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Role of Hydroxylamine Intermediates in the Phytotransformation of 2,4,6-Trinitrotoluene by *Myriophyllum aquaticum*

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Phytotransformation studies of 2,4,6-trinitrotoluene (TNT) were conducted using *Myriophyllum aquaticum* to clarify the role of initial intermediates of TNT transformation in the complex product distributions reported previously. 2-Hydroxylamino-4,6-dinitrotoluene (2HA46DNT) and 4-hydroxylamino-2,6-dinitrotoluene (4HA26DNT) were the initial intermediates of TNT phytotransformation. 2HA46DNT and 4HA26DNT were both abiotically transformed to 4,4′,6,6′-tetranitro-2,2′-azoxytoluene (2,2′azoxy) and 2,2′,6,6′-tetranitro-4,4′-azoxytoluene (4,4′azoxy) and also phytoreduced to the related amines 2-amino-4,6-dinitrotoluene (2A46DNT) and 4-amino-2,6-dinitrotoluene (4A26DNT). To further elucidate the initial steps of this TNT phytotransformation pathway, the transformations of known intermediates (including 2HA46DNT, 4HA26DNT, 2A46DNT, 4A26DNT, 2,2′azoxy, and 4,4′azoxy) were monitored in plant systems. The transformation rates were measured, and kinetic analysis using pseudo-first-order models was used to evaluate the relative rates of competing reactions. The formation of the azoxy products was determined to be more rapid than the formation of the amine products. Both the azoxy and amine products were subject to uptake and further transformation by the plant.

Introduction

As a result of munitions production and storage, 2,4,6-trinitrotoluene (TNT) and its byproducts are widespread and persistent contaminants of soil and groundwater at a number of government facilities. Since it is toxic and poses a serious environmental risk (*1*−*3*), contaminated soil and water are currently being remediated. During the evaluation of remediation processes, phytoremediation has been identified as a viable low-cost option for the cleanup of TNT-contaminated media (*4*, *5*). Phytoremediation processes rely on the ability of plants to take up and transform contaminants. Investigations of TNT phytoremediation have been conducted using aquatic and terrestrial plant systems (*5*−*12*). While TNT levels decreased, none of these studies showed significant mineralization of TNT. Still, considerable interest exists in the evaluation of TNT phytotransformation pathways to determine the intermediates and products formed and their potential to increase or decrease toxicity.

In studies of TNT metabolism by microorganisms, it has been widely demonstrated that the aryl nitro groups are the site of initial biological attack (*13*−*15*). Studies using several plant species have consistently showed the formation of two nitro-reduction products, 2-amino-4,6-dinitrotoluene (2A46-DNT) and 4-amino-2,6-dinitrotoluene (4A26DNT). However, these compounds generally account for a limited fraction (∼15 mol %) of the TNT transformed by the plant (*9*, *11*).

A previous study using the aquatic plant *Myriophyllum aquaticum* showed a rapid disappearance of TNT that was attributed to plant uptake and transformation (*9*). In this study, the use of axenic plants and plant tissue cultures confirmed that plant-associated bacteria were not responsible for TNT transformation. The accumulation of low concentrations of 2A46DNT and 4A26DNT were produced by plant metabolism and observed in the extracellular medium (*9*, *11*). Mass balances of ^{14}C showed that the TNT transformation products were distributed nearly evenly between the plant medium and the plant tissues. The ^{14}C associated with the plant were characterized as bound residues, as they could be assayed only through the combustion of plant samples. Metabolites other than the amines in the aqueous medium were characterized as conjugation metabolites and oxidation products (2-amino-4,6-dinitrobenzoate, 2,4-dinitro-6-hydroxybenzyl alcohol, 2-*N*-acetoxyamino-4,6-dinitrobenzaldehyde, and 2,4-dinitro-6-hydroxytoluene). The pathways leading to bound, conjugated, or partially oxidized forms are believed to involve initial reduction reactions, although the mechanism(s) for their formation is not well understood (*6*, *16*).

Little is known about the initial reduction reactions of TNT in plants. Ferredoxin $NADP^+$ reductase isolated from spinach leaves is known to reduce TNT to 4HA26DNT (*17*). Burken et al. state "hydroxylamines are postulated as a key intermediate in the transformation pathway of TNT, but conclusive research is needed" (*18*). The purpose of the studies presented was to evaluate the initial stages of the TNT phytoremediation pathway in *M. aquaticum* and to better understand how initial reactions may influence the formation of aminated products or other known metabolites that are not strictly the result of reduction reactions. This involved isolating and characterizing initial transformation intermediates, followed by studies in which these intermediates were exposed to the plants to evaluate their further transformation.

