



Chromatographic, spectroscopic and microscopic analyses reveal the impact of iron oxides and electron shuttles on the degradation pathway of 2,4,6- trinitrotoluene (TNT) by a fermenting bacterium  
by Thomas Borch

A dissertation submitted in partial fulfillment of requirement for the degree of Doctor of Philosophy in  
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**Abstract:**

Contamination of surface and subsurface environments with explosives such as 2,4,6-trinitrotoluene (TNT) is a worldwide problem. The fate and analysis of TNT were investigated in numerous artificially contaminated model systems. We developed a unique high performance liquid chromatography gradient elution method for the analysis of commonly observed TNT metabolites and EPA explosives. Column temperature was identified as the key parameter for optimal separation.

Iron (hydr)oxides play an important role in the reduction, sorption and fate of TNT in soil and sediment. Consequently, characterization of the nature and properties of natural and synthetic Fe (hydr)oxides is important for determining reaction mechanisms and surface-associated chemical processes. This work thus summarizes the potential applicability of imaging and spectroscopic techniques for eliciting chemical and physical properties of iron (hydr)oxides.

TNT is persistent in soils due to its low redox potential and sorption. Batch and column studies revealed some of the first results on TNT desorption behavior in two well-defined model soil systems. Biosurfactants were found to be the most promising technique for enhanced TNT desorption.

Batch studies with a *Cellulomonas* sp. in the presence of ferrihydrite and the electron shuttle anthraquinone-2,6-disulfonate (AQDS) were conducted to reveal biotic and abiotic mechanisms contributing to the degradation of TNT. Strain ES6 was found to reduce TNT and ferrihydrite with enhanced reduction in the presence of AQDS. Ferrihydrite stimulated the formation of more reduced TNT metabolites such as 2,4-diamino-6-nitrotoluene. Interestingly, a completely different degradation pathway was observed in AQDS-amended iron-free cell suspensions, showing a rapid transformation of TNT to 2,4-dihydroxylamino-6-nitrotoluene, which transformed into unidentified polar products.

The influence of iron phases (i.e. hematite, magnetite, and ferrihydrite) and secondary Fe mineral formation on the degradation of TNT was also evaluated. The initial reduction of TNT was fastest in the presence of hematite; however, the further reduction of hydroxylamino-dinitrotoluenes was fastest, in the presence of magnetite and ferrihydrite (no AQDS). The impact of AQDS was predominant in the presence of hematite resulting in the formation of 2,4,6-triaminotoluene. Ferrihydrite underwent reductive dissolution with the formation of secondary hematite. The enhanced TNT reduction in ferrihydrite-amended systems was therefore most likely due to redox-active Fe(II) rather than secondary Fe phases.

CHROMATOGRAPHIC, SPECTROSCOPIC AND MICROSCOPIC ANALYSES REVEAL  
THE IMPACT OF IRON OXIDES AND ELECTRON SHUTTLES ON THE  
DEGRADATION PATHWAY OF 2,4,6-TRINITROTOLUENE (TNT)  
BY A FERMENTING BACTERIUM

by

Thomas Borch

A dissertation submitted in partial fulfillment  
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in

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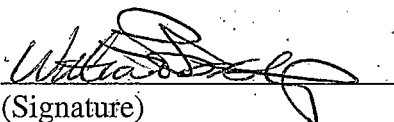
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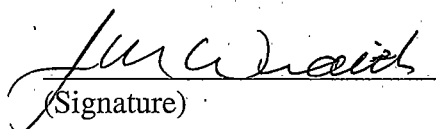
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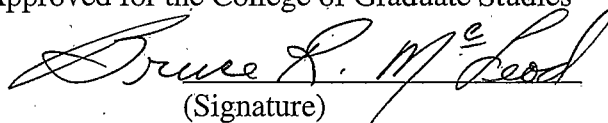
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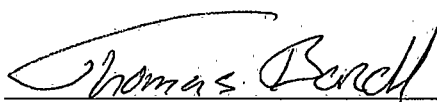
  
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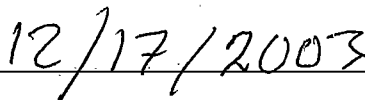
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## ABSTRACT

Contamination of surface and subsurface environments with explosives such as 2,4,6-trinitrotoluene (TNT) is a worldwide problem. The fate and analysis of TNT were investigated in numerous artificially contaminated model systems. We developed a unique high performance liquid chromatography gradient elution method for the analysis of commonly observed TNT metabolites and EPA explosives. Column temperature was identified as the key parameter for optimal separation.

Iron (hydr)oxides play an important role in the reduction, sorption and fate of TNT in soil and sediment. Consequently, characterization of the nature and properties of natural and synthetic Fe (hydr)oxides is important for determining reaction mechanisms and surface-associated chemical processes. This work thus summarizes the potential applicability of imaging and spectroscopic techniques for eliciting chemical and physical properties of iron (hydr)oxides.

TNT is persistent in soils due to its low redox potential and sorption. Batch and column studies revealed some of the first results on TNT desorption behavior in two well-defined model soil systems. Biosurfactants were found to be the most promising technique for enhanced TNT desorption.

Batch studies with a *Cellulomonas* sp. in the presence of ferrihydrite and the electron shuttle anthraquinone-2,6-disulfonate (AQDS) were conducted to reveal biotic and abiotic mechanisms contributing to the degradation of TNT. Strain ES6 was found to reduce TNT and ferrihydrite with enhanced reduction in the presence of AQDS. Ferrihydrite stimulated the formation of more reduced TNT metabolites such as 2,4-diamino-6-nitrotoluene. Interestingly, a completely different degradation pathway was observed in AQDS-amended iron-free cell suspensions, showing a rapid transformation of TNT to 2,4-dihydroxylamino-6-nitrotoluene, which transformed into unidentified polar products.

The influence of iron phases (i.e. hematite, magnetite, and ferrihydrite) and secondary Fe mineral formation on the degradation of TNT was also evaluated. The initial reduction of TNT was fastest in the presence of hematite; however, the further reduction of hydroxylamino-dinitrotoluenes was fastest in the presence of magnetite and ferrihydrite (no AQDS). The impact of AQDS was predominant in the presence of hematite resulting in the formation of 2,4,6-triaminotoluene. Ferrihydrite underwent reductive dissolution with the formation of secondary hematite. The enhanced TNT reduction in ferrihydrite-amended systems was therefore most likely due to redox-active Fe(II) rather than secondary Fe phases.

## CHAPTER 1

## INTRODUCTION

We are challenged with water, air and soil pollution from past and present discharge of domestic and industrial wastes, accidental spills and intentional releases. Nitroaromatic compounds are released into the environment almost entirely from anthropogenic sources, including chemicals used as pesticides, dyes and explosives. Sites used for manufacturing, loading and storage of munitions in the United States and Europe are often contaminated with explosives, especially from World War II [1]. 2,4,6-trinitrotoluene (TNT), is the primary explosive used in munitions manufacturing and the yearly production is estimated at 1000 tons [2,3]. TNT is mutagenic, acutely toxic and carcinogenic [4-9], consequently, the management of munitions waste products and the remediation of contaminated sites are vital to public health. The removal and or degradation of TNT from contaminated soils and waters has become an important problem where alternatives must be evaluated in terms of the acceptability of the formed metabolite, end-products, and cost [2].

The degradation of TNT by microorganisms has been evaluated under different environmental conditions and although biotransformation is common, complete mineralization to  $\text{CO}_2$  is generally not observed [10-12]. Consequently, the bioremediation of TNT must be understood in the context of specific degradation pathways and a careful evaluation of the fate of several possible pathway intermediates [13,14]. The most commonly used method for the analysis of explosives and

corresponding metabolites is high performance liquid chromatography (HPLC) with UV detection. However, the use of HPLC continues to present challenges [15] especially due to difficulties in separating isomers and metabolites with a wide range of polarities [16-20]. Improved methods of analysis of TNT pathway intermediates are necessary to evaluate mechanisms of TNT degradation. Therefore, one of the goals of the present study was to develop an HPLC method capable of separating the most commonly observed explosives and TNT transformation products. Specific objectives were focused on how column temperature and an ion-pair reagent (i.e. octanesulfonic acid) influence the chromatographic separation of TNT metabolites and explosives targeted in the Environmental Protection Agency (EPA) Method 8330. The improved analytical method developed here (Chapter 2) provided an important tool for analyzing the majority of the relevant TNT intermediates and was used in all subsequent experiments evaluating the degradation of TNT.

The impact of soil constituents such as soil organic matter (SOM) and mineral phases on the fate of TNT is still inadequately understood. Consequently, conclusions about the relative importance of binding interactions, redox-activity, and bioreduction of various mineral phases with respect to TNT transformation in the subsurface cannot yet be drawn. To this end, additional work is necessary to evaluate the complex interactions among microorganisms and mineral surfaces and the subsequent effects on TNT degradation. In particular, the presence of Fe(II) associated with Fe (hydr)oxide surfaces can play an important role in the reduction of a variety of organic and inorganic pollutants (e.g. U(VI)) in natural and engineered systems [21-25]. A host of Fe

(hydr)oxide minerals are thus used in research studies (e.g. [26,27]) despite the actual chemical and physical complexity of natural minerals [28]. Potential problems, such as poor estimation of sorptive properties and surface reactivity, can arise from the use of poorly characterized synthetic Fe (hydr)oxides as mineral analogs or from the use of well characterized mineral analogs to model very different natural minerals, as demonstrated by Perret et al. [29]. Although techniques exist to determine many chemical and physical properties of synthetic Fe (hydr)oxides [30-33], it is often difficult to represent naturally occurring Fe oxides. The highly sorptive and redox-active iron (hydr)oxide solids often develop surface layers with properties distinctly different from the bulk. Nevertheless, the comprehensive investigation of model Fe (hydr)oxide surfaces will improve our understanding of how different surface and structural properties of Fe (hydr)oxides influence the biodegradation of TNT. Consequently, prior to utilizing model Fe oxides in TNT degradation experiments, several analytical techniques were employed to compare two selected iron (hydr)oxide models used in a previous drinking-water study on biofilm control [34]. The two materials characterized were 1) corrosion products from a water system, and 2) a synthetic Fe (hydr)oxide thin film formed on glass surfaces (500- $\mu\text{m}$  beads and glass cover slips). Specifically, the objective under this goal was to evaluate the potential applicability of surface and bulk characterization techniques for eliciting physical and chemical properties of Fe (hydr)oxide analogs. Results from this study (Chapter 3) may be used to assist in the characterization of synthetic Fe phases (including thin films), identification of environmentally relevant Fe phases, and to reveal secondary mineralization pathways induced by microorganisms as demonstrated in Chapter 6.

Several bioremediation strategies for treating TNT contaminated soil and sediment have been evaluated both in the field and at the bench scale. The primary remediation alternatives include incineration, composting, chemical oxidation, alkaline hydrolysis, surfactant enhanced washing and biodegradation [35-39]. The high cost and modest effectiveness of several of these alternatives has prompted the need for improved remediation technologies [40]. *In situ* treatment is currently the most common strategy and generally meets the criteria established for successful application (i.e. implementability, performance and cost) [41]. Field bioremediation treatments such as excavation and *ex situ* composting are common, however, no *in situ* processes have been developed for remediation of TNT [42].

The limited bioavailability to microorganisms and their subsequent persistence in the subsurface [43] represents a major challenge for *in situ* treatment of TNT and its metabolites. The binding of TNT and its metabolites to soil constituents such as SOM has been well studied [19,44-47]. It was found that TNT binds to humic acids *via* hydrophobic interactions by a slow kinetic process and that the sorption capacity of SOM is significantly less than that of clay [46,48]. Conversely, it is generally well accepted that reduced metabolites of TNT such as the amino derivatives may bind covalently and in some cases irreversibly to SOM particularly under aerobic conditions [19,44,49,50]. It was further illustrated that the binding capacity of humic acid for TNT and its reduced metabolites was influenced by many factors, including humic acid concentration, pH, and ionic strength [46].

One technique for enhancing the desorption and potential bioavailability of TNT is the use of surfactants. Taha et al. [51] reported enhanced desorption of TNT from a contaminated soil when surfactants were added. In addition, the surfactant Tween 80 enhanced the mineralization of TNT by *Phanerochaete chrysosporium* [52]. However, there are potential limitations to the use of surfactants for *in* and *ex situ* remediation including the possible toxicity of surfactants to certain microorganisms and the bioavailability of surfactant micelle-phase contaminants [53]. For instance, some synthetic nonionic surfactants have been shown to inhibit mineralization of phenanthrene [54]. However, the microbial production of biosurfactant-like biomolecules (e.g. glycolipids) has been shown to enhance the solubility and bioavailability of pyrene [55]. In addition, exogenously added biosurfactants (i.e. rhamnolipids) were found to enhance the desorption of phenanthrene and to stimulate uptake of hexadecane by *Pseudomonas aeruginosa* [56,57]. Given the importance of surfactant-based remediation treatments and ionic strength, one of the goals of this study was to investigate the influence of ionic strength, primary cations, and biosurfactants on the desorption of TNT in two model soil systems. Specifically, important chemical-physical factors that influence the sorption of organic contaminants were identified based on a literature review; two model porous media using different combinations of quartz sand, goethite ( $\alpha$ -FeOOH) and humic acid were developed and the desorption of TNT studied under batch and column transport conditions. Results from this study (Chapter 4) improve our current understanding by revealing the affects of solution chemistry and surfactants on the equilibrium partitioning

of TNT in aqueous-solid phase environments and may be useful for optimizing bioremediation strategies.

Original studies on TNT biodegradability suggested that anaerobic degradation was not possible [58,59]; however, recent studies have shown that anaerobic biodegradation is more important than previously assumed [60]. One promising mechanism has been based on the full reduction of TNT to 2,4,6-triaminotoluene (TAT) under anaerobic conditions, which may bind irreversibly to soil, reducing bioavailability, mobility and subsequent exposure to biota [19]. Conversely, amino derivatives formed under aerobic conditions are more susceptible to electrophilic attack by dioxygenases than TNT and may be biodegraded [60]. Preliminary investigations have indicated that dissimilatory iron reducing bacteria (DIRB) might play a significant role in the transformation of nitroaromatics in the natural environment [22]. Hofstetter et al. [22] showed that a *Geobacter* sp. indirectly stimulated the transformation of 4-chloronitrobenzene to 4-chloroaniline. This conversion was thought to be the result of biologically produced Fe(II), which can reduce the nitro groups abiotically [22].

Alternatively, DIRB have been shown to reduce quinone moieties in humic acids to form hydroquinones [61,62]. Hydroquinones can also reduce TNT abiotically as shown recently in the presence of 8-hydroxy-1,4-naphthoquinone (juglone) [22,63]. The few studies investigating the impact of quinones (i.e. juglone) and DIRB (i.e. ferrogenic consortia) on the fate of TNT have all resulted in the formation of amino derivatives [22,63]. In general, most research has shown that TNT is easily transformed to numerous substituted metabolites without significant loss in aromatic character (no ring cleavage)

[42]. However, recent studies by Hughes et al. [64] demonstrated the possibility for the Bamberger rearrangement (e.g. the acid-catalyzed Bamberger rearrangement of 2,4-dihydroxylamino-6-nitrotoluene occurs with the hydroxyl addition *para* to the participating hydroxylamine resulting in the formation of 2-amino-4-hydroxylamino-5-hydroxyl-6-nitrotoluene) during TNT metabolism by *Clostridium acetobutylicum*. The formation of phenolic amines via the Bamberger rearrangement of 2,4-dihydroxylamino-6-nitrotoluene (TNT metabolite) is of particular interest since it may open the possibility for degradation through the o-aminophenol extradiol-like ring cleavage pathway [64-67].

The major component of my Ph.D. work was therefore inspired by these promising preliminary studies documenting degradation of TNT in systems containing either fermenting bacteria, DIRB, ferrous/ferric iron and quinone structures [22,63,64]. To understand potential mechanisms and pathways of TNT degradation in complex mineral-aqueous environments more research is needed to systematically evaluate affects of microbial processes, Fe surfaces, and humic-like substances. The objectives of my work were then focused on (i) the degradation of TNT by a novel fermenting (Gram-positive) bacterium (i.e. *Cellulomonas* sp. strain ES6) previously shown to reduce nitrilotriacetic acid chelated Fe(III), Cr(VI) and U(VI) [68], (ii) the role of Fe(II) on the fate of TNT under biotic and abiotic conditions, and (iii) the influence of anthraquinone-2,6-disulfonate (AQDS; herein used as a model compound for the quinone moieties in HA and an electron shuttle) and soil HA on the fate of 2,4,6-trinitrotoluene under biotic conditions. Results from this study (Chapter 5) provide a fundamental understanding of

how fermenting bacteria, ferrous iron, hydrous ferric oxide (HFO) and electron shuttles influence the degradation of TNT and the specific metabolites formed.

The bioreduction of dissolved Fe(III), and amorphous or crystalline Fe (hydr)oxides by DIRB such as Gram-negative *Shewanella* and *Geobacter* spp. has been studied extensively [69-77]. Furthermore, DIRB may play an important role in reducing toxic heavy metals and radionuclides, and in oxidizing aromatic compounds such as benzene [78-80]. *Geobacter* and *Shewanella* spp. have not been shown to reduce TNT, and *Geobacter metallireducens* was incapable of direct reduction of mononitroaromatic compounds [22]. The bioreduction of amorphous Fe(III) phases (i.e. ferrihydrite) by *Shewanella* and *Geobacter* spp. often leads to the formation of secondary Fe mineral phases such as goethite, siderite, and magnetite, depending on the chemical environment [69-71,77]. However, the contribution of abiotic versus biotic factors on biomineralization and the impacts of secondary phases on degradation processes are poorly understood [69,71].

It is now widely recognized that electron shuttling compounds such as humic acids (HA) and AQDS may enhance bioreduction of iron(III) minerals [61,81]. Consequently, quinone-mediated bioremediation strategies have been proposed due to the direct and indirect (by increased production of redox-active Fe(II)) reduction of pollutant metals and organics [82-85]. The impact of quinone moieties on secondary Fe mineral formation is not well understood, and can potentially impact subsequent degradation processes. For example, the formation of magnetite may sequester most of the Fe(III) in an aquifer to a form that is microbially unavailable and change the redox potential to a

point where contaminant reduction reactions may be less favorable [70]. One of the goals of this dissertation was therefore to evaluate the influence of three different Fe (hydr)oxides (i.e. ferrihydrite, hematite and magnetite) on the microbial (i.e. *Cellulomonas* sp. strain ES6) reduction of TNT. Specifically, the objectives of this study were to (i) determine the role of bio reducible Fe phases on the rate of TNT reduction and formation of metabolites in the presence and absence of AQDS, and (ii) assess the role of biomineralization processes on the reduction of TNT to gain insight into other potential consequences of using strain ES6 for bioremediation purposes (Chapter 5 and 6). Results from this study (Chapter 6) provide insights regarding the affects of Fe mineral bio reduction and secondary Fe phase formation on the fate of TNT.

Most of the dissertation work is either planned for submission to or already published in peer-reviewed journals. Chapter 2 has just been published in the Journal of Chromatography A (Borch, T and Gerlach, R. 2004, vol. 1022, pp. 83-94). Chapter 3 (Borch et al.) has been submitted for publication in the journal of Water Research. Chapter 5 (Borch et al.) and Chapter 6 (Borch et al.) will be submitted to the journal of Environmental Science and Technology. Parts of the dissertation have also been presented at several national and international conferences such as at the American Geophysical Unions Fall Meeting (2002) in San Francisco, California (please refer to *Eos Trans. AGU*, 83(47), Fall Meet. Suppl., Abstract B22E-11, 2002) and at the seventh international symposium on In Situ and On-Site Bioremediation (2003) in Orlando, Florida (please refer to session A4. Bioremediation of Energetic Compounds, in press).

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## CHAPTER 2

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

Introduction

The nitroaromatic compound 2,4,6-trinitrotoluene (TNT), has been found to contaminate soils at former and present munitions manufacturing facilities, storage depots, and former sites of explosives use [1,2]. Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) are also contaminants at these locations as they are routinely components of TNT-based explosive mixtures [1,3,4].

At these sites, TNT is often being biologically reduced stepwise to hydroxylaminodinitrotoluenes (HADNTs), aminodinitrotoluenes (ADNTs), diaminonitrotoluenes (DANTs), and 2,4,6-triaminotoluene (TAT) (Fig. 2.1). The HADNTs, which are intermediates in the transformation of TNT, include 2-hydroxylamino-4,6-dinitrotoluene (2-HADNT), 4-hydroxylamino-2,6-dinitrotoluene (4-HADNT), 2,4-dihydroxylamino-6-nitrotoluene (2,4-DHANT) and (not shown in Fig. 2.1) 2,6-dihydroxylamino-4-nitrotoluene (2,6-DHANT) [5]. The ADNTs are 2-amino-4,6-dinitrotoluene (2-ADNT) and 4-amino-2,6-dinitrotoluene (4-ADNT). The DANTs are

2,4-diamino-6-nitrotoluene (2,4-DANT) and 2,6-diamino-4-nitrotoluene (2,6-DANT) [1,3,6]. The azoxy compounds, such as 4,4',6,6'-tetranitro-2,2'-azoxytoluene (2,2'-azoxy) and 2,2',6,6'-tetranitro-4,4'-azoxytoluene (4,4'-azoxy) are believed to be the products of spontaneous abiotic hydroxylamino-nitroso condensation reactions (Fig. 2.1) [1,5,7].

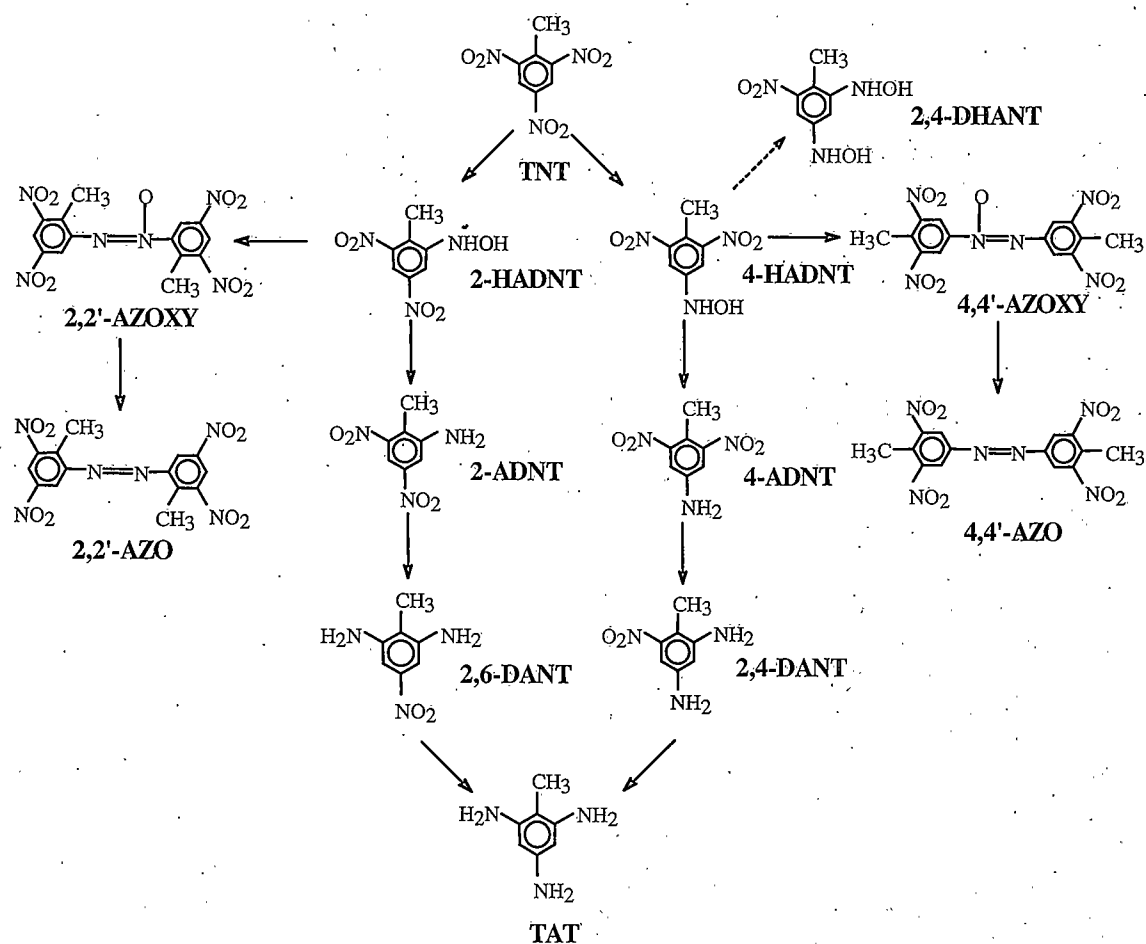


Figure 2.1. 2,4,6-trinitrotoluene (TNT) transformation pathways observed in soil and water.

Environmental concerns stem from the mutagenic, carcinogenic, and toxic effects of nitroaromatic and aminoaromatic compounds. TNT, 2-ADNT, 4-ADNT, 2,4-DANT, 2,6-DANT and TAT have been found to be cytotoxic presumably due to induced oxidative stress [8,9]. TNT, RDX, HMX, and the TNT-derived metabolites all demonstrate mutagenic capability [9] and the EPA classifies TNT and RDX as possible human carcinogens [10].

In order to develop accurate metabolic pathway maps, HPLC methods capable of analyzing and separating all explosives and their transformation products must be available. The reduced metabolites of TNT exhibit varying degrees of polarity and chemical stability making the chromatographic separation extremely challenging. The hydroxylamines are known to be unstable in aqueous solution in the presence of molecular oxygen potentially forming azoxy compounds [5]. Azoxy compounds can undergo further transformation to form 4,4',6,6'-tetranitro-2,2'-azotoluene (2,2'-azo) and 2,2',6,6'-tetranitro-4,4'-azotoluene (4,4'-azo) [11]. The formation of HADNTs, azoxy, and azo compounds may be the major cause of poor mass balances obtained in bioremediation systems, where only aminated products are monitored [5,12].

TAT is one of the most unstable reduced TNT metabolites in aqueous solutions. The three amino groups and the methyl group are all activating substituents, which increase the reactivity of the aromatic ring toward electrophiles such as oxygen. Consequently, TAT is highly oxygen sensitive and easily degraded under aerobic conditions [1]. However, even under anaerobic conditions, TAT can participate in oxydimerization and polymerization to form azo and poly-azo compounds [1,13].

Although GC, solid-phase microextraction (SPME)-GC-MS, CE-UV, and TLC have been used, HPLC has remained the major analytical tool for the detection and quantification of nitroaromatic compounds [1,3,7,11,12,14-18]. The most commonly used method for the analysis of nitroaromatics is HPLC with UV detection due to its widespread availability [3,15], while HPLC combined with MS and electrochemical detection (ED) are also viable but less frequently available methods of detection [13,14,19,20].

The analysis of explosives using HPLC continues to present challenges [3]. Previously published methods have focused on the use of C-18 RP-HPLC [3,5,20,21] and acetonitrile as the organic mobile phase. Acetonitrile is of significant greater health and environmental concern than methanol and therefore methods avoiding the use of acetonitrile are desirable. Many previously published methods had difficulties with the separation of the following isomer pairs: 2- and 4-HADNT, 2- and 4-ADNT, 2,4- and 2,6-DANT, 2,2'- and 4,4'-azoxy [11,12,14,15,21,22]. Consequently, the identification of the polar TAT and the less polar azoxy dimers in TNT degradation studies has often been performed in separate HPLC runs [23].

The goal of the present study was to separate TNT, HADNTs, 2,4-DHANT, ADNTs, DANTs, TAT, azoxy and azo compounds in a single HPLC run and to improve the chromatography of the 14 EPA Method 8330 compounds. The explosives targeted in EPA Method 8330 were included in the present studies due to the fact that they often occur as co-contaminants in environmental samples containing TNT. They could

therefore potentially interfere or co-elute with TNT or its reduced metabolites during HPLC analysis.

### Experimental

#### Chemicals and Sample Preparation

The TNT, ADNT, and DANT standards (1000 µg/ml in acetonitrile, purity > 99.0 %) were obtained from Supelco (Bellefonte, PA, USA). TAT\*3HCl and 4,4',6,6'-tetranitro-2,2'-azoxytoluene were obtained from Dr. R. J. Spanggord, SRI International, Menlo Park, CA. The chemicals included in EPA Method 8330 (all with a purity > 96.8 %), 4-hydroxylamino-2,6-dinitrotoluene (purity 96.0 %), 2-hydroxylamino-4,6-dinitrotoluene (purity 97.1 %), 2,2',6,6'-tetranitro-4,4'-azoxytoluene (purity 98.8 %), 2,2',6,6'-tetranitro-4,4'-azotoluene (purity 90.7 %) and 4,4',6,6'-tetranitro-2,2'-azotoluene (purity 94.7 %) were obtained from AccuStandard (New Haven, CT, USA). 2,4-dihydroxylamino-6-nitrotoluene was synthesized biochemically and kindly provided by Dr. J. B. Hughes, Rice University, Houston, TX [5]. TNT (neat; purity 98.0 %) for the degradation studies was obtained from Chem Service (West Chester, PA, USA). HPLC grade methanol (UV cutoff 205 nm) and Optima acetonitrile (UV cutoff 190 nm) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). HPLC grade dibasic sodium phosphate hepta hydrate and enzyme grade monobasic sodium phosphate were obtained from Fisher Scientific and Fisher Biotech, respectively (Fair Lawn, NJ, USA). The ion-pair reagent octanesulfonic acid (sodium salt) was obtained from ACROS (New Jersey, USA). The ion-pair reagent Low-UV PIC B8 was obtained from Waters

(Milford, MA, USA). The water used in the preparation of mobile phases and standards was obtained from a Barnstead NANOpure system (resistivity  $\geq 17.6 \text{ M}\Omega\text{-cm}$ ).

The TNT metabolite mixture was prepared in a glove box in the absence of oxygen (90 %  $\text{N}_2$ , 5 %  $\text{CO}_2$ , 5 %  $\text{H}_2$ ) and concentrated by rapid evaporation of acetonitrile. TAT standards have been found to easily degrade in  $\text{H}_2\text{O}$  solutions [24]. However, degradation of the oxygen and pH sensitive TAT standards was prevented when prepared in 0.025 M phosphate buffer (pH 7) as follows. The phosphate buffer was boiled after preparation and cooled down on ice under a constant purge of nitrogen to keep the solution free of oxygen. The oxygen free buffer solution was transferred into the anaerobic glove box and aliquots were added to 15 ml vials containing defined amounts of TAT\*3HCl powder. The vials were capped with polytetrafluoroethylene (PTFE) coated butyl rubber septa and crimp-sealed. The headspace was finally replaced with pure nitrogen. For analysis, aliquots of these solutions were transferred to HPLC vials in the glove box and analyzed immediately. The TAT concentration in the stock solutions was constant for at least 10 days (data not shown).

## HPLC

Apparatus. HPLC analyses were performed using a Hewlett-Packard 1090 Liquid Chromatograph equipped with an original autosampler and diode array detector. The Agilent ChemStation software (Rev. A.08.01 [783]) was utilized for instrument control, data acquisition, and analysis. Although multiple Supelcosil octyl (C-8) 150 x 4.6 mm (5- $\mu\text{m}$  particle size) columns were used during the method development, all data

published herein were obtained using the same column. Guard columns used for the method development included Supelcosil LC-CN, LC-ABZ+, and LC-8 (20 X 4.6 mm; 5  $\mu$ m), but only the LC-8 guard column was used for the final data collection. The injection volume was 10  $\mu$ l, with an initial syringe draw speed of 83  $\mu$ l/min.

Column Temperature, Diode Array Detection, and Mobile Phases. The column temperature was controlled using the block heater built into the HPLC system (circulating heated air around the column). Stainless steel tubing (35 cm long, 0.17 mm I.D.) was placed upstream of the column inside the oven compartment to ensure that the inlet solvent was acclimatized in order to minimize potential instrument-to-instrument variation [25,26]. Oven temperatures from 35 °C to 55 °C were investigated.

Chromatograms were extracted at absorbencies of 220, 230, 254, 360, and 370 nm. Peaks were scanned from 200 to 600 nm to obtain spectrochromatograms for compound characterization.

Two mobile phases were utilized to establish the gradient system. The organic mobile phase was HPLC-grade methanol. The aqueous mobile phase consisted of either 0.025 M sodium phosphate buffer (pH 7) with or without 0.1 % or 0.5 % w/w 1-octanesulfonic acid (ion-pair reagent), or of a phosphate buffer (0.0144 M Na<sub>2</sub>HPO<sub>4</sub>) amended with Low-UV PIC B8 resulting in a 0.1 % ion-pair reagent solution with a pH of 7. No significant differences were observed for the two different aqueous ion-pair reagent mobile phases. Since the use of the Low-UV PIC B8 ion-pair reagent resulted in significant time savings, all results presented herein were acquired using this phosphate

buffer solution. Mobile phases were kept oxygen free by purging them with helium for at least 30 minutes prior to the first sample run and continuously throughout analyses.

Gradient. The flow rate of the mobile phase was 1 ml/min. The mobile phase initially consisted of 99 % phosphate buffer (with or without 0.1 % ion-pair reagent) and 1 % methanol. By utilizing the Agilent ChemStation "narrow gradient range" option, the gradient was changed to 30 % methanol over 2 min, then to 43 % methanol over the next 13 min, finally increased to 100 % methanol over 12.5 min, and held constant for 0.5 min. The solvent ratio was returned to the initial conditions over 1 min and held for an additional 5 min before injection of the next sample. The total run time including conditioning time was 34 min.

#### Detection and Peak Performance Parameters

Performance assessment was based on calibration standards of authentic compounds and multipoint standard calibration curves. All compounds investigated had a linear detection range of at least 1 – 100 mg/l and were easily detected in the range of 5 – 10 ng per injection. Standard solutions with a concentration of 10 – 25 mg/l were used to generate the chromatograms with the exception of the azo compounds, which had a concentration of up to 40 mg/l.

Retention times ( $t_r$ ), retention time factors ( $k'$ ), and the chromatographic resolution ( $R_s$ ) were calculated for all compounds.  $k'$  was calculated using a void time which was determined from the column parameters supplied ( $k' = [t_r - (V_m / F)] / (V_m / F)$ ), where  $V_m$  is the column void volume [ $L^3$ ] and  $F$  the flow rate [ $L^3/t$ ]. The porosity of

the column packing material was 0.71 based on information from Supelco (Bellefonte, PA, USA).  $R_s$  and number of theoretical plates ( $N$ ) were calculated using the half-width method.

## Results and Discussion

### Analysis of TNT Metabolites

Separation of TNT and its reduced metabolites has been problematic due to the co-elution of the isomers 2-ADNT and 4-ADNT, difficulties with the quantification of ionizable intermediates such as TAT due to speciation, and poor retention [21]. Octanesulfonic acid has been used as an ion-pair reagent to increase the retention time of the highly polar TAT [13].

The development of a gradient elution method for the separation of the most commonly reported TNT metabolites was accomplished after extensive investigation of various types of chromatographic columns (including both C-8 and C-18 packing materials), guard columns, and mobile phases (at various pH values). This method was then optimized further by the choice of temperature and addition of an ion-pair reagent.

UV-Spectra of all investigated TNT metabolites were compared to published spectra [21,24] for positive identification, except for the UV-absorption spectra of 4,4',6,6'-tetranitro-2,2'-azotoluene and 2,2',6,6'-tetranitro-4,4'-azotoluene, which are to our best knowledge shown here for the first time (Fig. 2.2).

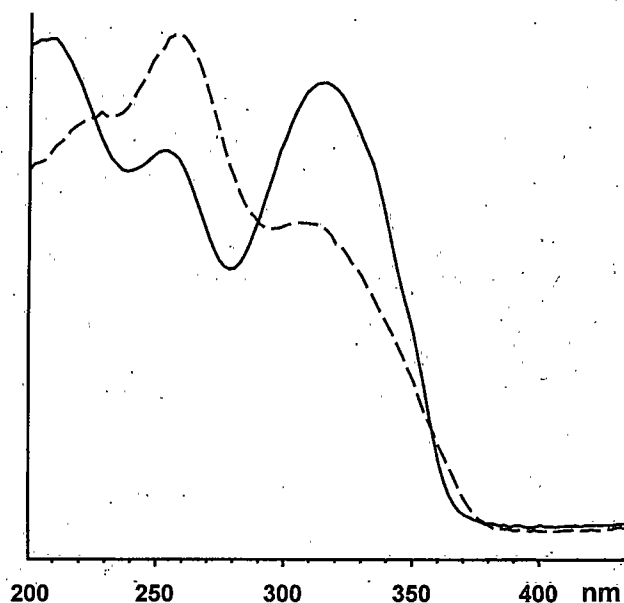


Figure 2.2. UV-Spectra of 4,4',6,6'-tetrinitro-2,2'-azotoluene (dashed line) and 2,2',6,6'-tetrinitro-4,4'-azotoluene (solid line).

The highest UV absorption was obtained at a wavelength of 230 nm for the majority of TNT metabolites. However, TAT, DANTs, 4,4'-azoxy, and 4,4'-azo had a higher absorption at 220 nm and 2,2'-azoxy and 2,2'-azo a slightly increased absorption at 254 nm (Fig. 2.2 and 2.3a) [21,24]. Most chromatograms shown herein are given at 254 nm unless otherwise noted, since it is the wavelength achievable on most HPLC-UV systems and the detector wavelength suggested by the EPA [27].

Influence of Temperature and an Ion-pair Reagent: The impact of temperature, on the separation of TNT metabolites in the absence of an ion-pair reagent is shown in Fig. 2.3a-b and in the presence of ion-pair reagent in Fig. 2.4. Although a temperature increase from 35 °C to 55 °C decreased the overall separation time by less than one

minute, the retention time of compounds such as the ADNTs decreased by approximately four minutes which resulted in a drastically improved overall separation of the compounds.

The isocratic retention as a function of temperature can often be described by the van't Hoff relationship ( $\log k' = a + b/T$ ;  $a$  and  $b$  are constants for a given compound and  $T$  is the absolute temperature) [28]. The following empirical relationship,  $t_r = a' + b'(1/T)$  where  $a'$  and  $b'$  are constants for a given solute as  $T$  is varied and all other conditions are kept constant, was derived by Zhu et al. [29] from the van't Hoff relationship based on assumptions such as  $k_0' \gg 1$  (value of  $k'$  at start of separation) and that  $S$  does not vary with temperature;  $S$  is a solute parameter (please refer to Zhu et al. [29] for further details).

A linear relationship ( $R^2 > 0.99$ ) was obtained when plotting the  $t_r$  as a function of  $1/T$  for the herein investigated compounds. The following order of temperature sensitivity was observed based on the calculated slopes ( $b'$ ): 4-ADNT ( $b' = 21738$ ) > 2-ADNT ( $b' = 21231$ ) > HADNTs >> TNT >> 2,4-DANT ( $b' = 3581$ ) > 2,6-DANT ( $b' = 2464$ ) (Fig. 2.3b). The linear relationship for the studied explosives can potentially be used to predict the retention time as a function of temperature and thereby help improving the separation of closely eluting compounds, which is in agreement with detailed studies by Zhu et al. [29,30].

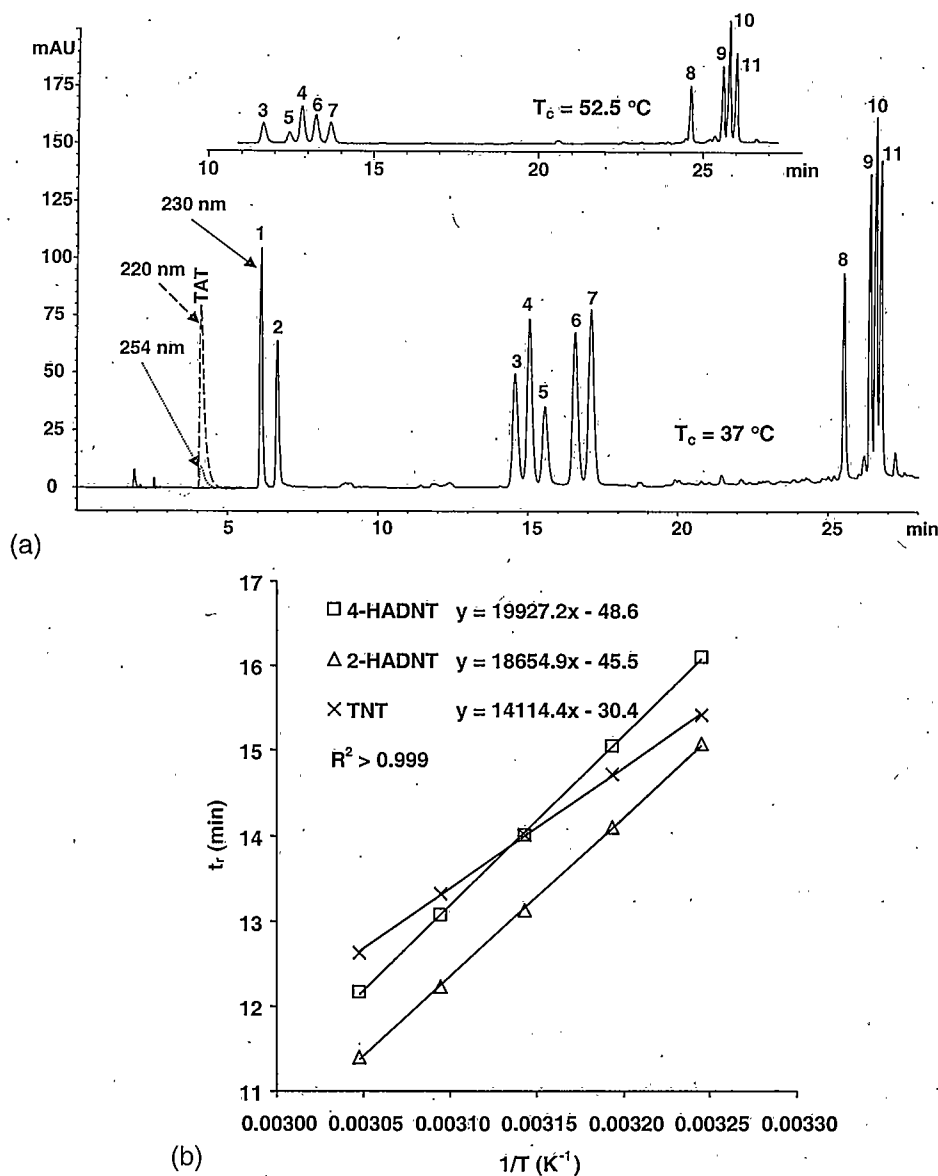


Figure 2.3. (a) Optimal separation of TNT metabolites in the absence of an ion-pair reagent at  $37\text{ }^{\circ}\text{C}$  (1 = 2,6-DANT; 2 = 2,4-DANT; 3 = 2-HADNT; 4 = TNT; 5 = 4-HADNT; 6 = 2-ADNT; 7 = 4-ADNT; 8 = 2,2'-Azoxo; 9 = 4,4'-Azoxo; 10 = 2,2'-Azo; 11 = 4,4'-Azo). Note the improved separation of the azoxy and azo compounds with increased temperature and baseline separation of all compounds at  $52.5\text{ }^{\circ}\text{C}$  (see inset).  $37\text{ }^{\circ}\text{C}$  was chosen as the best column temperature due to the potential for larger instrument-to-instrument variation at higher temperatures. (b) The retention time ( $t_r$ ) as a function of the absolute temperature for selected compounds.

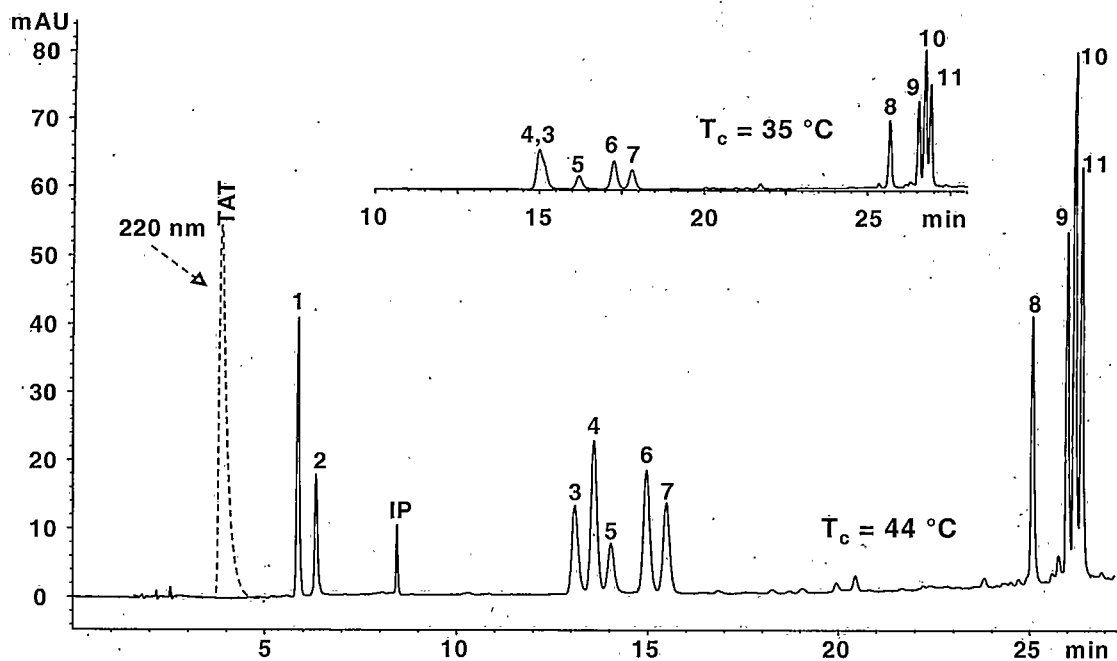


Figure 2.4. Optimal separation of TNT metabolites in the presence of an ion-pair reagent at 44 °C; the peak labeled "IP" is an inherent peak caused by the addition of the ion-pair reagent (1 = 2,6-DANT; 2 = 2,4-DANT; 3 = 2-HADNT; 4 = TNT; 5 = 4-HADNT; 6 = 2-ADNT; 7 = 4-ADNT; 8 = 2,2'-Azoxo; 9 = 4,4'-Azoxo; 10 = 2,2'-Azo; 11 = 4,4'-Azo). Note the co-elution of TNT and 2-HADNT at 35 °C (see inset).

