Environ. Sci. Technol. **1999**, *33,* 3354−3361

Characterization of Oxidation Products of TNT Metabolism in Aquatic Phytoremediation Systems of *Myriophyllum aquaticum*

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TNT transformation processes in sediment-free, "natural", aquatic phytoremediation systems of *Myriophyllum aquaticum* were investigated with specific interest in oxidation products. Extraction procedures combining liquid−liquid extractions and solid-phase extractions were developed for the isolation of the mostly acidic, oxidized TNT metabolites. Six compounds unique from the reduction products of TNT were isolated and characterized by UV−vis, ^{1}H, and ^{13}C NMR spectroscopy, by mass spectroscopy, and by chemical synthesis where feasible. These compounds include 2-amino-4,6-dinitrobenzoic acid, 2,4-dinitro-6-hydroxy-benzyl alcohol, 2-*N*-acetoxyamino-4,6-dinitrobenzaldehyde, 2,4-dinitro-6-hydroxytoluene, and two binuclear metabolites unique from the customary azoxytetranitrotoluenes. The monoaryl compounds show clear evidence of oxidative transformations, methyl oxidation and/or aromatic hydroxylation. It is possible that oxidative transformation(s) preceded nitro reduction since studies on exposure of *M. aquaticum* to either 2-amino-4,6-dinitrotoluene or 4-amino-2,6-dinitrotoluene did not yield any of the oxidation products identified here. The accumulation of oxidation products was significant: 2-amino-4,6-dinitrobenzoic acid, 4.4%; 2,4-dinitro-6-hydroxy-benzyl alcohol, 8.1%; 2-*N*-acetoxyamino-4,6-dinitrobenzaldehyde, 7.8%; and, 2,4-dinitro-6-hydroxytoluene, 15.6%. The binuclear metabolites accounted for an estimated 5.6%. This study is the first direct evidence for oxidative transformations in aquatic phytoremediation systems.

Introduction

The cleanup of contaminated soil and water at facilities involved in the manufacturing, handling, packaging, and testing of explosives continues to be a serious challenge due to the extent and recalcitrance of explosives in the environment (*1*). The primary culprit is the nitroaromatic compound 2,4,6-trinitrotoluene (TNT) and related synthesis byproducts. Lately, phytoremediation or plant-driven bioremediation has been viewed as a viable, low-cost option for the amelioration of TNT-contaminated media (*2*). In light of such interest, elucidating the metabolic fate of TNT in plants is relevant to determining environmentally acceptable end point(s) of such processes (*3*). Investigations into phytoremediation of TNT include a diverse array of plant systems: yellow nutsedge (*4*), bush beans (*5*), aquatic and wetland species (*6*−*9*), axenic root cultures (*6*, *10*), garden vegetables (*11*), hybrid poplar (*12*), and other terrestrial species (*13*). In all of these studies, the activity of the reductive pathway has been reported, customarily leading to the formation of 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene. Further, the formation of conjugates of 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene has been recently elucidated as a significant process of plant-catalyzed metabolic fate of TNT beyond early reduction products (*10*).

In contrast, the activity of oxidative pathway(s) of TNT metabolism in plants has remained largely uninvestigated. This could include transformation of the ring-substituted methyl group and the hydroxylation of the aromatic ring. Oxidative metabolism of herbicides is particularly well-documented as an initial reaction leading to activation or deactivation of the parent herbicide (*14*, *15*). A majority of these reactions in plants are dependent on mixed-function oxygenases (mfo) (*16*, *17*). Evidence of such transformations during plant metabolism of TNT could facilitate the elucidation of additional routes to conjugate formation and sequestration of xenobiotic explosives in plants and a greater understanding of metabolic and toxicology profiles during phytoremediation operations. The studies presented here were directed at the objective of isolating and characterizing products of oxidative metabolism of TNT in plants. This was accomplished by examining the product profile in the extracellular sediment-free, aqueous matrix of the aquatic plant *Myriophyllum aquaticum* exposed to TNT. *Myriophyllum* is capable of metabolizing TNT in "natural" systems (*6*, *9*), and the present analysis provides evidence for oxidative metabolism of TNT in its presence.

Materials and Methods

Chemicals and Materials. 2,4,6 Trinitrotoluene (TNT), 4-amino-2,6-dinitrotoluene, and 2-amino-4,6-dinitrotoluene from Chemservice (Westchester, PA) were used for amendment of *M. aquaticum* experimental systems. Reference standards employed for analytical quantification and/or product identification include the following: TNT, 2-amino-4,6-dinitrotoluene, and 4-amino-2,6-dinitrotoluene from Chemservice; 2,4-diamino-6-nitrotoluene, 2,6-diamino-4-nitrotoluene, 4-hydroxylamino-2,6-dinitrotoluene, 2-hydroxylamino-4,6-dinitrotoluene, 2,2′,6,6′-tetranitro-4,4′-azoxytoluene (4,4′-azoxy), 4,4′,6,6′-tetranitro-2,2′-azoxytoluene (2,2′-azoxy), and 2-amino-4,6-dinitrobenzoic acid gifted by Dr. R. J. Spanggord (SRI International, Menlo Park, CA); 2,4-dihydroxylamino-6-nitrotoluene by catalytic biosynthesis according to Hughes et al. (*18*); 2,4,6-triaminotoluene from Sigma-Aldrich (St. Louis, MO); and plant metabolites of TNT, namely, the 2-amino-4,6-dinitrotoluene-derived conjugates TNT-5.5 and 2A-5.6 and the 4-amino-2,6-dinitrotoluene-derived conjugates TNT-6.7 and 4A-6.6 purified from nitroaromatic-amended *C. roseus* axenic roots as in Bhadra et al. (*10*). For product analysis, [ring-U-^{14}C]-2,4,6-trinitrotoluene (Chemsyn, Lenexa, KS), with specific activity of 21.58 mCi/mmol and purity of 98%, was used. Solvents for NMR analysis, CD_3OD (99.8 atom %) and CD_3CN (99.8 atom %),

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10.1021/es990436i CCC: $18.00
Published on Web 08/13/1999

were obtained from Aldrich (Milwaukee, WI). HPLC grade 2-propanol, acetonitrile, methanol, hexane, and ethyl acetate supplied by EM Science (Gibbstown, NJ) were used for liquid−liquid extraction (LLE), solid-phase extraction (SPE), and HPLC. Supelclean LC-8 from Supelco (Bellefonte, PA) and silica were employed for solid-phase extraction. β-Glucuronidase from *Helix pomatia* (EC 3.2.1.31) and β-glucosidase from almonds (EC 3.2.1.21) supplied by Sigma Chemicals (St. Louis, MO) were used for enzymatic hydrolysis of purified analytes. Stock solutions were prepared in HPLC-grade methanol: TNT, 25 mg/mL, and ^{14}C-TNT, 3130 dpm/μL.