Materials and Methods

Chemicals. Nitroaromatic compounds used were TNT (99% purity: Chem Service, West Chester, PA); (U-ring-^{14}C)-TNT (Chemsyn Science, Lenexa, KS) purified (98.6%) as described previously (*13*); 2HA46DNT and 4HA26DNT synthesized and purified as described previously (*13*); 2A46DNT, 4A26DNT, and 2,4-diamino-6-nitrotoluene (24DA6NT) (Accustandard Inc., New Haven, CT); and 2,2′,6,6′-tetranitro-4,4′-azoxytoluene (4,4′azoxy) and 4,4′,6,6′-tetranitro-2,2′-azoxytoluene (2,2′azoxy) (Dr. Deborah Roberts, University of Houston, Houston, TX). HPLC grade solvents included ethyl acetate, methylene chloride, and acetonitrile (99.9%) (Fisher Scientific, Alanta, GA). Silica gel for column chromatography was 70−230 mesh (Fisher Scientific). NMR was conducted in methanol-*d* (99.8 atom %) (Aldrich, St. Louis, MO).

Plants. As described in our previous report (*9*), *M. aquaticum* was purchased from a local supplier. The plants were grown to between 10 and 30 g/L (wet wt) in outdoor

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pools recharged with rainwater and rooted in compost-amended sediment and gravel (no TNT was present in the sediments). Before use in experiments, the plants were gently uprooted and washed thoroughly with tap water to remove sediment and gravel.

Analytical Methods. The Waters system (Milford, MA) used for HPLC analysis consisted of a separation module (model 2690) and a diode-array UV−visible detector (model 996). The system was controlled by a PC-based workstation equipped with the Millenium Chromatography Manager software. Spectra were acquired continuously between 200 and 400 nm, and chromatograms were extracted at 230 nm for quantitation. Analytes were separated on a reverse-phase Waters Nova-Pak-C18 column (2 × 150 mm) at room temperature with gradient mobile phases consisting of water/acetonitrile (0−5 min, 75/25 (v/v); 5−12 min, 70/30 (v/v); 12−20 min, 40/60 (v/v); 20−25 min, 75/25) at 0.6 mL/min.

The measurement of ^{14}C in HPLC fractions and stock solutions was performed with a Beckman LS6500 scintillation counter after addition of samples to ReadyGel liquid scintillation cocktail (Beckman). ^{1}H NMR spectra were obtained on a Bruker AC-250 spectrometer. Chemical shifts are reported in ppm relative to methanol-*d* (3.31and 4.78 ppm).

TNT Transformation Studies. Batch experiments were performed in 3-L glass tanks at room temperature exposed to indoor lighting. A [^{14}C]TNT solution (3 L at 25 ppm and 1000 dpm/mL of ^{14}C) was added to the tanks. For phytotransformation studies, *M. aquaticum* (90 g wet wt per tank or 30 g/L) were added after washing the plants with tap water. Control systems were identical to phytotransformation systems except that no plants were added. The transformation process was monitored by HPLC analysis of samples of the TNT solution. Samples were analyzed immediately by HPLC. Samples were not diluted since the TNT concentration was 25 ppm or less. Plant systems and controls with no plants added were set up in duplicate. One set of plant systems was terminated at 9 d, and the other was maintained for a total of 15 d.

Isolation of Transformation Intermediates. Intermediates of TNT phytotransformation were obtained at different points in the transformation process. Transformation was stopped after either 9 or 15 d by removing the plants from the reaction mixture. The TNT intermediates were extracted from the solution with methylene chloride (200 mL) and then ethyl acetate (300 mL). The combined organic fractions were dried with anhydrous $MgSO_4$. After the $MgSO_4$ was filtered, the filtrate was evaporated, leaving a light yellow residue. Intermediates were then isolated by silica column chromatography (ethyl acetate/hexane = 1/10−1/2). The products eluted off the column in the following order: 4,4′azoxy, 2,2′azoxy, 4A26DNT, 2A46DNT, 2HA46DNT, and 4HA26DNT. These products were subjected to ^{1}H NMR analysis and HPLC analysis.

Synthesis of ^{14}C Isotope Intermediate Standards. ^{14}C isotope intermediate standards were synthesized biochemically using [^{14}C]TNT as a starting material (*13*, *19*). [^{14}C]-4HA26DNT was obtained from reduction of [^{14}C]TNT by *Clostridium acetobutylicum* (*13*). [^{14}C]-2HA46DNT, [^{14}C]-2A46DNT, and [^{14}C]-4A26DNT were obtained from [^{14}C]TNT transformation in plant systems using the same methods as for the isolation of transformation intermediates. [^{14}C]-2,2′azoxy and [^{14}C]-4,4′azoxy were obtained as described previously from the oxidation of [^{14}C]-2HA46DNT and [^{14}C]-4HA26DNT, respectively (*19*).

