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Dendroremediation of Trinitrotoluene (TNT). Part 2: Fate of TNT in Morphological Compartments of Trees

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Abstract

Background, Aim and Scope. The problem of the long-term existence of the environmental contaminant 2,4,6-trinitrotoluene (TNT) and the necessity for the use of trees ('dendroremediation') in sustainable phytoremediation strategies are described in the first part of this paper. As an integrated part of the dendroremediation research concept for TNT, described in part 1, the aim of the second part is the estimation of [¹⁴C]-TNT uptake, of localisation of TNT-derived radioactivity in mature tree tissues, and of TNT-degradation during the dendroremediation process.

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

Results. After 60 days, recovered ¹⁴C is accumulated in the root (70% for the sand variants, 34% for the AP-soil variant). 15-28% of ¹⁴C remained in sand and 61% in soil. For the aboveground portions (3.3 to 14.4%) differences occur between *Salix* and *Picea*. In *Salix*, nearly the half of the aboveground-¹⁴C is found in bark-free wood, whereas in *Picea* the main aboveground compartments are older needles (54-69%).

TNT is readily transformed in tree tissue. Approximately 80% of ¹⁴C is obviously nonextractable bound in roots, wood and leaves or needles. Only the quantitatively less important stem-bark and the May shoots showed higher extraction yields of up to 56%.

Discussion. The pulse application of [¹⁴C]-TNT provides evidence for the first time, that after TNT-exposure, in plant root extracts no TNT and none of the known metabolites, mono-amino-dinitrotoluenes (ADNT), diaminonitrotoluenes (DANT), trinitrobenzene (TNB) or dinitrotoluenes (DNTs) are present. The small extractable fractions contained for *Salix* at least three unknown metabolites (or groups) and for *Picea* four metabolites, where only one metabolite seems to be identical for *Salix* and *Picea*. All detected unknown metabolites were of a very polar nature.

Conclusions. The results of complete TNT-transformation in tree systems explain some previous findings with 'cold analytics', where no TNT and none of the ADNT-metabolites were found in tissues of *Salix* and *Populus* clones. It is concluded that 'cold' tissue extraction and analyses of tree organs or of herbaceous plant material are not suited for success control of phytoremediation applications *in situ*.

Recommendations and Outlook. Both, the fast growing *Salicaceae* trees, and conifer forests have a dendroremediation potential for TNT polluted soils. The large biomass of adult trees with their woody compartments of roots and stems may be utilized for the detoxification of 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

The 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 described in the first part of this report (1).

The main purpose of the present paper was to give an assessment support for other experiments, integrated in the described dendroremediation concept, which have the strategic goal to make dendroremediation more calculable for TNT and also for other nitroaromatic compounds (NAC).

Data regarding the fate and the compartmentation of radiolabelled TNT are restricted to juvenile trees (2). Up to now, no results are available for uptake and transformation of [¹⁴C]-TNT in mature woody plants for both, deciduous and coniferous trees.

1 Methods

1.1 Chemicals and Standards

Uniform ring-labelled [¹⁴C]-TNT (CAS-Nr. 118-96-7) was synthesised by Laszlo Vollner (International Isotopes Munich, Germany). The specific activity was 3.16 GBq g⁻¹ (19.47 mCi mmol⁻¹). Radiopurity was >95%, controlled by 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 obtained from a previous TNT growth screening project (3, 4). The trees show an apple-like leaf shape and a good branching tendency. The period for successive vegetative propagation is limited to the early spring. The moderately fast growing tree is drought resistant and exhibits a medium transpiration capacity which is in the same range as transpiration of *Picea abies*. *Salix* EW-20 is able to take up and to transform 'cold' TNT to ADNT (3, 4). The willow clone showed an 'absolute' growth tolerance to TNT, i.e. although growth was slightly inhibited by TNT, TNT-influenced growth is sufficient high and exceeded the growth of other clones which were showing a 'relative' TNT-tolerance, i.e. they were not subjected to a growth reduction by TNT (3, 4). *Picea abies* plants were available from a local origin (Forstbaumschule Luckenwalde, Germany).

Four years old trees of each, hybrid *Salix* EW-20 and *Picea abies*, respectively, 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 induce the formation of fresh 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. Immediately before potting the fresh weight of each tree was estimated and only plants with a similar initial weight were used for further experiments.

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 the former ammunition plant Stadtallendorf (State of Hesse, Germany). This ammunition plant soil (AP-soil) was loamy sand with 1.3-1.9% organic carbon, and a pH of 6.1. Prior

to planting, the AP-soil was homogenised and sieved to 2 mm particle size. Initial (background) soil contamination of AP-soil was 3.4 mg/kg nitroaromatics, preferentially TNT.

