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Incorporation Mechanisms of a Branched Nonylphenol Isomer in Soil-Derived Organo−Clay Complexes during a 180-Day Experiment

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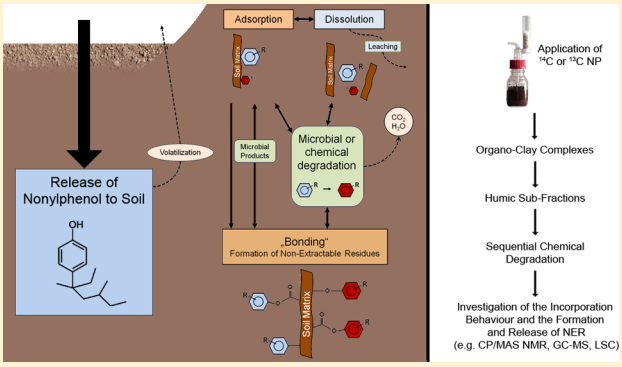
Supporting Information

ABSTRACT: The incorporation process of a defined ^{13}C- and ^{14}C-labeled nonylphenol isomer (4-(3,5-dimethylhept-3-yl)-phenol) into soil-derived organo−clay complexes was investigated. Isolated organo−clay complexes were separated into humic subfractions. Noninvasive (^{13}C−CP/MAS NMR) and invasive methods (sequential chemical degradation, pyrolysis) were applied to obtain detailed information about the mode of incorporation, chemical structure, and change of the incorporation character of nonextractable residues in course of incubation. ^{13}C−CP/MAS NMR measurements of humic acids revealed an increasing incorporation of phenolic compounds during the experimental time which was referred to residues of the introduced ^{13}C-labeled NP isomer. Detailed investigations by means of sequential chemical degradation indicated a predominant incorporation of nonextractable NP isomer residues via reversible ester (amide) bonds. In course of time, the amount of releasable compounds decreased, pointing to altering processes which affected the mode of incorporation. BBr_3-treatment, RuO_4 oxidation, and thermochemolysis released only low portions of nonextractable radioactivity giving evidence of strongly incorporated residues. With the comprehensive application of complementary methods (e.g., humic matter fractionation, ^{13}C−CP/MAS NMR, sequential chemical degradation) it was possible to provide a comparatively detailed insight into the incorporation behavior of the applied NP isomer.



INTRODUCTION

Soils are important sinks for anthropogenic contaminants in the environment. Release of organic compounds into the soil environment could be a consequence of conscious application as crop protection products, impurities of manure applied to soil, atmospheric deposition, or disposal of waste.[1] A major domain for the interaction of xenobiotics with soil is the soil organo−clay complexes, which are aggregates composed of clay minerals and organic moieties exhibiting comparatively high surface areas, high amounts of functional groups (e.g., hydroxyl moieties), and surface charges.[2] Anthropogenic contaminants incorporated in organo−clay complexes can be reduced in their extractability, leading to the formation of nonextractable or so-called "bound" residues. The formation of nonextractable residues (NER) is an important factor influencing the toxicity, bioavailability, transport, and volatility of anthropogenic pollutants.[3] The formation depends on the soil characteristics (e.g., organic matter composition, clay minerals, pH value) and the properties of the compounds and their metabolites (e.g., polar, nonpolar), as well as the influence of microorganisms (e.g., enzymatic-induced reactions). The mode of binding ranges from adsorptive and van-der-Waals forces, over ligand exchange and charge-transfer complexes, to reversible or irreversible covalent bonds.[1,4]

Generally, there are two principle approaches to elucidate the structure and incorporation mechanism of bound residues. In most cases, a site-specific labeling (e.g., ^{14}C, ^{13}C, ^{15}N) of the applied compounds facilitates their characterization. First, there are noninvasive, in situ spectroscopic methods, e.g. FT-IR or fluorescence spectroscopy. In addition to these, ^{13}C NMR as a noninvasive method has been used to examine the binding mechanisms of several compounds to humic subfractions.[5−7] However, application of noninvasive methods to natural samples is limited, because of interferences of compound-specific signals with background noise derived from numerous naturally occurring constituents.[8]

As a second approach, invasive methods are applicable to distinguish between different types of bonds by selective chemical degradation (e.g., alkaline hydrolysis, oxidation,

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pyrolysis). A release of xenobiotics occurs if either the macromolecular structure is degraded, leading to compounds which remain unaltered in the soil (e.g., by sequestration), or covalent bonds between the soil matrix and the incorporated compounds are cleaved. The latter process can produce artifacts of bound residues, and, thus, only an indirect elucidation of the chemical structure and binding mechanism is achievable. However, released compounds can be extracted, fractionated, cleaned, and measured by trace analysis methods such as GC-MS or HPLC-MS.[8]

