Microbial Release and Degradation of Nonextractable Anilazine Residues

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Humic substance fractions obtained from a degraded loess soil taken from a long-term lysimeter experiment with the fungicide anilazine were incubated in aerated liquid cultures together with native soil microorganisms. Biomineralization, remobilization of [U-*phenyl*-^{14}C]anilazine, respectively, its metabolites, and changes of the humic matrix were observed under variable nutrient conditions. Stimulated microbial activity favored the degradation of nonextractable ^{14}C-anilazine residues. However, nitrogen deficiency enhanced structural changes in the humic substances, which seemed to be used then as a nitrogen source. Along with the microbial degradation of the humic substances, parts of the bound anilazine residues became remobilized. Furthermore with the use of AMD−TLC, dihydroxy anilazine was detected within the nonextractable residues. The portion of rather weak bondings between the soil organic acids and the anilazine residues turned out to be considerably lower in the humic acids fractions than in the fulvic acids fraction.

Keywords: *Soil-bound residues; humic substances; bioavailability; triazine fungicide; AMD thin-layer chromatography*

INTRODUCTION

Nonextractable pesticide residues may constitute a potential hazard to agricultural soils since their ultimate fate is still unknown. A number of reviews describe the possible sorption and binding mechanisms of xenobiotics to soil organic matter or deal with their possible environmental impact (Kaufman, 1976; Khan, 1982; Führ, 1987; Calderbank, 1989; Bollag, 1992; Dec and Bollag, 1997; DFG, 1998). Soil bound residues may become biologically available to plants (Führ and Mittelstaedt, 1980; Roberts and Standon, 1981; Khan, 1982; Kloskowski and Führ, 1983; Nelson and Khan, 1990; Celi et al., 1997; Dec et al., 1997) and microorganisms (Khan and Dupont, 1987; Dec and Bollag, 1988; Alexander, 1995), especially when humus decomposition or enhanced co-metabolism are stimulated (Racke and Lichtenstein, 1985; MacRae, 1986).

Anilazine (4,6-dichloro-*N*-(2-chlorophenyl)-1,3,5-triazine-2-amine, Figure 1) is distributed as Dyrene (registered trademark of Bayer AG, Leverkusen, Germany). It is used to combat glume browning and glume blotch of wheat (Heitmann-Weber et al., 1994), a significant disease induced by *Septoria nodosum* and other fungal pests. Its fate in the soil is well-known from laboratory and lysimeter experiments. Only small amounts of anilazine are mineralized when applied to soil, whereas large amounts of nonextractable residues according to the IUPAC definition (Roberts et al., 1984) are formed very rapidly (Mittelstaedt et al., 1987). Coupling between anilazine and humic acids occurs via the intermediate metabolite monohydroxy-metabolite and takes place by the formation of covalent ether and/or ester bonds to the various functional hydroxyl groups of humic acids (Haider et al., 1993; Wais et al. 1995).

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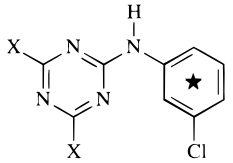

Figure 1. Chemical structure of anilazine (X = Cl) and dihydroxy anilazine (X = OH) (★ = position of ^{14}C-labeling).

The objective of this work was to study the potential microbial bioavailability and remobilization of nonextractable anilazine residues under conditions favoring microbial activity. Anilazine residues were characterized by a gradient development technique. This method has already been used to analyze pesticide residues in water (Vigne et al., 1991; Morlock, 1996) or to monitor carbohydrates or byproducts in beer (Brandolini et al., 1995) or wine fermentation (Romano et al., 1996), but it has not yet been used in connection with soil-bound residues.

MATERIALS AND METHODS

Nonextractable Residues. Nonextractable anilazine residues were obtained from a long-term lysimeter experiment (undisturbed soil profile: 1.1 m). The soil used in the lysimeter study was an orthic luvisol, a clayey silt derived from loess widespread in the Federal Republic of Germany, taken from a field plot near Merzenhausen, Rhineland, with the following characteristics for the A_P horizon (Heitmann-Weber et al., 1994): 1.2% organic carbon content, 6.4% sand, 78.2% silt, and 15.4% clay, pH 7.2. A total of 4 kg/ha [U-*phenyl*-^{14}C]anilazine (Figure 1) in the formulation of Dyrene was applied annually to the soil surface in five consecutive years. About 100% of the applied [^{14}C]anilazine was still detected in the soil 100 days after the fifth application with more than 90% in the 0−20-cm soil layer (Mittelstaedt and Führ, 1998). Soil samples (0−10 cm) from the fifth year of application were extracted with organic solvents and fractionated with basic and acidic agents

