DIFFERENCES IN THE BIOTRANSFORMATION OF 2,4,6-TRINITROTOLUENE (TNT) BETWEEN WILD AND AXENICALLY GROWN ISOLATES OF Myriophyllum aquaticum

Christopher F. Hoehamer, N. Lee Wolfe, and Karl Erik L. Eriksson

Department of Biochemistry and Molecular Biology and Center for Biological Resource Recovery, University of Georgia, Athens, Georgia, USA

The aim of this study was to demonstrate the potential for aquatic plants and their associated microbes to bioremediate wetland sites contaminated with 2,4,6-trinitrotoluene (TNT). The transformation of TNT was studied using both wild and axenically grown isolates of Myriophyllum aquaticum (parrot feather). Differences in TNT transformation rates and nitroaromatic metabolites were observed between different plants. The wild isolates, containing a consortium of associated microorganisms, transformed TNT into 2-amino-4,6-dinitrotoluene (2-A-DNT) and 4-amino-2,6-dinitrotoluene (4-A-DNT) via 2- and 4-hydroxylamino-dinitrotoluene, which were detected as intermediates. The wild M. aquaticum also converted the metabolites, 2-A-DNT and 4-A-DNT, into low levels of 2,4-diaminotoluene (2,4-DAT). The axenically grown plants, containing no cultureable microorganisms, also transformed TNT into 2-A-DNT and 4-A-DNT, but at a much lower rate than that observed for the wild isolates. Unlike the wild plants, axenically grown M. aquaticum could not transform either 2-A-DNT or 4-A-DNT into 2,4-DAT over the incubation period. The differences in the performance between these plants could indicate that plant-associated microorganisms assisted in the overall transformation of TNT. For each plant, unidentifiable metabolites were observed and the soluble monoamino-derivatives present in the wild and axenic medium accounted for 14 and 7% of the initial TNT concentration, respectively. Thus, the majority of nitroaromatic derivatives remained associated with the plant tissues. Furthermore, only 7 and 3% of the initial TNT concentration were extracted as monoamino-derivatives from the tissues of the wild and axenically grown plants, respectively.

KEY WORDS: Myriophyllum aquaticum, parrot feather, 2,4,6-trinitrotoluene (TNT), phytoremediation, bioremediation

INTRODUCTION

Over the past century, negligent handling and accidental release of waste effluents from munitions production in many countries have led to widespread contamination...
with 2,4,6-trinitrotoluene (TNT) (Medina and McCutcheon, 1996; Spain, 1995). TNT is persistent in soil and water and is commonly found around ammunition production facilities. Like many other nitroaromatics, TNT is toxic (Harter, 1985; Honeycutt, Jarvis, and McFarland, 1996; Won, Disalvo, and Ng, 1976; Won et al., 1974), carcinogenic (Baranek et al., 1982), and mutagenic (Kaplan and Kaplan, 1982). Therefore, plausible methods for the removal of this explosive from the environment are of interest. Mechanical treatments for detoxification of TNT-contaminated soil include land excavation and soil incineration, but these methods are expensive and often impractical due to the large quantities of TNT-contaminated soil requiring treatment (Funk et al., 1993). For polluted sites containing low concentrations of TNT, safe and inexpensive biological approaches to remediation are desired. Because both uptake and transformation of TNT by terrestrial and aquatic plants have been demonstrated (Bhadra et al., 1999; Hughes et al., 1997; Palazzo and Legget, 1986; Thompson, Ramer, and Schnoor, 1998), the use of plants as bioremediative agents has potential for the large-scale cleanup of contaminated sites. This type of biotechnology, known as phytoremediation, is an in situ process by which plants and their associated microorganisms detoxify a contaminated ecosystem by taking up and metabolizing hazardous anthropogenic compounds.