To understand the environmental fate of xenobiotics and to assess the risk of corresponding nonextractable residues, which can be remobilized, and, thus, become bioavailable, detailed investigations on the bonding characteristics and the chemical structures are required. This accounts also for nonylphenols (NP), environmental pollutants that are known endocrine disruptors. NP isomers are used for the production of resins and plastics or as stabilizer. The predominant type of application of NP is the synthesis of nonylphenol polyethoxylates (NPEO). The high consumption of NPEOs in several kinds of industries and products leads to an entry of these compounds into the environment either directly via, e.g. agricultural products or indirectly, via, e.g. surface active substances or cleaning products resulting in an accumulation in wastewater, soil, and sewage sludge. However, released NPEOs are biodegraded rather quickly to metabolites such as short chain ethoxylates, nonylphenoxy ethoxy acetic acids, or nonylphenols themselves, which are more toxic and persistent in the environment than the parent compounds.[9−11] Formation of nonextractable residues of nonylphenols has been already investigated. As for most xenobiotics, also nonylphenol forms usually higher amounts of NER in microbial active soils compared to NER formation in sterilized soils, but also the contrary has been reported.[12] The side-chain structure of nonylphenol determines not only the estrogenic potential and the degradation rate of xenobiotic isomers, but also the amount of NER formation.[13] Liu et al. [14] found that the amount of NER of a single nonylphenol isomer in paddy soils depends on both the clay and organic matter content, as well as on the treatment by nutrients. Nonextractable residues were associated mainly with the humin fraction of the soils. Further on, the bioavailability of nonylphenol residues in soils for plants is affected by sludge treatment: grass accumulates slightly higher amounts of the residues if sludge is nonconditioned compared to conditioned sludge.[15] The rapid and high uptake of nonylphenol in soils by earthworms seems to suggest that the source of the residues in the soil-feeding organisms is both the extractable and the nonextractable NP fractions in soils since the latter are quickly formed.[16]

Recently, we demonstrated that nonextractable residues of a specific nonylphenol isomer were preferentially located in soil-derived organo−clay complexes and, in particular, the corresponding humic acid fraction acted as the major sink for this isomer.[17] Furthermore, complementary studies revealed not only a rapid incorporation of the nonylphenol isomer[18] but also an influence of the incorporation process on the stereochemical properties of the contaminant indicating microbial assistance.[19] Following this course of investigations the present study focused on the incorporation processes of the labeled NP isomer in soil-derived organo−clay complexes. For this purpose corresponding incubation experiments of up to 180 days were performed and organo−clay complexes were separated from other soil constituents. To reveal information on the incorporation processes of NP isomer residues, ^{13}C−CP/MAS NMR analysis and a sequential chemical degradation of humic subfractions were executed. Released compounds were extracted and analyzed by means of liquid scintillation counting (LSC), HPLC radio detection, and GC-MS.

Chemical degradation techniques were carried out by several authors to investigate the structure of geomacromolecules or the incorporation of low molecular weight compounds such as PAHs or DDT.[20−22] In this study four degradation methods were executed sequentially. With each degradation step, the strength of the chemicals used to cleave and release incorporated residues was increased. Alkaline hydrolysis and BBr_3-treatment selectively cleave ester/amide and ether/ester bonds, respectively. RuO_4 oxidizes aromatic rings and functionalized carbon atoms with a high specificity under mild conditions.[21,23] RuO_4 has been applied in catalytic oxidation experiments of coals, oils, algae, lignite, and humic aids.[21,23−25] TMAH thermochemolysis combines efficient pyrolytical cleavage with subsequent methylation of functionalized groups. Oxidation and thermochemolysis are both abrasive methods which were carried out to degrade the macromolecular structure and, thus, release strongly incorporated residues.

The overall aim of the present study was to (i) balance the releasable portion of the residues during each degradation step, (ii) follow the dynamic properties of specific bonds during the incubation period, (iii) elucidate the structure of incorporated compounds, and (iv) compare percentages of released compounds calculated from radioactivity measurements with percentages measured via GC-MS in order to obtain information about the mode of incorporation.

MATERIALS AND METHODS

Soil. The experiments were conducted using a sandy loam soil obtained from an area close to Fuhrberg (Ap horizon), which is located 30 km north of Hannover, Germany. The soil was air-dried, sieved to pass a 2-mm mesh, and immediately used for the incubation experiments. Main soil characteristics were determined. Particle distribution was found to be 75.4% sand, 18.7% silt, and 5.9% clay. The organic carbon content for sand was 0.7%, silt 13.5%, clay 14.7%, and for the entire soil 3.9%. The pH value of the soil was 5.6 and the maximum water holding capacity was 0.42 g H_2O/g soil. The mineral composition of the clay fraction was determined by means of X-ray diffraction and amounted to 85.5% smectite, 8.4% kaolinite, 2.7% muscovite, and 3.4% quartz.

Chemicals. The ^{13}C- and ^{14}C-labeled NP isomer 4-(3,5-dimethylhept-3-yl)phenol was synthesized according to Russ et al.[26] A mixture of nonlabeled and [U]-ring-labeled phenol (60 mCi/mmol) was used for preparing the ^{14}C-labeled NP isomer resulting in a specific activity of 8.22 mCi/mmol, a radiochemical purity of 94% (HPLC), and a chemical purity of 90% (GC-MS), respectively. For the synthesis of the ^{13}C-labeled NP isomer, C1-labeled phenol (99% ^{13}C label, Isotec, Miamisburg, OH, USA) was used. The NP obtained showed a chemical purity of 90% (GC-MS).

