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Evaluation of the risk for soil organisms under real conditions

Development of a national position for amending downstream legislations of the new EU Plant Protection Products Regulation

Final report

For our Environment

Umwelt Bundesamt

TEXTE 201/2020

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Final report

by

Andreas Toschki (Coordination), Monika Hammers-Wirtz
gaiac -Research Institute for Ecosystem Analysis and Assessment, Aachen

Claudia Poßberg, Martina Roß-Nickoll, Andreas Schäffer, Burkhard Schmidt, Björn Scholz Starke
RWTH Aachen University, Aachen

Jörg Römbke, Adam Scheffzyk
ECT- Oekotoxikologie GmbH, Flörsheim am Main

Michael Klein, Udo Hommen
Fraunhofer IME, Schmallenberg

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gaiac-Research Institute for Ecosystem Analysis and Assessment e.V.
Kackertstr. 10
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Evaluation of the risk for soil organisms under real conditions

- Development of a national position in the context of the new European Plant Protection Regulation (EU 1107/2009)

FKZ 3710 67 410

Andreas Toschki (Coordination), Monika Hammers-Wirtz

gaiac -Research Institute for Ecosystem Analysis and Assessment, Aachen

Claudia Poßberg, Martina Roß-Nickoll, Andreas Schäffer, Burkhard Schmidt,

Björn Scholz Starke

RWTH Aachen University, Aachen

Jörg Römbke, Adam Scheffzyk

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In assessing the effects of plant protection products (PPP) on organisms in soil it is crucial to predict accurately the environmental concentration (PECsoil) which organisms are exposed to. The PECsoil is depending on the spatial and temporal distribution of the PPP, arising from characteristics of the chemical (e.g. Kow, water solubility, degradability) and from soil parameters (e.g. pH value, TOC, texture). The potential effects of PPP on soil organisms depend –besides the concentration of the chemical in the soil matrix- on the spatial and temporal distribution of the animals, i.e., their exposure as well as their specific sensitivity to the chemical. A new approach for deriving environmental concentrations in soil is currently under discussion, taking the preferred soil depth of the organisms into account. We conducted two different outdoor studies in Terrestrial Model Ecosystems (TMEs) to monitor (1) the movement of pesticides in soil over time and (2) the exposure and effects on soil organisms during the same time. Additionally, an indoor TME study was conducted to measure the fate of the radiolabelled pesticides and the formation of non-extractable residues in soil. In study [1] (outdoor) and [2] (indoor) Lindane (log Kow > 3) and Imidacloprid (log Kow < 1) were applied, two pesticides with different physico-chemical properties. In study [3] (outdoor), we investigated the effects of Carbendazim, a pesticide which is known as to be toxic for earthworms at certain concentrations. The effect analysis was conducted by means of different multivariate and univariate statistical methods. The synergistic conclusions based on the project results are proposed as recommendations for risk assessment concerning exposure and risk of soil organisms exposed to PPP under realistic conditions.</td></tr>
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Zur Erfassung der Effekte von Pflanzenschutzmitteln (PPP) auf Bodenorganismen ist eine korrekte Berechnung der initialen Konzentration (PECsoil) von wesentlicher Bedeutung. Die PECsoil ist abhängig von der räumlichen und zeitlichen Verteilung der PPP, die durch physiko-chemische (Kow, Wasserlöslichkeit, Abbaubarkeit) und Bodenkennwerte (pH-Wert, organischer Gehalt, Bodenart etc.) mitbestimmt werden. Die potentiellen Effekte auf Bodenorganismen werden zudem durch räumliche und zeitliche Verteilung der Bodentiere als auch durch ihre spezifische Sensitivität gegenüber der Chemikalie bestimmt. Eine neue Herangehensweise zur Bestimmung von relevanten Umwelt-Konzentrationen in Abhängigkeit von der bevorzugten Aufenthaltstiefe der Organsimen wird zur Zeit diskutiert. Zu Überprüfung dieser Herangehensweise wurden zwei Outdoor Terrestrische Mesokosmos Studien (TME) durchgeführt, um das Verhalten der Pestizide im Boden über die Zeit zu untersuchen und gleichzeitig die Exposition und die Effekte auf Bodenorganismen zu messen. Zudem wurde eine Indoor TME-Studie unter Verwendung radioaktiv markierter Substanzen durchgeführt, um den Gehalt an nicht-extrahierbaren Rückständen zu ermitteln. Für die Studie [1] (outdoor) und [2] (indoor) wurden die beiden Insektizide Lindan (log Kow>3) und Imidacloprid (log Kow<1) mit unterschiedlichen physiko-chemischen Eigenschaften ein-gesetzt. Für die Studie [2] wurden die gleichen Stoffe mit radioaktiver Markierung verwendet. In Studie [3] (outdoor) wurde das Pestizid Carbendazim verwendet, welches bei bestimmten Konzentrationen regenwurmtoxisch ist. Die Ermittlung der statistischen Signifikanz der Effekte erfolgte mit Hilfe unterschiedlicher univariater und multivariater statistischer Methoden. Aus der gemeinsamen und zusammenführenden Betrachtung der gesamten Ergebnisse werden Handlungsempfehlungen für die Risikobewertung von Bodenorganismen abgeleitet.</td></tr>
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1 Introduction

1.1 Background considerations

For the approval of active substances and for the authorization of Plant Protection Products (PPPs) in Europe (EU), it is necessary to test the active substances and products by using recent scientific standards to demonstrate that no unacceptable effects on the natural environment will occur following their intended use (Regulation (EC) No 1107/2009 (EU 2009), PflschG 2012). The effects of active substances and/or PPPs on the protection target are assessed by applying the criteria given in Regulation EU No. 546/2011(EU 2011). The thorough derivation of the environmental concentration (Predicted Environmental Concentration, PEC) of an applied PPP and the active substance(s) therein in the soil or water body is the crucial basis for the evaluation and assessment of possible detrimental effects on the non-target organisms that are exposed to it. The recent process in the development of guidelines and guidance documents (e.g. EFSA Guidance Documents and PPR Opinions 2010a and 2010b, 2012 and 2017) indicates that there is a need to follow up a new fundamental strategy to derive environmental concentrations in soil in the future.

Owing to the long-lasting EU debate about the assumptions concerning the distribution of the PPPs in the soil profile and the need of harmonisation when deriving the PECsoil between the different Member States, modifications in soil risk assessment are necessary. Both for the National authorization process and the approval of active substances at EU level it is assumed that there is an even distribution of PPPs after application in the upper soil layer. However, the different EU Member States and/or the three regulatory zones within the EU (EC 2014) SANCO/13169/2010) consider different soil depths for calculating the Predicted Environmental Concentrations in the soil (PEC_{soil}). The calcualted PEC is compared to ecotoxicological threshold concentrations that are derived in a first assessment step from laboratory test systems with standard test organisms (e.g. the chronic test with *Eisenia fetida*). With the background of the new guidance documents, this approach is open to criticism because the link between the fate of PPPs in soil and the effects on the soil community as the protection target and spatially distributed in the soil profile has not yet been clearly established.

In contrast to the risk assessment in the EU-regulations (see above), the current national approach in Germany when assessing the risk for soil organisms resulting from PPP use does not assume that all agents are homogenously distributed in the upper soil layer of 5 cm. For highly adsorbing agents (K_{OC}>500), slower vertical movement is postulated, based on data from Fent et al. (1999). In line with the current Guidance Document on Terrestrial Ecotoxicology (EC 2002, SANCO/10329/2002), the ecotoxicological results for tests with soil organisms exposed to active substances with a LogKow > 2 and performed in an artificial soil with 10 % peat are in most cases additionally recalculated by dividing the endpoint by 2. This is because in a test substrate with a high organic matter content, a lower bioavailability of the affecting agent can be assumed. This approach adresses the concerns that the *`realistic worse case´* concentrations could be higher when soil organisms living in the upper soil surface layer are exposed to a higher concentrations of persistent PPP in natural agricultural soils. Since the knowledge on the exposure of soil organisms' communities and the bioavailability of PPP's active substances in arable soils is poor, this approach has to be assessed.

The report of the project "Further development of a strategy for the assessment of the risk of plant protection products to soil organisms – considering different life form types and exposure scenarios" (Römbke et al 2010) suggests an adaption of soil communities – so called *focal communities* - to the land use type (arable land, orchards, grassland etc.) with repercussions for their exposure to PPP. Different focal communities are expected in different cultivars. Arable land focal communities might be seen in some cases as comparable to grassland communities but deprived in species diversity. In arable lands,the communities incude nevertheless life forms living in the uppermost soil layers (epigeic) and in deeper soil layers (endogeic) –even if in different shares than in grasslands. These findings are partially backing the new proposals on EU-level (e.g. EU Regulation 1107/2009/EC, Revision of Guidance documents EC (2000)`*Persistence in Soil´* and EC (2002) `*Terrestrial Ecotoxicology´*). The question arises whether specific spatial niches of soil organisms should be considered when defining the relevant soil layer for which the initial PEC_{soil} is calculated. Additionally, the physico-chemical properties of the active agent, i.e., the fate (DisT 50) and the resulting bioavailability should be considered.

These new conceptions and understandings of the ecological/ecotoxicological effects of PPP on soil organisms and of the active substance's fate and behaviour in soils should lead to a more realistic and relevant calculation of the predicted environmental concentrations (PECs). At the same time, it new concepts might result in a much more complex evaluation system than the one in place nowadays. Two crucial issues have to be addressed in the future:

- The spatial correlation between the toxic agent and the effects on soil organisms belonging to specific exposure types are not experimentally proven so far. The initial hypothesis that the behaviour and life form type of soil organisms in respect of their habitat preferences in the soil profile determine quality and duration of exposure is not yet scientifically confirmed.
- The resulting protection level for non-target soil organism of an approach differentiating for soil layers in comparison to the current practice is not consequently analysed, nor are the consequences for the risk assessment outcome acknowledged.

To support a national position for risk assessment of soil organisms in line with the new scientific and regulatory developments, it has to be determined whether the assumed relationship between spatial distribution of soil organisms, the distribution of PPP in soil and the ecotoxicological effects on soil organisms can be systematically observed. Moreover, it is crucial to evaluate the possible increased effort in the assessment and compare it to the resulting coverage of the protection goal so to develop the new assessment strategy with clear questions and aims.

1.2 Aims of the project

The aim of this project was to develop the technical basis in order to possibly adapt the exposure, effect and risk assessment of PPP in soil and for soil organisms according to newest scientific developments.

In addition to recording the state of knowledge about the relationship between the location of effects on soil organisms within the soil profile and the spatial and temporal distribution of PPPs, in particular, experimental investigations were conducted under controlled conditions, in order to provide a scientific basis for an adapted risk assessment strategy.

The project focussed on the following main questions:

- Can the assumed relationship between spatial distribution of a PPP in the soil profile and the location of ecotoxicological effects be confirmed?
- Is the exposure level and consequently the extent of ecotoxicological effects modulated by the preferred position and the behavior of soil organisms in the soil profile?
- Is the spatial transfer of the maximum concentration of a PPP into different soil layers over time accompanied by a sequence of effects in organism groups with different mode of exposure?
- Do active substances with different properties at a given time interfere with different groups of organisms, each representing a typical mode of exposure?

The results of the experimental studies should help refining the input parameters for current exposure models for soil organisms. The existing simulation models for exposure assessment in environmental risk assessment are able to calculate PECs for discrete soil depths. The aim of this project was to provide evidence on whether the average concentration of a PPP over different soil layers can be used for risk assessment of soil organisms or whether the concentration peak is determinative of the toxicity for soil organisms.

Finally, recommendations for the adaptation of risk assessment strategy for soil organisms were developed. Here a systematic and comprehensive comparison of the results of a risk assessment for soil organisms was performed with the currently established method and according to the specifications of a new adapted strategy. The aim was to document the achieved protection level of different strategies for the protection goal, that no unacceptable impacts on the subject of protection “soil and soil organisms” will occur, and to develop specific recommendations for the adjustment of the risk assessment.

1.2.1 Methodological requirements

To meet the above mentioned challenges within one study, appropriate methods and an adapted experimental design were required.

Study design

The test design should provide the possibility to measure toxicological effects on populations of different soil communities and the fate and behaviour of the toxicant at the same time and approximately at the same place. The test design should enable the analyses of different soil layers over time. Additionally, it had to be ensured that the statistical needs were met, i.e. that the sampling design and methods would take the sometimes high variability of soil organisms into

consideration in order to be able to detect statistically significant effects. Furthermore, the test system should be stable and mirror realistic conditions as in the field over a relevant period of time (at least one year).

Test items (PPP) and chemical analysis

The study was planned to study PPP with similar mode of action (insecticides), similar persistency but different sorption properties. It was decided to select one agent with a K_{OC} > 500 and another one with a K_{OC} < 500, assuming that one is retained in the upper soil centimetres, while the other is expected to be transported to deeper soil layers. Since the test items were to be measured in different soil layers, it had to be ensured that the analytical methods were standardised and able to detect the assumed small amounts of the agent in deeper soil layers.

Since studies with unlabelled test substances rely on the analysis of extractable fractions only, it was decided to establish studies with radiolabelled compounds in order to quantify the amount of non-extractable residues (NER). More effort was deemed to be necessary to identify the nature of such residues and their binding mode in the soil matrix. Recently, it has been shown that NER comprise three different types, i.e., type I containing xenobiotic residues entrapped in the voids of the inorganic and organic soil matter components, type II xenobiotic residues covalently bound to humic matter, and type II containing completely metabolized residues not distinguishable from natural organic matter, i.e. peptides, proteins, phospholipids etc. (biogenic residues) (Kästner et al., 2014). In the present project, though, we restricted our investigations on the quantitative aspects of NER in order to distinguish between readily and slowly desorbable and not bioavailable residues.

Investigated soil organisms

In the present study we aimed at considering representative groups of soil organisms of the macro- and mesofauna. The selection criteria were based on the respective sensitivity of the organism group to specific modes of actions of active substances in PPPs, the presence of the group in arable land habitats and the knowledge and practicability in dealing with these organisms in the process of risk assessment i.e. determination, classification of life form-type etc. A further advantage would be to select groups of soil organisms that have together a diverse structure - so that effects could be measured on different trophic levels and in different ecological niches, i.e. different exposure scenarios. Therefore, the populations of various animal groups, such as oribatid mites, collembolans, enchytraeids and earthworms were recorded in controlled model terrestrial ecosystems (TMEs) on species level and in different soil layers.

Exposure modelling

The experimental results with regard to the concentration gradient of the applied active substances in the soil profile and the analysis of soil water budget should serve as a basis for a model-based evaluation of the leaching behaviour of the active substances and of temporal and spatial distribution of the applied chemicals in the soil profile.

1.3 Assignment of tasks

The present project was divided into four work packages, which are drawn up in Figure 1. Furthermore, in Figure 1, the work packages are linked to the respective chapter in the present report.

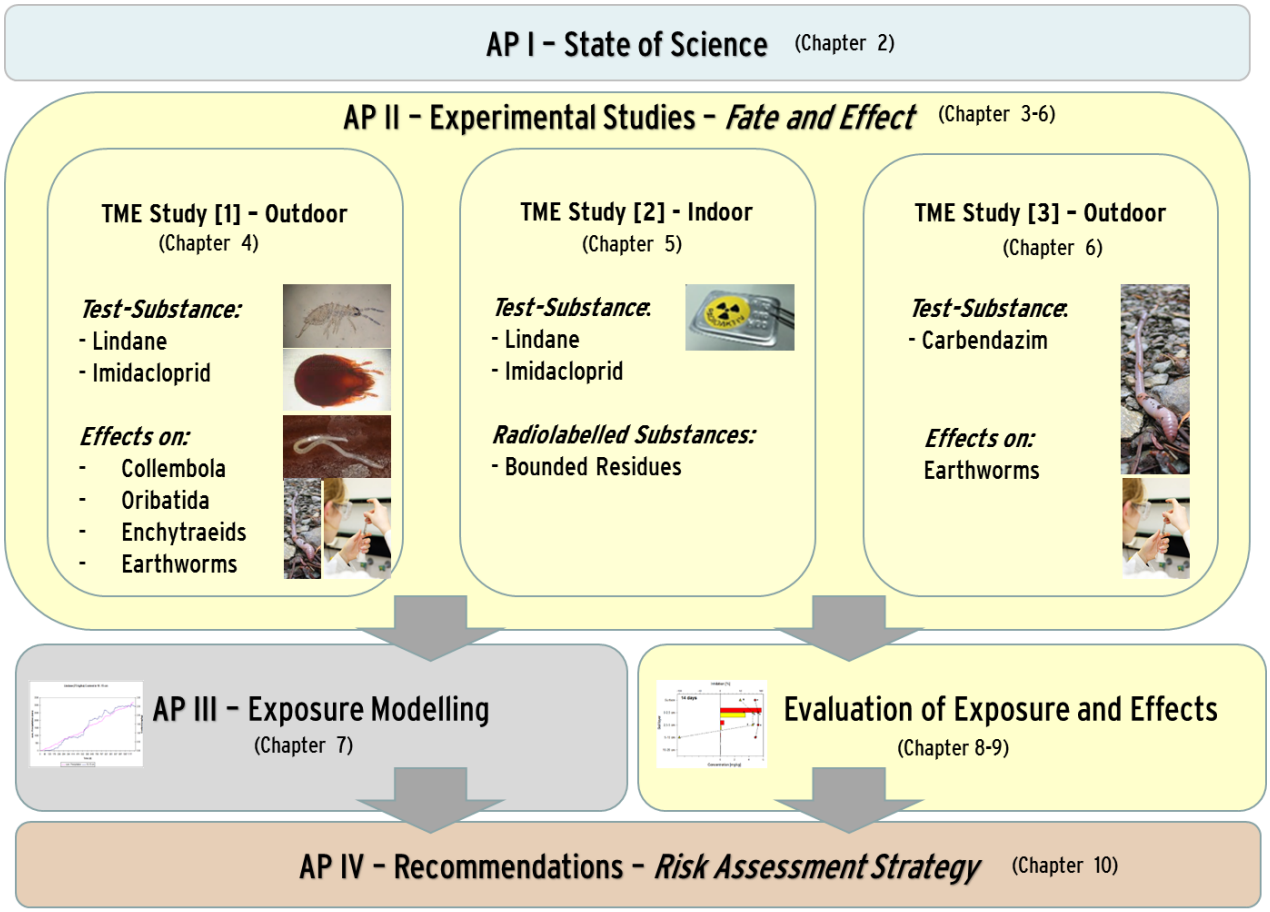

Figure 1 Schematic description of the project and report structure.

2 State of science

2.1 Exposure and PEC_{soil}

Based on the results of the UBA procurement report entitled "Further development of a strategy for the assessment of the risk of plant protection products to soil organisms – considering different life form types and exposure scenarios (FKZ 360 03 047; Römbke et al., 2010)" this Work Package compiles the existing knowledge on the relationship between the fate of pesticides (PPP) in soil and their effects on soil invertebrates, with particular attention to their vertical distribution within the soil profile. In order to do so, the current risk assessment procedure of PPPs for soil invertebrates is briefly summarized (Römbke et al., 2010).

The core of the risk assessment of pesticides (PPPs) is a comparison of exposure and effects. This means that in the case of the soil compartment those concentrations expected to appear in the field soil where the respective PPP will be applied are compared with those concentrations which cause effects in (usually laboratory) tests on a small range of organisms. Since the mid-Eighties of the last century, concentrations of PPPs in the soil are calculated using some simple assumptions: the amount of the test substance per hectare is distributed evenly within the uppermost 5 cm of a soil with a density of 1.5 g/cm³ dry weight ("standard" scenario; e.g. BBA 1986). Later modifications addressed mainly the question how much of the applied amount will reach the soil, e.g. by introducing vegetation interception fractions or by modelling spray drift (Ganzelmeier & Rautmann 2000). This approach has been used in the European Union for the registration of PPPs for 20 years (EU 1991) but is questioned more and more (Boesten et al. 2007). Critical issues are for example the considerable differences in soil properties in Europe as well as the ignorance of the composition of soil invertebrate communities, i.e. the goal to be protected.

The main problem regarding exposure estimations is the fact that pesticide active ingredients show a very different behaviour in soils, depending on the interaction between the physico-chemical properties of the compound (e.g. their binding affinity to organic matter in the soil or their water solubility) and the respective soil properties (e.g. organic matter content or pH-value). For instance, exposure of soil invertebrates differs considerably whether such a chemical is adsorbed in the uppermost millimetres of the soil or it is quickly leached towards deeper soil layers. However, biologists are well aware that soil invertebrate communities differ also in the various regions of Europe. In this context, differences on the species level (e.g. in taxonomy) might be less important than differences between life form types (e.g. in ecology). For example, exposure of organisms living in the litter layer of an orchard is completely different compared to the exposure of organisms living at the same site but in the mineral layer. Focusing on the fact that the ecology of soil invertebrates has been disregarded up to now, the following overview is divided into two parts:

1. A cursory literature review on the ecology, especially the vertical distribution and movement (and thus the potential exposure towards PPPs) of important soil invertebrate groups, in particular earthworms (Lumbricidae), potworms (Enchytraeidae), springtails (Collembola), mites (Acari: Oribatida and Gamasida) and wood lice (Isopoda) of agricultural soils. In this context, information from another UBA research project („Determination and analysis of the soil quality in the context of the implementation and further development of the National Strategy on Biodiversity"; FKZ 3708 72 201; Römbke et al., 2012) will be used, since the same invertebrate groups but isopods have been investigated there.

2. A summary of the discussion on the inclusion of the ecology of soil invertebrates when estimating the PECsoil (e.g. EFSA, 2009, 2010a, b).

2.1.1 Collembola

- Standardised sampling methods do exist for springtails, but the robustness of existing data regarding species presence and their abundances is affected by the usage of different extraction methods.
- Based on a literature review and EFSA data, "normal" mean values of abundance and species number of springtails can be given for Central European crop sites (41.000 ind./m^2 and 4 species) and for different grasslands (7.900 ind./m2 and 13 species).
- The taxonomic composition of collembolan communities at agricultural sites shows a wide variation, which is caused by a geographical component but also by crop type and crop management.
- Some species can be identified as frequent in crop sites, but the identification of species belonging to habitat specific "focal communities" is still difficult.
- In this respect, the relative percentage of the share of different ecological groups of springtail could be used for the identification of focal communities, but in terms of species richness crop sites and grasslands seem to differ only slightly.
- Springtail species can be classified into three ecological groups (epigeics, hemiedaphics, euedaphics) which differ in their vertical distribution and, thus, in their exposure towards PPPs. Based on this information, EFSA (2010) defines three rough exposure scenarios: soil surface /litter layer (if present) (ca. 1 cm), upper mineral soil (ca. 2.5 cm depth), mineral soil (5 cm depth).
- Without data on understanding of population dynamics and species composition of springtails at well characterised agricultural sites, the effects of anthropogenic stress cannot be accurately assessed.
- Further research is also needed to define the "borderlines" of the three springtail ecological groups and their movement in the soil profile.

2.1.1 Oribatida

- Standardised sampling methods do exist for mites, but the robustness of existing data is affected by the usage of different extraction methods.
- No recent overview on the abundance or species composition of oribatid mites at arable sites is available. However, intensive land management causes strong decreases in abundance and losses of species in the community.
- Referring to the data compiled in the database Bo-Info (just four sites), the mean abundance of oribatid mites in agricultural soils is very low: only 7 ind./m^2, belonging to approx. 7 species, have been found. In grasslands, these numbers are considerably higher: 5.800 ind. /m^2, belonging to 20 species.

- Oribatid communities are composed of few, highly abundant species with a wide ecological plasticity and a group of site-specific species which are highly diverse. Only very few species (*Liebstadia similis*, *Eupelops occultus*) do only occur in open landscapes as grasslands or, with even less individuals and constancy, at crop sites.
- Four ecological groups, based on their feeding habits, have been defined for oribatid mites, but the identification of the species belonging to focal communities is not yet possible due to a lack of data. Regarding their feeding modes, oribatid mites are often grazers and/or browser of fungal hyphae and do not ingest soil matrix as e.g. endogeic Annelida.In addition, it cannot be concluded on how much these groups differ in terms of their vertical distribution.
- Based on available information, differentiated exposure scenarios could not be defined. Thus, further research is needed to define the ecological groups for oribatid mites and in particular their depth distributions and movement in the soil profile and, thus, their exposure towards PPPs.

2.1.1 Enchytraeidae

The current knowledge on the taxonomy, biogeography and ecology (especially in terms of vertical distribution) of enchytraeids can be summarized as follows (EFSA, 2010b; Römbke et al., 2010, Römbke et al. 2013):

- The available data for enchytraeids are heterogeneously distributed over Germany.
- Standardised sampling methods for enchytraeids do exist and are widely used.
- Based on literature data and expert knowledge, "normal" means of abundance and species number of enchytraeids can be given for Central European crop sites and grasslands: e.g. at crop sites 20.000 ind./m^2 and fourteen species and at grasslands 14.000 ind./m^2 and twelve species. Because of the low number of studies performed so far, these numbers are considered to be preliminary.
- Due to the – until very recently – lack of a workable key species, species vary considerably.
- The structure of enchytraeid communities (i.e. species diversity) at different sites is less variable than abundance and biomass.
- The "typical" (i.e. occurring at more than 50 % of all crop sites) community (four species from the genus *Fridericia*, three species from the genus *Enchytraeus*, and one species from the genera *Enchytronia* and *Henlea*) is a "poor" grassland community, which typically contains four *Fridericia* species plus two *Henlea* species and one species from the genera *Buchholzia* and *Enchytraeus*.
- Temporal variability in abundance is high and clearly climate-driven. In temperate regions, spring and autumn are the most suitable sampling periods.
- Spatial and vertical distribution patterns differ strongly between species.

- Management practices (physical measures, organic matter supply) do influence enchytraeid communities, but due to their small size and quick reproduction these impacts seem to be less pronounced than in the case of earthworms.
- Enchytraeid species can be classified into three ecological groups (litter dwellers, intermediates, soil dwellers) which differ in their vertical distribution and, thus, in their exposure towards PPPs. Experience regarding the use of these groups for risk assessment is lacking.
- Based on this information, three exposure scenarios were distinguished in EFSA (2010b): litter layer (if present), upper mineral soil (ca. 2.5 cm depth), mineral soil (5 cm depth).

In the UBA project already mentioned, typical enchytraeid communities of crop and grassland sites (1. Level biotope classification) were identified (Römbke et al., 2012). Unfortunately, only few data are available in the literature which are useful for the differentiation of habitat subtypes (2. Level). These differences are discussed in the following.

For the habitat type arable land (no. 33 in Riecken et al. 2003) data for two subtypes are available (no. 33.03; 5 sites: „Farmed and fallow land on sandy soil" and "no. 33.04; 13 sites: „Farmed and fallow land on loess, loam or clay soil arable land on sandy soils) (Table 1). The mean abundance differs only by a factor of 1.5 and the mean species number is almost identical (14.2 vs. 14.4). Qualitatively there is also some overlap, since six species occur at both habitat types with >50% of all sites (*E. buchholzi, E. christenseni, E. minor, F. bulboides, F. christeri, H. perpusilla*). However, there are also clear differences in species composition (>50% occurrence in one habitat type, less than 20% in the other one):

- only in "farmed and fallow land on sandy soil" (no. 33.03): *A. aberrans, A. bibulba, E. norvegicus, E. annulata, E. parva, F. granosa* and *H. ventriculosa*;
- frequently only in „farmed and fallow land on loess, loam or clay soil, arable land on sandy soils (no. 33.04): *E. lacteus, E. minor* and *M. brendae*, plus several species of the genus Fridericia: *F. deformis, F. galba, F. isseli* and *F. paroniana*.

Keeping in mind that the number of sites (especially for habitat type no. 33.03) is very small, it would be premature to speculate which factors might be responsible for these differences.

For habitat level-1 type no. 34 ("natural dry grasslands and grasslands of dry and humid sites"), enchytraeid data are available for two subtypes: "Species-poor intensive grassland on moist sites" (no. 34.08; 6 sites) and "Trampled grass and park lawns" (no. 34.09; 7 sites) (Table 2). Clear quantitative differences in terms of species number were found (15 species in sites of no. 34.08 vs. 9 species in sites of no 34.09), but not regarding the mean total abundance: in both cases on average about 13,000 ind./m^2 were found. Only four species are frequently sampled at both grassland subtypes (i.e. at >50% of all sites): *B. appendiculata, F. bulboides, F. ratzeli, H. ventriculosa*).

Table 1 Species number and species composition as well as mean abundance of Enchytraeidae, separated according to two level-2 arable land habitat types, using the information from the Bo-Info data base (juveniles not included). Typical species (i.e. those with a frequency of more than 50% of all sites) are given in bold. 33.03 = Arable Land on sandy soils; 33.04 =Arable Land on clay/loam/loess soil

	33.03 (n = 5)	**33.04 (n = 13)**
Species	**Occurrence**	
Achaeta aberrans	**60.0%**	0.0%
Achaeta bibulba	**60.0%**	7.7%
Enchytraeus buchholzi	**80.0%**	**100.0%**
Enchytraeus bulbosus	20.0%	**69.2%**
Enchytraeus christenseni	**100.0%**	**100.0%**
Enchytraeus lacteus	20.0%	**69.2%**
Enchytraeus norvegicus	**60.0%**	7.7%
Enchytronia annulata	**60.0%**	0.0%
Enchytronia minor	**80.0%**	**53.8%**
Enchytronia parva	**60.0%**	7.7%
Fridericia bulboides	**80.0%**	**92.3%**
Fridericia christeri	**60.0%**	**84.6%**
Fridericia deformis	0.0%	**53.8%**
Fridericia galba	0.0%	**84.6%**
Fridericia granosa	**60.0%**	7.7%
Fridericia isseli	0.0%	**76.9%**
Fridericia paroniana	0.0%	**92.3%**
Henlea perpusilla	**100.0%**	**84.6%**
Henlea ventriculosa	**80.0%**	15.4%
Marionina brendae	0.0%	**76.9%**
Mean ind./m² ± SD	**28,924 ± 23,698**	**19,686 ± 11,242**
Mean species no./site ± SD	**14.2 ± 4.0**	**14.4 ± 4.5**

Typical for grassland sites belonging to "Species-poor intensive grassland on moist sites" (no. 34.08) are *E. parva, F. benti, F. galba* and *H. perpusilla*, while in grasslands belonging to Trampled grass and park lawns" (no. 34.09) *A. pannonica, E. buchholzi, E. christenseni, E. minor, F. bisetosa, F. christeri* and *F. lenta* commonly occur (Table 3). The species of the latter group were only rarely found (i.e. in less than 20%) in the sites belonging to no 34.08. This is not true vice versa: three of the four species (i.e. not *H. perpusilla*) typical for the sites belonging to no 34.08 sites were also found at more than 20 % at sites belonging to 34.09. In some cases, the difference between the two subtypes already becomes evident at the generic level: in sites belonging to no 34.08

species of the genus *Achaeta* were never found. This is an interesting result, since the species of this genus are not suitable for reference values for Level 1 habitat types, but very well for differentiating between subtypes of grassland (Table 2) and arable sites (Table 1). However, since the number of studied sites belonging to each subtype is still small (5 – 7), it is clear that these findings are just an indication for differences between these two habitat level-2 types. Clearly, more research is needed here.

Table 2 Species number and species composition as well as the mean abundance of Enchytraeidae, separated according to two grassland habitat Level-2 types, using the information from the Bo-Info data base (juveniles not included). Typical species (= those with a frequency of more than 50% of all sites) given in bold. 34.08 = Intensive Grassland ; 34.09 = Trampled grass /Parklawns.

	34.08 (n = 6)	34.09 (n = 7)
Species	Occurrence	
Achaeta pannonica	0.0%	**85.7%**
Buchholzia appendiculata	**83.3%**	**85.7%**
Enchytraeus buchholzi	33.3%	**57.1%**
Enchytraeus christenseni	16.7%	**100.0%**
Enchytraeus norvegicus	16.7%	**57.1%**
Enchytronia minor	0.0%	**71.4%**
Enchytronia parva	**83.3%**	28.6%
Fridericia benti	**83.3%**	28.6%
Fridericia bisetosa	16.7%	**71.4%**
Fridericia bulboides	**83.3%**	**85.7%**
Fridericia christeri	0.0%	**57.1%**
Fridericia galba	**66.7%**	42.9%
Fridericia lenta *	0.0%	**57.1%**
Fridericia ratzeli	**100.0%**	**71.4%**
Henlea perpusilla	**83.3%**	14.3%
Henlea ventriculosa	**66.7%**	**71.4%**
Mean ind./m² ± SD	12,480 ± 8,476	13,168 ± 11,347
Mean species no./site ± SD	9.5 ± 4.3	15.0 ± 4,0

* as *F. leydigi* in the Bo-Info database. According to Schmelz (2003) *F. lenta* is largely identical with *F. leydigi* sensu Nielsen & Christensen (1959), the identification guide used by most of the identifiers, whereas the identity of *F. leydigi* as originally described (Vejdovský 1878, 1879) is uncertain.

