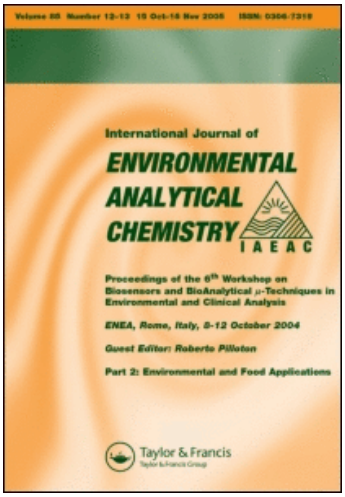

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Evaluation of the Binding Mechanism of Anilazine and its Metabolites in Soil Organic Matter

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EVALUATION OF THE BINDING MECHANISM OF ANILAZINE AND ITS METABOLITES IN SOIL ORGANIC MATTER

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Soil bound residues of organic xenobiotics or their metabolites can be partly extracted by diluted NaOH together with the humic and fulvic acids or remain in the humin. These residues, however, are only detected by their radioactivity after using ^{14}C- or ^{3}H-labeled xenobiotics.

A more detailed analysis of the binding character of xenobiotics was conducted with the fungicide Dyrene^{R} which contains anilazine as an active ingredient. This compound forms up to 80% of unextractable residues after a short incubation period in soil. ^{13}C NMR-spectroscopic analysis of the bound residues in the humic matrix of a soil could be significantly improved by application of the ^{13}C-enriched ingredient. Further improvement was gained by incubation of the fungicide in an artificial soil, obtained by humification from corn stalk material of plants previously grown in a $^{12}CO_2$ atmosphere. The results revealed that the binding of anilazine metabolites occurs in the form of ethers or possibly also of esters with various functional OH-groups of the humic molecules. Derivatization of the humus matrix by silylating reagents also provided additional information.

KEY WORDS: Soil bound residues, anilazine, ^{13}C-enrichment, ^{13}C NMR-spectroscopy, humic compounds, bound anilazine metabolites.

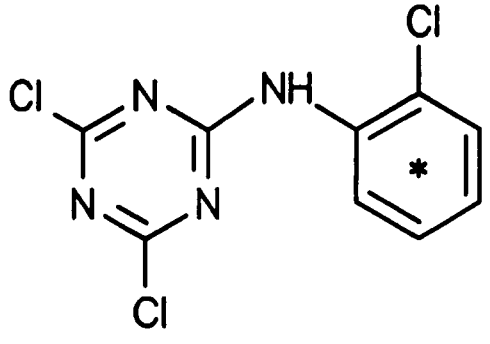
INTRODUCTION

Organic xenobiotics can be sorbed or bound to humic compounds by various types of

interactions such as van der Waal's forces, hydrogen bonds, formation of charge transfer complexes and covalent bonding[1]. Several of these mechanisms may operate in soil leading to either reversible sorption or to unextractable 'bound' residues. These residues, remaining after extraction of the treated incubated soil with various solvents, are only detectable by radioactivity measurements and therefore the studies must be conducted using ^{14}C- or ^{3}H-radiolabeled xenobiotics. These labeled residues can amount to 40% or more of the initially applied label[2]. The radioactivity is then partly bound to humic or fulvic acids or sometimes remains in the humin.

Several herbicides, insecticides, fungicides or their metabolites rapidly form non-extractable residues. An example is the fungicide Dyrene^{R} which contains anilazine as the active ingredient (Techn. Inf. Dyrene, Bayer AG, March 1990). Its fungicidal activity is presumably based on alkylation reactions of SH-groups in membrane proteins of fungal spores or mycelia[3]. Within a few days after application of ^{14}C-labeled anilazine to soils, only 20% or less of the radioactivity can be extracted with organic solvents[4]. The extractable activity contains small amounts of the parent compound, its dihydroxy- and monohydroxy-derivative and several unknown metabolites. Even during prolonged incubation, only a small portion of the radioactivity is evolved as $^{14}CO_2$, and this is observed for both the carbons of the triazine- and the aniline-moiety[4,5] (Figure 1). The release of $^{14}CO_2$ from the ^{14}C-labeled dihydroxy-derivative of anilazine was, however, 6 to 9 times higher than that of the ^{14}C-labeled anilazine[6].

According to several studies[4,5,7], it has been proposed that this strong binding occurs after biotic or abiotic removal of one or two of the chlorine atoms from the triazine ring to form a reactive intermediate which reacts with the soil organic matter. Extraction with dilute NaOH of the soil previously extracted with organic solvents, dissolves another 40% of the



*denotes position of the radiolabel

Molecular Weight: 275.5

Figure 1 Structural formula of anilazine, further chemical and physical data see: Haider *et al.* [7]

applied radioactivity in the form of humic and fulvic acids, whereas a significant amount remains in the humin[7].

Recently, an efficient extraction of humic compounds from soils including the humin has been achieved by derivatization of the soil organic matter with silylating reagents in organic solvents at room temperature[7]. Using this technique up to 80% or more of the bound radioactivity in the soil or the humin fraction can be extracted.