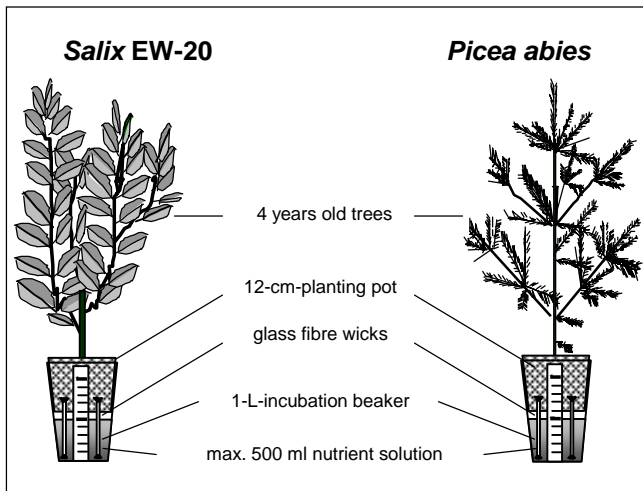


Fig. 1: Experimental design for the incubation of plants and for pulse application of [U-¹⁴C]-2,4,6-trinitrotoluene.

1.3 Incubation system

Immediately after tree potting, two 6-mm glass fibre wicks (Ortmann, Hilden, Germany) were inserted 3 cm into the basis of the growth substrates of the pots. The upper wick ends were fixed above the pot ground with a plastic clamp. The pots were placed in scaled 1-litre-beakers. The basal 10 cm wick ends reached to the beaker ground allowing a moderate basal water and nutrition supply (**Fig. 1**). The outer surface of the beakers was black coated to prevent photooxidative TNT-degradation and to avoid algae growth. The incubation system was a tree-adopted variant of the incubation systems, originally developed for herbaceous plants by Pestemer and Pucelik-Günther (1997) (5) which were also used by Gong et al. (1999) (6) for TNT-phytotoxicity testing of seedlings of *Lepidium*, *Brassica*, *Avena* and *Triticum*. The tree nutrient solution was prepared with a calcium-rich tap water and Osmosol 523 (NPK: 23+9+12+trace elements, Scotts Europe B.V., Helen, Nederland). The applied nutrient concentration was 0.1 g kg⁻¹ water, with a pH of 6.3. Nutrient solutions were only completed to the initial levels of 500 ml when the basal storage beakers were empty.

1.4 Transpiration Control

All trees were further cultivated in a temperature controlled greenhouse at 19°C. Additional artificial light was provided from high pressure sodium lamps with an 16 hr daily photoperiod. Intensity of the additional light in the photosynthetically active region (PAR) was 240 μmol m⁻² s⁻¹ at 40 cm plant height level. The spectral light composition allowed photomorphogenetic tree development, comparable to outdoor conditions.

From groups of 12-20 plants evapotranspiration of each tree was monitored at time intervals of 2-3 days for a five-month period. Consumption of nutrient solution was registered gravimetrically with a digital balance by weighting the whole incubation system.

1.5 Application of [¹⁴C]-TNT

After pre-cultivation, two trees with equal growth and comparable transpiration rates were selected for [¹⁴C]-TNT application for each experimental variant. In an aqueous volume of 37.5 ml, 3.12 mg TNT were added per tree (631 ± 12 kBq per vessel) leading to a calculated soil concentration of 5.2 mg TNT per kg dry soil. This single pulse

application was performed either, directly into the sand and AP-soil, or indirectly into the basal nutrient solution. The variants for [¹⁴C]-trinitrotoluene application are shown in **Table 1**.

1.6 Determination of Overall Radioactivity

For overall analysis of ¹⁴C the 'Biological Oxidizer OX 500' (Zinsser Analytik GmbH, Frankfurt (M.), Germany) was used. Usually, aliquots of 200 mg dry weight were oxidized for 4 min. Developed ¹⁴CO₂ was trapped in 10 ml of 'Oxisolve C-400' scintillation cocktail (Zinsser Analytic, Berkshire, UK) and transferred to 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. The combustion efficiency ranged from 97 to 101%. Blanks were subtracted.

Extracts of plant material were analysed directly by LSC, using 'Ultima Gold' scintillator for aqueous samples (Packard Bioscience B.V., Groningen, Nederland) and 'Quicksafe A' scintillator (Zinsser Analytic, Berkshire, UK) for organic solvents. The volumes were 5 ml solvent sample plus 5 ml scintillator.

1.7 Radio Thin Layer Chromatography

For separation of analytes from root tissue extracts, radio thin layer chromatography (TLC) was utilized. The mobile phase, used by Sens et al. (1999) (7) was applied, consisting of acetone/methanol/water in a volume ratio of 200:30:20. The stationary phases were 20x20 cm-plates of 'Silica Gel 60 F254' with a thickness of 0.25 mm (Merck, Darmstadt, Germany). Sample application to the plates was done automatically with a 'Linomat IV' (Camag, Muttenz, Switzerland). The application volumes were varied between 100 and 200 µl. Radioactivity distribution on the plates was evaluated with an 'Automatic TLC-Linear Analyser' (Berthold Technologies, Bad Wildbad, Germany). Co-chromatographed reference substances were detected under ultraviolet light.

