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Reactivity of Partially Reduced Arylhydroxylamine and Nitrosoarene Metabolites of 2,4,6-Trinitrotoluene (TNT) toward Biomass and Humic Acids

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Sequential anaerobic/aerobic treatment of 2,4,6-trinitrotoluene (TNT) generally results in the incorporation of residues into biomass and natural organic matter fractions of a system. To better understand the potential contribution of hydroxylamine and nitroso moieties in these reactions, studies were conducted using model systems taking advantage of the biocatalytic-activity of *Clostridium acetobutylicum* that does not produce aminated TNT derivatives. To evaluate binding to biomass only, systems containing cell-free extracts of *C. acetobutylicum* and molecular hydrogen as a reductant were employed. At the end of treatment, mass balance studies showed that 10% of the total ^{14}C was associated with an insoluble protein-containing precipitate that could not be extracted with organic solvents. Model reactions were conducted between a mixture of 2,4-dihydroxylamino-6-nitrotoluene (DHA6NT) and 4-hydroxylamino-2,6-dinitrotoluene (4HADNT) and 1-thioglycerol to test the involvement of the nitroso-thiol reaction in binding to biomass. It was demonstrated that DHA6NT formed a new and relatively polar product with 1-thioglycerol only in the presence of oxygen. The oxygen requirement confirmed that the nitroso functionality was responsible for the binding reaction. The reactivity of arylhydroxylamino and nitrosoarene functionalities toward International Humic Substance Society (IHSS) peat humic acid was evaluated under anaerobic and aerobic conditions, respectively. 4HADNT showed no appreciable reactivity toward peat humic acid. Conversely, the nitrosoarene compound, nitrosobenzene, showed rapid reactivity with peat humic acid (50% removal in 48 h). When tested with two other humic acids (selected on the basis of their protein content), it became apparent that the proteinaceous fraction was responsible at least in part for the nitrosoarene's removal from solution. Furthermore, the pretreatment of the humic acids with a selective thiol derivatizing agent had a considerable effect on their ability to react with nitrosobenzene. Finally, molecular modeling tools were used to compare the electrophilic characteristics of potential nitroso intermediates forming from the oxidation of arylhydroxylamino metabolites of TNT. Molecular modeling analysis demonstrated that the more reduced TNT derivative containing nitroso groups were more likely to react with nucleophiles in humic substances than the less reduced nitroso intermediates.

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Introduction

Over the years, numerous bench- and field-scale biotransformation studies conducted with polynitroaromatic contaminants, particularly 2,4,6-trinitrotoluene, have resulted in a disparity in the molar balance between the parent compound and identifiable reduced products (*1, 2*). The use of ^{14}C and ^{15}N radiolabeled nitroaromatic compounds has demonstrated this discrepancy mostly results from the binding of reduced metabolites to the solid matrix. Further investigations to determine the identity of the solid matrix fraction binding the TNT metabolites have pointed in the direction of biomass (*3*−*7*) and natural organic matter (NOM) (*3, 6, 8*−*11*). The observed binding has been deemed "irreversible" or covalent because a variety of extraction techniques with common organic solvents have yielded poor recoveries of the reduced metabolites (*8, 9*). Studies with radiolabeled TNT focusing on the NOM binding have shown the radiolabel to be associated predominantly with the humin and humic acid fractions (*10*−*13*). In recent studies, the existence of covalent linkages has been confirmed using ^{15}N NMR spectroscopy (*12*−*14*). The functional group (on the metabolite) that is typically held responsible for such reactions is the aromatic amino group, primarily because of evidence regarding its fate in model systems. From model studies, three pathways for the irreversible binding of aromatic amines to soil NOM have been elucidated: the nonenzymatic 1,4-nucleophilic or Michael addition of amino groups to quinoid rings of humic acid constituents (*15*−*17*); phenol oxidase (e.g., laccase)-mediated free radical addition reactions (*1, 18*); and metal catalyzed reactions between aromatic amines and humic substances (*19*).

Unfortunately, little is known about the role of nitroso and hydroxylamino intermediates generated from partial reduction of TNT, despite their recognized reactivity. Several nitroaromatic biotransformations conducted with pure cultures of common soil anaerobic microorganisms have demonstrated an inability to produce the completely reduced amine intermediates (*20*−*24*). Instead, these studies demonstrate the formation of only partially reduced hydroxylamino intermediates. Model studies, such as those conducted with arylamines, have not been attempted with nitrosoarene and arylhydroxylamine compounds in environmental systems. This is largely due to the instability of such compounds and to the difficulty of the synthesis of their standards. A recent study has implied that the reactive hydroxylamino groups of partially reduced TNT metabolites are responsible for initiating an abiotic covalent binding to humic acids under anaerobic conditions (*25*). Questions that remain unanswered at the current time regarding the binding of partially reduced TNT metabolites include the following: (1) specifically, which functional group or groups on TNT metabolites are responsible for initiating the covalent binding; (2) which specific chemical functional group(s) or site(s) on the sorbent organic matter participates in the binding reaction; (3) what is the mechanism of the chemical binding reaction; and (4) what external environmental (or system) parameters/conditions control the binding reaction.

Recent studies focusing on the conditions necessary to increase the extent of binding to NOM have indicated two

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important factors. These are the degree of reduction achieved in the initial anaerobic phase and the need for an aerobic stage following the initial anaerobic reduction phase (*8*−*10*). These findings are believed to strengthen the evidence for the nucleophilic addition mechanism because the greater reduction achieved in the anaerobic phase leads to greater nucleophilic character of the amino nitrogen produced. Furthermore, the aerobic stage requirement allows for the reoxidation/tautomerization of the hydroquinone back to the quinone, thereby promoting further nucleophilic additions possibly leading to the formation of nitrogen heterocycles (*15*, *19*). However, this mechanism does not adequately address the extensive binding observed in purely biological systems (*26*, *27*) or the binding occurring to biomass in engineered remediation systems (*3*, *4*, *6*, *7*). First of all, quinones make up the structure of electron-transfer proteins and oxidoreductases in biological systems, both of which are present in limited quantities in a microorganism (*28*). Second, studies that involve the recovery of humic substance fractions from soil NOM to establish a mass balance for the parent radiolabel, overlook the presence of coextracted and coprecipitated molecules (*29*−*31*). Soil NOM not only contains humic substances but also has two other major components: namely, decomposable organic residues from plant and animal decay and the heterotrophic organisms that feed on the organic residue (*30*, *32*). Since the various humic substance fractions (namely, fulvic acids, humic acids, and humin) are operationally defined on the basis of their aqueous solubility with respect to solution pH (i.e., following an alkaline extraction), they often contain varying degrees of biomolecular contaminants such as denatured proteins and carbohydrates that originate from the parent soil NOM (*29*). This point is further supported by evidence that the greatest degree of binding of reduced nitroaromatics occurs to the humin fraction (i.e., fraction insoluble at all pH) of soil NOM (*12*, *13*); structurally, humin is considered to be an aggregation of various organic and inorganic molecules that include humic acids and biomolecules (*33*).

In this study we report the reactivity of arylhydroxylamino and nitrosoarene functionalities toward biomass and humic acids in model environmental systems. The reactivity toward biomass is studied under sequential anaerobic-aerobic conditions in a "low-activity" and cell-free *Clostridium acetobutylicum* cell extract/H_2 (electron donor) model system. In this portion of the work, the ^{14}C distribution was examined in the context of the protein distribution in the reaction mixture. In addition, reactions of the partially reduced TNT metabolites with model thiols were conducted under different conditions to test possible mechanisms for the binding to biomass. The reactivity toward humic acids was investigated in model systems with International Humic Substance Society (IHSS) peat humic acid under anaerobic and aerobic conditions, respectively. Peat humic acid was selected because peaty soils tend to be water logged and, subsequently, their humic acids contain high concentrations of biological molecules due to a lack of aerobic decay (*34*). 4-Hydroxylamino-2,6-dinitrotoluene (4HADNT), a common metabolite found in the reduction pathway of TNT, was used as the model arylhydroxylamine. One of the only two relatively stable and commercially available C-nitrosoarenes, namely nitrosobenzene, was used as the model nitroso compound. Using nitrosobenzene, we explored the dependence of the nitroso group's reactivity on the proteinaceous content of humic acid by attempting to restrict the binding contribution from strong nucleophiles such as thiols by pretreating the humic acids with a selective thiol derivatizing agent. Finally, we employed molecular modeling tools to compare the electrophilic nature of unstable nitroso intermediates of TNT to determine whether the binding behavior of these intermediates via nucleophilic substitution reactions.

