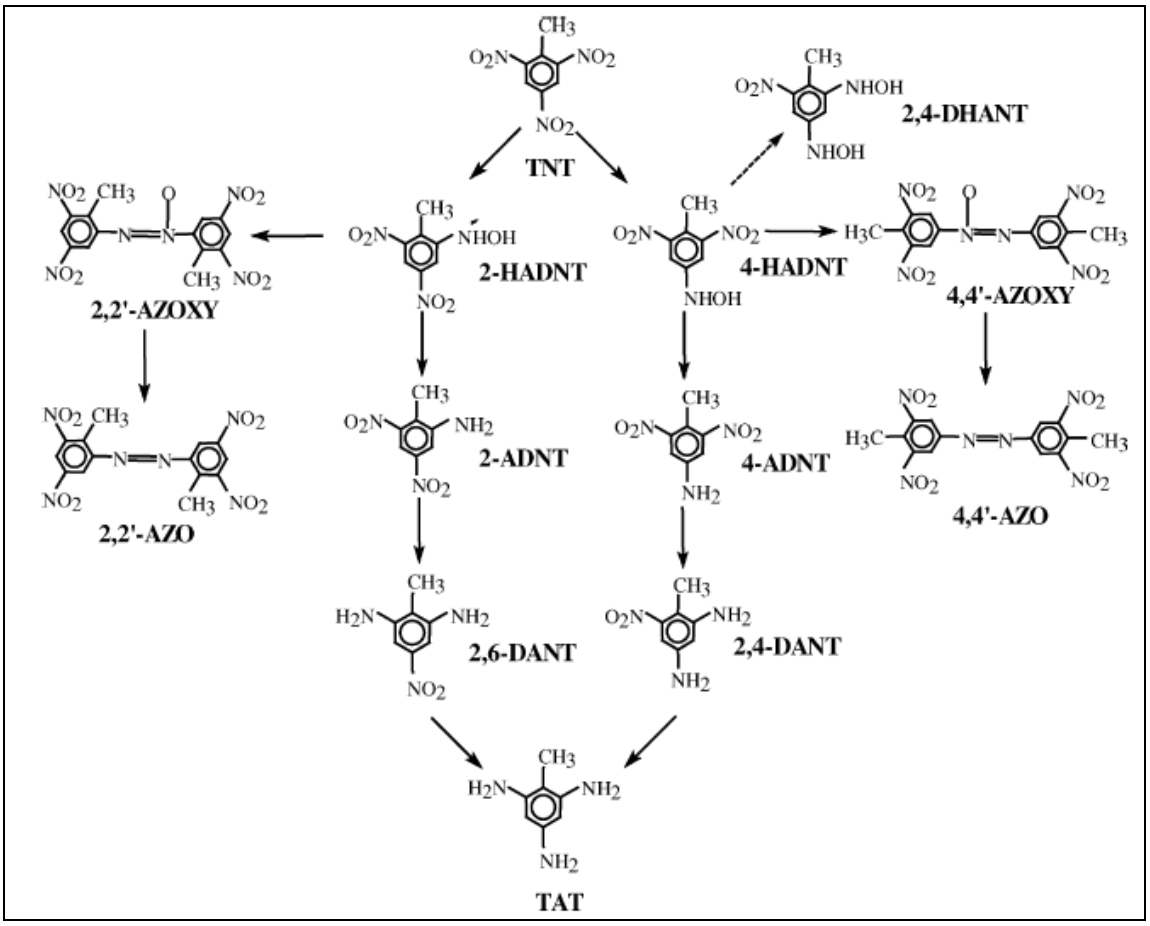


Figure 2: Pathways of biological TNT transformation. (from Borch and Gerlach , 2004).Key: 2,4,6-trinitroamtoluene (TNT); 2-amino-4,6-dinitrotoluene (2-ADNT); 4-amino-2,6-dinitrotoluene (4-ADNT); 2,4-diaminonitrotoluene (2,4-DANT); 2,6-diaminonitrotoluene (2,6-DANT); 2-hydroxylamino-4,6-dinitrotoluene (2-HADNT); 4-hydroxylamino-2,6-dinitrotoluene (4-HADNT); 2,4-dihydroxyl-6-aminonitrotoluene (2,4-DHANT); 4,4’,6,6’-tetranitro-2,2’-azotoluene (2,2’-AZO); 2,2’,6,6’-tetranitro-4,4’-azotoluene (4,4’-AZO); 4,4’,6,6’-tetranitro-2,2’-azoxytoluene (2,2’-AZOXY); 2,2’,6,6’-tetranitro-4,4’-azoxytoluene (4,4’-AZOXY); and 2,4,6-triaminotoluene (TAT).

2-ADNT and 4-ADNT (Bruns-Nagel et al., 1996; Claus et al., 2007; Conder et al., 2004; Oh and Kim, 1998; Park et al., 2003; Van Aken et al., 2004). Further reduction of a second nitro group was observed by the appearance of 2,4-DANT and 2,6-DANT along with nitrite released as NO_2^- (Bruns-Nagel et al., 1996; Claus et al., 2007; Kim et al., 2002; Van Aken et al., 2004). Other metabolites detected during aerobic transformation

of TNT included 4-N-acetylamino-2-amino-6-nitrotoluene (Bruns-Nagel et al., 1996), HADNTs (Kim et al., 2002), and 2,4- and 2,6-DNT (Park et al., 2003). Studies that monitored radiolabeled ^{14}C-TNT found up to 80% of TNT-associated carbon present in the cell pellet, some of which was identified as azoxy dimers of TNT (Claus et al., 2007; Conder et al., 2004). Unfortunately, in most experiments where certain amounts of carbon are found to be associated with cell pellets, it is not discernable whether the TNT-carbon has actually been incorporated into the cells or just externally bound to the cells (French et al., 2001). In a rare instance, Kim et al. (2002) reported TNT mineralization of 6% under aerobic conditions, though TNT was not used as a source of carbon or nitrogen.

Under anaerobic conditions, bacteria requiring an external source of both carbon and nitrogen to transform TNT included strains of *Clostridium, Desulfovibrio, Cellulomonas, Pseudomonas, Rhizobia,* and *E. coli* (Admassu et al., 1998; Borch at al., 2005; Esteve-Nunez et al., 2001; Kubota et al., 2008; Labidi et al., 2001; Lewis et al., 1996; McCormick et al., 1976), as well as mixed cultures from wastewater treatment plant enrichments (Daun et al., 1999; Kwon, 2000). These bacterial transformations produced many of the same metabolites observed under aerobic conditions, as well as reduced metabolites. However, the occurrence of TNT polymers (azo and azoxy dimers) was less frequent under anaerobic conditions, suggesting the prevalence of ring-cleavage products (Esteve-Nunez et al., 2001; McCormick et al., 1976). In strictly anoxic experiments under neutral pH, the most reduced TNT metabolite, TAT, was observed (Daun et al., 1999; Esteve-Nunez et al., 2001; Lewis et al., 1996). Other metabolites observed included limited amounts of aliphatic compounds (indicative of ring cleavage),

phenolic and more polar compounds, and carbon dioxide (<2% mineralization of TNT) (Esteve-Nunez et al., 2001; Labidi et al., 2001; Lewis et al., 1996).

When fungi are used to mediate co-metabolic TNT transformation, similar nitroaromatic metabolites are observed, though often with more extensive mineralization (where mineralization is defined as the percentage of TNT-carbon transformed to CO_2). Reports of mineralization of TNT by single strain fungi include: 18% by *Phanerochaete chrysosporium*, 36% by *Stropharia rugosoanulata*, and 42% by *Clitocybula dusenii* (Esteve-Nunez et al., 2001 and French et al., 2001). A mixed culture of bacteria and fungi was reported with 60% mineralization of TNT as a co-metabolic process (French et al., 2001). Fungal transformation of TNT has been found to proceed via ligninolytic enzymes, which suggests that fungi may not be suitable for in-situ soil remediation schemes due to competition with soil bacteria and lack of lignin-like substrates (French et al., 2001).

A more efficient approach to biological TNT degradation is the use of TNT as the sole source of nitrogen. In many cases when TNT served as the nitrogen source, growth conditions were aerobic, and removal of nitro groups (as opposed to reduction only) was more common than when an external source of nitrogen was provided. Strains of *Pseudomonas*, *Enterobacter*, *Desulfovibrio*, and *Stenotrophomonas* can more easily degrade mono- and dinitrotoluenes than trinitrotoluenes as the sole nitrogen source (Esteve-Nunez et al., 2001). Therefore, mixed cultures have shown more ring-cleavage products and TCA cycle intermediates than single strain cultures alone (Esteve-Nunez et al., 2001; Stenuit et al., 2005). In mixed bacterial cultures, mineralization of TNT was

less than 3% (Esteve-Nunez et al., 2001; Stenuit et al., 2005). Certain strains of *E. coli* using TNT as the sole nitrogen source produced nitrite, 2-hydroxylamino-6-nitrotoluene, 2-ADNT, 4-ADNT, and both 2,2'- and 4,4'-azoxy dimers (Gonzalez-Perez et al., 2007; Stenuit et al., 2006). Both Vorbeck et al. (1998) and French et al. (1998) identified H^--TNT hydride and $2H^-$-TNT dihydride Meisenheimer complexes as intermediates in the reaction solution, followed by the formation of unknown polar products.

The most desirable biological treatment for TNT transformation would be one where TNT is used as a source of both carbon and nitrogen. This is a major obstacle to bioremediation treatment processes, as reports of it are rare. French et al. (2001) and Stenuit et al. (2005) have noted that cultures able to use TNT for both carbon and nitrogen are likely to be mixed cultures continuously grown in the presence of TNT that eventually adapt to solely use TNT. One case of TNT being employed as the sole carbon, nitrogen, and energy source was reported by Tront and Hughes (2005) with a continuously grown mixed culture originally enriched on 2,4-DNT that had been maintained in their laboratory for 5 years. This report described a novel aerobic degradation pathway through 3-methyl-4,6-dinitrocatechol, which is typically a precursor to aromatic ring cleavage. Because this was a mixed culture, more than one pathway for TNT transformation was used, resulting in only a small amount of TNT being ultimately mineralized (3%). Approximately 17% of TNT-associated carbon was incorporated into the cell pellet, and 2- and 4-ADNT were detected as accumulated products.

Biological degradation of TNT is not a straightforward process. Different microorganisms and experimental growth conditions induce different transformation

pathways, intermediates, products, growth and degradation rates, and yields. In addition, the time (weeks to months) for complete transformation of TNT may be an issue when considering remediation treatments. With respect to these issues, the combination of abiotic and biological methods in one treatment system may be more favorable for the transformation of TNT to environmentally benign end products than the use of either abiotic or biological methods alone.

Nitrocellulose

Nitrocellulose (NC) is a nitrated form of cellulose, whose structure is given in Figure 3. NC is typically characterized by the degree of nitration (%N), which is the weight percentage contributed by the nitrogen incorporated as nitrate groups on the NC molecule. The fully nitrated form of NC, where the three hydroxyl groups of cellulose have each been replaced with nitrate, is 14.15% N. Considered a Class B explosive (US Department of Transportation), NC poses a significant fire hazard when dry, though it is stable when wet. Aside from its energetic properties, NC is non-toxic. Its primary use is in gunpowder and rocket propellant, which requires %N values greater than 12.5%. Many commercial products contain NC at %N values between 11.5 and 12.5%, including photographic film, filter membranes, inks, adhesives, and plastics. All NC whose %N is less than 11.5% is considered waste, as is NC with short fiber length (Kim et al., 1998).

The main generators of NC waste are ammunitions manufacturing facilities, of which there are 15 plants in the United States, some of which have accumulated upwards

of one million pounds of NC waste (often termed “legacy waste”). For safety reasons, this waste is typically kept in large holding ponds awaiting disposal.

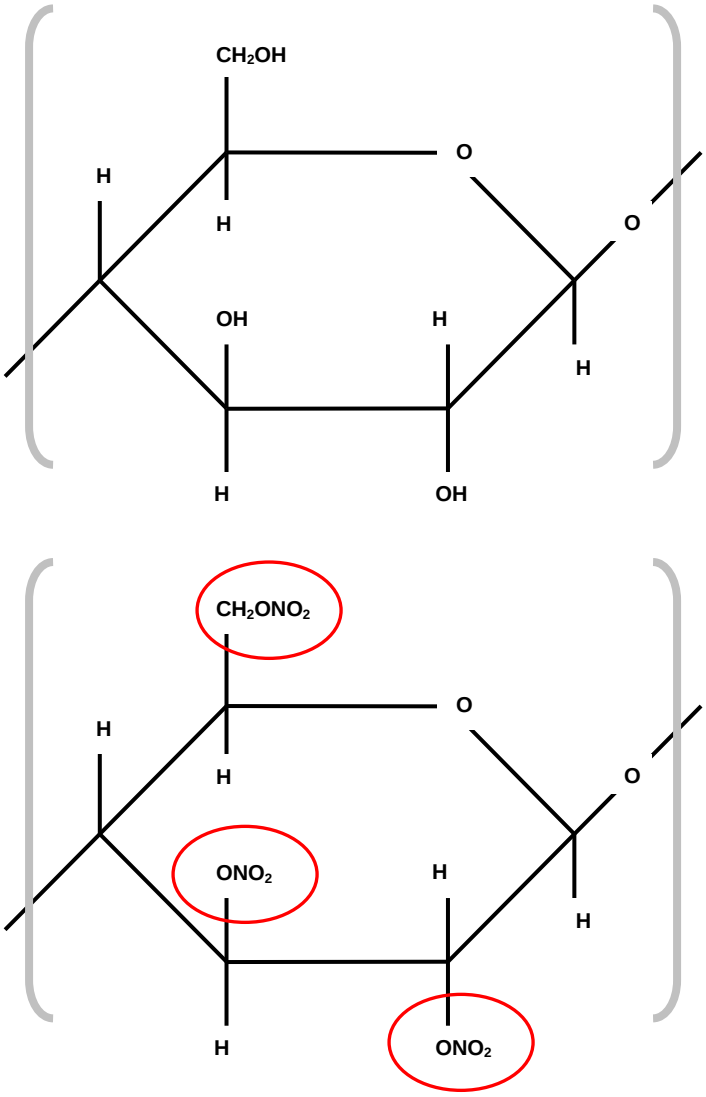


Figure 3: Chemical structure of cellulose (top) and fully nitrated nitrocellulose (bottom). Molecular mass of 14.15% N nitrocellulose = 297g/mole.

Because the explosive nature of NC prohibits land-filling as a means of disposal, other treatment methods have been investigated, including both abiotic and biological approaches. While the primary aim of NC disposal is to decrease the %N to below explosive levels (about 10%N), this less-nitrated NC would still be highly flammable. Therefore complete transformation of the NC molecule to non-hazardous and non-toxic products is preferred (Kim et al., 1998; Christodoulatos et al., 2001).

The most widely-used method for NC disposal is controlled open burning or closed-vessel incineration (Auer et al., 2005). However, this practice is becoming

increasingly unattractive from air quality and operation safety standpoints. Another common treatment for NC degradation is hydrolysis. Acid hydrolysis has been shown to reduce the % N (Christodoulatos et al., 2001), which removes the explosive hazard of the NC, though it still remains flammable. It has been found that alkaline hydrolysis results in a more pronounced degradation of the NC molecule where the cellulosic backbone is broken down, resulting in non-explosive, non-flammable products (Christodoulatos et al., 2001).

The first report of alkaline hydrolysis of NC was published by Kenyon and Gray (1936). Some of the identified products of NC hydrolysis included: nitrate, nitrite, cyanide, carbon dioxide, oxalic acid, malic acid, glycolic acid, trioxyglutaric acid, dioxybutyric acid, malonic acid, tartonic acid, various sugars, modified celluloses and their nitrates, and NC with lower %N. They found that up to 70% of the nitrogen originally present on NC was released as nitrite, and also that increasing the hydroxide concentration and/or the reaction temperature (from 30°C to 60°C) hastened the solubilization of NC. The NC concentration used by Kenyon and Gray (1936) was 10g/L, and hydroxide-to-NC molar ratios (OH^-:NC) tested ranged from 3:1 to 53:1. Some hydrolysis reactions were allowed to sit for as long as 46 days to ensure complete hydrolysis under conditions of lower OH^-:NC ratio and lower temperature.

More recently, Alleman et al. (1994) observed similar results for NC hydrolysis at 25, 35, and 50°C for OH^-:NC molar ratios between 12 and 50. The concentration of NC used was 6g/L. Comparison of KOH and NaOH showed approximately equal rates for degradation of NC. For all reaction conditions, less than 2% of NC carbon was

mineralized to CO_2. At 10g/L NaOH (OH^-:NC ratio of 12), 40% (by weight) of the original NC remained as suspended solids in solution, while nearly 80% of NC carbon was released as organic carbon, and 20% NC nitrogen was released as nitrate and nitrite. At 40g/L NaOH (OH^-:NC ratio 50), 17% (by weight) of the original NC remained as suspended solids in solution, while 100% of NC carbon was released as organic carbon, and 50% NC nitrogen was released as nitrate and nitrite. These numbers differ from Kenyon and Gray (1936) who observed 70% nitrogen released as nitrite at OH^-:NC ratios near 50.

Bunte et al. (1997) performed alkaline pressure hydrolysis of NC-containing propellants (made up of 56-97% NC having a nitrogen content of at least 12.5%), at 150°C and up to 30 bar. Large quantities of nitrogen-containing gaseous compounds were formed (NO_x compounds), as well as mono- and dicarboxylic acids in the hydrolysate solution. The highest OH^-:NC ratio in these experiments was 0.43, showing that the addition of pressure contributed greatly to hydrolysis at low hydroxide concentrations. However, the combination of high temperature and pressure presents safety concerns for disposal of munitions-grade propellants.

In 2001, Christodoulatos et al. performed a number of alkaline hydrolysis experiments with 10g/L NC, NaOH concentrations ranging from 1-150g/L, and temperatures from 30 to 90°C. Ratios of OH^-:NC ranged from 0.7-111 in this study. While higher hydroxide concentrations and temperatures increased the rate of the hydrolysis reaction, as had been shown previously (Kenyon and Gray, 1936; Alleman et al., 1994), the authors here confirmed that hydrolysis of >95% of the initial NC solids

was feasible in short reaction times (30-60 min) for 50°C or 70°C. It was found that nitrite and nitrate were released into solution at a consistent molar ratio of approximately 4:1, respectively. Depending upon the reaction conditions, up to 92% of the nitrogen from NC was released as nitrate and nitrite during hydrolysis.

Biological Treatments

The concept of a purely biological treatment to degrade NC seems applicable since NC is the cellulose molecule (which could be used by cellulose degraders) with nitro groups in place of the hydroxyl groups (which could be used by denitrifiers). In theory, mixed cultures of denitrifiers and cellulose degraders would be suitable for complete transformation of NC. Also, the availability of both carbon and nitrogen in the NC molecule make it an attractive candidate for efficient biological degradation, as supplementation of external carbon or nitrogen sources may not be necessary.

Unfortunately, it has been observed that even a small percentage of nitro-substituted hydroxyl groups on the cellulose molecule creates significant resistance to microbial degradation of the resulting cellulose derivative (Sui et al., 1949). Pfeil (1999) maintained that the nitro groups of NC created a major steric hindrance to enzymatic attack of the cellulosic backbone in a composting environment. In agreement with this statement, Freedman et al. (2002) attemped to use both denitrifying and sulfidogenic cultures to degrade NC, which was not used as the electron donor. Only reduction and removal of NC nitro groups was observed, though not to an adequate extent to render the initial munitions-grade NC (12-13.5% N) non-hazardous (i.e. %N below 10%).

One study with several types of lignocellulolytic and cellulolytic fungi showed some promise for biological NC degradation (Auer et al., 2005). Up to 43% of the inital NC (3-10g/L) was degraded, with some strains able to use the NC as a carbon and/or nitrogen source. Better degradation was observed when external carbon and nitrogen sources were supplied, and NC degradation by the fungi required several weeks. The requirement for supplemented carbon and nitrogen sources, as well as the prolonged reaction time are major obstacles to overcome to achieve an efficient biological process using fungi to transform the large back-log of accumulated NC waste.

Combined Hydrolysis and Biological Treatments: Based on the products of alkaline hydrolysis of NC that have been identified, a combined chemical and biological system may be feasible as a method of safe NC disposal. A few studies have been published with this intent. Wendt and Kaplan (1976) incorporated both denitrifying and aerobic activated sludge cultures to the treatment of NC hydrolysate. Incomplete hydrolysis of up to 20g/L NC was achieved with 30g/L NaOH (OH^-:NC molar ratio 11) at 95°C for 30 min. The resulting solution with residual NC solids was then subjected to a series of denitrifying and aerobic cultures. It was observed that the organic carbon from NC was not used as an electron donor, and no mineralization to carbon dioxide was observed. With the addition of an external carbon source, 88.6% of the biological oxygen demand (BOD), 55.2% chemical oxygen demand (COD) and 54.5% total organic carbon (TOC) was removed from the system. Since the starting NC concentration did not exceed 20g/L and hydrolysis was incomplete, concentrations of nitrate (170mg/L) and nitrite (2.4mg/L) in solution were not inhibitory to microbial growth. A combined hydrolysis

plus biological treatment process with the conditions used here would be acceptable for water treatment standards. The hazardous nature of the NC residual was not determined.

Alleman et al. (1994) and Kim et al. (1998) performed biodegradability tests on the hydrolysate of NC treated with NaOH. Hydrolysis was performed at 25°C with 6g/L NC and either 10 or 40g/L NaOH. Neither of the hydroxide concentrations tested was sufficient to completely hydrolyze the NC, as residual solids were observed after the reaction was finished. A municipal-industrial activated sludge culture, spiked with known nitrifying bacteria (which included ammonia and nitrite oxidizers) was used for biological treatment of the hydrolysate. The high nitrite concentrations measured, up to 340mg/L, were oxidized during BOD analysis and were responsible for misleadingly high BOD measurements. It was concluded that the organic compounds produced by NC hydrolysis were not susceptible to biological degradation. This may have been due to inhibition of microbial growth by the high nitrite present, which was not discussed as a possible reason for lack of change in the concentration of organics. The addition of denitrifying microorganisms, as opposed to nitrifying organisms, may have been beneficial for nitrite removal.

Though the studies of hydrolysis followed by biological degradation were not optimal for complete removal of NC, this process may still be effective given further characterization and optimization of both treatment steps.

Summary

In the case of TNT, exploring the effects of alkaline hydrolysis at more moderate hydroxide concentrations ($pH \leq 10$) combined with elevated temperature should be considered. At these pH levels, it may be feasible to add a biological component that can tolerate an alkaline environment and possibly also elevated temperatures. A combined treatment method having both abiotic and biological elements may be effective for safe conversion of TNT to environmentally benign end products. Similarly, a single-vessel treatment for NC that incorporates alkaline hydrolysis to degrade the parent molecule and biological denitrification to remove the resulting nitrite load would be beneficial for processing accumulated waste NC.

References

Admassu, W.; Sethuraman, A.V.; Crawford, R.; Korus, R.A. Growth kinetics of *Clostridium bifermentans* and its ability to degrade TNT using an inexpensive alternative medium. *Bioremediation Journal.* **1998**, *2*(1), 17-28.

Alleman, J.E.; Kim, B. J.; Quivey, D.M.; Equihua, L.O. Alkaline hydrolysis of munitions-grade nitrocellulose. *Wat. Sci. Tech.* **1994**, *30*, 63-72.

Auer, N.; Hedger, J.N.; Evans, C.S. Degradation of nitrocellulose by fungi. *Biodegradation.* **2005**, *16*, 229-236.

Bajpai, R.; Parekh, D.; Herrmann, S.; Popović, M.; Paca, J.; Qasim, M. A kinetic model of aqueous-phase alkali hydrolysis of 2,4,6-trinitrotoluene. *Journal of Hazardous Materials.* **2004**, *106B*, 55-66.

Bhushan, B.; Halasz, A.; Hawari, J. Effect of iron(III), humic acids and anthraquinone-2,6-disulfonate on biodegradation of cyclic nitramines by *Clostridium* sp. EDB2. *Journal of Applied Microbiology.* **2006**, *100*, 555-563.

Bishop, R.L.; Flesner, R.L.; Larson, S.A.; Bell, D.A. Base hydrolysis of TNT-based explosives. *Journal of Energetic Materials.* **2000**, *18*, 275-288.

Boopathy, R.; Gurgas, M.; Ullian, J.; Manning, J.F. Metabolism of explosive compounds by sulfate-reducing bacteria. *Current Microbiology.* **1998**, *37*, 127-131.

Borch, T.; Inskeep, W.P.; Harwood, J.A.; Gerlach, R. Impact of ferrihydrite and anthrquinone-2,6-disulfonate on the reductive transformation of 2,4,6-trinitotoluene by a gram-positive fermenting bacterium. *Environ. Sci. Technol.* **2005**, *39*, 7126-7133.

Brown, K.W. and Thomas, J.C. Degradation of explosive propellants by in-vessel composting. *BioCycle.* **1995**, *36*, 56-61.

Bunte, G.; Drause, H.H.; Hirth, T. Disposal of energetic materials by alkaline pressure hydrolysis and combined techniques. *Prop. Expl. Pyrotech.* **1997**, *22*, 160-164.

Bruns-Nagel, D.; Breitung, J.; von Löw, E.; Steinbach, K.; Gorontzy, T.; Kahl, M.; Blotevogel, K.-H.; Gemsa, D. Microbial transformation of 2,4,6-trinitrotoluene in aerobic soil columns. *Applied and Environmental Microbiology.* **1996**, *62*(7), 2651-2656.

Christodoulatos, C.; Su, T.-L.; Koutsospyros, A. Kinetics of the alkaline hydrolysis of nitrocellulose. *Water Environ. Res.* **2001**, *73*, 185-191.

Claus, H.; Bausinger, T.; Lehmler, I; Perret, N.; Fels, G.; Dehner, U.; Preuß, J.; König, H. Transformation of 2,4,6-trinitrotoluene (TNT) by *Raoultella terrigena*. *Biodegradation.* **2007**, *18*, 27-35.

Conder, J.M.; La Point, T.W.; Bowen, A.T. Preliminary kinetics and metabolism of 2,4,6-trinitrotoluene and its reduced metabolites in an aquatic oligochaete. *Aquatic Toxicology.* **2004**, *69*, 199-213.

Daun, G.; Lenke, H.; Knackmuß, H.-J.; Reuß, M. Experimental investigations and kinetic models for the cometabolic biological reduction of trinitrotoluene. *Chemical Engineering and Technology.* **1999**, *22*(4), 308-313.

Davis, E.P.; Boopathy, R.; Manning, J. Use of trinitrobenzene as a nitrogen source by *Pseudomonas vesicularis* isolated from soil. *Current Microbiology.* **1997**, *34*, 192-197.

Emmrich, M. Kinetics of the alkaline hydrolysis of 2,4,6-trinitrotoluene in aqueous and highly contaminated soils. *Environmental Science and Technology.* **1999**, *33*, 3802-3805.

Esteve-Núnez, A.; Caballero, A.; Ramos, J.L. Biological degradation of 2,4,6-trinitrotoluene. *Microbiology and Molecular Biology Reviews.* **2001**, *65*(3), 335-352.

Felt, D.R.; Larson, S.L.; Valente, E.J. UV-VIS spectroscopy of 2,4,6-trinitrotoluene-hydroxide reaction. *Chemosphere.* **2002**, *49*, 287-295.

Freedman, D.L.; Caenepeel, B.M.; Kim, B.J. Biotransformation of nitrocellulose under methanogenic conditions. *Wat. Sci. Tech.* **1996**, *34*, 327-334.

Freedman, D.L.; Cashwell, J.M.; Kim B.J. Biotransformation of explosive-grade nitrocellulose under denitrifying and sulfidogenic conditions. *Waste Management.* **2002**, *22*, 283-292.

French, C.E.; Nicklin, S.; Bruce, N.C. Aerobic degradation of 2,4,6-trinitrotoluene by *Enterobacter cloacae* PB2 and by pentaerythritol tetranitrate reductase. *Applied and Environmental Microbiology.* **1998**, *64*(8), 2864-2868.

French, C.E.; Rosser, S.J.; Bruce, N.C. Biotransformation of explosives. *Biotechnology and Genetic Engineering Reviews.* **2001**, *18*, 171-217.

Gonzalez-Perez, M.M.; van Dillewijn, P.; Wittich, R.-M.; Ramos, J.L. *Escherichia coli* has multiple enzymes that attack TNT and release nitrogen for growth. *Environmental Microbiology*. **2007**, *9*, 1535-1540.

Hess, T.F.; Schrader, P.S. Coupled abiotic-biotic mineralization of 2,4,6-trinitrotoluene (TNT). *Journal of Environmental Quality.* **2002**, *31*(3), 736-744.

Hwang, S.; Ruff, T.J.; Bouwer, E.J.; Larson, S.L.; Davis, J.L. Applicability of alkaline hydrolysis for remediation of TNT-contaminated water. *Water Research.* **2005**, *39*, 4503-4511.

Karasch, C.; Popovic, M.; Qasim, M.; Bajpai, R.K. Alkali hydrolysis of trinitrotoluene. *Applied Biochemistry and Biotechnology.* **2002**, *100*, 1173-1185.

Khachatryan, G.E.; Mkrtchyan, N.I.; Simonyan, N.V.; Khachatryan, T.V.; Tatikyan, S.Sh. Two unidentified aerobic bacterial strains that transform 2,4,6-trinitrotoluene. *World Journal of Microbiology and Biotechnology.* **2000**, *16*, 393-395.

Kim, B.J.; Alleman, J.E.; Quivey, D.M. (1998). *Alkaline hydrolysis/biodegradation of nitrocellulose fines.* (USACERL Technical Report 98/65). Champaign, IL: US Army Corps of Engineers Construction Engineering Research Laboratories.

Kim, H.-Y.; Bennet, G.N.; Song, H.-G. Degradation of 2,4,6-trinitrotoluene by *Klebsiella* sp. isolated from activated sludge. *Biotechnology Letters.* **2002**, *24*, 2023-2028.

Kubota, A.; Maeda, T.; Nagafuchi, N.; Kadokami, K.; Ogawa, H.I. TNT biodegradation and production of dihydroxylamino- nitrotoluene by aerobic TNT degrader *Pseudomonas* sp. strain TM15 in an anoxic environment. *Biodegradation*. **2008**, *19*, 795-805.

Kurinenko, B.M.; Denivarova, N.A.; Yakovleva, G.Yu. Sensitivity of various *Escherichia coli* strains to 2,4,6-trinitrotoluene. *Applied Biochemistry and Microbiology.* **2005**, *41*(1), 47-50.

Kwon, S.-H. A biological approach in the treatment of TNT wastewater. *Toxicological and Environmental Chemistry.* **2000**, *75*(1-2), 17-23.

Labidi, M.; Ahmad, D.; Halasz, A.; Hawari, J. Biotransformation and partial mineralization of the explosive 2,4,6-trinitrotoluene (TNT) by rhizobia. *Canadian Journal of Microbiology.* **2001**, *47*, 559-566.

Lewis, T.A.; Goszczynski, S.; Crawford, R.L.; Korus, R.A.; Admassu, W. Products of anaerobic 2,4,6-trinitrotoluene (TNT) transformation by *Clostridium bifermentans*. *Applied and Environmental Microbiology.* **1996**, *62*(12), 4669-4674.

Li, Z.M.; Comfort, S.D.; Shea, P.J. Destruction of 2,4,6-trinitrotoluene by Fenton oxidation. *Journal of Environmental Quality.* **1997**, *26*, 480-487.

McCormick, N.G.; Feeherry, F.E.; Levinson, H.S. Microbial transformation of 2,4,6-trinitrotoluene and other nitroaromatic compounds. *Applied and Environmental Microbiology.* **1976**, *31*(6), 949-958.

Mills, A.; Seth, A.; Peters, G. Alkaline hydrolysis of trinitrotoluene, TNT. *Physical Chemistry Chemical Physics.* **2003**, *5*, 3921-3927.

Oh, K.-H. and Kim, Y.-J. Degradation of explosive 2,4,6-trinitrotoluene by *s*-triazine degrading bacterium isolated from contaminated soil. *Bulletin of Environmental Contamination and Toxicology.* **1998**, *61*, 702-708.

Park, C.; Kim, T.-H.; Kim, S.; Kim, S.-W.; Lee, J.; Kim, S.-H. Optimization for biodegradation of 2,4,6-trinitrotoluene (TNT) by *Pseudomonas putida*. *Journal of Bioscience and Bioengineering.* **2003**, *95*(6), 567-571.

Pfeil, A. Microbial degradation of nitrocellulose in a composting environment. *Prop. Expl. Pyrotech.* **1999**, *24*, 156-158.

Popesku, J.T.; Singh, A.; El-Alawi, Y.; Ward, O.P. Trinitrotoluene removal in a soil slurry and soil box systems by an oil-degrading mixed bacterial culture. *World Journal of Microbiology and Biotechnology.* **2006**, *22*, 1075-1081.

Ro, K.S.; Venugopal, A.; Adrian, D.D.; Constant, D.; Qaisi, K.; Valsaraj, K.T.; Thibodeaux, L.J.; Roy, D. Solubility of 2,4,6-trinitrotoluene (TNT) in water. *Journal of Chemical Engineering Data.* **1996**, *41*, 758-761.

Saupe, A.; Garvens, H.J.; Heinze, L. Alkaline hydrolysis of TNT and TNT in soil followed by thermal treatment of the hydrolysates. *Chemosphere.* **1998**, *36*(8), 1725-1744.

