# Linkage and structure elucidation of non-extractable NP and MCPA residues in organo-clay complexes

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## Abstract
Organo-clay complexes represent an important biogeochemical interface and thus, play a major role in the immobilisation, bioavailability and persistence of xenobiotics. The ability of soil to incorporate xenobiotics varies from adsorption and sequestration phenomena to chemical reactions resulting in strong covalent linkages. In this study we want to investigate both, the characteristics of the linkages responsible for bound residues as well as the detailed structure of the non-extractable compounds. Therefore, soil samples were incubated with NP (4-(3,5-dimethylhept-3-yl)phenol) and MCPA ((4-chloro-2-methylphenoxy)acetic acid) over an incubation period of 180 and 120 days, respectively. After particle size separation humic fractions (fulvic acid, humic acid, humin) were sequentially chemical degradated to release non-extractable residues. First results showed that on incubation day one 100 % of incorporated nonylphenol derived radioactivity in FA and HA could be extracted after alkaline hydrolysis. Regarding the overall amount of applied radioactivity, on incubation day one 4 % and on day 180 7 % of applied $^{14}C$ was incorporated into the humic substances via ester bonds whereas ether bonds played only a minor role in the immobilization prozess of nonylphenol. Measuring solid-state NMR with humic acid fractions the aromatic moiety of nonylphenol was still intact after 180 days of incubation.

## Key Words
Nonylphenol, MCPA, Bound residue, Sequential chemical degradation, Ester bonds, Ether bonds.

## Indroduction
Regarding the incorporation behavior of anthropogenic substances the organo-clay complexes represent an important biogeochemical interface and thus, play a major role in the immobilization, bioavailability and persistence of such compounds. Klausmeyer *et al*. (not published) have shown that clay-humic complexes are a major sink for xenobiotics like 4(3',5'-dimethyl-3'-heptyl)-phenol (NP) and (4-chloro-2-methylphenoxy)acetic acid (MCPA). To assess the fate and behavior of such substances, one must distinguish between extractable and non-extractable (bound) residues. Bound anthropogenic residues are, basically, those that are not extractable by methods which do not alter their chemical structure (Khan 1982; Roberts 1984). The ability of soil to incorporate xenobiotics varies from adsorption and sequestration phenomena to chemical reactions resulting in strong covalent linkages. The amount of non-extractable residues in soil depends on time and microbial activity (Gaveo 2005). Most of the former studies dealing with bound residues used $^{14}C$-labeled compounds for investigating the amount and distribution in soil. For instance, Barriuso *et al.* (Barriuso 1991) demonstrated that bound radioactivity was located in soil size fractions as well as humic sub-fractions (humic acid, fulvic acid and humin).

The radioactive labeling on the one hand enables the quantification and distribution of compounds but on the other hand gives no information about the molecular structure and binding of incorporated substances. In this study we want to investigate both, the characteristics of the linkages responsible for bound residues as well as the detailed structure of the non-extractable compounds. Therefore, soil samples were incubated with NP and MCPA over an incubation period of 180 and 120 days, respectively. After particle size separation the clay fraction was fractionated into humic acid, fulvic acid and humin. Thereafter the humic fractions were sequentially chemical degradated to distinguish between types of linkages (e.g. ester, ether, C-C bonding) and to release non-extractable residues. The extracts here from received were measured via GC-MS. Additionally, in case of NP the humic acid fraction was measured via $^{13}C$-CP-MAS-NMR.

 
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**Methods**

*Chemicals*

$^{13}C$- and $^{14}C$-labeled 4-(3,5-dimethylhept-3-yl)phenol (NP, 304.14 MBq/mmol) was synthesized via Friedel–Crafts alkylation according to Russ *et al.* ( 2005). The [ring-U-$^{14}C$] (4-chloro-2-methylphenoxy)acetic acid (MCPA , 59.94 MBq/mmol) was provided by Prof. M. H. Gerzabek (University of Vienna) as a mixture of 92 % MCPA methylester and 8 % of the free acid.

*Spiking experiments*

Aliquots of 559 µg (0.167 MBq) of $^{14}C$-MCPA dissolved in 0.5 mL methanol were applied to approx. 100 g of air-dried, homogenized, and sieved ($\leq$ 2 mm) soil samples from Fuhrberg, Germany. In a second experiment 121 µg (0.167 MBq) of $^{14}C$-NP dissolved in 0.5 mL petrolether was similarly applied to a soil sample from the same location. Additionally 50 g of soil samples were spiked with 50 mg $^{13}C$-NP dissolved in petrolether. The solvent was evaporated and the flasks were shaken for 15 min in an overhead shaker. The water content of the samples was adjusted to 60% of maximum water holding capacity. The flasks were closed with an absorption device for $^{14}CO_2$ containing soda lime. The samples were incubated over a period of 180 days (NP) and 120 days (MCPA) at 20 °C in the dark.

