INTRODUCTION

During World War II, 2,4,6-trinitrotoluene (TNT) was the major component of explosives and ammunition. During both production and disarmament, many sites were contaminated heavily with TNT [1]. Due to its recalcitrance and toxicity, TNT may be a severe hazard to human health. Thus, the remediation of soils contaminated with TNT is of particular concern because these compounds endanger groundwater quality at many sites [1]. Bioremediation of TNT-contaminated soil is considered to be the most cost-effective and sustainable technique. Fungal treatment is one of the bioprocesses applied in soil and in control soil. Up to 17.5% of the 15N label was detected as 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene. Three percent was detected as 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene.

Radiolabeled [14C]TNT has been used in micro- and mesocosm studies for balancing the TNT turnover processes in soils [5–7]. Most examinations of the fate of TNT during bioremediation processes in soil indicated a bio-immobilization rather than a mineralization of the compound [8] and the organic carbon of the molecule mainly was incorporated into the organic soil matrix. However, a considerable mineralization of TNT in soil also was reported recently [9]. For drawing general conclusions regarding the behavior and fate of TNT, 14C is the appropriate label, but as yet very few reports on the fate of nitrogen from TNT exist. Furthermore, to the best of our knowledge, no mass balances of 15N in soil systems have yet been published. For tracing the degradation pathways of TNT, 15N mass balances may provide an improved understanding of the fate of TNT in bioremediation processes, including residue formation in complex soil systems. Evidence was presented by 15N nuclear magnetic resonance spectroscopy that TNT metabolites were bound covalently to soil organic matter [10–13]. Semiquinones, anilino(hydro)quinones, anilides, and imines derivatives of TNT were found to be the species that bind to humic matter [12–15].

The mineralization of TNT by bacterial activity generally is considered to be negligible [1,3–7]. Particularly with initial anaerobic treatment, the nitro groups of TNT mainly are reduced in soils to aminodinitrotoluenes (ADNT) and diaminonitrotoluenes (DANT) with accompanying formation of hydroxylaminodinitrotoluenes (HADNT), which may react via condensation to form azoxytetranitrotoluenes (for review see [5]). Abiotic reductions catalyzed by Fe(0) also may be involved in the reduction of TNT [16]. The activity of ligninolytic fungi results in higher mineralization of TNT and additional formation of N-formylated or acetylated ADNT metabolites [5]. After supplementation of readily degradable carbon sources and establishment of strictly anaerobic conditions in soil, a reduction of TNT to triaminotoluene and disappearance of the metabolites was observed [17,18]. Subsequent aerobic incubation of the soils treated by anaerobic processes was shown to decrease the overall amount of TNT metabolites drastically [17,18] but the carbon derived from [14C]TNT still remained in the soil. Nitrogen also was assumed to remain in the soil after the aerobic maturation phase [10]. The TNT transformation studies revealed in most cases overall 14C-label recoveries of 92 to 99% with about 90% nonextractable residues [7,18].

Several reports have been published on the cleavage of nitrogen from TNT by pure cultures of aerobic bacteria [5,19–23], anaerobic bacteria [24], and fungi [25]. The first report about NH4 release was published in 1989 [23] but the extent of N release in soils has not been analyzed until now. Additional reductive processes involving the TNT molecule also lead to reductive elimination of nitrite by the formation of Meisenheimer complexes with hydride addition to the aromatic ring, as shown a few years ago for picric acid (2,4,6-trinitrophenol) and 2,4-dinitrophenol [26]. The NO2 derived from

Keywords—[15N]2,4,6-Trinitrotoluene, Biodegradation, Fungi, Azoxytetranitrotoluene, Dinitrogen oxide

Abstract—The fates of the labels from [14C] and [15N] trinitrotoluene were analyzed in bioreactors under aerobic conditions in soil treated by a fungal bioremediation process with Stropharia rugosoannulata and in control soil. Up to 17.5% of the 15N label had a different fate than the 14C label. Three N-mineralization processes were identified in detailed experiments with [15N]TNT. About 2% of the 15N label was found as NO2 and NH3, showing simultaneous processes of direct TNT denitrification (I) and reduction with cleavage of the amino groups (II). The enrichment of NO2/NO3 (up to 7.5 atom% 15N abundance) indicates the formation of Meisenheimer complexes with a denitration of [15N]TNT. A 1.4% of the label was found distributed between N2O and N2. However, the 15N enrichment of the N2O (up to 38 atom%) demonstrated that both N atoms were generated from the labeled TNT and clearly indicates a novel formation process (III). We propose, as an explanation, the generation of N2O by cleavage from condensed azoxy metabolites. In addition, 1.7% of the 15N label was detected as biogenic amino acids in the wheat straw containing the fungus. Overall, 60 to 85% of the applied [15N]TNT was degraded and 52 to 64% was found as nonextractable residues in the soil matrix. Three percent was detected as 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene.

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TNT may then serve as a N source for various bacteria [26-28]. These processes result in a different fate for C and N from TNT in environmental systems. However, most authors do not consider the various fates of C and N derived from TNT in soil experiments. A cleavage of N from the TNT molecule would provide evidence for the decomposition of the compound and for the decrease in hazardous potential of non-extractable residues after biotransformation of TNT in soils. Therefore, the goal of the present work was to compare the mass balances of C- and N turnover in tracer experiments using [14C] and [15N]2,4,6-TNT in native soil and in soil treated by a fungal remediation process with the litter-decaying fungus *Stropharia rugosoannulata*. The question of whether the specific fate of N from [14N]TNT is different from the fate of C was studied together with the processes of N mineralization. During the soil experiments, the formation of [15N]NO2, -NO3, -NH4, -N2O, -N2, and the incorporation of the label into biomass were determined.

