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TRANSFORMATON OF 2,4,6-TRINITROTOLUENE BY THE AQUATIC PLANT *MYRIOPHYLLUM SPICATUM*

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Abstract—The ability of the aquatic plant Eurasian Watermilfoil (*Myriophyllum spicatum*) to transform 2,4,6-trinitrotoluene (TNT) was investigated in a series of batch assays. The TNT was added to plant cultures in single and multiple consecutive additions, at various initial concentrations, to determine its transformation kinetics, identify products formed, evaluate phytotoxic effects, and to determine the effect of light deprivation on the TNT transformation process. Rapid disappearance of TNT from the plant culture media was observed. The TNT disappearance rate was a function of both plant and TNT concentration (i.e., followed mixed, second-order kinetics). The TNT transformation occurred only in the presence of plants and was inhibited by the addition of sodium azide. Phytotoxicity leading to plant chlorosis was observed in batch plant cultures with an initial TNT concentration above 5.9 μM. Reductive transformation of TNT to aminodinitrotoluenes and lower levels of hydroxylaminodinitrotoluene and diaminonitrotoluenes detected in the plant culture media accounted for less than 10 to 20% of the total TNT mass added. Extraction of plant material at the end of batch incubations when all TNT was depleted from the media yielded low levels of TNT and aminodinitrotoluenes and accounted for only 3.4% of the initially added TNT mass. Light deprivation decreased both the rate and extent of the reductive transformation of TNT.

Keywords—Aquatic plants *Myriophyllum spicatum* Phytoremediation Phytotoxicity Trinitrotoluene

INTRODUCTION

2,4,6-Trinitrotoluene (TNT) has been widely used as the primary explosive for both military and civilian purposes. However, soil, surface water, and groundwater contamination have resulted because of past poor maintenance of TNT manufacturing and handling facilities as well as land disposal of munitions wastes. Because of the toxic and mutagenic effects of TNT and its derivatives [1–11], remedial action at many former military munitions facilities is required. Currently, soil excavation and incineration is the most common remediation technology, but it is energy intensive and costly [12]. As a result, less costly alternative technologies such as bioremediation have been tested [13,14].

The transformation pathways of TNT and other explosives commonly observed in microbial systems have recently been reviewed [15,16]. Although TNT mineralization has been observed, complete mineralization has not been demonstrated [17–22]. Although removal of a nitro group of TNT as nitrite has been observed [23–25], reductive transformation of TNT is the most often observed pathway. The reduction of a nitro group of nitroaromatic compounds proceeds in three steps, each requiring two electrons, as follows [26]: R-NO_2 → R-NO (nitrosamine) → R-HNOH (hydroxylamine) → R-NH_2 (amine). Progressive reduction of all three nitro groups of TNT leads to the formation of aminodinitrotoluene isomers (ADNT), then to diaminonitrotoluene isomers (DANT) and finally to triaminotoluene (TAT) [16]. An alternate reaction is the condensation of nitroso and hydroxylamino groups of different molecules resulting in the formation of azoxy isomers (tetranitro-azoxytoluenes) [27,28].

Relative to the wealth of information related to the microbial transformation of TNT, very little is known about the uptake and transformation of TNT in plants. Uptake of TNT by terrestrial plants and recovery of aminonitrotoluenes from plant tissues has been reported [8,29]. Only recently, disappearance of TNT from aqueous solutions in the presence of several species of aquatic plants and production of low levels of aminonitrotoluenes has been demonstrated [30–33]. It was suggested that a nitrate reductase enzyme isolated from both aquatic sediments and aquatic plants mediated the observed reductive transformation of TNT in laboratory systems containing either aquatic plants or sediments [30]. Similarly, enzymes extracted from aquatic sediments reduced TNT to mono- and diaminonitrotoluene isomers with nicotinamide adenosine dinucleotide phosphate (NADPH) as an electron donor under aerobic conditions [34].

The kinetics of TNT transformation as well as phytotoxicity effects were not delineated in the above-mentioned studies of TNT transformation in the presence of aquatic plants. The objectives of the work reported in this article were to assess the kinetics of TNT transformation and product formation by the aquatic plant *Myriophyllum spicatum*, as well as to examine potential phytotoxicity effects, and the effect of long-term light deprivation on the TNT transformation process.