Background Reactions

Although limited and sometimes conflicting information regarding the reactivity of partially reduced metabolites of TNT can be found in environmental literature, publications exist in chemistry and toxicology literature that review the various reactions of related functionalities (i.e., aryl-hydroxylamino and nitroso). These include publications on reactions with thiols (*35*, *36*), rearrangement reactions (*37*, *38*), and various enzyme-catalyzed reactions (*39*).

Figure 1 presents a summary of the reactions of arylhydroxylamino and nitrosoarene functionalities reported in the literature. Generally speaking, the nitrosoarene functionality undergoes reactions that are mostly spontaneous, whereas the arylhydroxylamino functionality requires catalysis to cleave the N−O bond to undergo further reaction. The nitroso and hydroxylamino functional groups exist in what can be considered a "pseudo redox equilibrium" due to the insignificant activation energy barrier for their interconversion (*39*). As a consequence, reductive conditions favor arylhydroxylamine formation, whereas oxidative conditions favor nitrosoarene formation. Although the presence of arylhydroxylamine reduction intermediates of nitroaromatic compounds is frequently reported in anaerobic remediation systems (*2*, *5*, *22*, *24*, *25*, *40*, *41*), the detection of nitrosoarenes is a rarity in natural and engineered systems, especially when arylhydroxylamines are also present. The presence of arylhydroxylamines in any system results in the scavenging of the nitrosoarenes to form relatively insoluble azoxy compounds via a rapid condensation reaction (*39*, *42*, *43*). Hence, the production of nitroso compounds in a system is typically inferred from the presence of the commonly detected and relatively insoluble azoxy compounds.

Experimental Section

Chemicals. The following chemicals were used in this study: 2,4,6-trinitrotoluene, 99% purity (ChemService, Westchester, PA); [U-ring-^{14}C]-2,4,6-trinitrotoluene, specific activity of 21.6 mCi/mmole, 99.5% purity (Chemsyn Science, Lenexa, KS); 2-hydroxylamino-4,6-dinitrotoluene (2HADNT), 4-hydroxylamino-4,6-dinitrotoluene (4HADNT), 2,2′,6,6′-tetranitro-4,4′-azoxytoluene, 2,4′,6,6′-tetranitro-2′,4-azoxytoluene, and 4,4′,6,6′-tetranitro-2,2′-azoxytoluene (Ron Spanggord, SRI International, Menlo Park, CA); 0.1 mg/mL analytical standard of 4HADNT in acetonitrile (AccuStandard Inc., New Haven, CT); 3-mercapto-1,2-propanediol (1-thioglycerol), 95% purity, and nitrosobenzene, 97% purity (Aldrich, Milwaukee, WI); EDTA (J. T. Baker, Phillipsburg, NJ); biotech grade bovine serum albumin lyophilized powder (Fisher Biotech, Fisher Scientific, Fair Lawn, NJ); protein assay dye reagent concentrate (Bio-Rad, Hercules, CA); monobasic and dibasic potassium phosphate (Sigma, St. Louis, MO); HPLC grade acetonitrile, methanol, methylene chloride, *n*-pentane, 1 N HCl solution, 1 N NaOH solution, ScintiSafe Plus 50% cocktail (Fisher Chemical, Fisher Scientific, Fair Lawn, NJ); carbon-14 Cocktail for R. J. Harvey biooxidizer (R. J. Harvey Instrument Corp., Hillsdale, NJ); monobasic and dibasic potassium phosphate, and 90% pure N-dansylaziridine (Sigma, St. Louis, MO); IHSS peat humic acid standard, leonardite humic acid standard, and Summit Hill reference humic acid (Paul Bloom, University of Minnesota, St. Paul, MN).

Preparation of Cell Extract. Crude cell extracts of *Clostridium acetobutylicum* ATCC 824 were prepared using the lysozyme/sonication procedure as described previously (*2*). However, one step was added to remove larger structural proteins by ultracentrifugation at 45 000*g*. Also, the anaerobically sealed cell extract vials were stored at −20 °C. Two batches of cell extract were produced. The first batch was kept in storage at −20 °C for a long duration (i.e., 6 months)

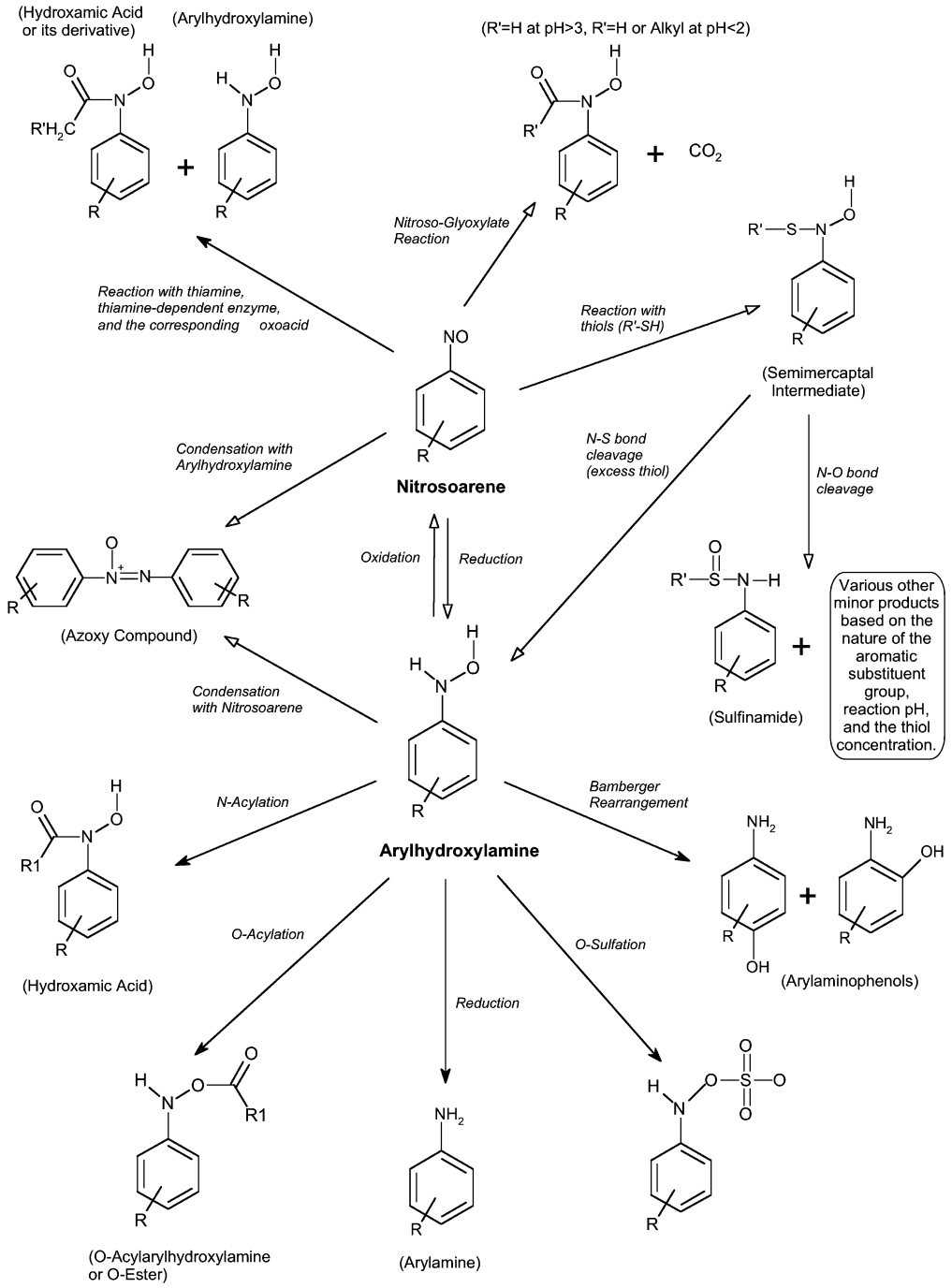

FIGURE 1. Reactions of arylhydroxylamines and nitrosoarenes reported in the literature. Reactions of arylhydroxylamines involving the heterolytic cleavage of the N−O bond (i.e., reduction, O-acylation, O-sulfation, Bamberger Rearrangement, and N-acylation) generally require catalysis. All reactions of nitrosoarenes are of a spontaneous nature, with the exception of reactions involving thiamine-dependent enzymes. Note that reactions requiring catalysis are depicted with solid arrowheads and spontaneous reactions are depicted with clear arrowheads.

prior to experimentation and had a very low TNT transformation activity (0.33 mg TNT/volume% cell extract/minute). The loss in enzyme activity in cell extracts of gram positive organisms can be substantial over long periods of time due to proteolytic degradation, even at storage temperatures of −20 °C (*44*). The low activity extract was used in the first transformation reaction in order to determine the fate of the radiolabeled TNT following anaerobic-aerobic treatment.

