

Research Articles

Dendroremediation of Trinitrotoluene (TNT)

Part 2: Fate of Radio-labelled TNT in Trees

Part 1: Literature Overview and Research Concept – Part 2: Fate of Radio-labelled TNT in Trees

Preamble. Phytoremediation technologies are of rapid growing concern for the cost effective sanitation of large areas polluted with low to middle or even an unknown degree of contamination. The interest in phytoremediation research is extensively mirrored by ecomed publisher's ScientificJournals (<http://www.scientificjournals.com>) [see JSS – J Soils & Sediments 3 (2) 72 (2003)]. Mainly, because the current number of phytoremediation reports from internet sources nearly exploded to an information jungle, the term 'dendroremediation' is preferred if trees are used or tested as sustainable remediation tools or if the role of trees in natural attenuation processes should be assessed. Using the example of the environmental TNT problem, a holistic approach was tried for the dendroremediation of trinitrotoluene and its concomitant hazards. The TNT dendroremediation research concept, presented in Part I, should be an interdisciplinary compromise between basic research and practical application. Part II reports on results regarding the fate of [¹⁴C]-TNT in compartments of older trees of willow and Norway spruce.

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Abstract

Background, Aim and Scope. Problems of long-term existence of the environmental contaminant 2,4,6-trinitrotoluene (TNT) and necessities for the use of trees ('dendroremediation') in sustainable phytoremediation strategies for TNT are described in the first part of this paper. Aims of the second part are estimation of [¹⁴C]-TNT uptake, localisation of TNT-derived radioactivity in mature tree tissues, and the determination of the degree of TNT-degradation during dendroremediation processes.

Methods. Four-year-old trees of hybrid willow (*Salix spec.*, clone EW-20) and of Norway spruce (*Picea abies*) were cultivated in sand or ammunition plant soil (AP-soil) in wick supplied growth vessels. Trees were exposed to a single pulse application with water solved [U-¹⁴C]-TNT reaching a calculated initial concentration of 5.2 mg TNT per kg dry soil. Two months after application overall radioactivity and extractability of ¹⁴C were determined in sand/soil, roots, stem-wood, stem-bark, branches, leaves, needles, and *Picea* May sprouts. Root extracts were analysed by radio TLC.

Results. 60 days after [¹⁴C]-TNT application, recovered ¹⁴C is accumulated in roots (70% for sand variants, 34% for AP-soil variant). 15–28% of ¹⁴C remained in sand and 61% in AP-soil. 3.3 to 14.4% of ¹⁴C were located in aboveground tree portions. Above-ground distribution of ¹⁴C differed considerably between the angiosperm *Salix* and the gymnosperm *Picea*. In *Salix*, nearly half of above-ground-¹⁴C was detected in bark-free wood, whereas in *Picea* older needles contained most of the above-ground-¹⁴C (54–69%).

TNT was readily transformed in tree tissue. Approximately 80% of ¹⁴C was non-extractably bound in roots, stems, wood, and leaves or needles. Only quantitatively less important stem-bark of *Salix* and *Picea* and May shoots of *Picea* showed higher extraction yields (up to 56%).

Discussion. Pulse application of [¹⁴C]-TNT provided evidence for the first time that after TNT-exposure, in tree root extracts, no TNT and none of the known metabolites, mono-amino-dinitrotoluenes (ADNT), diamionitrotoluenes (DANT), tri-

nitrobenzene (TNB) and no dinitrotoluenes (DNTs) were present. Extractable portions of ¹⁴C were small and contained at least three unknown metabolites (or groups) for *Salix*. In *Picea*, four extractable metabolites (or groups) were detected, where only one metabolite (or group) seemed to be identical for *Salix* and *Picea*. All unknown extractables were of a very polar nature.

Conclusions. Results of complete TNT-transformation in trees explain some of our previous findings with 'cold analytics', where no TNT and no ADNT-metabolites could be found in tissues of TNT-exposed *Salix* and *Populus* clones. It is concluded that 'cold' tissue analysis of tree organs is not suited for quantitative success control of phytoremediation *in situ*.

Recommendations and Outlook. Both short rotation *Salicaceae* trees and conifer forests possess a dendroremediation potential for TNT polluted soils. The degradation capacity and the large biomass of adult forest trees with their woody compartments of roots and stems may be utilized for detoxification of soil xenobiotics.

Keywords: Conifer; deciduous tree; dendroremediation; hybrid willow (*Salix spec.*); natural attenuation; nitroaromatic compounds; Norway spruce (*Picea abies*); phytoremediation; soil decontamination; TNT (2,4,6-trinitrotoluene)

Introduction

Problems of the recalcitrance of the soil pollutant 2,4,6-trinitrotoluene (TNT) and the necessity for the use of trees ('dendroremediation') in sustainable phytoremediation strategies were introduced in the first part of this report [1]. The main purpose of the present paper was to provide assessment support for other experiments integrated in our dendroremediation concept, which has the goal of making dendroremediation comparable for different tree species.

Data regarding tree uptake of [¹⁴C]-TNT are available from Dobner (2003) [2] for seedlings of *Pinus sylvestris* and for young aspen saplings. Mass balances and distribution of radiolabelled TNT in trees are restricted to juvenile poplar

cuttings [3]. Up to now, no results are available for uptake and transformation of [^{14}C]-TNT in mature woody plants for either deciduous or coniferous trees.

1 Methods

1.1 Chemicals and standards

Uniform ring-labelled [^{14}C]-TNT (CAS-Nr. 118-96-7) was synthesised by Laszlo Vollner (International Isotopes Munich, Germany). Specific activity of solid TNT was 3.16 GBq g^{-1} ($19.47 \text{ mCi mmol}^{-1}$). Certified radiopurity ($>95\%$) was confirmed by radio-TLC using an Instant Imager (Canberra Packard GmbH, Dreieich, Germany).