2.1.2 Earthworms (Lumbricidae):

The current knowledge on the taxonomy, biogeography and ecology (especially in terms of vertical distribution) of earthworms can be summarised as follows (Römbke et al., 2010, Jaensch et al. 2013):

- Earthworms have regularly been sampled in many but not all parts of Germany.
- Based on literature data, "normal" means of abundance and species number of earthworms can be given for Central Europe: at crop sites, on average 50 ind./m² and four species do occur, while at grasslands the respective numbers are 250 ind./m² and five species.
- Due to methodological differences and the heterogeneity of the sampled sites, these values vary by about 122 % (grassland) or 175 % (crop sites). The species diversity, measured as species per site, is less variable than abundance and biomass.
- The "normal" community of crop sites (*Aporrectodea caliginosa, Aporrectodea rosea, Lumbricus terrestris*) is a "poor" grassland community, which usually contains several endogeics, two epigeics and one additional anecic species.
- The juvenile to adult ratio differs between species: large anecics tend to reproduce seasonally, while the reproduction of small endogeics shows lower differences within one year, being governed by actual climatic factors and food availability.
- Temporal variability in abundance is high and clearly climate-driven. In temperate regions, spring and autumn are the most suitable sampling periods.
- Spatial and vertical distribution patterns differs strongly between species.
- Management practices (physical measures, organic matter supply) strongly influence earthworm communities, but the influence of crop types or crop rotations cannot be assessed due to a lack of information.
- Earthworm species can be classified into three ecological groups (epigeics, endogeics, anecics) which differ in their general vertical distribution and, thus, in principle, in their exposure towards PPPs. However, vertical movements between different soil layers have to be further addressed.
- Based on this information, EFSA (2010b) distinguishes three exposure scenarios: litter layer (if present), soil surface (ca. 1 cm depth), mineral soil (20 cm depth)

Above it has been stated that the earthworm communities at different crop site habitat types do differ. This will be exemplified in Table 3, using three second-level habitat types (Jaensch *et al.* 2013). On farmed and fallow land on shallow skeletal calcareous soil (habitat type 33.01), at least three endogeic species with a mean total adult abundance of 28.7 ind./m² should occur. In addition to *Aporrectodea caliginosa* and *A. rosea*, *Octolasion tyrtaeum* but not the anecic *Lumbricus terrestris* was most frequently present. *Dendrobaena octaedra* and *Dendrodrilus rubidus* are not expected to occur at this habitat type. On farmed and fallow land on sandy soil (habitat type 33.03), only *A. caliginosa* should always occur (100% of all 21 sites in the present data basis) with a total mean adult abundance of 18.9 ind./m². *D. rubidus*, *L. castaneus* and *O. tyrtaeum* should be absent. On farmed and fallow land on loess, loam or clay soil, (habitat type

33.04), at least four species can be expected: besides *A. caliginosa*, *A. rosea* and *L. terrestris*, the endogeic *A. chlorotica* was also frequently found at this habitat type. This habitat type thus showed the highest mean species richness and also by far the highest mean abundance of adults (93.2 ind./m²) of all crop-site types. Acido-tolerant epigeic species (in particular *D. octaedra* and *D. rubidus*) were almost totally missing here. Variability in abundance was high for all three habitat types.

Table 3 Species composition (relative frequency), average species number and mean total abundance of adult Lumbricidae, separated according to level-2 crop habitat types, using the information from the Bo-Info data base. Typical species (i.e. those with a frequency of more than 50% of all sites) are given in bold. SD – standard deviation, CV – coefficient of variation. 33.01 = Arable Land on limy soils; 33.03 = Arable Land on sandy soils; 33.04 = Arable Land on clayey/ loamy/ loess soils.

Species	33.01 (n = 16)	33.03 (n = 21)	33.04 (n = 31)
A. chlorotica	12.5%	14.3%	**54.8%**
A. caliginosa	**75.0%**	**100.0%**	**87.1%**
A. longa	6.3%	4.8%	41.9%
A. rosea	**75.0%**	14.3%	**87.1%**
D. octaedra	0.0%	9.5%	0.0%
D. rubidus	0.0%	0.0%	0.0%
L. castaneus	12.5%	0.0%	16.1%
L. rubellus	43.8%	9.5%	16.1%
L. terrestris	37.5%	33.3%	**83.9%**
O. tyrtaeum	**62.5%**	0.0%	12.9%
Mean Ind./m² ± SD	28.7 ± 37.2	18.9 ± 28.1	93.2 ± 126.1
	CV: 130%	CV: 149%	CV: 135%
Mean species no./site ± SD	3.4 ± 1.9	1.9 ± 1.2	4.4 ± 1.7
	CV: 56%	CV: 63%	CV: 39%

2.2 Overview of the vertical distribution of soil organisms

2.2.1 Introduction

Summarising the available information on the vertical distribution of soil invertebrates at German crop sites it can be stated that:

- Different soil invertebrate species prefer different soil layers, but are usually found either in the litter layer (if present) or in the uppermost 5 – 10 cm of the mineral soil. The most notable exceptions are anecic earthworms which can burrow several meters deep.
- Species living in the same soil layer often have common physiological or morphological properties, i.e. they can be classified into ecological groups. The best known example is the classification of earthworms into three groups (epigeic, endogeic and anecic species (Bouché 1977), but similar groups have also been defined for Enchytraeidae and Collembola (EFSA 2010b).
- Depending on the site properties (soil, climate, land use etc.) typical invertebrate communities consisting of species or ecological groups can be identified.
- Since species and, accordingly, ecological groups differ in their vertical distribution, they might also be differently exposed towards PPPs. The possible movement of the animals in the soil profile should however not be disregarded. PPPs are usually sprayed on the soil surface or on crop plants, meaning that a vertical concentration gradient of these chemicals is the normal exposure scenario.

Recently, the exposure and exposure pathways of PPPs and soil invertebrates has been reviewed by Peijnenburg et al. (2012).

2.2.2 Vertical distribution of collembola

Springtail species can be classified into three ecological groups (epigeics, hemiedaphics, euedaphics) which differ in their vertical distribution and, thus, in principle, in their exposure towards PPPs. Based on this information, three exposure scenarios can be distinguished according to EFSA (2010b): litter layer (if present) or soil surface layer (ca. 1 cm) , upper mineral soil (ca. 2.5 cm depth), mineral soil (5 cm depth). This vertical niche differentiation of collembolans is correlated to species-specific morphological traits. According to the “life form concept” (Gisin 1943; Christiansen 1964), springtails can be categorized based on morphological traits, i.e. the size of furca (springing organ) and antennae, the number of ocellae and their pigmentation, into epigeic, hemiedaphic and euedaphic species. Although some species are strictly confined to a certain soil layer, many species have a broader vertical niche and move in the upper soil profile. Since they do not have the ability to create burrows, springtails depend on the existing soil pore system and burrows made by, e.g. earthworms. The highest density of collembolans in openland habitats of central Europe can be expected in the upper 5 to 10 cm soil layer. Vertical migration regularly exists and is mainly induced by climatic factors or by food availability. In the following Table 4, the three ecological classes of Collembola are defined:

Table 4 Definition of the three ecological groups of Collembola, including some characteristic species for the specific group. For details see Römbke et al. (2010).

Life form class	Characteristics	Example species
Epigeic: fast dispersal species, living in soil surface	Most species with more than 5+5 ocelli; long to very long antennae; furca fully developed	*Parisotoma notabilis, Entomobrya multifasciata, Pogonognathellus flavescens*
Hemiedaphic: medium dispersal species, living down to 2.5cm layer	Variable number of ocelli; short antennae; furca reduced or short	*Megalothorax minimus, Micranurida pygmaea, Isotomiella minor,Folsomia quadrioculata, Folsomia candida*
Euedaphic: species with very low dispersal ability, living down to 5cm layer (in some case down to 10cm)	Blind species; very short antennae; furca absent or not well developed	*Protaphorura armata, Mesaphorura krausbaueri*

The preliminary description of the community based on functional traits determining life-form class, based on the data compiled so far (presence-absence data for 20 sites only embracing a limited number of crops) shows the similarity in the composition of life-form groups between crop and grassland areas (Table 5), despite the differences existing in terms of species composition (Römbke et al. 2010).

Table 5 Average values (and limits) for the number and percentage of species in each life-form class of Collembola for crop and grassland areas (Römbke et al. 2010)

	Euedaphic	Hemiedaphic	Epigeic
Crop areas (N=12)			
Species (N)	5 (1 – 14)	11 (0 – 24)	13 (1 – 29)
Species (%)	24 (9 – 50)	34 (0 – 67)	42 (17 - 57)
Grasslands (N=8)			
Species (N)	5 (0 – 15)	8 (2 – 16)	11 (4 – 24)
Species (%)	15 (0 – 31)	34 (24 – 50)	51 (44 - 71)

2.2.3 Vertical distribution of oribatid mites

No robust information is available on the vertical distribution of oribatid mites in grasslands or crop sites (Römbke et al., 2012). In addition, the species of this group have not been classified in

specific ecological groups depending on their vertical distribution. In contrast, oribatid mites are classified according to their feeding habits, i.e. their gut contents (Schuster 1956; Luxton, 1972). According to Weigmann (2006), the most abundant feeding guilds are: macrophytophagous (feeding on leaves, wood, pollen), microphytophagous (feeding on fungi, algae, bacteria) and panphytophagous (feeding on different sources, plants and humus). Oribatid mites are distributed along vertical gradients, following the feeding source e.g. in the litter-humus layers of forest soils, which can be interpreted as a succession of species compositions. Humus rich forest soils contain the most diverse and abundant Oribatid mite communities. For some species groups e.g. Brachythoniidae and Suctobelbidae, a correlation to fungi and bacteria presence can be observed (Weigmann 2006). At open land sites, i.e. arable land, grassland, highly organic habitats as in forests are not so well developed. It can be assumed that the vast majority of oribatid mites is living on or close to the soil surface. However, as described above, some species and individuals are following the feeding source, e.g. plant roots, holes of lumbricids etc. in deeper soil layers. Hence, some individuals could occur and be captured also in the mineral soil as well.

2.2.4 Vertical distribution enchytraeidae

In general, the vertical distribution of potworms shows a very steep decrease within the uppermost 10 cm of the soil. As an example, the percentage of potworms in the uppermost four layers of mineral soil of two German sites, a crop site and grassland, is presented (Figure 2). About 45% occur in the uppermost 2.5 cm, while, in the deepest mineral soil layer, 15% and 10%, respectively of the worms were found. Similar patterns are found in forests, with the highest percentage of enchytraeids in the litter layer, consisting of decaying leaves. The vertical distribution of enchytraeids at crop sites is strongly influenced by ploughing, since due to this practice organic matter is transported to deeper layers (Didden et al., 1997). As a result, the usual vertical distribution at sites without ploughing (i.e. high densities close to the surface with decreasing numbers in deeper layers) could be changed in a way that the occurrence of enchytraeids can be more or less even within the ploughing layer of the mineral soil – but only as long as food is available there. Vertical migration of potworms is probably mainly caused by climatic factors (temperature, moisture) (Lagerlöf et al. 1989), but it could also be caused by anthropogenic stress such as PPPs applied to the soil surface. In any case, individual species as well as ecological groups have clearly different vertical preferences.

Despite the mentioned uncertainties, especially about species-specific preferences, the following soil depths have been assigned to the enchytraeid ecological groups at agricultural sites (EFSA 2010b):

Litter layer (if available) or soil surface (ca. 1 cm): Litter-dwelling potworms

Mineral soil down to depth of 5 cm: Soil-dwelling potworms

Uppermost mineral soil layer: Intermediate worms.

At the same time, the potential exposure of enchytraeids differs in these three layers.

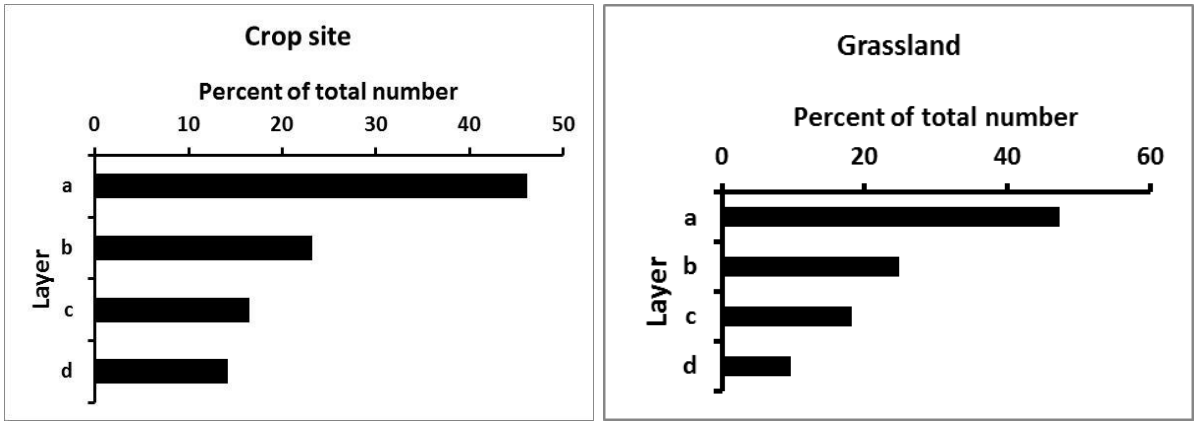

Figure 2 Vertical distribution of enchytraeids at a crop site (left) and a grassland site (right) in Northern Germany. Each layer consists of 2.5 cm. (Bo-Info database, unpublished results)

2.2.1 Vertical distribution of earthworms

Different species of lumbricids inhabit different depth zones in the soil, depending mainly on climatic conditions; thus considerable seasonal changes of the vertical distribution occur (Edwards and Bohlen, 1996). For example, Gerard (1967) showed that the vertical distribution of common earthworm species in England mainly depends on the moisture and temperature of the uppermost soil layers (*Aporrectodea caliginosa, Aporrectodea longa, Aporrectodea rosea, Lumbricus terrestris*). Nearly all cocoons of these species were found in the uppermost 15 cm, most being in the top 7.5 cm. The same observations were made in Sweden (Rundgren 1975) and Germany (Peters 1984). Anecics stay active as long as possible simply by retreating to the bottom of their burrows during extremes of heat or cold.

The availability of food is the second most important factor determining the vertical distribution of earthworms. Feeding on organic material contaminated with PPP residues is considered to be a relevant exposure pathway. Depending on the ecological group, earthworms prefer different soil layers and are thus in principle differently exposed to PPPs:

- Epigeics live close to the soil surface, either on it or within the first 2.5 cm depth. Since they cannot burrow they have to feed on litter. Thus, in case there is no organic layer on the soil surface these species will not occur or only in negligible numbers;

- Endogeics inhabit usually the uppermost 15 – 20 cm (adults of *Octolasion cyaneum* rarely go deeper). They feed on the organic material homogenously distributed within the mineral soil. Thus, their contact with sorptive PPP sprayed on the soil surface should be in principle lower. However, as described above, their vertical distribution migh change according to soil moisture and food availability in the soil profile, possible also in short time ranges. However, juvenile endogeics may feed more closely to the soil surface (for example, they are often found in or close to the turf layer at grasslands).

- Among anecics, *Aporrectodea longa* prefers the uppermost 45 cm and *Lumbricus terrestris* can go down to 2.5 m but being usually restricted to a depth of about 1 m (Edwards and Bohlen, 1996). The adults collect leaves or blades of grass close to the opening of their burrows. The food will be stored deep in the burrows until it is more palatable. Again, anecic juveniles also tend to feed more closely to the soil surface, while the adults show the “typical” behaviour of their ecological group (Briones & Bol 2002). Obviously, this behaviour means that anecics are exposed

to sprayed PPPs, either directly, via contact to residues or via contaminated food. The first possibility does not play an important role, since these worms usually leave their burrows in the night. They avoid daytime activities due to the high risk of predation. Contact to residues might occur during movement on the soil surface or during burrowing activities. The third possibility, on the other hand, is an oral exposure pathway because litter contaminated with persistent PPP residues will actively be sought and, after some time in the burrows, be eaten.

Summarising this section, there are three layers which can be distinguished by the occurrence of certain ecological groups of earthworms:

Litter layer (if available) or soil surface (ca. 1 cm):	Epigeic and anecic earthworms
Mineral soil down to depth of 15 – 20 cm:	Endogeic earthworms
Soil surface (ca. 1 cm) or within the vertical burrows:	Anecic earthworms.

At the same time, the potential exposure of earthworms differs in these three layers.

2.3 Exposure of soil organisms to PPP

This subchapter is based on a previous UBA project and is intended as background information. Please refer to Römbke et al. (2010).

2.3.1 Exposure pathways

Soil organisms are exposed to chemicals by a variety of pathways. Biologically speaking, morphology, physiology and behaviour of these organisms mainly determine how (and which amount of) a PPP is taken up. Most of the available information has been gained in standardised laboratory tests with only few selected species (e.g. the earthworm *Eisenia fetida* or the springtail *Folsomia candida*), meaning that the complexity of field situations (e.g. soils with hugely varying properties, climatic conditions or the biodiversity of invertebrates) has not been directly taken into account. The extrapolation from the assessment of few surrogate species to the situation in the field is achieved by means of assessment factors, that should be calibrated at least with results of field tests.

Soil invertebrates can be exposed towards PPPs via four pathways:

- Pore water.
- Contact soil.
- Ingestion of food (living or dead matter) and soil particles.
- Inhalation of air present in the soil pores.

In addition, direct contact with the spray is possible for organisms living on the soil surface, in the litter layer or in vertical burrows (e.g. anecic earthworms).

The relative importance of each of these uptake routes is determined by morphological (e.g. structure of the epidermis), physiological (e.g. mode of uptake of water [drinking versus uptake via the skin], mode of uptake of oxygen, feeding habits) and behavioural properties. A general sub-division may be made between so-called ‘soft-bodied’ organisms (like nematodes, earthworms, enchytraeids and some insect larvae) and ‘hard-bodied’ invertebrates (arthropods like spiders, some mites, insects, some collembolans, millipedes, centipedes, harvestman, isopods, and some other terrestrial crustaceans like some crab species). ‘Hard-bodied’ organisms

have evolved special organs for assimilation of oxygen and water, while for 'soft-bodied' biota uptake via the skin is the most important route of uptake of water and oxygen. Contaminants and nutrients may also be taken up via these distinct exposure routes while uptake of contaminants via food is possible for all biota. In this context also the uptake via "secondary poisoning", i.e. predators feeding on contaminated prey (e.g. predatory mites on worms). Consequently, soil dwelling organisms are exposed to chemicals by a variety of pathways. Most organisms share the feature that the relative contribution of each pathway varies. On top of ecological impacts, these contributions depend on factors like the hydrophobicity of the chemical and variations in environmental conditions like soil type, climate, etc.

Knowledge on uptake routes of organic contaminants by soil invertebrates is far from complete. Most information is available for earthworms, collembolans and isopods. The equilibrium partitioning theory appears to be valid for earthworms and collembolans in laboratory settings, although some uncertainties like food type need further investigation. The contribution of oral uptake may vary within a specific taxon but for soil organisms in close contact with the soil solution, pore water mediated uptake is in general the dominant pathway and it is commonly modified by soil specific ageing and speciation, and by specific factors of the organisms, like nutrition status. Here it must be mentioned that sorption properties of the soil-PPP combination are often difficult to determine (Peijnenburg et al. 2010).

Intra-species (especially between different life-stages) and inter-species variances (like size and ecological preferences) will most likely modify the actual contribution of potential exposure pathways. Uptake of nutrients and chemicals is possible for all invertebrates via their food and this may be an important route in case of food sources in which high concentrations of chemicals are present. The assimilation efficiency will however depend on species specific properties of the digestive tract and no general conclusions are to be generated in this respect.

2.3.2 Determinants of exposure related to the active substance properties and use

2.3.2.1 Properties of substances

The most important properties of active substances in PPPs determining their fate and behaviour in soil are water solubility, adsorption/mobility and persistence. Based on this information gained in standardised laboratory and field tests, the behaviour of the respective active substances in PPPs can be evaluated. For example, such an evaluation could be performed using the following criteria for the individual properties of active substances in PPPs, e.g.:

- Water solubility: AERU (2009)
- Adsorption (mobility): PSD (2005)
- Persistence: Beek et al. (2001); SANCO (2002)

The fate and behaviour of an active substance in soil should preferably be assessed (EFSA 2009).

New guidance on the assessment of fate and behaviour of active substances in soil is being currently developed by EFSA.

2.3.2.2 Application conditions: pattern and amount

The application pattern (i.e. how often but also at which time slot in the cropping cycle or the season) and the applied amount of the PPP surely determine the exposure situation in soil. Quantitatively, within an intended PPP use, the most important factor is whether the soil is covered by a vegetation layer or not: if yes, the concentration in soil is often lower since the PPP can be intercepted by the vegetation and adsorbed to the organic material. However hand, in such a case exposure is higher for those organisms living in or feeding on this organic material in the litter layer (EFSA 2010c). As in the current practice, it has to be distinguished between realistic worst case situations -usually applied on lower tiers of the environmental risk assessment (ERA) process- and those conditions relevant for a specific region or crop. As required by EFSA (2009), it is recommended to provide both concentration metrics for active substances in soil (i.e. total content and soil pore water concentrations) – but it is not necessary to measure both. Actually, it should be possible to determine one endpoint (e.g. total contents in soil) and to model the other one – as long as active substance properties and environmental conditions (soil, climate etc.) are known.

2.3.2.3 Site conditions

The properties of specific sites (or regions, depending on the tiered steps of the ERA process) have to be taken into account when estimating exposure concentrations of active substances for soil organisms (FOCUS 1997; 2006). This kind of information includes climatic properties, soil properties but also the crop type (as a minimum, whether it is an annual or permanent crop). Guidance on the data needed to estimate environmental concentrations after intended PPP use for ERA, in particular at higher tier assessment steps, are already available and is being currently updated (e.g. soil properties: texture (% sand, silt, clay), water holding capacity, organic matter content, C/N-ratios and the soil pH value.

2.3.3 Case study: Influence of PPPs on different ecological groups of earthworms (and, thus, on their vertical distribution)

Thirty standard earthworm field studies were evaluated regarding effects of the reference substances Benomyl and Carbendazim. The reference substances were usually applied at a rate of 4 or 8 kg a.s./ha to confirm the sensitivity and exposure of the earthworm community under the given test conditions. Benomyl and Carbendazim are systemic fungicides that are taken up by roots and leaves. Figure 3 and Figure 4 show boxplots of the relative abundances of the three ecological groups of earthworms in comparison to the untreated control 1, 4 to 6 and 12 months after application (maa) for all sites and for 16 grassland and 14 crop sites separately.

The observed effects pattern for the reference substances Benomyl and Carbendazim can be explained partly by the physico-chemical properties of these compounds. These two substances can be classified as intermediate regarding their behaviour in soil (i.e. low water solubility, moderate mobility and moderate persistence). Hence, one month after application, the substances will still be concentrated in the litter and upper soil layers explaining the pronounced effect on epigeic earthworms and also on anecic earthworms - as these feed on litter and the substances may also enter their vertical burrows (EFSA 2010c). However, also endogeic earthworms show pronounced effects from the start of the experiments on, pointing to an exposure to the upper layer containing the active substances. With time, the substances will increasingly enter the mineral soil layers but also start degrading which explains the continuous

effect on endogeic earthworms and the beginning recovery of epigeics 4 to 6 months after application. This trend will continue and degradation increase which leads to a possible complete recovery of epigeics at some sites and less effects on epigeics and anecics 12 months after application. The more pronounced long-term effects in grassland compared to crop sites may be explained by the higher organic content of the upper soil layers and thus a delayed relocation of the reference substances to deeper soil layers. Also, a different composition of the earthworm community might determined the observed recovery process. This means that especially for persistent and immobile actives, the layer must be taken into account when defining exposure scenarios (EFSA 2010c). However, experimental verification of the fate of PPPs is needed taking site specific characteristics into account.

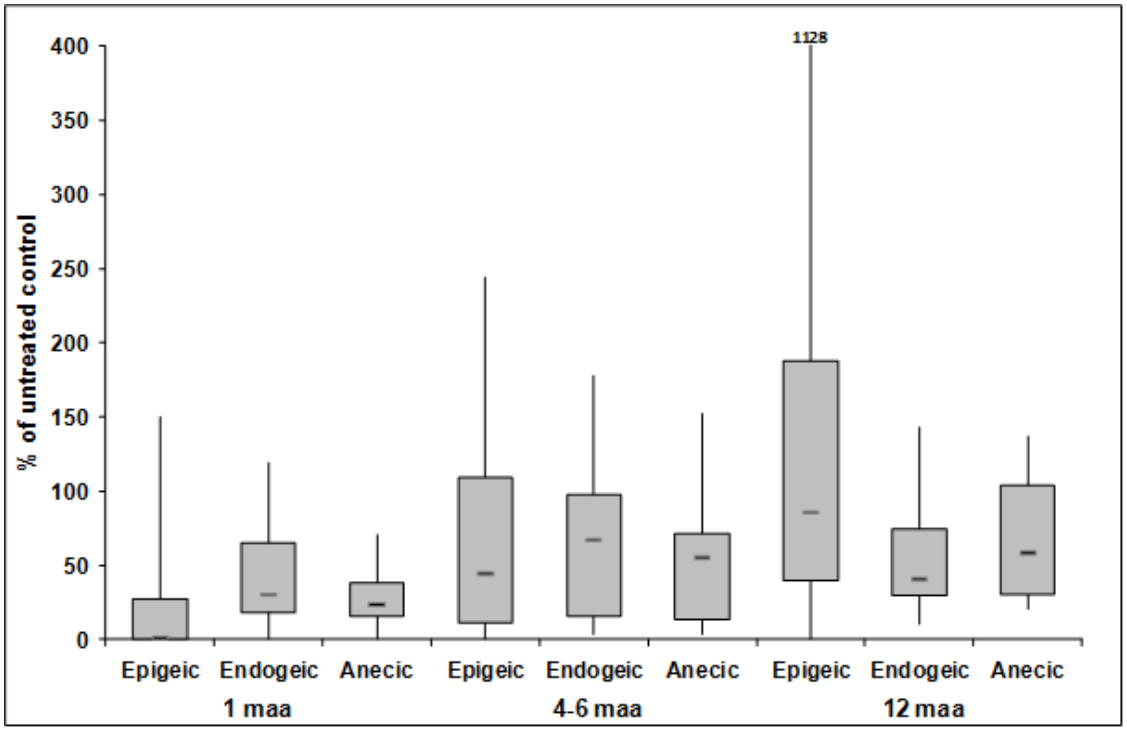

Figure 3 Boxplots of the relative abundance of the three ecological groups of earthworms at 16 grasslands treated with Benomyl or Carbendazim compared to the untreated control (maa = months after application) (Römbke et al. 2010).

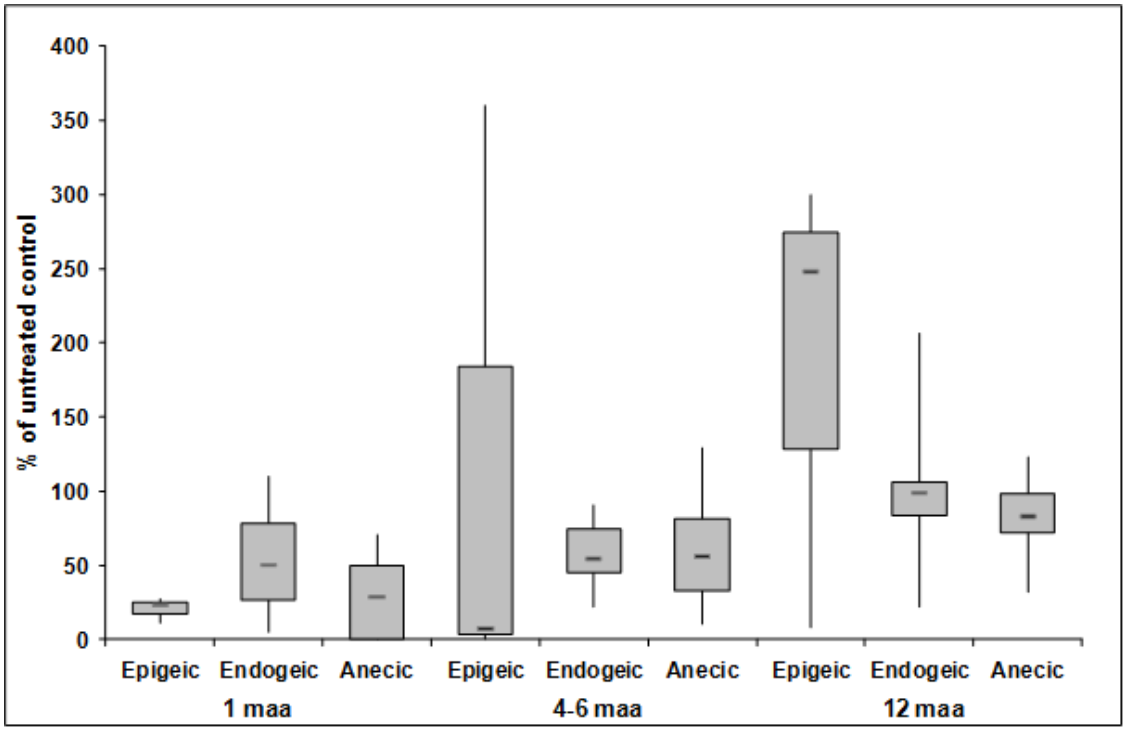

Figure 4 Boxplots of the relative abundance of the three ecological groups of earthworms at 14 crop sites treated with Benomyl or Carbendazim compared to the untreated control (maa = months after application) (Römbke et al., 2010).

3 Material and Methods

3.1 Terrestrial Model Ecosystems (TMEs)

The present study was designed to assess the effects of pesticides on soil organisms under realistic condition and exposure in soil. The study was conducted by means of Terrestrial Model Ecosystems (TMEs, Figure 5). These systems provide the possibility to study natural soil communities under standard conditions over a period up to one year (Schäffer et al., 2008; Scholz-Starke, 2013; Scholz-Starke et al., 2013). The advantage of these systems is that there are replicable and it is generally possible to measure and investigate different taxa at the same time (Sheppard, 1997). They provide the possibility to analyse the behaviour of pesticides over time for different soil layers by using adequate soil sampling approaches which are also suitable to link between laboratory (see study [2] with radiolabelled substances or ecotoxicological single species testing in lower tier risk assessment) and the real conditions in field (Odum, 1984, Scholz-Starke, 2013). In the present study, we used open TMEs that were cored in grassland and contained an undisturbed soil community typical for grassland habitats. For study [1] and [3] they were placed outside (outdoor) for study [2] they were placed in the laboratory (indoor).

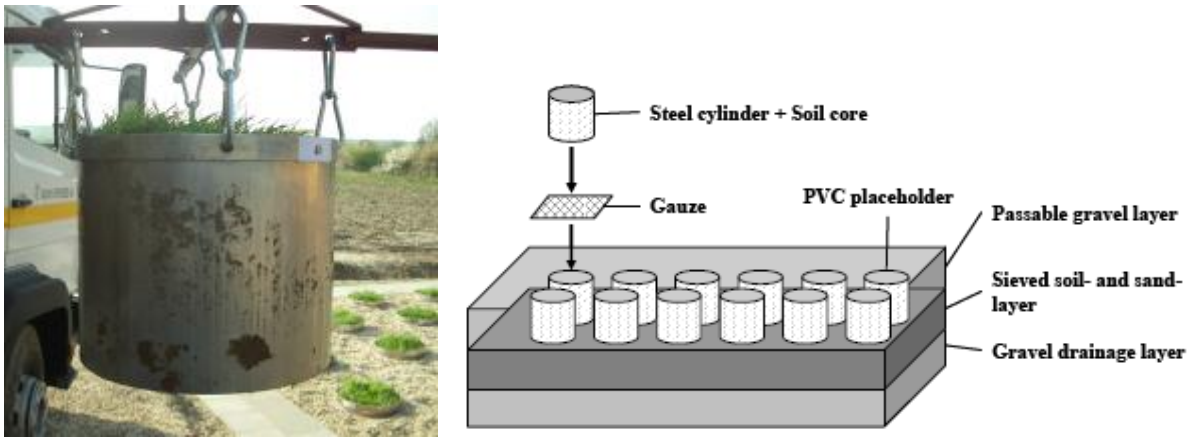

Figure 5 left: Terrestrial Model Ecosystem (TME) Ø 467 mm, height 400 mm; right: schematic picture of the study facility in Aachen

3.2 Coring Site

3.2.1 Selection of the coring site

To fulfil the necessary prerequisites to detect pesticide effects on soil organisms, a sufficient average number of animals with homogenous distribution in soil is crucial to meet statistical needs (Scholz-Starke et al., 2011). The variance in the untreated controls is one important measure for statistical power to detected effects. Because of these dependencies a preliminary-screening of the communities - Collembola, Oribatida, Enchytraeida and Earthworms- for different hay meadows in the region of Aachen was conducted for site selection. For the present study, it was important to have both sufficiently high average numbers of individuals for each taxon and preferably wide spread diversity to make the results representative for a larger number of species in agricultural soil communities. One of the most important groups of animals in soil is the group

of earthworms, comprising several species, some of them known as ecological engineers. So far the TME-Method was not assessed and developed to study earthworm communities, but it was required to involve them because of their outstanding function in soil. A core depth of 40 cm was needed in order to sample an adequate number of earthworms belonging to the typical traits i.e. endogeic, anecic etc. life form type in the TME. Based on a wider screening for oribatid mites, collembolans and enchytraeids, also the average earthworm number of two sites (extensively and intensively farmed, resp.) were surveyed. The number of earthworms in the soil of the intensively managed meadow were found to be high (426 ind./m^2) and represents a typical cross section of the earthworm community (see Table 6), so it was chosen to be the coring site for the present study (see Figure 6).

Table 6 Results of the pre-screening for Earthworms on two hay meadow sites, extensive and intensive farmed, in the Eifel region.

Summary		
	Number per m^2	
Species	Intensive	Extensive
Aporrectodea sp. sensu lato [1]	154.00	57.33
Allolobophora chlorotica	0.00	0.00
Aporrectodea caliginosa	108.00	49.33
Aporrectodea longa	0.00	0.00
Aporrectodea rosea	10.00	8.00
Lumbricus spp.	84.00	38.67
Lumbricus rubellus	8.00	0.00
Lumbricus castaneus	8.00	9.33
Lumbricus terrestris	12.00	2.67
Octolasion spp.	4.00	4.00
Octolasion cyaneum	0.00	2.67
Octolasion lacteum	0.00	0.00
Undetermined [2]	4.00	16.00
Total of endogeic species	118.00	60.00
Total of anecic species	12.00	2.67
Total of tanylobous species	112.00	53.33
Total of epilobous species	276.00	121.33
Total of adults	138.00	72.00
Total of juveniles	242.00	100.00
Total	426.00	188.00

[1] Not distinguished between juveniles of the closely related genera *Apporectodea* and *Allolobophora*

[2] Fragments of worms which could not be identified

3.2.2 Location and characterisation of the coring site

The coring site is located in the northern Eifel region near Höfen (Figure 6), approx. 30 km south of Aachen (North Rhine Westphalia). The secular annual mean temperature in this region is 8.1 °C, the secular annual mean precipitation 1112 mm (NLP-Eifel, 2012).