The second batch was prepared similarly; however, its storage time was considerably less (approximately 1 week). This extract was used to rapidly convert TNT to predominantly 2,4-dihydroxylamino-6-nitrotoluene (DHA6NT) for the reaction with the model thiol compound. The activity of this extract could not be measured with the TNT depletion assay, as TNT could not be measured within 2 min of cell extract addition (based on initial conditions the activity was >3.1 mg TNT/volume% cell extract/minute).

Analytical Methods. Temporal samples for TNT biotransformation reactions as well as thiol reaction samples were monitored using the Waters (Milford, MA) HPLC system. The system consisted of 2690 separations module, 996 photodiode array detector, and a Nova-Pak C8 analytical column (3.9 × 150 mm) and guard column assembly. The method used for all HPLC analysis was an acetonitrile/water

gradient method with a linear ramp from 35% to 75% acetonitrile in 12 min followed by a return to the original conditions in 6 min. Two TNT metabolites were identified based on UV spectra and relative retention times based on earlier work (*2*, *22*). These metabolites included 2,4-dihydroxylamino-6-nitrotoluene (DHA6NT) and the major aminophenol product resulting from the Bamberger rearrangement of DHA6NT.

Temporal samples for the reaction of humic acids with 4HADNT and nitrosobenzene were also monitored using the Waters (Milford, MA) HPLC system described earlier. The HPLC method used for analysis was an acetonitrile/water gradient method with a linear ramp from 40% to 70% acetonitrile in 10 min followed by a return to the original conditions in 4 min. 4HADNT experiments were monitored at a wavelength of 220 nm, whereas nitrosobenzene experiments were monitored at a wavelength of 305 nm. A nonpolar gradient method was used to analyze the dissolved precipitate produced in the aerobic 4HADNT/peat humic acid experiment. This method involved a linear ramp of acetonitrile from 50% to 100% in 12 min, followed by a 100% plateau for 1 min and a recovery to original conditions in 5 min. The mobile phase flow rate for all HPLC methods was 0.5 mL/min.

TNT Transformation. Both cell-extract-free transformation reaction solutions were degassed with nitrogen for 30 min followed by molecular hydrogen for 30 min. The cell extract was added after 30 min of hydrogen addition to mark the start of the anaerobic reaction phase. Temporal bulk aqueous samples were collected under gas purge (molecular hydrogen for anaerobic phase, air for aerobic phase, and nitrogen when anaerobic conditions were to be maintained without further reaction). The samples were analyzed for ^{14}C content by scintillation counting and for product formation by HPLC.

The starting conditions for the first TNT transformation reaction were 865 dpm/mL ^{14}C, 8% cell extract (v/v) concentration, and TNT at a concentration of 100 mg/L. The anaerobic phase or molecular hydrogen bubbling was continued for 22.5 h (1350 min), after which it was replaced with air for a period of 1 h. At the end of the anaerobic phase the reaction mixture consisted of DHA6NT and aminophenols (*2*) as the only identifiable products.

The initial conditions for the second reaction were 1493 dpm/mL ^{14}C, 8% cell extract (v/v) concentration, and TNT at a concentration of 50 mg/L. This reaction was carried out in 10 mM (pH 7) phosphate buffer. Temporal samples were collected after 20 and 40 min of hydrogen addition and analyzed by HPLC. Note that a trial reaction was run earlier to determine cell extract activity on the basis of TNT depletion. Fractions were also collected (one fraction/2 min) for the 40-min sample using a 100 μL injection. After 40 min of hydrogen addition all of the parent TNT had been transformed to DHA6NT with only traces of 4HADNT remaining. At this point, the reaction was stopped by flushing residual hydrogen out of the system with nitrogen gas.

^{14}C-Radiolabel Recovery and Protein Quantitation. At the end of the first transformation reaction (sequential anaerobic-aerobic conditions), a clear solution (i.e., without any visible precipitate) was decanted from the reaction vessel. Three milliliters of this solution was passed through a Gellman Sciences 0.2 micron PTFE filter. Both the filtrate and the prefiltered clear solution were subjected to liquid scintillation counting using ScintiSafe Plus 50% cocktail (10 mL cocktail/1 mL sample) on a Beckman LS6500 scintillation counter. Protein quantitation was also performed on both samples by the Bio-Rad protein assay (adapted from the Bradford Assay) which uses the Coomassie brilliant blue G-250 dye. Serial dilutions of a 2 mg/mL solution of bovine serum albumin were used with a 1:5 dilution of the dye reagent concentrate to generate a standard curve for the protein quantitation.

The long strands of the brown floc-like precipitate remaining in the reaction solution after decantation were removed using a sterile spatula. The collected precipitate was processed for ^{14}C analysis by combusting it at 900 °C in the R. J. Harvey Instrument Corporation Biooxidizer Model OX600, trapping the radiolabeled CO_2 produced in the Carbon-14 Cocktail and counting it by scintillation counting. A small fraction (0.12 gm wet weight) was removed to conduct protein analysis and solubility testing. Acid hydrolysis was performed on half of this fraction by first placing it in a microcentrifuge tube with DDI water (500 μL) and vortexing it for 30 s. Following the mixing, the tube was centrifuged (13 000 rpm for 5 min), and the supernatant solution above the pellet was discarded after the centrifugation. HCl (500 microliters at pH 2) was added to the pellet, and the tube was vortexed for 30 s to mix its contents, followed by centrifugation (13 000 rpm for 5 min). The acidic solution above the pellet was used for protein analysis. Solubility testing was carried out with the remaining precipitate by vortexing the precipitate with *n*-pentane and methylene chloride.

Reaction with 1-Thioglycerol. Molecular hydrogen addition for the second TNT transformation reaction conducted with the higher activity cell extract (concentration of 8% v/v) in phosphate buffer (10 mM, pH 7) was stopped after 40 min. At this point, the reaction was stopped by flushing residual hydrogen out of the system with nitrogen gas. The reaction was monitored by HPLC (100 μL injection), and 2-min fractions were collected for the 18-min HPLC method. Following sampling, 1-thioglycerol stock in acetonitrile (6 μL of 200 mM) was added to the still anaerobic reaction mixture to yield a molar ratio of thiol groups to potential nitroso groups (calculated on the basis of initial TNT concentration) of 1:2. This mixture was then allowed to react under anaerobic conditions for 20 min. A sample was collected from the reaction mixture under the nitrogen purge at the 20-min anaerobic reaction time mark and immediately analyzed by HPLC. Injection and fraction collection were conducted as earlier. The reaction mixture with thiols was then exposed to air, and a 20-min sample was collected and analyzed by HPLC with fraction collection performed as earlier.

Reactions with Humic Acids. All reactions were carried out in triplicate and in the dark in 10 mM phosphate buffer (pH of 7) with continuous stirring. 4HADNT was chosen as the model arylhydroxylamine compound and nitrosobenzene was chosen as the model nitrosoarene compound. Experiments were designed to keep a low sorbate (e.g., 4HADNTor nitrosobenzene) to sorbent (humic acid) mass ratio at initial conditions in order to better estimate potential second-order reactions with pseudo-first-order kinetics (*45*, *46*). The initial sorbate to sorbent mass ratio for 4HADNT experiments was approximately 0.02 and for nitrosobenzene experiments was approximately 0.01; the difference in the ratio between the two compounds reflects the approximately 2:1 ratio of molecular weights for 4HADNT:nitrosobenzene even though these molecules have potentially one mole of reactive groups per mole of either compound. The humic acid concentration was fixed at 625 mg/L. This concentration reflects a less than 10-fold increase (on the basis of carbon concentration) from maximum dissolved organic material concentrations of 50 mg carbon/L observed in nature (*47*). As in past studies (*19*, *47*), the higher than natural concentration of humic acid was selected to bring about an appreciable change in aqueous sorbate concentration over a reasonable period of time, which in this case was 48 h.

