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Elimination of racemic and enantioenriched metalaxyl based fungicides under tropical conditions in the field

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Abstract

The elimination has been studied of racemic and enantioenriched metalaxyl applied as an emulsifiable concentrate and wettable powder combined with copper in a Cameroonian field site. The kinetics of the degradation/dissipation of metalaxyl and its acid metabolite were investigated using reversed phase HPLC-MS/MS, while the enantiomeric ratios were measured by HPLC-MS/MS using a Chiralcel OD-H HPLC column. Some soil enzymes activities were determined concurrently for 120 d. The elimination of racemic metalaxyl was shown to be enantioselective, with the *R*-enantiomer being degraded more slowly than the *S*-enantiomer. Dissipation followed approximate square root first-order kinetics ($R > 0.98$) without lag phases. The enantiomers of metalaxyl have different elimination rates, with half-lives ranging from only 0.8 to 1.5 days. After application to soil, the elimination of metalaxyl in the copper containing formulation was slower. The activities of acid phosphatase, alkaline phosphatase, and alkaline glucosidase were monitored throughout the experiments. No significant influence of metalaxyl and copper could be observed on these parameters. The significantly shorter half-life values of all forms of metalaxyl under field conditions, compared to the previously reported laboratory derived ones, may have implications for the plant disease control with these fungicides in tropical rainforest areas.

Keywords: Enantioselective pesticide analysis; LC-MS/MS; Degradation behavior

1. Introduction

Metalaxyl [methyl *N*-(2,6-dimethylphenyl)-*N*-(methoxyacetyl)-DL-alaninate], an important acylanilide fungicide with residual and systemic activity, is used to protect crops against diseases caused by pathogens of the orders Peronosporales (*Peronospora*, *Pseudoperonospora*, *Plamopara*, and *Bremia*) and Pythiales (*Pythium* and *Phytophthora*) (Kerkenaar and Sijpestijin, 1981). It is apoplastically transported and selectively interferes with the synthesis of ribosomal RNA (Hassal, 1990). Metalaxyl is chiral because it contains a stereogenic centre in the carboxy alkyl moiety (Fig. 1) so it consists of a pair of enantiomers, *S*(+) and *R*(−) (Ariëns et al., 1988). It is a stable compound resistant to a broad range of pH, temperature, and light (Singh et al., 1982). On account of these properties and its broad-spectrum activity, this compound has been registered for use on a wide range of crops and in many countries and in both temperate and tropical regions.

Metalaxyl was initially marketed as a racemic product, even though its fungicidal activity is almost entirely due to the *R*-enantiomer. The product enriched with the *R*-enantiomer (trade names: Metalaxyl-M, Mefenoxam; M = minus; laevorotatory; Nuninger et al., 1996), typically comprises 97.5% of the *R*-enantiomer and 2.5% of *S*-enantiomer and has the same biological activity as racemic metalaxyl at ~50% of the application rate. Wettable powder combined with copper formulated *rac*-metalaxyl, which

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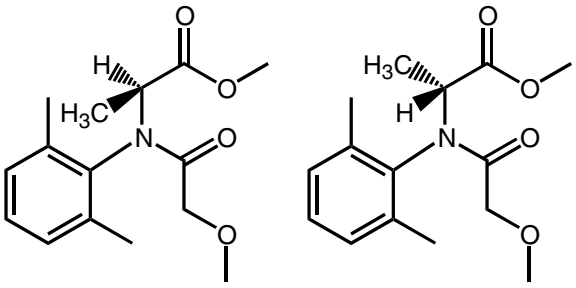

Fig. 1. Structures of *S*- and *R*-metalaxyl.

has been massively used for the protection of cash crops (cocoa) and subsistence crops in Cameroon (Monkiedje et al., 2000; Duguma et al., 2001; Fontem, 2001), is currently being replaced in the market by the enantioenriched substitute metalaxyl-M. Recent projections of higher cocoa production rates by 2010 in the main farming regions in Africa (FAO, 2003), mean that the consumption rate of this new product is likely to increase in the future in Cameroon.

Metalaxyl is readily biodegraded in soil, plants, and in animals by cleavage of the ester and by a concurrent series of oxidative biotransformations (*N*-dealkylation, alkyl and aryl hydroxylation Roberts and Hutson, 1999). The major metabolite of metalaxyl in soil is metalaxyl carboxylic acid {MX-acid; 2-[(2,6-dimethylphenyl) methoxyacetylamino] propionic acid}, with further metabolites also being formed from this primary hydrolysis product. MX-acid is also chiral (Buser et al., 2002).

Many reports have documented the microbially mediated degradation of metalaxyl in soils (Sukul and Spiteller, 2001), and the more rapid degradation of the *R*-metalaxyl in slightly acidic to alkaline soils in temperate climate under controlled laboratory conditions (Buser and Müller, 1995; Müller and Buser, 1995; Buser et al., 2002; Buerge et al., 2003). Further studies such as the correlation between the biodegradation rate of the compounds and enzyme activities and the influence of the type of formulations on degradation under tropical field conditions remain to be made. However, under specific field conditions, these compounds are applied as formulated products consisting of complex mixtures of a number of potentially hazardous and potentially interacting agents for which there is, as a whole, a lack of toxicity data relating to soil biology.

Recent work has dealt with the degradation behavior of metalaxyl in soils performed under controlled laboratory conditions. *S*-Metalaxyl was degraded more rapidly than *R*-metalaxyl in tropical soils (Sukul and Spiteller, 2001) and in aerobic soils with pH < 4 as well as in most anaerobic soils (Buerge et al., 2003), when these soils were spiked with racemic metalaxyl. These laboratory findings, if validated by field data, may have far reaching consequences for agricultural practice. The application rate as well as the application frequency of metalaxyl in the main regions of use, may have to be reassessed. Moreover, the replacement of racemic metalaxyl by enantioenriched metalaxyl means that knowledge of and data on the persistence of the latter and its effect on soil biology under field conditions have to be reassessed too. This is essential for use, management and registration of enantioenriched metalaxyl and it's copper based formulation, especially in the tropical regions of Africa. Knowledge concerning the elimination mechanism is a prerequisite for registration in new field applications.