Figure 6 left: Location of the coring site of TMEs in Höfen / Monschau (North Rhine Westphalia). No. 4 Intensive meadow (Coring Site), No. 5 adjacent Extensive Meadow involved in pre-screening of soil organisms; right: Placing of TMEs into the test facility

The coring site of the TMEs can be characterized as an intensive farmed and fertilised hay meadow in the mountainous Eifel region, 573 a.s.l. near Monschau/ Höfen (Coordinates: 50° 31' 18.05", 6° 17' 8.44"). The habitat type is classified as species-poor intensive grassland on moist sites (Code: 34.08.02.01 according to Riecken et al., 2003). The soil type is classified as brown soil, without stagnant moisture or groundwater soil wetness, with 30-100 cm soil depth (B32; B33; according to soil map of NRW (BK50 NRW; Figure 7, AG Boden 2005). The parent material is built by shale from silt- and clay stone. Soil samples were analysed by the Chemical & Pharmaceutical Laboratory Dr. Graner & Partner GmbH in Munich. The texture of the different soil layers can be classified as loamy silt to silty Loam (U,g,s,t according to DIN 4022). The pH-value is in the range between 6.1 and 6.5 depending on soil depth. The total Organic carbon content was in the upper layer 0-5 cm measured as 4.7 % DS, 3.1 % DS in the 5-10 cm layer and 2.7 % DS in the 15-25 cm layer. A screening for different pesticides showed no measurable content on any pesticide substance in the upper soil layer (see appendix 1).

Figure 7 (right side) Soil profile of the brown soil at the coring site in the Eifel (soil column approx. 80 cm high).

Further soil characteristics like total organic carbon content, total nitrogen content, total phosphor concentration were also analysed for the uppermost soil layer and are given in Table 7. Extended results to soil classification (e.g. grain size distribution) are given in the report of Dr. Graner & Partner GmbH (appendix 1).

Table 7 Results of the soil analysis of the coring site in the Eifel. TOC: Total organic carbon; Total N: concentration of total nitrogen; Total

layer	pH	TOC [% DS]	Total N [mg/kg DS]	Total P [mg/kg DS]	Sum PPP
0-5 cm	6.5	4.7	5700	1600	0
5-10 cm	6.1	3.1	-	-	-
15-25 cm	6.2	2.7	-	-	-

3.3 Experimental design

In this project, three separate studies were conducted in order to assess the exposure and effects of pesticides on soil organisms.

Study [1] The first outdoor study was conducted with two different pesticides to assess the effects on soil organisms under realistic conditions.

Study [2] The second study was conducted in the laboratory with the same two but radiolabelled pesticides to assess the fate of the actives but also the formation of non-extractable residues as part of the total exposition to soil organisms.

Study [3] The third study was additionally conducted as an outdoor experiment to assess the toxic effects on earthworms because acute effects could not be measured in the first study. In this third study it was used an agent that is known as earthworm toxic.

All three have in common that during the study the pesticide concentration and the toxic effect on different soil animal taxa were measured in different soil layers over time as well as the water inputs (precipitation) and outputs (leachate water) for the whole TME. This approach should provide the possibility to merge the specific data with one another and with the approaches and calculations that were made in the registration process for pesticides at present time.

3.3.1 Experimental design of study [1]

The experimental design of the outdoor study should meet the highly complex relationship structure of soil organism on the one hand and the exposition to pesticides i.e. the behaviour and fate of toxicants in soil on the other. Therefore, a test design was chosen with two different pesticides according to their behaviour in soil. The chosen pesticides were Lindane and Imidacloprid (characteristics see appendix 3). They were selected under consideration of following criteria:

- One substance with comparatively high mobility in soil (Imidacloprid), one substance with low mobility (Lindane) (see Figure 8)
- High persistence of the substances - thus only few metabolites
- High analytical sensitivity, analytical standard procedures available
- Access to radio labelled substances

- Both substances should be toxic for the focused soil organisms, particularly soil microarthropods and oligochaetes

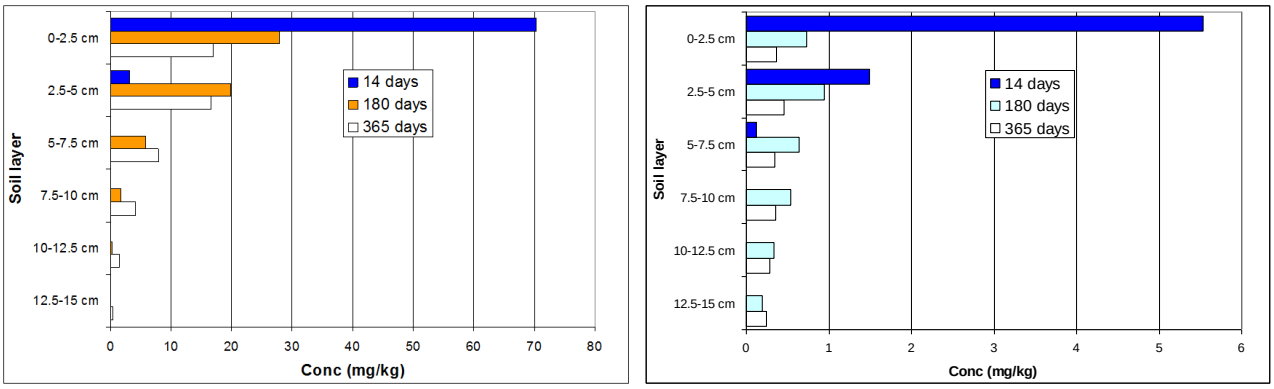

Figure 8 Calculated Lindane (left side) and Imidacloprid (right side) concentration in the soil 14 days and 180 days after application (pre-screening simulations with PELMO for Okehampton, rate: 2 kg/ha Imidacloprid). Further information is given in appendix 3.

Both pesticides were applied in two different concentrations (Table 8). The concentrations were chosen with the aim to produce significant effects on all considered soil animal groups while total erasure of the populations should be avoided. The feasibility and sensitivity of analytical approaches should be considered at the same time. For this purpose a literature survey took place and a prospective assessment with different scenarios (i.e. precipitation) was modelled via PELMO-model (see Figure 8, appendix 3).

Table 8 Application rates of the two pesticides involved in the first outdoor TME study [1] and sampling of different soil organisms

	(Conc. g a.s./ha)	Orib., Coll., Enchy.	Earthw.	Leach.
Imidacloprid				
Conc. low	750	x	x	
Conc. high	2000	x		x
Lindan				
Conc. low	7500	x	x	
Conc. high	20000	x		x

Conc. Earthw.= Concentration used for earthworm TME;

Leach. = Concentration used for Leached Water TME

To meet the highly sophisticated analytical, ecotoxicological and statistical requirements, a compromise was created to set the necessary replicates for a dose-response approach in the current study. For the microarthropods and enchytraeids, 5 sampling dates (T1-T5) with 5 replicates (TMEs) for each concentration and 10 controls were set. For the earthworms, three sampling dates were set with 5 replicates for the lower rate of each pesticide and the control. At

the end of the study, after all microarthropod, enchytraeid and analytical samples were taken, the soil of all TMEs i.e. two pesticides with two concentrations each, were sampled for earthworms. Thus, for the last sampling date also data for earthworms at the higher pesticide rates were available. To sample earthworms it was necessary to destructively sample the entire TME soil core at a time. More sampling dates or considered concentrations would increase the effort of coring, the test facility and sampling enormously, thus a reduced approach was chosen. In addition to those TMEs that were used for sampling replicates for animals additional analytical TMEs were installed that serve further methodological needs for the analytics i.e. sampling of deeper soil layers without disturbing the soil animal community. There were two TMEs installed for the measurement of soil temperature and soil moisture in two different soil layers over time. In a separate set-up nearby, two more TMEs used for measurement of leachate water were installed (see Chapter 3.5). Table 9 gives an overview of the number of samplings and the number of TMEs that were used as replicates or for the different measurements. Numbers of samples for earthworms and mesofaune differ since high efforts are needed when collecting lumbricids (i.e. a lack of resources), but at the same time the spatial distribution of earthworms is considered to be less variable than that of, for example, enchytraeids, due to their higher mobility. Accordingly, such a difference is also found when studying ISO guidelines for the sampling of these organism groups in monitoring programs (ISO 2006a,b; ISO 2007).

Table 9 Number of replicates (TMEs) and samplings for soil taxa, analytics, leachate water measurements and moisture/temperature-TMEs in the first outdoor study [1]

TME	number of samplings	Replicates					
		Control	Lindane 7.5 kg/ha	Lindane 20 kg/ha	Imidacl. 0.75 kg/ha	Imidacl. 2 kg/ha	no pesticide
Oribatida, Collembola, Enchytraeidae	5	10	5	5	5	5	-
Earthworms	3	5	5	-	5	-	-
Analytics	6	-	1	1	1	1	-
Percolation water	-	-	-	1		1	-
Moisture and Temperatur	-	-	-	-	-	-	2

The pesticides were applied in the following randomised pattern (see Figure 9) on the TME cores in the test facility in Aachen (cp. Chapter 3.3.1)

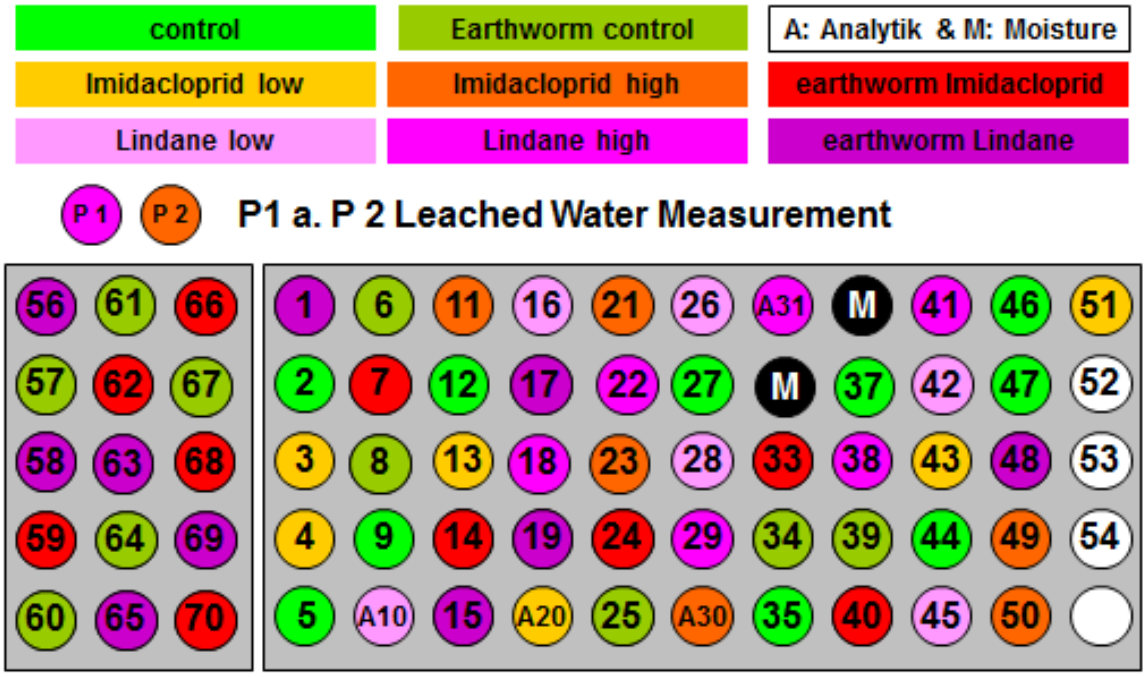

Figure 9 Randomised study design with controls, two pesticides (Lindane & Imidacloprid) in two application rates each and measurement of leachate water. The TMEs named control, Imidacloprid low/high and Lindane low/high were used to sample microarthropods and enchytraeids. Analytical measurement for the pesticide in the soil layers 0-2.5 cm; 2.5-5 cm and 5-10 cm were also taken within these TMEs. The "earthworm"-TMEs were used for sampling earthworms only. The four TMEs labelled with A were considered to measure the pesticide concentrations in deeper soil layers 10-20 cm

The following soil layers were considered separately: A: 0 – 2.5 cm, B: 2.5 – 5 cm, C: 5 – 10 cm, D: 10 - 20 cm (Figure 10). Due to practical reasons, no sampling of the layer 0-1 cm could be performed for soil organisms in the outdoor TMEs. By contrast, in the indoor TMEs with radiolabelled substances, also the 0-1 cm soil horizon was sampled separately (please refer to Table 11).

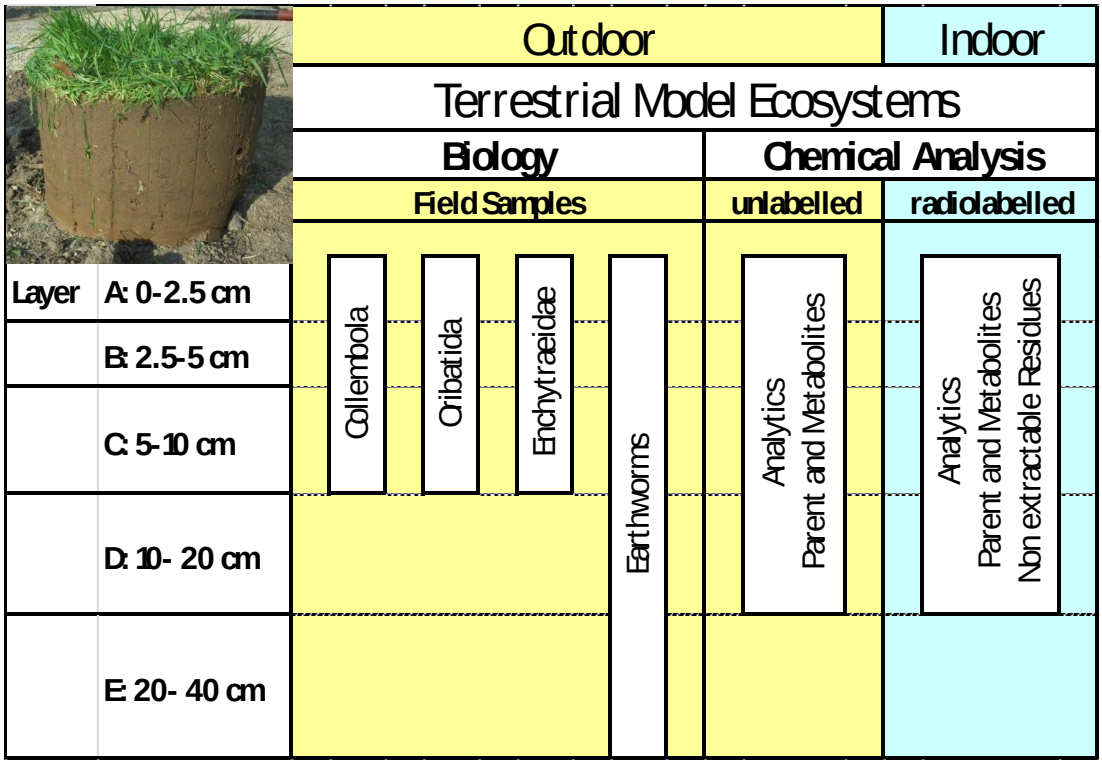

Figure 10 Link of analytical and biological data out of the field (outdoor study [1] and indoor laboratory study [2] by sampling in Terrestrial Model Ecosystems (TMEs).

Table 10 Time table for the first outdoor TME study [1]

timeline		
Coring of TMEs in the Eifel		03 May 2011
Placing TMEs in the facility in Aachen		03 May 2011
Application of Pesticides	Lindane, Imidacloprid	10 May 2011
Sampling T0	ana.	11 May 2011
Sampling T1 (14 d)	ana., Ori, Coll, Ench, Earthw	24 May 2011
Sampling T2 (42 d)	ana., Ori, Coll, Ench	21 June 2011
Sampling T3 (140 d)	ana., Ori, Coll, Ench, Earthw	27 September 2011
Sampling T4 (189 d)	ana.*, Ori*, Coll*, Ench*	15 November 2011
Sampling T5 (365 d)	ana., Ori, Coll, Ench, Earthw	8 May 2012

ana. = analytics; Ori = Oribatida; Coll = Collembola; Ench = Enchytraeidae; Earthw = Earthworms

* only layer 0-2.5 cm and 2.5-5.0 cm

71 TMEs were cored on 03 May 2011 in the hay meadow and placed in the TME-facility in Aachen (cp. Figure 6, Figure 9). Three TMEs were considered as backups and were not used within the study, so that overall 68 TMEs were used within the study [1]. The timeline of coring, applying of pesticides and sampling were given in Table 10.

3.3.2 Experimental design of the laboratory study [2] (use of radioactive test substances)

Radioisotope labelled substances cannot be applied to the field or in outdorr experiments because of regulatory issues. Therefore, numerous studies focusing on the in-field fate of pesticides are limited to the application of unlabeled compounds. This approach, however, only enables the analysis of compounds residues remaining in the soil using so-called 'cold' extractions methods. Furthermore, it is very difficult to identify the transformation products which differ structurally from the parent compound and its primary metabolites. Soil system is a very complex matrix, therefore isotope tracers are needed for a proper quantification and identification of a compound turnover, particularly the evolution of CO_2 or binding mechanisms to soil matrix (i.e. formation of non-extractable residues, NER). Therefore, radiotracers were used for elucidation of the fate of Lindane and Imidacloprid in our laboratory studies (^{14}C labelled Lindane and Imidacloprid).

For each test substance, five TMEs mimicking the field TMEs but of smaller size (400 mm deep and 100 mm in diameter) were installed in the radioisotope-laboratories of the Institute. The soil cores were sampled at the same coring site as the soil cores in the outdoor study (Eifel). They were treated with a mixture of radio labelled and unlabelled compound in the amounts corresponding to the high application rates of the outdoor study (20 kg/ha Lindane, 2 kg/ha Imidacloprid).

The laboratory TMEs were kept under similar soil moisture conditions as the outdoor TMEs, i.e. all precipitation and leaching events in the outdoor study were recorded and the laboratory TMEs were watered at the same amount and time. In the outdoor study, the soil cores were placed on a waterlogging preventing ground. In the laboratory studies, glass bottles were placed under each TME to collect the leachate. The sampling dates and the extraction of the samples comply with the dates of the extraction of soil samples from the outdoor experiment, lasting however only for 189 days. The soil extracts and the leachate were measured with Liquid Scintillation Counting (LSC) to quantify the residual radioactivity.

Subsequently, the extracted and the unextracted soil samples were combusted in a biological oxidizer in order to quantify the total radioactivity and the amount of NER. The analytical recovery comprises only the ^{14}C content in the soil extracts, leachate and NER; since any volatile products could not be trapped (i.e. it was an open system). The volatility (as parent insecticide, metabolite or CO2) was therefore calculated using the following equation:

% volatility = (100 % of applied amount of radioactivity) – (radioactivity in extracts) – (radioactivity in leaching water) – (radioactivity in NER).

Experimental setup:

Ten soil cores were sampled from the field on 1st of May 2012, as it was described previously in Chapter 3.2 Thereafter, they were installed in the radioisotope laboratory and incubated at 15°C (see Figure 11). Moreover, TMEs were exposed with two 400 W metal halide light sources (15 hours/day).

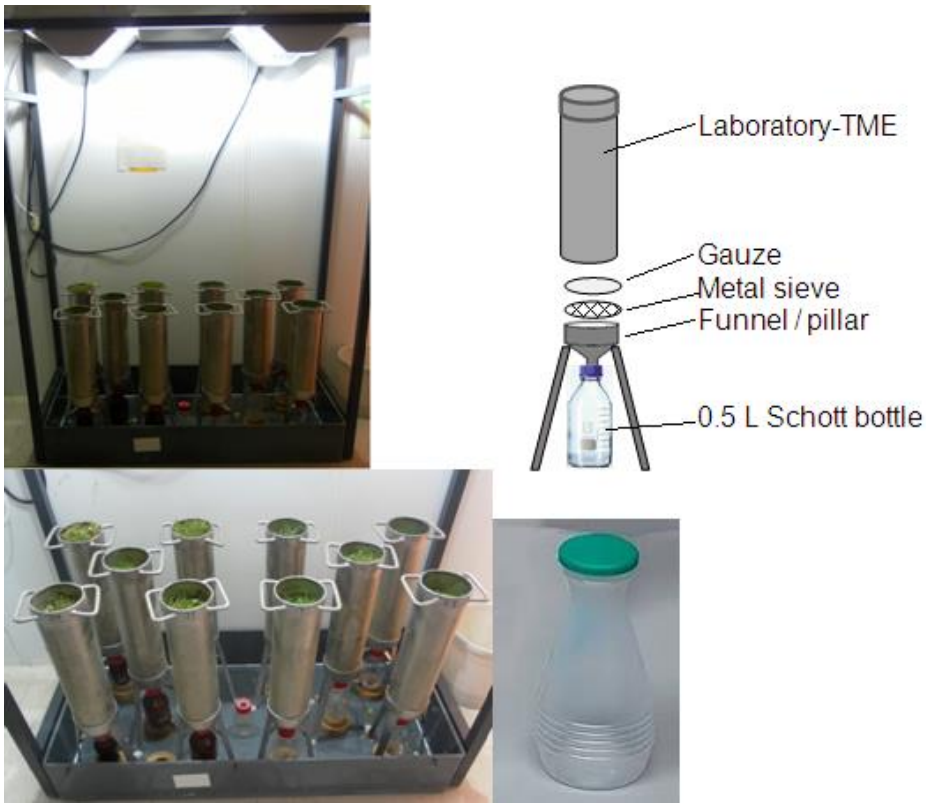

Figure 11 Left: Terrestrial Model Ecosystems (TMEs) Ø 100 mm, height 400 mm, in the radioisotope laboratory, study [2]. Top right: schematic picture of a laboratory-TME. Down right: sprayer used for watering.

The applied amount of the respective active substance (5 TMEs for each insecticide) was as follows:

Lindane: 20 kg/ha = 15.71 mg a.s./78.54 cm² (surface of the TME), this included 2.432 MBq radioactive labelled test substance.

Imidacloprid: 2 kg/ha = 1.57 mg a.s./78.54 cm² (surface of the TME), this included 2.595 MBq radioactive labelled test substance.

At each sampling date, one Lindane TME and one Imidacloprid TME were sampled completely. The soil cores were divided into five soil layers which were analysed separately (see Table 11).

Table 11 Dates of sampling and soil layers

Dates of sampling:			Sampled soil horizons:	
Application:	15. May 2012	Tag 0	Layer S	Grass cover
Sampling T0	16. May 2012	Tag 1	Layer A1	0 – 1 cm
Sampling T1	29. May 2012	Tag 14	Layer A2	1 – 2.5 cm
Sampling T2	26. June 2012	Tag 42	Layer B	2.5 – 5 cm
Sampling T3	02. October 2012	Tag 140	Layer C	5 – 10 cm
Sampling T4	20. November 2012	Tag 189	Layer D	10 – 20 cm

3.3.1 Experimental design of the additional earthworm study [3]

The aim of the additional experiment was to study acute toxic effects on earthworms which are those animals of the considered soil organisms with the highest mobility in soil and which are highly relevant in soil risk assessment at the time. Study [3] was perfomed after the results of study [1] were available, showing no pronounced initial effects on earthworm at the chosen application rates for Imidacloprid and Lindane. For the purpose of observing the profile of effects on earthworms, Carbendazim was chosen, since it is known as toxic reference for earthworms in numerous field studies (characteristics see appendix 2). Carbendazim persists in soil for a sufficient time (half-life 28-36 days at 15°C, EU 2007) and can be analysed accurately.

The study was set up according to the first study (study [1]) with five replicates and three samplings each (see Table 12). Just as in study [1], the temperature and moisture was measured in two additional TMEs. Furthermore, two TMEs were used for measurement of leachate water.

Table 12 Number of replicates and samplings for soil taxa (earthworms), leachate water measurements and moisture/temperature TMEs in outdoor study [3]

TME	number of samplings	Replicates			
		Control	Carbendazim low	Carbendazim high	no pesticide
Lumbricidae	3	5	5	5	-
Percolation water	-	-	-	-	2
Moisture and Temperatur	-	-	-	-	2

Carbendazim was applied in the following randomised pattern (see Figure 9) on the TME cores in the test facility in Aachen (cp. Chapter 3.3.1). The application rates were 7.5 kg/ha and 15 kg/ha. The application rates were chosen with the aim to produce significant effects on earthworms - while total erasure of the population should be avoided.

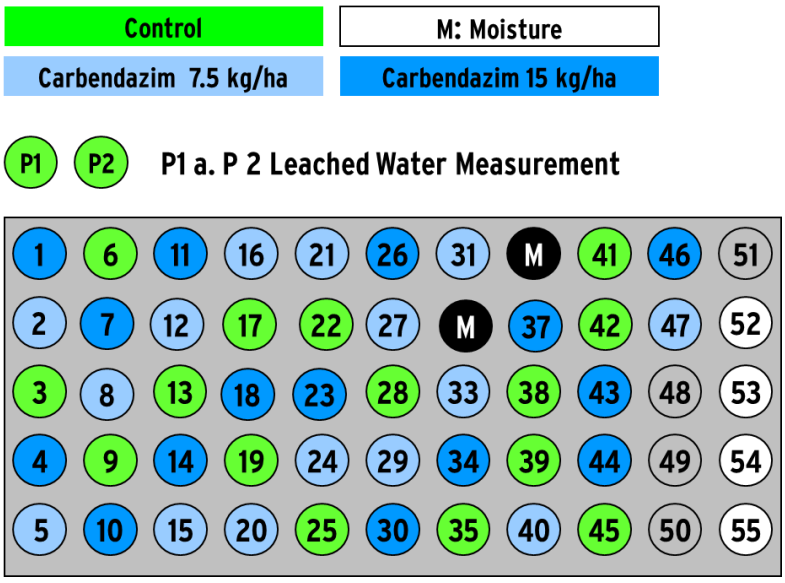

Figure 12 Randomised study design with controls and two pesticide application rates and study of leachate water in outdoor study [3]. For Earthworm sampling 5 TMEs of controls and 5 TMEs of each treatment were destructively sampled at each sampling date. M: TMEs for measurement of moisture and temperature.

The timeline of coring, applying of pesticides and sampling for outdoor experiment [3] are given in Table 13. Because of a long and strong winter 2012/2013, the coring of the TMEs took place in the early June of 2013. The application was on 10th of June and was followed up by three samplings 16, 114 and 148 days after application.

Table 13 Time table for study [3]

timeline		
Coring of TME in the Eifel		06 June 2013
Placing TME in the facility in Aachen		06 June 2013
Application of Pesticide	Carbendazim	10 June 2013
Sampling T0	ana.	11 June 2013
Sampling T1 (16 d)	ana., Earthw.	26 June 2013
Sampling T2 (114 d)	ana., Earthw.	02 October 2013
Sampling T3 (148 d)	ana., Earthw.	05 November 2013

ana. = analytics; Earthw. = Earthworms; * only layer 0-2.5 cm and 2.5-5.0 cm

Overall 53 TMEs were cored in the hay meadow and placed in the TME-facility in Aachen. Four TMEs were considered as backups and were not used within the study, so that all in all 49 TMEs were used within study [3].

3.4 Application

3.4.1 Application of Imidacloprid and Lindane in study [1]

In this study, both pesticides were applied as formulations. For Imidacloprid the formulation IMIDACLOPRID WG 70A W and for Lindane the formulation GAMMA-HCH EC 150 g/l were used, both provided by Bayer CropScience AG, Monheim, Germany. Information on the two test items used for this study is listed in Table 14 and Table 15 respectively.

Table 14 Information about the used Imidacloprid formulation

Trade name:	Imidacloprid WG 70 A W
Batch no.:	ID ED00077349
Active ingredient:	Imidacloprid
Active ingredient content:	70.3 %w/w

Table 15 Information about Lindane formulation

Trade name:	GAMMA-HCH EC 150 g/l
Batch no.:	2011-002215
Active ingredient:	GAMMA-HCH
Active ingredient content:	15.0 %w/w

Equipment:

- Two hand held sprayer (Gloria 172 RTG) with a conventional hydraulic nozzle
- Buckets and plastic foil to cover the other TMEs during application
- Measuring wind speed instrument
- Thermometer
- Magnetic stirrer
- Volumetric flasks (1L, 2L)
- Volumetric pipettes (50 ml, 100 ml)

3.4.1.1 Preparation of the application solutions

The following test application rates of Imidacloprid were tested in this study: 750 g a.s./ha and 2000 g a.s./ha. Based on the active ingredient content and the area of the TMEs the amount of formulation applied per TME was calculated (see Table 16). For each test variant a separate application solution was prepared. A volume of 100 mL application solution was applied on each TME.

For the application solutions of Imidacloprid an amount of 0.4873 g and 0.3655 g of the formulated product was weighed by means of a calibrated analytical balance, quantitatively mixed into tap water by using volumetric flasks, and made up to a volume of 1000 mL and 2000 mL respectively for both test concentrations (see Table 16). These application solutions were intensely stirred

resulting in homogeneous solutions of the formulation. The same procedure for preparation of the application solutions was applied for Lindane.

For Lindane the following test application rates were used in the study: 7.5 kg a.s./ha and 20 kg a.s./ha. Here, 22.838 g and 17.1287 g of the formulated product were used for preparation of the applications solutions (see Table 17).

Table 16 Calculation of the Imidacloprid concentration in the application solutions

Calculation of test concentrations - Imidacloprid					2000 g a.s./ha 1L-volumetric flask Conc [mg form/1L appl.solution]	750 g a.s./ha 2L-volumetric flask Conc mg Form/2L appl.solution
Conc [g a.s./ha]	Conc [g form/ha]	Conc [g Form/m2]	Conc [g form/TME]	Conc [mg form/TME]		
750	1066.86	0.1067	0.0183	18.27		365.48
2000	2844.95	0.2845	0.0487	48.73	487.30	
				Weighed portion [g]	**0.4873**	**0.3655**

Table 17 Calculation of the Lindane concentration in the application solutions

Calculation of test concentration - Lindane					20000 g a.s./ha 1L-volumetric flask Conc [mg form/1L appl.solution]	7500 g a.s./ha 2L-volumetric flask Conc [mg form/2L appl.solution]
Conc [g a.s./ha]	[g form/ha]	Conc [g Form/m2]	[g form/TME]	[mg form/TME]		
7500	50000.00	5.0000	0.8564	856.43		17128.67
20000	133333.33	13.3333	2.2838	2283.82	22838.23	
				Weighed portion [g]	**22.8382**	**17.1287**

3.4.1.2 Application method

For both substances tested in this study (Imidacloprid and Lindane) and the negative control (tap water), a spray application method was used for homogenous application of the test item on the soil surface. To the 15 control TMEs, equivalent volumes of tap water were added (plus volume of washing water, see below).

Prior to application, the grass layer was cut to a minimum to ensure that most of the test item would reach the soil core and actual weather conditions were checked (maximum wind speed, air temperature). The volumetric flasks containing the application solutions were placed on a magnetic stirrer. Three analytical samples (50 ml per sample) were taken from each well-mixed application solution by means of a 50 ml volumetric pipette immediately before application. All TMEs actually not under treatment were covered with buckets or plastic foils to avoid contamination (Figure 13).

Figure 13 Spray application performed in the study [1]

A hand held sprayer (Gloria 172 RTG) with a conventional hydraulic nozzle was used for application (see Figure 13). Firstly, 100 ml tap water was filled into the tank of the hand held sprayer, and then 100 ml application solution was taken out of the volumetric flask by a volumetric pipette and also transferred into the tank of the sprayer. For application, the hand held sprayer was set under a pressure of 1 bar. The content was sprayed carefully and evenly onto the surface of the TME, to minimize spray-drift. The nozzle was kept in a minimum distance of about 10 cm above soil surface. Afterwards the hand held sprayer was rinsed with 200 ml tap water, which was additionally added onto the respective TME. Thus, a total volume of 400 ml liquid was applied to each soil core. Application of the test item followed in order to an increasing application rate. The second test substance was applied with an additional hand held sprayer. To ensure a sufficient and homogenous infiltration of the test substance into the upper soil layer, an artificial rain treatment was conducted. Within 48 hours after application, a total amount of 1700 ml tap water per TME (volume of application inclusive) was applied. That leads to 10 mm precipitation per m^2. Artificial rain was applied by an irrigation device usually used for gardening purpose. At the day of application, the amount of water applied was limited to 800 ml per TME to avoid flooding; at the following day 900 ml were applied per TME; resulting in the total volume of 1700 ml.