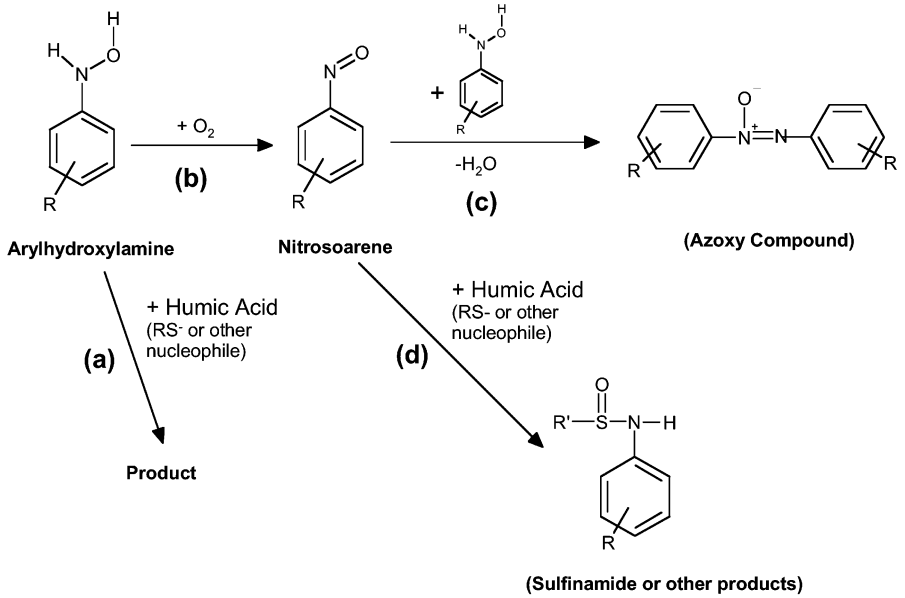

FIGURE 2. Reactions attempted during the course of this study. Note that arylhydroxylamines are stable only under anoxic conditions.

The reaction between 4HADNT and IHSS peat humic acid (Figure 2, reaction (a)), together with a control containing no sorbent, was conducted under anaerobic conditions. Freshly prepared 4HADNT stock in acetonitrile was added to vials containing degassed (sparged 30 min with Helium followed by 30 min with Nitrogen) buffer/humic acid solution to start the reaction. Temporal samples were collected under a nitrogen purge and passed through Accell Plus QMA anion exchange cartridges (Waters, Milford, MA) into HPLC vials that had been flushed with nitrogen. A needle attached to a disposable check valve (Cole-Parmer, Vernon Hills, IL) was used to allow the nitrogen displaced by the sample to exit the HPLC vials. Samples were analyzed by HPLC immediately upon collection. An additional reaction was run with 4HADNT and IHSS peat humic acid. This reaction was conducted with the same precautions as the earlier anaerobic reaction; however, the reaction mixture was exposed to the atmosphere in order to assess the ability of the humic acid to compete for the nitroso oxidation product of 4HADNT (Figure 2, reaction (b) followed by reactions (c) and (d) in parallel). The precipitate formed in this reaction was extracted using methylene chloride, followed by air-drying, redissolution in a 50/50 (v/v) acetonitrile/buffer mixture, and analysis by RP-HPLC/UV−visible detection.

Reactions between nitrosobenzene and various humic acids (Figure 2, reaction (d)) were conducted in a closed aerobic system (i.e., no prior degassing) due to the mild volatility/sublimation characteristics of nitrosobenzene. As before, temporal samples were collected and passed through Accell Plus QMA cartridges to remove humic acid and placed in HPLC vials for analysis. During sampling, creation of a vacuum in the reaction bottle was avoided by again using a needle/disposable-check-valve assembly to allow air in to replace the sample volume removed (note that the gas flow to equilibrate pressure was in the opposite direction when compared to Figure 2, reaction (a)). Additionally, controls with no sorbent were run for the experiment.

Thiol Derivatization and Recovery of Humic Acids. The thiol content of IHSS peat and Summit Hill humic acids was derivatized by adapting a thiol derivatizing procedure presented elsewhere (*48*−*51*). This procedure utilized N-dansylaziridine, a thiol derivatizing agent. A ratio of humic acid (10 mg) in 2.5 mL of derivatization buffer (67 mM phosphate/0.2 mM EDTA, pH = 8.2) to 0.25 mL of a solution of N-dansylaziridine in methanol (5 mg/mL) was used. The derivatization mixture was placed in a crimped top serum bottle and suspended in a water bath (60 °C) for 1 h. The bottle was then removed from the water bath, the top was removed, and its contents were allowed to cool under stirring for another hour. The cooled mixture was transferred into 500 Dalton (Molecular Weight Cutoff [MWCO]) cellulose ester dialysis membranes that were sealed using dialysis clips. The sealed membranes were placed in aluminum foil pouches containing 10 gm of Spectra/Gel absorbent powder to dewater membrane contents overnight (Note: all dialysis equipment and dewatering absorbents obtained from Spectrum Laboratories, Laguna Hills, CA). After overnight dewatering/volume reduction the contents were transferred into 500 Dalton MWCO cellulose ester Spectra/Por Dispodialyzers. The dispodialyzers were suspended in a DDI water reservoir for approximately 12 h. During this step the reservoir water was replaced with freshwater at least twice. The dispodialyzer contents were dewatered as before using the absorbent powder; however, this time they were left in the absorbent bed for only 6−8 h owing to their substantially higher surface area-to-volume ratio. The contents of the dewatered dispodialyzers were removed using a thin disposable pipet and transferred into preweighed aluminum weighing dishes. The weighing dishes were placed into a 60 °C oven for 6 h, the time used to reach a constant weight using this procedure. After 6 h the dishes were removed from the oven and allowed to cool in a desiccator overnight. The cooled dishes were weighed to calculate the weight of their contents (i.e., derivatized humic acid). The derivatized humic acid was ground using a pestle and mortar, transferred to a labeled vial, and stored in a desiccator.

Molecular Modeling. Chemical structures for nitrosobenzene, 4-nitroso-2,6-dinitrotoluene (4NDNT, potential oxidation product of 4HADNT), and 2-nitroso-4-hydroxylamino-6-nitrotoluene (2N4HA6NT, potential oxidation product of 2,4-dihydroxylamino-6-nitrotoluene) were created in CAChe software (Oxford Molecular/Pharmacopeia, Hunt Valley, MD) that utilizes semiempirical quantum mechanics routines to determine structural conformations with the lowest heat of formation. Eventually, two parameters were evaluated to assess the reactivity of the nitroso-nitrogen. These were the partial charge on the nitroso-nitrogen and the electrostatic potential averaged over a constant electron density molecular surface of the structure (this parameter correlates with a "susceptibilty to a nucleophilic attack" in CAChe (*52*, *53*)).

TABLE 1. ^{14}C Radiolabel Distribution and Protein Concentrations for Various Fractions

description	initial ^{14}C-dpm (% of total)	post-treatment ^{14}C-dpm (% of total initial)	initial protein concn (mg/mL)	post-treatment protein concn (mg/mL)
biooxidizer		8.1		0.34[a]
sampling losses		12.2		
filter reject		22.4		0.03
filtrate		53.7		0.07
total	100	96.2	0.18	

[a] Refers to a pH 2 hydrolysate.

Values for both these parameters were generated with MOPAC/PM3 wave function for each structure at a geometry determined by performing a preoptimization calculation in an empirical molecular mechanics force field model (an augmented version of Allinger at al.'s MM3 model (*54*) incorporated in CAChe), followed by an optimized geometry calculation in MOPAC using PM3 parameters (*55*).