MATERIAL AND METHODS

Chemicals

All chemicals except where otherwise specified were purchased in p.a. grade from Merck (Darmstadt, Germany) and Sigma Aldrich (Deisenhofen, Germany). The [U-15N]-labeled TNT with a purity of 95% was obtained from K. Steinbach (Department of Chemistry, University of Marburg, Marburg, Germany). Radiolabeled [U-14C]-TNT with a specific activity of 30 mCi mmol-1 was obtained from W. Fels (University of Paderborn, Paderborn, Germany).

Soil

Soil samples were collected from Werk Tanne, a former TNT production plant in Clausthal-Zellerfeld, Germany. The soil was mixed, sieved (< 2 mm), and stored at 4°C. The contamination of the native soil amounted to 1190 ± 420 mg TNT, 80 ± 3 mg 4-ADNT, and 53 ± 3 mg 2-ADNT kg-1 soil (dry wt). The composition of the soil was: Sand 47.5%, silt 39.7%, clay 12.9%, organic carbon 13.7%, carbonate 2.15%, nitrogen 4.2%, with a pH of 7.4.

Organisms and culture conditions

The litter-decaying fungus *S. rugosoannulata* was found to be capable of metabolizing and mineralizing TNT to a certain extent in liquid cultures [29]; it also can be grown in soil and has low nutritional requirements. For inoculation of the soil reactor, a malt agar culture plate of *S. rugosoannulata* (Deutsche Sammlung für Mikroorganismen, [DSM] 11372) was added to 100-g autoclaved oat seeds moistened with 50 ml of distilled H2O in 500-ml flasks that were incubated in the dark for 14 d at 22°C. The oat seeds containing the mycelia were then transferred to 200-g autoclaved wheat straw moistened with 500 ml of distilled H2O in plastic bags sealed with cotton plugs. After four weeks of incubation, the mycelia on the straw were transferred to the soil as described below.

Experimental setup

The soils for the comparison of 14C and 15N mass balances were spiked with 7.5 g [14C]TNT or with 7.5 g of [15N]TNT per kg soil (dry wt). The radiolabeled TNT was diluted with nonlabeled TNT, resulting in a specific activity of 81 µCi kg-1 soil (dry wt). Crystalline TNT was mixed thoroughly into air-dried native soil and the water content of the soil finally was adjusted to 30% (weight/weight; ≈ 60% of water-holding capacity). To age the added TNT, the soil was stored for 30 d at 4°C. Two kilograms of the aged soils were filled into soil bioreactors of 5.7 l volume and the wheat straw containing the mycelia of *S. rugosoannulata* was supplied to the soil in layers of 5 cm between three 10-cm layers of TNT-contaminated soil. The bioreactors were operated with a closed gas atmosphere pumped in a circulation flow of 2 l/h. Oxygen was provided to the reactor by a 10-l flexible gas bag and the dosage was performed by volume reduction of the gas atmosphere due to absorption of CO2 within two gas-washing bottles filled with 2 N potassium hydroxide. The bioreactors were operated at 18°C for 176 d.

The soil for the 15N tracer experiment was prepared by spiking the soil with 428.5 mg 15N-labeled and 428.5 mg nonlabeled TNT per kg resulting in a 15N abundance of 48 atom%. The soil finally was adjusted to 30% water content. Forty grams of the contaminated soil was filled into 650-ml soil bioreactors and was incubated under synthetic atmosphere of 80% helium and 20% oxygen in order to eliminate the natural N content of the atmosphere. Two sets of six reactors with native soil and two sets with fungal inoculation (10 g of straw-mycelia as a layer on top of the soil) were incubated at 18°C. The oxygen content of the atmosphere in the soil reactors was maintained at 20% by electrolysis of sulfuric acid in separate vessels using a modified apparatus for long-term biological oxygen demand measurement (Sapromat D 12, Voith, Heidenheim, Germany). The initial quantity of 15N in TNT per vessel amounted to 5,590 ± 20 µmol and was set to 100% for the turnover calculations. The overall 15N abundance in the soil amounted to 1.9 ± 0.008 atom%. The amount of gaseous N species was analyzed by gas sampling at several points of time. Two reactors were sacrificed for each sampling time up to 108 d and were analyzed for the content and isotopic composition of NO3, NO2, NH3, TNT, and metabolites.

Analytical procedures

For extraction of TNT and primary metabolites, 2 g of dry soil was extracted sequentially three times with 10 ml methanol in Hungate tubes (Belco International, Feltham, UK). The tubes were sonicated for 15 min in an ultrasonic water bath (Schmidbauer, Singen, Germany) and centrifuged at 8,000 rpm for 5 min; the supernatants were then combined and analyzed by gas chromatography mass spectroscopy (GC-MS). For analysis of NO3, NO2, and NH3, 20 g soil was extracted with 100 ml 1 N KCl-solution for 3 h on a rotary shaker at 20 rpm. Soil pellets were saved for elemental analysis. The concentrations of NO3, NO2, and NH3 were determined by anion exchange chromatography with an IonPac 4 × 250 mm column coupled to the AD 25 ultraviolet-detector at 212 nm ( Dionex DX-100, Idstein, Germany).

Hydrolysis of proteins and derivatization of amino acids were performed according to Richnow et al. [30]. A 1-g soil sample was heated for 22 h at 110°C with 5 ml of 6 M HCl in a closed vessel filled with N2. After cooling, the hydrolysate was filtered through a 0.2-µm GF6 sterile glass fiber filter (Schleicher & Schuell, Dassel, Germany) and the filtrate was dried completely in a rotary evaporator at 45°C under vacuum. For methylation of the carboxyl groups of the amino acids, 1 ml of iso-propanol and 250 µl acetyl chloride (both cooled to 4°C) were added to the dried residues and the reaction mixture was incubated for 10 h at 70°C under N2 atmosphere. This solution was then evaporated and 500 µl dichloromethane and 500 µl trifluoroacetic acid were added for the acetylation.
The isopropyltrifluoracetates of the amino acids were analyzed for structure and isotopic composition using GC-MS and GC-combustion-isotope ratio MS (GC-C-IR-MS) techniques as described below.