3.4.2 Application of Imidacloprid and Lindane in study [2]

a) ***Chemicals and Solvents***

- Ethyl acetate, distilled one time (EtAc)
- Acetonitrile (ACN), super gradient for HPLC (VWR, Germany)
- Purified water (H2O): MILLI-Q Water System (Millipore, Germany)
- Lindane formulation GAMMA-HCH EC 150 g/l: 15,0 % w/w Lindane, 146,3 g/l (Bayer CropScience Germany)
- [U-^{14}C] Lindane: specific radioactivity 5.39 MBq/mg, Radiochemical purity 98,78 %, chemical purity 100 % (Institute of Isotopes Co., Ltd., Budapest, Hungary)
- Imidacloprid (Imi) formulation IMIDACLOPRID WG 70A W: 70 % (w/w) Imidacloprid (Bayer CropScience AG, Germany)

- [methylene-^{14}C] Imidacloprid (^{14}C -Imi): specific radioactivity 4,44 MBq/mg, Radiochemical purity > 98 %, Chemical purity > 98 % (Bayer CropScience AG, Germany)
- Anhydrous sodium sulphate (Merck, Germany)
- Scintillation cocktail for liquid samples: Irga-Safe-PlusTM (PerkinElmer, USA)
- Scintillation cocktail for CO_2: Oxysolve C-400 (Zinsser Analytics, Germany)
- Radioactive standard: Spec-check-^{14}C (PerkinElmer, USA)

b) ***Material and methods***

Current laboratory material was used. Both pesticides were applied as formulation (the same as used in the field study), which was mixed with their radiolabelled counterparts. The volume of application solution was 4 ml and was thus proportional to the amount applied to the field. For each TME, 25 ml of application stock solution was prepared.

i. Preparation of the application solution of Lindane

An application of 20 kg/ha of Lindane corresponds to the application of 15.71 mg a.s./78.54 cm^2. This application solution should therefore have a concentration of 98.18 mg / 25 ml (15.71 mg/ 4 ml * 25 ml).

^{14}C-Lindane: The specific radioactivity of the compound is 5.39 MBq/mg. The substance was dissolved in ethyl acetate. The radioactivity of the solution was 9.50 MBq/ml. 256 µl of this solution have an activity of 2.432 MBq, therefore 1600 µl (256 µl/4 ml*25 ml) was used to prepare the application solution. 1600 µl of this solution contains 2.82 mg of ^{14}C-Lindane.

Formulation: 98.175 mg of Lindane are required for the application solution, thereof 2.82 mg of ^{14}C-Lindane. Hence, 95.355 mg of unlabelled Lindane was needed. As the concentration of Lindane in the formulation is 15 % w/w, 635.70 mg of the formulation was used (95.355/15*100).

635.70 mg of Lindane formulation was weighed in a 25 ml of volumetric flask and 1600 µl of ^{14}C-Lindane solution were pipetted into the solution using a glass pipette. The flask was then filled with tap water. The radioactivity of the final application solution was 0.624 MBq/ml (analysed four times, corresponded to 2.496 MBq/4 ml). The obtained data are summarised in Table 18.

Table 18 Preparation of the application solution Lindane. Each TME 4 ml solution was applied.

	Concentration	Radioactivity	used amount	Absolute content
Lindane formulation	15 g/100 g	-	635.70 mg	95.355 mg Lindane
^{14}C-Lindane-Solution	9.50 MBq/ml	5.39 MBq/mg Lindane	1600 µl	2.82 mg Lindane
Water	-	-	added to 25 ml	
Application solution	15.71 mg/ 4 ml	2.496 MBq/4 ml		

ii. Preparation of the application solution of Imidacloprid

An application of 2 kg/ha of Imidacloprid corresponds to an application of 1.57 mg a.i./78.54 cm2. The application solution should therefore have a concentration of 9.82 mg / 25 ml (1.57 mg/ 4 ml * 25 ml).

^{14}C-Imidacloprid: The specific radioactivity of the compound is 4.44 MBq/mg. This substance was dissolved in acetonitrile. The radioactivity of the solution was 3.81 MBq/ml. 816 µl of this solution have an activity of 2.595 MBq, therefore 5100 µl (816 µl/4 ml*25 ml) was used to prepare the application solution. 5100 µg of this solution contained 3.65 mg of ^{14}C -Imidacloprid.

Formulation: 9.82 mg of Imidacloprid are required for the application solution, thereof 3.65 mg of ^{14}C-Imidacloprid. Hence, 6.17 mg of unlabelled Imidacloprid was needed. As the concentration of Lindane in the formulation is 70 % w/w, 8.81 mg of the formulation was used (6.17/70*100).

8.81 mg of Imidacloprid formulation was weighed in a 25 ml of volumetric flask and 5100 µl of ^{14}C-Imidacloprid solution were pipetted into the solution. The flask was thereafter filled with tap water. The radioactivity of the final application solution was 0.555 MBq/ml (analysed four times, corresponded to 2.220 MBq/4 ml). The obtained data are summarised in Table 19.

Table 19 Preparing of the application solution of Imidacloprid. 4 ml were applied on each TME. "Imi" = Imidacloprid

	Concentration	Radioactivity	used amount	Absolute content
Imidacloprid formulation	70 g/100 g	-	8.81 mg	6.17 mg Imi
14C-Imidacloprid-Solution	3.81 MBq/ml	4.44 MBq/mg Imi	5100 µl	3.65 mg Imi
Water	-	-	added to 25 ml	
Application solution	1.57 mg/ 4 ml	2.220 MBq/4 ml		

iii. Application of the substances

In order to mimic the experimental setup of the outdoor study [1], the grass cover of each TME was cut to 2 cm prior to the application of the respective pesticide. 4 ml of the respective application solution containing either ^{14}C-Lindane or ^{14}C-Imidacloprid was added to each TME . This was accomplished using a 1 ml of Hamilton microliter syringe; the application solution of the respective pesticide was dropwise and equally distributed on the surface of each TME . The same syringe was also used for application of 4 ml of tap water in order to clean the syringe and to wash off the application solution from the vegetation. After the application with pesticide, the TMEs were watered with 40 ml of tap water, which is equivalent to a watering of 5 mm.

3.4.3 Application of Carbendazim in study [3]

In this study, Carbendazim was applied as Derosal 360g/L formulation (see Table 20) provided by ECT, Flörsheim, Germany.

Table 20 Information about Carbendazim formulation used in study [3]

Trade name:	Derosal 360 g/L
Batch no.:	GAB Code 2006 1089

Active ingredient:	Carbendazim
Active ingredient content:	28.90 %w/w

In line with study [1], a spray application was performed and the same procedure for preparation of the application solutions was used. The calculation of the application rates per TME and the amount of the formulated product used are summarized in Table 21. Here, 8.890 g and 17.781 g of the formulated product were used for preparation of the applications solutions (see Table 21) corresponding to 7500 and 15000 g a.s./ha respectively.

Table 21 Calculation of the Carbendazim concentration in the application solutions for outdoor study [3]

Calculation of test concentrations - Carbendazim

Conc [g a.s./ha]	Conc [g Form/ha]	Conc [g form/m2]	Conc [g form/TME]	Conc [mg form/TME]	Conc [mg form/L appl. solution]	Conc [g form/ 2L appl. solution]
7500	25951.56	2.5952	0.4445	444.52	4445.16	8.890
15000	51903.11	5.1903	0.8890	889.03	8890.31	17.781

Conc [g a.s./ha]	Weighed portion [g]
7500	8.890
15000	17.781

3.5 Sampling for pesticide analysis, of microarthropods, enchytraeids and earthworms

The timeline of sampling for study [1] and [3] is given in Table 10 and Table 13. T0 is the sampling date 1 day after application - which is used only for analytical sampling in this study - in order to determine the total recovery of the applied pesticides. For this purpose, only the two upper layers, A: 0-2.5 cm and B: 2.5-5 cm, were sampled. Thus the wording T0 equates in this given context not to the ecotoxicological meaning where T0 is known as the status before application. The sampling T4 is another special case. At this date only the two upper layers A and B were sampled for oribatid mites, collembolans and enchytraeids. The analytics were sampled as described above.

3.5.1 Sampling for pesticide analysis, study [1] outdoor study with Lindane and Imidacloprid

Soil samples were taken with a soil corer (Ø 5 cm, Figure 16 shows a similar model), using a calibration disk (see Figure 14) to define the coring positions. The soil was crashed and filled into plastic bottles. Sampling bottles were pre-weighed. Layer A and B (0-2.5 and 2.5-5 cm) were cored together and the core was separated with a knife on a tray. To prevent contaminations, the tray was covered with two pieces of aluminium foil, which were changed after every sample. Between every sampling soil corer, knife, tray and other tools were thorough cleaned with acetone. Afterwards, the Lindane samples were weighed again and 400 g/kg of soil anhydrous sodium sulphate were added. All samples (except samples of two control-TMEs) were stored at –20°C.
At all sampling dates in the analytic TMEs, two samples (diameter of soil core 5 cm) were taken in all soil layers up to 20 cm (layer A-D). Due to an additional sampling date at day 140 (T4) the analytical samples at day 140 and day 365 (T5) were taken with a smaller sample corer with a diameter of 3.5 cm. For each sample, two smaller samples were taken between the sampling positions shown in Figure 14 with a total surface of 19.2 cm^2 instead of 16.6 cm^2. The total amounts of Lindane and Imidacloprid in these samples were calculated for a surface of 19.6 cm^2.
At day 1 (T0) layer C in the mesocosm TMEs was not sampled, because the substances are not expected to reach this layer. Also at day 140 (T4) layer C was not sampled in the mesocosm TMEs (as discussed with UBA for this additional sampling). In the mesocosm TMEs, layer D (10 – 20 cm) was never sampled to minimise effects due to a destroyed soil core (like wash-out). Layers beyond the depth of 20 cm were not sampled to minimize wash-out effects and because the a metal corer could not be dig that deep into the soil without machines.

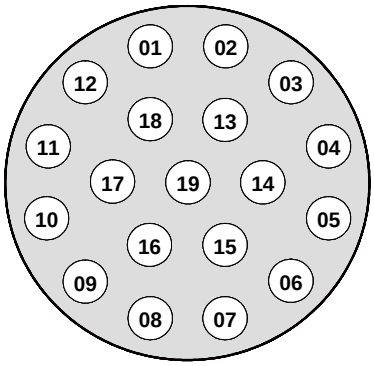

Figure 14 Left: Tray with 2 samples (Layer A and B; 0-2.5 and 2.5-5 cm) Right: Calibration disc for the identification of sampling positions (Ø 5 cm) 1 to 19 for microarthropods, enchytraeids and analytic sampling.

3.5.2 Sampling for pesticide analysis, study [2] indoor study with radiolabelled Lindane and Imidacloprid

During sampling, the soil core was pushed with a pressure tool through the bottom of the steal casing and cut into subsamples (Figure 15). Thereby a contamination of deeper soil layers with substance from the upper layers is avoided. The 40 cm deep soil core was first pushed 20 cm out of the column and cut off. This layer was discarded. In the next steps, the soil core were pushed out 10 cm and cut off, then 5 cm and cut off. The last 5 cm were pushed out and cut into the layers 0-1 cm, 1-2.5 cm and 2.5-5 cm. Each layer was crushed on a separate piece of aluminium foil to avoid contamination and was filled in pre-weighed plastic bottles (the same used at the outdoor study). The bottles with the soil were weighed again. A few gram of soil were used to determine the content of dry matter (as described in Chapter 3.7.2). The Lindane containing samples were weighed again and about 400 g/kg soil of anhydrous sodium sulphate were added. The samples were stored at -20°C until analysis.

3.5.3 Sampling for pesticide analysis, study [3] outdoor study with Carbendazim

The soil sampling for Carbendazim analysis was taken by means of a soil corer (Figure 16). For the determination of total recovery one day after application in every TME was taken one soil sample in layer 0-1 cm, 0-2.5 cm and 2.5-5 cm. The replicates (5 TMEs/sampling date) were mixed for each soil layer. The weight of the mixed samples was recorded. The samples also weighted right before the measurement after transport to the laboratory. All samples were analysed by the external laboratory Dr. Graner & Partner (DIN EN 14346 dry substance, DIN 38407-35 Carbendazim).

For the sampling dates T1-T3, three soil cores of each considered soil layer (0-1 cm, 0-2.5 cm and 2.5-5 cm. 5-10 cm, 10-20 cm, 20-40 cm) were taken and mixed and analysed by the above mentioned external laboratory (DIN EN 14346 dry substance, DIN 38407-35 Carbendazim). DIN 38407-35 described the principal measurement method to determine the concentration of the active substance via LC-MS/MS. According to this description, 20-30g of the soil samples, inclusive the root material of plants, were dried at 40°C over night. On the next day, the dried soil samples were finely pulverised by means of a mill (Pulverisette, Fa. Fritsch). 5g of the fine milled soil of each sample were weighted in and were shaken with 10 ml of acetonitril. After the extraction with acetonitril, the sample solution was diluted with destillated water 1:10, membrane filtered and analysed with LC-MS/MS.

Figure 15 The soil core was pushed out of the column through the bottom and cut into subsamples.

3.5.4 Sampling, extraction and determination of microarthropods and enchytraeids

The sampling to capture abundances of microarthropods and enchytraeids took place by mean of a soil corer (Ø 5 cm, see Figure 16) in given coring positions provided by the sample disk (Figure 11). In all replicates (TMEs) that are considered for these groups in study [1] (Figure 9) were cored two soil cores , 0-5 cm (layer A+B) to capture Oribatid mites and Collembolans, and one soil core, 0-5 cm (layer A+B) to capture Enchytraeids. Afterwards the soil cores were separated in layer A and B by cutting them into pieces with a knife. To sample layer C, further soil cores were taken in the same pattern and same sample positions for the layer 5-10 cm.

Oribatid mites and Collembolans were extracted in a modified McFadyen Extractor according to the ISO Guideline (ISO, 2003) for microarthropods. The soil cores were placed upside-up on a sieve (mesh width 2 mm). Underneath the sieve there is a capture vessel that contained a benzoeacid-ethanol-water mixture for conservation of the soil arthropods. After acclimatisation by room temperature (20°C), the temperature was increased stepwise daily by 3°C over 14 days. The temperature underneath the samples was constantly cooled down to 10°C, so that the temperature gradient increased daily. The soil arthropods moved due to the increasing dryness of the soil cores downwards and were captured in the vessels. After 2 weeks the animals were transferred in 70 % ethanol and provided for sorting and determination.

The determination of the oribatid mites species were conducted according to Weigmann (2006). Accordingly, the determination of the springtails is based on the “Synopses on Palaearctic Collembola“, e.g. for the Tullbergiinae (Zimdars & Dunger 1994), Symphypleona (Bretfeld 1999), Isotomidae (Potapow 2001) and Hypogastruridae (Thibaud et al. 2004). In all other cases, Schulz et al. (2003) was used for determination.

Figure 16 Soil corer with plastic cartridges (l: empty r: filled) used for sampling of microarthropods and enchytraeids.

The cored soil samples to capture the Enchytraeids were directly stored and cooled in adequate boxes and transported to ECT Ecotoxicology GmbH for determination. After arrival of the samples at the laboratory of ECT, the samples were stored in a cooled storage room (8°C) till they could be extracted via wet extraction. The extraction took place in two charges (for more details see ISO, 2007). Single species were transferred as specimen copy in pure alcohol or were fixed on microscope slides after coloration. The determination took place under consideration of The Guide by Schmelz and Collado (Schmelz and Collado, 2010).

3.5.5 Pitfall traps to capture epigeic arthropods

To capture the epigeic active species of the collembolans, pitfall traps (Ø 5 cm) were installed. The traps were set up in the given holes that were created by the sampling of microarthropods. The pitfalls were filled with approx. 60 ml of 70 % ethylene-glycol and were opened for a period of one week right after the sampling of the soil cores. After the capture duration, they were filled with sand to be opened up and filled with ethylene-glycol again for the next capture duration. Captured Collembolans were determined as mentioned above.

Figure 17 Separating of the individual soil layers of the TME for the sampling of earthworms: 1. Row: left ejection device, middle process of hand sorting; right slicing of top soil layer; 2. Row left and middle measurement of soil layer; right soil column of TME pressed out of steel cylinder 3. Row left soil layer with remaining holes from analytical sampling; middle slicing of deeper soil layer; right ejection device with loaded TME

3.5.6 Sampling of earthworms

To measure the impact of the pesticide on earthworms, the respective TME (Figure 9, Figure 12) were sampled via hand sorting of a complete TME column as a replicate. This destructive method is according to the relevant guidelines (ISO, 1999, 2006) as well as according to recommendations of the PERAS-Workshop (Schäffer et al., 2008). The TME cores were pressed out from underneath in total by means of a heaver which was constructed for this purpose (Figure 17). After pressing out the core successively, the different layers A: 0-2.5 cm; B: 2.5-5.0 cm; C: 5.0-10.0 cm; D: 10-20 cm were cut with a saw (cp. Figure 17) and were separately searched for earthworm individuals. All found individuals were directly fixed in 70 % ethanol. The fixation liquid was renewed parallel to determination and weighing of the species to provide a long lasting fixation of the animals.

The determination of the earthworm species was conducted according to standard determination literature (Graff, 1953; Bouché, 1972; Sims and Gerard, 1999; Blakemore, 2002). Accordingly, the endpoints were abundance, biomass, and species composition of earthworms. Juvenile individuals were determined up to the genus or especially for *Aporrectodea* / *Allolobophora* up to the genus-group.

3.6 Measurement of leachate water, precipitation and irrigation

3.6.1 Irrigation and measurement of leachate in the outdoor studies [1] and [3]

During the outdoor studies, precipitation was measured by means of rain gauges which were set up within the TME facilities. Additionally, it was possible to retrieve the precipitation data of a nearby weather station. Because the mobility of pesticides is amongst others strongly depending on the precipitation amount, the outdoor study conditions (resp. precipitation) were set up alike those used for the PELMO-simulation beforehand (see therefore Chapter 7). To create realistic data, the precipitation means per month for the region where the TMEs were cored were calculated (Monschau/Höfen, see Table 22). The annual mean precipitation for Monschau is 1250 mm and matches the amounts that were used for PELMO calculations. In the middle and end of each month, these guiding values were approximated via irrigation.

Table 22 Data for precipitation [mm] in Monschau for the years 2007 to 2011 (wetter.com, 2011). Months with no data availablewere not included in mean calculation

year	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
2007	-	-	-	-	-	149	129	179	164	41	161	141
2008	82	87	234	-	63	89	158	107	107	-	-	110
2009	43	157	137	53	71	63	110	52	48	84	107	91
2010	27	58	73	20	-	23	-	-	-	68	105	65
2011	121	33	-	-	-	-	-	-	-	-	-	-
mean	68.3	83.8	148	36.5	67	81	132	113	106	64.3	124	102

Two TMEs were equipped with an Equitensiometer (Model MNT-EQ3505) and with temperature sensors (MNT-BT02). The soil moisture and temperature were continuously measured in two different soil layers (0-6 cm und 6-12 cm). The 12 h mean values (moisture unit: Pascal, temperature unit: °C) were recorded by means of a data logger (MNT Advanced Data logger, Meier Nachrichtentechnik, Zwönitz, Germany). Low values of the matrix potential around 0 kPa indicated a high water saturation of the soil. High values up to -1500 kPa indicated an increasing dryness of the soil, whereas at -1500 kPa the permanent wilting point was reached, i.e. no water was available for plants anymore.

The regular control of the moisture values and the temporarily irrigation of the TMEs guaranteed optimal environmental conditions for the soil organisms during the study time.

To measure the amount of water which leaches through the TME over time, a fit-for-purpose facility was constructed (see Figure 18). The facility contained two TMEs that were set on a perforated plate. First the leached water was collected in a hopper, then conducted through drainage pipes into a catchment box. To avoid the intrusion of water from outside the TMEs, all junction points were sealed with silicon. The TMEs were embedded into gravel so that conditions were alike those in the TMEs sampled for soil organisms. After rainfall, the catchment boxes were controlled and the leachate water amount was documented. In study [1], samples were taken and frozen for pesticide analytics from the leachate water when the amount was above 10 ml.

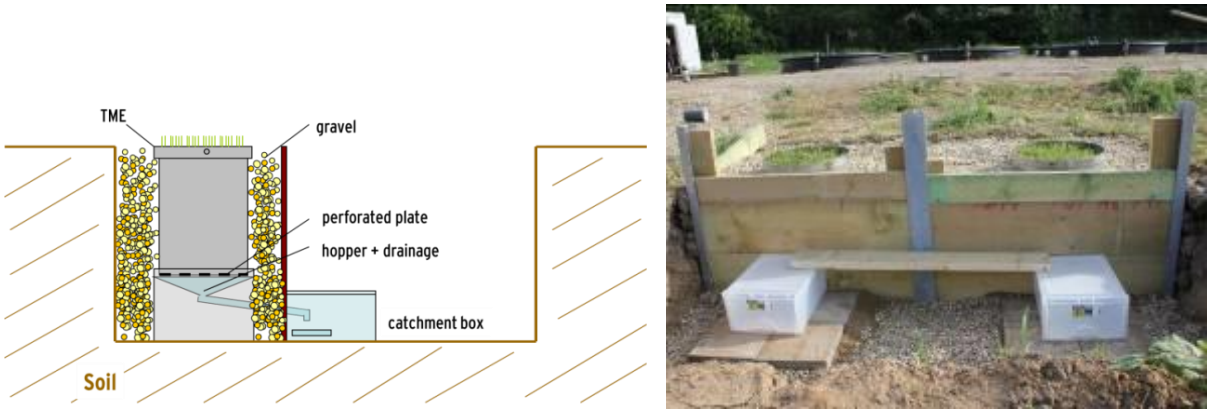

Figure 18 Schematic profile (left) and picture of the facility for measurement of leachate water (right)

3.6.2 Irrigation and measurement of leachate in study [2]

The indoor TMEs of study [2] were kept constantly at 15 °C. For watering, a 250 ml sprayer was used (Figure 11). The amount of watering had to meet three criteria:

- The soil cores should not dry out under the halogen bulb in order to avoid shrinkage of the soil core.
- The amount of leachate water observed at the outdoor-study should be simulated in the laboratory study (in proportion to the surface of the TME).
- The amount of water from rainfall and watering observed outside should be applied in the laboratory study.

The days and the amount of the watering are given in Apendix 2. Irrigation of the laboratory TMEs is presented in Table 23.

Table 23 Irrigation and leachate in the laboratory study [3]. For each month the rainfall situation outdoor, the calculated rainfall amount indoor and the final situation indoor are given.

	Precipitation and Irrigation			Leachate Water		
	Field study mm/m²	Target Laboratory ml/TME	Laboratory ml/TME	Field study ml/TME	Target Laboratory ml/TME	Laboratory ml/TME
May	61	479	270	11	1	0
June	89.5	703	480	316	14	90
July	78	612	605	184	8	8
August	134	1048	1300	3788	174	131
September	59	463	400	23	1	0
October	52	408	470	1631	75	77
November	0	0	380	22.5	1	0
Sum	473	3713	3905	5976	274	306

3.7 Chemical analysis

3.7.1 Preliminary remarks

The following section outlines the methods for the analyses of the three active substances chosen in this study (without and with radioactive labelling) in the soil and water matrix.

Lindane

Lindane (gamma- isomer of hexachlorocyclohexane) is known to volatilize rapidly with water as compared to other organochlorine chemicals (Ulmann, 1973; OSPAR Commission, 2002, Domsch, 1992). During the treatment of soil samples, measurable losses of Lindane were observed in past experiments. To prevent losses during the period of storage (frozen samples), the defrosting and the homogenisation with a mortar, the water contained in the soil was bound with anhydrous sodium sulphate.

The method of extraction and determination of Lindane in soil should be quantitative, reproducible, easy to implement at the laboratory and - regarding the large numbers of samples - fast. Therefore, in this study analyses were performed based on a method developed from Castro et al. (2001). A defined amount of soil is weighed in a small solid-phase-Extraction (SPE)-tube and extracted with ethyl acetate in an ultrasonic bath. The extract is quantified using gas chromatography with an electron-capture-detector (GC-ECD). With fortified samples Castro et al. (2001) obtained recovery rates between 93.1 ± 5.6 % and 105.0 ± 8.6 % for concentrations between 0.1 mg/kg and 1 mg/kg. Recovery rates for two different types of soil and with two different levels

of humidity were not significantly different. Additionally, the material of SPE-extraction-tubes (glass vs. polypropylene) were tested over a period of 30 days without influence of the recovery.

In the present report, recovery tests were conducted over a wider range of concentrations (36 µg/kg – 36 mg/kg) of a soil- sodium sulphate mixture. The samples were spiked with Lindane following methods of Mottaleb and Abedin (Mottaleb and Abedin, 1999) and of Tor et al. (2006). Soil spiked with Lindane was suspended with acetone, sonicated and then evaporated (rotary evaporator). All recoveries were between 82 % and 105 %.

For safety reasons, the extraction of radioactive labelled Lindane from soil could not be conducted with solid phase extraction (SPE) under vacuum. Aliquots of these samples were weighed into centrifugation tubes; the solvent was decanted after centrifugation.

Imidacloprid

Imidacloprid samples were analysed using LC-MS/MS. Several methods to extract Imidacloprid from soil were tested. The method developed by Bayer CropScience AG (M00790/M001) was rejected since the microwave extraction with acetonitrile/water was difficult to employ in our lab for higher numbers of samples. Besides, at higher concentrations and after incubation of a week, the recovery decreased to about 80 %.We applied ultrasonic extraction at 50°C using three extraction steps with acetonitrile/water. In all analyses, deuterated d_4-Imidacloprid were used as an internal standard. By this method, we achieved analytical recoveries of 99 -108 %.

Carbendazim

Carbendazim samples were analysed using LC-MS/MS. The analysies was carried by an external laboratory (Dr. Graner & Partner) following DIN 38407-35. Fot this, 20-30 g of the delivered samples were dried over night. Organic components like rootparts were included within the samples. The dried samples were finely ground by mill (Pulverisette; Fa. Fritsch). An aliquot of 5 mg was taken and shaked in 10 ml of Acetonitril over a period of 30 minutes. The extracted solvent was deluted 1:10 with destillated water, membrane-filtered and measured with LC-MS/MS.

3.7.2 Determination of dry matter content

All results of the analysis of Lindane and Imidacloprid in soil were expressed in mg/kg dry matter. The content of dry matter in soil samples of the Imidacloprid treatments were analysed in an aliquot of the sample before extraction. Because of the content of sodium sulphate, this was not possible with the soil samples of the Lindane treatments. The content of dry matter was therefore determined in samples of two randomly chosen control-TMEs. Each layer (A, B and C) was analysed separately.

Due to the high amount of grass roots, the top layer were used completely to determine the content of dry matter. The sample of the other layers were homogenised and three subsamples were used. Samples were dried over night at 104 ± 2 °C and the content of dry matter were calculated with Equation 1.

$$\textbf{Factor of dry matter} = \frac{(\textbf{Weight dry} - \textbf{Weight PD})}{(\textbf{Weight wet} - \textbf{Weight PD})} \qquad \text{(Equation 1)}$$

$Weight_{dry}$ = Weight of petri dish and dry soil in g
$Weight_{PD}$ = Weight of petri dish in g
$Weight_{wet}$ = Weight of petri dish and natural wet soil in g

3.7.3 Lindane analyses

a) ***Materials***

Chemicals and Solvents

- Technical grade Lindane, 99.9 % (Bayer, Germany)
- Anhydrous sodium sulphate (Merck, Germany)
- Ethyl acetate, once distilled
- Toluene, once distilled
- Scintillation cocktail for liquid samples: Irga-Safe-PlusTM (PerkinElmer, USA)
- Scintillation cocktail for CO2: Oxysolve C-400 (Zinsser Analytics GmbH, Germany)
- Radioactive standard: Spec-check-14C (PerkinElmer, USA)

Plastic bottles

The soil samples were stored in 250 ml plastic bottles of polypropylene (VWR, Germany).

Further material

Current laboratory material, in particular the following equipment:

- Empty SPE –Tubes, polypropylene, 20 ml (Sigma-Aldrich, Germany)
- Filter paper, grade 1, 20 mm (Whatman, UK)
- Micro test tubes, 1.5 ml (Eppendorf, Germany)
- Centrifugation tubes, 50 ml, polypropylene (Roth, Germany)
- Micro separator, own construction
- Glass cylinder with joint, 250 ml von VWR (Germany)
- Combustion Cones: Combusto-Cone, flexible (PerkinElmer, Germany)
- Scintillation vials (Polyvials® V, Zinsser Analytics GmbH, Germany)

b) ***Devices***

GC-ECD

Lindane analyses were performed with an Agilent Technologies 6890N Network Gas chromatograph, equipped with an Agilent 7683 Autosampler and 63Ni ECD. The extract was injected onto a fused silica capillary column (HP-5, 30 m by 0.32 mm) coated with HP-5 stationary phase (5% phenyl, 95% dimethyl polysiloxane, 0.25 µm film thickness). The initial oven temperature of 100°C was held for 0.5 min, temperature programming was from 100 to 300°C at 15°C min-1, which was held isothermal for 10 min. The injection port temperature was set at 250°C and the detector at 300°C. By using nitrogen as a carrier gas, the column head pressure was set at 0.8 bar to give a linear column and make-up velocity of 60 ml min-1. All injections (1 µl) were made split less.

Further devices:

- Scales: Sartorius LE323S and Sartorius CPA225P
- Ultrasonic bath: Transsonic 460 (Elma, Germany)
- 8-port vacuum manifold for Solid Phase Extraction (SPE) (own construction)
- Scintillation counter (LCS): LS 6500 Multi-Purpose Scintillation Counter (BeckmanCoulterTM, Germany)
- Centrifuge: J-20 XPI Avanti™ Centrifuge (BeckmanCoulterTM, Germany)
- Biological Oxidizer OX 501 (Zinsser Analytic GmbH, Germany)

c) ***Methods***

i. Standards and calibration solutions

A stock solution was prepared as follows: 10.28 mg of Lindane (technical grade) was weighed in a volumetric flask and dissolved with ethyl acetate to 10 ml. The concentration of the stock solution is 1.029 mg/ml (L1). From this, a working standard solution was prepared containing 1.029 µg/ml Lindane (L5n) in two dilution steps. Calibration solutions were made with ethyl acetate and Lindane-free soil extract. Care was taken to ensure that the calibration solutions had the same concentration of soil extract as the samples. To prepare the matrix, soil from control TMEs were extracted in the same way than samples. The resulting extract (matrix) is used for the preparation of the calibration standards. Calibration solutions of the range 309 – 4.1 ng/ml (Limit of Quantification, LOQ) were used.

ii. Fortification of soil samples with Lindane:

The following soils (free of Lindane) were fortified with Lindane:

- Moist soil: soil from control-TMEs, which was frozen after sampling. One soil sample each of the upper 3 layers were put together and homogenised with a mortar.
- Na_2SO_4 -soil: moist soil as described above containing 30 % sodium sulphate (43 g Na_2SO_4/100 g soil).
- Agriculture soil: air dried soil, passed through a 2-mm sieve.

Six fortifications were made as shown in Table 24.

Table 24 Fortification of soil samples with Lindane. The concentrations of the standards are as follows: L5n = 1.03 µg/ml, L4 = 10.29 µg/ml, L2 = 102.9 µg/ml and L1 = 1029 µg/ml

Soil	mg/kg	Type of soil	Preparation
1	0,036	Na_2SO_4 -soil	71,5 g sample mass (50.0 g soil) + 2.5 ml L5n + 60 ml acetone
2	0,072	Na_2SO_4 -soil	71.5 g sample mass (50.0 g soil) + 0.5 ml L4 + 60 ml acetone
3	3,594	Na_2SO_4 -soil	42.9 g sample mass (30.0 g soil) + 1.5 ml L2 + 50 ml acetone
4	35,970	Na_2SO_4 -soil	42.9 g sample mass (30.0 g soil) + 1.5 ml L1 + 50 ml acetone
5	5,145	Moist soil	30.0 g sample mass + 1.5 ml L2 + 50 ml acetone
6	5,142	Agriculture soil	30.0 g sample mass + 1.5 ml L2 + 40 ml acetone

30 g of soil, 42.9 g and 71.5 g of Na_2SO_4 –soil were weighed in a round-bottomed flask and fortified with the respective amount of Lindane and the solvent. The mixture was sonicated for 30 minutes. Afterwards the solvent was removed on the rotary evaporator. The dry soil was extracted as described in section iv.

iii. Fortification of water samples with Lindane:

1.029 µg (a) and 10.29 µg (b) of Lindane (diluted in 1 ml of ethyl acetate) were added to 100 ml of tap water. Three parallels of a) and one parallel of b) were extracted as described in section v and measured twice at GC-ECD.

iv. Extraction of Lindane from soil:

Two circles of filter paper were placed at the bottom of the SPE tubes and 2 g of anhydrous sodium sulphate were added, then 5 g of soil (thawed and thoroughly homogenised with a mortar) were weighed into the tubes. After adding 5 ml ethyl acetate, soil samples were sonicated for 15 min at room temperature. The tubes were closed with caps and supported upright in a tube rack during sonication.

The columns were then placed on the vacuum manifold where the solvent was filtered and collected in volumetric flasks (10 ml, 20 ml or 50 ml). The procedure was repeated once more with further 4 ml of ethyl acetate (15 min sonication). The soil samples were washed with 1 ml of solvent. The total extract collected was adjusted to 10 ml (Layer B – D). Layer A (0 – 2.5 cm) was extracted a third time, the extract was adjusted to 20 ml or 50 ml. The extracts were analyzed either diluted or undiluted by GC at the same day. Generally, soil samples were extracted twice, in the event of divergent results up to six parallel samples were extracted. All extracts were analysed twice with GC-ECD.

v. Extraction of Lindane from leachate:

The water samples (a maximum of 200 ml) were filled with 15 ml of toluene in a glass cylinder and roughly shaken for 2 minutes. When the phases have separated, the upper toluene phase was removed with a pasteur pipette in a beaker with some anhydrous sodium sulfate. After shaking, the dry toluene phase was transferred with a pipette into a 50 ml volumetric flask. This procedure

was repeated twice with 15 ml of toluene each. The beaker with sodium sulfate was washed twice with 2 ml of toluene. The graduated flask was filled up with toluene; the extract was then analyzed with GC-ECD. The equipment is shown in Figure 19.

