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Degradation of the fungicide metalaxyl and formation of non-extractable residues in soil particle-sized fractions of organo-clay and organo-silt

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ABSTRACT

The proportion of organic matter and mineral composition is an important factor determining the formation and type of non-extractable pesticide residue (NER) in soil. In this study we investigated the degradation and formation of NER of the chiral fungicide metalaxyl in soil size fractions of organo-silt and organo-clay. Microbial and extracellular enzyme activities during these processes were monitored using non-sterilized ("native") and sterilized samples, respectively. The temporal influence on the fate of the fungicide was noted by short- (10 days) and long-term (92 days) incubation experiments. Besides acquisition of quantitative data with GC/MS, stereoselective analyses were performed with chiral-GC/MS. Quantitative results pointed to a higher metabolism rate of the pesticide through microbial activity than extracellular enzyme activity. This was also confirmed by the enantioselective depletion of R-metalaxyl, and the subsequent formation of R-metalaxyl acid in microbial active native samples. The silt fraction containing a high amount of organic matter exhibited a significant hydrolyzable proportions of metalaxyl NER that are releasable under alkaline conditions. On the contrary, organo-clays showed enhanced affinity for covalently bound residues. Based on our results we recommend to differentiate between reversibly and irreversibly bound proportions of pesticides in the persistence and environmental risk assessment, as the reversible fraction pose potentially bioavailable amounts of residues that may be released under natural conditions.

Key Words: organo-minerals; pesticide degradation; enantioselectivity; extracellular enzymes; soil size fractions

INTRODUCTION

Controlling excessive contamination of the environment through the usage of pesticides is a huge challenge facing our future. These xenobiotics can be persistent and highly resistant to natural degradation processes without certainty of their re-accessibility for exposed organisms. So far, non-extractable residues (NER) of pesticides are considered as degraded and persistent (Gevao et al. 2000; Barraclough et al. 2005, Barriuso et al. 2008). Recently, a classification scheme of NER has been described differentiating three types. Type I NER are sequestered and entrapped residues containing

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either the parent substance or transformation products or both and having the potential to become released. Type II NER are those residues that are covalently bound to soil/sediment organic matter and that are considered as strongly bound with very low remobilisation rates. Noteworthy, harsh environmental as well as extraction conditions may release both types of xenobiotic NER but for type II this will rarely happen under normal physiological conditions. Type III NER comprises biogenic NER of no environmental concern, that are formed after degradation of the xenobiotics and anabolic formation of natural biomolecules. The mode of binding to soil and sediment can determine the extent of persistency and remobilization potential of these chemicals (Schäffer et al. 2018).

Adsorbed proportions can be extracted by organic solvent – water mixtures while covalent bonds need chemical degradation procedures to cleave the linkage (Ortega-Calvo et al. 2015). Depending on the strength of the degradation reagents these methods differentiate between reversible covalent bonds (ester, amide, ether) and very strong covalent bonds (C-C). In general, covalent bonds are considered to reveal very low remobilisation rates like those of humic matter degradation (half-lives of years to decades) (Dec et al. 1997; Gevao et al. 2000; Northcott & Jones 2000; Conant et al. 2011).

The incorporation processes of pesticides are also greatly influenced by the organic matter content of soil (Bollag et al. 1992). Similar to the humification processes organic contaminants are transformed and retained in the soil geopolymers through abiotic and biotic reactions. Further on, the amount of soil organic matter (SOM) has an influence on the activity of free and immobilized extracellular enzymes and microorganisms with viable enzymes in soil as it is their source of energy (Nannipieri et al. 1980; Edwards et al. 1992; Stevenson 1994; Saviozzi et al. 2001; Nannipieri 2006; Sinsabaugh et al. 2008; Leinweber et al. 2008). This leads to the assumption of higher biotic activity in high organic carbon containing soil fractions (Schnürer et al. 1985; Gianfreda & Rao 2008; Botterweck et al. 2014; Schäffer et al. 2015).

Considering abiotic reactions, clay minerals also have been associated with the formation of humic substances by different polymerization reactions involving free radicals engaged in oxidative coupling of phenolic or anilinic substructures (Bollag & Loll 1983; Senesi 1992). Accordingly, clay minerals, exhibiting large surface areas and forming predominantly complexes with soil organic matter, are also responsible for catalyzing the incorporation and thus the accumulation of xenobiotics into the soil matrices. Here not only the interlayer region of minerals but also the regions of clay, that are coated with organic matter, provide microporous areas for possible pesticide retention (Stevenson 1994; Li et al. 2003).

Besides abiotic binding processes biotic incorporation reactions depend mainly on the activities of microorganisms (involving intracellular enzymatic proteins) and extracellular enzymes (Gianfreda & Rao 2008). Previous studies showed the impact of microbial activity and additionally extracellular enzyme activity during pesticide degradation and formation of non-extractable metalaxyl residues (Botterweck et al. 2014; Kalathoor et al. 2015). However, stereochemical aspects have not been followed so fa, although stereoselective processes are often directly influenced by enzyme activities (Hühnerfuss et al. 1992; Pickel et al. 2010). Noteworthy, only very few information exist about their effect in xenobiotic retention processes especially with regard to the total organic carbon and mineral content of soil. The fungicide metalaxyl consists of S- and R- enantiomers the latter being the active ingredient. Researchers noticed favorable adsorption of metalaxyl on soil mineral surfaces especially in soils with less soil organic matter (Sukop and Cogger 1992). Kalathoor et al. (2015) studied the enantiomeric distribution of metalaxyl residues during the formation of NER in clay loam bulk soil from an agricultural field.

We here report studies on the effect of microbial and extracellular enzyme activities on the stereoselective degradation of metalaxyl including the formation of NERs in organo-silt and -clay fractions. Incubation experiments were conducted on native and sterilized size fractions isolated prior to incubation from the bulk soil. The effect of (free) water extractable enzymes was also studied as the size fractionation was done prior to incubation. The sterilization method used allowed to inhibit microbial activity but does not reduce extracellular activity significantly (Botterweck et al. 2014; Kalathoor et al. 2015). The size-fractions holding different amounts of organic matter and mineral substances were spiked with racemic metalaxyl and were incubated for 10 and 92 days. These periods are in accordance with our previous study on short- and long-term reactions of metalaxyl on the individual soil constituents and different biotic influence.