1.8 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 as already described. 60 days after application, the trees were removed from sand or AP-soil. The tree compartments and ambient materials were separated with forceps and pruning shears into the parts indicated in the respective figures or tables. The phloem bearing bark was peeled away from the xylem-wood of the stems. After cutting the woody parts to pieces of 1 cm, all plant and substrate samples were dried for 24 hours at 40-50°C and dry weight was estimated. The polypropylene pots and vessels were washed with water followed by rinsing with ethyl acetate. Amounts of rinsing solutions were determined gravimetrically and aliquots of samples were subjected to liquid scintillation counting (LSC).

All plant-derived samples were grinded to powder for 5 min with an oscillating steel disk mill (Type TS 250, Siebtechnik GmbH, Mühlheim/R., Germany) and final dry weight was controlled for losses during homogenisation. From each tissue sample 5-10 aliquots of 150-200 mg dry weight were separated for the determination of the overall radioactivity by combustion in the biological oxidizer.

1.9 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, respectively, were extracted with 50% v/v acetic acid, methanol, acetonitrile, ethyl acetate or dichloromethane. Each extraction lasted 15 min and was performed in a temperature controlled ultrasonic bath at 35°C with a frequency of 31.5 kHz (Type Sonorex Super Digital, Bandelin, Berlin, Germany). After extraction, plant residues and the paper filter used for purification

(Schleicher & Schüll, Dassel, Germany) were combusted readily in the biological oxidizer to compare summarised radioactivity of solvent extract plus extracted residues with those obtained by previous aliquot bio-oxidation. Thus, percentage of the extraction efficiency could be calculated.

1.10 Assessment of the Applied Method

Traditional planting and soil-spiking procedures are not applicable for elder trees. Planting of elder trees into spiked soil is leading to a planting shock, mainly caused by fine root damage. Conventional soil spiking by adding TNT, pre-solved in organic solvents or mixing soils with a known contaminant concentration interfered in other experiments with the 'after-effect of soil homogenisation' ('ASH-effect'), which leads to a unspecific, rapid initial decline of the soil TNT-content (Schoenmuth et al. 200X, in prep.). This effect is very common and may mask the remediation effect of plants and it disturbs ecotoxicologic assessments. Examples for the 'ASH-effect' may be found throughout the TNT research area, mostly known from sample storage. Ageing or weathering of disturbed soil for some months hides the effect. The benefits of the such 'ASH'-effects may be utilized for TNT- decontamination by mechanical treatment of TNT-soil (8) and other 'land farming' soil sanitation procedures. In our outdoor experiments with tree planted lysimeter pots the 'ASH'-effect led to an 85% TNT loss within a five-month period, while the trees were just establishing their dendroremediation potential (Schoenmuth and Pestemer 200X, in prep.) and our findings are in accordance with the 90% 'ASH'-effect within 6 month, found *in situ* in a former ammunition plant soil of the 'Tanne'-project (9, 10).

Although *Salicaceae* trees were shown to be able to transport oxygen down to the roots (11), flooding conditions should be strictly avoided in our experiments. Reductive soil conditions are not comparable to outdoor conditions of the vadose soil zone, where flooding only occasionally occurs. Furthermore, *Picea abies* is not known to be flooding tolerant and anaerobiosis destroys the conifer mycorrhiza.

Axenic tree cultures could not be applied for our purposes, since such conditions are not practicable with lignified older trees and extremely juvenile tree material is not comparable with the outdoor-cultivated trees. For instance, we found that axenic agar-cultivated seedlings of *Picea abies* were already inhibited in root growth and hypocotyl elongation by a 5 mg l⁻¹ substrate concentration of TNT, whereas soil cultivated four-years-old *Picea* tolerated at least 15 mg l⁻¹ TNT during a continuous application period of four weeks (Schoenmuth 200X, in prep.).

Our incubation design excludes accidental surface contact by splash water. The applied method further avoids the problems of spatial contaminants heterogeneity (12), of uncertain bioavailability (13) and of non-predictable 'ASH-effects', which seem to be the current bottlenecks for phytoremediation control, not only *in situ*. Moreover, the method separates the experimental contamination site from the remediation place by mimicking the natural soil solution supply of the trees with the bioavailable pollutant (TNT). The method is applicable for other water-soluble soil contaminants and for various planting substrates. It allows a single pulse application of the pollutant and the application velocity can be regulated by the controllable tree (evapo-)transpiration rate.

2 Results and Discussion

2.1 Evapotranspiration

Evapotranspiration rates are generally higher in *Salix* EW-20 than in *Picea abies* test plants (**Fig. 2**). The relative humidity of the ambient greenhouse air was never exceeding 45% thus ensuring high transpiration values. The slight reduction of the evapotranspiration rate of *Salix* after approx. one month indicates that the formation of new leaves and the leaf enlargement in willows has nearly ceased and differentiation of leaf hairs lowers transpiration.