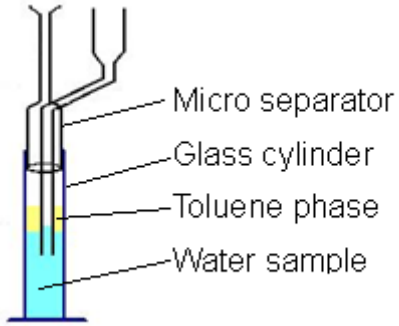

Figure 19 Shown is the micro separator, which was used to extract Lindane from leachate.

vi. Calibration: Comparison of the calibration with standard solved in ethyl acetate and in matrix extract

During evaluation of the Lindane analysis, also the matrix impact of the soil extract on quantification was tested. For this, two rows of calibration solutions in ethyl acetate and in matrix extract were prepared. Extracts of different spiked soil samples were analysed with both calibration curves. Differences in the recovery ranged between 3–5 %. For this purpose, calibration solutions were prepared with the same concentration of matrix as in the sample extracts. Results of these experiments are shown in appendix 2.

vii. Extraction and analysis of isotope-labelled Lindane containing soil samples

Samples with isotope-labelled Lindane were extracted similar to the samples without isotope-label. Following changes were made:

- For each sample, one 10 g (Layer A1) or 15 g subsample of the soil-sodium sulphate mixture was weighed in a 50 ml Falcon Tube. The samples were extracted three times with 15 ml ethyl acetate each in an ultrasonic bath at room temperature. After centrifugation at 30,000 g with an Avanti J-20 XPI for 10 min, the extracts were combined in a 50 ml graduated flask. For LSC measurment (see point 3.4.4.4) 1 ml of the soil extract was mixed with 3 ml Irga-Safe-PlusTM.
- Aliquots of the original soil and of the extracted soil were combusted in an oxidizer and analysed for radioactive labelled carbon (see point 3.4.4.5).

3.7.4 Imidacloprid

a) Material

Chemicals and solvents

- Imidacloprid (Imi): Pestanal, analytical standard, 99.9 % (Sigma-Aldrich, Germany)
- Imidacloprid –d_4 Pestanal (d4-Imi), 99.9 % (Sigma-Aldrich, Germany)

- Acetonitrile (ACN): super gradient for HPLC (VWR, Germany)
- Purified water (H2O): MILLI-Q Water System (Millipore, Germany)
- Scintillation cocktail for liquid samples: Irga-Safe-PlusTM (PerkinElmer, USA)
- Scintillation cocktail for CO2: Oxysolve C-400 (Zinsser Analytics GmbH, Germany)
- Radioactive standard: Spec-check-14C (PerkinElmer, USA)

Plastic bottles

The soil samples were stored in 250 ml plastic bottles of polypropylene (VWR, Germany).

Extraction:

- Micro test tubes, 1.5 ml (Eppendorf, Germany)
- Centrifugation tubes, 50 ml, polypropylene (Roth, Germany)
- Combustion Cones: Combusto-Cone, flexible (PerkinElmer, Germany)
- Scintillation vials: Polyvials® V (Zinsser Analytics GmbH, Germany)

b) Devices

HPLC-MS/MS

Imidacloprid analyses were performed with an Agilent Technologies 1200 Series High-Pressure-Liquid-Chromatograph (HPLC), equipped with a Thermo Scientific LTQ XL mass spectrometer (MS/MS). Samples were ionised with Electro-Spray-Ionisation (ESI) and quantified with high resolution Orbitrap XL. Two scan events were measured recording the mass spectrum and the MS/MS-spectrum of the mass 256, respectively. Quantification was based on the quotient of the peak areas of Imidacloprid and the internal standard (see 4.2.2). Parameter of HPLC, ionisation and mass spectrometry are listed in appendix 2.

Further devices:

- Ultrasonic bath: Transsonic 460 (Elma, Deutschland)
- Centrifuge, large: Hettich Centrifuge Universal 32 R (Hettich, Deutschland)
- Centrifuge, small: Eppendorf Centrifuge 5417 R (Eppendorf, Deutschland)

c) Methods

i. Standards and calibration solutions:

The concentration of the Imidacloprid stock solution was 98 µg/ml in acetonitrile. Two working standards of 9.8 g/ml and 0.98 µg/ml of Imidacloprid were prepared in acetonitrile/water (1/1, v/v). Similarly, three solutions of the internal standard, d_4-Imidacloprid, were prepared. The concentration of the stock solution was 48 µg/ml and the concentrations of the two working standards were 9.52 and 0.952 µg/ml.

Calibration solutions were prepared in Imidacloprid-free soil extract (matrix). Mainly two rows of calibration solutions were used, a) 10 ng/ ml to 150 ng/ml and b) 100 to 1000 ng/ml. Calibration solutions contain 40 ng/ml or 100 ng/ml internal standard (d_4-Imidacloprid).

ii. Fortification of soil samples

15 g of soil of control-TMEs (free of Imidacloprid, Layer 2.5-5 cm) were spiked with 2.5 µg and 10 µg of Imidacloprid in a 50 ml centrifugation tube and incubated for 30 minutes. Two further samples of 15 g soil were spiked with 2.45 and 49 µg of Imidacloprid and the samples were incubated for 1 day at room temperature in the dark. The concentrations were 166.7, 666.7, 163.3 and 3267 µg/kg fresh soil. The extraction is described in Section iii). All values are summarised in Table 25.

Table 25 Spiking of soil samples with Imidacloprid

Spiking of soil [µg/kg]	Incubation time	Addition of Imidacloprid to 15 g soil [µg]	Concentration in extract [ng/ml]
166.7	30 minutes	2.50	50
666.7	30 minutes	10.00	200
163.3	1 day	2.45	49
3267	1 day	49	980

iii. Analysis and recovery of Imidacloprid from leachate

An aliquot of 960 µl of leachate and 40 µl of internal standard (40 ng) were mixed and analysed at LC-MS/MS.

To determine the recovery, in a HPLC vial 900 µl of Imidacloprid free leachate were mixed with 40 µl internal standard (1 µg/ml) and 588, 98 or 19.6 ng of Imidacloprid. The volume was filled up with water to a total volume of 1000 µl. For each concentration 3 parallels were analysed on HPLC-MS/MS.

iv. Extraction of Imidacloprid from soil and preparing of matrix extracts

15 g of soil were weighed in a 50 ml centrifugation tube after thawing and homogenisation with a mortar. After addition of 20 ml of solvent 1 (ACN / H20; 30/70; v/v) and 200 µl or 500 µl of internal standard (≙2 µg and 5 µg of d_4-Imidacloprid), the samples were sonicated for 15 min at 50°C. The centrifugation tubes were then centrifuged for 7 min at room temperature (15,344 g), the supernatant was decanted in a 50 ml volumetric flask. 14 ml solvent 2 (100 % acetonitrile) were added to the soil and suspended by vigorous shaking for more than a minute. The tubes were sonicated for further 15 minutes, centrifuged and the extract decanted in the volumetric flask. This procedure was repeated another time with 15 ml of solvent 1. The volumetric flask was filled up with solvent 1 to the mark. The resulting extract had an acetonitrile-water ratio of about 1:1 (v/v). 1.5 ml of the extract was centrifuged at 11,000 g and the supernatant was measured at LC-MS/MS.

Soil of control-TMEs (Imidacloprid free) was extracted in the same way as the samples. The extract ("matrix") was used to prepare calibration solutions.

v. Calibration: Comparison of the calibration with standard - in matrix extract

Imidacloprid was analysed and quantified with LC-MS. Errors resulting from matrix effects were avoided by using an internal standard (d_4-isotope labelled Imidacloprid), which was added to the samples prior to the extraction. The added concentration of the internal standard was equal in extracts and calibration solutions (40 ng/ml or 100 ng/ml). A calibration curve was measured every day. Every 20 samples a calibration solution was measured. The analysis was performed with the area of m/z 256.055–256.065 and the area of the internal standard (IS) d_4-Imidacloprid of m/z 260.080–260.090. The MS-MS-spectrum of mass 256 was used to identify Imidacloprid (Imi). A spectrum and a calibration are shown as an example in appendix 2. Calculations were made according to following equations:

$$x = \frac{\text{Area}_{\text{Standard}}}{\text{Area}_{\text{Internal Standard}}} = \text{Intensity ratio} \qquad \text{(Equation 2)}$$

$$\text{Conc}_{\text{Dry soil}} = \frac{\text{Conc}_{\text{Analyte}} \cdot \text{Volume of Extraction}}{\text{Weight of sample}} \cdot \text{Factor of dry matter} \qquad \text{(Equation 3)}$$

$\text{Conc}_{\text{Analyte}}$ = Concentration of Imidacloprid in the extract

$\text{Conc}_{\text{Dry soil}}$ = Concentration of Imidacloprid in dried soil sample

vi. Extraction and analysis of isotope-labelled Imidacloprid soil samples

Samples of radioactive labelled Imidacloprid were analysed similar to the samples with not labelled substance. Following changes were made:

- Because of the lower density (high content of roots) only 7.5 g of soil from layer A1 (0-1 cm) was weighed and extracted with a total of 50 ml of solvent.
- The extracts were measured by liquid scintillation counting. Aliquots of the extracted and the not extracted soil were combusted on a biological oxidizer and analysed for radioactive labelled carbon dioxide.

3.7.5 Analysis of leachate and soil-extracts of samples with isotope-labelled substance (Imidacloprid and Lindane)

Radioactive disintegrations per minute, dpm, were measured from soil extracts and from leachate samples with radioactive-labelled insecticide. In scintillation vials 1 ml of the extract or 1 – 5 ml of the leachate was mixed with three times the volume of scintillation cocktail and radioactive disintegrations were measured for ten minutes. Matrix extract and tap water, respectively, were analysed in the same way to determine the blank. From these results, disintegrations of the total sample were calculated according to Equation 4 and Equation 5. In study [2], a mixture of labelled and not labelled substance was applied (see Chapter 3.4.1). 100 dpm correspond to 10.76 ng of Lindane and 1.01 ng of Imidacloprid, respectively. The amount of radioactivity, though, does not distinguish whether the original compound, a metabolite or any metabolised biomolecule is present in a sample. Therefore, the results are calculated as Lindane and Imidacloprid-equivalents.

$$\frac{\mathbf{dpm(Sample) - dpm(Blank)}}{\mathbf{Weighed\ sample\ [g]}} \times \frac{\mathbf{Volume\ of\ extract\ [ml]}}{\mathbf{Volume\ measured\ [ml]}} \times \frac{\mathbf{F(I)}}{\mathbf{F(DM)}} = \mathbf{c(IE)} \qquad \text{(Equation 4)}$$

F(I) = Factor, ng of insecticide per dpm

F(DM) = Factor of dry matter [see 3.3.3.2]

c(IE) = concentration of insecticide equivalent in µg/kg

$$\frac{\mathbf{dpm(Sample) - dpm(Blank)}}{\mathbf{Volume\ measured\ [ml]}} \times \mathbf{F(I)} = \mathbf{c(IE)} \qquad \text{(Equation 5)}$$

F(I) = Factor, ng of insecticide per dpm

c(IE) = concentration of insecticide equivalent in ng/ml

3.7.6 Determination of the total and the non-extractable Lindane and Imidacloprid residues in soil/vegetation

After extraction of the soil or vegetation, the remaining samples were homogenized and aliquots of about 2 g were removed and kept in a desiccator (Lindane samples) or dry cabinet (Imidacloprid samples) overnight. Then aliquots of about 0.4 g soil or 0.25 g vegetation were weight into combustion cones.

The soil or vegetation samples were filled into combustion-cones, which were inserted into the oxidizer and combusted in an oxygen atmosphere at a high temperature for 4 min. During this procedure, organic compounds are combusted to $^{14}CO_2$ or CO_2 and H_2O using a catalyst. The CO_2 formed was trapped using 16 ml of scintillator. The apparatus was cleaned by an N_2 stream after each combustion cycle. The samples were examined by liquid scintillation counting. In appendix 2, details of the calibration and the parameters of the combustion procedure are summarized.

3.8 Data management

The data management was conducted in a therefore built relational database in Microsoft-Access (Figure 20). The incoming data from all area e.g. analytics, oribatid mites, collembolans, leachate water, precipitation etc. were collected within this data-base. The use of this database provided a convenient, systematic, flexible and interdisciplinary evaluation of the data.

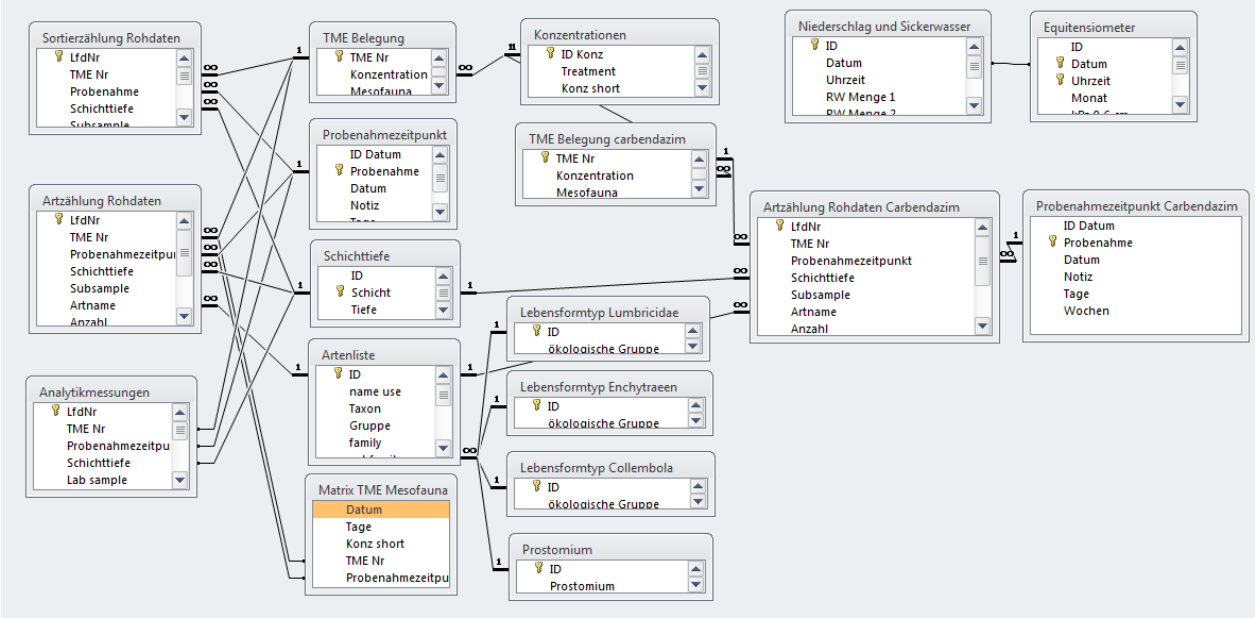

Figure 20 Screenshot of database structure for data management and evaluation

3.9 Statistical analysis

The biological data were analysed as follows: For each taxon (species or group of species if appropriate; total counts per sample) univariate statistics were used to test on differences between treatments and controls. At the community level, diversity and similarity indices as well as Principal Response Curves were used for analyses. The statistical methods are described in more detail hereafter. The program Community Analysis™ V4.3.14 was used for the pairwise comparison between treatment and control as well as for the calculation of the community parameters, except for the Principal Response Curves. A former version of the CA program is described in Hommen et al. (1994). The PRC analysis was performed with CANOCO™ 4.53 (DLO, Wageningen, NL), which represents the original program used in published papers describing the method. This is the commonly used standard software for multivariate evaluation of communities (Van den Brink & Ter Braak, 1998, 1999; Van den Brink et al., 1995).

3.9.1 Differences between treatments and controls

Differences between treatments and controls were statistically tested by means of the multiple t-test by Williams (1971, 1972). In case, if only one treatment was sampled, i.e. for lumbricides at intermediate sampling dates, the Student t-test was used instead. The abundance data of the organisms were log-transformed [y' = ln (ay+1)] before the analysis, in order to better approximate normality and homoscedascity (homogeneity of variances) requirements. All pairwise tests were performed one-sided with α = 0.05 (5 % level of significance) by using the software program

Community Analysis V14 (CA 4.3.14). Additionally the Minimum Detectable Differences (MDD) were calculated for selected endpoints in accordance to Brock et al. (2015) also by means of the software program Community Analysis V14 (CA 4.3.14).

3.9.2 Diversity analysis

The diversity of a community was described using three different measures. Firstly, the number of species (taxa richness) per treatment was plotted against time. Secondly, the Shannon-Index (sometimes also called Shannon-Wiener-Index, Streit 1980), a frequently used diversity measure, was calculated. This index is dependent on species richness and frequency distribution of the individuals of a species (see Boyle et al., 1990; please note typing error "Shannon-Weiner") and gets larger the more species are found and the more homogeneous the individuals are distributed on the species (Equation 6)

$$HS = -\Sigma pj \ln(pj) \quad \text{(Equation 6)}$$

with H_S = Shannon-Index, p_j = relative abundance of species j

The Evenness was calculated by dividing the Shannon-Index by the maximum possible value (Shannon-Index if all species are equally abundant). The maximum Evenness is 1 while dominant species result in lower Evenness values (Equation 7).

$$E = HS / HSmax = HS / \ln(n) \quad \text{(Equation 7)}$$

with E = Evenness, HS = Shannon-Index, n = number of species

3.10 Similarity analysis

To calculate the similarity of communities between different treatments, two indices of similarity were used:

Steinhaus' index (Smith, 1986, Boyle et al., 1990, Engels & Ratte, 1992) compares the absolute densities of the species in both samples (Equation 8).

$$SSteinhaus = 2 * W / (\Sigma nik + \Sigma njk) \quad \text{(Equation 8)}$$

with W= sum of the minor abundances (>0) in the compared two samples, nik, nηjk = absolute abundance of species k in sample i or j

Stander's index (Smith 1986, Boyle et al. 1990, Engels & Ratte 1992) considers the relative abundances of the single species in a community. For this reason, it is not that sensitive against differences in rare species as indices based on absolute data (e.g. Steinhaus' index).

Stander's similarities between samples were calculated according to Equation 9.

$$SStander = \Sigma(pik\ pjk) / \sqrt{(\Sigma pik2\ \Sigma pjk2)} \quad \text{(Equation 9)}$$

with i,j: samples to compare,
pik, pjk : relative abundance of species k in sample i or j

The TMEs of each treatment were compared to the controls. The mean of these similarities was plotted over time for each treatment to show dose response relationships of differences and recovery of communities.

3.11 Short explanation of Principal Response Curves (PRC) analysis

The PRC analysis is a multivariate approach to analyse and visualise effects on the community level which is described in detail in the papers of Van den Brink and Ter Braak (1998, 1999). It focuses on the difference between species composition in controls and treatments over time.

PRCs are calculated via the ordination technique Redundancy Analysis (RDA) which can be seen as a canonical form of a Principal Component Analysis (PCA), because RDA uses only the variance of the explanatory variables. Because PRCs are based on redundancy analysis (RDA) as a special form of PCA, the PCA will be explained first.

Usually original abundances are log-transformed prior to analysis, e.g. y'= ln y + 1. In the following, for the sake of simplicity, the term abundance is used also for the log-transformed data.

3.11.1 Principal Component Analysis (PCA)

PCA is the most commonly used multivariate technique to analyse ecological data sets. The objective is to find those factors that best explain differences in species composition between samples. PCA uses a linear model similar to a linear regression model. But in contrast to regression analysis explanatory variables are not measured but latent (constructed by the PCA itself): The abundance of a species k in sample i, yik, is modelled as a linear combination of mean abundance in all samples $\bar{y}k$ and the sample score xi, which can be seen as property of the sample i, valid for all species. Therefore, the rank 1 model of PCA can be written as

$$yik = \bar{y}k + bk\,xi + eik \qquad \text{(Equation 10)}$$

The regression coefficients bk are called species weights. They are specific for each species and depend on the sample. The term eik symbolises the error term with a mean of zero.

Sample scores and species weights can be displayed in an ordination diagram on the first axis (x-axis). The second latent variable can be extracted from the remaining variance (rank 2 model, y-axis in ordination diagram).

3.11.2 Redundancy analysis (RDA)

While PCA takes into account all of the variance of the data set, RDA is restricted to the explained variance only. For a pond study example, the explained variance is the variance which can be attributed to the treatment, the time and their interaction. RDA can be seen as a PCA in which the sample scores are constrained to be linear combinations of the explanatory variables. Or, in other words, such an RDA can be obtained from a PCA, in which the replicates are replaced by the treatment means. In a so-called biplot, sample with similar species composition are located closely together, while samples with very different species composition are far apart. Species weights can also be shown in a biplot diagram and allow to recalculate the relative abundance of species in each sample. However, this interpretation of the ordination diagram is not easy, especially for data sets with a lot of samples and species.

A permutation test at every sampling date allows to assess the statistical significance of effects, caused by the explanatory variables on species composition. Usually there are not enough replicates in a pond study to test every treatment against the control. In this case, the univariate Williams test (Williams 1972) can be applied to the first principal component (the sample scores) of a PCA at every sampling date. Thus, a $NOEC_{community}$ can be calculated.

3.11.3 Principal Response Curves

PRCs offer the possibility to overcome the shortcomings of the ordination diagrams. The PRC-approach focuses on the differences between species composition in control and treatments by modelling the abundance of a species k at dose d and replicate i as a sum of:

The mean abundance of species k in controls at time t $\bar{y}_{0tk}$, a date specific treatment effect (Tdtk = bk cdt), and the error ed(i)tk.

The PRC analysis fits Equation 9 using the whole data set:

$$y_{d(i)tk} = \bar{y}_{0tk} + b_k c_{dt} + e_{d(i)tk} \qquad \text{(Equation 11)}$$

with:

$y_{d(i)tk}$ = Log-transformed counts of taxon k, at time t, in treatment d and in replicate i

$\bar{y}_{0tk}$ = mean abundance of taxon k in control on sampling date t

c_{dt} = Principal Response Curves of the community in treatment d on sampling date t

b_k = weight of species k with PRC (= affinity of species k to the PRCs)

$e_{d(i)tk}$ = error term for replicate i of treatment d on date t for species k

Thus, the task is to calculate the principal responses cdt for each sampling date and treatment, as well as the species weights, valid for all sampling dates. This sort of least square estimation is done by partial RDA.

A plot of the cdt values over time gives a much clearer picture than a classical ordination diagram. The species weights can be shown in an additional diagram and allow an interpretation down to the species level. The higher the weight, the more corresponds the actual response pattern of the species to the PRC. High negative weights are obtained for species with an opposite response pattern as the PRC. Taxa with weights near zero show a response not related to the pattern of the PRC or no response at all. However, low species weights cannot be translated automatically into low susceptibility of the taxon to the stressor if the response pattern is different from the PRC.

The species weights (bk) and the principal response per sampling date and treatment (cdt) allow to calculate the predicted response for each taxon k at a given time and treatment by the term exp(bk cdt). The exponential function has to be used because the data are log-transformed (y'=ln a y +1) before the analysis (for the rationale see van den Brink et al. 1995).

To test if the PRC diagram as a whole displays a significant amount of the total variance a Monte Carlo permutation test following the PRC was performed (Van den Brink & Ter Braak 1998, 1999).

Therefore a F–type test statistic based on the eigenvalue of the component is used. The null-hypothesis of this test is that

$$b_k c_{dt} = 0 \quad \text{for all t, d, and k}$$

This permutation test is allowed to permute whole time series only. A calculated p-value below 0.05 was used as an indication of significance of the PRCs.

In addition, a redundancy analysis restricted to each sampling date, gave information if the treatments showed significant differences in community structure at this date. If this was the case ($p \leq 0.05$) a Principal Component Analysis was applied to the data of that sampling date. The resulting sample scores were used as inputs in a Student t-test in order to test the effects on the community level.

4 Results of the experimental outdoor study [1], testing the pesticides Lindane and Imidacloprid

4.1 Leachates and environmental conditions

The data for precipitation and irrigation as well as the leachate measurements are given in Table 26. Because of dryness in the months Mai, June and September, the TMEs required additional irrigation. Except for November 2011 and March 2012, the differences between the actual precipitation and the desired value were adjusted either by irrigation or by natural precipitation in the other months. In November 2011 there was hardly any precipitation (3 mm) at all, however, the soil was well moisturised. In March 2012, the precipitation was also low and because of the cold freezing weather situation it was not possible to irrigate and replace the missing rainfall. Due to this situation, the largest difference between the measured precipitation level and the desired value occurred in this month.

The highest precipitation occurred in December 2011 (151 mm), followed by January (137 mm) and August 2011 (133.5 mm). The total annual precipitation from Mai 2011 to April 2012 was 931 mm.

The amount of leachate was roughly similar between the two replicates measured in additional TMEs- except for October 2011. The reason for the large difference (TME 1: 384 ml and TME 2: 2877 ml, diff.: 2493 ml) in this month is unclear. The highest leachate level was recorded during winter (Dec. 25.2 L, Jan. 23.9 L, cp. Figure 21).

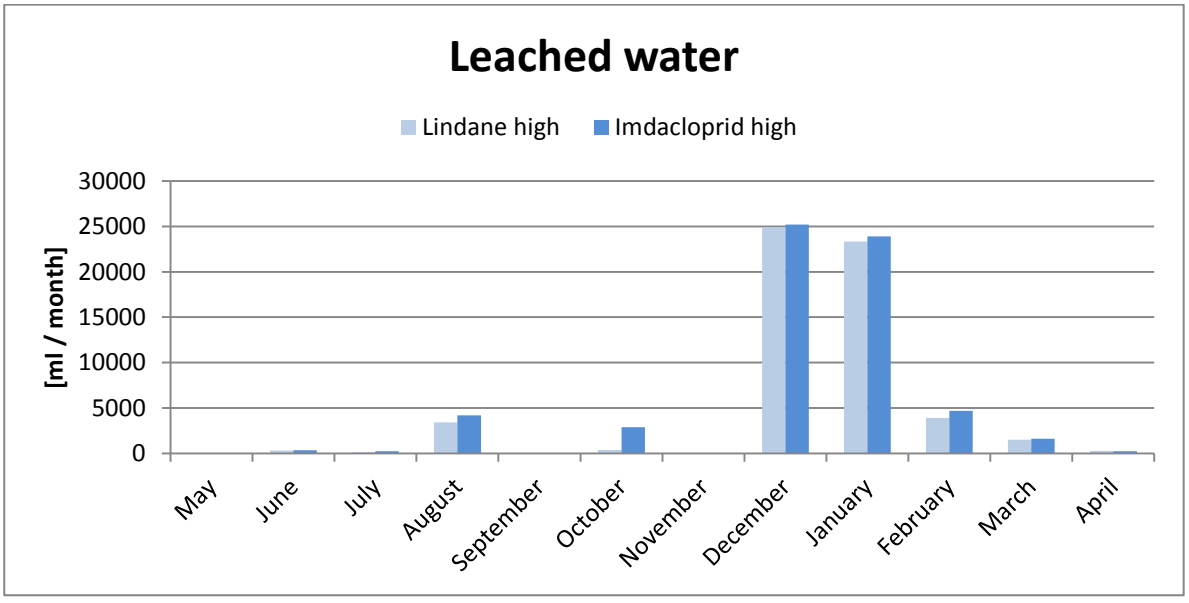

Figure 21 Measurements of leachate levels for the months May 2011 to April 2012 during the study.

Table 26 Precipitation, irrigation and the amount of leachate from the TMEs in the period of study [1] from May 2011 to April 2012. Differences (diff.) between measured data and desired values from Wetter.com (2011) for Monschau/Höfen were given.

	Irrigation [mm]	Precipitation [mm]	Sum [mm]	Leachate [ml] TME 1	Leachate [ml] TME 2
May	35	48	83	12	10
		Desired value	67		
		Difference	**16**		
June	0	77.5	77.5	307	324
		Desired value	81		
		Difference	**-3.5**		
July	24	54	78	143	226
		Desired value	132		
		Difference	**-54**		
August	0	133.5	133.5	3402	4175
		Desired value	113		
		Difference	**20.5**		
September	17	42	59	21	24
		Desired value	106		
		Difference	**-47**		
October	0	52	52	384	2877
		Desired value	64		
		Difference	**-12**		
November	46	3	49	0	0
		Desired value	124		
		Difference	**-75**		
December	0	151	151	24920	25200
		Desired value	102		
		Difference	**49**		
January	0	137	137	23350	23900
		Desired value	68		
		Difference	**36**		
February	0	39	39	3900	4690
		Desired value	84		
		Difference	**-45**		
March	0	14.5	14.5	1510	1611
		Desired value	148		
		Difference	**-133.5**		
April	0	57.5	57.5	0	0
		Desired value	37		
		Difference	**20.5**		
	Sum of irrigation and precipitation		**931**		
		Sum of leachate		**57949**	**63037**

In Figure 22, the data from continuous measurement of moisture and temperature in especially equipped TMEs (see chapter 3.3.1) is shown. The dryness which necessitated the additional irrigation in May, June and September 2011 is clearly visible. Although there was nearly no precipitation in November 2011, as previously stated, no dryness of the soil occurred. Soil dryness reoccurred in February 2012 which was quite likely related to the freezing temperature at that time.

The soil temperature measured in two TMEs in different soil layers showed nearly the same course over time (Fig. 2). The highest soil temperature of 29.8 °C was measured in June 2011 (29.06.2011), the lowest of -6.2 °C in February 2012 (21.02.2012), both in layer 0-6 cm. In September and December 2011 the temperature sensor for layer 6-12 cm was defective and was unable to record any data at all.

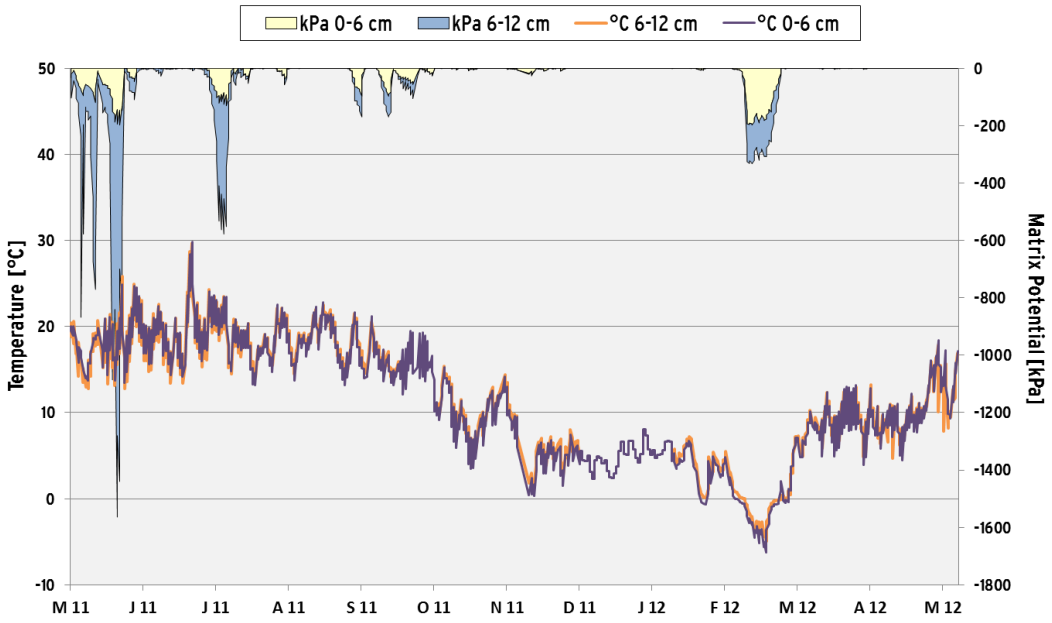

Figure 22 Data from continuous measurement of equitensiometer and temperature sensors in the TMEs of study [1] from May 2011 to May 2012.

4.2 Analyses in leachate

4.2.1 Recovery in spiked leachate: Lindane

The recovery of Lindane in water was determined in 100 ml of water which was extracted with 50 ml of toluene. The recovery was 90.2 ± 1.9 % and 96.9 ± 0.3 % at concentrations of 10 and 100 µg/L, respectively. All results are summarised in appendix 2. Analysed contents of Lindane in leachate were between „not detectable“ and 13 µg/L. Thus the method is also appropriate to analyse the low contents in the leachate.

4.2.2 Recovery in spiked leachate: Imidacloprid

Spiked samples were prepared with 800 µl of leachate (taken from the Lindane leachate TMEs, free of Imidacloprid), 40 µl of internal standard, 20 – 80 µl of Imidacloprid standard and 80 – 140

µl of acetonitile/water (1/1, v/v). The recovery found in these samples was 98.4 ± 6.9 %. All calculated data are shown in appendix 2.

4.2.3 Analysis of Lindane in leachate

Lindane containing leachate was collected from one TME (application rate 20 kg/ha of Lindane). During the incubation time, 24 leachate sampling events with a volume between 12 ml and 18 L took place. The Lindane concentration was measured in a subsample and ranged between 0.64 and 13.19 µg/L. In total 188 µg of Lindane were detected in the total volume of 52.3 L leachate. This corresponds to an average concentration of 3.8 ± 4.2 µg/L of Lindane (average of the measured concentrations). The total amount of Lindane found in the leachate constituted 0.05 % of the amount added initially. All results are shown in Table 27.

Table 27 Content of Lindane in the leachate of the TME of field study [1]. On 30th May, 2011, due to the low amount of water, the limit of quantitation was 0,04 µg. Soil sampling dates were May 16, May 29, June 26, October 02 and November 20."