For elemental analysis, the soil and the dried residues from the extractions were dried at 45°C and were combusted in an elemental oxidizer (EA 2000) coupled to a Balzer Quadstar, Germany) in order to determine the N isotopic composition in the soil. The fraction of nonextractable residues was calculated by subtraction of the extractable fractions from the total soil content. The peak area of the ion traces m/z 28 and 29 was used to calculate the isotopic composition and the concentration of nitrogen. The isotopic composition of a sample generally was measured as atom% abundance with relative standard deviations of <8% due to the inhomogeneity of the soil. The total N content was measured with the same system.

Both TNT and metabolites were analyzed in a GC-MS system (HP 6890, MS 5973, Hewlett-Packard, Avondale, PA, USA). Two µl of the methanol extracts was injected and separated on an SPB-5 column, 30 m × 0.32 mm, 0.25 µm film thickness (Supelco, Taufkirchen, Germany), and the split ratio was set to 1:50. The temperature program was: 3 min at 60°C, heating with 8°C/min to 182°C, then 3 min at 182°C, heating with 8°C/min to 300°C, and finally 6 min at 300°C. The detection limit of TNT and metabolites was <0.1 mg kg⁻¹, which corresponds to 0.013% of the applied [15N]TNT. The relative standard deviation amounted to <10% for the amounts of metabolites. The concentrations of gaseous N species such as N₂ and N₂O were analyzed on a GC-C-IR-MS system described below for compound-specific isotope analysis.

The [15N]-isotopic composition of TNT and metabolites was analyzed on the GC-MS system used for the identification and determination of the concentrations as described above. To determine the amount and the [15N]-isotopic composition of NO₃⁻, NO₂⁻, and NH₄⁺, a steam distillation according to Bremner [31] was applied. Duplicates (40 ml) of the filtered KCl extracts of each sample were distilled for 3 to 5 min depending on the N content. The two-step distillation procedures generated first the quantitative amount of NH₃ with MgO as reagent and second the amount of NO₂/NO₃ after addition of Devarda alloy. This procedure recovered 95 to 98% of the inorganic N. For isotopic ratio measurement, the dried distillates were combusted in the elemental analyzer described above. The [15N]-isotopic compositions of gaseous N species such as N₂ and N₂O were analyzed on a GC-C-IR-MS system (Finnigan Mat, Bremen, Germany) consisting of a gas chromatography unit connected to a Finnigan Mat combustion device coupled to a Finnigan Mat 252 mass spectrometer. Gaseous samples (100 µl for N₂, 2.5 ml for N₂O) were injected on-column and were separated on a Poraplot Q-HAT Plot FS capillary column (25 m + 2.5 m precolumn × 0.32 mm, Chrompack, Varian, Darmstadt, Germany). The oven temperature was set to 40°C. The instrument was calibrated using N₂ reference gas with an isotopic composition of 0.366 atom% against air. Samples were measured with relative standard deviations of ± 0.5%. The detection limit for N₂O was 0.12 pmol [15N]. The isotopic composition of amino acids was determined on the GC-C-IR-MS described above. A BPX 5 column (50 m × 0.32 mm, 0.5-µm film thickness, SGE, Weiterstadt, Germany) was used for the separation of these compounds. The temperature program was: 10 min held at 50°C, 2°C/min heating to 175°C, 10 min at 175°C, 4°C/min heating to 260°C, and finally 10 min at 260°C. These samples were measured with standard deviation of <4% and the detection limit in terms of isotopic compositions was 16 µg [15N]. Due to the much lower enrichment of the single amino acids, the isotopic ratios were reported as δ% (1,000 δ% = 0.75 atom% or 0.39 atom% excess).

The [14C]-radioactivity in liquid samples was quantified by β-scintillation spectroscopy in an LS 6500 counter (Beckman, Fullerton, USA). All determinations were performed in triplicate analyses with 10 ml optimized scintillation fluid (Opti-Fluor for aqueous phases and UltimaGold, for organic, alkaline and acid samples, both purchased from Packard Instruments, Dreieich, Germany). Solid samples were combusted in an oxidizer (OX 500, Zinsser-Analytik, Frankfurt, Germany) at 900°C. The CO₂ from oxidation was absorbed in Oxysolve 400-scintillation liquid (Zinsser-Analytik) and was analyzed by β-scintillation spectroscopy for the [14C] content. Calibration was performed with labeled TNT on quartz sand and standard deviations of the measurements amounted to <3%. Radioactivity of TNT and metabolites in the methanol extracts were analyzed on thin-layer chromatography plates by a thin-layer chromatography linear analyzer (LB Multitracemaster 20, Berthold SGE, Bad Wildbad, Germany) with a standard deviation <5%. The CO₂ in the exhaust gas of the soil bioreactors was absorbed continuously by a two-stage trapping system in 2 N potassium hydroxide that was then analyzed for radioactivity.