In the present study anilazine was applied with >90% ^{13}C-enrichment of the carbons of the triazine ring together with a small amount of the ^{14}C-labeled sample. The interactions between anilazine residues and humic compounds were investigated by measuring the ^{14}C-label and by ^{13}C NMR-spectroscopy. Furthermore, the ^{13}C-signals of the humus matrix could be largely suppressed by incubation of the ^{13}C labeled anilazine in an artificial soil, obtained by humification of cornstalk material from plants grown previously until maturity in a phytotrone in which the atmosphere was continuously supplied with $^{12}CO_2$

EXPERIMENTAL

Anilazine (4,6-dichloro-N-(2-chloro-phenyl)-1,3,5-triazine-2-amine, see Figure 1) the active ingredient of the fungicide Dyrene^R was applied with 90% ^{13}C-enriched carbons in the triazine ring together with ^{14}C-labeling in the chlorophenyl moiety. The ^{13}C anilazine samples added to soil or the sand/humified plant mixture, contained 5 μg of ^{14}C-labeled anilazine (6 MBq/mg) to follow the yields of the different extraction procedures by means of their radioactivity. Both samples were obtained from Bayer AG, Leverkusen. Several derivatives of anilazine were synthesized according to published procedures[8] and their ^{13}C NMR-spectra were used for identification of metabolites observed during incubation. They included its dihydroxy derivative (4,6-dihydroxy-N-(2-chloro-phenyl)-1,3,5 triazine -2-amine), its dimethoxy derivative (4,6-dimethoxy-N-(2-chloro-phenyl)-1,3,5-triazine -2-amine) and the N-trimethyl-dihydroxy derivative (2 - chloro - phenylimino) - 1,3,5- trimethyl-1,3,5-triazinane-4,6-dione. The corresponding structures and the ^{13}C NMR-spectra are shown in Figure 2.

Between 5 and 10 mg of the labeled anilazines were carefully mixed with samples of 100 g dry soil (Ap-horizon of a podzolic soil, Udalf, Luvisol from Bodenstedt near Hildesheim, FRG, 1.25% C, 0.13% N, pH 7.2, 13.3% clay, 83.8% silt, 2.9 % sand) and were incubated for 2 to 8 weeks and this corresponded to a 50 to 100 times higher application rate as recommended in the field trials. Similarly, a mixture of 200 g samples from pure quartz sand with humified ^{13}C-depleted cornstalk material was incubated with the anilazine samples.

The cornstalk material was obtained from maize plants grown for 11 weeks in a phytotrone continuously supplied with 300–500 vppm of 99,9 % $^{12}CO_2$ (Isotec Inc., Miamisburg, Ohio, USA) to its atmosphere[9]. The seedlings were grown, before their transfer into the growth chamber, for 2 weeks in a normal atmosphere, and the chamber was opened several times during the growth period. Therefore the corn stalks at maturity were not completely depleted of ^{13}C, but contained 0.3% ^{13}C instead of 1.1 % in a total carbon content of 45 % on an ash free base. Three grams of the material, milled to a fine mesh (0.7 mm), were mixed with 200 g of pure quartz sand (0.3–0.4 mm) in Erlenmeyer flasks and supplied