Date	Amount of water [ml]	Concentration of Lindane [µg/L]	Total amount of Lindane [µg]
30.05.2011	12	< 3.33	<0.04
06.06.2011	250	6.87	1.72
21.06.2011	21	2.17	0.05
29.06.2011	26	1.59	0.04
25.07.2011	120	13.19	1.58
28.07.2011	16	11.91	0.19
09.08.2011	27	3.38	0.09
19.08.2011	3350	7.87	26.35
26.10.2011	105	2.17	0.23
02.12.2011	6920	5.46	37.77
13.12.2011	18000	1.61	29.06
05.01.2012	3800	1.21	4.61
06.01.2012	1200	1.03	1.23
09.01.2012	2600	0.75	1.95
20.01.2012	4600	12.98	59.69
23.01.2012	4350	1.39	6.04
27.01.2012	1200	1.45	1.74
17.02.2012	1220	9.00	10.99
24.02.2012	2500	1.58	3.96
27.02.2012	180	1.08	0.19
19.03.2012	1500	0.29	0.43
12.03.2013	10	n.d.	n.d.
16.04.2012	190	0.64	0.12
26.04.2012	81	1.17	0.09
Sum	52278		188.15
Mean		3.78 ± 4.19	

4.2.4 Analysis of Imidacloprid in leachate

Imidacloprid containing leachate was analysed from a TME with the application rate of 2 kg/ha of Imidacloprid, that are equivalent to 34.26 mg/TME. The total volume of 54.8 L leachate was collected in 24 events, and the volume ranged between 10 ml and 18 L. The Imidacloprid concentration ranged between <5 to 165.51 µg/L. The highest concentrations were found in June and July 2011 and mostly decreased over time. The total amount of 124.6 µg of Imidacloprid found in the water contained 0.36 % of the initially applied amount. The average concentration in the leachate was 13.5 ± 34.8 µg/L of Imidacloprid (average of the measured concentrations). Values below the Limit of Quantifications (LOQ) of 5 µg/L were considered at 2.5 µg/L in the mean and in the sum. All results are shown in Table 28.

Table 28 Content of Imidacloprid in the TME leachate of the field study [1]. The limit of quantification was 5 µg/L, concentrations below 1.5 µg/L were not detectable (n.d.). *) analysed twice. Soil sampling dates were May 16, May 29, June 26, October 02 and November 20.

Date	Amount of water [ml]	Concentration of Imidacloprid [µg/L]	Total amount of Imidacloprid [µg/date]
30.05.2011	10	< 5.0	0.03
* 06.06.2011	260	165.51	43.03
21.06.2011	21	45.12	0.95
29.06.2011	31	< 5.0	0.08
25.07.2011	156	26.94	4.20
28.07.2011	59	42.07	2.48
09.08.2011	73	12.85	0.94
19.08.2011	4065	7.77	31.57
26.10.2011	85	< 5.0	0.21
02.12.2011	7200	< 5.0	18.00
13.12.2011	18000	n.d.	-
05.01.2012	4000	n.d.	-
06.01.2012	1200	n.d.	-
09.01.2012	2600	n.d.	-
20.01.2012	4800	n.d.	-
23.01.2012	4400	n.d.	-
27.01.2012	1300	n.d.	-
17.02.2012	2600	6.88	17.89
24.02.2012	1900	< 5.0	4.75
27.02.2012	190	n.d.	-
09.03.2012	1600	n.d.	-
19.03.2012	11	< 5.0	0.03
16.04.2012	190	< 5.0	0.48
26.04.2012	81	n.d.	-
Sum	54832		124.64
Mean		13.5 ± 34.8	

4.3 Chemical analyses in soil layers

4.3.1 Sample amounts and content of dry matter

Soil cores (5 cm diameter) were sampled with a height of 2.5 cm, 2.5 cm, 5 cm and 10 cm, named layer A, B, C and D respectively. In particular, the samples of layer A (0–2.5 cm) consisted mainly of roots and the biomass of roots increased during the course of the experiment. Also layer B (2.5–5 cm depth) was well rooted. Layer D (10–20 cm) contained some stones (up to 3 cm in diameter), which were pestled. Stones and roots were homogenised with the soil for subsequent analysis. The samples of layer A weighed about 38–60 g and contained 57–69 % of dry matter. Layers B and C (2.5–5 and 5–10 cm) weighed 37–61 g and 106–128 g, respectively, and contained about 62-76 % and 71–81 % of dry matter.

The weight of samples of layer D ranged between 147 and 229 g; the same content of dry matter as in samples of layer C was assumed. The diverging weights of the samples are mainly due to varying contents of moisture and of roots but of course also to the sampling itself. All sample weights and dry matter contents are summarized in appendix 2.

4.3.2 Lindane analysis

4.3.2.1 Recovery in spiked soil samples

To test the applicability of the method developed by Castro et al. (2001) in a wider range of concentrations, known amounts of Lindane were spiked to soil and extracted (each two extraction steps with 5 ml of ethyl acetate). Four concentrations of 0.036–36 mg/kg in Na_2SO_4–soil mixture were tested equalling to a concentration of Lindane of 0.051–51.5 mg/kg pure soil. Additionally soil samples from a control-TME without sodium sulphate (moist soil) and a sample of agriculture soil (sieved and air-dried) were tested. Table 29 summarises the obtained recoveries.

Table 29 Recovery (mean ± SD) of Lindane in spiked soils. Additionally soil samples from a control-TME without sodium sulphate (moist soil) and a sample of agriculture soil (sieved and air-dried) were tested.

Soil	Spiked concentration [mg/kg]	Type of soil	Recovery [%]
1	0.036	Na_2SO_4 - soil	89.00 ± 8.43
2	0.072	Na_2SO_4 - soil	99.95 ± 5.02
3	3.594	Na_2SO_4 - soil	94.00 ± 3.99
4	35.970	Na_2SO_4 - soil	82.08 ± 1.74
5	5.145	moist soil	104.68 (analysed once)
6	5.142	agricultural soil	88.41 ± 2.97

Recoveries were in all cases above 80 %. In the sample with the highest concentration of Lindane (soil 4) the recovery was the lowest (82 %). Therefore soil samples of the upper layer were extracted 3-fold. Overall the results were satisfactory and reproducible and demonstrate that the method can be used. The reasons of slightly lower recoveries than described in the literature are on the one hand a wider range of concentrations, and on the other hand perhaps the different method of spiking. Castro et al. (2001) added Lindane in one millilitre of solvent to the weighed soil sample in the extraction tube and extracted after incubation of 30 minutes (and up to 30 days). In the present study Lindane probably sorbed more strongly to the soil due to a different fortification method, resulting in slightly lower recovery.

4.3.2.2 Results of the outdoor study [1] – soil samples

Usually seven parallel sample cores have been examined. For layer D (10-20 cm) and on day 140 for layer C (5-10 cm) only two sample cores and on day 1 (T0) six replicates were used. A summary of the results of the analysis is presented in Table 30 and Table 31. Shown are the mean concentrations from the lower and from the higher application rate as well as the percentage of the absolute extractable Lindane content in one layer in relation to the total extractable amount in all layers.

In appendix 2, Table 29 the concentrations of Lindane in soil dry matter of all sample cores are given as well as the total amounts of Lindane in a sample core of 5 cm diameter (calculated from the Lindane concentration in fresh soil and the weight of the sample core). For this calculation, concentrations below the limit of quantification (LOQ, 0.01-0.02 mg/kg, varying with content of soil organic matter) were taken into account with half of the concentration of the LOQ; if Lindane was not detectable (below the limit of detection, LOD, 0.01 mg/kg) concentrations were set to zero. Additionally the arithmetic means of the concentrations and the medians are given in appendix 2.

With both application amounts and at all sampling times the major amount of the extractable Lindane was found in the upper soil layer (Layer A, 0–2.5 cm). The concentration of Lindane in this soil layer after application of 7.5 kg/ha decreased from 28.41 ± 23.24 mg/kg dry matter one day after application to 20.62 ± 10.76 mg/kg after 14 days and 7.99 ± 7.41 mg/kg after 42 days (Table 30). During summer and autumn the concentrations remained nearly stable (8.60 ± 4.31 mg/kg and 8.21 ± 3.51 mg/kg after 140 and 189 days, respectively). Over winter until sampling in May (365 days after application) the concentration decreased to 4.74 ± 0.70 mg/kg dry matter. In layer B (2.5–5 cm depth) concentrations between 0.31 ± 0.34 mg/kg (day 1) and 0.74 ± 0.49 mg/kg (day 365) were analysed, equal to 1.8 to 17 % of the amount extracted on this sampling date in the total soil depth analysed (0–20 cm). In Layer C (5–10 cm) Lindane is always detected in concentrations below the LOQ of 0.015 mg/kg. At later samplings, analysed concentrations were between 0.03 mg/kg and 0.15 mg/kg. Samples of layer D (10–20 cm) were analysed for the first time after 42 days. Afterwards the concentration was close to the LOQ or not detectable (< LOD, after 189 days).

Table 30 Application of 7.5 kg/ha of Lindane –Given are the mean concentrations of Lindane in different soil layers and percentage of the extracted amount (calculated in relation to the absolute content of Lindane in the total sample core (0-20 cm)). Standard deviations of the concentrations and the median are given in appendix 2.

	mg/kg dry matter						% of the extracted amount					
	Day											
Layer (cm)	1	14	42	140	189	365	1	14	42	140	189	365
A: 0 - 2.5	28.4	20.6	8.0	8.4	8.2	4.7	98.2	95.9	91.3	94.4	90.1	80.8
B: 2.5 - 5	0.31	0.53	0.43	0.36	0.70	0.74	1.8	2.0	6.3	4.2	8.8	16.6
C: 5 - 10	0.01	0.15	0.06	0.03	0.03	0.04	0.1	2.1	1.9	1.0	1.0	2.3
D: 10 - 20			0.01	0.01	n.d.	0.00			0.6	0.5	0.0	0.3
Sum							100	100	100	100	100	100

Table 31 Application of 20 kg/ha of Lindane – Given are the mean concentrations of Lindane in different soil layers and the percentage of the extracted amount (calculated in relation to the absolute content of Lindane in the total sample core (0-20 cm)). Standard deviations of the concentrations and the median are given in appendix 2.

	mg/kg dry matter						% of the extracted amount					
	Day											
Layer (cm)	1	14	42	140	189	365	1	14	42	140	189	365
A: 0 - 2.5	61.5	47.4	20.1	18.4	21.0	13.0	99.6	95.5	95.6	86.7	90.2	70.8
B: 2.5 – 5	0.16	0.48	0.46	0.74	1.27	3.19	0.4	1.2	2.6	4.5	6.9	22.6
C: 5 – 10	0.01	0.33	0.08	0.39	0.33	0.34	0.0	2.1	0.9	4.7	2.9	6.5
D: 10 – 20		0.08	0.05	0.20	0.04	0.00		1.2	0.8	4.1	0.0	0.1
Sum							**100**	**100**	**100**	**100**	**100**	**100**

The samples of the 20 kg/ha-application show a similar concentration pattern decreasing in the upper soil layer from day 0 (61.5 ± 24.7 mg/kg dry matter) to 47.4 ± 22.3 mg/kg after 14 days and to 20.1 ± 13.8 mg/kg after 42 days; this concentration level remained roughly constant during summer and autumn. After one year the concentration deceased to 13.0 ± 5.5 mg/kg. In the soil layer B (2.5–5 cm) the concentration of Lindane increased during the experiment from 0.16 ± 0.26 to 3.19 ± 1.74 mg/kg after one year and reached then 22.6 % of the extracted amount (Table 31). In layer C (5-10 cm), one day after application the concentration of Lindane was below the LOQ of 0.015 mg/kg and at the other dates it ranged between 0.08 ± 0.07 and 0.39 ± 0.36 mg/kg dry matter. In samples of layer D (10-20 cm) only low concentrations between 0.04 mg/kg and 0.2 mg/kg were recorded on days 14 and 189. After one year, the concentrations were below the LOQ of 0.008 mg/kg (no samples were taken one day after application). Please note that the limits of quantification vary over time due to different amounts of roots and humic substances in the soil samples.

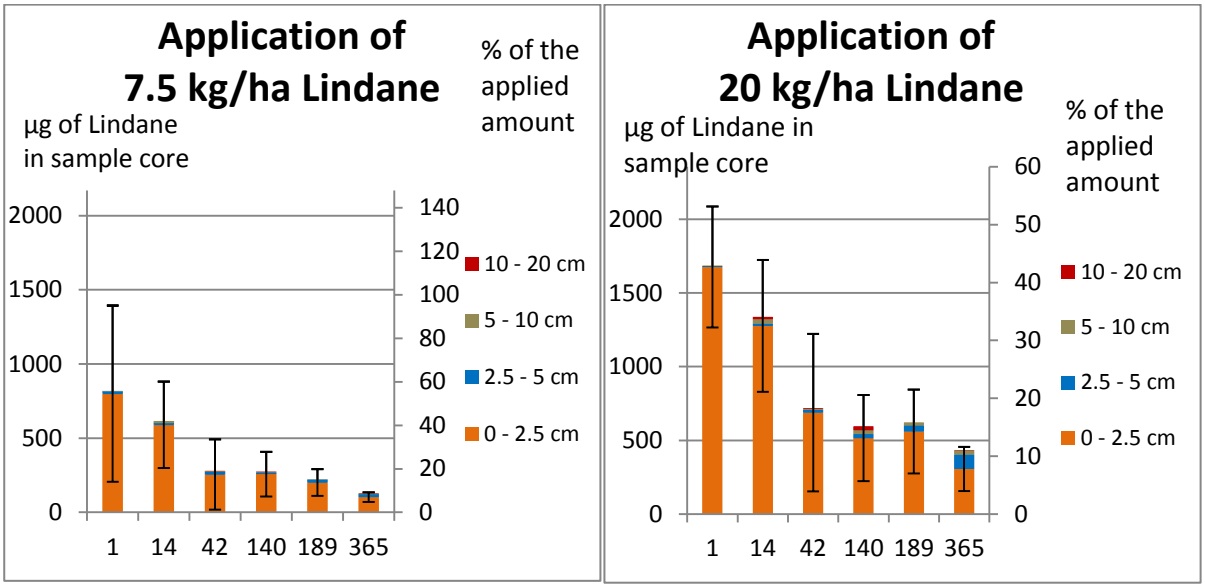

Figure 23 Mean amount of Lindane measured in the different soil layers given in µg/soil core (5cm diameter) and % of the applied amount. The applied amount on one soil core (5 cm in diameter, surface 19.635 cm²) is 1472.6 µg with an application rate of 7.5 kg/ha and 3927 µg with application rate of 20 kg/ha. For reasons of clarity only the error bars indicating the standard deviation for the upper layer (0 – 2.5 cm) are given.

The total amount of extractable Lindane in each sample core and the relative recovery in percent of the initially applied amount are graphically shown in Figure 23, Figure 24 and Figure 25. The diagrams show the concentrations of Lindane in the different soil layers. In addition in Figure 26 the concentrations of Lindane are shown separately for each application rate and sampling date.

An application of 7.5 kg/ha is equal to an application of 1.473 mg/19.64 cm² (surface of a sample core of 5 cm in diameter). An application of 20 kg/ha of Lindane equals the amount of 3.927 mg/19.64 cm². One day after application total amounts (summing up all soil layers) of 0.802 ± 0.595 mg and 1.677 ± 0.412 mg of Lindane, respectively, were analysed, equivalent to recoveries of 55.5 ± 41.5 % and 42.9 ± 10.8 %. Please note that the grass cover (about 2 cm in height) was not analysed; however, in the later described experiments with ^{14}C-labelled Lindane the above-ground plant parts were additionally analysed.

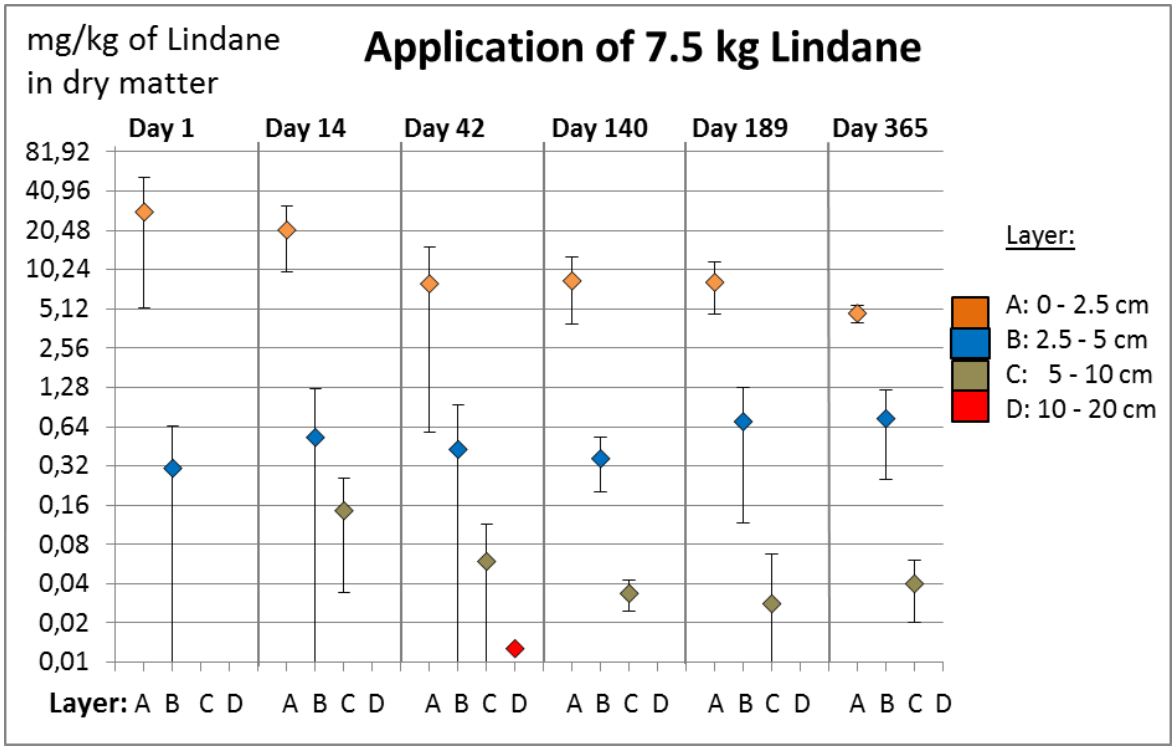

Figure 24 Mean concentrations of Lindane after application of 7.5 kg in mg/kg dry matter in the different soil layers on a logarithmic scale. Error bars indicating the standard deviation. Data points below the limit of quantification are not shown.

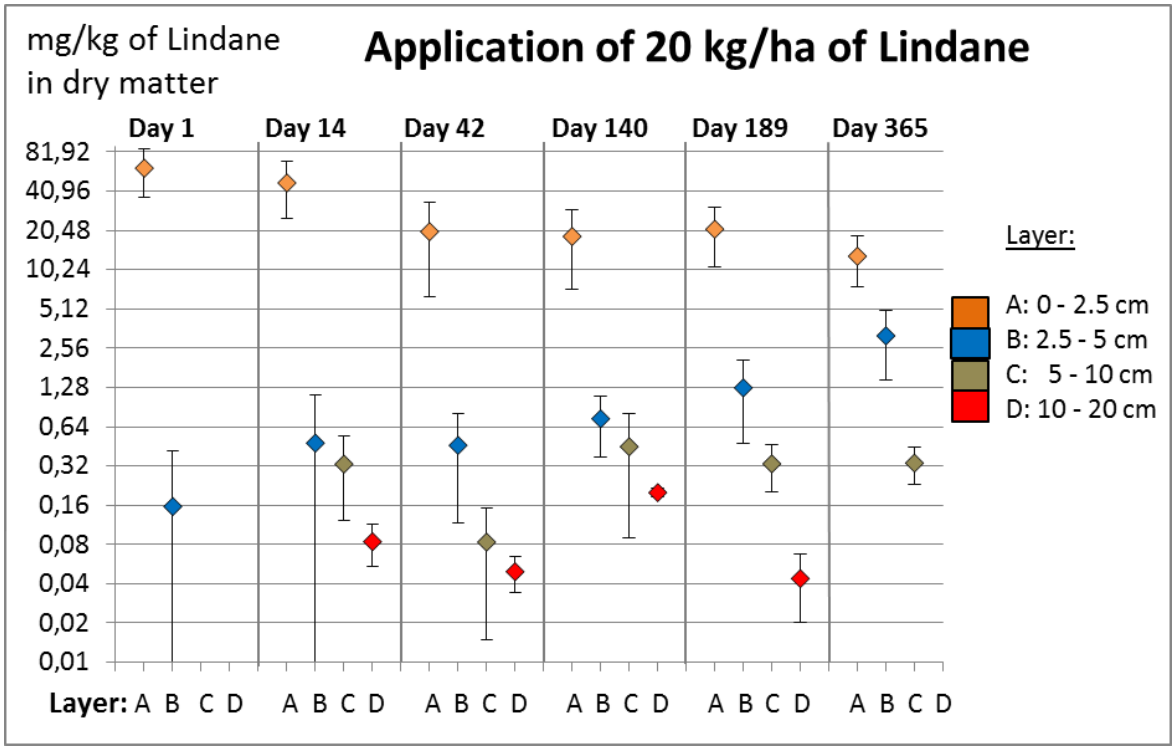

Figure 25 Mean concentrations of Lindane after application of 20 kg in mg/kg dry matter in the different soil layers on a logarithmic scale. Error bars indicating the standard deviation. Data points below the limit of quantification are not shown.

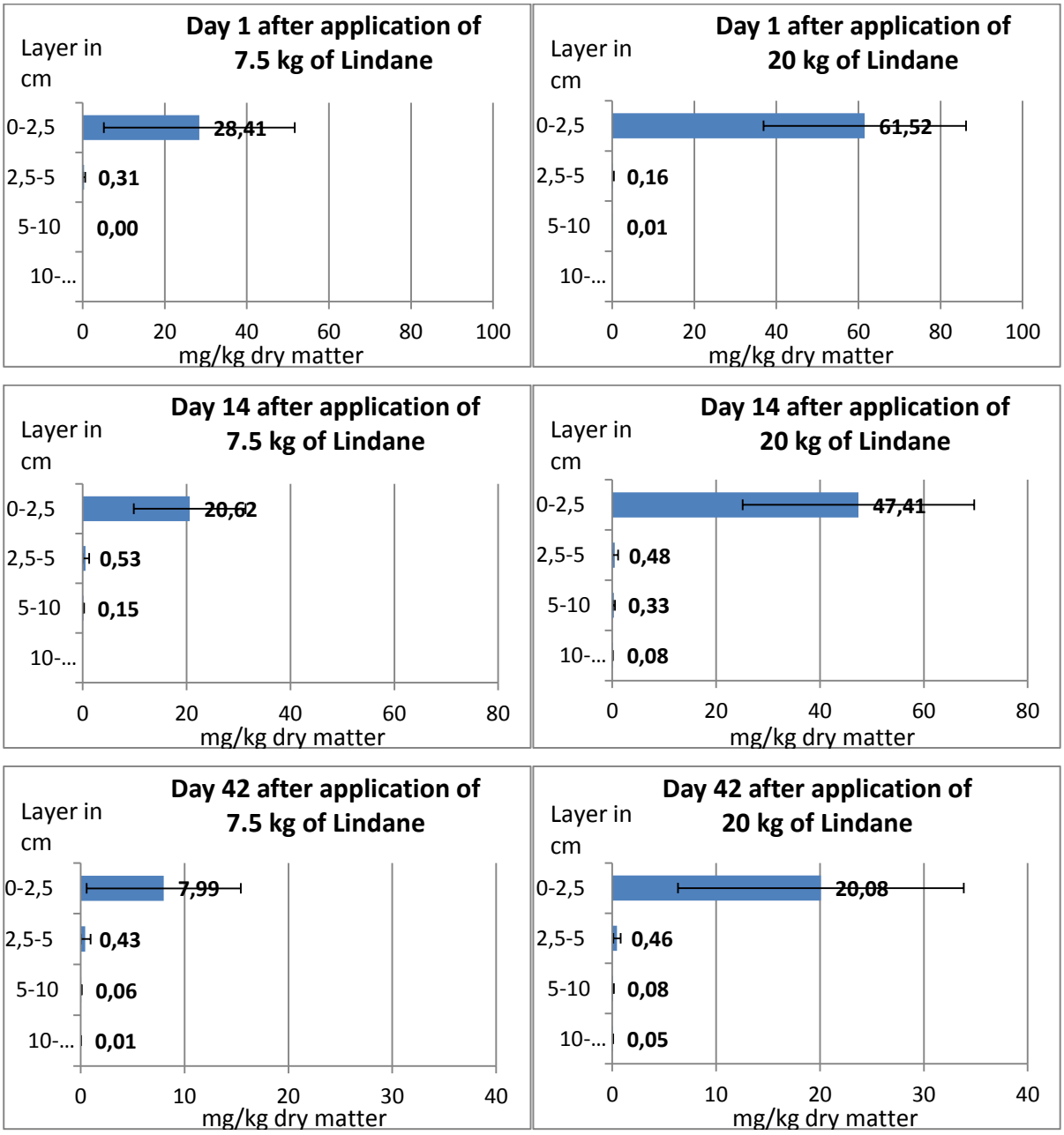

Figure 26 Caption see below

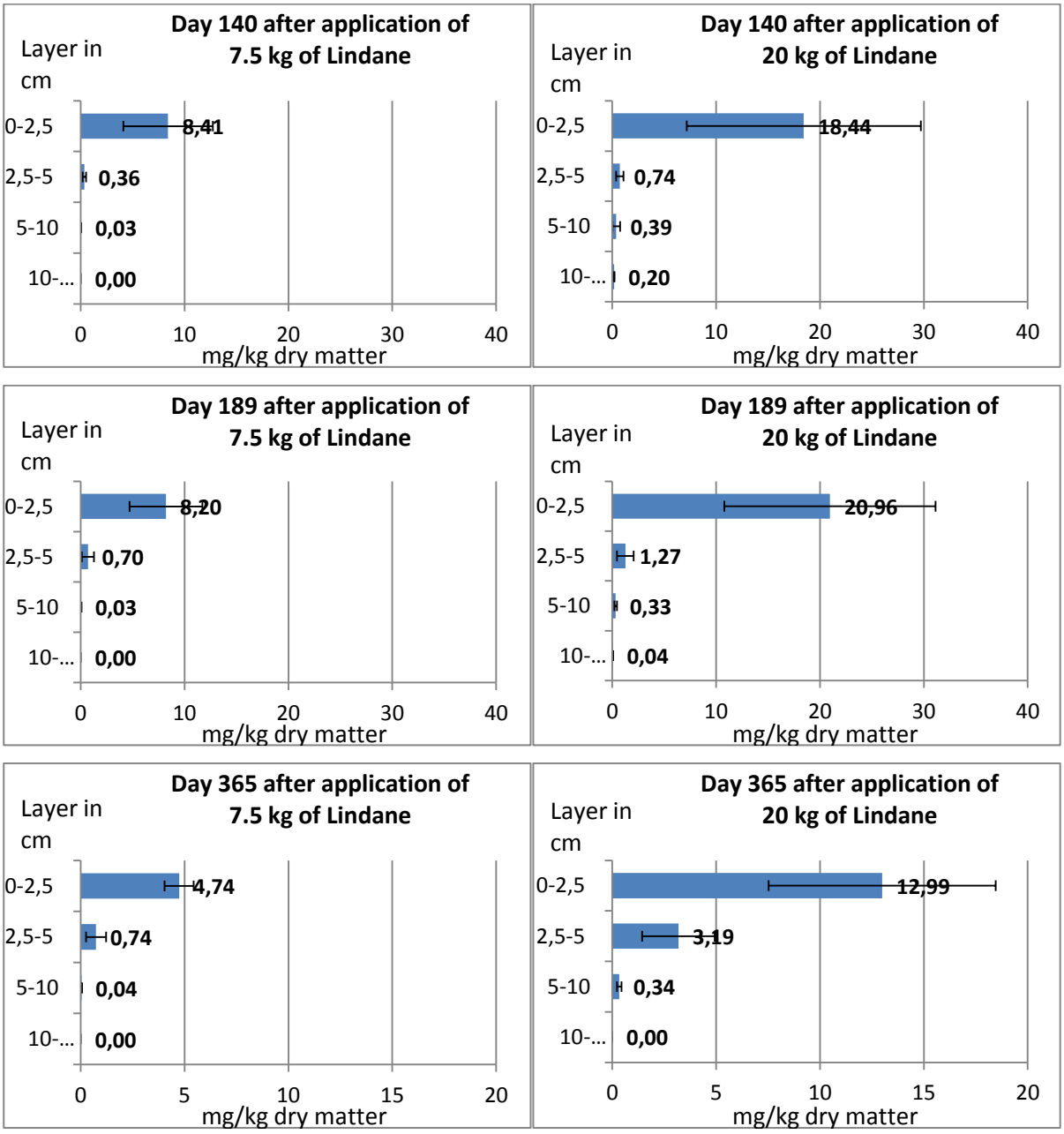

Figure 26 Mean concentrations of Lindane in analysed soil layers at different time points after application. Error bars indicating the standard deviation.

4.3.3 Imidacloprid analysis

4.3.3.1 Recovery in spiked soil samples

The analyses of soil samples which were spiked with known amounts of Imidacloprid ensure the applicability of the extraction method. The addition of an internal standard prior to the extraction compensates losses during the extraction. Recoveries of the four spiking experiments are shown in Table 32.

Table 32 Recovery (mean ± SD) of Imidacloprid in spiked soils.

Spiked concentration	Incubation	Recovery
0.16 mg/kg soil (49 ng/ml)	30 min	108.2 ± 2.2 %
0.16 mg/kg soil (49 ng/ml)	1 day	98.8 ± 7.0 %
0.67 mg/kg soil (196 ng/ml)	30 min	107.5 ± 4.3 %
3.27 mg/kg soil (980 ng/ml)	1 day	99.2 ± 0.5 %

In all cases the recovery ranged between 98 and 108 %.

The calibration solutions to analyse the recovery samples after one day of incubation (both 99 %) were prepared with matrix extract from exactly the same soil. The matrix extract, which was used to prepare the calibration solutions for the other samples (with 30 min incubation), derives from soil with a slightly higher content of roots. Differences in the matrix cause a differing suppression and enhancement during the electrospray ionisation and can therefore explain the higher recovery of 108 %. During sample analyses particular attention was paid that calibration solutions were prepared with matrix extract, which was derived from the same soil layer and the same sampling date.

4.3.3.2 Results of the outdoor study [1] – soil samples

For the analysis of Imidacloprid two (layer C, 5-10 cm and D, 10-20 cm depth) or five (other layers) parallel sample cores have been examined. A summary of the means and the distribution of the substance over the different soil layers is presented in Table 33 and Table 34. The detailed results of all soil samples including the arithmetic means of the concentrations and the median values are presented in appendix 2, Table 32. Additionally, the total amounts of Imidacloprid in a sample core of 5 cm diameter (calculated from the Imidacloprid concentration in fresh soil and the weight of the sample core) are given in the appendix. For this calculation, concentrations below the limit of quantification (LOQ, 0.02 mg/kg) were taken into account with half of the concentration of the LOQ; if Imidacloprid was not detectable (below the limit of detection, LOD) concentrations were set to zero.

Imidacloprid remains primarily in the upper layer (0-2.5 cm) of soil with distinct lower concentrations already in the following layer 2.5-5 cm. During the experiment of one year, only small amounts moved into layer C (5–10 cm), in deeper layers Imidacloprid was not quantifiable. After application of 7.5 kg/ha, the concentration of Imidacloprid in soil layer A (0–2.5 cm), decreased from 2.1 ± 0.4 mg/kg dry matter one day after application to 0.2 ± 0.1 mg/kg after one year (Table 33). In samples of layer B (2.5-5 cm), between 0.1 mg/kg and 0.3 mg/kg were extractable, equal to 3 to 40 % of the amount extracted on this sampling date in the total soil depth analysed (0–20 cm). Only traces of up to 0.06 mg/kg Imidacloprid were detectable in layer C and in layer D (10–20 cm), but only at one single sampling date after 189 days and not detectable at the other sampling dates.

Table 33 Application of 0.75 kg/ha of Imidacloprid – Given are mean concentrations of Imidacloprid in different soil layers and percentage of the extracted amount (calculated from the absolute content of Imidacloprid in the sample core in proportion to the absolute content in all layers of a sampling date). n.a.: not analysed, n.d.: not detectable. Standard deviations of the concentrations and the median are given in appendix 2.

	Concentration [mg/kg dry matter]						% of the extracted amount					
Layer (cm)	day 1	14	42	140	189	365	day 1	14	42	140	189	365
0 - 2.5	2.08	3.51	1.80	0.30	0.49	0.20	88.7	95.9	88.5	58.5	75.7	59.2
2.5 - 5	0.27	0.12	0.18	0.22	0.11	0.14	11.3	3.3	9.0	41.5	16.6	40.8
5 - 10	n.d.	0.03	0.05	n.d.	0.03	n.d.	0.0	0.8	2.5	0.0	4.6	0.0
10 - 20	n.a.	n.d.	n.d.	n.d.	0.02	n.d.		0.0	0.0	0.0	3.1	0.0
Sum							100	100	100	100	100	100

Table 34 Application of 2 kg/ha of Imidacloprid – Given are mean concentrations of Imidacloprid in different soil layers and percentage of the extracted amount (calculated from the absolute content of Imidacloprid in the sample core in proportion to the absolute content in all layers of a sampling date). *)below the Limit of Quantification (LOQ), calculated with half the value of the LOQ. Standard derivations of the concentrations and the median are given in appendix 2.

	Concentration [mg/kg dry matter]						% of the extracted amount					
Layer (cm)	day 1	14	42	140	189	365	day 1	14	42	140	189	365
0 - 2.5	9.06	5.73	4.18	0.58	1.19	0.75	97.0	90.7	76.5	70.6	74.2	56.4
2.5 - 5	0.28	0.52	1.15	0.21	0.35	0.49	3.0	8.3	21.0	25.4	21.6	36.5
5 - 10	n.d.	0.05	0.12	0.03	0.07	0.08	0.0	0.7	2.2	4.0	4.2	5.9
10 - 20	n.a.	< 0.03	< 0.03	n.d.	n.d.	n.d.	0.0	0.2*	0.3*	0.0	0.0	1.1*
Sum							100	100	100	100	100	100

A similar pattern can be seen after application of 2 kg/ha of Imidacloprid. The concentration in the top soil layer (0 – 2.5 cm) on day 1 (9.1 ± 3.0 mg/kg dry matter) decreased to 4.2 ± 1.4 mg/kg after 42 days and furthermore to 0.8 ± 0.1 mg/kg after one year. In the soil layer of 2.5 – 5 cm the concentration of Imidacloprid increased during the experiment from 0.3 ± 0.5 and 1.2 ± 0.7 mg/kg on day 42 to 0.5 ± 0.2 mg/kg after 365 days, equal to an increase of 3 to 37 % of the amount extracted on the corresponding sampling date in the total soil column analysed (0 – 20 cm).

