SOIL ORGANISMS Volume 81 (2) 2009 pp. 237–264

ISSN: 1864 - 6417

Field studies for the assessment of pesticides with soil mesofauna, in particular enchytraeids, mites and nematodes: Design and first results

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Abstract

Currently, earthworm field tests and litter-bag studies are regularly required as part of the environmental risk assessment of pesticides in soil. These tests give almost no indication if there is any structural impact, i.e. on the biodiversity of the soil organism community. Therefore, a design is presented here for such a structural study, which follows basically the design of litter bag studies. Our proposal is based on practical experience made in Southern Germany in 2007 / 2008. A new pesticide was tested, framed by a control (water) and a mix of two reference substances (the fungicide Benomyl and the insecticide Chlorpyrifos(ethyl)) of known effect, the latter in order to confirm the sensitivity of the studied soil organisms. For confidentiality reasons, the identity of the test substance cannot be given here. Six replicates were used per treatment (plot size: 7 m x 3 m). The study was performed on grassland and test chemical and reference substance were applied without soil incorporation. Grassland was chosen because diversity and abundance of enchytraeids, mites and nematodes (and soil mesofauna in general) is usually very low in agricultural soil. The duration of the study was one year and sampling was performed –2, 32, 89, 187 and 372 DAT (days after treatment). The taxonomic groups assessed were enchytraeids, soil-inhabiting mites, collembolans, and nematodes, but collembolans will not be covered in this contribution. All groups except nematodes were sampled using ISO standard methods. Specimens were sampled with soil corers (diameter and depth: 5 cm each). The enchytraeids were identified to the species level, but assessment is based on the genus level. Among mites only the oribatids were identified to species level, the rest to higher groups. The nematodes were only assessed quantitatively. The variance of the samples collected was small enough to detect a change in the taxonomic structure of the soil organism community. In this contribution results from the control, test substance and reference plots are presented. In comparison to the control, the number of enchytraeids on the reference plots was reduced by 60 % at individual sampling dates, thus validating the test design. Mites were not affected at all. The number of nematodes decreased by 48 % at most. The test substance itself showed no significant effect at all, except an unexplained reduction of enchytraeids at the 4th sampling date. Genera of enchytraeids behaved slightly differently, suggesting that in future studies the species level should be addressed. Referring to the experiences made in this study it is concluded that the use of soil mesofauna groups in field studies is a practical and promising tool in the environmental risk assessment of pesticides.

Keywords: ecotoxicology, standardisation, grassland, Benomyl, Chlorpyrifos(ethyl)

1. Introduction

In the European Union, any new pesticide must be registered before it can be marketed. In Directive 91/414/EEC (EC 1991), the respective requirements, in particular the environmental risk assessment of these chemicals, are described, including potential effects on soil organisms. Two years ago the European Food Safety Agency expressed its Opinion No. 461 (EFSA 2007) about changes that should be addressed in the revision process of Directive 91/414/EEC (Part A of Annexes II and III). One of the opinions on the present ecotoxicological testing was that especially in the terrestrial environment not enough structural endpoints are used, i.e. potential effects on the composition of organism communities (= biodiversity). Such endpoints should be included more in future testing, in particular in the field.

Currently, earthworm field tests (ISO 1999) and litter bag studies (OECD 2006) are regularly required as part of the environmental risk assessment of pesticides in soil. The former test focuses exclusively on the earthworm community while the latter test is designed to assess soil function (organic matter breakdown). Especially, the litterbag test gives no indication if there is any structural impact, i.e. on the biodiversity of the soil organism community. Therefore, a design is presented here for such a structural field study focussing on the biodiversity of selected soil organism groups. It follows basically the design of litter bag studies. The new method is supposed to include invertebrates with different life forms (i.e.-soft versus hard-bodied groups or different feeding habits). In addition, recommendations concerning the performance of such a study will be made. They are based on practical experience in a case study, which was performed in Southern Germany in 2007/2008. The dynamics of three soil organism populations including changes of the community structure was followed over the period of 12 months. The taxonomic groups assessed were enchytraeids, soil-inhabiting mites, collembolans, and nematodes; collembolans will not be covered in this contribution. Due to confidentiality reasons name and properties of the tested pesticide (i.e. the test item) cannot be given here. Reference substances are chemicals which are known to affect soil organisms negatively at a given concentration. Such a substance is used in order to verify the sensitivity of the soil organism community at a study site: If the reference substance does not cause an effect on the tested organisms, the results of the whole study are not valid. According to the Earthworm Field Test guideline, such a confirmation has to be made only once: If effects are found at the first sampling date, no further action has to be taken (ISO 1999).

The objectives of this contribution can be summarised as follows:

–Proposal of a new field test method using the abundance and biodiversity of invertebrate soil organisms as endpoints.

–Presentation of experiences with such a field test under routine conditions. Since the identity of the tested pesticide cannot be revealed, this presentation will focus on methodological issues but will also show data from the pesticide, reference and control plots.

2. Materials and methods

2.1. Study site

For the study, a plot of commercial grassland was used. Grassland was chosen because diversity and abundance of enchytraeids, mites and nematodes (and soil mesofauna in general) is usually very low in agricultural soil. The site selected was located in Kraichtal-Oberöwisheim, in the Southwest of Germany. The area had no slope and had not received any treatments with pesticides during the last three years. The environmental conditions were recorded continuously using a data logger system. The parameters measured were air temperature at 2 m height (°C), soil temperature at 5 cm depth (°C) and rainfall (mm). The rainfall at the site (May 2007 to May 2008) was 100 % of the long-term average (855 l m^{-2}) with variation for the individual month, while the average air temperature between April 2007 and May 2008 was 10.9 °C. Actually, the average soil temperature in the 0–5 cm horizon was 10.9 °C, too, but the individual monthly values could vary by up to 2 °C in both directions. A composite soil sample (10 samples) from the 0–20 cm horizon was taken from randomly selected locations at the edge of the plots. This soil sample was used for soil characterisation and determination of microbiological activity. Microbial biomass was determined by measuring short-term respiration activity of soil samples after the addition of glucose with the OxiTop Systemâ (= 61.8 mg C/100 g dry weight).

Soil characterisation was carried out for the following parameters: determination of particle size distribution, soil type, pH value ($CaCl_2$-method), WHCmax (maximum water holding capacity), CEC (cation exchange capacity) and total organic carbon content. The soil type at the site was sandy silt (3.4 % clay, 59.9 % silt and 36.7 % sand), the pH value 7.3, the total organic content 3.57 %, WHCmax 57.1 g H_2O/100 g soil dry weight and the CEC 25.3 mval Ba/100g soil.

2.2. Study design

The trial consisted of three treatments: one control, one test item treatment and one reference item treatment. Each treatment consisted of 6 replicate plots (labelled a–f) measuring 3.0 m x 7.0 m in a randomised complete block design (see Fig. 1). There was a separation of at least 3 m between replicate plots and the distance to the edge of the field was at least 5 m. A central sampling area of 2.0 m x 6.0 m was defined within each plot. The sampling area was divided into five rectangles. On each sampling date three soil cores were taken from each square (see Fig. 2).

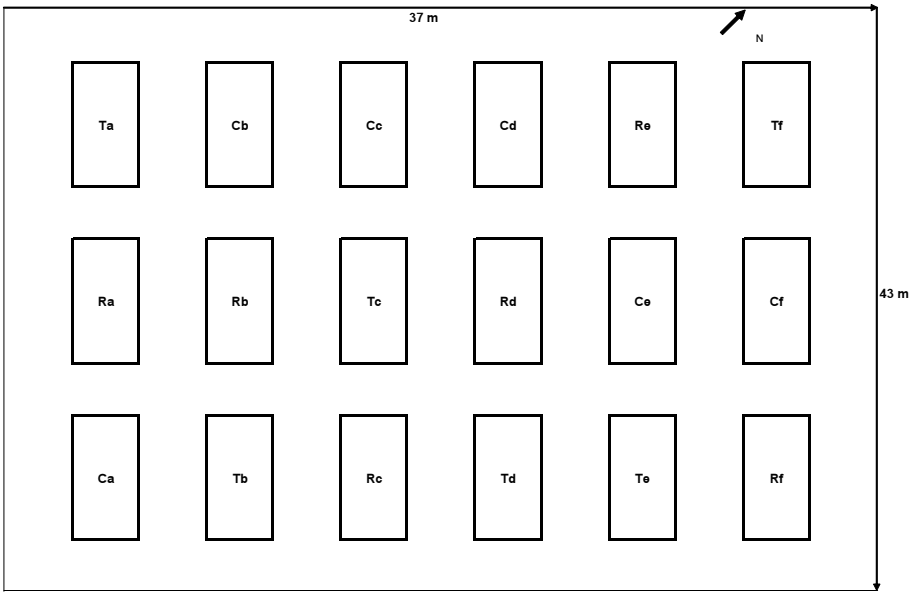

Fig. 1 General trial layout.

