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Soil Decontamination of 2,4,6- Trinitrotoluene by Alfalfa (*Medicago sativa*)

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Abstract: Present study investigate the toxicity effect of 2,4,6-trinitrotoluene (TNT) on a terrestrial plant, alfalfa (*Medicago sativa*) in artificial soils. In this study, TNT toxicity assessment was performed on spiked silica with this nitroaromatic compound by determination of the percent of emergence and shoots and roots biomasses at the concentration range of 3.2-10000 mg kg^{-1} Dry Weight (DW). The emergence was reduced by 22-32% after 5 days of exposure at TNT concentrations up to 100 mg kg^{-1} DW; shoot and root biomasses were reduced by 48-50 and 63-74%, respectively after 30 days exposure at TNT concentrations ≤32 mg kg^{-1} DW. Concentrations higher than 100 mg kg^{-1} DW can not be tolerated at all. Concentrations of TNT and its metabolites in silica, root and shoot were measured by High-Performance Liquid Chromatography (HPLC). Analyses of TNT spiked soil extracts reveal hat during alfalfa cultivation for 30 days, TNT was partially transformed at the extent of 15-27%. This transformation decreased at higher TNT soil concentrations. TNT is taken up and metabolized by plants to its downstream derivatives.

Key words: Phytoremediation, TNT, nitroaromatics, transformation, emergence, alfalfa

INTRODUCTION

Soil and groundwater at sites throughout the world were contaminated by manufacturing, processing and storage of explosives. Unlike many other nitroaromatic compounds, including pesticides and various feedstock chemicals, the energetic nitroaromatics and heterocyclic nitroamines are highly resistant to degradation and may persist in the environment for decades (Ekman *et al*., 2003). Field analyses have revealed that the concentrations of energetic contaminants are sufficiently high to adversely impact environmental health in most contaminated sites. 2,4,6-trinitrotoluene (TNT) is present in the environment as a result of decommissioning activities and through field usage and disposal activities such as open burning. So most toxicological studies of explosives have involved TNT. The toxic effects of TNT have been studied involving microorganisms, algae, fungi, tidepool copepods, oyster larvae, a mascroinvertebrate, insects, fathead minnows, earthworms, plants, mammalian cell cultures and rats (Frische, 2002; Renoux *et al*., 2000; Scheibner *et al*., 1997). Some studies have demonstrated the mutagenic and toxic effects of TNT on human and animal cells (White and Claxton, 2004). Moreover, liver damage and aplastic anemia in humans have been reported (McCutcheon and Schnor, 2003).

Certain plant species have the ability to accumulate TNT from their surroundings and thus offer a potential means for removing these compounds from the environment (Hannink *et al*., 2002). However, most species can not tolerate the contamination levels of the sites, which has to be cleaned. Thus, screening of the plant species with enhanced abilities to tolerate and remove TNT from soil upon phytoremediation, remains an area of the challenge. Phytoremediation is an innovative technology that uses trees, grasses and other plants to clean our environment and remediate hazardous waste sites.

To measure the susceptibility of terrestrial plants from contaminants, standardized toxicity assays including seed germination (observed as root emergence) and biomass assessment have been used (ISO, 1993, 1995; Best *et al*., 2004). However, the number of studies describing the phytotoxicity of explosives such as TNT on higher plants are limited (Talmage *et al*., 1999; Sunahara *et al*., 1999). Transpiration and biomass of hybrid poplar cuttings decreased when exposed to 22 μM TNT (Thompson *et al*., 1998), germination as well as root and shoot growth (length per day) of tall fescue decreased linearly with increasing TNT concentrations in solution (Peterson *et al*., 1996). At sublethal concentrations, TNT can induce morphologically abnormal development of roots. Gong *et al*. (1999) and Peterson *et al*. (1996) observed sparse, short and abnormal root hairs of plants grown in a TNT-amended soil and in hydroponic solutions. Compared to

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germination, seedling (shoot or root) growth is more sensitive, probably because the seeds use the energy reserves in the cotyledons for germination.