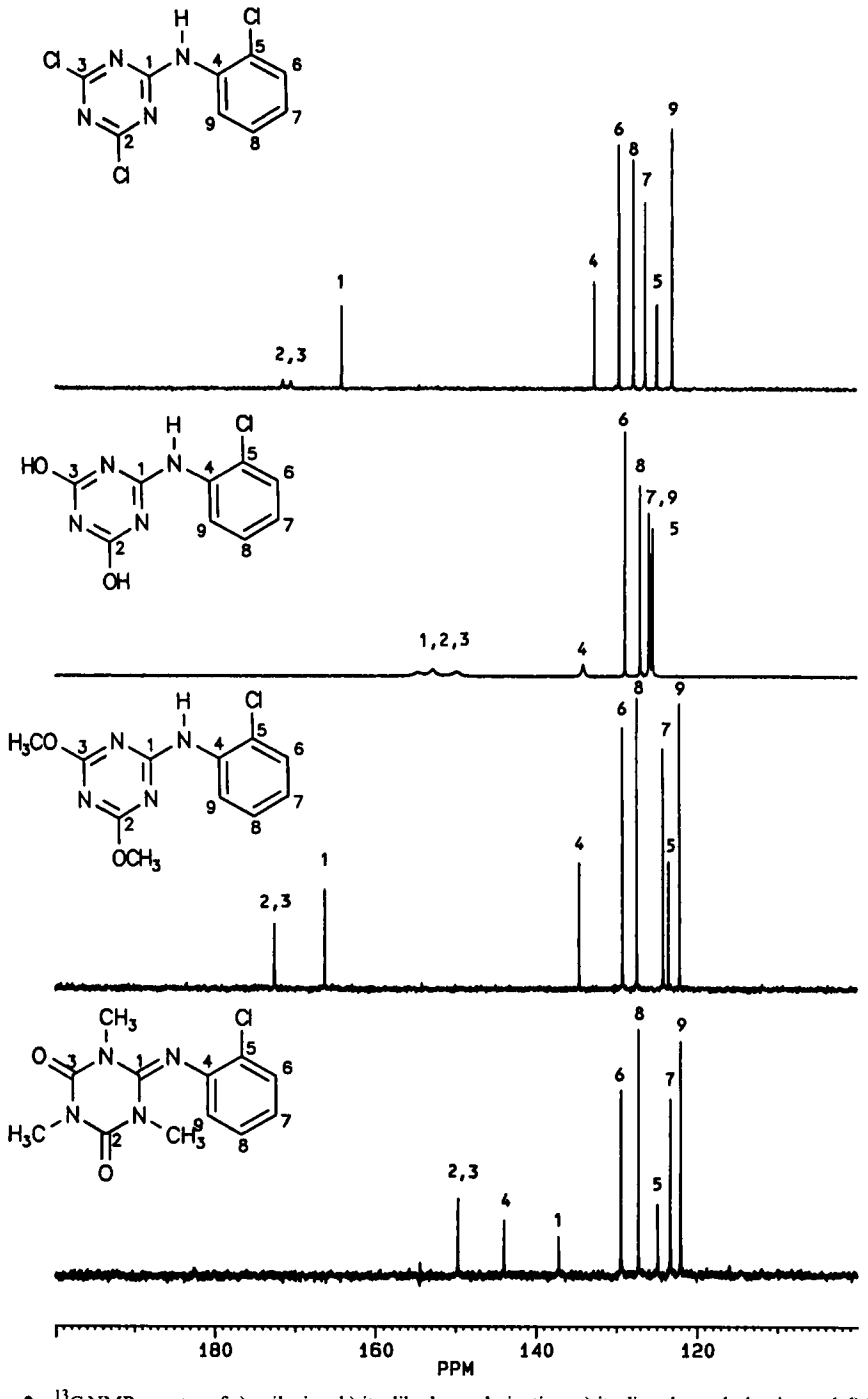


Figure 2 ^{13}C NMR-spectra of a) anilazine; b) its dihydroxy derivative; c) its dimethoxy derivative and d) its N-, N′-, N″-trimethyl derivative, a) – d) with ^{13}C at natural abundance.

with 25 ml water to 60 % WHC. The water contained 10 ml of a filtrate from an aqueous compost-extract, obtained from 1 g of wet compost, for inoculation. The plant material was humified under continuous aeration with CO_2-free air for 16 weeks at 25 °C and during this time about 60% of its carbon was mineralized to CO_2. The humified plant/sand mixture was then lyophilized and remoistened for incubation studies with the labeled anilazine.

After incubation of the soil or sand samples treated with the labeled anilazine they were exhaustively extracted by 4 h each under vigorous shaking with 200 ml isopropanol-H_20 1:1, two times with the same volume of pure isopropanol and then with 200 ml CH_2Cl_2 to remove solvent extractable anilazine or its free metabolites. The isopropanol-H_20 and the first isopropanol extract were plotted on silica gel precoated thin layer plates (KGF 254, Fa. Merck, Darmstadt) and developed with $CHCl_3$-ethanol-acetic acid (93:7:3).

Humic acids were obtained from aliquots of the solvent extracted soil or sand samples by extraction with 0.1 N NaOH under a blanket of N_2-gas and precipitated with HCl. After centrifugation they were dissolved in dilute NaOH to reach a neutral pH value and were then dialyzed against distilled water until the conductance was below 10 $\mu S\ m^{-1}$ Afterwards they were centrifuged at 20,000 g and finally lyophilized[10]. About 100 mg of the dry humic acids were dissolved in 2 ml 0.1 N NaOD and used for ^{13}C NMR spectroscopy.

Silylation of the solvent extracted soil or sand samples followed previously described methods[7]. Briefly, 5 g aliquots of soil or 10 g from sand were shaken for 2 hours with 100 ml 0.1 N NaOH under N_2 and then sonicated for 3 min with a 300 W Labsonic 1510 sonifier (Fa. Braun, Melsungen, FRG). The brown suspension was immediately frozen and lyophilized. Acetone, which was formerly described as a solvent for silylation[7], reacted sometimes with the inorganic components from soil in the presence of NaOH to form a slightly brownish polymer artifact. Therefore, in contrast to the previously described procedure, the lyophilized alkaline soil or sand samples were suspended in 80 ml of dry $CHCl_3$, and under cooling with ice water amended with 5 ml of freshly distilled chlorotrimethylsilane. The suspension was additionally supplied with sufficient dry solid NaOH to neutralize the HCl developed during the silylation procedure. It was shaken (100 rpm) for 24 h in the dark at room temperature and moisture was excluded. Four hours after the first addition another 5 ml chlorotrimethylsilane was added. The yellow-brown $CHCl_3$-extract was centrifuged and separated from the precipitate. The wet precipitate was immediately transferred into Soxhlet-tubes and exhaustively extracted with dry acetone. Both the yellow $CHCl_3$ and the dark brown acetone extracts were evaporated to complete dryness.