MATERIALS AND METHODS

Chemicals

The TNT was purchased from Chem Service (West Chester, PA, USA). Stock solutions of TNT were prepared by dissolving pure TNT in filtered tap water at about 80°C with rapid mixing in the dark. The solutions were filtered through 0.2-μm membrane filters and analyzed by high-performance liquid chromatography (HPLC). The following compounds were purchased from SRI International (Menlo Park, CA, USA): 4-

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hydroxyamino-2,6-dinitrotoluene (4HA26DNT); 2-amino-4,6-dinitrotoluene (2A46DNT); 4-amino-2,6-dinitrotoluene (4A26DNT); 2,4-diamino-6-nitrotoluene (24DA6NT); 2,6-diamino-4-nitrotoluene (26DA4NT); 4,4′,6,6′-tetranitro-2,2′azoxytoluene (2-2′ Azy); 2,4′,6,6′-tetranitro-2,4′azoxytoluene (2-4′ Azy); and 2,2′,6,6′-tetranitro-4,4′azoxytoluene (4-4′ Azy).

Plants

Myriophyllum spicatum plants and sediment were collected from shallow ponds at the Alabama Army Ammunition Plant, Childersburg, Alabama, USA. The plants with their associated sediment were placed in a 200-L glass aquarium that was then filled with activated carbon-filtered tap water. The plant aquarium was kept at room temperature (22 ± 2°C) and placed near a window that provided natural light. To supplement the natural light, two 48-inch long, 40-W cool-white fluorescent lamps were also used with a 16-h photoperiod and at a light intensity of 100 μ Einstein m^{-2} s^{-1}. Porous stones and compressed air were used to provide good mixing in the aquarium water column.

Batch assays

Several batch assays were performed following the same general procedure described below. Aliquots of 300 ml of an inorganic nutrient solution that contained 3.5 ml of Schultz's liquid nutrient media (10% N, 15% P_2O_5, and 10% K_2O by weight) per liter of chlorine-free tap water were added to 800-ml polycarbonate beakers. The pH of the nutrient solution was adjusted to 7.8 with 1 N $NaHCO_3$ to provide a carbon source for plant growth as well as to buffer the solution. Whole plants were removed from the plant aquarium after careful selection to provide a uniform population (i.e., similar proportion of roots, stems, and leaves), rinsed thoroughly with tap water, weighed, and placed in the beakers that contained the TNT-free nutrient solution. The plant concentration (wet weight basis) in the beakers was 33.3 g/L. The nutrient solution was aerated with compressed air delivered through glass Pasteur pipettes. A 7-d adjustment period was allowed before the beginning of the batch assay. Then, appropriate aliquots of stock TNT solution were added to the beakers to achieve the desired initial concentrations. All treatments were performed in triplicate. Contaminant-free plant controls as well as plant-free, contaminant-amended controls were also kept under the same experimental conditions to evaluate plant health and to determine any losses due to adsorption and/or photolytic breakdown of TNT. Clear plastic bags were used to cover the beakers and minimize evaporative losses of water during the incubation period. Periodically, liquid samples were removed, centrifuged, and analyzed following procedures described below. All batch assays were performed at room temperature (22 ± 2°C) and under the same light and photoperiod conditions used for the plant stock culture, unless otherwise specified.

Analyses

Liquid samples (2.5 ml) were removed from the experimental beakers using glass Pasteur pipettes and transferred into 3-ml polycarbonate centrifuge tubes and centrifuged at 16,000 *g* for 15 min using an Eppendorf model 5415C microcentrifuge. Then, 2 ml of the sample supernatant was transferred to a 2.5-ml autosampler vial, capped with a Teflon-lined septum, and aluminum crimped. The vials were either analyzed immediately or stored in the dark under refrigeration at 4°C until they were analyzed. Repeated analyses of the same sample over a storage period of a week did not show any losses of TNT or 4A26DNT (coefficient of variation less than 2%).

The TNT and its transformation products were analyzed on a Hewlett Packard 1050 series HPLC system equipped with a diode array detector operating in the 200- to 450-nm wavelength range and 25-cm LC-18-Db reverse-phase 5-μm silica column (Supelco, Bellefonte, PA, USA). A guard column (All-Guard Adsorboshpere C18-5V; Alltech Associates, Deerfield, IL, USA) was used for column protection and replaced regularly. The eluent consisted of 70% acetonitrile and 30% 0.005 M KCl solution for sample and eluent stability. Sample volumes of 20 μl were injected from 2.5-ml glass autosampler vials.

Using stock solutions, calibration curves and UV scans were prepared for the following compounds: TNT, 4HA26DNT, 4A26DNT, 24DA6NT, 2-2′ Azy, 2-4′ Azy, and 4-4′ Azy. Two compounds, 4A26DNT and 2A46DNT coeluted. Likewise, the compounds 24DA6NT and 26DA4NT also coeluted. However, for routine HPLC calibrations, only 4A26DNT and 24DA6NT were used and the HPLC data were reported in terms of 4A26DNT and 24DA6NT for both mono-amino- and diamino- compounds, respectively. As a result, the designations ADNT and DANT were used throughout this work. A mixed standard solution in acetonitrile and a blank were analyzed at the beginning and end of each 21-vial sequence for analytical quality control. The method detection limit for all analytes tested was 0.44 μM. Periodically, samples analyzed at the start of the sequence and stored in the autosampler tray were analyzed again after 24 h. Less than 2% difference in all analytes was observed, thus verifying analyte stability in the autosampler vials while awaiting analysis.