Schrader, P.S.; Hess, T.F. Coupled abiotic-biotic mineralization of 2,4,6-trinitrotoluene (TNT) in soil slurry. *Journal of Environmental Quality.* **2004**, *33*, 1202-1209.

Stenuit, B.; Eyers, L.; El Fantroussi, S.; Agathos, S.N. Promising strategies for the mineralisation of 2,4,6-trinitrotoluene. *Reviews in Environmental Science and Bio/Technology.* **2005**, *4*, 39-60.

Stenuit, B.; Eyers, L.; Rozenberg, R.; Habib-Jiwan, J.-L.; Agathos, S.N. Aerobic growth of *Escherichia coli* with 2,4,6-trinitrotoluene (TNT) as the sole nitrogen source and evidence of TNT denitration by whole cells and cell-free extracts. *Applied and Environment Microbiology.* **2006**, *72*(12), 7945-7948.

Sui, R.G.H.; Darby, R.T.; Burkholder, P.B.; Barghoorn, E.S. Specificity of microbial attack on cellulose derivatives. *Textile Res. J.* **1949**, *19*, 484-488.

Symons, Z.C.; Bruce, N.C. Bacterial pathways for degradation of nitroaromatics. *Natural Product Reports.* **2006**, *23*, 845-850.

Thorn, K.A.; Thorne, P.G.; Cox, L.G. Alkaline hydrolysis/polymerization of 2,4,6-trinitrotoluene: characterization of products by ^{13}C and ^{15}N NMR. *Environmental Science and Technology.* **2004**, *38*, 2224-2231.

Tront, J.M.; Hughes, J.B. Oxidative microbial degradation of 2,4,6-trinitrotoluene via 3-methyl-4,6-dinitrocatechol. *Environmental Science and Technology.* **2005**, *39*, 4540-4549.

Van Aken, B.; Yoon, J.M.; Schnoor, J.L. Biodegradation of nitro-substituted explosives 2,4,6-trinitrotoluene, hexahydro-1,3,5-trinitro-1,3,5-triazine, and octahydro-1,3,5,7-tetranitro-1,3,5-tetrazocine by a phytosymbiotic *Methylobacterium* sp. associated with poplar tissues (*Populus deltoides* × *nigra* DN34). *Applied and Environmental Microbiology.* **2004**, *70*(1), 508-517.

Vorbeck, C.; Lenke, H.; Fischer, P.; Spain, J.C.; Knackmuss, H.-J. Initial reductive reactions in aerobic microbial metabolism of 2,4,6-trinitrotoluene. *Applied and Environmental Microbiology.* **1998**, *64*(1), 246-252.

Won, W.D.; DiSalvo, L.H.; Ng, J. Toxicity and mutagenicity of 2,4,6-trinitrotoluene and its microbial metabolites. *Applied and Environmental Microbiology.* **1976**, *31*(4), 576-580.

Zupko, A.J.; Johnson, N.P.; Leibbert, J.M.; Empson, W.B.; Mroz, D.L.; Bales, F.E. Roy F.Weston, Inc. and United States Army Corps of Engineers, Kansas City District. Incineration of explosives-contaminated soil and reactive RCRA waste at the former Weldon Spring Ordnance Works, Missouri, USA. Technical paper (#9008) presented at the Incineration and Thermal Treatment Technology Conference. May 1999, Orlando, FL.

ENRICHMENT FOR A TNT-DEGRADING MICROORGANISM

Background

The occurrence of TNT soil and groundwater contamination at munitions manufacturing and testing sites is widespread in the Unites States. A method of safe disposal as an alternative to incineration, with the ultimate goal of transforming TNT to environmentally benign end products, is desired for remediation of these sites. While both alkaline hydrolysis and biological degradation of TNT have been investigated individually, a one-step combined treatment comprising both methods has not been reported to date. For this single-vessel system to work, lower hydroxide concentrations tolerable by microorganisms (i.e. pH $\leq$ 10) must be used for hydrolysis. To compensate for the decreased alkalinity, elevated temperatures may be used to promote hydrolysis of TNT. With these reaction conditions in mind, microorganisms able to grow at high temperature (>50°C) and relatively high pH (8-10) (known as thermoalkaliphiles) are needed.

Materials and Methods

To obtain bacteria capable of degrading TNT under alkaline conditions and at elevated temperatures, alkaline hot springs in the Heart Lake Geyser Basin of Yellowstone National Park were sampled. Water, sediment, and microbial mat samples from four alkaline hot springs were collected (Table 1).

Table 1: YNP spring samples.

Sample	GPS Coordinates	pH	Temperature
Spring 1	N 44°18' 15.9" W 110°31' 21.1"	8.72 - 9.10	77.5 - 81.1℃
Spring 1 effluent		9.02 - 9.36	53.6 - 62.8℃
Spring 2		8.58 - 9.13	83.3 - 88.7℃
Spring 3	N 44°18' 15.6" W 110°31' 20.7"	8.56 - 9.06	82.3 - 90.0℃
Spring 4	N 44°18' 14.4" W 110°31' 16.7"	8.96 - 9.06	66.0 - 68.8℃

Enrichments of each spring sample were carried out using a thermophile medium, adjusted to pH 9.0, adapted from Viamajala et al. (2007) (L^{-1}): 0.1g $C_6H_7NNa_2O_6$ (sodium nitrilotriacetic acid: a source of inorganic carbon, not used as carbon source for growth), 0.05g $CaCl_2 \cdot 2H_2O$, 0.125g $MgSO_4 \cdot 7H_2O$, 0.01g NaCl, 0.01g KCl, 0.05g $Na_2HPO_4 \cdot 7H_2O$, 0.03% (w/v) $FeCl_3$ solution, 1mL Nitsch's trace metal solution (composition (L^{-1}): 2.2g $MnSO_4 \cdot H_2O$, 0.5g $ZnSO_4 \cdot 7H_2O$, 0.5g H_3BO_3, 0.016g $CuSO_4 \cdot 5H_2O$, 0.025g $Na_2MoO_4 \cdot 2H_2O$, 0.046g $CoCl_2 \cdot 6H_2O$). An external carbon source, 1 g/L yeast extract, was added to the medium. An inoculum of 10% (v/v) of each spring sample was amended to the liquid medium, along with 20 mg/L TNT, and was incubated at both 60°C and 80°C for up to one month.

Detection and quantification of TNT and other nitroaromatics was done using an Agilent 1090 HPLC using a method developed by Borch and Gerlach (2004). Protein production was measured using the Bradford assay (Bradford, 1976).

To isolate single bacterial species from a mixed culture, liquid cultures originating from the Sp4 spring sample were transferred at least three times between TNT-containing (20 mg/L TNT) solid and liquid media. Noble agar plates (20 g/L) with the thermophile medium adapted from Viamajala et al. (2007) were streaked and incubated at 60°C. Individual colonies that grew were identical in morphology. DNA extraction of the colonies was performed using an Ultra Clean Microbial DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA), followed by PCR with PCR Master Mix (Promega Corp., Madison, WI), 8F forward and 1492R reverse primers (IDT, Inc., Coralville, IA). PCR product clean-up was done with an Exo-SapIT PCR Clean-up kit (USB Corp., Cleveland, OH) and cleaned PCR product was sent to the Idaho State University Molecular Research Core Facility for sequencing of the isolate's 16S rRNA gene. Results for fourteen forward sequences and fourteen reverse sequences were aligned using ClustalX to obtain consensus forward and reverse sequences. A sequence similarity search was performed using the Basic Local Alignment Search Tool (BLAST; found on the National Center for Biotechnology Information (NCBI) website ncbi.nlm.nih.gov).

Results and Discussion

Significant growth was observed at 60°C for the Spring 4 (Sp4) inoculum with yeast extract as the carbon source. In agreement with many reported biological TNT

degradation studies, no growth was observed in enrichments without an external carbon source.

Kinetic experiments with the Sp4 mixed culture grown on yeast extract at 60°C and pH 9.0 in the presence of 16mg/L TNT were performed. As seen in Figure 4, disappearance of TNT in the Sp4 inoculated treatment occurred in about 40h. Both the cell-free and yeast extract free cultures showed significant depletion of TNT during the course of this experiment, albeit at slower rates than that of the Sp4 inoculated treatment.

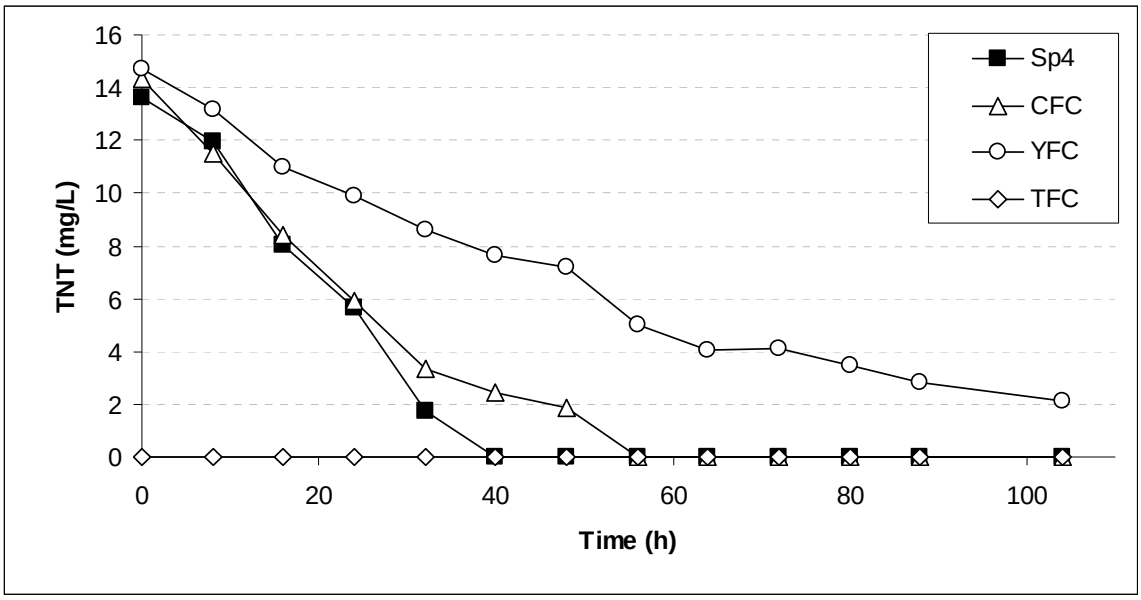


Figure 4: Biological treatment and controls for 16mg/L TNT degradation with 1g/L yeast extract at 60°C and pH 9. Error bars represent one standard deviation of triplicate experiments. Absence of error bars indicates error bars are smaller than data symbol. (CFC is cell-free control; TFC is TNT-free control; YFC is yeast extract free control)

Figure 5 shows the total protein production for this experiment. Comparison between Figures 4 and 5 suggests that microbial growth in the Sp4 treatment is inhibited until TNT has been completely degraded. However, the total amount of protein produced by

the Sp4 treatment reaches the same level as that of the TNT-free control suggesting the presence of TNT was not detrimental to biomass production.

Significant changes in a peak having a retention time of 9.4 min. were observed in the HPLC chromatograms during this experiment (data not shown). As shown in Figure 6, this degradation product reached a maximum concentration coincident with the disappearance of TNT (see Figure 4) and remained in solution throughout the experiment in the controls. Interestingly, in the Sp4 treatment, this product was quickly consumed.

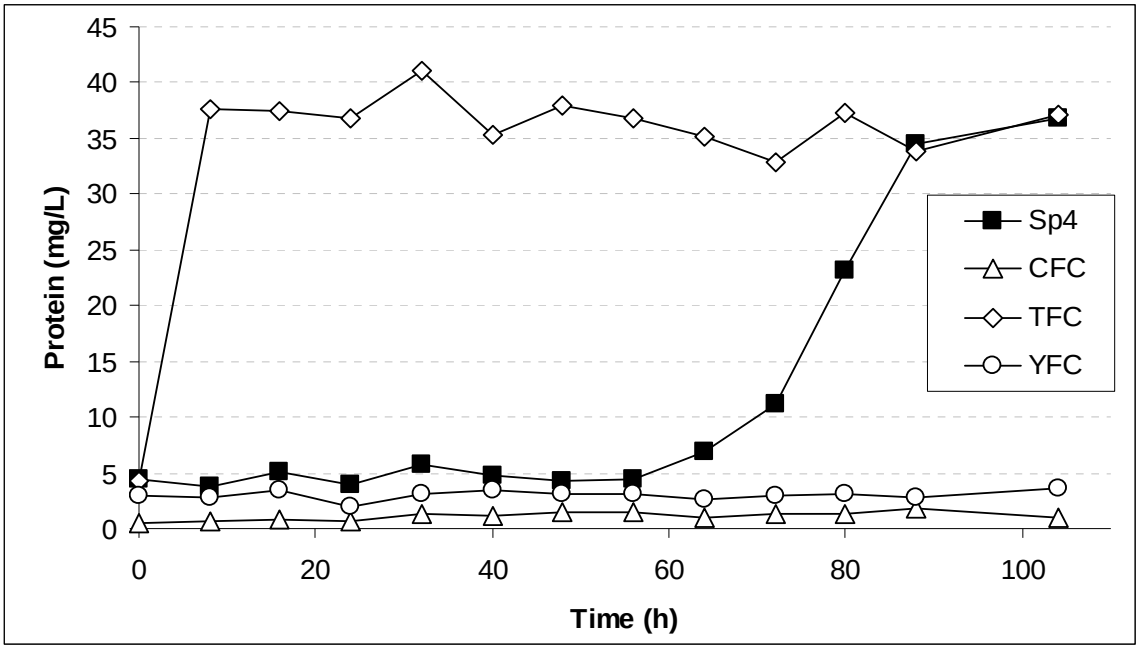


Figure 5: Protein production for 16mg/L TNT degradation and 1g/L yeast extract at 60°C and pH 9. Error bars represent one standard deviation of triplicate experiments. Absence of error bars indicates error bars are smaller than data symbol. (CFC is cell-free control; TFC is TNT-free control; YFC is yeast extract free control)

With the use of LC-MS and confirmation by HPLC standards, the product was identified as 1,3,5-trinitrobenzene (TNB) (see Figure 7). It was calculated that only one-third of the moles of TNT initially present were transformed to TNB.

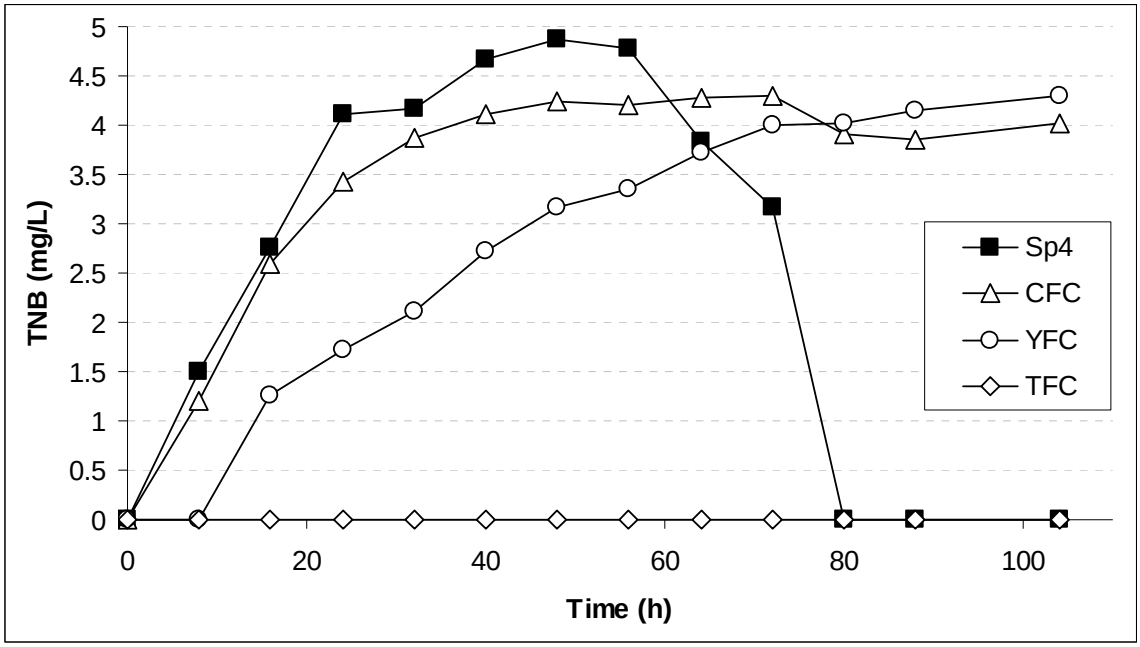


Figure 6: Occurrence of TNB as the abiotic product of 16mg/L TNT degradation at 60°C and pH 9. Error bars represent one standard deviation of triplicate experiments. Absence of error bars indicates error bars are smaller than data symbol. (CFC is cell-free control; TFC is TNT-free control; YFC is yeast extract free control)

Similar to TNT, TNB is also a Class A explosive (US Department of Transportation), though it is less sensitive to impact than TNT. Most TNB contamination results from the slow photo-oxidation of TNT and persists in the environment because it is not easily degraded (Hwang et al., 2000; Reddy, 1997). For this

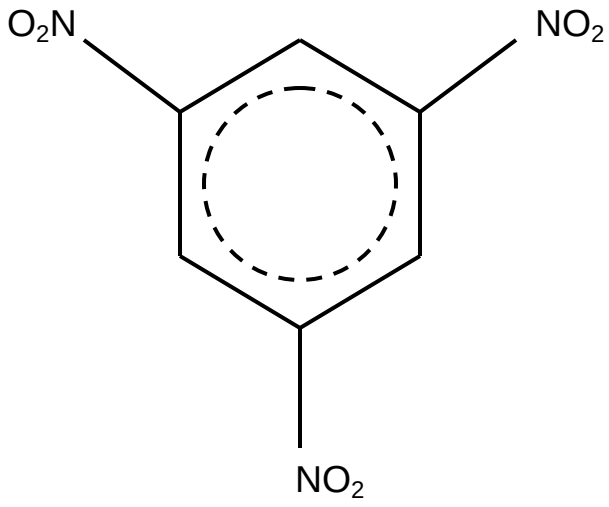


Figure 7: Chemical structure of TNB. Molecular mass = 213g/mole.

study, preliminary experiments were done with both foil-covered serum bottles and bottles exposed to light. No difference was observed between dark and light exposed bottles with respect to TNT degradation, TNB production, or microbial growth (data not shown).

TNB has a tendency to leach out of soil, thus causing groundwater contamination (Reddy, 1997). Few studies of biological degradation of TNB have been done. Boopathy et al. (1998) described TNB degradation by a bacterial consortium under sulfate reducing conditions. Davis et al. (1997) found that a strain of *Pseudomonas vesicularis* used TNB under aerobic conditions as the sole nitrogen source during growth, for which nitrobenzene (NB) and ammonia were the end products.

Isolation of the TNB-degrading organism and DNA extraction was performed as laid out in the Materials and Methods section. The BLAST results of the consensus sequence showed the TNB-degrading isolate to be a member of the *Anoxybacillus* genus, most closely related to the *Anoxybacillus kualawohkensis* with a 99% 16S sequence similarity.

To find an alternative to yeast extract and have a completely defined growth medium, aerobic carbon source screening was performed with *A. kualawohkensis* in the presence (5mg/L) and absence of TNB based on turbidity. Growth with TNB was observed to occur only with sugars (dextrose, fructose, galactose, maltose, sucrose) as the carbon source; no growth was observed with compounds such as lactate, acetate, butyrate, or methanol. Dextrose (1g/L) was used as the carbon source for all subsequent experiments. This bacterial isolate was screened for aerobic TNB tolerance and found to

grow in the presence of up to 35mg/L TNB. Growth was measured as optical density at 620nm. Anaerobic TNB tolerance was investigated, up to 50mg/L TNB. Interestingly, anaerobic bottles at high TNB concentrations became turbid after 1-2 days while aerobic bottles required significantly longer periods of time (1-2wks) to become cloudy, even at the lowest TNB concentration tested, 5mg/L (data not shown).

Considering that growth (based on turbidity) of *A. kualawohkensis* was faster under anaerobic conditions, further experiments were done to test TNB for use as the sole nitrogen source, including TNB-free and cell-free controls. Turbidity was observed for *both* inoculated bottles and cell-free controls, protein measurements confirmed that growth only occurred in inoculated bottles. HPLC analysis of the culture solution and cell-free control showed that TNB had been depleted in both. A number of other measures were taken to ensure there was no contamination of cell-free controls, however TNB disappearance and solution cloudiness were still observed.

A literature search attempting to find information about the interaction between dextrose and TNB that might be causing the observed cloudiness produced a patent describing the degradation of nitrogen-based explosives by combining alkaline solution ($pH \leq 13$) and a soluble carbohydrate (e.g. dextrose) with optional heating (Kornel, 2003). Figure 6 clearly showed that the Sp4 mixed culture was responsible for the disappearance of TNB produced from TNT when yeast extract was used as the carbon source. It may be that the amount of carbohydrates present in the yeast extract was low enough so as not to compete with the biological transformation of TNB by the culture. Based on this information and the results of the above experiments, it was concluded that

A. kualawohkensis was not metabolizing TNB when 1g/L dextrose was used as the carbon source. The disappearance of TNB was due to an abiotic reaction with the dextrose as stated within the patent, and was faster under anaerobic conditions.

Conclusions

Being unable to find a suitable thermoalkaliphilic culture for use in a single-step combined hydrolysis and biological treatment, it was decided to separate the two processes and focus on the rates and products of alkaline hydrolysis of TNT at concentrations where [TNT] > [OH^-]. The resulting TNT hydrolysate can then be screened for growth with readily available mesophilic and neutrophilic cultures, such as those enriched from a wastewater treatment plant.

References

Admassu, W.; Sethuraman, A.V.; Crawford, R.; Korus, R.A. Growth kinetics of *Clostridium bifermentans* and its ability to degrade TNT using an inexpensive alternative medium. *Bioremediation Journal.* **1998**, *2*(1), 17-28.

Bajpai, R.; Parekh, D.; Herrmann, S.; Popović, M.; Paca, J.; Qasim, M. A kinetic model of aqueous-phase alkali hydrolysis of 2,4,6-trinitrotoluene. *Journal of Hazardous Materials.* **2004**, *106B*, 55-66.

Bhushan, B.; Halasz, A.; Hawari, J. Effect of iron(III), humic acids and anthraquinone-2,6-disulfonate on biodegradation of cyclic nitramines by *Clostridium* sp. EDB2. *Journal of Applied Microbiology.* **2006**, *100*, 555-563.

Bishop, R.L.; Flesner, R.L.; Larson, S.A.; Bell, D.A. Base hydrolysis of TNT-based explosives. *Journal of Energetic Materials.* **2000**, *18*, 275-288.

Boopathy, R.; Gurgas, M.; Ullian, J.; Manning, J.F. Metabolism of explosive compounds by sulfate-reducing bacteria. *Current Microbiology.* **1998**, *37*, 127-131.

Borch, T. and Gerlach, R. Use of reversed-phase high-performance liquid chromatography - diode array detection for complete separation of 2,4,6-trinitrotoluene metabolites and EPA Method 8330 explosives: influence of temperature and an ion-pair reagent. *J. Chromatogr. A.* **2004**, *1022*, 83-94.

Borch, T.; Inskeep, W.P.; Harwood, J.A.; Gerlach, R. Impact of ferrihydrite and anthrquinone-2,6-disulfonate on the reductive transformation of 2,4,6-trinitotoluene by a gram-positive fermenting bacterium. *Environ. Sci. Technol.* **2005**, *39*, 7126-7133.

Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248-254.

Bruns-Nagel, D.; Breitung, J.; von Löw, E.; Steinbach, K.; Gorontzy, T.; Kahl, M.; Blotevogel, K.-H.; Gemsa, D. Microbial transformation of 2,4,6-trinitrotoluene in aerobic soil columns. *Applied and Environmental Microbiology.* **1996**, *62*(7), 2651-2656.

Claus, H.; Bausinger, T.; Lehmler, I; Perret, N.; Fels, G.; Dehner, U.; Preuß, J.; König, H. Transformation of 2,4,6-trinitrotoluene (TNT) by *Raoultella terrigena*. *Biodegradation.* **2007**, *18*, 27-35.

Conder, J.M.; La Point, T.W.; Bowen, A.T. Preliminary kinetics and metabolism of 2,4,6-trinitrotoluene and its reduced metabolites in an aquatic oligochaete. *Aquatic Toxicology.* **2004**, *69*, 199-213.

Daun, G.; Lenke, H.; Knackmuß, H.-J.; Reuß, M. Experimental investigations and kinetic models for the cometabolic biological reduction of trinitrotoluene. *Chemical Engineering and Technology.* **1999**, *22*(4), 308-313.

Davis, E.P.; Boopathy, R.; Manning, J. Use of trinitrobenzene as a nitrogen source by *Pseudomonas vesicularis* isolated from soil. *Current Microbiology.* **1997**, *34*, 192-197.

Davis, J.L.; Brooks, M.C.; Larson, S.L.; Nestler, C.C.; Felt, D.R. Lime treatment of explosives-contaminated soil from munitions plants and firing ranges. *Soil & Sediment Contamination.* **2006**, *15*, 565-580.

Emmrich, M. Kinetics of the alkaline hydrolysis of 2,4,6-trinitrotoluene in aqueous and highly contaminated soils. *Environmental Science and Technology.* **1999**, *33*, 3802-3805.

Emmrich, M. Kinetics of the alkaline hydrolysis of important nitroaromatic co-contaminants of 2,4,6-trinitrotoluene in highly contaminated soils. *Environmental Science and Technology.* **2001**, *35*, 874-877.

Esteve-Núnez, A.; Caballero, A.; Ramos, J.L. Biological degradation of 2,4,6-trinitrotoluene. *Microbiology and Molecular Biology Reviews.* **2001**, *65*(3), 335-352.

Felt, D.R.; Larson, S.L.; Valente, E.J. UV-VIS spectroscopy of 2,4,6-trinitrotoluene-hydroxide reaction. *Chemosphere.* **2002**, *49*, 287-295.

French, C.E.; Nicklin, S.; Bruce, N.C. Aerobic degradation of 2,4,6-trinitrotoluene by *Enterobacter cloacae* PB2 and by pentaerythritol tetranitrate reductase. *Applied and Environmental Microbiology.* **1998**, *64*(8), 2864-2868.

French, C.E.; Rosser, S.J.; Bruce, N.C. Biotransformation of explosives. *Biotechnology and Genetic Engineering Reviews.* **2001**, *18*, 171-217.

Gonzalez-Perez, M.M.; van Dillewijn, P.; Wittich, R.-M.; Ramos, J.L. *Escherichia coli* has multiple enzymes that attack TNT and release nitrogen for growth. *Environmental Microbiology.* **2007**, *9*, 1535-1540.

Hess, T.F.; Schrader, P.S. Coupled abiotic-biotic mineralization of 2,4,6-trinitrotoluene (TNT). *Journal of Environmental Quality.* **2002**, *31*(3), 736-744.

Hofstetter, T.B.; Heijman, C.G.; Haderlein, S.B.; Holliger, C.; Schwarzenbach, R.P. Complete reduction of TNT and other (poly)nitroaromatic compounds under iron-reducing subsurface conditions. *Environ. Sci. Technol.* **1999**, *33*, 1479-1487.

Hwang, H.-M.; Slaughter, L.F.; Cook, S.M.; Cui, H. Photochemical and microbial degradation of 2,4,6-trinitrotoluene (TNT) in a freshwater environment. *Bulletin of Environmental Contamination and Toxicology.* **2000**, *65*, 228-235.

Hwang, S.; Ruff, T.J.; Bouwer, E.J.; Larson, S.L.; Davis, J.L. Applicability of alkaline hydrolysis for remediation of TNT-contaminated water. *Water Research.* **2005**, *39*, 4503-4511.

Karasch, C.; Popovic, M.; Qasim, M.; Bajpai, R.K. Alkali hydrolysis of trinitrotoluene. *Applied Biochemistry and Biotechnology.* **2002**, *100*, 1173-1185.

Khachatryan, G.E.; Mkrtchyan, N.I.; Simonyan, N.V.; Khachatryan, T.V.; Tatikyan, S.Sh. Two unidentified aerobic bacterial strains that transform 2,4,6-trinitrotoluene. *World Journal of Microbiology and Biotechnology.* **2000**, *16*, 393-395.

Kim, H.-Y.; Bennet, G.N.; Song, H.-G. Degradation of 2,4,6-trinitrotoluene by *Klebsiella* sp. isolated from activated sludge. *Biotechnology Letters.* **2002**, *24*, 2023-2028.

Kornel, A. Decomposition of nitrogen-based energetic material. United States patent #PCT/US03/23602. Date issued September 15 **2003**.

Kubota, A.; Maeda, T.; Nagafuchi, N.; Kadokami, K.; Ogawa, H.I. TNT biodegradation and production of dihydroxylamino-nitrotoluene by aerobic TNT degrader *Pseudomonas* sp. strain TM15 in an anoxic environment. *Biodegradation*. **2008**, *19*, 795-805.

Kurinenko, B.M.; Denivarova, N.A.; Yakovleva, G.Yu. Sensitivity of various *Escherichia coli* strains to 2,4,6-trinitrotoluene. *Applied Biochemistry and Microbiology.* **2005**, *41*(1), 47-50.

Kwon, S.-H. A biological approach in the treatment of TNT wastewater. *Toxicological and Environmental Chemistry.* **2000**, *75*(1-2), 17-23.

Kwon, M.J.; Finneran, K.T. Microbially Mediated biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine by extracellular electron shuttling compounds. *Applied and Environmental Microbiology.* **2006**, *72*(9), 5933-5941.

Labidi, M.; Ahmad, D.; Halasz, A.; Hawari, J.. Biotransformation and partial mineralization of the explosive 2,4,6-trinitrotoluene (TNT) by rhizobia. *Canadian Journal of Microbiology.* **2001**, *47*, 559-566.

Lewis, T.A.; Goszczynski, S.; Crawford, R.L.; Korus, R.A.; Admassu, W. Products of anaerobic 2,4,6-trinitrotoluene (TNT) transformation by *Clostridium bifermentans*. *Applied and Environmental Microbiology.* **1996**, *62*(12), 4669-4674.

Li, Z.M.; Comfort, S.D.; Shea, P.J. Destruction of 2,4,6-trinitrotoluene by Fenton oxidation. *Journal of Environmental Quality.* **1997**, *26*, 480-487.

McCormick, N.G.; Feeherry, F.E.; Levinson, H.S. Microbial transformation of 2,4,6-trinitrotoluene and other nitroaromatic compounds. *Applied and Environmental Microbiology.* **1976**, *31*(6), 949-958.

Mills, A.; Seth, A.; Peters, G. Alkaline hydrolysis of trinitrotoluene, TNT. *Physical Chemistry Chemical Physics.* **2003**, *5*, 3921-3927.

Oh, K.-H. and Kim, Y.-J. Degradation of explosive 2,4,6-trinitrotoluene by *s*-triazine degrading bacterium isolated from contaminated soil. *Bulletin of Environmental Contamination and Toxicology.* **1998**, *61*, 702-708.

Park, C.; Kim, T.-H.; Kim, S.; Kim, S.-W.; Lee, J.; Kim, S.-H.. Optimization for biodegradation of 2,4,6-trinitrotoluene (TNT) by *Pseudomonas putida*. *Journal of Bioscience and Bioengineering.* **2003**, *95*(6), 567-571.

Popesku, J.T.; Singh, A.; El-Alawi, Y.; Ward, O.P. Trinitrotoluene removal in a soil slurry and soil box systems by an oil-degrading mixed bacterial culture. *World Journal of Microbiology and Biotechnology.* **2006**, *22*, 1075-1081.

Rainwater, K.; Heintz, C.; Mollhagen, T.; Hansen, L. *In situ* biodegradation of high explosives in soils: field demonstration. *Bioremediation Journal.* **2002**, *6*(4), 351-371.

Reddy, G.; Reddy, T.V.; Choudhury, H.; Daniel, F.B.; Leach, G.J. Assessment of environmental hazards of 1,3,5-trinitrobenzene. *Journal of Toxicology and Environmental Health.* **1997**, *52*, 447-460.

Ro, K.S.; Venugopal, A.; Adrian, D.D.; Constant, D.; Qaisi, K.; Valsaraj, K.T.; Thibodeaux, L.J.; Roy, D. Solubility of 2,4,6-trinitrotoluene (TNT) in water. *Journal of Chemical Engineering Data.* **1996**, *41*, 758-761.

Saupe, A.; Garvens, H.J.; Heinze, L. Alkaline hydrolysis of TNT and TNT in soil followed by thermal treatment of the hydrolysates. *Chemosphere.* **1998**, *36*(8), 1725-1744.

Schrader, P.S.; Hess, T.F. Coupled abiotic-biotic mineralization of 2,4,6-trinitrotoluene (TNT) in soil slurry. *Journal of Environmental Quality.* **2004**, *33*, 1202-1209.

Stenuit, B.; Eyers, L.; El Fantroussi, S.; Agathos, S.N. Promising strategies for the mineralisation of 2,4,6-trinitrotoluene. *Reviews in Environmental Science and Bio/Technology.* **2005**, *4*, 39-60.

Stenuit, B.; Eyers, L.; Rozenberg, R.; Habib-Jiwan, J.-L.; Agathos, S.N. Aerobic growth of *Escherichia coli* with 2,4,6-trinitrotoluene (TNT) as the sole nitrogen source and evidence of TNT denitration by whole cells and cell-free extracts. *Applied and Environment Microbiology.* **2006**, *72*(12), 7945-7948.