In Layer C Imidacloprid was quantifiable from day 14 onwards, but the concentrations ranged only between 0.03 and 0.12 mg/kg, corresponding to a gradual increase from 0 to 6 % of the extractable amount. On day 14, 42 and 365, in layer D Imidacloprid was detectable only in low concentrations below the LOQ. As in case of Lindane, the concentrations of Imidacloprid observed at day 140 were significantly lower than expected from the overall degradation profile (see Figure 27). The reasons were unclear but heavy rain before and during sampling may have influenced the results.

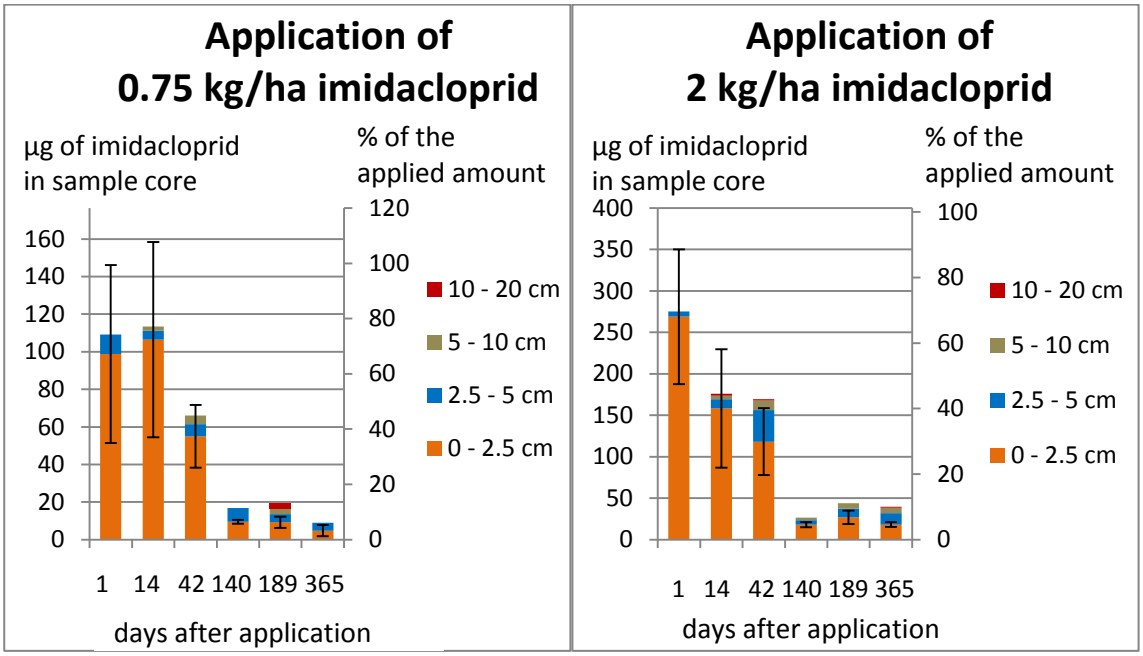

Figure 27 Mean amount of Imidacloprid measured in the different soil layers given in µg/soil core (5cm diameter) and in % of the applied amount. The applied amount on one soil core (5 cm in diameter, surface 19.635 cm²) is 147.3 µg with an application rate of 0.75 kg/ha and 392.7 µg with application rate of 20 kg/ha. For reasons of clarity only the error bars indicating the standard deviation for the upper layer (0 – 2.5 cm) are given.

The absolute extractable content of Imidacloprid in a single sample core and the relative recovery in percent of the applied amount are given in Figure 27, Figure 28 and Figure 29. The diagrams show the concentrations of Imidacloprid in mg/kg dry matter in the different soil layers. In Figure 30 the concentrations of Imidacloprid shown separately for each application rate and sampling date.

An application of 0.75 kg/ha is equal to an application of 147.3 µg/19.64 cm² (surface of a sample core of 5 cm in diameter). An application of 2.0 kg/ha of Imidacloprid equals the amount of 392.7 µg/19.64 cm². One day after application total amounts (summing up all soil layers) of 109.2 ± 56.5 µg and 275.4 ± 89.1 µg of Imidacloprid, respectively, were analysed, equivalent to recoveries of 67.0 ± 32.2 % and 68.5 ± 20.7 %. The grass cover (about 2 cm in height) was not analysed.

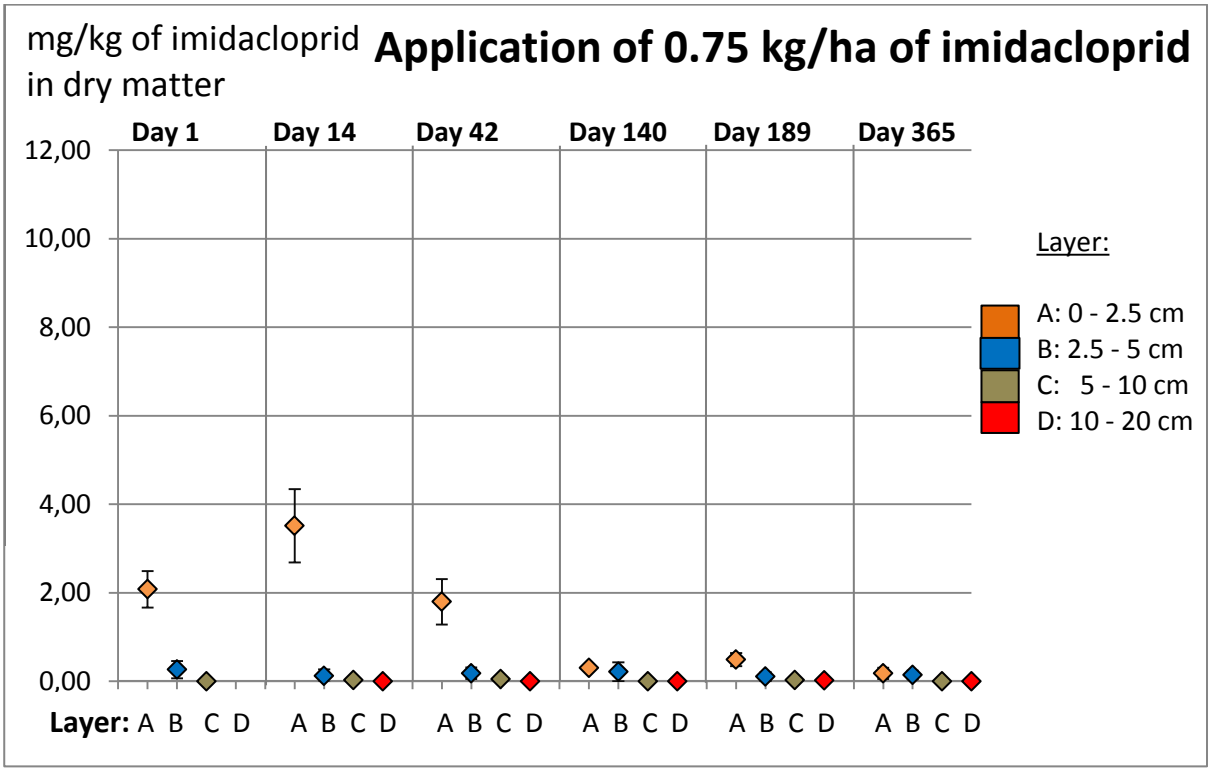

Figure 28 Mean concentrations of Imidacloprid after application of 0.75 kg/ha in mg/kg dry matter in the different soil layers. Error bars indicating the standard deviation. Data points beyond the limit of quantification are not shown.

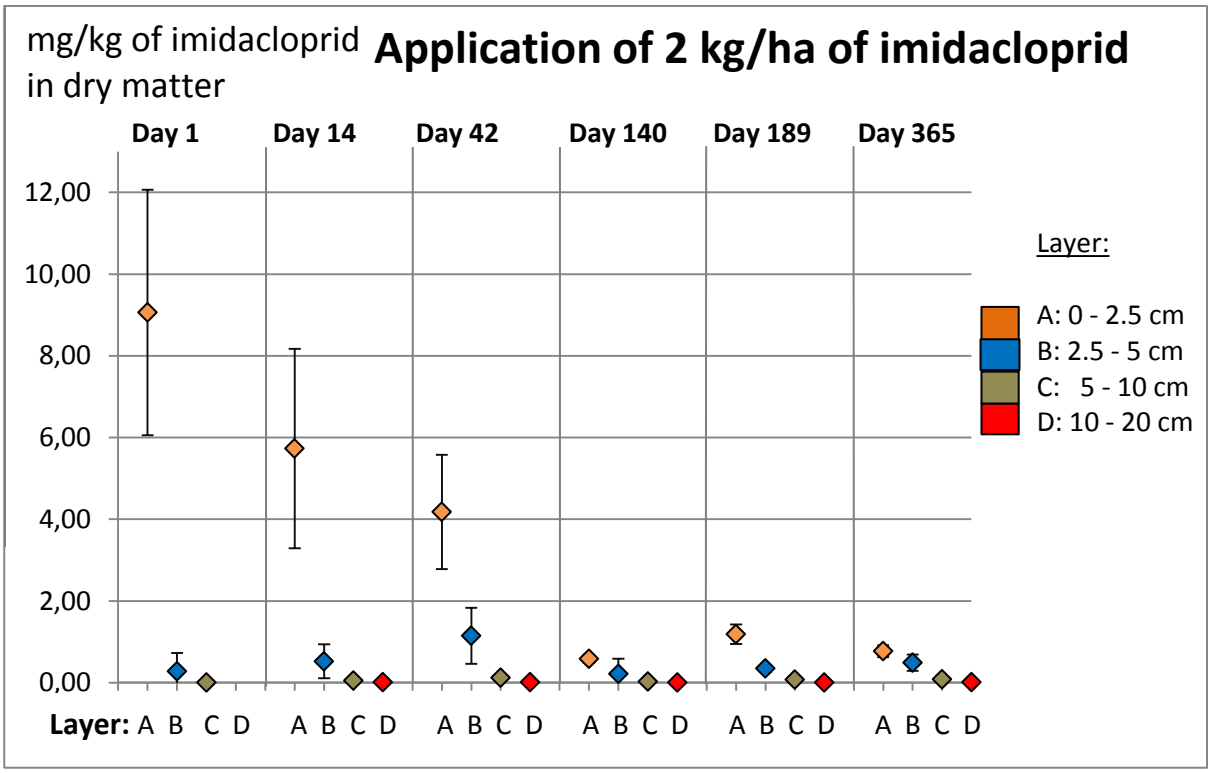

Figure 29 Mean Concentrations of Imidacloprid after application of 2 kg/ha in mg/kg dry matter in the different soil layers. . Error bars indicating the standard deviation. Data points below the limit of quantification are not shown.

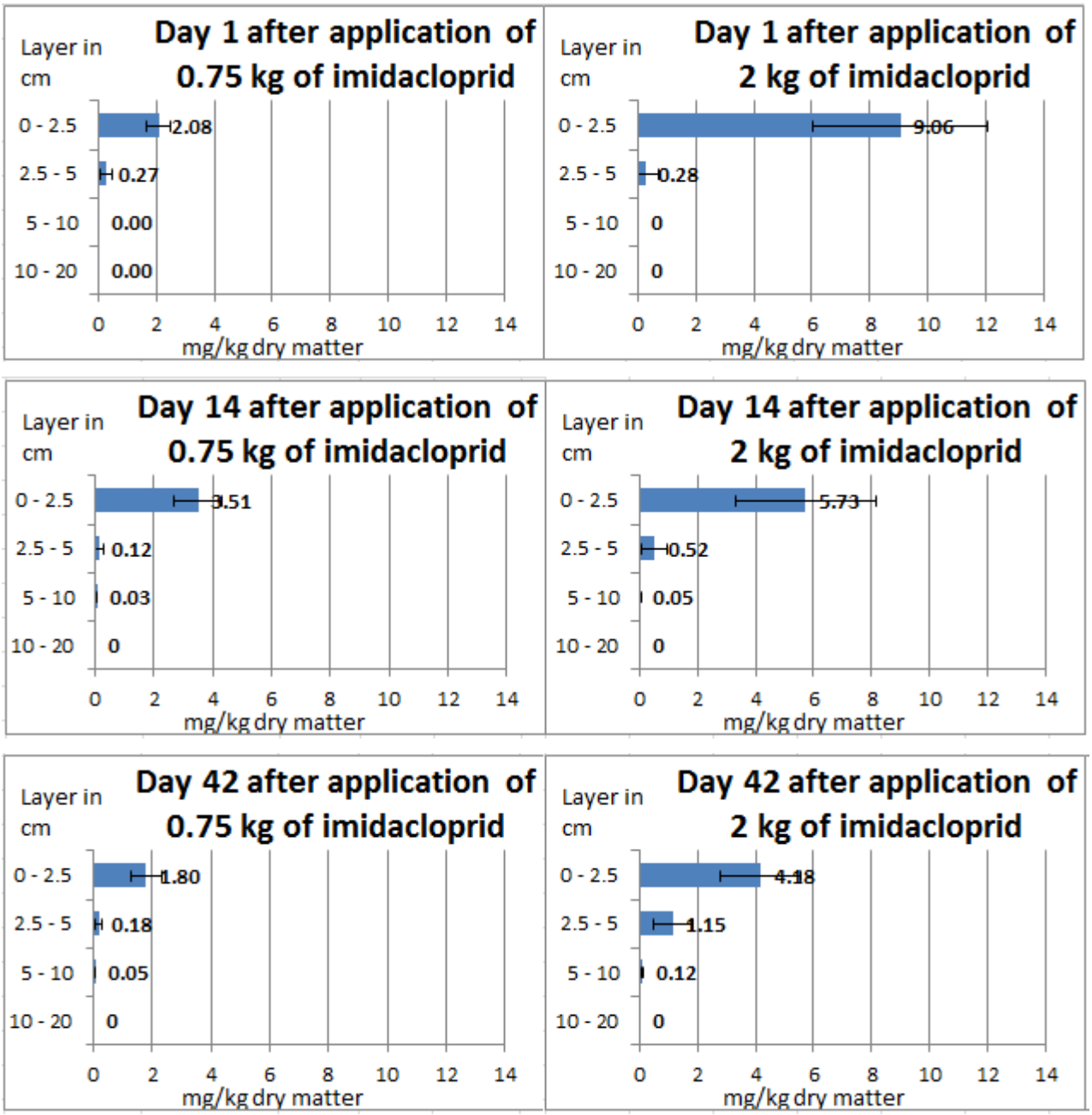

Figure 30 Caption see below

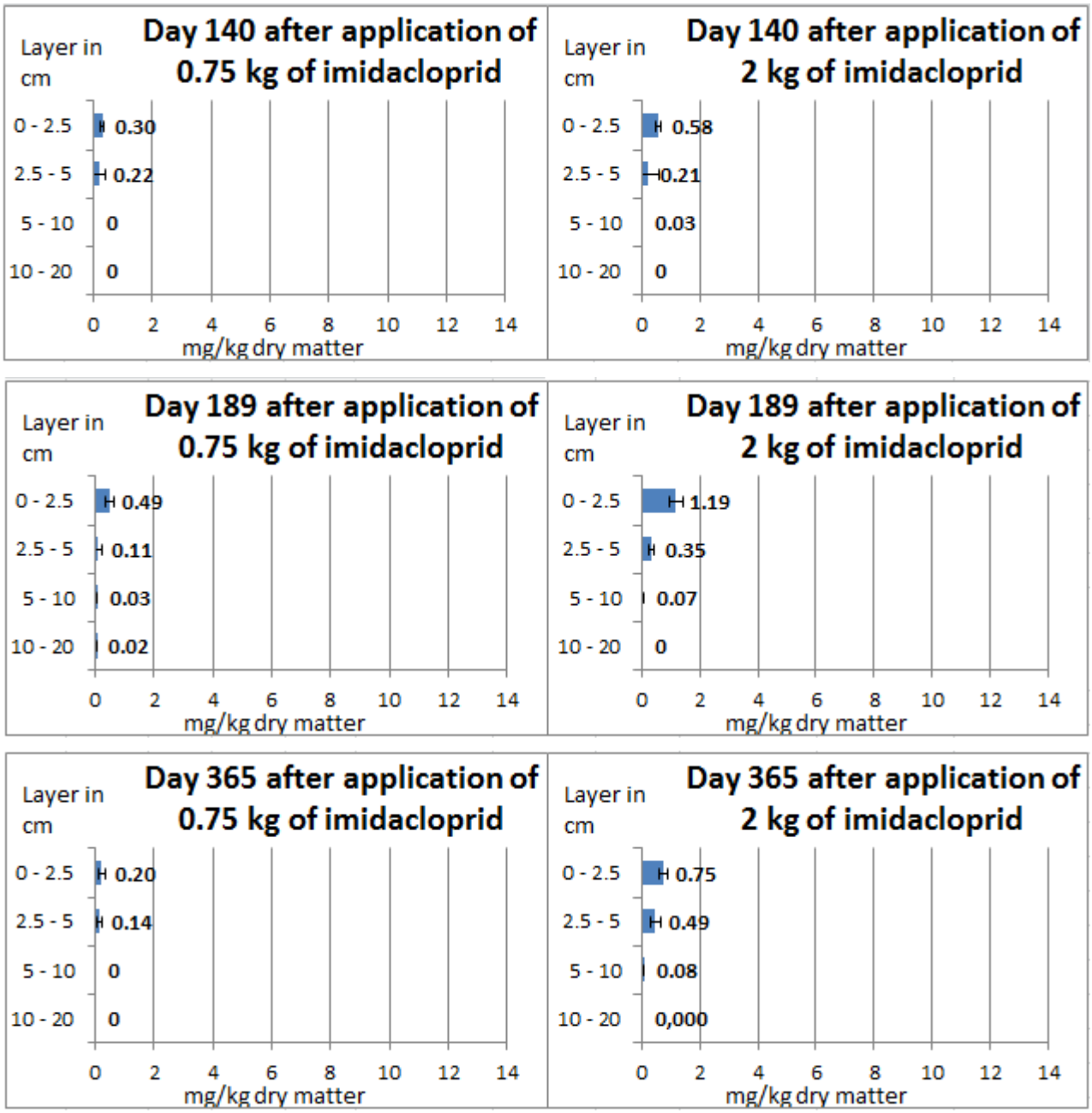

Figure 30 Mean concentrations of Imidacloprid analysed in soil layers at different time points after application. Error bars indicating the standard deviation.

4.4 Abundance and distribution of microarthropods, enchytraeids and earthworms in soil layers of the TMEs of study [1]

The first sub-chapter of this section discuss the consequences of the chosen design in the background of the statistic evaluation of the data (4.4.1). The second of the following sub-chapters presents the results of the control replicate TMEs (4.4.2). These results give an idea of the overall occurrence of the organisms, abundance of taxa and species over time and their distribution in the different soil layers of the TMEs. They provide the basis for the evaluations described in the following chapters, i.e. regarding vertical distribution, phenology and effects on the population and species level of soil organisms exposed to the two applied PPP in study [1].

When presenting the effects in Chapter 4.4.3 for Lindane and Chapter 4.4.4 for Imidacloprid, we follow a uniform four-step scheme, providing a sound evaluation of the highly diverse and complex pattern of results. The four steps are:

First step: Analysis of total abundance of soil organisms groups

We present a statistical description and analysis of the total numbers of individuals for the taxonomic groups in the different soil layers on different sampling dates. This analysis is presented by means of boxplot diagrams that provided insight into the mean numbers, standard deviations and significant differences to the control (cp. Chapter 3.9). Additionally, the percentage decrease in abundance in the treated TME soils is given for each treatment in comparison to the control for every soil layer and sampling date.

Second step: Community structure

By means of a summarizing table, the presence or lack of species in the different TME treatments in comparison to the control soils is demonstrated.

Third step: Analysis of species abundance

In the third step, we provide further information on the vertical distribution of species of soil organisms in the control TME soil layers. Therefore, some relevant species were chosen according to their dominance, ecological importance or their indicator function for the soil community. Furthermore, the percentage in-/decrease of the respective population of relevant species compared to the respective control treatment were provided for each layer at any sampling date.

Fourth step: Statistical community analysis

A summarizing overview of different diversity endpoints for the soil organisms community in the different TME treatments (number of taxa, Eveness and Shannon Index) is presented. Two similarity indices are used and demonstrate in detail the differences in the communities between control and treatments in the different layers (cp. Chapter 3.10).

Additionally, multivariate statistics are used in order to analyse the soil organisms' community response. Firstly the PRC (cp. Chapter 3.11), based on *RDA* and *PCA* calculations, is used to determine the significance of effects on the entire soil community, i.e. for the sum of individuals in all soil layers. In the following, only those PRC diagrams are presented in which significant effects have occurred.

4.4.1 Data analysis and interpretation: discussion of the methodology

In the present study [1], a test design was chosen using different numbers of replicates for the control (n=10) and each treatments (n=5). With this design it was possible to test two different substances each with two application levels and with a higher number of controls ensuring a higher statistical power for the univariate statistical analysis (Scholz-Starke 2013, Williams 1972). The uneven replicate numbers are also a consequence of resource limits for this particular project. There are some disadvantages in having an unequal number of replicates that need to be taken into consideration when interpreting the following results. This design is limited when the numbers of individuals tend to be very low, i.e. for all replicates where only single individuals can be recorded. In this case, the probability of having a single record in ten replicates is higher than in only five. Also regarding species with low constancy in the samples, underestimation of their presence in the treatments with lower replicates might occur. Consequently, abundance effects of the test items can be overestimated. In the present study, different soil layers were considered independently. Some soil layers have naturally low numbers of individuals regarding different species hence this overestimation cannot be excluded. To get an adequate assessment and interpretation of the results, the following issues had to be taken into account:

1. The results for the sum of all layers present the best results, in terms of statistical power. However, these result might not describe properly changes in the distribution profile of individuals. Movements of single species from top layers to lower layers in the TME would e.g. not necessarily result in changes in the allover sum of individuals.
2. When considering presence/absence of species, a cross evaluation of both treatment levels (as average of 5 + 5 =10 replicates) can help interpreting the results. Using the mean of both treatment levels, it must be considered that two different concentrations were mixed together, but without treatment-related effects, the mean value of both treatment levels (each 5 replicates) should theoretically be the same as the mean of 10 control replicates. For example, the mean abundance of *Eupelops occultus* (Table 35) in the control for layer A (0-2.5 cm) on day 140 is 0.44 whereas the mean of both treatments is 0.38 (0.54, 7.5 kg/ha; 0.22, 20 kg/ha), thus within the same range as the control. No statistically significant effects of Imidacloprid on oribatid mites were observed. For Lindane, which is known as highly toxic for oribatid mites, significant effects were observed even for these low numbers.
3. The above mentioned bias must be considered for all analyses using presence/absence data (Number of taxa, Similarity etc.). However, with regards to the results of effects of Imidacloprid on oribatid mites (chapter 4.4.4.2) as reference for “no-effects”, the influence of the different replicate numbers might be considered as negligible.
4. Not all rare species can be assumed to be captured within 10 replicates. To capture more species -and even more rare soil organisms species- it is necessary to use more than +/- 20 replicates. However – as long as no species similarities are considered -the relevance of this single captures in the background of toxicity testing can be assumed of minor importance.

Table 35 Detail of statistical analysis of the oribatid mite species *Eupelops occultus* in layer A (0-2.5 cm). *Eupelops* was chosen by way of example for a species showing no statistically significant treatment related effects for the substance Imidacloprid. The mean abundance of the control (n=10)and treatments (n=5) is given at every sampling date. Additionally, the decrease of abundance and the statistical significance is presented with the minimum detectable difference (MDD).

***Eupelops occultus* (layer A: 0-2.5 cm)**

Imidacloprid

	Mean abundance			Decrease of abundance		
Day	**Control**	**0.75 kg/ha**	**2 kg/ha**	**0.75 kg/ha**	**2 kg/ha**	**MDD**
14	0	0	0	0%	0%	-
42	0,38	0	0	100%	100%	115,08
140	0,44	0,54	0,22	-23%	50%	126,57
189	0,96	0,22	0,32	77%	66%	86,31
364	0,49	0,22	0,39	55%	20%	126,10

Lindane

	Mean abundance			Decrease of abundance		
Day	**Control**	**7.5 kg/ha**	**20 kg/ha**	**7.5 kg/ha**	**20 kg /ha**	**MDD**
14	0	0	0	0%	0%	-
42	0,38	0	0	100%	100%	115,08
140	**0,44**	**0**	**0**	**100%**	**100%**	**91,46**
189	**0,96**	**0**	**0**	**100%**	**100%**	**74,17**
364	**0,49**	**0**	**0**	**100%**	**100%**	**93,61**

Bold: Williams t-test: $p < 0.05$

4.4.2 Overview of abundance and distribution patterns in soil layers

Both in terms of overall abundance, species number and species composition, the soil organisms in the TMEs mirror a typical community in Central European grassland habitats on non-sandy soils (cp. Römbke et al., 2012 , Theißen, 2010, Toschki, 2008, Weigmann and Kratz, 1981, Southwood and Emden, 1967, Jänsch et al. 2013, Römbke et al. 2013). However, this statement is differently robust when comparing the available knowledge on the four organism groups: surely, the number of available data from such grasslands is highest for earthworms, and lowest for oribatids, while the respective number of enchytraeids and springtails is somewhere in the middle (Römbke et al., 2012). Overall 3642 individuals from 25 species were recorded for collembolans, 1656 individuals from 19 species for oribatid mites, 4524 individuals of 18 species for enchytraeids and 2547 individuals from six species for earthworms (cp. appendix 1).

The species belonging to the four groups mentioned above are vertically stratified in the soil column. Soil species were classified into different life-form types:

1. **Hyperedaphic species** living in the herb layer
2. **Epedaphic/ epigeic species** living on the surface and in the litter
3. **Hemiedaphic/ hypogeic species** living in the humus layer
4. **Euedaphic/endogeic species** living in the surface soil
5. **Anecic species** deep digging species

These classifications were chosen for the different species to provide a better connection between the expected (classification in literature) and the `real´occurrence (results of the studies) in the soil profile.

92 % of the collembolans (68.4 % in layer A of the TMEs, corresponding to 0-2.5 cm depth), 98 % of oribatid mites (90.8 % in layer A), 88 % of enchytraeids (60.3 % in layer A) and 58 % of the earthworms (35.9 % in layer A) were recorded in the top 5 cm of soil, i.e. layers A and B (Figure 31).

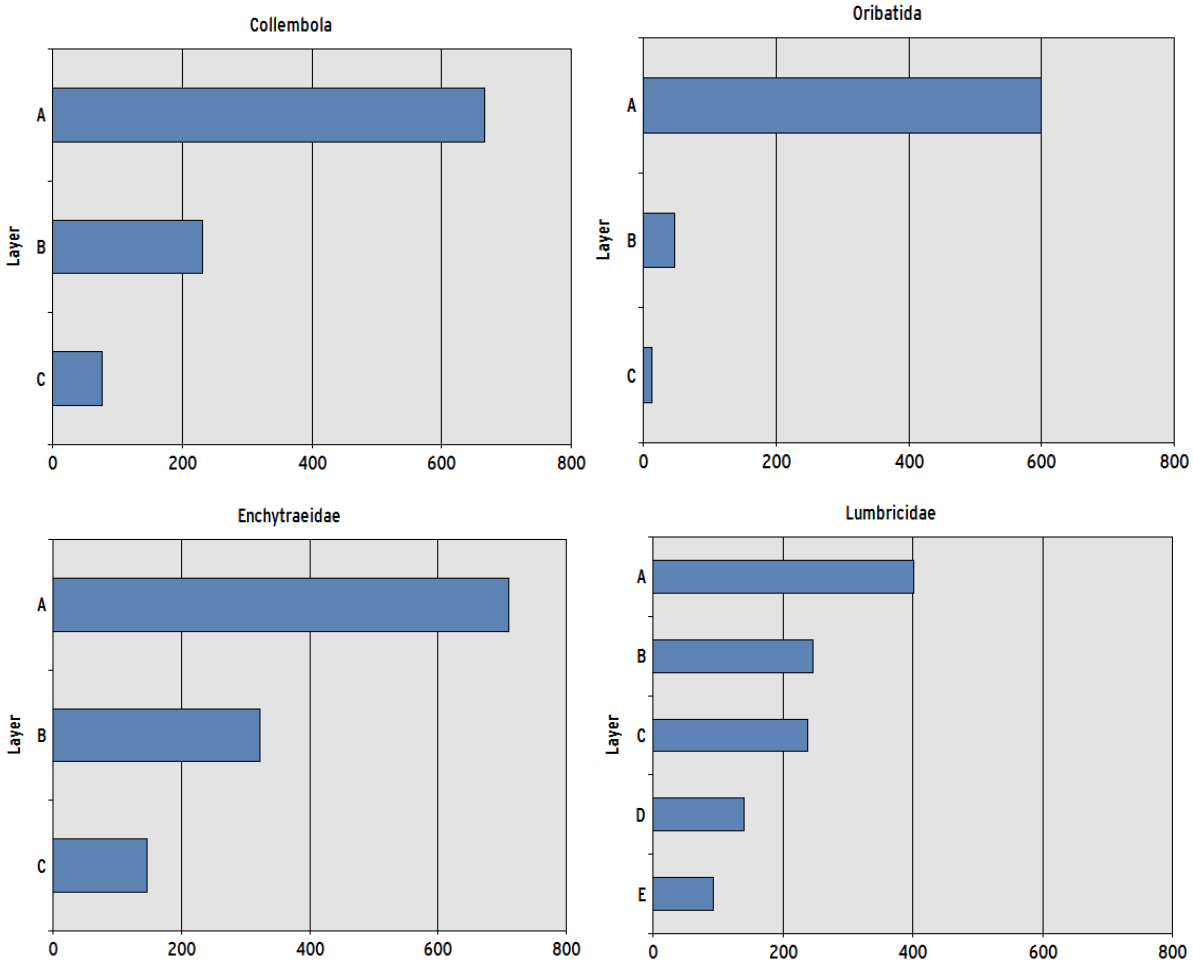

Figure 31 Sum of individuals of the four taxonomic groups (all species) in the three (five for earthworms) soil layers of the control TMEs (n=10). Layer A: 0-2.5 cm; layer B: 2.5-5 cm; layer C: 5-10 cm; layer D: 10-20 cm; layer E: 20-40 cm.

For collembolans and oribatid mites, the highest mean abundance was always found in layer A (see Table 36). Both groups showed a fluctuating abundance over time. For both, the highest mean abundance in the controls was recorded in November 2011 (on day 189 after application)(coll. 19.3 ind./two soil cores, orib. 21.0 ind./two soil cores). In contrast to the microarthropods, the highest mean abundance of enchytraeids and earthworms in layer A were found after one year in May 2012 (day 364, ench. 19.6 ind./soil core, earth. 31.4 ind./TME). Except for day 14, the highest mean abundance of enchytraeids was always found in layer A. The highest mean number of lumbricids was recorded 14 days after application in layer C (24.8 ind.), on day 140 in layer B (9.8 ind.) and on day 364 in layer A (31.4 ind.).

Table 36 Mean abundance ± 95 % confidence interval followed by (min-max) values of individuals of soil organisms belonging to different groups in the soil layers for different sampling dates in the control TMEs (n=10). For Collembola and oribatid mites the mean abundance is given as sum of individuals/2 soil cores (Ø 5 cm) for layer A-C, sum of individuals/pitfall trap (Ø 5 cm, one week) for layer O. For enchytraeids sum of individuals/soil core (Ø 5 cm) and for earthworms sum of individuals/TME; "-" means that no data were recorded (cp. Chapter 3.5).