In a recent review by Dolan [25] it was reported that a change of temperature especially influenced the selectivity of ionizable and polar compounds due to a possible concurrent change of the  $pK_a$ , which is consistent with the results observed in this study. The impact of temperature on the DANTs was attenuated compared to the ADNTs possibly due to a significantly higher  $pK_a$  value or other physical-chemical interactions, which were not further investigated as part of this study [25,29-32]. However, when comparing the relative shift in retention time of the polar TAT with TNT and 4-ADNT as a result of an increase in column temperature from 37 °C to 52.5 °C similar relative shifts were observed (i.e. 13 %, 16 %, and 19 %, respectively).

The increased temperature sensitivity of 2- and 4-HADNT, as compared to TNT, was used to optimize the peak separation (Fig. 2.3b). Most compounds were separated with baseline resolution ( $R_s \geq 1.5$ ) at a temperature of 37 °C except for 2,2'-azo and 4,4'-azo which had  $R_s$  values of 1.3 and 1.2, respectively (Fig. 2.3a). The resolution of the azo isomers was improved to  $R_s \geq 1.4$  by increasing the column temperature to 52.5 °C (inset in Fig. 2.3a and Table 2.1). However, 37 °C was chosen as the best-suited chromatography temperature due to potentially increased relative standard deviation, and potentially larger instrument-to-instrument variation at elevated column temperatures (Table 2.1) [26].

Increased temperature decreased the peak width ( $W$ ) of all peaks and increased the number of theoretical plates ( $N$ ) for the majority of peaks. In a few cases a decrease of  $N$  was observed. In the absence of an ion-pair reagent for instance, a temperature increase from 35 °C to 52.5 °C resulted in an approximately 33 % increase in  $N$  for 2,6-DANT, but a 13 % decrease in  $N$  for TNT.  $N$  should in theory increase as temperature increases, however, experimental studies have shown that this is not always the case in gradient elution [29].

An ion-pair reagent has often been used for the chromatography of TNT metabolites in order to prolong the retention time of the polar TAT [13]. The addition of an ion-pair reagent to the aqueous mobile phase in the present study resulted in the co-elution of 2-HADNT and TNT at the commonly used column temperature of 35 °C (inset in Fig. 2.4). However, the co-elution was completely avoided by increasing the temperature to 44 °C (Fig. 2.4).

Table 2.1. Retention time factor ( $k'$ ) and peak resolution ( $R_s$ ) calculated for the TNT metabolites (37 °C; Fig. 2.3a), EPA Method 8330 compounds (50.5 °C; Fig. 2.7a), and TNT metabolites combined with the EPA Method 8330 compounds (50.5 °C; Fig. 2.8a).

| Compound<br>(n = 6)    | TNT<br>metabolites <sup>a</sup> |         | EPA<br>compounds <sup>a</sup> |         | Metabolites and<br>EPA compounds <sup>a</sup> |         |
|------------------------|---------------------------------|---------|-------------------------------|---------|-----------------------------------------------|---------|
|                        | $k'^b$                          | $R_s^b$ | $k'^b$                        | $R_s^b$ | $k'^b$                                        | $R_s^b$ |
| TAT <sup>c</sup>       | 1.05                            | > 1.5   |                               |         | 0.83                                          | > 1.5   |
| 2,4-DHANT <sup>d</sup> | 1.95                            | n.a.    |                               |         |                                               |         |
| 2,6-DANT               | 2.05                            | > 1.5   |                               |         | 1.91                                          | > 1.5   |
| HMX                    |                                 |         | 1.89                          | > 1.5   | 1.91                                          | 0.0     |
| 2,4-DANT               | 2.32                            | > 1.5   |                               |         | 2.11                                          | > 1.5   |
| RDX                    |                                 |         | 2.74                          | > 1.5   | 2.74                                          | > 1.5   |
| 1,3,5-TNB              |                                 |         | 3.46                          | > 1.5   | 3.47                                          | > 1.5   |
| 1,3-DNB                |                                 |         | 4.17                          | > 1.5   | 4.19                                          | > 1.5   |
| NB                     |                                 |         | 4.94                          | > 1.5   | 4.97                                          | > 1.5   |
| 2-HADNT                | 6.27                            | > 1.5   |                               |         | 5.08                                          | 0.82    |
| Tetryl                 |                                 |         | 5.27                          | > 1.5   | 5.28                                          | > 1.5   |
| 4-HADNT                | 6.77                            | > 1.5   |                               |         | 5.49                                          | 1.4     |
| TNT                    | 6.51                            | > 1.5   | 5.61                          | > 1.5   | 5.63                                          | 0.94    |
| 2-ADNT                 | 7.27                            | > 1.5   | 5.89                          | > 1.5   | 5.91                                          | > 1.5   |
| 4-ADNT                 | 7.53                            | > 1.5   | 6.13                          | > 1.5   | 6.14                                          | > 1.5   |
| 2,4-DNT                |                                 |         | 6.36                          | > 1.5   | 6.38                                          | > 1.5   |
| 2,6-DNT                |                                 |         | 6.60                          | > 1.5   | 6.62                                          | > 1.5   |
| 2-NT                   |                                 |         | 7.52                          | > 1.5   | 7.55                                          | > 1.5   |
| 4-NT                   |                                 |         | 7.73                          | 1.3     | 7.75                                          | 1.3     |
| 3-NT                   |                                 |         | 8.06                          | > 1.5   | 8.08                                          | > 1.5   |
| 2,2'-Azoxy             | 11.7                            | > 1.5   |                               |         | 11.4                                          | > 1.5   |
| 4,4'-Azoxy             | 12.2                            | > 1.5   |                               |         | 11.8                                          | > 1.5   |
| 2,2'-Azo               | 12.3                            | 1.3     |                               |         | 11.9                                          | 1.4     |
| 4,4'-Azo               | 12.4                            | 1.2     |                               |         | 12.0                                          | > 1.5   |

<sup>a</sup> Ranges of relative standard deviation (R.S.D.) of the retention time ( $t_r$ ): T = 37 °C (0.01 – 0.06 %); T = 50.5 °C (0.01 – 0.17 %).

<sup>b</sup> Relative standard deviation (RSD) of  $k'$  and  $R_s$  << 1 % for all compounds.

<sup>c</sup> TAT was measured at 220 nm and run separately (n = 7).

<sup>d</sup> 2,4-DHANT was run separately because the chemical was obtained just before submission of this manuscript (n = 3).

A relatively low shift in retention time was observed in the presence of an ion-pair reagent compared to previously published results with Supelcosil C-18 and ABZ+ columns (25 cm by 4.6 mm; 5- $\mu$ m particles) [13,24]. The relatively low shift in  $t_r$  compared to these results could be due to a lower ion-pair reagent affinity to the (15 cm long) C-8 column packing material, differences in the mobile phase, or differences in the gradient elution. However, the exact reasons remain unknown and were beyond the scope of this investigation. The retention time of TNT decreased by 2.6 % in the presence of the ion-pair reagent possibly due to a change of polarity in either the mobile phase or the stationary phase. The temperature sensitivity in the presence of an ion-pair reagent decreased by 8 % to 17 % for all compounds based on comparisons of the calculated slopes ( $b'$ ) (4-ADNT ( $b' = 19985$ ) > 2-ADNT ( $b' = 19483$ ) > 4-HADNT ( $b' = 18103$ ) > 2-HADNT ( $b' = 16269$ ) > TNT ( $b' = 12014$ ) > 2,4-DANT ( $b' = 3031$ ) > 2,6-DANT ( $b' = 2054$ )) with the calculated slopes in the absence of an ion-pair reagent (see above and Fig. 2.3b).

The use of an ion-pair reagent for improved compound selectivity often has an unfavorable impact on various factors such as slow column equilibration and method ruggedness, however it is still one of the favored ways to increase the selectivity factor ( $\alpha$ ) of ionizable compounds in RP-HPLC [29]. The use of an ion-pair reagent in this study resulted in the appearance of an inherent peak (IP). In this case the inherent peak did not interfere with other peaks of interest (e.g. IP observed at 8.45 min in Fig. 2.4), however the presence of such an IP could complicate the detection of TNT related compounds in certain situations. Since the overall chromatography was not improved by

use of an ion-pair reagent in the aqueous mobile phase it is suggested to perform the chromatography in its absence, which will result in a significant cost reduction.

Method Performance. An acceptable level of reproducibility must be established before any separation method can be applied to the analysis of intricate environmental samples. Therefore, the chromatographic performance and reproducibility of the developed gradient method for the analysis of TNT and its metabolites was investigated at 37 °C and 52.5 °C (data not shown), which had demonstrated optimal separation in the absence of an ion-pair reagent, and at 44 °C (data not shown) in the presence of an ion-pair reagent. A mixture of TNT and its metabolites (solubilized in acetonitrile) was repeatedly injected and monitored for variability in retention time ( $t_r$ ), retention time factor ( $k'$ ), and peak resolution ( $R_s$ ) (Table 2.1). TAT was run separately, due to its poor solubility and stability in acetonitrile.

The retention time factors ( $k'$ ) for the less polar azoxy and azo compounds were significantly higher than for the other metabolites (Table 2.1). However, in order to obtain sufficient separation of the azoxy and azo compounds a slow increase of the methanol concentration was necessary. If the chromatography of the azo or both the azo and azoxy compounds is not desired the total run time can be significantly decreased by use of a steeper final gradient. If the chromatography of the azo compounds is not desired, the methanol concentration can be increased to 100 % over 8.5 minutes instead of 12.5 minutes, if neither azo nor azoxy compounds are of interest, the methanol concentration can be increased over 1 min.

Very good reproducibility and performance was obtained under all three conditions, however a slightly increased variation in the retention times was observed at 52.5 °C (range of RSD: 0.02 – 0.27 %;  $n = 3$ ). The retention time factors were less than 12.4 and the resolution was better than 1.2 for all compounds with baseline resolution ( $R_s \geq 1.5$ ) for most of the investigated compounds (Table.2.1).

Since sorption of an ion-pair reagent to the column packing material can change the characteristics of a column, the reproducibility of the presented results was tested by reconfirming the quality of separation with a new Supelcosil LC-8 column. The new column had never been exposed to ion-pair reagent. The retention times of the 14 EPA 8330 compounds at 50.5 °C changed by less than 2.2 % between the columns and the resolution was of the same quality as with the LC-8 column that had been exposed to ion-pair reagent. The slight change in  $t_r$  may be due to column aging rather than sorption of the ion-pair.

In order to test the developed method in a real case scenario samples were taken from a TNT biotransformation experiment [33] and analyzed using the developed gradient method at 37 °C. No significant retention time shift was observed over a period of 86 days after analysis of more than 500 samples from various TNT degradation studies using the same Supelcosil LC-8 chromatography column. The two chromatograms in Figure 2.5 demonstrate the degradation of TNT by strain ES6 in the presence of hydrous ferric oxide and the electron shuttling compound 9,10-anthraquinone-2,6-disulfonate (AQDS). TNT was completely transformed via the HADNTs and ADNTs metabolites to 2,4-DANT and TAT after 86 days.

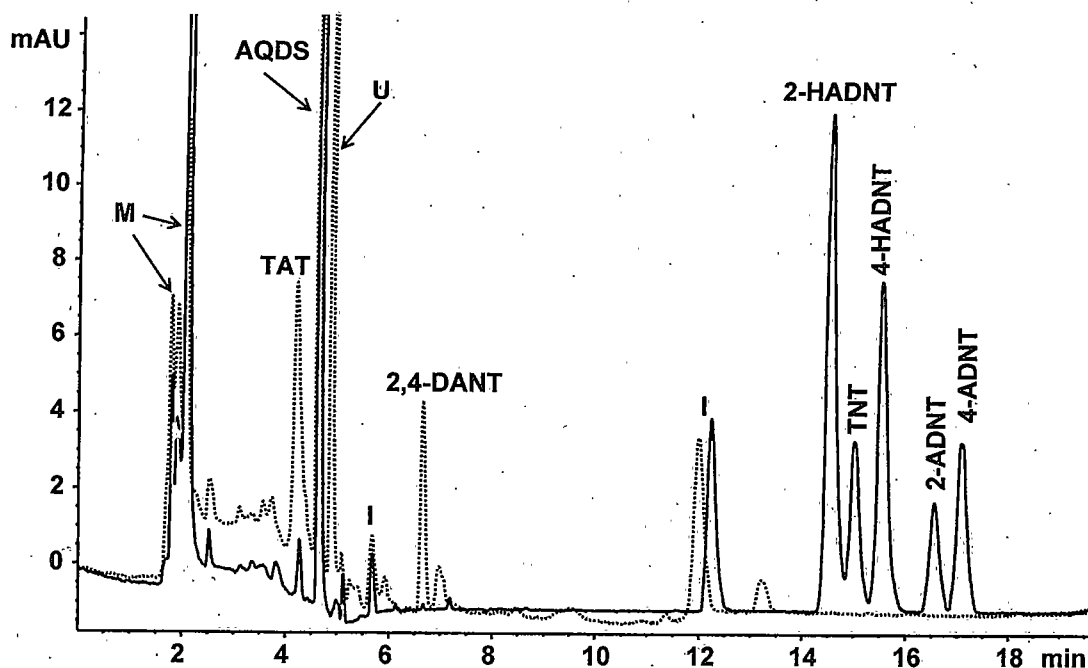


Figure 2.5. Randomly selected chromatograms from the analysis of aqueous samples of a TNT biodegradation experiment [33]. Test tubes contained synthetic ground water [34], carbonate buffer (pH 7), hydrous ferric oxide, 9,10-anthraquinone-2,6-disulfonate (AQDS), sucrose, and strain ES6, tentatively identified as a *Cellulomonas* sp. [33]. The test tubes were injected with 52  $\mu$ M of TNT after 14 days of inoculation and TNT transformation and metabolite patterns were observed over time. The solid line and the dotted line show TNT and its metabolites after 3 days and 86 days of incubation, respectively. The chromatograms are shown at 230 nm and were obtained using the developed gradient elution method at 37 °C. M: microbially related metabolites, U: unidentified peaks, I: sample inherent peaks.

#### Analysis of EPA Method 8330 Explosives

Fourteen explosives, octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), 1,3,5-trinitrobenzene (TNB), 1,3-dinitrobenzene (DNB), nitrobenzene (NB), TNT, 2,4-dinitrotoluene (2,4-DNT), 2,6-dinitrotoluene (2,6-DNT), 2,4,6-trinitrophenylmethylnitramin (Tetryl), 2-ADNT, 4-

ADNT, 2-nitrotoluene (2-NT), 3-nitrotoluene (3-NT), and 4-nitrotoluene (4-NT) are included in the EPA Method 8330 [35].

The main difficulty with the isocratic EPA Method 8330 is the reported co-elution of the DNTs and ADNTs when employing the recommended primary C-18 (250 X 4.6 mm, 5  $\mu$ m) RP-HPLC column [3,27,35]. Consequently a second confirming run is needed for the separation of the DNTs and ADNTs, which is commonly performed by utilizing a CN (250 X 4.6 mm, 5  $\mu$ m) RP-HPLC column [27,35]. The co-elution problem of DNTs and ADNTs was solved in a recent study by use of a two-phase approach in a single RP-HPLC run. Lang and Burns [3] used a CN guard column in series with a C-18 column, while keeping the EPA Method 8330 specifications. Lang and Burns [3] demonstrated that, although, the two-phase approach resulted in a total run time of approximately 32 min compared to approximately 24 min for the EPA Method 8330, it only required one HPLC run since the co-elution of the DNTs and ADNTs was avoided [3,35].

The goal of this part of the study was to develop an alternative method, that would decrease the total run time, reduce the consumption of solvents (mobile phases) compared to the discussed methods, and still baseline separate all 14 EPA Method 8330 compounds.

Influence of Temperature and an Ion-Pair Reagent. Baseline resolution was achieved for all compounds (except for 4-NT;  $R_s = 1.3$ ) by optimizing the column temperature of the described gradient elution method. Fig. 2.6 illustrates how the increased temperature sensitivity of the ADNTs can be used to separate them from the

DNTs. 4-ADNT and 2,4-DNT co-eluted at 42 °C but were completely separated at 50 °C. The higher temperature sensitivity of the ADNTs might be due to the ionizable character of these compounds [25]. An optimum temperature of 50.5 °C was found to rapidly (18 min), reliably (RSD < 0.2 %), and fully separate all the EPA Method 8330 compounds (Fig. 2.7a and Table 2.1).

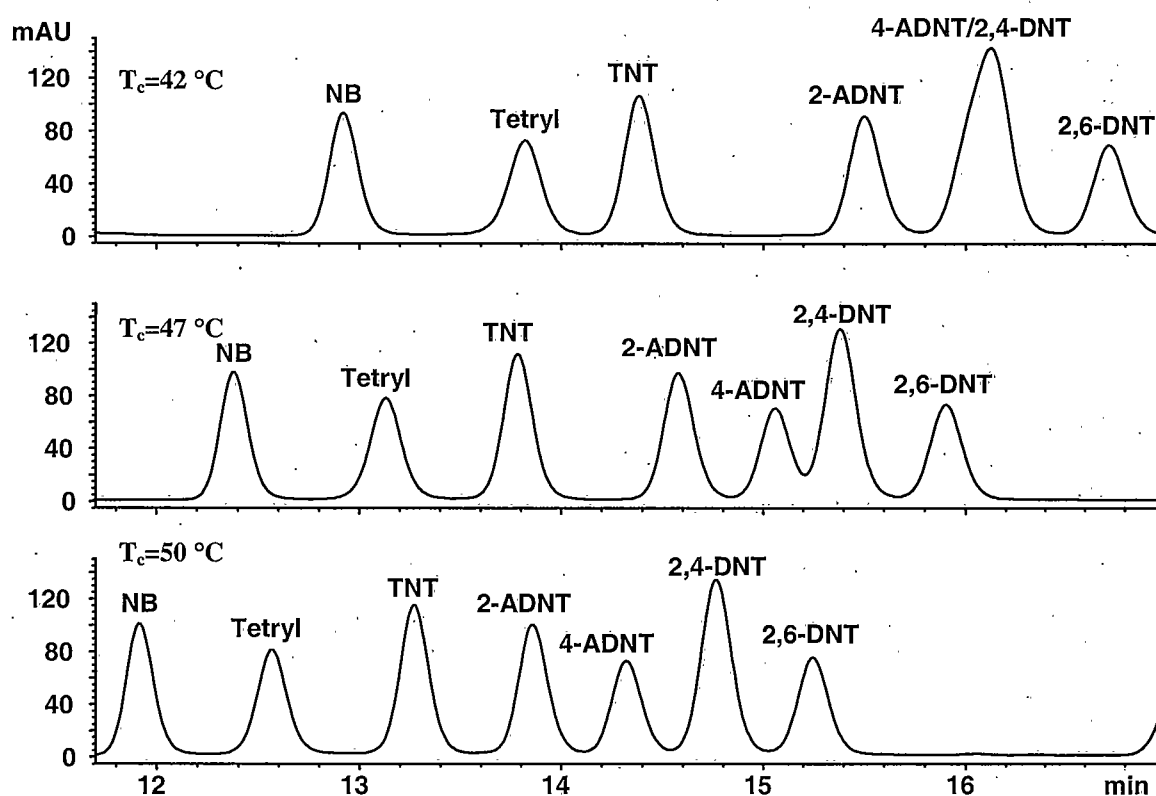


Figure 2.6. Influence of temperature (42, 47, and 50 °C) on the separation of selected EPA Method 8330 chemicals in the absence of an ion-pair reagent. Note the co-elution of 4-ADNT and 2,4-DNT at 42 °C and the improved separation with increased temperature.

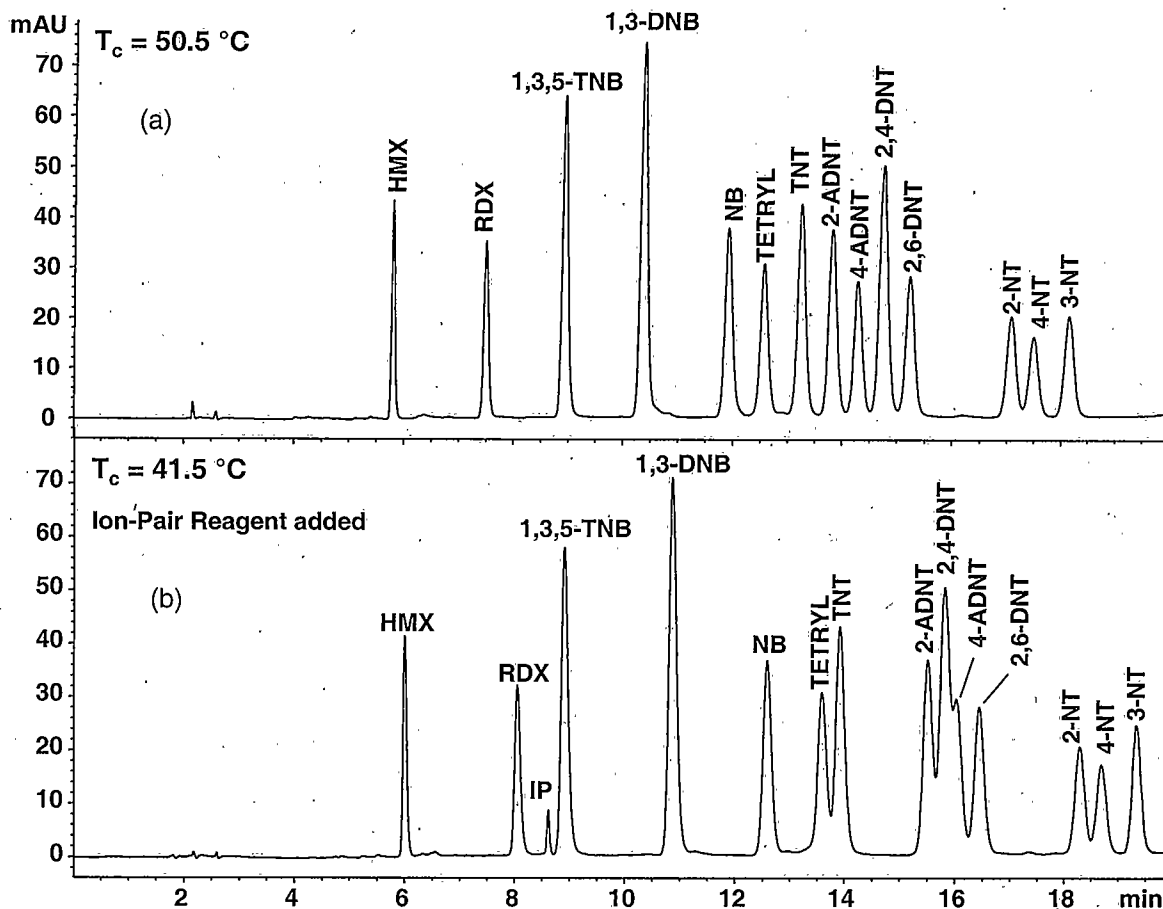


Figure 2.7. (a) Baseline separation of EPA Method 8330 compounds at the optimized temperature of  $50.5\text{ }^{\circ}\text{C}$  (no ion-pair reagent added). (b) Separation of the fourteen EPA Method 8330 chemicals in the presence of an ion-pair reagent at the optimized temperature of  $41.5\text{ }^{\circ}\text{C}$ . Note how the ion-pair reagent prevents baseline separation of several compounds.

The flow rate in this study was  $1\text{ ml/min}$  as compared to  $1.5\text{ ml/min}$  used in the EPA Method 8330 and the method proposed by Lang and Burns [3,34]. Additionally, the total run time in this study was reduced by approximately  $7\text{ min}$  ( $\approx 22\%$ ) compared to Lang and Burns [3], based on a seven minutes column conditioning time following the elution of 3-NT. The combined decrease in total run time and flow rate resulted in an

approximately 48 % reduction in solvent consumption as compared to Lang and Burns and a 30 % reduction compared to the EPA Method 8330 [3,34].

Optimal chromatographic separation in the presence of an ion-pair reagent was obtained at 41.5 °C (Fig. 2.7b). The temperature selectivity of the ADNTs relative to the DNTs was decreased in the presence of an ion-pair reagent, exemplified by the reverse elution order of 4-ADNT and 2,4-DNT (Fig. 2.7a-b). The affinity of the ion-pair reagent for the ADNTs resulted in increased retention times and consequently a poor separation of 2-ADNT, 2,4-DNT ( $R_s = 0.83$ ), and 4-ADNT ( $R_s = 0.63$ ) (Fig. 2.7b). Hence, it is not recommendable to use an ion-pair reagent for the separation of compounds included in the EPA Method 8330.

#### Combined Analysis of TNT Metabolites and EPA Method 8330 Compounds

Soils and water contaminated with TNT and its metabolites often contain co-contaminants such as the compounds included in EPA Method 8330 [18,27]. Thus, it is important to minimize the potential for co-elution of chemicals to prevent false positive results when analyzing complex environmental samples. It was therefore tested whether the developed gradient method would be capable of separating all 23 chemicals investigated in this study ranging from the very polar TAT to the less polar nitrotoluene, azoxy, and azo compounds in a single run.

Various temperatures were investigated (data not shown) and it was found that the same temperature used for the optimal separation of EPA Method 8330 compounds (50.5 °C) gave the most satisfying chromatographic separation (Fig. 2.8a and Table 2.1). 2,6-

DANT and HMX co-eluted and 2-HADNT and TNT had a peak resolution of less than 1. Nevertheless, reliable identification was obtained for the majority of the investigated compounds including the 7 pairs of isomers contained in the analyte mixture (Table 2.1).

Decreasing the steepness of the proposed initial methanol gradient can prevent the co-elution of 2,6-DANT and HMX. However, this will result in a poor separation of compounds with longer retention times (data not shown). The use of an ion-pair reagent in the aqueous mobile phase at 41.5 °C allowed for a slightly better separation of 2,6-DANT and HMX ( $R_s \sim 0.7$ ), however, it also resulted in an overall decreased chromatographic resolution (Fig. 2.8b).

The peak labeled IMP was an inherent impurity (IMP) originating from the 4-HADNT standard. The peak labeled A in the chromatogram is a possible transformation product, which accumulated over time. It is believed, based on previous studies [5,36], that the degradation product (A) originated from the spontaneous dimerization of the oxygen sensitive HADNTs potentially producing an additional azoxy-isomer (e.g. 2,4',6,6'-tetranitro-2',4'-azoxytoluene or 2',4,6,6'-tetranitro-2,4'-azoxytoluene). This was supported by an abiotic experiment investigating the resulting transformation product after oxygen-purging a standard mixture containing only 2- and 4-HADNT by comparison of the DAD spectra (data not shown).

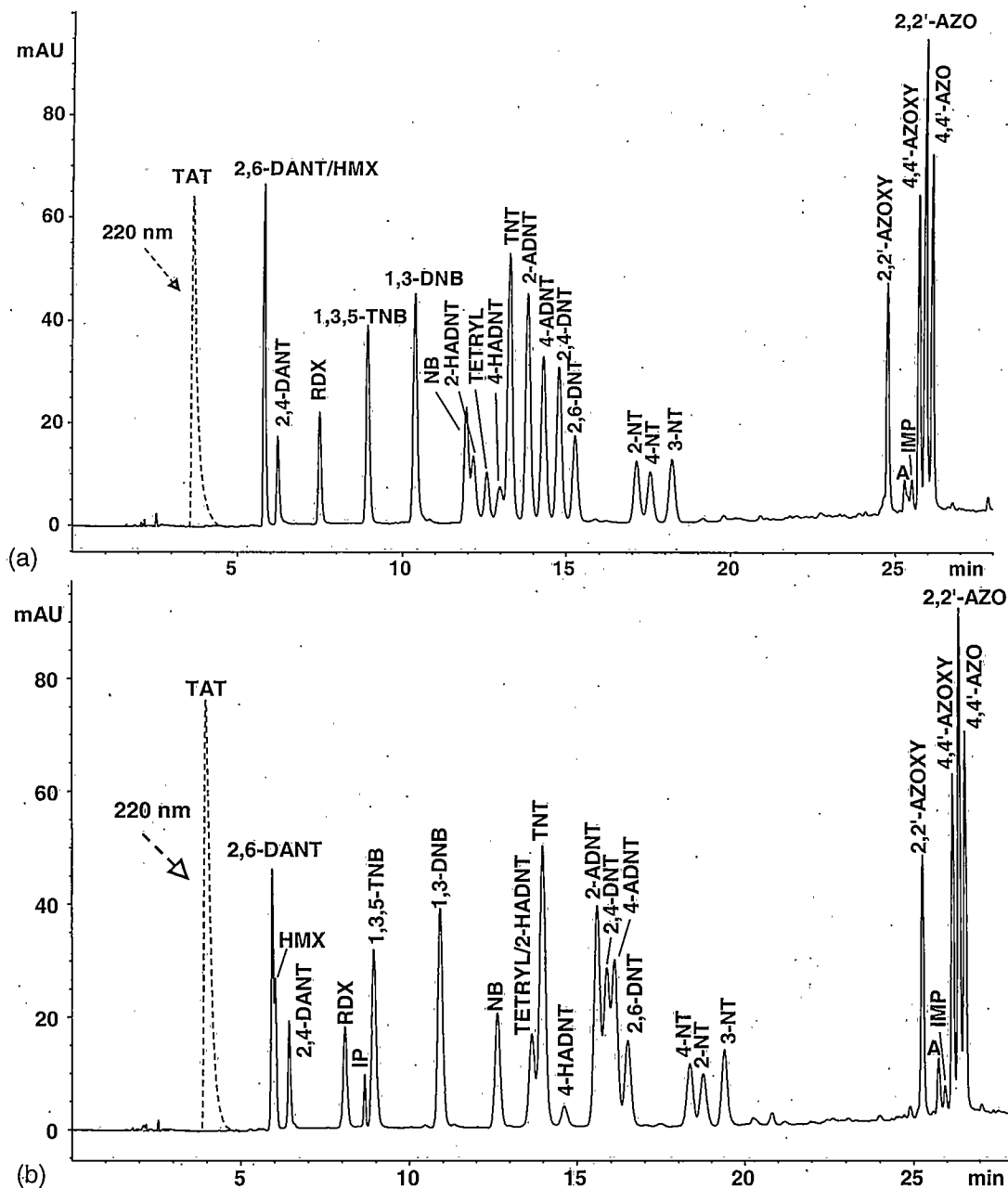


Figure 2.8. (a) Separation of the 23 TNT metabolites and EPA Method 8330 compounds at 50.5 °C in the absence of an ion-pair reagent. (b) Separation at 41.5 °C in the presence of an ion-pair reagent. Note the slightly improved separation of 2,6-DANT and HMX in the presence of an ion-pair reagent but the overall decreased chromatographic resolution. "A" is a possible transformation product (probably 2,4',6,6'-tetrinitro-2',4'-azoxytoluene, see text for discussion) and "IMP" is an inherent impurity contained in the 4-HADNT standard.

### Conclusion

This study demonstrates for the first time the importance of optimizing the temperature for the improved separation of complex mixtures containing explosives-related compounds. The findings herein evoke that column temperature should not always be used as the last parameter to optimize RP-HPLC methods. Additionally, this work is a supplement to research illustrating the importance of column temperature for the improved separation of compounds like chlorophylls, herbicides, peptides, and drugs (e.g. anticancer agents) [25,30,37,38].

The developed gradient method is unique because the same method at different temperatures can be used to completely separate TNT and 12 of its reduced metabolites as well as the compounds targeted in the EPA Method 8330. The TNT metabolites included 2,4,6-triaminotoluene (TAT), the DANTs, ADNTs, HADNTs, 2,4-DHANT, tetranitroazoxytoluenes, and tetranitroazotoluenes.

The proposed chromatographic method for the EPA Method 8330 compounds does not only provide improved separation of the 14 target compounds but also a significant reduction of total run time and solvent consumption when compared to previous studies [3,27,35].

The use of an ion-pair reagent for increased selectivity was also investigated. However, the use of the costly ion-pair reagent could be totally avoided by optimizing the column temperature.

Finally, the gradient method proved to be capable of separating all 23 explosives-related compounds, including the 12 reduced TNT metabolites tested, and the EPA

Method 8330 compounds in a single run except for 2,4-DANT and HMX, which co-eluted. Thus, the gradient elution method described here can become a valuable tool for the fast and reliable analysis of complex samples containing mixtures of nitroaromatics, aminoaromatics, and nitramines.

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## CHAPTER 3

CHARACTERIZATION AND COMPARISON OF IRON (HYDR)OXIDE ANALOGS  
FOR WATER RESEARCH BY USE OF IMAGING AND ANALYTICAL  
TECHNIQUESIntroduction

In the United States approximately 15 % of all pipelines supplying drinking water are unlined cast iron pipe [1]. Corrosion products (CP) easily form at the iron-water interface and can lead to undesirable changes in the drinking water quality within the distribution system. CP react with chlorine based disinfectants resulting in a reduction of disinfectant residual [2]. The presence of unlined cast iron pipe has been correlated with an increase in total coliforms [3], which are indicators of possible microbial contamination. It has been shown that biofilm that forms on the walls of pipelines can contain a number of undesirable organisms, and that biofilm forms more readily when CP and natural organic mater (NOM) are present [4]. The adsorption of NOM to CP is believed to promote biofilm growth. Therefore, it has been recognized that in order to understand the complex interactions between CP, biofilm, NOM and other water constituents, it is vital to characterize the minerals that comprise the CP [2,5].

Synthetic iron (hydr)oxides have often been used as models for iron CP for improved homogeneity in experimental systems [4], however, potential problems can arise from oversimplification of natural iron (hydr)oxide mixtures being modeled as demonstrated by Perret et al. [6]. In addition, application of synthetic iron (hydr)oxides to

granular filter media has been used for the removal of particulates [7] and organic matter [8,9] and for the immobilization of metals (e.g. arsenic) and radionuclides (e.g. strontium) from waste-, drinking-, and ground-water [10-12].

CP are composed primarily of iron (hydr)oxides [2]. The complexities of iron (hydr)oxide CP make it difficult to select appropriate analogs for elucidation of the interactions between CP, biofilm and NOM. The different (hydr)oxide phases comprising CP possess different chemical properties such as free energy of formation, crystal structure, morphology, and sorptive properties. The role of these different phases in biofilm formation and HA adsorption requires a better understanding of the properties of these phases. The highly reactive nature of iron (hydr)oxides often results in the formation of surface layers with properties distinctly different from the bulk. A systematic investigation of model iron (hydr)oxide surface properties should lead to a better understanding of how iron (hydr)oxides control biofilm and HA interactions with CP.

In the present investigation, a variety of bulk and surface analytical techniques were evaluated for the characterization of two iron (hydr)oxide CP analogs; crushed tubercles from the interior of a corroded drinking water pipe and a synthetic mineral analog. These results are important in interpreting the response of engineered and experimental systems where interactions between iron oxide phases and disinfectant, natural organic matter, and biofilm growth are investigated.

## Experimental Section

### Iron (Hydr)Oxide Analogs

The two iron (hydr)oxide analogs investigated were the same as those used in a water distribution study described elsewhere [4]. The first analog was crushed corrosion products (CP) from an unlined cast iron pipe, while the second consisted of a synthetic iron oxide deposited as a thin film on a glass substratum. CP were obtained from an iron pipe recovered from a water distribution system in the greater Boston area (Massachusetts, USA) that had been in operation for approximately 80 years. CP were dried under a flow of  $N_2$  and crushed. Particles analyzed passed a No. 10 (2.00-mm) sieve. CP were subsequently washed using Nanopure (Barnstead-Thermolyne, Dubuque, IA) reagent-grade water, dried and stored under  $N_2$ . During the removal, crushing, and sieving processes the CP were exposed to atmospheric oxygen and some oxidation may have occurred because of exposure to the ambient atmosphere. However, the aim was not to characterize the CP in its pure natural state as attempted previously by Sarin et al. [2], but rather to compare and characterize two iron (hydr)oxide CP models for simple use in the laboratory and total oxygen elimination was not feasible in the present studies. Glass beads (GB) with a nominal diameter of 0.5 mm (Biospec Products, Inc., Bartlesville, OK) were coated with iron oxides (IOCB) using a forced acid hydrolysis technique similar to the protocols of Rieke et al. [13] with exception of the sulfonated self-assembled monolayer. IOCB were rinsed with Nanopure water, dried at room temperature, and stored under  $N_2$ . The same iron oxide coating technique was applied to glass cover slips

(IOCC) (Fisher Scientific, 0.13-0.17 mm thickness). IOCC were analyzed by AFM and GID to avoid geometric constraints of spherical IOCB.

#### Reference Iron Oxides

Five iron oxides in powder form were used as reference compounds in this investigation: hematite ( $\alpha\text{-Fe}_2\text{O}_3$ ), goethite ( $\alpha\text{-FeOOH}$ ), magnetite ( $\text{Fe}_3\text{O}_4$ ) and maghemite ( $\gamma\text{-Fe}_2\text{O}_3$ ), all commercial products (Alfa Aesar, Ward Hill, MA), and 2-line ferrihydrite ( $\text{Fe}_{1.55}\text{O}_{1.66}(\text{OH})_{1.33}$ ) synthesized according to Cornell and Schwertmann [14].

#### Bulk Properties

The total iron content of the CP and IOCB was determined (Hach Method 8147–FerroZine Method) by digestion in concentrated HCl and subsequent spectrophotometric analysis [4]. Specific surface area of GB, IOCB, and CP samples was measured with a FlowSorb 2300 (Micromeritics, Norcross, GA) using a three-point Brunauer, Emmett and Teller (BET) N<sub>2</sub> sorption isotherm. An acid-base titration modified from Chang [15] was used to estimate the proton-binding capacity.

#### Scanning Electron Microscopy (SEM) and Energy Dispersive Spectroscopy (EDS)

SEM and EDS was applied to obtain high resolution 3D images and the elemental composition. The instrument used was a JEOL 6100 SEM with a LaB<sub>6</sub> source (JEOL USA Inc., Peabody, MA) coupled to a Noran Voyager X-ray detector for elemental analysis (1- $\mu\text{m}$  spot size, Thermo NORAN Inc., Middleton, WI) and a Rontec Xflash detector for elemental mapping (Rontec USA, Inc., Acton, MA). EDS spectra in the

range of 0-10 eV were obtained using an accelerating voltage of 15 kV. The working distance was 8-39 mm for high-resolution imaging and 39 mm for EDS analysis and elemental mapping. All samples were carbon-coated before SEM and EDS analysis. Digital imaging (using both secondary electron and backscattered electron signals), EDS spot analysis, and 2-dimensional elemental mapping were used to distinguish mineral phases and characterize their morphology, texture and composition.

#### Atomic Force Microscopy (AFM)

AFM was used to obtain 3D topographic images. Image analysis provided roughness and surface area information with sub-nanometer height resolution under both wet and dry conditions. IOCB and GB were carefully pressed into soft indium foil before they were mounted to the sample stage. CP proved too rough for our instrument. IOCC samples were mounted with double-sided tape to the sample disc, and an uncoated glass cover slip (UC) was used as a reference. All AFM images were obtained using a Nanoscope IIIa Extended Multimode AFM from Veeco Metrology (Santa Barbara, CA). Tapping mode images in air were obtained using silicon tips and cantilevers (TAP 300 HD with a nominal tip radius of less than 10 nm and a specified nominal cantilever spring constant of 40 N/m from Nano-Devices, Santa Barabara, CA). Contact mode images, in both air and liquid, were obtained using silicon nitride tips and cantilevers (TM microscopes (NPS 20) with a specified nominal cantilever spring constant of 0.3 N/m). Height images were minimally processed using a 1<sup>st</sup> order flatten procedure to prevent introduction of artifacts [16].

Depth Profiling by Time of Flight  
(Dynamic) Secondary Ion Mass  
Spectrometry (ToF-SIMS)/AFM

ToF-SIMS was used to recover fragments of ionized molecules from the outermost atomic layer of the surface. ToF-SIMS/AFM for depth profiling has the advantage of sample imaging, spatial resolution and high surface sensitivity [17]. The IOCC coating was depth profiled using a TRIFT I Time-of-Flight Secondary Ion Mass Spectrometer (ToF-SIMS, PHI-Evans, Eden Prairie, MN) with a pulsed gallium liquid metal ion gun fired at 25 keV with a 10-kHz repetition rate, serving as the primary ion source. IOCC and UC samples were sputter coated with gold (less than 10 nm) to prevent charge buildup and then introduced into a vacuum of less than  $1 \times 10^{-8}$  torr. The raster area for sputtering was  $40 \mu\text{m}^2$ , while the raster area for analysis of secondary ions was  $25 \mu\text{m}^2$  in the center of the sputtered area. Sputtering intervals for depth profiling were 15 s, and secondary ion collection intervals were 60 s. The boundary between the glass and the iron oxide coating of IOCC was located by sputtering the surface and monitoring iron and silicon peaks until the counts for silicon ceased to increase with further sputtering. The depth of the ablation pits was directly measured using the section analysis mode of the AFM.

Transmission Electron Microscopy  
(TEM) and Selected Area Electron  
Diffraction (SAED)

TEM-SAED was applied to obtain atomic-resolution images and crystal information on selected areas. Specimens of the synthetic coating were prepared by sonication of IOCB in deionized water and collection of the suspended material onto a C-

coated Cu grid with a formvar support film. CP were deposited directly onto the Cu grid. Imaging and analyses were performed using a JEOL 2010 high resolution TEM (JEOL USA Inc., Peabody, MA), equipped with a LaB6 filament, operated at 200 kV with a resolution of 1.9 Å. The JEOL 2010 was coupled to a Oxford EDS system using Link ISIS analytical software. Images were analyzed using Digital Micrograph software (Gatan Inc., Pleasanton, CA). Selected area electron diffraction (SAED) ring patterns formed by nanoparticles were collected and evaluated by Desktop Microscopist software (Lacuna Laboratories, Tempe, AZ). The nominal accuracy of the d-spacings was approximately 0.1 Å.

#### X-Ray Photoelectron Spectroscopy (XPS)

XPS was applied to achieve information of binding energies, elemental composition, and oxidation states. IOCC, UC samples, and the reference samples of hematite, goethite, magnetite, and ferrihydrite were analyzed directly. The CP were ground to a powder with a Diamonite (sapphire) mortar and pestle and inserted rapidly (< 5 min) under ambient conditions. Spectra were collected with a Model 5600ci spectrometer (Perkin Elmer Corp.) employing a monochromated Al  $K\alpha$  X-ray source (1486.6 eV) at 300 W and a 5 eV flood gun. Pass energies of 58.7 eV were used for broad scans and 5.85 (IOCC/UC) and 23.5 (CP) eV for high resolution scans. Binding energies were referenced to the adventitious carbon peak (C1s) at 285.0 eV. Component positions were determined by the location of individual Gaussian-Lorentzian peaks, obtained by deconvolution of the XPS spectra using a nonlinear least squares fitting routine with adjustable polynomial baseline using RBD AugerScan 3.0.1 software.

Powder and Grazing Incidence (GI)  
X-Ray Diffractometry (XRD)

XRD was applied for bulk characterization of crystalline materials. The X-ray diffraction apparatus used in this study was a Philips X'Pert MPD system. The X-ray source was operated at 40 kV, 50 mA ( $\text{LAMBDA} = \text{Cu K}\alpha_1$ , 1.5406 Å). The study specimen was examined in Bragg-Brentano para-focusing geometry on a 190-mm 2-theta goniometer radius using incident- and diffracted-beam Soller slits (0.04 radians), automatic divergence and anti-scatter slits, and a 0.2-mm receiving slit. Wavelength selection was achieved with a graphite diffracted beam monochromator, and the detector was a Xe-filled proportional counter. The specimen holder was a single-crystal quartz plate. The diffractometer was controlled using the Philips X'Pert software suite (X'Pert Data Collector, V1.3d). Data analysis was accomplished using Jade V6.5.7 (Materials Data, Inc., Livermore, CA) and the Powder Diffraction File database (PDF-2, 2002 Release, International Centre for Diffraction Data, Newtown Square, PA). The CP were ground to a powder with a Diamonite mortar and pestle. Scan Parameters were: 2-theta = 5.00°-75.00°, and the scan rate was 0.02°/5s. The iron oxide thin film was examined in grazing-incidence geometry on a 220-mm 2-theta goniometer radius. The incident-beam optic was a Göbel mirror (parallel beam). The receiving optics were a 0.27 radian parallel plate collimator and a flat graphite monochromator. The incident-beam angle (OMEGA) was fixed at 2.50°, and the scan axis was 2-theta. The scan range was 15.00°-75.00°, and the scan rate was 0.05°/45s. The beam spot was approximately 20 mm wide and, at OMEGA = 2.50°, approximately 27 mm long.