Other (nonlabeled) chemicals were purchased from Sigma-Aldrich (Taufenkirchen, Germany), Merck (Darmstadt, Germany), and ABCR (Karlsruhe, Germany).

Incubation Method. A detailed description of the incubation procedure has been published recently.[17] Briefly, to 100 g of air-dried soil, 125 μg (0.167 MBq) of ^{14}C-labeled NP isomer dissolved in petrolether was added (1.25 mg NP/kg soil). In case of the ^{13}C-labeled NP isomer, 50 mg dissolved in

petrolether was applied to 50 g of soil (1 g NP/kg soil). After application, the solvent was evaporated and the flasks were shaken for 15 min with an overhead shaker. All samples were adjusted to 60% of the maximum water holding capacity (WHC_{max}) using deionized water. The incubation was executed in the dark at 20 °C. Incubation experiments were conducted in triplicate (^{14}C) and duplicate (^{13}C). Samples were taken after 1, 7, 14, 30, 90, and 180 days of incubation.

Degradation Procedures. Degradation procedures were carried out using one replicate of each label. For achieving humic subfractions, organo−clay complexes were separated from soil by a wet sieving and ultrasonification procedure. The clay fraction was extracted with methanol and dichloromethane by means of Soxhlet apparatus. The remaining organo−clay complexes were fractionated into humic acids, fulvic acids, and humin (minerals plus organic) according to the classical alkaline separation method. More details of the former mentioned procedures were previously described.[17] Chemical degradation of the separated humic subfractions was performed sequentially. Although the applied methods are widely accepted redissolution and redistribution of NP between soil constituents upon particle size fractionation and upon base/acid treatment for humic substances, fractionation could not be fully excluded but was tried to keep as low as possible. The workflow is displayed in Figure 1.

Alkaline Hydrolysis. To the preseparated and freeze-dried humic subfractions, 15 mL of an alkaline mixture (2 M) of methanol, water, and potassium hydroxide (1:1.2:250 v/v/w) was added. The mixture was heated for 24 h at 105 °C. After the solution was cooled, it was acidified with HCl to pH = 2. To extract the released NP isomer residues, 15 mL of diethyl ether was added and the vessels were shaken for 10 min on a horizontal shaker. After centrifugation (3 min at 1800 rpm) the organic layer was decanted. Remaining precipitate was resuspended in 15 mL of diethylether, shaken, and the supernatant was decanted. The extraction procedure was then repeated twice using 15 mL of ethyl acetate each. The combined organic extracts were dried with anhydrous sodium sulfate, filtered, and concentrated to a volume of approximately 0.5 mL. The crude extracts were separated into three fractions by column chromatography (Baker, 2 g of silica gel 40 μm) using *n*-pentane/dichloromethane (60:40 v/v), dichloromethane, and methanol (5 mL each).

In case of the four executed degradation steps all fractions were analyzed by unspecific LSC analysis, whereas specific methods (e.g., GC-MS) were used only for those fractions containing significant amounts of radioactivity.

Boron Tribromide Treatment. The solid humic subfractions, pretreated by alkaline hydrolysis, were separately suspended in deionized water and neutralized by adding NaOH solution. After the sample was dried (40−50 °C in an oven), 15 mL of a 1 M BBr_3 solution in dichloromethane was added. The suspension was treated for 15 min in an ultrasonic bath and shaken for 24 h on a horizontal shaker at room temperature. The suspension was cooled in an ice bath and 10 mL of deionized water was added. After 10 mL of diethyl ether was added, the mixture was shaken for 10 min. After centrifugation (3 min at 1800 rpm) the organic layer was decanted and the aqueous suspension was extracted another two times with 15 mL of diethyl ether. The combined organic extracts were dried, filtered, and concentrated. The crude extracts were separated into three fractions by column chromatography using *n*-pentane/dichloromethane (95:5 v/v), dichloromethane, and methanol (5 mL, each) prior to analysis.

Ruthenium Tetroxide Oxidation. Residual samples remaining from the BBr_3-treatment were dried in an oven. Ten mg of RuO_4 and 500 mg of $NaIO_4$, 8 mL of CCl_4, 8 mL of acetonitrile, and 1 mL of deionized water were added. The vessels were closed and shaken for 4 h on a horizontal shaker. For reaction termination, 50 μL of methanol and 2 drops of concentrated H_2SO_4 were added. The samples were centrifuged (3 min at 1800 rpm) and the supernatant was decanted. The precipitate was washed using 8 mL of CCl_4, centrifuged, and the supernatant was decanted. To the combined organic layers, 5 mL deionized water was added, and the mixture was immediately shaken for 3 min. Thereafter, the water layer was removed and extracted three times with 5 mL of diethyl ether. The ether extracts were combined with the CCl_4 fraction. The mixture was dried and concentrated to a volume of 0.5 mL. A saturated $Na_2S_2O_3$ solution (0.5 mL) was then added. The organic layer was removed and dried, filtered, and concentrated. The crude extracts were separated into two fractions by column chromatography using 5 mL of dichloromethane and a mixture of 2 mL of diethyl ether and 3 mL of methanol prior to analysis.