The silylated humic compounds from soil or sand samples were generally dissolved in CH_2Cl_2 and were agitated for less than one minute with ice water to remove silylated heavy metal oxides or other paramagnetic compounds interferring with the ^{13}C NMR measurements. The CH_2C1_2 layer was then separated, immediately dried with Na_2SO_4 and evaporated to dryness. The residue was dissolved in $CDCl_3$ and used for ^{13}C NMR measurements. Humic acids obtained by conventional extraction from the soil with NaOH were treated with chlorotrimethylsilane in the same way, but rinsing with ice water to remove paramagnetic impurities was not necessary. HPLC-analysis of the silylated humic extracts was conducted as described before[7]. Bigger amounts of extracts were chromatographed on TLC-plates using $CHCl_3$/ethanol/acetic acid (95:5:1) as a solvent system for development. Distinct zones from parallel plates were eluted and subsequently used for ^{13}C NMR spectroscopic analysis.

The ^{13}C NMR-spectra were measured in 5 or 10 mm tubes with a Bruker AC 200 NMR spectrometer operating at 50.3 MHz under ^{1}H broad band decoupling conditions (acquisition time 1.376 sec, pulse width 30°, delay time 0 sec, line broadening 1 Hz ≈ 40,000 scans). Spectra of humic acids in NaOD with dioxane as external standard, and also of several silylated extracts in $CDCl_3$, were measured under inverse-gated decoupling[11] (acquisition time 0.172 sec, pulse width 45 °, delay time 1.0 sec, decoupler off, line broadening 40 Hz, number of scans ≈ 100,000).

RESULTS

The ^{13}C NMR-spectra of anilazine, of its dihydroxy-derivative, and of two derivatives used as model compounds for interaction of anilazine metabolites with the soil organic matrix, dissolved in $CDCl_3$ or D_6-DMSO, are shown in Figure 2. The three carbons of the triazine ring from anilazine give rise to one sharp signal (C_1 at 164.1 ppm) and two relatively weak and broad absorptions for C_2 at 171.6 and C_3 at 170.6 ppm. The chemical shift values of the dihydroxy-derivative at δ = 155.5, 153.7 and 150.5 for C_1, C_2 and C_3 indicate that this compound is predominately present in the keto-form. This is also confirmed by its ^{1}H NMR-spectrum with a broad signal at 7.9 ppm of an aromatic proton connected by a hydrogen bond to one of the N-atoms in the triazine ring (Figure 2 b). Absorptions of the triazine ring carbons of the dihydroxy-derivative dissolved in aqueous NaOD (together with small amounts of DMSO) were shifted down field with δ = 157.7 for C_1 and for C_2 and C_3 coinciding to one signal at δ = 156.1. Signals from carbons of the aromatic moiety are only slightly shifted towards the higher ppm-region.

Since the triazine-ring of the dimethoxy-derivative of anilazine can rotate freely (Figure 2 c) C_2) and C_3 coincide as one signal at δ = 172.3, with C_1 at δ = 167.3. Compared to anilazine, the absorptions of this compound are shifted slightly down field. Alkaline conditions scarcely influence the shift values to δ = 173.6 and 168.3. The dimethylmercapto-analogue of the dimethoxy-derivative of anilazine has shifts at δ = 180.9 for C_2/C_3 and with C_1 at δ = 160.9. Another model compound, the N-trimethyl-dihydroxy derivative for possible sorptive interaction of anilazine metabolites with the humus matrix is shown in fig. 2 d. It is only present in the keto-form and has shift values of δ = 150.2 for C_2 and C_3 and δ = 137.8 for C_1. Other possible metabolites such as cyanuric acid and 2-chlorophenyl-urea have shifts for the C=0 carbons of δ = 149.9 and δ = 155.2, respectively.

Due to the Nuclear Overhauser Effect (NOE) and the described tautomerism, the signals from carbons at natural ^{13}C abundance from the phenyl ring of anilazine and of its dihydroxy-derivative are more intense than those of the triazine moiety. These triazine signals however, became largely intensified when the carbons were enriched with ^{13}C. The spectra from anilazine and its dimethoxy-derivative with ^{13}C enriched carbons in the triazine ring are shown in Figures 3 a and b. They have enlarged signals at the same positions as the non-enriched components. The dihydroxy-derivative (dissolved in D_6-DMSO) shows sharp but narrow doublet signals of δ = 152.4 and 151.6 (C_1 and $Ci2C_2$) and one weaker signal of δ = 148.6 for C_3. These double signals are better separated and more intense if the spectrum is measured at elevated temperature (70 °C vs. room temperature).