Transformation of Observed Intermediates. Complementary experiments using methods similar to those described for TNT transformation studies were carried out for observed intermediates. These batch experiments were carried out in 1L glass containers at room temperature and exposed to indoor light. *M. aquaticum* was added to obtain a wet weight plant concentration of 30 g/L after washing with tap water. Intermediates 2HA46DNT, 4HA26DNT, 2,2′ azoxy, 4,4′ azoxy, 2A46DNT, and 4A26DNT were added individually to a concentration of 25 mg/L. The transformation processes were monitored over various time periods (120 h−21 d) depending on the disappearance rate of individual compounds added. Samples were analyzed immediately by HPLC. During the control test using hydroxylamines, the products were recovered by extraction with methylene chloride. The extract was dried with anhydrous $MgSO_4$, filtered, and evaporated to yield white solids. These compounds were verified as azoxy compounds by HPLC retention time, UV spectra, and ^{1}H NMR as compared to standards.

Kinetic Analysis. Data obtained from plant transformation studies were evaluated using a second-order kinetic mechanism as described by Pavlostathis et al. (*10*) and shown in eq 1:

$$\frac{dC_i}{dt} = -k_i C_i P \quad (1)$$

where C_i is the concentration (mM) of the compound of interest, t is time (h), k_i is a second-order rate coefficient (L g^{-1} t^{-1}), and P is plant concentration (g L^{-1}). Under the conditions of this study, plant growth/decay was assumed to be negligible (i.e., plant concentration was constant during the tests) causing the analysis to become pseudo-first-order.

To analyze the rate of abiotic transformation in controls amended with 4HA26DNT and 2HA46DNT, disappearance profiles were evaluated with the first-order model in eq 2:

$$\frac{dC_i}{dt} = -kC_i \quad (2)$$

where k is the first-order rate coefficient (time^{-1}) and C_i is the concentration of the parent compound. This model is a simplification of a more complex mechanism that would describe the oxidation of a hydroxylamine to a nitroso functionality followed by a reaction with a separate hydroxylamine to form an azoxy compound. The use of a simple first-order relationship was predictive, however, as the rate-controlling step is the oxidation of hydroxylamine to form a nitroso intermediate.

Results

TNT Transformation Studies. Six intermediates (2HA46DNT, 4HA26DNT, 2A46DNT, 4A26DNT, 2,2′azoxy, and 4,4′azoxy) were extracted and isolated from the TNT phytotransformation solution on day 9. The identities of these compounds were confirmed by HPLC retention time, UV spectra, and ^{1}H NMR as compared to standards. Figure 1 illustrates the HPLC separation and UV spectra of the six compounds. The chemical shifts in ^{1}H NMR spectra of 2HA46DNT, 4HA26DNT, 2A46DNT, and 4A26DNT are summarized in Table 1 and correspond to values published previously (*13*). Only three metabolites could be isolated on the 15th day and were confirmed as 2A46DNT, 4A26DNT, and 24DA6NT.

The results from temporal sampling and HPLC analysis of TNT transformation in *M. aquaticum* are shown in Figure 2. In the negative control, TNT was stable in the water solution. In the plant system, TNT disappeared nearly completely after 15 d. During the transformation of TNT, 2HA46DNT and 4HA26DNT were the primary products initially observed by HPLC analysis. The hydroxylamines reached their maximum observed combined levels on day 9 and then dissipated to only trace levels by day 15. Following the production of the hydroxylamine intermediates, aminodinitrotoluenes (2A46DNT and 4A26DNT) and azoxy compounds (2,2′azoxy and 4,4′azoxy) were detected. 2A46DNT