Symons, Z.C.; Bruce, N.C. Bacterial pathways for degradation of nitroaromatics. *Natural Product Reports.* **2006**, *23*, 845-850.

Thorn, K.A.; Thorne, P.G.; Cox, L.G. Alkaline hydrolysis/polymerization of 2,4,6-trinitrotoluene: characterization of products by ^{13}C and ^{15}N NMR. *Environmental Science and Technology.* **2004**, *38*, 2224-2231.

Tront, J.M.; Hughes, J.B. Oxidative microbial degradation of 2,4,6-trinitrotoluene via 3-methyl-4,6-dinitrocatechol. *Environmental Science and Technology.* **2005**, *39*, 4540-4549.

Van Aken, B.; Yoon, J.M.; Schnoor, J.L. Biodegradation of nitro-substituted explosives 2,4,6-trinitrotoluene, hexahydro-1,3,5-trinitro-1,3,5-triazine, and octahydro-1,3,5,7-tetranitro-1,3,5-tetrazocine by a phytosymbiotic *Methylobacterium* sp. associated with poplar tissues (*Populus deltoides* × *nigra* DN34). *Applied and Environmental Microbiology.* **2004**, *70*(1), 508-517.

Viamajala, S.; Richards, L.; Peyton, B.M.; Petersen, J.N. Solubilization, solution equilibria, and biodegradation of PAHs under thermphilic conditions. *Chemosphere*. **2007**, *66*, 1094-1106.

Vorbeck, C.; Lenke, H.; Fischer, P.; Spain, J.C.; Knackmuss, H.-J. Initial reductive reactions in aerobic microbial metabolism of 2,4,6-trinitrotoluene. *Applied and Environmental Microbiology.* **1998**, *64*(1), 246-252.

Won, W.D.; DiSalvo, L.H.; Ng, J. Toxicity and mutagenicity of 2,4,6-trinitrotoluene and its microbial metabolites. *Applied and Environmental Microbiology.* **1976**, *31*(4), 576-580.

Zupko, A.J.; Johnson, N.P.; Leibbert, J.M.; Empson, W.B.; Mroz, D.L.; Bales, F.E. Roy F. Weston, Inc. and United States Army Corps of Engineers, Kansas City District. Incineration of explosives-contaminated soil and reactive RCRA waste at the former Weldon Spring Ordnance Works, Missouri, USA. Technical paper (#9008) presented at the Incineration and Thermal Treatment Technology Conference. May 1999, Orlando, FL.

ALKALINE HYDROLYSIS OF TNT

Introduction

The need for TNT (a major component of several types of explosive mixtures) remediation is a worldwide concern. Unreacted TNT contaminates many commercial and residential areas in Europe as a result of World War II (Reiger and Knackmuss, 1995). In the United States, this contamination is localized to TNT manufacturing facilities and munitions testing areas. Due to the observed toxicity and mutagenicity of TNT, even at concentrations less than 10mg/L (Won et al., 1976; McCormick et al., 1976), both soil and groundwater contamination poses a significant risk to certain plants, fish, algae, and a number of other organisms. Prolonged exposure to TNT among humans has shown to cause liver damage (causing black urine and yellow skin), anemia, infertility, and is suspected to cause cancer (Emmrich, 1999; Bruns-Nagel et al., 1996; Esteve-Nunez et al., 2001; Symons and Bruce, 2006). The most widely employed method for destruction of TNT is incineration (Reiger and Knackmuss, 1995). This method has been discouraged in recent decades due to environmental issues with air quality as well as safety issues related to TNT's Class A explosive characteristics. However, the current acceptable limit for TNT in post-incineration soil is 57ppm, which still serves a significant toxic risk for soil and marine biota (Won et al., 1976; Zupko et al., 2000). Additionally, public opposition continues to hinder consistent use of incineration, especially for commercial and residential contaminated areas in Europe (Li et al., 1997; Reiger and Knackmuss, 1995).

The synthesis of TNT is performed under acidic conditions, by sequentially nitrating toluene with nitric acid. Therefore a practical approach for TNT degradation is to subject it to alkaline conditions. The electron-withdrawing effect of the three nitro groups on TNT makes it susceptible to nucleophilic attack by OH^-. The use of alkaline hydrolysis as a chemical treatment process has been previously reported for hydroxide concentrations in excess of the concentration of TNT being hydrolyzed (Bajpai et al., 2004; Bishop et al., 2000; Davis et al., 2006; Emmrich, 1999; Felt et al., 2002; Hwang et al., 2005; Karasch et al., 2002; Mills et al., 2003; Saupe et al., 1998; Thorn et al., 2004). What has not been reported, however, is alkaline hydrolysis of TNT at lower pH combined with elevated temperature. It has been stated that "alkaline hydrolysis occurs only if the base concentration exceeds that of TNT" (Emmrich, 1999). This has been demonstrated in the literature as alkaline hydrolysis studies involving TNT have only covered conditions of pH $\geq$ 11, and higher pH has been correlated to faster rates of TNT disappearance. Alkaline hydrolysis at elevated temperatures (>20°C) has also been shown to increase the degradation rate of TNT, though only in combination with excess hydroxide concentrations. Further, the high hydroxide concentration necessitates considerable dilution and/or neutralization prior to downstream biological treatment. Therefore the current study investigated the rates and products of TNT alkaline hydrolysis where $[OH^-] < [TNT]$ and with elevated temperature, as well as the biological susceptibility of the resulting TNT hydrolysate solution.

Materials and Methods

Chemicals

2,4,6-trinitrotoluene (TNT) and 1,3,5-trinitrobenzene (TNB) was obtained from ChemService (West Chester, PA). All chemicals used were of reagent grade. All water used was nanopure (17.0MΩ). All other solvents used were of HPLC or HPLC-MS grade. Standards were obtained from Sigma-Aldrich / Supelco (St. Louis, MO) and included: 1,3-dinitrobenzene; nitrobenzene; 2-amino-4,6-dinitrotoluene; 4-amino-2,6-dinitrotoluene; 2,6-dinitrotoluene; 2,4-dinitrotoluene; 2-nitrotoluene; 3-nitrotoluene; 4-nitrotoluene; 2,4-diaminonitrotoluene; 2,6-diaminonitrotoluene; 2-hydroxylamino-4,6-dinitrotoluene; 4-hydroxylamino-2,6-dinitrotoluene; 4,4',6,6'-tetranitro-2,2'-azotoluene; 2,2',6,6'-tetranitro-4,4'-azotoluene; and 2,2',6,6'-tetranitro-4,4'-azoxytoluene.

Hydrolysis Experiments

Experiments were performed in triplicate in sealed 150mL serum bottles. Aqueous stock solutions of 80mg/L TNT were stored at 4°C in the dark. For the initial screening experiments either 12mM phosphate (for pH 7-8) or 12mM borate (for pH 9-11) was added to 100mL of 45mg/L TNT solution. For all subsequent experiments, 12mM borate was added to 100mL of 80mg/L TNT solution. The buffered solution was immediately pH-adjusted (with either 6N HCl or 10M NaOH) to the appropriate pH value (7-11). Bottles were incubated at 20, 60, or 80°C and shaken at 100 rpm.

Sampling and Storage

At regular intervals 1.5mL samples were taken from each replicate and immersed in an ice-saltwater bath (-5°C) to stop the hydrolysis reaction or neutralized with HCl. Samples neutralized with HCl showed no significant differences for hydrolysis product detection or quantity from frozen samples (data not shown), therefore quick freezing was used for all subsequent samples. Samples were stored at -20°C for ion chromatography (IC), high performance liquid chromatography (HPLC), and liquid chromatography-mass spectrometry (LC-MS) analyses.

Analyses

Samples for IC analysis were thawed and filtered (0.2μm). Both nitrite (NO_2^-) and nitrate (NO_3^-) were detected on a Dionex DX500 IC (Sunnyvale, CA) fitted with a CD20 conductivity detector, AS9-HC (4 x 250mm) column and 100μL sample loop. The mobile phase was 9.0mM Na_2CO_3 with a helium overpressure. The detection limit for each nitrite and nitrate was approximately 1mg/L.

Prior to HPLC analysis, samples were filtered with 0.2μm Spartan regenerated cellulose syringe filters (Whatman Inc., Piscataway, NJ). Nitroaromatic losses from filtration have been reported as less than 2% with these filters (Hofstetter et al., 1999). This was tested prior for the present study and was found to be in agreement with the <2% loss of nitroaromatic compounds (data not shown). Samples (10μL injection volume) were run on an Agilent HPLC 1090 equipped with a Supelco LC-8 column, operated at 36°C. The method used is described in detail elsewhere (Borch and Gerlach, 2004). Briefly, the mobile phase flow rate was 1mL/min with a gradient of 25mM

phosphate buffer to methanol. TNT and other nitroaromatic compounds were detected using a diode array detector (DAD) at 230nm. Concentrations as low as 0.5mg/L were detectable for nitroaromatic standards, while the linear detection range was 1-100mg/L.

Preparation of samples for LC-MS was the same as that for HPLC analysis. An Agilent HPLC 1100 was used in line with an Agilent Ion Trap 6300, model G2440DA mass spectrometer (Agilent Technologies Inc., Liberty Lake, WA). Atmospheric Pressure Chemical Ionization (APCI) was used in both positive and negative modes.

Radiolabeled TNT Experiments

To close the mass balance, uniformly ring labeled (U-ring) ^{14}C-TNT (9 x 10^5 dpm/mL; radiochemical purity >99%; ChemSyn Laboratories, Lenexa, KS) was used to repeat the pH 10, 60°C hydrolysis experiment. Samples were prepared for HPLC analysis, from which the eluent was collected using a Spectra/Chrom CF-1 Fraction Collector (Spectrum Chromatography, Houston, TX) at the frequency of one fraction every 0.5 min. Each fraction was added to 10mL Ultima Gold Scintillation Cocktail (Packard BioScience B.V., Groningen, Netherlands) and radiolabeled ^{14}C was analyzed using a 1900CA Tri-Carb Liquid Scintillation Analyzer (Packard Instrument, Meriden, CT).

Biological Screening

Biological utilization of TNT hydrolysate was tested using the 3rd generation (i.e. transfer) of a denitrifying culture enriched from a sample obtained from the Waste Water Treatment Plant (WWTP) in Bozeman, MT. The sample was enriched for denitrifying

microorganisms with methanol as the carbon source in the following modified minimal medium for denitrifying bacteria (Atlas, 2004) (L^{-1}): 5g KNO_3, 1g $(NH_4)_2SO_4$, 0.66g K_2HPO_4, 0.54g KH_2PO_4, 0.2g $MgSO_4 \cdot 7H_2O$, 0.02g $CaCl_2 \cdot 2H_2O$, 0.01g $FeSO_4 \cdot 7H_2O$, 0.005g $MnSO_4 \cdot H_2O$, 0.001g $CuSO_4 \cdot 5H_2O$, 0.001g $Na_2MoO_4 \cdot 2H_2O$, 10mL 0.1N HCl. The final pH of this medium was 6.80. A nitrogen headspace was used for all denitrifying enrichment cultures, and a 10% (v/v) inoculum was used for each transfer. TNT hydrolysate from the pH 10, 60°C experiments were amended with the following components as modified from a culture medium described by Viamajala et al. (2007) (L^{-1}): 0.1g $C_6H_7NNa_2O_6$ (sodium nitrilotriacetic acid: a source of inorganic carbon, not used as carbon source for growth), 0.05g $CaCl_2 \cdot 2H_2O$, 0.125g $MgSO_4 \cdot 7H_2O$, 0.01g NaCl, 0.01g KCl, 0.05g $Na_2HPO_4 \cdot 7H_2O$, 0.03% (w/v) $FeCl_3$ solution, 1mL Nitsch's trace metal solution (composition (L^{-1}): 2.2g $MnSO_4 \cdot H_2O$, 0.5g $ZnSO_4 \cdot 7H_2O$, 0.5g H_3BO_3, 0.016g $CuSO_4 \cdot 5H_2O$, 0.025g $Na_2MoO_4 \cdot 2H_2O$, 0.046g $CoCl_2 \cdot 6H_2O$). This solution was then pH-adjusted to pH 7.0 using 6N HCl. Cultures were screened with and without an external nitrogen source of 0.3g/L NH_4Cl. No additional carbon source was added to any culture. Both aerobic (air headspace) and denitrifying (N_2 headspace) treatments were performed. A 10% (v/v) inoculum was used for all treatments. Cultures were incubated at 30°C in sealed serum bottles and shaken at 100rpm.

Results and Discussion

Previous work with alkaline hydrolysis of aqueous solutions of TNT (~88mg/L solubility, 20°C) has focused only on conditions where the $[OH^-] >> [TNT]$, which occurs

at pH values ≥ 11 (Bajpai et al., 2004; Bishop et al., 2000; Emmrich, 1999; Felt et al., 2002; Hwang et al., 2005; Karasch et al., 2002; Mills et al., 2003; Ro et al., 1996; Saupe et al., 1998; Thorn et al., 2004). It has been assumed that there is virtually no hydrolysis activity when $[OH^-] \leq [TNT]$, which explains the lack of TNT hydrolysis studies at lower pH values. Table 1 compares molar concentration of TNT and OH^- at varying pH values.

Table 2: Comparison of molar TNT and hydroxide concentrations at various pH values.

pH	Hydroxide Concentration $[OH^-]$ (µM)		$[OH^-]$: [TNT] molar ratio	[TNT] : $[OH^-]$ molar ratio
7	0.1	80mg/L TNT = 352µM	0.000284	3520
8	1		0.00284	352
9	10		0.0284	35.2
10	100		0.284	3.52
11	1000		2.84	0.352

Initial screening experiments were performed to observe the extent of alkaline hydrolysis on TNT at lower hydroxide concentrations with pH values less than 11 and elevated temperature. Figure 8 shows the significant influence of both elevated pH and temperature on the disappearance of TNT. Emmrich (1999) reported a rate constant of zero for pH 10 at 20°C, however, this reaction was only monitored for 4 days. Given ample time (i.e. days to months as opposed to hours to days) it can be seen that TNT concentration does not remain constant, and the rate of disappearance is considerably faster with higher temperature (see Figure 8). Applying elevated temperatures to the alkaline hydrolysis of TNT at pH ≥ 11 has been previously studied (Bajpai et al., 2004; Bishop et al., 2000; Mills et al., 2003; Saupe et al., 1998). It has been stated that “higher temperatures may help completely hydrolyze nitroaromatic compounds that are only

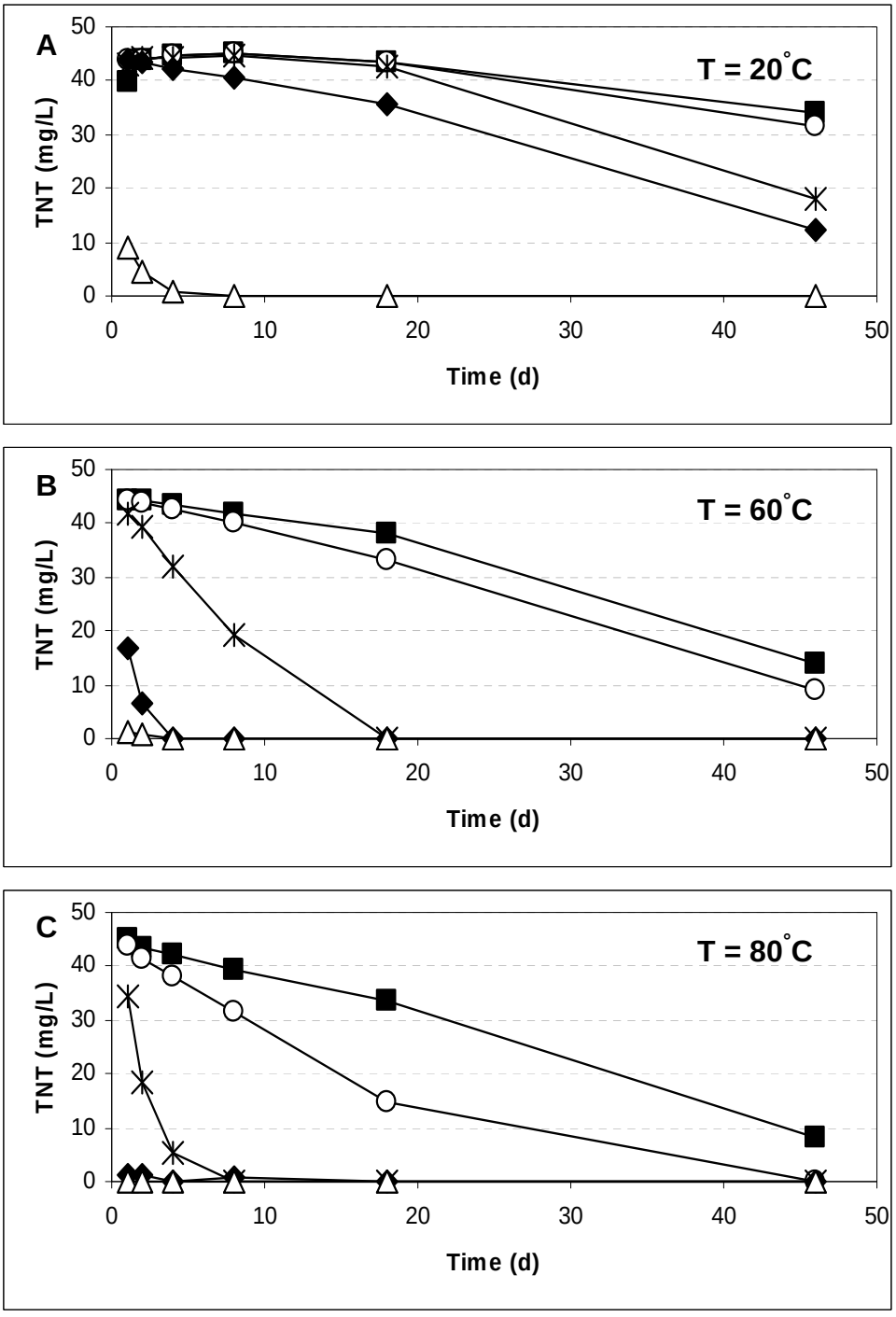


Figure 8: Effect of pH and temperature on TNT concentration. (A) 20°C, room temperature; (B) 60°C; (C) 80°C. Legend: ■, pH 7; ○, pH 8; ∗, pH 9; ◆, pH 10; △, pH 11. Error bars represent ± one standard deviation of triplicate experiments. Absence of error bars indicates error bars are smaller than data symbol.

partially hydrolyzed at room temperature" (Emmrich, 2001). Reports of alkaline hydrolysis of TNT have only focused on reaction conditions when the concentration of hydroxide exceeds that of TNT. Figure 8 clearly shows that hydrolysis of TNT is significant, especially at elevated temperatures, even under hydroxide limited conditions. While it is clear from Figure 8 (A) that alkaline hydrolysis of TNT has very slow rates at 20°C when $[OH^-]$: [TNT] < 1, plots (B) and (C) show that elevated temperatures (60°C and 80°C) considerably improve the hydrolysis reaction rate under hydroxide-limited conditions (pH ≤ 10). These results prompted a more detailed study of the rates of alkaline hydrolysis at pH 9-11, 60°C and 80°C.

Kinetics of Hydrolysis

Figures 9, 10, and 11 show depletion of 80mg/L (0.352mM) TNT with concurrent production of nitrite and nitrate at pH 9, 10, and 11, respectively. At pH 9, the time of

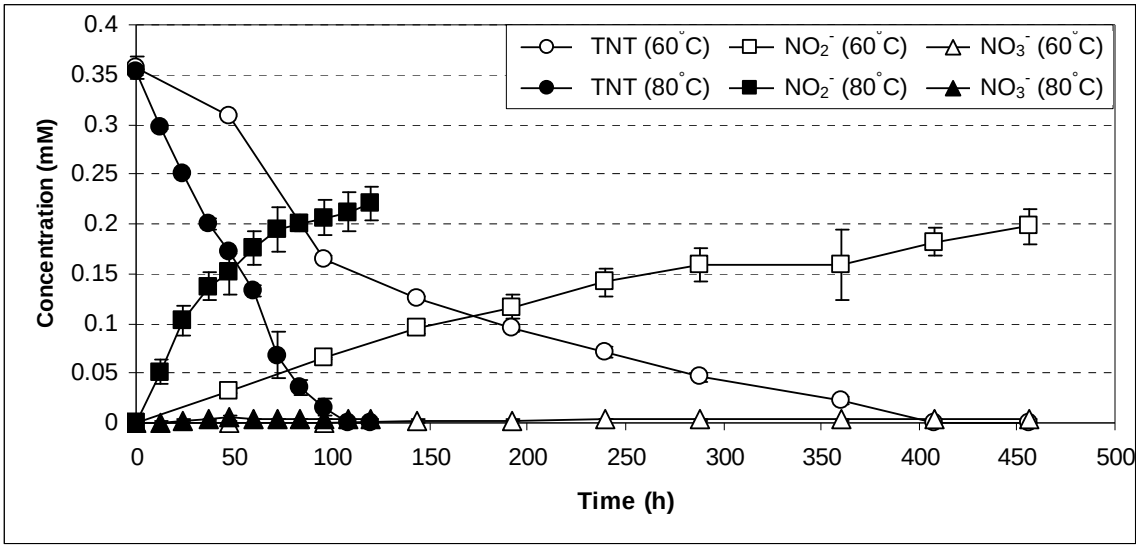


Figure 9: TNT degradation with production of nitrite and nitrate at pH 9. Error bars represent ± one standard deviation of triplicate experiments. Absence of error bars indicates error bars are smaller than data symbol.

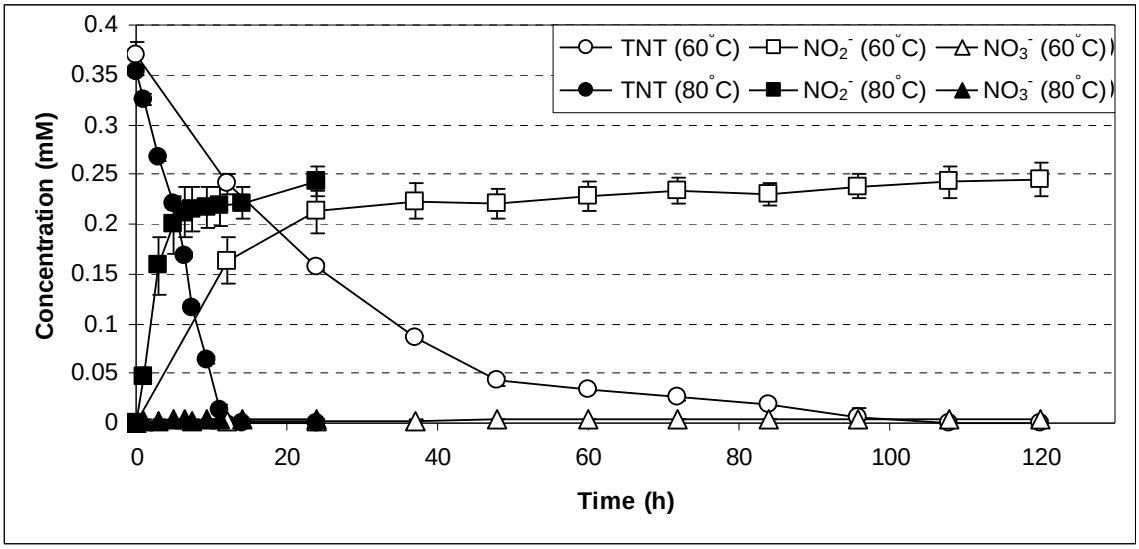


Figure 10: TNT degradation with production of nitrite and nitrate at pH 10. Error bars represent ± one standard deviation of triplicate experiments. Absence of error bars indicates error bars are smaller than data symbol.

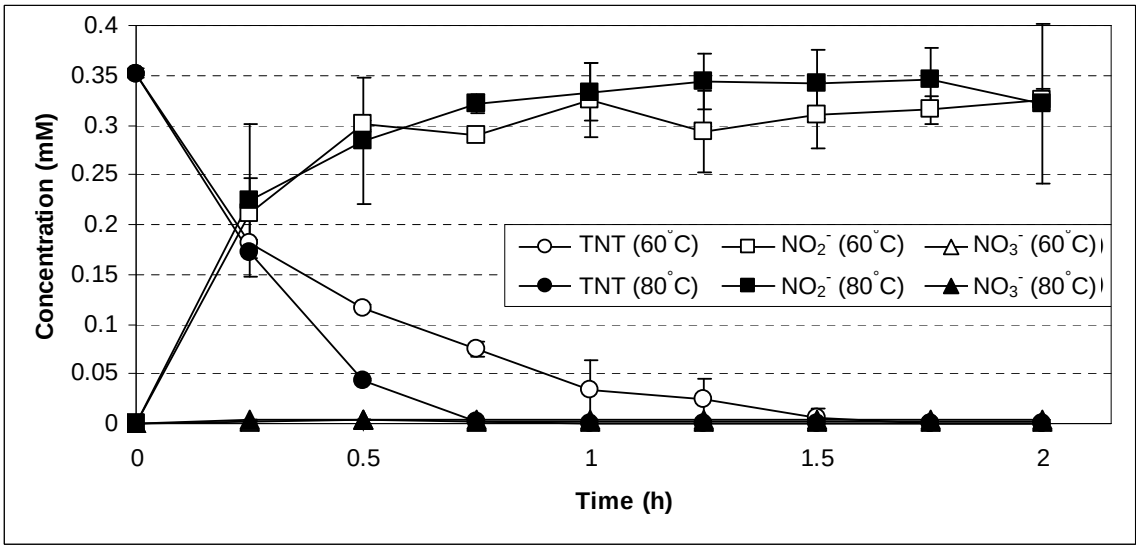


Figure 11: TNT degradation with production of nitrite and nitrate at pH 11. Error bars represent ± one standard deviation of triplicate experiments. Absence of error bars indicates error bars are smaller than data symbol.

disappearance of TNT was decreased nearly 75% by increasing the temperature from 60°C (400h) to 80°C (100h). A similar significant time decrease, by nearly 85%, was seen at pH 10 where TNT in 60°C treatments was depleted in 100h while TNT in 80°C treatments was depleted in approximately 15h. At pH 11, TNT disappearance was much closer in magnitude for both 60°C and 80°C treatments, which was significantly faster than at pH 9 and 10, due to the molar ratio of $[OH^-]$: [TNT] being greater than one (see Table 2). TNT degradation was complete at pH 11 in less than 2h for both 60°C and 80°C treatments.

For kinetic analysis of alkaline hydrolysis of TNT, previous studies ($pH \geq 11$) assumed a pseudo-first order rate of TNT degradation because $[OH^-] >>$ [TNT] and/or because the pH was controlled throughout the experiment (Bajpai et al., 2004; Emmrich, 1999; Hwang et al., 2005; Karasch et al., 2002; Mills et al., 2003). In this study, where $[OH^-] <$ [TNT], a pseudo-first order rate was assumed because a borate buffer was used to stabilize the pH of the hydrolysis solution. Independent of reaction temperature, an average final pH of 8.9, 9.7, and 10.6 was measured for initial pH values of 9, 10, and 11, respectively. Pseudo-first-order rate constants (k') were determined according to Equation 1. The rate constant $k' = k[OH^-]$, where k is the actual rate constant and $[OH^-]$ is the initial hydroxide concentration that was assumed to remain constant throughout the experiment.

$$-d[TNT] / dt = k' [TNT] \qquad \text{Equation 1.}$$

Equation 1 was integrated and rearranged to obtain

$$\ln [TNT / TNT_0] = -k't \qquad \text{Equation 2.}$$

where TNT_0 was the initial concentration of TNT. Plotting t (time) versus ln [TNT / TNT_0] gave a straight line with slope –k'. Table 3 gives pseudo-first-order (with respect to TNT concentration) reaction rate constants for this study as well as other reports of alkaline hydrolysis of TNT. From Table 3 it can be seen that few kinetic studies have been done above ambient temperature.

Table 3: Comparison of pseudo-first-order kinetic rate constants, k' (h^{-1}). Rate constants for this study are given with ± one standard deviation. The concentrations listed at the top of each column indicate the initial TNT concentration used for that study.

pH	[OH^-] (mM)	This study 80mg/L	Emmrich (1999) 77mg/L	Bajpai et al. (2004) 35mg/L	Karasch et al. (2002) 25mg/L	Mills et al. (2003) 50mg/L	Hwang et al. (2005) 25mg/L
9	0.01	0.00110 (60°C) ±0.00042 0.0309 (80°C) ±0.0004	-	-	-	-	-
10	0.1	0.0406 (60°C) ±0.0005 0.275 (80°C) ±0.029	0.000 (25°C)	-	-	-	-
11	1	2.54 (60°C) ±0.05 9.35 (80°C) ±1.32	0.026 (20°C)	-	-	-	0.0192 (25°C)
12	10	-	0.361 (20°C)	0.185 (25°C) 0.200 (40°C)	0.61 (25°C)	-	0.114 (25°C)
13	100	-	-	-	-	1.908 (25°C) 4.32 (40°C) 27.72 (60°C)	-

The rate constants of TNT disappearance for pH 9 and 80°C ($0.0309h^{-1}$) as well as pH 10 and 60°C (0.0406) are of the same magnitude as rate constants for hydrolysis at pH 11 ($0.026h^{-1}$ and $0.0192h^{-1}$) as reported by Emmrich (1999) and Hwang et al. (2005), respectively. The rate constant calculated for pH 10 and 80°C was on the same order of

magnitude as those for other hydrolysis studies at pH 12. For reactions at pH 11 in this study, the elevation of temperature increased the TNT first order rate constant by two orders of magnitude above those for pH 10 and 60°C, on the same order as hydrolysis rates determined for pH 13 by Mills et al. (2003). These results show that by increasing the reaction temperature for TNT hydrolysis, the hydroxide concentration can be reduced 1-2 orders of magnitude and the total reaction time will remain relatively constant.

The energy of activation (E_a) of the TNT hydrolysis reaction was calculated using all rate constants given in Table 3. To incorporate initial hydroxide concentration, each actual rate constant, k, was calculated from each k' in Table 3 and its corresponding reaction pH. The Arrhenius equation and its linearized form are shown as Equations 3 and 4, respectively. These were used to find both E_a and A (the Arrhenius constant, unique for a given reaction) where R is the universal gas constant (8.314 J/mol·K), k is the actual rate constant, and T is temperature (in Kelvin). Figure 12 shows the linear plot of (1/T) vs. ln k, for which E_a was calculated from the slope ($-E_a/R$) and A was calculated from the y-intercept (ln A).

$$k = A\exp(-E_a/RT) \qquad \text{Equation 3.}$$

$$\ln k = \ln A - (E_a/RT) \qquad \text{Equation 4.}$$

The activation energy (and standard deviation) calculated for hydrolysis of TNT was 75.8 ± 8.10 kJ/mol. The only activation energy found in the literature for alkaline hydrolysis of TNT was by Mills et al. (2003), who reported an activation energy of 64 ± 12 kJ/mol for pH 12 and 13 at reaction temperatures of 25, 40, and 60°C. The activation energy range (70.4 - 83.1 kJ/mol) for this study does overlap with the range

(52 – 76 kJ/mol) given by Mills et al. (2003). The higher activation energy range found in this study may be attributed to the inclusion of hydrolysis data from hydroxide-limited conditions (pH 9 and 10), whereas the activation energy found by Mills et al. (2003) only considered reaction conditions of excess hydroxide. Further kinetic analyses at varying temperatures and hydroxide concentrations will contribute to the accuracy of the calculated activation energy for TNT hydrolysis.

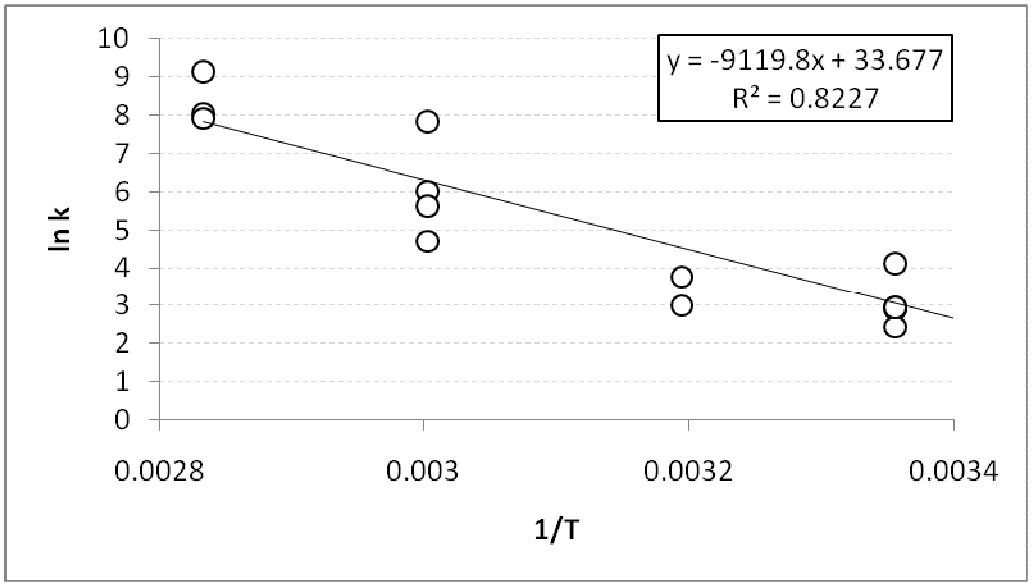


Figure 12: Linearized Arrhenius plot for determination of activation energy of the alkaline hydrolysis of TNT. Data points were calculated from pseudo-first-order rate constants (k') from Table 3.