Unlabelled 2,4,6-TNT was obtained from Promochem (Wesel, Germany). 2,4-dinitrotoluene (2,4-DNT) and 2,6-dinitrotoluene (2,6-DNT) were from Sigma-Aldrich (Seelze, Germany). 2-amino-4,6-dinitrotoluene (2-ADNT), 4-amino-2,6-dinitrotoluene (4-ADNT), and 2,4-diamino-6-nitrotoluene (2,4-DANT) were from Promochem. 1,3,5-trinitrobenzene (1,3,5-TNB) was from Ehrenstorfer (Augsburg, Germany). Solvents were HPLC-grade. Acetone (Pestanal) and ethyl acetate (Pestanal) were purchased from Sigma-Aldrich. Methanol (LiChrosolv), acetonitrile (LiChrosolv), dichloromethane (LiChrosolv) and acetic acid (puriss.) were obtained from Merck (Darmstadt, Germany).

1.2 Trees, selection and pre-cultivation

The male hybrid *Salix* EW-20 was derived from previous TNT growth screening experiments [4,5]. *Salix* EW-20 is a drought-resistant willow clone and its transpiration capacity is comparable to Norway spruce (*Picea abies*). *Salix* EW-20 was able to take up 'cold' TNT and TNT-reduction products (ADNTs) were found in roots [4,5]. *Picea abies* plants were obtained from local origin (Forstbaumschule Luckenwalde, Germany).

Four-year-old trees of hybrid *Salix* EW-20 and *Picea abies* were pre-selected for growth uniformity in our nursery field stand. Initial tree height chosen was approximately 50 cm for spruce and 100 cm for willow. Tree roots were heavily pruned to enhance formation of lateral roots and to allow planting into 12-cm-diameter polypropylene pots (TEKU 12 LC, Pöppelmann, Lohne, Germany). Stems of willows were trimmed to 35 cm in height and all lateral branches were removed. Before potting fresh weight of each tree was estimated and plants with a similar initial weight were used. Potting substrate was either 600 g (DW) of thoroughly washed quartz sand (Type 'Dorsilit 8', particle size 0.3–0.8 mm, Herford, Berlin, Germany) or 600 g of soil originated from a former ammunition plant at Stadallendorf (State of Hesse, Germany). This ammunition plant soil (AP-soil) was loamy sand with 1.3–1.9% organic carbon, and pH of 6.1 (CaCl_2). Prior to planting, AP-soil was homogenised and sieved to a 2 mm particle size. Initial (background) soil contamination of AP-soil was 3.4 mg kg^{-1} nitroaromatics, preferentially TNT.

1.3 Incubation system and transpiration control

Immediately after tree potting, two 6-mm glass fibre wicks (Ortmann, Hilden, Germany) were inserted 3 cm into the basis of growth substrates. Upper wick ends were fixed above pot ground with plastic clamps and pots were placed in scaled

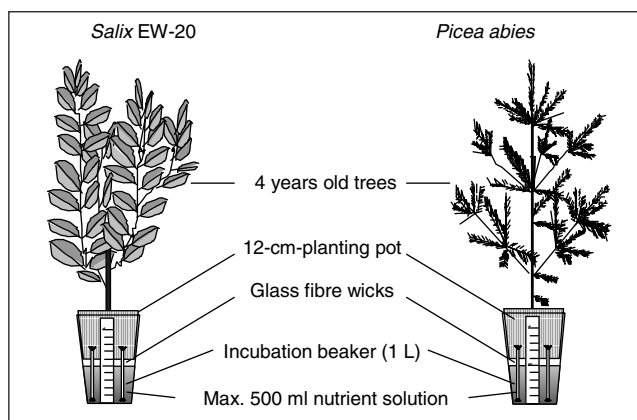


Fig. 1: Experimental design for incubation of plants and for pulse application of [^{14}C]-2,4,6-trinitrotoluene

1-litre-beakers (Fig. 1). Outer surfaces of beakers were coated black to prevent photooxidative TNT-degradation and to avoid algae growth. Tree nutrient solution was prepared with calcium-rich tap water and tree fertiliser Osmosol 523 (NPK: 23+9+12+trace elements, Scotts Europe B.V., Helen, Netherlands). Applied nutrient concentration was 0.1 g kg^{-1} water (pH 6.3). Nutrient solutions were completed to initial levels of 500 ml when basal storage beakers were empty. Groups of 12–20 potted trees were pre-cultivated in a temperature-controlled greenhouse at 19°C for five months. Additional light was provided from high pressure sodium lamps with a 16 hr daily photoperiod. Photomorphogenetic tree development was comparably to outdoor growth. Evapotranspiration of each tree pot was registered gravimetrically by weighting the whole incubation system.

1.4 Application of [^{14}C]-TNT

After pre-cultivation, two trees with equal growth and comparable transpiration rates were selected for [^{14}C]-TNT application for each experimental variant. In an aqueous volume of 37.5 ml, 3.12 mg TNT were added per tree ($631 \pm 12 \text{ kBq}$ per vessel) leading to a calculated soil concentration of $5.2 \text{ mg TNT per kg dry soil}$. This single pulse application was performed either, directly into sand and AP-soil, or indirectly into basal nutrient solution. Variants for [^{14}C]-trinitrotoluene application are listed in Table 1.

1.5 Determination of radioactivity

For overall analysis of ^{14}C in substrates and tree tissues, sample aliquots of 200 mg dry weight were combusted for 4 min (Biological Oxidizer OX 500, Zinsser Analytik GmbH, Frankfurt/M, Germany). Developed $^{14}\text{CO}_2$ was trapped in 10 ml of 'Oxisolve C-400' scintillation cocktail (Zinsser Analytic, Berkshire, UK) and radioactivity was measured in a Multipurpose Liquid Scintillation Counter (LSC, Model LS 6500, Beckman Instruments GmbH, Munich, Germany). Oxidizer efficiency was controlled by oxidation of standards with a known radioactivity. Combustion efficiency ranged from 97 to 101%. Blanks were subtracted. Extracts of plant material and substrates were analysed directly by LSC, using an 'Ultima Gold' scintillator for aqueous samples (Packard Bioscience B.V., Groningen, Netherlands) and a 'Quicksafe A' scintillator

(Zinsser Analytic, Berkshire, UK) for organic solvents. Volumes were 5 ml sample plus 5 ml scintillator.