In a wider context, such experiments address the consequences of certain agricultural management practices such as tillage, resulting in partial breakdown of soil particle aggregates and potential uncovering of releasable pesticide residues. Furthermore, understanding the interdependence of microbial activity and extracellular enzymatic activity can be related to the impact of climatic conditions such as drought on the degradation potential of soils.

MATERIALS AND METHODS

Chemicals

Non-labeled rac-metalaxyl (methyl-N-(methoxyacetyl)-N-(2,6-dimethylphenyl)-DL-alaninate) (rac-MX) with chemical purity > 99.3% was obtained from Dr. Ehrenstorfer GmbH, Germany. Metalaxyl-acid was synthesized as described by Kalathoor et al. (2015). Metaxalyl stock solutions of 2 µg/mL were used. The surrogate standards d_{10}-benzophenone and d_4-bis(2-ethylhexyl)phthalate (d_4-DEHP) (concentrations: 5 ng/µL) were provided by Sigma Aldrich, USA. All further chemicals and solvents (ethylacetate, dichloromethane, pentane, anhydrous sodium sulfate, $HgCl_2$) were purchased from Sigma LAdrich at analytical purity grade.

Soil sample and sterilization

The soil sample used for incubation experiments was taken in 2010 from a long-term agricultural field experiment site in Ultuna, Sweden, from depths of 0-17 cm (Ah horizon). The samples have been dried at room temperature and sieved (2mm mesh) directly after sampling and kept at 4°C in the dark. Shortly before the experimental usage the soil was allowed to warm up to 20°C. Sterilization was carried out according to Alef and Nanniperi (1995) with mercuric chloride treatment followed by chloroform fumigation. This method allowed to obtain soil exclusively with extracellular enzyme activity after inhibiting microbial activity. Detailed description of soil sterilization is published elsewhere (Kalathoor et al. 2015). Some relevant soil characteristics are given in Tab. 1.
Unspiked soil samples have been checked for any metalaxyl residues.

TABLE I

Characteristics and properties of the Ultuna (Sweden) soil used in this experiment Kirchmann et al. 1994; Gerzabek et al. 2006). TOC: total organic carbon, WHCmax: maximum water holding capacity.

Particle distribution (%)			TOC (%)				WHC_{max} (g H_2O/g soil)	$pH_{(0.01M\ CaCl2)}$
sand	silt	clay	bulk soil	sand	silt	clay		bulk soil
27	51	23	2.6	0.3	1.7	0.5	0.17	5.7

Silt and Clay incubation experiments

A schematic overview on the experimental set-up and relevant subfractions are given in Fig. 1. Particle-size fractionation of bulk soil was carried out to isolate silt and clay as formerly described (Kalathoor et al. 2015). Low energy sonication (0.17 kJ/g output energy) allowed to disperse the soil-water suspension yet preserve the microaggregates of the silt and clay fraction as well as the enzymatic activities (Stemmer et al. 1998). Metalaxyl-free sea sand in each sample was added for better handling of silt and clay, respectively. For each individual set-up 10 µg of racemic metalaxyl (rac-MX) dissolved in 5 mL deionized water was added to 20 g of air-dried silt with 30 g annealed sea sand (resulting in approx. 0.5 mg rac-MX/kg silt). The same amount of metalaxyl in deionized water (5 mL) was added to 10 g of air-dried clay with 40 g metalaxyl-free sea sand (approx. 1 mg rac-

MX/kg clay). The applied amount water adjusted the moisture content to 60% of the maximum water holding capacity. After spiking the samples were homogenized by mixing extensively with a glass rod for 10 min. All experiments were carried out in duplicates and spiked samples were kept in the dark to avoid photodegradation (at 15°C). To provide an insight to short- and long-term processes duplicates of each clay and silt samples were taken after 10 and 92 days of incubation, respectively.

Noteworthy, due to the extensive experimental set-up as well as the high effort in sample treatment and analytical methods, only duplicates of each incubation experiment were accomplished and analyzed. Since a detailed statistical analysis of this low number of replicates is not suitable, all data presented here are calculated only as average of the duplicates and the corresponding standard deviation.