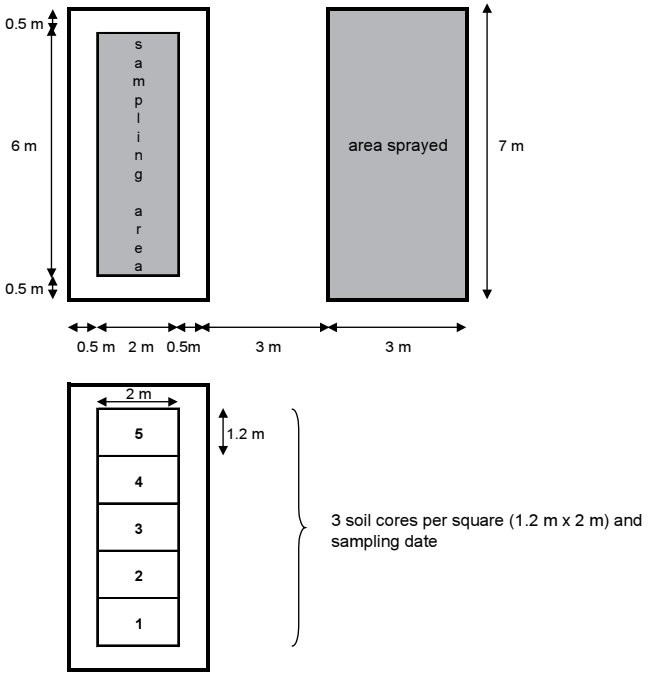

Fig. 2 Detailed plot design.

For maintenance the grassland was cut with a lawnmower 2 days before the first application and again on the day of the application. The plant material was removed. A second cut was performed immediately before the second application and the plant material stayed in the plots. The grass was cut as close to the ground as possible. Furthermore, the plots were cut several times during the study period and the grass material stayed in the plots. During the experimental period, no fertiliser and no additional pesticide treatments were applied. The vegetation cover was assessed on every sampling date throughout the test period. The vegetation cover in the individual plots was 60 to 100 % throughout the whole study period.

2.3. Application

To simulate a realistic exposure scenario, two applications of the test item with a 14-day interval were performed. The control was treated at the same time with water. The reference item was only applied once in parallel to the first application of the test item. It was sprayed as a tank mix, consisting of two pesticides (the fungicide Benomyl and the insecticide Chlorpyrifos(ethyl)). Benomyl is the required reference substance in standard earthworm field studies (ISO 1999) while the insecticide Chlorpyrifos(ethyl) is known to affect micro-arthropods, in particular Collembola, in laboratory tests and field studies (Frampton et al. 2006, Jänsch et al. 2006). The reference substances were applied together as a tank mixture in a rate of 8 kg benomyl ha^{-1} and 900 g chlorpyrifos ha^{-1} All applications were made with a 3-m boom sprayer and a water rate of 800 l ha^{-1}. The first application took place on 10 May 2007, the second application on 24 May 2007. The concentration of the test item in the soil was confirmed with analytical analysis of the treated soil. Neither the identity (including their physico-chemical properties) nor the concentrations of the test item can be given here due to confidentiality reasons.

2.4. Sampling

The study lasted for 12 months. Samples were taken before application to confirm the distribution of invertebrates in the soil and to produce a species list (Series 1: May 2007). Afterwards, four additional samplings were made mainly at favourable times, i.e. spring and autumn (series 2: June 2007; series 3: August 2007; series 4: November 2007; series 5: May 2008). Enchytraeids, soil mites (Acarina) and nematodes were sampled destructively using a soil corer. In total 15 soil cores were taken in each plot on each sampling occasion. Each soil core had a diameter of 5 cm with a surface area of 19.6 cm¸ and a depth of 5 cm. One sampling destroyed 0.25 % of the total sampling area in each plot. All samplings together took 1.25 % of the surface area away. For the soil mites 5 soil cores (5 cm in diameter) were collected to a soil depth of 5 cm, i.e. those layers in which the bulk of the micro-arthropods are living. Variation was supposed to be higher for the worms (enchytraeids and nematodes). Therefore 10 soil cores (5 cm in diameter) were collected to a soil depth of 5 cm, i.e. those layers in which the bulk of the enchytraeids and nematodes are living. This design resulted in 5 respective 10 individual assessment results for each replicated plot. Samples were taken before application (DAT –2), after 1 month (DAT 32), 3 (DAT 96), 6 (DAT 187) and 12 months (DAT 372) after application (DAT = days after application). All samples of one sampling date were taken during one day (except for sampling No. 5)

2.5. Extraction of enchytraeids and nematodes

The soil samples containing enchytraeids and nematodes were stored at temperatures of < 10 °C in a storage room and later transferred to a wet extractor within 4 weeks after arrival. According to the literature, this period is well within the range of two months during which no significant reduction of the numbers of Enchytraeidae occurs (Abrahamsen 1972). Enchytraeidae and Nematoda were extracted from the soil by wet extraction. Due to the necessity to determine enchytraeids in vivo, the samples were divided in three to four groups in random order. Each sample was kept in the extraction vessels for four days (duration determined based on the experience made in a pre-series collected in April 2007) to allow all worms to leave the soil. In principle, the extraction of the worms from the soil is caused by their active movements in the water-saturated sample. Plastic bowls containing sieves [mesh size 0.5–1 mm] were filled up with tap water until the empty sieves were completely covered by water. The samples (i.e. soil cores) were put into the sieves and carefully broken apart by hand. The bottom of the sieves did not reach the bottom of the bowls.

At the end of the extraction procedure the sieves were removed, the soil was discarded and disposed of. The water was slowly and carefully decanted from the bowl. The sediment at the bottom of the bowls was not disturbed, meaning that a small amount of water (up to a height of 5 mm to 10 mm) remained in the bowls. Subsequently this debris was placed in a Petri dish and briefly stored until soil particles had settled and the water became clear. Since the whitish worms are heavier than water but are rarely able to hide themselves in the narrow sediment layer they could easily be collected out of the Petri dish under a dissecting microscope. For this transfer a soft steel forceps was used. Due to their white colour the worms were clearly visible against the usually brownish soil particles. The animals were transferred to small glass vessels (about 20 ml). This extraction method is known to provide a good estimate of enchytraeid numbers (Didden et al. 1995) and follows recent international guidelines (ISO 2007).

2.6. Extraction of arthropods

At the testing facility samples were transferred inverted into a high gradient extractor within 7 hours after arrival. The arthropods were extracted from the soil using a high gradient extractor (MacFadyen 1961), a modified Berlese-Tullgren funnel. This apparatus consists of a light source mounted over a funnel containing a wire mesh that fits the circumference of the funnel. Soil invertebrates moved away from the heat and light, fell through the mesh and were funnelled into a jar of preservation fluid that killed and preserved the specimens. The fluid was either 59 % ethylene glycol or 5 % sodium benzoate in a jar at the bottom of the funnel. The extraction ran for 7 days with a starting temperature of 25 and an end temperature of 50° C.

2.7. Taxonomy

The following main taxonomic references were used to identify the collected enchytraeid specimen to genus level: Nielsen & Christensen (1959, 1961, 1963), Dózsa-Farkas (1992), Rota (1995), Schmelz (2003). Identification to species level was performed for the mite suborder Oribatida; all other adult mites were identified on the suborder level: Astigmata, Prostigmata, Uropodina and Gamasina **(**Balogh & Balogh (1992), Karg (1993), Schaefer (2000), Weigmann (2006), Wunderle et al. (1990)). In the case of the nematodes only the total abundance was determined, i.e. no further identification took place (Bongers 1988).

2.8. Statistics

Only taxa of a certain minimal abundance were included in the final statistical analysis because invertebrate taxa with very low abundances do not give any information on possible treatment effects, as chance effects become especially likely. The criteria for inclusion were an average of 1 individual per soil core calculated from the total numbers of the taxon in the control. Since multiple observations were available per plot, the mean abundance per plot was calculated prior to any analysis (the multiple observations came from the soil samples used for the extraction of arthropods/worms). The plot mean was then considered as the observed abundance for the plot. Observed abundances were transformed onto the log10 (abundance + 1) scale for statistical analysis. Reference and control were evaluated independently from each other.

For analysis, normality of the distribution was tested using the Shapiro-Wilk test ($p > 0.05$). Homogeneity of variance was tested with the F-test ($p > 0.05$). If the two groups evaluated had the same variance, the pooled-variance *t*-test was used. Otherwise, when the variances are not assumed to be equal, an approximate *t*-test (Satterthwaite's approximation) was used. If neither homogeneity of variance nor normality were observed, a Wilcoxon test was used on the data set. Comparisons were made between each treatment (test item (T) and reference (R)) and the untreated control (C). All such tests were carried out at the five per cent probability level. All statistical analyses of the data were carried out using SAS® for Windows, release 9.1.3 (SAS, Inc., Cary, NC, USA (2003)).

Results of the statistical analysis are summarised in tables (not shown for individual mite species) and graphs. Asterisks have been used to indicate statistically significant differences in abundance between the treatments and the control ($p \leq 0.05$). The figures for each taxon show the results for each test item plot compared to the range of the respective control over time. The reference is not graphically presented because only 2 samplings took place. Additionally, from the non-transformed values the average numbers of total mites, enchytraeids and nematodes were calculated for each treatment and assessment date. Using these values, the effect of the test item and the reference substance was determined according to Abbott (1925). This formula was used because of statistically equal numbers at the pre-application evaluation.

3. Results

An overview on the number of sampled invertebrates is given in Tab. 1.

Tab. 1 Total numbers of Acarina, Enchytraeidae and Nematoda in all samplings.