1.6 Radio thin layer chromatography

For radio TLC of root tissue extracts, a mobile phase used by Sens et al. (1999) [6] was applied, consisting of acetone/methanol/water in a volume ratio of 200:30:20. Stationary phases were 20 x 20 cm-plates of 'Silica Gel 60 F254', thickness 0.25 mm (Merck, Darmstadt, Germany). Sample application was performed automatically with a 'Linomat IV' (Camag, Muttens, Switzerland). Application volumes varied from 100 to 200 μ l. TLC plates were evaluated with an 'Automatic TLC-Linear Analyser' (Berthold Technologies, Bad Wildbad, Germany). Co-chromatographed reference substances were detected by means of ultraviolet light.

1.7 Tree tissue preparation

At 5 and 14 days after addition of radiolabelled TNT, samples of apical branch tips were taken from each tree to control their radioactivity by ^{14}C -oxidation. 60 days after application, trees were harvested rapidly. Tree compartments and ambient materials were separated into the parts indicated in the respective figures or tables. Phloem bearing bark was peeled away from xylem-wood of stems. Plant and substrate samples were dried for 24 hours at 40–50°C and dry weight was estimated. Polypropylene pots and vessels were washed with water and rinsed with ethyl acetate. Amounts of liquids were determined gravimetrically and aliquots were subjected to LSC.

Plant samples were grinded to powder for 5 min with an oscillating steel disk mill (Type TS 250, Siebtechnik GmbH, Mühlheim/R, Germany). Final dry weight was controlled for losses during homogenisation. 5–10 aliquots of 150–200 mg dry weight were separated from each tissue sample for determination of overall radioactivity by oxidizer combustion.

1.8 Extraction of soil and plant material

Triplicate samples of 5 g dry weight of sand or AP-soil and of 200 mg of powdered tree tissue were extracted with 50% v/v acetic acid, methanol, acetonitrile, ethyl acetate or dichloromethane. Ultrasonic extraction at 35°C lasted 15 min (frequency 31.5 kHz, Type Sonorex Super Digital, Bandelin, Berlin, Germany). Remnants and paper filters used for purification (Schleicher & Schüll, Dassel, Germany) were combusted in the biological oxidizer. Summarised radioactivity of solvent extracts and residues was compared with values of previous aliquot bio-oxidation. Thus, extraction efficiency could be calculated.

2 Results and Discussion

2.1 Evapotranspiration

Evapotranspiration rates were generally higher in *Salix* EW-20 than in *Picea abies* test plants (Fig. 2). Relative humidity of ambient greenhouse air never exceeded 45%, thus ensuring high transpiration values. A slight reduction of evapotranspiration rate of *Salix* after approx. one month indicated that the formation of new leaves and leaf enlargement

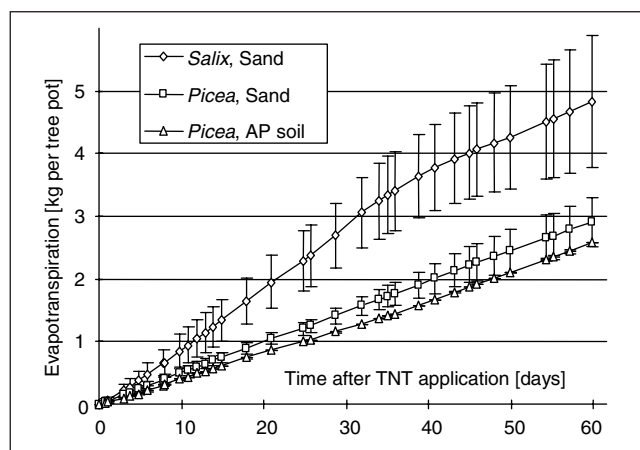


Fig. 2: Time course of cumulative evapotranspiration as a control indicator for vitality of *Salix* EW-20 and *Picea abies*. For overview reasons, substrate application variants and basal application variants were combined

in willows had ceased and that the differentiation of leaf hairs lowered transpiration.

Cumulative evapotranspiration (see Fig. 2, Table 1) shows that initial nutrient solution volumes of 500 ml had to be replaced nine times for *Salix* and five times for *Picea*, respectively. Thus, water streams, essential for the pollutant transport through planting substrates, were very intensive throughout the experiment and allowed a thorough upward 'leaching' of sand and of AP-soil.

2.2 General recovery

After 60 days, the mean recovery of [^{14}C]-TNT-radioactivity was $82.0 \pm 4.2\%$, which is comparable with 78% of Sens et al. (1998) [7] for *Phaseolus* and with 74–86% of Thompson et al. (1998) [3] for long-term incubation of *Populus*. Because apical wick parts were intensively rooted, their radioactivity was added to root portions. Radio portions of ambient materials, like vessels, nutrient and cleaning solutions were never exceeding 0.5% (see Table 1).

2.3 Appearance of radioactivity in apical tree parts

Although transpiration was slightly lower for AP-soil grown spruces, than for sand cultivated *Picea* trees (see Fig. 2, Table 1), higher concentrations of radioactivity were found in branch tips and apical needles in this variant (Fig. 3). Higher TNT-bioavailability should be expected in sand, but radioactivity reaches a twofold value five days after application in AP-soil grown spruces. This higher radioactivity in spruces cultivated in AP-soil indicates that plant uptake of radioactivity is more rapid from AP soil. It may be concluded that microbial consortia of the rhizosphere as well as microorganisms of bulk soil had already established a high TNT-degrading activity in AP-soil during the pre-cultivation time. Soil microbial communities are able to adapt to TNT toxicity [8] and it may be assumed that they had already produced TNT-metabolites that are taken up much easier than the parent compound TNT. For instance, uptake of 2-ADNT was very rapid in sand potted cuttings of willow clone EW-13, since applied 2-ADNT was measurable within 24 hours in apical branch tips and leaves (Schoenmuth 1995, unpubl. data).