Plants. *M. aquaticum* (parrot feather) plants were maintained outdoors, rooted in a bottom layer of compost and gravel, in 20-gal containers that were recharged periodically with water. Prior to the start of each study, plants were removed from outdoor storage and acclimated to indoor conditions for 24 h.

Analytical Methods. C_8 reverse-phase HPLC with PDA detection as described in Hughes et al. (*6*) was employed for the quantification of the following: TNT; and the reduction derivatives, 2-amino-4,6-dinitrotoluene, 4-amino-2,6-dinitrotoluene, 4-hydroxylamino-2,6-dinitrotoluene, 2-hydroxylamino-4,6-dinitrotoluene, 2,4-diamino-6-nitrotoluene, 2,6-diamino-4-nitrotoluene, and 2,4-dihydroxylamino-6-nitrotoluene. A simple modification of the same method was employed for the HPLC analysis of oxidized TNT metabolites isolated in this study, ion-suppression HPLC on C_8 stationary phase using a mobile phase of 82/18 (% v/v) 10 mM H_3PO_4/2-propanol. The azoxy-tetranitrotoluenes, 4,4′-azoxy and 2,2′-azoxy, were analyzed by C_{18} reverse-phase HPLC with PDA detection as described in Bhadra et al. (*10*). The TNT conjugate metabolites, TNT-5.5 and 2A-5.6, and TNT-6.7 and 4A-6.6 were analyzed by C_{18} reverse-phase HPLC with PDA detection as described in Bhadra et al. (*10*). Peak identification in all cases was based on comparisons to retention times and UV-vis spectra of authentic standards as well as spiking with authentic standards as necessary.

^{1}H NMR spectra were acquired on a Bruker AC-250 MHz spectrometer relative to either CD_3OD (^{1}H: δ, 3.31 and 4.87 ppm) or CD_3CN (^{1}H: δ, 1.94 ppm). ^{13}C NMR spectra were acquired with the same spectrometer relative to CD_3OD (^{13}C: δ, 49.15) or CD_3CN (^{13}C: δ, 118.69 and 1.39). Chemical shifts are reported in ppm and coupling constants in hertz (Hz). Mass spectroscopy was performed by direct probe chemical ionization mass spectroscopy (MS-CI) on a Finnigan MAT 95 spectrometer with CH_4 as the ionizing gas. Radioactivity was detected and quantified using a Beckman LS 6500 scintillation counter as described in Hughes et al. (*6*).

Extraction and Separation of Transformation Products. Intracellular unbound metabolites of nitroaromatic transformation were determined as described in Bhadra et al. (*10*), starting with cold extraction of freeze-dried biomass aliquots with methanol. Extracellular products of TNT transformation by *M. aquaticum* were extracted from the residual aqueous milieu of exposed plants in the large-scale aquarium study (described below in Materials and Methods). The aqueous mixture was extracted by LLE (liquid−liquid extraction) in 1-L batches with equal volumes of ethyl acetate (HPLC-grade) following an initial pH adjustment to ~2 by the addition of H_3PO_4 (1 *M*). LLEs were conducted in 2-L separatory funnels; for each batch, the aqueous phase was discarded and the organic phase collected. After the entire aqueous solution was extracted in this manner, the organic extracts were pooled and concentrated (without heating) to 100 mL under strong vacuum (10^{-6} mmHg) using a rotary evaporator (Buchler model RE-121). This initial LLE stage enabled the preferential separation of neutral analytes (including TNT and reduction derivatives) and acidic TNT metabolites from the aqueous milieu. The concentrated crude extract was stored at 4 °C, and in subsequent separation stages, aliquots treated to further LLE followed by solid-phase extraction (SPE) as described below.

In the next stage, approximately half the volume of the crude extract was diluted to 500 mL by adding ethyl acetate, and extracted thrice with 350-mL batches of 1% (w/v) aqueous $NaHCO_3$. The objective of this LLE stage was to separate acidic metabolites from neutral analytes (TNT and its customary monoaminodinitrotoluene derivatives) that remained in the organic phase. The aqueous phase from the extractions was pooled, its pH adjusted to ~2, and extracted with ethyl acetate to obtain protonated acidic TNT metabolites in organic solution. The organic phase from the final LLE was collected and concentrated to ~100 mL for finer separation by several stages of SPE.

The final organic extract from the LLE separations described above was further fractionated by a series of C_8 solid-phase extractions (SPE). Visual observation of bands and HPLC analysis were employed to monitor eluant fractions. The first SPE stage was elutions on a C_8 stationary phase with mixtures of H_3PO_4 (10 mM) and 2-propanol. The column was hand-packed with ~40 g solids in a glass tube of dimensions, 250 (L) × 90 mm (ϕ). The elution gradient was from H_3PO_4 to 79/21 (% v/v) H_3PO_4/2-propanol; final washes include MeOH followed by ethyl acetate. Six previously unreported TNT metabolites were separated by this procedure, either pure or in mixtures with TNT, monoamines, and/or each other. One TNT-metabolite eluted as a pure fraction with 95/5 H_3PO_4/2-propanol, and a second eluted as a pure fraction with 90/10 H_3PO_4/2-propanol. A third TNT metabolite,which eluted in 85/15 H_3PO_4/2-propanol with TNT as the major impurity, was purified by silica-SPE as outlined in Bhadra et al. (*10*). The remainder three metabolites coeluted as mixtures in elutions of 83/17 to 81/19 H_3PO_4/2-propanol. TNT and monoamines were removed from these fractions with silica−SPE as previously performed. Beyond that, an additional re-elution on C_8−SPE with H_3PO_4 (10 mM)/2-propanol was sufficient to purify one of these TNT metabolites. Another C_8−SPE method, which used gradient elutions of H_3PO_4 (10 mM)/CH_3CN from 80/20 to 75/25 (% v/v), was formulated for the separation of the remainder two analytes. Except the first C_8−SPE of the crude extract, subsequent SPE fractionations were executed in 30-mL glass syringes with 20-mL bed volume. All pure fractions/TNT metabolites were dried without heating under vacuum as previously described and redissolved in MeOH for chemical analysis.