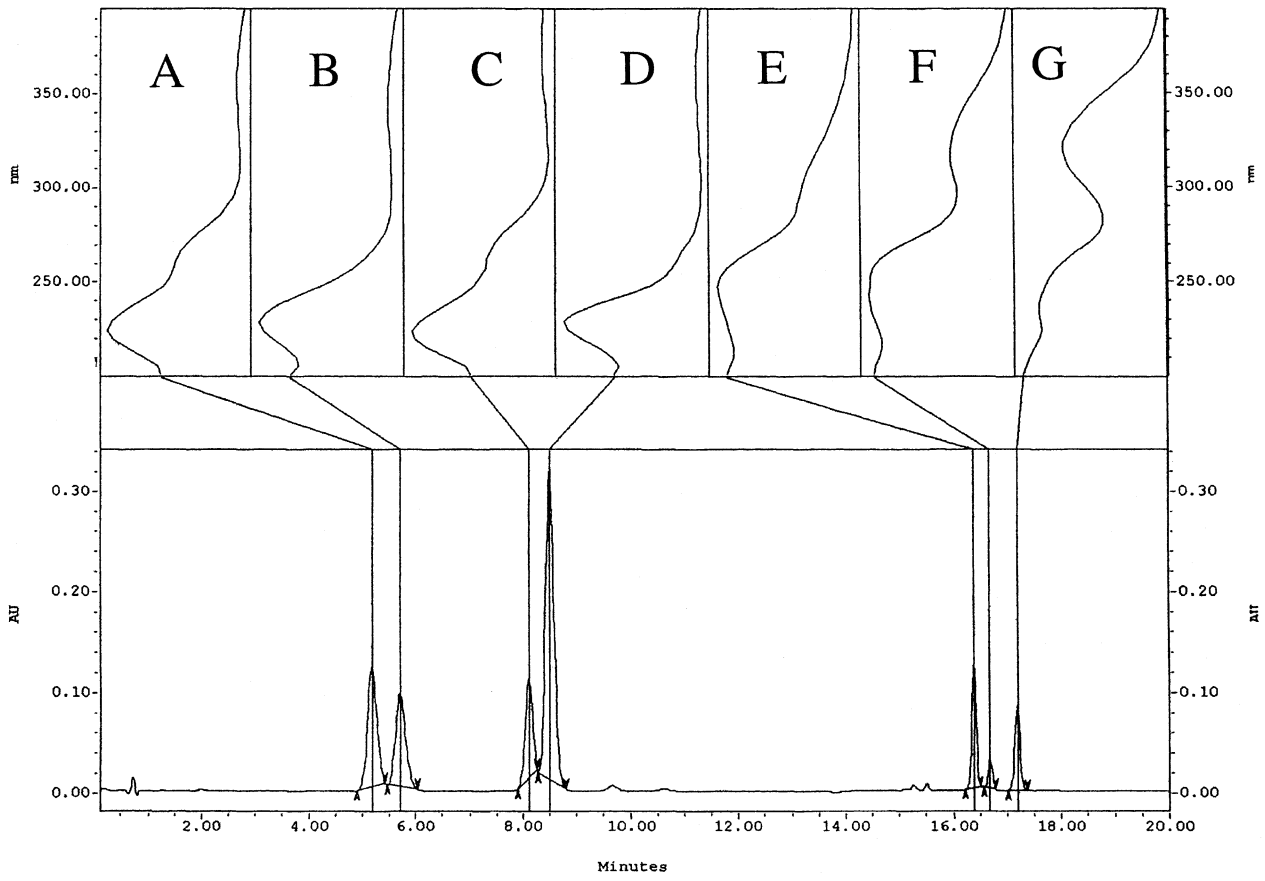
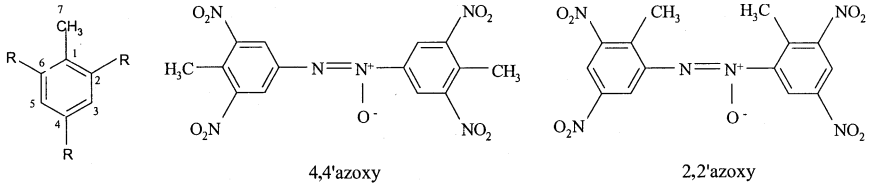


FIGURE 1. HPLC separation and UV spectra of 2HA46DNT (A), 4HA26DNT (B), 2A46DNT (C), 4A26DNT (D), 2,2′azoxy (E), 2,4′azoxy (F), and 4,4′azoxy (G).

TABLE 1. ^{1}H NMR and ^{13}C NMR Spectra of TNT and Isolated Intermediates



4,4'azoxy 2,2'azoxy

compound	H-3	H-5	H-7	C-1	C-2	C-3	C-4	C-5	C-6	C-7
TNT	8.95 (s)		2.64 (s)	134.9	153.0	123.6	147.5			15.7
2A46DNT	7.7 (d)	7.8 (d)	2.22	126.2	150.1	117.2	147.4	108.7	150.9	13.6
4A26DNT	7.21 (s)		2.21	112.8	153.8	113.1	149.6			13.8
4HA26DNT	7.49 (s)		2.32	116.6	153.5	112.3	152.9			14.0
24DA6NT	6.21 (d)	6.45 (d)	2.04	106.5	149.6	101.4	148.0	106.1	153.8	12.0