## Results and Discussion

Characterization of the CP and IOCB surfaces was done for two reasons. The first was to provide information on the nature of these two iron oxides using a suite of tools to reveal similarities and differences in composition and reactivity. Secondly, the data were useful in giving insight on the manner in which these two materials interacted with environmental conditions used to test the ability of bacteria to form biofilms in simulated drinking water systems. Knowledge of the material characteristics allowed for comparison and contrast of the results obtained in these experiments and provides a basis for selection of relevant analogs for investigating iron oxide interactions in other complex environmental systems.

### Bulk Properties

The iron content of the IOCB was 7.35 mg Fe (coating)/m<sup>2</sup> beads. This result is similar to that reported for sand coated by baking with FeCl<sub>3</sub> sludges (9.33 mg/m<sup>2</sup>) [9] and three times lower than that reported for the adsorption of goethite crystals to sand (24.2 mg/m<sup>2</sup>) [18]. Iron content of the CP was 22.4 mg Fe/m<sup>2</sup> CP, approximately 3 times greater than that of the IOCB. Reactive surface areas for IOCB and GB were 0.068 ± 0.01 m<sup>2</sup>/g and 0.064 ± 0.02 m<sup>2</sup>/g, respectively, based on BET analysis. On a per gram basis, BET areas for IOCB are forty times lower than those reported for sand coated by baking with FeCl<sub>3</sub> sludges [9]. The measured surface area of 26.2 ± 0.69 m<sup>2</sup>/g for CP fell within the range of values reported from various studies of natural and synthetic iron (hydr)oxide crystals as reviewed by Cornell and Schwertmann [14] and comparable to the

area ( $21.3 \text{ m}^2/\text{g}$ ) of goethite reported by Scheidegger et al. [18], which was later observed to be the major iron phase. These results were just below the range of 32 to  $82 \text{ m}^2/\text{g}$  reported for corrosion scales from old iron drinking water pipes in the Boston area [2]. The higher surface area observed by Sarin et al. [2] might be due to the pulverization process during sample preparation; this study selected particles of a certain sieve size.

The surface site density estimated by acid titration to pH 3.00 was  $14 \text{ meq/g Fe}$  for IOCB and  $0.714 \text{ meq/g Fe}$  for CP, indicating that IOCB had approximately 20 times the capacity to accept protons under these titration conditions on a per gram of iron basis. The usefulness of this titration experiment was to demonstrate that IOCB had between one and two orders of magnitude greater proton binding capacity per unit mass of iron than CP over a time scale of several days. Normalization of IOCB and CP site densities to their respective BET areas shows that IOCB have an approximately 6.5 times greater site density per unit area. Caution should be used in extrapolating such bulk results since major differences in sorptive capacity may exist for other sorbates and conditions [19]. This was evident in the experiments where these materials were exposed to humic substances and disinfectants where the CP had a higher reactivity to both. [4].

#### Scanning Electron Microscopy (SEM) and Energy Dispersive Spectroscopy (EDS)

Representative secondary electron images are shown in Figure 3.1. Comparison of the GB image (Fig. 3.1a) with the IOCB image (Fig. 3.1b) shows limited impact of the coating on the overall physical structure of the surface (due to the inherent roughness of the GB), which is consistent with the BET results. For CP, most images could be

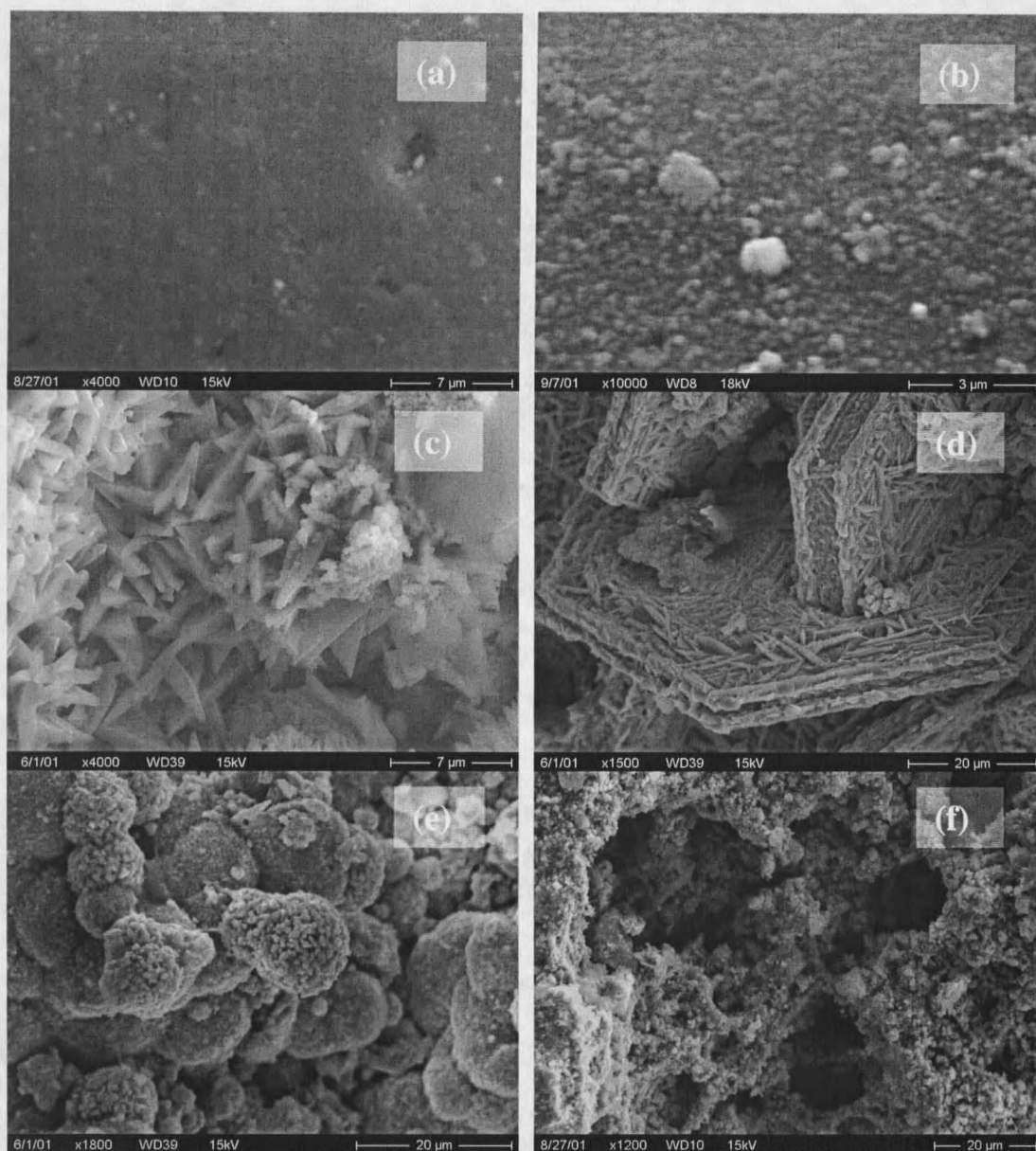


Figure 3.1. Scanning electron microscopy (SEM) secondary electron images of (a) an uncoated glass bead (GB), (b) an iron oxide-coated glass beads and (c-f) the four dominant morphologies found in CP: (c) tabular crystals with some rhombohedral forms evident; (d) hexagonal plates organized into various microstructures coated with needle shaped crystals likely goethite; (e) spherical (or globular) nodules; (f) micro- or crypto-crystalline materials that may include amorphous compounds.

divided into four distinct morphological types (Fig. 3.1c-f), which were similar to those found in other published SEM images of corroded iron pipes [20].

Visual identification of the morphologies present in the CP proved challenging, however, the crystals observed in Fig. 3.1c appear like multidomainic or bipyramidal crystals (e.g. goethite) or the structures could alternatively be octahedral crystals (e.g. magnetite) [14]. The hexagonal structures in Fig. 3.1d may be green rust and appears to be covered with needle shaped goethite [14,21,22]. The globular structures in Fig. 3.1e are likely magnetite [14,23]; interestingly, cocci-shaped bacteria were found inside some of these globules (data not shown) similar to studies by Dong et al. [23]. Fig. 3.1f seems to represent micro-crystalline materials or amorphous iron such as hydrous ferric oxides [14]. SEM proved to be a useful tool for visual comparison of natural and synthetic iron (hydr)oxide surfaces. CP appeared very rough, with a large density of macropores, while IOCB had much less variation in particle size; although SEM is not an ideal tool to elucidate roughness. CP clearly contained a large portion of crystalline material, while IOCB coating appeared as a thin scattering of spheres on the order of 100-500 nm in diameter. Although SEM was able to document the presence of several distinct crystal morphologies on the surface of CP, such images cannot be used independently for mineral identification.

EDS elemental analysis results are shown in Table 3.1 for CP, IOCB, and GB. The CP results were similar to those reported by Sarin et al. [2] for corrosion scales in old iron pipes from the Boston area. Additionally, the authors reported between 1 and 4 atom % Al and between 1 and 11 atom % Si as well as sporadic findings (< 2 atom %) of Ca,

Cu, and Zn. In this study Ca, Al, Si, Na, and Mg were all present in less than 1 atom %. It is known that the aqueous composition and other factors impact the formation of crystals, which may explain some of the observed differences [13,14]. Recent water quality data for the two systems are comparable, with the exception that the CP used in this study were from a system with higher mean sulfate levels (32.0 mg/l compared to 5.6 mg/l). The sulfate form of green rust was later suggested by XRD.

Table 3.1. Electron Dispersive Spectroscopy (EDS) elemental composition of corrosion products (CP), iron oxide coated beads (IOCB), and a glass bead (GB). All values are in atom %. Reported values are normalized and values of less than 1% are reported in the text. Numbers of replicates analyzed are based on variability found in preliminary investigations:

| Sample | Replicates | Ca  | Fe   | Na  | Mg  | Al  | Si   | S   |
|--------|------------|-----|------|-----|-----|-----|------|-----|
| CP     | 8          | -   | 92.3 | -   | -   | -   | -    | 7.7 |
| IOCB   | 3          | 7.7 | 3.5  | 9.9 | 5.1 | 2.3 | 71.5 | -   |
| GB     | 1          | 8.7 | -    | 9.2 | 6.0 | 1.9 | 74.3 | -   |

EDS of GB showed typical glass composition including a majority of Si with small amounts of Na, Mg, Al, and Ca. EDS of IOCB showed a similar composition to GB, with the addition of a small amount of Fe, demonstrating that the 1 to 2  $\mu\text{m}$  excitation depth under the electron beam exceeded the thickness of the iron oxide coating or suggesting that the Fe surface coverage was incomplete.

Rastering of the EDS beam (1  $\mu\text{m}$  spot size) across the sample surfaces yielded elemental maps that exhibited little spatial variation in elemental composition for any of the samples. For IOCB this result indicated the presence of a well-distributed coating of relatively uniform thickness.

### Atomic Force Microscopy (AFM)

Atomic force microscopy is suitable for quantifying roughness of relatively smooth, flat surfaces (e.g. thin films). Scans of various randomly selected regions on UC ( $n > 45$ ) were obtained with good data agreement (i.e. similar roughness of the regions (see below)); scans of randomly chosen areas on IOCC ( $n > 150$ ) also resulted in good reproducibility. Therefore, the AFM images and their corresponding cross-sections shown are representative of the sample surfaces of UC and IOCC, respectively (Fig. 3.2a-b). Comparisons of an UC (Fig. 3.2a) with an IOCC (Fig. 3.2b) show how the coating physically alters the relatively flat glass surface. The AFM image of an IOCC (Fig. 3.2b) and the SEM image of an IOCB (Fig. 3.1b) both suggest that the coating covered the surface with a scattering of micron and sub-micron domains. It is apparent, based on the section analysis, that the domains had a substantial range in height and diameter (Fig. 3.2b). The surfaces of the GB and IOCB samples proved to be near the limit of our AFM capabilities with regard to vertical relief ( $\sim 6 \mu\text{m}$ ), and only a portion of the selected regions yielded good-quality scans (data not shown). Shellenberger and Logan [24] presented AFM scans of glass beads, however it was not specified whether they were universally successful or, if they were forced to find viewable areas by trial and error.

Additional capabilities of AFM include calculation of roughness parameters and estimation of the 3-dimensional surface area. These parameters are presented for UC and IOCC in Table 3.2. The root mean square (RMS) roughness is the standard deviation of the Z values (height measurements) within a given area, and mean roughness ( $R_a$ ) represents the arithmetic average of deviations from the center plane [16]. The iron

coating increased the roughness over uncoated glass by a factor of approximately 27. Estimates of the 3-dimensional surface area from the AFM images show an increase of 6 % after iron coating, which agrees well with the 6 % increase estimated by BET for the coating of the GB. The IOCC were also analyzed in dry and fluid contact mode for comparison to the above-mentioned dry Tapping mode analysis with good data agreement (Tab. 3.2).

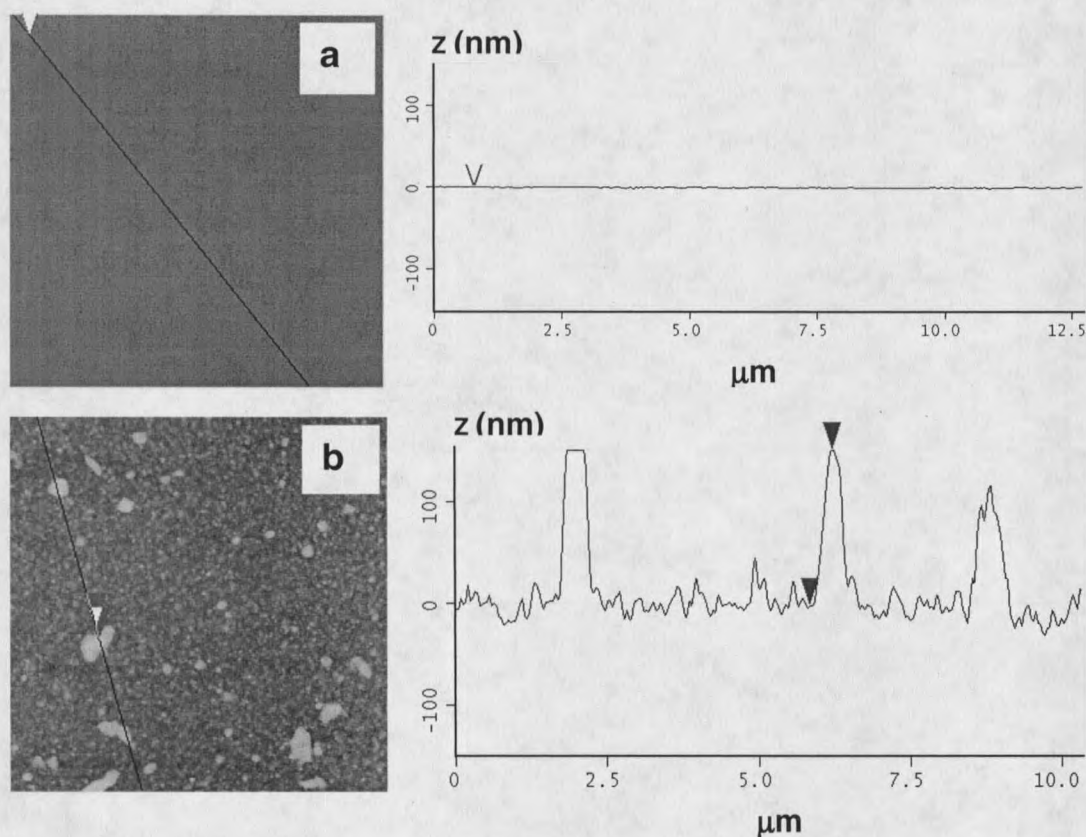


Figure 3.2. Atomic Force Microscopy (height) images (10\*10 μm) illustrating the surface roughness before (a) and after coating of a cover slip (b) using the AFM section analysis tool.

Table 3.2. Surface area and roughness of a glass cover slips before (UC) and after iron oxide coating (IOCC). IOCC were analyzed in three different AFM modes for comparison: dry Tapping, dry contact, and fluid (in NanoPure water) contact. n = number of analyzed areas, RMS (root mean square) is the roughness and Ra is the average roughness.

| Sample | n  | AFM mode      | 2D Area ( $\mu\text{m}^2$ ) | 3D Area ( $\mu\text{m}^2$ ) | RMS (nm)       | Ra (nm)        |
|--------|----|---------------|-----------------------------|-----------------------------|----------------|----------------|
| UC     | 45 | Dry TAP       | 100.00                      | 100.03 $\pm$ 0.01           | 0.9 $\pm$ 0.2  | 0.6 $\pm$ 0.2  |
| IOCC   | 45 | Dry TAP       | 100.00                      | 105.9 $\pm$ 0.9             | 27.3 $\pm$ 5.2 | 16.2 $\pm$ 3.1 |
| IOCC   | 10 | Dry Contact   | 100.00                      | 106.6 $\pm$ 0.7             | 24.3 $\pm$ 7.0 | 14.1 $\pm$ 2.8 |
| IOCC   | 5  | Fluid Contact | 100.00                      | 105.9 $\pm$ 0.4             | 22.8 $\pm$ 4.2 | 13.2 $\pm$ 2.2 |

The AFM images contained a high level of detail and the image analysis software provided powerful analysis tools. Data such as those shown in Figure 3.2a-b and Table 3.2 is of great value for comparison and for improved knowledge of iron (hydr)oxide thin films. For methods to combine the strengths of SEM and AFM techniques in surface studies refer to Russell et al. [25].

#### Depth Profiling by ToF-SIMS/AFM

Fourteen depth measurements of the coating on IOCC resulted in an average thickness of  $124.6 \pm 1.3$  nm (mean  $\pm$  95 % confidence) including the Au layer. Subtracting the Au sputter coat of 6.4 nm as determined by analysis of a gold coated UC, the estimated thickness of the iron oxide coating formed by 9 hours of forced thermal hydrolysis on a glass cover slip was approximately 118 nm. The combination of ToF-

SIMS and AFM offered a powerful method to measure the depth of the iron (hydr)oxide coating with high surface sensitivity and spatial resolution. Alternative techniques for depth measurement of thin films include XPS and grazing-angle X-ray analysis [26].

#### Transmission Electron Microscopy (TEM)

In addition to SEM and AFM, TEM offered another method to visually inspect the synthetic coating and the CP (Fig. 3.3a-b and 3.4a-c). TEM images of the coating revealed the occurrence of domains of approximately 5–50 nm, typically 10–20 nm in size (Fig. 3.3b). Estimates obtained by using the AFM particle analysis tool showed that the majority of the domain sizes ranged from 30–120 nm (data not shown). Some larger domains may have been destroyed in the process of abrading the coating for deposition on the carbon coated Cu grid. Rieke et al. [13] reported domain sizes ranging from 10–70 nm using TEM on samples of synthetic iron (hydr)oxides, a result which is comparable to ours. Lattice fringing in thicker particles was indicative of a crystalline thin film (Fig. 3.3b). Elemental analyses of the coatings were similar to the results from SEM-EDS (data not shown).

The electron diffraction pattern (Fig. 3.3c) indicates crystalline and potential amorphous phases, however, the measured d-spacing ( $\text{\AA}$ ) revealed the presence of hematite (Fig. 3.3d). The presence of ferrihydrite was also suggested by particularly the diffuse broad nature of the diffraction pattern at  $1.49 \text{ \AA}$  (Fig. 3.3c). Comparison of the sample diffraction data with standard data for maghemite, magnetite, lepidocrocite, and goethite suggests that these minerals are absent. This is in contrast to

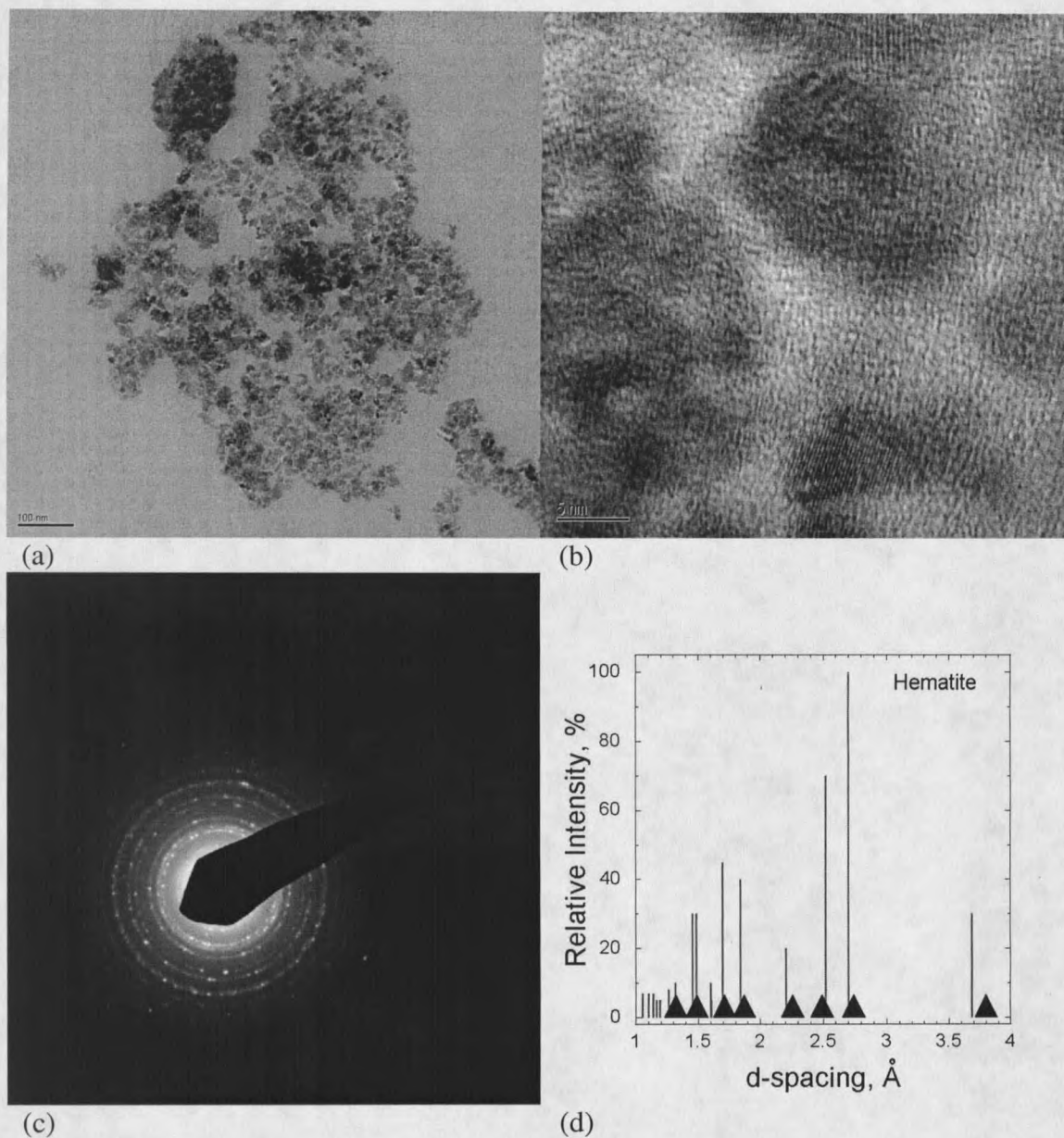


Figure 3.3. Low resolution (a) and high resolution TEM image (b) of the synthetic iron oxide thin film removed from the IOCB. (c) Resulting electron diffraction pattern; bright spots indicate crystalline material while diffuse rings indicate poorly crystalline material. (d) Measured d-spacing ( $\text{\AA}$ ) of the electron diffraction pattern (triangles) compared with the standard pattern (lines) for hematite. Triangle width denotes average uncertainty.

the results of Rieke et al. [13] who used the same coating procedure but on a silicon wafer with a sulfonated self-assembled monolayer and found electron diffraction patterns consistent with goethite. However, a thermodynamic analysis by those authors showed that the expected products of this procedure might include ferrihydrite, goethite, and hematite, depending on the solution conditions and the precipitation sites.

TEM revealed that the CP consisted of a heterogenous mixture of iron (hydr)oxides (Fig. 3.4a). Lattice fringing, d-spacings based on SAED, and TEM images suggested for example the presence of  $\alpha$ -FeOOH (Fig. 3.4b) and ferrihydrite (Fig. 3.4c). Resin embedded thin sections (70 nm thick) of the CP did not provide further information (data not shown).

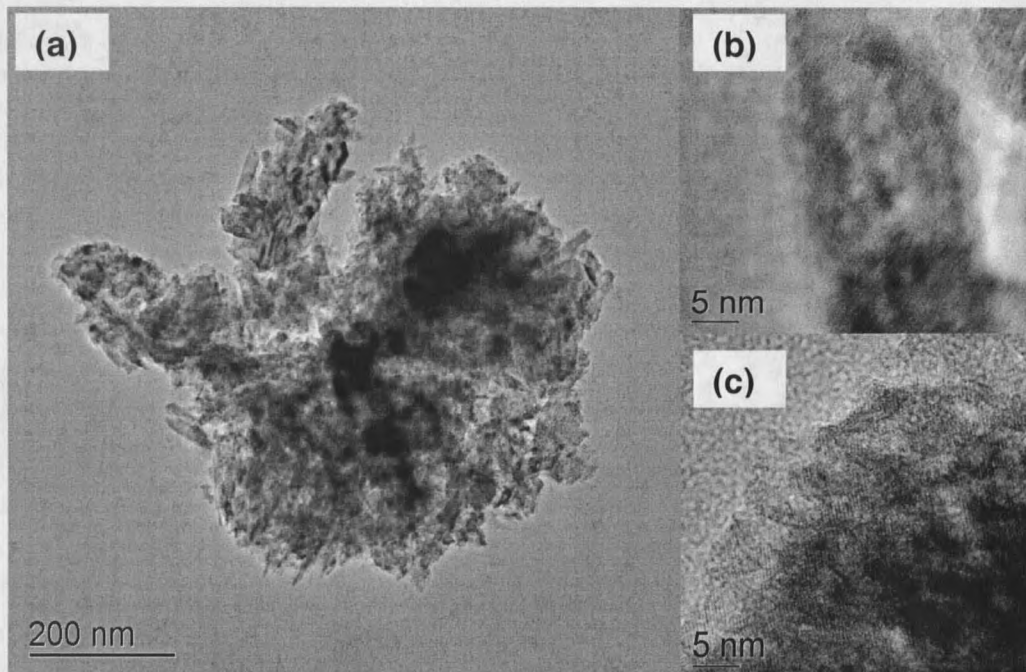


Figure 3.4. TEM images of the CP with low resolution (a) and high resolution of selected areas (b-c). Fig. 3.4b and 3.4c shows lattice fringing suggesting the presence of  $\alpha$ -FeOOH (b) and ferrihydrite ( $\text{Fe}(\text{OH})_3 \cdot n\text{H}_2\text{O}$ ) (c) based on calculations of the d-spacing from their respective electron diffraction patterns.

### X-ray Photoelectron Spectroscopy (XPS)

The Fe(2p<sub>3/2</sub>) and O(1s) core regions proved difficult to use for iron (hydr)oxide characterization since the binding energies for the iron (hydr)oxides are very similar. Even though the O(1s) region of oxyhydroxides is unique due to the presence of two different oxygen binding energies (i.e. O<sub>2</sub><sup>-</sup> and OH<sup>-</sup>), care should be taken to prevent confusion between OH<sup>-</sup> from physically adsorbed H<sub>2</sub>O and FeOOH [27]. Thus, the Fe3p region from IOCC samples was studied and a spectral line value of 55.62 eV was measured. According to McIntyre and Zetaruk [27], both hematite and maghemite possess Fe3p spectral lines at  $55.7 \pm 0.15$  eV, while goethite has its spectral line at  $56.6 \pm 0.2$  eV. Both Fe(2p) and Fe(3p) spectra for the CP indicated the presence of Fe<sup>3+</sup> (~712.0, 710.8, and 56.6 eV), Fe<sup>2+</sup> (~708.0 and 54.0 eV), and Fe<sup>0</sup> metal (~706.6 and 53.0 eV) due to peak-broadening and consequently the need for more bands to fit the spectral peak envelopes (data not shown). The observed binding energies of the major core lines are in agreement with published values for  $\alpha$ -FeOOH, Fe<sub>3</sub>O<sub>4</sub>, and Fe metal [27]. However, to characterize the mineralogy of CP by XPS is not an easy task without some knowledge of the sample history and good assumptions. An elemental survey scan indicated furthermore the presence of sulfur and a minor contribution of calcium.

### Powder and Grazing Incidence XRD

Powder XRD patterns of both intact and crushed IOCB and IOCC samples were negative for crystalline minerals, showing only the amorphous "hump" characteristic of silica glass. However, the presence of hematite as the sole crystalline phase was revealed by analyzing the thin film with a slow scan rate in grazing incidence mode (Fig. 3.5a).

Powder XRD analysis of CP showed a complex mixture of three different iron (hydr)oxides: goethite ( $\alpha$ -FeOOH orthorhombic), lepidocrocite ( $\gamma$ -FeOOH face-centered orthorhombic), and magnetite ( $\text{Fe}_3\text{O}_4$  isometric) and likely green rust ( $\text{Fe}_{3.6}\text{Fe}_{0.9}(\text{O},\text{OH},\text{SO}_4)_9$ ) and calcite ( $\text{CaCO}_3$  trigonal) (Fig. 3.5b).

The presence of green rust has previously been reported in CP [21,28]. The weight percent of mineral phases can be semi-quantitatively determined based on their reference intensity ratio (RIR); however, no RIR reference data exist for green rust. This furthermore prevents semi-quantitative analysis of the other phases since one cannot ignore the contribution to the X-ray diffractogram from green rust. Nevertheless, a rough estimate of the weight percent indicated the relative abundance of the following phases:  $\alpha$ -FeOOH  $69 \pm 14$  %,  $\text{Fe}_3\text{O}_4$   $18 \pm 4$  %,  $6 \pm 1$  % ( $\gamma$ -FeOOH), and  $7 \pm 1$  %  $\text{CaCO}_3$  where the contribution of green rust was ignored. The observed iron (hydr)oxide minerals and their weight percents were in good agreement with results obtained previously from both iron and steel water pipes [2,29]. It should be noted that these CP samples, though dried and stored under  $\text{N}_2$ , were at some times exposed to air during handling and analysis, and that reduced or mixed-state iron oxide forms can oxidize with exposure to atmospheric air. However, complete oxygen-free conditions are not feasible when conducting studies of the kind shown herein.

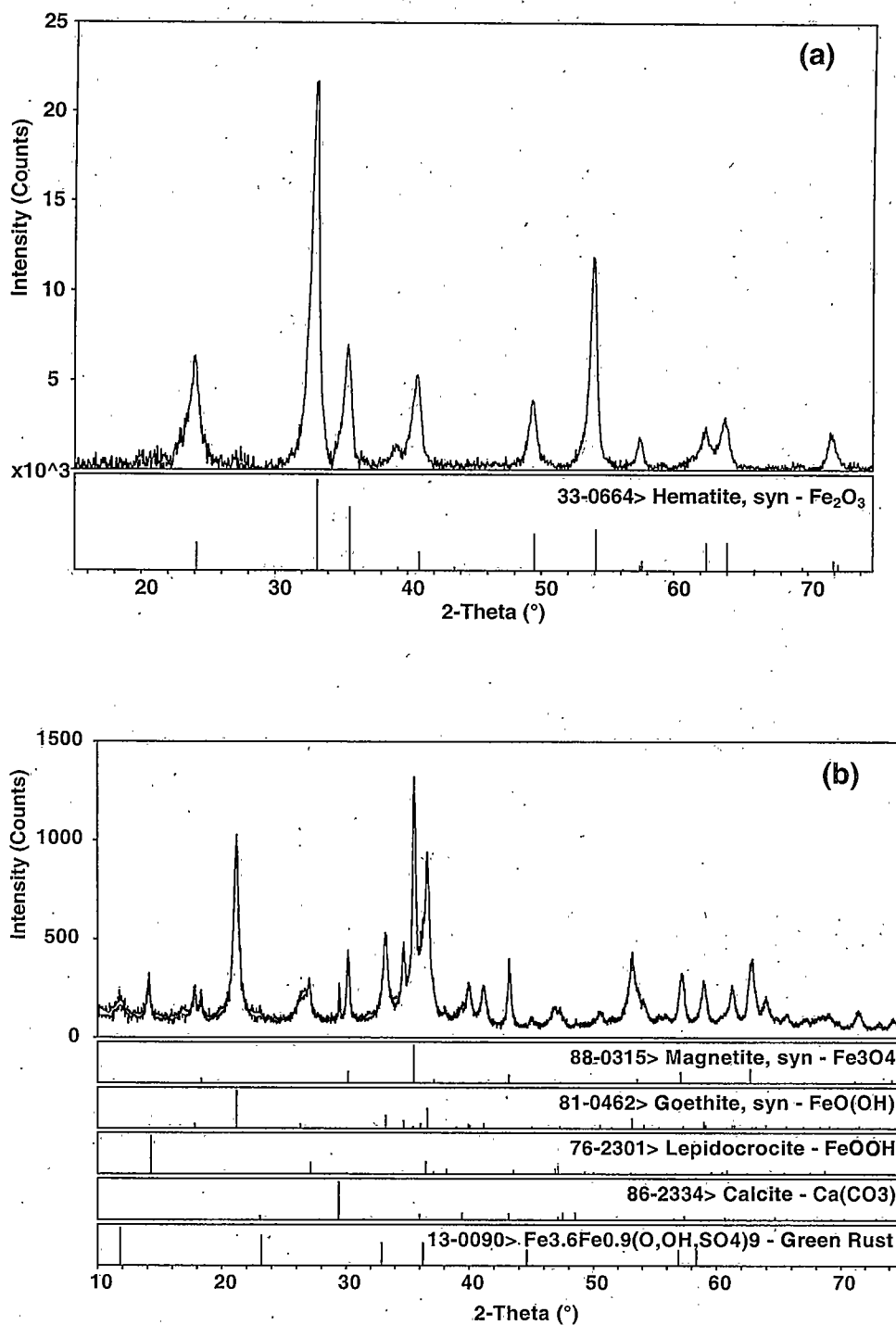


Figure 3.5. (a) GI-XRD pattern of the 118 nm thick synthetic iron oxide thin film (IOCC) after background subtraction of the amorphous glass. (b) Powder XRD pattern of the CP compared to the five most probable minerals based on a database investigation.

Surface Analytical Techniques and their Relevance  
for Characterization of Iron (Hydr)Oxide CP Analogs

This study focused on the application of bulk and surface analytical techniques for the characterization and comparison of iron (hydr)oxide analogs. Table 3.3 summarizes the key results of these techniques and suggests their appropriate application towards either mineral identification or evaluation of physico-chemical properties of iron (hydr)oxides.

The BET surface area, total iron content, and reactivity measured by acid-neutralizing capacity were simple to obtain, though caution should be used in extrapolating these results. For instance, we found that the BET area ( $0.068 \text{ m}^2/\text{g}$ ) and the Fe surface cover ( $7.4 \text{ mg Fe}/\text{m}^2$ ) of the IOCB were significantly smaller than for the CP (BET =  $26.2 \text{ m}^2/\text{g}$ ;  $22.4 \text{ mg Fe}/\text{m}^2$ ), but that the proton-active surface-site density of the IOCB was larger than for the CP. These results illustrate the need for assessing reactivity by a variety of techniques. As mentioned earlier, in a parallel study, it was found that the CP had a higher capacity for adsorption of humic substances and a greater reactivity with chlorine than the IOCB even though the latter had greater surface site density [4].

XRD, XPS, EDS, and TEM-SAED were capable of identifying several crystalline phases, oxidation states, and the elemental composition of the corrosion products without extensive sample preparation. XRD proved to be the most useful technique for crystal phase identification of the CP and revealed the presence of goethite and magnetite as major phases and lepidocrocite as a minor phase. These results are similar to those of

Table 3.3. Summary of the key results that each technique provided for the study of iron oxide coated beads (IOCB) or iron oxide coated cover slips (IOCC) and corrosion products from a water distribution system (CP).

| Technique              | IOCB/IOCC results                                                                | CP results                                                                                                                                      | Applicability and expected results                                        |
|------------------------|----------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------|
| Fe by ferrozine method | 7.4 mg Fe/m <sup>2</sup> ; IOCB                                                  | 22.4 mg Fe/m <sup>2</sup>                                                                                                                       | widely applicable, iron content                                           |
| Acid-base titration    | 72 sites nm <sup>-2</sup> ; IOCB                                                 | 11 sites nm <sup>-2</sup>                                                                                                                       | widely applicable, proton-active surface-site densities                   |
| BET                    | 0.068 m <sup>2</sup> /g; IOCB                                                    | 26.2 m <sup>2</sup> /g                                                                                                                          | widely applicable, surface area                                           |
| SEM                    | Thin scattering of spheres 100 – 500 nm in diameter; IOCB                        | Rhombohedral, hexagonal plates, needle, globular and amorphous morphologies                                                                     | widely applicable imaging tool                                            |
| EDS                    | Ca, Fe, Na, Mg, Al, and Si; IOCB                                                 | Fe and S                                                                                                                                        | widely applicable, elemental information                                  |
| AFM                    | Ra (nm) = 16.2±3.1 and uniform coating thickness = 118 nm; IOCC                  | too rough for analysis                                                                                                                          | limited applicability, precise morphology of smooth surfaces              |
| XRD                    | coating too thin for analysis; IOCC                                              | major phases: $\alpha$ -FeOOH, Fe <sub>3</sub> O <sub>4</sub> ; minor phase: $\gamma$ -FeOOH; probable phases: green rust and CaCO <sub>3</sub> | identification of crystalline materials, semi-quant. analysis             |
| GID                    | $\alpha$ -Fe <sub>2</sub> O <sub>3</sub> ; IOCC                                  | phases identified by XRD                                                                                                                        | identification of crystalline thin films                                  |
| XPS                    | probable phases: hematite or maghemite; IOCC                                     | probable phases: $\alpha$ -FeOOH, Fe <sub>3</sub> O <sub>4</sub> , and Fe metal                                                                 | difficult for mixtures of iron minerals. Oxidation state/binding energies |
| TEM/ED                 | domain size 5-50 nm; phase ID of $\alpha$ -Fe <sub>2</sub> O <sub>3</sub> ; IOCB | mixture of iron (hydr)oxides (e.g. $\alpha$ -FeOOH)                                                                                             | HR-imaging and d-spacing of crystals                                      |
| ToF-SIMS               | depth profiling determined coating thickness in combination with AFM; IOCC       | not applicable                                                                                                                                  | spatial surface composition (m/z), depth profiling                        |

Lin et al. [29] and Sarin et al [2] who determined that their corrosion products consisted of goethite, magnetite and lepidocrocite. In our study, green rust and calcite were suggested as a probable phases. The presence of green rust and calcite could be important since green rust is a very redox-active phase [30] and calcite is a good sorbent for many contaminants such as lead [31]. The presence of carbonates on Fe-oxides can also modify the activity of the oxide surfaces [5]. The use of XPS and SAED for CP characterization is not a simple task based on the complexity of CP and therefore not recommended. The nano-crystalline hematite structure of the iron oxide thin film was revealed distinctively using GI-XRD and this result was partly supported by TEM-SAED, which identified hematite and potentially ferrihydrite, and XPS, which identified hematite or maghemite. Consequently, it is recommended to use the grazing incidence mode of XRD for phase identification of nanometer thin iron oxide coatings.

Imaging techniques including AFM, SEM and TEM were useful in providing gross comparisons of crystallinity, roughness, domain size, and surface area (Table 3.3). SEM may be used on a variety of samples (e.g. IOCB and CP) for bulk comparison, while AFM can provide detailed information such as roughness in the nano-scale range of relatively smooth coatings with a vertical relief of a few micrometers. TEM requires a thin sample made either by sectioning or by deposition of small particles onto a Cu grid. TEM proved to be a powerful tool for detailed imaging particularly in combination with SAED for phase identification and EDS for identification of the elemental composition. If the thickness of a very thin coating is of interest, it may be measured with high surface sensitivity and spatial resolution by the combined application of ToF-SIMS and AFM;

this depth profiling technique revealed the presence of a 118 nm thick Fe coating on the glass cover slips.

#### Relating Surface Analysis Results to Iron Oxide Behavior in Simulated Drinking Water Conditions

A common problem faced by many drinking water studies is the choice of a material that will best simulate iron pipes in older municipal drinking water systems. There has been significant interest in understanding interactions between CP in pipelines and chlorine disinfectants [5]. In this and other drinking water related studies [7-12] it has become important to characterize the iron (hydr)oxides if relevant research is to be performed. Yet, when considering actual CP from cast iron pipes one must take into account the varying water environment and assess the characteristics of the action CP. This paper serves to describe and compare a number of techniques available for characterization and provide a guide for future research by indicating the techniques most appropriate for the desired result.

The reductive approach of using a single Fe (hydr)oxide phase is typical in environmental investigations where the native material is complex and the use of defined materials leads to quantitative information and better understanding of reaction mechanisms. For example, Vikesland and Valentine [5] used pure iron oxide phases to study a surface-catalyzed oxidation of ferrous iron by monochloramine. However, it is well recognized that this approach may lead to erroneous extrapolations when complex Fe (hydr)oxides are present.

To compare and contrast the results obtained using a model oxide and complex oxides from the natural environment in a simulated drinking water system [4], it was necessary to use a suite of tools capable of describing the nature and reactivity of the two surfaces. IOCB (hematite) was used as a defined iron oxide analog for comparison to the heterogeneous CP (magnetite, goethite, lepidocrocite, green rust and calcite, Table 3.3). The corrosion products were rougher and had higher surface BET surface area than the analog, but lower proton active surface area. However, the CP had higher chlorine demand and capacity for humic adsorption than the IOCB [4], demonstrating that proton active surface area alone is not predictive of these behaviors. When biofilm accumulation in the presence of chlorine with and without the addition of a phosphate corrosion inhibitor was evaluated, growth on the IOCBs was less pronounced, suggesting that this surface was impacted by the inhibitory action of phosphate to a greater degree than the CP. These results illustrate that the complexity or type of oxide will have an impact on the results. However, it was also of interest to note that the simple presence of an iron oxide, either as the analog or in the corrosion products, had a profound impact on the ability of a biofilm to grow with humic substances as the sole carbon and energy source. These results and those of others illustrate the need for both approaches; defined materials to understand results in depth and complex materials to simulate reality.

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## CHAPTER 4

THE EFFECT OF CATIONS AND SURFACTANTS ON 2,4,6-TRINITROTOLUENE  
DESORPTION IN TWO MODEL SOILSIntroduction

Soil contamination by 2,4,6-trinitrotoluene (TNT) and its amine metabolites has been identified by the Department of Defense as one of their most critical environmental concerns. TNT is a mutagen, listed as a suspected carcinogen, and is toxic at concentrations as low as 2.5 mg/L [1-5]. TNT contamination has occurred primarily through production, loading, and disposal of explosives at U.S. Army ammunition sites [6]. The U.S. Army Environmental Center has identified many TNT contaminated sites in the U.S. [7]. Soil contaminated with TNT is, however, even more problematic in other countries such as Germany where explosives manufacturing sites were demolished at the end of World War II. Most of these German sites have since been used for industry and housing [8,9].

Hydrophobic organic contaminants often exhibit limited bioavailability to microorganisms and can persist in the subsurface for extended periods [10]. The limited bioavailability may be due to binding to soil organic matter (SOM) since SOM is one of the primary adsorbents for organic chemicals in the environment [11]. The most commonly studied fractions of SOM are humic and fulvic acid, which are isolated from soils and sediments by simple extraction techniques [12]. The main reason for the persistence of TNT in soil environments is due in part to its low redox potential and or

sorption to organomineral surfaces [13-15]. The binding of TNT and its metabolites to soil constituents such as SOM has been well studied and it was found that TNT binds to humic acid in a slow kinetic process and that the sorption capacity of SOM is less significant than that of clay and iron oxides [15-20]. Conversely, it is generally well accepted that reduced metabolites of TNT such as the amino derivatives can bind covalently and in some cases irreversibly to SOM particularly under aerobic conditions [16,17,21,22]. Nitroaromatics bind to SOM mainly through covalent binding, ion binding or *via* hydrophobic interactions [19,23]. It was further reported that the binding capacity of humic acid for TNT and its reduced metabolites was influenced by several factors, including humic acid concentration, pH, and ionic strength [19]. However, binding of TNT and especially its amino derivatives (e.g., 2,4,6-triaminotoluene) to mineral surfaces happens through dipole-dipole interactions, London van der Waals forces, hydrogen bonding, and other intermolecular interaction mechanisms [23].

Various remediation strategies for treating TNT contaminated soil and sediment have been evaluated both in the field and at the bench scale. The primary remediation strategies include incineration, composting, chemical oxidation, electrochemical treatment, alkaline hydrolysis, surfactant-enhanced washing, and bioremediation [24-31]. The associated cost and modest effectiveness has prompted the need for improved remediation technologies [13,32]. Field bioremediation treatments such as excavation with subsequent *ex situ* composting are common, however, no *in situ* processes have been developed for the remediation of TNT contaminated soils [33]. The limited bioavailability of TNT and its metabolites to microorganisms and their subsequent

persistence in the subsurface [34] represents a major challenge for *in situ* treatment. One technique for enhancing the desorption and potential bioavailability of TNT and or TNT metabolites is the use of surfactants. Taha et al. [35] reported enhanced desorption of TNT from a contaminated soil when surfactants were added. In addition, the surfactant Tween 80 enhanced the mineralization of TNT by *Phanerochaete chrysosporium* [25]. However, there are potential limitations to the use of surfactants for *in* and *ex situ* remediation including the possible toxicity of surfactants to specific populations and the bioavailability of surfactant micelle-phase contaminants [36,37]. For instance, some synthetic nonionic surfactants have been shown to inhibit mineralization of phenanthrene [36,38]. However, the microbial production of biosurfactant-like biomolecules (e.g. glycolipids) has been shown to enhance the solubility and bioavailability of pyrene [39]. In addition, exogenously added biosurfactants (i.e. rhamnolipids) were found to enhance the desorption of phenanthrene and to stimulate uptake of hexadecane by *Pseudomonas aeruginosa* [40,41].