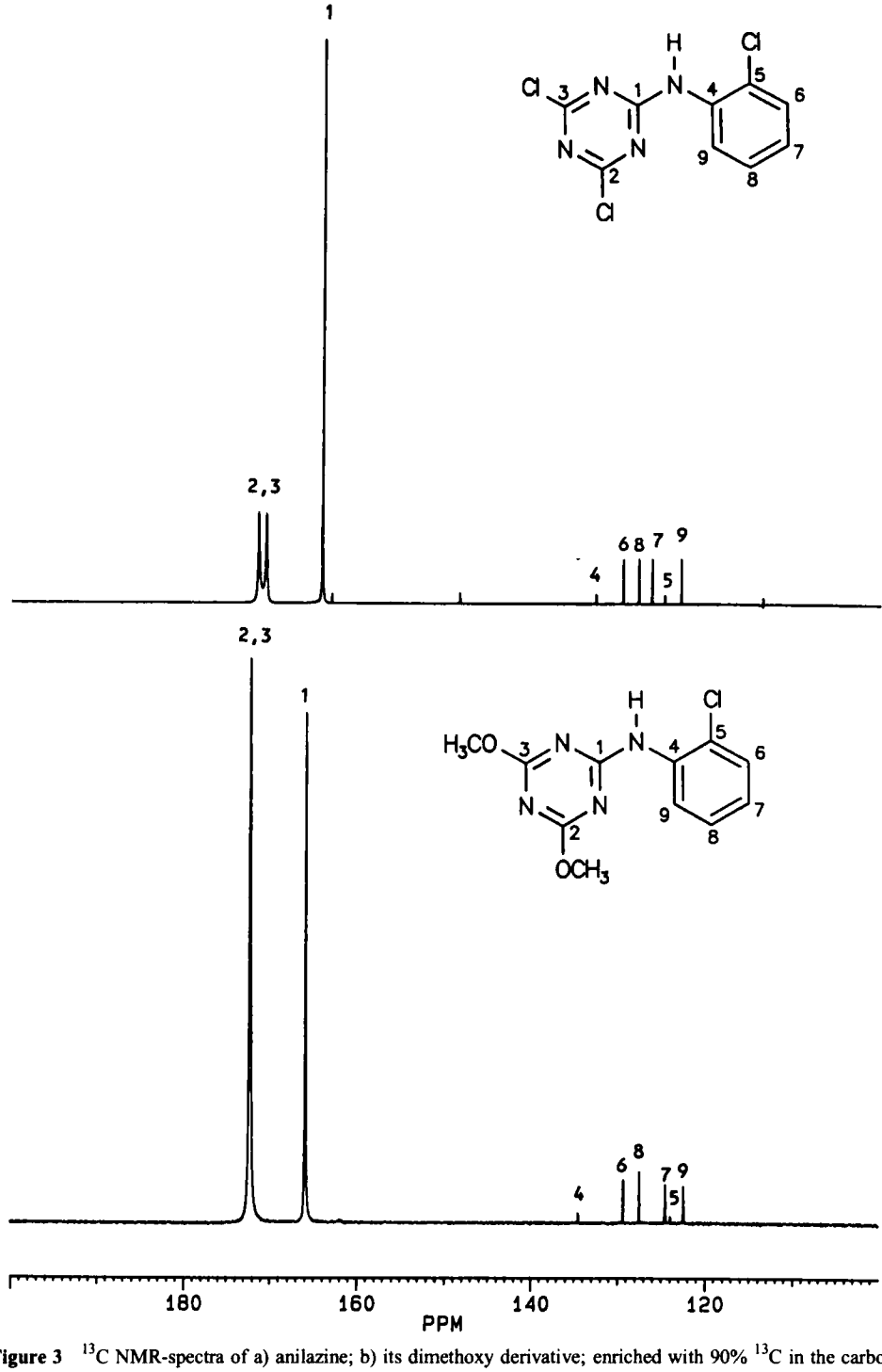


Figure 3 ^{13}C NMR-spectra of a) anilazine; b) its dimethoxy derivative; enriched with 90% ^{13}C in the carbon atoms of the triazine ring.