The elimination of pesticides in soil does not always follow first-order kinetics, but shows a pattern where the pesticide concentration declines more rapidly in an initial phase. The decline of pesticide residues in soil was investigated in 420 experiments in order to validate possible non-linear behavior. Specifically the square root 1st order model showed a better fitting than the linear model in 35% of the investigations (Timme et al., 1986). Several studies have substantiated the relevance of the square root first order kinetic (Korpraditskul et al., 1993; Di et al., 1998; Banerjee et al., 2006).

The purpose of the present work was to investigate the behavior of racemic and enantioenriched metalaxyl in two different formulations under field conditions in tropical rainforest soil, and to verify and monitor any changes in the enantiomeric ratio (ER) of the racemic pesticide formulations. Indeed, a stereo specific biodegradation may lead to changes in the enantiomeric ratios of parent compounds and metabolites. Further, in light of the fact that enzymes play a role in the biological degradation of metalaxyl, we monitored the effect of these compounds on the activities of selected soil enzymes with a view to determining the relationship between the elimination rate of various forms of metalaxyl and the activities of these enzymes.

2. Experimental section

2.1. Experimental site

The study was conducted in Yaounde (Cameroon) on an agricultural plot which had not received any pesticide application for at least ten years. Selected physicochemical properties of the soil are reported in Table 1. This site has

Table 1
Selected physicochemical properties of the soils investigated

Texture analysis (USDA)	Value
Clay (<2 μm) (%)	51.2
Silt (2–50 μm) (%)	16.1
Sand (50–2000 μm) (%)	32.7
pH (water, ratio 1:2.5)	4.75
pH (0.01 M $CaCl_2$, ratio 1:2.5)	3.7
Corg. (% dry soil)	5.01
C/N	17.5
P (mg P_2O_5 Kg^{-1} dry weight)	21.3
Cation exchange capacity (meq g^{-1} dry weight)	0.04
Maximum water holding capacity (MWHC) (%)	52.6
Bulk density (g ml^{-1})	1.27
Field capacity (cm^3 water cm^{-3} soil)	0.40
Available water (cm^3 water cm^{-3} soil)	0.12

been under continuous cultivation since 1990. The crops grown, irrespective of seasons, have included maize, peanut, sweet potato, and cassava. This site, which is located in the equatorial rainforest region, receives an annual rainfall of 1400–1600 mm, while the annual temperature varies between 19 and 28 °C. The study site was divided into four blocks. Each block included replicates of all fungicide treatments and a control plot. Plots within the blocks were organized according to the "Latin Square" model of randomized blocks (Rohrmoser, 1986). This arrangement of the plots represents complete randomization, as both treatments and controls were distributed at random in blocks and in columns. In order to control the undesired influences such as soil differences amongst blocks and border effects, each treatment occurred once in each soil block. Each plot measured 1 m × 1.5 m. The experiments began at the onset of the rainy season and ended at the beginning of the following dry season.

2.2. Chemicals

The standards racemic metalaxyl [(*R*,*S*)-metalaxyl] (chemical purity >99.3%) and enatioenriched metalaxyl [*R*(−) enantiomer (chemical purity, 96.5%)], were purchased from Dr. Ehrenstorfer GmbH (Germany). The commercial products Fonganil Neu (formulated racemate metalaxyl) and Fonganil Gold (formulated *R*-enantiomer enriched metalaxyl) were obtained as emulsifiable concentrate formulations containing 24% and 45% of racemic and enantiomer enriched metalaxyl, respectively, from Syngenta Agro, Frankfurt, Germany. The compounds that make up the remaining 76% and 55% of the respective emulsifiable concentrate formulations include a petroleum-derived solvent, a glycol-derived solvent, naphthalene, trimethylbenzene, alkyl-aryl-poly-oxy-ethylenate, and emulsifiers whose exact structures are kept confidential by the company. Ridomil Plus 72 (formulated racemic metalaxyl combined with copper) and Ridomil Gold Plus/Copper (formulated enantiomer enriched metalaxyl combined with copper) were obtained from a local vendor as wettable powder formulations containing 12% and 6% of racemic and enantioenriched metalaxyl and 60% copper oxide, respectively,. Metazachlor, used as internal standard, was purchased as analytical grade from Riedel-de Haen (Germany). C18 SPE cartridges (500 mg, 6 ml) were from Baker, Deventer, Netherlands. Formic acid,, hydrochloric acid, and the HPLC grade solvents (ethyl acetate, *n*-hexane, 2-propanol, acetonitrile, and methanol) were purchased from Merck, Darmstadt, Germany.

2.3. Application of the fungicides on the field

The dose calculations were based on the assumption of a penetration depth of 0.05 m and a soil density of 1.5 g cm^{-3}. Calculated amounts, corresponding to recommended field application rates of roughly 1 kg ha^{-1} of racemic metalaxyl and 0.5 kg ha^{-1} of enantioenriched metalaxyl in both formulations were pipetted or weighed and mixed thoroughly and separately in 1500 ml of distilled water. The rates of application of the racemic metalaxyl were 1085 g a.i. ha^{-1} of the emulsifiable formulation and 1096 g a.i. ha^{-1} of the wettable powder formulation. Application rates of the enantioenriched metalaxyl were 585 g a.i./ha of the emulsifiable formulation and 550 g a.i. ha^{-1} of the wettable powder formulation. These solutions were uniformly applied once by hand spray (7-l compression sprayer, Intereko 7) to the prepared surface of the experimental plots. No attempt was made to incorporate the chemicals into the soil by mechanical mixing. The plots were managed with minimum tillage. The plots were not cultivated and weed control was performed by manual weeding.

