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Urea-facilitated uptake and nitroreductase-mediated transformation of 2,4,6-trinitrotoluene in soil using vetiver grass

Padmini Das [a], Dibyendu Sarkar [a], Konstantinos C. Makris [b], Rupali Datta [c,*]

[a] Department of Earth and Environmental Studies, Montclair State University, NJ, USA
[b] Cyprus International Institute for Environmental and Public Health in association with Harvard School of Public Health, Cyprus University of Technology, Limassol, Cyprus
[c] Department of Biological Sciences, Michigan Technological University, MI, USA

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ABSTRACT

Limited bioavailability of hydrophobic nitroaromatic compounds such as 2,4,6-trinitrotoluene (TNT) is a major challenge toward developing an effective *in situ* bioremediation method for active or former military sites. A greenhouse-scale study evaluated the efficiency of a stimulative phytoremediation method using urea, a common nitrogen fertilizer, as a solubilizing agent to facilitate TNT uptake by vetiver grass (*Chrysopogon zizanioides* L.). Kinetics of TNT removal by vetiver from the TNT-spiked soil (100 mg kg^{-1}) was fast (up to 0.004 kg d^{-1} g^{-1}), following a pseudo first-order reaction rate. Vetiver showed high affinity for TNT (>80% removal within 22 days), and significant root-to-shoot TNT translocation (average 37%). Soil TNT removal rates by vetiver were significantly ($p < 0.0001$) enhanced by urea. Urea application at agronomically-recommended nitrogen rates (125–350 mg kg^{-1} soil) was optimum for TNT uptake by vetiver grass. Monoaminodinitrotoluenes and 1,3,5-trinitrobenzene were the main TNT metabolites detected in plant tissues, posing little, if any, influence on plant health. Enhanced activity of nitroreductase enzyme (NR) in TNT treated vetiver plants was observed, which coincides with the prevalence of amino-based TNT metabolites within plant tissues, indicating an effective biochemical defense mechanism against TNT toxicity.

Introduction

2,4,6-Trinitrotoluene has been historically the most widely used secondary explosive. It is a potential mutagen and a group C human carcinogen [1,2]. Due to its persistence in the environment, the removal of TNT from contaminated military and non-military sites became high priority for environmental agencies worldwide [1]. Increasing urban population has been driving the unprecedented sprawling of cities toward peripheral areas, sometimes close to former military sites. Residential expansion toward such military land is often impeded by the high remediation cost of large military areas contaminated with relatively low residual TNT concentrations (<150 mg kg^{-1}) [3]. Search for ecologically-viable and cost effective environmental remediation/restoration methods for such contaminated sites has identified novel *in situ* bioremediation techniques, such as bioaugmentation, and phytoremediation [1,4]. One of the major challenges in developing a successful *in situ* biological remediation technique lies in the limited bioavailability of nitroaromatic compounds due to their hydrophobic nature [5]. To address this problem, our group has proposed a TNT remediation method called *in situ* stimulative phytoremediation, which uses the synergistic combination of phytoremediation using both vetiver grass (*Chrysopogon zizanioides* L.) and a solubilizing agent, *i.e.*, urea, which is commonly used as a crop fertilizer [6].

Soil amendment using various alkaline materials is one of the more promising approaches for degradation of energetic compounds like TNT, RDX, and HMX. Application of hydrated lime, quicklime, and Class C fly ash has been reported to remediate TNT, RDX, and HMX from a mixed system by transforming them to inorganic salts and soluble organic compounds [7]. However, addition of caustic materials can cause very high soil pH, which is hazardous. Using urea as a soil amendment may also lead to increased soil pH, but our experiments have demonstrated that application of urea within the environmentally safe application rates are not sufficient to cause substantial increase in soil pH. Urea acts as a solubilizing agent, which increases the release of TNT in soil solutions, making it more available for the plants to take up [8]. In biological studies, urea is routinely used as a chaotropic agent to increase solubility of

* Corresponding author at: Biological Sciences Department, Michigan Technological University, Houghton, MI 49931, USA. Tel.: +1 906 487 1783; fax: +1 906 487 3167.
E-mail address: rupdatta@mtu.edu (R. Datta).

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hydrophobic molecules. Urea disrupts the secondary and tertiary structures of proteins to solubilize membrane proteins, dissociate antigen–antibody complexes, *etc.* [9]. Chaotropic agents modify the water structure around aggregated biomolecules such as proteins or sugars and increase the solubility of their hydrophobic regions in aqueous environments [10]. Our previous studies using urea as a TNT-extractant were encouraging; urea enhanced TNT solubility in aqueous media, significantly increasing the phytoextraction of TNT by vetiver and wheat in hydroponic settings [11,12]. A pilot experiment using a soil with minimal TNT retention capacity demonstrated a significant ($p < 0.001$) increase in TNT removal rates by vetiver grass in the presence of a high urea application rate (1000 mg kg^{-1}) [13].

However, the performance of urea at agronomically-recommended application rates (<1000 mg kg^{-1}) in enhancing soil residual TNT uptake is yet to be evaluated. Optimum agricultural crop guidelines recommend use of urea at >125 mg kg^{-1} urea [8]. A consistent yield depression of agricultural crop like maize was observed after a single urea application rate of 350 mg kg^{-1} [8]. Higher than 1000 mg kg^{-1} urea application rates exhibited strong toxic effects on earthworms, often considered soil ecotoxicological indicators [8]. Hence, 1000 mg kg^{-1} is the highest level of urea that can be used in soil without affecting the soil health.