### Table 1. Recovery of [14C] and [15N] after microbial degradation of [14C]- and [15N]-labeled 2,4,6-TNT in soil inoculated with *Sphingobium chemicus* on straw

<table>
<thead>
<tr>
<th>Recovery</th>
<th>[14C]</th>
<th>[15N]</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNT</td>
<td>19.8</td>
<td>20.3</td>
</tr>
<tr>
<td>Mineralization</td>
<td>(CO₂)</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td></td>
<td>(O₂)</td>
<td>6.3</td>
</tr>
<tr>
<td></td>
<td>NO₃⁻</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>NH₄⁺</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>NO₂/NO₃</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td>(N₂, 1.7)</td>
<td></td>
</tr>
<tr>
<td>Metabolites</td>
<td>16.9</td>
<td>21.4</td>
</tr>
<tr>
<td>Nonextractable</td>
<td>52.3</td>
<td>30.2</td>
</tr>
<tr>
<td>residues</td>
<td>&lt;0.1</td>
<td>11.2</td>
</tr>
<tr>
<td>Straw</td>
<td>89.1</td>
<td>89.4</td>
</tr>
<tr>
<td>TNT metabolized</td>
<td>69.3</td>
<td>69.1</td>
</tr>
<tr>
<td>Soil</td>
<td>89.0</td>
<td>72.4</td>
</tr>
</tbody>
</table>

* a [14C]TNT = 3,268 kBq/kg (dry wt); [15N]TNT = 8,500 mg/kg ≈ total TNT.
* Aminodinitrotoluenes and diaminonitrotoluenes.
* Soil without straw; determined by combustion and elemental analysis.

### RESULTS AND DISCUSSION

The comparison of the mass balances of [14C] and [15N] from labeled TNT in soil bioreactors showed significant differences in the fate of nitrogen and carbon. Radiolabeled [14C]TNT was mineralized to <0.1% and was metabolized to 69.3% after fungal treatment of the soil over a period of 176 d. About 16.9% was metabolized to ADNT and DANT and 52.3% was converted to nonextractable soil residues (Table 1). The transformation activity in the native soil was much lower in comparison to the fungal treatment (data not shown). The reactor with [15N]TNT metabolized 21.4% to ADNT and DANT but only 30.2% to nonextractable soil residues. In comparison to the reactor with [14C], this reactor clearly showed a much higher...
N mineralization and 6.3% was converted to NO$_3^-$, NH$_3$, NO$_2^-$, and N$_2$. Surprisingly, 11.2% of the $^{15}$N were converted to biogenic $^{15}$N compounds or unknown metabolites in the straw layer containing the fungus, which indicates the use of TNT as nitrogen source. Thus, the first comprehensive $^{15}$N mass balance of TNT transformation in soil showed at least 17.5% of the $^{15}$N label to be metabolized via different pathways in comparison to the $^{14}$C-label (Table 1). Very few authors have proposed in previous papers that nitrogen from TNT may undergo a different fate in comparison to the carbon from the same compound [4,10].

Therefore, detailed $^{15}$N-turnover experiments were conducted in order to investigate the processes governing the specific fate of C and N derived from TNT in soil. With addition of the litter-decaying fungus S. rugosoannulata pregrown on straw, the concentrations of $[^{15}$N]$\text{TNT}$ decreased during 108 d of incubation from 857 mg TNT kg$^{-1}$ to 127 mg kg$^{-1}$ soil (dry wt), which corresponds to a degradation of 85% of the applied TNT in the treated soil. The amount of TNT decreased only to 335 mg kg$^{-1}$ (63% degradation) in the presence of the autochthonous microflora of the native soil (Tables 2 and 3). The total formation of nonextractable $^{15}$N residues in soil amounted to 64% after fungal treatment and to 52% in the native soil. The metabolites 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene that generally are observed in similar soil experiments were detected only transiently in amounts $\leq$3% of the applied TNT. During anaerobic treatments of TNT-contaminated soils, ADNT and DANT initially were detected in amounts one order of magnitude higher than after aerobic treatments [6,7]. Formylated or acetylated metabolites known from fungal metabolism of TNT [5], as well as HADNT, were not detected in the present experiments. However, traces of azoxytetranitrotoluenes could be identified (M. Kröger, University of Paderborn, Germany, personal communication). Significant amounts of the $^{15}$N label (up to 3%) were detected as water-soluble N-mineralization products NO$_2^-$/NO$_3^-$, and NH$_3$ (see also Table 4), up to 1.5% as gaseous compounds N$_2$O and N$_2$, and about 1.7% as certain biomass components in the straw (see also Table 5). These data prove for the first time that a significant mineralization of N from TNT occurred and that several processes are involved. Nitrile was released directly from TNT and the compound or the oxidation product NO$_3^-$ may be consumed as terminal electron acceptors to form N$_2$O and N$_2$. The TNT also served as a nonspecific electron acceptor for the microorganisms leading to the release of NH$_3$. In ad-

### Table 2. Speciation of $^{15}$N derived from $[^{15}$N]$\text{TNT}$ in soil inoculated with Stropharia rugosoannulata on straw. ND = Below detection limit