Taxon	Control (C)		Reference (R)		Treatment (T)	
	n	%	N	%	N	%
Acarina juv./damaged	23	0.44	8	0.32	19	0.31
Gamasina	1907	36.25	832	33.04	1857	30.44
Uropodina	7	0.13	4	0.16	5	0.08
Prostigmata	583	11.08	86	3.42	516	8.46
Astigmata	68	1.29	50	1.99	81	1.33
Achipteria coleoptrata	87	1.65	31	1.23	128	2.1
Scheloribates laevigatus	382	7.26	176	6.99	482	7.9
Scheloribates latipes	19	0.36	15	0.6	42	0.69
Scheloribates initialis	24	0.46	29	1.15	39	0.64
Ceratozetes mediocris	124	2.36	101	4.01	215	3.52
Eupelops occultus	390	7.41	137	5.44	368	6.03
Liebstadia similis	122	2.32	47	1.87	130	2.13
Tectocepheus velatus sarekensis	26	0.49	6	0.24	58	0.95
Nanhermannia sp. (juv.)	46	0.87	102	4.05	64	1.05
Nanhermannia nana	–	–	2	0.08	3	0.05
Minunthozetes semirufus	–	–	2	0.08	1	0.02
Protoribates capucinus	–	–	1	0.04	–	–
Oppiella nova	3	0.06	15	0.6	8	0.13
juvenile Oribatida	1449	27.55	874	34.71	2085	34.17
Oribatida total	**2671**	**–**	**1538**	**–**	**3623**	**–**
Acarina total	**5260**	**100**	**2518**	**100**	**6101**	**100**
Achaeta	522	4.66	68	7.23	393	4.52
Buchholzia	1902	16.96	24	2.55	1501	17.27
Enchytraeus	2113	18.84	200	21.25	1773	20.4
Enchytronia	197	1.76	17	1.81	122	1.4
Fridericia	3274	29.2	370	39.32	2452	28.22
Henlea	301	2.68	30	3.19	198	2.28
Marionina	2435	21.72	197	20.94	1860	21.4
unidentified Enchytraeidae	469	4.18	35	3.72	391	4.5
Enchytraeidae total	**11213**	**100**	**941**	**100**	**8690**	**100**
Nematoda	**20291**	**–**	**2793**	**–**	**20370**	**–**

n – sum of all individuals caught over all assessments

3.1. Enchytraeidae

The following 19 species were found at the study site: *Achaeta* sp. 1 *'*dzwilloi*'*, *A. eiseni, A. pannonica*, *Buchholzia appendiculata*, *Cernosvitoviella* sp.*, Cognettia sphagnetorum*, *Enchytraeus buchholzi* agg. sp. 1, *Enchytraeus buchholzi* agg. sp. 2*, Enchytronia* sp., *Fridericia galba*, *F. bisetosa*, *F. connata*, *F. isseli*, *F. paroniana*, *F. perrieri*, *F. ratzeli*, *Fridericia* sp. 1, *Henlea perpusilla*, *Marionina argentea*, *M. brendae*, *M. communis***.**

The most abundant enchytraeid genera in descending order were *Fridericia*, *Marionina*, *Enchytraeus* and *Buchholzia*. In the control and the test item plots, 11 213 and 8690 enchytraeids were found, respectively (Tab. 1). The total number of Enchytraeidae, the genera *Buchholzia*, *Henlea*, *Fridericia*, and *Marionina* were statistically significantly affected through the toxic reference application (1 month after application). The effect of the reference substances on the total number of Enchytraeidae was also confirmed by the calculations according to Abbot (1925). Since an effect of the reference substance on the enchytraeids could be proven in the first sampling after application, no further samples were taken from the reference plots (Fig. 3 and Tab. 2). The mean number of Enchytraeidae and the genera *Buchholzia*, *Enchytraeus* and *Marionina* were statistically significantly reduced by the test item at the fourth sampling date. No significant differences were observed at the first two and the last sampling 12 months after application. The other three genera *Achaeta*, *Fridericia* and *Henlea* were never affected by the test item.

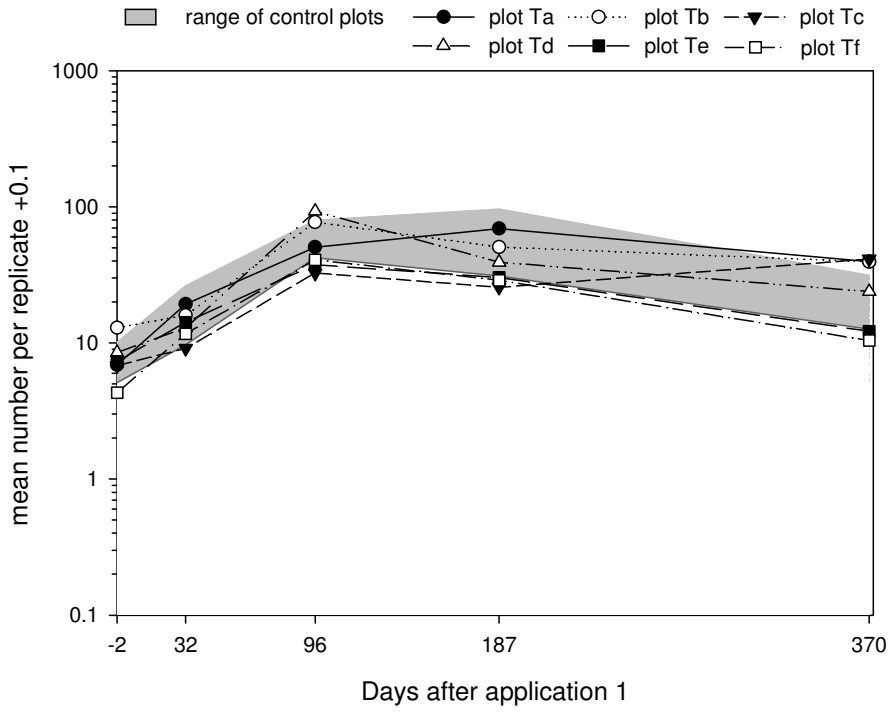

Fig. 3 Abundance of Enchytraeidae (all taxa pooled) in the test item plots.

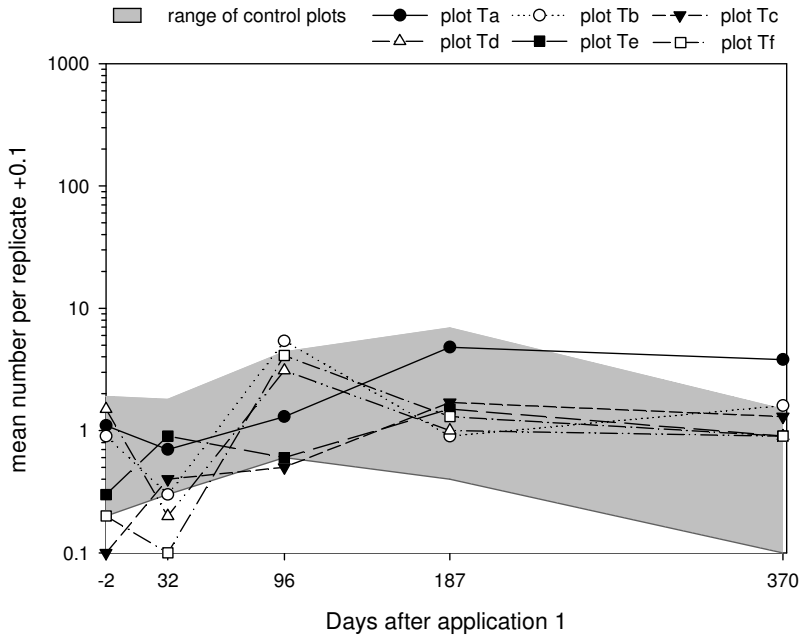

Fig. 4 Abundance of the genus *Achaeta* (Enchytraeidae, all species pooled) in the test item plots.

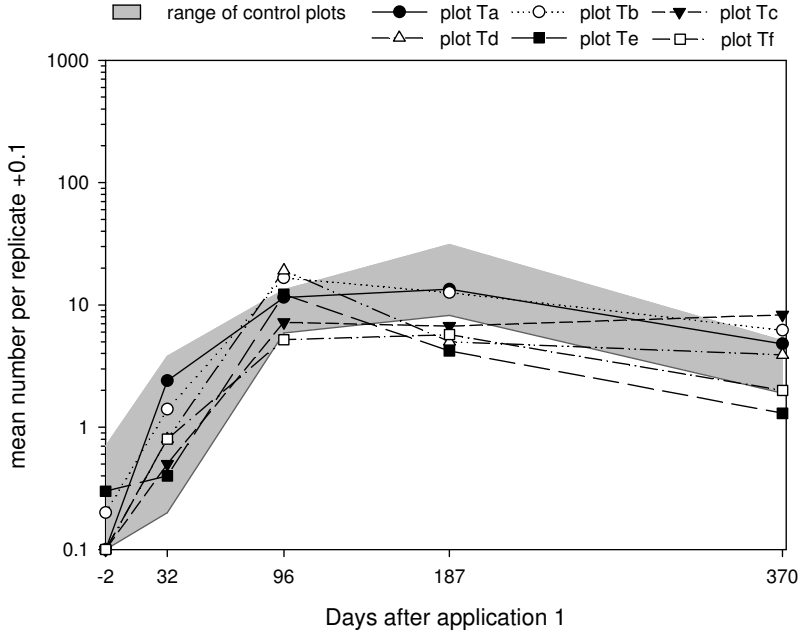

Fig. 5 Abundance of the genus *Buchholzia* (Enchytraeidae, all species pooled) in the test item plots.