A generalized kinetic expression for the first order rate of TNT degradation by alkaline hydrolysis was constructed from the parameters calculated above. To our knowledge, this is the first model for the hydrolysis reaction of TNT and is shown in Equation 5, where [TNT] is TNT concentration at time t (h), T is temperature in Kelvin, $[TNT_0]$ is initial TNT concentration, and $[OH^-_0]$ is initial hydroxide concentration.

$$d[TNT] / dt = (4.224x10^{14})\exp(-9119.8/T)[TNT_0][OH^-_0] \quad \text{Equation 5.}$$

Because this model assumes a constant hydroxide concentration throughout the reaction (first order), additional TNT hydrolysis testing where hydroxide concentration is closely monitored will be necessary to determine the actual reaction order. This will improve the accuracy of this kinetic model for the TNT alkaline hydrolysis reaction.

Hydrolysis Products

Nitrate (NO_3^-) release was minimal with concentrations near the 1mg/L (0.016mM) IC detection limit and was consistently low across all pH and temperature treatments. This was consistent with Bishop et al. (2000) and Saupe et al. (1998) who reported limited amounts of nitrate from TNT hydrolysis, though significant amounts of nitrite were detected.

In this study, the concentration of nitrite (NO_2^-) was observed to increase with pH; however, the different temperature treatments at the same pH value did not significantly change the maximum nitrite concentrations. Solutions at pH 9 reached a maximum NO_2^- concentration of 0.20mM (9.2mg/L). This corresponded to 0.57 moles nitrite released per mole TNT added. Average maximum nitrite concentrations reached at pH 10 were slightly higher, 0.25mM (10.4mg/L), and corresponded to 0.64 moles nitrite per mole TNT added. At pH 11, the molar ratio of nitrite to TNT approached unity. Bishop et al. (2000) reported 1 mole nitrite released per mole TNT for pH 14 and 90-150°C. Karasch et al. (2002) reported approximately 0.5 mole nitrogen ($NO_2^- + NO_3^-$) per 1 mole TNT at 25°C and pH 12. At 20°C and pH 13 Emmrich (1999) reported 2 moles nitrogen (NO_2^- +

NO_3^-) released per mole TNT. Saupe et al. (1998) reported 1.2 moles nitrite per 1 mole TNT for pH 14 and 80°C.

In this study, approximately 2/3 of the total nitrogen from TNT was accounted for as nitrite (0.5-1moles per mole TNT) and 1,3,5-trinitrobenzene (TNB) (0.3mole TNB = 0.9moles N per mole TNT). Other nitrogen is most likely present in other nitroaromatic products and possibly as ammonia (which was not measured here) (Saupe et al., 1998).

Products of TNT alkaline hydrolysis detected included: nitrate, nitrite, TNB, 1,3-dinitrobenzene, 4-amino-2,6-dinitrotoluene, 4-nitrotoluene, and aminobenzene (data not shown). TNB production reached a maximum concentration of approximately 25mg/L (0.117mM) which was one-third of the initial concentration of TNT. Only limited amounts (< 5mg/L) of these identified nitroaromatics were detected. The resulting brown color of the reaction solution suggested the presence of TNT polymers, which was supported by HPLC peaks in the less-polar region of the chromatogram where the TNT dimers (e.g. 2,2',6,6'-tetranitro-4,4'-azotoluene and 2,2',6,6'-tetranitro-4,4'-azoxytoluene) appear, though these peaks remained unidentified. Incidence of these polymerized TNT products was consistent with the findings of Bishop et al. (2000), Felt et al. (2002), Hwang et al. (2005), Mills et al. (2003), Saupe et al. (1998), and Thorn et al. (2004). There were also peaks detected in the more-polar region of the chromatogram, which suggested some ring cleavage products, similar to the results of Bishop et al. (2000), Hwang et al. (2005), and Saupe et al. (1998) who reported aliphatic organic compounds such as acetate, oxalate, and formate. Karasch et al. (2002) reported a complete loss of aromaticity in the final hydrolysate as evidenced by the UV spectrum as

well as the loss of color from the solution. It is possible that the smaller amount of TNT hydrolyzed (initial TNT concentration used by Karasch et al. (2002) was 25mg/L) prevented significant amounts of TNT polymers from being formed.

Radiolabeled ^{14}C-TNT Experiments

To close the mass balance, radiolabeled TNT hydrolysis was performed at pH 10 and 60°C with ^{14}C-TNT. Figures 13-15 show that all TNT-carbon (TNT-C) was recovered (97-100%) in the reaction solution, suggesting no carbon-containing gaseous products were formed. After one day, TNT-C was widely distributed among a variety of compounds as shown from the number of HPLC fractions containing ^{14}C. The majority, 12%, of TNT-C was contained in the remaining TNT (retention time 14-15 min), 5% was associated with TNB (retention time 9-10 min), and 10% of TNT-C was associated with the region of the chromatogram where TNT dimers are generally found (retention time 25min), although it was not detectable by DAD at 230nm. After 9 days, when the TNT had been completely hydrolyzed, the distribution of carbon-containing fraction was more pronounced than at day 1. Figures 13-15 show that the products of hydrolysis became increasingly more polar and that no single fraction contributed more than 4% of the total carbon. These results are encouraging for the prospect of using the hydrolysate as a carbon source for subsequent biological treatment.

The wide distribution of hydrolysis products suggests that any remaining nitroaromatics would not be present in concentrations high enough to inhibit microbial growth. Also, the majority of products were more polar than the parent TNT molecule

indicating ring cleavage products, which would be more easily used for microbiological growth.

French et al. (2001) and Emmrich (2001) noted that certain transient hydrolysis products including aromatic amines and hydroxylamines act as nucleophiles under alkaline conditions, competing with the hydroxide ion, and may contribute to TNT transformations initiated by the alkaline hydrolysis reaction. This may explain why

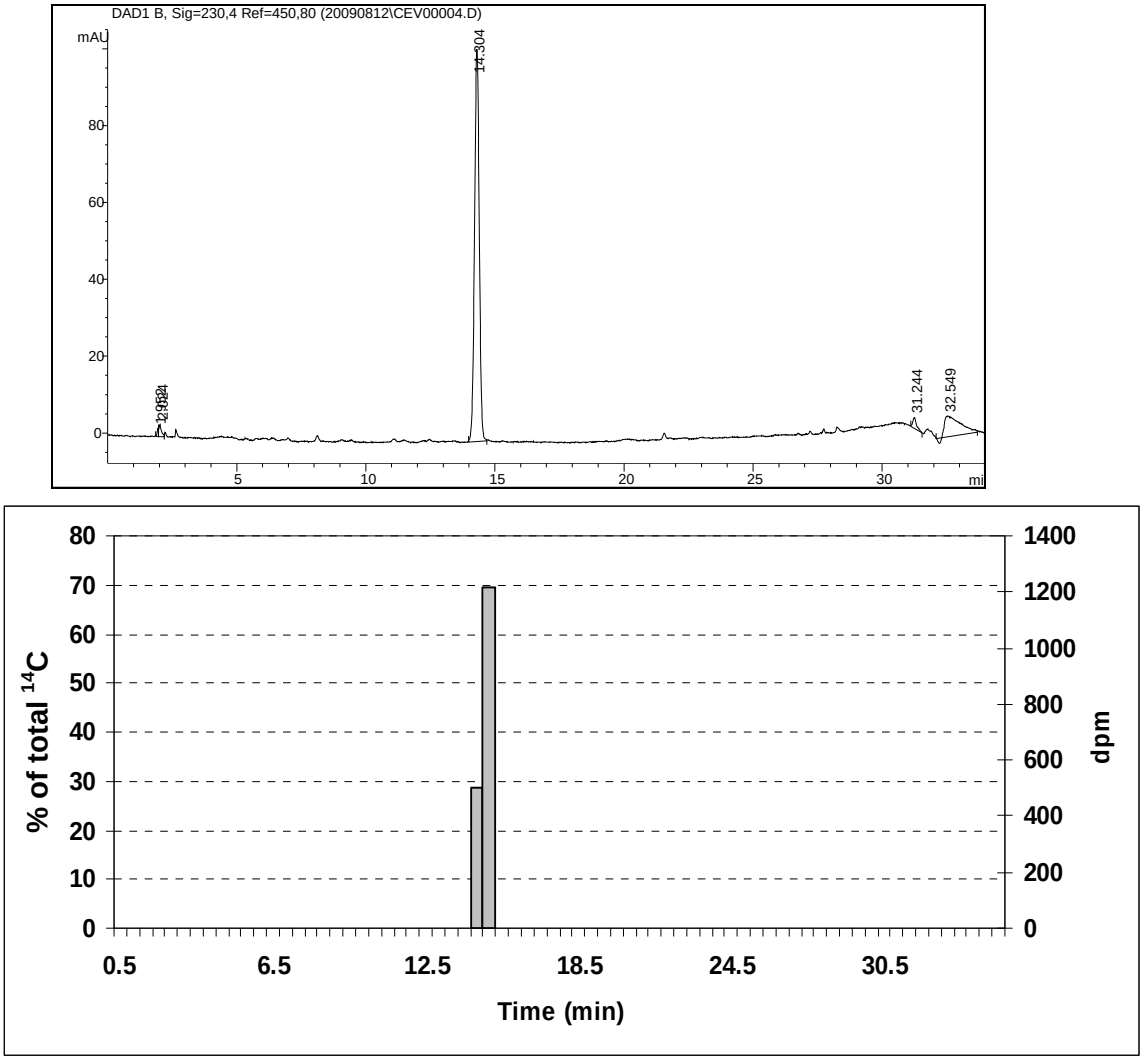


Figure 13: Initial (day 0) distribution of ^{14}C-TNT (bottom) and corresponding HPLC chromatogram (top).

degradation of TNT was observed for [OH^-] : [TNT] molar ratios less than one (pH 9 and 10).

From the results obtained in this study, it is apparent that a stoichiometric (1:1) amount of hydroxide is not required to observe complete transformation of the parent TNT molecule to collectively less energetic and more polar end products that may be amenable to further degradation using biological treatment.

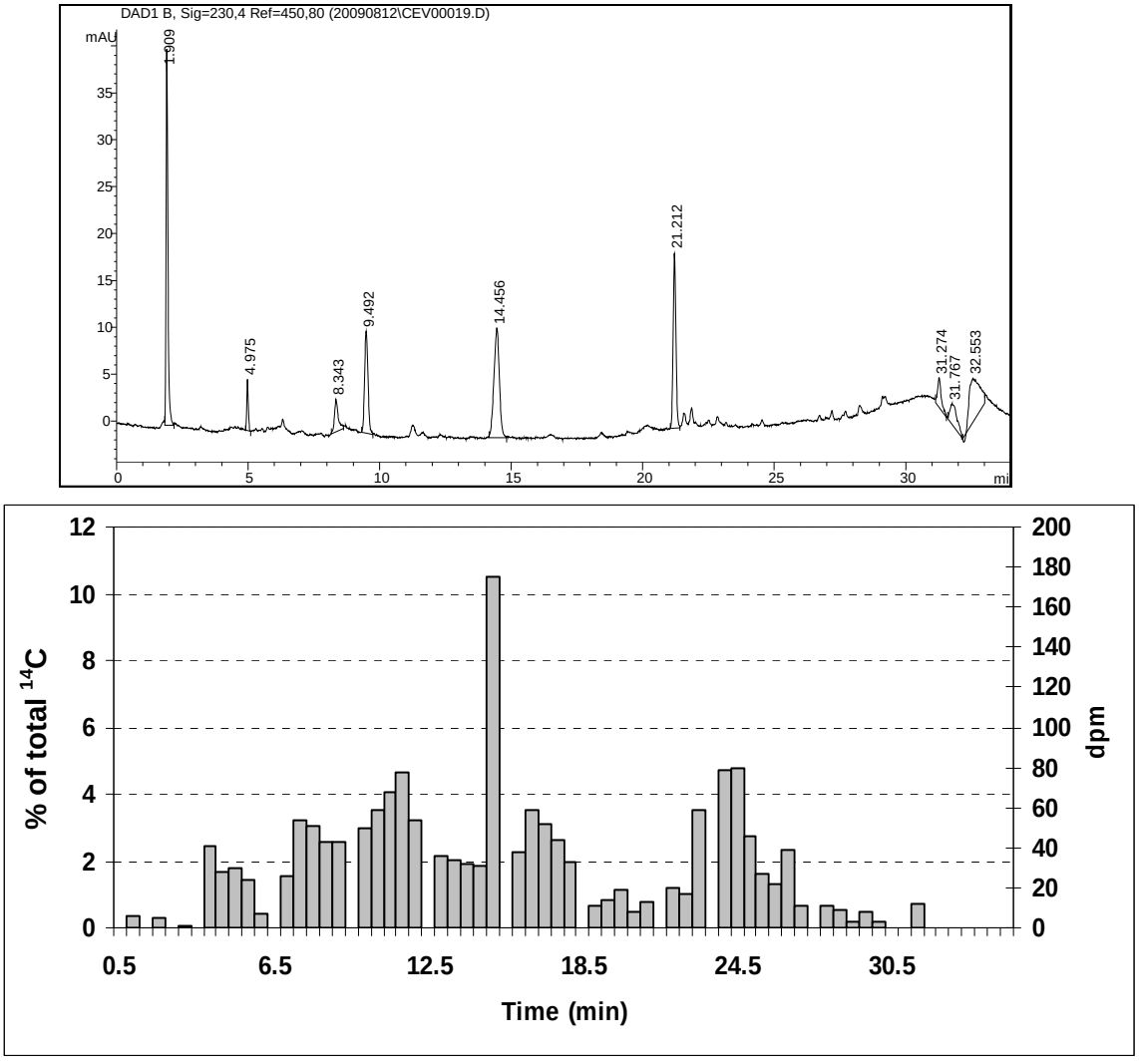


Figure 14: Day 1 distribution of ^{14}C from TNT treated at pH 10 and 60°C (bottom) and corresponding HPLC chromatogram (top).

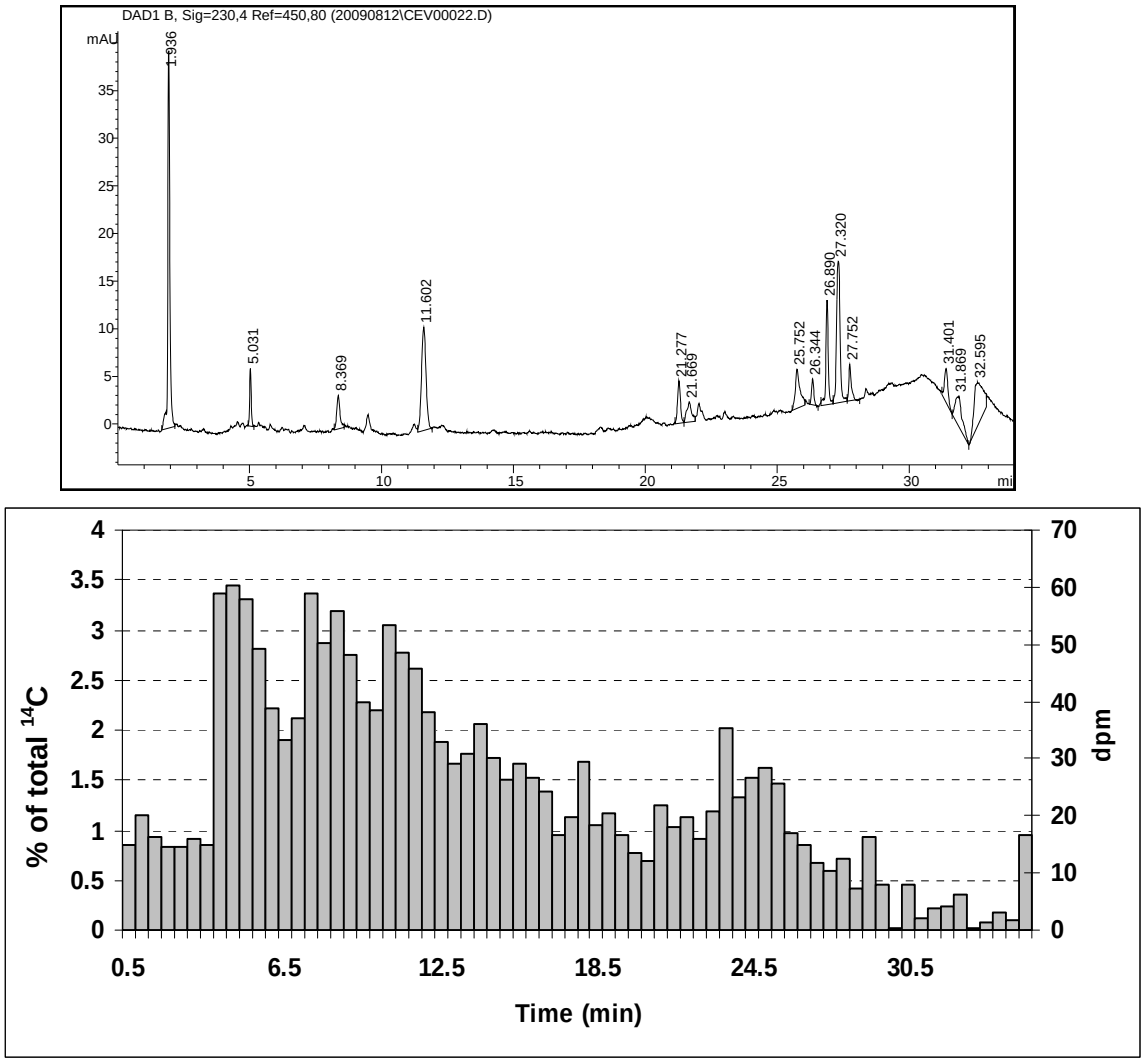


Figure 15: Day 9 distribution of ^{14}C from TNT treated at pH 10 and 60°C (bottom) and corresponding HPLC chromatogram (top).

Biological Degradation of TNT Hydrolysate

To demonstrate the potential for biological utilization of the TNT hydrolysate as a carbon source and nitrogen source, a denitrifying culture enriched from a wastewater treatment plant (WWTP) sample (Bozeman, MT, WWTP) was inoculated into pH 7.0 hydrolysate. The hydrolysate used was from pH 10 and 60°C experiments, and was

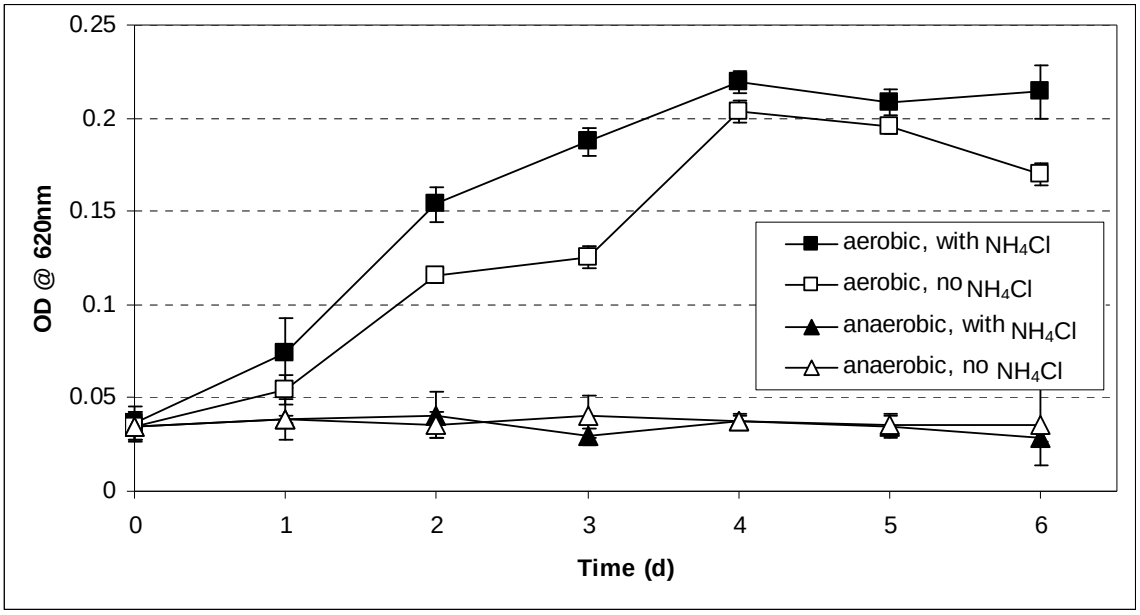


Figure 16: Growth of WWTP denitrifying culture on TNT hydrolysate. Error bars represent ± one standard deviation of triplicate experiments. Absence of error bars indicates error bars are smaller than data symbol.

neutralized using 6N HCl. The solution was also amended with nutrients to support growth, as detailed in the Materials and Methods section. Figure 16 shows aerobic cultures both with and without an external nitrogen source exhibited growth over a period of four days. A maximum biomass absorbance (620nm) of 0.219 was observed, which corresponds to a total protein concentration of 19 mg/L as calculated from absorbance vs. protein standard data (see Appendix D). Cultures given a nitrogen headspace to encourage denitrification showed no growth. This was not surprising since 80mg/L TNT would have only released 11mg/L nitrite (0.25mM) based on data from Figure 10 (neither nitrate nor nitrite was measured for these biological experiments). This small amount of theoretical nitrite would not likely support growth of a denitrifying culture. Also, nitrate production from hydrolysis was minimal and would not have supported growth as an electron acceptor. For alkaline hydrolysis, since the solubility of TNT in aqueous solution

is low (~88mg/L at 20°C), the amount of nitrite initially present in the hydrolysate (11 mg NO_2^-/L) is not a concern for inhibition of biological growth. If all nitrogen from TNT was released as nitrite, this concentration would still not exceed 60 mg/L, which falls below the general 100mg/L limit of acceptable nitrite concentration for nitrite-related bacterial growth inhibition (Beccari et al., 1983). Figure 16 shows that growth occurred in solution without ammonium chloride as an added nitrogen source, suggesting that the culture used nitrogen released from TNT.

Whether or not there was an external nitrogen source available, the culture used the carbon-containing hydrolysis products of TNT as an electron donor. According to Cox (2004) and Kubitschek and Friedman (1971), a general mass for bacteria is 9.5×10^{-13}g biomass / cell, and a general concentration of protein for bacteria is 660×10^{-15} g protein / cell. Assuming that approximately 50% of cell mass is carbon, then 13.7 mg/L of the total protein (19mg/L as given above) is carbon. Of the 80mg/L TNT originally present, 30mg/L is carbon ($C_7H_5N_3O_6$). It can be concluded that 46% of TNT-associated carbon was used for growth.

Conclusions

From the results of this study, it is clear that alkaline hydrolysis of TNT occurs even when $[OH^-] < [TNT]$, and the rate of disappearance of TNT is accelerated at elevated temperatures. In addition to the first report of TNT hydrolysis under hydroxide-limited conditions, this is also the first report of a generalized kinetic model for TNT degradation by alkaline hydrolysis. The major intermediate observed was TNB, which

had not been frequently observed for hydrolysis at pH values of 11 and greater. This result suggests a different mechanism of TNT transformation for lower hydroxide concentrations. TNB was subsequently transformed to an aminobenzene, which accumulated in the reaction medium. Other unidentified products of hydrolysis appeared in the TNT dimer region of HPLC chromatograms, as did compounds more polar than TNT. The more polar compounds represented the majority of final hydrolysis products as shown with ^{14}C analysis. A screening for microbial growth in the hydrolysate solution was positive for aerobic growth using an undefined mixed culture, illustrating the non-toxic nature of the hydrolysate from hydrolysis of TNT under hydroxide-limited conditions. This may eliminate the need for dilution prior to biological treatment. The application of lower pH (9-10) and elevated temperature (60 and 80°C) followed by biological degradation of the hydrolysate is a promising method for complete transformation of 80 mg/L TNT to environmentally benign products.

References

Admassu, W.; Sethuraman, A.V.; Crawford, R.; Korus, R.A. Growth kinetics of *Clostridium bifermentans* and its ability to degrade TNT using an inexpensive alternative medium. *Bioremediation Journal.* **1998**, *2*(1), 17-28.

Bajpai, R.; Parekh, D.; Herrmann, S.; Popović, M.; Paca, J.; Qasim, M. A kinetic model of aqueous-phase alkali hydrolysis of 2,4,6-trinitrotoluene. *Journal of Hazardous Materials.* **2004**, *106B*, 55-66.

Bhushan, B.; Halasz, A.; Hawari, J. Effect of iron(III), humic acids and anthraquinone-2,6-disulfonate on biodegradation of cyclic nitramines by *Clostridium* sp. EDB2. *Journal of Applied Microbiology.* **2006**, *100*, 555-563.

Bishop, R.L.; Flesner, R.L.; Larson, S.A.; Bell, D.A. Base hydrolysis of TNT-based explosives. *Journal of Energetic Materials.* **2000**, *18*, 275-288.

Boopathy, R.; Gurgas, M.; Ullian, J.; Manning, J.F. Metabolism of explosive compounds by sulfate-reducing bacteria. *Current Microbiology.* **1998**, *37*, 127-131.

Borch, T. and Gerlach, R. Use of reversed-phase high-performance liquid chromatography - diode array detection for complete separation of 2,4,6-trinitrotoluene metabolites and EPA Method 8330 explosives: influence of temperature and an ion-pair reagent. *J. Chromatogr. A.* **2004**, *1022*, 83-94.

Borch, T.; Inskeep, W.P.; Harwood, J.A.; Gerlach, R. Impact of ferrihydrite and anthrquinone-2,6-disulfonate on the reductive transformation of 2,4,6-trinitotoluene by a gram-positive fermenting bacterium. *Environ. Sci. Technol.* **2005**, *39*, 7126-7133.

Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248-254.

Bruns-Nagel, D.; Breitung, J.; von Löw, E.; Steinbach, K.; Gorontzy, T.; Kahl, M.; Blotevogel, K.-H.; Gemsa, D. Microbial transformation of 2,4,6-trinitrotoluene in aerobic soil columns. *Applied and Environmental Microbiology.* **1996**, *62*(7), 2651-2656.

Claus, H.; Bausinger, T.; Lehmler, I; Perret, N.; Fels, G.; Dehner, U.; Preuß, J.; König, H. Transformation of 2,4,6-trinitrotoluene (TNT) by *Raoultella terrigena*. *Biodegradation.* **2007**, *18*, 27-35.

Conder, J.M.; La Point, T.W.; Bowen, A.T. Preliminary kinetics and metabolism of 2,4,6-trinitrotoluene and its reduced metabolites in an aquatic oligochaete. *Aquatic Toxicology.* **2004**, *69*, 199-213.

Cox, R.A. Quantitative relationships for specific growth ratios and macromolecular compositions of *Mycobacterium tuberculosis, Streptomyces coelicolor* A3(2) and *Escherichia coli* B/r: an integrative theoretical approach. *Microbiology*. **2004**, *150*, 1413-1426.

Daun, G.; Lenke, H.; Knackmuß, H.-J.; Reuß, M. Experimental investigations and kinetic models for the cometabolic biological reduction of trinitrotoluene. *Chemical Engineering and Technology.* **1999**, *22*(4), 308-313.

Davis, E.P.; Boopathy, R.; Manning, J. Use of trinitrobenzene as a nitrogen source by *Pseudomonas vesicularis* isolated from soil. *Current Microbiology*. **1997**, *34*, 192-197.

Davis, J.L.; Brooks, M.C.; Larson, S.L.; Nestler, C.C.; Felt, D.R. Lime treatment of explosives-contaminated soil from munitions plants and firing ranges. *Soil & Sediment Contamination.* **2006**, *15*, 565-580.

Emmrich, M. Kinetics of the alkaline hydrolysis of 2,4,6-trinitrotoluene in aqueous and highly contaminated soils. *Environmental Science and Technology.* **1999**, *33*, 3802-3805.

Emmrich, M. Kinetics of the alkaline hydrolysis of important nitroaromatic co-contaminants of 2,4,6-trinitrotoluene in highly contaminated soils. *Environmental Science and Technology.* **2001**, *35*, 874-877.

Esteve-Núnez, A.; Caballero, A.; Ramos, J.L. Biological degradation of 2,4,6-trinitrotoluene. *Microbiology and Molecular Biology Reviews.* **2001**, *65*(3), 335-352.

Felt, D.R.; Larson, S.L.; Valente, E.J. UV-VIS spectroscopy of 2,4,6-trinitrotoluene-hydroxide reaction. *Chemosphere.* **2002**, *49*, 287-295.

French, C.E.; Nicklin, S.; Bruce, N.C. Aerobic degradation of 2,4,6-trinitrotoluene by *Enterobacter cloacae* PB2 and by pentaerythritol tetranitrate reductase. *Applied and Environmental Microbiology.* **1998**, *64*(8), 2864-2868.

French, C.E.; Rosser, S.J.; Bruce, N.C. Biotransformation of explosives. *Biotechnology and Genetic Engineering Reviews.* **2001**, *18*, 171-217.

Gonzalez-Perez, M.M.; van Dillewijn, P.; Wittich, R.-M.; Ramos, J.L. *Escherichia coli* has multiple enzymes that attack TNT and release nitrogen for growth. *Environmental Microbiology*. **2007**, *9*, 1535-1540.

Hess, T.F.; Schrader, P.S. Coupled abiotic-biotic mineralization of 2,4,6-trinitrotoluene (TNT). *Journal of Environmental Quality*. **2002**, *31*(3), 736-744.

Hofstetter, T.B.; Heijman, C.G.; Haderlein, S.B.; Holliger, C.; Schwarzenbach, R.P. Complete reduction of TNT and other (poly)nitroaromatic compounds under iron-reducing subsurface conditions. *Environ. Sci. Technol.* **1999**, *33*, 1479-1487.

Hwang, H.-M.; Slaughter, L.F.; Cook, S.M.; Cui, H. Photochemical and microbial degradation of 2,4,6-trinitrotoluene (TNT) in a freshwater environment. *Bulletin of Environmental Contamination and Toxicology.* **2000**, *65*, 228-235.

Hwang, S.; Ruff, T.J.; Bouwer, E.J.; Larson, S.L.; Davis, J.L. Applicability of alkaline hydrolysis for remediation of TNT-contaminated water. *Water Research.* **2005**, *39*, 4503-4511.

Karasch, C.; Popovic, M.; Qasim, M.; Bajpai, R.K. Alkali hydrolysis of trinitrotoluene. *Applied Biochemistry and Biotechnology.* **2002**, *100*, 1173-1185.

Khachatryan, G.E.; Mkrtchyan, N.I.; Simonyan, N.V.; Khachatryan, T.V.; Tatikyan, S.Sh. Two unidentified aerobic bacterial strains that transform 2,4,6-trinitrotoluene. *World Journal of Microbiology and Biotechnology.* **2000**, *16*, 393-395.

Kim, H.-Y.; Bennet, G.N.; Song, H.-G. Degradation of 2,4,6-trinitrotoluene by *Klebsiella* sp. isolated from activated sludge. *Biotechnology Letters.* **2002**, *24*, 2023-2028.

Kornel, A. Decomposition of nitrogen-based energetic material. United States patent #PCT/US03/23602. Date issued September 15 **2003**.

Kubitschek, H.E. and Friedman, M.C. Chromosome replication and the division cycle of *Escherichia coli* B/r. *J. Bacteriol*. **1971**, *107*, 95-99.

Kubota, A.; Maeda, T.; Nagafuchi, N.; Kadokami, K.; Ogawa, H.I. TNT biodegradation and production of dihydroxylamino-nitrotoluene by aerobic TNT degrader *Pseudomonas* sp. strain TM15 in an anoxic environment. *Biodegradation*. **2008**, *19*, 795-805.

Kurinenko, B.M.; Denivarova, N.A.; Yakovleva, G.Yu. Sensitivity of various *Escherichia coli* strains to 2,4,6-trinitrotoluene. *Applied Biochemistry and Microbiology.* **2005**, *41*(1), 47-50.

Kwon, S.-H. A biological approach in the treatment of TNT wastewater. *Toxicological and Environmental Chemistry.* **2000**, *75*(1-2), 17-23.

Kwon, M.J.; Finneran, K.T. Microbially Mediated biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine by extracellular electron shuttling compounds. *Applied and Environmental Microbiology.* **2006**, *72*(9), 5933-5941.

Labidi, M.; Ahmad, D.; Halasz, A.; Hawari, J.. Biotransformation and partial mineralization of the explosive 2,4,6-trinitrotoluene (TNT) by rhizobia. *Canadian Journal of Microbiology.* **2001**, *47*, 559-566.

Lewis, T.A.; Goszczynski, S.; Crawford, R.L.; Korus, R.A.; Admassu, W. Products of anaerobic 2,4,6-trinitrotoluene (TNT) transformation by *Clostridium bifermentans*. *Applied and Environmental Microbiology.* **1996**, *62*(12), 4669-4674.

Li, Z.M.; Comfort, S.D.; Shea, P.J. Destruction of 2,4,6-trinitrotoluene by Fenton oxidation. *Journal of Environmental Quality.* **1997**, *26*, 480-487.

McCormick, N.G.; Feeherry, F.E.; Levinson, H.S. Microbial transformation of 2,4,6-trinitrotoluene and other nitroaromatic compounds. *Applied and Environmental Microbiology.* **1976**, *31*(6), 949-958.

Mills, A.; Seth, A.; Peters, G. Alkaline hydrolysis of trinitrotoluene, TNT. *Physical Chemistry Chemical Physics.* **2003**, *5*, 3921-3927.

Oh, K.-H. and Kim, Y.-J. Degradation of explosive 2,4,6-trinitrotoluene by *s*-triazine degrading bacterium isolated from contaminated soil. *Bulletin of Environmental Contamination and Toxicology.* **1998**, *61*, 702-708.