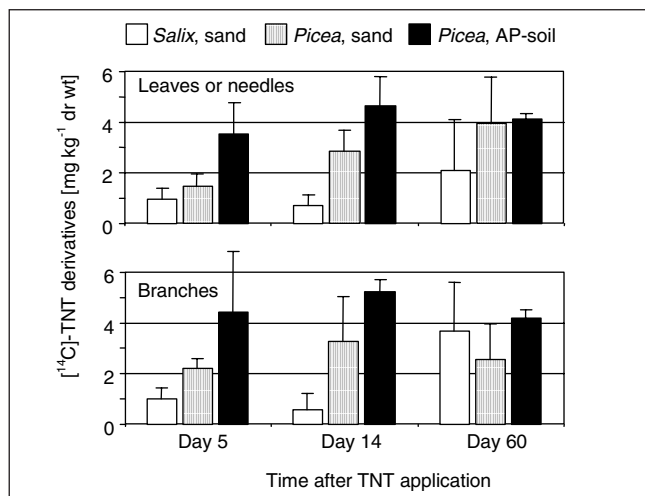


Fig. 3: Concentration of radioactivity in leaves, needles and branches after 5, 14, and 60 days

2.4 General distribution of recovered radioactivity

Main portions of recovered ¹⁴C-TNT-radioactivity for all sand cultivated trees were measured in roots, where 69.9% ± 0.8 were found. For *Picea*, grown in AP-soil 39% of recovered ¹⁴C were detected in roots (Table 1, variant 3). 14.9–27.7% of recovered ¹⁴C remained in sand and 61.4% in AP-soil. Above-ground tree parts contained 3.3–14.9% of recovered ¹⁴C.

The very high value of 14.9% for above-ground portions of sand-cultivated *Salix* in the basal TNT-application variant may be caused by high cumulative evapotranspiration of 5.1 kg water per tree (Table 1, variant 1A).

At least partly, higher AP-soil content of ¹⁴C, combined with lower root content results from technical separation problems. After sand culture, only a small portion of roots re-

mained in substrate. But from dried loamy AP-soil a great portion of visible fine roots was not separable by mechanical means under radioactive labour conditions.

The root accumulation of TNT-radioactivity after pulse TNT-application is in accordance with earlier 'cold extraction' findings for *Salix* EW-27, using excessive and long-term TNT/ADNT delivery of an AP-soil for six months. Soil-to-root accumulation factors have to consider both, approximately five-fold higher physical density (g cm⁻³) of dried soil or sand, if compared with dry root density, and concentrations of TNT-radioactivity on a dry weight basis (Fig. 4). Although TNT-supply was limited, soil to root accumulation was approximated to be ten-fold for *Salix* EW-20 and *Picea* in sand, and six-fold for *Picea* in AP-soil.

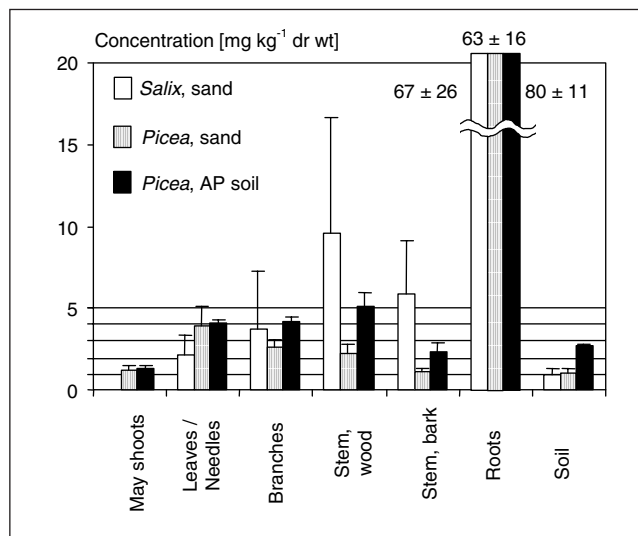


Fig. 4: Soil and tree compartment concentrations, calculated from [U-¹⁴C]-TNT label. Error bar = + standard deviation for n = 3

Table 1: Percentage of recovery of applied ¹⁴C-TNT-radioactivity, transpiration and distribution of radioactivity. AP = ammunition plant soil. Two trees were used per variant

Variant No.	1A	1B	2A	2B	3
Tree	<i>Salix</i> EW-20	<i>Salix</i> EW-20	<i>Picea abies</i>	<i>Picea abies</i>	<i>Picea abies</i>
Substrate	Sand	Sand	Sand	Sand	AP-soil
Application site	Nutrient solution	Substrate	Nutrient solution	Substrate	Substrate
A. Recovery [%]:					
Plant tissue	68.60 ± 1.92	59.64 ± 8.92	66.22 ± 8.21	57.96 ± 0.83	34.22 ± 3.35
Soil	12.00 ± 1.79	22.71 ± 13.3	18.07 ± 6.86	18.90 ± 1.29	54.25 ± 2.33
Ambient material (sum), thereof:	0.08	0.34	0.28	0.21	0.47
Residual nutrient solution	0.02 ± 0.00	0.06 ± 0.07	0.15 ± 0.11	0.13 ± 0.14	0.19 ± 0.12
Rinsing water	0.03 ± 0.01	0.04 ± 0.02	0.06 ± 0.02	0.06 ± 0.01	0.15 ± 0.01
Vessel cleaning ethyl acetate	0.02 ± 0.02	0.01 ± 0.00	0.05 ± 0.03	0.01 ± 0.01	0.03 ± 0.03
Polypropylene pot + beaker	0.00 ± 0.00	0.23 ± 0.33	0.03 ± 0.04	0.00 ± 0.00	0.10 ± 0.13
Overall sum of recovery [%] (Mean = 82.0 ± 4.2)	80.68 ± 3.74	82.69 ± 4.00	84.58 ± 1.46	77.07 ± 1.96	88.94 ± 0.72
B. Distribution of recovered radioactivity					
Sand or AP-soil	14.9%	27.7%	21.3%	24.6%	61.4%
Plant (sum)	85.1%	72.3%	78.7%	75.4%	38.7%
(Above-ground parts)	(14.4%)	(3.3%)	(8.3%)	(5.9%)	(5.0%)
(Roots)	(70.7%)	(69.0%)	(70.4%)	(69.5%)	(33.7%)
Recovered sum	100.0%	100.0%	100.0%	100.0%	100.0%
C. Cumulative evapotranspiration in 60 days					
	5.1 kg per tree	4.5 kg per tree	3.2 kg per tree	2.6 kg per tree	2.6 kg per tree