Thermochemolysis (TMAH). Residual samples from RuO_4-treatment were separately placed in digestion bombs containing a Teflon tube. Five mL of a 25% methanolic tetramethylammonium hydroxide (TMAH) solution was added and the mixture was suspended by ultrasonification. Methanol was removed by a gentle stream of nitrogen. The mixture was

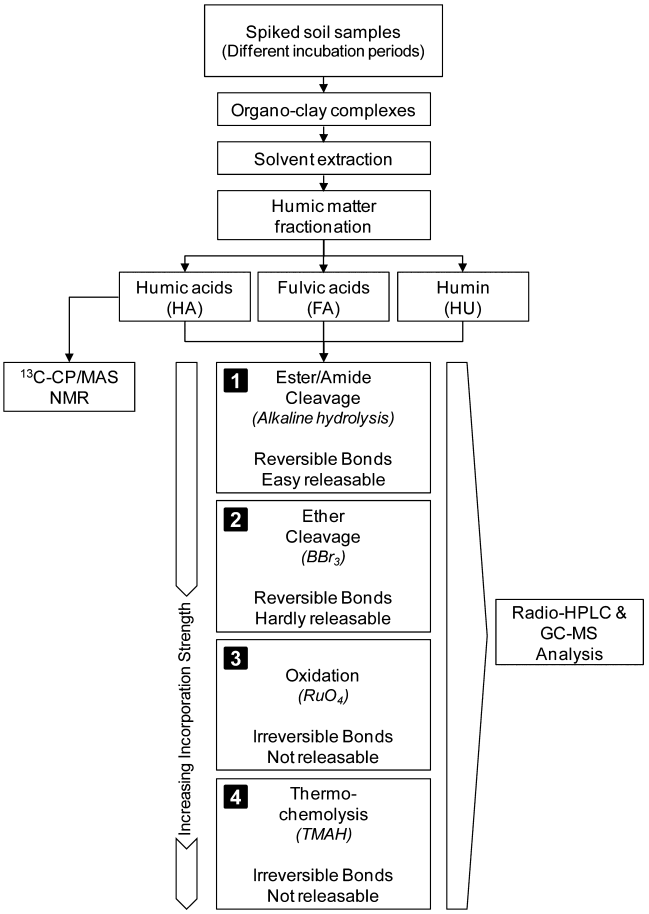

Figure 1. Workflow of sample preparation and applied chemical degradation methods.

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heated for 2 h at 270 °C and thereafter cooled to −18 °C. Diethyl ether (10 mL) was added and the mixture was treated for 5 min in an ultrasonic bath. The extract was decanted and the precipitate was sonicated again with diethyl ether. After decanting, the latter steps were repeated with dichloromethane and *n*-hexane. All organic solutions were combined, dried with anhydrous sodium sulfate, filtered, and concentrated. The crude extracts were separated into three fractions by column chromatography using *n*-pentane/dichloromethane (95:5 v/v), dichloromethane, and methanol (5 mL, each) prior to analysis.

RESULTS AND DISCUSSION

Structural Characterization of NP Isomer Residues Bound to Humic Acids Derived from Organo−Clay Complexes by ^{13}C−CP/MAS NMR. In a first step the binding character of nonextractable NP residues was investigated by means of a nondestructive method, the ^{13}C−CP/MAS NMR spectroscopy. For this purpose the aromatic ring of the NP isomer used for the incubation experiment was specifically ^{13}C-labeled at the carbon atom directly bond to the hydroxyl moiety (C1). Analyses were performed only on humic acid fractions isolated from soil-derived organic−clay complexes, since these subfractions showed the highest portion of incorporated residues.[17] Results obtained from the ^{13}C NMR measurements are displayed in Figure 2. Generally, the entire

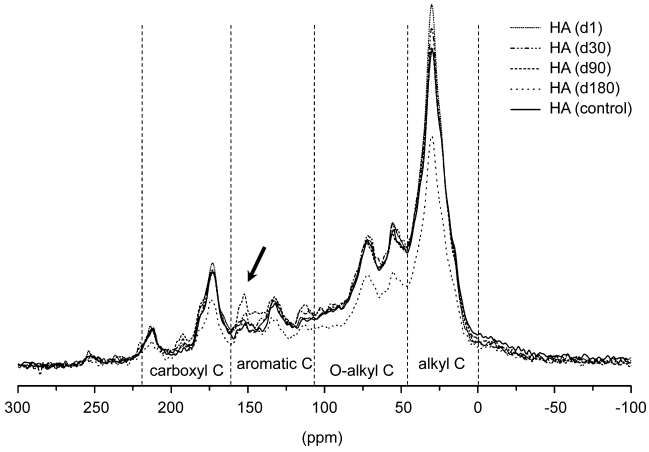

Figure 2. Overlay of spectra derived from measurements of the humic acids after 1, 30, 90, and 180 days of incubation (dashed lines) including the spectra of the control humic acid without NP isomer application (solid line).