Hydrolysis of TNT Metabolites. To evaluate the formation of hydrolyzable metabolites of TNT, such as conjugates and sugar adducts, metabolites that were isolated were treated by enzymatic hydrolysis as described in Bhadra et al. (*10*). The enzymes used include β-glucuronidase and β-glucosidase.

Transformation Studies. Studies on nitroaromatic metabolism in natural aquatic systems of *M. aquaticum* were conducted at three scales: TNT-exposure studies in a reaction volume of 250 mL in 500-mL wide-mouth Erlenmeyer flasks; TNT-exposure studies in a reaction volume of 24 L in a 30-L aquarium; and aminodinitrotoluene-exposure studies in a reaction volume of 6 L in 10-L aquariums. At the start of each study, *M. aquaticum* plants, previously acclimated to room temperature for 1 day, were washed thoroughly to remove adherent soil, gravel, and microflora; patted dry; weighed; and introduced into the aqueous nitroaromatic solution in each reactor. Initial biomass density in these studies were as follows: 250 mL scale, 100 g/L fresh weight (FW); 24 L scale, ~40 g/L FW; and 6 L scale, ~30 g/L FW. All reaction systems were either maintained in darkness or cloaked adequately from light to prevent photodegradation reactions. Aquarium volumes were calibrated and marked at 1-L intervals, and each aquarium was mounted on wooden blocks so that the

aqueous solution could be mixed using Teflon-covered stir bars driven by several magnetic stirrers placed beneath the tank.

The initial concentrations of nitroaromatic reactants in these studies were as follows: 250 mL scale, 75 mg/L TNT, and 18 900 dpm/mL ^{14}C-TNT; 24 L scale, 64 mg/L TNT and 18.5 dpm/mL ^{14}C-TNT; and 6 L scale, 23 mg/L 2-amino-4,6-dinitrotoluene or 22 mg/L 4-amino-2,6-dinitrotoluene. For the small-scale study (in 500-mL shake flasks), stock solutions were added to tap water in shake flasks to attain initial target levels of TNT and ^{14}C-TNT. A different approach was adopted for the larger-scale tank studies since methanol that was used as solvent in stock solutions, supported microbial growth causing progressive cloudiness. For these, the appropriate mass of nitroaromatic compound (TNT, 2-amino-4,6-dinitrotoluene or 4-amino-2,6-dinitrotoluene)—calculated from the product of the reaction volume (24 or 6 L) and the initial target concentration—was dissolved in 10 mL of HPLC-grade ethyl acetate and applied across the bottom of the tank. The solution was then air-dried, resulting in the nitroaromatic being plated across the bottom of the aquarium. The appropriate volume of tap water was then poured into the tank and stirred for 3−4 days. Dissolution was monitored visually (absence of flakes) as well as by measuring aqueous concentration with HPLC; 2−3 days were sufficient for complete dissolution and to reach the initial target concentration.

The purpose of the small-scale study (in 500-mL shake flasks) was to examine the pH dependence of the extractability of plant metabolites of TNT into ethyl acetate. Further, this study segment was aimed at formulating an optimal approach for separating TNT metabolites from the dilute aqueous phase of TNT-exposed experimental plant systems. TNT-derived ^{14}C-label was employed to evaluate the extractability of TNT metabolites, since the possibility of unidentified ones being formed appeared significant (*6*). One TNT-amended flask was sacrificed at each sampling event, and the extractability of aqueous-phase ^{14}C-label into ethyl acetate measured following pH adjustment of 25-mL aliquots to 1, 4, 8, or 13. The control in this situation was no pH adjustment. The purpose of the aquarium-scale nitroaromatic-exposure studies was to monitor nitroaromatic metabolism by plants and in addition, to harvest the aqueous phase milieu for TNT metabolites. For these studies, nitroaromatic-containing aqueous controls devoid of plants were maintained to monitor abiotic degradation, evaporation, and transpiration. The controls were performed in a reaction volume of 6 L in 10-L aquariums for monoamine exposure studies and in a reaction volume of 12.5 L in 20-L aquariums for TNT-exposure studies. Samples were removed periodically to monitor extracellular concentrations of known nitroaromatic compounds, detectable products, and ^{14}C-label. Prior to each sampling event, the aqueous volume in the control tank was measured, and the appropriate volume of water added to each tank to compensate for evaporation losses. At the end of each aquarium-scale experiment, the plants were separated from the aqueous phase, rinsed with freshwater, and frozen at −20 °C. The aqueous phase volume was measured, combined with the rinsate, and the combined rinsate was stored at 4 °C. Subsequently, it was utilized for separation and analysis of TNT metabolites.

Results

pH Dependence of Extractability of TNT Metabolites. The initial characterization of TNT metabolites in aquatic systems of *M. aquaticum* was performed in 500-mL shake flasks as described in the previous section. The pH dependence of the extractability of aqueous-phase TN metabolites into ethyl acetate was examined. At the start of the exposure study, when only unmetabolized TNT was present in the aqueous phase, pre-extraction pH adjustment appeared to have little effect (Figure 1). The initial data are also an estimate of extraction efficiencies. Over time, the levels of nitroaromatics (●) and ^{14}C-label (□) in the extracellular aqueous phase decreased, with the former being lower than the latter as reported previously (*6*). This provided strong evidence for *M. aquaticum*-mediated transformation to products other than the reduction derivatives of TNT. A profile of transformants with pK_a-dependent hydrophobicities was also evident from the pH dependence (at pH of 1, 4, 8, or 13) of the extractability of aqueous-phase TNT metabolites into ethyl acetate. Beyond the initial time point, ^{14}C extractabilities at most pH were higher than the level of TNT and monoamine derivatives and were favored by pre-extraction adjustment to acidic pH. The extractability outcome at pH 1 was significant from that with no pH adjustment ($\alpha = 0.02$, paired *t*-test) and also from the extractability outcome at pH 13 ($\alpha = 0.01$, paired *t*-test) over the duration of the study.