Results and Discussion

Fate of TNT Following Anaerobic/Aerobic Treatment. The anaerobic phase was terminated when HPLC analysis showed that the initial TNT was in the form of DHA6NT and aminophenols, products that have been reported previously (*2*). During the aerobic phase, creamy white strands formed that slowly turned into a brown floc-like precipitate. Unlike behavior expected from azoxy compounds, no dissolution of the precipitate was observed in either *n*-pentane or methylene chloride. However, the precipitate appeared to partially dissolve in acidic (pH = 2) solution, turning the solution to a cloudy color. No recognizable products (with the exception of the polar front) were observed on the reverse phase (RP) HPLC chromatogram following the aerobic stage. The final pH of the reaction mixture was estimated to be approximately 5.5.

An appreciable drop in the bulk aqueous ^{14}C concentration was not observed over the entire course of the anaerobic/aerobic treatment. Upon completion of the experiment an overall mass balance of 96.2% was obtained on the basis of the total initial ^{14}C count (Table 1). At the end of the anaerobic/aerobic treatment the remaining ^{14}C in the system was distributed as follows: 9.6% precipitate (biooxidized fraction), 26.6% aqueous filter reject, and 63.8% aqueous filtrate. The protein assay established that the bulk of the protein mass was in the form of—or associated with—the precipitate. Binding of the Coomassie blue G-250 dye to protein becomes quite inefficient in acidic solutions resulting in a subsequent loss of sensitivity of the assay (*44*). Still, a high value of 0.34 mg/mL was obtained for the pH 2 hydrolysate of the precipitate (Table 1).

The precipitate contained approximately 10% of the remaining ^{14}C after the experiment. The bulk of the ^{14}C was in the aqueous phase, perhaps associated with the soluble protein fraction (Table 1). It must be noted, however, that the ultracentrifugaton step during the preparation of the cell extract excluded most of the larger structural proteins that could provide much higher levels of material for the binding and precipitation of ^{14}C. An alternative explanation for the absence of RP-HPLC/UV−Vis analyzed products without a corresponding loss in ^{14}C is the possible loss of aromatic character of the products formed upon aeration. This phenomena appears to be unlikely because the reaction mixture contained DHA6NT in addition to the aminophenols; although unstable under aerobic conditions, DHA6NT has never been reported to undergo ring fission upon exposure to air. Regardless of the explanation for this unusual phenomenon, a significant portion of the radiolabel was found to be associated with insoluble proteins.

Reaction of Anaerobically Biotransformed TNT with a Model Thiol. The second TNT transformation reaction yielded predominantly DHA6NT (retention time of 4.1 min) and trace levels of 4HADNT (retention time of 8.1 min). The chromatogram and its percent ^{14}C distribution obtained from fraction collection are presented in parts (a) and (b), respectively, of Figure 3. As expected, the radiolabel was concentrated in the peaks of the reduced metabolites of TNT. The addition of approximately 1.2×10^{-3} mmole of 1-thioglycerol followed by 20 min of anaerobic incubation produced no significant change in the percent ^{14}C distribution of the chromatogram (Figure 3c,d). The molar ratio of thiols to potential nitroso functionalities was deliberately kept below 1:1 because the presence of excess thiol reduces the nitroso functionalities back to hydroxylamino (*56*). This sets up an undesirable situation where both nitroso and hydroxylamino functional groups are present in the system and a rapid condensation of azoxy products ensues (especially at alkaline to mildly acidic pHs). Following 20 min of aerobic incubation, a new more polar peak (retention time of 3.3 min) was identified (Figure 3(e)) that had a virtually identical UV spectrum as DHA6NT. A corresponding shift in the percent ^{14}C distribution of the chromatogram to the new peak was also observed (Figure 3(f)). The reaction profile matches that of the 1-thioglycerol/nitrosobenzene reaction reported in the literature (*35*, *56*). This reaction generates an acid labile and more polar sulfinamide as the dominant product. No new products having spectra similar to 4HADNT were identified. This could be the result of the inability of 1-thioglycerol to act as a competing nucleophile for the strongly ring deactivated 4HADNT molecule (A more detailed discussion of this phenomenon is presented later in the molecular modeling subsection.). Upon aerobic incubation, the peak area for 4HADNT decreased, and only 57% of the ^{14}C associated with this peak was conserved (as compared to 98% percent conservation of ^{14}C between chromatographic retention times of 2 and 6 min), indicating that perhaps the remaining nucleophilic hydroxylamino groups were outcompeting the thiol nucleophile to produce azoxy compounds. In conclusion, the model reaction demonstrated the feasibility of the nitroso-thiol reaction for nitrosoarenes produced from the oxidation of DHA6NT; however, a similar result was not obtained for 4HADNT.

The formation of a new product by the reaction of oxidized DHA6NT with 1-thioglycerol indicates that thiols do act as competing nucleophiles for partially reduced TNT metabolites in sequential anaerobic/aerobic biotransformation systems. Such reactions can allow the thiol-containing cysteine amino acid residues of proteins to become potential sinks for the parent nitroaromatic contamination. In addition, if the protein undergoing binding is involved as an enzyme in the reduction pathway, then binding can pose an inhibition threat for further transformation. In any case, the evaluation of the thiol concentration at any given time in the system may be of importance in determining the fate of the nitroaromatic contamination. This could be especially true for TNT bioremediation processes where the biomass, and subsequently the protein concentrations, are considerably higher than the low concentrations utilized in this study. Furthermore, the pH of such anaerobic processes is typically quite acidic and consequently does not favor azoxy formation. Such conditions can allow other nucleophiles such as thiols to react with any nitrosoarene metabolites in the system. Finally, in light of the findings of this study, it becomes critical to determine the nature of the covalent bond(s) formed from sequential anaerobic-aerobic treatment before such binding can be proposed as a means of immobilizing nitroaromatic contamination (*10*, *40*). As Table 2 demonstrates with the