<table>
<thead>
<tr>
<th>Fraction</th>
<th>$t_0$</th>
<th>0</th>
<th>2</th>
<th>7</th>
<th>15</th>
<th>28</th>
<th>108 [d]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol extracts</td>
<td>$[^{15}$N]TNT</td>
<td>99.7 ± 8.5</td>
<td>98.8 ± 7.5</td>
<td>98.0 ± 10</td>
<td>88.7 ± 9.3</td>
<td>76.6 ± 6.8</td>
<td>57.8 ± 6.3</td>
</tr>
<tr>
<td>2-amino-4,6-dinitrotoluene-N</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>1.23 ± 0.1</td>
<td>1.74 ± 0.1</td>
<td>1.43 ± 0.1</td>
<td>1.78 ± 0.2</td>
</tr>
<tr>
<td>4-amino-2,6-dinitrotoluene-N</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.68 ± 0.1</td>
<td>0.51 ± 0.1</td>
<td>0.56 ± 0.1</td>
<td>1.33 ± 0.2</td>
</tr>
<tr>
<td>NO$_2^-$/NO$_3^-$</td>
<td>0.05 ± 0.0</td>
<td>0.05 ± 0.0</td>
<td>1.56 ± 0.2</td>
<td>1.34 ± 0.1</td>
<td>1.24 ± 0.1</td>
<td>1.57 ± 0.2</td>
<td>2.09 ± 0.3</td>
</tr>
<tr>
<td>KCl extracts</td>
<td>NH$_3$</td>
<td>0.10 ± 0.0</td>
<td>0.05 ± 0.0</td>
<td>0.16 ± 0.0</td>
<td>0.48 ± 0.1</td>
<td>0.43 ± 0.1</td>
<td>0.38 ± 0.0</td>
</tr>
<tr>
<td>Biomass on straw</td>
<td>N$_2$O</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.12 ± 0.0</td>
<td>0.64 ± 0.1</td>
<td>0.74 ± 0.1</td>
</tr>
<tr>
<td>Volatiles</td>
<td>N$_2$</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.03 ± 0.0</td>
<td>0.04 ± 0.0</td>
<td>0.10 ± 0.0</td>
</tr>
<tr>
<td>Residual $[^{15}$N] in soil</td>
<td>N$_2$</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.01 ± 0.0</td>
<td>0.04 ± 0.0</td>
<td>0.66 ± 0.1</td>
</tr>
<tr>
<td>Recovery</td>
<td>120 ± 12</td>
<td>119 ± 12</td>
<td>120 ± 13</td>
<td>115 ± 13</td>
<td>112 ± 10</td>
<td>108 ± 11</td>
<td>109 ± 13</td>
</tr>
<tr>
<td>Loss of label</td>
<td>-0.23</td>
<td>0.71</td>
<td>0.27</td>
<td>5.28</td>
<td>8.09</td>
<td>12.2</td>
<td>11.9</td>
</tr>
</tbody>
</table>

a Recovery of $^{15}$N after spiking of the soil.
b Recovery of $^{15}$N after four weeks of aging at 4°C.
c 5.590 µmol $[^{15}$N]$\text{TNT}$ = 100% was applied initially.
d The initial content of natural $^{15}$N compounds in the soil was 20.4%.
0.0 = <0.02.

### Table 3. Speciation of $^{15}$N derived from $[^{15}$N]$\text{TNT}$ in the native soil. ND = Below detection limit

<table>
<thead>
<tr>
<th>Fraction</th>
<th>$t_0$</th>
<th>0</th>
<th>2</th>
<th>7</th>
<th>15</th>
<th>28</th>
<th>108 [d]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol extracts</td>
<td>$[^{15}$N]TNT</td>
<td>98.8 ± 8.5</td>
<td>99.8 ± 7.5</td>
<td>94.3 ± 9.5</td>
<td>92.8 ± 9.4</td>
<td>86.8 ± 7.0</td>
<td>65.0 ± 6.6</td>
</tr>
<tr>
<td>2-amino-4,6-dinitrotoluene-N</td>
<td>ND</td>
<td>ND</td>
<td>0.14 ± 0.0</td>
<td>0.76 ± 0.1</td>
<td>1.23 ± 0.1</td>
<td>0.36 ± 0.0</td>
<td>ND</td>
</tr>
<tr>
<td>4-amino-2,6-dinitrotoluene-N</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>KCl extracts</td>
<td>NO$_2^-$/NO$_3^-$</td>
<td>0.05 ± 0.0</td>
<td>0.05 ± 0.1</td>
<td>0.89 ± 0.1</td>
<td>2.1 ± 0.3</td>
<td>0.81 ± 0.8</td>
<td>0.37 ± 0.0</td>
</tr>
<tr>
<td>Volatiles</td>
<td>NH$_3$</td>
<td>0.10 ± 0.0</td>
<td>0.09 ± 0.0</td>
<td>0.23 ± 0.0</td>
<td>0.41 ± 0.1</td>
<td>0.22 ± 0.1</td>
<td>0.41 ± 0.0</td>
</tr>
<tr>
<td>Volatiles</td>
<td>N$_2$O</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Volatiles</td>
<td>N$_2$</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Residual $[^{15}$N] in soil</td>
<td>N$_2$</td>
<td>20.4 ± 3.2</td>
<td>20.4 ± 3.1</td>
<td>20.0 ± 3.0</td>
<td>19.1 ± 3.0</td>
<td>27.5 ± 2.6</td>
<td>48.6 ± 4.6</td>
</tr>
<tr>
<td>Recovery</td>
<td>119 ± 12</td>
<td>120 ± 11</td>
<td>116 ± 13</td>
<td>115 ± 13</td>
<td>117 ± 10</td>
<td>115 ± 11</td>
<td>114 ± 10</td>
</tr>
<tr>
<td>Loss of label</td>
<td>0.62</td>
<td>-0.29</td>
<td>4.41</td>
<td>4.83</td>
<td>3.38</td>
<td>5.20</td>
<td>5.68</td>
</tr>
</tbody>
</table>

a Recovery of $^{15}$N after spiking of the soil.
b Recovery of $^{15}$N after four weeks of aging at 4°C.
c 5.590 µmol $[^{15}$N]$\text{TNT}$ = 100% was applied initially.
d The initial content of natural $^{15}$N compounds in the soil was 20.4%.
0.0 = <0.02.
dition, the N was assimilated by microbial biomass, which clearly indicates the use of TNT as N source.