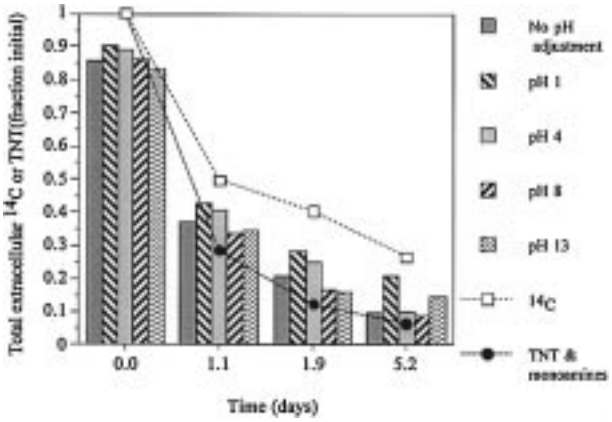


FIGURE 1. Levels of TNT-derived ^{14}C-label and nitroaromatic compounds (TNT, 2-A-4,6-DNT and 4-A-2,6-DNT) in the aqueous phase of sediment-free *M. aquaticum* exposed to TNT, and the extractability of ^{14}C-label into ethyl acetate. Extractability analysis was preceded either by no pH adjustment or adjustment to pH 1, 4, 8, or 13. Scale of study, 250 mL of reaction volume in a 500-mL Erlenmeyer flask. Initial concentrations: TNT, 75 mg/L; ^{14}C-TNT, 18,900 dpm/mL.

***Myriophyllum*-catalyzed Biosynthesis and Separation of TNT Metabolites.** A 24-L sediment-free, aquatic system of *M. aquaticum* was employed to generate TNT metabolites for subsequent separation. The levels of TNT, its reduction products 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene, and ^{14}C-label in the extracellular aqueous phase of the TNT-exposed plants are depicted in Figure 2. TNT (▲) and its aminated derivatives (□, ■) combined for a total residual level of 18% initial moles following exposure for 12 days. At the same time, the level of TNT-derived ^{14}C-label (●) in the aqueous phase was 64% of initial, the difference presumably representing unidentified products.

TNT-derived plant metabolites were separated from the extracellular aqueous phase of the large-scale aquarium reactor study as described in Materials and Methods following the exposure of *M. aquaticum* to TNT for 12 days. Ninety percent of the aqueous-phase TNT-derived ^{14}C-label or 58% of initial ^{14}C-label was separated by liquid−liquid extraction with ethyl acetate after initial pH adjustment to ~2. The final LLE-stage extract (base extracted) contained several UV-active, TNT-derived analytes of interest. As shown in Figure 3, these were eluted by ion-suppression C_8−HPLC at retention times of 5.1, 7.3, 8.4, 10.2, 11.6, and 14.5 min. These analytes were separated and purified by C_8 column chromatography as described in Materials and Methods and subjected to further analysis. Some 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene was also found in the extract at

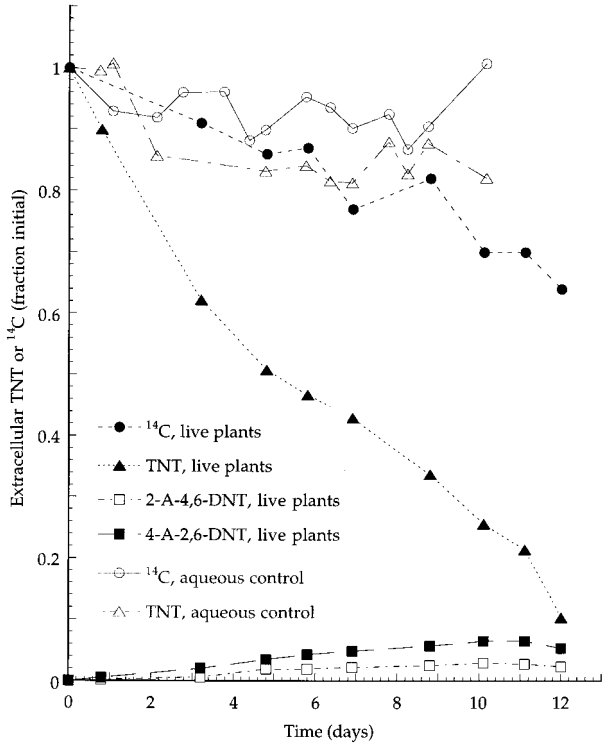


FIGURE 2. Levels of TNT-derived ^{14}C-label, TNT, 2-A-4,6-DNT, and 4-A-2,6-DNT in the extracellular aqueous phase of sediment-free *M. aquaticum* and biomass-free aqueous control over a 12-day period. Live plants were exposed to initial levels of 64 mg/L TNT and 18.5 dpm/ml ^{14}C-TNT in a 24-L reaction volume in a 30-L aquarium. The biomass free aqueous control was conducted with initial levels of 25 mg/L TNT and 33 dpm/mL ^{14}C-TNT in a 12.5-L reaction volume in a 20-L aquarium.

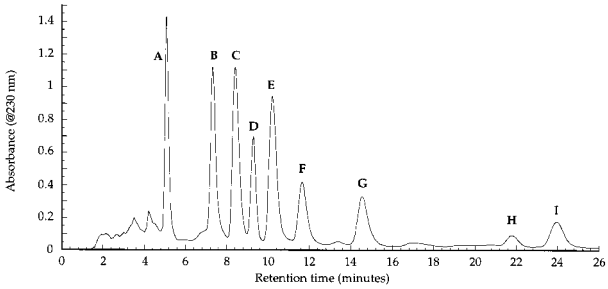


FIGURE 3. Chromatogram (at 230 nm) of ion-suppression C_8−HPLC analysis of the extracted aqueous phase of *M. aquaticum* exposed to 64 mg/L TNT for 12 days in a 30-L aquarium. Extraction includes two LLE steps: an initial extraction into ethyl acetate after pH adjustment to ~2 and a second step of base extraction with 1% (w/v) $NaHCO_3$. The HPLC method employed a Novapak-HR column (7.8 × 300 mm, Waters) and a mobile phase of 82/18 (% v/v) 10 mM H_3PO_4/2-propanol at 5 mL/min. Peak labels are: A, 2-amino-4,6-dinitrobenzoic acid; B, 2,4-dinitro-6-hydroxybenzyl alcohol; C, 2-*N*-acetoxyamino-4,6-dinitrobenzaldehyde; D, TNT; E, 2,4-dinitro-6-monohydroxytoluene; F, binuclear metabolite 1; G, binuclear metabolite 2; H, 2-A-4,6-DNT; I, 4-A-2,6-DNT.

retention times of 21.8 and 24 min, respectively, due to the slight solubility of ethyl acetate in water.