2.4. Soil sampling and preparation

Six soil cores were taken at random from each plot at each incubation period (0, 1, 3, 15, 30, 60, 90 and 120 d) from 0 to 5 cm depth using a plastic coring tube (5 cm diameter). The samples were taken at different positions within the plot to get better statistical distribution. No sampling was done at the border of the experimental plots (20 cm minimum distance). The sampling areas were marked with wooden sticks and refilled with untreated soil in order to avoid preferential flow. The samples were bulked and homogenized in disposable plastic buckets. Whenever necessary, the soil was gently air-dried for a few days to reduce the moisture content of the soil and make it suitable for sieving to a maximum particle size of <2 mm. Enzyme activities were determined immediately after sampling of field moist and sieved sub-samples. The soils for fungicide residue analysis were kept field moist and stored frozen (−20 °C) until analysis. A determination of gravimetric soil moisture content was performed concurrently with each parameter analysis.

2.5. Preparation of the metalaxyl acid metabolite

N-(2,6-Dimethylphenyl)-*N*-(methoxyacetyl)alanine (metalaxyl acid) was synthesized according to a previously described procedure (Sukul and Spiteller, 2001). Racemic metalaxyl (100 mg, purity >99%) was added to 2 N KOH (5 ml) and the mixture was refluxed (110–115 °C) for 1 h before cooling to ambient temperature. 2 N HCL (7 ml) was added to the reaction mixture, which was then stored in a freezer overnight. The resulting white crystals were collected and washed with ice-cold water. When poor crystallization was observed, the whole solution was extracted three times with ethyl acetate (50 ml aliquotes). The combined extracts were gently evaporated to dryness and the resulting white crystals were collected. The identity of the metalaxyl acid metabolite obtained was confirmed from NMR data. The NMR spectra were recorded using a Varian 200 MHz spectrometer equipped with a 5 mm probe.

^{1}H NMR (CD_3OD, 200 MHz): $\delta = 7.13$ (m, 3H, H-3,H-4,H-5), 4.37 [q, $J = 7.5$ Hz, 1H, H-2′], 3.45 [m, 2H, H-2″],

3.16 [s, 3H, H-3″], 2.35 (s, 3H, H-8), 2.06 (s, 3H, H-7), 0.91 (d, $J = 7.5$, Hz, 3H, H-3′).

2.6. Extraction and clean-up of treated samples

The procedure described previously was employed (Monkiedje et al., 2003). Here, extraction of the acidified soil (100 g) with 200 ml methanol was carried out in Erlenmeyer flasks containing the incubated soil and the internal standard (IS) Metazachlor (5 μg abs.) on an overhead shaker. After allowing the sediment to settle, the clear supernatant was decanted through a glass fibre filter. After a second extraction step, the pooled methanolic extracts were evaporated to approx. 10 ml on a rotary evaporator and cleaned up using C_{18} SPE cartridges. The final methanolic eluates were concentrated to about 5 ml and aliquots were subjected to HPLC-MS analysis.

2.7. Quantitation via reversed phase HPLC-MS/MS

The quantitation of metalaxyl and the acid metabolite was performed by reversed phase HPLC-MS/MS using a Survior HPLC system (Thermo Finnigan, San Jose, CA, USA). A Luna C_{18} 100 Å column from Phenomenex (Torrance, CA, US; 150 × 2 mm, 3 μm particle size, free exposed silanols virtually eliminated by complete bonding and end-capping) was used for non-chiral separations. The mobile phase (flow rate = 0.25 ml min^{-1}) consisted of water and acetonitrile, both HPLC grade (Baker, Deventer, Netherlands) with 0.1% formic acid. The gradient was programmed as follows: 0–2 min: 10% acetonitrile; 2–3.5 min: 10 → 30%; 3.5–8 min: 30 → 100%; 8–15 min: 100%, 15–16 min: 100 → 10%, and equilibration for another 8 min. All separations were performed at 22 °C. The calibration was performed as a multilevel internal standard calibration (IS metazachlor). The ratio of peak areas (analyte/IS) was linear within the range 0.04–100 μg ml^{-1} (0.002–5 μg g^{-1} soil). The procedure gave recoveries of 114.0% with 9.3% RSD for metalaxyl and 104.0% with 6.2% RSD for its acid metabolite (recovery rates obtained from spiked soil; ~1 μg g^{-1} dry soil). The limit of determination was 0.002 μg g^{-1} soil for both metalaxyl and its acid metabolite. Blanks were found to be below the limit of detection. The data reported are uncorrected for recoveries.

The identity of the compounds was confirmed by comparing retention times and multiple reaction monitoring in tandem mass spectrometry (TSQ quantum ultra AM equipped with an APCI source, Thermo Finnigan, San Jose, Ca, USA). For (+)APCI, the ionization conditions were optimized for every compound and two selected product ions were monitored for each precursor compound. Nitrogen was used as both drying and nebuliser gas. The drying gas temperature was set at 200 °C and a pressure of 124 kPa. The collision gas (argon) pressure was set to 0.2 Pa. For the selected reaction monitoring (SRM) scans, a dwell time of 0.15 s was applied. MS/MS parameters for every SRM chromatogram are reproduced in Fig. 2.