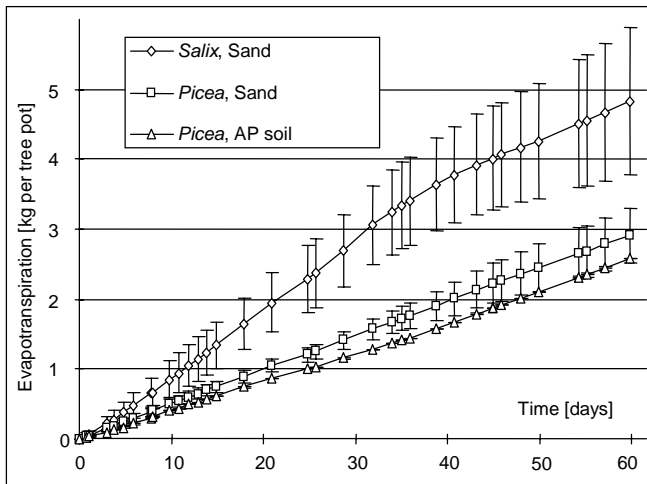


Fig. 2: Time course of cumulative evapotranspiration as a control indicator for vitality of *Salix* EW-20 and *Picea abies*

The cumulative evapotranspiration (**Fig. 2, Table 1**) shows that the initial nutrient solution volume of 500 ml had to be replaced nine times for *Salix* and five times for *Picea*, respectively. Thus the water stream, essential for the pollutant transport through the planting substrate, was very intensively throughout the experiment and allowed a thoroughly upwards 'leaching' in the sand and the 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) (21) for *Phaseolus* or 74-86% of Thompson et al. (1998) (2) for long-term incubation of *Populus*. Because the apical wick parts were intensively rooted, their radioactivity was added to the root portion. The radio portion of ambient materials, like vessels, nutrient and cleaning solutions was not exceeding 0.5% (**Table 1**).

2.3 Appearance of Radioactivity in Aboveground Portions

Although transpiration is slightly lower for AP-soil grown spruces, than for the respective sand cultivated *Picea* trees (**Fig. 2, Table 1**), higher levels of radioactivity are found in branch tips and apical needles in this variant (**Fig. 3**). A higher TNT-bioavailability should be expected in sand, but five days after application the radioactivity reaches the twofold value in the AP-soil variant. This higher radioactivity in AP-soil cultivated spruces is obviously indicating, that the 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 the bulk soil had already established a high TNT-degrading activity in AP-soil during the five month' pre-cultivation time. The soil's microbial communities are able to adapt to TNT toxicity (14) 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, the uptake of 2-ADNT was very rapid in sand potted cuttings of the willow clone EW-13, since applied 2-ADNT was measurable within 24 hours in apical branch tips and leaves (Schoenmuth 1995, unpubl. data).

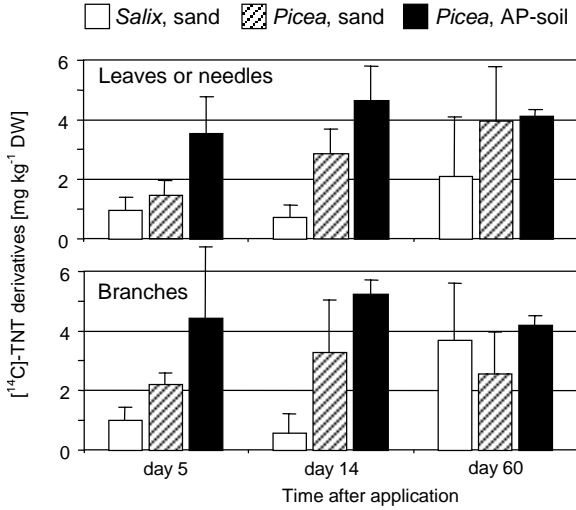


Fig. 3: Concentration of radioactivity in leaves, needles and branches after 5, 14 and 60 days

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 (all)	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 soil	14.9%	27.7%	21.3%	24.6%	61.4%
Plant (sum)	85.1%	72.3%	78.7%	75.4%	38.7%
(Aboveground 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

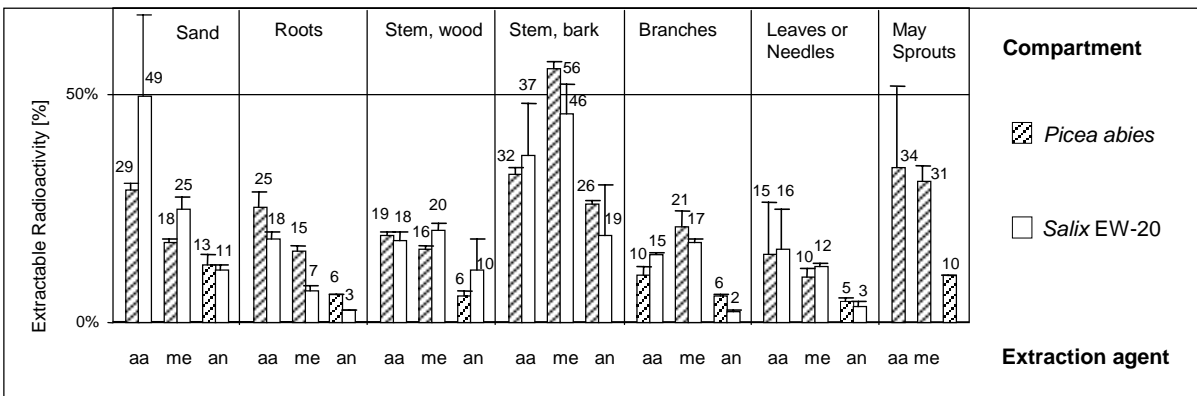


Fig. 4: Extractability of radioactivity from sand and tree compartments. aa = 50% (v/v) acetic acid, me = methanol, an = acetonitrile. Error bar: + Standard deviation for three extractions.