Characterization and/or Identification of TNT Metabolites. The characterization of TNT metabolites detected initially by HPLC−UV, resulted in the identification of several mono- and diaryl compounds. The monomers include (refer to Figure 3) 2-amino-4,6-dinitrobenzoic acid (HPLC retention time, 5.1 min.); 2,4-dinitro-6-hydroxybenzyl alcohol (HPLC retention time, 7.3 min); 2-*N*-acetoxyamino-4,6-dinitrobenzaldehyde (HPLC retention time, 8.4 min); and 2,4-dinitro-6-hydroxytoluene (HPLC retention time, 10.2 min). Further, the list of TNT metabolites characterized also includes two binuclear (or diaryl) metabolites unique from the customarily detected azoxy-tetranitrotoluene derivatives of TNT; these have HPLC retention times of 11.6 and 14.5 min. The assignments are supported by ^1H and ^{13}C NMR spectroscopy (Tables 1−3), mass spectroscopy (Table 4), and wherever feasible, such as in the case of 2-amino-4,6-dinitro benzoic acid, independently by chemical synthesis.

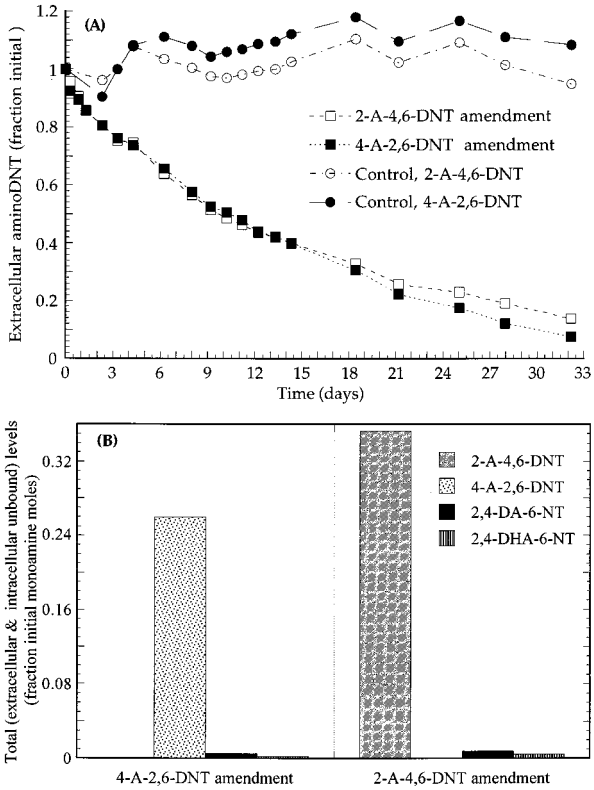
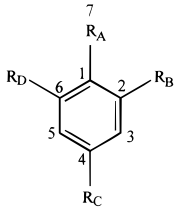


FIGURE 4. Levels of 2-A-4,6-DNT or 4-A-2,6-DNT and products in aquatic systems of *M. aquaticum* exposed to either monoamine: (A) levels in the extracellular aqueous phase over a 32-day exposure period; (B) total levels in the system (aqueous and unbound biomass phase) after exposure for 32 days. Scale of study, 6-L reaction volume in a 10-L aquarium. Initial concentrations: 2-amino-4,6-dinitrotoluene, 23 mg/L; 4-amino-2,6-dinitrotoluene, 22 mg/L.

On the basis of ^1H NMR, each monomer derivative of TNT isolated in this study has two magnetically nonequivalent aromatic protons (Table 1). Similar to the nonequivalent aromatic protons of 2-amino-4,6-dinitrotoluene, the nonequivalent aromatic protons of monomers isolated in this study exhibited long-range coupling of the order 2−2.25 Hz. With respect to nonaromatic protons, two benzylic protons (at $\delta = 4.97$ ppm) were detected in the metabolite identified as 2,4-dinitro-6-hydroxy-benzyl alcohol, close to Schoolery's predictions ($\delta = 4.64$ ppm) for an internal methylene group attached to terminal phenyl and hydroxy residues (*19*). Another proton resonance detected in the same metabolite at 9.6 ppm is that of the ring-substituted hydroxyl proton, close to the reported value ($\delta = 9.25$ ppm) of the hydroxyl proton in phenol. Alkyl protons corresponding to methyl groups were detected: for ring-substituted methyl in the metabolite identified as dinitrohydroxytoluene ($\delta = 2.61$ ppm) and for the acetoxymethyl ($\delta = 2.6$ ppm) in the metabolite identified as *N*-acetoxyaminodinitrobenzaldehyde.

The intact aromatic ring of the metabolites identified as 2-amino-4,6-dinitrobenzoic acid and 2,4-dinitro-6-hydroxy-

TABLE 1. Summary of ^{1}H NMR Resonances of Mono-aryl TNT-Metabolites Isolated from the Extracellular Aqueous Phase of *M. aquaticum* Exposed to TNT, Compared to Nitroaromatic Standards