compound	H-3	H-3′	H-5	H-5′	H-7	H-7′
2,2′azoxy	9.32 (d)	9.19 (d)	9.02 (d)	8.80 (d)	2.65	2.76
4,4′azoxy	8.99 (s)		9.15 (s)		2.65	2.68

and 4A26DNT accumulated to a combined maximum concentration on day 14, while the azoxy compounds had a maximum concentration on day 5 and then decreased to trace levels by day 15. The total initial ^{14}C in the extracellular medium decreased to 60% by day 9. At the completion of the study, 41% of the initial ^{14}C in the extracellular media was in forms that could not be confirmed using HPLC protocols and were likely polar oxidation products previously identified (*16*).

Transformation of 2HA46DNT and 4HA26DNT. The results from temporal sampling and HPLC analysis of 2HA46DNT and 4HA26DNT transformation are shown in Figures 3 and 4. In the control test of 4HA26DNT (Figure 3A) and 2HA46DNT (Figure 4A), an abiotic transformation of hydroxylaminonitrotoluenes in aqueous solution occurred at a relatively rapid rate. Initially the yellow solution turned clear, and then a white precipitate was observed. By comparing the retention time and UV spectra with standards, the white precipitate was confirmed as 2,2′azoxy and 4,4′azoxy, which were the same compounds as those observed during TNT phytotransformation. Following the extraction of the precipitates, the yields of 2,2′azoxy and 4,4′azoxy products were 91% and 89%, respectively.

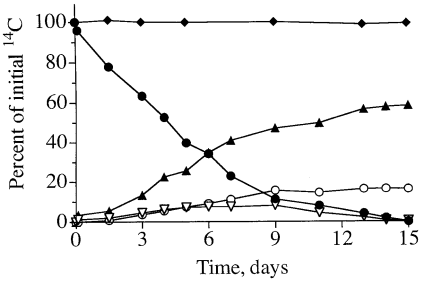


FIGURE 2. Results obtained from the HPLC fractionation of TNT phytotransformation. TNT (●), 2HA46DNT and 4HA26DNT (▽), 2A46DNT and 4A26DNT (○), TNT (control) (◆), and water-soluble ^{14}C in extracellular medium (▲).

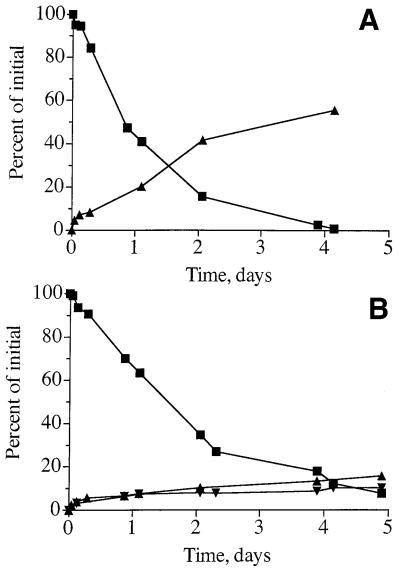


FIGURE 3. (A) Results obtained from the HPLC fraction of 4HA26DNT control test: 4HA26DNT (■) and 4,4′azoxy (▲). (B) Results obtained from the HPLC fraction of 4HA26DNT phytotransformation: 4HA26DNT-(■), 4,4′azoxy (▲), and 4A26DNT (▼).

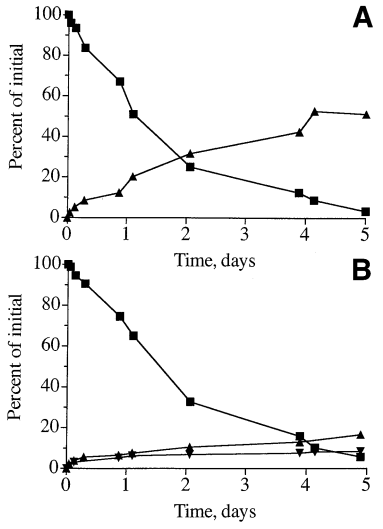


FIGURE 4. (A) Results obtained from the HPLC fraction of 2HA46DNT control test: 2HA46DNT (■) and 2,2′azoxy (▲). (B) Results obtained from the HPLC fraction of 2HA46DNT phytotransformation: 2HA46DNT (■), 2,2′-azoxy (▲), and 2A46DNT (▼).