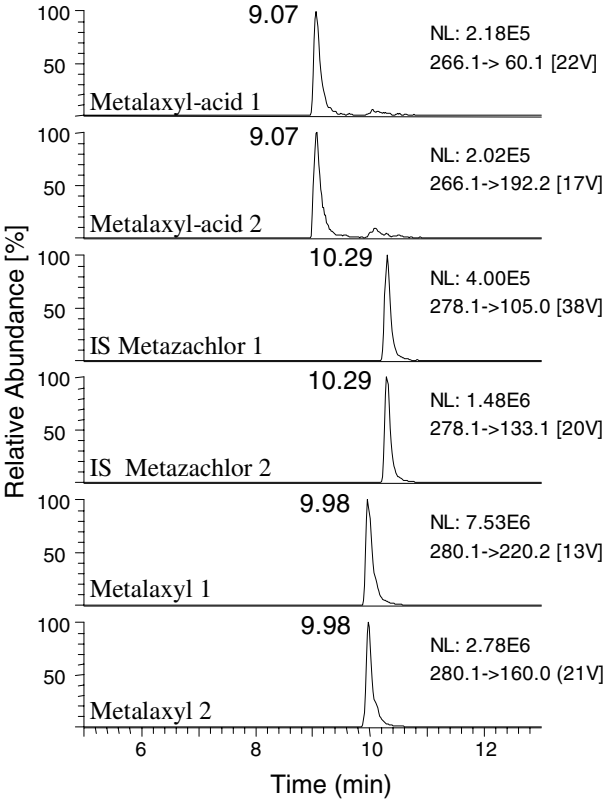

Fig. 2. SRM chromatograms of metalaxyl, the acid metabolite and the internal standard (IS) Metazachlor of a soil extract after 15 days of incubation; quantifier and qualifier traces are shown.

2.8. Enantioselective analysis by normal phase HPLC-MS/MS

Aliquots of the extracts prepared for RP-HPLC (0.5 ml) were conditioned for chiral HPLC by solvent exchange to *n*-hexane/2-propanol by means of careful evaporation to dryness at 60 °C under vacuum using an Eppendorf Concentrator 5301 (Eppendorf, Hamburg, Germany). The residues were then re-dissolved in 0.1 ml of *n*-hexane:2-propanol (80:20). A 5 μl aliquot of this solution was then analyzed by chiral normal phase HPLC-MS/MS using a Gynkotek/Dionex HPLC system consisting of a degassing unit (ERC-3822), a Gina 50 automatic sampler, and a Dionex P 580 pump. Separations were performed at room temperature which was maintained at 22 °C.

The enantiomers of racemic metalaxyl, and those of the corresponding racemic MX-acid, were separated on a Chiralcel OD-H HPLC (5 μm, 25 cm × 4.6 mm i.d.; Daicel Chemical Industries, Tokyo, Japan) column. The mobile phase consisted of *n*-hexane:2-propanol (50:50) with 0.3% formic acid (isocratic elution). The flow rate was set at 0.3 ml min^{-1}. The calibration was performed by using high purity metalaxyl and metalaxyl-M standards prepared in HPLC grade *n*-hexane:2-propanol (80:20).

Mass spectra were obtained using a Varian 1200L LC-MS (Varian, Inc. Palo Alto, CA, USA) equipped with an ESI source operating in positive mode. For ESI, the operating

conditions were as follows: needle voltage at 4 kV, shield at 600 V and tube lens at 4 V. The drying and nebuliser gas was nitrogen, which was set at 200 °C and a pressure of 18 psi. The acid metabolite was detected as both the sodium adduct $[M + Na]^+$ and, after fragmentation, as the product ion $(288 \rightarrow 113)$. Intensive fragments of the $[metalaxyl + Na]^+$ were not observed; so the sodium adduct only was monitored for this compound. The selectivity of the mass spectrometric detection was high, no additional peaks with the same precursor/product ion could be observed in the range of the expected retention time in all soil extracts.

The enantiomer ratios in the samples were determined from their peak area ratios. Racemic metalaxyl acid yields an exact peak area ratio of 1:1 in both standard solutions and spiked to soil extracts. But the ratio differed for metalaxyl on account of peak broadening at the higher retention time of the *R*-metalaxyl on normal phase separation. To ensure a matrix-independent result, the effect of the soil-matrix was checked by injection of racemic metalaxyl standards and soil extracts spiked with racemic metalaxyl. Samples from all four experiments (both formulations, both metalaxyl and enantioenriched metalaxyl) were checked by this procedure and confirmed the correction factor for the ER of metalaxyl which ranged from 1.2 to 1.3 (average 1.25). This factor of 1.25 was applied to all samples to yield corrected enantiomeric ratios (racemates corrected to the ratio of 1:1). All concentrations of the chiral separation were tested for concentration dependencies concerning the peak area ratios. Ratios (*R*:*S*) were determined for both the racemic mixtures (metalaxyl and acid metabolite) for the entire range of concentrations analyzed. Ratios were also constant for different injection volumes of soil extracts.

2.9. Determination of enzyme activities

Acid and alkaline phosphatase activities were determined according to methods described in the literature (Tabatabai and Bremmer, 1969; Eivazi and Tabatabai, 1982) with slight modifications. Toluene was not included in the procedure as it has been shown to increase the observed activities of both acid and alkaline phosphatases and can be used as source of carbon by most soil microorganisms. β-Glucosidase activity was measured using a published method (Eivazi and Tabatabai, 1988). The results of the enzyme activity studies are reported on an oven dry weight basis, determined by drying the soils at 105 °C for 24 h.

3. Results and discussion

3.1. Elimination kinetics

Many published degradation studies assume simple first-order degradation kinetics. Often, however, the elimination of pesticides in soil does not follow first-order kinetics, but shows a pattern where the pesticide declines at an initial phase rapidly and in a second phase less rapidly. To describe the non-linear decline of the pesticide residues in soil, a number of non-linear equations, like the square root 1st order model have been developed (Timme et al., 1986; Matsumura, 1989; Korpraditskul et al., 1993; Di et al., 1998). In this study the 1st order model has been compared with the square root 1st order model.