It has been reported that a variety of plants, including Phaseolis vulgaris (Harvey et al., 1990), Cyperus esculentus (Legget and Palazzo, 1986), and Populus deltoides X nigra, DN34 (Thompson et al., 1998) can take up and transform TNT from soil or water. In general, these studies showed that the transformation of TNT produced low levels of reduced transformation products such as 2-A-DNT and 4-A-DNT in the growth medium, but a majority of the TNT metabolites remained associated with the plant tissues. In addition to the reduced TNT metabolites, unidentifiable TNT products in the growth medium and tissue extracts were also detected. Because TNT and its reduced metabolites contaminate numerous wetlands and other water bodies, aquatic plants are of obvious interest for use in phytoremediation.

A number of aquatic plants have been tested for their ability to degrade TNT. Among them, plants from genera Nitella (stonewort), Charaphyta (green algae), and Myriophyllum (parrot feather) were all investigated, with M. aquaticum being the favored plant for phytoremediation because of its rapid growth and ability to transform TNT (personal communication, T. Y. Ou and N. L. Wolfe). Similarily, Hughes et al. (1997) demonstrated the uptake and transformation of TNT by axenically grown cultures of M. aquaticum, native plants of M. spicatum, and Catharanthus roseus hairy root cultures. In that study, mass balance determinations were used to investigate the fate and deposition of [U-14C]-TNT in each type of plant over a period of 1 wk. An analysis of their data indicates that, in all plant species, a rapid disappearance of TNT was observed, but the formation of 2-A-DNT and 4-A-DNT in the medium of axenically grown M. aquaticum appeared much slower than that observed in the media for both native plants of M. spicatum and C. roseus. Although, the Myriophyllum plants used are different in species, the native isolates of M. spicatum were capable of transforming TNT into significantly higher levels of TNT metabolites than was observed for the axenically grown M. aquaticum. In that same study, neither native nor axenic Myriophyllum produced 2,4-DAT in the medium or in tissue extracts and mineralization of [U-14C]-TNT to 14CO2 was not observed (Hughes et al., 1997). Thus, this report concluded that axenic M. aquaticum was only capable of transforming TNT into 2-A-DNT or 4-A-DNT via nitroso and hydroxylamino derivatives, but after the production of the monoamino metabolites, plant-mediated nitroreduction of TNT ended. Pavlostathis et al. (1998) reported that TNT was transformed by wild M. spicatum into low levels of 2,4-DAT through a similar reduction pathway, forming similar monoamino metabolites.
Furthermore, there are not only differences in TNT degradation between the native and axenically grown plants of *Myriophyllum* in the study by Hughes et al. (1997), but there also appears to be a more extensive transformation of TNT by the *M. spicatum* plants used in the later report. Although the plants for each study were obtained from different sources, these variances in data may possibly suggest that plant-associated microorganisms contributed in the overall degradation of TNT.

Preliminary studies performed at the Environmental Protection Agency (EPA) laboratory in Athens, Georgia, revealed significant differences in TNT transformation between wild and axenically grown *M. aquaticum*. The wild isolates, containing a variety of culturable microbes, repeatedly demonstrated faster TNT transformation rates, a higher production of reduced metabolites, and the presence of 2,4-DAT, as well as additional, unidentified products, in the medium. In this article, we demonstrate that wild plants, containing a associated consortia of unknown microorganisms, are much more efficient in the overall transformation of TNT than axenically cultivated isolates of *M. aquaticum*.

### MATERIALS AND METHODS

#### Chemicals and Reagents

Analytical grade acetonitrile, methanol, and boric acid were purchased from Chem Service (West Chester, PA). Sodium dodecyl sulfate (SDS), sodium hydroxide, Murashige and Scoog (MS) medium, potato dextrose agar (PDA), tryptic soy agar (TSA), and yeast extract were purchased at Sigma (St. Louis, MO). High-pressure liquid chromatography (HPLC) external standards for 2,4,6-trinitrotoluene (TNT), 2-amino-4,6-dinitrotoluene (2-A-DNT), 4-amino-2,6-dinitrotoluene (4-A-DNT), 2,4-diamino-6-nitrotoluene (2,4-DAT), 4-hydroxylamino-2,6-dinitrotoluene (4-OH-A-DNT), 2-hydroxylamino-4,6-dinitrotoluene (2-OH-A-DNT), and 2,4,6-triaminotoluene (TAT) were purchased from Accustandard (New Haven, CT).