Given the importance of surfactant-based remediation treatments and ionic strength, we hypothesized that biosurfactants would result in enhanced desorption and that the concentration and valence state of cations would affect the equilibrium partitioning of TNT in aqueous-solid phase environments. Experiments were conducted to evaluate the influence of ionic strength, primary cations, and biosurfactants on the desorption of TNT in two model systems. Important chemical-physical factors that influence the sorption of organic contaminants were identified based on a literature review; two model porous media using different combinations of quartz sand, goethite

( $\alpha$ -FeOOH) and humic acid were developed and the desorption of TNT studied in batch and column systems. Specifically, a batch and a column study were designed which included the two developed model-soils; humic acid (HA) coated goethite was used in batch studies and quartz sand coated with both HA and goethite was used in column studies. The desorption-potential of TNT was investigated in these systems under a variety of chemical conditions (i) low ( $I = 0.005$  mol/kg) versus high ( $I = 0.1$  mol/kg) ionic strength, (ii) monovalent ( $\text{Na}^+$ ) versus divalent cations ( $\text{Ca}^{2+}$ ), and (iii) presence versus absence of biosurfactants (rhamnolipids). Desorption of TNT in the flow-through column experiment was further modeled using the convective-dispersion equation (CXTFIT) and the results of this modeling exercise is discussed.

### Background

#### Sorption of Organic Contaminants

Sorption of organic contaminants with soil phases is referred to as sorption (either adsorption onto a two-dimensional surface, or absorption into a three-dimensional matrix). Sorption processes involve an array of phenomena that can alter the distribution of contaminants among the constituent phases and interfaces of subsurface systems [42]. Low solubility organic contaminants may partition into hydrophobic domains such as soil organic matter. Additionally, contaminants may displace water near the mineral surface to some extent and thereby be bonded to the surface via dipole-dipole interactions, van der Waals forces, hydrogen bonding and other intermolecular interaction mechanisms [23].

In general, most soil minerals are polar and expose a combination of hydroxy- and oxy-moieties at their surfaces (e.g.,  $\text{FeOOH}$  and  $\text{SiO}_2$ ). These polar surfaces favor polar interactions, which allow them to form hydrogen bonds. Replacing water molecules from a mineral surface by non-polar organic contaminants is not energetically favorable. Organic contaminants can also diffuse into the pores of a soil mineral as illustrated in Figure 4.1. Soil mineral pore diffusion can occur in pore liquids or along pore wall surfaces. Liquid and surface diffusion may act at the same time and are difficult to distinguish [42,43]. Diffusion into mesopores (2 – 50 nm) can be retarded by sorption to surfaces or partitioning into SOM coatings. Tortuosity, variable pore diameter and dead end pores, can give lower apparent diffusivity [44]. Diffusion into micropores (< 2 nm) will have the same factors applied as diffusion into mesopores, but tortuosity is more severe. Additionally, diffusion in micropores will be retarded by steric hindrance from pore walls and sorption may be increased by simultaneously binding with adjacent surfaces [44].

Hydrophobic interactions comprise the primary motivation for a large class of sorption reactions in the subsurface. The association of neutral, relatively non-polar organic contaminants with soils often results in quasi-linear equilibrium sorption patterns, and the magnitude of the associated coefficients often vary with the organic carbon content of the soil [42]. Diffusion of organic contaminants into hydrophobic domains of organic matter (e.g. humic substances) does not require displacement of tightly bound water molecules. SOM is pictured to exist in large part as organic polymers coiled into

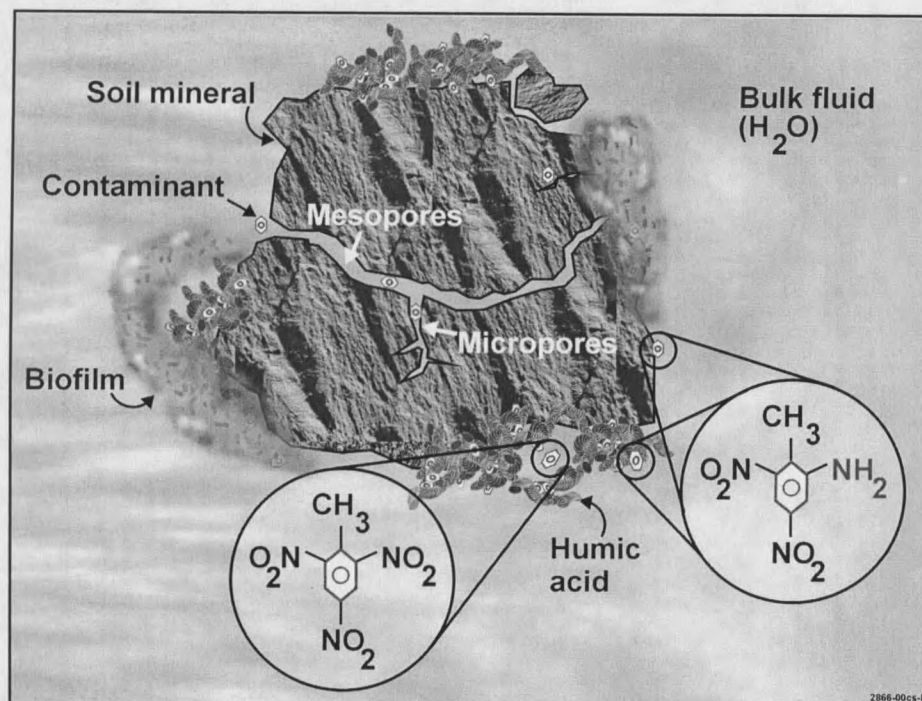


Figure 4.1. Organic contaminant (e.g. TNT and 2-ADNT) interaction with a model soil aggregate.

globular units (with carboxyl, phenolic hydroxyl, alcoholic hydroxyl and carbonyl as the principal polar functional groups) and to occur in somewhat isolated patches coating mineral solids. Because of the “porous” nature, nonpolar sorbates can physically penetrate between the chains and find themselves dissolved into or absorbed onto a non-aqueous phase [23] (Figure 4.1).

The sorption and desorption rate for an organic contaminant may differ significantly from each other in the same solid-solute-solution system; this phenomenon is called hysteresis [45-48]. Differences in the sorption and desorption stages of contaminant interactions with soils are commonly referred to as “aging” [46,49-51], because the desorption rate is often decreasing over time. Alexander [52] reported that

some abiotic processes influenced the SOM structure (time dependent processes) with a consequently decreased bioavailability of phenanthrene.

Soil organic matter evolves from oxidized amorphous biopolymers (e.g., cellulose) to relatively reduced and condensed humic macromolecules and then further to highly reduced and condensed kerogens [53-55]. These different types of SOM can have a significant influence on the diffusion rate of organic contaminants. Diffusion is generally slower in "glassy" (condensed) than in "rubbery" (amorphous) polymers [56]. Sorbing molecules can diffuse freely into and out of highly oxidized local SOM regions, regions that are completely hydrated, swollen, and amorphous. Pores within such amorphous matrices are sufficiently flexible to accommodate hydrophobic organic solute molecules (e.g., phenanthrene) as their aqueous phase concentration increase [49]. Conversely, more reduced and condensed local SOM domains favor solute-sorbent interactions dominated by van der Waals forces rather than interactions with polar water molecules [49].

#### Chemical Factors that Influence the Sorption of Organic Contaminants

Much research has shown that changes in the aqueous chemical environment surrounding the soil organic matter can significantly affect the sorption kinetics of contaminants. Chemical factors such as ionic strength, divalent cations (e.g.,  $Mg^{2+}$ ,  $Ca^{2+}$ ) and the presence of surfactants have been recognized to have major impact on the sorption behavior of organic contaminants (e.g. TNT) and thus the focus of this chapter [19,25,35,40,57].

Humic acid is considered a flexible, linear polyelectrolyte at low concentration ( $< 3.5$  g/L), above a certain pH ( $\text{pH} > 3.5$ ), and at moderate ionic strength ( $< 50$  mM). Beyond these conditions, HAs behave like rigid, uncharged colloids [58,59]. Under conditions where HA behaves as a linear polyelectrolyte, the configuration of HAs is strongly affected by its concentration, pH, and ionic strength [58-61].

Li et al. [19] observed a nearly 2-fold increase in binding of trinitrotoluene (TNT) to HA for a 5-fold increase in ionic strength of phosphate buffer. Since solvated inorganic ions would not be expected to interfere or compete with the penetration of non-polar organic compounds into SOM, one can reasonably assume that salts affect the value of the organic matter - water partition coefficient ( $K_{\text{om}}$ ) primarily through the contaminants aqueous activity coefficient ( $\gamma_{\text{w}}$ ) [23]. Changes in salt concentration may also impact other factors like the coiling of SOM and the contaminants solvency for SOM (i.e., changing the contaminants organic matter activity coefficient,  $\gamma_{\text{om}}$ ) and thereby increase or decrease the sorption of an organic contaminant. This postulate agrees with Murphy et al. [57], who observed, that peat humic acid (PHA) was coiled in solution at high ionic strength and elongated at low ionic strength. Li et al. [19] speculated that the presence of significant levels of carboxyl functional groups and phenolic structures in HAs were likely involved in some of the binding reactions with 2,6-diamino-4-nitrotoluene (2,6-DANT). Thus, the charge, configuration, and aggregation of HA will be different depending on its ionic and pH environment and its own concentration. At high HA concentration, HAs tend to be coiled and aggregated [58], leading to a decrease in the available exposed binding sites, which can explain the binding increase with decreasing

HA level. Experimental evidence shows that the intrinsic viscosity of HAs and fulvic acids (FA) is more sensitive to changes in salt level than to changes in pH [59,62]. Reuter [63] used a combination of viscometry and gel permeation chromatography to demonstrate that the size of dissolved humic substances is reduced with increased salt content of the water. At high pH, the HA molecules may shift to a more open configuration in the solution due to the charge repulsion between ionized functional groups [57,64]. Li et al. [19] observed that the binding level of TNT to HA was higher at pH 6.8 than pH 4.6, however, the opposite pH dependence was observed for 2,6-DANT.

The structures of the contaminants have also been shown to have important effects upon binding. Li et al. [19] observed a decreasing HA binding capacity for the following three compounds  $2,6\text{-DANT} > 2\text{-aminodinitrotoluene (2-ADNT)} \geq \text{TNT}$ . The measurements are consistent with TNT being uncharged while 2-ADNT and 2,6-DANT possess partially uncharged amines. Potential binding sites for these positive charges are located at the numerous negatively charged carboxylic acid-derivatized phenolic rings found in HA.

Soil organic matter may also control the metal ion concentration in soils and waters thereby affecting the mobility of cations in soils and aquifers [65,66], although the exact impact of metal ions (e.g.,  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$ ) on the structure of HA is not well understood. However, Engebretson and Von Wandruszka [67] reported a cation-enhanced formation of hydrophobic domains in aqueous humic acids. They observed that added divalent cations (e.g.,  $\text{Mg}^{2+}$ ) first associate with anionic groups on the HA polymer, partially neutralizing them and causing bridging interactions between different

parts of the HA chains. This drew the humic acid molecules together and enhanced the formation of hydrophobic pseudomicelles as illustrated in Figure 4.2.

The hydrophobic pseudomicelles were however of a transitory nature, and they disintegrated as the metal ions gradually migrated to locations where they formed stable inner-sphere complexes with functional groups on the HA molecule. In addition, Schlautman and Morgan [68] showed that the addition of divalent cations (e.g.,  $\text{Ca}^{2+}$ ) generally increased the binding of polycyclic aromatic hydrocarbons (PAHs) to Suwannee River humic substances at pH 7 and 10, but little effect was observed at pH 4.

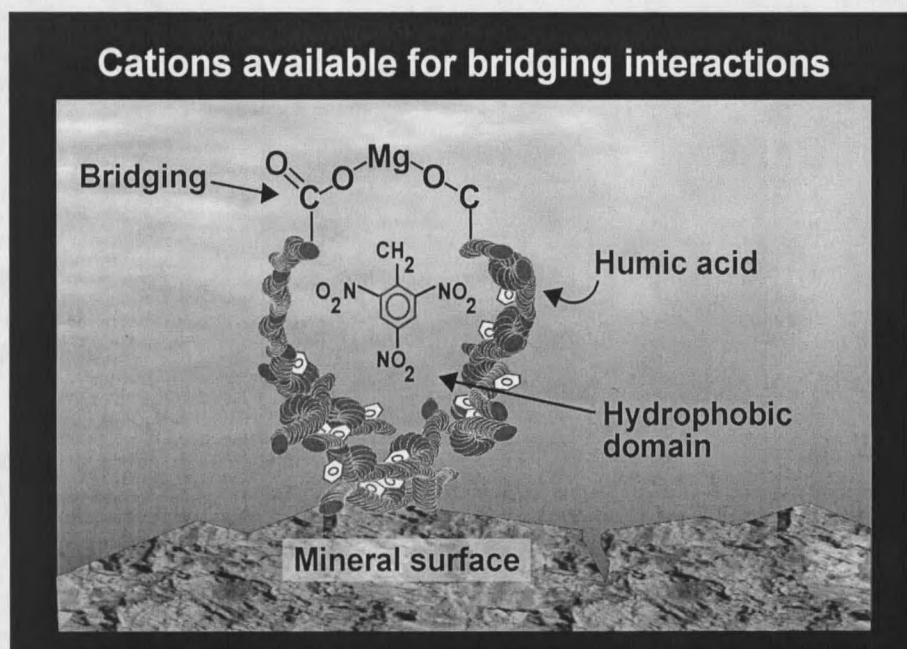


Figure 4.2. Hypothetical interaction between a divalent cation (i.e.  $\text{Mg}^{2+}$ ) and humic acid functional groups (i.e. carboxyl- and hydroxyl groups) potentially creating a hydrophobic domain. These hydrophobic domains might increase the sorption potential of hydrophobic contaminants such as TNT.

The ability of surfactants to modify soil surface chemical properties may also have an important impact on sorption of organic contaminants. One of the fundamental properties of surfactants is their binding at the solid-aqueous interface [69], resulting in the formation of interfacial aggregates with a variety of structures [70,71]. At the solid-water interface, surfactants tend to orient themselves with the hydrophilic part into the aqueous phase and the hydrophobic portion at the interface. In a concentrated surfactant environment a surfactant bilayer may be formed as illustrated in Figure 4.3.

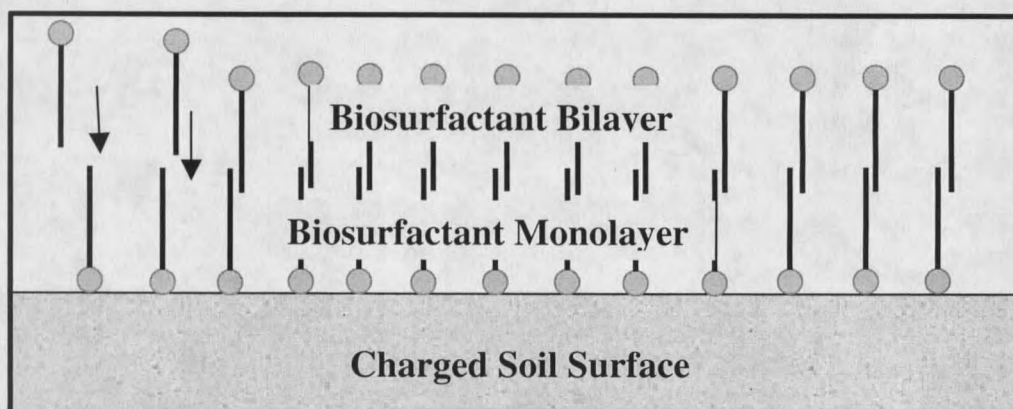


Figure 4.3. Monolayer and bilayer formation of surfactants on a charged model soil surface.

Bowman et al. [72] reported that the soil sorbed surfactant molecules create an organic environment into which hydrophobic organic chemicals (HOCs) can partition. Surfactants were suggested as a technique for decreasing interfacial tension and phase partitioning between HOCs and soil [73-75]. Surfactant amendment may thus enhance HOC mobility and potentially increase HOC bioavailability. Laha and Luthy [76] proposed that nonionic surfactants may interact with SOM in three ways: 1) through

hydrophobic surface interactions between the hydrocarbon chains of surfactant molecules and the hydrophobic regions of humic matter, 2) through hydrogen bonding between surfactant ethoxylate groups and polar groups of humic matter, (e.g., hydroxyl and phenolic groups), and 3) through partitioning of the nonionic surfactant into bulk organic matter. In aqueous solution, surfactant molecules form micelles if the concentration exceeds the critical micelle concentration (CMC). When the surfactant concentration is less than the CMC, only monomers exist in solution [77]. Solubilization of HOCs by aqueous surfactant solutions is proposed to be a micellar phenomenon [78] as illustrated in Figure 4.4.

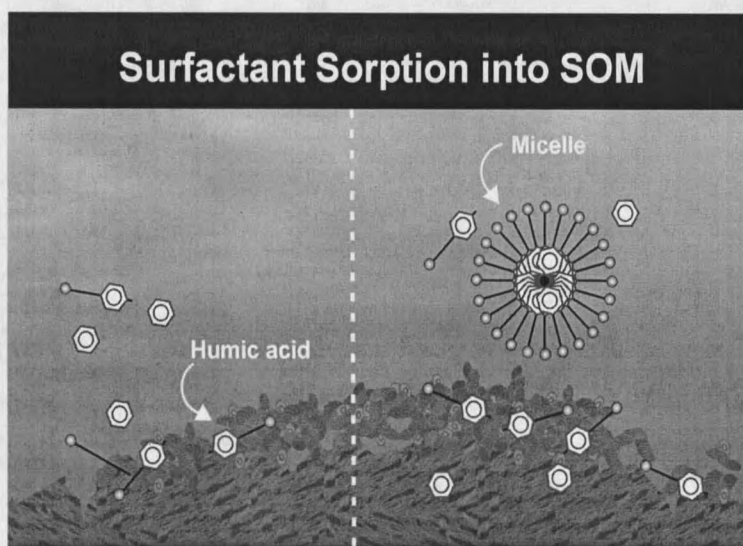


Figure 4.4. Contaminant (here illustrated with benzene rings) interaction with aqueous surfactants. The left hand side and the right hand side illustrate situations below and above the CMC, respectively.

## Materials and Methods

### Batch Experiment

The objective was to coat goethite (irreversibly) with humic acid (HA) and to use this two-component solid to represent naturally occurring Fe (hydr)oxides for batch studies of TNT desorption. The desorption-potential of TNT was investigated as a function of (i) low versus high ionic strength, (ii) monovalent versus divalent cations, and (iii) the presence versus the absence of biosurfactants in batch vessels containing the humic-coated goethite.

Initial preparations for the coating of humic acid to goethite required the removal of background carbon from all labware. Glassware was first dishwashed, then soaked (24 hrs) in a Nochromix<sup>®</sup> 36N sulfuric acid solution from Godax Laboratories. Residual acid was thoroughly rinsed from the glassware using reverse osmosis purified water at 17.4 M $\Omega$ -cm from a Barnsted Nanopure system. The glassware was covered with aluminum foil and carbon contamination was removed by heating to 550°C (3 hrs) using a Cress Electric Furnace. The baked glassware was allowed to cool down slowly (5 hrs), then stored in a low-dust area.

Carbon removal from less durable labware required alternate carbon elimination techniques. Carbon was removed from the glass pipettes, plastic syringes, and stainless steel needles by purging with 30 mL of 1 % HCl followed by three 30 mL rinses with nanopure water. The removal of carbon from the syringe filters was done by purging

30 mL of nanopure water through the 0.2  $\mu$ m filter based on a carbon flushing study (Figure 4.5).

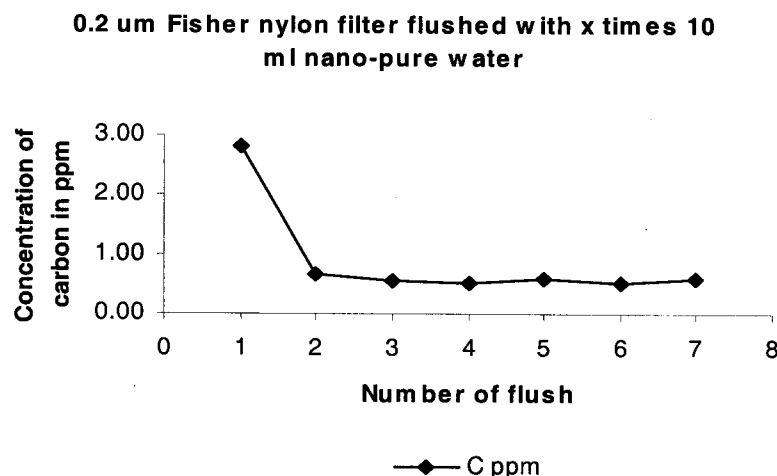


Figure 4.5. Carbon removal from a nylon filter. Illustrating that the carbon removal ceases after flushing with 30 mL of nano-pure water.

The removal of organic carbon from 250 ml Nalgene Teflon FEP bottles (Fisher Scientific) first involved filling the bottles with 1% HCl. Caps made of Tefzel ETFE were then screwed onto the top of the Teflon coated bottles and the solution was shaken vigorously. The 1% HCl solution was decanted and the residual HCl was removed through repeated rinses of nanopure water. Sterile Teflon coated stir bars (35 x 6 mm) from Fisher scientific were added to two FEP bottles. Dry weights of the capped Teflon bottles with Teflon coated stir bars were measured using a Metler Toledo AE200 Scale with an accuracy of  $\pm 0.0001$ g.

Goethite Preparation. Twenty-five grams of goethite powder ( $\alpha$ -FeOOH) from Alfa Aesar Co. was added to the two FEP bottles. Calcium perchlorate ( $\text{Ca}(\text{ClO}_4)_2$ ) was mixed with nanopure water to give an electrolyte with an ionic strength of 0.001 mol/kg (important background electrolyte for optimal results). The unbuffered electrolyte was adjusted to a pH of 7.8 using drop-wise additions of a 0.2M NaOH solution. All pH measurements were made using a calibrated Corning pH Probe connected to a Model 50 Accumet Transmitter. Both FEP bottles, each containing a stir bar and 25 g of  $\alpha$ -FeOOH, received 200 ml of electrolyte solution. A stir plate was used to keep the  $\alpha$ -FeOOH-electrolyte mixture homogenized. This solution was adjusted to a pH 6.8 using drop-wise additions of concentrated (50 % by weight) NaOH solution.

Humic Acid Adsorption. One gram of Elliott soil humic acid from the International Humic Substances Society (IHSS) was added to 100 ml of ( $\text{Ca}(\text{ClO}_4)_2$ ) electrolyte to create a 10,000 mg/L HA stock solution. The HA stock solution was adjusted to a pH of 6.8 and was allowed to equilibrate overnight and readjusted after 24 hrs.

The technique used to adsorb HA onto  $\alpha$ -FeOOH first involved centrifuging the FEP bottles containing the equilibrated  $\alpha$ -FeOOH-electrolyte mixture with a Sorvall RC5C Centrifuge at 2980 RPM for 20 minutes. A carbon-free glass pipette was used to extract 8 mL of supernatant from each FEP bottle. This extraction was then filtered through a pre-cleaned 0.2  $\mu\text{m}$  syringe filter to remove  $\alpha$ -FeOOH and non-soluble carbon particles. This filtrate was stored in a carbon free scintillation vial and used as a measure

for the carbon background. The first humic acid addition involved the addition of 8 mL of the agitated HA stock solution (34.17 mg HA-C) to both FEP bottles. The bottles were tightly capped, and the solution was agitated by hand to homogenize the solution. A Glas-Col Laboratory Rotator (Model 099A) at 18 rotations per minute was used to equilibrate the  $\alpha$ -FeOOH/HA solutions. The first HA addition was allowed to equilibrate for two hours based on studies by Murphy et al. [57]. The procedure for the next four humic acid additions proceeded in the same manner, and each extraction was saved in a scintillation vial for carbon analysis.

Humic Acid Desorption. Soluble and reversibly bound HA was removed from the systems by centrifuging the bottles and then fully decanting the supernatant. The wet-mass of the bottles was measured to determine the residual water saturation. Each bottle was then filled with 200 mL of electrolyte and rotated for two hours. The procedure for the next two carbon desorptions proceeded in the same manner and 10 mL samples were saved in scintillation vials for carbon analysis.

Carbon Analysis. A Shimadzu TOC-5000A Carbon Analyzer was used to determine the amount of HA carbon (HA-C) in solution.

$^{14}\text{C}$ -TNT Adsorption. The preparation of the 2,4,6-trinitrotoluene solution involved mixing of  $^{14}\text{C}$ -labeled TNT (20,000 dpm/mL) from ChemSyn Laboratories with "cold" TNT (38 mg/L) from Chem Services in 500 mL of nanopure water.  $^{14}\text{C}$ -TNT was used to provide measurements of the TNT bonded to the humic-coated goethite by use of a biological oxidizer and a scintillation analyzer. Cold TNT was incorporated in this

solution to create a realistic TNT concentration close to what can typically be observed at contaminated sites around the U.S. and to provide a sufficient concentration so that HPLC could be used to verify the potential formation of metabolites during experimentation [79].

The FEP bottles containing waterless HA coated  $\alpha$ -FeOOH were filled with 225 mL of the  $^{14}\text{C}$ -TNT solution, the bottles were capped, fully agitated, and the mixtures were allowed to rotate for 24 hours. Upon equilibration, the slurry was set on a stir plate at high speed, and 9 mL of this slurry (containing 1.0 g of TNT-HA coated  $\alpha$ -FeOOH) was equally delivered by glass pipette to 10 mL centrifuge vials (in triplicates). HPLC analysis verified the presence of TNT and the absence of TNT metabolites during the entire experiment.

$^{14}\text{C}$ -TNT Desorption Experiment. Twenty-four centrifuge vials were used to test the impact of adding salt solutions of 0.1 mol/kg and 0.005 mol/kg ionic strengths, and by adding 2.5 and 25 times the critical micelle concentrations (CMC  $\sim$  40 mg/L) of rhamnolipid biosurfactant on the desorption of  $^{14}\text{C}$ -TNT. The rhamnolipid (microbially produced by *Pseudomonas aeruginosa*; 50 g/L) was obtained from Aventis Research and Technologies, Frankfurt, Germany, for further details please refer to the review by Lang and Wullbrandt [80]. One mL-test solutions containing nanopure water (as reference/control),  $\text{Ca}^{2+}$ ,  $\text{Na}^{+}$ , and biosurfactants were added individually to each of the vials that contained 9 mL (1.0 g TNT-HA coated  $\alpha$ -FeOOH) of slurry. The samples were then equilibrated for 12 hours and the presence of aqueous  $^{14}\text{C}$ -TNT and cold TNT were

evaluated on a Packard scintillation analyzer and by a Hewlett Packard 1090 HPLC with a diode array detector (see chapter two and five for further experimental details). The amount of  $^{14}\text{C}$ -TNT on the solid phase was measured by combusting subsamples in a Harvey Instruments OX500 Biological Oxidizer. The  $^{14}\text{C}$  released during oxidation was trapped in a basic Harvey's  $^{14}\text{C}$  cocktail and counted on a 1900 TR Liquid Scintillation Analyzer (Packard Instrument Co., Downers Grove, IL).

### Column Experiment

The objective was to coat quartz sand (irreversibly) with first goethite and then HA and to use this three-component solid to represent naturally occurring porous media for column studies of TNT desorption. The desorption-potential of TNT was investigated as a function of (i) ionic strength, (ii) divalent cations (i.e.  $\text{Ca}^{2+}$ ), and (iii) the presence and the absence of biosurfactants under flow conditions in columns containing the goethite-HA coated quartz sand. Desorption of TNT in the column experiment was modeled using the CXTFIT model.

Carbon removal of the glassware, Teflon bottles, and plastics was performed using the same procedures as described in the materials and methods section for the batch experiment. The 1000 ml glass pressure flask used in this experiment was only acid washed (not baked) by washing the interior of the bottle with 1 % HCl for five minutes then rinsing with nanopure water four times.

Preparation of Column Packing Material. Industrial quartz sand from Unimen Corporation with an effective size of 0.1 mm and 30 % retained on 70 Mesh or coarser

was prepared for use in the column experiments as follows. Quartz sand was first soaked and agitated in 2M HCl for one hour in a baked 1000 ml glass flask to remove organic and inorganic contamination. The HCl was decanted and the quartz sand was repeatedly rinsed with nanopure water to remove the residual acid. The acidity of the quartz sand slurry was measured and then adjusted to a pH of 7.0 using a solution of 0.8M NaOH. The quartz sand was dried at 110 °C for three days in a Fisher Scientific (Model 655 F) Isotemp Oven.

The goethite coating procedure was a modified version of a previously published method [81]. Goethite preparation for the quartz coating experiment was done in the cleaned pressure flask by adding 26.4 g of  $\alpha$ -FeOOH to 132 mL of a 0.01M NaNO<sub>3</sub> solution. The solution was adjusted to a pH of 7.0 with a concentrated solution of NaOH, and equilibrated on a shaker tray at 60 cycles/minute for 24 hours at  $21 \pm 3$  °C. After equilibration, 220 g of quartz sand was added to the suspension, pH adjusted to 7.0, and was further equilibrated on the shaker tray for 24 hours. The tightly sealed pressure flask (providing a pressure above 1 atm) was then put into the Isotemp oven at 120 °C for 24 hours, and the flask was occasionally inverted to ensure optimal adsorption of  $\alpha$ -FeOOH to the quartz sand.

The pressure flask was removed from the oven and allowed to cool down slowly. The slurry contents of the pressure flask were emptied out on a 75  $\mu$ m stainless steel sieve, and the  $\alpha$ -FeOOH-coated quartz was rinsed with nanopure water until the unbound fraction of  $\alpha$ -FeOOH was removed. The coated sand was then rinsed with a one liter NaNO<sub>3</sub> solution (1.0M, pH 3.0) to remove weakly bound  $\alpha$ -FeOOH from the quartz sand

surface, and was finally rinsed with nanopure water until all (visible) reversibly bound  $\alpha$ -FeOOH was removed. The sieve tray containing the  $\alpha$ -FeOOH-coated quartz was covered with aluminum foil and dried at 110 °C for 24 hours in the Isotemp oven.

Scanning electron microscopy (SEM) was performed on a JEOL JLM-6100 Scanning Microscope (see chapter three for experimental details) to confirm that the characteristic crystal structure of  $\alpha$ -FeOOH was maintained after sorption to the quartz sand. A spectrophotometer (Spectronic Instruments Genesys 5) determined the iron content (e.g. sorbed  $\alpha$ -FeOOH) by use of the Ferrozine method (see chapter 5 and 6 for experimental details). The  $\alpha$ -FeOOH-coated quartz was divided into four FEP bottles and then coated with HA using similar procedures as described in the materials and methods section for the batch experiment.

Preparation of Column System. Omnifit columns from Supelco were used for the column experiments. The borosilicate glass column (100 mm long, 25 mm inner diameter) had PTFE end-fittings, viton O-rings, and PTFE frits with a nominal pore size of 10  $\mu$ m. A HPLC pump (Accuflo Series III) was used to pump liquid through the column to a programmed Spectra/Chrom CF-1 Fraction Collector. TFE tubing (1/32" inner diameter, 1/16" outer diameter) was used to connect the pump to the column and the column to the fraction collector. A simplified version of the porous media column apparatus is illustrated in Figure 4.6.

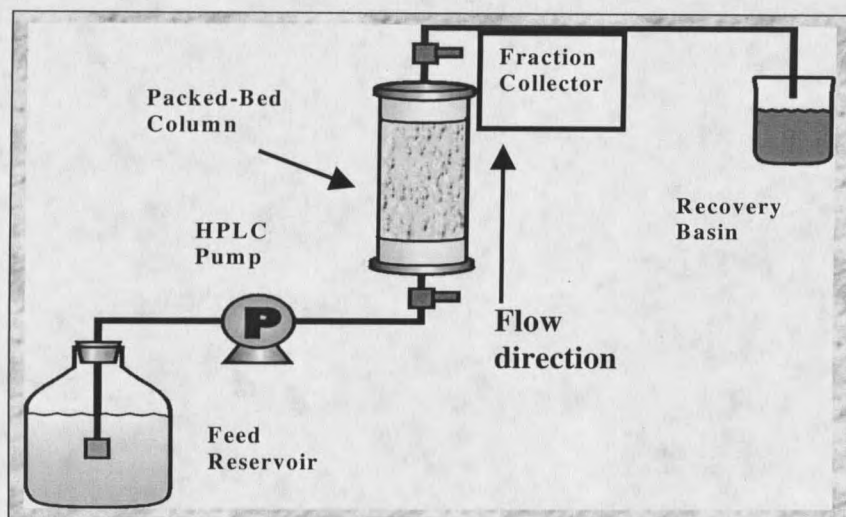


Figure 4.6. Schematic of the porous media column apparatus (simplified).

Rinsing of the column system,  $^3\text{H}_2\text{O}$  (tracer) adsorption,  $^3\text{H}_2\text{O}$  desorption, and TNT adsorption were conducted using the following procedures: column preparation involved soaking the borosilicate glass and end-fittings in a mixture of 70 % ethanol and 30 % nanopure water, and then thoroughly rinsing with nanopure water. The frits were placed in their respective end-fittings, and the end-fittings were cooled in a freezer to  $-15^\circ\text{C}$  to allow insertion into the glass column. The column was oriented so that liquid would flow up through the column. The total volume of the unpacked column and tubing apparatus was 33 mL. Sterilization of all internal column components was done by pumping 150 ml of 70 % ethanol through the apparatus at 5 mL/min. The column was emptied of ethanol, and 150 ml of electrolyte ( $0.0017\text{M Ca}(\text{ClO}_4)_2$ ;  $I = 0.005$ ; pH 6.8) solution was pumped through the system at 5 mL/min to remove the residual ethanol. The electrolyte was decanted and the column was loaded with 50 g of  $\alpha\text{-FeOOH-HA-}$

coated quartz in 1 cm increments to ensure uniform packing of the column. Two pore volumes (40 mL) of electrolyte were then pumped through the packed-column at 0.1 mL/min to remove loose particulate matter and to standardize the system before use.

Tritiated H<sub>2</sub>O and <sup>14</sup>C-TNT Breakthrough Curves. Tritiated water (<sup>3</sup>H<sub>2</sub>O; 4.0 x 10<sup>6</sup> dpm/mL) from Perkin Elmer LifeSciences was used as a conservative column tracer, and introduced to the column at 0.1 mL/min for approximately 400 minutes. The fraction collector collected 1.0 mL samples with a 10-minute resolution, and the amount of <sup>3</sup>H<sub>2</sub>O in effluent fractions was determined using scintillation analysis. Electrolyte (Ca(ClO<sub>4</sub>)<sub>2</sub>; I = 0.005; pH 6.8) was then introduced to the column at 0.1 mL/min for approximately 400 minutes to flush out the <sup>3</sup>H<sub>2</sub>O and to obtain information of the desorption behavior of the conservative tracer. The solvent in the feed reservoir was then changed to a solution containing <sup>14</sup>C-TNT (7300 dpm/mL). The column was loaded with <sup>14</sup>C-TNT at 0.1 mL/min for 800 minutes while the fraction collector collected 1.0 mL samples with a 10-minute resolution.

<sup>14</sup>C-TNT Desorption. The <sup>14</sup>C-TNT desorption procedures were different for each of the four columns. Desorption of TNT from the first and second column was done by pumping Ca<sup>2+</sup>-electrolyte solutions with ionic strengths of 0.001 mol/kg and 0.1 mol/kg, respectively, at 0.1 mL/min for 800 minutes through the columns. Desorption of TNT from the third and fourth columns was done by pumping solutions containing 2.5 and 25 times the CMC (CMC ~ 40 mg/L), respectively, of rhamnolipid biosurfactant at 0.1

mL/min for 800 minutes through the columns. Aqueous concentrations of  $^{14}\text{C}$ -TNT and cold TNT for each column were evaluated on the scintillation analyzer and HPLC, respectively. Samples of solid phase bound  $^{14}\text{C}$ -TNT from each column were burned off using the biological oxidizer and the produced  $^{14}\text{CO}_2$  was captured in a basic scintillation cocktail and determined using the scintillation analyzer.

## Results and Discussion

### Batch Experiment

The amount of HA-C adsorbed to  $\alpha$ -FeOOH was determined from an isothermal plot, which indicated that approximately 8 mg of HA-C was adsorbed per gram of goethite. This corresponds to a fraction of organic carbon ( $f_{oc}$ ) of 0.8 % (wt/wt) in this two-component solid, which is within the range of commonly observed  $f_{oc}$  in clay minerals and some topsoils (Figure 4.7) [22,82]. The sorbed HA was apparently irreversibly bound based on desorption data showing that the same amount of HA-C was adsorbed to the  $\alpha$ -FeOOH surface even after washing with an electrolyte solution (Figure 4.7 and data not shown). The determination of maximum humic acid loading was an important parameter to ensure that the fraction of organic matter on the  $\alpha$ -FeOOH was optimized for TNT adsorption.

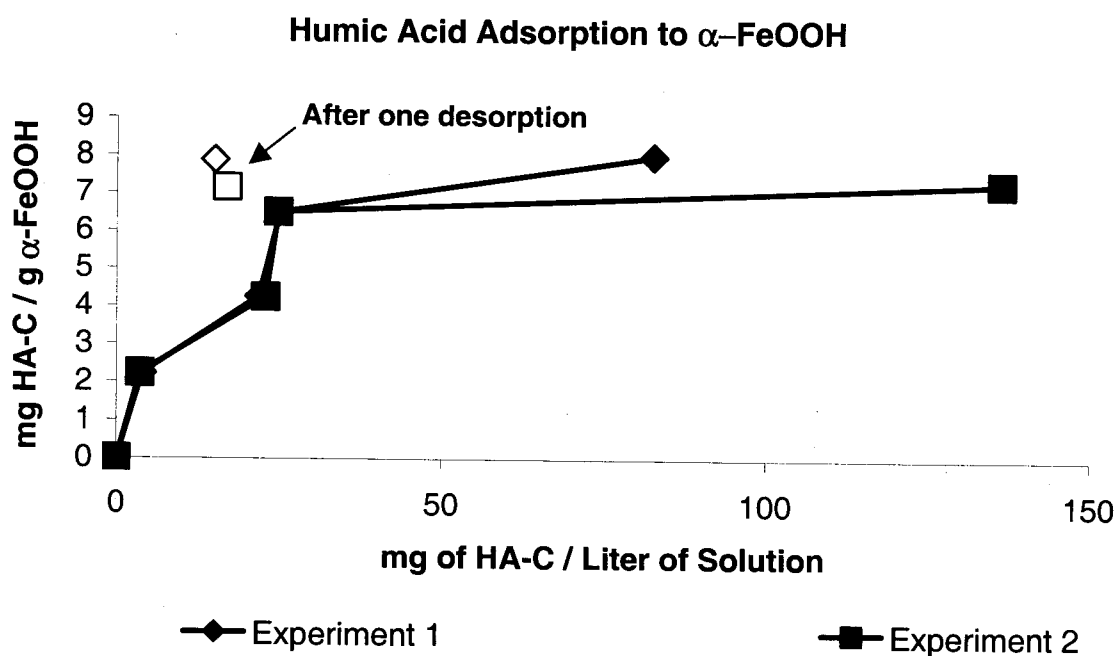


Figure 4.7. Sorption isotherm of soil humic acid to goethite.

Approximately 26 % of the added TNT was sorbed to the HA-coated  $\alpha$ -FeOOH after one day of reaction time. The desorption of  $^{14}\text{C}$ -TNT from the HA-coated  $\alpha$ -FeOOH was observed to be most significant with the addition of rhamnolipid biosurfactant compared to the increased sorption induced by the addition of  $\text{Ca}^{2+}$  and  $\text{Na}^{+}$  relative to the aqueous controls. Figure 4.8 represents aqueous  $^{14}\text{C}$ -TNT fractions (normalized to the aqueous reference sample) after 12 hours of equilibration in the presence of biosurfactants,  $\text{Na}^{+}$  or  $\text{Ca}^{2+}$  using a scintillation analyzer. Figure 4.9 represents the physically bound fraction of  $^{14}\text{C}$ -TNT on the HA-coated  $\alpha$ -FeOOH (normalized to the reference sample), which was measured by use of a biological oxidizer with subsequent quantification of the produced  $^{14}\text{CO}_2$  on a scintillation analyzer.

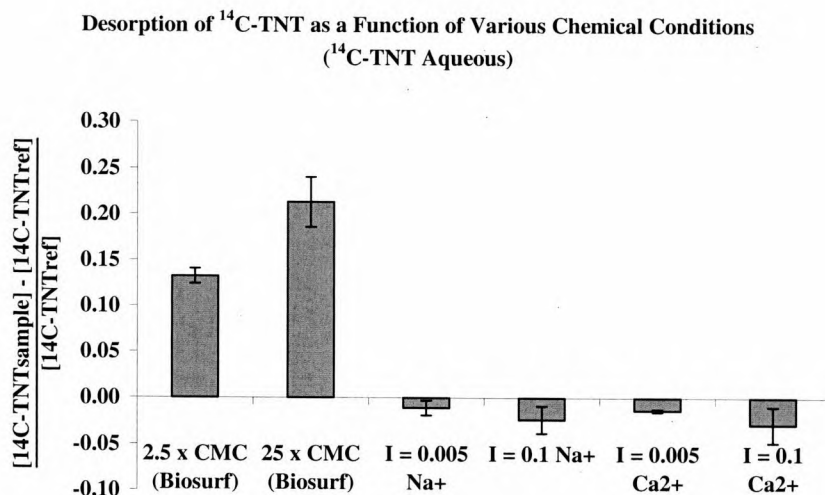


Figure 4.8. Relative desorption of  $^{14}\text{C}$ -TNT from the humic acid coated goethite after 12 hours of reaction with Rhamnolipids,  $\text{Na}^+$ , and  $\text{Ca}^{2+}$  in different concentrations. Based on measured  $^{14}\text{C}$ -TNT in the aqueous phase by use of a scintillation analyzer. Triplicate analysis  $\pm$  one standard deviation.

Figures 4.8 and 4.9 show that the addition of biosurfactants at 2.5 times the CMC increases the TNT desorption from the HA-coated  $\alpha\text{-FeOOH}$  by approximately 13 % compared to the TNT desorption in the aqueous reference. The figures also indicate that desorption is slightly enhanced when the biosurfactant concentration is increased from 2.5 to 25 times the CMC. The addition of  $\text{Ca}^{2+}$ - and  $\text{Na}^+$ -ions to the HA-coated  $\alpha\text{-FeOOH}$  resulted in less TNT desorption compared to the aqueous reference. Increased ionic strength ( $I = 0.1$  vs.  $0.005$  mol/kg) resulted in a further decrease of TNT desorption. Based on the results in Figure 4.9, a slightly increased sorption of TNT was observed in the presence of divalent ions versus monovalent ions.

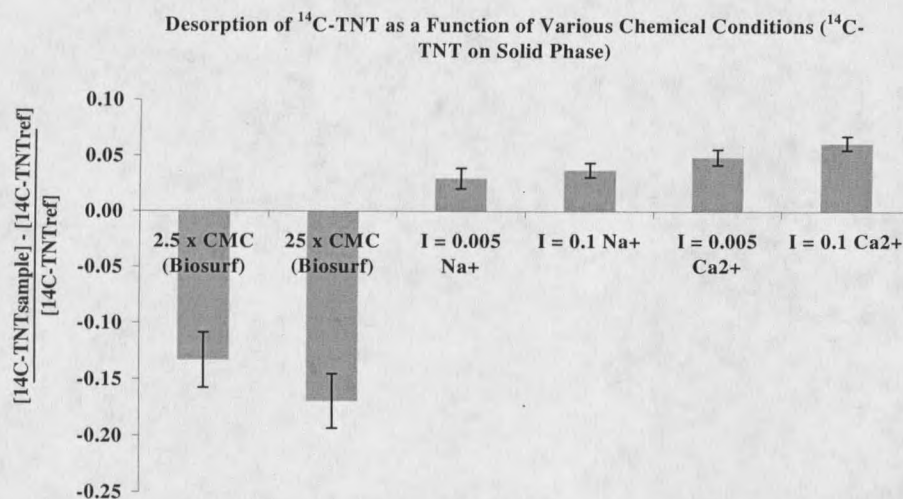


Figure 4.9. Relative desorption of  $^{14}\text{C}$ -TNT from the humic acid coated goethite after 12 hours of reaction with Rhamnolipids,  $\text{Na}^+$ , and  $\text{Ca}^{2+}$  in different concentrations. Based on residual  $^{14}\text{C}$ -TNT on the solid phase measured by use of a biological oxidizer in combination with a scintillation analyzer. Triplicate analysis  $\pm$  one standard deviation.