Park, C.; Kim, T.-H.; Kim, S.; Kim, S.-W.; Lee, J.; Kim, S.-H.. Optimization for biodegradation of 2,4,6-trinitrotoluene (TNT) by *Pseudomonas putida*. *Journal of Bioscience and Bioengineering.* **2003**, *95*(6), 567-571.

Popesku, J.T.; Singh, A.; El-Alawi, Y.; Ward, O.P. Trinitrotoluene removal in a soil slurry and soil box systems by an oil-degrading mixed bacterial culture. *World Journal of Microbiology and Biotechnology.* **2006**, *22*, 1075-1081.

Rainwater, K.; Heintz, C.; Mollhagen, T.; Hansen, L. *In situ* biodegradation of high explosives in soils: field demonstration. *Bioremediation Journal.* **2002**, *6*(4), 351-371.

Reddy, G.; Reddy, T.V.; Choudhury, H.; Daniel, F.B.; Leach, G.J. Assessment of environmental hazards of 1,3,5-trinitrobenzene. *Journal of Toxicology and Environmental Health.* **1997**, *52*, 447-460.

Ro, K.S.; Venugopal, A.; Adrian, D.D.; Constant, D.; Qaisi, K.; Valsaraj, K.T.; Thibodeaux, L.J.; Roy, D. Solubility of 2,4,6-trinitrotoluene (TNT) in water. *Journal of Chemical Engineering Data.* **1996**, *41*, 758-761.

Saupe, A.; Garvens, H.J.; Heinze, L. Alkaline hydrolysis of TNT and TNT in soil followed by thermal treatment of the hydrolysates. *Chemosphere.* **1998**, *36*(8), 1725-1744.

Schrader, P.S.; Hess, T.F. Coupled abiotic-biotic mineralization of 2,4,6-trinitrotoluene (TNT) in soil slurry. *Journal of Environmental Quality.* **2004**, *33*, 1202-1209.

Stenuit, B.; Eyers, L.; El Fantroussi, S.; Agathos, S.N. Promising strategies for the mineralisation of 2,4,6-trinitrotoluene. *Reviews in Environmental Science and Bio/Technology.* **2005**, *4*, 39-60.

Stenuit, B.; Eyers, L.; Rozenberg, R.; Habib-Jiwan, J.-L.; Agathos, S.N. Aerobic growth of *Escherichia coli* with 2,4,6-trinitrotoluene (TNT) as the sole nitrogen source and evidence of TNT denitration by whole cells and cell-free extracts. *Applied and Environment Microbiology.* **2006**, *72*(12), 7945-7948.

Symons, Z.C.; Bruce, N.C. Bacterial pathways for degradation of nitroaromatics. *Natural Product Reports.* **2006**, *23*, 845-850.

Thorn, K.A.; Thorne, P.G.; Cox, L.G. Alkaline hydrolysis/polymerization of 2,4,6-trinitrotoluene: characterization of products by ^{13}C and ^{15}N NMR. *Environmental Science and Technology.* **2004**, *38*, 2224-2231.

Tront, J.M.; Hughes, J.B. Oxidative microbial degradation of 2,4,6-trinitrotoluene via 3-methyl-4,6-dinitrocatechol. *Environmental Science and Technology.* **2005**, *39*, 4540-4549.

Van Aken, B.; Yoon, J.M.; Schnoor, J.L. Biodegradation of nitro-substituted explosives 2,4,6-trinitrotoluene, hexahydro-1,3,5-trinitro-1,3,5-triazine, and octahydro-1,3,5,7-tetranitro-1,3,5-tetrazocine by a phytosymbiotic *Methylobacterium* sp. associated with poplar tissues (*Populus deltoides* × *nigra* DN34). *Applied and Environmental Microbiology.* **2004**, *70*(1), 508-517.

Viamajala, S.; Richards, L.; Peyton, B.M.; Petersen, J.N. Solubilization, solution equilibria, and biodegradation of PAHs under thermphilic conditions. *Chemosphere*. **2007**, *66*, 1094-1106.

Vorbeck, C.; Lenke, H.; Fischer, P.; Spain, J.C.; Knackmuss, H.-J. Initial reductive reactions in aerobic microbial metabolism of 2,4,6-trinitrotoluene. *Applied and Environmental Microbiology.* **1998**, *64*(1), 246-252.

Won, W.D.; DiSalvo, L.H.; Ng, J. Toxicity and mutagenicity of 2,4,6-trinitrotoluene and its microbial metabolites. *Applied and Environmental Microbiology.* **1976**, *31*(4), 576-580.

Zupko, A.J.; Johnson, N.P.; Leibbert, J.M.; Empson, W.B.; Mroz, D.L.; Bales, F.E. Roy F. Weston, Inc. and United States Army Corps of Engineers, Kansas City District. Incineration of explosives-contaminated soil and reactive RCRA waste at the former Weldon Spring Ordnance Works, Missouri, USA. Technical paper (#9008) presented at the Incineration and Thermal Treatment Technology Conference. May 1999, Orlando, FL.

NC TREATABILITY STUDY

Background

The propellant NC must have a minimum fiber length and percent nitration (%N) to be considered munitions-grade. NC having inadequate fiber length or %N < 12.5 is considered waste. Large amounts of waste NC have accumulated in holding ponds at Radford Army Ammunition Plant (RAAP) awaiting safe disposal. While NC is a non-toxic compound, its energetic properties prevent disposal in landfills, and open burning has been banned by the EPA due to air quality considerations. Contained incineration is a widely used, though expensive, disposal process (Spain et al., 2000; Kim et al., 1998). The current treatment method used specifically at RAAP is a combination of NC hydrolysis and biodegradation from soil microorganisms, carried out in the named Tank B472. However, the extent of NC transformation due to biodegradation is not known. Therefore, this study was intended to serve as a treatability study for determination of NC biodegradation. In addition, experiments were performed with a thermoalkaliphilic inoculum to test their contribution to NC biodegradation.

Analysis of microbial degradation of NC was done under conditions relevant to those in Tank B472. Results of NC hydrolysis and biodegradation tests are presented, as well as recommendations for potential operational changes in the currently used NC treatment processes at RAAP.

Materials and Methods

Waste NC and Tank B472 water + sludge were obtained from RAAP and refrigerated upon arrival at MSU. Settled waste NC in water was received from a RAAP holding pond, and contained some foreign matter (e.g. twigs, dirt particulates). The pH of the tank water as received was 10.98. Tank water was poured off the sludge and the pH of the tank water was adjusted from 10.98 to 9.50 with 6N HCl. To use the tank water, which contained very fine particulates, as the medium for abiotic control experiments, a portion of the tank water was centrifuged five times at 10,000rpm (8400 x g) before vacuum filtering with 0.2µm filter. Experiments were prepared and labeled according to Table 4.

Table 4: Composition of NC experiments at both room temperature (RT) and 60°C.

	200mL total volume pH 9.5	filtered tank water	unfiltered tank water	10% (v/v) tank sludge	60% (v/v) waste NC	10% (v/v) mixed thermophile inoculum
60°C	a,b,c = triplicate treatments		x	x	x	x
	d (control)	x		x	x	x
	e (control)		x	x		x
	f (control)		x		x	x
	g (control)		x	x	x	
	h (control)	x			x	
RT	a,b,c = triplicate treatments		x	x	x	
	d,e,f = triplicate controls	x			x	

The thermophile inoculum consisted of the following hot spring samples taken

from the Heart Lake Geyser Basin in Yellowstone National Park: PKF-09 (60°C) INFL sediment 20080914, PKF-09 (60°C) sediment Effl 20080914, PKF-32 (55°C) slurry mat 9-14-08, and PKF-9 (60°C) sediment INFL 20080914.

At room temperature (RT, 19-21°C), dried waste NC was added to 250mL serum bottles for a final concentration of approximately 60% (v/v) or 280 g/L. Sterile 250mL serum bottles were filled to a total volume of 200mL, sealed with butyl rubber septa, crimped with aluminum seals, incubated at either 20°C or 60°C (± 1°C), and shaken at 100 rpm. Treatments consisted of tank water, waste NC, and sludge from Tank B472 which served as inoculum. Controls consisted of filtered (0.2μm) tank water and waste NC only. Room temperature and 60°C treatments and controls were performed in triplicate.

At 60°C, treatments were similar to those at room temperature with the addition of a thermoalkaliphile inoculum obtained from a mixture of water and sediment/mat samples from alkaline hot springs in Yellowstone National Park.

Samples were taken with disposable serological pipets every 2 days in a laminar flow hood. Bottles were shaken immediately prior to sampling to obtain homogeneous 2mL samples. Each sample was divided as follows: 1mL for DNA analysis, 1mL for %N (solids) and IC (liquid). From the 1mL designated for %N and IC, 0.1mL of liquid was removed and diluted up to 1mL which was filtered (0.2μm) for IC analysis. Samples were stored in 1.5mL microcentrifuge tubes with the exception of the DNA samples which were stored in Axygen low protein binding 1.5mL microcentrifuge tubes. %N samples were refrigerated at 4°C, while DNA and IC samples were frozen at -20°C.

Samples for %N determination were dried, dissolved in concentrated H_2SO_4, and titrated with a 1.15M $FeSO_4$ solution in 25% (v/v) concentrated H_2SO_4. The $FeSO_4$ solution had been standardized for nitrogen content with KNO_3, so that a given volume of solution was known to reduce a certain amount of NO_3^-.

DNA analysis was prompted by the unsuccessful results of both protein analysis (Bradford, 1974) and cell counting due to the complexity of the sample matrix (data not shown). DNA was extracted from designated samples with a PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA). DNA samples were stored at -20°C until quantified using a Qubit fluorometer (Invitrogen Corporation, Carlsbad, CA).

IC samples (stored at 1:10 dilution) were diluted to a final 1:100 concentration to fall within the 0-100mg/L nitrite and nitrite IC calibration range. Samples were run on an Ion Chromatograph (Dionex Corporation, Sunnyvale, CA) fitted with an AS9-HC column and 100uL sample loop, using a 9mM Na_2CO_3 mobile phase.

Results and Discussion

The colorimetric titration method used to determine %N works well for relatively clean NC in water, however this analysis was very insensitive for the dark mixtures containing Tank B472 water or sludge. Also, the dried weight of NC used for %N calculations was skewed by non-NC debris from the waste NC and sludge particulates. As seen in Figures 17 and 18, the %N actually appeared to increase slightly in some bottles. Measurements of %N over the 60 day treatments showed that at pH 9.5 and RT the slope for a linear fit was -0.010 ± 0.005 (standard deviation). This slight overall

decrease of 0.0102% N corresponds to a 2.04mM increase of NO_2^-+NO_3^- in solution. This increase does not correspond with IC results given in Figures 18 and 19 where there was a combined decrease of nitrate and nitrite of more than 50mM. The discrepancy between the %N and IC results may be due to some denitrifying activity, though it is apparent that no significant hydrolysis was observed for NC in RT treatments. The RT control %N data was calculated to have an overall upward trend with a slope of 0.043 ± 0.007. This illustrates the unreliability of the %N titration for tracking hydrolysis since an increase in %N is improbable under alkaline conditions. Because NC hydrolysis was not adequately represented by the %N titration analysis, ion chromatography (IC) was used to measure changes in nitrogen content of the solution.

Figures 18 and 19, 23 and 24 show that high concentrations of nitrate and nitrite were initially present in the tank water itself. Separate experiments were carried out to

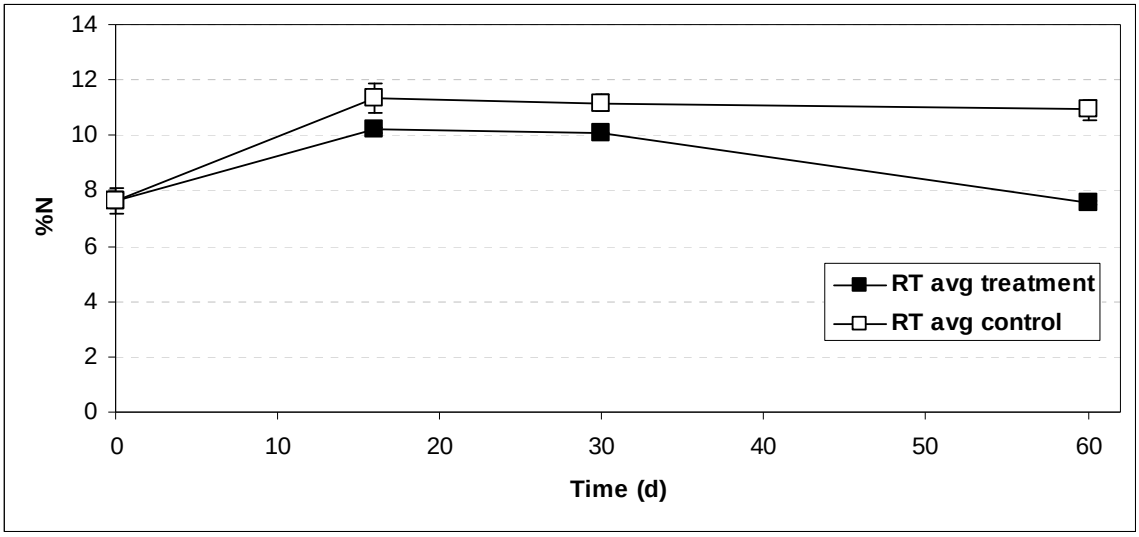


Figure 17: Percent nitration of solid phase residual (NC and tank sludge) in room temperature treatments determined by titration method. Error bars represent one standard deviation of triplicate experiments. Absence of error bars indicates error bar is smaller than data point symbol.

observe changes in nitrite and nitrate concentrations due to elevated pH alone (no added NC or tank sludge). Experiments were done with 15mM nitrate and 80mM nitrite added to filtered tank water at pH 9.5, as well as with the same nitrate and nitrite concentrations added to nanopure water at pH 9.5. Both the filtered tank water treatments and water-only treatments were performed in triplicate at RT and 60°C (data not shown). In the filtered tank water treatments, a gradual decrease of both nitrate (~5mM) and nitrite (~30mM) was observed over a period of 10 days for both temperatures. In contrast, the nitrate and nitrite in water only showed no significant change at either temperature over 10 days. These results suggest that there is some abiotic reaction of nitrate and nitrite with constituents in the tank water itself. Decreases greater than 5mM for nitrate or 30mM for nitrite may be attributed to the added sludge and/or NC.

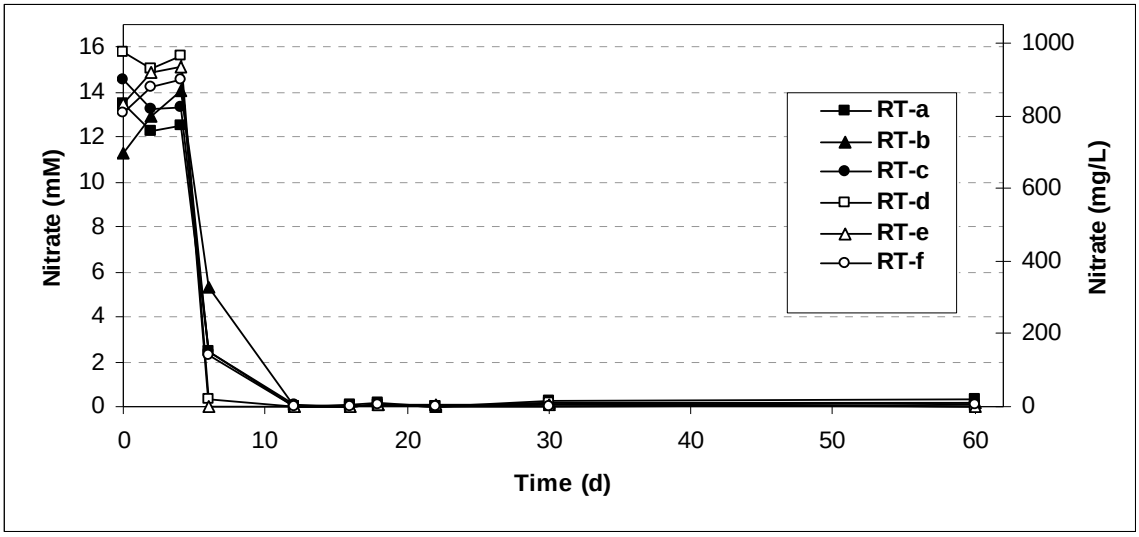


Figure 18: Nitrate concentrations for room temperature experiments as determined by IC (see Table 4 for letter key).

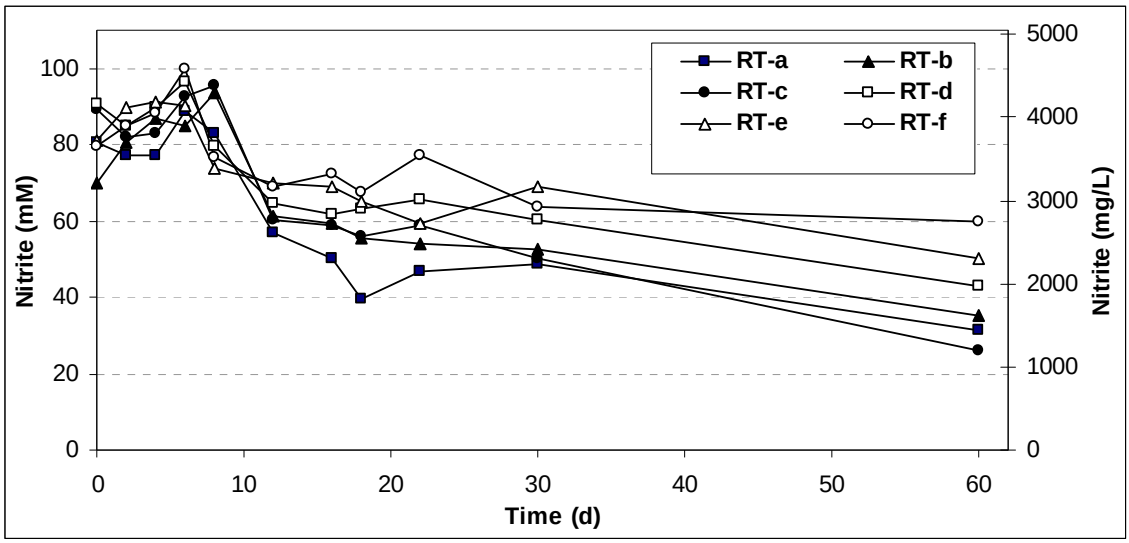


Figure 19: Nitrite concentrations for room temperature experiments as determined by IC (see Table 4 for letter key).

In Figures 18 and 19 (the room temperature tests), the relatively small increases in nitrate (~ 2-3mM) and nitrite (~ 15-20mM) seen over the first four days may be due to the initial, rapid hydrolysis of NC.

Given a starting NC concentration of 280g/L at 12% N, a decrease of 0.5% N would increase nitrate and nitrite in solution collectively by 100mM. There was a marked overall decrease in NO_2^- and NO_3^- at room temperature starting between days 6 and 10. This decrease does not support the occurrence of significant NC hydrolysis where a net *increase* in solubilized nitrogen would have been observed. However, there was an increase in nitrite concentration around six days which coincided with the disappearance of nitrate, potentially indicative of biological denitrification. Since this trend was seen in the treatments as well as the controls, it suggests that denitrifying microorganisms would be present in the waste NC and/or in the unfiltered tank water or sludge.

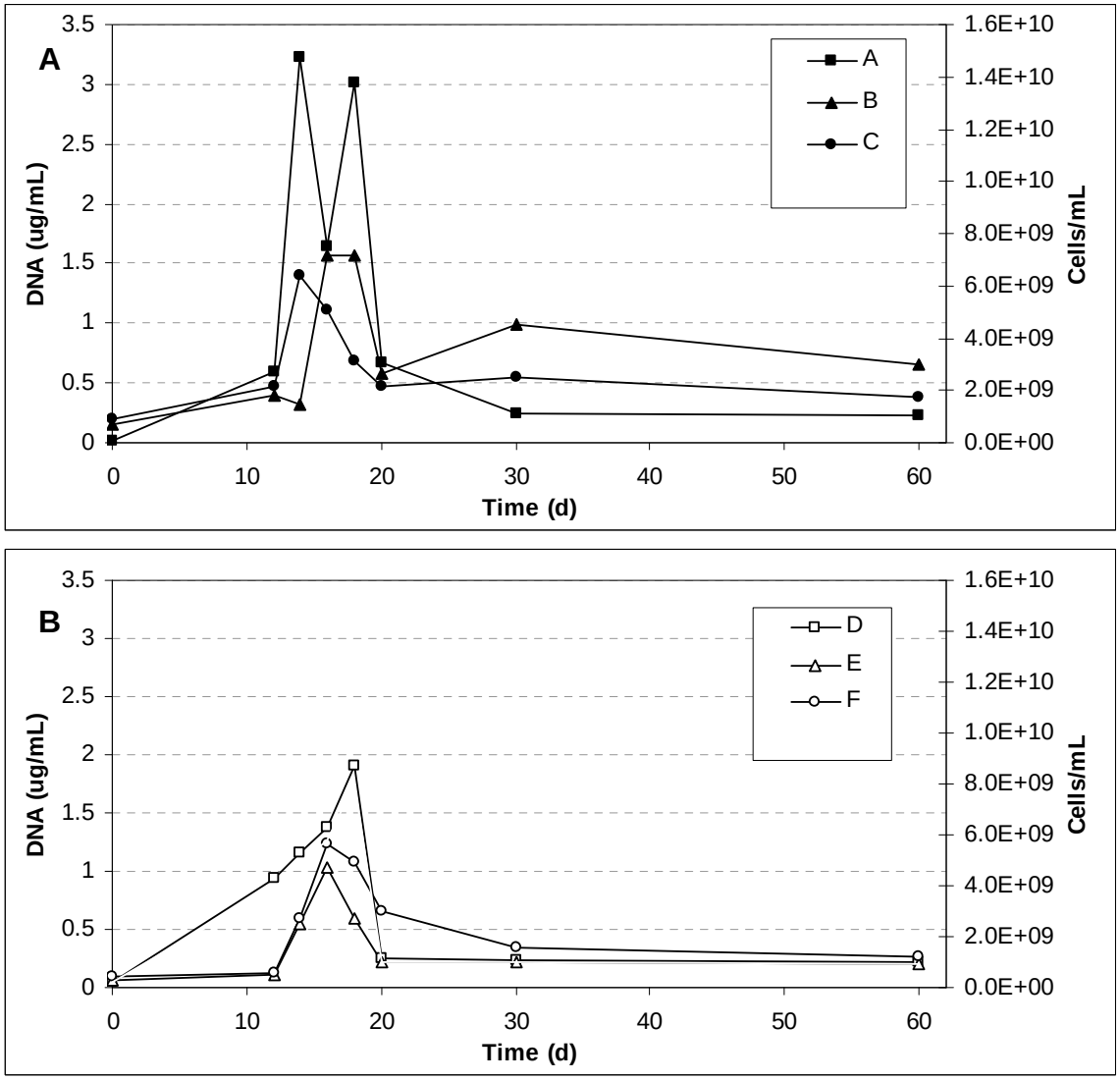


Figure 20: DNA and equivalent cell concentrations for (A) room temperature treatments and (B) controls (see Table 4 for letter key).

To supplement observations of the presence or absence of biological activity, DNA extractions were performed. The RT tests showed a significant increase around day 14 of the experiment as seen in Figures 20. Conversion of DNA concentration to cell/mL was calculated according to Kubitschek and Friedman (1971) who reported 4.2×10^{15} g DNA per *E. coli* cell. These data generally coincided with the decreases in nitrate and nitrite

concentrations that occurred around day 12 (Figures 18 and 19), suggesting biological denitrification activity may have occurred with a slight lag in biomass production. The RT DNA data also support the idea that microbial biomass was introduced with the waste NC, since DNA concentration in controls increased along with those of the treatment bottles.

Samples from experiments performed at 60°C were initially analyzed in the same manner as the RT experimental samples to gauge the extent of alkaline NC hydrolysis, by the %N titration (Figure 21). The overall linear trend for 60°C treatments had a slope of –0.001 ± 0.048, indicating that there was no significant change in %N.

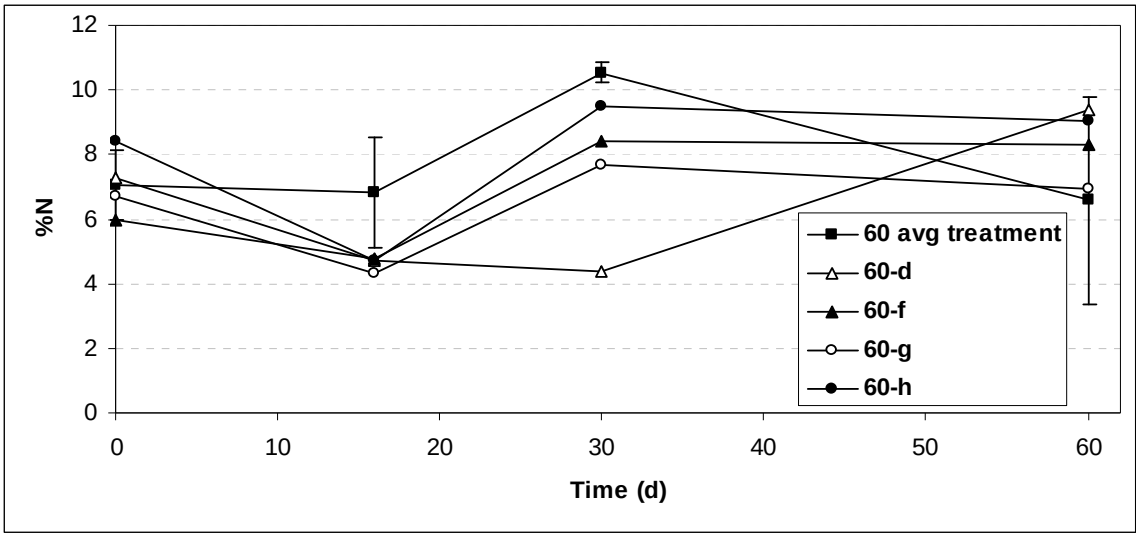


Figure 21: Percent nitration of solid phase residual in 60°C treatments determined by titration method. Error bars represent one standard deviation for triplicate experiments of the treatment only (see Table 4 for letter key). Absence of error bars indicates error bar is smaller than data point symbol.

To the contrary, IC results for nitrate and nitrite (Figures 23 and 24) both show large increases in concentration over the 60 day experimental period. Like the RT controls, the 60°C controls showed a slight overall increase in %N having a slope of 0.037 ± 0.014. Again, these %N results are unreliable since increases in %N are unlikely under experimental conditions of high pH. In addition, Figures 23 and 24 show that 60°C controls, like the 60°C treatments, had a large increase in soluble nitrogen, which would only correspond to a decrease in %N.

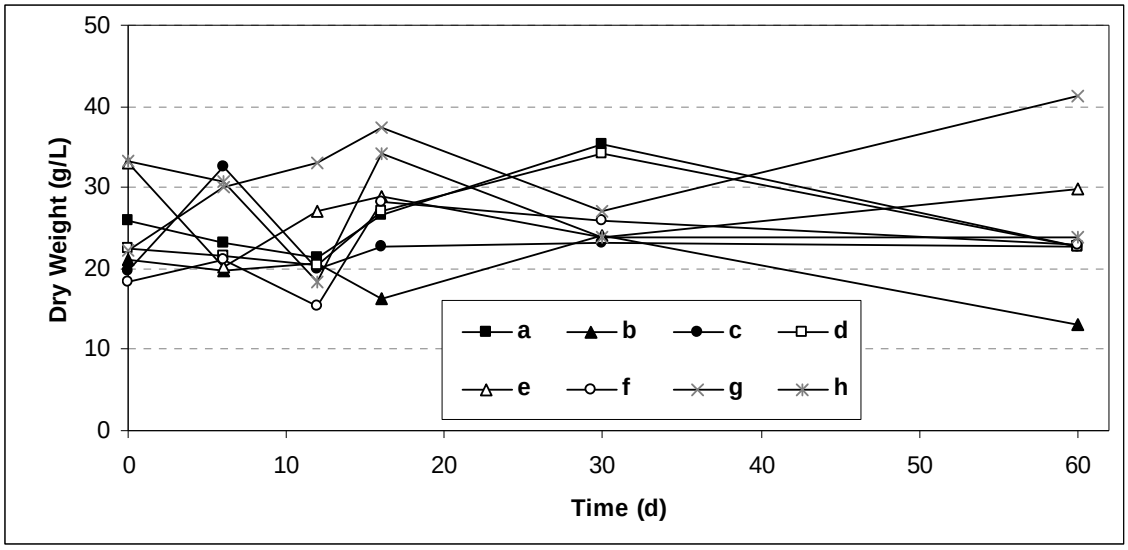


Figure 22: Total suspended solids for 60°C experiments (see Table 4 for letter key).

The change in mass of total suspended solids (TSS) is often used as a secondary indicator of NC hydrolysis (Christodoulatos et al., 2001). Data shown in Figure 22 did not show a statistically significant downward trend with time for either treatments or controls. A linear trend applied to the 60°C treatments gave a slope of -0.036 ± 0.062, and control data had a slope of 0.049 ± 0.124. The standard deviations of these slopes were

each approximately twice as large as the slope values themselves, indicating the inconsistency of TSS as a measure of NC hydrolysis. In these experiments, the matrix of tank sludge and NC in tank water made %N and TSS measurements much more difficult than NC in water alone. Neither %N nor TSS analyses were suitable indicators of hydrolysis. As with the RT results of negligible NC hydrolysis as measured by %N, analytical focus was shifted to the more sensitive measure of nitrate and nitrite.

For the 60°C pH 9.5 experiments shown in Figures 23 and 24, data show that both nitrate and nitrite concentrations increased significantly over the reaction period due to release of these species during NC hydrolysis. These results support the trend described in Christodoulatos et al. (2001) where an increased temperature greatly improved hydrolysis rates for a given hydroxide concentration.

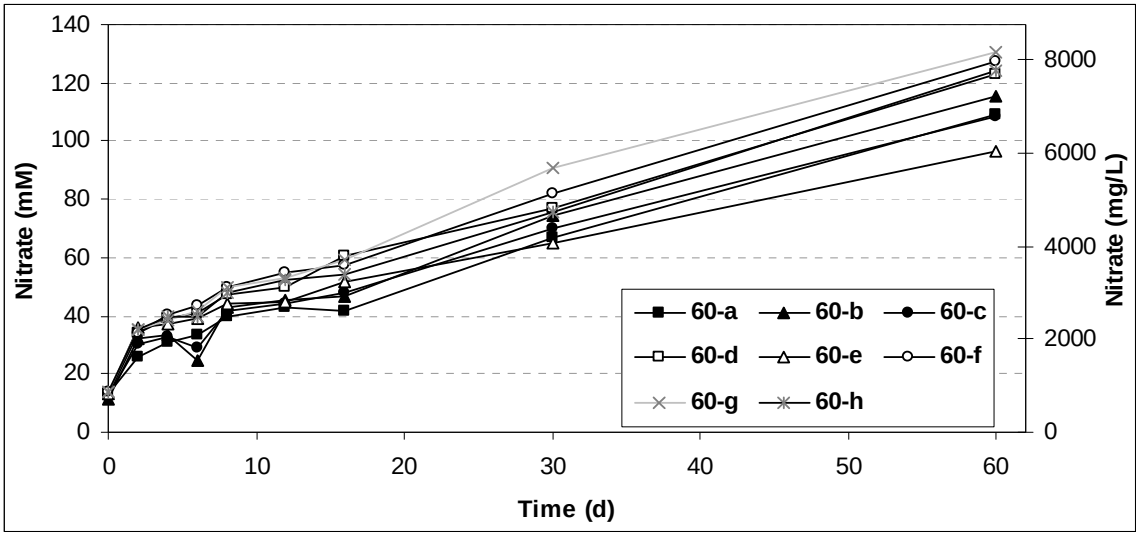


Figure 23: Nitrate concentrations for 60°C experiments as determined by IC (see Table 4 for letter key).

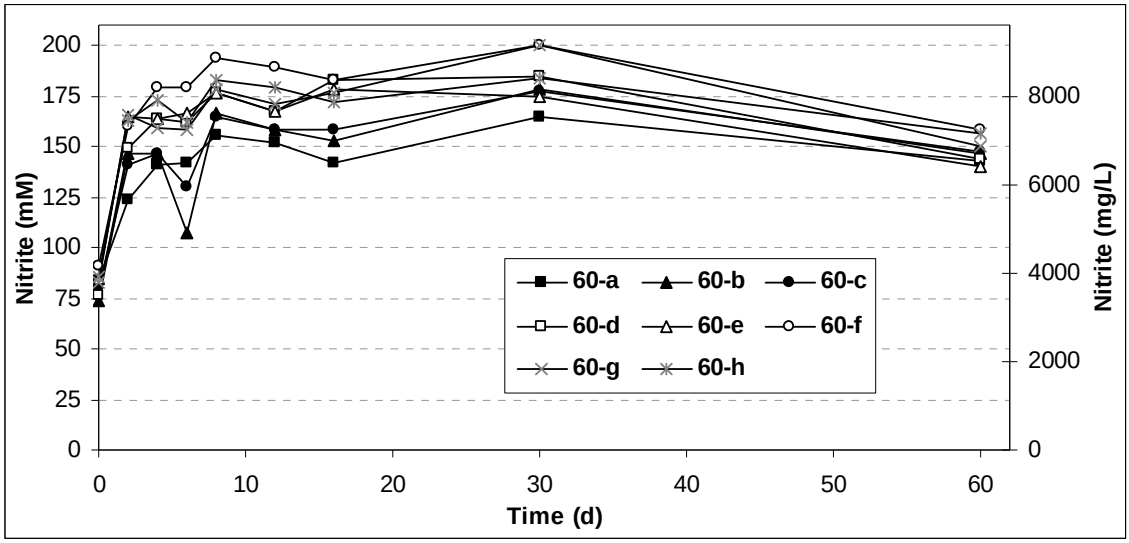


Figure 24: Nitrite concentrations for 60°C experiments as determined by IC (see Table 4 for letter key).