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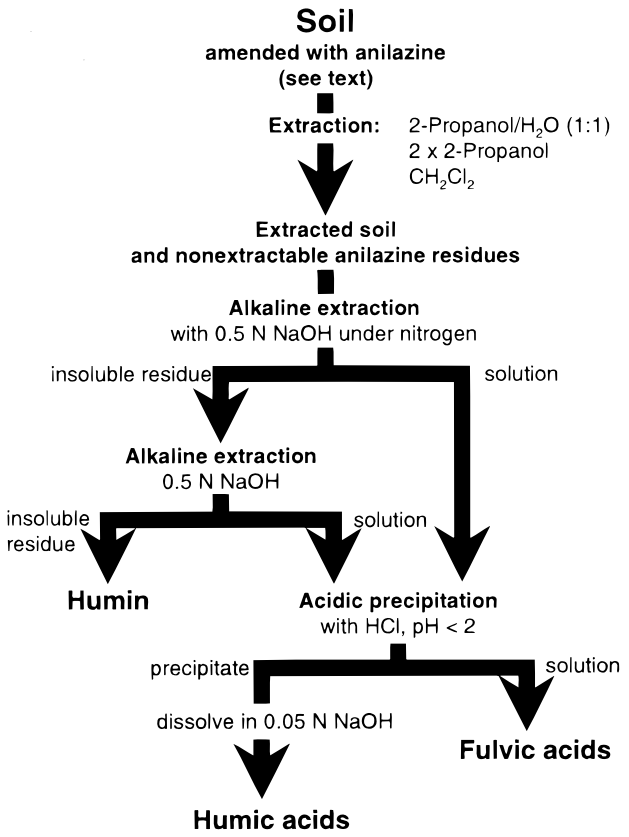

Figure 2. Schematic diagram of humic substances fractionation.

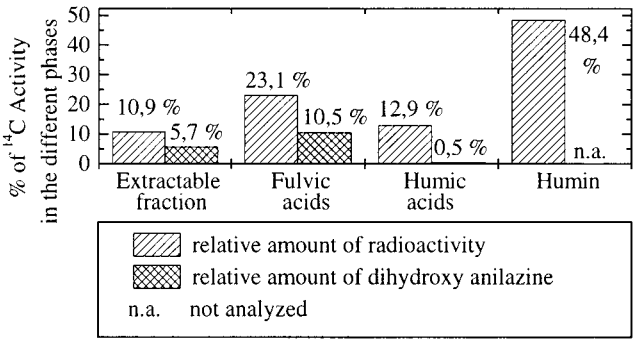

Figure 3. Distribution of ^{14}C activity and dihydroxy anilazine in the different fractions in the soil after the 5-year treatment as described (data on top of columns indicate relative amount of radioactivity, data on the right indicate the part of radioactivity identified as dihydroxy anilazine).

(Figure 2) as described by Khan (1982) and Wais et al. (1996). Figure 3 gives an overview about the mass balance of ^{14}C in the different phases of the extraction procedure.

Remobilization Studies. Soil organic matter fractions were neutralized (fulvic acids with 4 M NaOH; humic acids with 1 M H_3PO_4) and supplemented with mineral salts according to Focht (1994). Humin media were prepared by adding 10 g of extracted soil containing humin-bound anilazine residues to 50 mL of a mineral medium prepared as described by Focht (1994). The solutions were then neutralized with 1 M H_3PO_4. Additionally 25 mg L^{-1} yeast extract was added to all batches to provide complex nutrients. Media with an additional carbon source contained 0.1% glucose, and media with a nitrogen source were supplemented with $(NH_4)_2SO_4$ to a final concentration of 10 mM (Table 1). A 10-g soil (orthic luvisol) sample was shaken with 100 mL of $Na_4P_2O_7$ (0.2%) for 30 min. Media were inoculated with 1 mL of this soil suspension. An overview of treatments examined is given in Table 1. The culture solutions containing nonextractable

Table 1. Overview of Treatments with Organic Matter-Bound Residues

abbreviation of treatment	organic matter fraction	N source (10 mM)	C source (0.1%)
FANG	fulvic acids	$(NH_4)_2SO_4$	glucose
FAN	fulvic acids	$(NH_4)_2SO_4$	no
FAG	fulvic acids	No	glucose
FA	fulvic acids	No	no
HANG	humic acids	$(NH_4)_2SO_4$	glucose
HAG	humic acids	no	glucose
HRNG	humin residue	$(NH_4)_2SO_4$	glucose
HR	humin residue	no	no

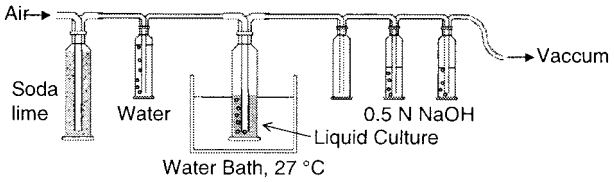

Figure 4. Experimental design of remobilization studies.

anilazine residues were incubated at 27 °C in aerated liquid cultures in the dark for 8 weeks (Figure 4). During the incubation period, the pH values of the media were maintained within the range of 7.0−7.5 by applying 0.87 M H_3PO_4.