#### Plants and Tissue Cultures

Wild isolates of *M. aquaticum* were purchased from the Jim Price Nursery (Fairburn, GA) after being cultured outdoors in an aquatic system. Axenically grown *M. aquaticum* were obtained from Dr. Micheal Kane (University of Florida, Gainsville) and cultivated on one-half strength MS media containing 1.5% (w/v) tissue culture agar and 3% (w/v) sucrose (pH 5.7). Axenic plants were maintained at 28°C in phytotrays (Sigma, St. Louis, MO) under 40-watt cool white and broad spectrum bulbs for a 16-h photoperiod at a light intensity of 100 µEinstein/m². Axenic plants were grown in the laboratory for 10 wk to obtain sufficient biomass for the experiments. Before the TNT transformation studies were performed, axenic plants were harvested, cut into 1-inch segments, and plated on a variety of microbial growth media including potato dextrose agar (PDA), yeast extract, and tryptic soy agar to ensure that no fungal or bacterial contaminants were present on the surface or interior of the plants.

#### Transformation Studies

Transformation studies were conducted to investigate the metabolism, kinetics, product formation, and terminal fate of TNT by both wild and axenically grown isolates of *M. aquaticum*. Because the wild isolates of *M. aquaticum* were partially covered with
sediment and other pond debris, the plants were washed as follows before the TNT transformation experiments were carried out. This was not a sterilization procedure. Briefly, the wild isolates were rinsed thoroughly and subsequently soaked in distilled water containing 1% (v/v) clorox bleach and 0.1% (v/v) Tween 20 for 15 min followed by three washes with distilled water. Once the sediment and other natural materials were removed, the plants were blotted dry and weighed.

Reaction flasks were prepared by adding 50 g of plants to 1000-ml Erlenmeyer flasks containing 450 ml of filter-sterilized (0.22 \( \mu m \)) water and 100 mg/L solubilized TNT. The top of each flask was then covered with plastic wrap to control evaporation. The reaction flasks were incubated for 168 h at 25°C in a temperature-controlled growth chamber. Triplicate samples (each 5 ml) of the medium were taken every 12 h over the incubation period. Each sample was then filtered and stored in an amber vial at 4°C for identification and quantification of TNT and its derivatives. Control flasks were made by autoclaving the plants in water before adding the TNT.

**Analytical Methods**

Identification and quantification of TNT and its derivatives were determined by reverse-phase high-pressure liquid chromatography (RP-HPLC) using a Hewlett Packard series 1100 quaternary pump with a PRP-1 column (Hamilton, 5 mm, 4.6 \( \times \) 250) and autosampler. The nitroaromatics were separated on the column using a 10-mM KH2PO4 buffer (pH 7.1): acetonitrile 30:70 (v/v) at a flow rate of 1 ml/min. Nitroaromatics were monitored at 235 nm using a diode-array detector with a detection limit of 1 mg/L. For the quantitation and separation of 2-A-DNT and 4-A-DNT, micellar electrokinetic capillary electrophoresis (MECE) was performed using a Hewlett Packard 3-D capillary electrophoresis instrument equipped with a diode-array detector. Samples were injected on a 50-\( \mu m \) capillary column (Hewlett Packard) using a 20-mM borate buffer (pH 8.6) containing 50 mM SDS. While spectra were obtained from 190 to 400 nm, the peaks of interest were quantitated at 238 nm. This method can detect nitroaromatics as low as 0.5–1 mg/L. After each sample, the capillary was flushed with 1 M NaOH and was re-equilibrated with running buffer.