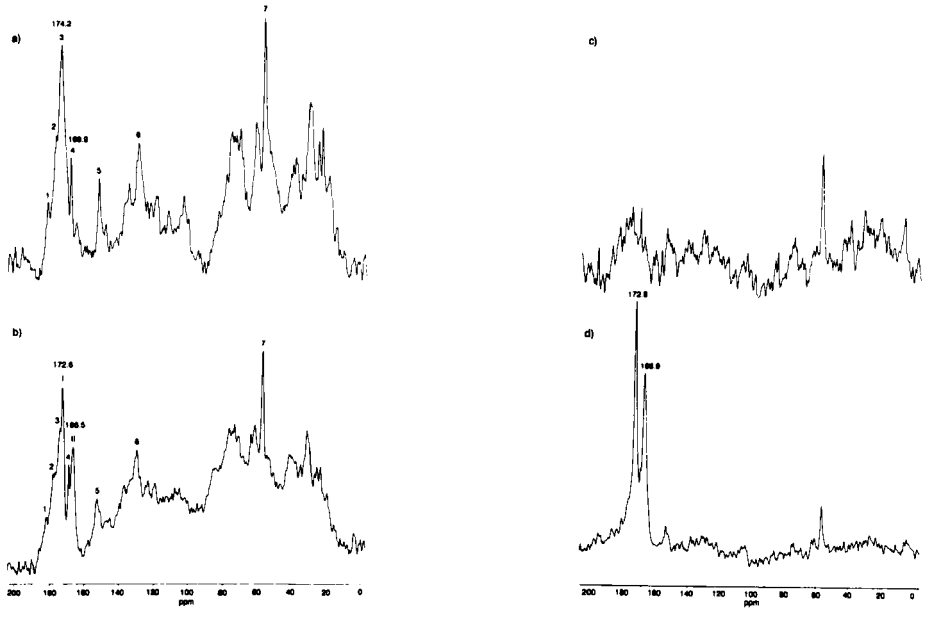


Figure 4 ^{13}C NMR-spectra of purified humic acids in NaOD from a) Ap-horizon of podzolic soil without anilazine amendment; b) with $^{13/14}C$-anilazine amendment after 2 weeks of incubation (residues corresponding to 0.6 mg of original anilazine); c) from the humified ^{13}C depleted corn stalk/sand mixture, without anilazine amendment; d) with $^{13/14}C$-anilazine amendment after 2 weeks of incubation (residues corresponding to 1.6 mg).

Samples from the podzolic soil or from the sand/humified corn stalk-mixture were incubated for 2 weeks with $^{13/14}C$-anilazine (10 mg per 100 g soil or 200 g sand mixture) and humic acids were extracted with NaOH and purified. By previous exhaustive extraction with organic solvents of the incubated soil or sand mixture, 20 and 24 % of the remaining ^{14}C-activity could be removed, respectively. Thin layer chromatography from both the isopropanol-H_20 and the first isopropanol extracts on silica gel plates showed spots from anilazine (faint) and more intense ones from its mono-and dihydroxy-derivatives.

The radioactivity in the humic acids, isolated by NaOH-extraction, corresponded to 12 and 16% of the initially applied ^{14}C. The ^{13}C NMR-spectrum of the soil humic acid without anilazine (Figure 4 a) measured in NaOD under inverse gated decoupling conditions had the usual relatively broad signals in the ranges of humic acid absorptions[12,13]. The spectrum of the humic acid from the ^{13}C-anilazine treated soil (Figure 4 b) shows two additional signals which culminate at 172.6 (I) and 166.5 ppm (II). These two absorptions are similar to those of the dimethoxy-derivative from anilazine (Figures 2 and 3 b) with signals at 172.3 and 167.3 ppm (in DMSO) or 173.6 and 168.3 ppm (in NaOD). The corresponding humic acid spectra from the sand/humified ^{13}C-depleted cornstalk material are shown in figs. 4 c and d. Due to the low ^{13}C-content of the humic acid, obtained from the humified ^{13}C-depleted cornstalk material, the absorptions are not typical for normal humic acids. The corresponding

spectrum of the humic acid which contains anilazine metabolites, has two strong additional peaks at 166.9 and 172.9 ppm. These absorptions agree well with the additional peaks of the humic acid from the anilazine treated soil (Figure 4 b) and resemble those of the dimethoxy-derivative. Signals from the ^{13}C-labeled dihydroxy-derivative dissolved in alkaline solution are well separated from these additional peaks and appear at $\delta = 156$ and 158.

Humic compounds generally contain various aliphatic and aromatic oxygen containing functions. Therefore the broad signals in Figures 4b and d can cover signals from different binding sites of the humic acid. After silylation, soil humic acids, however, with or without anilazine residues showed in their ^{13}C NMR-spectra such numerous signals in the region from 120–200 ppm which made it difficult to attribute distinct absorptions to either soil humic acid or anilazine residues. The silylated humic acids from the sand/^{13}C-depleted humified cornstalk mixture without anilazine treatment (Figure 5 a) have nearly no signals in this region. Rather broad signals do appear in the silylated humic acid spectra from the sand mixture after one and four weeks of incubation with ^{13}C-anilazine (Figures 5b and c) and occur around 174 to 172 and 169 to 166 ppm ppm in the region of the dialkoxy-derivative. A further broad signal around 153 ppm originates from the dihydroxy-derivative of anilazine, probably released during the silylation procedure by splitting of ether (or ester) linkages. Further broad signals in the range between 134–109 ppm arise from carbons of the aniline moieties in different chemical environments.

By silylation of the previously solvent extracted soil or sand samples, 10 to 20 % of the anilazine residues were detected in the $CHCl_3$- and 40 to 60 % in the acetone-extracts, respectively. This was somewhat less than by direct silylation in acetone with removal of more than 80%[7]. The spectrum from the silylated sand/humified ^{13}C-depleted plant mixture (Figure 6a) again shows relatively weak absorptions in the range between 120 to 200 ppm. The spectrum of the sample with anilazine treatment after one week of incubation (Figure 6b) has several sharp signals between $\delta = 175$ to 165 indicating the alkylation of anilazine metabolites by several binding sites of the humic compounds. A signal at 154 ppm is probably caused by the free dihydroxy-derivative of anilazine and those around 130 ppm by carbons of the aniline moiety. Signals from the free dihydroxy-derivative also appeared even after prolonged incubation of the soils with anilazine. A signal around 109 ppm appears in both the spectra with or without anilazine amendment and should not originate from metabolic products of this fungicide.

Due to the NOE, signals from carbons of the aniline moiety in NMR spectra measured by the ^{1}H broad band decoupling method, are relatively enlarged against those from the triazine ring. By registration of the spectrum under inverse-gated decoupling conditions (Figure 6c), these latter signals get somewhat, but not significantly enlarged, with their absorptions still at the same positions.