During the phytotransformation of 4HA26DNT (Figure 3B) and 2HA46DNT (Figure 4B), most of the initial compounds had disappeared within 120 h, with the production

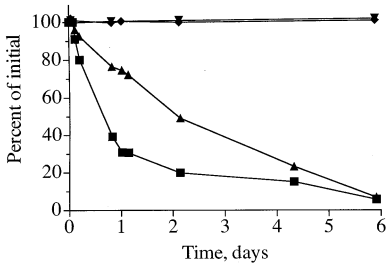


FIGURE 5. Results obtained from the HPLC fractionation of azoxy compounds phytotransformation: 4,4′azoxy (■), 2,2′azoxy (▲), 4,4′azoxy control (▼), and 2,2′azoxy control (◆).

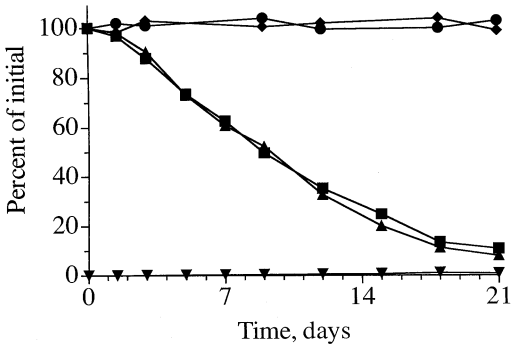


FIGURE 6. Results obtained from the HPLC fractionation of amino compounds phytotransformation: 2A46DNT (■), 4A26DNT(▲), 24DA6NT (▼), 2A46DNT control (◆), and 4A26DNT control (●).

of both amine compounds (2A46DNT and 4A26DNT) and both azoxy compounds (2,2′azoxy and 4,4′azoxy). In contrast to studies with TNT as the parent compound, the phytotransformation of hydroxylamines resulted in higher levels of azoxy compound formation and at a faster rate.

Transformation of 2,2′azoxy and 4,4′azoxy. The results of HPLC analysis of the 2,2′azoxy and 4,4′azoxy transformations are shown in Figure 5. Both compounds were stable in the control systems, but both were rapidly removed from solution when plants were present. Both 2,2′azoxy and 4,4′azoxy were rapidly depleted within 140 h of exposure, and no degradation products from either azoxy transformation were observed to accumulate according to HPLC analysis.

Transformation of 2A46DNT and 4A26DNT. The results of HPLC analysis of the 2A46DNT and 4A26DNT transformations are shown in Figure 6. While 2A46DNT and 4A26DNT were stable in the controls, both 2A46DNT and 4A26DNT were slowly transformed in the plant systems. After 21 d of exposure, a small amount of the original 2A46DNT and 4A26DNT remained in the aqueous phase of plant systems, but only 1% of the products could be identified as 24DA6NT. The identities of the other products were unconfirmed.

Kinetic Analysis. Data taken from the plant studies and the abiotic transformation of 4HA26DNT and 2HA46DNT were analyzed using the integrated form of eqs 1 and 2 yielding eq 3:

$$\ln\frac{C}{C_o} = -k^{obs}t \qquad (3)$$

where C_o is the initial concentration (mM), C is the concentration (mM) at any time t (h), and k^{obs} is the observed rate coefficient determined by linear regression. Results of this analysis are presented in Table 2. In the case of biotic systems, it is assumed that the k^{obs} obtained is a function of P (g/L). Therefore, Table 1 also presents the k^{bio} (L h^1 P^{1-}) by dividing the k^{obs} by P in experimental systems.

TABLE 2. Calculated Pseudo-First- and Second-Order Rate Constants Observed for Various Parent Compounds in Biotic and Abiotic Systems[a]

compound	k^{obs} (h^{-1})	r^2	k^{bio} ($L\ h^{-1}\ P^{1-}$)
TNT			
biotic	$1.1 \times 10^{-2} \pm 0.1 \times 10^{-2}$	0.97	3.6×10^{-4}
abiotic	nobsd	na	na
4HA26DNT			
biotic	$2.1 \times 10^{-2} \pm 0.1 \times 10^{-2}$	0.99	0.7×10^{-3}
abiotic	$4.5 \times 10^{-2} \pm 0.2 \times 10^{-2}$	0.98	na
2HA46DNT			
biotic	$2.6 \times 10^{-2} \pm 0.1 \times 10^{-2}$	0.98	8.6×10^{-4}
abiotic	$2.6 \times 10^{-2} \pm 0.1 \times 10^{-2}$	0.98	na
4A26DNT			
biotic	$4.7 \times 10^{-3} \pm 0.3 \times 10^{-3}$	0.98	1.5×10^{-4}
abiotic	nobsd	na	na
2A46DNT			
biotic	$5.3 \times 10^{-3} \pm 0.3 \times 10^{-3}$	0.97	1.7×10^{-4}
abiotic	nobsd	na	na
4,4′azoxy			
biotic	$1.9 \times 10^{-2} \pm 0.2 \times 10^{-2}$	0.89	6.3×10^{-4}
abiotic	nobsd	na	na
2,2′azoxy			
biotic	$1.7 \times 10^{-2} \pm 0.1 \times 10^{-2}$	0.97	5.7×10^{-4}
abiotic	nobsd	na	na

[a] nobsd, not observed; na, not available.