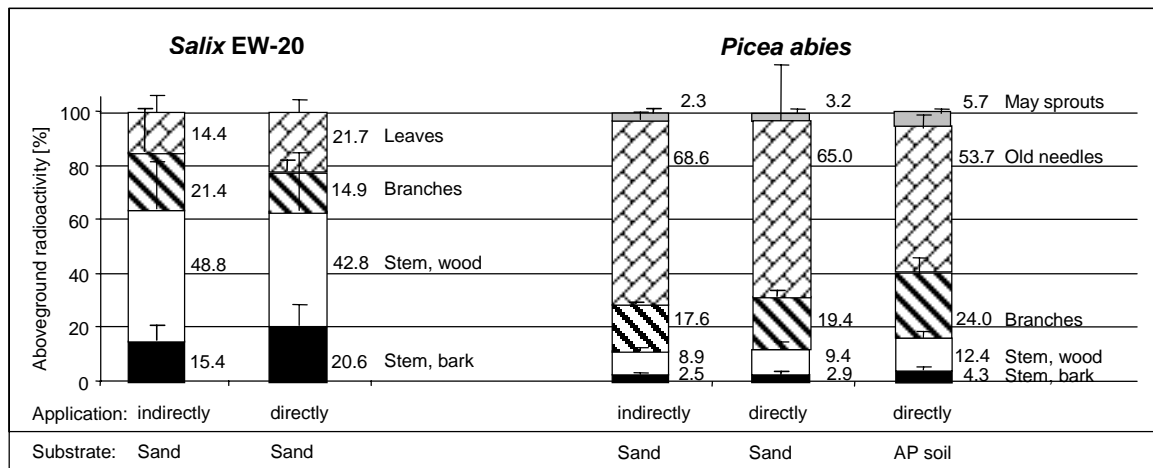


Fig. 5: Compartmentation of radioactivity in the aboveground plant parts at different modes of [^{14}C]-TNT application. Data calculated from [^{14}C]-oxidizer analyses of 10 aliquots of the respective tree compartments. Error bar: + standard deviation for $n = 10$.

2.4 General Distribution of Recovered Radioactivity

The main portion of recovered [^{14}C]-TNT-radioactivity for all sand cultivated trees was measured in the roots, where $69,9\% \pm 0.8$ were found. For *Picea*, grown in AP-soil 39% of recovered ^{14}C were detected in roots (**Table 1**, variant 3). 14.9-27.7% of recovered ^{14}C remained in sand and 61.4% in AP-soil. Aboveground tree parts contained 3.3-14.9% of recovered ^{14}C .

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

At least partly, higher AP-soil content of ^{14}C , combined with lower root content results from technical separation problems. After sand culture, only a small portion of roots remained in the substrate. But from the 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 the excessive and long-term TNT/ADNT delivery of an AP-soil for a six-month period (3). The soil-to-root accumulation factor has to consider both, the approximately five-fold higher physical density (g cm^{-3}) of dried soil or sand, when compared with dry root density, and the concentration of TNT-radioactivity on a dry weight basis (**Fig. 6**). Although, the TNT-availability was limited, the soil to root accumulations were approximated to be ten-fold for *Salix EW-20* and *Picea* in sand and six-fold for *Picea* in AP-soil.

Above all, the root accumulation results derived from AP-soil grown *Picea* (**Fig. 6**) indicate that roots may extract soil TNT or TNT-derived compounds. This phytoextraction is strongly supported by the transpiration force of the trees. The importance of the plant transpiration stream in mobilising organic pollutants in soil was also emphasized by Harvey et al. (2002) (15), referring to results of Liste and Alexander (2002a,b) (16, 17), who found, that pollutant transport from bulk soil to the rhizosphere even occurs for the hydrophobic PAHs pyrene and phenanthrene.

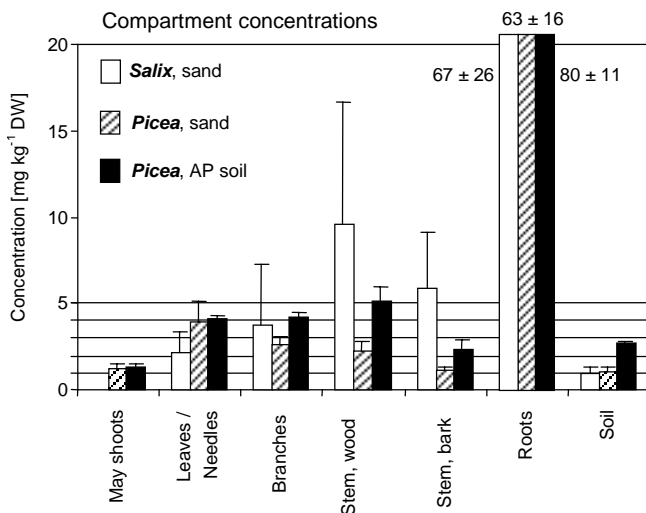


Fig. 6: Soil and tree compartment concentrations, calculated from [U-¹⁴C]-TNT label. Error bar = + standard deviation for n = 3.