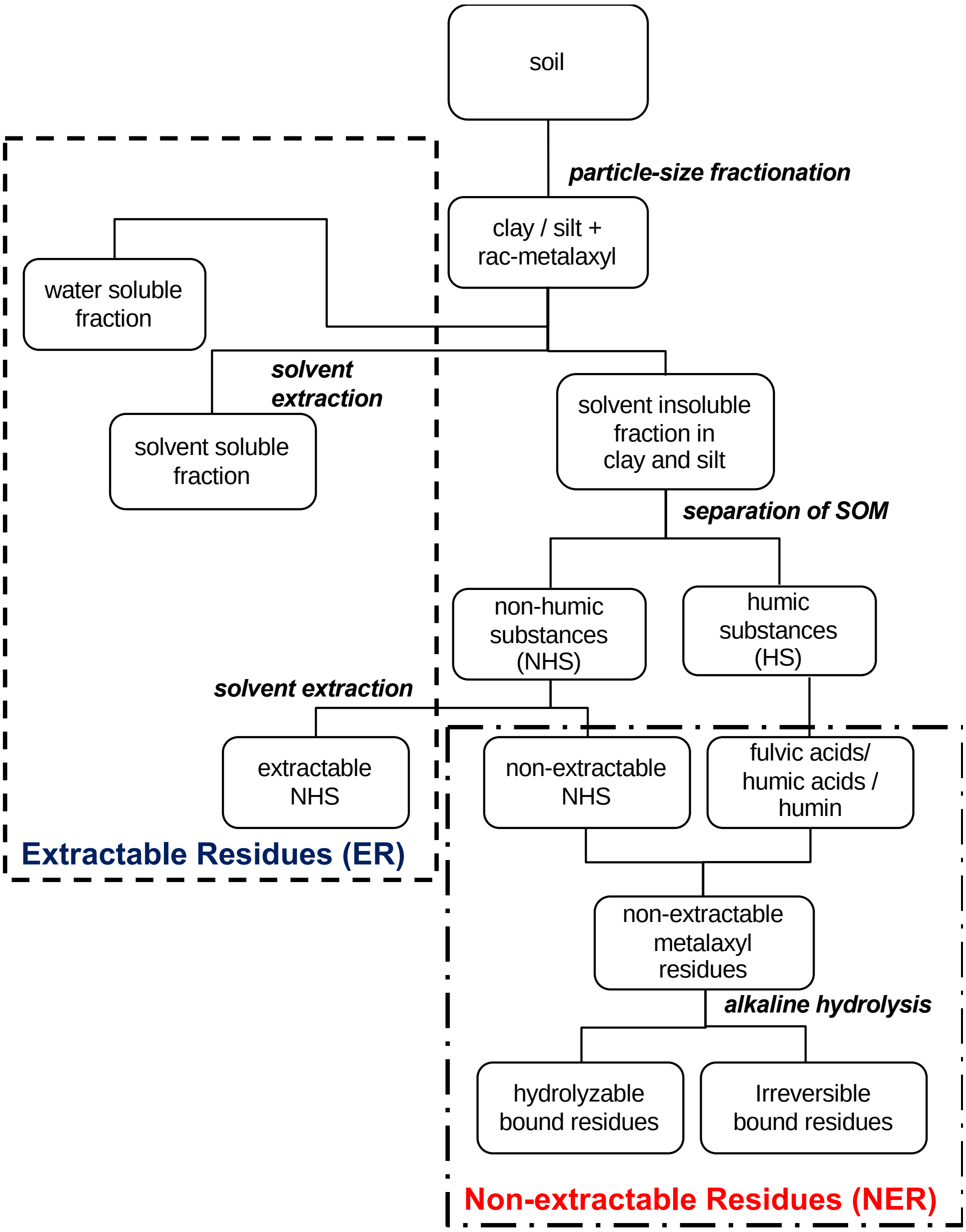

Fig. 1 Experimental workflow. Differentiation of non-extractable and extractable proportions of the fungicide. SOM: soil organic matter.

Extractable fraction

Around 200 mL of Millipore water was added to each sample and the suspensions were dispersed using an ultrasonic disaggregator (Sonopuls HD200, Bandelin, Berlin) with 0.17 kJ/g output energy for 6 minutes as a pre-extraction procedure with water. The additional sea sand was separated through wet sieving and centrifugation, respectively. The water-soluble proportion of clay and silt samples was obtained after liquid-liquid extraction of this fractionation water with organic solvents. Metalaxyl was extracted with 3x40 mL ethyl acetate while pH was slightly above 7 and metalaxyl-acid was subsequently extracted (also with 3x 40 mL ethyl acetate) with acidified water (pH 2). Each combined solvent fraction was dried over anhydrous sodium sulfate and concentrated to a final volume of 1 mL. Recovery rate for the total procedure was 83.2±0.5% of the applied radioactivity.

Sokol et al. (1988) verified the complexation of metalaxyl with $HgCl_2$. When using $HgCl_2$ as a sterilization agent for soil, this should be taken in account. The formation of a secondary complex with the metabolite metalaxyl acid as the ligand for $HgCl_2$ inhibits the extractability of the acid metabolite with dichloromethane. In this study, we used ethyl acetate as extraction solvent due to its ability to form coordination complexes with metal ions.

Solvent soluble proportion of metalaxyl residues was obtained by exhaustive extraction of the freeze-dried particulate matter with 200 mL methanol for 6h by means of Soxhlet apparatus. Extractable proportions of metalaxyl residues were also obtained from non-humic subfractions after dilute HCl-treatment and subsequent liquid-liquid extraction (non-humic extractable residue (NH-ER)). Thereafter, all pre-extracted silt and clay samples were fractionated into the corresponding fulvic acids, humic acids and humin-mineral complexes using alkaline separation method according to (Stevenson 1994). Detailed extraction procedure of non-humic subfraction and humic fractionation is formerly described (Kalathoor et al. 2015). All solvent soluble extracts and extractable non-humin fractions were dried over anhydrous sodium sulfate and concentrated to a volume of 100µL.

Non-extractable metalaxyl residues

Alkaline hydrolysis Alkaline hydrolysis were conducted on all experimental set-ups and humic matter fractions. A variable volume of 5 to 30 mL of a 2 M solution of potassium hydroxide was added to the neutralized and dried clay and silt humic acids, fulvic acids, humin-mineral complex and non-humic NER fractions, respectively and heated at 105 °C for 24 h in a closed vessel. The cooled aqueous phases were acidified (pH 2) and after sonication for 15 minutes extracted with ethyl acetate (3x15 mL). Combined organic phases were dried over anhydrous sodium sulfate and reduced to a volume of 200 µL. The hydrolysates of each sample were fractionated into five fractions by micro column chromatography as formerly described (Kalathoor et al. 2015b). Fractions four and five of the micro column chromatography were combined and concentrated to a volume 100µL. Recovery rates for micro column fractionation of metalaxyl were 98.5±0.3%.

HPLC fractionation The combined polar fractions containing metalaxyl and metalaxyl-acid were further fractionated on a Nucleosil 100-5 C18 AB column (5 µm x 4.6 mm x 250 mm, Macherey-Nagel, Germany) with Nucleosil C8 ec pre-column using an HPLC-UV device (254 nm). The chromatographic conditions and further sample preparation are described in detail elsewhere (Kalathoor et al. 2015a). Fractions containing metalaxyl-acid were derivatized with diazomethane and concentrated to a volume of 25µL. Unhydrolyzed metalaxyl containing fractions were also reduced to a final volume of 25 µL. Recovery rates for HPLC-fractionation were at 70.5±0.5%.

GC/MS analysis GC/MS analyses were performed on a Trace MS mass spectrometer linked to a Trace GC equipped with a ZB-5 capillary column (30 m x 0.25 mm ID x 0.25 µm film). An example of GC/MS measurements is given in Fig. 2. Chiral GC/MS analyses were performed on the same Instrument with an enantioselective column of the same dimensions BGB-172 (20% tert-butyldimethylsilyl-β-cyclodextrin dissolved in 15% phenyl-, 85% methylpolysiloxane (BGB-15); BGB-Analytik, Adliswil, Switzerland). Chromatographic and mass spectrometric conditions were as formerly described (Kalathoor et al. 2015a). Prior to GC/MS analysis all samples were reduced to dryness by evaporation at room temperature under ambient pressure and reconstituted to their previous volume in a surrogate standard solution containing 10 ng/µL of d_{10}-benzophenone and 9 ng/µL of d_4-DEHP.