The effects of TNT in the concentration range of 25 to 1,600 mg kg^{-1}soil on four plant species were tested, among them two dicotyledonous species, cress (*Lepidium sativum* L.) and turnip (*Brassica rapa* Metzg.), presented higher sensitivity to TNT than did two monocotyledonous species, oat (*Acena sativa* L.) and wheat (*Triticum aestivum* L.) (Gong *et al*., 1999). The lowest observable effect concentration (LOEC) of TNT on germination and seedling growth of cress and turnip was 50 mg kg^{-1} soil. However, oat can tolerate up to 1,600 mg TNT per kg soil (Gong *et al*., 1999) indicating that phytotoxic effects of TNT varies with plant species (Friedl and Picka, 2004). In other studies, alfalfa (*Medicago sativa*) was not able to grow in soil contaminated with 100 mg TNT kg^{-1}, whereas wheat and bush bean (*Phaseolus vulgaris*) could develop at 500 mg TNT kg^{-1} soil (Scheidemann *et al*., 1998).

TNT is taken up and metabolized by plants to 2-amino-4,6- dinitrotoluene (2-ADNT), 4-amino-2,6-dinitrotoluene (4-ADNT), 2,4-dinitrotoluene (2,4-DNT) and 2,6-dinitrotoluene (2,6-DNT) (Sens *et al*., 1998, 1999). This study is describing the effects of TNT on a dicotyledonous higher plant; alfalfa (*Medicago sativa*) by evaluation of the emergence and shoot and root biomass. As a second objective of this study, the soil contamination removal efficacy by alfalfa is estimated through transformation analysis which was carried out upon tracing of the TNT down-products in both root and shoot.

MATERIALS AND METHODS

TNT and other explosive standards including; 2,4,6-Trinitrotoluene (2,4,6-TNT), 2,4-Dinitrotoluene (2,4-DNT), 2,6-Dinitrotoluene(2,6-DNT), Nitrobenzene (NB), 2-Nitrotoluene (2-NT), 4-Nitrotoluene (4-NT) are obtained from domestic military pilots. Glassware was washed with phosphate-free detergent followed by rinses with acetone, nitric acid (10%, v/v) and deionized water. Alfalfa (*Medicago sativa* var. Ghara-Yuonja) seeds used for the assays were obtained from the agriculture research center.

Artificial soil and the sand covering consisted of silica 24 and 16 meshes, respectively. Appropriate 24 mesh silica on Dry Weight (DW) basis at 100 g was placed in Petri dishes (150-15 mm). Due to low aqueous solubility of TNT (130 mg L^{-1} at 20°C; Ryon, 1987), it was dissolved in acetonitrile as carrier solvent to prepare TNT solutions which were added to the silica sand to obtain final concentrations of explosive in the concentration range of 0-10^4 mg kg^{-1} dry weight of soil. Using a solvent, that was evaporated later in the chemical hood, allowed to obtain higher concentrations of explosives in soil samples than the field-contaminated soils. After 2 days of evaporation the residual solvent remained at low level at 0.001-0.04 μL g^{-1} dry soil (Robidoux *et al*., 2003). Nevertheless, to limit any possible toxicity interference caused by residual acetonitrile concentrations, the tested soils spiked with a limited volume of acetonitrile solution (35 mL), with or without TNT, were evaporated and aged for 4 week in the dark in a chemical hood prior to exposure. A solvent control (acetonitrile only, without TNT) and a negative control (water only) were also tested.