In comparison to the processes of N release, the carbon mineralization of [14C]TNT by microbial activity generally is considered to be very low and the labeled carbon from [14C]TNT remained in the soil [3–8,27]. Thus, the question of why N was mineralized and C was not, remained to be answered. Obviously, the reduced TNT metabolites and the residual carbon skeleton of the molecules after cleavage of the N appear to be highly reactive in coupling reactions to soil organic matter [14,18,32] so that the microbes do not have access to the carbon. However, even after coupling, the nitro or amino groups of the molecules may still be available to cleavage reactions. Nonextractable residues are suspected to contain still toxic primary TNT metabolites but are resistant to further microbial degradation, with a turnover <2% per year [3]. A cleavage of nitro groups from TNT or of amino groups from primary reduction products ADNT or DANT improves the biodegradability of the compounds [28] and thus can be considered as a detoxification mechanism. Inorganic N products released from TNT in the experiments showed a dramatic increase in the 15N enrichment of up to 7.6 atom% abundance for NO2−/NO3− and up to 1 atom% for NH3 during incubation in comparison to the natural 15N abundance of 0.37 atom% (Table 4). Considering an initial 15N abundance of 48 atom% in the applied TNT and predominantly oxic conditions in the soil, it can be seen that the major process of N release was a result of the cleavage of the nitro groups from TNT and only smaller amounts were released by cleavage of NH3-groups after reduction of TNT. However, the released NO3− must be oxidized to NO2− immediately by microbial or abiotic processes. The results show for the first time that the release of inorganic N species from 15N-labeled organic compounds in complex soil systems can be assessed semiquantitatively by analyzing the isotope enrichment of the products.

Gaseous compounds such as N2O and N2 also were found in significant amounts of up to 1.5% (Tables 2 and 3). The amounts released show that N2O formation and denitrification were not the predominant processes of TNT mineralization in the soil. However, N2O was found to be generated in the experiments with exceptional 15N abundances of 12 to 30 atom% (Table 4) and up to a maximum of 38 atom% at day 36 in the soil treated by the fungal process. The high 15N enrichment indicates that both N atoms of the N2O are derived from the parent [15N]TNT. Therefore, we propose that N2O must have been formed by a novel process extracting both labeled N from TNT (see below) and that N2O obviously is a relevant metabolite of TNT degradation in soil.

Several reports about nitrite release by pure bacterial cultures in aerobic liquid media are available, but a denitrification of TNT in soils has not been reported until now. A denitrification of polynitratred aryls already has been described two decades ago [23]. The release of NO3− and the formation of the corresponding hydroxylated catechols were observed at a later date in pure cultures of Pseudomonas fluorescens that used TNT as sole source of N [19]. The denitrification product 2,4-DNT led to the conclusion that nitrite was cleaved from the ring via a formation of a TNT-Meisenheimer complex [20]. The Meisenheimer complex is formed after hydride addition to the aromatic ring as shown for di- and trinitrophenols [26] and leads to the reductive cleavage of nitrite. The pentaerythritol reductase isolated from Enterobacter cloacae was shown to be the enzyme responsible for the reduction of TNT to the complex and for the release of nitrite [33]. A Staphylococcus sp. [34] and a Pseudomonas savastanoi strain [21] were able to release nitrate from TNT, generating 2A–4NT, by a similar

<table>
<thead>
<tr>
<th>Sample</th>
<th>Amino acid</th>
<th>Gly</th>
<th>Thr</th>
<th>Ser</th>
<th>Val</th>
<th>Leu</th>
<th>Ile</th>
<th>Asn</th>
<th>Phe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonlabeled</td>
<td>Standard</td>
<td>−4.0</td>
<td>3.0</td>
<td>6.3</td>
<td>3.2</td>
<td>−0.6</td>
<td>−0.9</td>
<td>−4.8</td>
<td>−3.4</td>
</tr>
<tr>
<td></td>
<td>Reference soil</td>
<td>ND</td>
<td>−3.3</td>
<td>−3.0</td>
<td>14.9</td>
<td>4.9</td>
<td>2.0</td>
<td>7.1</td>
<td>7.6</td>
</tr>
<tr>
<td></td>
<td>Reference straw</td>
<td>5.2</td>
<td>10.2</td>
<td>−5.6</td>
<td>1.7</td>
<td>0.7</td>
<td>3.9</td>
<td>5.6</td>
<td>7.6</td>
</tr>
<tr>
<td>Native soil</td>
<td>248</td>
<td>20.0</td>
<td>11.2</td>
<td>13.1</td>
<td>6.0</td>
<td>12.1</td>
<td>4.7</td>
<td>40.5</td>
<td>ND</td>
</tr>
<tr>
<td>Soil + Fungus</td>
<td>304</td>
<td>29.3</td>
<td>21.6</td>
<td>23.9</td>
<td>42.9</td>
<td>40.7</td>
<td>210</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Straw 28 d</td>
<td>236</td>
<td>139</td>
<td>236</td>
<td>262</td>
<td>315</td>
<td>310</td>
<td>250</td>
<td>234</td>
<td></td>
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<tr>
<td>Straw 108 d</td>
<td>1,004</td>
<td>815</td>
<td>1,140</td>
<td>1,093</td>
<td>1,118</td>
<td>1,067</td>
<td>978</td>
<td>989</td>
<td></td>
</tr>
</tbody>
</table>

*Gly = glycine; Thr = threonine; Ser = serine; Val = valine; Leu = leucine; Ile = isoleucine; Asn = asparagine; Phe = phenylalanine.
*1,000 δ permille ≈ 0.75 atom% ≈ 0.39 atom% excess.
type of reaction. NO$_3^-$ derived from TNT then serves as N
source for several bacteria (e.g., Pseudomonas sp. [20,27,28]
or Nocardiooides sp. [26]). The only known pathway that
releases NO$_3^-$ from TNT is the generation of a Meisenheimer complex; therefore, we propose that the cleavage of $^{15}$N from
TNT proceeds via this reaction (Fig. 1). Whereas until now
the Meisenheimer complex only has been monitored in liquid
media [3,26], we present indirect evidence here for the rele-
vance of this reaction in soil, namely the isotopic enrichment
of NO$_3^-$/NO$_3^-$. Denitrated metabolites could not be detected in
the present soil experiments. This lack of detection is consid-
ered to be caused by a rapid coupling of the products to soil
organic matter or by microbial degradation of such compounds
as described for soil slurries [28]. However, if the bacterial
mineralization of denitrated compounds were to play a rele-
ant role in this soil, a much higher mineralization of the $^{14}$C label
should have been observed and thus coupling reactions to soil
organic matter are much more likely.

