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Treatment of trinitrotoluene by crude plant extracts

Victor F. Medina [a,*], Steven L. Larson [a], Lovell Agwaramgbo [b], Waleska Perez [c], Lynn Escalon [d]

[a] *Environmental Engineering Branch, EP-E, Environmental Laboratory, ERDC, US Army Corps of Engineers (USACE), 3909 Halls Ferry Road, Vicksburg, MS 39180, USA*
[b] *Department of Chemistry, Dillard University, New Orleans, LA 70122, USA*
[c] *Department of Chemistry, Purdue University, West Lafayette, IN 47907, USA*
[d] *Analytical Services, Inc., Vicksburg, MS 39180, USA*

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Abstract

Crude plant extract solutions (spinach and parrotfeather) were prepared and spiked with 2,4,6-trinitrotoluene (TNT) (20 mg l^{-1}). 90-h TNT removal by these solutions was compared to controls. Spinach and parrotfeather extract solutions removed 99% and 50% of the initial TNT, respectively; TNT was not eliminated in the controls or in extract solutions where removal activity was deactivated by boiling. A first-order removal constant of 0.052 h^{-1} was estimated for spinach extract solutions treating 20 mg l^{-1} TNT concentrations, which compared favorably to intact plant removal. Concentration variation was described by Michaelis–Menton kinetics. Detectable TNT degradation products represented only a fraction of the total TNT transformed, and the transformation favored the formation of 4-aminodinitrotoluene. The results indicated that crude plant extracts transform TNT, without the presence of the live plant.

Keywords: Phytoremediation; Trinitrotoluene; Plant extracts

1. Introduction

Phytoremediation of organic compounds is promising for the treatment of a wide range of environmental contaminants in soil and water matrices (Dushenkov et al., 1995; Newman et al., 1997; Hughes et al., 1998; Nzengung et al., 1999). The majority of applications described in the literature involve the growth of plants in the zone of contamination, and having the plant uptake and accumulate or uptake and transform the contaminant. Although effective in many cases, these approaches suffer from the several inherent drawbacks, including: (1) the treatment is limited to areas where the plant roots can absorb and uptake the contaminant; (2) the system cannot work in areas where the contaminant concentration is toxic to the plant; and (3) the treatment rate is limited by the rate of contaminant uptake by the plant. If contaminants could react with extracted plant chemicals, in the absence of the plant itself, applications of phytoremediation could be expanded.

Because it has been well studied and is transformed and degraded by plants, 2,4,6-trinitrotoluene (TNT) is a model organic compound for phytoremediation studies. Previous studies have focused on in vivo transformation processes (Hughes et al., 1998; Thompson et al., 1998; Larson et al., 1999); however, there is much evidence that transformations occur outside the plant (in vitro) as well. For example, studies with *Myriophyllum* sp. reported the recovery of transformation products (aminodinitrotoluenes (ADNT), diaminonitrotoluene, and hydroxylaminodinitrotoluenes) in culture medium (Hughes et al., 1998; Pavlostathis et al., 1998; Rivera et al., 1998; Medina et al., 2000a,b, 2002). The plant either exuded these

* Corresponding author. Tel.: +1-601-634-4283.
E-mail address: victor.f.medina@erdc.usace.army.mil (V.F. Medina).

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metabolites after uptake and transformation, or they were formed from parent TNT in solution. Root-associated bacteria may have also formed the metabolites, but removal of periphyton from *Myriophyllum* cultures did not eliminate the appearance of the metabolites, and transformation products were not detected in the non-planted controls. Photolytic reactions could be ruled out, as the transformation products were not found in the controls.

The goal of this study was to determine if plants could transform TNT in aqueous solution external to the plant. The study focused on crude plant extracts released from the plant during submersion and agitation in aqueous solution.