Plant toxicity tests: The effects TNT were assessed using standard methods (USEPA, 1997). Toxicity tests were carried out using the artificial soil. Assays were conducted in triplicate using similar test conditions. The 40 alfalfa seeds were placed on the contaminated substrate (100 g of artificial soil-24 mesh-spiked with the appropriate substance). At the beginning of each experiment, the soil (dry weight) was rehydrated to 85% of the water-holding capacity (37.4 mL/100 g silica). The water-holding capacity was determined by saturating the soil with water and measuring the water content. Water content was determined by the loss of soil weight during with drying (Robidoux *et al*., 2003).

Then 100 g cover sand (16 mesh silica) was placed on top of the hydrated test soil. Each Petri dish was randomly placed into growth chamber with corresponding conditions (25°C, 16 h light). The emergence of alfalfa shoot was recorded after 5 days of exposure. The plants feeding with nurture solution (Hoagland and Arnon, 1950) was started at the day five. The wet and dry biomasses of the roots and shoots were separately weighed after 30 days of exposure. The results obtained from the treated groups were statistically compared to that of control groups (negative control and solvent vehicle controls). All toxicity tests were done in triplicate and included solvent and negative controls (without added toxicant). Plant emergence and growth endpoints were verified against our laboratory in-house control data.

Chemical analyses: TNT and its metabolites were extracted into acetonitrile from soil samples at the beginning (t = 0) and at the end (t = 30 days) of the exposure process by using the sonication followed by analytical High-Performance Liquid Chromatography (HPLC) based on the method (USEPA, 1997). The soil samples spiked with TNT concentrations at higher than 100 mg kg^{-1} DW were disregarded throughout of the analysis due to harsh declined growth of the plant. Thus, concentrations: 3.2, 10, 20, 32, 50, 100 mg kg^{-1} DW were used in these analyses. Acetonitrile (10 mL) was added to a portion of a weighed soil sample (2 g) and then vortexed

for 1 min which followed by continuous ultrasonication for 18 h at 8°C. $CaCl_2$ solution (5 g L^{-1}) was added at volume of 1:1 to soil extract. The mixture was then filtered through a 0.45 μm membrane (Millex-H, Millipore) to be made ready to load.

Plants were also clipped into small pieces and were homogenized by grinding in liquid nitrogen. TNT and its metabolites were extracted from root and shoot samples from single Petri dish after 30 days using the same acetonitrile-sonication method which followed by filtration step. In brief, root and shoot samples dried in 37°C and dark condition for 12 h and suspended in 10 mL acetonitrile followed by vigorous vortexing for 1 min. The sample suspension, were ultrasonicated as previously described. The extracts were released from particles by centrifugation for 10 min at 2,000 g. Filtration of the supernatants through a 0.45 μm membrane (Millex-H, Millipore) was then carried out. The filtered solutions was analyzed using a Waters HPLC chromatographic system composed of a model Knauer Wellchrom pump k-1001, UV detection k-2600, Smartline autosampler 3800, optimal ODS-H Supelco LC-18 reverse phase HPLC column with guard column. The mobile phase comprised of 1:1 (v/v) methanol/organic-free reagent water and flow rate was 1 mL per min. The sample volume injected was 10 μL with a run time of 40 min and finally analyses were detected by UV absorption at 254 nm (Best *et al*., 2004).

Data analysis: Statistical analyses were conducted with the software SPSS.12 (SPSS Inc. Chicago, IL. USA) for Windows XP. Linear correlation between nominated and experimentally evaluated concentrations of the explosives was assessed. Normal distribution of the data was tested with the Duncan's test.

Analysis of variance of the data also was done. This analysis was expanded with a multiple range test using the Fisher's least significant difference procedure. The p-value in the ANOVA is a measure of the significance of the analysis; it was set at a 95% confidence level.