Discussion

The purpose of these studies was to evaluate how initial reduction reactions may contribute to the product distribution of TNT phytotransformation using the aquatic plant *M. aquaticum*. Experimental results of TNT phytotransformation were consistent with previous reports demonstrating the plant's ability to transform TNT resulting in both extracellular and plant associated fractions (*11*). In this research, however, six transformation products were observed in the extracellular medium of the plant system, including 2HA46DNT, 4HA26-DNT, 2A46DNT, 4A26DNT, 2,2′azoxy, and 4,4′azoxy.

From this study, 2HA46DNT and 4HA26DNT are established as the initial intermediates, in contrast to previous studies using diverse plant systems that have generally cited aminodinitrotoluenes as the primary intermediates in TNT phytotransformation (*6*, *9*, *11*, *16*). Low concentrations of 2A46DNT and 4A26DNT were observed in *M. aquaticum* and *Catharanthus roseus* hairy root culture (*10*). Other primary products that have been reported include conjugated compounds (*6*) and oxidation compounds (*16*). To the best of our knowledge, 2HA46DNT from TNT phytotransformation has not previously been identified, although the role of hydroxylamines as important intermediates was proposed earlier (*11*).

The hydroxylamines can be further reduced to aminodinitrotoluenes (2A46DNT and 4A26DNT) or undergo oxidation to nitroso intermediates that then form azoxy compounds (2,2′azoxy and 4,4′azoxy). The abiotic experimental results of 2HA46DNT and 4HA26DNT transformations in aqueous solution demonstrate that hydroxylamines are unstable in the presence of oxygen and form azoxy compounds at a rapid rate with near stoichiometric yields (*19*). The oxidation reaction was more rapid than the reduction reaction, but aminodinitrotoluenes were observed in the extracellular medium. The formation of aminodinitrotoluenes and azoxy dimers requires partial reduction via hydroxylamine intermediates (*20*). Experimental results of the transformation of 2A46DNT and 4A26DNT indicated that plants take up and transform aminodinitrotoluenes at a slower rate than other reactions. 24DA6NT was a minor product after 2A46DNT and 4A26DNT were completely transformed, indicating that further reduction is possible but that the formation of a second amine group is not favorable. The identification of the compounds in this study was facilitated by the HPLC method employed, and care was taken to avoid decomposition. Critical to the HPLC approach is the ability to resolve hydroxylamines from corresponding amines since they have some similar spectral properties (*6*). Decomposition was avoided by minimizing storage times during sample handling; otherwise the hydroxylamines would have decomposed, leaving only low levels of stable amines (*21*).

Previous studies that used TNT as the starting material to identify phytotransformation intermediates have not recovered azoxy compounds. According to the research presented here, 2,2′azoxy and 4,4′azoxy were very stable in plant-free controls but were taken up and transformed by plants at rapid rates. The rapid uptake of the azoxy compounds observed in this study explains why the concentration of azoxy compounds was extremely low in the TNT phytotransformation system and perhaps why they have not been monitored as a phytotransformation product. Extraction of plant biomass did not recover the azoxy compounds, implying that they are subject to further transformation by the plant. This hypothesis is supported by previous studies that specifically investigated the nature of plant associated residues and found that the bulk of metabolites in the plant biomass were in a "bound residue" form (*11*).

An important consideration that was not evaluated in this study is the effect of plant concentration (or net activity) on the relative proportions of azoxy compound formation as compared to reduced aminated derivatives. On the basis of the control studies, oxidation of hydroxylamines is independent of the metabolic activity of the plant. Reduction of hydroxylamines is a plant-mediated reaction, which may be enhanced relative to abiotic transformations by increasing the plant concentration. Differences in plant concentration (or net activity) may explain why the formation of azoxy compounds is not always reported and may also influence the degree to which aminated forms accumulate.