Extraction of plant material was performed at the end of several batch assays to recover TNT and transformation products. Plants were removed from the assay beakers, blotted dry, placed in plastic bags, and frozen until extraction was performed. The plants were removed from the freezer, placed in a large ceramic mortar, and exposed to liquid nitrogen. The frozen plants were then ground with a pestle, weighed, and placed in a 300-ml glass jar. Acetonitrile was then added to the glass jar at a ratio of 5 ml/g of plant material. The glass jar was sealed and shaken on a shaker table for 24 h. Supernatant was then drawn off and passed through a Supelclean LC-18 6-ml cartridge tube (Supelco) to remove plant pigments that interfered with the HPLC analysis of plant extracts. The recovery of TNT and other nitroaromatic compounds in spiked samples subjected to the same cartridge separation procedure varied between 95 and 100%.

Data analysis

Based on experimental observations (reported here and other preliminary assays), it was concluded that the kinetics of TNT disappearance could be described by a mixed, second-order rate expression as follows:

$$-\frac{dC}{dt} = KPC \tag{1}$$

where C = TNT concentration (μM), t = time (h), P = wet plant concentration (g/L), and K = second-order TNT disappearance rate constant (L/g h). By assuming the plant concentration constant throughout the incubation period, Equation 1 reduces to

Table 1. Extent of 2,4,6-trinitrotoluene (TNT) removal, pH variation, and observed TNT disappearance rate constants in *Myriophyllum spicatum* cultures at six initial TNT concentrations

Initial TNT concentration (μM)	TNT removal (%)[a]	Final pH[b]	First-order rate constant (k, per h)	r^2	Plant-normalized rate constant (K, ml/g h)
5.8 ± 0.1[c]	100	6.1 ± 0.3[c]	0.142 ± 0.028[d]	0.850	4.26 ± 0.84[d]
22.7 ± 0.3	100	6.0 ± 0.2	0.080 ± 0.016	0.856	2.40 ± 0.48
49.0 ± 0.5	100	6.7 ± 0.9	0.065 ± 0.030	0.630	1.95 ± 0.90
127.4 ± 1.2	98.2 ± 3.2	7.0 ± 0.4	0.017 ± 0.005	0.807	0.51 ± 0.15
259.5 ± 2.4	79.4 ± 4.6	7.8 ± 0.2	0.013 ± 0.002	0.955	0.39 ± 0.06
500.4 ± 2.4	57.0 ± 12.3[e]	8.0 ± 0.2	0.029 ± 0.013	0.754	0.87 ± 0.39

[a] After 123 h of incubation.
[b] Initial pH was 7.8 ± 0.1.
[c] Mean ± standard deviation.
[d] ±95% confidence interval.
[e] Not measured at 123 h (reported value is at 22 h of incubation).

$$-\frac{dC}{dt} = kC \quad (2)$$

where $k = KP$ = first-order TNT disappearance rate constant (per h) for a given and constant plant concentration. In turn, because $K = k/P$, K can be considered as the plant-normalized TNT disappearance rate constant. Integration of Equation 2 leads to

$$\ln\left(\frac{C}{C_0}\right) = -kt \quad (3)$$

Equation 3 was used to determine the value of k based on TNT concentration data over the incubation period using linear regression.

RESULTS AND DISCUSSION

Effect of initial TNT concentration on phytotoxicity and TNT transformation

Following the above-described standard assay protocol, triplicate plant samples at a concentration of 33.3 g/L (wet weight) were exposed to a wide range of initial TNT concentrations ranging from 5.9 to 500 μM. Six plant-free controls containing only the TNT solution at six TNT concentrations were also used to determine any adsorptive losses and potential photolytic breakdown of TNT. A control containing *M. spicatum* plants in a TNT-free nutrient solution was also used for plant health comparisons in the evaluation of phytotoxic effects.