The increased TNT desorption from HA-coated  $\alpha\text{-FeOOH}$  caused by the addition of biosurfactant could be due to the arrangement and orientation of the surfactant molecules at the solid-water interface. The surfactant orientation and competition for hydrophobic sites on the solid surface may have created a shift in the TNT equilibrium that ultimately resulted in the observed increased TNT desorption. Biosurfactant concentrations above their critical micelle concentration may solubilize organic contaminants. Thus the formation of micelles in the present study may consequently have increased the solubility of TNT or changed the hydrophobicity at the solid-water interface in favor of TNT desorption. Very few studies have investigated the effects of surfactants on the desorption of TNT and none of them have involved the use of

biosurfactants [25,35,83]. However, surfactant enhanced desorption of TNT from contaminated soils at a military site was recently reported by Taha et al. [35] who investigated, anionic (SDS and DOWFAX 8390), cationic (CTAC and CTAB), and nonionic (Tween 80 and Brij 35) surfactants at concentrations ranging from 0.1 to 1%. Anionic surfactants provided significantly increased desorption of TNT from the soil, but there was no increase in TNT desorption for the nonionic or cationic surfactants at concentrations ranging between 0.1 to 1 % [35]. The competition of the negatively charged soil surfaces for the positively charged cationic and the neutral nonionic surfactants was postulated as a potential reason for this phenomenon. TNT was significantly desorbed when the concentrations of Tween 80, DOWFAX 80 and SDS were increased to 10 % and they emphasized the importance of surfactant concentrations above the CMC for significant desorption enhancements. Rhamnolipids consist of multiple anionic components [71] and was amended in concentrations of 1 % and 10 % in this study which reinforces that anionic surfactants are promoting TNT desorption.

Monovalent and divalent cations can impact the structure and sorption behavior of humic acid [57]. At low ionic strengths, charge repulsion occurs between humic acid functional groups, resulting in an uncoiled humic structure, which may increase the adsorption of compounds such as the amino derivatives produced during the biotransformation of TNT. At high ionic strengths charge neutralization occurs between humic acid functional groups, resulting in a coiled humic structure and the possible formation of divalent cation induced bridging between functional groups [84]. These bridging complexes may form hydrophobic domains that can potentially enhance the

adsorption characteristics of hydrophobic contaminants such as TNT (Figure 4.2). In contrast, Murphy et al. [57] indicated that a coiled humic acid structure occupies half the mineral surface area at an ionic strength of 0.1 mol/kg compared to the humic acid structure at an ionic strength of 0.001 mol/kg, and that this coiling might decrease the sorption of organic contaminants. In addition, Schlautman and Morgan [68] have shown that the addition of divalent cations (i.e.  $\text{Ca}^{2+}$ ) generally increased the binding of PAH to humic substances, which was hypothesized to be caused by the formation of hydrophobic cavities. Conversely, increases in pH and ionic strength (i.e.  $\text{Na}^+$  concentration) generally decreased the PAH binding [68].

At least two types of TNT binding might have occurred in the investigated batch study binding to soil HA and or to the  $\alpha\text{-FeOOH}$ , however it is not possible to identify the exact underlying mechanisms for the increased sorption in the present study based on the obtained data. Previous sorption studies by us showed that sorption of approximately 0.03 mg TNT per gram of  $\alpha\text{-FeOOH}$  was possible, which indicates that up to 8 % of the TNT in the present study may have sorbed directly to goethite. The high ionic strength in the system will decrease the aqueous activity of TNT and may result in an ionic strength-controlled phase partitioning of TNT. In addition, Li et al. [19] hypothesized that the less hydrophilic configuration of HA with increasing ionic strength would bind the hydrophobic ligand TNT more effectively. Thus, the binding level is related to one factor being more dominant than another (e.g. HA hydrophilicity vs HA coiling/aggregation). Our results indicate that the mode of TNT adsorption to the HA-coated  $\alpha\text{-FeOOH}$  may be a combination of phase partitioning and hydrophobic sorption.

### Column Experiment

Three milligrams of  $\alpha$ -FeOOH was bound to each gram of quartz sand. For comparison, this corresponds to one order of magnitude more Fe(III) compared to the acid extractable (0.5N HCl) amount of Fe(III) reported for an aquifer sediment at the U.S. Geological Survey Cape Cod Research Site [85]. The crystal phase of  $\alpha$ -FeOOH was presumed intact based on detailed studies by Scheidegger et al. [81] and SEM micrographs (Figure 4.10).

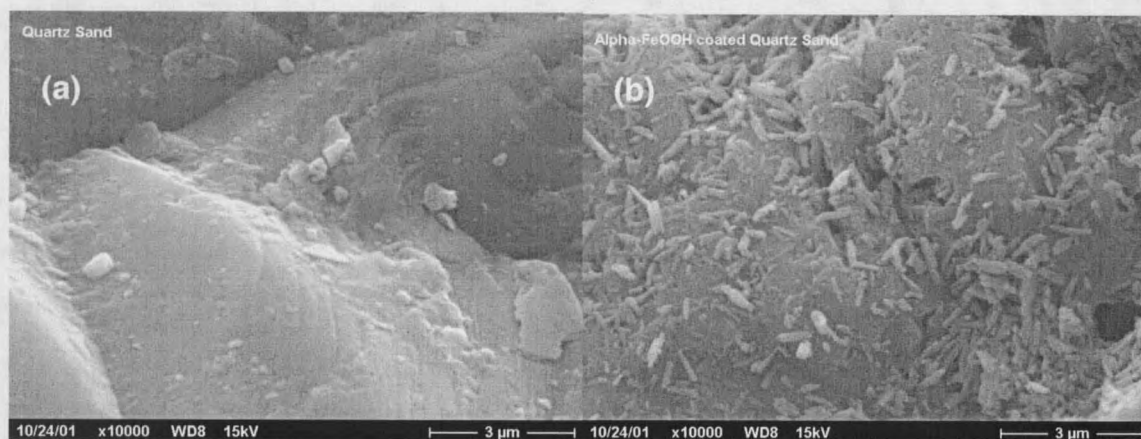


Figure 4.10. (a) SEM image of pure quartz sand (before goethite coating). (b) SEM image of goethite coated quartz sand (showing the characteristic “needle-shaped” goethite particles).

An adsorption isotherm (Figure 4.11) showed that approximately 0.3 mg of HA-C was adsorbed to each gram of  $\alpha$ -FeOOH coated quartz sand. This ( $f_{oc} = 0.03\%$ ) corresponds to the observed organic carbon fraction in typical aquifer sediments or a sandy subsoil [71,85].

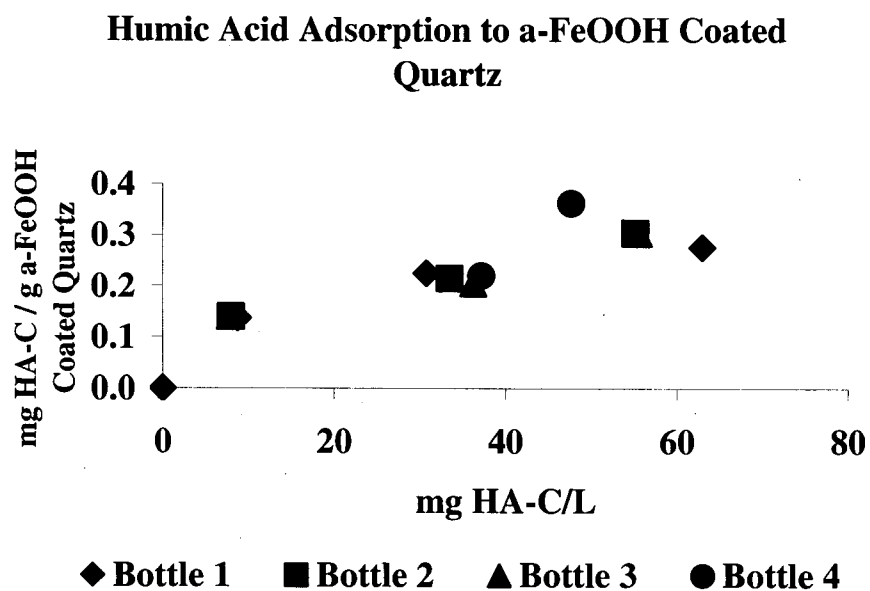


Figure 4.11. Adsorption isotherms for humic acid adsorption onto  $\alpha$ -FeOOH-coated quartz sand. This porous media was used in all column studies.

Tritiated water breakthrough curves (BTCs) were used to obtain column parameters (Figure 4.12). The calculated column parameters were based on a column loaded with 50 g of quartz sand with a particle density ( $\rho_p$ ) of approximately  $2.65 \text{ g/cm}^3$ , a bulk density ( $\rho_b$ ) of  $1.52 \text{ g/cm}^3$  [ $\rho_b = \text{mass}_Q/V$ ], and a porosity ( $\eta$ ) of 0.43 [ $\eta = 1 - (\rho_b/\rho_d)$ ]. The average linear velocity ( $v$ ) of the mobile phase was  $2.87 \text{ cm/h}$  [ $v = q/(A*\eta)$ ] at a flow rate ( $q$ ) of  $0.1 \text{ mL/min}$ . This system had flow and soil (sediment) characteristics comparable with the U.S. Geological Survey Cape Cod Research Site [85].

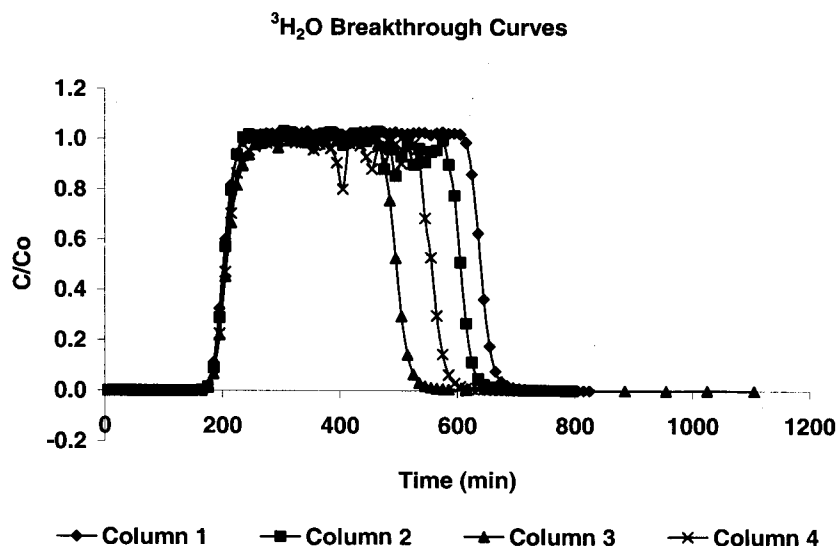


Figure 4.12. Tritium breakthrough curves for all four columns; electrolyte ( $\text{Ca}(\text{ClO}_4)_2$ ;  $I = 0.005 \text{ mol/kg}$ ;  $\text{pH } 6.8$ ) was added after 405 (C1), 425 (C2), 294 (C3), and 355 (C4) minutes to flush out tritium in the columns.

The conservative  $^3\text{H}_2\text{O}$  tracer indicated that breakthrough for all columns occurred at 205 minutes (corresponding to a pore volume of 20.5 mL). The FORTRAN based program CXTFIT Version 2.1 [86] was used to fit  $^3\text{H}_2\text{O}$  BTCs to physical equilibrium and physical non-equilibrium models based on the advection-dispersion equation (ADE, equation 1):

$$R_f (\partial c / \partial t) = D (\partial^2 c / \partial x^2) - v (\partial c / \partial x) \quad (\text{equation 1})$$

which describes the change in solute concentration ( $c$ ) as a function of time ( $t$ ) and depth ( $x$ ), where  $D$  = dispersion coefficient ( $\text{cm}^2 \text{ hr}^{-1}$ ),  $R_f$  = retardation factor (unitless), and  $v$  = average linear velocity ( $\text{cm hr}^{-1}$ ). The CXTFIT models transport phenomena using a nonlinear least-squares parameter optimization method. The physical equilibrium models of the  $^3\text{H}_2\text{O}$  BTCs produced dispersion coefficients ( $D$ ) (Table 4.1) for each of the columns (Figure 4.13).

Table 4.1. CXTFIT-estimated dispersion coefficients (D) for the columns.

| Tracer Results    | Fixed $R_f$ | D (cm <sup>2</sup> /h) | R <sup>2</sup> |
|-------------------|-------------|------------------------|----------------|
| Tritium Tracer C1 | 1.0         | 0.0551                 | 0.994          |
| Tritium Tracer C2 | 1.0         | 0.0572                 | 0.987          |
| Tritium Tracer C3 | 1.0         | 0.0727                 | 0.996          |
| Tritium Tracer C4 | 1.0         | 0.0706                 | 0.992          |

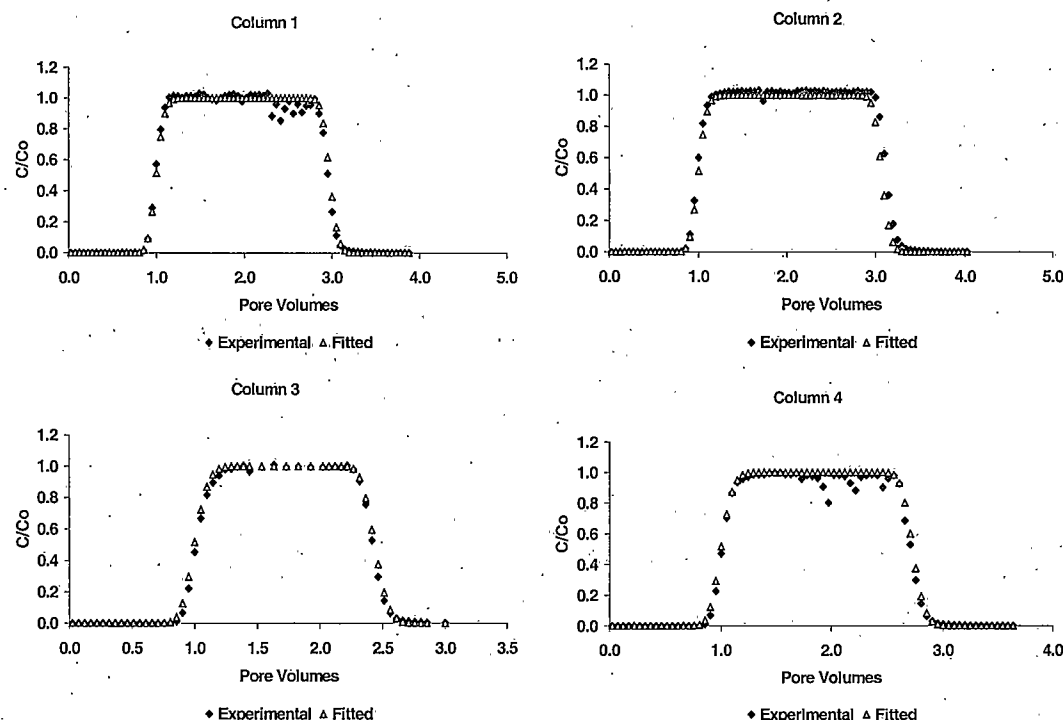


Figure 4.13. Modeled tritium tracer curves fitted to experimental data for each column. Desorption of tritium with  $\text{Ca}^{2+}$ -electrolyte was started after approximately 1.95 pore volumes (see figure 4.12 for details; one pore volume corresponds to 205 min).

Mechanical dispersion in a homogeneously packed column includes hydrodynamic dispersion and molecular diffusion [87]. Dispersion coefficients from conservative tracers can be applied to dilute solutions when hydrodynamic dispersion, influenced by turbulent eddies, dominates over molecular diffusion.

A physical nonequilibrium CXTFIT model was used to obtain mass transfer coefficients for the various test solutions used for desorption of  $^{14}\text{C}$ -TNT in the saturated column systems. A logistic approach using the dispersion coefficients from the  $^3\text{H}_2\text{O}$  BTCs and retardation factors ( $R_f$ ) from a physical equilibrium model based on experimental TNT data were used to find the best data fit. Correlation coefficients indicated that better modeling was obtained by a non-equilibrium fit (Figure 4.14) rather than equilibrium based fit (data not shown). The increase in  $^{14}\text{C}$ -TNT concentration for column 2 was a physical phenomenon that occurred due to a two hours lag time between solvent reservoir changes. The solvent changes for the other columns took place within 20 minutes.

TNT desorption characteristics for low and high ionic strength calcium perchlorate solutions (Figure 4.15) are similar to their respective desorption curves of tritium. Results from the batch experiment can be correlated to the discussion of TNT desorption behavior in the column experiments. The high ionic strength test solution consistently resulted in lower TNT effluent concentrations compared to desorption using the low ionic strength solution. This phenomenon could be due to increased formation of hydrophobic domains or a lower aqueous activity of TNT because of the higher ionic strength.

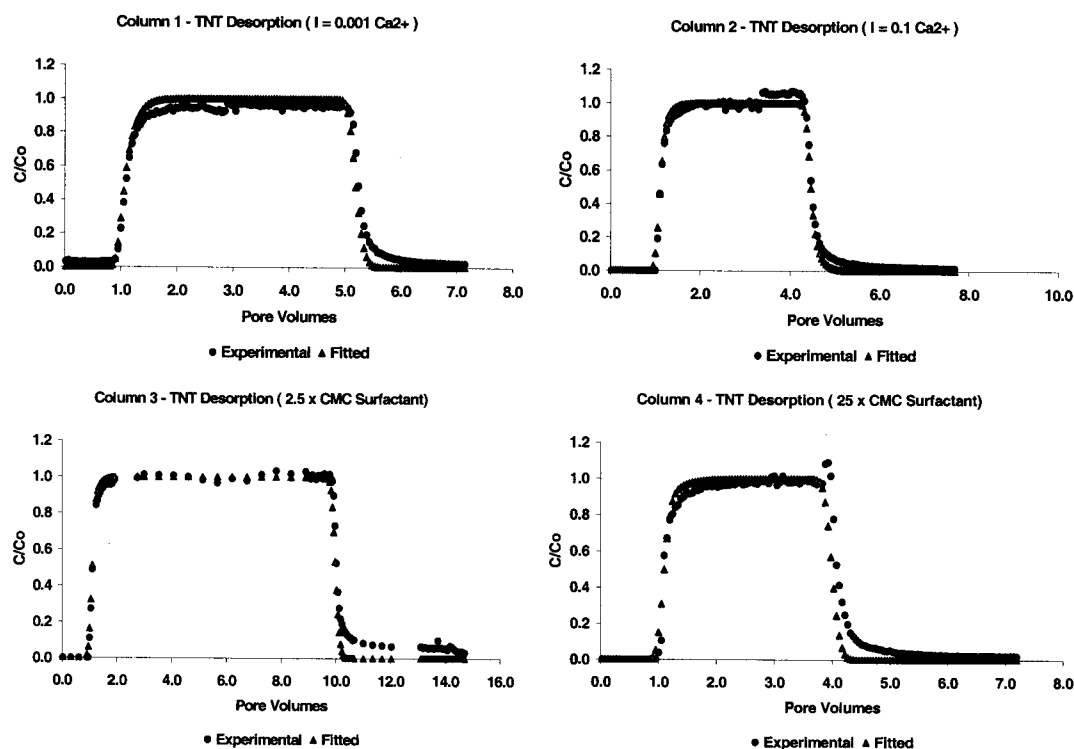


Figure 4.14. Step curves for experimental and fitted TNT adsorption and desorption using various solvents for each column.

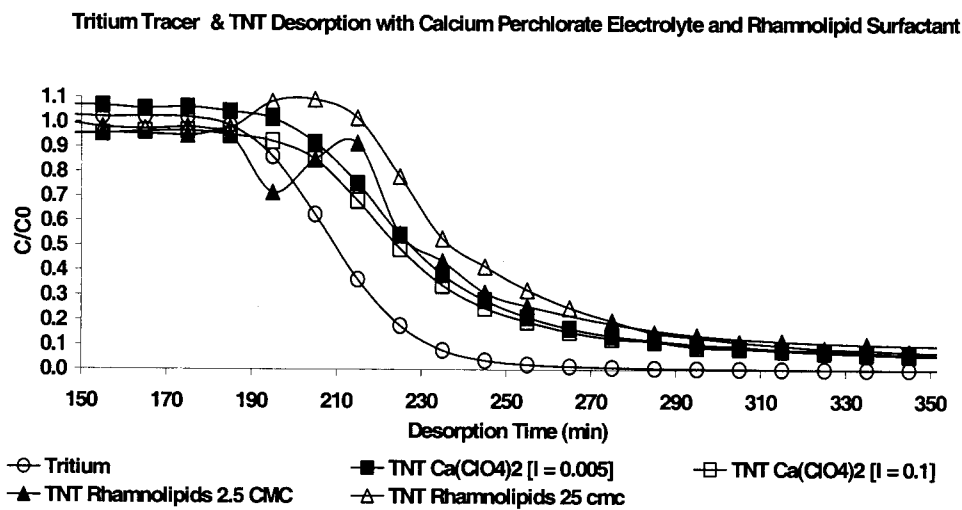


Figure 4.15. Tritium desorption curve from column two, and TNT desorption curves for various test solutions. One pore volume corresponds to 205 min.

Figure 4.15 indicates that the addition of 2.5 and 25 times the CMC of rhamnolipid biosurfactants produced a TNT desorption phenomena different from the columns added  $\text{Ca}^{2+}$ -electrolyte. TNT desorption using biosurfactant at 2.5 times the CMC produced an initial decrease in TNT effluent concentration, followed by a rise in TNT effluent concentration. This fall and rise phenomenon could have been attributed to dispersed surfactants first forming a TNT sorbing monolayer on the solid phase (Figure 4.3). The subsequent rise in TNT concentration occurred near the first pore volume (195 minutes). This indicates that as surfactant concentration increased in the column the critical micelle concentration was reached resulting in the formation of a surfactant bilayer and micelles. This change in surface and aqueous surfactant chemistry appears to favor TNT desorption from the column systems.

An increase in initial TNT effluent concentration was observed with the addition of biosurfactant at 25 times the CMC. A potential rapid formation of a surfactant bilayer with a concurrent production of micelles may have accelerated the solubilization of TNT in the aqueous phase and resulted in the observed increased desorption kinetics. The observed desorption characteristics were in agreement with phenanthrene studies by Noordman et al. [40]. Who showed that the removal time of 90 % of the initially added phenanthrene from artificially contaminated soil columns was reduced up to 3.5-fold when elution was performed with an electrolyte solution containing 500 mg/L ( $\sim 13 \times$  CMC) rhamnolipid as compared to the treatment without rhamnolipid.

First-order desorption mass transfer rate coefficients ( $k$ ) for TNT were determined from the non-equilibrium transport parameters (Table 4.2). The obtained transport

parameters included  $R_f$  (retardation factor; see definition above),  $\beta$  (the distribution of soil water between mobile and immobile domains; when  $\beta = 1$ , then no preferential flow), and  $\omega$  (the relative mass transfer rate between the mobile and immobile domains; when  $\omega = 100$ , then indicative of equilibrium). Equation 2 was used in determining  $k$  for the various test solutions used in the desorption of TNT ( $v$  = average linear velocity (i.e. 2.87 cm hr<sup>-1</sup>;  $L$  = characteristic length of column (i.e. 6.7 cm)) [86]:

$$k = \omega * v / ((1 - \beta) * R_f * L) \quad (\text{equation 2, [86]})$$

Table 4.2. CXTFIT estimated first-order desorption mass transfer rate coefficients and non-equilibrium parameters for experimental columns (values of  $R_f$  and  $D$  obtained independently).

| Solvent Use in TNT                  | Fixed D.             | Fixed $R_f$ | Beta  | Omega  | $R^2$ | $k$ (h <sup>-1</sup> ) |
|-------------------------------------|----------------------|-------------|-------|--------|-------|------------------------|
| Desorption                          | (cm <sup>2</sup> /h) |             |       |        |       |                        |
| Ca <sup>2+</sup> ; I = 0.001 mol/kg | 0.0551               | 1.126       | 0.949 | 0.433  | 0.99  | 3.2                    |
| Ca <sup>2+</sup> ; I = 0.1 mol/kg   | 0.0572               | 1.103       | 0.907 | 3.294  | 0.97  | 13.5                   |
| Rhamnolipid; 2.5 x CMC              | 0.0727               | 1.117       | 0.895 | 5.336  | 0.96  | 19.3                   |
| Rhamnolipid; 25 x CMC               | 0.0706               | 1.123       | 0.891 | 10.740 | 0.94  | 36.9                   |

Desorption rate coefficients indicate that the addition of biosurfactants decrease the removal time of TNT from the saturated porous medium up to 10-fold as compared to the treatments containing various ionic strengths of calcium-ions (Table 4.2). The estimated desorption rate coefficients of TNT in the presence of biosurfactants appears overestimated when compared to the observed increase in TNT desorption (Figure 4.15).

Several factors may have contributed to the apparent variations between experimental and fitted TNT BTCs (Figure 4.14). The first factor is that  $^3\text{H}_2\text{O}$  dispersion coefficients were similar for columns one and two and 25 % higher for columns three and four. In theory, the dispersion coefficients should be the same for similar columns when using a conservative tracer. This 25 % deviation in dispersion constants may be attributed to an unintentional difference in the way each column was packed and might be a source of error.

Figure 4.14 illustrates that CXTFIT was especially not capable of accurately fitting the tailing resolution when desorbing TNT for all columns, and did not fit the rise and fall characteristics of the TNT desorption when using biosurfactants. These inaccuracies influence the correctness of the transport parameters  $R_f$ ,  $\beta$ , and  $\omega$  that were used to calculate  $k$ . Noordman et al. [40] reported that the effect of rhamnolipid on the removal of phenanthrene was satisfactorily simulated using independently obtained parameters with a two-component advective-dispersive model accounting for micellar solubilization and admicellar sorption. They concluded that rhamnolipid enhanced the removal of phenanthrene mainly by micellar solubilization and by influencing sorption kinetics.

### Conclusion

This study demonstrated the impact of various solution chemistries on desorption of 2,4,6-trinitrotoluene from HA-coated  $\alpha\text{-FeOOH}$  and  $\alpha\text{-FeOOH}$ -HA-coated quartz sand. The batch experiment demonstrated that biosurfactants increased TNT desorption

from HA-coated  $\alpha$ -FeOOH by approximately 20 %. Solutions containing divalent and monovalent cations (i.e.  $\text{Ca}^{2+}$  and  $\text{Na}^{+}$ ) decreased the amount of TNT desorbed from the HA-coated  $\alpha$ -FeOOH relative to controls, indicating a cation/ionic strength-controlled phase partitioning. Additionally, the column experiments indicated that TNT mass transfer from  $\alpha$ -FeOOH-HA-coated quartz sand is increased in the presence of biosurfactants compared to mass transfer in ionic solutions.

The CXTFIT program was a useful tool in defining transport parameters for desorption of TNT from the porous medium in packed columns. However, a more sensitive experimental system is needed to provide results that are more significant.

In summary, desorption characteristics of TNT from HA-coated  $\alpha$ -FeOOH and  $\alpha$ -FeOOH-HA-coated quartz sand is dependent on the surrounding aqueous chemistry. Phase partitioning was the dominant mode of TNT mass transfer when adding ionic solutions to the model systems. Rhamnolipid (biosurfactant) enhanced TNT desorption was observed and was probably a result of competitive sorption or micelle solubilized TNT. The small fraction of HA on the solid phases may have caused the relative short retardation of TNT thus making it difficult to compare the desorption kinetics as a function of solution chemistry.

### Perspectives

Future investigations should involve solid phases or real soil (e.g. topsoil) with a significantly higher TNT sorption capacity to increase the sensitivity of the experimental system towards changes in solution chemistry. The objective of this study was to

investigate the influence of cations and surfactants on the (de)sorption behavior of TNT under abiotic conditions. However, extrapolation of the findings herein to an actual contaminated site has to include an evaluation of the potential influence of microorganisms.

Therefore, the hypothetical role of biofilm is briefly evaluated in the following paragraphs based on a literature study. Nutrient flux across the biofilm-water interface [88] and slow mass transport in the biofilm matrix [89] results in the accumulation of ionic species that increase the ionic strength in the microenvironment at the solid-water interface. This could induce structural changes in SOM that could affect the association of contaminants with soil surfaces (and thus, their subsequent bioavailability) [19,84,90,91].

Biofilms are complex physical structures [92] that maintain their architecture by a matrix of both inorganic and organic constituents that include exopolysaccharides, or EPS [93]. EPS has a tremendous capacity for cation exchange and is responsible for the accumulation of divalent metals in biofilms [94]. Consequently, biofilm activity promotes the accumulation of divalent metal cations (e.g.,  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ) at the soil-water interface. Soil organic matter can also form strong complexes with divalent cations [95]. Engebretson and von Wandruszka [67] investigated the effects of  $\text{Mg}^{2+}$  on the kinetics of aggregation of dissolved humic acid, as discussed earlier (see above). Cation effects of SOM structure could likewise influence the bioavailability of SOM-associated contaminants.

A characteristic of various microorganisms is their ability to produce biosurfactants (e.g. *Pseudomonas aeruginosa*). Not all microbes have this ability, and those that do may produce surface-active compounds whose functions are dramatically different. Surfactants possess both hydrophilic and hydrophobic structural moieties, which impart unusual properties, including an ability to lower the surface tension of water and the emulsification of water with water-immiscible substrates [96]. The production of biofilm surfactants may solubilize hydrophobic compounds and change their bioavailability [70]. Major classes of biosurfactants include: 1) glycolipids, 2) phospholipids and fatty acids, 3) lipopeptide/lipoproteins, 4) polymeric surfactants, and 5) particulate surfactants. These classes are differentiated based on their biochemical nature and the microbial species producing them. Surfactin, a cyclic lipopeptide produced by *Bacillus subtilis*, is an example of a very effective biosurfactant that reduces surface tension at very low concentrations [96]. Finally, recent studies by us [39] indicated that HAs might have surfactant-like behavior. Our study [39] showed that the presence of Elliot soil humic acid resulted in significantly increased bioavailability of pyrene as compared to unsaturated systems containing the bare magnetite mineral.

Though the findings in this study suggest increased bioavailability in the presence of biosurfactants, one would have to test that hypothesis in a biodegrading system to confirm that micelle-solubilized TNT is bioavailable.

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## CHAPTER 5

BIOTIC AND ABIOTIC MECHANISMS CONTRIBUTE TO THE DEGRADATION  
OF 2,4,6-TRINITROTOLUENE BY A GRAM-POSITIVE FERMENTING  
BACTERIUM IN THE PRESENCE OF HYDROUS FERRIC OXIDE AND  
ELECTRON SHUTTLES

Introduction

Contamination of groundwater and soil with explosives, such as 2,4,6-trinitrotoluene (TNT), is a widespread environmental problem. Concerns stem from the mutagenic, carcinogenic, and toxic effects of TNT and its metabolites [1-4]. TNT is extremely recalcitrant against microbial mineralization in the environment [5], however, many studies have reported that TNT biotransforms to amino derivatives [6]. Hydroxylaminodinitrotoluenes (HADNTs) are commonly observed intermediates during the transformation of TNT to aminodinitrotoluenes (ADNTs) and the reduction of all three nitro groups to form triaminotoluene (TAT) has been shown to occur under strictly anaerobic conditions [6]. The reduction of TNT is often regioselective, leading to the preferential formation of 4-amino-2,6-dinitrotoluene (4-ADNT) and 2,4-diamino-6-nitrotoluene (2,4-DANT) and may be due to the more negative character of the 4-substituted nitro group [7,8]. Understanding the degradation pathway is important, since some metabolites (e.g. 2,4,6-triaminotoluene (TAT)) may bind strongly to soils and others may be even more toxic than TNT (e.g. 2,2'-6,6'-tetranitro-4,4'-azoxytoluene (4,4'-Azoxy)) [2,9-13]. Given that the complete mineralization of TNT by microorganisms does not readily occur in natural environments, immobilization of

reduced TNT metabolites is one mechanism for minimizing transport and enhancing the natural attenuation of TNT [11]. However, soils represent very heterogeneous systems containing organic matter of various origins, different mineral phases including iron (hydr)oxides, and a variety of microorganisms. In these complex contexts, the mechanisms and pathways of TNT biotransformation are not well studied.

The reduction of Fe(III) (hydr)oxides by dissimilatory metal reducing bacteria (DMRB) has received considerable attention in recent years since the ferrous iron (Fe(II)) generated during the biological reduction of solid phase Fe(III) (hydr)oxides has proven to be an effective reductant of highly toxic metals such as Cr(VI) and U(VI) [14-16]. The reduction rate of Fe(III) (hydr)oxides by DMRB can increase in the presence of humic acids (HA) and anthraquinone-2,6-disulfonate (AQDS). Both, HA and AQDS, have been shown to function as electron shuttles, thus increasing the rate and extent of Fe(III) reduction by diminishing the need for direct contact between microorganisms and Fe(III) surfaces [15,17-21].

While the influence of electron shuttling compounds in Fe(III) mineral reduction has been investigated intensively, very few studies have examined their influence on the reduction of organic pollutants. Kappler and Haderlein [22] recently reported the reduction of chlorinated compounds by reduced humic acids (HA) and the reduced form of AQDS (anthrahydroquinone-2,6-disulfonate, AHQDS). Hofstetter et al. [23] demonstrated that TNT can be reduced to its amino derivatives by Fe(II) present at the surface of  $\alpha$ -FeOOH or, less efficiently, by hydroquinones in the presence of  $H_2S$ . Lastly, they demonstrated that *Geobacter metallireducens* and a mixed Fe(III)-reducing

consortium can indirectly contribute to the reduction of nitroaromatics via production of Fe(II) in ferrogenic columns [23].

The majority of research investigating the effect of electron shuttles and iron mineral reduction on the fate of environmental contaminants has been conducted with the two highly studied, metal-reducing genera *Shewanella* and *Geobacter*. However, little is known regarding Fe mineral reduction mediated by other common soil organisms. Benz et al. [24] demonstrated that a number of Gram positive and Gram negative bacteria can reduce Fe(III) minerals in the presence of electron shuttling compounds and suggested that these organisms could have a significant influence mediating electron transfer in soil environments. Sani et al. [25] recently described a number of isolates, identified as *Cellulomonas* spp., that were the predominant organisms obtained in Cr(VI)-reducing enrichment cultures from aquifer samples from the US Department of Energy's Hanford site. *Cellulomonas* sp. strain ES6 was recently shown to reduce Cr(VI), U(VI), Fe(III)-NTA, goethite, maghemite, magnetite, hematite, HFO, TNT and AQDS [3,25,26].

Given the importance of reduced Fe(III) (hydr)oxides and AQDS on the fate of TNT, the primary goal of the present study was to utilize strain ES6 to investigate the contribution of i) direct microbial, ii) electron shuttle-mediated, and iii) Fe(III, II)-mediated nitroaromatic reduction on the fate of TNT in multi-component systems.

## Experimental Section

### Chemicals

Compounds used in this study and their suppliers were the same as described previously [3]. Additionally, [U-ring- $^{14}\text{C}$ ]TNT was purchased from ChemSyn Laboratories (KS, USA), HPLC grade methanol,  $\text{FeCl}_3$ , and sucrose from Fisher Scientific (NJ, USA). Tryptic soy broth (TSB) was obtained from Difco Laboratories (MD, USA),  $\text{FeCl}_2$ , ferrozine, and anthraquinone-2,6-disulfonate from Sigma-Aldrich (MO, USA). Soil humic acids were ordered from the International Humic Substances Society (MN, USA). All chemicals were of analytical grade or higher purity. The deionized (DI) water was obtained from a Barnstead NANOpure system.

### Preparation of ES6 Cultures

The *Cellulomonas* strain designated strain ES6 was maintained in frozen stock cultures containing TSB with 20 % glycerol and were stored at  $-70\text{ }^{\circ}\text{C}$  [25]. Cells were pre-cultured in TSB (30 g/L) for 24 hours shaken at 150 rpm at  $21 \pm 2\text{ }^{\circ}\text{C}$ , transferred into fresh TSB, and grown again for 18 hours in TSB. Cultures were harvested by centrifugation at  $5,860 \times g$  for 20 minutes at  $4\text{ }^{\circ}\text{C}$ , washed once in  $\text{O}_2$ -free synthetic groundwater, and resuspended in SGW to obtain the desired cell concentration.

### Media Composition

A synthetic ground water medium (SGW) of the following composition was used in all experiments [27] (all concentrations in mg/L):  $\text{Na}_2\text{SiO}_3 \cdot 9\text{H}_2\text{O}$  (455),  $\text{Na}_2\text{CO}_3$  (160),  $\text{Na}_2\text{SO}_4$  (6), yeast extract (50), casamino acids (100), KCl (26.5),  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (8),

Mg(OH)<sub>2</sub> (33.5), NaHCO<sub>3</sub> (2519.7), LiCl (0.021), CuSO<sub>4</sub>·5H<sub>2</sub>O (0.08), ZnSO<sub>4</sub>·7H<sub>2</sub>O (0.106), H<sub>3</sub>BO<sub>4</sub> (0.6), Al<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub>·18H<sub>2</sub>O (0.123), NiCl<sub>2</sub>·6H<sub>2</sub>O (0.11), CoSO<sub>4</sub>·7H<sub>2</sub>O (0.109), Na<sub>2</sub>O<sub>4</sub>Se (0.05), MnCl<sub>2</sub>·4H<sub>2</sub>O (0.629), KBr (0.03), KI (0.03), Na<sub>2</sub>MoO<sub>4</sub> (0.01), and FeSO<sub>4</sub>·7H<sub>2</sub>O (0.3). The Na<sub>2</sub>SiO<sub>3</sub>·9H<sub>2</sub>O, Na<sub>2</sub>CO<sub>3</sub>, Na<sub>2</sub>SO<sub>4</sub>, yeast extract, and casamino acids were dissolved in DI water and autoclaved. The autoclaved solution was boiled for 10 minutes, cooled to room temperature under an O<sub>2</sub>-free atmosphere of N<sub>2</sub>/CO<sub>2</sub> (80:20) and sterile, O<sub>2</sub>-free stock solutions of KCl, NaHCO<sub>3</sub>, CaCl<sub>2</sub>·2H<sub>2</sub>O, Mg(OH)<sub>2</sub>, and the trace minerals were added using purged syringes and needles. The medium was dispensed under the N<sub>2</sub>/CO<sub>2</sub> atmosphere to maintain O<sub>2</sub>-free conditions.

#### Iron Synthesis and Analysis

Hydrous ferric oxide (HFO) was synthesized by dissolving 10.8 g FeCl<sub>3</sub> in 100 mL of DI water and slowly adjusting the pH to 7.0 using dropwise addition of 6 N NaOH over a period of one hour [28]. An aliquot of 0.25 mL of this stock solution was added to the HFO containing treatments described below. Total iron (extraction with 2.5N HCl + 0.25N NH<sub>2</sub>OH for 24 hours), extractable Fe(II)<sub>0.5N HCl</sub> (extraction with 0.5N HCl for 2 hours) and aqueous Fe(II)<sub>aq</sub> (filtered through a 0.2 μm filter) were determined using the Ferrozine method [29].

#### TNT Transformation Experiments

Reaction solutions (25 mL) containing O<sub>2</sub>-free SGW, sucrose (10 mM), and unlabeled TNT (55-65 μM) were prepared anaerobically. SGW and sucrose were dispensed under the N<sub>2</sub>/CO<sub>2</sub> atmosphere, and TNT was added in a glove box (90 % N<sub>2</sub>; 5

% CO<sub>2</sub>; 5 % H<sub>2</sub>) to 30 mL test tubes (Bellco Glass, NJ, USA) in the presence and absence of 6 mM HFO and 100  $\mu$ M AQDS. The test tubes were crimp sealed with polytetrafluoroethylene-faced butyl rubber septa (West Pharmaceutical Services, PA, USA) and the treatments were inoculated with strain ES6 at initial population densities of  $3.8 \times 10^8$  (pre-incubated systems) or  $1.8 \times 10^9$  (fresh cell systems) colony forming units (CFU) per mL. The tubes were pressurized with 10 psi of N<sub>2</sub>/CO<sub>2</sub> (80/20) to achieve a media pH of 7. The vials were incubated in the dark on a rotator at  $21 \pm 2$  °C and triplicate sets of vials were sampled periodically in the O<sub>2</sub>-free glove box and analyzed for dissolved TNT and metabolites. Several experiments were conducted using <sup>14</sup>C-TNT ( $9 \times 10^5$  dpm/mL) at an initial cell density of  $1.5 \times 10^7$  CFU/mL to track the fate of TNT in the presence of AQDS where we observed that some TNT reaction products were not identifiable using an exhaustive analytical protocol for determination of TNT metabolites [3].

#### TNT Metabolite Analysis

Aliquots of 500  $\mu$ L were withdrawn from the vials over time and filtered (0.2  $\mu$ m, Spartan 13/A, regenerated cellulose, Schleicher & Schuell MicroScience, Inc., FL, USA). Losses of nitroaromatics due to filtration were small (< 2 %) [23] and all HPLC samples were analyzed immediately as described by us previously [3]. Briefly, TNT and its metabolites were analyzed by a Hewlett-Packard 1090 Liquid Chromatograph equipped with a diode array detector. A Spectra/Chrom CF-1 Fraction Collector (Spectrum Chromatography, TX, USA) was used to collect radiolabeled fractions of the HPLC eluent. TNT, 4,4',6,6'-tetranitro-2,2'-azoxytoluene (2,2'-Azoxy), 4,4',6,6'-tetranitro-

2,2'-azotoluene (2,2'-Azo), 2,2',6,6'-tetranitro-4,4'-azotoluene (4,4'-Azo), and metabolites shown in Figure 5.1 were fully separated on a Supelcosil C-8 column with a Supelcoguard C-8 guard column from Supelco, PA, USA. The details of the HPLC method are available in [3]. Calibration standards were obtained from authentic compounds and multipoint standard calibration curves were established for quantification of the compounds. The measurements of  $^{14}\text{C}$  in HPLC fractions and stock solutions were performed with a Packard scintillation analyzer (Tri-Carb 1900CA) after addition of samples to Ultima Gold scintillation cocktail (Packard Instrument, CT, USA).

### Results and Discussion

#### Influence of HFO and AQDS on the Microbial Transformation of TNT

TNT was reduced within 24 hours in the presence of strain ES6 resulting in the concurrent production of 4-HADNT (step 1.1) and smaller amounts of 2-HADNT (step 1.2) and 4,4'-Azoxy (step 4, Figure 5.1 and 5.2a). The production of the dimer 4,4'-Azoxy is likely a result of the spontaneous abiotic hydroxylamino-nitroso condensation reaction [3,6,30]. Further reduction of 4-HADNT lead primarily to 2,4-DHANT (step 2), 4-ADNT (step 6) and one unidentified compound (Figure 5.1). Based on an abiotic experiment described previously [3], in which a standard mixture containing 2- and 4-HADNT was allowed to react, this compound is believed to be either the 4',6',2,6-tetranitro-2',4-azoxytoluene or the 4,6,2',6'-tetranitro-2,4'-azoxytoluene. 2-HADNT, 4-HADNT, and their nitroso counterparts are known precursors of azoxy compounds [30,31] and we demonstrated in a previous study that one of the products of this reaction

had the same retention time and UV-spectrum as the unknown compound observed in this study [3].

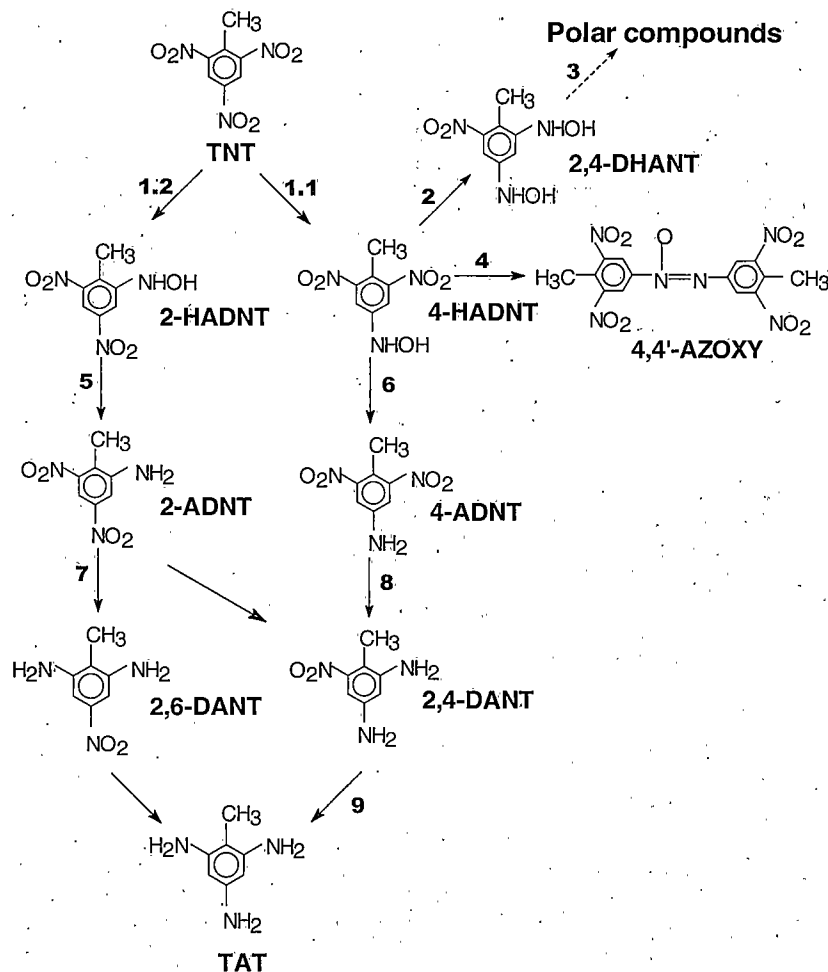


Figure 5.1. Possible TNT degradation pathways and metabolites formed under biotic and or abiotic conditions. Strictly abiotic reduction via Fe(III) (hydr)oxide associated Fe(II) has been shown in previous studies [23]. Biotic pathways often lead to the initial preferential reduction of the nitro group *para* to methyl group [7]. Details of how strain ES6, HFO and AQDS influenced the fate of TNT are discussed in text.