spectra correlated well with results published by other authors.[27] The most characteristic peak in all spectra was found for the aliphatic carbons (0−45 ppm). In the aromatic carbon section (110−160 ppm) the peak around 130 ppm resulted from C- and H-substituted aromatic carbons and around 150 ppm from O-substituted aromatic carbons such as phenols. The carboxyl section (160−220 ppm) showed a peak around 175 ppm which could be traced back to carboxylic, ester, or amide carbons.[28,29]

Due to different concentrations of ^{13}C-atoms in the samples (especially at day 180) the percentage of the peak area at 154 ppm to the total peak area of the spectra was calculated. In the control sample (without application) the percentage was found to be 2.3%. In course of incubation the percentage increased from 2.8% after 1 day to 4.7% after 180 days. This specific shift is to be expected from the C1 carbon of phenolic compounds. We thus concluded that the ^{13}C NMR peak at 154 ppm was due to residues of the introduced [1-^{13}C]-NP isomer containing an intact aromatic ring.[30] Moreover, after 180 days the aromatic ring appeared to be still intact and incorporated as such into the humic acid fractions of the organo−clay complexes. Similar results were recently obtained during incubation experiments with ^{13}C-sulfadiazine (1 g/kg soil) and soil with manure amendment.[31]

Regarding incorporation processes of phenolic compounds into humic acids, noncovalent mechanisms such as adsorption, hydrogen bonds, or charge transfer complexes are possible.[4] In this study, solvent extraction of the organo−clay complexes prior to humic matter fractionation was executed. Thus, concerning the incorporation as NP and its possible transformation products into humic materials, processes producing more stable associations such as sequestration or the formation of covalent linkages were more likely. Covalent coupling of NP isomer residues may be thought to occur mainly via the hydroxyl moiety resulting in the formation of ester or ether bonds. This coupling should lead to an alteration of the electronic environment of the NP ring carbon C1 and, thus, to a modified chemical shift in the NMR spectra as compared to the parent substance ^{13}C-NP. However, due to the fact that the samples were measured in the solid state, the peaks are relatively broad. This aspect together with the low amount of NP in the investigated samples led to a signal at 154 ppm of considerably low intensity.[5] A calculation of the chemical shifts of possible binding modes (e.g., ester, ether) of the NP to the humic substances showed that the expected maximum modification of the chemical shift of the C1 carbon amounted to about 10 ppm at around 154 ppm. Due to the overcrowded ^{13}C NMR spectrum and the broad peaks, this change of 10 ppm in the chemical shift was consequently too low for an unequivocal assignment to a specific binding mechanism.

Generally, the results indicate that the aromatic moiety of NP remained (partly) unalterated during incorporation processes and the resulting residues persist over time.

Incorporation Processes of Nonextractable (Bound) NP Isomer Residues As Indicated by Sequential Chemical Degradation. A second approach to reveal insights into the incorporation processes used destructive methods based on the sequential application of chemical degradation techniques. The resulting subfractions released by degradation methods of increasing decomposition power were investigated by (i) radioactive balancing for following the temporal incorpoation behavior, (ii) qualitative analysis for structure elucidation of the nonextractable residues, (iii) chemical classification of incorporation mode, and (iv) comparison of radioactivity with quantitative data of chemically released residues for estimation the alteration of NP during incorporation.

Radioactive Balancing of Released Residues and Temporal Incorporation Behavior. Portions of radioactivity released after each chemical degradation treatment (alkaline hydrolysis, BBr_3-treatment, thermochemolysis) are summarized in Table 1. Percentages refer to the initial amount of radioactivity in the individual humic subfractions.

In terms of alkaline hydrolysis, the entire radioactivity incorporated in FA and HA associated with the organo−clay complexes was released in case of the samples obtained after one day of incubation. During the following six days of incubation, releasable ^{14}C decreased to 49% (FA) and 70%

Table 1. Percentage of Released Radioactivity after Chemical Treatment (Percentages Refer to the Initial Amount of ^{14}C in the Individual Pre-Extracted Humic Subfractions)[a]

		step 1 hydrolysis	step 2 BBr_3	step 3 oxidation	step 4 pyrolysis	
humin fraction	incubation period (days)	ester/amide (%)	ether/ester (%)	macromolecular structure degradation (%)		recovery (%)
FA	1	109	-	-	-	109
	7	49	6	2	2	59
	14	38	7	3	1	49
	30	43	7	2	2	54
	90	19	11	2	3	35
	180	36	5	1	8	50
HA	1	107	-	-	-	107
	7	70	1	1	2	74
	14	77	2	1	1	81
	30	57	2	2	2	63
	90	37	6	1	5	49
	180	56	2	1	2	61
HU	1	60	10	3	2	75
	7	30	7	5	2	44
	14	44	12	5	3	64
	30	52	13	3	7	75
	90	31	7	3	3	44
	180	25	6	1	1	33

[a](-) Indicates no radioactivity was observed (< LOD).