Photometric Analysis. Changes in the amount of humic substances in the filtered (0.45 μm) culture solutions were determined as changes of absorption from $\lambda = 230$ to $\lambda = 380$ nm in comparison to unincubated media (control media). All photometric recordings were performed in triplicates against double-distilled H_2O as the background. Unincubated medium of each single soil organic fraction was defined to have a relative humic substance concentration of 100%. Controls for media containing the humin fraction were shaken for 4 h. Additionally, the behavior of the media was observed at the following wavelengths: 665, 465, 380, 300, 265, 240, and 220 nm. Changes in absorption were documented as relative absorption as compared to the unincubated medium (see above).

Assay of Radioactivity. [^{14}C]Carbonate from the NaOH traps was determined in a liquid scintillation counter. In addition, samples of the culture media were filtered (0.45 μm) to distinguish between dissolved and suspended radioactivity before and after the experiment. The residue was oxidized first and then analyzed in a liquid scintillation counter.

Thin-Layer Chromatography. Soluble metabolites of [^{14}C]anilazine in the soil fractions and in the media were analyzed by radio-TLC. One hundred microliters of the centrifuged culture media was applied together with 5 μL of anilazine and dihydroxy anilazine standard (1 mg mL^{-1} acetone) on HPTLC plates (Merck, Darmstadt, Germany). Development of HPTLC plates was done with an AMD (automated multiple development) system (CAMAG Muttenz, Switzerland). A polarity gradient with 25 steps was established with a sequence of different solvent mixtures, which were composed of methanol/H_2O, ethyl acetate, acetic acid, and chloroform (Figure 5a). Before each step, the plates were preconditioned with methanol by flushing the chamber with methanol-enriched nitrogen. After each step, the plates were vacuum-dried for 3 min at room temperature. Additionally, a second gradient was performed with a small selection of samples to confirm the obtained results. The plates were conditioned with formic acid before each step, and each solvent mixture contained 1% (v/v) formic acid. After the 5th step, the mixer was emptied to remove the polar components methanol and H_2O (Figure 5b). Results were compared to those obtained by conventional TLC development methods previously used to characterize anilazine and its metabolites without establishing a polarity gradient (Kloskowski et al., 1986; Mittelstaedt et al., 1987). The developing solution was a mixture of chloroform:ethyl acetate:acetic acid (95:10:5, v/v/v). A bioimager system (Raytest Bioimager, Fuji, Tokyo, Japan) was used to evaluate thin-layer chromatograms.

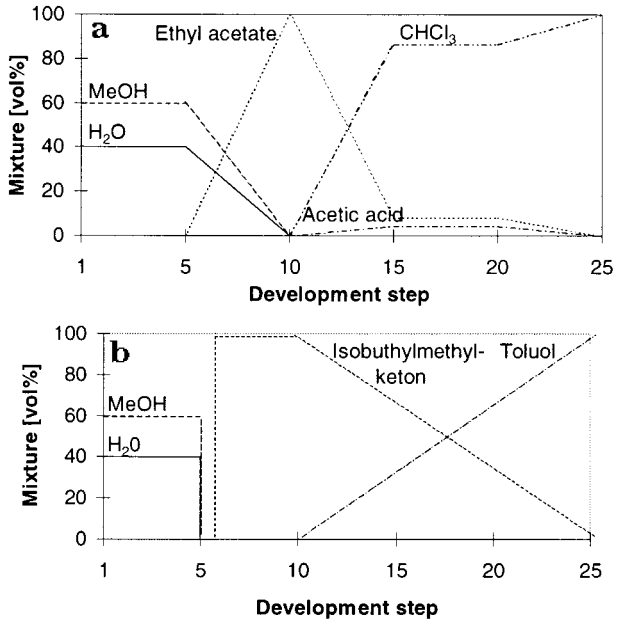

Figure 5. (a) AMD−gradient diagram for TLC analyses of culture media. (b) AMD−gradient diagram for consolidation TLC analyses.