2. Experimental methods

2.1. Basic experimental approach

Fig. 1 is a schematic showing the basic experimental approach. Plants were placed in autoclaved 200-ml screw-top jars, and deionized (DI) water was added to create a biomass concentration of 50 $g\,l^{-1}$. The jars were placed on a shaker table for 24 h. The plants were then removed from the jars, and the remaining solutions were filtered through a 0.22-μm filter to remove all particulate matter and microorganisms. There are several studies that indicate that 0.22-μm membrane filters are effective at removing microorganisms from aqueous solution (Logan et al., 1993; Madaeni, 1999; Hijnen et al., 2000; Ohtani et al., 2000). Then, a 100-$mg\,l^{-1}$ aqueous TNT solution was added to the filtered solutions in the jars to form the target concentration (usually 20 $mg\,l^{-1}$). The jars were incubated in the dark at 20 °C, sampled, and analyzed for TNT and its breakdown products. We assumed that, for a given plant, the extract concentration would be about the same for a given extraction period. However, we did not take measurements to confirm this.

Concentrations of TNT, ADNT, and other breakdown products were determined directly from the aqueous solutions using high-pressure liquid chromatography. The methods have been previously described (Rivera et al., 1998; Medina et al., 2000a,b). The method detection limit for TNT was determined to be 0.003 $mg\,l^{-1}$ and the laboratory reporting limit was 0.020 $mg\,l^{-1}$.

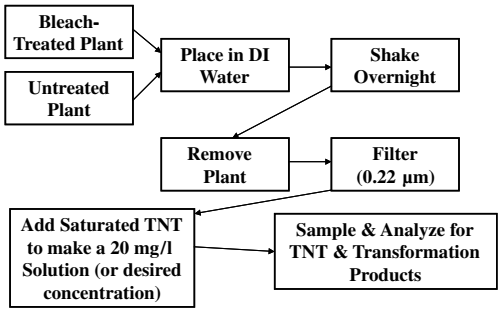

Fig. 1. Schematic of the experimental design.

Controls were prepared using the same jars as the experimental reactors and consisted of DI water with no plants added. They underwent identical filtering and shaking as the experimental reactors.

Several experiments were conducted using this approach: screening study, boiled extract study, and kinetics study. In addition, an intact plant experiment was conducted for comparison. A concentration variation study was conducted to develop additional understanding of the extract reaction kinetics.

2.2. Plants

Two plant species were tested: parrotfeather (*Myriophyllum aquaticum*) and spinach (*Spinicia oleracea*). Parrotfeather was chosen because it has been widely tested for the treatment of munitions (Hughes et al., 1998; Rivera et al., 1998; Larson et al., 1999; Medina et al., 2000a,b, 2002). Parrotfeather was purchased at a local nursery and was cultivated in hydroponic tanks in tap water. Spinach was chosen because it is commonly used in botanical studies, is widely available, and is easy to grow. Some of it was purchased from a local supermarket and was used within a day of purchase. Spinach was also grown in the laboratory, particularly for the intact plant uptake study. The plants were thoroughly washed, first with tap water and then in deionized water.

2.3. Plate counting for heterotrophic bacteria

Standard Method 9215D, which is a filtration-based plate counting method, was used to enumerate heterotrophic bacteria from solution (Greenberg et al., 1992). Methods for extracting microorganisms from the plant surfaces were discussed in Medina et al. (2000b).

A bleach treatment method (described in Medina et al. (2000b)) was used to remove microorganisms living on the plant surfaces. The method has been documented to remove 96–100% of bacteria from parrotfeather and spinach, respectively (Medina et al., 2000b).

2.4. Screening experiment

Parrotfeather and spinach were separated into two batches: bleach-treated (see above) and untreated. Untreated plants did not undergo the bleach-treatment process. The experiment involved the following: one control, one bleach-treated spinach, one untreated spinach, one bleach-treated parrotfeather, and one untreated parrotfeather. The control and the untreated parrotfeather were triplicated. The jars were incubated in the

dark at 20 °C for 90 h, sampled, and analyzed for TNT and its breakdown products.