(HA) in the individual humic fraction. Thereafter, a slight decrease until 180 days of incubation could be observed. Hydrolysis of the HU fraction released a lower portion of ^{14}C (60%) after one day of incubation. This indicated a qualitatively different initial incorporation as compared to the radioactivity associated with FA and HA fractions. During the entire incubation period, the highest portion of releasable radioactivity was found for the HA fraction (average of 67%).

Subsequent cleavage of ether or ester bonds by BBr_3-treatment [32] released a comparatively lower amount of radioactivity as alkaline hydrolysis. An average portion of 9% in the individual fraction was released from HU, 7% from the FA, and 3% from the HA during the entire incubation period. The lowest releasable portions of radioactivity were found after 180 days of incubation, pointing to a low significance of this bound fraction not only in terms of relative proportion but also with time. However, the results of BBr_3-treatment indicated not only that ether bonds are of minor importance for the formation of nonextractable NP residues but also that the preceding cleavage of ester bonds by means of alkaline hydrolysis was already almost complete.

The final oxidation reaction and thermochemolysis released the lowest portions of radioactivity among the executed degradation methods. In the course of incubation time, thermochemolytical cleavage showed no distinct trend in terms of releasable radioactivity. However, oxidative releasable ^{14}C decreased until day 180 indicating also aging or altering of the incorporated residues.

Schwarzbauer et al. investigated the linkages of DDT and several metabolites to sediment taken from the Teltow Canal in Germany. In contrast to our results, they released significant portions of DDT and DDT derived metabolites after BBr_3 and

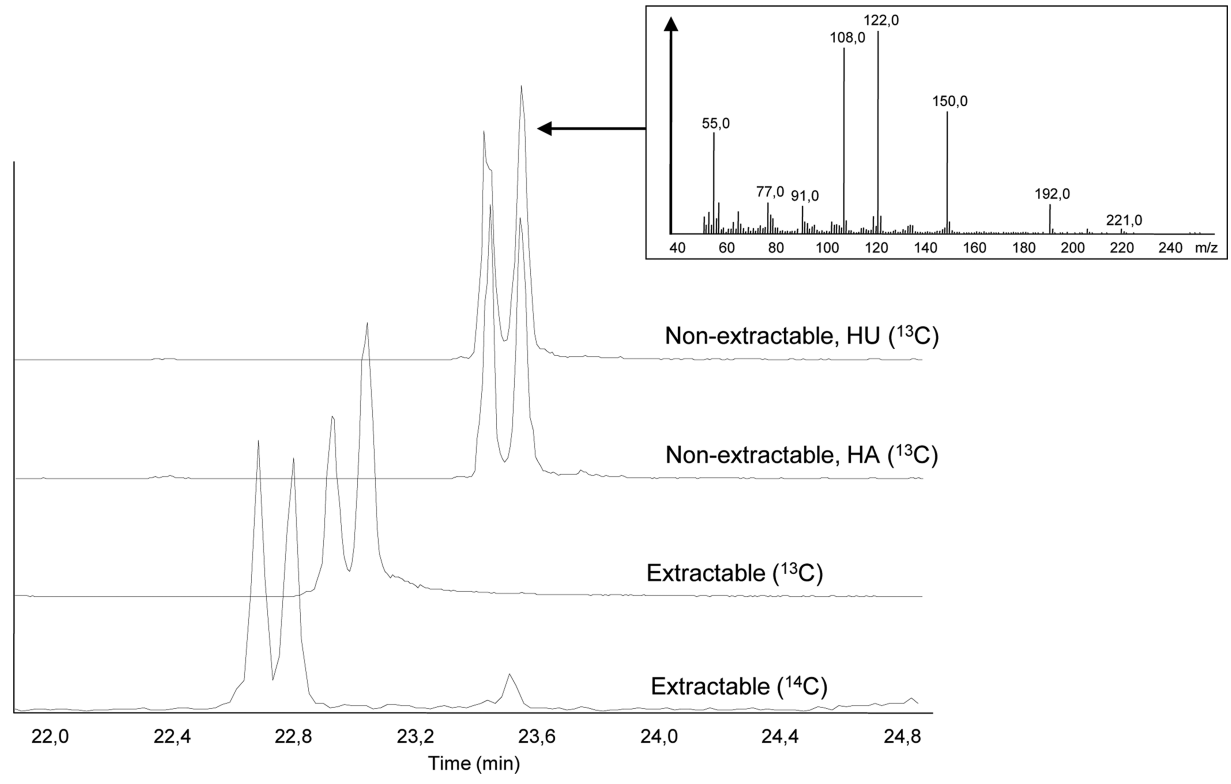

Figure 3. Examples of gas chromatograms (stacked) of the extractable portions after Soxhlet extraction and the nonextractable portion released after alkaline hydrolysis from humic acids (HA, fraction 3) and humin (HU, fraction 3). Peaks were characterized as the applied NP isomer by their retention times (cochromatography with nonlabeled NP isomer standard) and mass spectra. A shift in the retention times may be a result of matrix effects.