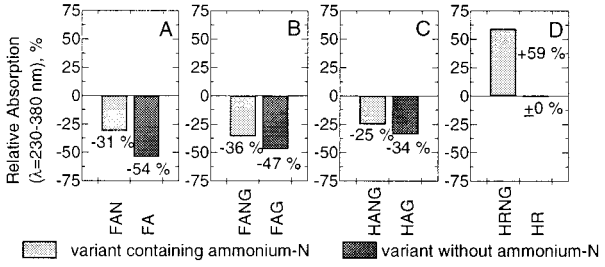

Figure 6. Changes of relative absorption (λ = 230−380 nm) with and without nitrogen in the media as compared to a control without incubation. (A) Fulvic acids. (B) Fulvic acids plus glucose. (C) Humic acids plus glucose. (D) Humine residue plus glucose.

RESULTS

Photometric Analyses. Absorption of culture media at wavelengths from 230 to 380 nm decreased in most incubated batches (Figure 6). Minor differences of pH values in the parallels did not affect the general results and were always within the range of variation. Greatest changes were observed with media containing fulvic acids (FA) and humic acids (HA) without a nitrogen source.

Different results were obtained with the humin assays. Only minor differences as compared to the control medium were observed with nitrogen-free batches (HR). However, an increase of absorption values with nitrogen and glucose (HRNG) could be observed.

Considering the different wavelengths, the greatest changes of absorption values were observed in almost each case at longer wavelengths (Figure 7). Only humin residue treatments containing nitrogen showed an increase of absorption by 60−70% at each wavelength.

Mineralization of ^{14}C-Labeled Nonextractable Anilazine Residues. The amount of radioactivity remaining on the filter was around 1% of applied radioactivity [Applied radioactivity is considered as the amount of radioactivity used in the described remobilization studies.] in batches with fulvic acids after 8 weeks of incubation, 1−4% in those with humic acids,

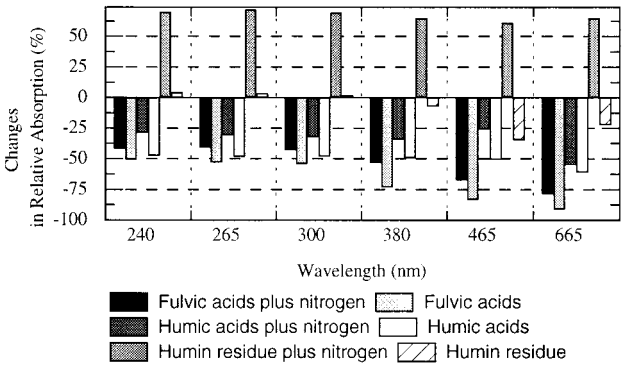

Figure 7. Relative absorption of media at different wavelengths after 8 weeks of incubation compared to not incubated media (control).

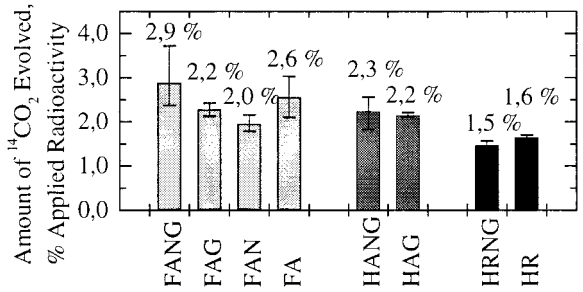

Figure 8. Mineralization of nonextractable [U-*phenyl*-^{14}C]-anilazine residues within an 8-week incubation period (applied radioactivity = 100%; error bars indicating variation of parallels).

and around 39% in batches with humin. Only small amounts of added [U-*phenyl*-^{14}C]anilazine residues were mineralized (1.5−2.9%, Figure 8). The largest amount of $^{14}CO_2$ was evolved within the first 7−14 days of incubation. After this period only minor $^{14}CO_2$ evolution could be measured. In case of an additional amendment of glucose during the experiment, the $^{14}CO_2$ evolution increased again significantly.

The largest quantity of $^{14}CO_2$ was evolved from residual ^{14}C activity in those batches with fulvic acids that had been treated with glucose and ammonia (Figure 8). No differences between the treatments were observed with humic acid media and with media containing humin-bound anilazine residues.

Thin-Layer Chromatography. With the described method, only the dihydroxy metabolite of anilazine was found in extracts and media. The R_f values for dihydroxy anilazine were 0.87 for the first gradient, 0.68 for the second gradient, and 0.10 for the conventional radio-TLC (Figure 9). The same quantitative results were obtained by the two gradient chromatographs. Dihydroxy anilazine could only be detected by AMD chromatography in the humic acid fraction but not by conventional radio-TLC. No unchanged anilazine was present in any sample analyzed. The results shown in Figure 3 were obtained with the first gradient described.