Based on the measured increases in both nitrite and nitrate, the %N of the original NC was calculated to have decreased by 1% over the 60 day experiment. While there was evidence of some NC hydrolysis at 60°C (Figures 23 and 24), there was no evidence of significant biological transformation of NC or of biological denitrification (as indicated by any decrease in nitrate or nitrite concentrations).

Only very small amounts of DNA were detected in the 60°C samples (data not shown). This concentration did not change over the course of the experiment, which also supports our observation that little or no biological activity was present in the 60°C tests.

Conclusions

The key findings were: 1) No significant biological activity was observed which would contribute to NC removal in Tank B472, though it may be possible that some

denitrifying microorganisms are present, 2) No significant NC hydrolysis was observed at pH 9.5 and ambient temperature, 3) No significant biological activity was observed at 60°C, 4) A significant increase in soluble nitrogen species at 60°C suggested that elevated temperature increased NC hydrolysis. From these findings it was concluded that the combination of NC alkaline hydrolysis and biological denitrification in the same tank is most likely ineffective and requires modification to obtain complete digestion of NC to environmentally benign end products.

At room temperature, no *significant* hydrolysis or biodegradation of the waste NC was observed at pH 9.5. This observation correlates well with the abiotic results published by Christodoulatos et al., (2001). While our tests concluded that biological activity does not play a significant role in the degradation of NC in Tank B472, some potential biological denitrification was observed. Data that support this observation include 1) all of the nitrate originally present disappeared, and 2) a temporary increase in DNA concentration.

For degradation of the waste NC, it has been shown previously that elevated base concentrations (high pH) and temperatures both contribute to the hydrolysis of NC (Alleman et al., 1994; Kim et al., 1998). For example, in only 1 hour a 95% decrease in total solids was observed for hydrolysis of 10g/L NC in water at 50°C with hydroxide concentrations equivalent to pH 13 (Christodoulatos et al., 2001). In our study, significant increases in nitrate and nitrite concentrations suggested hydrolysis of the waste NC at pH 9.5 and 60°C. Unfortunately, the complex nature of the experimental systems (i.e. fine particulate sludge and darkly colored tank water) prevented reproducibly sensitive

measurement of changes in total suspended solids or %N. By assuming the total change in nitrogen concentration (as NO_2^- and NO_3^-) as wholly contributing to a change in %N, the %N was calculated to decrease by 1% over the 60 day experiment. From the 60°C experiments with a thermoalkaliphilic inoculum, no discernable change in DNA concentration was observed. The high nitrite concentrations (30-100mM) of the tank water were likely a key inhibitor to microbial growth (Beccari et al., 1983).

Recommendations

To improve nitrocellulose (NC) removal, it is recommended to separate the processes of NC hydrolysis and biological denitrification. Hydrolysis at high OH^-:NC ratios and elevated temperatures would rapidly digest the NC and produce a solution high in nitrate and nitrite. Neutralization and subsequent addition of this solution to a denitrifying culture would allow for removal of nitrate and nitrite species.

Based on these results, we suggest that to obtain significant NC degradation, the NC hydrolysis process should be separated from the biological denitrification process. NC hydrolysis results in production of high concentrations of nitrate and nitrite. High pH values and high nitrate and nitrite concentrations, however, are not amenable to biological denitrification. We suggest a separate denitrification step to be used for the biological conversion of nitrate and nitrite to nitrogen gas. Biological denitrification would be performed ideally at circumneutral pH values (pH 6-8), which can be achieved by neutralization prior to addition of the hydrolysate to a wastewater treatment plant.

By establishing these two processes as distinct entities, higher pH values and temperatures necessary for rapid hydrolysis could be optimized. For the denitrification step, there would be no demand for a hydrolyzing environment, so that near complete biological removal of nitrate and nitrite could be achieved, potentially with the carbon compounds produced through NC hydrolysis (Bunte et al., 1997). Separation of hydrolysis and denitrification would allow the use of denitrifying cultures obtained from a wastewater treatment plant or even the use of the existing wastewater treatment plant facilities at RAAP for denitrification.

Conditions of high pH and high temperature have been shown to drastically increase hydrolysis rates of NC, which is a critical first step to biological or physicochemical NC treatment processes. Based on the results of NC hydrolysis reported by Christodoulatos et al. (2001), we suggest that a OH^-:NC molar ratio of at least 10 and temperature of at least 50°C be used in the NC hydrolysis tank to achieve reasonable rates of NC degradation. Alternatively, larger OH^-:NC ratios could be used at lower temperatures or lower OH^-:NC ratios at higher temperatures. It should be noted, however, that hydrolysis experiments done by Christodoulatos et al. (2001) used only 10g/L NC, which is not a realistic concentration for the purpose of processing large amounts of waste NC produced at RAAP.

References

Alleman, J.E.; Kim, B.J.; Quivey, D.M.; Equihua, L.O. Alkaline hydrolysis of munitions-grade nitrocellulose. *Wat. Sci. Tech.* **1994**, *30*, 63-72.

Atlas, Ronald M. Handbook *of Microbiological Media*, 3rd ed.; CRC Press LLC: Boca Raton, FL, 2004; p.1176.

Auer, N.; Hedger, J.N.; Evans, C.S. Degradation of nitrocellulose by fungi. *Biodegradation.* **2005**, *16*, 229-236.

Beccari, M.; Passino, R.; Ramadori, R.; Tandoi, V. Kinetics of dissimilatory nitrate and nitrite reduction in suspended growth culture. *J. Water Pollut. Control Fed.* **1983**, *55*, 58-64.

Brown, K.W. and Thomas, J.C. Degradation of explosive propellants by in-vessel composting. *BioCycle.* **1995**, *36*, 56-61.

Bunte, G.; Drause, H.H.; Hirth, T. Disposal of energetic materials by alkaline pressure hydrolysis and combined techniques. *Prop. Expl. Pyrotech.* **1997**, *22*, 160-164.

Christodoulatos, C.; Su, T.-L.; Koutsospyros, A. Kinetics of the alkaline hydrolysis of nitrocellulose. *Water Environ. Res.* **2001**, *73*, 185-191.

Freedman, D.L.; Caenepeel, B.M.; Kim, B.J. Biotransformation of nitrocellulose under methanogenic conditions. *Wat. Sci. Tech.* **1996**, *34*, 327-334.

Freedman, D.L.; Cashwell, J.M.; Kim B.J. Biotransformation of explosive-grade nitrocellulose under denitrifying and sulfidogenic conditions. *Waste Management.* **2002**, *22*, 283-292.

Kenyon, W.O. and Gray, H.LeB. The alkaline decomposition of cellulose nitrate. I. Quantitative studies. *Am. Chem. Soc. J.* **1936**, *8*, 1422-1427.

Kim, B.J.; Alleman, J.E.; Quivey, D.M. (1998). *Alkaline hydrolysis/biodegradation of nitrocellulose fines.* (USACERL Technical Report 98/65). Champaign, IL: US Army Corps of Engineers Construction Engineering Research Laboratories.

Kubitschek, H.E. and Friedman, M.C. Chromosome replication and the division cycle of *Escherichia coli. J. Bacteriol.* **1971**, *107*, 95-99.

Pfeil, A. Microbial degradation of nitrocellulose in a composting environment. *Prop. Expl. Pyrotech.* **1999**, *24*, 156-158.

Wendt, T.M. and Kaplan, A.M. A chemical-biological treatment process for cellulose nitrate disposal. *J. Water Pollut. Control Fed*. **1976**, *48*, 660-668.

ALKALINE HYDROLYSIS OF NC

Introduction

Nitrocellulose (NC) is a primary component of gunpowder and rocket propellants, as well as a number of commonly-used commercial products such as films, inks, filters, and plastics (Kim et al., 1994). The application of NC depends on its nitrogen content by weight (%N), where %N greater than 12.5 is considered munitions grade and 11.5-12.5 %N is considered commercial grade. Waste NC is any NC with inadequate %N or fiber length for the respective application. At the Radford Army Ammunitions Plant (RAAP) in Radford, VA, the accumulation of waste NC is a growing concern due to lack of safe disposal methods. When wet, NC is very stable and the approximate 15 million pounds of 'legacy waste' is kept in large holding ponds at RAAP and other ammunitions manufacturing facilities (Alleman et al., 1994; Kim et al., 1998). Although NC is a non-toxic compound because of its aqueous insolubility and consequent immobility in the environment, its energetic properties as a Class B explosive (when dry) prohibit land-filling operations. The most common method for NC removal is incineration which is becoming increasingly less popular based on safety issues and air quality concerns by the EPA. As an alternative to incineration, alkaline hydrolysis is a possible method for the transformation of NC to environmentally-friendly end products since this type of hydrolysis results in a more complete degradation of the cellulosic backbone than acid hydrolysis.

The alkaline hydrolysis of NC has been previously reported (Alleman et al., 1994; Christodoulatos et al., 2001; Kim et al., 1998; Wendt and Kaplan, 1976). It has been found that a number of factors influence the rate and extent of hydrolysis, including hydroxide concentration, OH^-:NC ratio, and temperature. Kenyon and Gray (1936) reported the first experimental results for alkaline hydrolysis of NC where large amounts of nitrite (~70%) were released from NC solubilization. Though little mineralization to carbon dioxide was observed, a wide variety of organic carbon compounds were produced, including acids and sugars. Kenyon and Gray noted that increased temperatures and increased hydroxide concentrations were necessary to increase the rate of hydrolysis. Higher OH^-:NC ratios were required to increase the extent of hydrolysis. Alleman et al. (1994), Kim et al. (1998), and Christodoulatos et al. (2001) published similar findings where the rate and extent of hydrolysis was improved with higher reaction temperatures and alkali concentrations. Alleman et al. (1994) showed that nearly all of the carbon from NC was solubilized as organic carbon for sodium hydroxide concentrations of at least 40g/L NaOH and 6g/L NC, which corresponds to a OH^-:NC molar ratio of 50. Nitrogen release from NC varied significantly among these reports. Kenyon and Gray (1936) saw up to 70% nitrogen release from NC for a OH^-:NC ratio of 50, while Alleman et al. (1994) noted only 20-50% nitrogen released from NC for that same ratio. Christodoulatos et al. (2001) observed up to 92% of nitrogen released from NC, which included hydrolysis testing at OH^-:NC ratios up to 111. Christodoulatos et al. (2001) also noted that complete solubilization occurred when the OH^-:NC was at least 22.

Of these reports for alkaline hydrolysis of NC, the initial NC concentration was typically 20g/L NC or less and did not exceed 70g/L. A concentration of 200g/L NC is more representative of the large volume of legacy waste of NC at RAAP. This necessitates a higher OH^- concentration for hydrolysis to destroy accumulated NC waste in a timely manner. The present study investigated the alkaline hydrolysis of NC at hydroxide and NC concentrations ten times higher than those previously reported, representing more realistic initial NC concentrations. The OH^-:NC molar ratio was initially set to 15 or 30 to observe the extent of solubilization based on a guideline of at least 22 set by Christodoulatos et al. (2001). Use of the hydrolysate as a carbon source for a denitrifying culture was tested, as well as using adding methanol (a commonly-used substrate for denitrifying cultures) as the carbon source. These experiments of separate NC hydrolysis and biological denitrification determined rates of NC hydrolysis at high NC concentrations (100-200g/L) which, to our knowledge, have not yet been reported. This data will be beneficial to Radford for the development of waste NC disposal strategies.

Materials and Methods

Clean NC (i.e. not waste NC) was obtained from Radford Army Ammunition Plant (RAAP) in Radford, VA, with a nitrogen content (% N) of 12.56% N as nitrate by weight. NC was stored in water at 4°C and air-dried overnight in a fume hood prior to use in experiments. For 100g/L or 200g/L NC experiments performed at 10mL total volume, dry NC was weighed to 1g or 2g, respectively. Stock solutions of 400g/L NaOH were

stored at room temperature in plastic containers. For experiments at 60°C, 10mL aliquots of NaOH solution were heated prior to combining it with NC. Throughout each experiment, serum bottles remained open (for escape of any evolved gases (Wendt and Kaplan, 1976)) and were shaken at 100rpm at either 20°C or 60°C. All chemicals used were of reagent grade. All experiments were performed in triplicate.

Due to the difficulty in obtaining homogeneous samples from the same treatment bottle over time, destructive sampling was employed for these NC hydrolysis experiments. Each set of conditions had three bottles (replicates) for each time point, such that data collection from six time points required 18 separate serum bottles. When sampled, the entire contents of each bottle were immediately vacuum filtered through a filter crucible fitted with a pre-weighed Whatman 934-AH glass microfiber filter (1.5µm, Whatman Inc., Clifton, NJ). The filter was then removed and air-dried. Once dry, the filter was re-weighed to determine total suspended solids (TSS). The filtrate was cooled in an ice-saltwater bath (-5°C) and subsequently stored at 4°C.

Filtrate samples were prepared for IC analysis by filtering (0.2µm) and diluting 1:1000 into the reliable working range of the instrument (1-100mg/L nitrite or nitrate). Both nitrite (NO_2^-) and nitrate (NO_3^-) were detected on a Dionex DX500 IC (Sunnyvale, CA) fitted with a CD20 conductivity detector, AS9-HC (4 x 250mm) column and 100µL sample loop. The mobile phase was 9.0mM Na_2CO_3 with a helium overpressure. The detection limit for NO_2^- and NO_3^- was 1 mg/L.

A water sample was obtained from the wastewater treatment plant (WWTP) in Bozeman, MT. This sample was enriched for denitrifying activity using methanol as the

carbon source in the following modified minimal medium for denitrifying bacteria (Atlas, 2004) (L^{-1}): 5g KNO_3, 1g $(NH_4)_2SO_4$, 0.66g K_2HPO_4, 0.54g KH_2PO_4, 0.2g $MgSO_4 \cdot 7H_2O$, 0.02g $CaCl_2 \cdot 2H_2O$, 0.01g $FeSO_4 \cdot 7H_2O$, 0.005g $MnSO_4 \cdot H_2O$, 0.001g $CuSO_4 \cdot 5H_2O$, 0.001g $Na_2MoO_4 \cdot 2H_2O$, 10mL 0.1N HCl. The final pH of this medium was 6.80. Cultures were prepared in sterile serum bottles, sealed with butyl rubber septa and crimped aluminum seals. The headspace was replaced with N_2, bottles were incubated at 30°C, and shaken at 100rpm. When transferred to fresh media, 10% (v/v) inoculum was used. Inoculum for triplicate experiments had been previously transferred at least three times, but not more than six times. For experiments, the denitrifying culture was grown with KNO_3 as the initial electron acceptor. Once the KNO_3 was depleted (near the end of the exponential growth phase), subsequent spikes of neutralized (pH 6.80) hydrolysate were added to the denitrifying culture so that the final nitrite concentration was approximately 100mg/L, 150mg/L, and 200mg/L nitrite for the first, second, and third spikes, respectively.

Biomass was monitored as optical density at 620nm using a Multiskan Spectrum spectrophotometer (Thermo Scientific Corporation, Waltham, MA). Samples were filtered (0.2μm) prior to IC analysis. Both nitrite (NO_2^-) and nitrate (NO_3^-) were quantified as described above.

Results and Discussion

Hydrolysis of NC

Because previous reports of the alkaline hydrolysis of NC were performed at

concentrations of 70g/L or less (Christodoulatos et al., 2001; Kim et al., 1998), more realistic concentrations (100-200g/L) for an industrial scale treatment were tested. Christodoulatos et al. (2001) observed that for hydrolysis of 10g/L NC at 50°C and 70°C, the reaction time required to degrade > 95% NC was approximately 30-60min. Based on those results, where the OH^-:NC ratio was 15 or 30, the initial concentration of NC in the present study was increased ten-fold (from 10 to 100g/L NC). In keeping the OH^-:NC ratio constant, the hydroxide concentration was also scaled up 10-fold (from 40 to 400g/L NaOH). The results for total suspended solids (TSS) of these experiments at 60°C are shown in Figure 25.

Reaction times similar to Christodoulatos et al. (2001) were expected for NC hydrolysis: 25 min for OH^-:NC ratio of 30, and 45 min for OH^-:NC ratio of 15. Figure 25 shows that for a ten-fold increase in both NC and hydroxide concentrations, the time needed to consume > 95% NC was drastically reduced from 25 min down to 2 min. At 60°C, both the 100g/L and 200g/L NC treatments at 400g/L NaOH (30 and 15 OH^-:NC ratios, respectively) showed less than 5% of the initial NC remaining as TSS after just 1 to 2 min. Once the dried NC was combined with the NaOH at 60°C, it took about 1-1.5min for the 100g/L NC to become well mixed, and at that point, the solid NC almost instantaneously dissolved. Similarly, the 200g/L NC took 1.5-2min to become well mixed, then it immediately dissolved. Therefore data could not be collected at times less than 2 min for the 100g/L NC, or less than 2.5 min for 200g/L NC. Filtration of residual NC required a well-mixed solution to avoid clumps of NC remaining in the bottle when the reaction solution was poured onto the filter. In the case of the room temperature (RT)

control, the reaction solution became very viscous making it difficult to filter the NC solids at each time point. The filter appeared to be clogged with the thick NC-hydroxide mixture, creating large errors for TSS measurements over the 50 min experiments. Visual inspection of these RT controls left for 24h showed no NC residual.

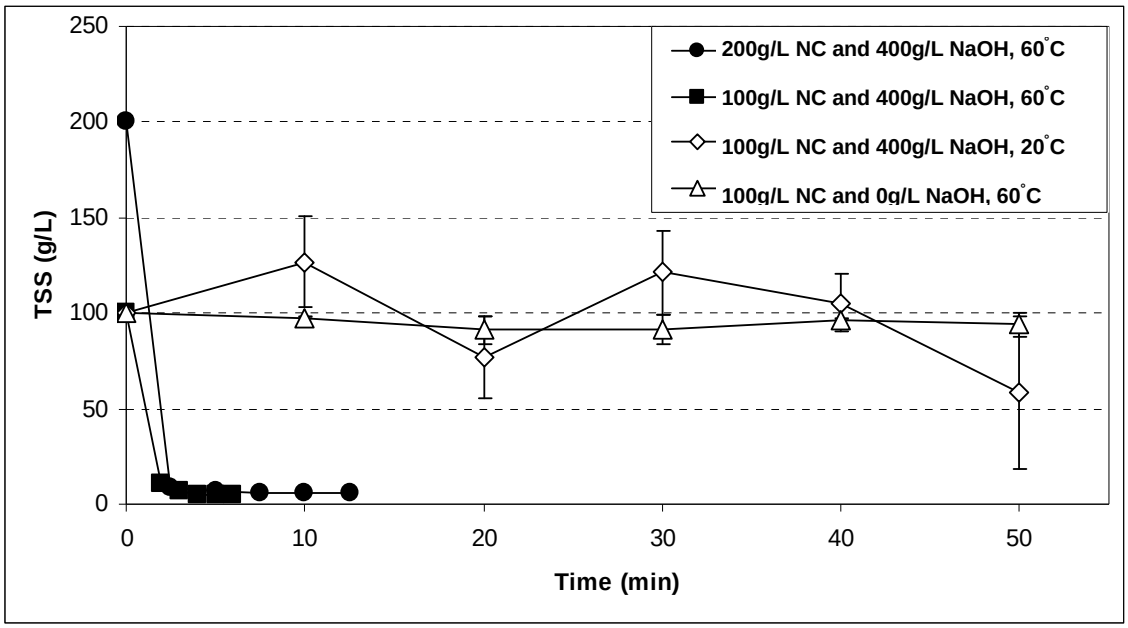


Figure 25: Total suspended solids (TSS) for treatments (solid symbols) and controls (hollow symbols). Error bars represent ± one standard deviation of triplicate experiments. Absence of error bars means error smaller than data point symbol.

A generalized kinetic equation for NC alkaline hydrolysis was constructed by Christodoulatos et al. (2001) to calculate final NC concentration, as TSS, with time. The calculation was based on the initial NC concentration, initial hydroxide concentration, and reaction temperature. Equation 6 is the kinetic model given by Christodoulatos et al. (2001), where C_{NC} is NC (g/L) at time t (min), C^0_{NC} is NC (g/L) at time 0 min, C^0_{NaOH} is NaOH (g/L) at time 0 min, and T is reaction temperature in $^{\circ}$C. It agreed well with RT observations of the current study, where it took approximately 20-24h to degrade 95% of

the initial 100g/L NC at 400g/L NaOH and 20°C. Equation 6 predicted that 22.5h would be required for these conditions.

$$C_{NC} = C^0_{NC} \exp[(-4.73x10^{13}(C^0_{NaOH})^{1.5}\exp(-12141.4/T))t] \qquad \text{Equation 6.}$$

A comparison of the TSS results from 100 and 200g/L NC at 60°C and 400g/L NaOH with Eqn. 6 is given in Figure 26.

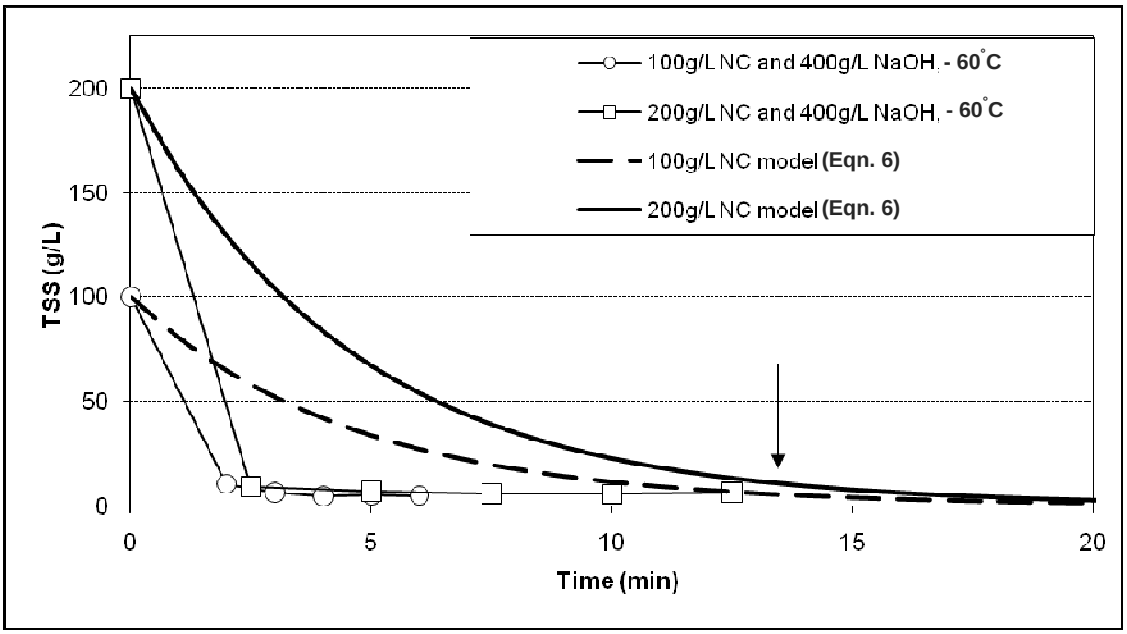


Figure 26: TSS for this study and predicted TSS from Christodoulatos et al. (2001) model Equation 6. The arrow indicates the point at which 95% of the initial NC for both 100g/L and 200g/L would be digested according to Equation 6 (~13.5min).

It can be seen that the Christodoulatos et al. (2001) kinetic model does not accurately predict the rapid rate of NC digestion that was observed here in laboratory experiments. The reaction time predicted for 95% reduction of initial NC was around 13.5 min for both 100g/L and 200g/L NC, which was more than 10 min later than the

actual time of 1 to 2 min. This model was developed from data at 10g/L NC, 70°C, and hydroxide concentrations ranging from 1-50g/L as NaOH. While extrapolated TSS data for 100 or 200g/L NC and 400g/L NaOH may not be presumed to agree extremely well with actual data, the almost immediate solubilization of NC in this study was unexpected.

The general mechanism of alkaline hydrolysis of NC is believed to be hydroxide attack on outer layers of the NC which is degraded and solubilized, exposing the next inner layer to the hydroxide ions, and so on. It has been stated that diffusion of hydroxide into the solid matrix of NC does not affect overall degradation rate, though this is only true above a given hydroxide concentration (0.75M or 30g/L NaOH) where complete solubilization of NC is observed (Kim et al., 1998; Wendt and Kaplan, 1976; Christodoulatos et al., 2001). However, this hydroxide concentration was determined using relatively low initial NC concentrations (≤70g/L). When scaling up the hydrolysis process to accommodate NC concentrations of 100-200g/L, the OH^-:NC molar ratio was assumed to be a more reliable parameter for design of treatments. Ratios of at least 40 appeared to be necessary for achieving 95% reduction of NC solids at ambient temperature, while a ratio as low as 5 may be suitable for NC degradation at 90°C (Wendt and Kaplan, 1976; Christodoulatos et al., 2001).

Assuming a first order reaction with respect to NC concentration, a conservative rate constant, k (min^{-1}), for 100 and 200g/L NC at 400g/L NaOH at 60°C can be calculated from the slope between the first two data points of Figure 26. For 100g/L NC (ratio of 30), k was 0.448 ± 0.0206 min^{-1} (standard deviation), and k for 200g/L NC (ratio of 15) was 0.765 ± 0.000937 min^{-1}. When compared to results from Christodoulatos et al.

(2003), these values of k illustrate the drastic increase in NC degradation rate for a given OH^-:NC ratio. Christodoulatos et al. (2003) calculated k values of 0.0122 min^{-1} (50°C) and 0.0616 min^{-1} (70°C) for a ratio of 15. For a ratio of 30, k was 0.0242 min^{-1} (50°C) and 0.166 min^{-1} (70°C). From this rate data, it appears that the OH^-:NC ratio cannot be used to effectively compare NC hydrolysis when the hydroxide concentration is on the order of 400g/L. Results obtained in this study are contrary to the belief that diffusion of hydroxide into the solid matrix of NC does not affect overall degradation rate above a given hydroxide concentration.

Amounts of NO_3^- and NO_2^- released over time from the hydrolysis of NC are given in Figure 27. These data show that approximately 2 moles of nitrite are released for every 1 mole of nitrate. This 2:1 ratio differs from that of Christodoulatos et al. (2001) who reported a molar ratio of 4:1, consistent across time points for experiments at 10g/L NC, 30-90°C, and hydroxide concentrations from 1-150g/L NaOH. Because both NO_3^- and NO_2^- are found in the reaction solution, the hydrolysis of NC releases NO_3^- and NO_2^- directly from the NC molecule by the interaction with hydroxide (Wendt and Kaplan, 1976). The constant ratio of nitrite to nitrate suggests the independence of hydrolysis from temperature and hydroxide concentration (above a certain minimum temperature and OH^- concentration). In this study, the amount of nitrogen originally present in 12.56%N NC released as [NO_2^-+NO_3^-] was 83.5% for 100g/L and 92.4% for 200g/L NC. The OH^-:NC ratios for 100g/L and 200g/L NC with 400g/L NaOH were 30 and 15, respectively. These numbers are comparable with Christodoulatos et al. (2001) who reported up to 92% of the nitrogen in NC was released during hydrolysis of 10g/L NC.

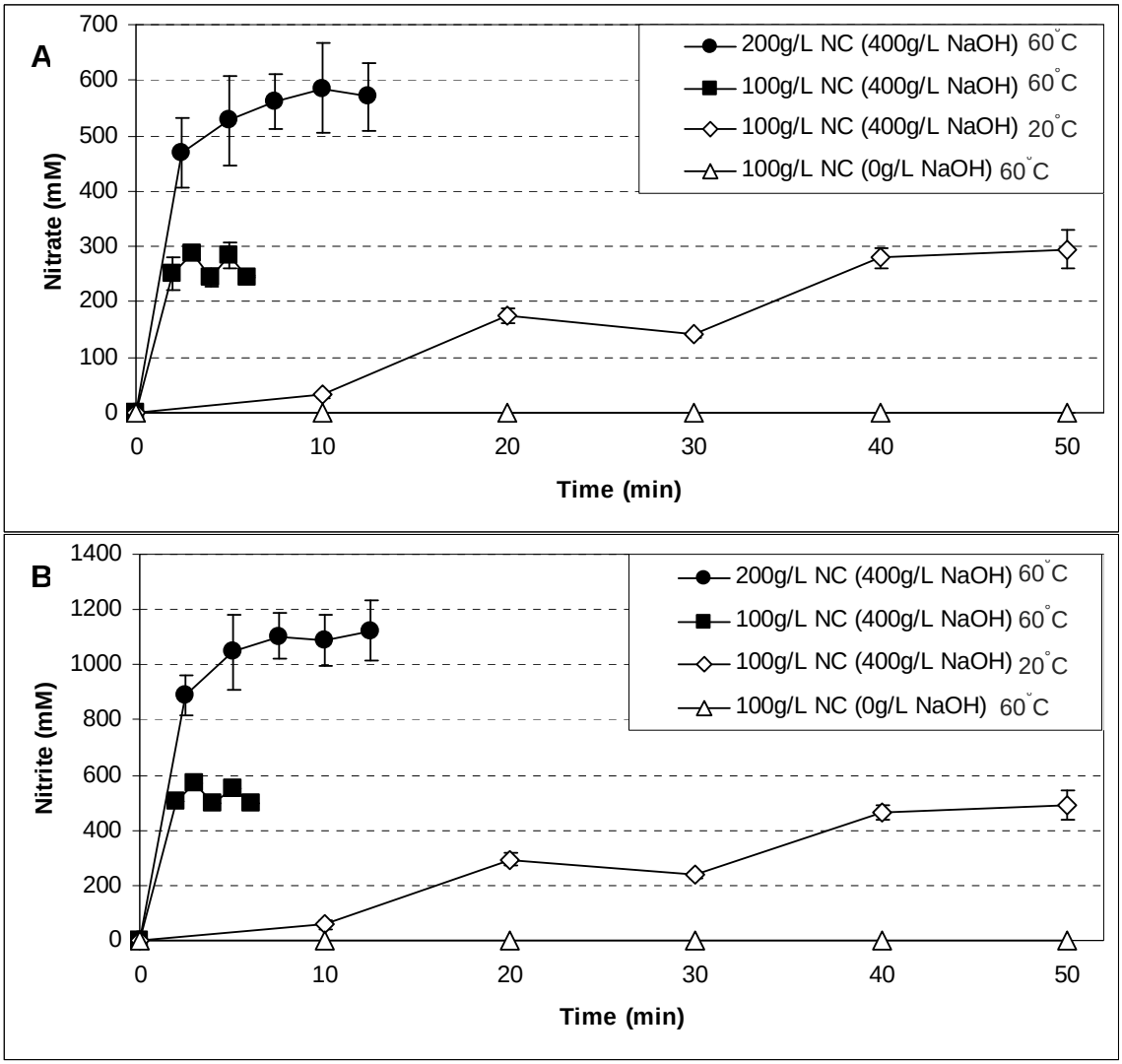


Figure 27: Nitrate (A) and nitrite (B) concentrations for NC hydrolysis treatments and controls. Error bars represent ± one standard deviation of triplicate experiments. Absence of error bars means error bar is smaller than data point symbol.

Biological Degradation of NC Hydrolysate

To determine if the resulting hydrolysate was biodegradable, the NC hydrolysate was tested for use as a carbon source and terminal electron acceptor for an un-characterized WWTP denitrifying culture. Once the culture reached the exponential growth phase and had depleted the initial KNO_3 added, the neutralized (pH 6.80)

hydrolysate from the 100g/L NC, 400g/L NaOH, 60°C experiments was spiked into the culture. Growth experiments were performed to test the hydrolysate as both the electron donor (carbon source) and the electron acceptor. Alternatively, cultures were also grown with methanol, a typical carbon source used for biological denitrification. Growth (as determined by a significant increase in biomass as compared to controls) was not observed in methanol-free cultures after 10 days (data not shown). There was also no change in nitrate or nitrite levels in the methanol-free cultures, suggesting that the NC hydrolysate was not a suitable carbon source for the denitrifying culture. Nitrate, nitrite, and biomass (optical density, OD) for the culture with methanol are shown in Figure 28.