The results indicate that the binding of anilazine in the humic compounds occurs probably as a dialkoxy-derivative by exchange of the chlorine atoms from anilazine with functional hydroxy groups in the humic matrix. The presence of free anilazine is unlikely, since this compound is already hydrolyzed into its dihydroxy-derivative during the treatment with dilute NaOH for humic acid extraction. Also the presence of other possible functional metabolites from anilazine (Figures 2 and 3 and others) can be excluded.

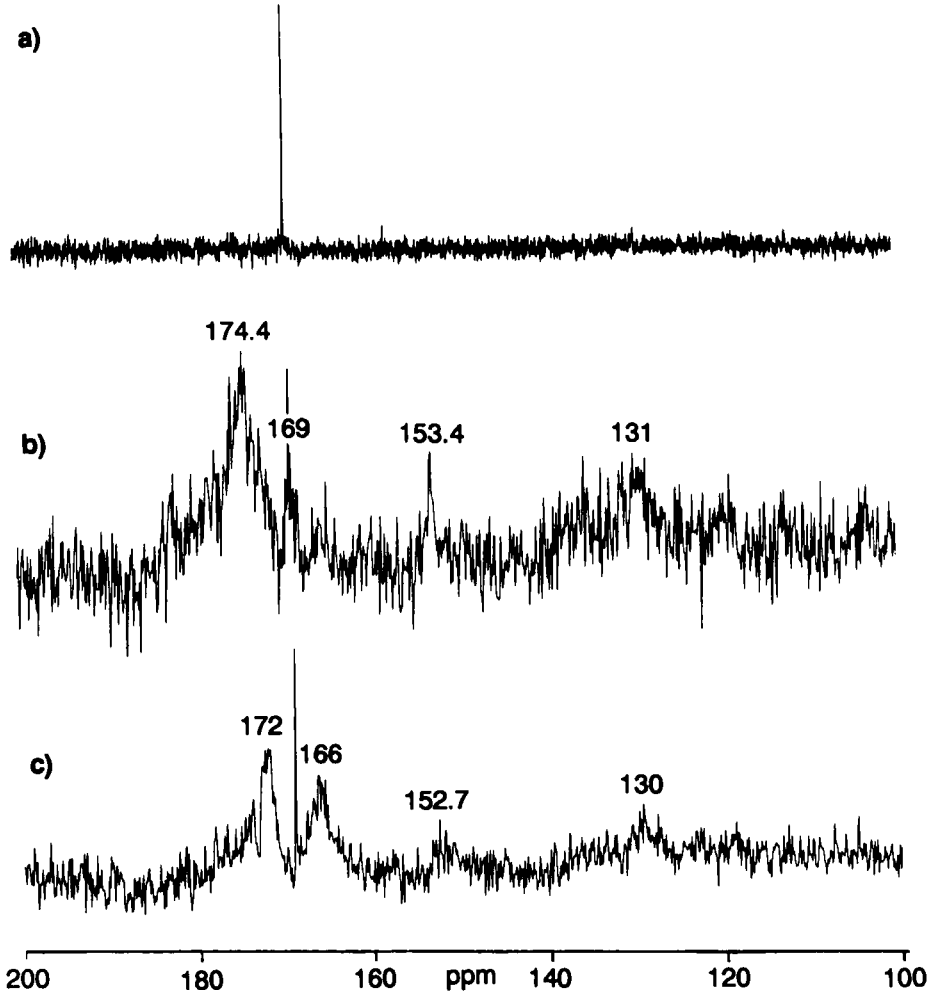


Figure 5 ^{13}C NMR-spectra of silylated humic acid in $CDCl_3$ from the humified ^{13}C-depleted corn stalk/sand mixture, a) without anilazine amendment; b) with $^{13/14}C$-anilazine amendment after 2 weeks of incubation (residues corresponding to 0.8 mg of original anilazine); c) with $^{13/14}C$-anilazine after 4 weeks of incubation (residues corresponding to 0.7 mg).

DISCUSSION

Anilazine is an example of a plant protection agent which forms rapidly unextractable residues in soil which are intimately connected to the humic matrix[14,15]. By means of ^{13}C-enriched anilazine and subsequent ^{13}C NMR-measurements of the humic acid fractions it could be shown that this fungicide after soil incubation was bound by exchange of chlorine groups at the triazine moiety with oxygen containing functional groups of the soil organic