Independent of the incubation time, about 46% of applied radioactivity could be identified as dihydroxy anilazine in variants with fulvic acids. In media containing humic acids, 4% of applied radioactivity was identified as dihydroxy anilazine. After 8 weeks of incubation, this value doubled to 8%. Differences did occur depending on nitrogen availability. Between 4% and 11% of dihydroxy anilazine could be detected in the humin fraction. The large variation is caused by the inhomogeneous nature of the media.

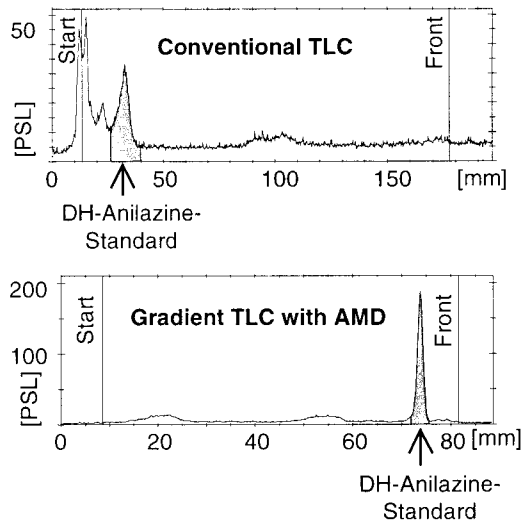

Figure 9. Comparison of performance of a fulvic acids extract after soil fractionation (Figure 2) in conventional and AMD−TLC (gradient used for quantitative analyses, details see text).

DISCUSSION

In general, the turnover of humic matter is very slow and can be observed by changes in the absorption of fractionated soil organic matter. Spectral absorption of humic substances is due to conjugated π-bond systems (chromophores) and additional auxochromes (e.g., hydroxyl or amino groups), which are responsible for a shift of λ_{max} to higher wavelengths (Bloom and Leenheer, 1989). Due to microbial activity, the π-bonds present in humic substances might be ruptured in such a manner that either the length of the conjugated system becomes shorter or the auxochromes are altered or lost. Furthermore, anaerobic conditions may lead to a microbial reduction of humic matter (Lovley et al., 1996) and thus to changes in the spectroscopic behavior.

The decrease of absorption might have several reasons. In experiments with the white rot fungus *Phanerochaete chrysosporium*, Haider and Martin (1988) observed mineralization of more than 30% of ^{14}C-labeled humic substances. As humic substances were decomposed, the solution became decolorized. Hence, it can be assumed that the decrease of relative spectral absorption in the liquid cultures during incubation in the present investigations is also due to mineralization or substantial degradation of the humic matrix.

The changes of absorption during incubation (Figure 6) are correlated to a decrease in the content of humic substances in the media. A higher bioaccessibility of fulvic acids to soil microorganisms might be due to their lower complexity (Stevenson, 1985) and higher oxygen content (Haider, 1995) as compared to humic acids or the humin residue resulting in a larger decrease of absorption. Differences in the nitrogen content of humic substances (1.6−4.1% in humic acids and 0.7−2.8% in fulvic acids; Haider, 1995) could also be a reason for varying the extent of metabolism.

Comparing the three fractions, the greatest changes were observed in the fulvic acids fraction, followed by the humic acids and the humin residue (Figure 6). Presuming that nitrogen in the humic substances provides a nitrogen source for the microorganisms, the different C/N contents of the fractions may be a reason for the different rates of turnover: less humic acids—as compared to fulvic acids—need to be turned over to obtain the same amount of nitrogen to support microbial activity. However, microbial activity under nitrogen deficiency enhanced structural changes of soil organic matter, as indicated by the decrease of spectral absorption (Figure 6). Nonextractable anilazine residues are presumed to be decomposed along with the humic matrix (Kloskowski et al., 1986; Heitmann-Weber et al., 1994). Subsequent structural changes in humic substances might then lead to a release of nonextractable anilazine residues as was observed in the humic acids fraction, where the portion of dihydroxy anilazine was very low before incubation.

Spectral absorption in the humin residue, after treatment with ammonia, increased from $\lambda = 230$ to $\lambda = 380$ nm by approximately 60%. This can be explained by solubilization of parts of the humin residue during 8 weeks of incubation as compared to only 4 h shaking for the control. This could be due to the long period of incubation, which led to further extraction of sequestered material. On the other hand, it is conceivable that microbial exoenzymes attacked suspended humic substances, which then became solubilized and caused an increase of relative spectral absorption (Figure 6). This assumption is confirmed when the results of those treatments without nitrogen are considered, where no increase was observed. If the incubation time had the same influence as an extended period of extraction, the values of relative spectral absorption of about 100% implied an actual reduction from the possible 160%. This means that—as with the other organic fractions—the humin residue was changed most effectively under conditions of nitrogen deficiency.