2.5 Different Modes of [¹⁴C]-TNT Application

A good accordance was found in the aboveground distribution of radioactivity between the two application modes, [¹⁴C]-TNT addition to substrate and basal solution application of [¹⁴C]-TNT, respectively (Fig. 5). This had the consequence that the simpler basal application method could be further developed as a standard method for continuous and repeated pollutant application of TNT and other NAC in dynamical dendrotoxicity experiments (Schoenmuth et al. 200X, in prep.) which were delivering dendrotolerance limits as one of the essential components for the quantification of tree specific dendroremediation potential (1).

2.6 Aboveground Tree Compartmentation of ¹⁴C

Detailed analysis of aboveground tree portions is necessary because these parts are easily accessible also *in situ*. Bark removal from stem wood may additional exclude the strong bark matrix potential of *Salicaceae* trees. Stem peeling can also prevent analytical interferences with non-removable surface contaminations on the resinous bark of *Picea abies* trees, observable *in situ* (Schoenmuth et al. 200X, in prep.).

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

Higher distribution percentages in the wood of *Salix* and of *Picea*, compared with bark, are not only due to wood's mass dominance in the tree architecture, but mainly result from the higher concentrations of radioactivity in wood (Fig. 6). Thereby, at least 80% of the wood-located radioactivity is nonextractable bound, showing no differences in extractability between *Salix* and *Picea* (Fig. 4). As expected, in branches, where bark was not separated from wood, intermediate concentrations of radioactivity were found (Fig. 6) and differences between *Salix* and *Picea* are disappearing here. Also, higher needle concentrations of ¹⁴C (Fig. 6) are mainly responsible for the higher needle portion of ¹⁴C in *Picea*, when compared with the leaves of *Salix* (Fig. 5).

The higher concentrations in stem wood of both, *Salix* and *Picea*, compared with bark, indicate that at the time of tree harvest, the bulk of plant radioactivity is already incorporated in the wood, and the 'supply' with available TNT-derivatives in the wood compartment is nearly exhausted.

When compared with *Picea* the higher wood deposition capacity for ¹⁴C in *Salix* is explainable by the principal differences of xylem morphology of their stems (18). In the '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 (19)). Furthermore, the 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 should not be discussed in this paper, because this was done excessively by others (e.g. (20), see also **Fig. 8**).

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. For instance, after application of [^{14}C]-TNT, for *Phaseolus* 50% (21) and for *Triticum* 57% (7) of the roots' ^{14}C were located in the cell wall fraction. Besides 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 (21).

It is assumed, that the bulk of nonextractable ^{14}C in tree compartments is also localised in the cell wall of trees. Here, we consider lignines and hemicelluloses as main targets for ^{14}C deposition (see **Fig. 8**). The mechanism of binding seems to be simple for hemicelluloses, since the amino groups of TNT-derived ADNTs or DANTs are possibly conjugated with free carboxyl groups. For lignins, which are of different compositions in dicot *Salix* trees and in conifers, the binding mechanisms could be sequestration into the three-dimensional lignin molecules or introduction of TNT metabolites into the lignin synthesis pathways as already postulated by Lyr (1993, pers. comm.).

We agree with Trapp et al. (2001) (22), which 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 the main disposal site for metabolised TNT.

Readily transformation of TNT is apparently 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 aboveground plant compartments, when *in situ* transpiration was extremely high and thus the high upwards transport velocity of TNT derivatives could temporarily exceed the metabolisation rates for TNT (Schoenmuth and Pestemer 200X, in prep.).

2.8 Extractability of ^{14}C from Tree Tissue

In most cases of tree tissue extraction, 50% acetic acid showed the best efficiency, if compared with methanol and acetonitrile (**Fig. 4**). 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 (23) could also not enhance the extraction yield.

In general, in the tree tissues, the cortex (bark) of stems (*Picea* and *Salix*) and the May sprouts (*Picea*) show the highest degree of extractability of TNT-derived radioactivity. Cortex and May sprouts are bearing many cytoplasm-rich cells in which differentiation is just on the way. The highest metabolic activity and a high 'sink' potential should be expected here for the cambium-near area of the cortex and for the rapid growing May shoots. For younger roots, similar or higher extraction results are anticipated, which are hidden in the results by the dry weight dominance of older roots in the overall root mass (**Fig. 4**). The vacuoles in these young cells may only serve as a temporary disposal site for the extractable TNT-derivatives, since fully differentiated (vacuolated) cells of the mature leaves (*Salix*) and older needles (*Picea*) show lower extraction percentages (**Fig. 4**), although the overall ^{14}C concentration is higher in old *Picea* needles than in May shoots (**Fig. 6**). Thus we may follow, that extractable ^{14}C in *Picea* and *Salix* could be mainly located in the cytoplasm.