Under anaerobic conditions, Pseudomonas strain JLR11
was able to release nitrite from TNT with subsequent reduction to
NH$_4^+$ [27]. The sequential reduction of the nitro groups of
TNT to ADNT and DANT also involves the cleavage of
NH$_3$ from the aromatic ring. Pure cultures of obligate anaer-
obic bacteria [5] were found to be able to eliminate NH$_3$ from
reduced primary TNT metabolites. A Pseudomonas fluores-
cens strain also was found able to release NH$_3$ from
TNT under such conditions [23]. In the present experiments, a cleav-
age of NH$_3$ from TNT in soils was proven for the first time by
the enrichment of $^{15}$N from $^{15}$NH$_3$ up to 0.8 atom% (Table 4).
Hydroxylaminoylases belong to the nitroreductase enzymes and
may cause a release of NH$_3$ from hydroxylaminophenols [22].
Boopathy et al. [24,35] observed the release of NH$_3$ under
anaerobic conditions by Desulfovibrio sp., but some authors
argued whether a denitrification or a deamination of TNT was
the initial cleavage process for the generation of the dinitro-
toluene [14]. Oxidative deamination of ADNT or DANT by mono-
or dioxygenases may provide another pathway leading to the
formation of NH$_3$ [5,23]. Even a disintegration of TNT by
abiotic reactions with Fe$_0^+$ may result in a release of NH$_3$
[36]. After the reduction of TNT to a HADNT, an additional
hydroxylation reaction may lead to a rearrangement forming
phenolic compounds. This reaction is called Bamberger re-
arrangement and makes nitrated aryls more bioavailable [37].
Once the ring is hydroxylated, oxygenases degrade these prod-
ucts more readily. Hawari et al. [25] found the white rot fungus
Phanerochaete chrysosporium able to degrade TNT via the
Bamberger rearrangement in liquid cultures. However, despite
the formation of more readily degradable metabolites, the C-
mineralization of TNT did not exceed 1% [25], which suggests
that TNT only acts as N source. The low $^{15}$N enrichment of
NH$_3$ (=0.8 atom%) observed in the present experiments shows
that NH$_3$ release is a relevant but not the dominant process of
$^{15}$N release. No other processes of N release with formation
of hydroxylamine have been reported and no information on
fungal denitrification capabilities is available. Therefore, the most
likely major process of $^{15}$N release from TNT with formation of
NO$_2^-$/NO$_3^-$ is summarized in Figure 1.

Considering the time course of the occurrence of $^{15}$N[TNT
mineralization products and the $^{15}$N abundance, we can provide
evidence for the relevant degradation pathways of microor-
ganisms coping with the toxic substrate TNT. The measured
amounts and $^{15}$N abundances in NO$_2^-$/NO$_3^-$ and NH$_3$ are
presented in Figure 2 and in Table 4. During the first few days
of incubation, an immediate increase of $^{15}$N[NO$_2^-$/NO$_3^-$ was
observed in both native soil and with the fungal treatment.
Although the increase was slower with the fungal treatment,
It remained on a high level during the experiment whereas the
release slowed down after 7 d in the native soil. The highest
release was reached at day 7, when almost 7.6 atom% abun-
dance of $^{15}$N was detected. The increase in the $^{15}$N abundance
occurred in parallel to the general increase of NO$_2^-$/NO$_3^-$ release
in the native soil, but with the fungal treatment a continuously
increasing release of NO$_2^-$/NO$_3^-$ was observed. A second in-
crease of NO$_2^-$/NO$_3^-$ release after 20 d of incubation in the
native soil of about 5 to 6 atom% indicates an additional pro-
cess of N release that may be related to the turnover of no-
extractable soil residues (see also Table 3). We detected only
a release of NO$_2^-$ but not of NO$_3^-$, which also was observed in
experiments with Fenton’s reagents [38]. Thus, the released
NO$_2^-$ has to be oxidized immediately to NO$_3^-$. The amount of
released $^{15}$NO$_2^-$/$^{15}$NO$_3^-$ represents 1.5 to 2% of the applied $^{15}$N
from $^{15}$N[TNT both in the native soil and in the soil treated
by the fungal process, respectively. The inoculation of the soil
with the fungus S. rugosoannulata did not result in a signifi-
cantly higher release of NO$_3^-$ compared to the autochthonous
microflora (Tables 1 and 2) although a much higher amount of
TNT was metabolized. This is probably due to the assim-
ilation of released N compounds by the fungal biomass.

The release of NH$_3$ started immediately within the first few
days, but at a lower level compared to the NO$_2^-$/NO$_3^-$ release,
and remained nearly constant during the incubation period. The maximum 15N abundance of 1.2 atom% was reached by the fungal treatment on day 7. The release of NO3-/NO2- and NH4+ occurred simultaneously, suggesting at least two processes of 15N release. The 15NH4+ is considered to be cleaved from the molecule after the reduction of TNT to ADNT or DANT; this may explain the fact that the initial amount of 15N released as NH4+ was only a quarter of the amount released as NO3-/NO2-. Thus, denitration must be considered to be the dominant pathway of 15N release from TNT. The time courses of NH4+ release in native soil and with fungal treatment were similar, indicating that the effect of the fungal-mediated NH4+ release was not very pronounced.