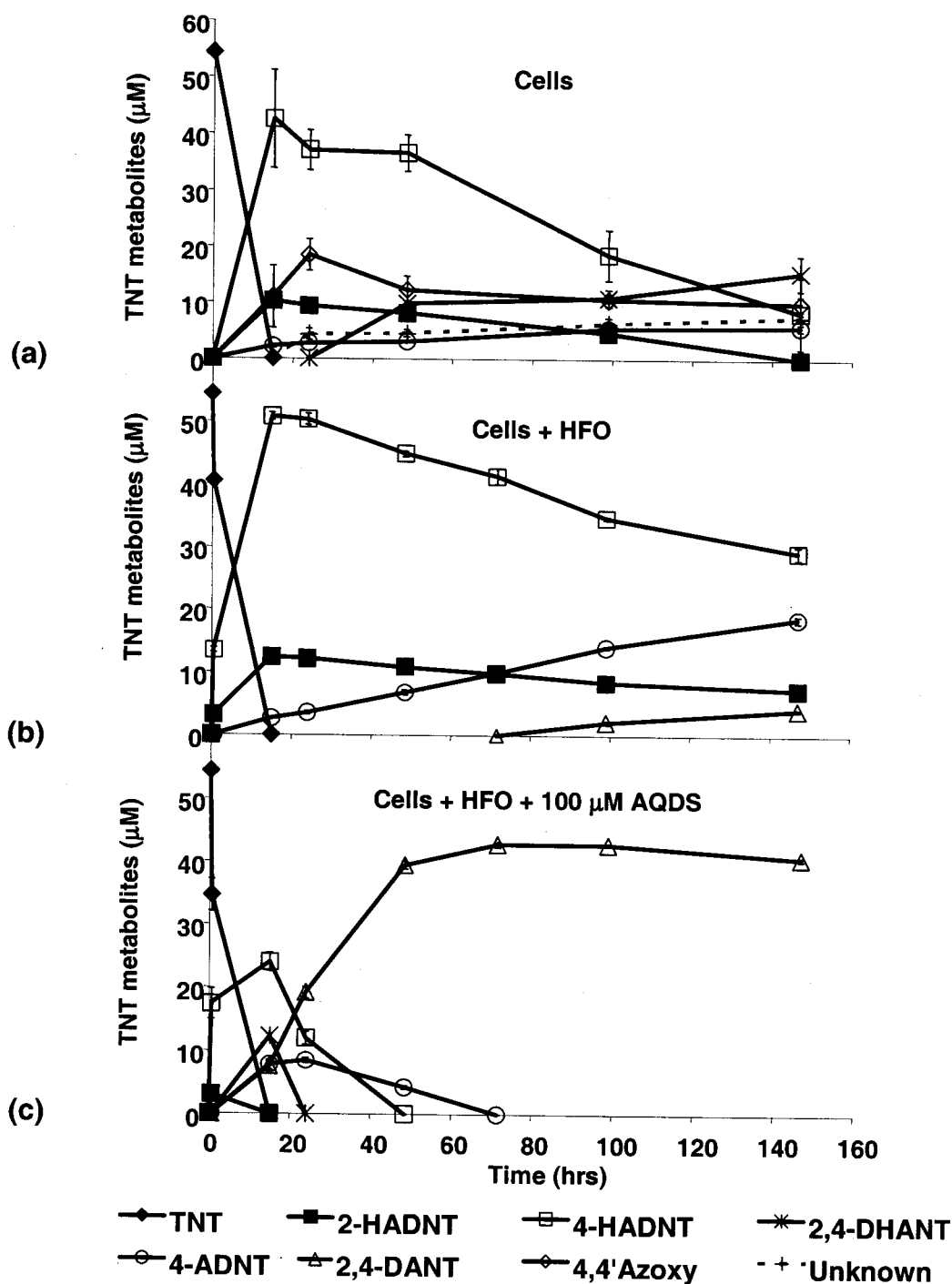


Figure 5.2. Transformation of 2,4,6-trinitrotoluene (TNT) with strain ES6 (a), ES6 + hydrous ferric oxide (HFO) (b), and ES6 + HFO + anthraquinone-2,6-disulfonate (AQDS) (c). The unknown compound observed in Fig. 5.2a may be 2',4- or 2,4'-Azoxy (see text). Inoculation and TNT addition occurred at day 0. Error bars represent one standard deviation of triplicate analyses.

The observed TNT degradation pattern in the presence of ES6 is similar to other fermenting bacteria although we detected azoxy derivatives, which had not been reported in previous studies. Lewis et al. [32] showed that *Clostridium bifermentans* was capable of completely reducing TNT to TAT with a transient appearance of HADNTs, 4-ADNT, 2,4-DHANT, 2-hydroxylamino-4-amino-6-nitrotoluene (2-HA4ANT), and 2,4-DANT. *Clostridium thermoaceticum* and *C. acetobutylicum* were both reported to degrade TNT to the Bamberger rearrangement product of 2,4-DHANT via 2- and 4-HADNT [33-36]. It was hypothesized that the capacity of *C. bifermentans* for rapid TNT nitro reduction was due to the accumulation of strong reductants (e.g. ferredoxin and hydrogenase) during glucose fermentation [32]. Conversely, it was recently shown that purified CO dehydrogenase (CODH) from *C. thermoaceticum* catalyzes the reduction of TNT in the presence of CO in the absence of ferredoxins or viologens [33].

The addition of hydrous ferric oxide (HFO) to ES6 cultures together with 54  $\mu$ M TNT caused a dramatic shift in the TNT degradation pathway (Figure 5.2b). No 2,4-DHANT or azoxy compounds were observed resulting in a higher initial concentration of 2- and 4-HADNT, which were then further reduced to 4-ADNT and 2,4-DANT within 150 hours (step 1.1, 1.2, 6 and 8, Figure 5.1). The influence of HFO may be a result of biogenically produced Fe(II) or a change in the metabolic state of ES6 in the presence of HFO. Hofstetter et al. [23] investigated the fate of TNT in Fe(III)-reducing aquifer columns and stated that surface-bound Fe(II) was responsible for TNT reduction. Thus, the production of surface-associated Fe(II) in batch systems could have influenced the TNT reduction. However, it is also possible that the metabolic state of strain ES6 changed in the presence of HFO. Figure 5.3 shows the association of ES6 cells with HFO

surfaces, indicating the presence of biofilms. Adhesion of cells can trigger the expression of a sigma factor that derepresses numerous genes such that biofilm cells are clearly phenotypically distinct from their planktonic counterparts. Sessile cells respond to micro environmental gradients and often differ fundamentally from planktonic cells of the same species [37].

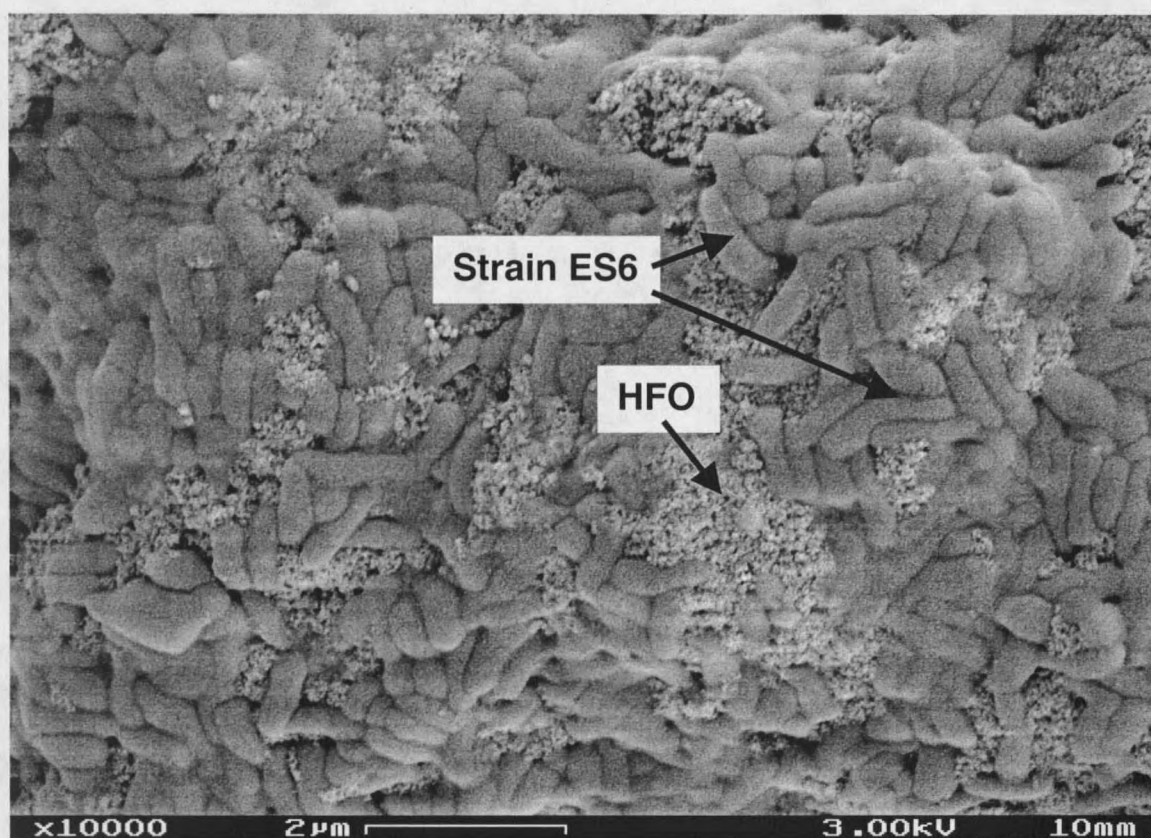


Figure 5.3. Scanning electron micrograph (SEM) of strain ES6 colonizing hydrous ferric oxide (HFO).

If the increased reduction capacity was due to the presence of Fe(II), one would expect a further enhancement in the presence of an electron shuttle such as AQDS, based on past work with *Geobacter* sp. and *Shewanella* sp., where it has been shown that

AQDS catalyzes the reduction of HFO. Results in the presence of HFO *and* 100  $\mu$ M AQDS (Figure 5.2c) support this hypothesis as demonstrated by increased transformation of TNT to 4-HADNT, 4-ADNT, and finally 2,4-DANT (step 1.1, 6, and 8, Figure 5.1), compared to systems without AQDS (Figure 5.2b). Only traces of 2-HADNT and 2,4-DHANT were observed (step 1.2 and 2, Figure 5.1); 2,4-DANT was the only detectable transformation product after 80 hours and a small decrease in the mass balance was noted. The absence of 2-ADNT and 2,6-DANT is noteworthy (step 5 and 7, Figure 5.1) because these metabolites were associated with TNT degradation observed in ferrogenic aquifer columns, due to a hypothesized abiotic reaction with surface-bound Fe(II) species. Electron shuttles such as AQDS may however influence the fate of TNT directly (e.g. reduction of TNT with the reduced form of AQDS (AHQDS)) or via multiple step mechanisms including both biotic and abiotic components. In one scenario, AQDS may enhance the biogenic production of Fe(II) and subsequently increase the abiotic signature (i.e. formation of 2-ADNT and 2,6-DANT) of the TNT degradation pathway. The electron shuttle AQDS increased the production rates of the amino derivatives in these systems, but the loss of mass balance also indicates that AQDS can cause the production of metabolites not identifiable with the HPLC-DAD method utilized in this study. Due to the loss in mass balance and the potential for direct reduction of TNT by AHQDS,  $^{14}\text{C}$ -labeled TNT degradation studies were conducted using  $[^{14}\text{C}]\text{TNT}$  in the absence of HFO (described below).

The transformation patterns described in Figure 5.2 (a-c) indicate that both, HFO and AQDS, have an influence on the transformation of TNT. In nature, it is expected that

bacteria and Fe minerals have already been present for an extended period of time before contaminants are introduced. Thus, it is likely that surface-associated Fe(II) is already present and available for reaction with contaminants such as heavy metals, radionuclides, or TNT [38]. To simulate such a situation, ES6 was incubated in the presence of HFO for 14 days to allow the production of surface-associated Fe(II) prior to TNT injection. Similar to the experiments without pre-incubation (Figure 5.2a-c), three treatments were established: A) cells only, B) cells + HFO, and C) cells + HFO + AQDS. The addition of AQDS was intended to accelerate Fe(II) production by strain ES6 during the 14 day pre-incubation before addition of TNT. Pre-incubation with strain ES6 resulted in the production of 0  $\mu\text{M}$ , 176  $\mu\text{M}$  and 704  $\mu\text{M}$  Fe(II)<sub>0.5N HCl</sub> for treatments A, B, and C, respectively. In the absence of HFO (Figure 5.4a), the TNT transformation pattern was similar to the transformation pattern with fresh ES6 cells (Figure 5.2a) although the rates of TNT and metabolite reduction were lower. The lower reduction rates might be explained by an approximately 7 times lower cell count at time of TNT addition in the pre-incubated systems. Both, 4- and 2-HADNT were observed, however, a higher concentration of 2-HADNT relative to 4-HADNT was observed in the systems where TNT was added after 14 days of pre-incubation (step 1.2 vs. 1.1, Figure 5.1). The higher relative concentration of 2-HADNT may be due to the presence of biologically produced reductants in the cell suspension capable of nitroaromatic reduction after 14 days of pre-incubation. For example, it has been reported that the accumulation of reductants such as ferredoxins, hydrogenases, and carbon monoxide dehydrogenase produced by fermenting

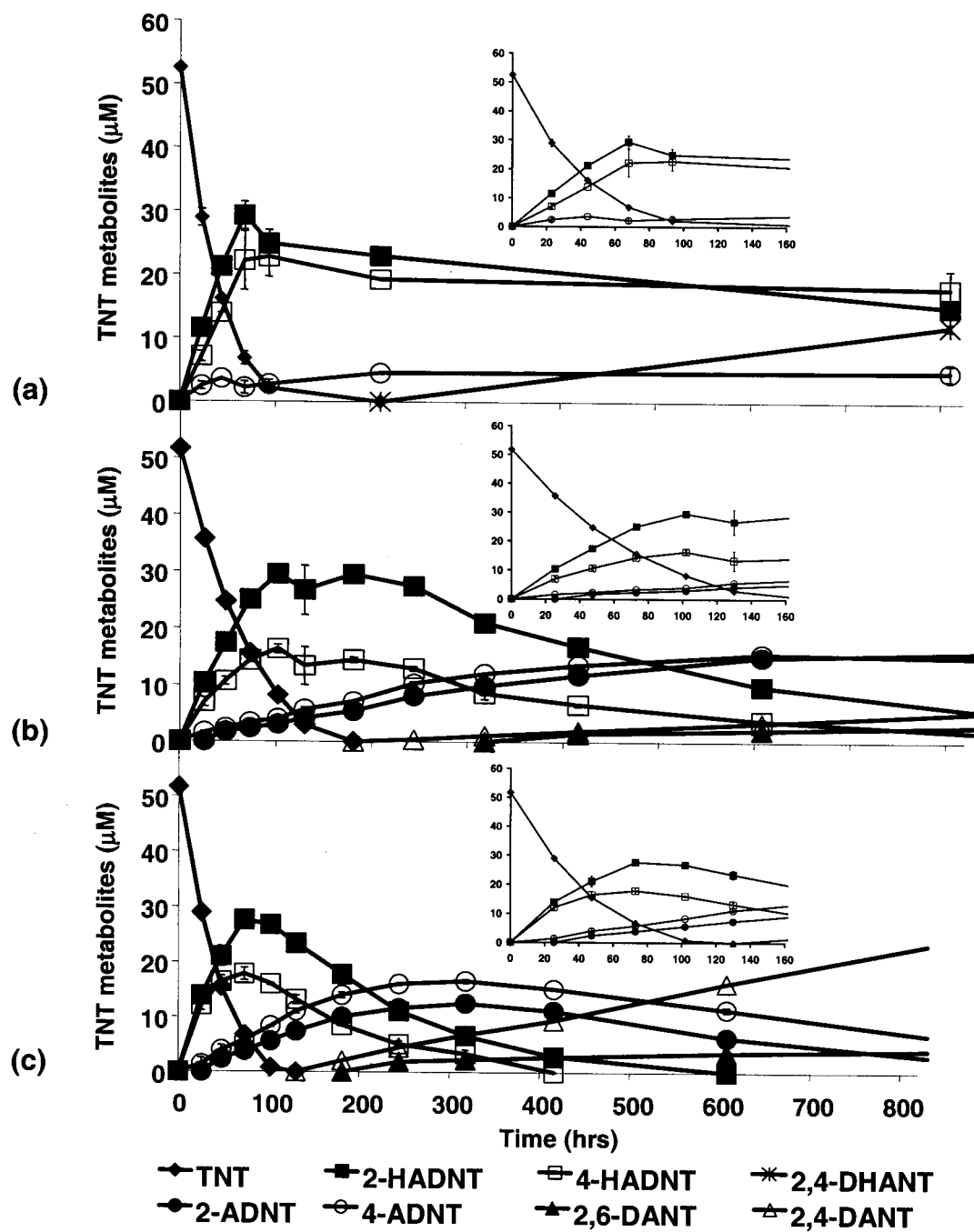


Figure 5.4. Transformation of 2,4,6-trinitrotoluene (TNT) with strain ES6 (a), ES6 + hydrous ferric oxide (HFO) (b), and ES6 + HFO + anthraquinone-2,6-disulfonate (AQDS) (c). The systems were pre-incubated with strain ES6 two weeks prior to injection of TNT. The experiments (a-c) contained (at time of TNT injection) 0, 176 and 704  $\mu\text{M}$  of  $\text{Fe(II)}_{0.5\text{N HCl}}$ , respectively. Insets show a timeframe comparable to Figures 5.2a-c. Experiments were conducted in triplicate and error bars correspond to one standard deviation.

Clostridia can rapidly reduce TNT [32,33]. The presence of such reductants could also explain the reduced formation of 2,4-DHANT and azoxy compounds compared to the fresh cell suspensions (Figure 5.2a).

When comparing Figure 5.2b (fresh cell suspensions in the presence of HFO) with Figure 5.4b (TNT addition after 14 days of ES6 pre-incubation in the presence of HFO), it is apparent that the TNT degradation pattern shifts from the *para*-substituted metabolites (4-HADNT/4-ADNT) toward the *ortho*-substituted metabolites (2-HADNT/2-ADNT) (step 1.2 and 5, Figure 5.1). TNT degradation patterns also shifted dramatically in pre-incubated treatments containing HFO and AQDS (Figure 5.4c) compared to fresh cell suspensions (Figure 5.2c). Additionally, the presence of AQDS during pre-incubation increased the TNT and metabolite reduction rates compared to systems that were pre-incubated in the absence of AQDS (compare Figure 5.4c with Figure 5.4b). For example, forty-two percent of the TNT was reduced to TAT (step 9, Figure 5.1) in the presence of AQDS after 83 days (data points not shown in Figure 5.4c) in contrast to no observable TAT in the absence of AQDS.

To verify that AQDS promoted Fe(III) reduction rates and formation of reduced TNT metabolites, additional studies were conducted where the production of Fe(II) and DANTs were analyzed as a function of AQDS concentration. The impact of AQDS on the formation of DANTs was investigated in systems containing ES6 and HFO with and without AQDS, which were pre-incubated for 14 days prior to the injection of TNT. To eliminate the influence of initially different Fe(II) concentrations in the 100  $\mu$ M and 200  $\mu$ M AQDS treatments, the 200  $\mu$ M AQDS systems were established by spiking three pre-

incubated 100  $\mu\text{M}$  AQDS treatments with an additional 100  $\mu\text{M}$  AQDS at the time of TNT addition. In parallel experiments, the impact of AQDS on the reduction of HFO was investigated by establishing systems in which ES6 was incubated in the presence of HFO and different AQDS concentrations (0 – 500  $\mu\text{M}$ ).

After 13 days (315 hrs), the formation of 2,4-DANT and Fe(II) were highly correlated with increasing AQDS concentration (Figure 5.5a-b). Increases in 2,6-DANT were also observed, but not to the same extent as observed for 2,4-DANT, indicating that AQDS causes accelerated TNT reduction via 2,4-DANT as opposed to 2,6-DANT (step 8 vs. 7, Figure 5.1). This observation is in agreement with abiotic regioselective studies in the presence of  $\text{H}_2\text{S}$  and the electron shuttle juglone showing preferential reduction of the nitro group *para* to the methyl substituent on the TNT molecule [7]. However, in contrast to our study, the addition of juglone significantly reduced the yield of monoamino products during bisulfide-mediated reduction of TNT [7]. There was no clear evidence for a direct correlation between the production of 2,6-DANT and increased AQDS concentration, which indicates that the formation of this isomer occurs via a different mechanism. To determine whether the presence of surface associated Fe(II) truly promotes the production of *ortho* substituted metabolites, abiotic studies were conducted in which Fe(II) was added to cell-free batch systems in the presence and absence of HFO.

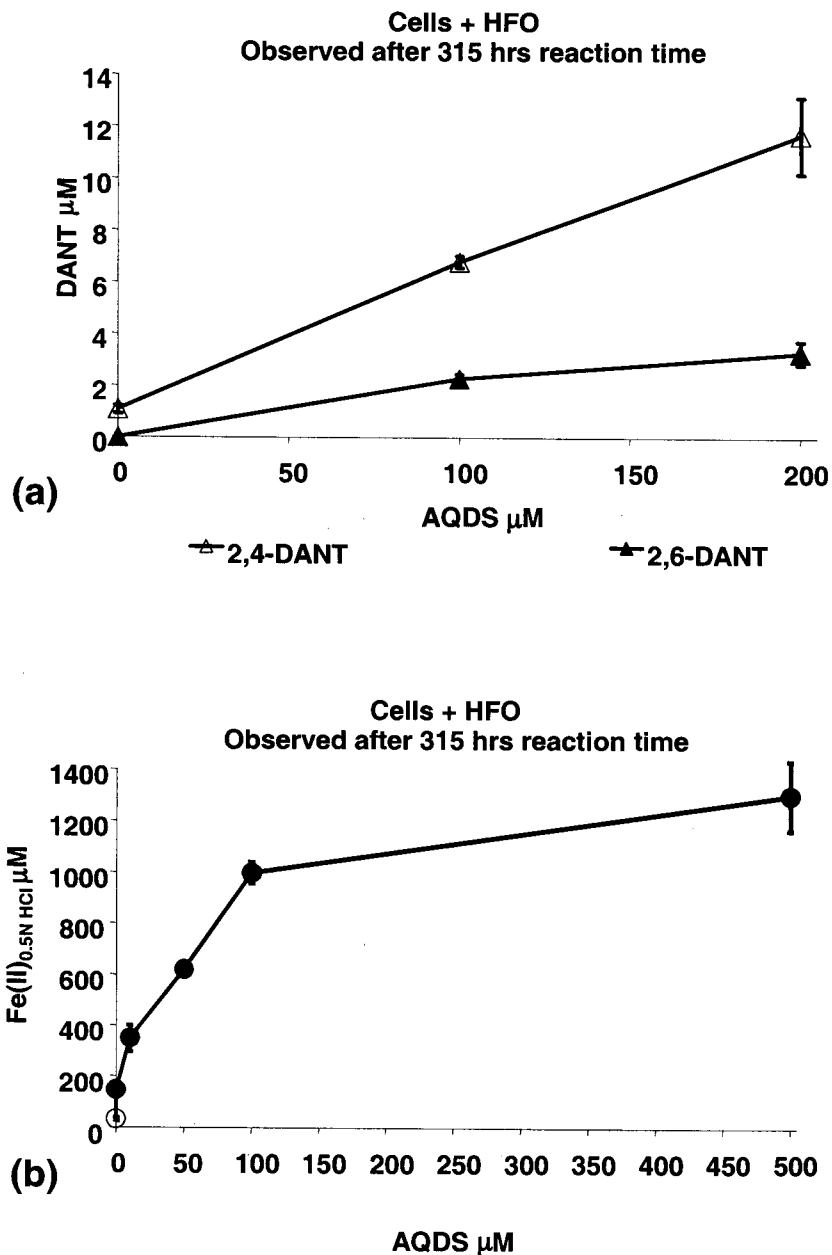


Figure 5.5. Formation of 2,4-DANT and 2,6-DANT in the presence of HFO by ES6 as a function of AQDS (a). Reduction of HFO (6 mM) by ES6 as a function of AQDS (b). The open ring (in b) shows the presence of Fe(II) in a sterile control with 500  $\mu\text{M}$  AQDS. Experiments were conducted in triplicate and error bars correspond to one standard deviation.

### Influence of Surface-Associated Fe(II) on the Transformation of TNT

Three abiotic TNT treatments were established under anaerobic conditions at a pH of 7: (i) 6 mM HFO in SGW, (ii) 0.89 mM Fe(II)<sub>aq</sub> in DI water (to prevent reaction between SGW and Fe(II)), and (iii) 6 mM HFO + 0.89 mM Fe(II)<sub>aq</sub> in SGW. Most (92 %) of the Fe(II) added was sorbed to the HFO surface after 24 hours when TNT was injected (data of sorption isotherms are shown in Chapter 6, Figure 6.4a). While no TNT transformation was observed in systems containing only HFO or only dissolved Fe(II), TNT was degraded rapidly (within 5 h) in the combined Fe(II)-HFO system (Figure 5.6).

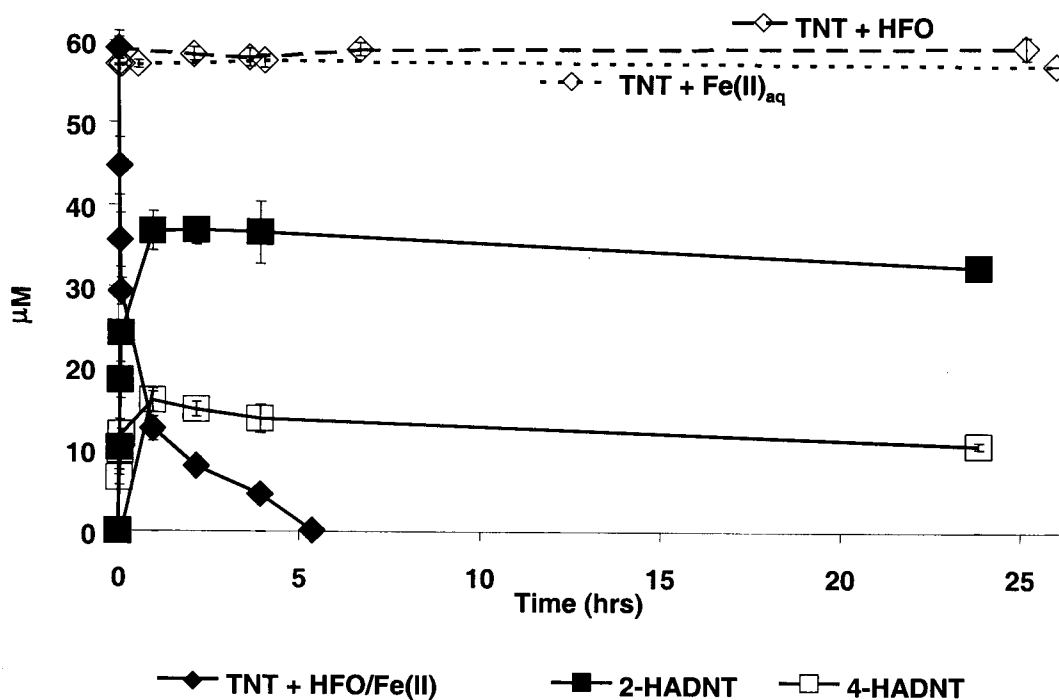


Figure 5.6. Abiotic transformation of TNT in the presence of either hydrous ferric oxide (6 mM HFO), ferrous iron (0.89 mM Fe(II)<sub>aq</sub>), or HFO and Fe(II)<sub>aq</sub> at pH 7. Several metabolites (i.e. 2-ADNT, 4-ADNT, 2,6-DANT, and 2,4-DANT) appeared in concentrations below 7 μM (not shown for clarity). Experiments were conducted in triplicate and error bars correspond to one standard deviation.

The *ortho* substituted isomer 2-HADNT was preferentially formed over the *para* isomer 4-HADNT. Traces of 2-ADNT (5.2  $\mu\text{M}$ ), 4-ADNT (5.9  $\mu\text{M}$ ), 2,6-DANT (2.7  $\mu\text{M}$ ), and 2,4-DANT (3.7  $\mu\text{M}$ ) were also observed after 24 hours (for clarity of Figure 5.6, these data are not shown). These observations are in agreement with Hofstetter et al. [23] who reported that TNT was only reduced by Fe(II) if it was associated with goethite surfaces. The pseudo-first-order rate constant ( $k_{\text{obs, Fe(II)/HFO}}$ ) for TNT reduction was estimated to be  $2.4 \pm 0.3 \times 10^{-3} \text{ s}^{-1}$  (in the presence of 0.89 mM Fe(II)) using the initial-rate method and is comparable with the TNT reduction rate constant ( $k_{\text{obs, Fe(II)/goethite}}$ ) of  $4.6 \pm 1.2 \times 10^{-3} \text{ s}^{-1}$  (in the presence of 1.5 mM Fe(II)) reported by Hofstetter et al. [23]. The rate constants when normalized to Fe(II) are very similar, suggesting that surface associated Fe(II) is the important reactive species. The reduction of the nitro group *ortho* to the methyl-substituent was induced in the presence of surface-associated Fe(II). Thus, the formation of 2-HADNT, 2-ADNT, and 2,6-DANT in the TNT transformation studies in which ES6 was allowed to pre-reduce HFO for 14 days prior to TNT addition (Figures 5.4b and 5.4c) are likely to be a result of the abiotic reduction of TNT by biologically produced Fe(II).

However, both HFO and AQDS were present in these studies (Figure 5.4bc). Thus, to separately evaluate the effect of AQDS on the transformation of TNT by ES6 and due to the observed loss in mass balance (in Figure 5.2a and 5.2c), studies were conducted with  $^{14}\text{C}$ -labeled TNT in the absence of HFO.

### Role of AQDS on the Fate of TNT

It is well known that electron shuttles such as AQDS can enhance the biological reduction of Fe(III) minerals by dissimilatory metal-reducing bacteria such as *Shewanella* spp. and *Geobacter* spp. [15,18]. However, the enhancement is not limited to DMRB as we illustrated in Figure 5.5b for ES6. This observation agrees with studies by Benz et al. who reported increased Fe(II) production and modified fermentation patterns by *Propionibacterium freudenreichii*, *Lactococcus lactis*, and *Enterococcus cecorum* in the presence of electron shuttles [24]. Since the direct reductive transformation of TNT by strain ES6 most likely involves the transfer of electrons obtained during the fermentation of sucrose from ES6 to TNT, the presence of an electron shuttling compound such as AQDS could have an influence on the transformation of TNT.

The impact of AQDS on TNT reduction in the presence of ES6 was investigated in a series of experiments with both unlabeled and  $^{14}\text{C}$ -TNT. Increasing AQDS concentrations resulted in increasing rates of TNT reduction (Figure 5.7) and the observed transformation of TNT in the presence of 1000  $\mu\text{M}$  AQDS was best modeled using zero order kinetics ( $k_{\text{obs}} = (4.5 \pm 0.1) \mu\text{M h}^{-1}$ ;  $R^2 = 0.998$ ;  $1.5 \times 10^7 \text{ CFU mL}^{-1}$ ). Moreover, Figure 5.8a shows that the TNT transformation in the presence of AQDS resulted in the production of both 4-HADNT and 4,4'-Azoxy, which were subsequently converted to 2,4-DHANT (step 1.1, 2 and 4, Figure 5.1). During the first 22 hours of the experiment, the HPLC-DAD analysis accounted for about 90 % of the added TNT. After about 30 hrs. however, unknown metabolites were produced which were not detectable using the HPLC-DAD method. The use of  $^{14}\text{C}$ -TNT allowed us to track the fate

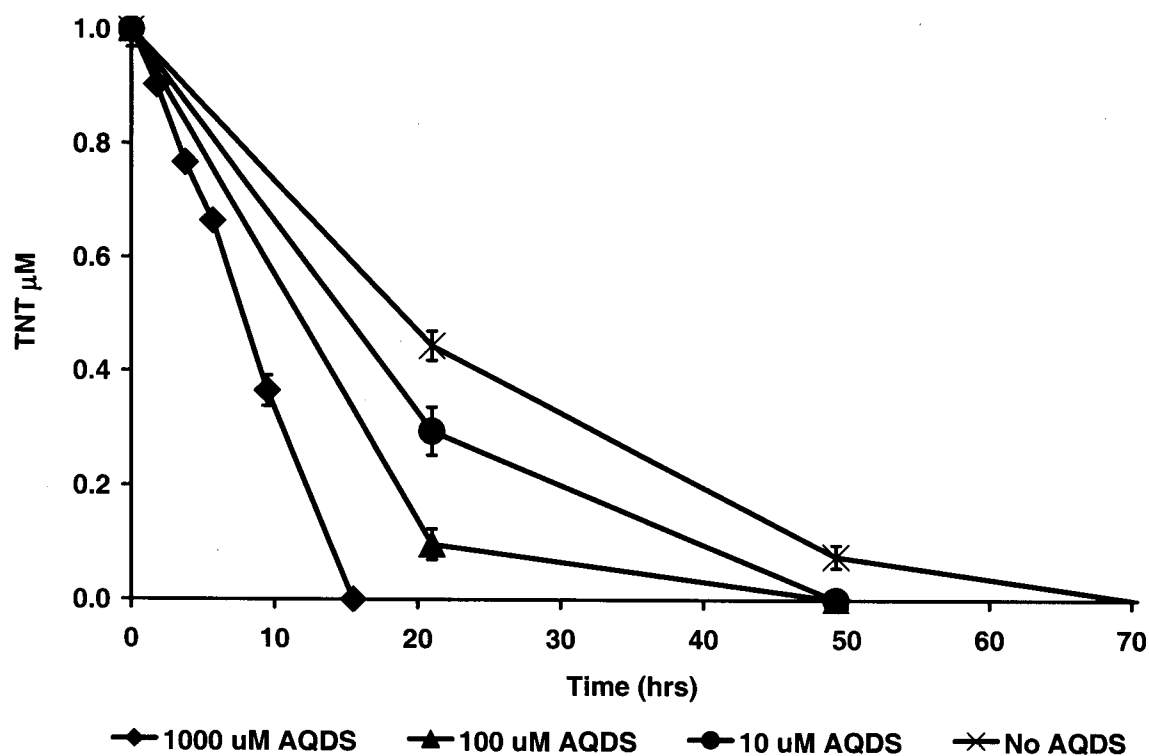


Figure 5.7. Reduction of TNT by ES6 as a function of AQDS concentration. Experiments were conducted in triplicate and error bars correspond to one standard deviation.

of TNT in these experiments thus the column effluent from the HPLC was collected using a fraction collector and subjected to separate liquid scintillation analysis. After 240 hours of reaction time, the majority of  $^{14}\text{C}$  was associated with compounds eluting between approximately 2.7 and 8 minutes (Figure 5.8b). This retention time range corresponds to products with a polarity most likely higher than that of 2,4-DANT ( $t_r$  of 6.7 min) and 15% of the products appeared to have polarities similar to or higher than TAT ( $t_r$  of 4.1 min). A significant percentage of recovered  $^{14}\text{C}$  was associated with HPLC peaks, which eluted at or around the same time AQDS eluted ( $t_r \sim 4.6$  min; Figure 5.8b).

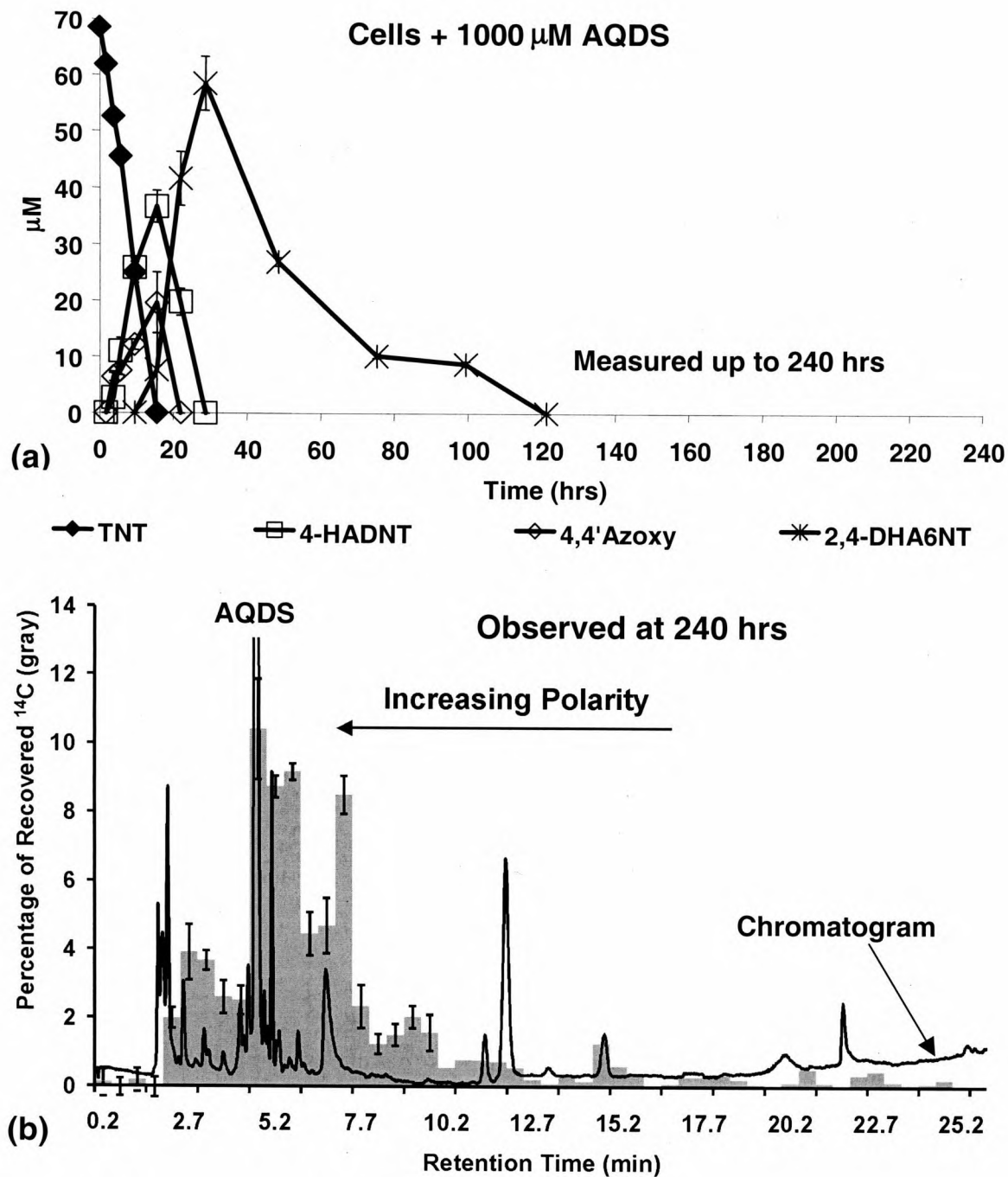


Figure 5.8. Degradation of  $^{14}\text{C}$ -radiolabeled TNT in the presence of ES6 and 1000  $\mu\text{M}$  AQDS (a). Superimposed HPLC chromatogram and  $^{14}\text{C}$  radiogram (gray bars) obtained after 240 hours of reaction time showing the recovered  $^{14}\text{C}$ -fractions collected from the HPLC eluent as a function of the chromatographic retention time (b).

Major binding of metabolites to AQDS appeared less likely in the present study based on the mass balance obtained in the concurrent presence of AQDS and HFO and the fact that the chromatographic peak of AQDS appeared constant throughout the entire experiment. This is in agreement with previous studies showing no significant sorption of nucleophilic compounds, such as 2,4-DANT, to organic matter in anoxic sediments [8]. Elovitz and Weber [8] suggested that under reducing conditions the quinone moieties are reduced (or partially reduced) and therefore unavailable for nucleophilic attack by the amine. In addition, Ahmad and Hughes [34] demonstrated that hydroxylamines (e.g. 4-HADNT) showed no appreciable reactivity with humic acids and that 2,4-DHANT only reacted with 1-thioglycerol (HA analog) in the presence of oxygen. The possibility for metabolite binding to AQDS in the current study cannot be completely excluded due to the possibility that different metabolites were formed.

Fiorella and Spain [39] also reported that TNT was converted to unidentified (polar) end products via the hydroxylamino intermediates 2,4-DHANT and 2-HA4ANT by strain JS52. Later, Hughes et al. [35] reported that *Clostridium acetobutylicum* was able to reduce TNT to 2,4-DHANT and then to hydrophilic phenolic products (e.g. 2-amino-4-hydroxylamino-5-hydroxyl-6-nitrotoluene) via the Bamberger rearrangement. The UV spectrum (UV  $\lambda_{\max}$  [nm] = 221.7 and 290.4) of the Bamberger rearrangement product described by Hughes et al. [35] was similar to one of the metabolites observed in this study (UV  $\lambda_{\max}$  [nm], 215 and 284;  $t_r$  = 2.5 min). The observed *blue shift* of approximately 6.5 nm might be due to a solvent effect (i.e. increased solvent polarity). In addition, one of the unidentified chromatographic peaks with a retention time of 6.7 min

(UV  $\lambda_{\text{max}}$  [nm], 221: 329; Figure 5.8b) was produced concomitant with the disappearance of 2,4-DHANT (i.e. past 16 hours; data not shown).

There was no significant sign of  $^{14}\text{C}$ -TNT mineralization (production of  $\text{CO}_2$ ) based on a recovery of  $> 90\%$   $^{14}\text{C}$  in the aqueous phase. Only 78 % of the  $^{14}\text{C}$  in the aqueous samples was recovered in the HPLC effluent after 240 hrs of reaction. The filtration prior to HPLC analysis was found to be responsible for the loss of approximately 22 %  $^{14}\text{C}$  from the aqueous phase, indicating binding or precipitation of unknown metabolites to bacterial cells or biomass since losses of (poly)nitroaromatic compounds due to filtration were expected to be less than 2 % [23]. Consequently, a 0.2-micron-pore Nucleopore (polycarbonate) filter was used to estimate the amount of cell/biomass-bound/precipitated  $^{14}\text{C}$  and showed that approximately 12 % of the  $^{14}\text{C}$  remained on the filter after ample purging with a phosphate buffer (25 mM; pH of 7). These results suggest that the nature of the unidentified metabolites caused some of them to be incorporated, precipitated, or bound to ES6 or other macromolecules in the experimental system. No significant chemical binding to the Nucleopore filter was observed. The potential binding of metabolites produced via hydroxylamino intermediates such as 2,4-DHANT and 2-HA4ANT is in agreement with previous studies showing that a fraction of the formed polar metabolites clearly became associated with cells of strain JS52 [39].

AQDS caused significant changes in the fate of TNT. Consequently, control studies with soil humic acid (HA) were established to simulate soil conditions more properly. The HA concentration was 29.5 mg/L (17.1 mg C/L) which is within the concentration range of DOC (1-50 mg C/L) typically observed in natural ecosystems

[40]. HA considerably enhanced the formation of the most reduced amino derivatives (i.e. 4-ADNT, 2,6- and 2,4-DANT; see Table 5.1 and step 6, 7 and 8, Figure 5.1). The production of 2,6-DANT might be due redox-active constituents such as humic-metal-complexes in the soil HA [22,24]. The overall production of 4-HADNT and especially 2,4-DHANT was considerably decreased as compared to pure cell suspensions (Table 5.1). Most (93 %) of the metabolites were accounted for after 1270 hours of

Table 5.1. Distribution (in %) of TNT and its metabolites after 1270 hours in the presence and absence of *Cellulomonas* sp. strain ES6 (ES6), AQDS, and soil HA.

| %                         | Media<br>(control) | AQDS<br>(control) | ES6 | ES6 +<br>AQDS | ES6 +<br>Soil HA |
|---------------------------|--------------------|-------------------|-----|---------------|------------------|
| TNT                       | 101                | 95                | 0   | 0             | 0                |
| 4-HADNT                   | 0                  | 0                 | 31  | 0             | 24               |
| 2-HADNT                   | 0                  | 0                 | 3   | 0             | 0                |
| 2,4-DHANT                 | 0                  | 0                 | 27  | 0             | 14               |
| 4-ADNT                    | 0                  | 0                 | 13  | 0             | 24               |
| 2-ADNT                    | 0                  | 0                 | 0   | 0             | 0                |
| 2,4-DANT                  | 0                  | 0                 | 8   | 0             | 24               |
| 2,6-DANT                  | 0                  | 0                 | 0   | 0             | 7                |
| <sup>a</sup> Mass Balance | 101                | 95                | 82  | 0             | 93               |

<sup>a</sup>Mass balance is based on unlabeled metabolite analysis using HPLC [3]. Mass balance was closed (~ 100 %) when using [14C]TNT and scintillation analysis.

incubation using HPLC analysis, which is in contrast to the results obtained in the presence of AQDS (Table 5.1). No significant binding of arylhydroxylamines (i.e. 4-HADNT and 2,4-DHANT) to HA was observed which is in agreement with previous studies showing no reactivity of 4-HADNT toward peat HA (625 mg/L) in the absence of oxygen at a sorbate/sorbent mass ratio of 0.02 [34]. On the contrary, Achtnich et al. [41] reported a complete removal of 36  $\mu$ M HADNT isomers within 2 hrs when humic acid (7500 mg/L) was present. However, the high removal of HADNT by HA might have been due to the high concentration of HA, which can result in a change of the HA structure and behavior as well as an unsolubilized fraction of HA [34,42,43].