Further, because TNT has been proven highly recalcitrant to biological degradation, it is important to assess the role of enzyme-mediated detoxification pathway in those plants which show an innate ability to cope with high TNT concentrations [14]. TNT does not contain the functional group required for conjugation; direct conjugation of TNT to plant macromolecules is unlikely [15]. TNT tolerant plants showed enhanced resistance either by nitroreductase (NR) mediated transformation of TNT to aminodinitrotoluenes (ADNTs) that allows subsequent conjugation to plant molecules and/or by glutathione transferase (GST) catalyzed formation of TNT-glutathione product that are more amenable to degradation [14]. The assessment of the enzyme mediated detoxification pathway is required to evaluate the effectiveness of our phytoremediation technique [15].

This study is aimed to fully characterize the proposed stimulative phytoremediation method that showed promise in our earlier results obtained in laboratory and hydroponic set-ups under more realistic greenhouse conditions. The effectiveness of vetiver grass in removing soil residual TNT is further evaluated in presence of urea at its agronomically recommended application rates. The specific objectives of this study were to: (i) determine the kinetics of TNT removal from soil by vetiver grass in the presence of added urea, (ii) evaluate the effectiveness of urea, as a solubilizing agent, within the range of environmentally-relevant and agronomically-recommended fertilizer N rates in catalyzing soil TNT uptake by vetiver grass, (iii) measure the magnitude of plant TNT uptake and monitor both TNT and its metabolites in root and shoot tissues, while measuring the activity of nitroreductase (NR) enzyme responsible for the transformation of TNT to amino-based metabolites within vetiver grass, which is required for detoxification.

Experimental methods

Materials

2,4,6-Trinitrotoluene (TNT) was purchased from Chem Service (West Chester, PA, USA) in an aqueous slurry form. It was air-dried, dissolved in acetonitrile, and stored in dark at 4 °C. HPLC-grade standards of TNT and its potential metabolites (as listed in the USEPA 8330 method), 1,3-dinitrobenzene (1,3-DNB); 2,4-dinitrotoluene (2,4-DNT); 2,6-dinitrotoluene (2,6-DNT); 2,4-di-amino-nitrotoluene (2,4-DANT); 2,6-di-amino-nitrotoluene (2,6-DANT); nitrobenzene (NB); 3-nitrotoluene (3-NT); 4-nitrotoluene (4-NT), tetryl, 1,3,5-trinitrobenzene (1,3,5-DNB); 2-amino-4,6-dinitrotoluene (2-ADNT); and 4-amino-2,6-dinitrotoluene (4-ADNT) were purchased from AccuStandard (New Haven, CT, USA) [16]. Urea was purchased from Fisher Scientific. HPLC grade solvents and nano-pure quality water were used for preparing solutions.

TNT and urea treatments

Our study used 100 mg kg^{-1} soil TNT concentration, which is much higher than the benchmark of 30 mg kg^{-1} TNT toxicity limit for terrestrial plants [3]. Four urea concentrations (0, 125, 350, 1000 mg kg^{-1}) were chosen to evaluate the performance of urea as a solubilizing agent at environmentally-relevant (0–1000 mg kg^{-1}) and agronomically-recommended (125–350 mg kg^{-1}) application rates to a TNT-contaminated soil [8].

Soil selection and preparation

The Millhopper soil, which is a sandy loam, with a pH of 6.4 and relatively low organic matter content (4.38%), was chosen based on our previous batch experiments conducted in the absence of plants to investigate its TNT-sorption characteristics [8]. Hysteretic sorption of TNT by Millhopper soil suggested irreversible adsorption of TNT in soils and thus indicated that the adoption of an extractant to increase soil-bound TNT bioaccessibility would be beneficial [8]. Effective enhancement of bioaccessibility was observed within the agronomically-recommended urea application rates in Millhopper soil, allowing for a significant ($p < 0.001$) increase in the extraction of pre-adsorbed TNT by urea (56%), when compared with that of water-based TNT extraction (36%) [8].

Millhopper soil samples were collected from the surface horizon (0–30 cm) at the University of Florida campus at Gainesville, FL, USA. The soil was spiked with TNT stock solution, reaching 100 mg kg^{-1} soil-TNT concentrations. TNT-spiked soil was poured in polyethylene plastic bags, placed in three pots for each treatment and kept 7 days for equilibration before planting vetiver.

Greenhouse set-up

Vetiver (*C. zizanioides* L.) plants were purchased from Florida Farms and Nursery, Florida. Plants were carefully selected to provide uniform distribution of shoot and root biomass for all experimental units and were allowed to acclimatize in potting soil for 2 weeks at 25 °C and 16 h photoperiod in a state-of-the-art greenhouse located within the premises of Montclair State University. At the beginning of the experiment (day 0), plants were washed with tap water, weighed and placed in the pots, containing 2 kg TNT-spiked soil. Vetiver plants, uniformly weighing 100 g were placed in each pot. On day 1, urea solution was prepared in half-strength Hoagland solution [17] and added to the pots. The volumes of tap water-based solutions added to the pots were frequently adjusted to maintain soil at 70% water holding capacity throughout the experimental period. The pots were covered with aluminum foil to avoid possible photo-degradation reactions with TNT. All treatments were performed in triplicates. Three TNT-free (control) soil pots were set up with vetiver grass. Three plant- and urea-free, TNT-amended soil pots were also kept as controls to capture TNT losses due to indigenous soil biodegradation processes.

Sampling and extraction

Soil samples were collected after 2, 5, 9, 14, and 22 days to evaluate TNT removal kinetics from soil. Three grams of soil were