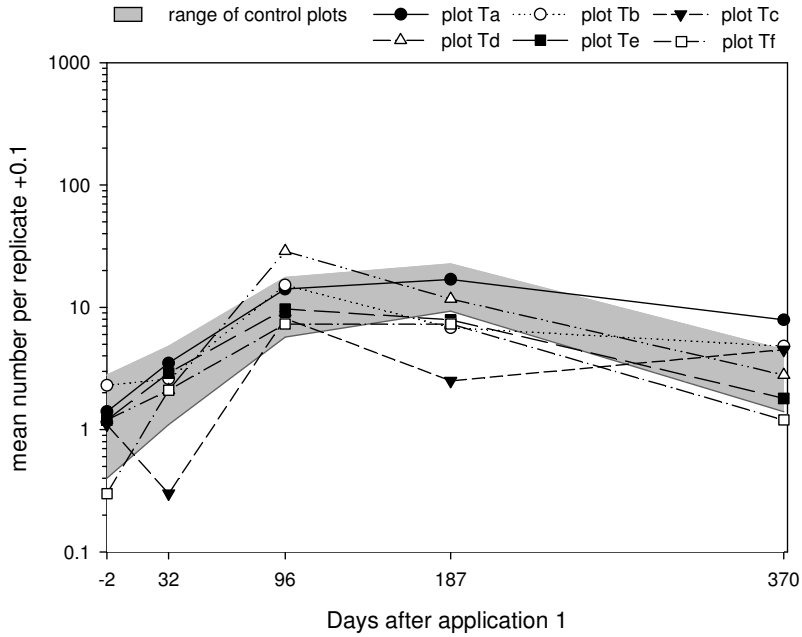

Fig. 6 Abundance of the genus *Enchytraeus* (Enchytraeidae, all species pooled) in the test item plots.

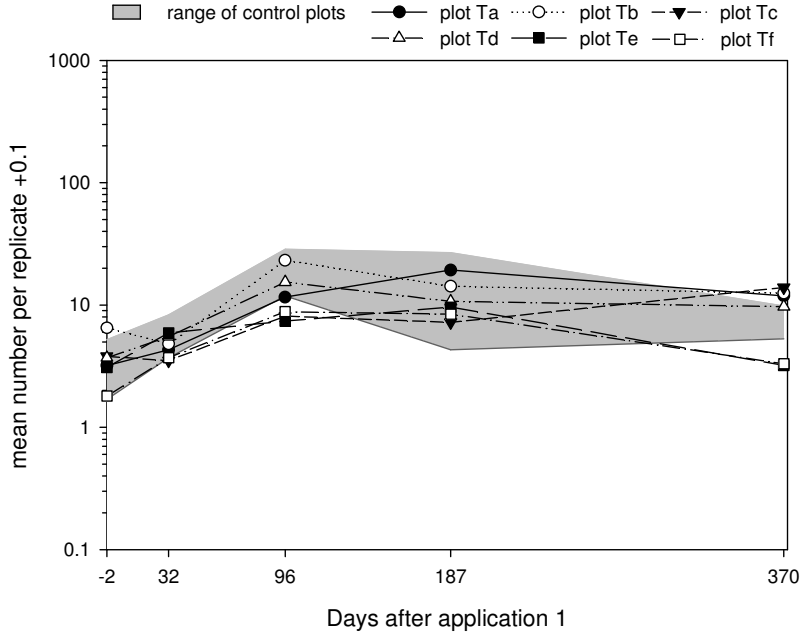

Fig. 7 Abundance of the genus *Fridericia* (Enchytraeidae, all species pooled) in the test item plots.

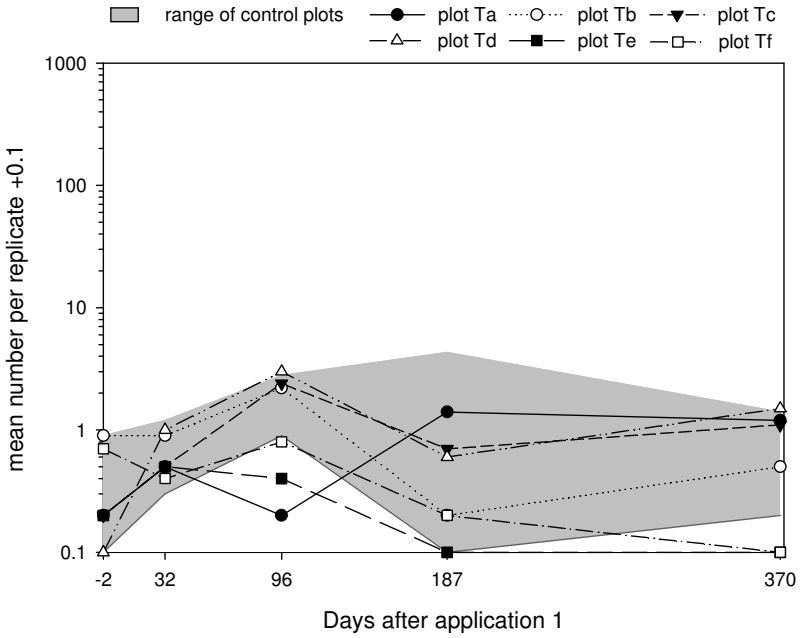

Fig. 8 Abundance of the genus *Henlea* (Enchytraeidae, all species pooled) in the test item plots.

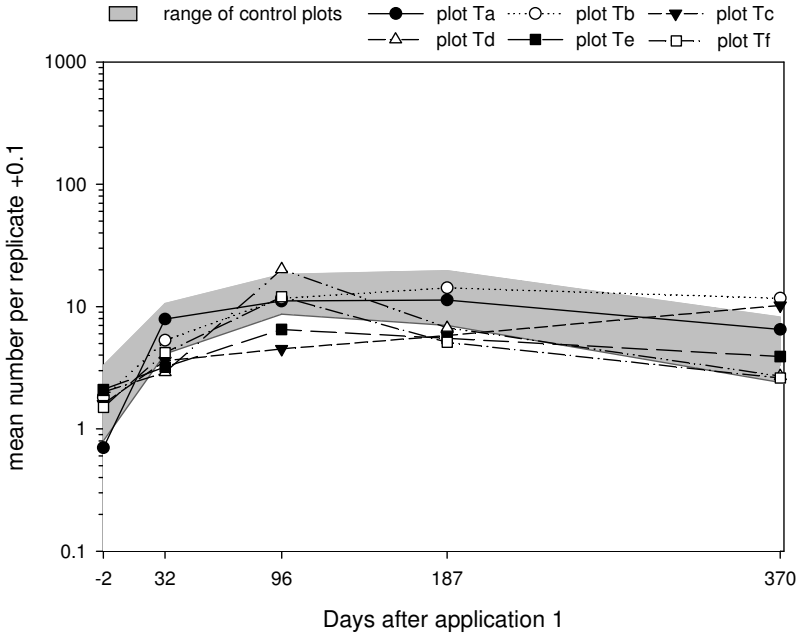

Fig. 9 Abundance of the genus *Marionina* (Enchytraeidae, all species pooled) in the test item plots..

Tab. 2 Mean number of Enchytraeidae (all taxa pooled) in all soil cores (Series 1: May 2007; series 2: June 2007; series 3: August 2007; series 4: November 2007; series 5: May 2008).

taxa	sampling	treatment	mean/plot						mean/ sampling	SD	CV
			a	b	c	d	e	f			
total Enchytraeidae	1	Control	10	5	9.8	9.2	7.4	6.5	8	2.01	0.25
		R	7.3	7	8.6	10.1	14.1	4.5	8.6	3.27	0.38
		T	6.8	12.8	6.7	8.4	7.2	4.2	7.7	2.86	0.37
	2	Control	14.3	26.2	23	20	15.9	9.6	18.2	6.08	0.33
		R	9.5	4.8	8.5	5	10.3	4.4	7.1*	2.64	0.37
		T	19.2	15.9	9	12.8	14.1	11.6	13.8	3.54	0.26
	3	Control	42.2	77.8	79.8	69.2	47.1	55.4	61.9	15.97	0.26
		T	50.4	77.1	32.5	92.6	37.5	40.7	55.1	24.25	0.44
	4	Control	73.2	95.4	96.1	91	78.2	30.9	77.5	24.65	0.32
		T	69.2	50.6	25.5	39	30.2	28.8	40.6*	16.71	0.41
	5	Control	20.2	31.2	23.8	12.5	22.2	18.2	21.4	6.22	0.29
		T	39.5	39.4	41.2	23.7	12.1	10.3	27.7	14.29	0.52

* significantly different to control ($p \leq 0.05$)

Tab. 3 Mean number of *Achaeta* in all soil cores.

taxa	sampling	treatment	mean/plot						mean/ sampling	SD	CV
			a	b	c	d	e	f			
Achaeta (Echytraeidae)	1	Control	1.8	0.7	0.9	0.1	0.1	0.8	0.7	0.63	0.86
		R	1.8	0.4	1	0.2	1.7	0.3	0.9	0.72	0.8
		T	1	0.8	0	1.4	0.2	0.1	0.6	0.57	0.97
	2	Control	0.2	0.4	0.3	0.5	1.7	0.2	0.6	0.58	1.05
		R	0.4	0	0.1	0.1	0.7	0.1	0.2	0.27	1.15
		T	0.6	0.2	0.3	0.1	0.8	0	0.3	0.31	0.92
	3	Control	0.5	2.7	4.1	4.3	3.3	1.8	2.8	1.45	0.52
		T	1.2	5.3	0.4	3	0.5	4	2.4	2.02	0.84
	4	Control	3.4	4	5.8	6.8	3.6	0.3	4	2.25	0.56
		T	4.7	0.8	1.6	0.9	1.4	1.2	1.8	1.47	0.83
	5	Control	1.4	0	0.7	0.6	0.2	1	0.7	0.51	0.79
		T	3.7	1.5	1.2	0.8	0.8	0.8	1.5	1.13	0.77