The elution order of the chiral analyses showed S- prior to R-metalaxyl, with Rs = 0.95. The peak areas of the enantiomers were corrected using the tailing factor at 5% peak height. Enantiomeric fractions were calculated using the corrected peak areas with EF = AS/(AS+AR), were AS and AR as the peak areas of S- and R-metalaxyl respectively.

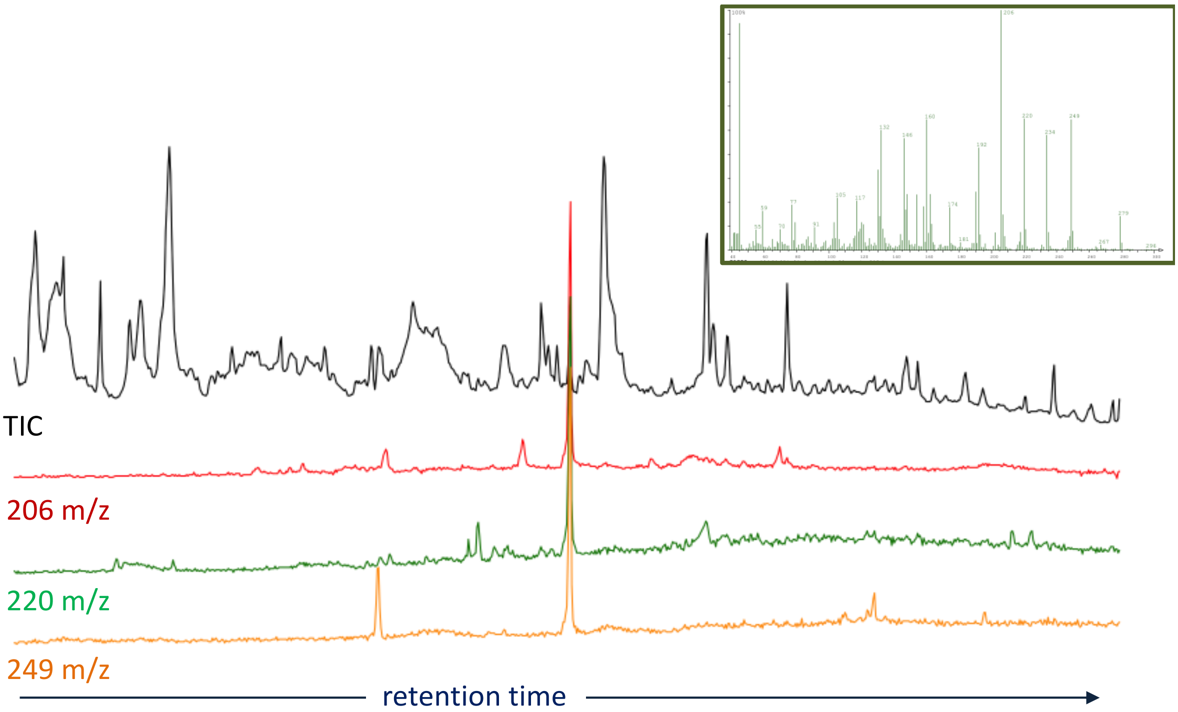

Fig. 2: Exemplifying GC/MS measurement of metalaxyl in an extract obtained from silt sample after 10 days. Beside the total ion chromatogram TIC, the specific ion traces of m/z 206, 220 and 249 are illustrated as well as the mass spectrum of metalaxyl.

RESULTS

This study differentiated microbial and predominantly extracellular enzymatic assisted processes using non-sterilized (native) soil and sterilized soil, respectively, according to Botterweck et al. (2014). A particle size-fractionation was performed after a short (10 days) and long term (92 days) incubation period to examine the distribution of the pesticide and its metabolites in the corresponding silt and clay fractions. Focus of the present study was to investigate the enantioselectivity of the degradation and NER formation of metalaxyl in the pre-separated soil organo-silt and -clay fractions. Kalathoor et al. (2015a) gave detailed description of incubation experiments with rac-metalaxyl conducted on bulk soil samples of the same sampling area used in this experiment.

Extractable metalaxyl residues (ER)

Three subsequent extraction steps revealed three different fractions of extractable residues as shown in Fig.1: (i) extractable residues from water-soluble fraction, (ii) a particle associated extractable fraction and (iii) extractable resdieus from the non-humin fraction (NH-ER).

In the short-term experiment (day 10) both water soluble as well as solvent soluble residues of the organo-clay as well as the organo-silt fractions contained high concentrations of metalaxyl with each around 30 % of the total applied amount (except the solvent soluble silt extract of the non-sterilized soil containing 11%). The missing proportions were detected in the NH-ER fraction (around

21% of the total applied metalaxyl as shown in Table II). The silt and clay fractions of the sterilized soil delivered higher solvent extractable proportions than the corresponding non-sterilized fractions, whereas the latter yielded higher amounts in the NH-ER fraction (Fig. 3). Such residues of the non-ionic fungicide metalaxyl are considered to rapidly adsorb to soil organic surfaces and, on the other hand, to have a high affinity to the polar non-humic substances (Riefer et al. 2011b).