We observed a stronger reduction in relative absorption at longer wavelengths than at shorter ones (Figure 7), indicating a stronger decrease of longer fragments. Long fragments might be divided into shorter ones, or auxochromes might be split off leading to an increase in the amount of shorter fragments. Since a decomposition as well as a formation of rather short fragments might occur this way, we suppose that the decomposition of humic substances is independent of their size.

Stimulated microbial activity—due to availability of ammonia and glucose at the same time—was observed to be the most effective treatment for the biomineralization of nonextractable anilazine residues (Figure 8). However only slightly less of the residual ^{14}C activity was mineralized without these supplements. This leads to the conclusion that both ammonia and glucose were used up very rapidly for cell growth, followed by conditions of deficiency, which were self-induced. In particular, the absence of a readily accessible nitrogen source seemed to have a positive effect on biomineralization. The deficiency of nitrogen was probably remedied by that derived from the humic substances or the anilazine residue. Both are possible, and Cook and Hütter (1981) propose that microorganisms may utilize triazines as a nitrogen source. Decomposition of dihydroxy anilazine by splitting off the phenyl ring is conceivable, considering that bacteria may utilize triazines as nitrogen sources by an initial N-dealkylation step (Mougin et al., 1997). On the other hand, it is known that dihydroxy anilazine is only slowly mineralized (Kloskowski et al., 1986; Heitmann-Weber et al., 1994) leading us to infer that this may not be the only pathway.

Low amounts of dihydroxy anilazine observed in the humic acids fraction (Figure 3) confirm the results

obtained by Haider et al. (1993) and Wais et al. (1995). Wais et al. (1995) found covalent bonds between anilazine metabolites and humic acids. On the other hand, 46% of applied radioactivity in the fulvic acids fractions was identified as dihydroxy anilazine (Figure 3), indicating that many of the interactions between soil organic matter fractions and the main anilazine metabolite are represented by weak bonds, such as hydrogen bonds, formation of charge-transfer complexes, or by ligand exchange. Therefore, the formation of covalent ether and/or ester bonds may not be the only binding mechanism. After incubation of media containing humic acid-bound anilazine residues for 8 weeks, the portion of dissolved dihydroxy anilazine was doubled. Similar results were also obtained by Dec and Bollag (1988) and Dec et al. (1990) with humic acid-bound residues of catechol and chlorophenols, although lower portions were released. Possibly release of metabolites occurs in an analogues manner to the utilization of humic substances as a nitrogen and/or energy source by the involved microbiota. At the same time, biomineralization did not increase (Figure 8), and this makes it clear that soil organic matter might be altered by microbial activity while dihydroxy anilazine is only slowly metabolized.

In general, the remobilization potential of bound residues of [^{14}C]anilazine is small. Bioavailability and remobilization were closely connected to the turnover of soil organic matter. Nevertheless, it was observed that these were dependent on the specific humus fraction and the nutritional status of the liquid culture. While humic substances reveal greatest structural modifications in nitrogen deficiency situations (Figure 6), anilazine residues are decomposed more easily when microbial activity is stimulated by an easily accessible carbon source such as glucose (Figure 8). Glucose enhances microbial activity. After depletion of glucose, humic substances and anilazine metabolites are degraded with no general difference in bioavailability of either one of the considered soil organic matter fractions. The presumption that anilazine residues associated with fulvic acids are more available to plants and soil fauna (Khan, 1982) might still be true, because fulvic acids are dissolved in the soil solution under natural conditions (Stevenson, 1976).

Large amounts of the anilazine residues—particularly those bound to fulvic acids—seem to be loosely bound to humic substances (e.g., sorbed or sequestered) and may be readily remobilized in the form of the unchanged metabolite, dihydroxy anilazine. Therefore co-metabolism along with the humic matrix is not the only pathway for the degradation of nonextractable anilazine residues. Contrarily strong bonding (e.g., covalent ones) occurs frequently between anilazine metabolites and humic acids. Increased turnover of soil organic matter released bound metabolites of anilazine, which were then susceptible to further metabolism.

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LITERATURE CITED

Alexander, M. How toxic are chemicals in soil? *Environ. Sci. Technol.* **1995**, *29*, 2713−2717.