By combining previously reported TNT phytotransformation end products and the intermediates observed in this study, the initial steps of the TNT phytotransformation pathway in *M. aquaticum* can be deduced, as shown in Figure 7. In plant systems, TNT was taken up and transformed to 4HA26DNT and 2HA46DNT through a plant-mediated reduction. Then two different reactions compete for the hydroxylamines. 4HA26DNT and 2HA46DNT can be abiotically oxidized to reactive nitroso derivatives that undergo bimolecular nucleation to form azoxy compounds, or they can undergo a slower, plant-mediated reduction to form aminodinitrotoluenes (4A26DNT and 2A46DNT). Under the experimental conditions used in these studies, it appears that the further reduction of the hydroxylamines to form mono- and diaminonitrotoluenes was unfavorable, although they were produced to varying degrees. Under these conditions, it appears that the oxidation pathway would constitute the majority of the metabolite flux. However, the rapid uptake of azoxy compounds by the plants results in their low levels in solution when TNT is fed directly to the plants. Clearly, both the azoxy and aminated products need to be investigated in order to account for further transformation products.

Certain similarities can be identified when comparing the results of TNT phytotransformation by plants to the hydroxylamine pathways of TNT transformation by anaerobic microorganisms that we have previously reported (*13*, *22*). Both pathways begin with the formation of hydroxylaminodinitrotoluene intermediates. However, while anaerobic fermentor pathways lead to the accumulation of high concentrations of primary monohydroxylamine intermediates, the hydroxylaminodinitrotoluenes in plant systems

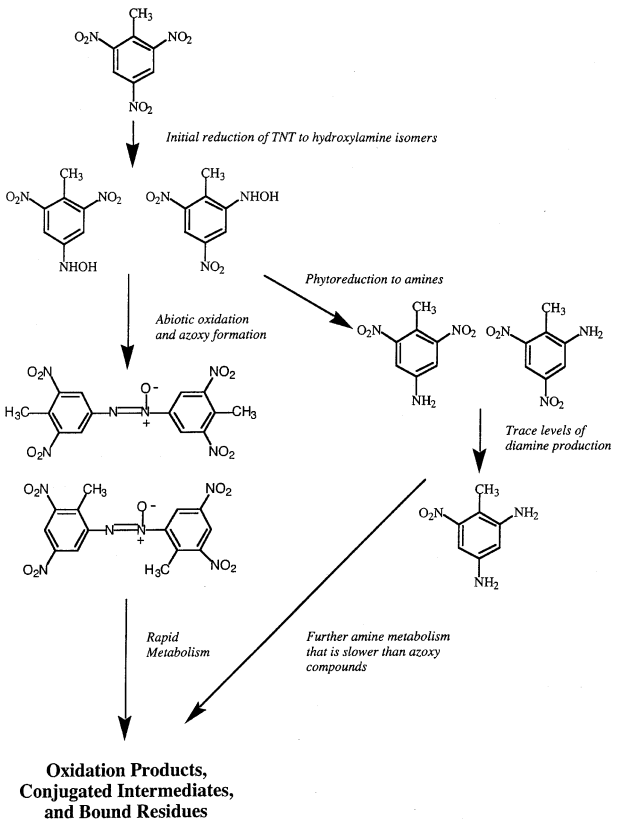


FIGURE 7. Initial steps of theTNT phytotransformation pathway in *M. aquaticum* and previously identified end products.

undergo further reduction to the central metabolite 2,4-dihydroxylamino-6-nitrotoluene and other products. An obvious difference between the TNT phytotransformation and anaerobic microbial TNT transformation is the presence of oxygen in the plant system. The presence of oxygen and the slow reduction rate of the hydroxylamine group make oxidation of the hydroxylamine to a nitroso group favorable and results in the scavenging of other hydroxylamines to form azoxy metabolites. These azoxy compounds can be rapidly taken up by the plant and apparently transformed. To what extent the transformation of azoxy compounds may contribute to the formation of bound residues, conjugated intermediates, or oxidation products is not clear at this time, and the characterization of that process requires further study.

The pathway to diverse products of TNT metabolism (i.e., conjugated compounds, bound residues, and oxidation products) carried out by plants becomes complex even in the first several steps. Additionally, the competing rates of early metabolite formation may differ based on experimental conditions, resulting in varying results from study to study. On the basis of the slow transformation rates of 2A46DNT and 4A26DNT as compared to the relatively fast transformation rates of azoxy compounds observed in this work, it may be advantageous to consider operational conditions that favor hydroxylamine oxidation via abiotic mechanisms. This would minimize the extent to which the washout of aminodinitrotoluenes may occur from phytoremediation systems and promote further metabolism by plants. Also, the oxidation process is facilitated by lower levels of plant activity and/or density. Further studies are required to characterize the transformation process of azoxy dimers and to determine their role in the formation of bound and water soluble transformation endpoints.

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