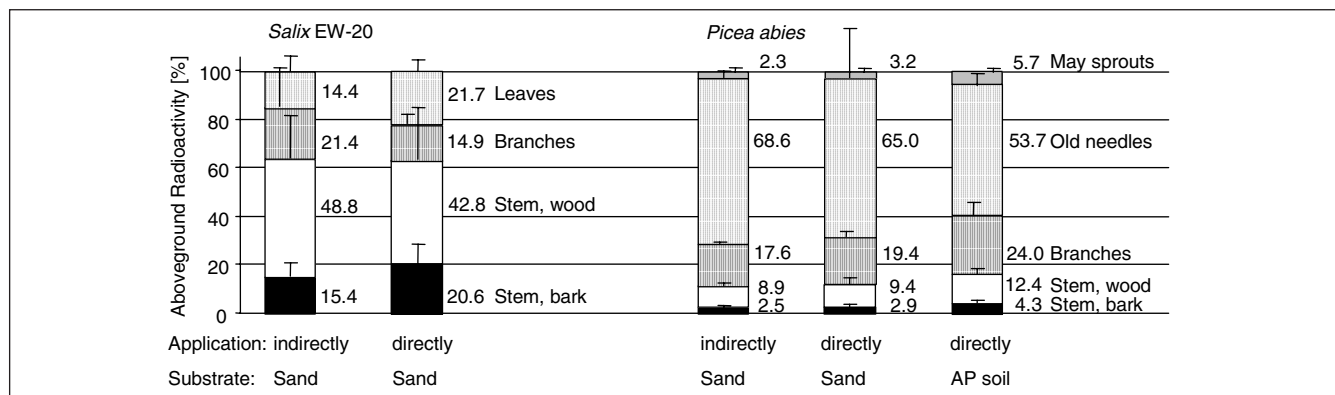


Fig. 5: Distribution of radioactivity in aboveground plant parts after different modes of [¹⁴C]-TNT application. Data are calculated from [¹⁴C]-oxidizer analyses of 10 aliquots of tree compartments. Error bar: + standard deviation for n = 10

Root accumulation results derived from AP-soil grown *Picea* (see Fig. 4) indicate that roots may extract TNT or TNT-derived compounds from soil. This phytoextraction is strongly supported by transpiration forces of trees. Importance of plant transpiration stream in mobilising organic pollutants in soil was also emphasised by Harvey et al. (2002) [9], referring to results of Liste and Alexander (2002a,b) [10,11], who showed that pollutant transport from bulk soil to the rhizosphere occurs even for the hydrophobic PAHs pyrene and phenanthrene.

2.5 Different modes of [¹⁴C]-TNT application

In the above-ground distribution of radioactivity, conformity was found between the two modes of application, [¹⁴C]-TNT addition to substrate and basal solution application of [¹⁴C]-TNT (Fig. 5). As a consequence, the simpler basal application method could be developed as a standard method for dendrotolerance experiments, where the application of TNT and other nitroaromatic compounds (NAC) was performed repeatedly or continuously and the responses of growth and transpiration were evaluated dynamically. Tree-specific dendrotolerance limits and quantification of the fate of 'cold' pollutants in the soil/tree system could be used here as essential components for the comparison of dendroremediation potentials (Schoenmuth and Pestemer 200X, in prep.).

2.6 Above-ground tree compartmentalisation of ¹⁴C

Detailed analysis of above-ground tree portions is necessary because these parts are easily accessible *in situ*. Bark removal from stem wood may additionally exclude the strong bark matrix potential of *Salicaceae* trees, which is extremely disturbing to the 'cold' analytics of NAC. Stem peeling can also prevent analytical interferences with non-removable surface contamination on the resinous bark of *Picea abies* trees which we observed *in situ*.

Above-ground distribution of ¹⁴C shows substantial differences between broadleaf trees and conifers. For *Salix* the main above-ground portion of ¹⁴C was found in bark-free wood (42–48%) and for *Picea* most of the above-ground ¹⁴C (54–69%) was detected in older needles (see Fig. 5). This high disposal of TNT derived radioactivity in needles has to be considered in long-term TNT-fate experiments, where microbial litter degradation should be examined.