* significantly different to control ($p \leq 0.05$)

Tab. 4 Mean number of *Buchholzia* in all soil cores.

taxa	sampling	treatment	mean/plot						mean/ sampling	SD	CV
			a	b	c	d	e	f			
Buchholzia (Echytraeidae)	1	Control	0.2	0.2	0.6	0.3	0	0	0.2	0.22	1.03
		R	0.1	0.1	0	0	0.2	0.4	0.1	0.15	1.13
		T	0	0.1	0	0	0.2	0	0.1	0.08	1.67
	2	Control	0.7	3.7	1.5	1.1	1.1	0.1	1.4	1.24	0.91
		R	0.6	0.2	0.3	0.1	0.4	0	0.3*	0.22	0.81
		T	2.3	1.3	0.4	0.7	0.3	0.7	1	0.75	0.79
	3	Control	5.8	8.8	13.2	8.5	6.4	6	8.1	2.8	0.35
		T	11.4	16.5	7.1	19	12.1	5.1	11.9	5.31	0.45
	4	Control	20.1	20.6	31.1	18.1	15.6	8.1	18.9	7.5	0.4
		T	13.3	12.5	6.6	4.9	4.1	5.6	7.8*	4.02	0.51
	5	Control	3.5	5	2.9	1.8	3.1	2.1	3.1	1.14	0.37
		T	4.7	6.1	8.2	3.8	1.2	1.9	4.3	2.62	0.61

* significantly different to control ($p \leq 0.05$)

Tab. 5 Mean number of *Enchytraeus* in all soil cores.

taxa	sampling	treatment	mean/plot						mean/ sampling	SD	CV
			a	b	c	d	e	f			
Enchytraeus (Echytraeidae)	1	Control	0.4	0.5	2.7	1.1	2.3	0.3	1.2	1.04	0.85
		R	3.1	0.8	1.8	1.2	3.4	1.2	1.9	1.09	0.57
		T	1.3	2.2	1	1.1	1.1	0.2	1.2	0.64	0.56
	2	Control	1.9	4	4.7	4	2.5	1	3	1.44	0.48
		R	2.1	0.2	1.6	0.9	3	0.7	1.4	1.03	0.72
		T	3.4	2.5	0.2	2	2.8	2	2.2	1.09	0.51
	3	Control	5.6	16.9	17.5	14.2	8.9	10.7	12.3	4.71	0.38
		T	14	15.1	8	28.7	9.6	7.2	13.8	7.98	0.58
	4	Control	10	15.1	15.2	21.5	22.5	9.2	15.6	5.57	0.36
		T	16.8	6.7	2.4	11.6	7.8	7.2	8.8*	4.91	0.56
	5	Control	2.8	4.4	3.5	1.3	3.7	2.9	3.1	1.06	0.34
		T	7.8	4.7	4.4	2.7	1.7	1.1	3.7	2.45	0.66

* significantly different to control ($p \leq 0.05$)

Tab. 6 Mean number of *Fridericia* in all soil cores.

taxa	sampling	treatment	mean/plot						mean/ sampling	SD	CV
			a	b	c	d	e	f			
Fridericia (Echytraeidae)	1	Control	3.5	2.3	3.7	5.1	3	1.6	3.2	1.21	0.38
		R	1	4.2	3.6	4.4	5.2	1.7	3.4	1.65	0.49
		T	3.1	6.4	3.7	3.6	3	1.7	3.6	1.55	0.43
	2	Control	5.9	8.2	4.8	6.8	4.5	3.6	5.6	1.68	0.3
		R	2.9	2.3	4.1	1.7	4.1	1.8	2.8*	1.08	0.38
		T	4.2	4.7	3.4	5.4	5.8	3.6	4.5	0.96	0.21
	3	Control	13.7	28.4	22.4	22.6	11.8	15.1	19	6.45	0.34
		T	11.5	23.1	8	15.3	7.3	8.7	12.3	6.05	0.49
	4	Control	22.4	26.6	24.1	20.2	20	4.2	19.6	7.93	0.41
		T	19.2	14.1	7.1	10.6	9.5	8.3	11.5	4.48	0.39
	5	Control	6.4	9.7	8	5.2	6.8	6.8	7.2	1.54	0.22
		T	11.8	12.4	13.8	9.6	3.1	3.2	9	4.72	0.53

* significantly different to control ($p \leq 0.05$)

Tab. 7 Mean number of *Henlea* in all soil cores.

taxa	sampling	treatment	mean/plot						mean/ sampling	SD	CV
			a	b	c	d	e	f			
Henlea (Echytraeidae)	1	Control	0.8	0	0.1	0.4	0.3	0.2	0.3	0.28	0.94
		R	0	0.3	0.3	1	1	0	0.4	0.46	1.06
		T	0.1	0.8	0.1	0	0.1	0.6	0.3	0.33	1.17
	2	Control	0.3	0.2	0.6	1.1	0.5	0.5	0.5	0.31	0.59
		R	0.1	0	0.2	0	0	0.1	0.1*	0.08	1.22
		T	0.4	0.8	0.4	0.9	0.4	0.3	0.5	0.25	0.47
	3	Control	1.7	2	2.6	2.7	0.8	2.4	2	0.71	0.35
		T	0.1	2.1	2.3	2.9	0.3	0.7	1.4	1.18	0.84
	4	Control	0.6	4.2	1.7	1.9	1.1	0	1.6	1.46	0.92
		T	1.3	0.1	0.6	0.5	0	0.1	0.4	0.49	1.13
	5	Control	1.3	0.4	0.5	0.2	0.1	0.9	0.6	0.45	0.8
		T	1.1	0.4	1	1.4	0	0	0.7	0.6	0.92

* significantly different to control ($p \leq 0.05$)

Tab. 8 Mean number of *Marionina* in all soil cores.

taxa	sampling	treatment	mean/plot						mean/ sampling	SD	CV
			a	b	c	d	e	f			
Marionina (Echytraeidae)	1	Control	3	0.7	1.6	1.2	1.3	3.2	1.8	1.03	0.56
		R	0.6	0.8	1	3.1	2	0.6	1.4	1	0.74
		T	0.6	1.7	1.5	1.9	2	1.4	1.5	0.5	0.33
	2	Control	4.5	8.5	10.5	5.4	5.1	4	6.3	2.58	0.41
		R	3	1.9	1.8	2.2	1.4	1.3	1.9*	0.62	0.32
		T	7.8	5.2	3.5	2.8	3.1	4.1	4.4	1.86	0.42
	3	Control	8.6	14.4	14.7	12.8	14.8	18.2	13.9	3.15	0.23
		T	11	11.6	4.4	20.1	6.4	11.9	10.9	5.45	0.5
	4	Control	11.3	19.6	15.9	17.6	10.6	6.9	13.7	4.83	0.35
		T	11.2	14.1	5.7	6.6	5.4	5	8.0*	3.75	0.47
	5	Control	3.3	8.1	5.3	2.3	6.6	3.5	4.9	2.22	0.46
		T	6.4	11.6	10.1	2.6	3.8	2.5	6.2	3.92	0.64

* significantly different to control ($p \leq 0.05$)

3.2. Micro-arthropods (Acarina)

The following higher mite taxa and oribatid species were found at the study site: Gamasina, Uropodina, Prostigmata, Astigmata, *Achipteria coleoptrata, Scheloribates laevigatus, Scheloribates latipes, Scheloribates initialis, Ceratozetes mediocris, Eupelops occultus, Liebstadia similis, Tectocepheus velatus sarekensis, Nanhermannia* sp. (juv.), *Nanhermannia nana, Minunthozetes semirufus, Protoribates capucinus* and *Oppiella nova* (see Tab. 1). In total, 13 879 Acarina were extracted from the soil cores. More than half of the Acarina collected (50.8 %) in the control plots belonged to the suborder Oribatida. Other abundant mite taxa were the Gamasina (36.3 %) and the Prostigmata (11.1 %). Five Acarina taxa were collected in numbers higher than 30 specimens (at least subdominant: 3.2 %) in at least two sampling occasions during the study period in the control and were therefore statistically analysed.

Mean numbers of total Acarina, total Oribatida and the juvenile Oribatida in the toxic reference plots were statistically significantly higher in the first sampling before the applications. No differences occurred in the second sampling 1 month after application. No significant differences were observed for all Acarina taxa statistically evaluated between the control and the reference substances or the test item treatments, respectively, throughout the whole study period. The single taxa further evaluated were the mite orders Gamasina, Prostigmata, Oribatida and its juveniles as well as the two species *Scheloribates leavigatus* and *Eupelops occultus.* Details are shown in Tab. 9 and in Figs 10–16.