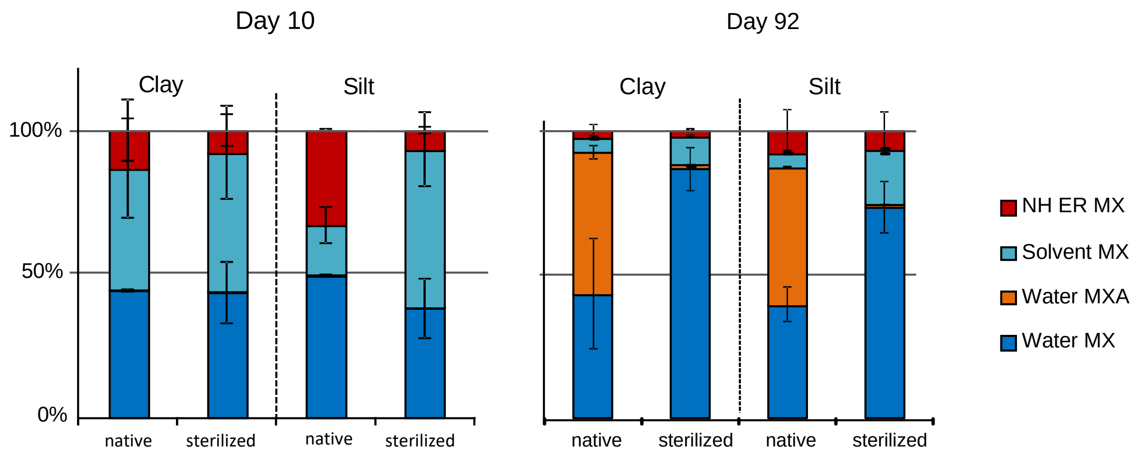

Fig. 3 Relative distribution of extractable metalaxyl residues in native and sterilized clay and silt soil fractions after 10 and 92 days of incubation. MX: metalaxyl, MXA: metalaxyl acid, NH-ER: non-humin extractable residue.

TABLE II

Quantitative data and standard deviations of extractable and alkaline hydrolysis releasable non-extractable metalaxyl residues after 10 days and 92 days of incubation (a) Clay (b) Silt. MX: metalaxyl, MXA: metalaxyl acid, NH: non-humic substances, ER: extractable residue, NER: non-extractable residue, FA: fulvic acids, HA: humic acids, HU: humin, *nd:* not detected (below the limit of quantitation)

a) Clay	**Day 10**		**Day 92**	
	native	sterilized	native	sterilized
	Extractable proportion [%]			
Water - MX	26±0	28±7	23±10	50±4
Water - MXA	0.3±0	0.4±0.1	26±1	1±0
Solvent - MX	24±10	31±10	3±0	6±0
NH-ER - MX	8±6	5±4	1±1	1±0
Total	**58**	**64**	**53**	**58**
	Hydrolyzable non-extractable proportion [%][†]			
NH-NER - MXA	*n.q.*	*n.q.*	*n.q.*	*n.q.*
FA - MXA	9	0.83	8	0.5
HU - MXA	0.2	*n.q.*	0.1	0.1
HA - MXA	*n.q.*	*n.q.*	*n.q.*	*n.q.*
Total	**9.2**	**0.83**	**8**	**0.6**

b) Silt	**Day 10**		**Day 92**	
	native	sterilized	native	sterilized
	Extractable proportion [%]			
Water - MX	32±0	26±7	26±4	52±7

Water - MXA	0.4±0	0.4±0	31±1	3±0
Solvent - MX	11±4	37±10	3±0	13±0
NH-ER - MX	21±0	5±4	5±4	5±4
Total	**64**	**68**	**65**	**71**
	Hydrolyzable non-extractable proportion [%]†			
NH-NER - MXA	*n.q.*	*n.q.*	*n.q.*	*n.q.*
FA - MXA	40	18	16	8
HU - MXA	0.8	n.q.	0.4	0.3
HA - MXA	*n.q.*	*n.q.*	*n.q.*	*n.q.*
Total	**40.8**	**18**	**16.4**	**8.3**

The elevated amounts of metalaxyl residues in sterilized clay and silt particulate matter (especially the latter) in comparison to that of the non-sterilized soil point to the microbially and enzymatically mediated binding. Under all conditions metalaxyl acid was only detected in the water-soluble fraction in small amounts (< 0.5% of the applied amount). The extractable fractions (water, solvent and NH-R) in total accounted for around 60-70% of the initially applied metalaxyl. The non-sterilized and sterilized silt fractions contained higher residue concentrations than their clay counterparts. All quantitative values with standard deviations are shown in Tab. 2.

The enantiomer fractions (EF) of the three extractable fractions (water, solvent, NH-R) showed an unchanged racemic mixture of metalaxyl identical to the applied rac-metalaxyl with Rs values around 0.48. However, analysis of the main metabolite metalaxylic acid revealed a stereoselective degradation. In the water-soluble fractions of the non-sterilzed soil, R-metalaxly acid had an EF value of around 0.23. On the contrary, the sterilized soil subfractions still contained the racemic mixture of the metabolite. This correlated with earlier findings in which stereoselective degradation of metalaxyl was associated with microbial activity (Kalathoor et al., 2015a). However, as there was no corresponding depletion notable in the extractable proportions of the parent compound and due to the low concentrations of the acid this enrichment affected the overall enantiomeric distribution only marginally.

Comparing the short term to the long-term experiment (day 92) total extractable proportions of the fungicide were similar (50-70 %) with slightly lower amounts for the sterilized and non-sterilized clay compared to the silt fractions (Table II). The formation of the main metabolite metalaxyl acid increased after 92 days of incubation to 26% and 30% of the applied amount in the non-sterilized clay and silt fractions, respectively. Only very low concentrations of metalaxyl acid were detected in sterilized fractions (1-3%). Solvent soluble as well as NH-ER extractable metalaxyl was clearly reduced after three months of incubation (1-13%). Higher particle associated but extractable proportions were obtained in the sterilized samples of clay (7%) and silt (18%) pointing to an increased microbially assisted retention of the fungicide onto organo-mineral, especially organo-clay mineral matter (Fig. 3).

In the samples of the soil incubated for 92 days, also slightly higher amounts of metalaxyl residues were extracted from the silt fraction rather than from the clay fraction. Ultuna soil containes predominantly illite as its main clay mineral (Kirchmann t al. 1994; Gerzabek et al. 2006), which are nonexpanding 2:1 layered clays. Therefore, the extractable metalaxyl residues were likely sequestered in the micropores of the organo-clay complexes and/or in voids of humic matter. The presence of expanding clay minerals like montmorillonite may also allow intercalation of the fungicide or its metabolite in the interlamellar spaces of the clay minerals (Stevenson 1994).