2.5. Boiled extracts experiment

Heat is an effective means for removing enzymatic activity. Medina et al. (2000a) found that intact plants did not remove TNT at a temperature of 54 °C and higher. Budge and Parrish (1999) established boiling as an effective means for removing lipolytic enzymatic activity, and van Beelen and Burris (1995) used boiling to inactivate nitroreductase enzymes isolated from soil. Therefore, boiling was used to deactivate enzymatic activity in this study. Extract solutions were heated on a hot plate and boiled for 5 min. A water-cooled condenser was used to recover evaporated liquid. The solution was then allowed to cool to room temperature.

A portion of spinach extract solution underwent the boiling procedure to deactivate enzymatic activity. Both untreated and boiled extract solutions were then spiked with TNT to form 20-mg l^{-1} concentrations. In addition, controls without extracts were prepared. Triplicate reactors were prepared for each condition. Removal of TNT and generation of amino-transformation products were compared at 90 h.

2.6. Kinetic experiment

Spinach extracts were prepared and used to treat a 23-mg l^{-1} TNT solution, and their performance were compared to control solutions. Triplicate reactors were prepared for each condition (extract and control). Measurements of TNT and aminonitro-transformation products were taken at 0, 3, 4, 5, 18, 40, 75, 97, and 147 h, enabling calculation of kinetic rates. These rates were compared to intact plants.

A first-order rate equation was used to analyze kinetic rates of TNT removal, and the pseudo-first-order rate constant, k_1, was then determined graphically. In actuality, the removal of TNT was expected to be second-order, in relation to enzyme (or extract) concentration (Pavlostathis et al., 1998; Medina et al., 2000a). However, for this study, we assumed this to be in excess and therefore could be considered a constant.

2.7. Removal of TNT by intact plants comparison study

For comparison, removal of TNT by intact spinach and parrotfeather plants was conducted using methods previously described (Medina et al., 2000a). A plant density of 50 g l^{-1} was used. Triplicate reactors were prepared for each plant. This removal was compared to that of triplicated extract solutions generated by identical plant densities.

2.8. Concentration variation comparison study

To investigate the effect of initial contaminant concentration, the experimental procedure was repeated using crude spinach extract solutions on TNT concentrations of 0.05, 2.0, 5.5, 10.5, and 21.0 mg l^{-1}.

Michaelis–Menton kinetics was then used to relate removal kinetics to concentration. The Michaelis–Menton equation can be rearranged to yield the Eadie–Hofstee equation:

$$v_0 = V_m - \frac{v_0}{S_0} K_m$$

By graphing v_0 versus v_0/S_0, the y-intercept gives V_m and the slope of the line gives K_m (Zubay, 1988). Because the same term (v_0) is in both the x and y term, it is expected that a high correlation coefficient will result. However, the method allows for more even spacing of data points compared to Lineweaver–Burke plots and has been commonly used in recent published literature (Iraburu et al., 1994; Chalot et al., 1996; Kajikawa et al., 1997; Javelle et al., 1999).

3. Results

3.1. Screening study

Concentrations of TNT were reduced during 90 h of exposure to crude plant extracts from both spinach and parrotfeather but remained unchanged in the control solution (Fig. 2). Spinach-based extract solutions were more effective, reducing over 95% of the initial TNT concentration compared to about 50% removal for parrotfeather extract solution. Plate counts indicated that the bleach treatment removed 100% of heterotrophic bacteria from spinach and 97% of the bacteria from the parrotfeather. In addition, plate counts indicated that filtration using a 0.22-μm membrane removed 100% of heterotrophic bacteria.

Small amounts of ADNT (1–3 mg l^{-1}) were detected in both the spinach and parrotfeather extract solutions following 90 h but not in the control. Although the ADNT concentrations were relatively low, the concentrations were well above the method detection limit.