analyte	H-3[b]	H-5[b]	H-7[b]	other ^{1}H[b]
2,4,6-trinitrotoluene[a] ($R_A = CH_3$; $R_B = R_C = R_D = NO_2$)	8.95(s)	*c*	2.64 (s)	
4-amino-2,6-dinitrotoluene[a] ($R_A = CH_3$; $R_B = R_D = NO_2$; $RC = NH_2$)	7.21 (s)	*c*	2.21 (s)	
2-amino-4,6-dinitrotoluene[a] ($R_A = CH_3$; $R_B = NH_2$; $R_C = R_D = NO_2$)	7.7 (d) $J = 2.25$ Hz	7.8 (d) $J = 2.35$ Hz	2.22 (s)	
2-amino-4,6-dinitrobenzoic acid[a] ($R_A = COOH$; $R_B = NH_2$; $R_C = R_D = NO_2$)	7.6 (d) $J = 2.13$ Hz	7.8 (d) $J = 2.13$ Hz		
2,4-dinitro-6-hydroxy-benzyl alcohol[a] ($R_A = CH_2OH$; $R_B = R_C = NO_2$; $R_D = OH$)	8.13 (d) $J = 2.25$ Hz	7.85 (d) $J = 2.25$ Hz	4.97 (s)	9.6 (s)
2-*N*-acetoxyamino-4,6-dinitrobenzaldehyde[a] ($R_A = CHO$; $R_B = NHOCOCH_3$; $R_C = R_D = NO_2$;)	9.45 (d) $J = 2.18$ Hz	8.93 (d) $J = 2.22$ Hz		2.62 (s)
4-*N*-acetoxyamino- 2,6-dinitrobenzaldehyde[a]	8.85 (s)	*c*		
2,4-dinitro-6-hydroxytoluene[a] ($R_A = CH_3$; $R_B = R_C = NO_2$; $R_D = OH$)	9.64 (d) $J = 2.1$ Hz	8.97 (d) $J = 2.1$ Hz		2.61 (s)
2,6-dinitro-4-hydroxytoluene[a]	8.93 (s)	*c*		

[a] Chemical shifts relative to CD_3OD (^{1}H: δ, 3.31 and 4.87) and/or CD_3CN (^{1}H: δ, 1.94). [b] s, singlet; d, doublet. [c] Chemical shift equivalent to H-3 due to aromatic symmetry.

TABLE 2. Summary of ^{13}C NMR Resonances of Mono-aryl TNT-Metabolites Isolated from the Extracellular Aqueous Phase of *M. aquaticum* Exposed to TNT, Compared to Nitroaromatic Standards

analyte	C−1	C−2	C−3	C−4	C−5	C−6	C−7	other C
2,4,6-trinitrotoluene[a]	134.9	153.0	123.6	147.5	*c*	*d*	15.70	
4-amino-2,6-dinitrotoluene[a]	112.8	153.8	113.1	149.6	*c*	*d*	13.76	
2-amino-4,6-dinitrotoluene[b]	126.2	150.1	117.2	147.4	108.7	150.9	13.6	
2-amino-4,6-dinitrobenzoic acid[a]	114.8	150.4	106.0	153.0	106.4	151.7	~179[b]	
2,4-dinitro-6-hydroxy-benzyl alcohol[a]	129.5	148.9	114.6	152.4	111.0	159.3	55.8	
2-*N*-acetoxyamino- 4,6-dinitrobenzaldehyde	130.4[b]	141.2[b]	122.6[b]	155.2[b]	114.9[b]	150.4[b]	203.3	165.96, 15.3
4-*N*-acetoxyamino- 2,6-dinitrobenzaldehyde	122.7[b]	150.4[b]	122.6[b]	146.0[b]	*c*	*d*		
2,4-dinitro-6-hydroxytoluene	125.8[b]	150.9[b]	110.8[b]	147.5[b]	116.8[b]	157.8[b]	15.6	
2,6-dinitro-4-hydroxytoluene	119.8[b]	150.9[b]	116.8[b]	154.3[b]	*c*	*d*		

[a] Chemical shifts relative to CD_3OD (^{13}C: δ, 49.15) and/or CD_3CN (^{13}C: δ, 118.69 and 1.39). [b] Chemical shift estimated from Ewing (*24*). [c] Chemical shift equivalent to C-3 due to aromatic symmetry. [d] Chemical shift equivalent to C-2 due to aromatic symmetry

TABLE 3. Summary of ^{1}H NMR Resonances of Binuclear TNT-Metabolites Isolated from the Extracellular Aqueous Phase of *M. aquaticum* Exposed to TNT, Compared to Azoxy Standards

analyte	aromatic protons	non-aryl protons
4,4′,6,6′-tetranitro-2,2′-azoxytoluene	9.32(d), 9.19 (d) 9.02 (s), 8.8 (d)	2.65 (s) 2.76 (s)
2,2′,6,6′-tetranitro-4,4′-azoxytoluene	8.99 (s) 9.15 (s)	2.65 (s) 2.68 (s)
binuclear metabolite 1 (rt in ion-suppression C_8-HPLC, 11.6 min)	8.75 (d), $J = 2.5$ Hz; 8.44 (d), $J = 2.5$ Hz 7.58 (d), $J = 2$ Hz 7.44 (d), $J = 1.75$ Hz	2.18 (s) 1.29 (s)
binuclear metabolite 2 (rt in ion-suppression C_8-HPLC, 14.5 min)	8.26 (s) 7.78 (d), $J = 1.75$ Hz; 7.46 (d), $J = 1.75$ Hz	2.58 (s) 1.27 (s)

benzyl alcohol were confirmed by the six aromatic carbons in the ^{13}C NMR spectra of both isolates (Table 2) in the proximity of estimated values. A ^{13}C-resonance detected in the latter at $\delta = 55.8$ ppm was that of the benzylic carbon. The molecular ions observed by desorption chemical ionization MS (Table 4) verified the molecular masses of both proposed assignments: 2-amino-4,6-dinitrobenzoic acid, 227.0; and 2,4-dinitro-6-hydroxybenzyl alcohol, 214.1. In addition, characteristic fragments, less a molecule of water, were observed for both. In the case of 2-amino-4,6-dinitrobenzoic acid, the identity was also confirmed by independently synthesizing the analyte.

TABLE 4. Summary of Mass Spectral Fragments of TNT-Metabolites Isolated from the Extracellular Aqueous Phase of *M. aquaticum* Exposed to TNT

analyte	observed molecular ion (M + 1)	primary fragment ion observed	observed reagent ions[a]
2-amino-4,6-dinitrobenzoic acid	228.0	210.0	256.1, 268.1
2,4-dinitro-6-hydroxy-benzyl alcohol	215.1	197.1	243.1
N-acetoxyamino dinitrobenzaldehyde	270.1	228.1	298.1, 310.1
dinitro monohydroxy toluene	199.2	181.1	227.2, 239.2
binuclear metabolite 1 (rt in ion-suppression C_8-HPLC, 11.6 min)		364.1, 336.1, 292.1, 248.1, 198.1	
binuclear metabolite 2 (RT in ion-suppression C_8-HPLC, 14.5 min)		332.1, 320.1, 292.1	

[a] Corresponds to M + $C_2H_5^+$ or M + $C_3H_5^+$.