Phytotoxic effects. Throughout this experiment, plant appearance was qualitatively evaluated by comparing the conditions of the plants in each TNT-amended plant culture to that of the plants in the TNT-free samples. Increased chlorosis (i.e., plants turning yellow to brown), leaf loss, and lack of new growth were used as indicators of phytotoxic effects. Prior to the beginning of this experiment, all of the plants were allowed to adjust to their new environment for 7 d before the addition of TNT. At the end of this adjustment period, all plants appeared healthy (i.e., the plants had green leaves and shoots). All plants at the higher concentrations of TNT (262 and 500 μM) showed chlorosis after 7 to 27 h of incubation. Several of the plants at the 49 μM and 127 μM initial TNT concentrations remained green and healthy for about 51 to 98 h; however, chlorosis was evident by 123 h of incubation. Three of the plant samples (two at 5.9 μM and one at 20 μM initial TNT concentration) showed new growth during this experiment. After 123 h of incubation, all of the 5.9 μM, two of the 23 μM, and one of the 49 μM initial TNT plant samples had green, living plants, whereas the remainder of the plants in the TNT-amended samples were yellow-brown. The control plants were green with new growth, indicating that TNT and/or TNT transformation products had caused the observed phytotoxic effects. In order to verify further these phytotoxic effects, all plant samples were incubated for another 2 weeks (330 h) and plant appearance was evaluated again. The only living plants after a total incubation time of 460 h were those exposed to an initial TNT concentration of 5.9 μM and the control (i.e., TNT-free) plants. A drop of the initial pH values was observed in plant samples that remained green, possibly through the utilization of bicarbonate (Table 1). The TNT-free plant control also had a lower pH (5.9) at the end of the experiment, further suggesting that the pH decrease was not caused by the TNT transformation but rather was plant mediated. As a result of lack of plant growth, the pH values in the reactors with high initial TNT concentrations remained practically unchanged compared to the initial pH values (Table 1).

Based on the results of this experiment, TNT concentrations between 5.9 and 23 μM (and higher) were considered toxic to *M. spicatum.* The TNT has been determined to be toxic to a number of photosynthetic organisms. The TNT inhibited the growth of freshwater algae at concentrations of 2 to 15 mg/L [1–3] and was toxic to the aquatic plant duckweed (*Lemna minor*) at concentrations above 1 mg/L [35] and to the terrestrial plant yellow nutsedge (*Cyperus esculentus* L.) at TNT concentrations of 5 mg/L and higher [8].

It is noteworthy that TNT transformation in the present study continued in those samples containing plants with demonstrated phytotoxic effects. Such an observation raises questions as to the role of plant growth, adsorption, and sequestration of TNT and/or transformation products within the plant matrix or even microbial processes in the observed disappearance and transformation of TNT. Additional batch experiments described below addressed these questions.

TNT transformation. All plant samples showed a decrease in TNT concentration over the incubation period. Figure 1A shows the mean TNT profile in the triplicate plant samples with a mean initial TNT concentration of 49 μM. The first-order TNT disappearance rate constants were calculated based on Equation 3 and are reported in Table 1. At all initial TNT concentrations, and with the same plant concentrations (33.3 g/L), the TNT disappearance followed first-order kinetics with respect to the TNT concentration. Although identical plant weights were used, with similar proportions of leaves, stems, and roots, the TNT disappearance rates were not consistent

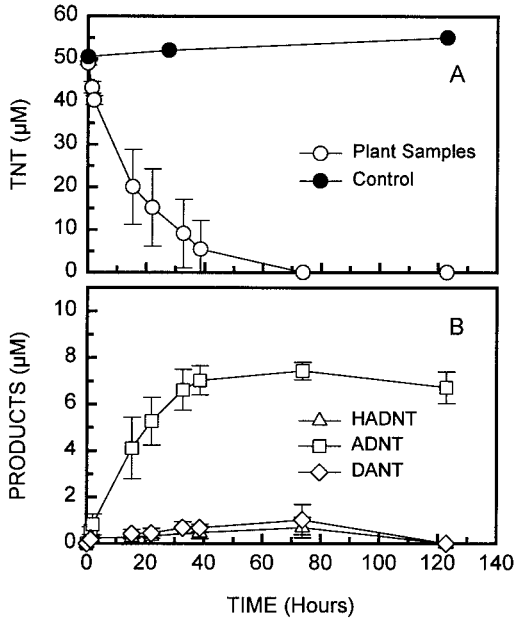

Fig. 1. Concentration profiles of 2,4,6-trinitrotoluene (TNT) (**A**) and transformation products (**B**) during the incubation of triplicate plant samples with an initial TNT concentration of 49 μM (error bars represent one standard deviation of the means).

among the three replicate plant samples at the same initial TNT concentration (Fig. 2A). The rate variation among triplicate plant samples may be attributed to differences in plant viability and/or stored reducing power within otherwise similar plants. Due to the observed variability in the TNT disappearance rates among the plant triplicates, regression of all data points was used to arrive at the overall TNT disappearance rate constant for each triplicate plant set (Fig. 2B).