After the initial bursts of NO3-/NO2- and NH4+ release, a lower release was observed between 30 and 107 d of incubation. Based on the release within this period, the average release of 15N can be estimated to 280 μmol TNT-N per year (76% as nitrite-N and 24% as ammonia-N) with fungal treatment and as 380 μmol TNT-N per year (46% as nitrite-N and 54% as ammonia-N) in the native soil. These data would represent an N mineralization of 5 to 7% of the applied TNT-N and/or of nonextractable residues per year under these incubation conditions. These rates are higher than the turnover of 2% calculated for the carbon-derived residues from [14C]TNT [36].

Both NO3-/NO2- and NH4+ are known to be products of TNT-Meisenheimer complexes or of reduced metabolites such as HADNT, ADNT, or DANT. In a combined anaerobic/aerobic soil slurry experiment, HADNT and azoxytetranitrotoluenes also appeared in concentrations up to 33 mg/kg soil in the initial incubation phase but were nearly undetectable after the final aerobic incubation period [17]. Fast processes of binding 2-HADNT or 4-HADNT to soil organic matter were proposed as the reasons for the disappearance of these compounds at the end of the experiments [3]. Under aerobic conditions, the lignonolytic fungus Phanerochaete chrysosporium degraded azoxytetranitrotoluenes quickly; other fungi are considered to be able to degrade such azo-compounds as well [5]. However, to the best of our knowledge, N2O never has been described as a product of primary TNT degradation until now. With a 15N abundance of around 30 atom% (Table 4) and a maximum of up to 38 atom% determined at day 36 in soil subjected to the fungal treatment, we present evidence that N2O is a metabolite of TNT and that both N atoms of the molecule must have been derived from the parent [15N]TNT (48 atom%).

The different processes that theoretically may generate N2O from TNT are depicted in Figure 3. Bold arrows represent more probable routes, whereas thin-lined arrows are considered to be unlikely. It is not possible for N2O to have been generated by incomplete oxidation of NH4+ (nitrification) cleaved from reduced TNT metabolites. In this process, the 15N abundance of <1.2 atom% in the NH4+ cannot lead to the formation of N2O carrying a higher abundance. On the other hand, NO3-/NO2- could have been the origin of N2O in a denitrification process. If NO2-/NO3- with an 15N enrichment of 8 atom% cleaved from the labeled TNT were to be used for an incomplete denitrification process, a similar 15N enrichment of N2O and N2 should occur (Fig. 3a). However, the oxic conditions of incubation and the fact that two labeled molecules would have to be combined clearly indicate against this possibility. Indeed, the process of denitrification would result in a decrease rather than an increase of the abundance due to dilution with N species derived from native N pools with natural abundance (0.37 atom%). If NO3- derived from [15N]TNT were to react with amino groups from soil organic matter according to the van Slyke reaction [40], the 15N abundance of N2O would be much smaller. This also would apply for the reaction with soil-derived hydroxylamines. The possibility that TNT-derived NO3-/NO2- could react with TNT-derived hydroxylamines, ADNT, or DANT is not very likely in the presence of natural N compounds in the soil that would dilute the 15N content significantly.

The most probable explanation of N2O formation is the hydrolysis or a hydroxyl radical attack at azoxytetranitrotoluenes with subsequent hydrolysis. Azoxytetranitrotoluenes were detected in trace amounts in the described experiments; they generally are present under anoxic degradation conditions [18] as well as under oxic conditions and were even found with the lignonolytic fungus Phanerochaete chrysosporium [5]. The proposed cleavage of azoxytetranitrotoluenes leads to N2O and two denitrated toluenes: 2,4-dinitro-6-hydroxytoluene and 2,6-dinitro-4-hydroxytoluene (Fig. 3b). Because both 15N atoms would stem from a single molecule, the reaction clearly explains the observed 15N abundance of N2O of up to 38 atom%, which almost reaches the initial abundance of the parent labeled TNT of (48 atom%).
In addition, the TNT-derived $^{15}$N transferred into the straw—mycelia layer amounted to up to 1.7% of the applied TNT (Table 2). Therefore, we compared the incorporation of the $^{15}$N label into amino acids in soil and straw after 28 d and at the end of incubation to nonlabeled reference materials (Table 5). The $^{15}$N enrichment of certain amino acids shows for the first time that the transformation of TNT-N in soil is coupled to the formation of biomass. The amount incorporated into the biomass was found to be in the same range as the amount of TNT-derived N recovered in the inorganic N species NO$_3^-$/NO$_2^-$ and NH$_3$ ($\sim 2\%$).

The isotopic signature of amino acids in soil increased only slightly during the experiment, whereas a pronounced enrichment was observed in the straw—mycelium layer. This indicates hyphal growth into the soil and an active transport of the $^{15}$N label into amino acids in soil and straw after 28 d and at the end of incubation to nonlabeled reference materials (Table 5). The $^{15}$N enrichment of certain amino acids shows for the first time that the transformation of TNT-N in soil is coupled to the formation of biomass. The amount incorporated into the biomass was found to be in the same range as the amount of TNT-derived N recovered in the inorganic N species NO$_3^-$/NO$_2^-$ and NH$_3$ ($\sim 2\%$).

Our investigations with [15N]TNT have shown the different fates of C and N from TNT and have elucidated processes that as that detected for ammonia in both soil types (Tables 2 and 3). This indicates that the uptake of NH$_3$ was the dominant pathway of N assimilation, which is consistent with the observations on other soil fungi [41]. However, only small amounts of $^{14}$N were found in the amino acids of the native soil, indicating that the fungus itself plays a decisive role in making the N from TNT bioavailable to serve as N source for the growth of the fungus* *S. rugosaomnulata.*

Acknowledgement—The work was funded by the German Federal Ministry of Education and Research (BMBSF Grant 14810925) and by a grant from the Centre for Environmental Research. We thank Matthias Gehre for assistance with the GC-C-IR-MS analyses and Mario Kröger, University of Paderborn for the determination of azoxytetenitrotoluenes still have to be investigated in additional experiments.

**REFERENCES**