*Particle size and humic substances fractionation*

Sand, silt and clay fractions were obtained by an initial manual wet sieving for separation the sand from silt and clay. Silt and clay were separated by centrifugation according to Stemmer *et al.* (Stemmer *et al.* 1998). Further on, the clay fraction containing dominantly organo-clay complexes was extracted by means of Soxhlet extraction with methanol as well as dichloromethane. The Soxhlet extracted clay was shaken with 0.5 M NaOH for 24 h and centrifuged for separating the insoluble humin fraction (HU) from humic acid fraction (HA) and fulvic acid fraction (FA). The supernatant containing HA and FA were acidified with HCl to pH 1. The precipitated HA was separated from FA by centrifugation. All fractions were dried before chemical treatment.

*Sequential chemical degradation and analytical methods*

FA, HA and HU fractions were separately subjected to the following degradation steps:

Step 1: Ester/amide cleavage (Alkaline hydrolysis)

Separated humic fraction was added to a mixture of methanol, water and potassium hydroxide (2 M) in a glass vessel and closed hermetically. The mixture was heated for 24 hours at 105 °C. After cooling, the solution was acidified to pH-value between 2-3 and extracted three times with diethyl ether. The combined organic layers were dried and concentrated before fractionation on silica gel columns with hexan/dichloromethane (40:60), dichloromethane and methanol.

Step 2: Ether cleavage ($BBr_3$)

Boron tribromide solution in dichloromethane was added to the dried sample residues from step one. The suspension was ultrasonically treated for 2 hours, then stirred at room temperature for 24 hours and finally ultrasonically treated again. The mixture was extracted three times with diethyl ether. Further work up was the same as in step 1.

Step 3: Oxidation ($RuO_4$)

To sample residues from step 2, $NaIO_4$, $RuO_4$, $CCl_4$, acetonitrile and water were added. The mixture was stirred for 4 hours at room temperature. After the reaction has been quenched with methanol and concentrated sulphuric acid, the mixture was decanted and the residues three times washed with $CCl_4$. After addition of water the, the $CCl_4$-phase was separated and the remaining aqueous solution washed three times with diethyl ether and the washing solution was combined with the $CCl_4$-phase. The combined organic layers were concentrated and iodine was removed by addition of sodium thiosulfate. The aqueous layer was carefully removed by pipettation and the remaining solution was dried, concentrated and fractionated with dichloromethane and methanol.

Step4: Thermochemolysis (TMAH)

The pre-extracted samples were transferred into digestion bombs and tetramethylammonium hydroxide (TMAH) in methanol (25 %) was added. After ultrasonification, methanol and oxygen were removed by a gentle stream of nitrogen. After closing, the bombs were heated for 2 h at 270 °C, and thereafter, cooled to -18 °C. The bombs content was extracted with diethyl ether, DCM and n-hexane two times. The combined organic layers were dried, concentrated and fractionated with a mixture of pentane, dichloromethane and methanol.

The achieved radioactive extracts were measured by liquid scintillation counting for quantification. The extracts containing $^{13}$C-residues will be measured by GC-FID and GC-MS for structure elucidation. Additionally $^{13}$C-CP-MAS-NMR was measured with $^{13}$C-containing humic acid fractions to investigate the structure of the incorporated compounds.

## Results

*Chemical Degradation*

Figure 1 presents the first results of the $^{14}$C-labeled nonylphenol degradation experiments. The amount of released radioactivity is referred to the percentage of radioactivity in the specific humic fraction before chemical treatment. The percentage of released $^{14}$C is the highest after cleavage of ester/amide bonds. We investigated that on incubation day one 100 % of incorporated radioactivity in FA and HA could be extracted after alkaline hydrolysis which was in detail 0.6 µg $^{14}$C in FA and 2.3 µg $^{14}$C in HA. However, after two weeks of incubation the percentage of released $^{14}$C decreased which points to an alteration of the binding character of incorporated residues and the humic matrix, respectively. Regarding the overall amount of applied radioactivity, at day one 4 % and at day 180 7 % of applied $^{14}$C was incorporated into the humic substances via ester/amide bonds. In contrary to the alkaline hydrolysis the amount of radioactivity released after further degradation steps remained approx. steady during the entire incubation period. Reaction with $BBr_3$ leads to a cleavage of ether and ester linkages. The low amount released indicating that the cleavage of ester bonds via alkaline hydrolysis was nearly complete.