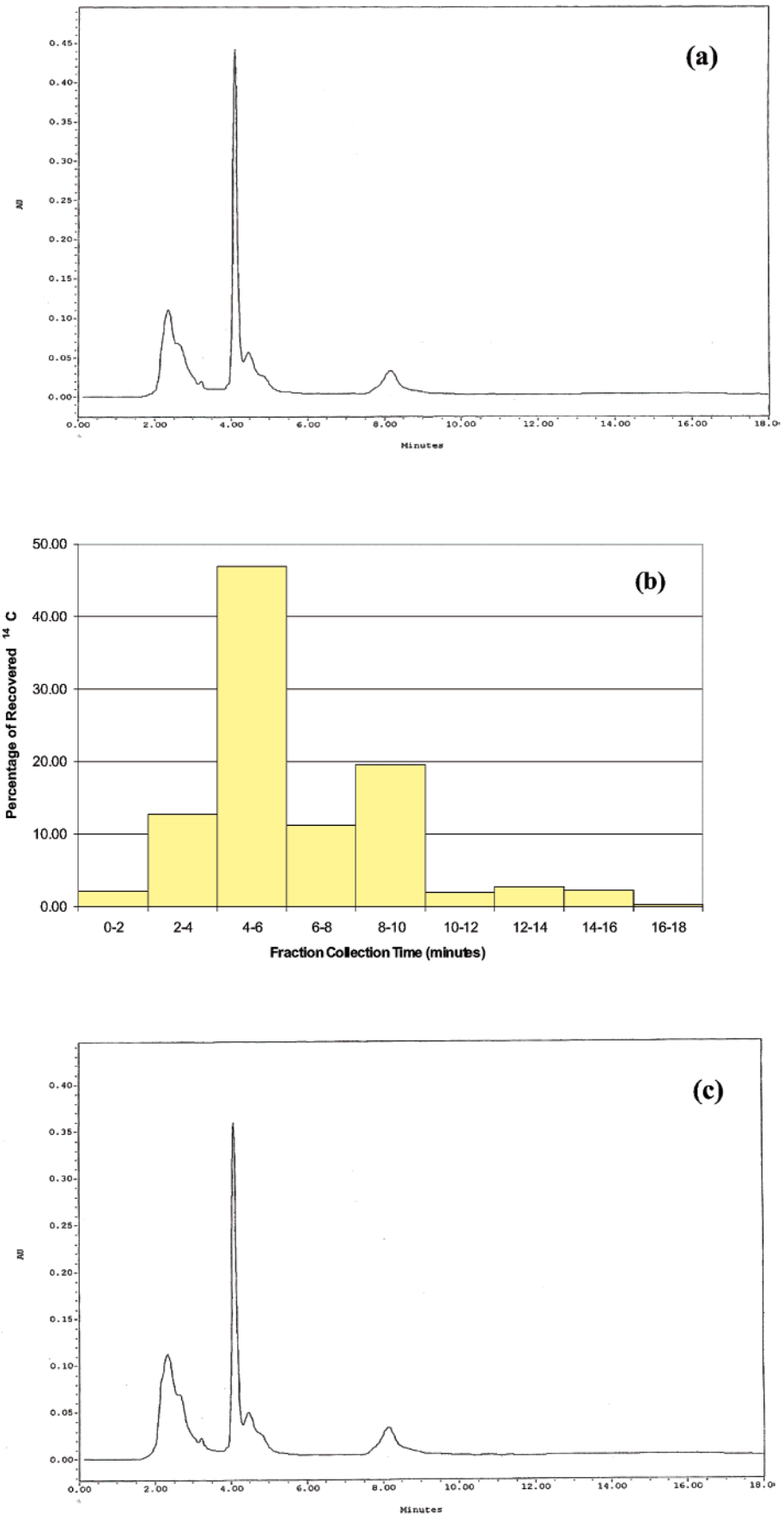

FIGURE 3. Parts (a) and (b) show the chromatogram and ^{14}C fraction distribution, respectively, under anaerobic conditions prior to thiol addition. Parts (c) and (d) show the chromatogram and ^{14}C fraction distribution, respectively, under anaerobic conditions after thiol addition

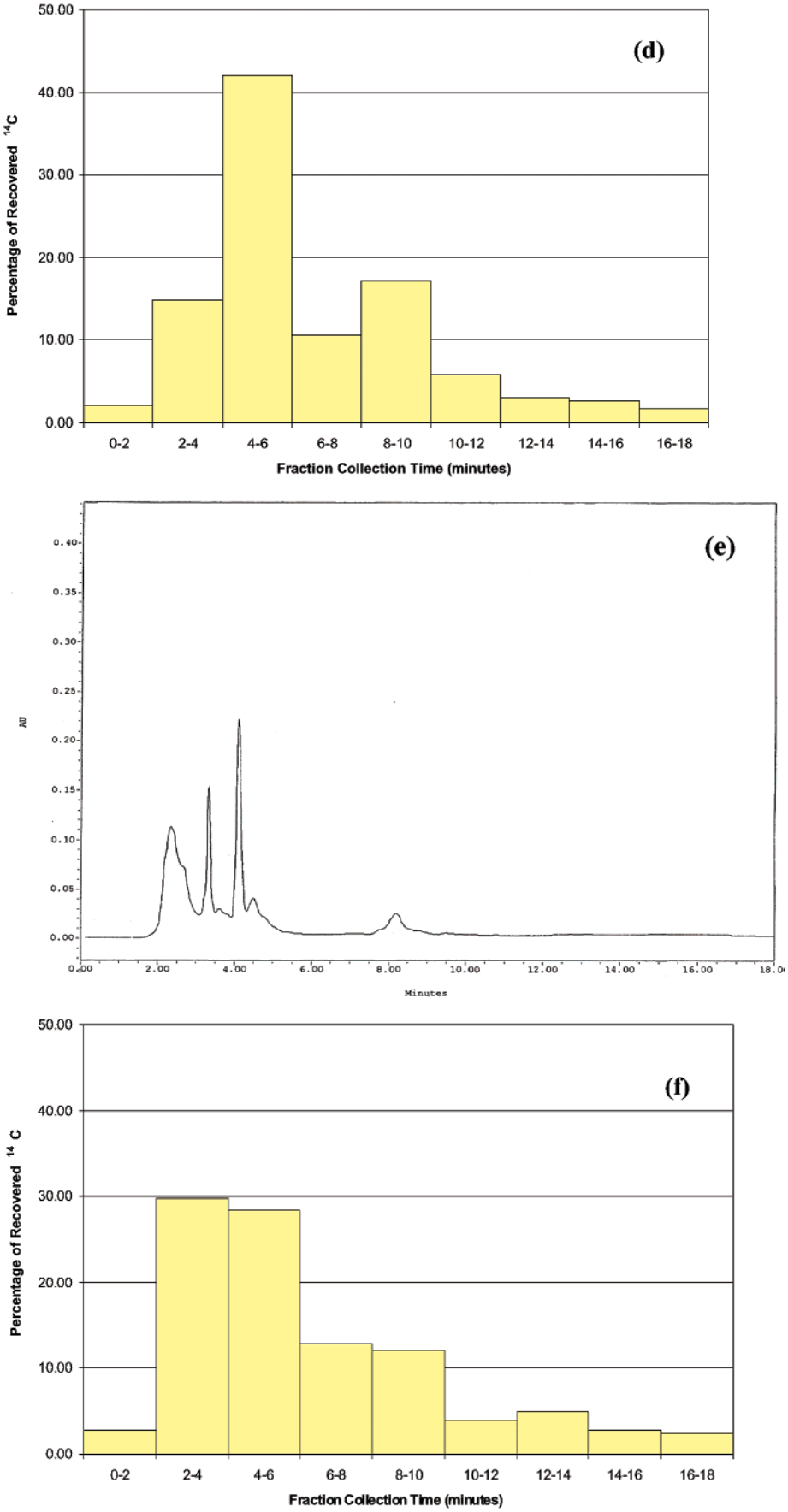

FIGURE 3. (Continued) (20 min anaerobic incubation time). Parts (e) and (f) show the chromatogram and ^{14}C fraction distribution, respectively, under anaerobic conditions after thiol addition (20 min aerobic incubation time).

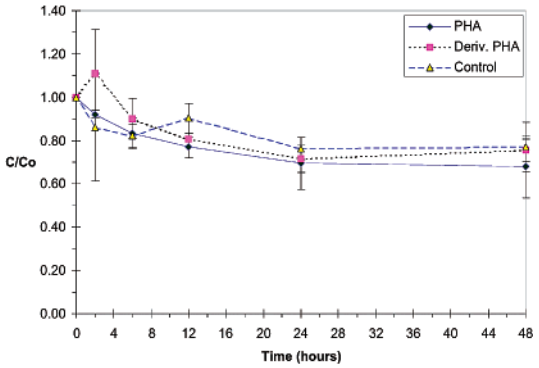

FIGURE 4. Reaction of 4HADNT with nonderivatized (PHA) and thiol-derivatized (deriv. PHA) IHSS peat humic acid standard under anaerobic conditions. The plot also shows data for an anaerobic control of 4HADNT in the absence of any humic acid.

TABLE 2. Bond Dissociation Energies at 298 K (*59*)

molecule	dissociation energy (kJ/mol)	molecule	dissociation energy (kJ/mol)
C−H	338	N−O	631
C−N	770	N−S	464

bond dissociation energy values of diatomic molecules, the strength of a N−S bond is only 60% the strength of a C−N bond.

Reactivity of 4HADNT with IHSS Peat Humic Acid. The anaerobic experimental series run to test the reactivity of 4HADNT toward IHSS peat humic acid at a sorbate/sorbent mass ratio of 0.02 yielded an approximately 30% loss in concentration over a 48-h duration (Figure 4). However, the difference between this removal and the removal observed in nonsorbent-containing controls was less than 6%. Loss of 4HADNT in the humic acid-free anaerobic control series was approximately 24%. The experiment was repeated and produced similar results (data not shown). Anaerobic reactivity of 4HADNT was also tested with nucleophile-derivatized IHSS peat humic acid in anticipation of the reactivity between 4HADNT and peat humic acid. This series also demonstrated virtually no difference between experimental systems and controls.

The lack of reactivity of the model arylhydroxylamino compounds toward humic acid can be explained in terms of its bioorganic chemistry in natural systems. The arylhydroxylamino nitrogen has reasonable nucleophilic characteristics. In all its observed reactions (with the exception of azoxy formation) it behaves as an extremely strong electrophile by first forming a nitrenium ion intermediate. However, arylhydroxylamines can form the electrophilic nitrenium ion only when assisted by catalytic conditions to overcome the kinetic barrier for the N−O bond cleavage. Therefore, in the absence of catalysis, either acidic or biological, it is unlikely that arylhydroxylamines will react with the multitude of potential nucleophiles present in the humic acid.