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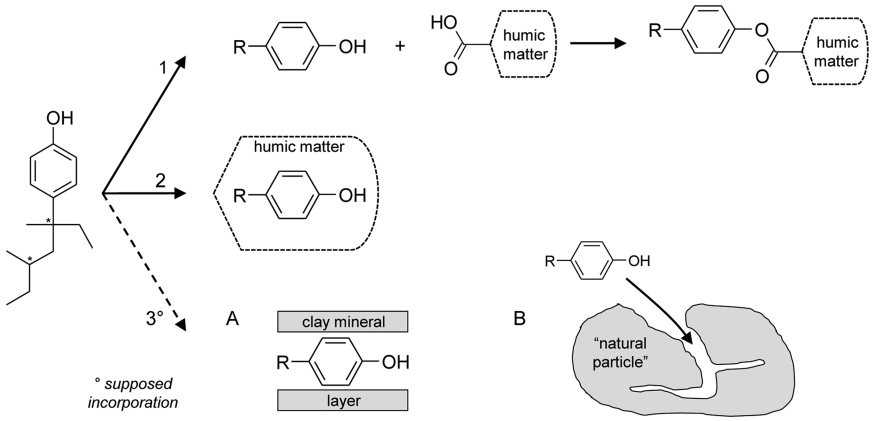

Figure 4. Incorporation processes of the analyzed NP isomer: (1) Formation of covalent ester bonds between the NP isomer and functional groups of humic matter. Released directly via ester bond cleavage. (2) Sequestration of the NP isomer in cavities of humic matter. Released indirectly via ester bond cleavage of humic matter. (3A) Intercalation of the NP isomer in interlayers of the clay minerals. (3B) Slow intramicroporosity diffusion into natural particles. Both rather unaffected by chemical treatment.

RuO_4-treatment.[22] It should be noted that the incorporation of the xenobiotic into sediments occurred under anoxic conditions, and that these sediments were contaminated with DDT and synthesis byproducts for decades. Compounds within these sediments underwent long aging processes, which required harsh methods in order to release residues. Our investigations derived from incubation experiments of up to 180 days may indicate that strongly incorporated residues are formed only after long-term altering and aging. This hypothesis is supported by our study showing a decrease of releasable ^{14}C during the entire incubation time.

To elucidate whether the degradation sequence performed covers the whole spectrum of possible modes of incorporation of NP isomer derived residues into humic fractions, ^{14}C recoveries for the entire chemical degradation procedure (Table 1) were determined by comparing the amount of radioactivity of the individual humic fractions before treatment with amounts released during the degradation steps. Total recoveries ranged from 33% to 109% with an average of 63%. Disregarding losses of ^{14}C during sample preparation, we found that high percentages of the initial radioactivity were not released by the sequential chemical degradation and therefore still remained incorporated into the organo−clay subfractions.

Structure Elucidation of Nonextractable NP Isomer Residues Derived from Organo−Clay Complexes. Radioactive balancing provides no information on the chemical structure of the detected bound moieties. Hence, qualitative analysis based on GC-MS was performed to obtain structural information on the nonextractable residues as released after the individual degradation steps. In case of the ester (amide) bond cleavage procedure, only the parent NP isomer was identified in the HA and HU fractions (Figure 3). Nontarget screening of the GC-MS analysis revealed no liberated transformation products of the NP isomer. Possible metabolites were either not detectable due to structural similarity with natural components of soil organic matter (e.g., phenolic moieties),[33] or their concentration was below the limit of detection. In the extracts derived from ether bond cleavage, oxidation, and thermochemolysis, neither the parent compound nor metabolites could be identified. We have shown recently that in the solvent-extractable fraction only the parent compound was detectable.[17] In contrast to this finding, a nitro-metabolite as transformation product after application of NP to soil or sediment was identified by several authors.[34−36] However, they did not differentiate between individual particle and humic fractions or address nonextractable residues.

With respect to our experiments the qualitative results indicated no significant formation of metabolites in either the extractable or the bound fraction.

Mode of Incorporation of NP Isomer Residues and Their Environmental Significance. A sequential application of chemical degradation techniques attacking different chemical bonds allows insights into the mode of incorporation. As already described, ether bond cleavage (average releasable amount of 7% of the initial ^{14}C within humic fractions), oxidative (2%), and thermochemical degradation (3%) were of minor importance for the release of nonextractable NP isomer derived residues. The highest portion of radioactivity was released after cleavage of ester (amide) bonds with an average amount of 52%. This high amount could be evidence that besides sequestration the formation of covalent bonds played a major role in the formation of nonextractable residues.[37] This conclusion is highly supported by stereochemical changes observed in the same course of experiment indicating a high microbial influence on the formation of the hydrolyzable residues.[19] Incorporation into the humic fractions was detected already after one day of incubation. In FA and HA, radioactivity was released completely after ester (amide) bond cleavage, whereas in humin fraction (HU) the amount released was significantly lower. The HU consists of organic material and clay minerals. X-ray diffractometry showed that 85% of these clay minerals belonged to the smectite group. Smectites are swellable 2:1 layered silicates. In addition to functional groups on their surfaces, these minerals contain exchangeable cations within the interlayers. Ionic as well as polar, nonionic xenobiotics can interact with the cations either directly or through water bridges.[37−39] Another possibility may be slow intramicroporosity diffusion into channels and cavities of natural particles (e.g., clay minerals, organo−clay complexes, or aggregates of smaller grains cemented together by organic or inorganic maeterials) as proposed by Pignatello and Xing.[40] Aging processes could result in the decrease of releasable ^{14}C observed in the further course of incubation. Such process comprises, e.g. movement of residues into cavities of the organic material, a change of the character of bonds, or microbial degradation and incorporation of transformation