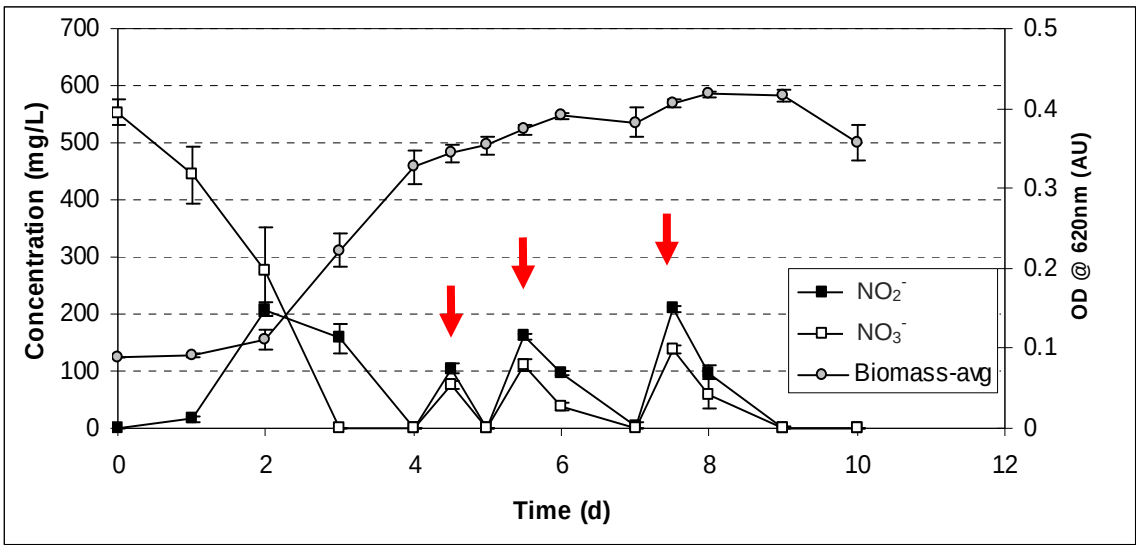


Figure 28: Biomass (OD_{620}), nitrate, and nitrite concentrations for denitrifying culture initially grown on KNO_3 as electron acceptor. Methanol was the carbon source. Arrows indicate when culture was spiked with neutralized NC hydrolysate to a final concentration of 100, 150, and 200mg/L nitrite, respectively. Error bars represent ± one standard deviation of triplicate experiments. Absence of error bars means error bar is smaller than data point symbol.

Kim et al. (1998) reported trace amounts of cyanide in the final reaction solution following hydrolysis of up to 70g/L NC. Because of the high nitrite concentration in the hydrolysate, the extent of dilution required to reach nitrite concentrations compatible with microbiological growth most likely also diluted any cyanide or similar problematic compounds that may have been produced during hydrolysis.

The reduction of both nitrate and nitrite spikes can be seen for the three successive hydrolysate spikes. The rates of denitrification for both nitrate and nitrite in the culture medium are given in Table 5.

Table 5. Denitrification rates (mg NO_x / h · mg biomass) with ± one standard deviation of triplicate experiments for WWTP culture spiked with NC hydrolysate.

Parameter	Initial Denitrification	1st Spike (4.5d)	2nd Spike (5.5d)	3rd Spike (7.5d)
dNO_3^-/dt	1.98 ± 0.144	0.694 ± 0.076	0.292 ± 0.025	0.349 ± 0.026
dNO_2^-/dt	1.12 ± 0.068	0.952 ± 0.087	0.436 ± 0.027	0.527 ± 0.017

From Table 5 it can be seen that the rates of nitrate and nitrite consumption was highest during the degradation of KNO_3 used to initiate growth of the culture. Over the 10 day course of these experiments, these rates of denitrification remained within the typical range (0.044-2.66 mg / d · mg biomass) for denitrifying bacteria as reported by Peyton et al. (2001). These results demonstrate that the diluted solution of NC hydrolysis using high initial NC and NaOH concentrations can be effectively denitrified by a WWTP culture.

Conclusions

The results of this study suggest that NC hydrolysis with NC concentrations more realistic for large scale operation, i.e. 100-200g/L is a very rapid process for transforming munitions-grade NC to non-energetic products. While using lower NC (10g/L) and lower hydroxide (40g/L) concentrations is effective for complete hydrolysis, long reaction times (hours to days) would be required to process the back-log of NC waste accumulated at NC manufacturing facilities. Alternatively, using high initial concentrations of NC (e.g. 100g/L) and high hydroxide concentrations has the advantage of being a very rapid process. Specifically with respect to RAAP, if a caustic stream from another process at the munitions facility was to be recycled for hydrolysis, the alkalinity of that caustic stream would be an important factor in designing the NC hydrolysis treatment. Following hydrolysis, the neutralized reaction solution was amenable to biological denitrification once diluted to non-inhibitory nitrite levels. The WWTP culture used for denitrification was able to degrade both nitrate and nitrite added at subsequently higher concentrations. The results of this study demonstrate that NC hydrolysis at high NC concentrations (100-200g/L) and high NaOH concentration (400g/L) is effective for completely and quickly degrading NC. The NC hydrolysate is suitable for biological denitrification by a typical WWTP culture.

References

Alleman, J.E.; Kim, B.J.; Quivey, D.M.; Equihua, L.O. Alkaline hydrolysis of munitions-grade nitrocellulose. *Wat. Sci. Tech.* **1994**, *30*, 63-72.

Atlas, Ronald M. Handbook *of Microbiological Media*, 3rd ed.; CRC Press LLC: Boca Raton, FL, 2004; p.1176.

Auer, N.; Hedger, J.N.; Evans, C.S. Degradation of nitrocellulose by fungi. *Biodegradation.* **2005**, *16*, 229-236.

Beccari, M.; Passino, R.; Ramadori, R.; Tandoi, V. Kinetics of dissimilatory nitrate and nitrite reduction in suspended growth culture. *J. Water Pollut. Control Fed*. **1983**, *55*, 58-64.

Brown, K.W. and Thomas, J.C. Degradation of explosive propellants by in-vessel composting. *BioCycle.* **1995**, *36*, 56-61.

Bunte, G.; Drause, H.H.; Hirth, T. Disposal of energetic materials by alkaline pressure hydrolysis and combined techniques. *Prop. Expl. Pyrotech.* **1997**, *22*, 160-164.

Christodoulatos, C.; Su, T.-L.; Koutsospyros, A. Kinetics of the alkaline hydrolysis of nitrocellulose. *Water Environ. Res.* **2001**, *73*, 185-191.

Freedman, D.L.; Caenepeel, B.M.; Kim, B.J. Biotransformation of nitrocellulose under methanogenic conditions. *Wat. Sci. Tech.* **1996**, *34*, 327-334.

Freedman, D.L.; Cashwell, J.M.; Kim B.J. Biotransformation of explosive-grade nitrocellulose under denitrifying and sulfidogenic conditions. *Waste Management.* **2002**, *22*, 283-292.

Kenyon, W.O. and Gray, H.LeB. The alkaline decomposition of cellulose nitrate. I. Quantitative studies. *Am. Chem. Soc. J.* **1936**, *8*, 1422-1427.

Kim, B.J.; Alleman, J.E.; Quivey, D.M. (1998). *Alkaline hydrolysis/biodegradation of nitrocellulose fines.* (USACERL Technical Report 98/65). Champaign, IL: US Army Corps of Engineers Construction Engineering Research Laboratories.

Peyton, B.M.; Mormile, M.R.; Petersen, J.N. Nitrate reduction with *Halomonas campisalis:* kinetics of denitrification at pH 9 and 12.5% NaCl. *Wat. Res*. **2001**, *35*, 4237-4242.

Pfeil, A. Microbial degradation of nitrocellulose in a composting environment. *Prop. Expl. Pyrotech.* **1999**, *24*, 156-158.

Wendt, T.M. and Kaplan, A.M. A chemical-biological treatment process for cellulose nitrate disposal. *J. Water Pollut. Control Fed*. **1976**, *48*, 660-668.

SUMMARY AND FUTURE WORK

For the explosive compounds studied, TNT and NC, the initial focus of this research was to develop and optimize a single-vessel treatment incorporating both chemical and biological components as a safe alternative to incineration as the primary disposal method. Research focus was shifted to separate chemical (alkaline hydrolysis) and biological process steps due to lack of a suitable thermoalkaliphile in the case of TNT, or due to lack of adequate hydrolysis at biologically relevant pH levels in the case of NC. In each instance, an advantage of separating the biological component lies in the fact that no specialized microorganisms are required. An undefined mixed culture enriched from a wastewater treatment facility was used aerobically for growth on TNT hydrolysate and as a denitrifying culture for NC hydrolysate. The hydrolysis-only step with TNT was beneficial in that lower hydroxide concentrations could be used; meaning that less neutralization and no dilution were required to prepare the hydrolysate for later biological treatment. The hydrolysis-only step with NC was beneficial in that large quantities of NC could be treated under high hydroxide conditions in a very short reaction time.

TNT

Future work with respect to TNT degradation should focus on both abiotic and biological aspects. Since the alkaline hydrolysis of TNT at limited (i.e. not excess) hydroxide concentrations has not been covered in the peer-reviewed literature prior to this study, more information should be acquired to further characterize this process.

Additional radiolabeled, ^{14}C as well as ^{15}N, hydrolysis experiments should be performed to determine the fate of each. To our knowledge, the generalized kinetic model for TNT hydrolysis reaction rate is the first that has been constructed. Based on results of previous studies as well as the current study, it was developed based on pseudo-first-order kinetics with respect to TNT, and assumed the hydroxide concentration to remain constant throughout the experiment. Further experiments designed to obtain TNT concentration as well as hydroxide concentration over time should be carried out with the intent of forming a more detailed kinetic model for predicting TNT degradation rates. Rates of TNT disappearance at various pH values (other than just 9, 10, and 11) and temperatures (other than 60 and 80°C) will be necessary to calculate a more accurate activation energy of the TNT hydrolysis reaction. Also, changing the initial concentration of TNT will contribute to rate information. Ultimately, a mathematical model for prediction of TNT degradation rates which accounts for changes in both TNT *and* hydroxide concentration over time may be developed.

For the biological component of TNT degradation, samples from YNP alkaline hot springs should be re-enriched for TNB-degraders. Carbon sources other than sugars/carbohydrates should be used to avoid the abiotic interaction given in a patent by Kornel (2003). Also, the isolated *Anoxybacillus kualawohkensis* should be revisited for TNB degradation using yeast extract and other carbon sources that were not originally tested. Growth experiments using radiolabeled TNT should be done to determine to what extent TNT-associated carbon or nitrogen is incorporated into biomass.

NC

Future work for NC degradation primarily involves more hydrolysis experiments at varying NC and hydroxide concentrations. Realistic initial NC concentrations (at least 100g/L) and corresponding high hydroxide concentrations (e.g. 400g/L NaOH) should be tested and given a detailed kinetic analysis. Experimenting across a range of temperatures will be necessary to construct a new mathematical model to calculate NC degradation rates relevant to these high NC concentrations. This additional data would show the experimental conditions (NC and OH^- concentrations) under which NC degradation rates would be correctly predicted for both high and low NC concentration models. If feasible, carbon and nitrogen radiolabeled hydrolysis experiments would be very useful in determining the distribution of both among hydrolysis products.

With respect to biological NC degradation, waste NC from RAAP holding ponds should be enriched for denitrifying cultures. It is possible that some microorganisms have become adapted to growth in NC holding ponds, and may be able to use NC as a source of carbon and nitrogen. These cultures may happen to be better suited to NC hydrolysate denitrification than typical WWTP enrichments.

The experimental outlook for TNT and NC should include further characterization of the reacted hydrolysate solution with respect to carbon and nitrogen fate. This information would contribute to the optimization of the biological step for remediation of the individual hydrolysates to environmentally friendly products, which is the ultimate goal of explosives disposal.

APPENDICES

APPENDIX A

CONSENSUS SEQUENCE FOR *Anoxybacillus kualawohkensis*

Sequence:

catgcagtcgagcggacgattcaaaagcttgcttttgaatcgttagcggcgcccttgagagtaacacgtgggcaacctgccctgt
agacggggatatcaccgagaaatcggtgctaataccggataacacgaaagaccgcatggtttttcgttgaaaggcggcgcaag
ctgtcgctacaggatgggcccgcggcgcattagctagttggtgaggtaacggctcaccaaggcgacgatgcgtagccgacctg
agagggtgatcggccacactgggactgagacacggcccagactcctacgggaggcagcagtagggaatcttccgcaatgga
cgaaagtctgacggagcaacgccgcgtgagcgaagaaggccttcgggtcgtaaagctctgttgttagggaagaacaagtacc
gcagtcactggcggtaccttgacggtacctaacgagaaagccacggctaactacgtgccagcagccgcggtaatacgtaggtg
gcaagcgttgtccggaattattgggcgtaaagcgcgcgcaggcggttccttaagtctgatgtgaaagcccacggctcaaccgtg
gagggtcattggaaactgggggacttgagtgcagaagaggagagcggaattccacgtgtagcggtgaaatgcgtagagatgt
ggaggaacaccagtggcgaaggcggctctctggtctgtaactgacgctgaggcgcgaaagcgtggggagcaaacaggatta
gataccctggtagtccacgccgtaaacgatgagtgctaagtgttagagggtatccacccttagtgctgtagctaacgcattaagc
actccgcctggggagtacgctcgcaagagtgaaactcaaaggaattgacgggggcccgcacaagcggtggagcatgtggttt
aattcgaagcaacgcgaagaaccttaccaggtcttgacatcccctgacaacccgagagatcgggcgttcccccttcgggggga
cagggtgacaggtggtgcatggttgtcgtcagctcgtgtcgtgagatgttgggttaagtcccgcaacgagcgcaaccctcgacc
ttagttgccagcattcagttgggcactctaaggtgactgccggctaaaagtcggaggaaggtggggatgacgtcaaatcatcatg
ccccttatgacctgggctacacacgtgctacaatgggcggtacaaagggtcgcgaacccgcgaggggagccaatcccaaaa
agccgctctcagttcggattgcaggctgcaactcgcctgcatgaagccggaatcgctagtaatcgcggatcagcatgccgcggt
gaatacgttcccgggccttgtacacaccgcccgtcacaacgcgagagtttgcaacacccgaagtcggtgaggtaacccttacg
ggagccagccgcc

1426 base pairs

BLAST RESULTS

Accession	Description	Max score	Total score	Query coverage	Max ident
DQ401072.1	*Anoxybacillus kualawohkensis* strain KW 12 16S ribosomal RNA gene, partial sequence	2573	2573	100%	99.23%
EU621360.1	*Anoxybacillus* sp. DR02 16S ribosomal RNA gene, partial sequence	2562	2562	100%	99.09%
EU621362.1	*Anoxybacillus* sp. DR04 16S ribosomal RNA gene, partial sequence	2556	2556	100%	99.02%
EU621359.1	*Anoxybacillus* sp. DR01 16S ribosomal RNA gene, partial sequence	2556	2556	100%	99.02%
AM902721.1	*Anoxybacillus tunisiense* partial 16S rRNA gene, type strain A06	2551	2551	99%	99.09%

APPENDIX B

RAW DATA FOR TNT/TNB EXPERIMENTS

TNT (Absorbance @ 230nm)

Time (h)	TNT (AU)	CFC (AU)	YFC (AU)	TFC (AU)
0	364.1	383.1	393.5	0
8	319.2	306.3	351.5	0
16	215.2	224.2	293.5	0
24	150.3	157.7	264.9	0
32	46	89.3	229.9	0
40	0	65.1	204.6	0
48	0	49.6	192.2	0
56	0	0	133.0	0
64	0	0	107.4	0
72	0	0	109.5	0
80	0	0	93.2	0
88	0	0	74.8	0
104	0	0	56.7	0

TNT (mg/L)

Time (h)	Sp4 (mg/L)	CFC (mg/L)	YFC (mg/L)	TFC (mg/L)
0	13.6	14.3	14.7	0
8	11.9	11.4	13.1	0
16	8.05	8.39	10.9	0
24	5.62	5.90	9.92	0
32	1.72	3.34	8.61	0
40	0	2.43	7.66	0
48	0	1.85	7.19	0
56	0	0	4.98	0
64	0	0	4.02	0
72	0	0	4.10	0
80	0	0	3.49	0
88	0	0	2.80	0
104	0	0	2.12	0

TNB (Absorbance @ 230nm)

Time (h)	Sp4 (AU)	CFC (AU)	YFC (AU)	TFC (AU)
0	0	0	0	0
8	32.8	26.3	0	0
16	60.6	56.9	27.9	0
24	90.6	75.5	37.7	0
32	91.8	85.1	46.4	0
40	102.7	90.3	59.7	0
48	107.1	93.4	69.7	0
56	105	92.3	73.9	0
64	84.3	94.3	81.9	0
72	69.6	94.6	87.9	0
80	0	85.8	88.3	0
88	0	84.7	91.3	0
104	0	88.6	94.4	0

TNB (mg/L)

Time (h)	Sp4 (mg/L)	CFC (mg/L)	YFC (mg/L)	TFC (mg/L)
0	0	0	0	0
8	1.49	1.19	0	0
16	2.75	2.58	1.26	0
24	4.11	3.43	1.71	0
32	4.17	3.86	2.10	0
40	4.66	4.10	2.71	0
48	4.86	4.24	3.16	0
56	4.77	4.19	3.35	0
64	3.83	4.28	3.72	0
72	3.16	4.30	3.99	0
80	0	3.90	4.01	0
88	0	3.85	4.15	0
104	0	4.02	4.29	0

Protein Standards (Absorbance @ 595nm)

Protein Standard (mg/L) for CFC and YFC	#1	#2	#3	Average	Corrected Avg
0	0.304	0.302	0.311	0.305	0
2.5	0.350	0.360	0.356	0.355	0.049
5	0.417	0.413	0.419	0.416	0.110
10	0.519	0.523	0.517	0.519	0.214
15	0.599	0.595	0.610	0.601	0.295
20	0.703	0.706	0.715	0.708	0.402

Protein Standards (Absorbance @ 595nm)

Protein Standard (mg/L) for Sp4 and TFC	#1	#2	#3	Average	Corrected Avg
0	0.305	0.300	0.307	0.304	0
2.5	0.352	0.349	0.355	0.352	0.048
5	0.396	0.403	0.401	0.400	0.096
10	0.474	0.508	0.518	0.500	0.196
15	0.571	0.549	0.598	0.572	0.268
20	0.658	0.667	0.648	0.657	0.353

Protein (Absorbance @ 595nm)

Time (h)	Sp4 (AU)	CFC (AU)	TFC (AU)	YFC (AU)
0	0.380	0.317	0.377	0.364
8	0.369	0.320	0.947	0.362
16	0.392	0.322	0.943	0.374
24	0.372	0.320	0.934	0.345
32	0.401	0.331	1.007	0.368
40	0.386	0.330	0.907	0.375
48	0.377	0.335	0.954	0.369
56	0.381	0.336	0.933	0.368
64	0.422	0.327	0.905	0.358
72	0.494	0.332	0.867	0.367
80	0.699	0.332	0.942	0.370
88	0.894	0.343	0.883	0.362
104	0.933	0.326	0.939	0.379

Corrected Protein (Absorbance @ 595nm)

Time (h)	Sp4 Corrected	CFC Corrected	TFC Corrected	YFC Corrected
0	0.076	0.011	0.073	0.058
8	0.065	0.014	0.643	0.056
16	0.088	0.016	0.639	0.068
24	0.068	0.014	0.630	0.039
32	0.097	0.025	0.703	0.062
40	0.082	0.024	0.603	0.069
48	0.073	0.029	0.650	0.063
56	0.077	0.030	0.629	0.062
64	0.118	0.021	0.601	0.052
72	0.190	0.026	0.563	0.061
80	0.395	0.026	0.638	0.064
88	0.590	0.037	0.579	0.056
104	0.629	0.020	0.635	0.073

Protein (mg/L)

Time (h)	Sp4 (mg/L)	CFC (mg/L)	TFC (mg/L)	YFC (mg/L)
0	4.44	0.56	4.26	2.88
8	3.80	0.70	37.60	2.78
16	5.14	0.80	37.36	3.38
24	3.97	0.70	36.84	1.94
32	5.67	1.25	41.11	3.08
40	4.79	1.20	35.26	3.43
48	4.26	1.45	38.01	3.13
56	4.50	1.50	36.78	3.08
64	6.90	1.05	35.14	2.59
72	11.11	1.30	32.92	3.03
80	23.09	1.30	37.30	3.18
88	34.50	1.84	33.85	2.78
104	36.78	1.00	37.13	3.63

APPENDIX C

RAW DATA FOR ^{14}C-TNT EXPERIMENTS

Start point (0 days)			
Time (min)	Fraction #	Count (dpm)	% of total count
0.5	1	0	0
1	2	0	0
1.5	3	0	0
2	4	0	0
2.5	5	0	0
3	6	0	0
3.5	7	0	0
4	8	0	0
4.5	9	0	0
5	10	0	0
5.5	11	0	0
6	12	0	0
6.5	13	0	0
7	14	0	0
7.5	15	0	0
8	16	0	0
8.5	17	0	0
9	18	0	0
9.5	19	0	0
10	20	0	0
10.5	21	0	0
11	22	0	0
11.5	23	0	0
12	24	0	0
12.5	25	0	0
13	26	0	0
13.5	27	0	0
14	28	0	0
14.5	29	505.2	29.71
15	30	1216.7	71.57
15.5	31	0	0
16	32	0	0
16.5	33	0	0
17	34	0	0
17.5	35	0	0
18	36	0	0
18.5	37	0	0
19	38	0	0
19.5	39	0	0

Start point (0 days)			
Time (min)	Fraction #	Count (dpm)	% of total count
20	40	0	0
20.5	41	0	0
21	42	0	0
21.5	43	0	0
22	44	0	0
22.5	45	0	0
23	46	0	0
23.5	47	0	0
24	48	0	0
24.5	49	0	0
25	50	0	0
25.5	51	0	0
26	52	0	0
26.5	53	0	0
27	54	0	0
27.5	55	0	0
28	56	0	0
28.5	57	0	0
29	58	0	0
29.5	59	0	0
30	60	0	0
30.5	61	0	0
31	62	0	0
31.5	63	0	0
32	64	0	0
32.5	65	0	0
33	66	0	0
33.5	67	0	0
34	68	0	0
34.5	69	0	0
35	70	0	0
Totals	x	1721.9	101.28

Time point (1 day)			
Time (min)	Fraction	Count (dpm)	% of total count
0.5	1	0	0
1	2	5.7	0.33
1.5	3	0	0
2	4	5.0	0.29
2.5	5	0.1	0.00
3	6	0.8	0.05
3.5	7	0	0
4	8	40.5	2.37
4.5	9	27.7	1.62

Time point (1 day)			
Time (min)	Fraction	Count (dpm)	% of total count
5	10	29.4	1.72
5.5	11	23.7	1.39
6	12	7.2	0.42
6.5	13	0	0
7	14	25.7	1.50
7.5	15	53.6	3.14
8	16	50.6	2.96
8.5	17	42.7	2.50
9	18	42.5	2.49
9.5	19	0	0
10	20	49.4	2.89
10.5	21	59.0	3.45
11	22	67.4	3.94
11.5	23	78.0	4.56
12	24	54.0	3.16
12.5	25	0	0
13	26	36.1	2.11
13.5	27	33.3	1.95
14	28	32.1	1.88
14.5	29	31.1	1.82
15	30	175.1	10.25
15.5	31	0	0
16	32	38.0	2.23
16.5	33	59.0	3.45
17	34	52.1	3.05
17.5	35	43.4	2.54
18	36	33.1	1.94
18.5	37	0	0
19	38	11.2	0.65
19.5	39	13.6	0.80
20	40	19.3	1.13
20.5	41	7.7	0.45
21	42	12.9	0.75
21.5	43	0	0
22	44	20.3	1.19
22.5	45	16.6	0.97
23	46	58.2	3.41
23.5	47	0	0
24	48	78.2	4.58
24.5	49	79.4	4.65
25	50	46.2	2.70
25.5	51	27.2	1.59
26	52	22.0	1.29
26.5	53	38.5	2.25

Time point (1 day)			
Time (min)	Time (min)	Time (min)	Time (min)
27	54	11.2	0.65
27.5	55	0	0
28	56	11.2	0.65
28.5	57	9.2	0.54
29	58	2.5	0.15
29.5	59	8.0	0.46
30	60	3.3	0.19
30.5	61	0	0
31	62	0	0
31.5	63	11.7	0.68
32	64	0	0
32.5	65	0	0
33	66	0	0
33.5	67	0	0
34	68	0	0
34.5	69	0	0
35	70	0	0
Totals	x	1706.9	100

End point (9 days)			
Time (min)	Fraction #	Count (dpm)	% of total count
0.5	1	14.8	0.86
1	2	20	1.16
1.5	3	16.2	0.94
2	4	14.6	0.85
2.5	5	14.6	0.85
3	6	15.8	0.91
3.5	7	15	0.87
4	8	59	3.43
4.5	9	60.4	3.51
5	10	57.8	3.36
5.5	11	49.2	2.86
6	12	38.8	2.25
6.5	13	33.4	1.94
7	14	37.2	2.16
7.5	15	59	3.43
8	16	50.4	2.93
8.5	17	55.8	3.24
9	18	48.2	2.80
9.5	19	40	2.32
10	20	38.4	2.23
10.5	21	53.2	3.09
11	22	48.4	2.81

End point (9 days)			
Time (min)	Fraction #	Count (dpm)	% of total count
11.5	23	45.8	2.66
12	24	38	2.21
12.5	25	32.8	1.90
13	26	29.2	1.70
13.5	27	31	1.80
14	28	36	2.09
14.5	29	30	1.74
15	30	26.2	1.52
15.5	31	29.2	1.70
16	32	26.6	1.54
16.5	33	24.4	1.42
17	34	16.8	0.97
17.5	35	19.8	1.15
18	36	29.6	1.72
18.5	37	18.2	1.05
19	38	20.4	1.18
19.5	39	16.6	0.96
20	40	13.4	0.78
20.5	41	12	0.69
21	42	21.8	1.26
21.5	43	18	1.04
22	44	19.6	1.14
22.5	45	15.8	0.91
23	46	20.8	1.21
23.5	47	35.2	2.04
24	48	23.2	1.35
24.5	49	26.6	1.54
25	50	28.4	1.65
25.5	51	25.8	1.50
26	52	17	0.98
26.5	53	14.8	0.86
27	54	11.8	0.68
27.5	55	10.4	0.60
28	56	12.6	0.73
28.5	57	7.4	0.43
29	58	16.4	0.95
29.5	59	7.8	0.45
30	60	0.2	0.01
30.5	61	7.8	0.45
31	62	2.2	0.12
31.5	63	3.8	0.22
32	64	4	0.23
32.5	65	6.4	0.37
33	66	0.4	0.02

End point (9 days)			
Time (min)	Fraction #	Count (dpm)	% of total count
33.5	67	1.4	0.08
34	68	3.2	0.18
34.5	69	1.8	0.10
35	70	16.6	0.96
Totals	x	1717.4	100

APPENDIX D

RAW DATA FOR BIOLOGICAL DEGRADATION OF TNT HYDROLYSATE

Optical Density (OD, absorbance @ 620nm)

Time (d)	a	b	c	w/nutrients, no NH_4Cl, air headspace (avg)	stdev
0	0.034	0.044	0.026	0.034	0.009
1	0.072	0.056	0.035	0.054	0.018
2	0.105	0.123	0.118	0.113	0.009
3	0.117	0.132	0.128	0.127	0.007
4	0.198	0.202	0.21	0.208	0.006
5	0.188	0.197	0.201	0.191	0.006
6	0.165	0.159	0.187	0.170	0.014
Time (d)	a	b	c	w/nutrients and NH_4Cl, air headspace (avg)	stdev
0	0.036	0.033	0.041	0.036	0.004
1	0.087	0.069	0.067	0.074	0.011
2	0.151	0.162	0.149	0.154	0.007
3	0.199	0.187	0.176	0.187	0.011
4	0.223	0.217	0.218	0.219	0.003
5	0.21	0.202	0.214	0.208	0.006
6	0.205	0.198	0.239	0.214	0.021
Time (d)	a	b	c	w/nutrients, no NH_4Cl, N_2 headspace (avg)	stdev
0	0.028	0.043	0.033	0.034	0.007
1	0.045	0.041	0.029	0.038	0.008
2	0.038	0.037	0.031	0.035	0.003
3	0.034	0.045	0.042	0.040	0.005
4	0.044	0.036	0.033	0.037	0.005
5	0.035	0.032	0.04	0.035	0.004
6	0.041	0.029	0.036	0.035	0.006
Time (d)	a	b	c	w/nutrients, and NH_4Cl, N_2 headspace (avg)	stdev
0	0.033	0.029	0.043	0.035	0.007
1	0.041	0.037	0.038	0.038	0.002
2	0.055	0.032	0.036	0.041	0.012
3	0.034	0.026	0.028	0.029	0.004
4	0.038	0.041	0.033	0.037	0.004
5	0.029	0.041	0.033	0.034	0.006
6	0.028	0.027	0.031	0.028	0.002

Conversion data (OD to Biomass)

OD (620nm)	Biomass (mg/L)
0.050	4.51
0.100	9.24
0.150	14.62

APPENDIX E

RAW DATA FOR TNT HYDROLYSIS EXPERIMENTS

pH 9, 60°C

pH 9, 60°C			peak area			mg/L		
Time (h)	TNT-a	TNT-b	TNT-b	TNT-a	TNT-b	TNT-b	avg TNT	TNT-stdev
0	1790.1	1707	1688.8	84.0	80.1	79.3	81.1	2.5
48	1486.1	1500.8	1496.7	69.7	70.4	70.2	70.1	0.3
96	772.2	793.3	810	36.2	37.2	38.0	37.1	0.8
144	607.8	615.3	595.2	28.5	28.8	27.9	28.4	0.4
192	484.7	444.3	454.2	22.7	20.8	21.3	21.6	0.9
240	337	376.1	322.5	15.8	17.6	15.1	16.2	1.3
288	212.9	250	209.9	9.9	11.7	9.8	10.5	1.0
360	104.8	124.8	95.4	4.9	5.8	4.4	5.0	0.7
408	0	0	0	0	0	0	0	0
456	0	0	0	0	0	0	0	0

pH 9, 60°C			peak area			mg/L		
Time (h)	NO_2^- (a)	NO_2^- (b)	NO_2^- (c)	NO_2^- (a)	NO_2^- (b)	NO_2^- (c)	avg NO_2^-	NO_2^- stdev
0	0	0	0	0	0	0	0	0
48	368532	365937	355564	1.42	1.41	1.37	1.40	0.02
96	794161	758183	760398	3.06	2.96	2.98	2.98	0.02
144	1095215	1109910	1215295	4.22	4.30	4.41	4.41	0.01
192	1271695	1345866	1543554	4.90	5.21	5.98	5.36	0.01
240	1492296	1753968	1790903	5.75	6.80	6.94	6.49	0.01
288	1731063	2104901	1837812	6.67	8.16	7.12	7.32	0.01
360	1956854	2277105	1444335	7.54	8.82	5.59	7.32	0.01
408	2130739	2335098	2007858	8.21	9.05	7.78	8.34	0.01
456	2128144	2536170	2389194	8.20	9.83	9.26	9.10	0.01

pH 9, 60°C

pH 9, 60°C			peak area			mg/L		
Time (h)	NO_3^- (a)	NO_3^- (b)	NO_3^- (c)	NO_3^- (a)	NO_3^- (b)	NO_3^- (c)	avg NO_3^-	NO_3^- stdev
0	0	0	0	0	0	0	0	0
48	0	0	0	0	0	0	0	0
96	0	0	9623	0	0	0.04	0.01	0.02
144	43715	13805	19581	0.19	0.06	0.09	0.12	0.06
192	43720	25465	24998	0.19	0.12	0.12	0.14	0.03
240	43717	40026	39124	0.19	0.20	0.19	0.19	0.00
288	43722	34716	46798	0.19	0.17	0.23	0.20	0.03
360	43721	39109	29920	0.19	0.19	0.14	0.18	0.02
408	43721	39809	38981	0.19	0.19	0.19	0.19	0.00
456	43713	39798	39241	0.19	0.19	0.19	0.19	0.00

pH 9, 60°C

Std (mg/L)	Peak area NO_2^-	Peak area NO_3^-
0	0	0
5	1215180	918362
10	2461266	1866856
25	6510260	5056886

pH 9, 60°C

TNT Standard Concentration (mg/L)	TNT Peak Area at 13.1min (AU)
0	0
20	386.6
40	909
60	1288.1
80	1677

pH 9, 80°C

pH 9, 80°C			peak area			mg/L		
Time (h)	TNT-a	TNT-b	TNT-b	TNT-a	TNT-b	TNT-b	TNT-avg	TNT-stdev
0	1705.6	1717	1712.9	80.0	80.6	80.4	80.3	0.2
12	1437.6	1426.8	1444	67.5	67.0	67.8	67.4	0.4
24	1215.7	1209.9	1213.8	57.0	56.8	56.9	56.9	0.1
37	958.3	999.7	949.3	45.0	46.9	44.5	45.5	1.2
48	842.2	824.3	830.5	39.5	38.7	38.9	39.0	0.4
60	660.1	657.9	612.3	30.9	30.8	28.7	30.2	1.2
72	400.4	202.6	383.3	18.8	9.5	17.9	15.4	5.1
84	170.4	134.5	204.8	8.0	6.3	9.6	7.9	1.6
96	123	54.4	45.2	5.7	2.5	2.1	3.4	1.9
108	0	0	0	0	0	0	0	0
120	0	0	0	0	0	0	0	0

pH 9, 80°C			peak area			mg/L		
Time (h)	NO_2^- (a)	NO_2^- (b)	NO_2^- (c)	NO_2^- (a)	NO_2^- (b)	NO_2^- (c)	avg NO_2^-	NO_2^- stdev
0	0	0	0	0	0	0	0	0
12	478015	746737	601724	1.85	2.89	2.33	2.36	0.52
24	1080055	1162165	1422170	4.18	4.50	5.51	4.73	0.69
37	1506393	1813197	1558233	5.83	7.02	6.04	6.30	0.64
48	1488306	1989966	1880953	5.76	7.71	7.29	6.92	1.02
60	1870718	2237425	2151214	7.24	8.67	8.34	8.08	0.74
72	2002495	2532566	2377401	7.75	9.81	9.21	8.93	1.06
84	2268633	2461410	2420998	8.78	9.54	9.38	9.23	0.40
96	2214372	2533741	2593186	8.57	9.82	10.053	9.48	0.79
108	2250546	2605625	2677333	8.71	10.10	10.38	9.73	0.89
120	2392659	2727370	2733968	9.26	10.57	10.59	10.14	0.76