Higher distribution ¹⁴C percentages in wood of *Salix* and of *Picea*, compared with bark, are not only due to the mass dominance of wood in tree architecture, but mainly result from higher concentrations of radioactivity in wood (see Fig. 4). At least 80% of wood-located radioactivity is non-extractably bound, showing no differences in extractability between *Salix* and *Picea* (Fig. 6). As expected, in branches where bark was not separated from wood, intermediate concentrations of

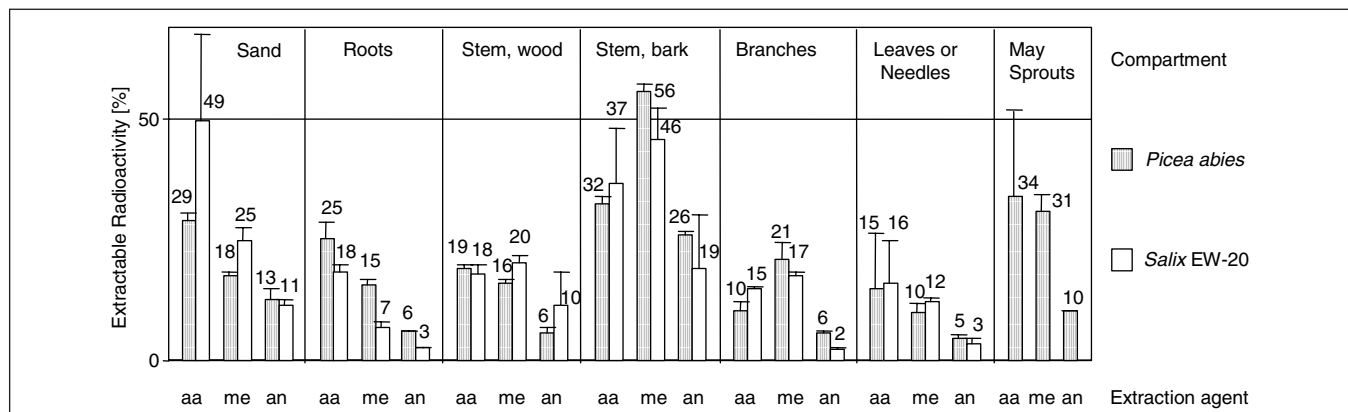


Fig. 6: Extractability of radioactivity from sand and tree compartments. aa = 50% (v/v) acetic acid, me = methanol, an = acetonitrile. Error bar: + Standard deviation for three extractions

radioactivity were found (see Fig. 4) and differences between *Salix* and *Picea* are seen to disappear here. Also, higher needle concentrations of ^{14}C (see Fig. 4) are mainly responsible for a higher needle proportion of ^{14}C in *Picea*, if compared with leaves of *Salix* (see Fig. 5).

Higher concentrations of ^{14}C in stem wood of both *Salix* and *Picea*, compared with bark, indicate that the bulk of plant radioactivity is already incorporated in wood at the time of tree harvest, and the 'supply' of wood compartments with available TNT-derivatives is nearly exhausted.

If compared with *Picea* the higher wood deposition capacity for ^{14}C in *Salix* is explainable by the principal differences

of xylem morphology of their stems [12]. In 'diffuse porous wood' of *Salix* simply more tree rings are used for water flow than in the 'nonporous wood' of *Picea* (For illustration, see Chaney 2003 [13]). Furthermore, physical density of dry willow wood is lower, thus contributing to higher 'DW-concentrations' of ^{14}C .

2.7 Possible binding sites for TNT metabolites in trees

Binding of reduced TNT metabolites to soil organic materials and sorption to clay minerals should not be discussed in this paper, because this has been done excessively by others (e.g. [14], see Fig. 7).