Bloom, P. R.; Leenheer, J. A. Vibrational, electronic, and high-energy spectroscopic methods for characterizing humic substances. In *Humic Substances II*; Hayes, M. H. B., MacCarthy, P., Malcolm, R. L., Swift, R. S., Eds.; John Wiley & Sons Ltd: Chichester, 1989; pp 409−446.

Bollag, J.-M. Decontaminating soil with enzymes: An in situ method using phenolic and anilic compounds. *Environ. Sci. Technol.* **1992**, *26*, 1876−1881.

Brandolini, V.; Menziani, E.; Mazzotta, D.; Cabras, P.; Tosi, B.; Lodi, G. Use of AMD-HPTLC for carbohydrate monitoring in beers. *J. Food Compos. Anal.* **1995**, *8*, 336−343.

Calderbank, A. The occurrence and significance of bound pesticide residues in soil. *Rev. Environ. Contam. Toxicol.* **1989**, *108*, 71−103.

Celi, L.; Gennari, M.; Schnitzer, M.; Khan, S. U. Extractable and nonextractable (bound) residues of acifluorfen in an organic soil. *J. Agric. Food Chem.* **1997**, *45*, 3677−3680.

Cook, A. M.; Hütter, R. s-Triazines as nitrogen sources for bacteria. *J. Agric. Food Chem.* **1981**, *29*, 1135−1143.

Dec, J.; Bollag, J.-M. Microbial release and degradation of catechol and chlorophenols bound to synthetic humic acid. *Soil Sci. Am. J.* **1988**, *52*, 1366−1371.

Dec, J.; Bollag, J.-M. Determination of covalent and noncovalent binding interactions between xenobiotic chemicals and soil. *Soil Sci.* **1997**, *167*, 858−874.

Dec, J.; Shuttleworth, K. L.; Bollag, J.-M. Microbial release of 2,4-dichlorophenol bound to humic acid or incorporated during humification. *J. Environ. Qual.* **1990**, *19*, 546−551.

Dec. J.; Haider, K.; Rangaswamy, V.; Schäffer, A.; Fernandes, E.; Bollag, J.-M. Formation of soil-bound residues of cyprodinil and their plant uptake. *J. Agric. Food Chem.* **1997**, *45*, 3514−3520.

DFG. *Pesticide Bound Residues in Soil*; Senatskommission zur Beurteilung von Stoffen in der Landwirtschaft, Ed.; Wiley-VCH: Weinheim, 1998.

Focht, D. D. Microbiological procedures for biodegradation research. In *Methods of Soil Analysis. Part 2, Microbiological and Biochemical Properties;* Soil Science Society of America Book Series 5; Weaver, R. W., Ed.; Soil Science Society of America, Inc.: Madison, WI, 1994; pp 407−426.

Führ, F. Non-extractable pesticide residues in soil. In *Pesticide Science and Biotechnology*; Greenalgh, R., Roberts, T. R., Eds.; Blackwell Scientific Publications: London, 1987; pp 381−389.

Führ, F.; Mittelstaedt, W. Plant experiments on the bioavailability of unextracted (carbonyl-^{14}C)methabenzthiazuron residues from soil. *J. Agric. Food Chem.* **1980**, *28*, 122.

Haider, K. *Biochemie des Bodens*; Enke: Stuttgart, 1995.

Haider, K. M.; Martin, J. P. Mineralization of ^{14}C-labelled humic acids and of humic-acid bound ^{14}C-xenobiotics by *Phanerochaete chrysosporium*. *Soil Biol. Biochem.* **1988**, *20*, 425−429.

Haider, K.; Spiteller, M.; Wais, A.; Fild, M. Evaluation of the binding mechanism of anilazine and its metabolites in soil organic matter. *Int. J. Environ. Anal. Chem.* **1993**, *53*, 125−137.

Heitmann-Weber, B.; Mittelstaedt, W.; Führ, F. The degradation of anilazine and dihydroxy-anilazine at various soil depths of an orthic luvisol. *J. Environ. Sci. Health* **1994**, *B29*, 247−264.

Kaufman, D. D. Bound and conjugated pesticide residues. In *Bound and Pesticide Residues*; Kaufman, D. D., Still, G. G., Paulson, G. D., Bandal, S. K., Eds.; ACS Symposium Series 29; American Chemical Society: Washington, DC, 1976; pp 1−10.

Khan, S. U. Bound pesticide residues in soil and plants. *Residue Rev.* **1982**, *84*, 1−25.

Khan, S. U.; Dupont, S. Bound pesticide residues and their bioavailability. In *Pesticide Science and Biotechnology*; Greenalgh, R., Roberts, T. R., Eds.; Blackwell Scientific Publications: London, 1987; pp 417−420.