**Plant Tissue Extraction**

After 168 h incubation with TNT, the plants were harvested to identify and quantify the nitroaromatics associated with their tissues. Plants were rinsed with deionized water and then wiped dry. The stems, roots, and leaves were chopped with cutting shears and ground in liquid nitrogen using a mortar and pestle. The plant tissues were subsequently lyophilized for 5 h, and the dry material was sonicated for 8 h in a covered ultrasonic bath (Branson, Danburg, CT) containing 100 ml of methanol at 30°C. The sample was centrifuged at 10,000 \( \times \) g to separate the insoluble plant residue while the supernatant was collected and filtered through a 0.22-\( \mu m \) filter. The filtrate containing the soluble nitroaromatics was concentrated by evaporation of the methanol in a fume hood. TNT and its metabolites were then quantified using HPLC analysis.

**RESULTS**

Both wild and axenic cultures of *M. aquaticum* were observed to take up TNT. The initial TNT concentration decreased to undetectable levels within 96 and 144 h of
DIFFERENCES IN THE BIOTRANSFORMATION OF TNT

incubation, respectively (Figures 1 and 2). Sampling of both plant media over a 1-wk incubation period revealed low concentrations of 2-OHAm-DNT, 4-OHAm-DNT, 2-A-DNT, 4-A-DNT, and 2,4-DAT, albeit to a lesser extent.

After TNT was added to the wild plant medium, the formation of hydroxylamino and monoamino derivatives was immediately detected and, within 12 h of incubation, these metabolites, each approximately 6 mg/L, were present in the growth medium (Figure 1). After 120 h, the level of hydroxylamino metabolites became undetectable as they were likely further transformed into either 2-A-DNT or 4-A-DNT. At this same time point, 2 mg/L of 2,4-DAT were detected in the wild plant medium. Therefore, it appears that the wild plants were able to produce 2,4-DAT from TNT via 2-A-DNT and 4-A-DNT. The initial transformation of TNT into 2-A-DNT or 4-A-DNT was significantly faster than the conversion of these metabolites into 2,4-DAT (data not shown). In addition, it was likely that other TNT transformation products were formed but not detected. The control flasks containing autoclaved native plant tissue decreased the initial TNT concentration in the medium by 1–3%, but no reduced metabolites were detected.

Figure 1 Transformation of TNT into reduced metabolites within the medium of wild *M. aquaticum*. Triplicate samples of the medium were taken every 12 h over an incubation period of 168 h. The samples were then identified and quantitated by HPLC. Each data point is the average concentration of either TNT or nitroaromatic metabolite.

Figure 2 Transformation of TNT into reduced metabolites within the medium of axenically cultivated *M. aquaticum*. Triplicate samples of the medium were taken every 12 h over a period of 168 h. The samples were then identified and quantitated by HPLC. Each point is the average concentration of either TNT or nitroaromatic metabolite.
Table 1  Nitroaromatics present in the medium and in plant tissue after 168 h of incubation with TNT in the wild and axenically cultured *M. aquaticum*

<table>
<thead>
<tr>
<th>Plant</th>
<th>Sample</th>
<th>TNT (% added)</th>
<th>4-A-DNT (% added)</th>
<th>2-A-DNT (% added)</th>
<th>2,4-DAT (% added)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Isolates</td>
<td>Medium</td>
<td>Not detected</td>
<td>9.8 ± 2.1</td>
<td>4.6 ± 0.9</td>
<td>2.0 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>Tissue</td>
<td>Not detected</td>
<td>3.8 ± 0.6</td>
<td>3.5 ± 0.7</td>
<td>Not detected</td>
</tr>
<tr>
<td>Axenic plants</td>
<td>Medium</td>
<td>Not detected</td>
<td>5.4 ± 1.1</td>
<td>2.1 ± 1.1</td>
<td>Not detected</td>
</tr>
<tr>
<td></td>
<td>Tissue</td>
<td>Not detected</td>
<td>1.9 ± 0.1</td>
<td>1.3 ± 0.3</td>
<td>Not detected</td>
</tr>
</tbody>
</table>

After 168 h, the TNT metabolites detected in the tissue extracts of the wild plants were 2-A-DNT and 4-A-DNT. The total amount of both monoamino-products extracted from the tissues only accounted for only 7% of the TNT added initially (Table 1), and neither TNT nor 2,4-DAT were detected in these extracts. While 2,4-DAT was only observed in the wild plant medium, its fate was undetermined. Similar to that observed in the medium, the more polar compounds, which were detected and suggested to be unidentifiable nitroaromatics, were also observed in the tissue extracts. Control extracts of autoclaved plant tissue contained 1–2% TNT, but no TNT metabolites were detected.