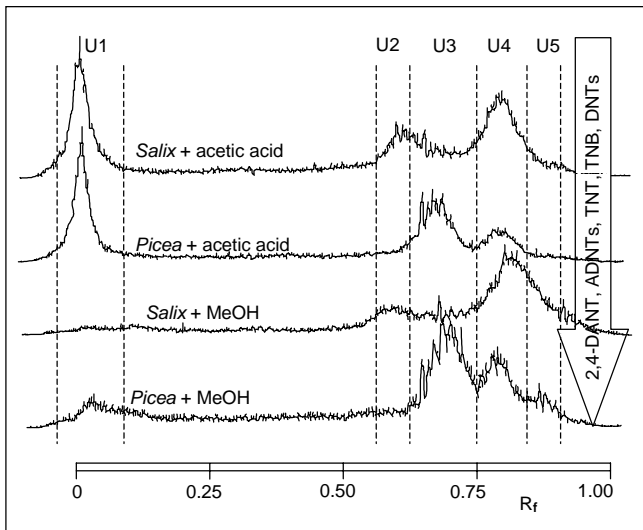


Fig. 7: Separation of root extracts by thin layer radio chromatography. U1 – U5 = unknown metabolites or metabolite groups

2.9 Polar Root Extractables

Beside polar unknowns, Thompson et al. (1998) (2) detected TNT and ADNTs in root extracts of hydroponically grown young *Populus*. The authors also found TNT, 4-ADNT, 2-ADNT and probably additionally 2,4-Diamino-6-nitrotoluene (DANT) in roots and stems, grown in [^{14}C]-TNT-spiked soil. Also, Thorne (1999) (24) found 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 the 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 found in the submersed plant *Myriophyllum aquaticum* after TNT-exposure (25). Wayment et al. (1999) (26) identified TNT-derived conjugates in aquatic *Myriophyllum* and also conjugate formation via ADNT after ADNT-feeding. Already earlier phytohormone research 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 (27, 28, 29), cytokinins (30), gibberellines (31), and abscisic acid (32).

In the 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. 7**).

Instead, five unknown, very polar ^{14}C -compounds or metabolite groups were found (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. The detection of U5 is uncertain in *Picea*, because of the weak scanner signal. We assume, that mild acidic extraction with acetic acid is suited to obtain such TNT-metabolites, which are not covalently bound to the tissue. Schoenmuth (1996) (3) showed that hydrolysis with hot sulphuric acid with the Görge-method (33, 34) could double the acetic acid extraction yield of ADNTs (and few TNT) in root tissue of hybrid *Salix* EW-13 and *Populus* ZP-007, thus extracting covalent bound ADNTs.

Although we have no evidence at this time, we assume that the polar unknowns, extracted from roots of *Salix* and *Picea* (**Fig. 7**) are mainly represented by oxidised polar metabolites of TNT, ADNTs and other intermediates of TNT-degradation pathways or by water-soluble conjugates of TNT, ADNTs and other intermediates of TNT degradation processes.

Oxidised, highly polar TNT metabolites were water extracted from soil (e.g. trinitrobenzoic acid, TNBA and 2-amino-dinitrobenzoic acid, 2-ADNBA) (35) and *Myriophyllum aquaticum* plants (36). Plant root surface peroxidases, which were proven to oxidise phenolics (37), 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 (25). Reversible low molecular weight conjugation could temporarily detoxify TNT metabolites and these conjugates might serve as transport forms for apoplasmatic long distance plant transport, together with oxidized metabolites. Also for airborne PAH (pyrene) besides oxidation, the formation of conjugates was detected in tree leaves of *Ginkgo*, *Acer* and other woody plants (38).

2.10 Conformation experiments

Most laboratory experiments with trees are 'low number' experiments, especially radiotracer investigations. Therefore those experiments demand for general validation. The effect of TNT-disappearance was similar in additional experiments using four-week application periods with repeated 'cold' TNT-supply (every 2-3 days) followed by a shorter (two-week) 'recovery' phase. TNT removal was proven quantitatively for adult conifers *Picea abies*, *Picea glauca* and *Pinus sylvestris*, adult *Salix* EW-13 and juvenile *Populus* ZP-007. Using juvenile *Salix* EW-13, additionally to TNT, we could quantify the degradation of 4-ADNT, TNB, 2,4-DNT and 2,6-DNT in the soil/tree system (Schoenmuth and Pestemer 200X, in prep.).