Racemic and enantiomer enriched metalaxyl in both formulation types were degraded in soils to levels of <2% of their initial concentration within 30 d of application. The concentrations of four replicate applications of *R- and S*-metalaxyl and *R*- and *S*-metalaxyl acid remaining in the soils are summarized in Tables 2 and 3. Because of the very low concentrations of the fungicide after 60 days and more, the uncertainty in measurement is high. Thus, regression analysis was only performed from time 0 up to 30 days. In contrast to further investigations of laboratory experiments (Buser et al., 2002; Monkiedje et al., 2003; Buerge et al., 2003) approximate first order kinetics were not observed after field application. The biodegradation of the parent compounds (both formulations) in soil appears to follow square root first order kinetics over the first 30 days. Fig. 3 shows the superior linear fit for $\ln(Ao/A_t)$ versus $\sqrt{t}$ (Timme et al., 1986) than for $\ln(Ao/A_t)$ versus t of *R*- and *S*-metalaxyl after application of the racemic metalaxyl to soil in emulsifiable concentrate. The comparison of 1st order model and Square root 1st order model is shown in Fig. 3 using the application of emulsifiable concentrates of racemic metalaxyl as an example. The following Eq. (1) (Timme et al., 1986) allows the calculation of the residue concentration over the time of the field experiment, here $[A]_t$ is the concentration (μg kg^{-1} soil) of A at time t (days) and $[A]_0$ is the initial concentration of A at time 0.

$$[A]_t = \frac{[A]_0}{k^{\sqrt{t}}} \tag{1}$$

Rate constants for each experiment were derived from "ln (A_t/A_0) versus $\sqrt{t}$" plots by linear regression analysis (fitting without any weighting). The half-life ($t_{1/2}$, days) was calculated from

$$t_{1/2} = \left(\frac{\ln 2}{\ln k}\right)^2 \tag{2}$$

The enantiomeric ratio (ER) is defined by the relation (3) where *R* and *S* are the concentration of *R*- and *S*-enantiomers, respectively. The ER values define a range from 0 ($R = 0$, $S = 100\%$) to >1 ($R > S$).

$$ER = \frac{[R]}{[S]} \tag{3}$$

Thus, elimination rate constants were determined using regression plots of ln (Ao/A_t) versus square root of the time $(\sqrt{t})$ (Fig. 3). Correlation coefficients are in the range from 0.984 to 0.999 for *R*- and *S*-metalaxyl in racemic formulations, and for *R*-metalaxyl in the enantiomer enriched formulations. Elimination kinetics of *S*-metalaxyl in the enantioenriched formulations was not determined, because

Table 2
Concentrations of *R*- and *S*-metalaxyl, together with the *R*- and *S*-enantiomer of its metabolite, in experimental plots treated with emulsifiable concentrate after the various soil sampling intervals; values represent means ± standard deviation of 4 independent samples; concentration (μg/100 g of dry soil)

Day	Total metalaxyl	*R*-metalaxyl	*S*-metalaxyl	Total MX acid	*R*-MX acid	*S*-MX acid
Emulsifiable concentrate of racemic metalaxyl						
0	249 ± 38	120 ± 10	129 ± 11	12 ± 2.8	7.1 ± 0.3	4.9 ± 0.4
1	133 ± 16	64 ± 3.2	68 ± 5	6.6 ± 1.4	3.5 ± 0.7	3.1 ± 0.7
3	77 ± 20	36 ± 5	40 ± 4	5.3 ± 1.6	3.1 ± 0.4	2.2 ± 0.05
15	17 ± 4.5	10.3 ± 1.1	6.4 ± 1.0	2.0 ± 0.8	1.3 ± 0.1	0.8 ± 0.05
30	5.3 ± 5.1	3.1 ± 0.7	2.1 ± 0.2	1.6 ± 1.1	1.2 ± 0.4	0.8 ± 0.05
60	2.5 ± 2.1	1.1 ± 0.05	1.3 ± 0.05	1.9 ± 1.3	1.1 ± 0.05	0.7 ± 0.05
90	0.7 ± 0.2	0.3 ± 0.05	0.3 ± 0.05	1.5 ± 1.0	0.8 ± 0.05	0.7 ± 0.05
120	0.6 ± 0.3	0.4 ± 0.05	0.15 ± 0.05	0.3 ± 0.25	0.19 ± 0.05	0.13 ± 0.05
Emulsifiable concentrate of enantioenriched metalaxyl						
0	147 ± 5	133 ± 6	13.2 ± 2.0	4.6 ± 0.5	4.2 ± 0.5	0.46 ± 0.05
1	86 ± 12	73 ± 7	12.7 ± 3.1	3.0 ± 0.3	2.7 ± 0.1	0.36 ± 0.05
3	49 ± 10	43 ± 6	5.9 ± 2.3	2.9 ± 0.2	2.6 ± 0.05	0.32 ± 0.05
15	10 ± 3.0	8.5 ± 2	1.6 ± 0.05	2.2 ± 0.6	2.0 ± 0.6	0.18 ± 0.05
30	6.2 ± 2.4	5.4 ± 1.5	0.8 ± 0.05	1.9 ± 0.8	1.9 ± 0.8	<0.02 ± 0.05
60	2.5 ± 0.9	1.8 ± 0.5	0.7 ± 0.05	1.8 ± 0.7	1.8 ± 0.6	<0.02 ± 0.05
90	1.7 ± 0.5	1.3 ± 0.4	0.3 ± 0.05	1.4 ± 0.6	1.4 ± 0.7	<0.02 ± 0.05
120	0.9 ± 0.3	0.8 ± 0.05	0.1 ± 0.05	0.7 ± 0.2	0.7 ± 0.2	<0.02 ± 0.05