RESULTS AND DISCUSSION

This study describes the phytotoxic responses to TNT in spiked soils along with the phytoremediation approach to remove and degrade TNT from soil samples. TNT had a significant number of sub-lethal effects on dicotyledonous alfalfa (*Medicago sativa*) including plant emergence or biomass. Plant under stress by TNT together with control samples were examined based on standardized toxicity tests (ASTM, 1999; USEPA, 1997). *Medicago sativa* has a wide geographical distribution, rapid growth and profuse generative reproduction. In addition, its seeds germinate simultaneously within several days and the species can be cultivated in testing environment. This species is relatively insensitive to organic contaminants and is widely used as a bioaccumulation indicator to assess soil contamination by some organic micropollutants (Best *et al*., 2004).

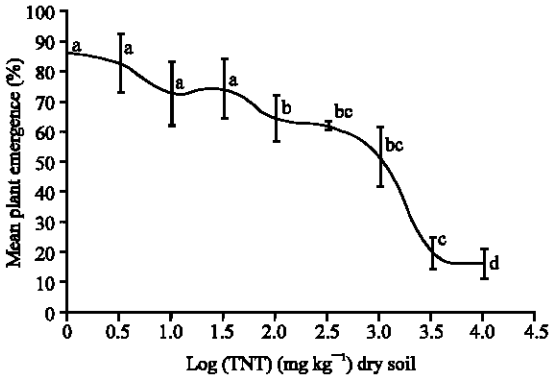

Fig. 1: Inhibitory effect of TNT on alfalfa emergence after 5 days of exposure to different nominal TNT spiked-soil concentrations (t = 0) in artificial soil (silica; n = 3 replicates of 40 seeds). Data are Mean±SD of 3 independent experiments. Letters a, b, c, d represent a significant inhibition of emergence by TNT exposure compared each to the other

Exposure to TNT-spiked artificial soil caused significant effects on *M. sativa* emergence which has been presented in a constructed semi-logarithmic plot (Fig. 1). At TNT nominal concentrations <100 mg kg^{-1} DW, plant emergence was reduced by 22-32% but at TNT concentrations ≥100 mg kg^{-1}DW, it was reduced by 49-80% as compared to the negative control groups at 5 days. It seems that TNT cause a delay in the plant emergence in the wide-range of concentrations. We observed that there is a correlation between delay in plant emergence and quantified TNT concentrations in artificial soil sample. Robidoux *et al*. (2003) have recently reported inhibitory effects of TNT on lettuce (*Lactuca sativa*) and barley (*Hordeum vulgare*) emergence up to 18 and 15.5% versus the control group, respectively (Robidoux *et al*., 2003). The mechanism of the seed emergence deceleration by nitoraromatics has not been yet understood. Meanwhile the aberrational effects of TNT on cell division steps especially in anaphase (White and Claxton, 2004) or on energy providing metabolic processes involved in shoot meristematic cells probably can be assumed.

Phytotoxic effects of TNT on plants in spiked artificial soil also were studied through evaluation of root and shoot biomass analyses again in a constructed semi