3.2. Boiled extract solution study

Using unboiled extract solutions, TNT was removed; however, the chemical was not removed from the controls (Fig. 3). Only a small amount of TNT (1.6 mg l^{-1}) was removed from boiled extract solutions (boiled before introduction of TNT), compared to 24.85 mg l^{-1} removed from the unboiled extract solution. The error bars, depicting the variation associated between different

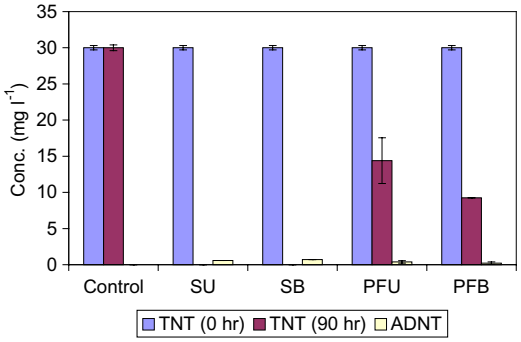

Fig. 2. TNT removal after 90 h of exposure to plant extracts released in water (SU = untreated spinach extract solution, SB = bleach-treated spinach extract solution, PFU = untreated parrotfeather extract solution, PFB = bleach-treated parrotfeather extract solution). Error bars represent standard deviation of measurements on triplicate reactors.

replicates, were relatively small, indicating the process is repeatable.

In each case, a small amount of 2ADNT was formed at the end of the 90-h period. The 2ADNT concentrations were about equal for each reactor. In addition, 4ADNT was detected at a level of about 5 mg l^{-1} in the extract reactors. While 4ADNT was found in both the boiled extract and the control reactor, the concentrations were more than two orders of magnitude lower than what was found in the unboiled extract solution reactors.

Analysis of variance (ANOVA) was conducted on the triplicate reactor data collected during this experimental set, using an error allowed, α, of 0.01. Comparing the initial concentration with the ending concentrations of the three test conditions (boiled, unboiled extract, and control), each had a statistically significant difference. The ANOVA comparison of the initial concentration, the boiled extract solution, and the control also showed a statistically significant difference. Comparisons between the initial concentration and the boiled extract, between the initial concentration and the control, and between the control and the boiled extract all had no statistical significant differences. All comparisons with the unboiled extract solution had statistically significant differences.

3.3. Kinetic study

Kinetic studies revealed that the removal of TNT by spinach extracts was first-order with a rate constant of 0.052 h^{-1} ($r^2 = 0.96$) (Fig. 4). The extracts degraded TNT to 2ADNT and 4ADNT, with 4ADNT being formed at levels 10 times higher than that of 2ADNT. Whole plant studies indicated that spinach and parrot-

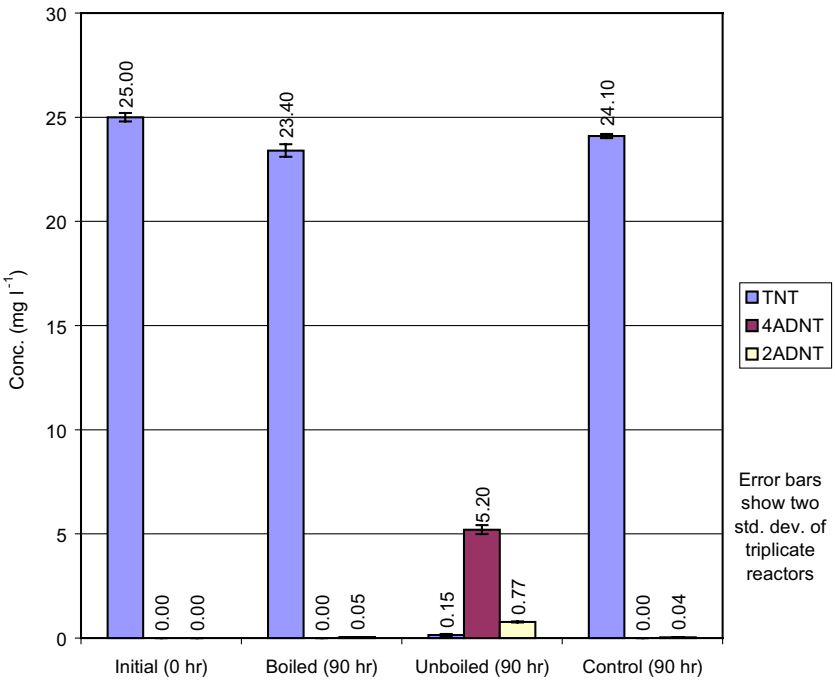

Fig. 3. Comparison of TNT removal after 90 h of exposure to spinach extracts in water, with comparison to control (no exudates) and boiled extracts. Initial is the starting TNT concentration. Error bars represent standard deviation of measurements on triplicate reactors.