Table 3
Concentrations of *R*-and *S*-metalaxyl, together with the *R*- and *S*-enantiomer of its metabolite, in experimental plots treated with copper containing wettable powder, material after the various soil sampling intervals; values represent means ± standard deviation of 4 independent samples; concentration (μg/100 g of dry soil)

Day	Total metalaxyl	*R*-metalaxyl	*S*-metalaxyl	Total MX acid	*R*-MX acid	*S*-MX acid
Copper containing wettable powder of racemic metalaxyl						
0	249 ± 31	130 ± 23	119 ± 32	14 ± 3.3	5.7 ± 0.3	8.2 ± 0.2
1	131 ± 10	74 ± 6.2	57 ± 6.3	12 ± 4.3	5.7 ± 0.4	6.2 ± 0.2
3	77 ± 12	42 ± 7.3	34 ± 19	5.5 ± 4.0	2.4 ± 0.2	3.2 ± 0.2
15	20 ± 7.9	12.7 ± 2.4	7.2 ± 0.9	5.2 ± 1.7	2.4 ± 0.4	2.9 ± 0.4
30	9.5 ± 3.6	6.2 ± 1.1	3.3 ± 0.3	2.9 ± 0.6	1.4 ± 0.05	1.5 ± 0.05
60	3.4 ± 1.7	2.2 ± 0.6	1.1 ± 0.05	2.8 ± 1.5	1.1 ± 0.05	1.7 ± 0.1
90	2.6 ± 1.2	1.0 ± 0.7	1.5 ± 0.05	2.8 ± 1.0	1.2 ± 0.05	1.6 ± 0.05
120	1.1 ± 0.6	0.7 ± 0.7	0.4 ± 0.05	0.8 ± 0.6	0.4 ± 0.05	0.4 ± 0.05
Copper containing wettable powder of enantioenriched metalaxyl						
0	105 ± 4.5	99 ± 1.9	5.9 ± 0.3	5.5 ± 0.6	4.9 ± 0.5	0.6 ± 0.05
1	64 ± 9.0	59 ± 3.4	5.0 ± 0.05	5.0 ± 0.1	4.5 ± 0.1	0.5 ± 0.05
3	43 ± 9.3	39 ± 3.5	4.1 ± 0.05	3.2 ± 0.1	2.6 ± 0.05	0.5 ± 0.05
15	12 ± 7.8	9.3 ± 1.4	2.8 ± 0.05	2.0 ± 0.7	1.7 ± 0.2	0.3 ± 0.05
30	6.2 ± 2.5	4.6 ± 0.6	1.5 ± 0.05	1.8 ± 0.85	1.6 ± 0.05	0.2 ± 0.05
60	1.7 ± 0.6	1.3 ± 0.2	0.4 ± 0.05	1.0 ± 0.21	0.9 ± 0.05	0.1 ± 0.50
90	1.7 ± 0.6	1.2 ± 0.05	0.5 ± 0.05	0.5 ± 0.05	0.4 ± 0.05	0.1 ± 0.05
120	0.6 ± 0.2	0.3 ± 0.05	0.3 ± 0.05	0.2 ± 0.05	0.2 ± 0.05	<0.02 ± 0.50

of the very low concentrations of *S*-metalaxyl in the soil samples (even immediately after application).

Considerably higher reaction constants and shorter half-lives (0.8–1.5 days) were observed in the field studies (Table 4) compared with those measured in the earlier laboratory experiments (Monkiedje et al., 2003: 17–39 days; Buerge et al., 2003: 3–60 days). This may be the result of the more complex processes of elimination in the field (degradation, hydrodynamic flow, runoff), compared to degradation alone in laboratory experiments. An inconsistent relationship between elimination rates of pesticides, including metalaxyl, measured in the laboratory or in the field has already been reported (Di et al., 1998). Environmental factors, such as variations in temperature, moisture (Matsamura, 1989) and light, which have a strong influence on microorganisms in their degradation preferences for metalaxyl enantiomers in experimental plots, may account for the observed differences in elimination rates, especially since these products were applied to the surface of the experimental plots without manual incorporation into deeper soil layers. One reason for the shorter half-lives in the field compared to the laboratory experiments may be the wash out of the fungicides by rain. Leaching into deeper soil must also be considered, especially as sampling was only performed until a depth of 5 cm.