Tab. 9 Mean number of Acarina (all taxa pooled) in all soil cores.

taxa	sampling	treatment	mean/plot						mean/ sampling	SD	CV
			a	b	c	d	e	f			
total Acarina	1	Control	23.6	25.2	31.6	24.8	27	22	25.7	3.34	0.13
		R	29.6	27.6	33.4	31.2	28	37.6	31.2*	3.78	0.12
		T	35.8	12.6	42.6	25.6	29.2	22	28	10.53	0.38
	2	Control	48	35	59.6	26.6	33.4	26.2	38.1	13.16	0.35
		R	53.8	71.2	63.2	69.2	27	31.8	52.7	19.1	0.36
		T	24.4	57	63.4	30.2	42.2	28.6	41	16.16	0.39
	3	Control	23.2	31.4	18.8	19.4	27.6	19	23.2	5.25	0.23
		T	33.6	45.4	24.6	23	25.8	28.4	30.1	8.35	0.28
	4	Control	66.4	91	58.4	30.4	42.8	32.8	53.6	23.12	0.43
		T	60.6	71.2	74	19.4	70	60	58.8	20.33	0.35
	5	Control	46.2	66	23	22.6	33.4	16.6	34.6	18.57	0.54
		T	37.4	69.4	44.4	58.8	47.6	39	49.4	12.4	0.25

* significantly different to control ($p \leq 0.05$)

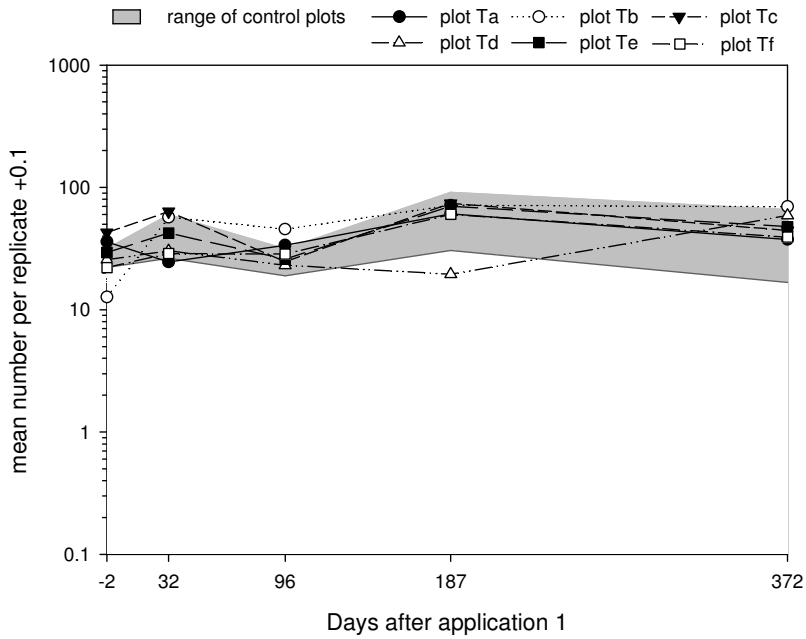

Fig. 10 Abundance of the Acarina (all taxa pooled) in the test item plots.

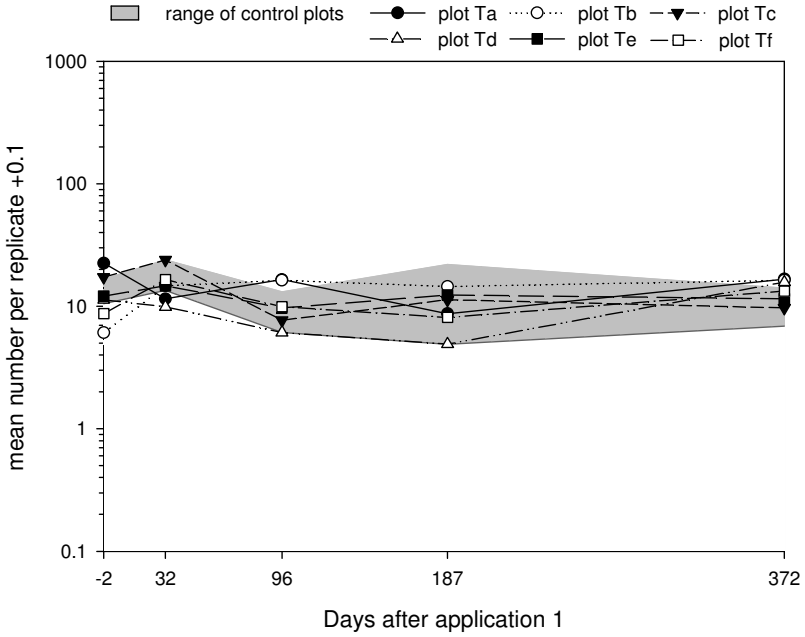

Fig. 11 Abundance of the Gamasina (all taxa pooled) in the test item plots.

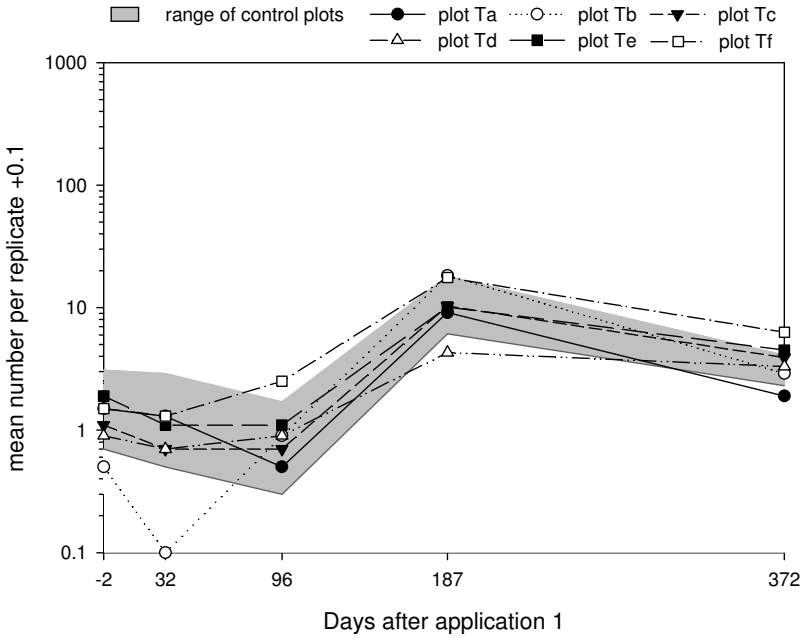

Fig. 12 Abundance of the Prostigmata (all taxa pooled) in the test item plots.

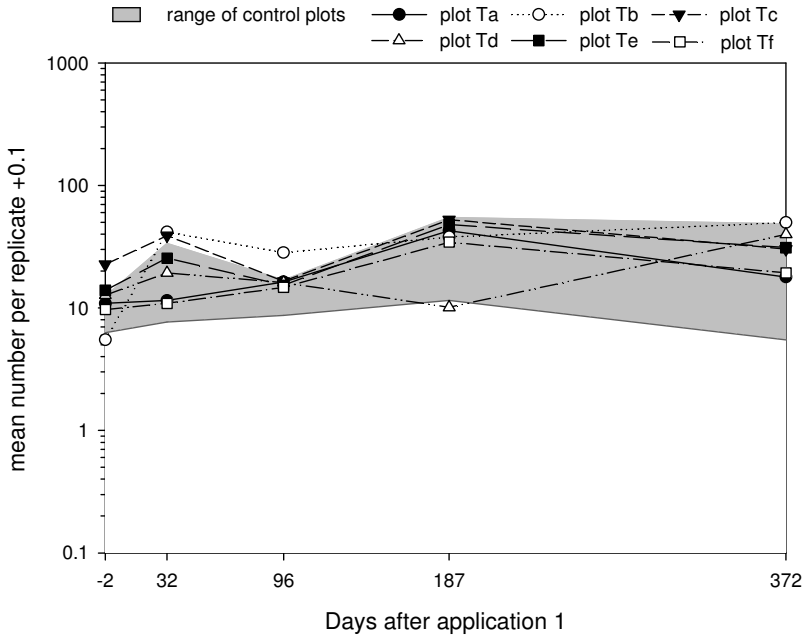

Fig. 13 Abundance of the Oribatida (all taxa pooled) in the test item plots.

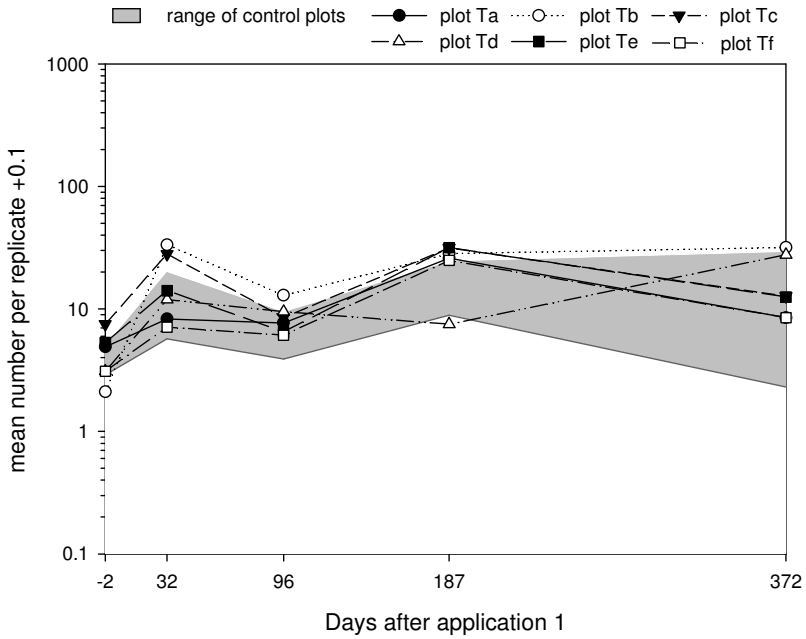

Fig. 14 Abundance of the juvenile Oribatida (all taxa pooled) in the test item plots.