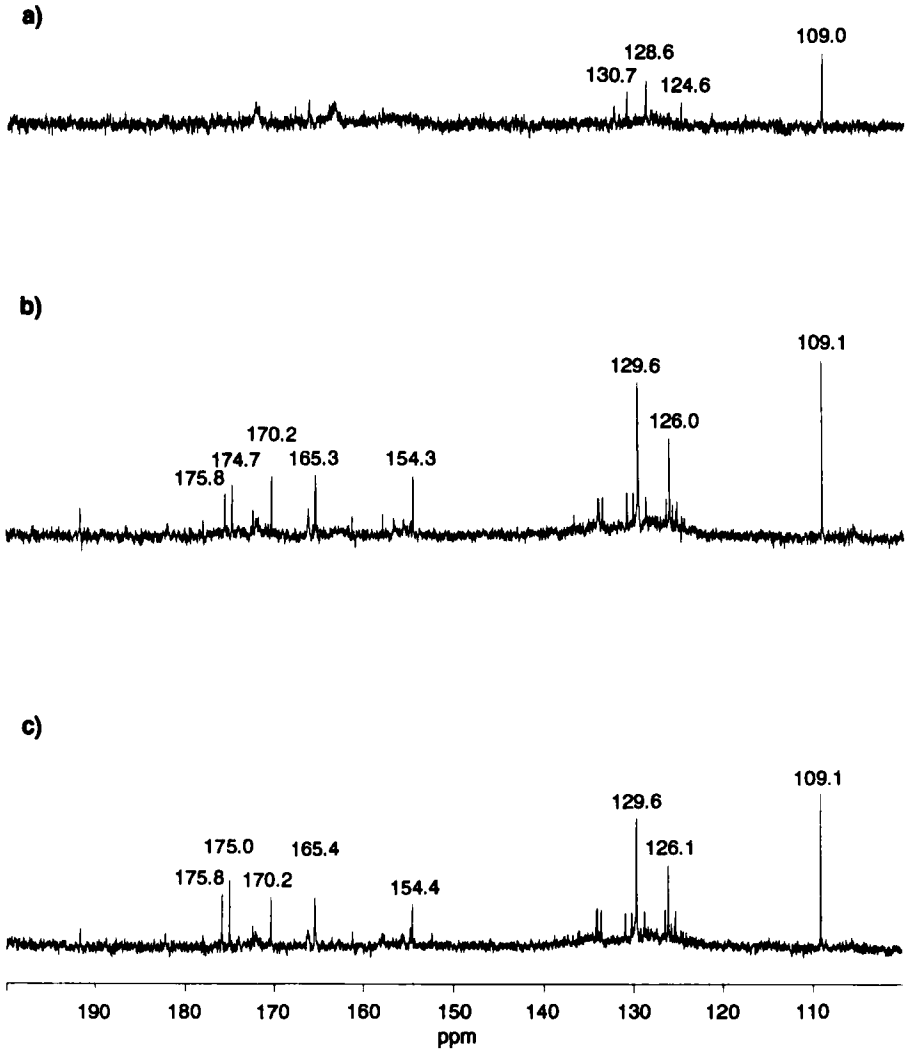


Figure 6 ^{13}C NMR-spectra of the silylated organic matter from the humified ^{13}C-depleted corn stalk/sand mixture a) without anilazine amendment; b) with $^{13/14}C$-anilazine after 1 weeks of incubation (residues corresponding to 0.8 mg of original anilazine), measurements under ^{1}H broadband decoupled conditions; same spectrum under inverse-gated decoupled conditions.

matter. A covalent binding by alkylation of the NH-groups in anilazine metabolites or by reaction with SH-groups in humic compounds is unlikely, since the corresponding NMR signals could not be detected.

The silylation may have led to a cleavage of some of the linkages of the bound molecules to the humic matrix and to a release of the dihydroxy-derivative of anilazine. Since chlorotrimethylsilane is a rather mild silylating reagent and splitting of ether or ester linkages

during the silylating procedure by this reagent have been only reported for temperatures above 100°C[16], several of these linkages are probably not very strong, whereas others are more stable. This cleavage is possibly facilitated by the conjugated system of double bonds in an oxygen substituted triazine-ring, easily shifting from the enol- into the keto-form.

Previous[7] and presently conducted HPLC-studies on ultrastyragel columns with silylated extracts from soils after incubation with ^{14}C-labeled anilazine, showed that the majority of silylated humic compounds had molecular weights between 300 to 4000 d and the ^{14}C-activity appeared as a discrete fraction in the range from 2,500 to 3,000 d. This indicated that most of the anilazine metabolites that were no longer extractable with organic solvents, were incorporated in the humic matrix.

A partial release of the dihydroxy-derivative of anilazine during the silylation procedure of humic compounds could be shown by thin-layer chromatography of bigger amounts of the derivatized humic compounds, developed with $CHCl_3$/ethanol/acetic acid. A faint zone corresponded to the R_f-value of the dihydroxy derivative, whereas the strong brown radioactive zone above corresponded neither to this derivative nor to any known free metabolite and was combined with silylated humic material. ^{13}C NMR-spectroscopy of the combined eluted material from the dark brown zone of several parallel thin-layer plates showed two prominent signals at $\delta = 172.3$ and 167.3 and indicated therefore the presence of an alkylated ^{13}C-enriched dihydroxy derivative bound to the humic matrix.

Several spontaneous or enzyme catalyzed coupling reactions have been reported to occur between phenols or aromatic amines and humic compounds[17,18,19]. The authors also tried to develop a plausible covalent binding mechanism of these molecules with the humic materials without, however having much experimental evidence. The present study combines isotopic labeling with ^{13}C NMR spectroscopy. This technique should be promising in further studies of possible binding mechanisms between pesticide residues and humic compounds.

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