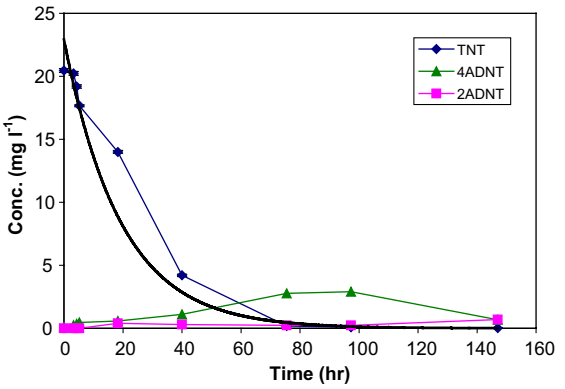

Fig. 4. Kinetic study results for spinach exudate removal of TNT. Error bars (representing standard deviation of triplicate reactors) are given for TNT but are quite small.

feather had first-order rate constants of 0.141 and 0.036 h^{-1} respectively. Therefore, the TNT removal rate constant by crude spinach extracts was about 1/2 that of the intact spinach plant and was greater than that of intact parrotfeather. This indicates that removal rates by the crude extracts are reasonably fast and could be competitive with that of intact plants.

3.4. Concentration variation study and relationship to Michaelis–Menton kinetics

A concentration variation experiment was conducted in which crude spinach extract solutions were used to treat initial concentrations of TNT varying from 0.5 to 21.5 $mg\,l^{-1}$. The measured pseudo-first-order rate constants decreased with increasing TNT concentrations (Fig. 5), a similar pattern to what was found with whole plant studies (Medina et al., 2000a). Although the pseudo-first-order rate constant decreased with increasing concentration, the reaction velocity increased (Table 1). These patterns might be explained by saturation of the

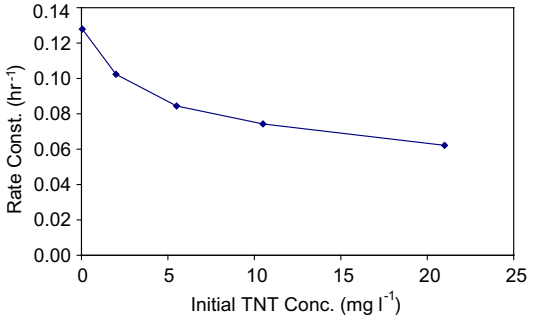

Fig. 5. Change of first-order rate constants with respect to initial TNT concentration for a series of spinach extract experiments.

Table 1
Comparison of measured removal rates versus predicted values using Michaelis–Menton parameters ($V_m = 59.75\ mg\,l^{-1}\,h^{-1}$ and $K_m = 5375\ mg\,l^{-1}$)

Initial TNT concentration ($mg\,l^{-1}$)	Measured TNT removal rate ($mg\,l^{-1}\,h^{-1}$)	Calculated TNT removal rate based on Michaelis–Menton kinetics ($mg\,l^{-1}\,h^{-1}$)
0.05	0.0006	0.0006
2.0	0.022	0.022
5.5	0.061	0.061
10.5	0.117	0.117
21.0	0.232	0.233

enzymes involved in the reaction. Michaelis–Menton kinetics (discussed in "Experimental Section") is a commonly used theory for enzyme saturation kinetics.

Fig. 6 is an Eadie–Hofstee plot of the data giving the Michaelis–Menton constants for the reaction ($V_m = 59.75\ mg\,l^{-1}\,h^{-1}$ and $K_m = 5375\ mg\,l^{-1}$). Comparing the measured reaction rates (or reaction velocities) with those predicted using the Michaelis–Menton constants indicate that the values are extremely close (Table 1).