Desorption chemical ionization MS of TNT-derived metabolites identified as 2-*N*-acetoxyamino dinitrobenzaldehyde and 2,4-dinitro-6-hydroxytoluene, yielded molecular masses of 269.1 and 198.2, respectively (Table 4); characteristic fragment ions were obtained, less an acetyl residue and a molecule of water, respectively. The ^{13}C NMR spectra (Table 2) supported the following in these isolates: a ring-substituted methyl carbon in the dinitrohydroxytoluene ($\delta = 15.6$ ppm); acetoxy carbon ($\delta = 165.9$ ppm), acetoxy methyl carbon ($\delta = 15.3$ ppm), and ring-substituted carbonyl carbon ($\delta = 203.3$ ppm) in the *N*-acetoxyamino dinitrobenzaldehyde. In addition, isomers of both compounds were possibly coeluted during separation as observed from the appearance of 12 resonances in the aromatic region of ^{13}C NMR spectra, and of two symmetric aromatic protons as well in ^1H NMR spectra. The spectral evidence supports the proposed structures. Positive identifications will need comparative data from authentic standards.

The two remainder TNT-derived analytes were designated as diaryl metabolites based on a combination of evidence. Their molecular masses are considerably greater than that of TNT and monoamine derivatives, and monomer analytes isolated in this study (Table 4). Both analytes have resonances for four aryl protons in their ^1H NMR spectra as well as for six nonaryl protons (Table 3). The six nonaromatic protons occur as a pair of singlet resonances, one corresponding to an intact ring-substituted methyl-group ($\delta = 2.2-2.6$ ppm). Both are composed of dissimilar aromatic units: binuclear metabolite 1 is composed of two nonsymmetric aryl units inferred from the two pairs of nonequivalent protons with long-range coupling and binuclear metabolite 2 is composed of one symmetric and one nonsymmetric aromatic unit. It is unlikely that these compounds are conjugates with sugar adducts since characteristic resonances were neither observed in the ^1H NMR spectra, nor did enzymatic hydrolysis with β-glucuronidase and β-glucosidase result in their decomposition. Comparison of proton resonances (Table 3) with those of commonly reported azoxytetranitrotoluenes also establishes their difference from those compounds.

Contribution of New Metabolites to Mole Balance. An additional analysis was performed to quantify total levels of the freshly identified TNT-derived metabolites in the 24-L aquatic system of *M. aquaticum* by employing ^{14}C-based response factors for HPLC areas at 230 nm. For monoaryl TNT-metabolites, the molar ^{14}C activity per mole of isolated metabolite was assumed to be the same as that of the ^{14}C-TNT and for binuclear (or diaryl) TNT-metabolites, double that of the ^{14}C-TNT. After exposure to TNT for 12 days, the extracellular aqueous milieu contained significant levels of these metabolites, 35.9% of initial TNT moles as the characterized monomers and 5.6% as the binuclear metabolites. The breakdown of the former group comprises 2-amino-4,6-dinitrobenzoic acid, 4.4%; 2,4-dinitro-6-hydroxy-benzyl alcohol, 8.1%; 2-*N*-acetoxyamino-4,6-dinitrobenzaldehyde, 7.8%; 2,4-dinitro-6-hydroxytoluene, 15.6%.

Exposure of *M. aquaticum* to 2-A-4,6-DNT and 4-A-2,6-DNT. To investigate the sequence of transformations, *M. aquaticum* plants were exposed separately to 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene in 6-L aquatic systems of roughly the same biomass density as with TNT-exposure studies. The depletion of both monoamines followed each other closely with apparent pseudo-first-order kinetic rates (normalized to biomass) of 0.00195 and 0.0025 L gFW^{-1} day^{-1}, respectively. The depletion of either monoamine in biomass-free, aqueous controls was marginal. Detectable transformation products that emerged from a total analysis (extracellular and intracellular unbound) of each aquatic plant system after exposure for 32 days, include low levels (<1% of initial) of 2,4-diamino-6-nitrotoluene and 2,4-dihydroxylamino-6-nitrotoluene. These were accumulated in the extracellular aqueous phase only; none was detected in the biomass phase. Only in the case of exposure to 2-amino-4,6-dinitrotoluene, the TNT conjugate metablite 2A-5.5 (*10*) accumulated extracellularly to a maximum level of 0.05%. The transformation of 4-amino-2,6-dinitrotoluene also appeared more complete than that of 2-amino-4,6-dinitrotoluene, 26 and 35%, respectively, remained untransformed. A larger residual fraction accumulated intracellularly in both cases, about 60−70% of the total. Related compounds that were identified in TNT-exposure studies, namely, 2-amino-4,6-dinitrobenzoic acid, 2-*N*-acetoxyamino-4,6-dinitro benzaldehyde, and the binuclear metabolites, were not detected when the starting material was either amino-dinitrotoluene.

Discussion

The objective of this study was to investigate oxidative transformation as a possible biocatalytic route for the metabolism of TNT by plants. A growing body of investigations have reported the reductive transformation of TNT by plants (*4*−*9*, *11*−*13*); the resultant list of TNT metabolites includes 2-amino-4,6-dinitrotoluene; 4-amino-2,6-dinitrotoluene; 2,4-diamino-6-nitrotoluene; 4-hydroxylamino-2,6-dinitrotoluene; and azoxytetranitrotoluenes. Oxidative transformations of TNT, however, remain comparatively unreported but are of interest from the perspectives of diversity of plant processes, of environmental fate in natural systems, and of acceptable "designed" fate in phytoremediation systems. The results of studies reported herein, conducted in sediment-free, natural aquatic systems of *M. aquaticum*, provide evidence for an array of metabolic products of TNT arising from plant-catalyzed oxidative metabolism, including oxidation of the ring-substituted methyl group and aromatic hydroxylation.