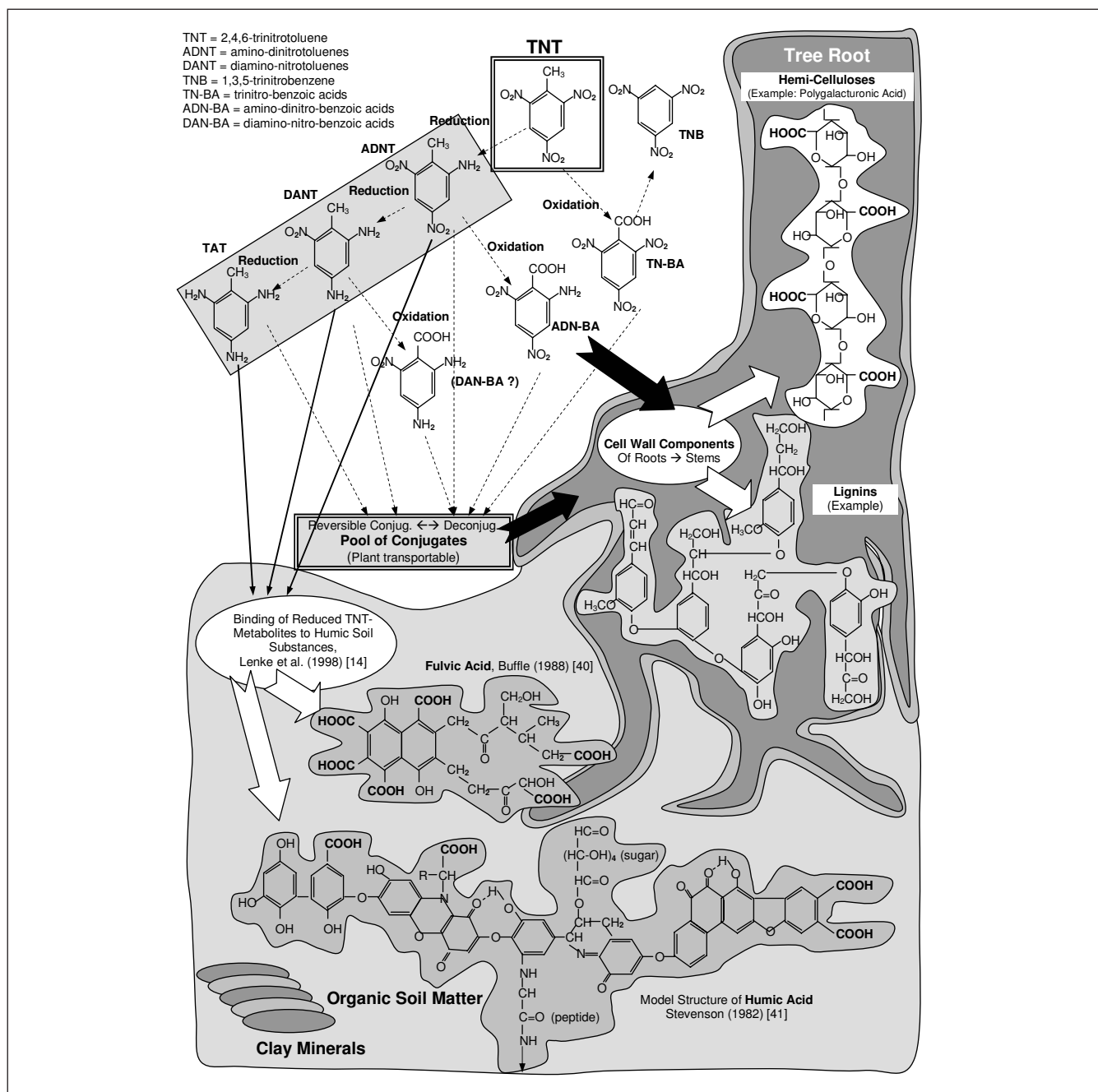


Fig. 7: Main TNT-degradation steps and possible binding sites for TNT-metabolites in tree roots and soil

For plants, it is generally accepted that the cell wall is one of the major detoxification/disposal sites for endogenous plant substances and for xenobiotic organics as well. After the application of [^{14}C]-TNT, for instance, 50% for *Phaseolus* [7], and 57% for *Triticum* [6] of the roots' ^{14}C was located in cell wall fractions. Aside from 48% cytoplasm- ^{14}C in *Phaseolus*, 20% of [^{14}C]-TNT root-radioactivity was detected in the lignin fraction, followed by 14% in hemicelluloses and 5% in pectins, but no ^{14}C was found in cellulose [7].

It is assumed that the bulk of non-extractable ^{14}C in tree compartments is also localised in the cell wall of trees. Here, we consider lignins and hemicelluloses as main targets for ^{14}C deposition (see Fig. 7). The mechanism of binding seems to be simple for hemicelluloses, since amino groups of TNT-derived ADNTs or DANTs are possibly reacting with free carboxylic groups. For lignins, which are of different compositions in 'hardwood' *Salix* trees and in 'softwood' conifers, binding mechanisms could be sequestration into three-dimensional lignin molecules or introduction of TNT metabolites into lignin synthesis pathways as already postulated by Lyr (1993, pers. comm.).

We agree with Trapp et al. (2001) [15], who concluded from experiments with excised branch segments of *Quercus robur* and *Salix viminalis* that 'wood can serve as a safe sink for environmental chemicals'. However, we have to transport this finding downwards to the soil located wood of roots as a main disposal site for metabolised TNT.

Rapid transformation of TNT is obviously confirmed by our *in-situ* analyses of stem wood of old trees, growing for 50 years on a highly TNT polluted 'Tanne' AP-area. Besides very high root contents of TNT, both in *Salix caprea* and in *Picea abies* trees, ADNTs were only found in above-ground plant compartments if *in situ* transpiration was extremely high and, thus, high upward transport velocity of TNT derivatives could temporarily exceed binding or metabolism rates for TNT.

2.8 Extractability of ^{14}C from tree tissue

In most cases of our tree tissue extractions, 50% acetic acid showed the best efficiency, if compared with methanol and acetonitrile (see Fig. 6). Nonpolar extraction agents (acetyl acetate and dichloromethane) showed root extraction efficiencies beneath the values for acetonitrile. Mixtures of acetonitrile/water in a 50/50 (v/v) ratio, recommended for TNT-metabolite extraction from plant tissue [16], could also not enhance extraction yield.

In tree tissues, in general, cortex (bark) of stems (*Picea* and *Salix*) and May sprouts (*Picea*) show the highest degree of extractability of TNT-derived radioactivity. Cortex and May sprouts bear many cytoplasm-rich cells in which differentiation is just beginning. The highest metabolic activity and a high 'sink' potential should be expected here for the cambium-near area of the cortex and for rapid growing May shoots. For younger roots, similar or higher extraction results are anticipated, which are hidden in our present results by the dry weight dominance of older roots in overall root mass (see Fig. 6). Vacuoles in these young cells may only serve as a temporary disposal site for extractable TNT-derivatives, since fully differentiated (vacuolated) cells of ma-

ture leaves (*Salix*) and older needles (*Picea*) show lower extraction percentages (see Fig. 6), although overall ^{14}C concentration is higher in old *Picea* needles than in May shoots (see Fig. 4). Thus, we may conclude that extractable ^{14}C in *Picea* and *Salix* could be mainly located in cytoplasm.

2.9 Polar root extractables

Beside polar unknowns, Thompson et al. (1998) [3] detected TNT and ADNTs in root extracts of hydroponically grown young *Populus*. The authors also detected TNT, 4-ADNT, 2-ADNT and probably additionally 2,4-Diamino-6-nitrotoluene (DANT) in poplar roots and stems, which had been grown in [^{14}C]-TNT-spiked soil. Also, Thorne (1999) [17] identified TNT, 2-ADNT and 4-ADNT in acetonitrile extracts of roots of the fibre plant *Hibiscus cannabinus* after irrigation with TNT-water or after cultivation in TNT-soil. Hydrolysis of extracted residues with 0.5 N NaOH and 50% H_2SO_4 yielded further ADNTs and also 2,4-DANT and 2,6-DANT. The conclusion was drawn that ADNTs and DANTs were conjugated. Both isomers of ADNT were shown in submersed *Myriophyllum aquaticum* plants after TNT-exposure [18]. Wayment et al. (1999) [19] identified TNT derived conjugates in *Myriophyllum* and also conjugate formation via ADNT was observed after ADNT-feeding. Earlier phytohormone research already revealed that various types of conjugation of endogenous plant substances or xenobiotics to low molecular compounds are common mechanisms in plants, because they could be found for very different chemical compound groups, for auxins [20, 21, 22], cytokinins [23], gibberellins [24], and abscisic acid [25].

In root extracts of *Salix* and *Picea*, none of the following known TNT metabolites or TNT-related compounds could be detected: TNT, ADNTs, DANTs, 1,3,5-trinitrobenzene (TNB) 2,4-dinitrotoluene and 2,6-dinitrotoluene (Fig. 8).