Table 2 gives the concentrations of 4ADNT and 2ADNT found in the concentration variation study. The

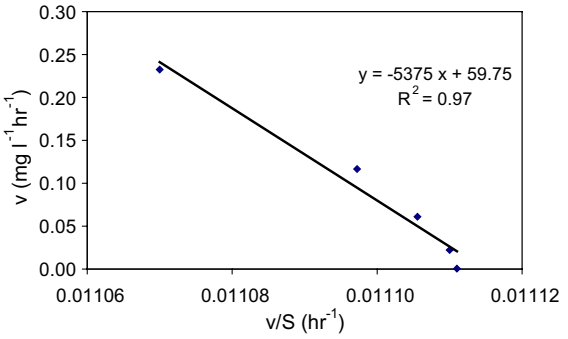

Fig. 6. Eadie–Hofstee plot for concentration variation study. V_m is the y-intercept ($59.75\ mg\,l^{-1}\,h^{-1}$), and K_m is the absolute value of the slope ($5375\ mg\,l^{-1}$).

Table 2
Final concentrations of 4-amino-2,6-dinitrotoluene (4ADNT) and 2-amino-4,6-dinitrotoluene in comparison to initial TNT concentrations after 90 h of reaction with spinach extract solutions

Initial TNT concentration ($mg\,l^{-1}$)	Final 4ADNT concentration ($mg\,l^{-1}$)	Final 2ADNT concentration ($mg\,l^{-1}$)
0.05	0.023	0.000
2.0	0.74	0.00
5.5	1.49	0.05
10.5	2.21	0.11
21.0	3.15	0.37

concentrations of both transformation products increased with increasing initial TNT concentration. The concentration of 4ADNT was considerably higher in each case than the concentration of 2ADNT.

4. Discussion/conclusions

The method developed for the experiment was designed to isolate and eliminate competing variables for TNT removal (e.g., photo-reactions, adsorption, microbial reactions), leaving released extracts as the most likely agent responsible for TNT transformation in solutions. Table 3 summarizes the isolation of variables. The literature indicates that filtration by a 0.22-μm filter should remove most bacteria from the extract solutions (Logan et al., 1993; Madaeni, 1999; Hijnen et al., 2000; Ohtani et al., 2000), and our own measurements indicated that filtration removed 100% of heterotrophic bacteria. Therefore, we conclude that microbial uptake and metabolism played a negligible role in the transformation. It might be possible that microbial enzymes passed through our filtration step. However, the dissipation of TNT was approximately equal for bleach-treated (which have their surface microorganisms removed) plants when compared to untreated plants, further supporting that microbial degradation was minimal in these experiments.

The released factor responsible for transformation may have been a nitroreductase enzyme (Schnoor et al., 1995; van Beelen and Burris, 1995) or a combination of nitrate- and nitrite-reductases that plants use to process nutrients (Larcher, 1995; Taiz and Zeiger, 1998). Nitrate-, nitrite-, and nitroreductases have been isolated from spinach (Baysdorfer and Robinson, 1985; Nakagawa et al., 1985; Shah and Spain, 1996; Shah and Campbell, 1997), and these have been demonstrated to react with tetryl (Shah and Spain, 1996) and nitrobenzene (Shah and Campbell, 1997). Presumably, spinach extracts removed TNT more completely than parrotfeather extracts because the spinach extracts had more nitroreductase activity than the parrotfeather extracts. However, plant protein or enzymes were not directly measured in this study.

Some plant chemical production and release is induced by the presence of the contaminant or by environmental conditions. For example, induced production of metal-interacting phytochelating agents has been observed in several studies, usually in nutrient limited conditions (Römheld, 1991; Salt et al., 1997), and nitrate availability induced the synthesis and regulation of NAD(P)H nitrate reductase from the bryophyte *Sphagnum magellanicum* (Deising and Rudolph, 1987). However, this does not appear to be the case in our studies. The living plant was removed before the TNT was added to the solution, indicating that the plant must have released the factor responsible for the transformation in the absence of the contaminant. Therefore, the presence of the released factor does not appear to induced by TNT. This is consistent with intact plant studies, where previous exposure to munitions does not seem to increase removal rates (Comstock, 1996; Pavlostathis et al., 1998; Medina et al., 2000b). In fact, some intact plant studies suggest the rate is even decreased after previous TNT exposure (Medina et al., 2000b).