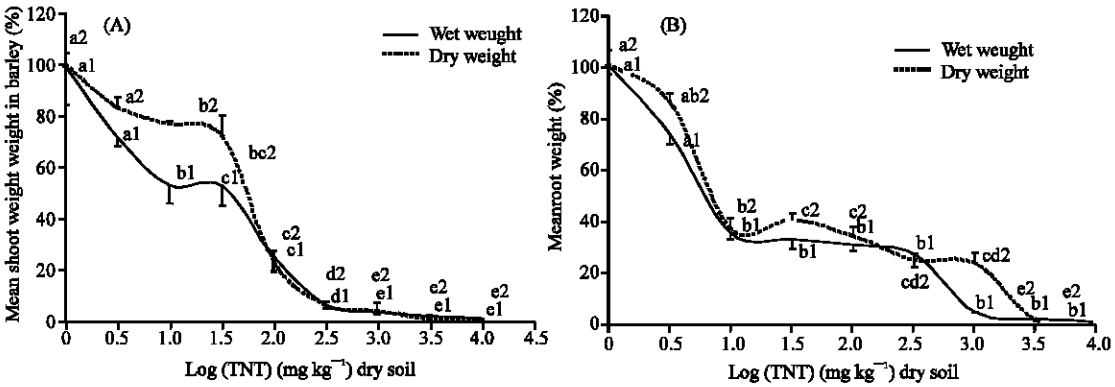

Fig. 2: Inhibitory effects of TNT on alfalfa. Shoot (a) and root growth (b) after 30 days of exposure to different nominal TNT spiked-soil concentrations in artificial soil (silica; n = 3 replicates of 40 seeds). Data are based on total wet and dry weights. Data are Mean±SD of 3 independent experiments. Letters a, b, c, d and e represent a significant reduction of biomass compared together

logarithmic plot for a nominal TNT concentration range of 0-10^4 mg kg^{-1} DW (Fig. 2). The effect of TNT on shoot growth was significant, using fresh- and dry-weight parameters (Fig. 2a). Fresh- and dry- shoot biomass were reduced, respectively by 48-50 and 30-32% compared to water controls at TNT concentrations ≤32 mg kg^{-1} DW. However, shoot biomass was reduced by 72-75% compared to water controls at TNT concentration ≥100 mg kg^{-1} DW. The growth of plants were halted at TNT concentrations >100 mg kg^{-1} DW. The effects of TNT on root growth were significant over a wide range of administered TNT concentrations at mg kg^{-1} DW, using fresh- and dry-weight assessments (Fig. 2b). Fresh- and dry- root biomasses were reduced by 63-74% compared to water controls at TNT concentrations ≤10 mg kg^{-1} DW. However root biomass was reduced by 75-96% compared to water control at TNT concentrations ≥100 mg kg^{-1} DW. These observations are supported by reports dealing with observed growth inhibitory effects of TNT to decrease fresh shoot biomass of cress and turnip at concentrations ≥54 mg kg^{-1} DW in field collected soils, whereas wheat and oat shoot biomasses were decreased at ≥158 and 311 mg TNT kg^{-1} DW of soil, respectively (Gong *et al*., 1999).

Ratio alteration style of shoot growth versus root growth under exposing with increasing concentrations of TNT is documented for thirty days cultivated plant samples (Fig. 3). Three phasic course of affection can be comprehended from figure. Root vulnerability in comparing with shoot is increased at administered concentrations of TNT under 3.2 mg kg^{-1} which followed by constant phase in the concentration range of 3.2-32 mg kg^{-1} DW. At TNT concentrations >32 mg kg^{-1} dry soil the greater toxic effect exerted on shoot growth than on root growth which was assessed to be

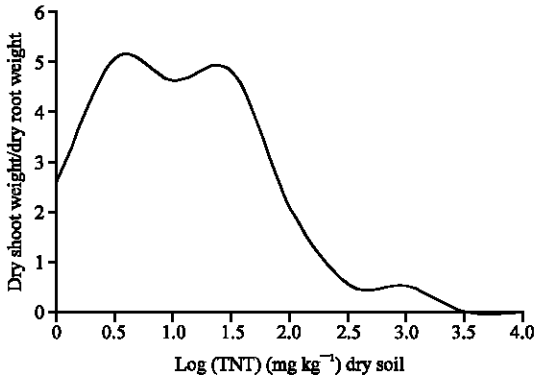

Fig. 3: Ratio alteration style of the shoot growth versus root growth exposed with increasing concentrations of TNT spiked-soil for 30 days

continued constantly to nominal TNT concentrations of 10000 mg kg^{-1} DW. It probably can be deduced that lower concentrations of TNT up to 32 mg kg^{-1} can influence root growth in much more extent than shoot. However at supplied concentration of TNT at higher concentrations (>32 mg kg^{-1} dry soil) shoot-part will be affected more than root-part.