Kloskowski, R.; Führ, F. Versuche zur Charakterisierung und Bioverfügbarkeit von Nicht-Extrahierbaren Simazin-Rückständen im Boden. *Chemosphere* **1983**, *12*, 1557−1574.

Kloskowski, R.; Führ, F.; Mittelstaedt, W. Plant availability of bound anilazine residues in a degraded loess soil. *J. Environ. Sci. Health* **1986**, *B21*, 487−505.

Lovley, D. R.; Coates, J. D.; Blunt-Harris, E. L.; Phillips, E. J. P.; Woodward, J. C. Humic Substances as Electron Acceptors for Microbial Respiration. *Nature* **1996**, *382*, 445−448.

MacRae, I. C. Formation and degradation of soil bound [14C]-fenitrothion residues in two agricultural soils: *Soil Biol. Biochem.* **1986**, *18*, 221−225.

Mittelstaedt, W.; Führ, F. Residue formation in soil after repeated application of anilazine and bioavailability of benzene ring carbon. In *Pesticide Bound Residues*; Deutsche Forschungsgemeinschaft; Wiley-VCH-Verlag: Weinheim, Germany, 1998; pp 138−144.

Mittelstaedt, W.; Führ, F.; Kloskowski, R. Anilazine-formation of bound residues in a degraded loess soil. *J. Environ. Sci. Health* **1987**, *B22*, 491−507.

Morlock, G. E. Analysis of pesticide residues in drinking water by planar chromatography. *J. Chromatogr. A* **1996**, *754*, 423−430.

Mougin, C.; Laugero, C.; Asther, M.; Chaplain, V. Biotransformation of s-triazine herbicides and related degradation products in liquid cultures by the white rot fungus *Phanerochaete crysosporium*. *Pestic. Sci.* **1997**, *49*, 169−177.

Nelson, S. D.; Khan, S. U. Effect of endomycorrhizae on the bioavailability of bound ^{14}C residues to onion plants from an organic soil treated with [^{14}C]fonofos. *J. Agric. Food Chem.* **1990**, *38*, 894−898.

Racke, D. R.; Lichtenstein, E. P. Effects of soil microorganisms on the release of bound ^{14}C residues from soils previously treated with [14C]Parathion. *J. Agric. Food Chem.* **1985**, *33*, 938−943.

Roberts, T. R.; Standon, M. E. Further studies of the degradation of the pyrethroid insecticide cypermethrin in soils. *Pestic. Sci.* **1981**, *12*, 285−296.

Roberts, T. R.; Klein, W.; Still, G. G.; Kearney, P. C.; Drescher, N.; Desmoras, J.; Esser, H. O.; Aharonson N.; Vonk J. W. Non-extractable pesticide residues in soils and plants. *Pure Appl. Chem.* **1984**, *56*, 945−956.

Romano, P.; Suzzi, G.; Brandolini, V.; Menziani, E.; Domizio, P. Determination of 2,3-butanediol in high and low acetoin producers of *Saccharomyces cerevisiae* wine yeasts by automated multiple development (AMD). *Lett. Appl. Microbiol.* **1996**, *22*, 229−302.

Stevenson, F. J. Organic matter reaction involving pesticides in soil. In *Bound and Conjugated Pesticide Residues;* Kaufman, D. D., Still, G. G., Paulson, G. D., Bandal, S. K., Eds.; ACS Symposim Series 29; American Chemical Society: Washington, DC, 1976; pp 180−207.

Stevenson, F. J. Geochemistry of soil humic substances. In *Humic Substances in Soil, Sediment, and Water*; Aiken, G. R., McKnight, D. M., Wershaw, R. L., MacCarthy, P., Eds.; John Wiley & Sons: New York, 1985; pp 13−52.

Vigne, U. de la; Jänchen, D. E.; Weber, W. H. Application of high-performance thin-layer chromatography and automated multiple development for the identification and determination of pesticides in water. *J. Chromatogr.* **1991**, *553*, 489−496.

Wais, A.; Haider, K.; Spiteller, M.; de Graaf, A. A.; Burauel, P.; Führ, F. Using ^{13}C-NMR spectroscopy to evaluate the binding mechanism of bound pesticide residues in soils. 1. Solution high-resolution NMR spectroscopy. *J. Environ. Sci. Health* **1995**, *B30*, 1−25.

Wais, A.; Burauel, P.; de Graaf, A. A.; Haider K.; Führ, F. Using ^{13}C-NMR spectroscopy to evaluate the binding mechanism of bound pesticide residues in soils. 2. Investigations of model humic substances. *J. Environ. Sci. Health* **1996**, *B31*, 1−24.

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