#### Environmental Significance

The results of this study indicate that the transformation of TNT by strain ES6 is significantly influenced by the presence of hydrous ferric oxide (HFO) and AQDS. The presence of HFO decreased the initial rate of TNT reduction likely due to the competition between HFO and TNT as electron acceptors. However, the biogenic production of surface-associated Fe(II) resulted in reduction of the nitro group *ortho* to the methyl group, and promoted the formation of more reduced TNT metabolites with time. The presence of AQDS in HFO-free cell suspensions resulted in an increase in initial TNT reduction rates and a significant change in metabolite trajectory. However, the combined presence of HFO and AQDS did not result in a cumulative metabolite pattern. Although AQDS increased the reduction rates of TNT and its metabolites in HFO containing cell suspensions, it did not cause the change in degradation pattern that was observed in HFO-free cell suspensions. Clearly, AQDS increased the rate of HFO reduction, resulting

in a higher concentration of surface-associated Fe(II), then enhancing TNT transformation along the same pathway observed for systems containing surface-associated Fe(II).

Figure 5.9 shows the proposed electron transport scheme for the reduction of TNT based on the reported observations. Strain ES6 was shown to be capable of using TNT,

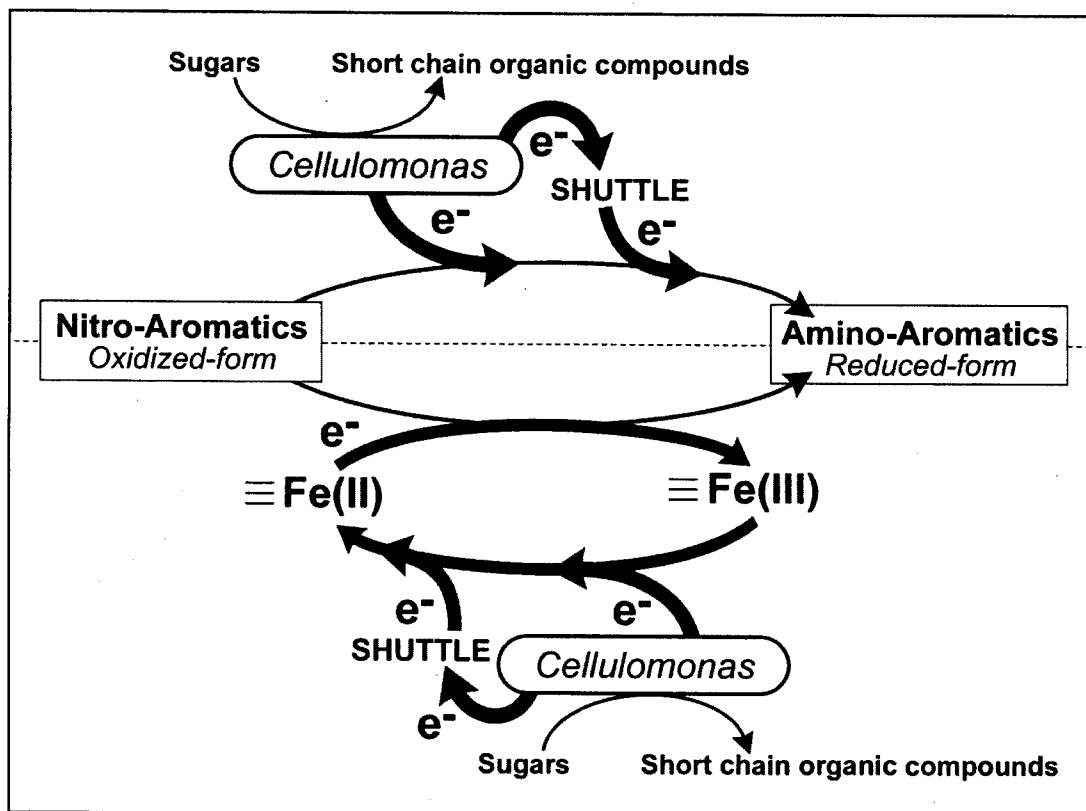


Figure 5.9. Simplified scheme showing a hypothetical electron transport for the reduction of TNT in the presence and absence of Fe(III) (hydr)oxide and electron shuttles (i.e. AQDS).

HFO, and AQDS as external electron acceptors during sucrose fermentation. While the mechanism of electron transfer from the cell is not clear, we would not expect the fermenting strain ES6 to be using these constituents in the same manner as in

dissimilatory reduction [44-46]. A minimum of three electron transfer pathways must be considered to explain the fate of TNT observed under the different treatment conditions: direct electron transfer between ES6 and TNT, electron transfer between microbially reduced AQDS (AHQDS) to TNT, and or via transfer from surface associated Fe(II). Direct electron transfer from ES6 cells to TNT was slow, but was accelerated in the presence of AQDS. Additionally, ES6 reduced HFO to produce surface-associated Fe(II), which then reduces nitroaromatics. The rate of Fe(II) production from HFO increased in the presence of AQDS, which also enhances the reduction of nitroaromatics (Figure 5.9). Each of the mentioned electron transfer pathways contributed to the degradation and fate of TNT, however the reduction mechanisms for the reactions including AQDS remain somewhat unclear.

It has been reported previously that AQDS can enhance the reduction of solid phase Fe(III) by DIRB [15,17,18]. Conversely, that AQDS also enhances the reduction and changes the degradation pathway of dissolved compounds such as TNT is surprising. However, based on the work by Newman and others it is plausible since AQDS has been shown to diffuse into and out of the membrane of the dissimilatory metal-reducing bacterium *Shewanella oneidensis* MR-1 [47]. If the electron transfer in the presence of AQDS and absence of HFO occurs preferentially from AQDS and not directly from the cell to the nitroaromatics, it is reasonable to observe a difference in the TNT transformation rate and pattern. In the presence of HFO and AQDS however, the electron transfer from the cells appears to go preferentially to AQDS, which in turn favors the reduction of HFO over TNT. Interestingly, it was recently reported that the fermenting bacteria *Propionibacterium freudenreichii*, *Lactococcus lactis*, and *Enterococcus*

*cecorum* all shifted their fermentation patterns towards more oxidized products when humic acids or AQDS were present both in the presence and absence of HFO [24,48]. This offers a hypothetical rationale for increased electron transport from cells to HFO or TNT due to the potential influence of HA and AQDS on the fermentation pattern, and suggests a means for increased TNT reduction.

Reduced amino derivatives are most commonly formed during the biotransformation of TNT (as observed in this study) and TAT has often been reported as a "dead-end" metabolite under strictly anaerobic conditions [49]. However, our studies show that the addition of an electron shuttle (i.e. AQDS) may stimulate a different metabolic pathway of TNT resulting in the initial formation of hydroxylamino derivatives that ultimately transform into polar compounds (step 3, Figure 5.1). Based on research by Nadeau et al. [50] and Hughes et al. [35], it is reasonable to believe that these hydrophilic compounds might be phenolic amines or protocatechuates. This could be of particular interest since compounds such as 2-aminophenol and 2-amino-4-methylphenol can be degraded through the o-aminophenol extradiol-like ring cleavage pathway [51], thereby opening the possibility for mineralization. The influence of electron shuttling compounds such as AQDS on the fate of environmental contaminants such as TNT might be greater than described previously since electron shuttling compounds can influence the transformation pattern in multiple ways. This study also shows that the combined effects of electron shuttles and iron minerals cannot be predicted based on single component experiments and that well-designed studies addressing the combined influence are necessary to make predictions about the fate of contaminants in the environment.

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## CHAPTER 6

THE INFLUENCE OF FE (HYDR)OXIDE PHASES AND THE ELECTRON  
SHUTTLE ANTHRAQUINONE-2,6-DISULFONATE ON THE  
REDUCTION OF 2,4,6-TRINITROTOLUENE BY  
A FERMENTING BACTERIUM

Introduction

The bioreduction of Fe(III) present as amorphous (i.e. ferrihydrite) or crystalline Fe (hydr)oxides (i.e. hematite, goethite and magnetite) by dissimilatory Fe reducing bacteria (DIRB) has been studied extensively, especially by the Gram-negative *Shewanella* and *Geobacter* spp. [1-9]. However, the bioavailability of Fe(III) and extent of Fe(III) reduction by DIRB differs significantly among Fe(III) (hydr)oxides (e.g. ferrihydrite >> hematite) and is a function of parameters such as crystal structure; surface area, and the presence of sorbed ions (e.g. Fe(II)) [10,11]. DIRB may also play an important role in reducing toxic heavy metals and radionuclides, and in oxidizing aromatic compounds such as benzene [12-14]. Despite significant research on Fe(III) reduction by *Shewanella* and *Geobacter* spp., the reduction of Fe(III) and toxic metal ions by other environmental relevant microorganisms is not well studied. For example, organisms isolated from Cr(VI)-reducing enrichment cultures obtained from subsurface samples of the Hanford site in Washington were predominantly Gram-positive bacteria identified as *Cellulomonas* spp. based on 16S rDNA analysis and phospholipid profiling [15,16]. These environmental isolates were recently reported to reduce 2,4,6-

trinitrotoluene (TNT), Cr(VI), U(VI), soluble Fe(III), and hydrous ferric oxide (HFO) [15-18].

The reductive dissolution of HFO by microorganisms may result in the formation of secondary Fe mineral phases such as goethite, siderite, vivianite, green rust and magnetite depending on the chemical environment [1-3,9], and the subsequent effect of these phases on contaminant fate can vary with specific metal/organic/Fe combinations. The current understanding of how secondary mineral formation impacts degradation processes of contaminants is inadequately understood. For example, immobilization of metals such as Sr and Zn during biomineralization of Fe(III) (hydr)oxides has been reported [19,20]. Conversely, mobilization (i.e. solubilization) of coprecipitated Co(III) and Ni(II) from goethite ( $\alpha$ -FeOOH) during dissimilatory iron reduction and concurrent secondary Fe phase formation has also been demonstrated [21]. The formation of reduced iron minerals such as magnetite results in a decrease in DIRB activity, directly impacting the potential transformation of contaminants [2,4,11,22,23].

It is now widely recognized that electron shuttling compounds such as humic acids (HA), anthraquinone-2,6-disulfonate (AQDS) and menaquinones enhance the rate of reductive Fe dissolution [24,25]. In addition, microorganisms such as *Deinococcus radiodurans* effectively coupled the reduction of AQDS to the reduction of U(VI) and Tc(VII) but no reduction was observed in the absence of electron shuttles [26]. Consequently, quinone-mediated bioremediation strategies have been proposed due to the possible direct and indirect (by increased Fe(II) production) reduction of pollutant metals and organics [17,27-30]. However, little is known about the impact of AQDS on organic

contaminants under biotic conditions and no studies have investigated the possible concomitant contributions of microorganisms, electron shuttles and crystalline Fe(III) phases on the fate of nitroaromatic compounds.

TNT and its metabolites represent a worldwide environmental problem, since they exhibit considerable toxicity to microorganisms and humans [31-34]. A variety of microorganisms have been shown to biotransform TNT into its hydroxylamino (-NHOH) and amino (-NH<sub>2</sub>) derivatives [35]. In a recent study, we demonstrated that hydrous ferric oxide (HFO), humic acid and the electron shuttle 2,6-anthraquinone disulfonate (AQDS) all had a significant impact on the reduction rate and degradation pathway of TNT in the presence of the fermenting bacterium *Cellulomonas* sp. strain ES6. Strain ES6 proved capable of reducing Fe(III), and abiotic studies showed that sorption of Fe(II) to HFO was essential for the subsequent abiotic reduction of TNT [17]. This is in agreement with other studies showing the importance of surface-associated Fe(II) for reduction of both inorganic (e.g. U(VI)) and organic compounds such as polyhalogenated methanes [12,36,37]. Consequently, the primary goal of the present study was to evaluate the reduction of TNT mediated by strain ES6 as a function of different Fe (hydr)oxide phases (i.e. ferrihydrite, hematite and magnetite). Specifically, the objectives of the current study were to (i) determine the affect of different Fe (hydr)oxides on the TNT reduction patterns by strain ES6, (ii) examine the impact of AQDS on Fe(III) reduction rates and subsequent TNT degradation in the presence of different Fe mineral phases, and (iii) evaluate the formation of secondary Fe phases in the presence of strain ES6.

## Materials and Methods

### Strain and Culture Conditions

*Cellulomonas* sp. strain ES6 (from hereon referred to as ES6), maintained in frozen stock culture containing TSB (Difco Laboratories, MD, USA) with 20 % glycerol at  $-70^{\circ}\text{C}$  [15], was pre-cultured in TSB (30 g/L) for 24 hours shaken at 150 rpm at  $21 \pm 2^{\circ}\text{C}$ , transferred into fresh TSB, and grown again for 18 hours in TSB. Studies were conducted with cells harvested during late log phase of growth. Cultures were centrifuged at  $5,860 \times g$  for 20 minutes at  $4^{\circ}\text{C}$ , washed once in  $\text{O}_2$ -free synthetic groundwater (SGW, described below), and resuspended in SGW to desired cell concentration.

### Media Composition

A carbonate-buffered synthetic ground water medium (SGW) containing (in mg/L of deionized (DI) water from a Barnstead NANOpure system)  $\text{Na}_2\text{SiO}_3 \cdot 9\text{H}_2\text{O}$  (455),  $\text{Na}_2\text{CO}_3$  (160),  $\text{Na}_2\text{SO}_4$  (6), yeast extract (50), casamino acids (100), KCl (26.5),  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (8),  $\text{Mg}(\text{OH})_2$  (33.5),  $\text{NaHCO}_3$  (2519.7), LiCl (0.021),  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (0.08),  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  (0.106),  $\text{H}_3\text{BO}_4$  (0.6),  $\text{Al}_2(\text{SO}_4)_3 \cdot 18\text{H}_2\text{O}$  (0.123),  $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$  (0.11),  $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$  (0.109),  $\text{Na}_2\text{O}_4\text{Se}$  (0.05),  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$  (0.629), KBr (0.03), KI (0.03),  $\text{Na}_2\text{MoO}_4$  (0.01), and  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (0.3) was used in all experiments [38].  $\text{Na}_2\text{SiO}_3 \cdot 9\text{H}_2\text{O}$ ,  $\text{Na}_2\text{CO}_3$ ,  $\text{Na}_2\text{SO}_4$ , yeast extract, and casamino acids were dissolved in DI water and autoclaved. The autoclaved solution was boiled for 10 minutes and cooled to room temperature under an  $\text{O}_2$ -free atmosphere by a constant purge with a mixed gas ( $\text{N}_2/\text{CO}_2$ ; 80:20). Sterile,  $\text{O}_2$ -free stock solutions of KCl,  $\text{NaHCO}_3$ ,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ,  $\text{Mg}(\text{OH})_2$ , and the

trace minerals were then added. The medium was dispensed under an  $N_2/CO_2$  atmosphere to maintain  $O_2$ -free conditions.

### Fe(III) (hydr)oxides and Fe Analysis

Three Fe phases were chosen based on their crystal structure and valence state: hematite, a crystalline Fe(III) oxide ( $\alpha\text{-Fe}_2\text{O}_3$ ); magnetite, a mixed valence Fe(III,II) oxide ( $\text{Fe}_3\text{O}_4$ ); and ferrihydrite, a poorly crystalline Fe phase ( $\text{Fe}(\text{OH})_3 \cdot n\text{H}_2\text{O}$ ). Ferrihydrite was synthesized by dissolving 10.8 g  $\text{FeCl}_3$  (Fisher Scientific, NJ, USA) in 100 mL of DI water, slowly (1 hour) adjusting the pH to 7.0 using NaOH [39], and 0.25 mL of this stock solution was included in selected reaction solutions. The ferrihydrite phase formed using this procedure exhibited intermediate characteristics of 2- and 6-line ferrihydrite (to be shown, [40]). Hematite (purity > 99.8%) and magnetite (purity > 97 %) were obtained from Alfa Aesar (MA, USA) and were used as purchased. Specific surface areas of hematite ( $\sim 18 \text{ m}^2 \text{ g}^{-1}$ ), magnetite ( $6.5 \text{ m}^2 \text{ g}^{-1}$ ), and ferrihydrite ( $300 \text{ m}^2 \text{ g}^{-1}$ ) were measured with a FlowSorb 2300 (Micromeritics, Norcross, GA) using a three-point Brunauer, Emmett and Teller (BET)  $N_2$  sorption isotherm. Total Fe (extraction with 2.5N HCl + 0.25N  $\text{NH}_2\text{OH}$  for 24 hours), extractable Fe(II)<sub>0.5N HCl</sub> (extraction with 0.5N HCl for 2 hours) and aqueous Fe(II)<sub>aq</sub> (filtered through a 0.2  $\mu\text{m}$  filter) were determined using the Ferrozine method [19]. Aliquots of these treatments were added to a solution of 1 g ferrozine (Sigma-Aldrich, MO, USA) in 1 L HEPES buffer at pH 7, and the absorbance was determined spectrophotometrically at 540 nm using an EL 808 Ultra Microplate Reader (Bio-Tek Instruments, Inc., VT, USA).

### TNT Transformation Experiments

A 20 mL test solution containing O<sub>2</sub>-free SGW and sucrose (10 mM; Fisher Scientific, NJ, USA) was added anaerobically to 30 mL test tubes (Bellco Glass, NJ, USA) in the presence and absence of hematite (6 mM; 14.1 m<sup>2</sup> L<sup>-1</sup>), ferrihydrite (6 mM; 150 m<sup>2</sup> L<sup>-1</sup>), magnetite (12 mM; 14.5 m<sup>2</sup> L<sup>-1</sup>) and AQDS (100 µM; Sigma-Aldrich, MO, USA). The test tubes were crimp sealed with polytetrafluoroethylene-faced butyl rubber septa (West Pharmaceutical Services, PA, USA) and all samples except for the controls were inoculated with strain ES6 14 days before adding 60 µM TNT. The bacterial concentrations in the vials at the time of TNT injection (day 14) were: 1.1 x 10<sup>8</sup> colony forming units (CFU) per mL (mineral free), 2.1 x 10<sup>7</sup> CFU/mL (with hematite), and 1.8 x 10<sup>8</sup> CFU/mL (with both ferrihydrite and magnetite). The vials were given a positive pressure (10 psi) of N<sub>2</sub>/CO<sub>2</sub> (80/20; pH 7) and incubated in the dark on a rotator at 21 ± 2 °C. Triplicate sets of vials were sampled periodically in a glove box (90 % N<sub>2</sub>; 5 % CO<sub>2</sub>; 5 % H<sub>2</sub>) and analyzed for dissolved TNT and its metabolites.

TNT and its metabolites were purchased and analyzed by high performance liquid chromatography (HPLC) with diode array detection (chromatograms extracted at 230 nm) as described by us previously [17,18]. Briefly, the mobile phase initially consisted of 99 % phosphate buffer (0.025 M Na phosphate; pH 7) and 1 % HPLC-grade methanol, and the gradient was changed to 30 % methanol over 2 min, then to 43 % methanol over the next 13 min, finally increased to 100 % methanol over 12.5 min, and held constant for 0.5 min. The solvent ratio was returned to the initial conditions over 1 min and held for

an additional 5 min before auto-injection of the next sample. The column temperature was 37 °C and the flow rate was 1 mL/min.

#### Abiotic Sorption of Fe(II) to Ferrihydrite and Subsequent TNT Reduction

Abiotic TNT transformation experiments were conducted in the presence of ferrihydrite preloaded with Fe(II), based on a sorption isotherm for the Fe(II)-HFO system. Sorption experiments were performed in triplicate in 30 mL test tubes containing oxygen-free SGW, ferrihydrite (6 mM), and varying concentrations of  $\text{Fe}^{\text{II}}\text{Cl}_2$  (0 to 2.18 mM; Sigma-Aldrich, MO, USA). Samples were extracted anaerobically using sterile syringes after 1.5 and 24 hours and analyzed for Fe(II). The experiments were terminated after 10 days, the mineral phases dried in a glovebag in the absence of  $\text{O}_2$  and analyzed by X-ray diffractometry.

#### Analysis of Secondary Phases in Presence of Ferrihydrite

The formation and properties of secondary mineral phases were examined in experimental treatments containing ferrihydrite in the presence and absence of ES6 and AQDS. Aliquots of these suspensions were subsequently analyzed by scanning electron microscopy (SEM), energy dispersive spectroscopy (EDS), transmission electron microscopy (TEM), selected area electron diffractometry (SAED), and powder X-ray diffractometry (XRD). Samples for SEM were placed on a SEM aluminum stub with double-sided C tape and dried in the absence of  $\text{O}_2$  (glovebox). The dried samples were sputter-coated with a thin layer of C, and examined in a field emission scanning electron

microscope (FESEM) LEO 982 operating at 3 kV. The elemental analyses were obtained at 20 kV using an Oxford electron dispersive spectroscopy (EDS) system coupled to the SEM. Oxford's Link ISIS software was used to identify and quantify elements present in samples.

Samples for TEM and SAED were also prepared in an anaerobic glovebox to avoid oxidation. Cell-mineral suspensions were washed twice in oxygen-free deionized water and mounted on Cu mesh grids with a Formvar support film. The samples were sealed in airtight containers and exposed to aerobic conditions for less than 1 min while being transferred to the high-vacuum sample chamber of the JEOL 2010. Samples were observed directly without staining to visualize electron-dense deposits (i.e. Fe-bearing phases) using an acceleration voltage of 200 kV. The nominal accuracy of the d-spacing measurements was approximately 0.1 Å.

Samples for XRD were prepared in an anaerobic glovebox to avoid oxidation while drying. The dried samples were placed in single-crystal quartz specimen holders and analyzed in a Philips X'Pert MPD system. The X-ray source was operated at 40 kV and 40 mA (LAMBDA = Cu K $\alpha$ 1, 1.5406 Å). The study specimen was examined in Bragg-Brentano parafocusing geometry on a 220-mm 2-theta goniometer radius using incident- and diffracted-beam soller slits (0.04 radians), automatic divergence and anti-scatter slits, and a 0.2-mm receiving slit. The diffractometer was controlled using the Philips X'Pert software suite (X'Pert Data Collector, V1.3d), from 5-75° 2 $\theta$  at a scan rate of 0.04°/30 s (for samples with AQDS) and 0.04°/150 s (for samples without AQDS). Data analysis was accomplished using Jade V6.5.7 (Materials Data, Inc., Livermore, CA)

and the Powder Diffraction File database (PDF-2, 2002 Release, International Centre for Diffraction Data, Newtown Square, PA).

### Results and Discussion

#### Influence of Iron Phases on the Fate of TNT

In the absence of mineral phases, strain ES6 rapidly transformed TNT to hydroxylamino derivatives (HADNTs) and traces of 4-ADNT (Table 6.1 and Figure 6.1).

Table 6.1. Percentage distribution of TNT and its metabolites in the presence of *Cellulomonas* strain ES6 (Cells). Numbers for AQDS amended systems are given in brackets [ ].

| Hours                         | Sterile  |          | Cells |     | Cells/Hematite |         | Cells/Magnetite |         | Cells/Ferrihydrite |         |
|-------------------------------|----------|----------|-------|-----|----------------|---------|-----------------|---------|--------------------|---------|
|                               | 67       | 1270     | 68    | 810 | 74             | 1070    | 74              | 1070    | 74                 | 1070    |
| <i>Compound</i>               |          |          |       |     |                |         |                 |         |                    |         |
| <b>TNT</b>                    | 105 [98] | 101 [95] | 13    |     | 17             |         | 48 [15]         |         | 30 [10]            |         |
| <b>2-HADNT</b>                |          |          | 45    | 23  | 50 [13]        | 36      | 23 [27]         | 15      | 39 [43]            | 15      |
| <b>4-HADNT</b>                |          |          | 34    | 27  | 25 [39]        | 17      | 13 [51]         | 6       | 22 [28]            |         |
| <b>2,4-DHANT</b>              |          |          |       | 23  | [18]           | 17      |                 |         |                    |         |
| <b>2-ADNT</b>                 |          |          |       |     |                | 5       | 3 [4]           | 24      | 5 [7]              | 32      |
| <b>4-ADNT</b>                 |          |          | 4     | 9   | [17]           | 14      | 4 [12]          | 29      | 6 [11]             | 28 [6]  |
| <b>2,6-DANT</b>               |          |          |       |     |                |         |                 | 4       |                    | 7 [9]   |
| <b>2,4-DANT</b>               |          |          |       |     |                | 3 [23]  |                 | 9 [70]  |                    | 13 [56] |
| <b>TAT</b>                    |          |          |       |     |                | [50]    |                 |         |                    |         |
| <sup>a</sup> mass balance (%) | 105 [98] | 101 [95] | 95    | 82  | 92 [88]        | 93 [72] | 90 [109]        | 88 [70] | 102 [100]          | 95 [70] |

<sup>a</sup>based on HPLC analysis

### 2,4,6-Trinitrotoluene Reduction: Function of Iron Phase

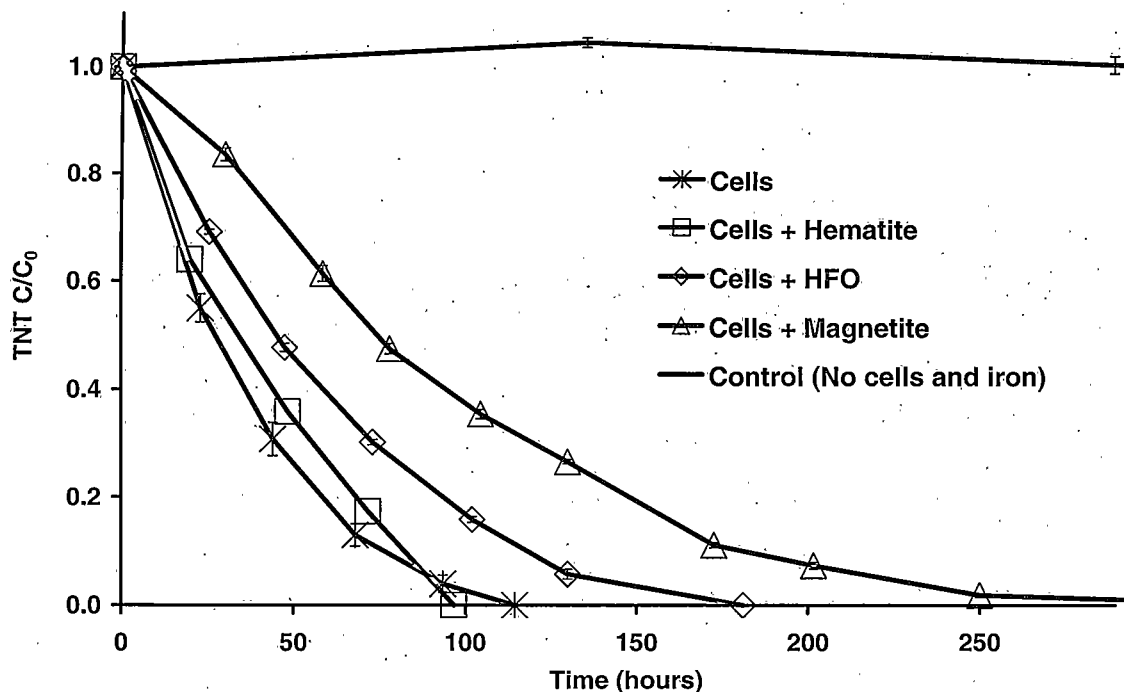


Figure 6.1. Reduction of 2,4,6-trinitrotoluene by strain ES6 in the presence of hematite, ferrihydrite (HFO) or magnetite. Triplicate analysis  $\pm$  one standard deviation.

Based on the molar distribution, 2-HADNT is further reduced to 2,4-DHANT (step 2.1, Fig. 6.2) whereas 4-HADNT is reduced primarily to 4-ADNT (step 4, Fig. 6.2 and Table 6.1). Although ES6 transformed TNT rapidly, the subsequent reduction of the HADNT's was very slow. The presence of hematite resulted in an initial rate of TNT degradation similar to systems without mineral amendment (Figure 6.1); however, a small increase in the more reduced metabolites (i.e. 2-ADNT, 4-ADNT, and 2,4-DANT) was observed with longer reaction time (1070 hrs) (Table 6.1 and step 4, 5 and 1, Figure 6.2). In the presence of magnetite and HFO, the initial TNT degradation was considerably slower (37 % to 60 %) than in hematite and mineral free suspensions (Figure 6.1 and Table 6.1).

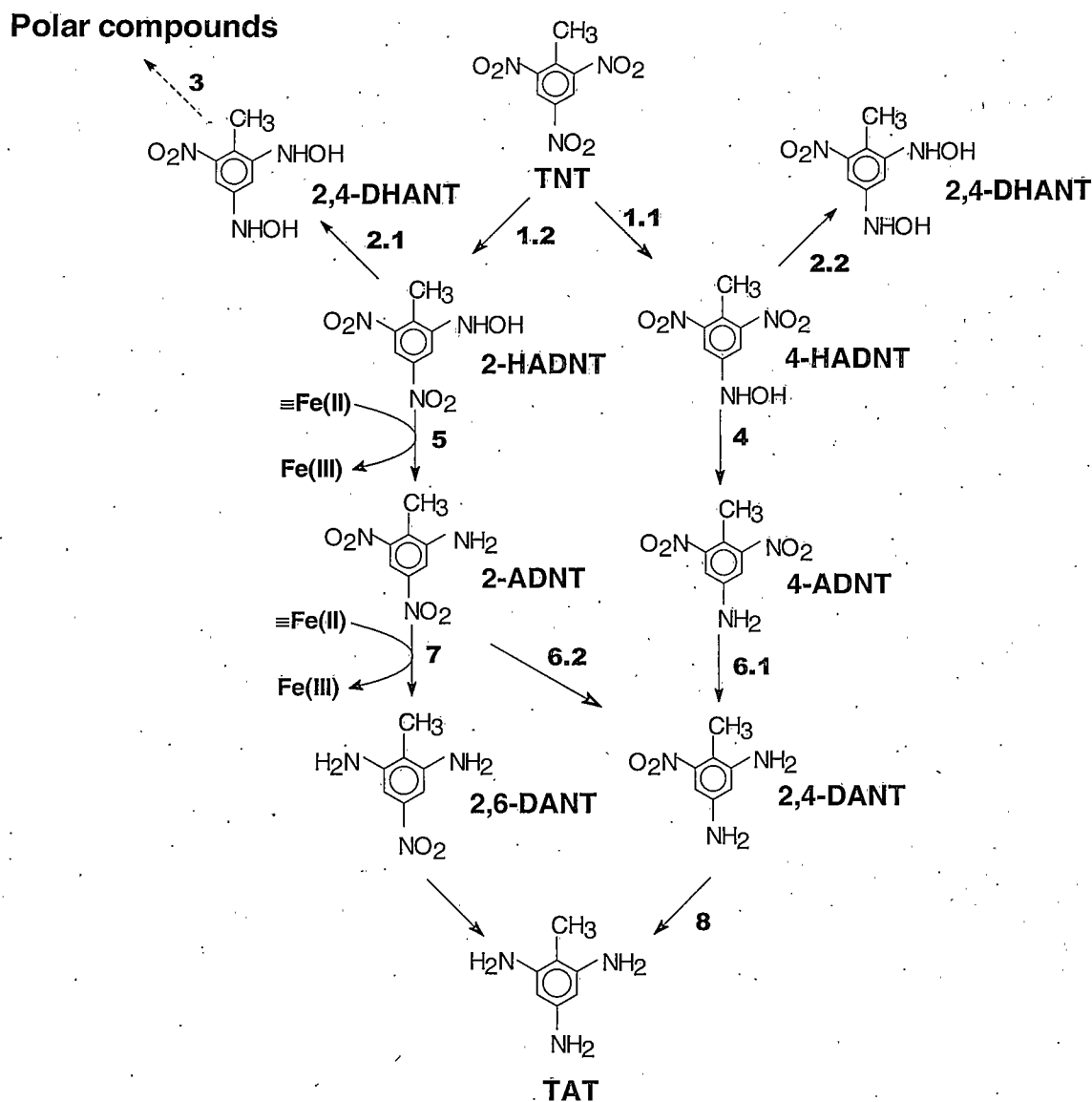


Figure 6.2. Transformation of TNT as observed in this study; each step is described in the text.

The decrease in initial TNT biotransformation rates to HADNT in the presence of magnetite and HFO may be due to competition between Fe(III) and TNT for electrons produced during the fermentation of sucrose by strain ES6. As will be discussed below,

the magnetite and HFO phases consumed a significant number of electrons compared to hematite, which potentially may have been available for direct reduction of TNT. These observations agree with those made by Wielinga et al. [41] who demonstrated that bacterially (*Shewanella algae* BrY) promoted uranium reduction was slower in the presence of ferrihydrite due to the competition between U(VI) and ferrihydrite as a terminal electron acceptor. Wielinga et al. [41] observed a similar influence of Fe phases on the U(VI) reduction rate as we did on TNT reduction rates with hematite suspensions demonstrating the highest U(VI)-reduction rates followed by mineral-free cell suspensions, and by ferrihydrite suspensions.

Despite the differences in initial TNT degradation rates, the metabolite trajectory in magnetite and ferrihydrite-amended systems was quite different than hematite and mineral free suspensions in two ways. First, the presence of magnetite or ferrihydrite resulted in higher concentration of 2-ADNT and subsequent reduction to 2,6-DANT (step 5 and 7, Fig. 6.2). Second, magnetite and ferrihydrite also resulted in accelerated production of 4-ADNT, which leads to greater formation of 2,4-DANT (step 1, Fig. 6.2 and Table 6.1). Consequently, in contrast to systems with little to no reducible Fe(III) (i.e. hematite) the presence of magnetite or ferrihydrite stimulated the further reduction of hydroxylamino compounds to amino derivatives as opposed to the formation of 2,4-DHANT (Table 6.1). The concentration of surface-associated Fe(II) in the magnetite and ferrihydrite suspensions was significantly higher than in the hematite suspensions and was found to correlate with a higher formation of the 2-substituted isomer of the aminodinitrotoluenes (2-ADNT) (Figure 6.3). No 2-ADNT was observed in the mineral-

free controls and very little 2-ADNT was observed in the presence of the hematite. Consequently, the increased reduction of the nitro group *ortho* to the methyl group was observed in the presence of more reducible Fe(III) (hydr)oxides (i.e. magnetite and ferrihydrite), which yield greater amounts of biogenic Fe(II) [17]. The formation of 2-ADNT due to abiotic reduction *via* surface-associated Fe(II) is an interesting observation, considering that most other studies have shown preferential formation of 4-ADNT and 2,4-DANT with yields up to 100 % [42,43]. Our observations are, however, in agreement with studies by Hofstetter et al. [37] who showed a preferential formation of 2-ADNT over 4-ADNT under Fe reducing conditions, albeit this finding was not further discussed.

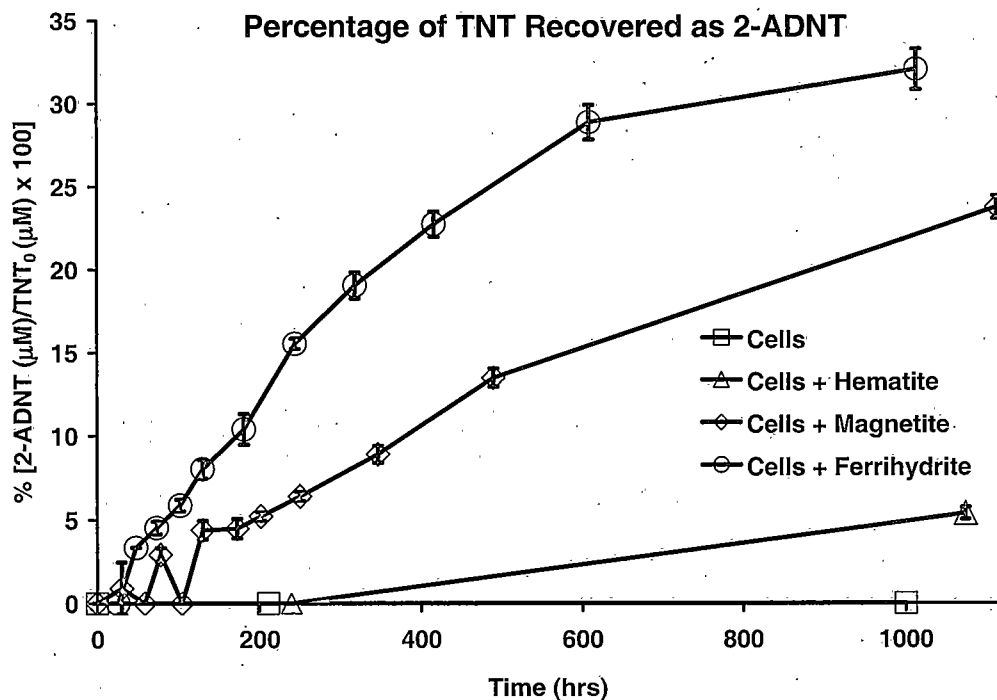


Figure 6.3. Formation of 2-amino-4,6-dinitrotoluene (2-ADNT) in the presence of strain ES6 and hematite, magnetite or ferrihydrite given as a recovery percent of added TNT. The amount of Fe(II)<sub>0.5N HCl</sub> at time of TNT addition was  $0 \pm 1.8$   $\mu$ M (hematite),  $65 \pm 16$   $\mu$ M (magnetite), and  $198 \pm 21$   $\mu$ M (ferrihydrite). Triplicate analysis  $\pm$  one standard deviation.

No abiotic degradation was observed in the presence of hematite and ferrihydrite (data not shown). Less than 2.5 % of the TNT was transformed under abiotic conditions in the presence of the mixed valence magnetite after 1200 hours indicating that no redox-active Fe(II) was present at the magnetite surface (data not shown). Consequently, the TNT reduction in the presence of Fe minerals required the presence of strain ES6, which was responsible for producing variable amounts of Fe(II).

The increased reduction capacity and the change in metabolite trajectory in the presence of magnetite and ferrihydrite suggest that surface-associated Fe(II) may play an important role governing the electron transfer to TNT and its metabolites [17,37]. This hypothesis is supported by the measured values of (surface associated) Fe(II) in systems containing hematite, magnetite, and ferrihydrite. The amount of extractable Fe(II)<sub>0.5N HCl</sub> produced after the 2 weeks incubation with strain ES6 ranged from  $0.0 \pm 1.8 \mu\text{M}$  for hematite,  $65.1 \pm 15.8 \mu\text{M}$  for magnetite and  $197.7 \pm 20.8 \mu\text{M}$  for ferrihydrite. The production of Fe(II)<sub>0.5N HCl</sub> was linear ( $R^2 > 0.9$ ) within the first 2-3 weeks of incubation, but leveled off thereafter (data not shown). Consequently, strain ES6 was capable of producing a significant quantity of Fe(II) in the presence of magnetite and ferrihydrite, which is then capable of serving as an important reductant in TNT degradation [37]. To evaluate the reduction capacity of surface associated Fe(II) in the absence of strain ES6, abiotic experiments were conducted using Fe(II)-loaded ferrihydrite.

#### Role of Ferrihydrite Sorbed Fe(II) on TNT Reduction

The Fe(II) sorption capacity of ferrihydrite is shown after 1.5 and 24 hours of reaction time (Figure 6.4a). Increased sorption was observed after 24 hours of reaction in

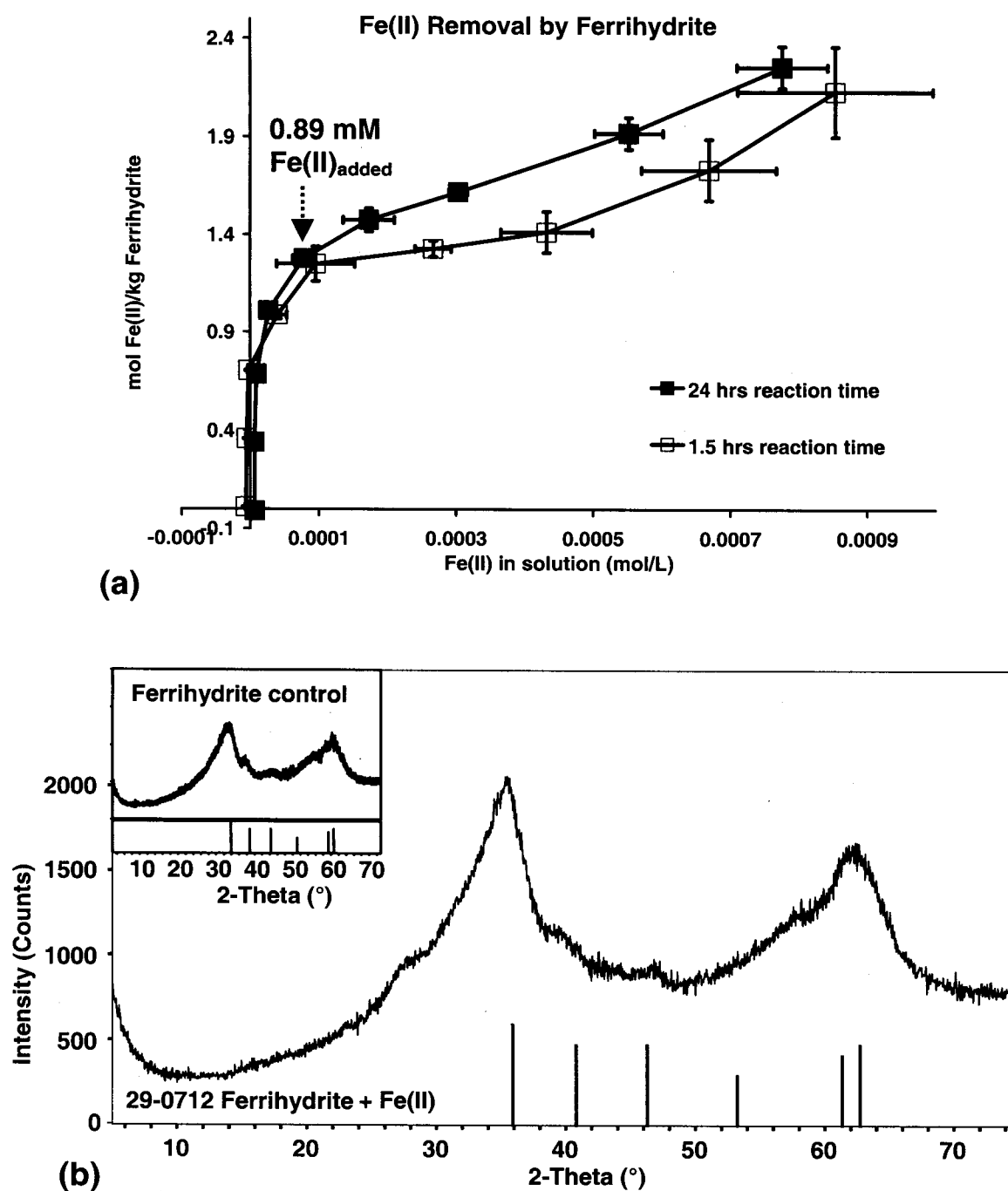


Figure 6.4. Removal of Fe(II) by ferrihydrite in C-buffered synthetic groundwater. Triplicate analysis  $\pm$  one standard deviation (a). X-ray diffractogram of the ferrihydrite sample added 2.18 mM Fe(II) after 10 days of reaction indicating that no Fe phases were formed (inset shows ferrihydrite control) (b).

samples containing  $\geq 1.11$  mM Fe(II) [1,44], but the sorption capacity clearly leveled off at approximately 1.25 mol Fe(II)/kg ferrihydrite (Figure 6.4a). Samples from this experiment were analyzed by XRD after 10 days of reaction time to assess the potential formation of secondary Fe phases. Ferrihydrite was the sole Fe phase in both the presence of low and high (2.18 mM) Fe(II) concentrations (Figure 6.4b). This is in contrast to abiotic studies by Hansel et al., [1] and Fredrickson et al. [45], showing the transformation of 2-line ferrihydrite into a mixture of Fe phases such as goethite, lipidocrocite, magnetite and siderite after 9 to 30 days of reaction time, respectively. Differences in experimental conditions among these studies, may explain the variable formation of secondary phases in Fe(II)-treated ferrihydrite. For example, Hansel et al. [1] used ferrihydrite-coated sand and 1,4-piperazinediethanesulfonic acid (PIPES) buffered water compared to ferrihydrite in carbonate buffered SGW. The presence of PIPES may influence the nucleation and growth of Fe phases as has been illustrated in a number of studies where surface sulfonation can influence the kinetics and type of Fe minerals formed [46-48]. Specifically, Rieke et al. [47,48] illustrated that sulfonic acid terminated self-assembled monolayers resulted in faster growth and deposition kinetics of goethite by thermal hydrolysis of  $\text{Fe}(\text{NO}_3)_3$ .