These results show toxicity of TNT on both of plant emergence and growth using *M. sativa*. Both of root and shoot growth were significantly reduced due to TNT toxicity. However the extent of TNT toxicity effect on them is switched from root to shoot at around 32 mg kg^{-1}. Interestingly, the fresh shoot weight endpoint was more sensitive than the dry shoot weight endpoint.

HPLC analysis showed that the measured TNT concentrations at time zero (t = 0) were usually similar with corresponding nominal concentrations (i.e., spiked concentrations). However, the former set was sometimes

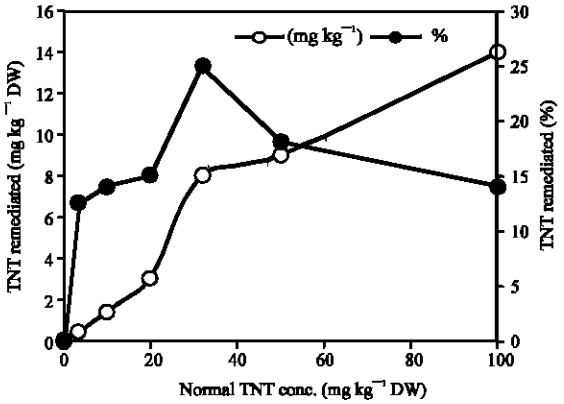

Fig. 4: Transformation of TNT in artificial soil (silica) by alfalfa for different TNT spiked-soil nominal concentrations; the values are different of mean measured TNT concentrations (n = 3) at 0 and 30 days for the range of 0-100 mg kg^{-1} nominal TNT concentrations

lower than the later one. Since analyses for TNT metabolites showed that TNT was not degraded during the evaporation period and soil samples (taken at t = 0) were not mixed with cover sand, the difference between initial and nominal concentrations were probably due to a dilution error. However, nominal concentrations were used to describe the toxicity data.

Figure 4 shows TNT remediation (mg kg^{-1} DW) against nominal TNT-spiked concentrations. There is an elevating correlation between nominal TNT-spiked concentrations and the extent of remediation throughout of the examined range up to 100 mg kg^{-1} DW. Analyses of TNT-spiked soil extracts results 15-27% transformation of TNT in artificial soil by alfalfa in which triplicate measured concentrations at the days 0 to 30 were subtracted to provide us net remediation extent for each of the nominal administered TNT concentrations.

The treatment of organic contaminants often results in the transformation of the contaminants to other compounds of unknown toxicity. In many cases, the transformation products are difficult to identify. Therefore, it is not surprising that the toxicities of transformation products are relatively unknown for many cases. Studies identifying these products and determining the phytotoxicity would enhance treatment system design. Thus, we studied the amount of TNT uptake in plant (root and shoot) cultured in TNT contaminated silica as artificial soil. Four nominated TNT metabolites: DNT (2,4-Dinitrotoluene and 2,6-Dinitrotoluene), NB (Nitrobenzene), 2-NT (2-Nitrotoluene), 4-NT (4-Nitrotoluene) were traced by HPLC. The results have been presented separately for root and shoot in Fig. 5. The results indicate that an uptake of TNT by roots which is followed by its transferring to shoot and its transformation to TNT's down metabolites during 30 days processing function of both root and shoot. Therefore, TNT detoxification is done to result in dominancy of NT products especially ortho- isomer in root in addition NB as the next main product in shoot. DNT also have been detected in both root and shoot HPLC analysis profiles. However estimated root total nitroaromatics are several folds (at maximum 6 fold) higher than shoot total nitroaromatics in ppm. Also it can be concluded that transferred and/or transformed TNT degradation products are in lesser extent than the root probably due to time delay effect during 30 days cultivation of the *M. sativa* seeds on TNT contaminated artificial soil. Comparative view on presence pattern of the metabolites in root and shoot may provide a new sight on TNT degradation path.