Interestingly, our results of no significant removal of 4HADNT with humic acids are in direct contrast to findings reported by others (*25*). Achtnich et al. reported a complete removal 0.04 mM 4HADNT (8.5 mg/L) in less than 2 h when humic acid was present at a concentration of 7500 mg/L. It is quite possible that the difference in outcomes for the two experiments could be attributed to a significantly difference in experimental design. The most obvious of these differences was the humic acid concentration; our experiments used a 625 mg/L concentration of IHSS peat humic acid that was approximately 56% carbon by mass (See Table A, Supporting Information, for the detailed characteristics of the IHSS humic acids used in this study.). This value translates into an effective carbon mass concentration of 350 mg/L, a value that is seven times the maximum DOM concentration typically observed in nature (*47*). Similarly, the humic acid concentration used by Achtnich et al. translates into a DOM concentration of 3750 mg carbon/L if a conservative carbon content of 50% by mass is assumed for their humic acid. This value is 75 times the maximum concentration of DOM in nature and probably exceeded the humic acid's aqueous solubility. At our relatively low humic acid concentrations of 625 mg/L we observed an average drop in pH of 0.4 units over the course of the 48-h experiment duration even though the experiments were conducted in pH 7, 10 mM phosphate buffer. Not having any data on their humic acid's carboxylic and phenolic acidity components, we can only speculate that the drop of pH in their pH 7.3, 50 mM buffered systems was most likely greater. A significant drop in pH can produce conditions for the acid-catalyzed cleavage of the arylhydroxylamine's N−O bond. Although the concentration of the nitrenium ions produced may not be significant when compared to the total concentration of arylhydroxylamine available, the presence of strong nucleophiles in the humic acid could lead to the depletion of arylhydroxylamines from the system.

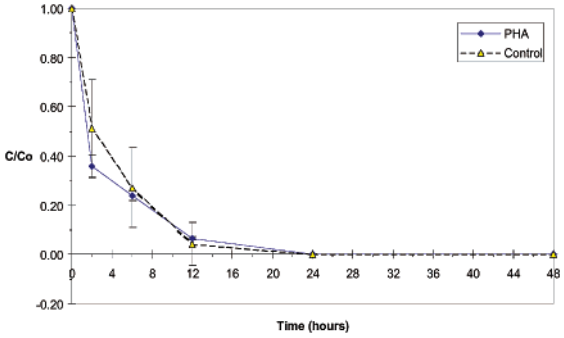

FIGURE 5. Aerobic reaction of 4HADNT with IHSS peat humic acid standard (PHA). The plot also shows data for a control reaction of 4HADNT performed under the same conditions but without any humic acid.

The second major difference between the two 4HADNT experimental systems may have been anaerobic "integrity". Our controls showed a 24% loss in concentration over 48 h, whereas Achtnich et al.'s control showed a 75% concentration loss in less than 20 h. The presence of trace levels of oxygen in the system could promote oxidation to the electrophilic nitroso functionalities that can subsequently be trapped by the nucleophilic nitrogen of the remaining arylhydroxylamines in solution to generate azoxy compounds.

In a second experiment with 4HADNT, the effects of converting the system from anaerobic to aerobic conditions were analyzed in the presence of IHSS peat humic acid. The purpose of this experiment was to determine whether the nucleophiles in the humic acid could compete with the arylhydroxylamines for the relatively electrophilic nitroso compounds produced upon exposure of 4HADNT to air (Figure 2, reaction b followed by reactions c and d in parallel). No appreciable difference in the rates of removal of 4HADNT from the aqueous phase could be observed between the humic acid series and the control series (Figure 5). Both series resulted in the formation of precipitate that was identified as predominantly 4,4′,6,6′-tetranitro-2,2′-azoxytoluene. The 4HADNT depletion/azoxy formation followed pseudo-first-order kinetics with a rate constant of 0.21/h. From the results it was evident that the nucleophiles present in humic acid were unable to compete for the nitroso intermediates resulting from the oxidation of 4HADNT. Earlier in the reaction of TNT metabolites with the model thiol, 1-thioglyc-

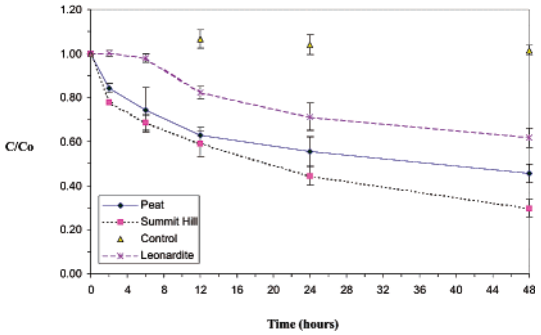

FIGURE 6. Data for the reaction of nitrosobenzene with nonderivatized IHSS peat and leonardite humic acid standards and IHSS Summit Hill reference humic acid in a closed aerobic system. The plot also shows data for an aerobic closed system control of nitrosobenzene in the absence of any humic acid.

erol, we demonstrated that thiols reacted more readily with the more ring destabilized (or electron rich) 2,4-dihydroxylamino-6-nitrotoluene (DHA6NT) than they did with the more strongly ring deactivated 4HADNT. It is very likely that the cause for this phenomenon is the aromatic substituent effects of the nitroso compound formed.

Reactivity of Nitrosobenzene with Various IHSS Humic Acids. Three IHSS humic acids were picked for their widely varying amino acid/proteinaceous content (based on the available analytical data for 13 amino acids) as well as their relatively limited range of sulfur content. (Total elemental sulfur content was 0.64, 0.71, and 0.76% by mass for Summit Hill, Peat, and Leonardite IHSS Humic Acids, respectively; see Table A, Supporting Information.) The organic sulfur content of the humic acids (based on the available information on the concentration of the sulfur-containing amino acid, methionine) correlated well with the protein content of the humic acids used. Nitrosobenzene was exposed to the three different humic acids, and the trends in reactivity were compared to nonsorbent containing controls. All three humic acids displayed the ability to remove nitrosobenzene from the buffer solution (Figure 6). Moreover, the extent of removal achieved over the 48-h period appeared at least in part to be dependent on the proteinaceous content of the humic acid. Going from highest to lowest protein content, Summit Hill, peat, and leonardite humic acids (1420, 373, and 11 nmole total amino acid/mg humic acid) showed nitrosobenzene removals of 70%, 50%, and 35%, respectively.

The results for the removal of nitrosobenzene with leonardite was somewhat unusual because it displayed an initial slow removal followed by a rapid increase in removal after the 6-h sampling mark. This trend in the leonardite data may be explained by the poor initial solubility of the leonardite humic acid observed in the initial phase of the experiment.

Biphasic kinetic analyses were performed on the peat and Summit Hill data using a "curve peeling" procedure (*45*). Different pseudo-first-order rate constants for the later slow removal (0.019, 0.009, and 0.008/h for Summit Hill, Peat, and Leonardite, respectively) and the initial fast removal (0.05 and 0.04 for Summit Hill and Peat, respectively) of aqueous nitrosobenzene were obtained using this procedure. The slower binding reaction showed reasonable least-squares fits with correlation coefficients generally over 0.96. The data in the faster phase, adjusted to remove any contributions from the slower phase, did not demonstrate as good fit on this first-order reaction analysis in large part due to the limited number of data points available for this phase of the reaction.

Reactivity of Nitrosobenzene with Thiol Derivatized IHSS Humic Acids. IHSS peat and Summit Hill humic acids were selected for derivatization. Both of these sorbents

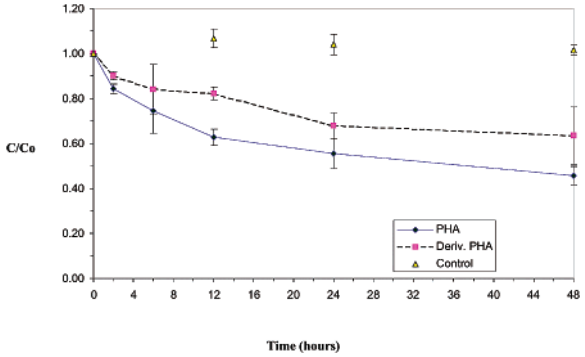

FIGURE 7. Effect of thiol-derivatization on IHSS peat humic acid's (PHAs) capacity to remove nitrosobenzene from the aqueous phase. Results for nonderivatized (PHA) and derivatized (deriv. PHA) peat humic acid as well as a nonhumic-acid-containing control of aqueous nitrosobenzene are shown.