The TNT reduction capacity of Fe(II)-ferrihydrite was determined in an experiment discussed in the previous chapter (Chapter 5, Figure 5.6). Specifically, the reduction of TNT was determined in experiments where 0.89 mM Fe(II) was added to 6 mM ferrihydrite after 24 hours equilibration time. The majority (92 % or 0.82 mM) of the added Fe(II) was sorbed to ferrihydrite at the time of TNT injection. TNT was reduced to

HADNTs, then ADNTs, and finally DANTs with a sequential decreasing rate of reaction [17]. TNT ( $59.1 \mu\text{M}$ ) was completely reduced to 2-HADNT ( $32.5 \mu\text{M}$ ), 4-HADNT ( $10.6 \mu\text{M}$ ), 2-ADNT ( $5.19 \mu\text{M}$ ), 4-ADNT ( $5.9 \mu\text{M}$ ), 2,6-DANT ( $2.7 \mu\text{M}$ ) and 2,4-DANT ( $3.7 \mu\text{M}$ ) after 24 hours of reaction time. These observations were similar to the biotic experiments in the presence of ferrihydrite- and magnetite-associated Fe(II) (Table 6.1) and in agreement with studies by Hofstetter et al. [37]. In theory, the reduction of  $60 \mu\text{M}$  TNT to this suite of metabolites would require  $0.32 \text{ mM}$  of Fe(II) suggesting that 39 % of the sorbed Fe(II) was oxidized over 24 hours. The incomplete reduction of TNT to DANTs during the 24 hr reaction time suggests that a fraction of the sorbed Fe(II) was not available or reactive with TNT, or that Fe(II) was oxidized by compounds other than the TNT metabolites [49]. Based on the abiotic reduction studies, the formation of  $17.3 \mu\text{M}$  (32 %) 2-ADNT and  $3.8 \mu\text{M}$  (7 %) 2,6-DANT in the biotic ferrihydrite-amended experiment (Table 6.1) would require approximately  $374 \mu\text{M}$  of  $\text{Fe(II)}_{0.5\text{N HCl}}$  indicating that renewal of Fe(II) must have taken place during the TNT degradation in these systems.

#### AQDS Enhances Fe(III) Bioavailability and TNT Degradation

Given the important role of surface-associated Fe(II) in reducing TNT, one would expect a further enhancement in the presence of an electron shuttle, where rates of Fe(II) production have been shown to increase [17].

Batch systems containing hematite, magnetite, or ferrihydrite were incubated with strain ES6 in the presence of  $100 \mu\text{M}$  AQDS for two weeks prior to injection of TNT,

comparable to treatments in the absence of AQDS. Uninoculated controls containing AQDS showed a very slight decrease ( $\leq 5\%$ ) in TNT concentrations over approximately 1000 hours (Table 6.1). Since no degradation products were observed in these systems, the loss is assumed to be due to increased sorption of TNT to the experimental systems in the presence of AQDS. However, the possible abiotic reduction of TNT by the reduced form of AQDS (AHQDS) after addition of AQDS from an  $O_2$ -free stock solution cannot be completely excluded.

The bioreduction of hematite increased in the presence of AQDS resulting in the formation of  $19.4 \pm 3.5 \mu\text{M Fe(II)}_{0.5\text{N HCl}}$  after 21 days of incubation. A more pronounced effect was observed in magnetite and ferrihydrite-amended systems, where the addition of AQDS resulted in a 10 fold increase in  $\text{Fe(II)}_{0.5\text{N HCl}}$  for magnetite ( $717.7 \pm 59.8 \mu\text{M}$  at day 21) and a 3 fold increase for ferrihydrite ( $665.8 \pm 5.4 \mu\text{M}$  at day 15), compared to systems without AQDS. Comparatively, the  $\text{Fe(II)}$  produced in hematite systems appears trivial compared to that produced in the magnetite and ferrihydrite systems. However, the presence of AQDS in the hematite-amended systems dramatically influenced the TNT metabolite trajectory and extent of reduction (Figure 6.5a) relative to treatments in the absence of AQDS (Table 6.1). For example, TNT was fully degraded within 48 hours with an initial, but transient appearance of 4-HADNT and 2-HADNT that were reduced to both 2,4-DHANT and 4-ADNT, then ultimately to 2,4-DANT and TAT after 1070 hrs. The degradation pathway suggests that TAT was being produced *via* 4-ADNT and 2,4-DANT (step 4, 6.1, and 8, Figure 6.2). The production rate of TAT in hematite-amended systems was approximately double the rate previously reported in the presence of

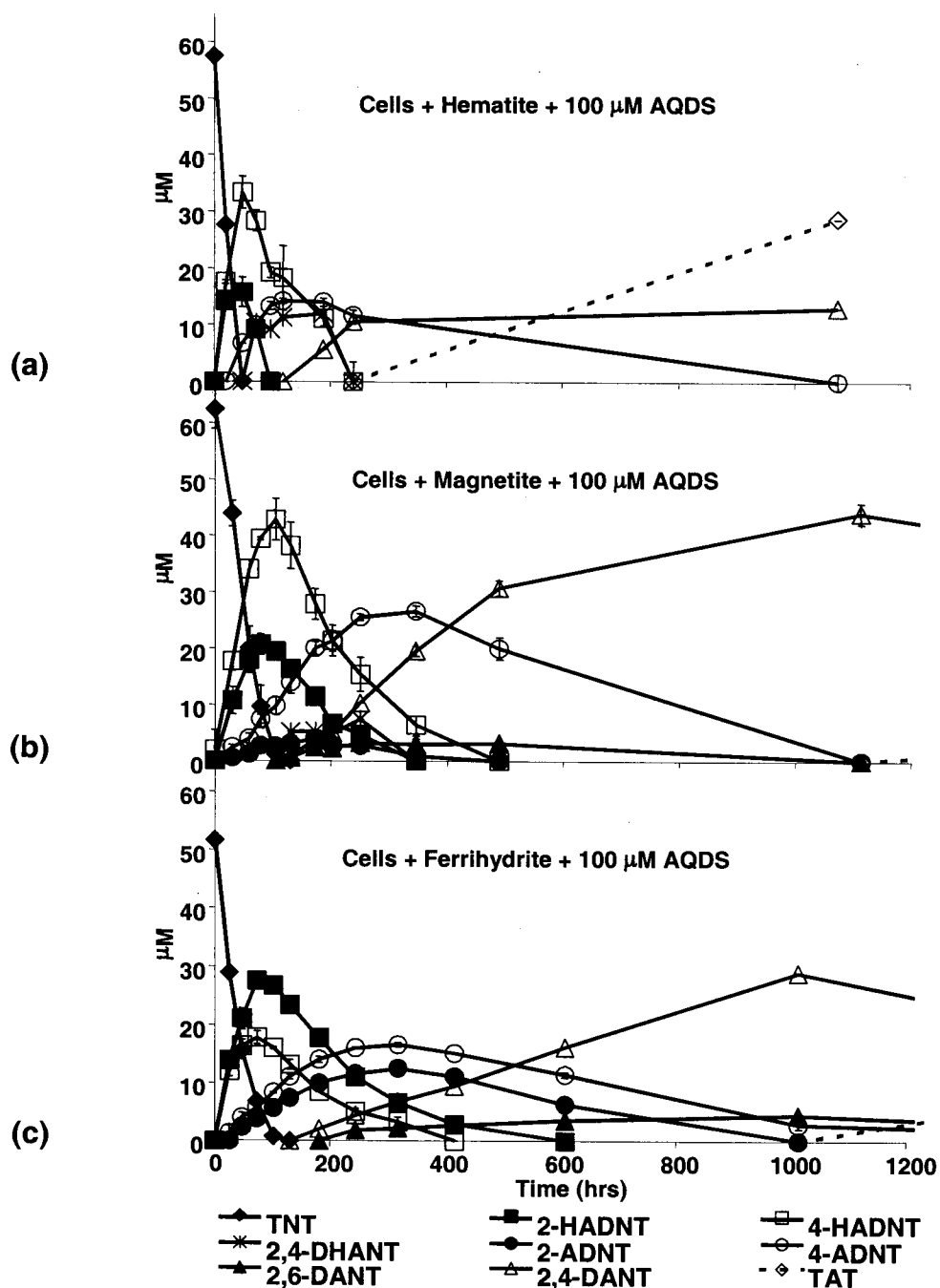


Figure 6.5. Transformation of 2,4,6-trinitrotoluene (TNT) with strain ES6 + hematite (a), ES6 + magnetite (b), and ES6 + ferrihydrite (c). Inoculation with ES6 and TNT injection was performed at day 0 and day 14, respectively. The experiments (a-b) contained 19  $\mu\text{M}$  and 718  $\mu\text{M}$   $\text{Fe(II)}_{0.5\text{N HCl}}$  (after 21 d inoculation), respectively, and experiment (c) 666  $\mu\text{M}$   $\text{Fe(II)}_{0.5\text{N HCl}}$  (after only 15 d of incubation). Experiments were conducted in triplicate and error bars correspond to one standard deviation.

ferrihydrite [17]. The lack of full mass balance (72 %) using HPLC analysis after 1070 hours reaction time was likely due to the transformation of 2,4-DHANT to polar metabolites, which have been shown to be important in the presence of AQDS [17,18].

The addition of AQDS to magnetite-amended systems (Figure 6.5b) resulted in a significant initial production of the hydroxylamino derivatives, particularly 4-HADNT. Sequential degradation of 4-HADNT to 4-ADNT was then followed by the accumulation of predominantly 2,4-DANT by 1070 hrs (step 4 and 6.1, Figure 6.2). Very little 2-ADNT and 2,6-DANT were observed, indicating a preferential reduction of the nitro group *para* to the methyl group. The addition of AQDS to ferrihydrite treatments also accelerated the reduction of TNT, resulting in the initial formation of both 2-HADNT (43 % of initial TNT) and 4-HADNT (28 % of initial TNT), followed by conversion to 2-ADNT and 4-ADNT, and then primarily to 2,4-DANT, with a minor, but notable production of 2,6-DANT (Figure 6.5c).

The TNT reduction patterns in the presence of the different Fe minerals were completely reversed in the presence of AQDS. In the absence of AQDS, a greater production of Fe(II) in the ferrihydrite treatment lead to formation of more reduced TNT metabolites (i.e. 2,4-DANT and 2,6-DANT), and the abiotic reduction of TNT with Fe(II)-ferrihydrite confirmed the role of surface-associated Fe(II). However, in the presence of AQDS, the hematite and magnetite treatments actually resulted in a greater production of reduced metabolites, especially 2,4-DANT and TAT (for hematite). The increased production of reduced TNT metabolites in AQDS-hematite and AQDS-magnetite treatments may be due to the rapid formation of reduced AQDS

(anthrahydroquinone-2,6-disulfonate; AHQDS), followed by the direct reduction of TNT by AHQDS. The elevated levels of Fe(II) produced in AQDS-ferrihydrite systems may be a consequence of reaction with AHQDS where bioavailable Fe(III) effectively competes with TNT, or its metabolites, as electron acceptors. In AQDS-hematite treatments, the lack of bioavailable Fe(III) would make the reduction of TNT and metabolites more favorable. The preferential reduction of the 4-substituted nitro group in AQDS-hematite and AQDS-magnetite treatments is in agreement with abiotic studies in the presence of the electron shuttling compound juglone (5-hydroxy-1,4-naphthoquinone) [42]. Consequently, in the presence of both Fe (hydr)oxides and AQDS, the TNT reduction pathways are determined by contributions from surface-associated Fe(II) and direct effects by the electron shuttle AQDS.

#### Secondary Phase Formation in the Presence of Ferrihydrite by *Cellulomonas* sp. strain ES6

It is well recognized that secondary mineral phases often form during the reductive dissolution of ferrihydrite [2,45,50] and the formation of highly redox-active Fe(II) or Fe(III, II) phases may also play an important role in the fate of TNT.

In contrast to abiotic experiments containing sorbed Fe(II), the biotically mediated reductive dissolution of ferrihydrite resulted in secondary mineral formation in the presence and absence of AQDS. Consequently, the formation of a secondary phase may contribute to the reduction pattern of TNT observed in ferrihydrite systems. Scanning electron microscopy (SEM) confirmed the association of strain ES6 with the

ferrihydrite surface (Figure 6.6a-b) and showed the formation of a secondary Fe phase (Figure 6.6c), with spheroidal geometry (diameter  $\sim 100 - 150$  nm) (Figure 6.6d).

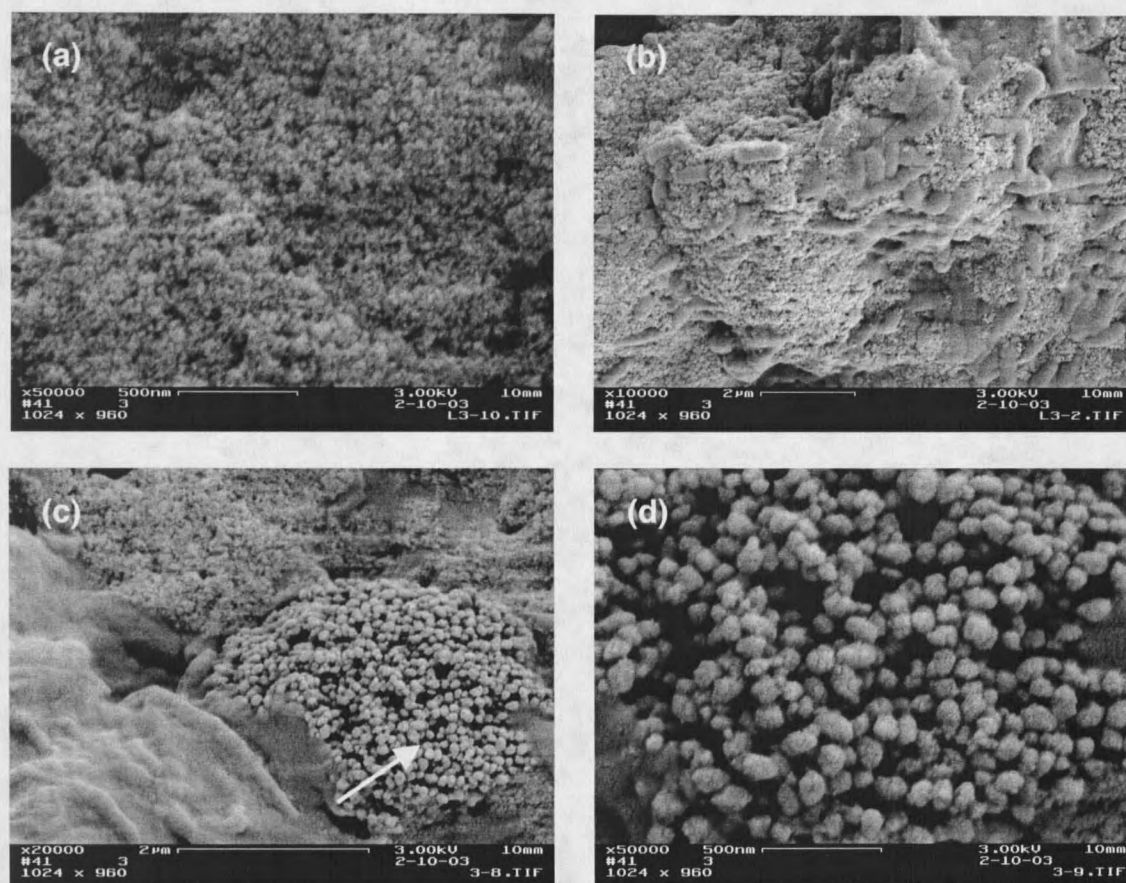


Figure 6.6. SEM micrographs of samples containing ferrihydrite (a) and *Cellulomonas* sp. strain ES6 (b) after 14 days of inoculation. The formation of a secondary morphology (an Fe phase based on EDS analysis) is indicated with the white arrow (c). High resolution SEM indicates that the secondary Fe phase has a spheroidal structure with a diameter of approximately 100 – 150 nm (d).

Transmission electron microscopy (TEM) confirmed the SEM observations that individual cells were in direct contact with ferrihydrite (Figure 6.7a) and that the secondary Fe phase was associated with ferrihydrite surfaces as opposed to cell walls

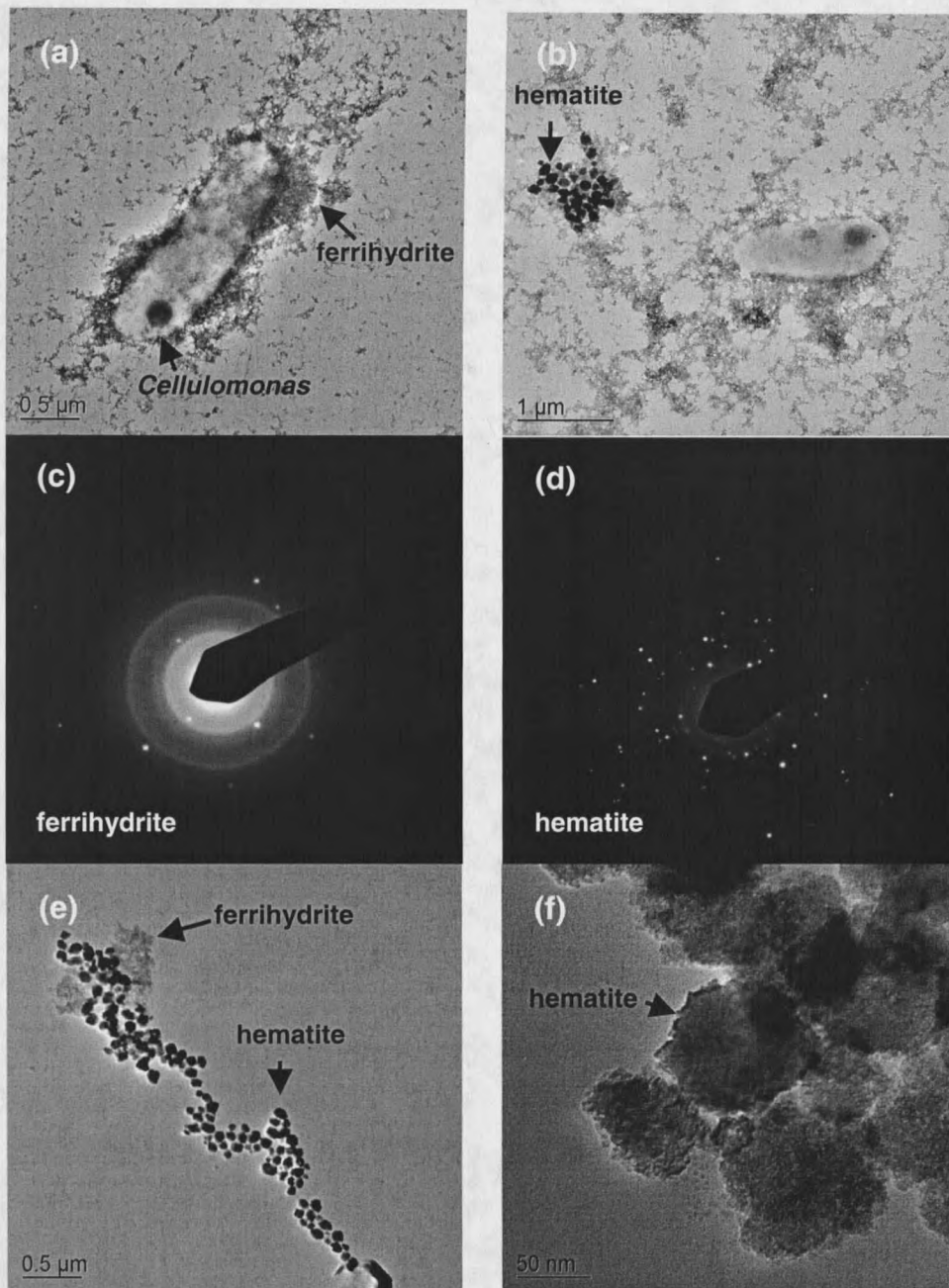


Figure 6.7. TEM images of *Cellulomonas* sp. strain ES6 after 14 days of inoculation in the presence of ferrihydrite. TEM micrographs show whole-mount of strain ES6 encrusted in ferrihydrite (a) and the formation of secondary hematite (b). Selected area electron diffraction indicated that ferrihydrite (c) and hematite (d) was the most likely Fe phases. A higher fraction of hematite particles was formed in the presence of AQDS some of which were linked as chains (e). High resolution TEM revealed that the hematite particles often had a rounded hexagonal shape with a typical diameter of 50 – 150 nm (f).

(Figure 6.7b). Selected area electron diffraction (SAED) was used to differentiate ferrihydrite (Figure 6.7c) from the secondary crystalline Fe phase, and d-spacings were consistent with values reported for hematite (Figure 6.7d). In some cases, the d-spacings suggest that siderite may have been a minor component (data not shown). A higher fraction of hematite was observed in systems amended with AQDS (Figure 6.7e-f), with particle sizes ranging from 50 – 150 nm (Figure 6.7f). Hematite phases were associated with the ferrihydrite surface and not the microbial cell envelope (Figure 6.7b, 6.7e and 6.7f), suggesting that strain ES6 was only indirectly responsible for hematite mineralization following the biogenic dissolution of ferrihydrite.

X-ray diffraction (XRD) was employed to characterize and confirm the identity of secondary Fe phase(s) in the presence and absence of AQDS. X-ray diffractograms of abiotic controls showed no transformation of ferrihydrite within 14 days of reaction time (Figure 6.8a). However, the presence of hematite was evident in biotic samples (Figure 6.8b) and to a higher extent in AQDS amended systems (Figure 6.8c). Ferrihydrite and hematite were the only Fe containing phases identified by XRD (Figure 6.5c). The presence of minor concentrations (< 1 to 2 % w/w) of other secondary phases (e.g. siderite) cannot be excluded based on the XRD analysis. The weight percent of the Fe phases (i.e. ferrihydrite and hematite) was determined semi-quantitatively based on their reference intensity ratio (RIR). The formation of hematite was 3.3 times higher in the presence of AQDS, consistent with the 3.4 times higher concentration of  $\text{Fe(II)}_{0.5\text{N HCl}}$  in these samples (Table 6.2).

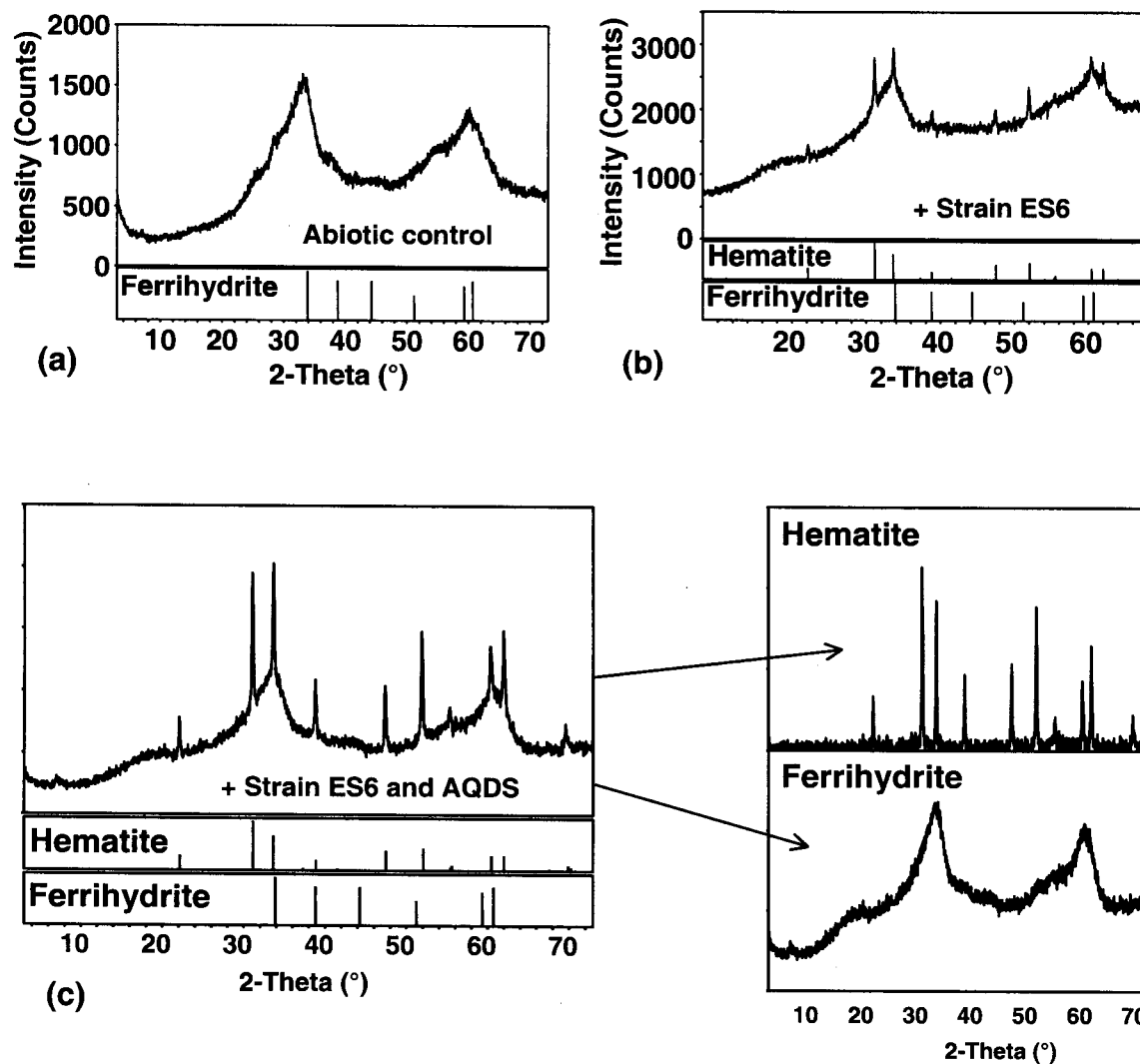


Figure 6.8. X-ray diffractograms of samples with *Cellulomonas* sp. strain ES6 after 14 days of inoculation in the presence of ferrihydrite. No secondary mineralization was observed under abiotic conditions (a), but hematite was formed in the presence of strain ES6 (b). The addition of an electron shuttle (AQDS) to the biological samples increased the formation of hematite (subtracted diffractograms are included for clarity) (c).

Table 6.2. Fe phases present after 14 days of reaction based on semi-quantitative XRD analysis.

| System                                | Ferrihydrite | Hematite |
|---------------------------------------|--------------|----------|
| <b>Ferrihydrite (abiotic control)</b> | 100 %        | 0 %      |
| <b>Ferrihydrite + Cells</b>           | 97 %         | 3 %      |
| <b>Ferrihydrite + AQDS + Cells</b>    | 90 %         | 10 %     |

Secondary mineralization in studies with Gram negative DIRB has often resulted in the formation of goethite, and reduced Fe phases such as vivianite, siderite, green rust and magnetite depending on the experimental conditions [1-3,9]. On the other hand, Brown et al. [51] reported precipitation of hematite by a natural microbial consortium including both Gram negative (e.g. *Shewanella* spp. were thought to be present) and Gram positive bacteria, and found that different Fe phases (i.e. hematite, ferrihydrite, ferrous hydroxide, and vivianite) were formed depending on the Fe:C ratio. Secondary mineralization pathways are most likely influenced differently depending on the microbial species present based on the recognized role of extracellular organic ligands such as oxalate and siderophores for increased dissolution and bioavailability of Fe(III) ([52] and references herein). For instance, Cornell and Schneider [53] showed how the presence of L-cysteine caused ferrihydrite to rapidly transform to goethite; on the contrary, hematite was the sole phase formed in its absence. Cysteine has also been hypothesized to function as an electron carrier for the transfer of electrons from *Geobacter sulfurreducens* to Fe(III) oxides [54]. The specific mineralization products of microbial Fe(III) reduction are also dependent on the crystalline state of the initial Fe

phase, the presence of coprecipitated or sorbed ions, and the solution chemistry [2,3,9,11]. For example, studies have reported the preferential formation of secondary hematite from ferrihydrite in the presence of sorbed ions, most likely due to inhibition of ferrihydrite dissolution or nucleation of goethite ([55] and references herein). The formation of secondary hematite in the present study may have contributed to the degradation rate and fate of TNT in the ferrihydrite-amended systems based on results obtained from hematite-supplemented systems. Also, the high concentration of biogenic Fe(II) in the ferrihydrite-amended systems will allow sorption of Fe(II) to the hematite-surfaces forming a hematite-Fe(II) redox couple that may impact the fate of TNT different from the ferrihydrite-Fe(II) redox couple [56].

#### Environmental Significance

The results of this study show that Fe(III) (hydr)oxides can influence the rate and extent of TNT reduction. The impact of the tested Fe phases depended on the ability of strain ES6 to produce surface-associated Fe(II). The presence of bio-reducible Fe(III) phases decreased the initial rate of TNT reduction likely due to competition for electrons, conversely, the presence of surface associated Fe(II) catalyzed the extent and pathway of TNT transformation. Addition of the electron shuttle AQDS increased the reduction rates of TNT particularly in the presence of hematite where Fe(III) is less available. The much lower impact of AQDS on the reduction of TNT in the presence of ferrihydrite indicates that the reduced form of AQDS (AHQDS) preferentially reduces bioavailable Fe(III) over TNT and its metabolites. Additionally, AQDS also increased the formation of secondary hematite from ferrihydrite. This indicates that the addition of AQDS to natural

systems rich in amorphous Fe will result in an initial slow reduction rate of nitroaromatics due to competition between TNT and ferrihydrite as electron acceptors; however, reduction of TNT may ultimately increase as less bioavailable Fe phases form. The impact and potential of quinone-mediated reduction of organic pollutants might therefore be evaluated based on the Fe phases and bacteria present as well as the natural turnover process of bioreducible Fe phases. Iron cycling is influenced by the O<sub>2</sub> content and the types of microorganisms present, since they affect the pH and redox status of natural waters [51]. We propose that quinone-mediated reduction of nitroaromatics will be most efficient in soil containing small amounts of bioavailable Fe(III) phases. However, based on previous studies [17] the addition of AQDS may lead to the formation of various yet unidentified polar metabolites especially in Fe-free systems. The formation of these unidentified polar metabolites would only be appreciated if they were less toxic or more biodegradable than their parent molecule. An electron shuttling promoted formation of secondary Fe was also observed and could have important implications in natural environments due to solubilization of co-precipitated heavy metals or an exhaustion of the pool of bioavailable Fe(III) [21]. Understanding what limits TNT reduction and how it may be stimulated are important for improving remediation strategies. The addition of AQDS stimulated TNT transformation, however the mechanism(s) and the environmental implications of this enhancement must be understood to make an intelligent evaluation of its potential for *in situ* bioremediation.

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## CHAPTER 7

## SUMMARY

The research performed in this dissertation was designed to develop chromatographic tools for the advanced analysis of explosives and 2,4,6-trinitrotoluene (TNT) metabolites and to evaluate imaging and spectroscopic techniques for the detailed analysis of environmentally relevant iron (hydr)oxides. The ultimate goal of this research was to investigate the concomitant and complex influence of bacteria, soil organic matter, electron shuttling compounds, and iron phases on the fate of TNT.

Five important aspects for the comprehensive understanding of TNT behavior under iron reducing conditions were investigated: (I) chromatography, to reveal degradation pathways of TNT (Chapters 2, 5 and 6); (II) high resolution imaging and spectroscopy, for in-depth analysis of iron(hydr)oxides and their impact on TNT transformation (Chapter 3, 5 and 6); (III) desorption, to reveal the impact of solution chemistry (i.e. presence of surfactants and cations) on binding interactions between TNT and humic acid coated iron (hydr)oxides (Chapter 4); (IV) biodegradation, to gain fundamental knowledge of the impact of a common soil bacterium (i.e. *Cellulomonas* sp. strain ES6) on the TNT transformation in the presence and absence of electron shuttles and iron phases (Chapters 5 and 6); (V) bioavailability of iron phases, to reveal the influence of three different iron minerals in the presence and absence of electron shuttles on the degradation pathway of TNT by strain ES6 (Chapters 5 and 6). The objectives listed in the Introduction were addressed in five separate but interrelated research projects, which are summarized in the five main chapters of this dissertation. This chapter

summarizes the key findings, discusses their implications and identifies future research needs.

Chapter 2 investigated the effects of column packing material, mobile phases, pH, flow rates, different gradient elution systems, ion-pair reagents and column temperature ( $T_c$ ) on the separation of TNT metabolites and EPA method 8330 explosives in high performance liquid chromatography (HPLC). The  $T_c$  and an ion-pair reagent proved to have the highest impact on the chromatographic separation of especially isomer pairs. Consequently, these parameters were chosen for detailed studies. The  $T_c$  was identified as the key parameter for optimal baseline separation. Increased temperature resulted in shorter retention times ( $t_r$ ) and better peak resolution particularly for the aminoaromatics investigated. A linear relationship was found when plotting the  $t_r$  as a function of the absolute temperature ( $1/T_c$ ) for the investigated compounds and the following temperature sensitivity was observed based on the calculated slopes ( $b'$ ): ADNTs > HADNTs >> TNT > DANTs (refer to Chapter 2 for further details). The linear relationship for the studied explosives may be used to predict the  $t_r$  as a function  $T_c$  and thereby help improving the separation of closely eluting compounds. The change of  $T_c$  especially influenced the selectivity of ionizable and polar compounds most likely due to a concurrent change of the  $pK_a$  value. Good separation was also achieved in the presence of an ion-pair reagent; however, since the overall chromatography was not improved by the use of an ion-pair reagent it is suggested to perform the chromatography in its absence. Finally, the developed gradient method is unique because (at a different column temperature) it is capable of separating 23 explosive-related compounds, including both

the most commonly observed TNT metabolites and the EPA Method 8330 compounds in a single HPLC run. Thus, the gradient elution method can become a valuable tool for the fast and reliable analysis of complex samples containing mixtures of nitroaromatics, (hydroxyl)aminoaromatics and nitramines. Chapter 2 is reprinted from Journal of Chromatography A, 1022, Thomas Borch and Robin Gerlach, 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, pp. 83-94, Copyright (2003), with permission from Elsevier.

Chapter 3 evaluated the use of bulk and surface analytical techniques for a comprehensive characterization and comparison of two environmentally relevant iron (hydr)oxide models used in a previous drinking-water study on biofilm control in the presence of humic acids. The two materials characterized were 1) corrosion products (CP) from a water system, and 2) a synthetic Fe (hydr)oxide thin film (SIOF) formed on glass surfaces (500- $\mu\text{m}$  beads and glass cover slips). Multiple techniques were applied, including the use of AFM, SEM-EDS, TEM-SAED, ToF-SIMS (for depth profiling), XRD (as well as grazing incident (GI) diffractometry), XPS, BET  $\text{N}_2$  adsorption, and a proton-binding capacity titration. The CP had a specific surface area of about  $26 \text{ m}^2 \text{ g}^{-1}$  and consisted of a mixture of ordered crystalline Fe(II, III) (hydr)oxides (goethite, magnetite, lepidocrocite), calcite, and a poorly ordered iron(II-III)-bearing phase tentatively identified as a sulfate green rust. The SIOF consisted of hematite with an average thickness of 2 nm when formed on glass beads (specific surface area of about 82

$\text{m}^2 \text{g}^{-1}$ ). Much thicker (118 nm) SIOF formed on glass cover slips and yielded a mean roughness ( $R_a$ ) of 16 nm. The two materials differed significantly in their proton-active surface-site densities, with CP having about 11 sites  $\text{nm}^{-2}$ , and SIOF about 72 sites  $\text{nm}^{-2}$ . The BET surface area, total iron content, and reactivity measured by acid-neutralization capacity were found simple to obtain. XRD, XPS, EDS and TEM-SAED were capable of identifying several crystalline phases, oxidation states and the elemental composition of the corrosion product without extensive sample preparation. XRD proved to be the most useful technique for phase identification of the CP, whereas the use of XPS and SAED for CP characterization proved challenging due to the complexity of CP. GI-XRD revealed the nano-crystalline structure of the SIOF and this result was partly supported by TEM-SAED (hematite and probable ferrihydrite) and XPS (hematite or maghemite) analysis. The imaging techniques were useful for comparisons of crystalline morphologies (SEM and TEM), roughness (AFM), domain size (AFM and TEM) and surface area (AFM). This work summarizes the potential applicability of these techniques to elicit physical and chemical properties of these iron (hydr)oxide models. This research may also become a valuable tool for the fast and reliable analysis of other complex samples containing mixtures of iron (hydr)oxides such as biomineralization products formed in environmental systems. Chapter 3 has been submitted to the journal *Water Research* for publication.

In Chapter 4, I investigated the effect of cations and biosurfactants on TNT (de)sorption in the presence of multi-component solid phase systems. The model systems developed for the use in the batch and column studies consisted of soil humic acid (HA)

coated goethite ( $\alpha$ -FeOOH), and quartz sand coated with both goethite and HA, respectively. Based on a literature review, mono- and divalent cations (including their ionic strength) and biosurfactants were found to be key factors that may influence the sorption kinetics of organic compounds. Batch studies were therefore conducted in the presence of rhamnolipid biosurfactants at both 2.5 and 25 times the critical micelle concentration (CMC) and showed up to 20 % improved TNT desorption as compared to aqueous controls. The enhanced TNT desorption may be caused by solubilization in the surfactant micelle structure. On the contrary, the addition of both mono- and divalent cations increased the sorption of TNT to the HA-coated  $\alpha$ -FeOOH relative to aqueous controls, indicating an equilibrium-controlled system. Column studies were subsequently setup to study the TNT desorption behavior under flow conditions. Chemical changes in the mobile phase of flow-through column studies showed little impact on the desorption of TNT. The lack of sensitivity in the column studies might be due to the low fraction of sorbed humic acid ( $f_{oc} = 0.03$  %) thus resulting in a low TNT sorption capacity of the  $\alpha$ -FeOOH-HA-coated quartz. However, minor trends were observed and the results were fitted by the CXTFIT model to obtain desorption kinetic data. Desorption rate coefficients obtained by the CXTFIT model indicated, that the removal of TNT from the saturated column system was faster in the presence of rhamnolipids than in the presence of divalent cations ( $\text{Ca}^{2+}$ ;  $I = 0.005 - 0.1$  mol/kg). However, the CXTFIT was not capable of accurately fitting the tailing resolution when desorbing TNT for all columns, and did not fit the rise and fall characteristics of the TNT desorption when using biosurfactants. The results from these studies may impact future remediation strategies due to a better

understanding of how solution chemistry can impact the binding interactions between TNT and humic acid coated iron (hydr)oxides/quartz.

In Chapter 5, batch studies were conducted to reveal differences in the transformation pathways during the reduction of 2,4,6-trinitrotoluene (TNT) by a novel Gram positive fermenting bacterium (strain ES6) in the presence and absence of hydrous ferric oxide (HFO) and the electron shuttling compound anthraquinone-2,6-disulfonate (AQDS). Strain ES6 was capable of TNT and HFO reduction with increased reduction rates in the presence of AQDS. In the presence of microbially reduced HFO, the reduction of the nitro group *ortho* to the methyl substituent and the amino derivatives were promoted and the formation of 2,4-dihydroxylamino-6-nitrotoluene (2,4-DHANT) and tetranitroazoxytoluenes was not observed. Enhanced and more complete TNT reduction was observed when both, microbially reduced HFO and AQDS, were present simultaneously resulting in the complete reduction to the corresponding aromatic polyamines, including 2,4,6-triaminotoluene (TAT). Abiotic studies revealed that Fe(II) had to be associated with the HFO surface to facilitate TNT reduction. Reduction of TNT by HFO-Fe(II) lead to the reduction of both the nitro group *para* and *ortho* to the methyl group in contrast to the preferential reduction of the *para* nitro group in mineral-free cell suspensions. In the absence of HFO, AQDS promoted the fast initial formation of arylhydroxylamines such as 2,4-DHANT, which were ultimately transformed into a number of unidentified polar products. The drastic AQDS-induced change in the degradation pathway of TNT could be an important finding, since the resulting polar metabolites might be compounds such as phenolic amines, which open the possibility for

TNT mineralization. This research emphasizes the significance and intricate interaction of *Cellulomonas* spp., redox-active soil HA, electron shuttling, and iron phases that may contribute to a hitherto unknown reactivity toward the fate of TNT in natural systems. This fundamental study could also originate a novel remediation concept based on quinone-enhanced degradation of nitroaromatics. Chapter 5 will be submitted to the journal Environmental Science and Technology for publication.

Chapter 6 investigated the impact of three environmentally relevant Fe phases, hematite, magnetite and ferrihydrite in the presence and absence of the electron shuttling compound anthraquinone-2,6-disulfonate (AQDS) on the degradation pathway of the explosive 2,4,6-trinitrotoluene (TNT). The studies were conducted with a Gram-positive sucrose fermenting bacterial strain isolated from the Hanford (WA) subsurface and tentatively identified as a *Cellulomonas* species (designated strain ES6). Strain ES6 reduced both, TNT and the Fe minerals at increased rates in the presence of AQDS. The production of surface-associated Fe(II) and changes in Fe mineralogy were found to influence the rate, extent, and transformation pathway of TNT reduction. While the initial reduction of TNT to the hydroxylaminodinitrotoluenes (HADNTs) was faster in the presence of the less bioreducible hematite, more reduced metabolites of TNT reduction were observed in the presence of ferrihydrite and magnetite in the absence of AQDS. These observations indicate that (bio)available iron phases compete with TNT as electron acceptors resulting in slower initial TNT reduction rates. However, ferrihydrite and magnetite both showed higher concentrations of surface associated Fe(II) than hematite that stimulated the further reduction of HADNTs to amino derivatives. The TNT

reduction patterns in the presence of the different Fe minerals were completely reversed in the presence of the electron shuttle AQDS. In the presence of AQDS, the hematite and magnetite treatments actually resulted in a greater production of reduced metabolites, especially 2,4-diaminonitrotoluene and TAT (for hematite) than ferrihydrite-amended treatments. The increased production of reduced TNT metabolites in AQDS-hematite and AQDS-magnetite treatments may be due to the rapid formation of reduced AQDS (anthrahydroquinone-2,6-disulfonate; AHDS), followed by the direct reduction of TNT by AHDS. The elevated levels of Fe(II) produced in AQDS-ferrihydrite systems may be a consequence of reaction with AHDS where bioavailable Fe(III) effectively competes with TNT, or its metabolites, as electron acceptors. In AQDS-hematite treatments, the lack of bioavailable Fe(III) would make the reduction of TNT and metabolites more favorable. The biotically mediated reductive dissolution of ferrihydrite also resulted in secondary mineral formation in the presence and absence of AQDS. Secondary hematite formation was revealed by use of SEM-EDS, TEM-SAED and XRD. Hence, the enhanced reduction of TNT was most like due to AHDS or the higher concentration of redox-active Fe(II) rather than the formation of redox-reactive secondary iron phases. However, one will have to consider the potential change in redox-activity due to formation of the hematite-Fe(II) redox couple in ferrihydrite treatments. Our results therefore indicate that despite the influence of electron shuttling compounds or Fe minerals on the fate of a reducible contaminants such as TNT might be known, the presence of both might have unexpected effects. Chapter 6 is in preparation for publication in the journal *Environmental Science and Technology*.

The author of this dissertation is convinced that the research summarized herein contributes towards an improved understanding of the complex influence of biogeochemical processes on the fate of TNT. The following paragraphs will however address some important future needs. Research focusing on the development or optimization of analytical tools such as HPLC-UV/MS might lead to a more complete understanding of the numerous degradation pathways of TNT and reveal the potential for mineralization. Future research should in particular address the metabolites formed under fermenting conditions (e.g. Bamberger rearrangement products) and metabolites formed in the presence of electron shuttling compounds (e.g. AQDS).

Factors influencing the biomineralization processes and thereby the fate of inorganic and organic pollutants are still poorly understood. Research should include new techniques for the improved understanding of issues such as factors controlling the nucleation of iron phases and the interfacial chemistry of inorganic and organic pollutants at Fe (hydr)oxide surfaces. Based on preliminary and current studies by the author performed at The Advanced Light Source (ALS) (Proposal Number: ALS-00839), it is suggested to include the use of synchrotron radiation (SR) based scanning transmission X-ray microscopy (STXM), since it opens the possibility for *in situ* (wet) spectromicroscopy. STXM can provide both high-resolution imaging (~30 nm) and near edge X-ray absorption fine structure (NEXAFS). NEXAFS has particular application to chemisorbed compounds, and can be used to determine the orientation and intramolecular bond lengths of a molecule sorbed to a surface. Other preliminary studies by the author (e.g. Environ. Sci. Technol., 2002, 36, 1276-1280 and TNT research under proposal:

ALS-00839) suggest the need for further exploration of infrared-based techniques such as SR-FTIR. The strength of SR-FTIR is the opportunity for *in-situ* investigation of (TNT) biodegradation and bioavailability processes in unsaturated environments.

Future research of TNT (de)sorption kinetics should involve numerous chemical and biological conditions and a wide range of model soils. These model soils should be developed with emphasis on high TNT sorption capacity for increased sensitivity of the experimental systems toward changes in solution chemistry. When a fundamental understanding of TNT desorption behavior has been established one must address questions regarding the potential biocompatibility, since TNT desorption is only desirable, if the desorbed fraction becomes available to degrading microorganisms. Based on studies herein and in the literature it is highly recommended to further investigate the potential for biosurfactant e.g. rhamnolipid-enhanced TNT degradation.

The novel research on the role of electron shuttling compounds (i.e. AQDS) in biodegradation of nitroaromatic compounds calls for future studies. It is important to identify the entire range of metabolites produced in the presence of AQDS to confirm or reject the hypothesis of potentially formed phenolic amines. Furthermore, mechanistic studies of how electron shuttling compounds enhance the reduction of solubilized pollutants must be conducted. To this end, it is recommended to investigate if the AQDS enhanced TNT reduction was primarily due to bio-reduced AQDS in the solution or for instance an increased electron transport over the cell membrane due to cell-surface associated AQDS. AQDS is often used as a model compound for the quinone moieties in humic acids; however, studies in this dissertation showed that soil humic acid stimulates

a radically different degradation pathway of TNT than AQDS. Future research should therefore re-examine the value of AQDS as a model compound for HA, especially in experimental systems, where direct AQDS-pollutant reduction is possible.

The importance of different iron minerals and biomineralization processes on the fate of TNT was emphasized in this dissertation. Future work must consider to a greater degree the potential disadvantage of microbial and electron shuttle accelerated iron phase reduction for enhanced remediation purposes. This research should focus on the potential risk of exhausting the amorphous iron pool in the environment, dissolution of sorbed heavy metals/radio nuclides, fate of organic contaminants and changes in the microbial ecology.

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