A clear stereoselective shift from the initial enantiomeric distribution (EF 0.48) was observed after metabolism of metalaxyl to metalaxyl acid in the non-sterilized fractions (Table III). While EF values of sterilized samples of clay and silt subfractions (including water soluble metalaxyl acid) remained unchanged, non-sterilized samples showed a clear preference for one enantiomer. Water, solvent soluble and extractable non-humic matter of non-sterilized clay and silt samples had EF values of around 0.77–0.93 showing an enrichment of S-metalaxyl in these samples. Complementary, EF values of metalaxyl acid show a distinct preference for the faster degraded R-metalaxyl (0.04 and 0.06 for native clay and silt fraction, respectively). This priority for one specific enantiomer during degradation was also an indicator for microbial assistance in the degradation process. Only a weak stereochemical effect was noticed by the formation of metalaxyl acid in the water extract of sterilized soil (EF around 0.45). Quantitative data of total extractable metalaxyl proportions were combined with enantiomer fractions to demonstrate the stereochemical effect in the degradation and metabolism of metalaxyl after 92 days (Fig. 4).

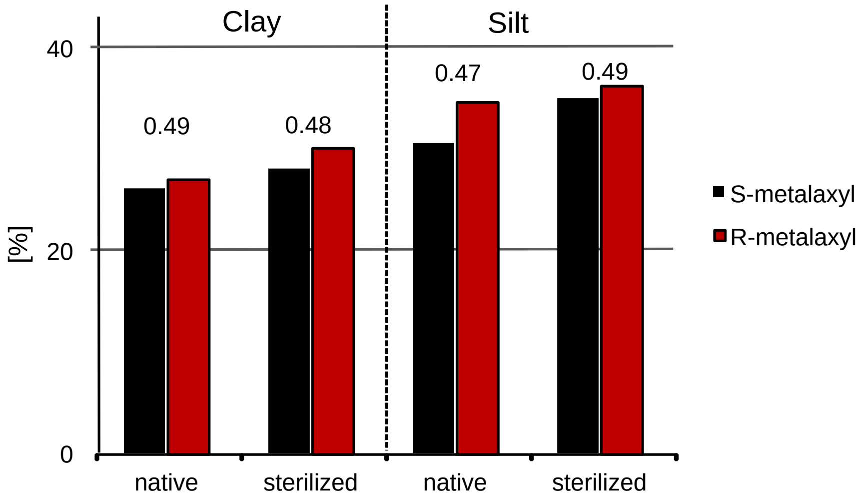

Fig. 4 Total extractable residues day 92. Chiral data linked with quantitative data: enantiomeric distribution of total extractable metalaxyl residues after 92 days of incubation. The corresponding total Enantiomeric fraction (EF) values are given over each column pair.

Non-extractable metalaxyl residues (NER), hydrolyzable under alkaline conditions

For all samples, conditions and incubation periods (silt and clay, sterilized and native, 10 days and 92 days) the highest amount of hydrolyzable NER was found in the fulvic acids fractions (Fig. 5). For both time periods native alkaline hydrolysis applied to the silt fractions released by far the highest amounts of metalaxyl residues (40 % after 10 days and 16 % after 92 days). Also in the sterilized silt fraction higher amounts were released than in their clay counterparts (8-18 % vs 1 %). While the hydrolyzable NER proportions halved in the silt fractions comparing incubations for 10 day and 92 day, respectively, the amounts of the corresponding clay fraction remained nearly constant. Correspondingly, after 92 days of incubation the proportion of metalaxyl and metalaxyl acid in the water-soluble fractions increased while the particle associated and strongly incorporated proportions decreased (Table II).

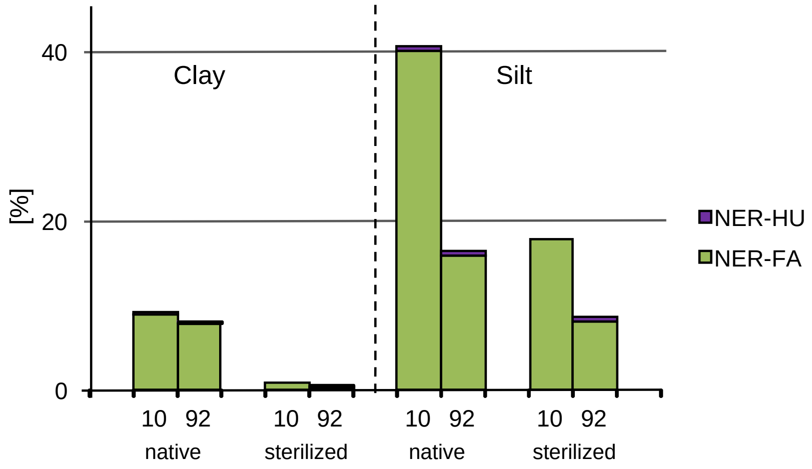

Fig. 5 Hydrolyzable residues. Total ester/amide bound non-extractable metalaxyl residues (NER) after alkaline hydrolysis of clay and silt size-fractions after 10 and 92 days of incubation. FA: fulvic acids, HU: humin.

The humin-mineral associated metalaxyl residues accounted for only one tenth (92 day incubation) – even less for the 10 day incubation – of the hydrolyzable NER amounts. No metalaxyl residues were detectable in the humic acids subfractions or in the non-humic-NER fractions – neither after 10 days nor after 92 days of incubation.

Alkaline hydrolysis of the pre-extracted samples cleaved predominantly the covalently bound main metabolite metalaxyl acid from the humic substance of soil as demonstrated by Kalathoor et al. (2015b). In the present study we performed a long term experiment to analyze the effect of ageing by the formation of covalent ester bonds between acidic pesticide metabolites and organic matter of soil. The release data point to such linkages in addition to further binding interactions such as hydrogen bonds as well as entrapment of metalaxyl residues in the voids of the organo-minerals. The amounts of residues that could be released by alkaline hydrolysis increased with incubation time, which corresponds to the kinetics of NER formation described by Barriuso et al. (2008) with rapid “flash” NER formation followed by a slower “maturation stage” or ageing.

Any stereoselective preference in NER formation could not be shown by chiral analysis. EF values of the residues released by alkaline hydrolysis were around 0.48 for sterilized samples and 0.75 to 0.9 for native samples. These values correspond well with the EFs of the extractable fraction (Tab. 3). Thus, microbial activities which were clearly responsible for the enantioselective degradation and the amounts of NER formation - especially after 92 days (Botterweck et al. 2014) - did not influence the stereoselectivity in the incorporation process. As the NER releasable after alkaline hydrolysis for sterilized sample also had no enantiomeric preference, extracellular enzymes are also not accounted for the stereoselectivity in NER formation. Both microbial and enzyme assisted reactions lead to ester bond formation which could be readily cleaved by the alkaline degradation step (Kalathoor et al. 2015b).