3 Conclusions

- (i) The results of complete TNT-transformation in tree systems explain some previous findings with 'cold analytics', where no TNT, and no ADNT-metabolites were found after TNT feeding in various *Salix* and *Populus* clones. (Schoenmuth 1994, unpubl.).
- (ii) The results also indicate, that 'cold' tissue extraction and analyses of tree organs (or herbaceous plant material) are not suited for quantitative success control of *in-situ* applications or experimental phytoremediation for TNT, since only the *status quo* of the dynamics of TNT-uptake and the proceeding TNT degradation can be reflected. Considering the spatial inhomogeneity of NAC in soil core samples, for a realistic success control for *in situ* phytoremediation pollution site and remediation site should be separated whenever possible.
- (iii) Both, the fast growing *Salicaceae* trees, and conifer woodlands have a dendroremediation potential for TNT, because of the large biomasses of the woody compartments of roots and stems and because of their enormous deposition possibilities for xenobiotics.

4 Recommendations

The morphological tree compartmentation 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) (24, 39, 40, 41, 42), RDX (43, 44, 41, 45, 42) and tetryl (46). Biochemical compartmentation 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 the long-term fate of tree-incorporated derivatives of explosives.

5 Outlook

Once established, trees will be superior to herbaceous plants and to laboratory-selected microorganisms in sustainable 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 suited to confront the longevity of the TNT problem. When the tree specific quantification of the dendroremediation potential for the plant degradable TNT will be calculable, site-specific application of dendroremediation will be roughly assessable.

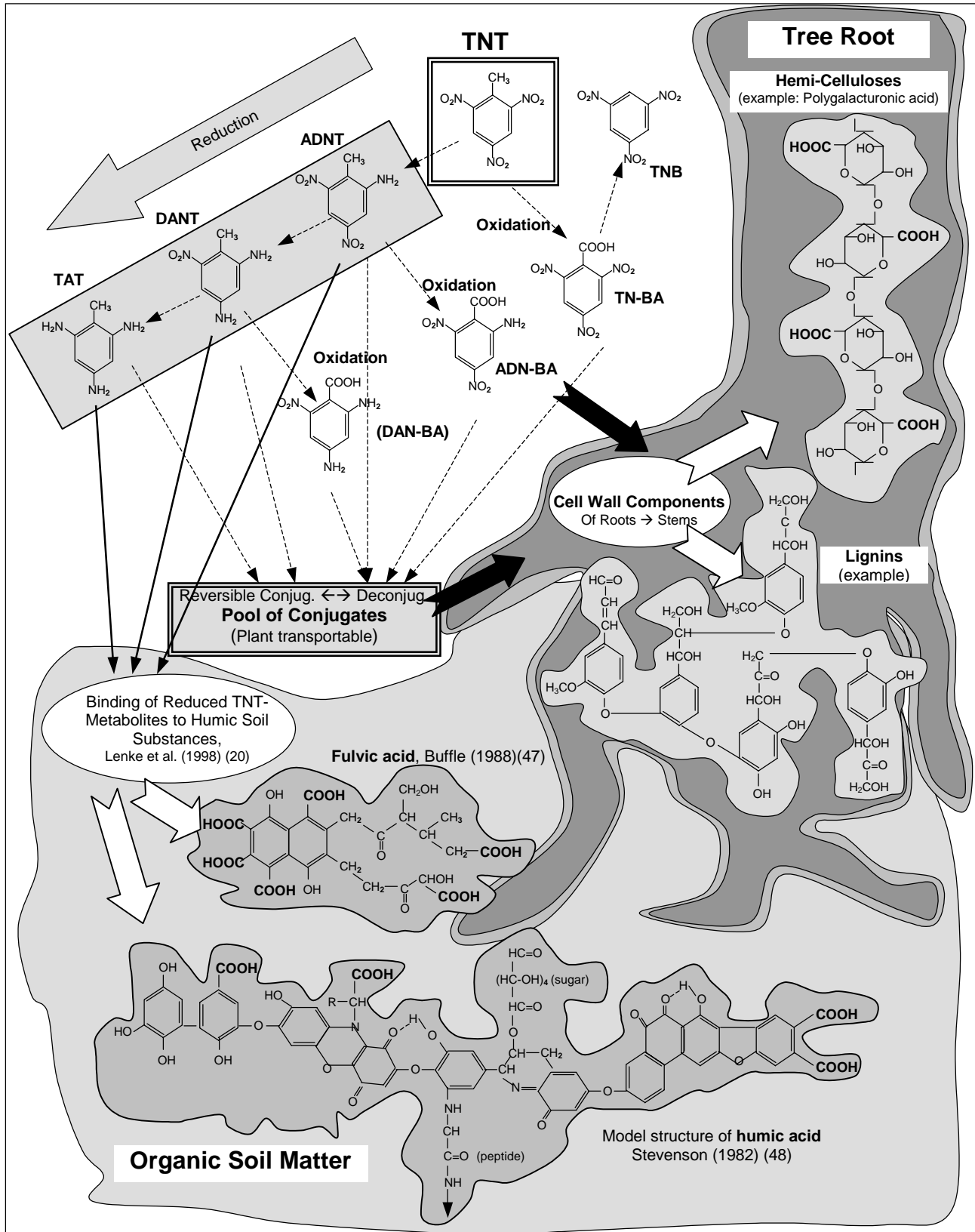


Fig. 8: Main TNT-degradation steps and possible binding sites for TNT-metabolites in tree roots and soil

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