products. Figure 4 displays the most significant incorporation processes.

It is highly obvious that the applied NP isomer was predominantly incorporated via ester (amide) bonds. Due to the rather reversible character of hydrolyzable bonds as compared to ether or C−C-linkages, biogenic degradation or the change of soil pH could lead to a release of such incorporated nonextractable (bound) residues into the environment and, thus, they may still be considered a potential risk.

Comparative Quantitation of Extractable and Nonextractable Residues. As a last step we combined the findings from radioactive balancing and quantitative GC-MS analyses of chemically released residues. Quantitative differences of radioactivity and NP content in the individual fractions would point to a chemical alteration of the NP during the incorporation. Therefore, the percentage of accordance of the actual amount of NP as determined by GC-MS ($NP_{GC\text{-}MS}$) and the amount calculated from radioactive balancing[17] as maximum identifiable NP (NP_{MAX}) in the respective sample was calculated. In the following this accordance will be designated as ($NP_{GC\text{-}MS}/NP_{MAX}$) percentage. Values of about 100% would point to an incorporation of radioactivity mainly via the parent NP isomer, whereas values significantly below would indicate that only a part of the radioactivity was due to the parent NP isomer. Previous work showed that the concentration of the applied ^{13}C-labeled NP influenced the microbial activity of soil, but as compared to recently published data the activity of these samples was still in the range of natural soil samples.[17,41] Hence, we supposed a similar behavior of the ^{13}C and ^{14}C-labeled NP during the incubation experiments. Generally, ($NP_{GC\text{-}MS}/NP_{MAX}$) percentages were found to be considerably low. Moreover, values showed a high inconsistency and no distinct trend during 180 days of incubation. ($NP_{GC\text{-}MS}/NP_{MAX}$) percentages from the ^{14}C-labeled extractable samples (Supporting Information (SI) Table SI-1) ranged from 5% to 13% and from the ^{13}C-labeled extractable samples (SI Table SI-2) ranged from 14% to 71%. The lowest ($NP_{GC\text{-}MS}/NP_{MAX}$) percentages were determined with the ^{13}C-labeled nonextractable samples derived from the alkaline hydrolysis (SI Table SI-3), ranging from 1% to 4%.

The low ($NP_{GC\text{-}MS}/NP_{MAX}$) percentages in our experiments correspond with results published by Kästner et al.[42] The authors investigated the formation of bound residues of ^{14}C-anthracene in soil. Only small amounts of nonextractable residues could be traced back to the PAH. They suggested different pathways leading to the incorporation of ^{14}C into humic subfractions. First, the applied parent compound or identifiable metabolites may have been incorporated biotically or abiotically into the humic matrix either by sequestration or by formation of bound residues, which can be released by chemical treatment. In our experiments, this portion represents the residues clearly identified. Second, Kästner et al. suggested an incorporation of unidentified structures as residues complying to the definition of bound residues or as (parts of) structures integrated into humic matter or assimilated into biomass.[43] A third possible incorporation process can be the fixation of microbially produced $^{14}CO_2$ into the soil matrix.[42] In addition to the above-mentioned processes, we assumed that the radioactivity extracted consisted of residues not released as freely available compounds but incorporated into oligomers of the humic matrix as consequence of the cleavage of several binding sites in the organic matter during chemical treatment. This assumption was supported during the necessary sample preparation for GC-MS analysis. Initially dissolved ^{14}C was found in the precipitate after concentration. Further extraction with organic solvents did not release incorporated radioactivity. Besides this precipitation process, which possibly removed bound NP residues from the extracts, the incorporation into oligomeric but soluble matter may have prevented the final detection via GC-MS due to, e.g., low volatility. Hence, these NP residues led to the high discrepancies in the amounts of detectable defined compounds and the measured radioactivity. Helling and Krivonak stated that residues remaining fixed to solubilized fulvic or humic acids still have to be considered as bound, since they are not in a discrete and chemically identifiable form.[44]

Generally, our results and the ongoing discussion in the literature indicate a high demand for further research on the nature of nonextractable residues.

■ ASSOCIATED CONTENT

Supporting Information

Detailed description of the analytical methods and tables containing information about the quantitation of extractable and nonextractable residues. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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