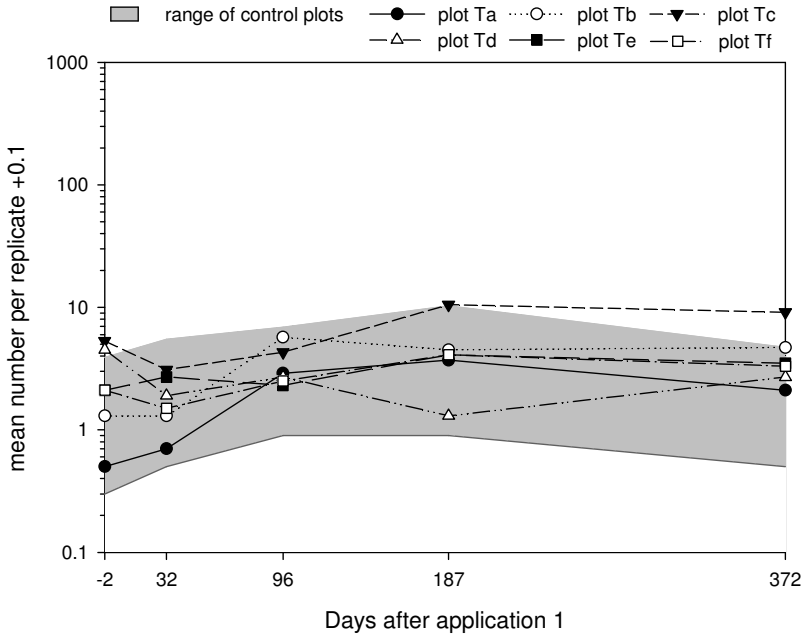

Fig. 15 Abundance of the species *Scheloribates laevigatus* (Acarina, Oribatida) in the test item plots.

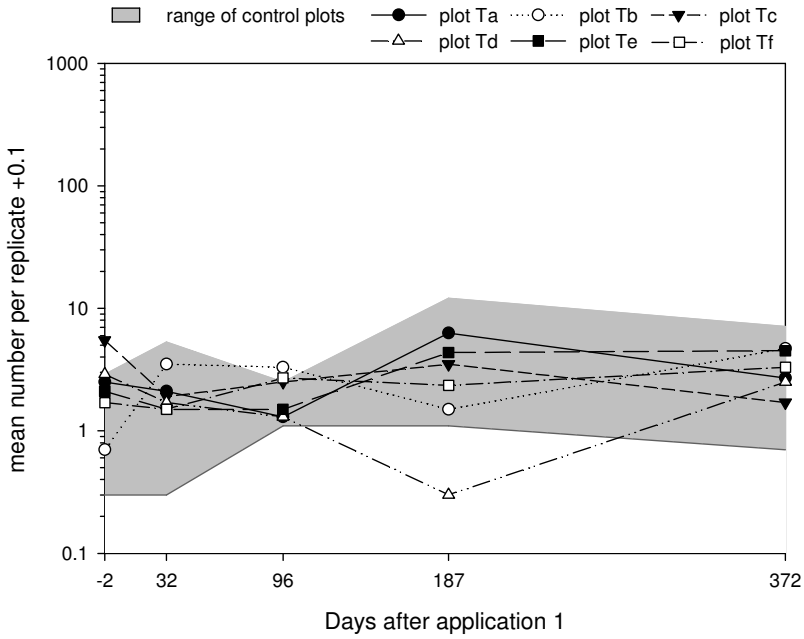

Fig. 16 Abundance of the species *Eupelops occultus* (Acarina, Oribatida) in the test item plots.

3.3. Nematoda

43 453 Nematoda were extracted from soil cores taken on 5 sampling occasions (Tab. 10). Out of this total number 20 291 Nematoda were collected in the control plots. The nematodes were assessed quantitatively; no further identification was carried out. The Nematoda showed a statistically significant reduction in mean numbers in the toxic reference compared to the control in the second sampling (1 month after application). Compared to the other groups, the variability of nematode numbers over time was very low (Fig. 17).

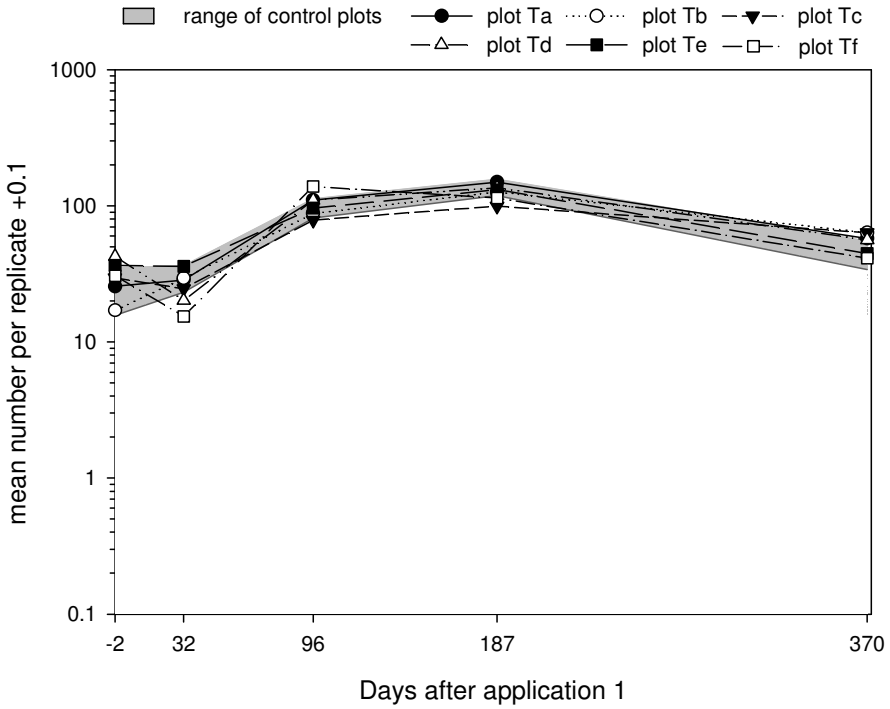

Fig. 17 Abundance of total Nematoda (all taxa pooled) in the test item plots.

No significant differences were observed between the test item and the control. Similar results were gained when using Abbott's formula (1925) where the highest effect was a reduction of 14 % (Tab. 11).

Tab. 11 Effects of reference and test items according to Abbott (1925).

Taxa	Sampling	Reference	Test item
total Acarina	1	–21.4	–8.9
	2	–38.3	–7.6
	3	–	–29.7
	4	–	–9.7
	5	–	–42.8
total Enchytraeidae	1	–7.5	3.8
	2	61	24.2
	3	–	11
	4	–	47.6
	5	–	–29.4
total Nematoda	1	–23.4	–15.7
	2	51.7	14.1
	3	–	–7.4
	4	–	9.3
	5	–	–15.5

4. Discussion

4.1. Enchytraeidae

In many soils world-wide, enchytraeids (Annelida: Oligochaeta) play a major role in soil functions like nutrient cycling. By feeding on bacteria they control the population dynamics of the microflora, thus they are indirectly involved in decomposition processes. In addition, standardised sampling methods are available and taxonomic as well as ecological knowledge is rapidly increasing. Enchytraeidae belong to the saprophagous mesofauna of the litter layer and the upper mineral soil. Through their feeding activity, the soil assumes a fine-grained 'crumb' structure with an often higher stability than that of the bulk soil (Didden 1990).

The number of Enchytraeidae at the five sampling dates differed by about a factor of 10, first of all due to seasonal reasons (Fig. 3). Surprisingly, their number increased already in August – more commonly such an increase is observed in September (Didden 1993). The relatively low absolute numbers of Enchytraeidae at this grassland site, varying between 4000 and 35 000 ind. m^{-2}, are within the range known for German grassland sites (2700–49 000 ind. m^{-2}) (Didden 1993). In Central Europe, the average number, based on about 15 studies, is 20 600 ± 12 600 ind. m^{-2} (Römbke et al. 1997). No differences in enchytraeid abundance were found at the various plots before application (DAT –2; mean 4000 ± 1500 ind. m^{-2}). After application their number decreased by 60 % in the plots with the reference substances but no distinct effect of the test item was observed (except, for unknown reasons, at the fourth sampling date). No other field studies about the effects of Benomyl on enchytraeids are known but severe effects of closely related fungicides such as carbendazim on enchytraeids have been found in laboratory tests and field studies (Didden & Römbke 2001, Jänsch et al. 2006). At the last sampling date, still no difference between control and test item plots occurred (the reference plots were not sampled any further). As expectable, the variability in numbers between individual plots and dates is higher on the genus level compared to the population level (Figs 4–9). In accordance, variability is also higher for genera low in absolute numbers. In one case, there may have been an effect of the test substance: The genus *Buchholzia*, represented by the single species *B. appendiculata*, was almost missing at DAT –2, while getting frequent and abundant at later dates (DAT 96 and after). This pattern may be explained by the fact that this species reproduces by fragmentation (i.e. very quickly). Therefore, it is known as an indicator species for 'disturbed' habitats, e.g. in terms of anthropogenic stress (Jänsch et al. 2005).