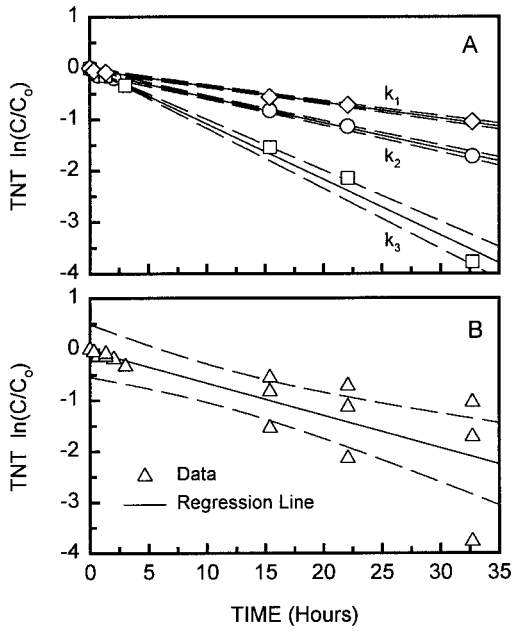

Fig. 2. Regression of 2,4,6-trinitrotoluene (TNT) concentration data over the incubation period in the three replicate plant samples individually (**A**: k_1 = 0.031 ± 0.004 per h, r^2 = 0.995; k_2 = 0.051 ± 0.004 per h, r^2 = 0.998; k_3 = 0.111 ± 0.023 per h, r^2 = 0.987) and collectively (**B**: k = 0.065 ± 0.030 per h, r^2 = 0.630) (initial TNT concentration 49 μM; broken lines and rate constant ± values represent 95% confidence intervals).

Table 2. Compounds identified in plant extracts at the end of a 123-h 2,4,6-trinitrotoluene (TNT) batch transformation assay

Initial TNT concentration (μM)	Compounds in plant extracts (μM)[a]	
	TNT	Aminodinitrotoluene
5.8 ± 0.1[b]	BDL[c]	0.3
22.7 ± 0.3	BDL	0.7
49.0 ± 0.5	BDL	1.5
127.4 ± 1.2	BDL	3.2
259.5 ± 2.4	2.9	3.5
500.4 ± 2.4	11.1	5.9

[a] Extraction concentrations are normalized to reactor volume.
[b] Mean ± standard deviation.
[c] BDL = below detection limit (0.44 μM).

The observed first-order TNT disappearance rate constants followed a trend related to initial TNT concentration. Higher initial TNT concentrations resulted in a lower rate constant, as shown in Table 1. It is noteworthy that initial TNT concentrations above 5.9 μM were toxic to *M. spicatum* plants. Therefore, the lower TNT disappearance rates associated with the higher initial TNT concentrations may be attributed to phytotoxic effects of TNT and/or transformation products. Although the TNT disappearance rate constants at higher initial TNT concentrations were low, the plants removed a larger mass of TNT over time as compared to those plants exposed to relatively low initial TNT concentrations. Plant samples with an initial TNT concentration of 262 μM showed an average decrease of 77 μM TNT over the first 22 h of incubation, whereas those with an initial TNT concentration of 500 μM had an average decrease of 286 μM TNT over the same period.

The TNT transformation products were not detected in the TNT solutions at the beginning of the experiment. However, as the TNT concentration decreased during the incubation period, the concentration of ADNT in the plant culture media increased (Fig. 1B). Lower concentrations of 4HA26DNT and DANT were detected, but by the end of the incubation period these compounds were not detected in the culture media. Traces (i.e., less than the equivalent of 0.4 μM of TNT) of the 2-2′ Azy compound were occasionally detected in the media and also vanished by the end of the incubation period. On a molar basis, the amount of ADNT formed ranged from 12 to 19% of the initially added TNT in the 500 μM and 5.9 μM initial TNT concentration samples, respectively. After the TNT was depleted from the culture media, ADNT also gradually disappeared but at a much lower rate (over 150-fold) than the TNT. Hughes et al. [32] also observed low levels of ADNTs in the liquid media as a result of TNT transformation by *M. spicatum*, *Myriophyllum aquaticum*, and *Catharanthus roseus* hairy root cultures.