After 12 h, the axenically grown plants decreased the initial TNT concentration by 17% and approximately 5 mg/L of soluble 2- and 4-hydroxylamino-derivatives were detected in the medium (Figure 2). Not until 24 h were these intermediates converted into detectable levels of 2-A-DNT and 4-A-DNT. After 1wk, the only identifiable metabolites detected in the medium were the monoamino derivatives, and these products only accounted for 7.5% of the TNT initially added (Table 1). In the axenic tissue extracts, only 3.2% of the initial TNT added were detected as either 2-A-DNT or 4-A-DNT. At the end of the incubation period, unidentifiable products were detected and neither TNT nor 2,4-DAT was observed in the medium or tissue extracts of axenic plants. From this study, we concluded that axenic *M. aquaticum* could only transform TNT into low levels of 2-A-DNT or 4-A-DNT. Using 2-A-DNT and 4-A-DNT as initial substrates, the axenic plants did not produce 2,4-DAT even after 1 wk. The controls containing autoclaved axenically grown plant material had little effect on the initial TNT concentration. Approximately 1–3% of the initially added TNT was extracted from the plant tissue control and no TNT metabolites were detected.

**DISCUSSION**

Because phytoremediation of organic contaminants will occur in the presence of a variety of plant-associated microorganisms, the aim of this study was to demonstrate that TNT could be degraded synergistically by a combination of plants and their associated microbes. Axenically grown cultures of *M. aquaticum* were obtained and used in this study to investigate the plant’s capacity to transform TNT in the absence of its normally associated microorganisms. Although TNT transformation by *M. aquaticum* had previously been demonstrated, this study shows that microbes associated with *M. aquaticum* can significantly enhance the overall degradation of TNT even after 1 wk.

Although TNT disappeared from the medium of the wild isolates at a faster rate, the initial uptake of TNT by each plant was rapid and comparable due to the equal amounts of plant biomass and roots used in each study. The autoclaved control plants were dead, and
still low quantities of TNT were associated to this plant matrix. This binding of TNT to the plant biomass was not considered active uptake, but more likely affinity partitioning to hydrophobic plant materials.

The initial product formation differed between the two different plant samples. While TNT metabolites were immediately detected in the medium of the wild isolates, a 24-h lag phase was observed before either 2-A-DNT or 4-A-DNT was produced by the axenically grown plants. Similarly, Hughes et al. (1997) did not observe the formation of either 2-A-DNT or 4-A-DNT in the medium of the axenically grown cultures until after 48 h of incubation with TNT. Thus, in both TNT transformation studies using axenically grown plants of *Myriophyllum*, a lag in the formation of monoamino products was observed. This delay in product formation probably does not reflect the time needed for the plant to take up TNT, but possibly the time required for induction of endogenous reductases.

For each isolate of *M. aquaticum*, the hydroxylamino and monoamino derivatives of TNT were able to pass in and out of the plant tissue. Therefore, these metabolites were localized in both the medium and in the tissue extracts, but could not be detected in stoichiometric quantities. After 1 wk, the identifiable transformation products in the medium and in tissue extracts of the wild and axenically grown plants accounted for approximately 22 and 10% of the TNT initially added, respectively. Thus, the principle metabolites from TNT degradation by both *M. aquaticum* plants are either soluble metabolites in unidentifiable form or undetermined tissue-bound residues. Because a majority of the degradation products appear to be unidentified, it is logical to suggest that additional degradation pathways were involved in the metabolism of TNT. While oxidation of TNT has not been observed in any degradation pathway, it is known that the nitroso and hydroxylamino derivatives can condense to form insoluble azoxy metabolites, such as 2,2′,6,6′-tetranitro-4,4′-azoxytoluene and 4,4′,6,6′-tetranitro-2,2′-azoxytoluene. Indeed, traces of these compounds have been detected in studies with *Myriophyllum* (Hughes et al., 1997), but these products did not account for the substantial gap in mass balances. However, in the present TNT degradation experiments, formation of these azoxy complexes was not detected. Unlike the azoxy compounds, the unidentifiable products were water soluble and more polar than the identifiable TNT metabolites.