At the study site, the dominant genus was *Fridericia* with about 40 % of all specimens. No clear differences in the dominance spectrum occurred after application of the reference substance or the test substance. The composition of the enchytraeid community on the genus level was comparable to other German meadow sites, meaning that genera like *Fridericia* and *Enchytraeus* are dominant. However, the number of species (19) is higher than the average number reported from the same Central European grasslands mentioned above, where 10.7 ± 8.2 species were found (Römbke et al. 1997). This may be explained as follows:

– In the past, it was difficult to separate individual species of the complex genus *Fridericia*. Only recently this situation has improved (Schmelz 2003);

– some of the rarely recorded species are usually found only in wet soils (e.g. *Cognettia sphagnetorum, Cernosvitoviella* sp.);

– they do not belong to the 'normal' inhabitants of grassland sites. Their occurrence may be explained by the adjacent ditch. However, due to their low numbers, they did not have a relevant influence on the whole community.

Methodologically, sampling and extracting of enchytraeids were not problematic, which is probably due to the fact that these actions followed the standard ISO guideline (ISO 2007). In addition, the determination of genera (and, in fact, species) was not problematic since the enchytraeid fauna of Central European grassland sites is relatively well known, i.e. keys working on the genus level (e.g. Nielsen & Christensen 1959) or, at least for the most important genus *Fridericia*, on the species level (Schmelz 2003) were available. Due to the very high number of samples which had to be investigated (720 in total) it was not possible to address the species level routinely, but this would certainly be possible assuming that resources are sufficient.

The enchytraeid part of the study has to be considered as valid since the reference substances (most probably Benomyl) decreased the number of these small annelids significantly one month after application. In general, the variability at the individual sampling dates was small, meaning that the overall test design (i.e. the number of individual samples per plot (10) and the number of plots per date (six) are considered to be sufficient for getting reliable results. However, one must note that taxa occurring in low numbers show considerably higher variability, for example in the case of the genera *Henlea* (Fig. 8) and *Achaeta* (Fig. 4). Maybe this high variability is an indication of ecological differences between the individual species belonging to these genera: For example, in *Achaeta* such differences are known between species, e.g. concerning preferences for specific soil properties of soil layers, while the ecological requirements of *Henlea*-species seem to be quite similar (Graefe & Schmelz 1999). These results suggest that in further studies the species level should be addressed. No explanation can be given why at the fourth sampling date the mean numbers of Enchytraeidae and the genera *Buchholzia*, *Enchytraeus* and *Marionina* were statistically significantly reduced by the test item. However, with respect to the absolute differences this reduction was relatively small. Since this effect occurred only at one date and did not last, it is considered to be ecologically irrelevant. Information of the sensitivity of different enchytraeid genera towards pesticides is rare, but it is sure that on this level differences can be identified: In a field study with the fungicide Carbendazim species of the genera *Fridericia* and, partly, *Henlea*, reacted stronger than species of the genera *Buchholzia*, *Enchytraeus* and *Marionina* (Moser et al. 2007). Clearly, such differences depend on the respective test substance, making any comparison difficult if not impossible when their identity is not known.

4.2. Acarina

The Acarina community was dominated by the Oribatida (armoured or moss mites) and the Gamasina (predatory mites). The Oribatida usually occur in high numbers in the upper soil layer, feeding mainly on decaying plants, fungi etc., thus having a central role in the soil food web (Walter & Proctor 1999). Oribatid mites generally have low metabolic rates, slow development and low fecundity. Adults live for a relatively long time; for example, estimates of development time from egg to adult vary from several months to two years in temperate soils. The role of oribatids for bioindication in agricultural soils was recently reviewed by Behan-Pelletier (1999). She used single species as indicators for soil quality after having integrated their life-history tactics, but the oribatid community is also valuable for assessing the impact of pesticides on oribatids (Siepel 1995). However, the sensitivity of an oribatid species *(Platynothrus peltifer*) in laboratory tests was found to be low, meaning that it was not standardised by OECD (Van Gestel & Doornekamp 1998). In addition, the taxonomy of these mites is difficult, but a new key has recently become available (Weigmann 2006). Predatory mites are also taxonomically quite diverse. They do not have a direct influence on the decomposition of organic material in the soil, but by preying on saprophagous mesofauna species such as enchytraeids they affect nutrient cycling considerably (Heal & Dighton 1985).

Numbers of Acarina were relatively constant throughout the whole study period. For meadows in Central Europe abundance between 10 000 and 40 000 ind. m^{-2} is typical (Römbke et al. 1997). At the field site numbers of Oribatida varied between 4600 and 19 300 ind. m^{-2}. Therefore, the community represents a typical meadow site in central Europe. Mean numbers were only reduced in the control and in the test item plots at the beginning of the study and at the third sampling. In contrast to the other taxa analysed the Acarina were not negatively affected by the toxic reference substances. Therefore, this part of the field study, referring to mites, cannot be considered as being valid. The non-sensitivity for this order was also confirmed for the juveniles and the two single species that were present in sufficient numbers for analysis. An additional hint confirming their often low sensitivity is the fact that up to now a test with oribatid mites has not been included in pesticide registration (Løkke & Van Gestel l998). However, a test with predatory mites was standardised recently and is regularly required (OECD 2008). In summary, the inclusion of mites in such field studies can be recommended, but a standardised sampling and extraction method should be used (ISO 2006).

4.3. Nematoda

Free-living terrestrial nematodes are the most abundant and also diverse metazoa in soils, reaching densities up to 20 millions per square metre (Bongers 1988). They interact closely with other soil organisms and contribute considerably to soil biological processes (e.g. decomposition of organic matter) which constitute them as important members of the soil fauna (Ettema 1998). The nematode fauna possesses a high potential to serve as an instrument for the environmental risk assessment of chemicals because of its high density and diversity, high colonisation rate, slight active movement and ease of isolation. Additionally, nematodes are in direct contact with soil pore water and dissolved xenobiotics (Bongers & Ferris 1999).

As in enchytraeids the abundance of nematodes followed a clear seasonal cycle, with high numbers in autumn and lower numbers in spring. No detailed information is available about the normal cycles or the absolute numbers to be expected at grassland sites. When sampling seven German grasslands once the total abundance of all nematode groups (except plant parasites) ranged between 60 000–130 000 ind. m^{-2}, but at one disturbed grassland site (more or less a fallow) only 24 000 nematodes m^{-2} were sampled (Römbke et al. 2002). At the study site the absolute abundance differed between 15 000 and 65 000 ind. m^{-2}. However, data cannot be compared because in the former study nematodes were extracted with a specific method while here only those nematodes were counted which were found in the enchytraeid extraction samples. In other words, the 'real' number of nematodes at the study site is probably higher. This difference however does not impede the evaluation of this study, since the methodological bias is similar at all plots. The results of the nematode part of the study are valid, since the reference substances caused a reduction up to 48 %. No effect of the test item on nematodes was detectable while at the same time the variability in abundance was extremely small, probably due to their high numbers. These results indicate that nematodes can be a valuable part of such field studies. However, in order to improve the comparability of the results they should be sampled and extracted according to standardised methods (ISO 2008). In addition, it is recommended to use their high indicative potential by determining the maturity index, which is based on their trophic diversity (Bongers 1990). This taxonomical level (i.e. genus to family) has been proven to be very sensitive in other nematode field studies (Yeates 1994).

4.4. Recommendations and outlook

The results of the case study confirm the approach chosen for the study design. Since the number of enchytraeids and nematodes were clearly reduced on the reference plots, it was confirmed that the spray application on grass is a valid exposure route. In addition, these effects confirm the validity of the study as far as enchytraeids and nematodes are concerned. However, the selected reference substances (the fungicide Benomyl and the insecticide Chlorpyrifos(ethyl)) did not affect the mites, meaning that this part of the study is not valid and that, in general, another compound has to be identified for this purpose. Although there are no methodological problems it seems questionable whether (oribatid?) mites as a group are well suited for such studies due to their low sensitivity. In general, on the group level the number of individuals was sufficiently high in order to detect possible effects, but on the species and (partly) genus level the variation was high. However, it would be very difficult to increase the number of samples in order to lower variability. Probably, the usage of multivariate statistical methods would allow an improved effect assessment even without more sampling (Moser et al. 2007). In addition, the usefulness of a field study for an environmental risk assessment is clearly higher if the study follows a dose-effect design, thus allowing the determination of EC50 or NOEC values.

The soil organisms reacted differently to the chemicals applied, i.e. risk assessment and risk management could be improved by the inclusion of these organism groups. No lasting effect of the test item was found, indicating that the applied concentration does not cause concern for soil mesofauna. However, more ecological information on soil organisms is needed in order to improve the evaluation of field-study results. In order to verify these first experiences it is needed to broaden the information on the reaction of mesofauna groups to pesticide

application under standardised conditions of a field study, including supporting residue analysis. In this respect the optimal identification level (species, genus, even higher?) has to be critically (and group-specifically) discussed in order to find a compromise between resources needed and information gained. In addition, it is recommended to perform more studies such as the one described here, maybe most efficiently in parallel to earthworm or litterbag studies, in order to improve study design and performance, so that this study type can be internationally standardised and validated. In the long run, more guidance (e.g. De Jong et al. (2006)) would be needed in order to assure the compilation of data sets useful for the risk assessment of pesticides.

5. Acknowledgements

We thank the industrial sponsor of this study for permission to present first results here. In addition, we want to thank all those people helping in sampling, extracting, counting and assessing these little beasts.

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Accepted 6 August 2009