At the end of this assay, extractions of the plant material were performed in an attempt to provide a mass balance and determine if TNT or transformation products were being accumulated within the plant material. Only TNT and ADNT were detected in the plant extracts (Table 2). From these extraction results, it is concluded that very little of the initial TNT was accounted for, possibly remaining within the plants in an unextractable form and/or existing in a form undetectable by the employed HPLC method (in the culture media and/or plant extracts). In a similar study, after the complete disappearance of TNT from *M. spicatum* cultures, 4A26DNT and 2A46DNT were detected in the medium but accounted for less than 14% of the initially added TNT moles [32]. These two

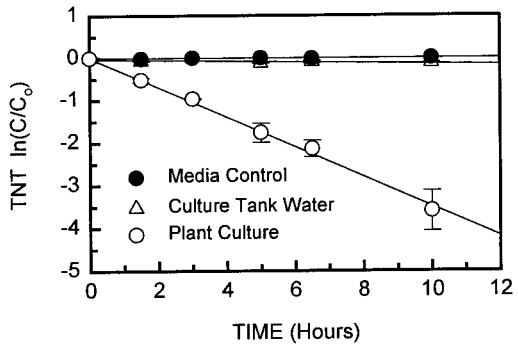

Fig. 3. Regression of 2,4,6-trinitrotoluene concentration data over the incubation period in triplicate, plant-free samples with culture tank water (k = 0.010 ± 0.004 per h; r^2 = 0.855), plant samples (k = 0.355 ± 0.010 per h; r^2 = 0.995), and plant-free media controls (error bars represent one standard deviation of data means; rate constant ± values represent 95% confidence intervals).

products and TNT were also identified in plant extracts but accounted for less than 4% of the added TNT moles.

In a related and concurrent study of TNT transformation by the aquatic plants *M. spicatum* and *M. aquaticum*, by the use of [ring-U-^{14}C]TNT it was demonstrated that TNT mineralization did not take place [32]. After the complete disappearance of TNT from the medium, about half of the label remained in the medium in a soluble form and the balance was in the plant matrix, only made available by the oxidation of the plant material. It is noteworthy that only about half of the label recovered by plant oxidation was obtained by plant lyophilization and methanol extraction, thus providing evidence of sequestration (perhaps conjugation) of TNT and/or its transformation products with the plant matrix [32]. Transformation of [U-ring-^{14}C]TNT by *M. aquaticum* plants resulted in the accumulation of approximately 30% of the initially added ^{14}C-labeled TNT mass as products in the culture medium, and the remaining fraction was retained inside the plant matrix, mostly in the roots [33].

In another study on the uptake and transformation of ^{14}C-TNT in hydroponic cultures of bush bean plants (*Phaseolus vulgaris*), TNT and its transformation products (mainly ADNT isomers) were transported from the plant roots to the plant stem and leaves where they were further transformed to more polar metabolites [29]. A significant fraction (19%) of the TNT metabolites in bush bean plants leaf tissue was determined to be hydrochloric acid-hydrolyzable conjugates, whereas about 40% of the radiolabel could not be extracted from the plant tissue and was considered to be conjugated.

Role of plants in the transformation of TNT

In order to assess the relative rate of TNT disappearance in the water column alone as compared to that associated with *M. spicatum*, three sets of triplicate plant cultures were prepared with an initial TNT concentration of 11 μM: one set having rinsed *M. spicatum* in inorganic nutrient solution; another set containing water taken from the plant culture tank and amended with the inorganic nutrient solution; and the third set containing plant-free, inorganic nutrient solution (control).

The TNT depletion rate in the plant cultures was much higher than that in the culture tank water (Fig. 3). At the conclusion of the 24-h incubation, 3 and 83% of the initial TNT concentration remained in the plant culture and culture tank water samples, respectively. Depletion of TNT was not evident in the inorganic nutrient solution control. Because microorganisms are associated with *M. spicatum* [36], it is difficult to determine the relative contribution of plants and attached epiphytic microorganisms to the observed TNT depletion. However, microscopic observations of plant sections did not reveal a significant amount of epiphytic biomass compared to the plant biomass. In contrast, microscopic observations of culture tank water revealed bacterial and algal populations at a concentration much higher than that of the epiphytic microorganisms. Therefore, most of the observed TNT disappearance in the plant cultures was plant associated. Recent studies have demonstrated the potential of axenic plant cultures to transform and sequester TNT and/or products [32,33]. It is noteworthy, that although the delineation of the relative contribution of plants and microorganisms to the observed TNT transformation rates is important, the plant/microbial systems evaluated in the present study are realistic and representative of natural systems.