TABLE III

Enantiomer fraction (EF) and their standard deviations of extractable proportion and hydrolyzable non-extractable metalaxyl residues after 92 days of incubation. MX: metalaxyl, MXA: metalaxyl acid, NH: non-humic substances, ER: extractable residue, NER: non-extractable residue, FA: fulvic acids, HA: humic acids, HU: humin, *nd:* not detected (below the limit of quantitation).

Day 92	**Clay**		**Silt**	
	native	sterilized	native	sterilized
	EF of Extractable proportion			
Water - MX	0.93 (± 0.02)	0.48 (± 0.01)	0.85 (± 0.00)	0.49 (± 0.01)
Water - MXA	0.04 (± 0.02)	0.45 (± 0.00)	0.06 (± 0.00)	0.46 (± 0.00)
Solvent - MX	0.88 (± 0.01)	0.49 (± 0.01)	0.91 (± 0.00)	0.50 (± 0.00)
NH-ER - MX	0.89 (± 0.02)	0.49 (± 0.01)	0.74 (± 0.01)	0.47 (± 0.03)
	EF of Hydrolyzable non-extractable proportion†			
NH-NER - MXA	*n.q.*	*n.q.*	*n.q.*	*n.q.*
FA - MXA	0.49	0.49	0.48	0.48
HU - MXA	0.48	0.48	0.49	0.48
HA - MXA	*n.q.*	*n.q.*	*n.q.*	*n.q.*

† single replicate

The yields in the clay fraction remained unchanged throughout the experiment, indicating a fast incorporation through covalent linkage with organic matter. Overall hydrolyzable NER and extractable proportion after 10 as well as 92 days revealed higher (irreversible) NER in organo-clay (40%) samples than in the silt fractions (Fig. 5). According to other studies (Sukop & Cogger 1992; Sukul & Spiteller 2001; Fernandes et al. 2003; McAllister & Semple 2010) metalaxyl has a preference for sorption to mineral surfaces. Especially the interlamellar spaces of clay minerals as well as diffusion of metalaxyl to cavities of microaggregates and organo-clay complexes offer sites protected from enzymatic or microbial degradation; such residues are considered to release to the environment also under natural conditions.

Comparison of experiments with bulk soil and with isolated size-fractions of silt and clay

Results from a previous study (Kalathoor et al. 2015a) with bulk soil differed from current findings in soil size fractions. Short term incubation (10 days) of bulk soil yielded around 60-90 % of the initially applied metalaxyl amount in the water-soluble fraction, i.e., three to four times more than in the experiments with pre-isolated silt and clay fractions. This is in accordance with the hypothesis of rapid association of metalaxyl and its metabolites on the more exposed macromolecular and mineral surfaces of the isolated subfractions. Also, formation of metalaxyl acid was higher in the bulk soil compared to that in the individual size-fraction. One reason for the reduced metabolism in the isolated fractions may be the removal of water extractable enzymes during the size fractionation process.

In contrast, long term incubation (92 days) delivered similar amounts of extractable residues in both bulk soil and soil size fractions. However, a significant difference was the amount of metalaxyl acid recovered in the fractionation water of the sterilized samples (ca. 1% of the applied amount of metalaxyl *vs* 23% in non-sterilized size fractions). This, again, points to the importance of microbial activity, since in the sterilized samples only extracellular enzyme activity is remaining. The proportion of metalaxyl residues hydrolyzable under alkaline conditions in clay was higher than in silt in the former experiment with bulk soil, but opposite in the current study with higher amounts in the organo-clay (Fig. 6).

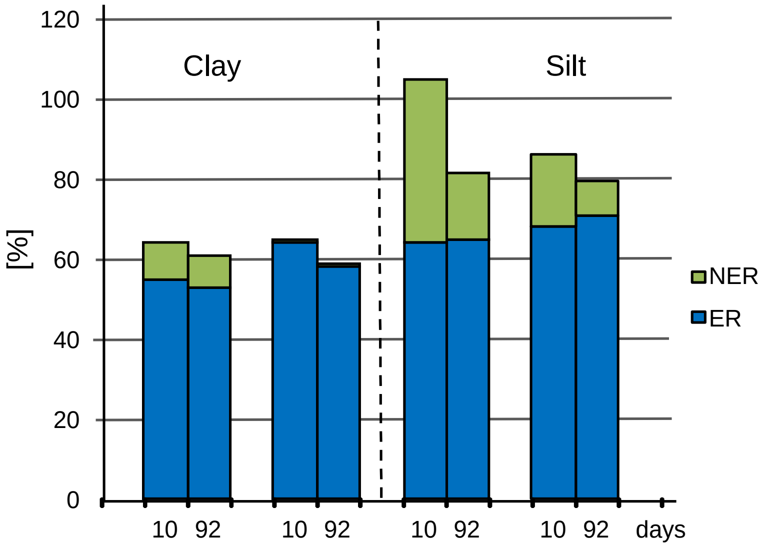

Fig. 6 Total releasable metalaxyl residues of clay and silt after the short term (10 days) and long term (92 days) experiment. ER: extractable residue, NER: (hydrolyzable) non-extractable residue.

DISCUSSION

Findings of our study fit well with results from other studies on the sorption and degradation of metalaxyl in soils. Sorption of metalaxyl to soil is influenced both by soil organic matter and clay minerals (Sukop and Cogger 1992). Binding constants based on Freundlich adsorption isotherms were highest in soils with high organo-clay contents with corresponding lowest mobility in such soils (Sharma and Awasthi 1997). Similar results were reported by Fernandes et al. (2003) who compared 15 agricultural soils performing batch equilibration studies. Besides the clay content, organic matter (OM) was the most important soil property for metalaxyl sorption which could even be enhanced when OM was increased by organic amendments like biochar (Gamiz et al. 2016a). Organo-clay amendment of soils reduced the bioavailability of metalaxyl enantiomers and their leaching in the soils, mitigating the particularly high leaching potential of the more persistent S enantiomer (Lopez-Cabeza et al. 2016). Enantioselective sorption of metalaxyl in soil was demonstrated by Gamiz et al. (2016b). R-metalaxyl, with a K_d of1.73 L kg^{-1} in the studied soil, was more strongly sorbed and showed less leaching in column experiments compared to S-metalaxyl with a K_d of 1.15 L kg^{-1}.