Instead, five unknown, very polar ^{14}C -compounds or metabolite groups were detected (U1–U5). U1 is present in both *Salix* and *Picea*, but it is only extractable with acetic acid. U2 was found only in acetic acid extracts of *Salix*. U3 is only present in *Picea*. U4 seems to be present in all variants, with a lower extraction efficiency for methanol. Detection of

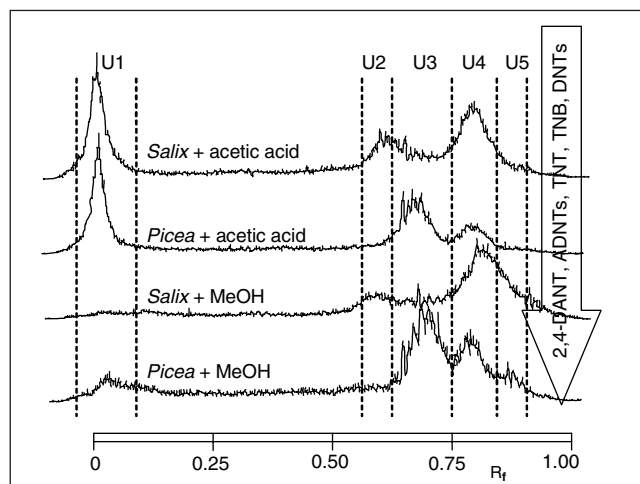


Fig. 8: Separation of root extracts by thin layer radiochromatography. U1 – U5 = unknown metabolites or metabolite groups

U5 is uncertain in *Picea*, because of weak scanner signals. We assume that mild acidic extraction with acetic acid is suitable to obtain such TNT-metabolites, which are not covalently bound to tissues. Schoenmuth (1996) [4] showed that hydrolysis with hot sulphuric acid with the Gorge-method [26, 27] could double acetic acid extraction yields of ADNTs (and few TNT) in root tissue of hybrid *Salix* EW-13 and *Populus* ZP-007, thus additionally extracting covalently bound ADNTs.

Although we have no evidence at this time, we assume that polar unknowns, extracted from roots of *Salix* and *Picea* (see Fig. 8), are mainly represented by oxidised polar metabolites of TNT, ADNTs and other intermediates of TNT-degradation pathways. Water-soluble conjugates of TNT, ADNTs and other TNT transformation products could additionally contribute to the small extractable portion of ¹⁴C compounds.

Oxidised, highly polar TNT metabolites, e.g. trinitrobenzoic acid (TNBA) and 2-amino-dinitrobenzoic acid (2-ADNBA) were water extracted from soil [28] and from *Myriophyllum aquaticum* plants [29]. Plant root surface peroxidases, which were proven to oxidise phenolics [30], could also be responsible for TNT and/or ADNT oxidation in tree roots. High molecular weight conjugates, derived from TNT and RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) were characterized in several aquatic and terrestrial plant tissues by gel permeation chromatography [18]. Reversible low molecular weight conjugation could temporarily detoxify TNT metabolites and these conjugates might serve as transport forms for long distance transport within the apoplast, together with oxidized metabolites. For airborne PAH (pyrene) aside from oxidation, the formation of conjugates was detected in leaves of woody plants as well [31].

2.10 Confirmation experiments

Most laboratory experiments with trees are 'low number' experiments and demand a general validation. Applying comparable wick-application methods, effects of TNT/ADNT-disappearance were similar in additional experiments using four-week application periods with repeated 'cold' TNT-supply (every 2–3 days) followed by a two-week 'recovery' phase for juvenile *Salix* EW-13 (98.8%). Disappearance of TNT/ADNT was proven quantitatively for adult conifers of *Picea abies* (97.4%), *Picea glauca* (96.7%), and *Pinus sylvestris* (97.5%), and for adult *Salix* EW-13 (99.4%).

Using juvenile *Salix* EW-13, in addition to TNT, we could quantify the disappearance of 4-ADNT (96.2%), TNB (97.8%), 2,4-DNT (88.4%) and 2,6-DNT (95.9%) in soil/tree systems (Schoenmuth and Pestemer 200X, in prep.).

3 Conclusions

(i) Results of complete TNT-transformation in tree systems explain some of our previous findings with 'cold analytics', where no TNT and no ADNT-metabolites were could be detected in *Salix* and *Populus* clones after TNT feeding.

(ii) Our results also demonstrate that 'cold' tissue extraction and analyses of tree organs (or herbaceous plant material) are not suited for quantitative success control of TNT phytoremediation *in-situ*, since only the *status quo* of over-

lapping dynamics of TNT-uptake and proceeding TNT transformation can be reflected here.

(iii) Both fast growing *Salicaceae* trees, and conifer forests have a dendroremediation potential for TNT, because of their TNT degrading capacity and because of the large biomass of roots and stems as deposition compartments for metabolised xenobiotics.

4 Recommendations

Morphological tree compartmentalisation experiments have to be completed for other common explosives, for which plant uptake has been shown, e.g. for HMX (octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine) [17,32–35], RDX [34–38] and tetryl [39]. Biochemical compartmentalisation of soil-derived explosives in deciduous and coniferous trees tissues is also required. Unknown polar metabolites have to be identified, to assess their ecotoxicological potential. Long-lasting, composting experiments should elucidate long-term fate of tree-incorporated derivatives of explosives.

5 Outlook

After establishment, trees will be superior to herbaceous plants and to soil inoculation with laboratory-selected microorganisms in sustainable *in situ* remediation strategies for explosives and other organic soil pollutants. With their minimum soil requirements, low maintenance costs and their long-term bioindication potential, trees are enabled to confront the longevity of the TNT problem. When tree specific quantification of dendroremediation potential for plant degradable TNT will be comparably, site-specific application of dendroremediation will be roughly assessable.

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