A decade studies points out on down degradation of TNT to its reduction (Hawari *et al*., 2000; Spain *et al*., 2000) as well as its mono- and di-denitration products (Best *et al*., 2004; Sens *et al*., 1999). The results of the present study alongside with previous reports (Gong *et al*., 1999; Peterson *et al*., 1996) suggest that the observed toxic effects may not be caused by TNT alone. TNT metabolites can also inhibit germination and seedling growth (Peterson *et al*., 1996), so sub-lethal toxicity effects of TNT on alfalfa in certain concentrations could be due in part to the toxicity of its metabolites. In focus on DNT, alfalfa did not grow at 0.55 mM (100 mg kg^{-1}) of 2,4-DNT in soil (Dutta *et al*., 2003) and lettuce was more sensitive than wheat, mustard and lentil, (Friedl and Picka, 2004). The highest Non-Observed Adverse Effect Concentrations (NOAEC) for the growth of lettuce were 20 mg kg^{-1} for TNT, in addition 2 mg kg^{-1} DW for 2,4 -DNT and 10 mg kg^{-1} DW for ,2,6-DNT. In soil, 2,4-DNT will be slightly mobile and based on aqueous biodegradation tests, 2,4-DNT may biodegrade in both

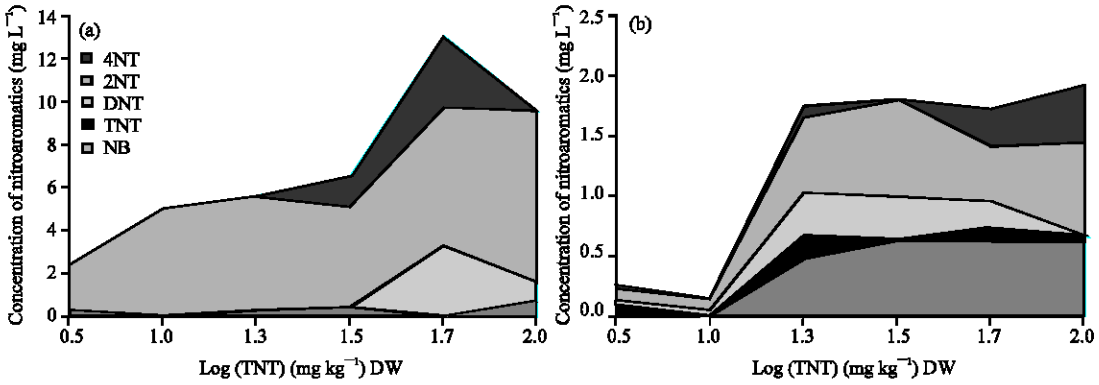

Fig. 5: Mean concentrations of the nitroaromatic compounds; TNT (2,4,6-Trinitrotoluene), DNT (2,4-Dinitrotoluene and 2,6-Dinitrotoluene), NB (Nitrobenzene), 2-NT (2-Nitrotoluene), 4-NT (4-Nitrotoluene) in root (a) and shoot (b) extracts from alfalfa after 30 days exposure with different nominal concentrations of TNT

aerobic and anaerobic zones of soil. From di-denitration products 2NT has a strong tendency to migrate to air and water. Scientific Committee on Health and Environmental Risks (SCHER) from European Commission (EC) has released on September 2006 that the vapor pressure (28 Pa) and a Henry's constant (1.2 Pa m^3 mol^{-1}) of the 2NT indicate a strong affinity for the atmospheric compartment. Nevertheless literature survey was not resulted in any more documents on phytotoxicity of the observed TNT derived compounds through denitration process.

In conclusion TNT was successfully removed from contaminated artificial soil (at <100 mg kg^{-1} DW) at the maximum range of 15-25% by alfalfa during a period of the plant cultivation for 30 days. It was transferred from soil to root and than metabolized and/or transferred from root to shoot and then transformed to degradation down products, emphasizing on denitration ones. TNT goes together with its down metabolites decreases the capacity of plant emergence and the growth of shoots and roots. Here, these effects have been analyzed in detail.

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