Masbou et al. (2018) combined compound specific isotope analysis and enantioselective analysis to study the degradation of metalaxyl in soil. While R-metalaxyl degradation was fast (half-life time ca. 10 days), concomitant enrichment in heavy isotopes of the more persistent S-metalaxyl enantiomer occurred after 200 days of incubation. In contrast, initial racemic ratios and isotopic compositions were conserved in sterilized soil samples, which indicates the predominance of microbial degradation in soils. The authors suggested hydroxylation as major enantioselective degradation pathway of metalaxyl in soil. Degradation of metalaxyl in soil was enhanced after repeated applications. The accelerated rates lead to the discrimination of the two enantiomers: degradation of the biologically active R-enantiomer was enhanced, whereas that of the non-active S-metalaxyl enantiomer was retarded. Soil column studies revealed that the leachate became increasingly enriched in S-enantiomer as the number of fungicide applications was increased (Celis et al. 2015).

Fungi have been shown to be able to degrade metalaxyl in soil (Martins et al. 2017). Two mucorales strains, previously isolated from soil and identified as *Gongronella sp*. and *Rhizopus oryzae*, were able to use metalaxyl as the main carbon and energy source and to degrade metalaxyl in polluted soils.

Regarding the effect of metalaxyl on enzymatic activities in soil, Yue et al. (2016) showed that urease, invertase, and catalase were not only related to the concentration of the enantiomers and soil incubation time, but also to the chiral configuration, i.e., effects were enantioselective. Effects on urease and catalase activities of R-metalaxyl were stronger than those of S-metalaxyl at the same concentration, but the opposite enantioselecte effect was observed on the activity of invertase.

In our study, total organic carbon in the isolated clay fraction was about 0.5% and about 1.7% in the silt fraction. Therefore, large amounts of metalaxyl residues were reversibly chemically bound to organic macromolecules in the silt fraction, and less in the clay fraction (Fig. 5). At the same time, this indicates a high sorption affinity of the residues to clay minerals compared to silt confirming earlier mentioned preference to minerals with larger surface area and coated organic matter.

Stereoselective reactions in both experimental set-ups (bulk soil vs. size fractions) showed some similarities but also some discrepancies. While extractable fractions of sterilized bulk soil samples showed stereoselective degradation of metalaxyl in the bulk soil, this was not noticed in the current study. Interestingly, in contrast to the trend in the previous study with bulk soil, EF values of hydrolyzable metalaxyl residues, in the current study for both sterilized and non-sterilized samples no enantiomeric preference in the incorporation process was observed. A recovery of some microbial and enzyme activity in the sterilized bulk soil sample after 92 days of incubation was reported (Botterweck 2014) which might explain the stereoselectivity and higher metalaxyl acid quantities in the sterilized soil sample compared to the sterilized size-fractions of clay and silt in our study. The only differing factor besides the total applied metalaxyl amount is the water extractable enzyme proportion which was removed in the current study through prior particle size-fractionation. This points to an influence of dissolved extracellular enzymes (as decribed by Botterweck 2014) in the stereoselectivity during the incorporation process for metalaxyl residues. Additionally, these results also support the idea of abiotically influenced formation of non-extractable metalaxyl residues through organo-mineral-pesticide interaction.

CONCLUSIONS

This study gave insight in the effect of biotic and specific abiotic effects during degradation of metalaxyl and the formation of its non-extractable residues in silt and clay subfractions of soil. Degradation and mineralization of this xenobiotic is linked to the microbial and enzymatic activity of the matrix (Botterweck et al. 2014). On the other hand, extracellular enzymes showed only a minor contribution in the metabolic formation of metalaxyl acid as well as in the mineralization process (Botterweck 2014). Nevertheless, both activities are relevant for the incorporation of the fungicide. Hydrolyzable metalaxyl residues showed a fast association of the fungicide to the particulate humic substances pointing to not only covalently ester or amide bound acid metabolite but also to a high amount of entrapped residues. However, these proportions decrease in the long run directing to a temporary diffusion of the fungicide into micropores of soil aggregates including humic substances protecting the xenobiotic from microbial and enzymatic degradation. Remobilization of entrapped residues can become bioavailable which indicates the significance for elucidating reversibly bound transformation products of pesticides.

The affinity of metalaxyl and its metabolites to form irreversibly bound non-extractable residues onto organo-clay surfaces was confirmed by our study - especially in contrast to more soil organic matter containing silt fraction. This showed that organo-clay complexes are major sinks for irreversibly bound non-extractable residues. Agricultural practices that alter the aggregation of soil particles, i.e. tillage, lead to reduced soil structural stability and increase of erosion. Disaggregation of silt and clay agglomerates after such actions will uncover pesticide residues from these fractions which we showed to have different binding strength to silt and clay particles. The confirmation of the potential release of pesticide non-extractable residues, especially those entrapped in the matrix, after mechanically disturbing the soil structure calls for future research.

Chiral analyses displayed an increased stereochemical effect during metabolism of metalaxyl in the long-term and supports preliminary observations (Riefer et al. 2011a). Particularly microbial activity promoted the enantioselective process in a much higher rate than extracellular enzymes. However, in contrast to our previous study with bulk soil, lack of enantioselective incorporation of the fungicide in pre-isolated soil size fractions indicated less biotic influence and higher abiotic binding interactions with no enantioselective preference during this process. Furthermore, our study points to the significance of water extractable extracellular enzymatic activity in soils with regard to the stereoselectivity of bound metalaxyl residues. Our findings can be related to the impact of natural stressors such as drought or water logging on the degradation potential of soils. The impact of such conditions on the microbial and extracellular enzyme activities and thus pesticide degradation should be further investigated.

As environmental implication it must be considered that the biological degradation of metalaxyl proceeds enantioselectively leading to non-racemic residues in soils shortly after application of the fungicide as a racemic mixture. The differentiation of sequestered non-extractable residues, prone to slow remobilization and thus to be included in persistence assessment, and covalently bound residues should be included in fate studies of chemicals in soil and sediment.

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