The fact that the extracts removed TNT without the physical presence of the plant suggests that the activity is relatively stable. This is consistent with the literature in

Table 3
Summary of isolation of competing variables for TNT removal

Variable	Means of isolation
Volatilization	1. TNT is relatively non-volatile (vapor pressure at 80 °C = 0.05 mm Hg) 2. Experiments conducted in screw-top jars 3. Accounted for by control
Adsorption on glass surfaces	Accounted for by control
Uptake by plant	Plant removed before TNT was added, eliminating this possibility
Reactions with light	1. Jars were incubated in the dark 2. Accounted for by control
Adsorption with solid plant particles	Particles were removed by filtration of liquid before TNT addition
Microbial degradation associated with the solutions and glassware	1. All glassware and solutions were autoclaved before use 2. Accounted for by control
Microbial degradation associated with the plants themselves	1. Use of bleach treatment to kill microbes before the plant was placed in the hydroponic system (verified by plate counting) 2. Microbes removed by filtration (0.22 μm) before TNT spiking (verified by plate counting)

which stable enzymatic activity has been described (Skujins, 1967). The sources of these enzymes can be difficult to determine, and generally, they have been assumed to have microbial origins. However, plant origins are possible (Skujins, 1967). In fact, stable enzymes capable of transforming TNT were isolated from aquatic sediments (van Beelen and Burris, 1995). Although the source of these enzymes was not determined, the authors noted that the sediments contained plant roots. Furthermore, the enzymes seemed ubiquitous; active enzymes were found in 10 of 11 freshwater sites sampled. In a similar study, stable peroxidase enzymes were extracted and purified from soils (Bollag et al., 1987). Once again, the source was not isolated, but the enzyme had similar properties to those isolated from horseradish.

The kinetic rates of removal by the crude extract solutions were relatively fast, comparing well to the removal rates of whole plant studies. A significant portion of this activity may be from released compounds associated with the plant's life, or even released during the death of plant tissue. If so, then a significant amount of treatment of TNT phytosystems may actually occur outside the plant.

The amount of transformation products formed was minimal, and they were largely degraded over time. It is not clear if some buildup of breakdown products would occur, as found in some continuous flow-through reactor studies (Rivera et al., 1998). These studies also indicate that the extracts preferentially form 4ADNT as compared to 2ADNT. This could be used as a tool to understand if these types of reactions are occurring, at least compared to adsorption reactions.

Removal also followed Michaelis–Menton kinetics. The maximum velocity (V_m) calculated for the crude spinach extracts was actually about a factor of ten higher than that calculated for intact parrotfeather studies (Medina et al., 2000a). Also, the half saturation constant (K_m) was also much higher for the spinach extract solutions compared to intact parrotfeather (94.2 $mg\,l^{-1}$, from Medina et al. (2000a)). An important consequence of the high K_m value is that it is much higher than the actual solubility of TNT in water (125 $mg\,l^{-1}$). This indicates that the reaction would be essentially pseudo-first-order for all possible concentrations. As such, this result is analogous to that found in intact plant studies (Medina et al., 2000a).

Stable enzymatic activity may allow for the development of extract-based remediation technologies. Such treatment systems would have several advantages over the traditional approach of growing the plants in the zone of contamination. Plants would not uptake the contaminant, and the potential for exposure of herbivores to contaminants is eliminated. Further, crude plant extracts can be applied to highly contaminated soils where the plants cannot live. Although research is required to test the efficacy of these ideas, treatment systems based on enhancing the treatment potential of plant extracts have the potential to greatly enhance the application of phytoremediation.

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