pH 9, 80°C

pH 9, 80°C			peak area			mg/L		
Time (h)	NO_3^- (a)	NO_3^- (b)	NO_3^- (c)	NO_3^- (a)	NO_3^- (b)	NO_3^- (c)	avg NO_3^-	NO_3^- stdev
0	0	0	0	0	0	0	0	0
12	0	12219	7259	0	0.06	0.03	0.03	0.03
24	38693	22462	25718	0.19	0.11	0.12	0.14	0.04
37	38695	39272	32095	0.19	0.19	0.16	0.18	0.02
48	38694	107440	41874	0.19	0.53	0.20	0.31	0.19
60	38693	38027	44638	0.19	0.19	0.22	0.20	0.02
72	38692	35526	37525	0.19	0.17	0.18	0.18	0.01
84	40730	49946	44042	0.20	0.25	0.22	0.22	0.02
96	38694	39773	42590	0.19	0.19	0.21	0.20	0.01
108	40729	42547	43011	0.20	0.21	0.21	0.20	0.01
120	40733	46732	46681	0.20	0.23	0.23	0.22	0.01

pH 9, 80°C

Std (mg/L)	Peak area NO_2^-	Peak area NO_3^-
0	0	0
5	1215180	918362
10	2461266	1866856
25	6510260	5056886

pH 9, 80°C

TNT Standard Concentration (mg/L)	Peak Area at 13.1min (AU)
0	0
20	386.6
40	909.0
60	1288.1
80	1677.0

pH 10, 60°C

pH 10, 60°C			peak area			mg/L		
Time (h)	TNT-a	TNT-b	TNT-b	TNT-a	TNT-b	TNT-b	avg TNT	TNT-stdev
0	1753.1	1748.3	1863.5	82.3	82.0	87.5	83.9	3.0
12	1192.5	1106.7	1185.5	55.9	51.9	55.6	54.5	2.2
24	782.2	755	745.3	36.7	35.4	34.9	35.7	0.8
37	425.9	400.1	413.4	20.0	18.7	19.4	19.4	0.6
48	233.7	184.9	191.8	10.9	8.6	9.0	9.5	1.2
60	151.8	159.7	168.8	7.1	7.4	7.9	7.5	0.3
72	137.9	109	127.8	6.4	5.1	6.0	5.8	0.6
84	85.2	88.4	98.6	4.0	4.1	4.6	4.2	0.3
96	0	76.4	0	0	3.5	0	1.1	2.0
108	0	0	0	0	0	0	0	0
120	0	0	0	0	0	0	0	0

pH 10, 60°C			peak area			mg/L		
Time (h)	NO_2^- (a)	NO_2^- (b)	NO_2^- (c)	NO_2^- (a)	NO_2^- (b)	NO_2^- (c)	avg NO_2^-	NO_2^- stdev
0	0	0	0	0	0	0	0	0
12	1228300	2336078	2194182	4.79	9.11	8.55	7.48	2.34
24	1848860	2752361	2903383	7.21	10.73	11.32	9.75	2.22
37	2102726	2899897	2876237	8.20	11.31	11.21	10.24	1.76
48	2179655	2731015	2880470	8.50	10.65	11.23	10.12	1.44
60	2261713	2940851	2888673	8.82	11.47	11.26	10.51	1.47
72	2369413	2988752	2903190	9.24	11.65	11.32	10.74	1.31
84	2379671	2774143	2975877	9.28	10.82	11.60	10.57	1.18
96	2433521	2996574	2989941	9.49	11.68	11.66	10.94	1.25
108	2397621	3033110	3154144	9.35	11.83	12.30	11.16	1.58
120	2382235	3134024	3150458	9.29	12.22	12.28	11.26	1.71

pH 10, 60°C

pH 10, 60°C			peak area			mg/L		
Time (h)	NO_3^- (a)	NO_3^- (b)	NO_3^- (c)	NO_3^- (a)	NO_3^- (b)	NO_3^- (c)	avg NO_3^-	NO_3^- stdev
0	0	0	0	0	0	0	0	0
12	37845	25558	24199	0.1925	0.13	0.12	0.14	0.03
24	38533	30855	31372	0.196	0.15	0.16	0.17	0.02
37	38534	33664	28169	0.196	0.17	0.14	0.17	0.02
48	38926	35729	35294	0.198	0.18	0.18	0.18	0.00
60	38929	38273	38345	0.198	0.19	0.19	0.19	0.00
72	38828	39134	34092	0.1975	0.20	0.17	0.19	0.01
84	39614	38099	38045	0.2015	0.19	0.19	0.19	0.00
96	39320	39504	40057	0.2	0.20	0.20	0.20	0.00
108	39123	41080	42083	0.199	0.21	0.21	0.20	0.01
120	39611	31196	44034	0.2015	0.15	0.22	0.19	0.03

pH 10, 60°C

std (mg/L)	Peak area NO_2^-	Peak area NO_3^-
0	0	0
5	1183367	885059
10	2440535	1838865
25	6477953	4947764

pH 10, 60°C

TNT Standard Concentration (mg/L)	Peak Area at 13.1min (AU)
0	0
20	386.6
40	909
60	1288.1
80	1677

pH 10, 80°C

pH 10, 80°C			peak area			mg/L		
Time (h)	TNT-a	TNT-b	TNT-c	TNT-a	TNT-b	TNT-b	avg TNT	TNT-stdev
0	1689.9	1717.6	1720.2	79.3	80.6	80.7	80.2	0.7
1	1597.1	1567.2	1543.7	74.9	73.5	72.4	73.6	1.2
3	1301.1	1277.4	1289	61.0	59.9	60.5	60.5	0.5
5	1043.5	1055.5	1097	49.0	49.5	51.5	50.0	1.3
6.5	822.6	798.1	830.5	38.6	37.4	38.9	38.3	0.7
7.5	553.7	576.8	550.7	26.0	27.0	25.8	26.3	0.6
9.5	122.3	120.8	151.6	5.7	5.6	7.1	6.1	0.8
11	34.5	56.7	89.9	1.6	2.6	4.2	2.8	1.3
14	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0

pH 10, 80°C			peak area			mg/L		
Time (h)	NO_2^- (a)	NO_2^- (b)	NO_2^- (c)	NO_2^- (a)	NO_2^- (b)	NO_2^- (c)	avg NO_2^-	NO_2^- stdev
0	0	0	0	0	0	0	0	0
1	371814	784656	515033	1.45	3.06	2.00	2.17	0.81
3	1003898	2362097	2236784	3.91	9.21	8.72	7.28	2.92
5	1516745	2679256	2854911	5.91	10.45	11.13	9.16	2.83
6.5	1760347	2879732	2863653	6.86	11.23	11.17	9.75	2.50
7.5	1884712	2849439	2860871	7.35	11.11	11.15	9.87	2.18
9.5	1938561	2833388	2869398	7.56	11.05	11.19	9.93	2.05
11	2007796	2865506	2844304	7.83	11.17	11.09	10.03	1.90
14	2130879	2899269	2799281	8.31	11.30	10.91	10.17	1.63
24	2424484	3050202	3114501	9.45	11.89	12.14	11.16	1.48

pH 10, 80°C

pH 10, 80°C			peak area			mg/L		
Time (h)	NO_3^- (a)	NO_3^- (b)	NO_3^- (c)	NO_3^- (a)	NO_3^- (b)	NO_3^- (c)	avg NO_3^-	NO_3^- stdev
0	0	0	0	0	0	0	0	0
1	39257	0	14394	0.189	0	0.07	0.08	0.09
3	39880	22848	27185	0.192	0.11	0.13	0.14	0.03
5	40087	30031	34905	0.193	0.15	0.17	0.17	0.02
6.5	40295	34971	31165	0.193	0.17	0.15	0.17	0.01
7.5	40503	32003	30055	0.195	0.16	0.15	0.17	0.02
9.5	40510	33064	33716	0.195	0.16	0.17	0.17	0.01
11	40512	31785	35269	0.195	0.16	0.18	0.17	0.01
14	40502	35090	33882	0.195	0.17	0.17	0.18	0.01
24	41957	34465	33980	0.202	0.17	0.17	0.18	0.01

pH 10, 80°C

Std (mg/L)	Peak area NO_2^-	Peak area NO_3^-
0	0	0
5	1183367	885059
10	2440535	1838865
25	6477953	4947764

pH 10, 80°C

TNT Standard Concentration (mg/L)	Peak Area at 13.1min (AU)
0	0
20	386.6
40	909
60	1288.1
80	1677

pH 11, 60°C

pH 11, 60°C			peak area			mg/L		
Time (h)	TNT-a	TNT-b	TNT-b	TNT-a	TNT-b	TNT-b	avg TNT	TNT-stdev
0	1696.3	1687	1711.3	79.6	79.2	80.3	79.7	0.5
0.25	886	859.4	890.1	41.6	40.3	41.7	41.2	0.7
0.5	544.8	570.9	575	25.5	26.8	27.0	26.4	0.7
0.75	389.8	323.1	379.9	18.3	15.1	17.8	17.1	1.6
1	0	233.9	262	0	10.9	12.3	7.7	6.7
1.25	0	155.9	187.8	0	7.3	8.8	5.3	4.7
1.5	0	0	79.3	0	0	3.7	1.2	2.1
1.75	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0

pH 11, 60°C			peak area			mg/L		
Time (h)	NO_2^- (a)	NO_2^- (b)	NO_2^- (c)	NO_2^- (a)	NO_2^- (b)	NO_2^- (c)	avg NO_2^-	NO_2^- stdev
0	0	0	0	0	0	0	0	0
0.25	2663087	2001164	2804154	10.38	7.80	10.93	9.70	1.67
0.5	3484077	3548797	3597231	13.58	13.84	14.03	13.81	0.22
0.75	3391716	3458345	3423746	13.22	13.49	13.35	13.35	0.13
1	4346117	3640786	3512086	16.94	14.20	13.69	14.94	1.74
1.25	2919646	3677482	3808989	11.38	14.34	14.85	13.52	1.87
1.5	3209559	3848231	3930439	12.51	15.01	15.33	14.28	1.54
1.75	3532824	3786305	3847094	13.77	14.76	15.00	14.51	0.65
2	3817605	3741623	3973838	14.88	14.59	15.50	14.99	0.46

pH 11, 60°C

pH 11, 60°C			peak area			mg/L		
Time (h)	NO_3^- (a)	NO_3^- (b)	NO_3^- (c)	NO_3^- (a)	NO_3^- (b)	NO_3^- (c)	avg NO_3^-	NO_3^- stdev
0	0	0	0	0	0	0	0	0
0.25	0	28647	43303	0	0.14	0.22	0.12	0.11
0.5	39696	53148	47874	0.194	0.27	0.24	0.23	0.03
0.75	39901	36081	39610	0.195	0.18	0.20	0.19	0.01
1	39701	42592	38750	0.194	0.21	0.19	0.20	0.01
1.25	40208	46986	45758	0.1965	0.24	0.23	0.22	0.02
1.5	40719	43954	46943	0.199	0.22	0.24	0.22	0.02
1.75	40412	39683	44778	0.1975	0.20	0.22	0.20	0.01
2	40725	42893	42174	0.199	0.21	0.21	0.21	0.01

pH 11, 60°C

Std (mg/L)	Peak area NO_2^-	Peak area NO_3^-
0	0	0
5	1183367	885059
10	2440535	1838865
25	6477953	4947764

pH 11, 60°C

TNT Standard Concentration (mg/L)	Peak Area at 13.1min (AU)
0	0
20	386.6
40	909
60	1288.1
80	1677

pH 11, 80°C

pH 11, 80°C			peak area			mg/L		
Time (h)	TNT-a	TNT-b	TNT-b	TNT-a	TNT-b	TNT-b	avg TNT	TNT-stdev
0	1678.3	1718.2	1712.8	78.8	80.6	80.4	79.9	1.0
0.25	851.4	822.2	809.7	39.9	38.6	38.0	38.8	1.0
0.5	211.9	204.3	217.6	9.9	9.5	10.2	9.9	0.3
0.75	11.3	15.4	0	0.5	0.7	0	0.4	0.3
1	0	0	0	0	0	0	0	0
1.25	0	0	0	0	0	0	0	0
1.5	0	0	0	0	0	0	0	0
1.75	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0

pH 11, 80°C			peak area			mg/L		
Time (h)	NO_2^- (a)	NO_2^- (b)	NO_2^- (c)	NO_2^- (a)	NO_2^- (b)	NO_2^- (c)	avg NO_2^-	NO_2^- stdev
0	0	0	0	0	0	0	0	0
0.25	1612511	3084409	3244767	6.29	12.03	12.65	10.32	3.50
0.5	2479011	3802894	3775500	9.67	14.83	14.72	13.07	2.95
0.75	3689035	3893690	3792541	14.39	15.18	14.79	14.79	0.39
1	4319683	3714769	3753567	16.85	14.49	14.64	15.32	1.32
1.25	4440173	3863869	3866551	17.32	15.07	15.08	15.82	1.29
1.5	4491445	3829822	3754249	17.52	14.93	14.64	15.70	1.58
1.75	4509390	3938783	3769514	17.59	15.36	14.70	15.88	1.51
2	4886241	3211625	3286686	19.06	12.52	12.82	14.80	3.68

pH 11, 80°C

pH 11, 80°C			peak area			mg/L		
Time (h)	NO_3^- (a)	NO_3^- (b)	NO_3^- (c)	NO_3^- (a)	NO_3^- (b)	NO_3^- (c)	avg NO_3^-	NO_3^- stdev
0	0	0	0	0	0	0	0	0
0.25	37701	36917	29904	0.193	0.18	0.15	0.17	0.02
0.5	37798	41519	35065	0.193	0.21	0.17	0.19	0.01
0.75	37896	29566	22262	0.194	0.15	0.11	0.15	0.04
1	38091	0	18351	0.195	0	0.09	0.09	0.09
1.25	37802	24166	17528	0.193	0.12	0.08	0.13	0.05
1.5	37818	25629	17840	0.193	0.13	0.09	0.13	0.05
1.75	37505	33846	23813	0.192	0.17	0.12	0.16	0.03
2	38287	0	14746	0.196	0	0.07	0.09	0.09

pH 11, 80°C

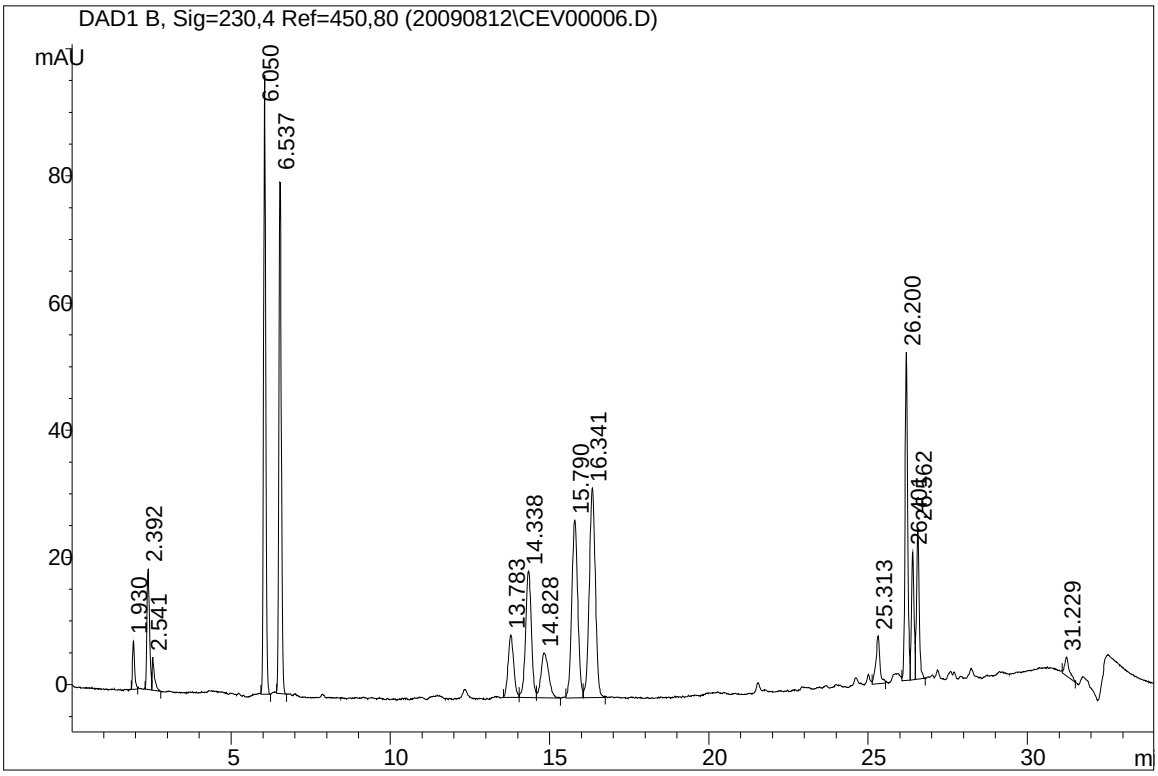
Std (mg/L)	Peak area NO_2^-	Peak area NO_3^-
0	0	0
5	1183367	885059
10	2440535	1838865
25	6477953	4947764

pH 11, 80°C

TNT Standard Concentration (mg/L)	Peak Area at 13.1min (AU)
0	0
20	386.6
40	909
60	1288.1

APPENDIX F

HPLC 1090 CHROMATOGRAM: “10 MIX” STANDARDS

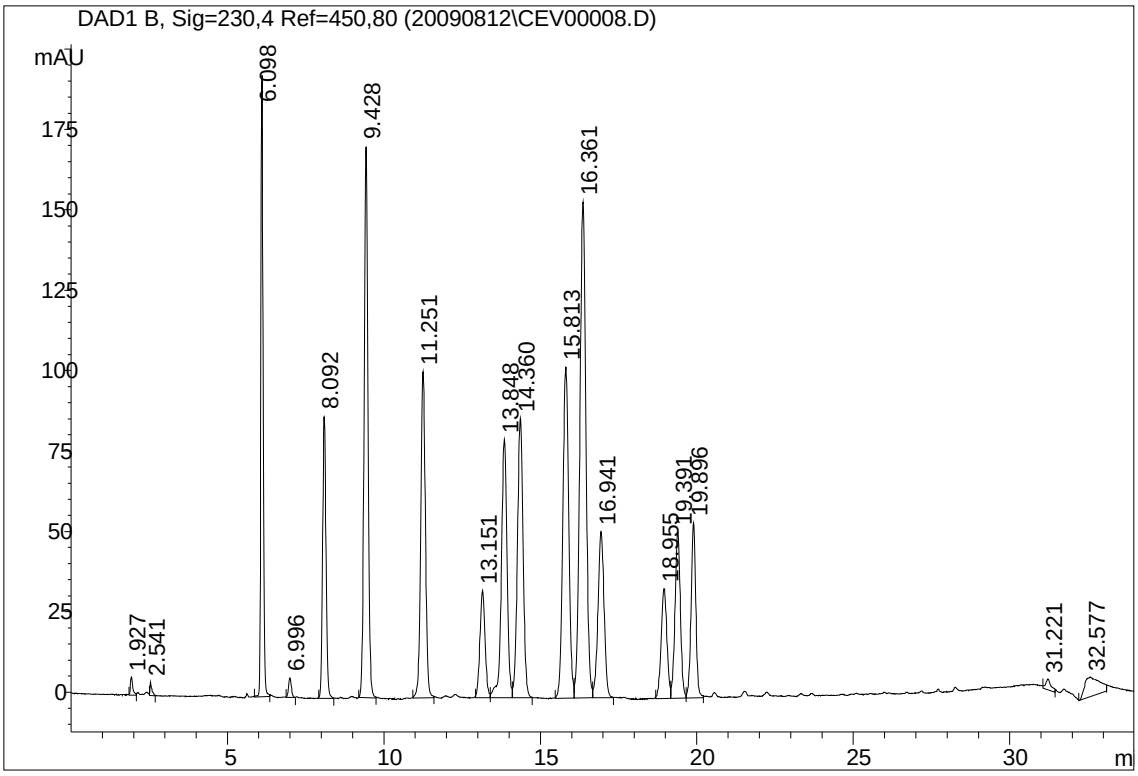


HPLC 1090 Chromatogram: “10 Mix” Standards

Retention Time (min)	Standard
6.050	2,6-DANT
6.537	2,4-DANT
13.783	2-HADNT
14.338	TNT
14.828	4-HADNT
15.970	2-ADNT
16.341	4-ADNT
26.200	4,4’-AZOXY
26.401	2,2’-AZO
26.562	4,4’-AZO

APPENDIX G

HPLC 1090 CHROMATOGRAM: “14 MIX” STANDARDS



HPLC 1090 Chromatogram: “14 Mix” Standards

Retention Time (min)	Standard
6.098	HMX
8.092	RDX
9.428	TNB
11.251	1,3-DNB
13.151	NB
13.848	Tetryl
14.360	TNT
15.813	2-ADNT
16.361	4-ADNT / 2,6-DNT
16.941	2,4-DNT
18.955	2-NT
19.391	4-NT
19.896	3-NT

APPENDIX H

RAW DATA FOR NC TREATABILITY STUDY

%N for RT						
Time (d)	RT-a	RT-b	RT-c	RT-d	RT-e	RT-f
0	7.51	8.11	7.22	7.90	7.38	7.64
16	10.26	10.10	10.25	11.93	11.01	11.00
30	10.06	10.068	10.06	11.50	11.06	10.97
60	7.61	7.63	7.48	11.12	11.12	10.51

%N for 60°C							
Time (d)	60°C -a	60°C -b	60°C -c	60°C -d	60°C -f	60°C -g	60°C -h
0	5.96	8.11	7.08	7.29	5.95	6.70	8.39
16	8.25	7.25	4.93	4.69	4.79	4.31	4.71
30	10.41	10.30	10.89	4.40	8.41	7.70	9.50
60	8.29	8.57	2.85	9.37	8.28	6.91	9.01

TSS for 60°C (g/L)								
Time (d)	a	b	c	d	e	f	g	h
0	25.942	21.202	19.762	22.442	33.122	18.302	22.182	33.342
6	23.062	19.722	32.462	21.542	20.262	21.122	29.942	30.802
12	21.402	20.702	19.882	20.302	27.162	15.402	33.142	18.322
16	26.542	16.322	22.802	27.082	28.822	28.122	37.422	34.202
30	35.322	24.062	23.182	34.142	23.822	26.002	26.962	23.842
60	22.742	12.982	22.782	22.722	29.882	22.882	41.322	23.802

NITRITE (NO_2^-) (mg/L)						
Time (d)	RT-a	RT-b	RT-c	RT-d	RT-e	RT-f
0	3714	3226	4102	4161	3732	3669
2	3544	3714	3767	3896	4133	3906
4	3557	3984	3809	4123	4187	4067
6	4089	3914	4266	4447	4141	4599
8	3823	4305	4401	3672	3400	3534
12	2621	2819	2776	2968	3217	3169
16	2305	2735	2712	2843	3181	3328
18	1825	2543	2571	2905	2996	3117
22	2155	2475	2698	3013	2722	3546
30	2240	2422	2306	2784	3173	2918
60	1451	1610	1202	1972	2301	2747

NITRITE (NO_2^-) (mg/L)

Time (d)	60°C -a	60°C -b	60°C -c	60°C -d	60°C -e	60°C -f	60°C -g	60°C -h
0	3847	3389	3683	3515	3894	4192	3792	3913
2	5685	6728	6486	6875	7551	7348	7599	7500
4	6476	6747	6735	7533	7516	8247	7317	7926
6	6540	4930	5974	7445	7642	8233	7261	7450
8	7166	7641	7582	8132	8132	8889	8190	8407
12	6979	7257	7270	7680	7706	8689	7865	8236
16	6506	7019	7270	8392	8198	8396	8114	7916
30	7552	8212	8163	8508	8047	9193	9200	8447
60	6582	6721	6787	6607	6435	7284	6897	7205

NITRATE (NO_3^-) (mg/L)

Time (d)	RT -a	RT -b	RT -c	RT-d	RT-e	RT-f
0	835	697	901	976	838	808
2	759	802	820	931	924	880
4	777	871	826	968	937	903
6	149	328	154	20.92	0	141
8	0	0	4.66	0	0	0
12	6.93	2.31	2.42	0	0	0
16	10.24	6.37	6.46	4.88	5.08	5.88
18	0	0	0	0	4.58	0
22	3.75	0	3.39	0	0	0
30	15.52	10.04	5.78	8.46	5.69	0
60	20.61	8.18	9.07	0	0	5.46

NITRATE (NO_3^-) (mg/L)

Time (d)	60°C -a	60°C -b	60°C -c	60°C -d	60°C -e	60°C -f	60°C -g	60°C -h
0	826	698	780	784	827	877	863	871
2	1609	1974	1861	2115	2224	2115	2198	2201
4	1931	2065	2024	2436	2302	2512	2402	2457
6	2084	1535	1812	2555	2428	2702	2585	2466
8	2462	2649	2563	2933	2737	3102	3081	2965
12	2639	2818	2719	3088	2768	3400	3287	3235
16	2580	2888	2959	3757	3202	3575	3691	3359
30	4151	4607	4336	4785	4009	5082	5641	4686
60	6766	7147	6713	7619	5982	7908	8084	7682

RT DNA (µg/mL)						
Time (d)	A	B	C	D	E	F
0	0.013	0.150	0.201	0.054	0.055	0.087
12	0.587	0.390	0.474	0.936	0.109	0.124
14	3.230	0.312	1.400	1.150	0.541	0.595
16	1.64	1.56	1.11	1.38	1.03	1.23
18	3.01	1.56	0.68	1.9	0.591	1.08
20	0.662	0.576	0.478	0.245	0.212	0.653
30	0.247	0.99	0.545	0.241	0.219	0.346
60	0.235	0.655	0.378	0.222	0.202	0.267

Sample	60°C DNA (µg/mL)	Avg	Stdev
60°C-d0	0.145		
60°C-d0 (2)	0.0824	0.1137	0.044265
60°C-d16	0.186		
60°C-d16 (2)	0.167	0.1765	0.013435
60°C-d30	0.223		
60°C-d30 (2)	0.149	0.186	0.052326
60°C-d60	0.144		
60°C-d60 (2)	0.208	0.176	0.045255

APPENDIX I

RAW DATA FOR NC HYDROLYSIS EXPERIMENTS

TSS (g/L), 100g/L NC and 400g/L NaOH, 60°C			
Time (min)	a	b	c
0	1	1	1
2	0.0559	0.1218	0.1319
3	0.0742	0.0641	0.0523
4	0.0496	0.0446	0.0458
5	0.0494	0.0488	0.0565
6	0.0469	0.0472	0.0493

TSS (g/L), 200g/L NC and 400g/L NaOH, 60°C			
Time (min)	a	b	c
0	2	2	2
2.5	0.0898	0.0895	0.0856
5	0.0997	0.0569	0.0483
7.5	0.0556	0.0606	0.0524
10	0.0564	0.0521	0.0545
12.5	0.0634	0.0606	0.0575

TSS (g/L), 100g/L NC and 0g/L NaOH, 60°C			
Time (min)	a	b	c
0	1	1	1
10	0.9717	0.9831	0.9722
20	0.8948	0.844	0.991
30	0.9696	0.825	0.9503
40	0.9621	0.9692	0.9696
50	0.9902	0.9542	0.8737

TSS (g/L), 100g/L NC and 400g/L NaOH, RT			
Time (min)	a	b	c
0	1	1	1
10	1.456	1.3542	0.998
20	0.554	0.7804	0.982
30	0.968	1.3835	1.29
40	1.226	0.949	0.981
50	0.55	0.208	1.001

	peak area (1:1000)			actual (mg/L)		
NO_2^-, 100g/L NC and 400g/L NaOH, 60°C						
Time (min)	a	b	c	a	b	c
0	0	0	0	0	0	0
2	5629659	5458065	6702266	21.95	21.29	26.14
3	6843952	7127585	7266233	25.43	26.49	27.00
4	5610745	5617370	6277595	21.88	21.91	24.48
5	6373152	7313387	6840157	23.68	27.18	25.42
6	5757027	5864459	5932606	22.45	22.87	23.14
10	6213728	6680006	5841256	24.23	26.05	22.78

	peak area (1:1000)			actual (mg/L)		
NO_3^-, 100g/L NC and 400g/L NaOH, 60°C						
Time (min)	a	b	c	a	b	c
0	0	0	0	0	0	0
2	3827656	3474438	4371100	15.34	13.92	17.52
3	4401079	4544505	4718350	17.30	17.86	18.54
4	3686926	3587773	4049966	14.77	14.38	16.23
5	4246178	4892830	4283898	16.69	19.23	16.84
6	3817764	3765863	3785837	15.30	15.09	15.17
10	4902732	4526243	3725942	19.65	18.14	14.93

	peak area (1:1000)			actual (mg/L)		
NO_2^-, 200g/L NC and 400g/L NaOH, 60°C						
Time (min)	a	b	c	a	b	c
0	0	0	0	0	0	0
2.5	10217258	10869269	11936694	37.97	40.40	44.36
5	13471143	14317650	11033552	50.07	53.21	41.01
7.5	13989774	12504823	14447623	51.99	46.47	53.70
10	14216389	14088308	12109406	52.84	52.36	45.00
12.5	14846587	12325134	14505979	55.18	45.81	53.91

	peak area (1:1000)			actual (mg/L)		
NO_3^-, 200g/L NC and 400g/L NaOH, 60°C						
Time (min)	a	b	c	a	b	c
0	0	0	0	0	0	0
2.5	6638375	7016683	8543272	26.09	27.58	33.58
5	8716913	9337790	6901332	34.26	36.71	27.13
7.5	9221531	7952420	9375064	36.25	31.26	36.85
10	10064989	9862914	7747933	39.56	38.77	30.45
12.5	9767506	7900594	9319459	38.39	31.05	36.63

	peak area (1:1)			actual (mg/L)		
NO_2^-, 100g/L NC and 0g/L NaOH, 60°C						
Time (min)	a	b	c	a	b	c
0	0	0	0	0	0	0
10	2021865	2121691	2016370	7.88	8.27	7.86
20	282918	208962	547693	1.10	0.81	2.13
30	501656	568357	499981	1.95	2.21	1.95
40	419952	466983	448198	1.63	1.82	1.74
50	740497	2304538	2059946	2.88	8.98	8.03

	peak area (1:1)			actual (mg/L)		
NO_3^-, 100g/L NC and 0g/L NaOH, 60°C						
Time (min)	a	b	c	a	b	c
0	0	0	0	0	0	0
10	1749276	1822017	1701126	7.01	7.30	6.81
20	363427	585498	1199565	1.45	2.34	4.80
30	1053675	1247969	1108957	4.22	5.00	4.44
40	974765	1036353	87798	3.90	4.15	0.35
50	1093167	1844763	1605699	4.38	7.39	6.43

	peak area (1:1000)			actual (mg/L)		
NO_2^-, 100g/L NC and 400g/L NaOH, RT						
Time (min)	a	b	c	a	b	c
0	0	0	0	0	0	0
10	907037	567175	600087	3.53	2.21	2.34
20	3325826	3685708	3879129	12.36	13.69	14.41
30	2831795	2904369	3079297	10.52	10.79	11.44
40	5352842	5875113	6032775	19.89	21.83	22.42
50	5093851	6213728	6091652	19.86	24.23	23.76

	peak area (1:1000)			actual (mg/L)		
NO_3^-, 100g/L NC and 400g/L NaOH, RT						
Time (min)	a	b	c	a	b	c
0	0	0	0	0	0	0
10	600862	418094	440352	2.40	1.67	1.76
20	2516988	2792327	2950724	9.89	10.97	11.60
30	2134969	2183272	2326219	8.39	8.58	9.14
40	4097041	4525615	4640318	16.10	17.79	18.24
50	3943865	4902732	4810754	15.80	19.65	19.28

APPENDIX J

RAW DATA FOR NC HYDROLYSATE BIOLOGICAL SCREENING

Optical Density (OD, absorbance @ 620nm)

Time (d)	OD (a)	OD (b)	OD (c)
0	0.089	0.089	0.088
1	0.091	0.089	0.093
2	0.111	0.123	0.099
3	0.212	0.247	0.209
4	0.303	0.336	0.341
4.5	0.352	0.332	0.349
5	0.367	0.347	0.348
5.5	0.380	0.367	0.374
6	0.388	0.390	0.395
7	0.361	0.389	0.398
7.5	0.401	0.41	0.407
8	0.421	0.419	0.413
9	0.418	0.409	0.422
10	0.332	0.369	0.370

Nitrite (mg/L)

Time (d)	NO_2^-(a)	NO_2^-(b)	NO_2^-(c)
0	0	0	0
1	21.2	14.8	11.5
2	222.7	203.6	197.8
3	157.6	132.4	182.4
4	0	0	0
4.5	115.4	101.9	98.0
5	0	1.6	0
5.5	156.1	158.8	167.4
6	97.5	94.4	100.7
7	10.2	0	2.3
7.5	212.0	203.9	211.5
8	111.8	87.3	95.4
9	0	0	4.5
10	0	0	0

Nitrate (mg/L)

Time (d)	NO_3^-(a)	NO_3^-(b)	NO_3^-(c)
0	580	537	543
1	405.4	424.3	500.4
2	201.4	356	267.9
3	0	0	0
4	0	0	0
4.5	85.5	70.3	72.4
5	0	0	0
5.5	100.1	113.8	117.2
6	34.6	31.9	45.6
7	0	0	0
7.5	137.9	143.4	131.2
8	43.7	88.2	48.9
9	0	0	0
10	0	0	0