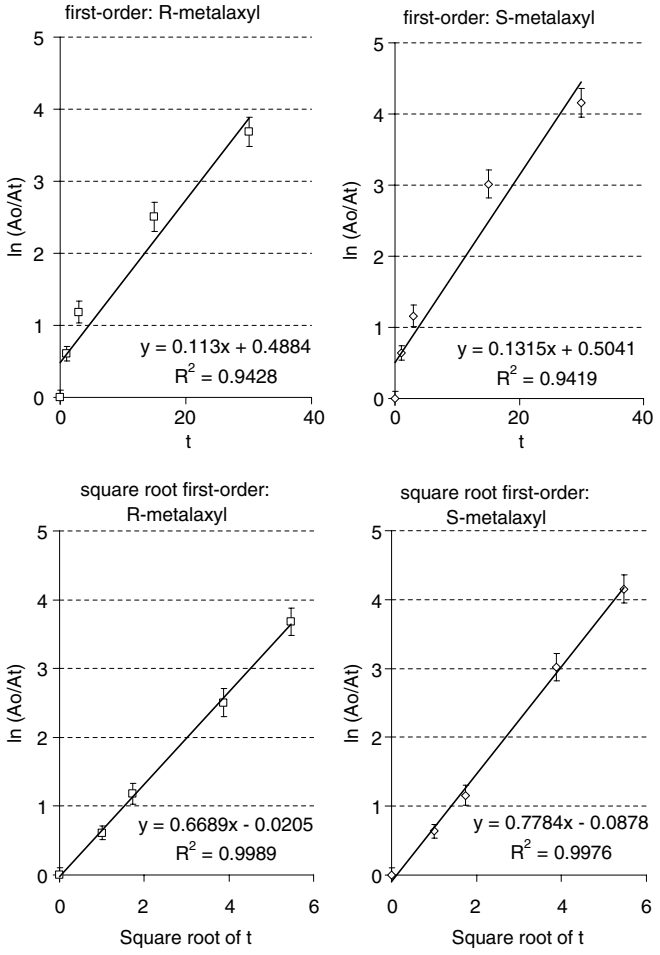

Fig. 3. Linear fit of $\ln(Ao/A_t)$ versus $\sqrt{t}$ as well as $\ln(Ao/A_t)$ versus t (days) of *R*- and *S*-metalaxyl after 0–30 days of application of racemic metalaxyl in emulsifiable concentrate.

3.2. Formation of acid metabolite

In all formulations, the acid metabolite was detected almost immediately after application of the parent compound (Tables 2 and 3). It is not possible to speculate on the kinetics of elimination of the metabolite on account of the continued degradation of metalaxyl and simultaneous elimination of the acid metabolite. Biological processes, especially the initial attack of compounds can be very rapid. Thus, the degradation of metalaxyl may be biologically mediated. The non-racemic composition of metalaxyl acid immediately after application of racemic metalaxyl in particular is a strong indication that the formation of the acid metabolite is at least partly biologically mediated. Given the nature of the soil studied (clay and acidic, Table 1), reactions occurring in the soil pores (e.g. acid-catalysis) or on the surfaces of clay minerals (heterogeneous surface catalysis) may also contribute to the hydrolytic degradation of these compounds as observed for other pesticides (Korpraditskul et al., 1993). In this soil, and with both formulations tested, the production of the acid metabolite was less pronounced, thus confirming our previous laboratory findings (Monkiedje et al., 2003). A maximum acid metabolite concentration of ~6% the initial concentration of metalaxyl was observed at the start of the experiment (0 d). The concentration of the metabolite decreased with incubation time (Tables 2 and 3). Possibly, the metalaxyl was transformed to metabolites other than the acid metabolite or was mineralized in the soils. Nevertheless, no other metabolites could be found using HPLC-MS in full scan mode as well as in precursor ion scan mode (LC-MS/MS) of typical metalaxyl fragments. These field observations of metalaxyl elimination contrast with those made from our previous laboratory incubation study using racemic and enantioenriched metalaxyl with German and Cameroonian soils (Monkiedje et al., 2003), where a gradual formation of MX-acid (0% → <7%) occurred in the course of 14 d incubation in a similar Cameroonian soil. Under field conditions with continuous rainfall it can be assumed that the highly polar metabolite will be leached into deeper soil.

3.3. Soil hydrolase activities

Many studies have reported microbial metabolism of pesticides in soil as a result of the broad-spectrum enzymes (hydrolases, oxidases, etc.) (Matsamura, 1989) generally present. In order to evaluate the relationship between the elimination of the various forms of metalaxyl and the activities of soil enzymes, we monitored the effect of these compounds on the activities of selected soil hydrolase enzymes. The results for soil enzyme activities (acid phosphatase, alkaline phosphatase, and β-glucosidase) in untreated and treated experimental plots differed only within the range of the uncertainty of measurement and sampling. No significant changes were observed for the parameters monitored; which were chosen to reflect the general microbial activity of the soil samples.

Table 4
Degradation rate constants (k) and half-lives ($t_{1/2}$) as derived from the regression line from the $\ln(C_0/C_t)$ versus $\sqrt{t}$ plot plus correlation coefficient (R^2) values for the degradation of total metalaxyl and its enantiomers in soil with two different formulations

Formulation	*R*-metalaxyl			*S*-metalaxyl		
	ln(k)	R^2	$t_{1/2}$ (d)	ln(k)	R^2	$t_{1/2}$ (d)
Emulsifiable concentrate of racemic metalaxyl	0.669	0.999	1.07	0.778	0.998	0.79
Emulsifiable concentrate of enantioenriched metalaxyl	0.614	0.984	1.27	–		–
Copper containing wettable powder of racemic metalaxyl	0.576	0.994	1.45	0.680	0.992	1.04
Copper containing wettable powder of enantioenriched metalaxyl	0.564	0.995	1.51	–		–

3.4. Enantioselective investigation

The ratios of *R*:*S* enantiomers in field plots were investigated in order to compare the fate of the two enantiomers of metalaxyl and of their respective acid metabolites. The ERs of the racemic metalaxyl standard and of the racemic acid metabolite were both 1.0.

The enantio enriched products investigated were highly enriched in *R*-metalaxyl (*R*:*S*; 96:4 and 94:6) with an enantiomeric ratio of 15.7 and 24.0. The ER of these products is not discussed because of the differing initial concentrations of *R*-metalaxyl and *S*-metalaxyl in the pesticide formulations used. Nevertheless, the elimination of the *S*- and *R*-metalaxyl is also presented in the Tables 2 and 3. The degradation rate constants and half-lives of the applied metalaxyl are reported in Table 4.

In the experimental plots, *S*-metalaxyl was degraded much more quickly ($t_{1/2}$ 0.8 and 1.0 days) than *R*-metalaxyl ($t_{1/2}$ 1.1 and 1.5 days) when racemic metalaxyl was applied in both formulations (Table 4). This is in agreement with previous reports on the behavior of metalaxyl in aerobic soils with pH < 4 and in most anaerobic soils (Buerge et al., 2003). After application to soil the *S*-metalaxyl degraded much faster than *R*-metalaxyl, independent of the type of formulation applied. The elimination of both, the *R*- and *S*-enantiomer in emulsifiable concentrate was faster than in the copper containing (Table 4). This suggests that copper exerts some toxic effect on soil micro-organisms. The toxic effects of copper on soil micro-organism activity are well known (Varlet and Berry, 1997). This is in agreement with a previous report (Klodka and Nowak, 2004) showing adverse effects on soil enzyme activities after application of pesticides in formulations.