Plant adsorption of TNT may account for part of the observed TNT depletion in previous experiments. In order to assess the degree of TNT depletion associated with adsorption, an experiment using sodium azide (NaN_3) was conducted. Azide blocks the electron transport in microorganisms and stops ATP synthesis [37]. Photodegradation of chlorophyl and inhibition of photosynthesis in the alga *Dunaliella bardawil* was observed with the addition of azide [38]. Low concentrations (1 μM) of sodium azide did not directly affect the process of photosynthetic oxygen evolution by mesophyll protoplasts of pea (*Pisum sativum*) but increased the extent of photoinhibition as a result of a decrease in their dark respiration rate [39]. Although the effect of sodium azide on *M. spicatum* was not known, it was assumed that 2 g/L of sodium azide would inhibit biological activity associated with the plants (at a wet plant concentration of 33.3 g/L) as well as algae and other microorganisms. Two sets of plants were prepared for this experiment. The first set consisted of triplicate plant cultures and one plant-free control amended with TNT, incubated for 10 h, drained, and amended with TNT in fresh nutrient solution for the second time. The second set consisted of triplicate plant cultures and one plant-free control amended with TNT and carried through two consecutive incubations, similar to that of the first set, except that sodium azide was added only at the beginning of the second incubation period. The plant-free control for the second set also contained 2 g/L of sodium azide to test for possible interaction between azide and TNT. The initial TNT concentration in all samples tested was approximately 11 μM.

During the first incubation period, both plant sets achieved similar TNT disappearance rates, whereas during the second incubation period, the TNT disappearance rate was dramatically reduced in the azide-amended plant cultures (Fig. 4). These results indicate that the observed TNT removal in the absence of azide was biologically mediated and not the result of adsorption. Therefore, the TNT disappearance from the plant culture media may have been the result of a combination of processes such as uptake, reaction, and sequestration (perhaps conjugation).

Effect of light deprivation on the transformation of TNT

Plant photosynthesis uses light as the energy source leading to the production of biochemical energy (e.g., ATP) and reducing power (NAD[P]H), which in turn are used for carbon fixation. The contribution of this electron source to the ob-

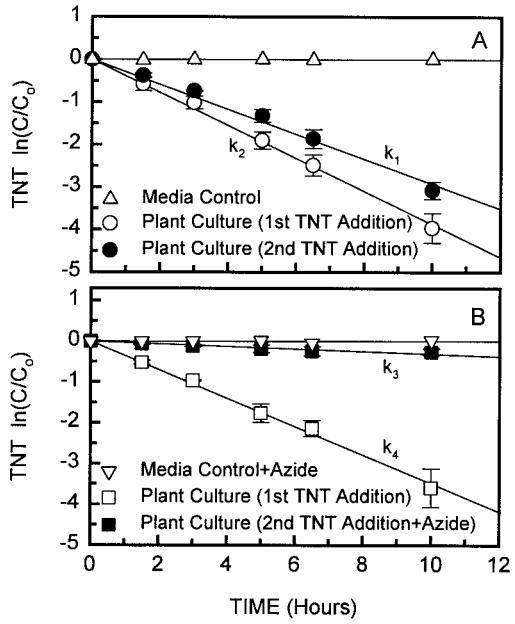

Fig. 4. Regression of 2,4,6-trinitrotoluene concentration data over the incubation period in triplicate, azide-amended and unamended plant samples (k_1 = 0.335 ± 0.011 per h, r^2 = 0.993; k_2 = 0.397 ± 0.094 per h, r^2 = 0.998; k_3 = 0.027 ± 0.004 per h, r^2 = 0.924; k_4 = 0.308 ± 0.013 per h, r^2 = 0.996) (error bars represent one standard deviation of the data means; rate constant ± values represent 95% confidence intervals).

served transformation of TNT in plant cultures was assessed by comparing the rate and extent of TNT transformation achieved in normally illuminated and light-deprived cultures of *M. spicatum*. Two sets of triplicate *M. spicatum* cultures, one set kept under the normal 16/8-h light/dark cycle, and another kept completely in the dark were used. Two plant-free samples were also prepared and served as the normal light/dark cycle and extended dark phase controls, respectively. All experimental groups (i.e., plant cultures and controls) were maintained at the same temperature (22 ± 2°C). All plant cultures were kept for 2 d under the normal light/dark cycle without any TNT addition, and then, half of the cultures were incubated under complete darkness. Four, sequential TNT additions were made, each at an initial concentration of 10.4 μM TNT. Half of the plant cultures were in the dark for a total of 56 h (16 h of which were prior to the first TNT addition).

Table 3. Extent of 2,4,6-trinitrotoluene (TNT) removal and observed TNT disappearance rate constants in *Myriophyllum spicatum* cultures kept under the normal light/dark cycle and in the dark over four consecutive TNT additions

Plant culture set TNT addition	Light/ dark	TNT removal (%)	First-order rate constant (k, per h)	r^2
Normally illuminated				
First TNT addition	Light	87.8	0.251 ± 0.033[a]	0.954
Second TNT addition	Dark	77.0	0.121 ± 0.043	0.871
Third TNT addition	Light	85.0	0.164 ± 0.035	0.916
Fourth TNT addition	Dark	64.5	0.080 ± 0.028	0.799
Light-deprived				
First TNT addition	Dark	76.6	0.169 ± 0.022	0.957
Second TNT addition	Dark	68.6	0.105 ± 0.026	0.854
Third TNT addition	Dark	49.6	0.077 ± 0.028	0.793
Fourth TNT addition	Dark	48.1	0.061 ± 0.019	0.838

[a] ±95% confidence interval.