TNT degradation studies with *Catharanthus roseus* hairy root cultures (Bhadra et al., 1999) revealed that unidentifiable products found in the medium and in tissue extracts were reduced TNT metabolites conjugated with sugars like glucose, galactose, or malonate. The addition of glucose or other conjugates to a metabolite molecule has been observed in the degradation of certain herbicides and plant hormones (Hatzios and Penner, 1982). Although the physiological function of the conjugated sugar is undetermined, glycosylated metabolites have been observed to be dead-end products in the degradation of many xenobiotic compounds (Carter, 1975).

In previous reports, it was demonstrated that a diversity of plant-associated fungi (Smith et al., 1989) and bacteria (Chand, Harris, and Andrews, 1992) can proliferate and accumulate on wild isolates of *M. spicatum*. Some of the plant-associated fungi that were isolated include, *Calletotrichum gloeosporioides*, *Acremonium curvulum*, *Cladosporium herbarum*, and *Aerobasidium pullulans* (Smith et al., 1989). Although there is no published study demonstrating that any one of these fungi can transform TNT, numerous fungi have been shown to be capable of TNT transformation to a greater or lesser extent (Scheibner et al., 1997). In addition to fungi, there are some well-studied bacteria isolated from *M. spicatum* (Chand et al., 1992), including the genera *Enterobacter* and *Pseudomonas*, which have already been shown to be capable of transforming TNT (Bryant and DeLuca,
In particular, Enterobacter cloacae, an enteric bacterium capable of producing an oxygen-insensitive nitroreductase, can reduce a variety of nitroaromatic compounds, including TNT and 2,4-dinitrotoluene (Bryant and DeLuca, 1990). Similarly, Pseudomonas pseudocaligenes and Pseudomonas fluorescens have been isolated from M. spicatum and have been reported to extensively transform TNT in shake-flask cultures (Somerville et al., 1995). Furthermore, a recent study by Siciliano and Greer (2000) reported that there is plant–bacterial cooperation in the phytoremediation of soils when exposed to high concentrations of TNT. With this finding and the works reported by Smith et al. (1989) and Chand et al. (1992), it appears that it is very possible for plant-associated microbes to assist in TNT transformation.

In the initial TNT transformation studies, wild isolates of M. aquaticum were washed and subjected to a surface sterilization protocol. This treatment removed many insoluble materials and microorganisms that were associated with the plant exterior. Thus, this study showed that only a portion of the plant’s microbial assimilative capacity was influencing TNT degradation. In nature, when this wash treatment is not performed on plants, a higher level of TNT degradation can be predicted. Because plants, fungi, and bacteria are all independently capable of TNT degradation, it appears likely that, in nature, the phytoremediation of organic compounds is a synergistic process of co-metabolism performed by plants and a consortia of associated microorganisms. Collectively, all these organisms could have great potential in decontaminating TNT-polluted ecosystems so, to accurately assess whether or not a plant is suitable for phytoremediation, its associated microbial and rhizobial populations should also be investigated. Future work in the bioremediation of TNT will focus on the microbes associated with the wild isolates of M. aquaticum to identify and isolate plant-associated microorganisms that are capable of transforming TNT and its metabolites.

ACKNOWLEDGMENTS

This research was funded by a NNEMs fellowship from the Environmental Protection Agency. I would like to thank Chris Mazur, Dr. Tse Yuan Ou, and Dr. Jianping Gao for their help with HPLC and capillary electrophoresis. We are also grateful to Dr. Jeffrey F. D. Dean for editorial advice on this manuscript.

REFERENCES


DIFFERENCES IN THE BIOTRANSFORMATION OF TNT