In this study, a number of unique plant-based products were isolated from the extracellular, aqueous milieu of TNT-

exposed *M. aquaticum*—2-amino-4,6-dinitrobenzoic acid; 2,4-dinitro-6-hydroxybenzyl alcohol; 2-*N*-acetoxyamino-4,6-dinitrobenzaldehyde and isomer; 2,4-dinitro-6-hydroxytoluene and isomer; and two binuclear metabolites. This analysis is supported by a suit of analytical evidence: UV-vis (Figure 3), ^{1}H and ^{13}C NMR spectroscopy (Tables 1−3), mass spectroscopy (Table 4), and where feasible, such as in the case of 2-amino-4,6-dinitrobenzoic acid, independent chemical synthesis. The formation of 2-amino-4,6-dinitrobenzoic acid likely involves multistep alkyl hydroxylation, formation of the benzyl group followed by that of the benzoyl group, as in the metabolism of the herbicide oxadiazon in rice (*20*) and peanut (*21*) plants. The metabolites 2-*N*-acetoxyamino-4,6-dinitrobenzaldehyde and 2,4-dinitro-6-hydroxybenzyl alcohol may represent modified intermediates/byproducts of this pathway. As isolated, these compounds also exhibit additional modifications, such as acetoxy addition at the aryl amino group and aromatic hydroxylation, respectively. From the monoamine exposure studies conducted here, it appears that methyl oxidation most likely precedes nitro reduction, since neither amino dinitrobenzoic acids nor acetoxyaminodinitrobenzaldehydes were detected when either 2-amino-4,6-dinitrotoluene or 4-amino-2,6-dinitrotoluene were the starting material for plant exposure. A cautionary note in this matter is the possible noninduction of the relevant enzyme system, direct *N*-conjugation of the amine, or partial to complete methyl hydroxylation followed by rapid conjugation in the monoamine-feed studies. The product profile for exposure of *M. aquaticum* to 2-amino-4,6-dinitrotoluene suggests the second possibility since the corresponding conjugate, 2A-5.6 was detected; 2A-5.6 is a conjugate metabolite that is formed by the addition of a six-carbon sugar to 2-amino-4,6-dinitrotoluene and is a hypothesized precursor of bound TNT metabolites in plants (*10*). Another interesting aspect of oxidative metabolism in *M. aquaticum* is the ring hydroxylation of TNT evidenced by the formation of 2,4-dinitro-6-hydroxybenzyl alcohol and 2,4-dinitro-6-hydroxytoluene. In both cases, aryl hydroxylation was accompanied by the elimination of a nitro group. Aromatic hydroxylation of the herbicide 2,4-D is known to occur (reviewed by Hatzios (*16*)), but the elimination of ring-substituted chlorine is a minor reaction; for this herbicide, the "NIH shift" mechanism was determined to be the dominant enzyme catalyzed pathway in which ring hydroxylation is accompanied by chlorine migration and the formation of 4-OH-2,3-D and/or 4-OH-2,5-D. The difference in aryl hydroxylation outcomes between TNT and 2,4-D may lie among other possibilities, in the degree of ring substitution of the parent compound. With respect to the binuclear metabolites, it is evident that their resolution in C_8−HPLC was pK_a dependent, achieved only by ion-suppression using 10 m*M* H_3PO_4. This leads to the possibility of constituent aryl units bearing aromatic hydroxyl group(s) that exhibit low pK_a in the presence of electronegative ring substituents, in this case one or more nitro-group of the parent compound (TNT). At this point, however, with the analytical evidence at hand, the aryl hydroxylation of binuclear metabolites, isolated in this study and, consequently, their relationship to oxidative metabolism of TNT, can only be hypothesized.

In our study, the products were isolated exclusively from the aqueous phase. This is in agreement with the conceptualized "green liver" metabolism of organic xenobiotic compounds in plants; products of the initial phase (phase I and/or phase II) of xenobiotic detoxification are more hydrophilic and less bioavailable than the parent molecule (*17*, *22*). Our isolation of oxidized metabolites of TNT from the aqueous phase of TNT-exposed plants does not, however, preclude their occurrence within TNT-exposed plant biomass. In studies with herbicides, products of oxidative metabolism, such as ring-hydroxylated and alkyl-hydroxylated derivatives often occurred as the phenolic β-glucoside, β-*O*-glucoside, glycosyl ester, or amino acid conjugate (*14*). It is similarly possible that the oxidized metabolites of TNT could occur intact within the biomass, or as conjugates that would require hydrolysis prior to the analysis performed in this study. Studies are presently ongoing that are examining this aspect of nitroaromatic fate in *M. aquaticum*.

The oxidized metabolites appeared in the aquatic system of TNT-exposed *M. aquaticum* together with the customarily reported reduction products: 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene. After exposure to TNT for 12 days, the residual aqueous phase contained 10% TNT, 2.3% 2-amino-4,6-dinitrotoluene, 5.3% 4-amino-2,6-dinitrotoluene, and an estimated total of 36% monomeric oxidized metabolites. It demonstrates that aquatic plants exposed to TNT, in this case *M. aquaticum*, are capable of both types of metabolism, reductive and oxidative. From our current state of understanding of herbicide metabolism in plants, the most plausible enzyme candidates for the oxidative metabolism of TNT are mixed function oxygenases (mfo). In most general terms, these are cytochrome P-450 groups of enzymes that are localized primarily in the microsomes (endoplasmic reticulum) of plant cells, requiring molecular oxygen as a second substrate, and NADPH or NADH as cofactors (*17*, *23*). Our present findings may be evidence for the activity of such enzymatic processes during TNT metabolism in plants. To further improve our understanding of the activity of oxidative metabolism relative to nitro reduction, temporal analyses of TNT-exposed plant systems, where both reductive and oxidative metabolites are monitored over time, will be necessary. Clearly, our investigation of oxidative metabolites in TNT-exposed plant systems is important to the total analysis of metabolic fate of TNT and related nitroaromatic xenobiotics in nature and in phytoremediation processes.

Acknowledgments

We would like to express our appreciation to Dr. Terry Marriot, Director, Mass Spectrometry Center at Rice University and Ms. Anne Bellatore of University of Texas, Health Science Center, for their assistance with mass spectrometric analysis of samples. This material is based in part upon work supported by the Texas Advanced Technology Program under Grant 003604-045, by NSF Young Investigator Award BES-9257938 to J.V.S. and by DSWA Project 01-97-1-0020.

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Received for review April 19, 1999. Revised manuscript received June 28, 1999. Accepted June 30, 1999.

ES990436I