All plants remained green throughout the incubation period. Figure 5 shows the TNT concentrations in each plant culture during the entire incubation period. Overall, the plants kept under the normal 16/8-h light/dark cycle achieved a higher extent of TNT removal than those kept in the dark (Table 3). For both plant sets, the TNT disappearance rate decreased with each subsequent TNT addition. However, the mean TNT disappearance rates observed in the normally illuminated plant cultures were consistently higher than those in the light-deprived plant cultures. The TNT concentration in the plant-free light control did not decrease over the incubation period, indicating that the difference in the observed TNT disappearance

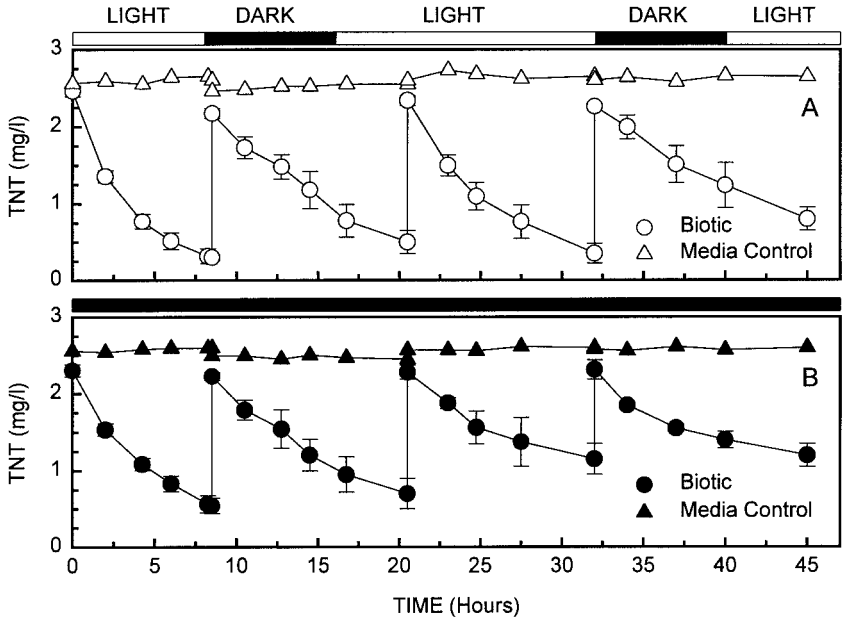

Fig. 5. 2,4,6-Trinitrotoluene concentrations over four consecutive additions in plant cultures kept under the normal light/dark cycle (**A**) and an extended dark incubation period (**B**) (error bars represent one standard deviation of the means).

rates between the two plant sets was not the result of TNT photodecomposition. For the first and third TNT addition, the difference in the mean TNT disappearance rate constants between the normally illuminated and the light-deprived plant sets was statistically significant ($\alpha = 0.05$). However, for the second and fourth TNT additions, the difference in the mean TNT disappearance rate constants between the two plant groups was not statistically significant ($\alpha = 0.05$).

The ability of the plant cultures to transform TNT even after 56 h in the dark indicates that either the plants' reducing power (produced during the light phase) was not completely exhausted or that it was regenerated via respiration and use of reverse electron flow while the plants were kept in the dark. It is also conceivable that the reducing power requirements for the reductive transformation of TNT may have been very low as compared to the pool of available reducing equivalents.

CONCLUSIONS

Rapid depletion of TNT from *M. spicatum* culture media was observed and the TNT disappearance rate was a function of both TNT and plant concentration. However, at a constant plant concentration the TNT disappearance followed first-order kinetics in batch systems. The TNT transformation occurred only in the presence of plants while TNT transformation in plant-free culture media obtained from the plant stock culture was insignificant. Use of sodium azide resulted in inhibition of the TNT transformation, indicating that the transformation was of a biological nature. In batch assays, approximately 10 to 20% of the initial TNT mass was transformed to ADNTs. The unaccounted for mass of TNT and/or transformation products was not detectable in the plant culture media by the HPLC method used nor was it extractable from the plant matrix. Phytotoxicity of *M. spicatum* by either the TNT and/or its transformation products was observed in batch systems with an initial TNT concentration above 5.9 μM. The fate and potential phytotoxicity of the TNT transformation products are not known and their evaluation is deemed necessary for phytoremediation applications of TNT-laden materials using aquatic plants.

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