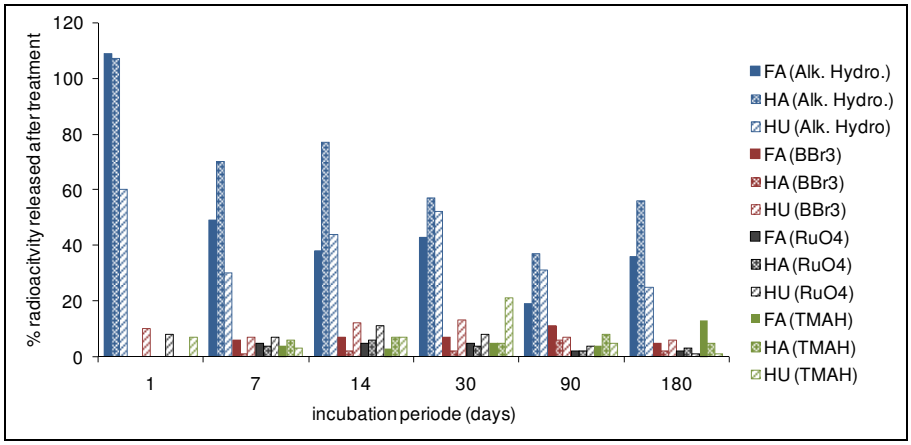


**Figure 1. Percentage of released radioactivity after sequential chemical degradation. Ester/amide linkages are predominatly responsible for the formation of non-extractable NP-residues.**

*$^{13}$C-CP-MAS-NMR*

Solid-state NMR provides information about the constitution of soil and sub-fractions with respect to specific functional groups. In our measurements we used humic acid fractions isolated from organo-clay complexes after different incubation days. In addition a control sample, without NP application, was also measured. The spectra show that the HA is dominated by alkyl C (45-10 ppm) followed by O-alkyl C (110-45 ppm). An overlay of all spectra shows a rise of the aromatic peak (154 ppm) over the incubation time.

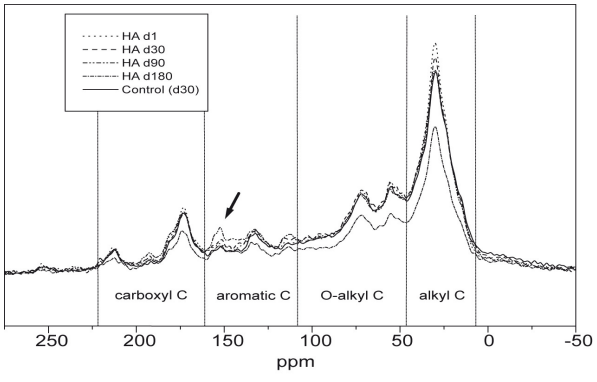


**Figure 2. Overlayed solid-state NMR spectra of humic acid fraction separated after different incubation days. The marked peak at 154 ppm indicates an accumulation of the aromatic moiety during the incubation time.**

To get information about an increase of the relative amount of the aromatic carbon the area ratio of the signals at 154 ppm compared with the area of the entire spectra was calculated. With increasing incubation time the ratio of the aromatic carbon peak increases. That indicates an incorporation of nonylphenol derived residues into the humic acid fraction with the aromatic moiety still intact after 180 days of incubation.

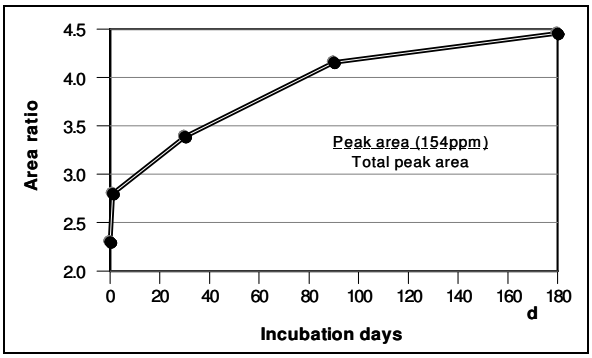


**Figure 3. Enrichment of aromatic carbon in the humic acid fraction during the incubation time as shown by an increase of the area ratio of peak area (154 ppm) normalized to the peak area of the total spectra. Incubation day 0 indicates the background signal measured with the control sample.**

## Conclusion

Within soil sub-fractions organo-clay complexes play a major role in the immobilisation, bioavailability and persistence of anthropogenic compounds. Looking beyond radioactive balancing, sequential chemical degradation, gas chromatography coupled with mass spectrometry and solid-state NMR provide tools investigating the binding and structure characteristics of extractable and non-extractable residues in soil. For the first time we could show that nonylphenol was mainly incorporated into the humic fractions via ester/amide bonds. Moreover, the $^{13}$C-CP-MAS-NMR leads to the result that the aromatic moiety of our applied nonylphenol is still intact and the amount increases over 180 days of incubation. Further investigations are still in progress to get more information about the chemical structure of bound and non-bound residues. Similar methodology will be employed with a second anthropogenic compound (MCPA).

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