The elimination behavior of the *R*-and *S*-enantiomers, as well as those of *R*- and *S*-metalaxyl acid, was assessed by plotting the ER at each sampling time from 0 up to 30 days after application of racemic mixtures (Fig. 4). After application to field plots, the ER of racemic pesticide formulations changed, showing that the two enantiomers are present in different proportions. This indicates that there is enantioselective degradation of metalaxyl in these soils in the same sense as in our previous study (Monkiedje et al., 2003).

On account of the more rapid degradation of *S*-metalaxyl, the ER of Metalaxyl is >1 for both formulations and increases with time. A significant increase in the ER is first apparent at day 15 for the emulsifiable concentrate and at day 1 for the copper containing metalaxyl. On the other hand, differences were observed for the formation of the acid metabolite. ER > 1 was measured with emulsifiable concentrate but ER < 1 was observed with the copper containing powder. The *S*-metalaxyl may generally be degraded more quickly in the emulsifiable concentrate. Thus, more of the *S*-metalaxyl-acid is formed and if the degradation of both the enantiomers of the acid metabolites is in the same range, more *S*-metalaxyl-acid is the result (Fig. 4).

One reason for the different ranking order of degradation rate constants of the two enantiomers of metalaxyl could be different microbial populations in the soil studied. These produce different enzymes, which preferentially degrade the different enantiomers. Our findings show that the degradation of racemic metalaxyl in the soil studied is clearly enantioselective, with *S*-metalaxyl being eliminated more rapidly than *R*-metalaxyl (residues enriched in *R*-metalaxyl). The physicochemical behavior, such as sorption, mobility and leaching, ought to be identical for *R*- and *S*-metalaxyl, as well as for *R*- and *S*-metalaxyl acid. Metalaxyl seems to be configurationally stable in soil under field conditions, since there is no evidence from the ER values to suggest interconversion of *R*- to *S*-enantiomer, which had already been reported for earlier reported laboratory studies (Buser et al., 2002). The influence of the possible leaching of metalaxyl and metalaxyl acid ought to be similar for both the enantiomers (*R*- or *S*-) on account of the similarity in their physicochemical properties.

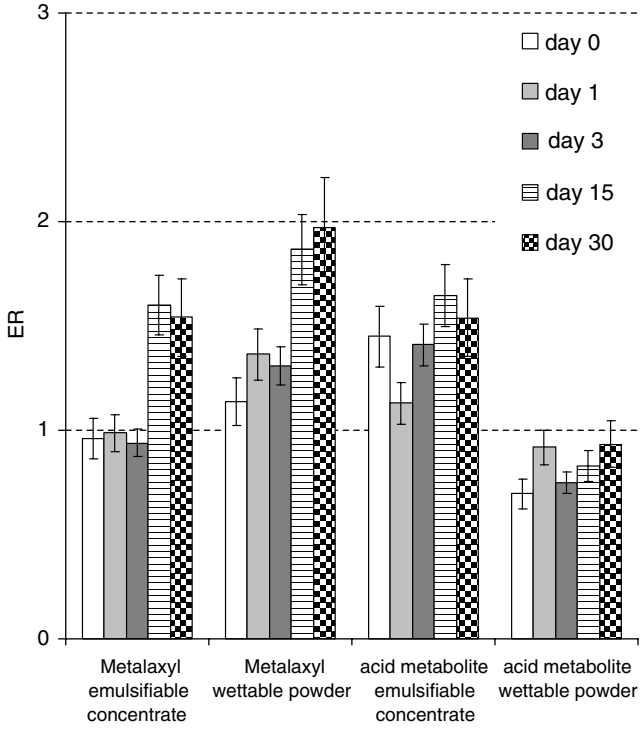

Fig. 4. Enantiomeric ratios (ER) of metalaxyl and metalaxyl acid after application of different formulations containing racemic metalaxyl to soil; day 0–30; values represent means ± standard deviation of 4 independent samples.

The field study presented here yielded in significantly shorter half-lives for metalaxyl than those previously reported from laboratory incubation studies (Buser et al., 2002; Monkiedje et al., 2003; Buerge et al., 2003). The application rates and frequencies of application employed by farmers are much higher than those recommended by the makers of metalaxyl-based products (Varlet and Berry, 1997; Fontem, 2001). The rapid field dissipation of these products in the field reported here may justify this heavy application in part. As a consequence, cases of metalaxyl resistance of fungal pathogens have been reported (Fontem et al., 2005).

4. Conclusion

The elimination has been studied of racemic and enantioenriched metalaxyl applied as emulsifiable concentrate or as a wettable powder combined with copper to a Cameroonian field site. Analysis of the degradation/dissipation of metalaxyl and its acid metabolite were investigated using reverse phase HPLC-MS/MS, while the enantiomeric ratios were measured by normal phase HPLC-MS/MS. The dissipation of racemic metalaxyl is enantioselective, with the *R*-enantiomer being degraded more slowly than the *S*-enantiomer. Elimination followed square root first-order kinetics with different with half-lives of the enatiomers ranging from only 0.8 to 1.5 days. Metalaxyl was eliminated more slowly after application of the copper containing formulation to soil. The activities of acid phosphatase, alkaline phosphatase, and alkaline glucosidase revealed no significant influence of metalaxyl and copper. The significantly shorter half-lives of all forms of metalaxyl under field conditions, compared to those previously reported for laboratory investigations, may have implications for the plant disease control with these fungicides in tropical rainforest areas.

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