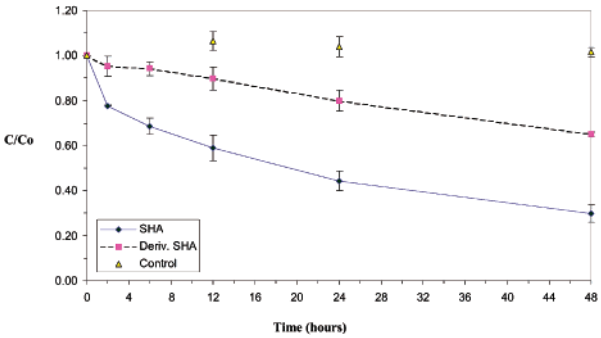

FIGURE 8. Effect of thiol-derivatization on IHSS Summit Hill humic acid's capacity to remove nitrosobenzene from the aqueous phase. Results for nonderivatized (SHA) and derivatized (deriv. SHA) Summit Hill humic acid as well as a nonhumic-acid-containing control of aqueous nitrosobenzene are shown.

showed a greater capacity for the removal of aqueous nitrosobenzene than the IHSS leonardite humic acid (Figure 6). N-Dansylaziridine was used as the thiol derivatizing agent. N-Dansylaziridine reacts only with strong nucleophiles such as thiols by forming thioether linkages. Unlike other thiol derivatizing compounds (e.g., N-substituted maleimides), N-dansylaziridine does not react with functionalities having weaker nucleophilic properties such as phenols, alcohols, and amines (*50*, *51*). Figures 7 and 8 illustrate the effects of the thiol derivatization on aqueous nitrosobenzene removal by IHSS peat and Summit Hill humic acids, respectively. Pretreatment of the two humic acids showed an obvious difference in the effectiveness of nitrosobenzene removal, with the change being more prominent for the Summit Hill humic acid. Incidentally, the Summit Hill humic acid had approximately four times the estimated amino acid concentration than the peat humic acid. (See Table A, Supporting Information.) However, since the cysteine concentrations were not available a value for the thiol concentration in each system could not be accurately determined. The thiol concentration values were inferred from the mass fraction of total sulfur, organic sulfur (i.e., methionine), and the protein content (i.e., sum of the concentrations of the 13 amino acids that were analyzed by the IHSS) of the humic acids. Based on the estimated total amino acid concentration of the Summit Hill humic acid alone, it appeared that a more drastic effect on nitrosobenzene removal was observed than could be explained by only the blocking of thiols. This observation could be explained by the possibility of reactions involving N-dansylaziridine and strong nucleophiles in the humic acids (other than thiols) that might have also bound the electrophilic nitrosobenzene.

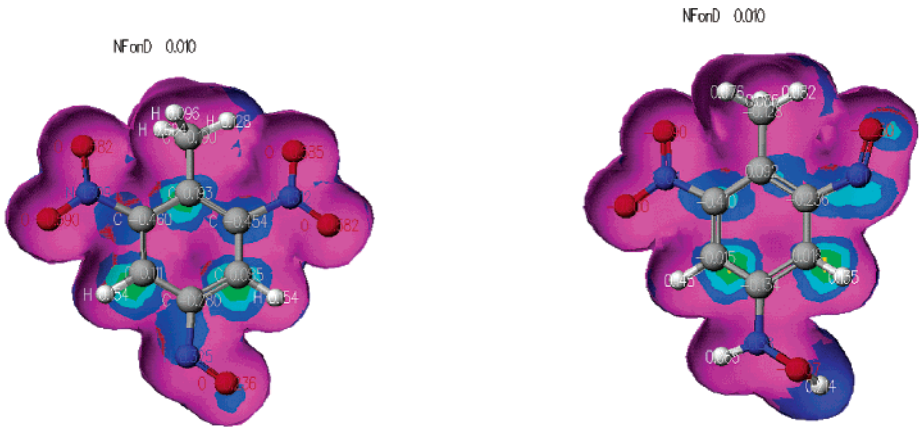

FIGURE 9. (a) Gas-phase molecular model of 4-nitroso-2,6-dinitrotoluene (4NDNT) in its lowest energy conformation, surrounded by its electron density isosurface. The colors of the surface depict the molecules susceptibility to nucleophilic attack (yellow > green > light blue > dark blue > pink). The red, blue, gray, and white atom colors represent oxygen, nitrogen, carbon, and hydrogen, respectively. (b) Molecular model of 2-nitroso-4-hydroxylamino-6-nitrotoluene (2N4HA6NT) in its lowest energy conformation, surrounded by its electron density isosurface.

Comparison of the Reactivity of Potential Nitroso Intermediates of TNT with Nitrosobenzene Using Molecular Modeling. Molecular models were created for nitroso intermediates that can potentially form from the oxidation of arylhydroxylamine metabolites of TNT (i.e., from the oxidation of 4HADNT and DHA6NT). The molecular modeling was performed to compare the two molecules' electrophilic properties so that patterns for their reactions with strong nucleophiles (e.g., thiol) could be predicted. Pictorial representations of the molecular models for 4NDNT (potential oxidation product of 4HADNT) and 2N4HA6NT (potential oxidation product of DHA6NT) in their lowest energy conformations are presented in parts (a) and (b), respectively, of Figure 9. The figures also show the color-coded depiction of the surface electrostatic potential on the electron density isosurface for the three molecules.

The partial charges on the nitroso-nitrogen (nitroso-N) of 4NDNT and 2N4HA6NT obtained from the lowest energy optimization were 0.325 and 0.320, respectively. These values showed limited utility in predicting the electrophilic nature of the three compounds because of their narrow range. On the other hand, the calculated surface electrostatic potentials as depicted in Figure 9 produced more interesting results. This parameter has been shown in the literature to be an effective parameter for the prediction of nucleophilic/electrophilic processes (*52*, *53*). Thus, the surface maps in Figure 9 show the locations where the molecules are most susceptible to a nucleophilic attack (yellow > green > light blue > dark blue > pink). Solvation effects were not accounted for in the molecular models; however, such effects in a polar solvent like water are expected to enhance the electrophilic nature of the nitroso-N of these molecules (*57*).

Comparing the electrostatic surface potentials above the nitroso-Ns it becomes clear that the nitroso group in 4NDNT (Figure 9(a)) has a very different electrophilic character than the one in 2N4HA6NT (Figure 9(b)). The nitroso group in 2N4HA6NT is a stronger electrophile than the one present in 4NDNT. The difference in electrophilic character can be explained by the aromatic substituent effects of the two molecules. The 4NDNT molecule has two very strong ring deactivating substituents in the form of nitro groups. The effect of these electron-withdrawing nitro groups is also visible in the electrostatic surface potentials above its C3 and C5 ring positions, which show a strong electron deficiency. Conversely, the surface plot of the more reduced 2N4HA6NT molecule shows that the replacement of the nitro group at the C4 position with a less electron withdrawing hydroxylamino group allows the nitroso nitrogen at the C2 position to display a much stronger electrophilic character than the less reduced 4NDNT molecule. Therefore, it would be expected that the more reduced hydroxylamino intermediates of TNT are favored to react via the nitroso-thiol reaction upon exposure of the reaction mixture to air. In addition, when more electron donating substituents are present on the ring, the increased electrophilic characteristic of the nitroso nitrogen is accompanied by a concomitant decrease in nucleophilic characteristics of the hydroxylamino nitrogen. This phenomenon may allow stronger nucleophiles such as thiols to outcompete arylhydroxylamines for the more reduced nitrosoarene intermediates, allowing reactions alternative to the azoxy reaction to occur.

Collectively, the studies presented herein demonstrate reactions that contribute to the binding of TNT reduction products to both biomass and natural organic matter. In both cases, the reactions center around the nitroso group formed directly through reduction reactions or indirectly through oxidation of amino or hydroxylamino forms with the reduced organic sulfur contained in either class of macromolecules. These findings help to explain the extent of binding of TNT that is often observed and can not be explained by reactions involving amino groups in organic matrices.

Acknowledgments

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Supporting Information Available

Chemical characteristics of IHSS peat and leanoardite humic acid standards and IHSS Summit Hill reference humic acid (Table A) and products resulting from the N−O bond cleavage of the semimercaptal intermediate formed by the nitroso-thiol reaction (Figure A). This material is available free of charge via the Internet at http://pubs.acs.org.

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