	Days after application	Layer O Surface	Layer A (0 - 2.5 cm)	Layer B (2.5 – 5 cm)	Layer C (5- 10 cm)	Layer D (10 – 20 cm)	Layer E (20 - 40 cm)
Collembola	14	6.6 ± 3.3 (1-16)	12.2 ± 11.6 (2-64)	6.2 ± 5.2 (0-28)	2 ± 0.9 (0-5)	-	-
	42	63.2 ± 18.1 (28-118)	16.4 ± 7 (3-40)	2.2 ± 0.6 (0-4)	2.7 ± 1.3 (0-7)	-	-
	140	21.7 ± 14.9 (2-74)	6.4 ± 2.5 (0-15)	2.25 ± 0.9 (0-5)	2.3 ± 1 (0-5)	-	-
	189	-	19.3 ± 15.2 (2-85)	10.9 ± 10 (1-56)	-	-	-
	364	49.1 ± 12.4 (19-84)	13 ± 7.4 (3-37)	3.2 ± 1.8 (0-7)	2.75 ± 1 (0-6)	-	-
Oribatida	14	-	6.3 ± 2.5 (0-12)	1.4 ± 0.3 (0-2)	1.5 ± 0.3 (0-2)	-	-
	42	-	10.1 ± 5.3 (4-33)	1.4 ± 0.5 (0-3)	0 ± 0 (0-0)	-	-
	140	-	10.7 ± 2.5 (3-16)	1.5 ± 0.4 (0-2)	0 ± 0 (0-0)	-	-
	189	-	21 ± 12.5 (1-64)	3.5 ± 3 (0-13)	-	-	-
	364	-	12.4 ± 4.4 (5-26)	2 ± 0.6 (0-3)	1 ± 0 (1-1)	-	-
Enchytraeidae	14	-	12.7 ± 5.4 (2-29)	14.3 ± 4.2 (4-27)	10.9 ± 9.4 (0-48)	-	-
	42	-	18.5 ± 8.1 (1-42)	4.4 ± 2.5 (0-14)	3.9 ± 1.6 (0-7)	-	-
	140	-	16.8 ± 7.7 (0-41)	6.8 ± 2.9 (0-14)	2.4 ± 0.9 (0-5)	-	-
	189	-	11.5 ± 5.8 (0-28)	8.8 ± 4.4 (0-18)	-	-	-
	364	-	19.6 ± 10.6 (2-43)	4.7 ± 2.5 (0-12)	3.2 ± 0.7 (0-5)	-	-
Lumbricidae	14	-	9.6 ± 3.4 (5-19)	19.4 ± 5.4 (4-25)	24.8 ± 3.9 (18-34)	14.6 ± 2 (10-18)	4.4 ± 1.2 (2-7)
	140	-	7.8 ± 2.9 (1-13)	9.8 ± 6.1 (3-27)	8.4 ± 2.8 (5-16)	6.2 ± 1 (4-8)	4.8 ± 1 (3-6)
	364	-	31.4 ± 18.3 (8-107)	10 ± 6.2 (1-36)	7.2 ± 4.2 (2-25)	4.5 ± 1 (3-8)	4.7 ± 1.3 (1-8)

The dominant collembolan species (i.e. those with more than 10 % abundance in all samples) were *Parisotoma notabilis* (12.4 %), *Isotoma anglicana* (11.5 %), *Brachystomella parvula* (10.6 %) and *Entomobrya lanuginosa* (10.4 %) (Figure 32). *P. notabilis* is a widespread hemiedaphic species (Römbke et al., 2012) that occurs in arable land as well as in grassland and forest habitats (cp. Edaphobase, 2013). The species is euryoecious and eurytopic with no significant environmental preferences. *Isotoma anglicana* is also a widespread euryoecious species whereas *B. parvula* and *E. lanuginosa* are known as species that are typical for open landscapes such as arable land and grassland habitats (Edaphobase, 2013). The life-forms taken from the literature (mainly Stierhoff, 2003 and Theißen, 2010) were substantiated by the findings in this study, i.e. most of the different collembolan species were found in the expected soil layers (Figure 33). These findings demonstrate that the soil community in the TME is widely undisturbed and represents a natural community comparable to the field community. The group of hemiedaphic collembolans contains the most abundant species, followed by the epedaphic life-form types.

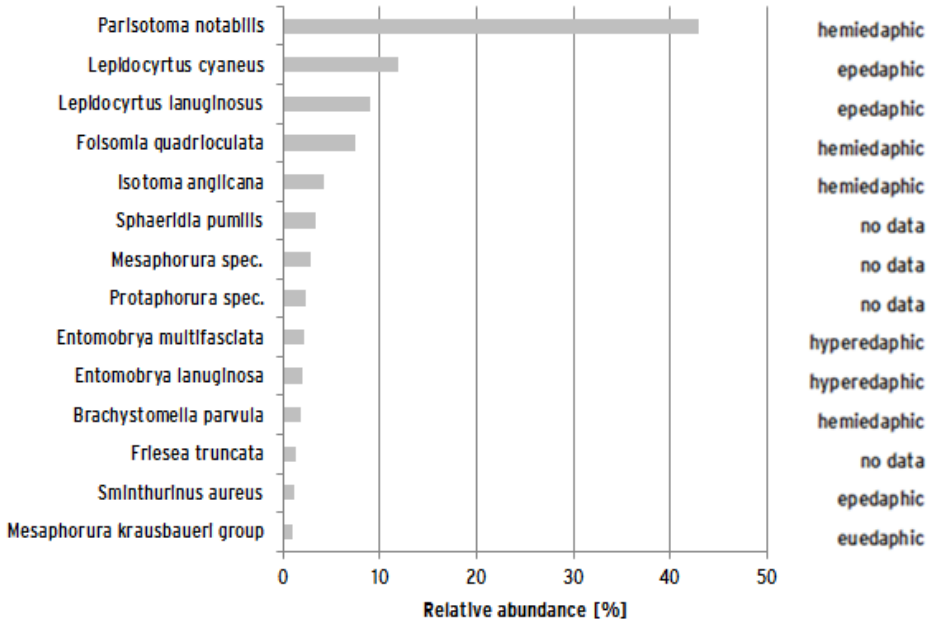

Figure 32 Dominance spectrum of collembolans over all sampling dates in the control TMEs (n=10). All taxa of collembolans with more than 1 % of total abundance and the classification to the ecological life-form types (according to Stierhoff 2003 and Theißen 2010) are shown.

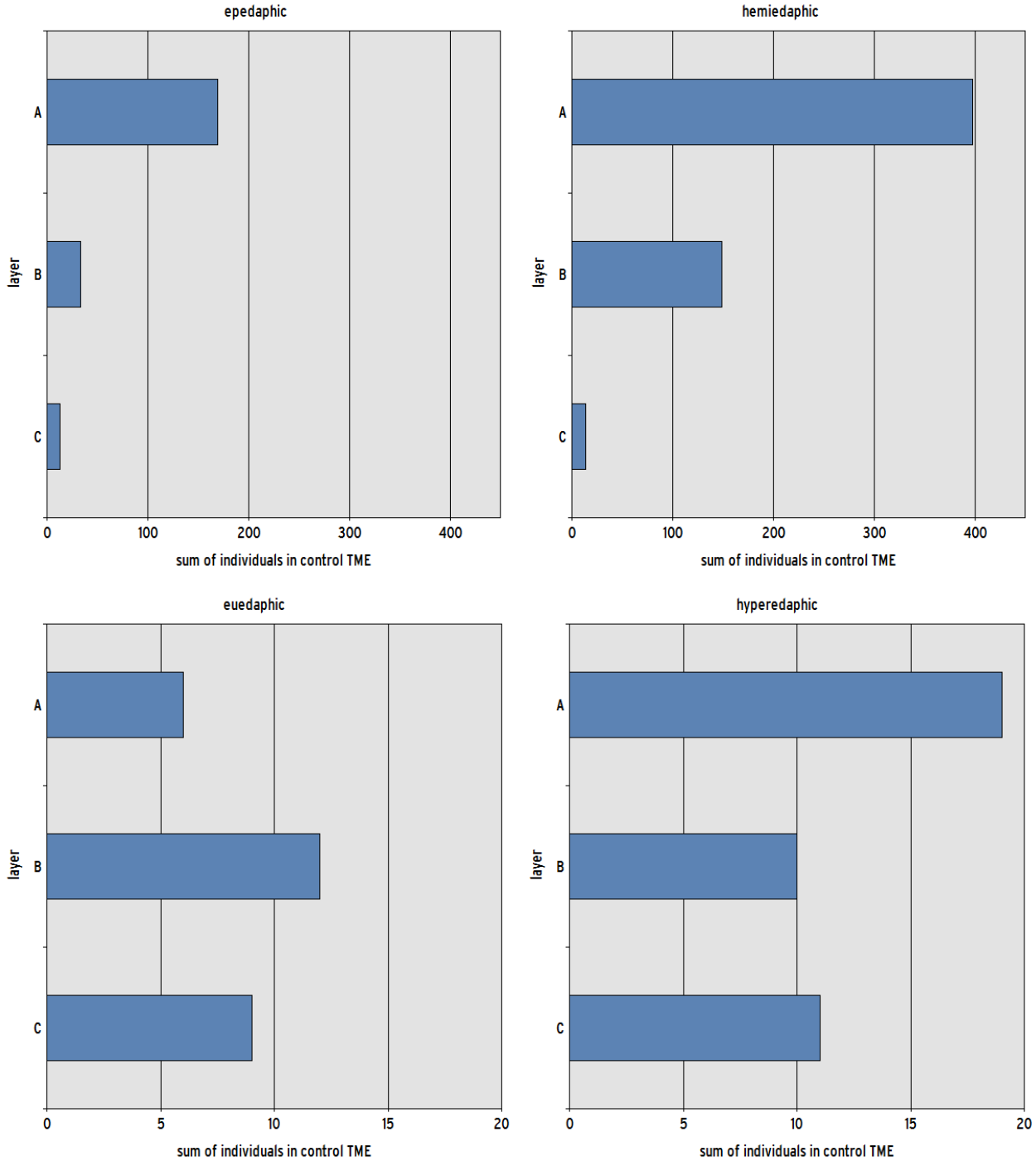

Figure 33 Abundance of collembolan species captured in the different soil layers of control TMEs (n=10) at 5 different sampling dates classified in four different ecological life form types after Stierhoff 2003 and Theißen 2010. Layer A: 0-2.5 cm; layer B: 2.5-5 cm; layer C: 5-10 cm.

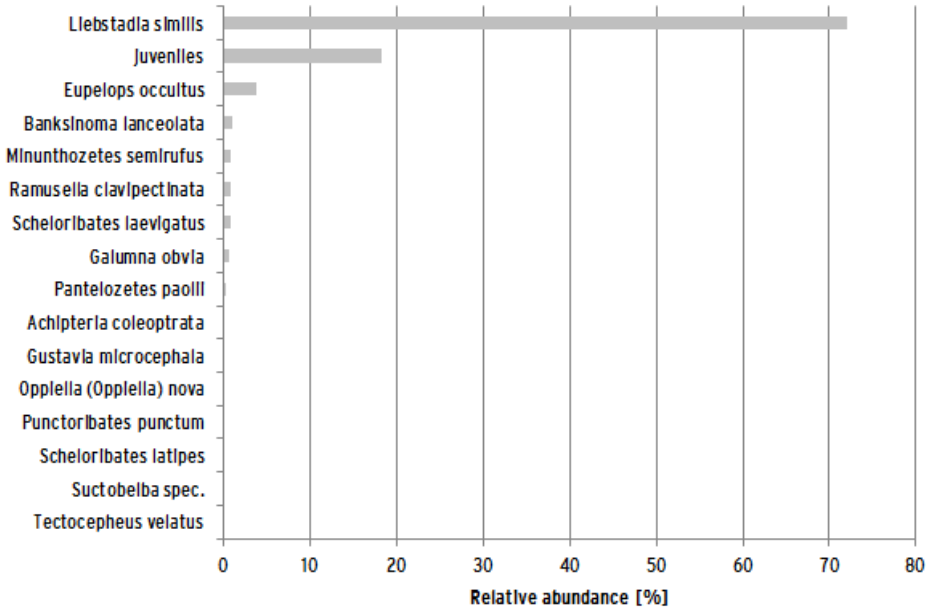

Figure 34 Dominance spectrum of oribatid mites over all sampling dates in the control TMEs (n=10)

The most dominant oribatid mite species by far was *Liebstadia similis*, showing 74 % of abundance of all those recorded (Figure 34). A high number of juveniles, whose species could not be determined, presumably belong to this dominant species. *Liebstadia similis* is a typical hemi- to epedaphic grassland species which is distributed widespread in Germany (Toschki, 2008; Edaphobase, 2013; Weigmann and Kratz, 1981). The second dominant species among the oribatid mites was *Eupelops occultus*. This species is as the former also typically known as highly characteristic for grassland habitats and is also distributed widespread in Germany (Edaphobase 2013, Römbke et al. 2012, Toschki 2008).

Within the group of enchytraeids, *Achaeta "dzwilloi"* and *Fridericia connata* were the most abundant species (34.9 % and 13.9 %, Figure 35). While the former (whose taxonomic status is not yet clear) is almost unknown in terms of its ecology, the latter can be classified as an endogeic worm, which prefers crop and grassland sites, especially those with a median range of organic matter content (Römbke et al. 2013).

Among the Lumbricidae, *Aporrectodea caliginosa*, a very widespread endogeic species, was the dominant species (21.3 %, Figure 36). It prefers crop and grassland sites with slightly acid to neutral soils, but is very tolerant in terms of soil texture or organic matter content. A high number of juveniles, determined only to the genus level as group of *Aporrectodea juveniles* (42.1 %), presumably belong to this species.

The current study reflects the classification of the different species to the ecological groups of earthworms in accordance with the literature (Bouché 1977, Jänsch et al. 2013) (Figure 37). This statement is especially true for the anecic vertical burrowers, but the difference between endogeic and epigeic worms regarding their vertical distribution is small. However, this comparison might be biased by the fact that on this grassland site the number of epigeics is very low (by a factor of 20 lower than that of the endogeics). Another factor influencing this comparison is the fact that juvenile endogeics prefer an almost epigeic lifestyle (i.e. they are often found in the root layer of the grass plants), while the adults are living in the upper mineral soil.

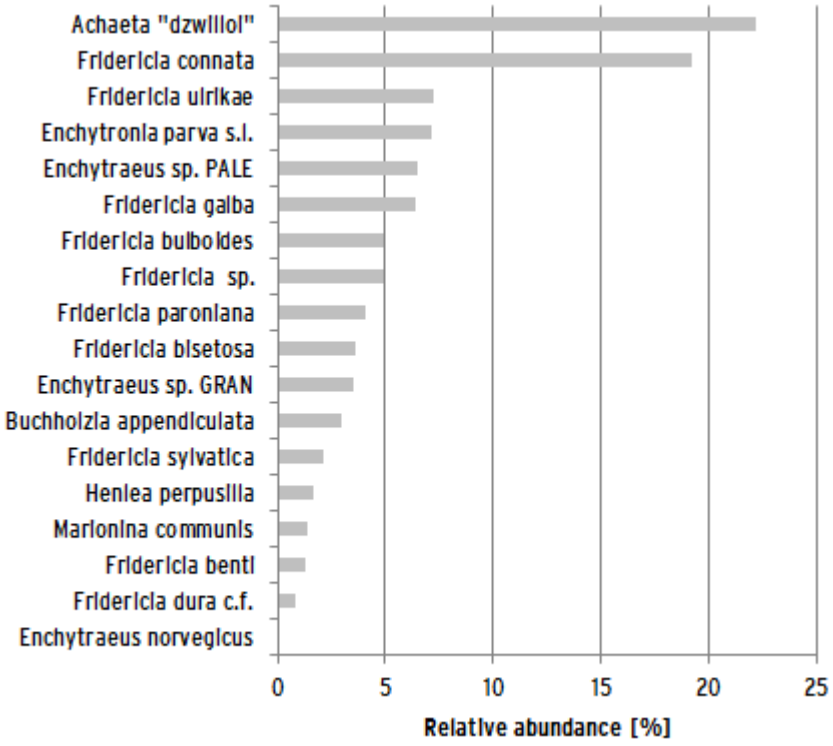

Figure 35 Dominance spectrum of echytraeids over all sampling dates in the control TMEs (n=10).

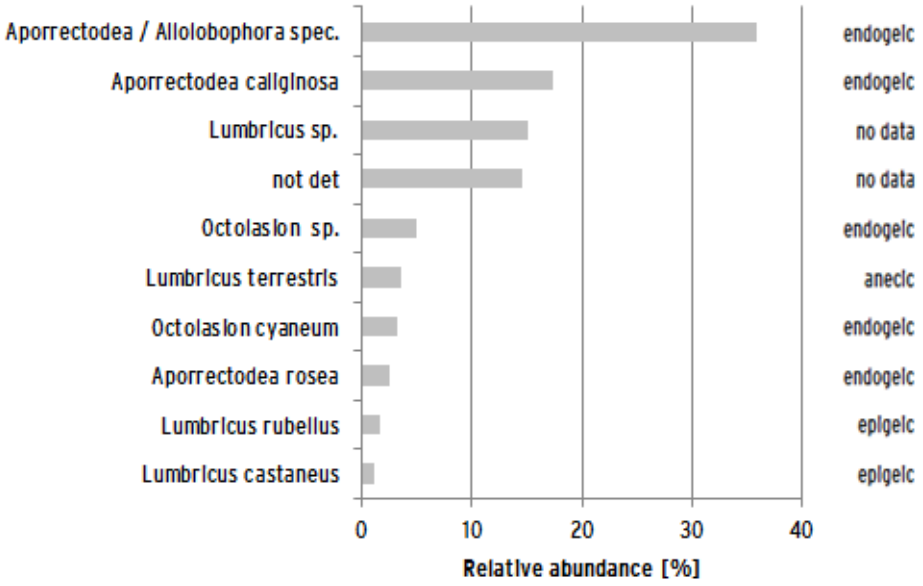

Figure 36 Dominance spectrum of lumbricids over all sampling dates in the control TMEs (n=10) and the classification to the ecological life form types after Bouché (1977).

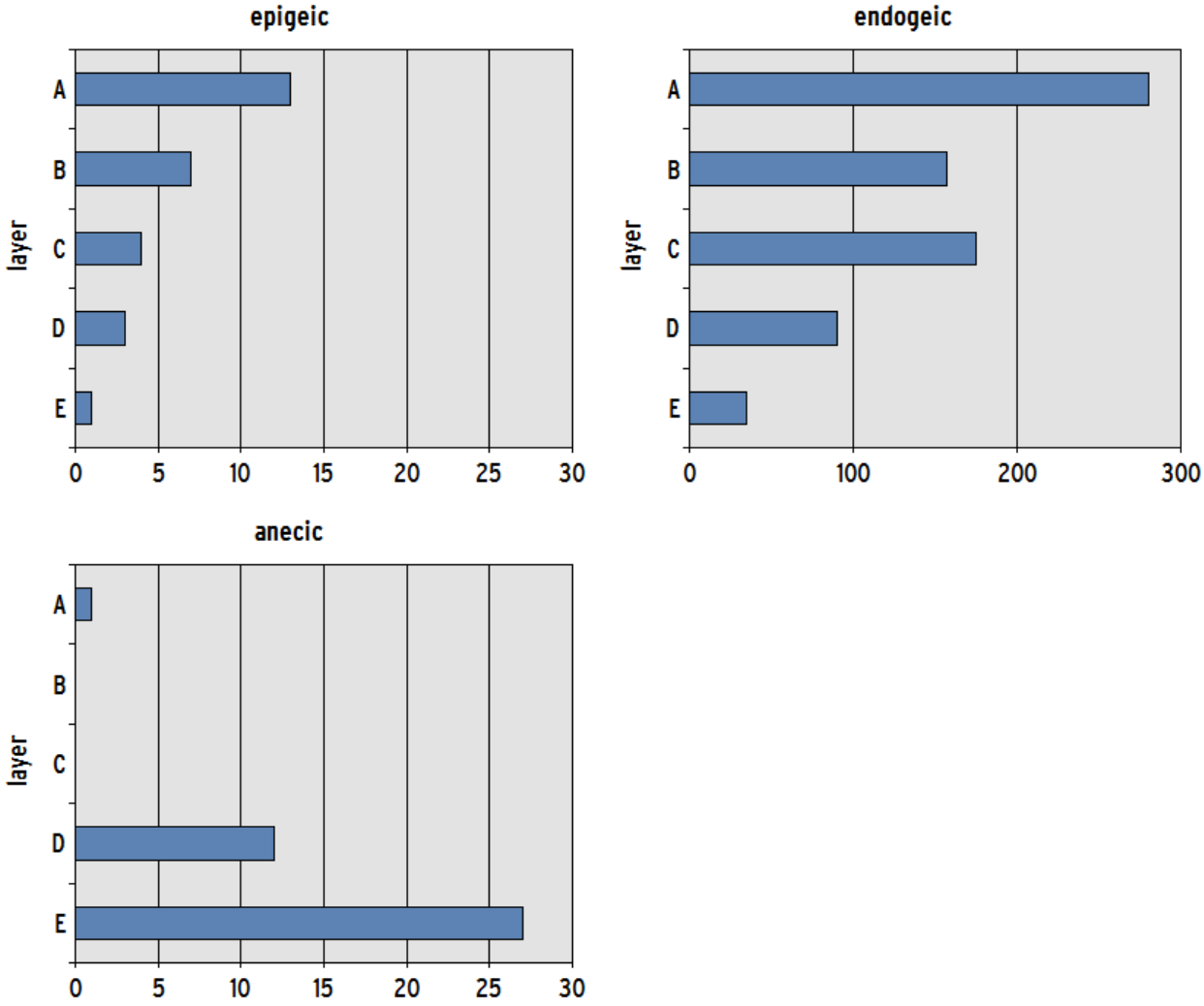

Figure 37 Total abundance of earthworms captured in the different soil layers of control TMEs (n=10) classified in three different ecological life-form types. Layer A: 0-2.5 cm; layer B: 2.5-5 cm; layer C: 5-10 cm; layer D: 10-20 cm; layer E: 20-40 cm.

4.4.3 Effects of Lindane

4.4.3.1 Effects on collembola

The abundance of the collembolans was markedly affected by Lindane (Figure 38, Table 37). In most cases a dose–response relationship was observed, i.e. the higher dose of Lindane induced the highest effect.

On each sampling date, in at least one layer, a significant reduction in total abundance was observed (Figure 38, Table 37). 14 days after application, the total abundance of collembolans in all layers (A-C) was clearly reduced by 90 % on average at the lower Lindane application rate and even by 95 % at the higher application rate. This indicates that the intended effects of the applied substance on collembola are in the 'low treatment' higher than planned by the test set-up. Furthermore, the collembolans on the surface (named layer O) were significantly affected resulting in a reduction of 73 % at the lower and 94 % at the higher application rate. On the subsequent sampling dates in the surface layer and layer A (0-2.5 cm) of the treated TMEs, total abundances slightly increased. After one year (364 days), a reduced abundance was still observed at both application rates. However, the effect seemed to be reduced in comparison to the previous sampling dates, especially at the surface for both application rates (52 % and 76%) and for the uppermost soil layer at the lower application rate (51 %). In contrast at the higher application rate the total abundance in layer A was continuously affected by 97-100%.

Effects were less pronounced in the lowest layer C, which is presumably due to the low number of individuals that were recorded in this layer. At the lower application rate on day 140, no statistically significant reduction was measured for the surface layer and layer A. This can be explained by the fact that at this time only small numbers of individuals were recorded even in the control (Figure 38).

The statistical analysis on the population level resulted in significant effects on at least one single date and one soil layer for 12 species out of 25 species (Williams t-test $p<0.05$, cp. appendix 1), i.e.in 48 % of all species,. This is remarkable, since the number of individuals is reduced when focusing on a single layer, meaning that the statistical identification of effects is therewith often impeded. Nevertheless, the highest effects were detected for the total number of individuals, i.e. when summing up the layers A-C (Table 37). When comparing the pattern of significant effects on different species, it seems that these effects were found most often in the uppermost soil layer (not the litter layer). In addition, the reaction is species-specific (e.g. in terms of the time and duration of effect).

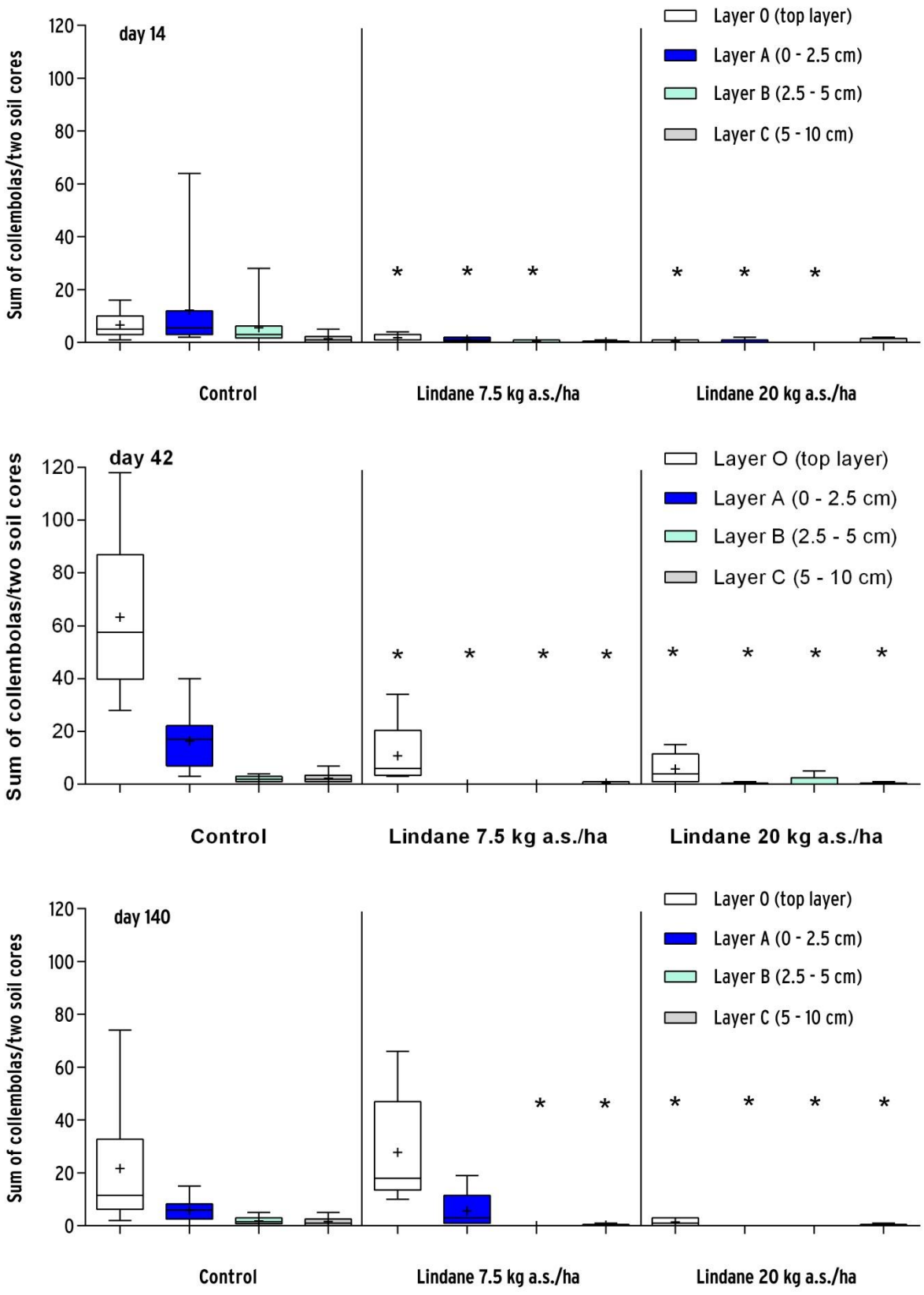

Figure 38 Caption see below

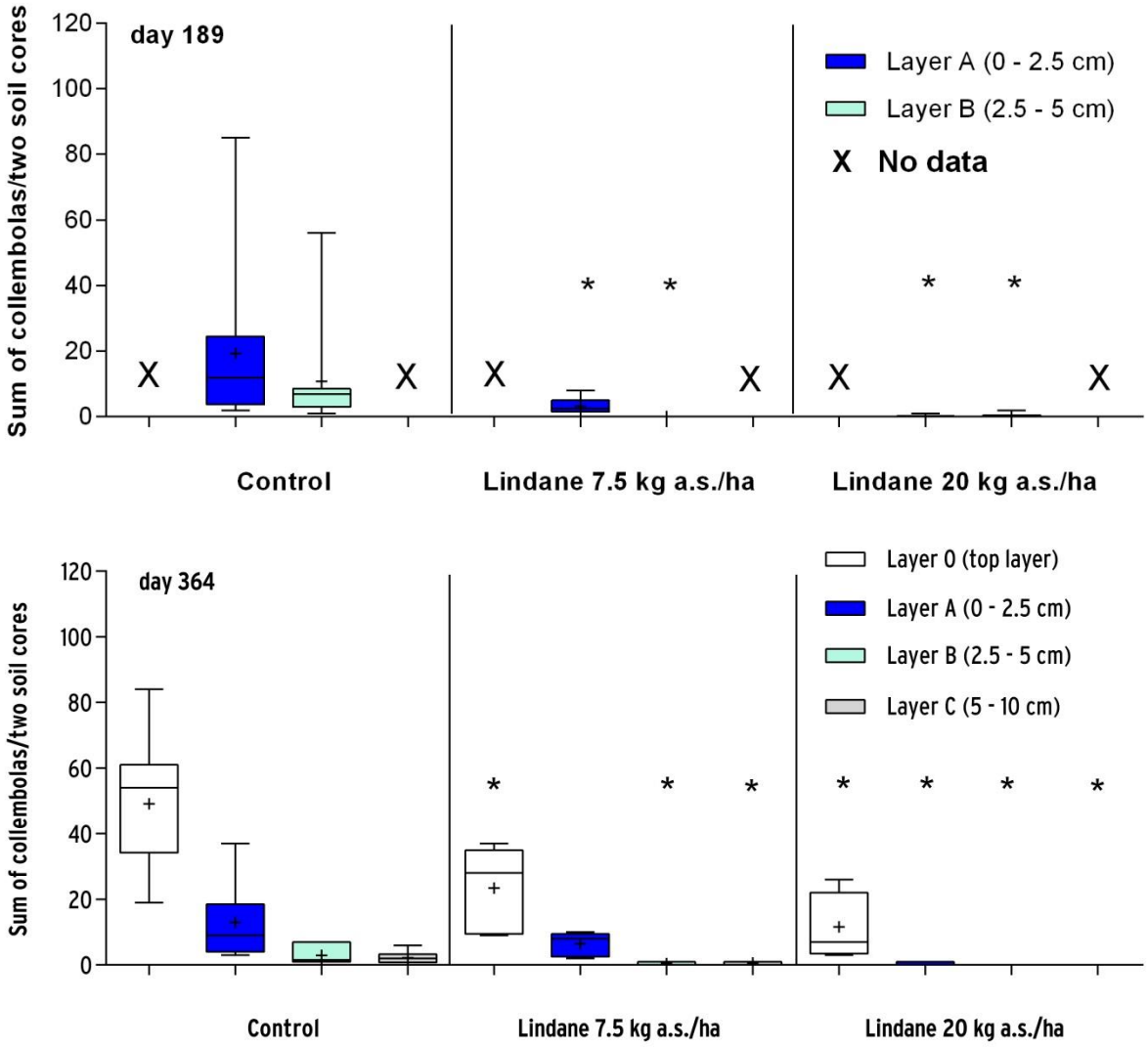

Figure 38 Total abundance of collembolans in the soil layers of the TMEs in study [1] (10 replicates for control; 5 replicates for lindane treatments). X: no data; cross: mean; *: significance (p-value Williams-test < 0.05). Sampling dates at day 14-364 from top to bottom

Table 37 Decrease of total abundance [%] of collembolan species in the different soil layers at different sampling dates, 14-364 days after application compared to control abundance. Red: decrease of more than 50% in comparison to the control; grey dots: less than 50% decrease in comparison to the control; X: no data available; Bold: significant effects (p-value Williams-test < 0.05); calculation is based on 10 replicates for the control and 5 replicates for the respective treatment.

Lindane 7.5 kg a.s./ha

		days after application				
	layer	14	42	140	189	364
O	Surface	**73**	**83**	-28	X	**52**
A	0 - 2.5 cm	**92**	**100**	3	**80**	51
B	2.5 - 5 cm	**89**	**100**	**100**	**100**	**86**
C	5 - 10 cm	86	**83**	**88**	X	**73**
	all layers	**90**	**98**	37	**87**	**59**

Lindane 20 kg a.s./ha

		days after application				
	layer	14	42	140	189	364
O	Surface	**94**	**91**	**94**	X	**76**
A	0 - 2.5 cm	**97**	**99**	**100**	**99**	**97**
B	2.5 - 5 cm	**100**	**50**	**100**	**97**	**100**
C	5 - 10 cm	57	**92**	**88**	X	**100**
	all layers	**95**	**93**	**98**	**98**	**98**

Table 38 Presence and cumulative mean abundance of captured collembolan species over time in the control TMEs (10 replicates) and the two different application rates of the treatment TMEs (5 replicates). The treatment mean is calculated as the mean abundance of collembolan species in all treatment TMEs (10 replicates), 5 with higher and 5 with lower pesticide rate.

	Control	Lindane		
		Treatment mean	*7.5 kg a.s./ha*	*20 kg a.s./ha*
Number of replicates	**10**	**10**	**5**	**5**
Soil cores				
Parisotoma notabilis	41.8	13	12	14
Lepidocyrtus cyaneus	11.5	0.4	0.6	0.2
Lepidocyrtus lanuginosus	8.7	1.0	1.4	0.6
Folsomia quadrioculata	7.2	0.2	0.2	0.2
Isotoma anglicana	4.1	0.3	0.6	-
Sphaeridia pumilis	3.3	-	-	-
Entomobrya multifasciata	2.1	-	-	-
Entomobrya lanuginosa	1.9	1.2	2.2	0.2
Brachystomella parvula	1.8	5.0	10.0	-
Friesea truncata	1.3	0.1	0.2	-
Sminthurinus aureus	1.2	0.3	0.6	-
Mesaphorura macrochaeta	0.8	-	-	-
Folsomides parvulus	0.7	-	-	-
Isotomurus fuciola/graminis	0.3	0.1	0.2	-
Stenaphorura quadrispina	0.3	0.3	0.4	0.2
Folsomia fimetaria	0.1	-	-	-
Lepidocyrtus lignorum	0.1	-	-	-
Pseudosinella alba	0.1	-	-	-
Sminthurus viridis	0.1	-	-	-
Stenaphorura denisi	0.1	-	-	-
Pitfall traps				
Bourletiella hortensis	x	x	x	x
Deuterosminthurus pallipes	x	x	x	x
Heterosminthurus bilineatus	x	-	-	-
Isotoma viridis	x	-	-	-
Isotomurus graminis	x	-	-	-
Number of all taxa	25	13	13	9

When considering the properties of the community i.e. presence/absence of species and structure of the community, the effects of the applied Lindane rates on the population level affected the whole community structure of collembolans. In Table 38 all species that were recorded are shown with their mean abundance in the respective treatment. To compare the species abundances of the control with the ones in the treatments, the different number of replicates for the control (n=10) and each treatment (n=5) had to be considered. The probability for the occurrence of rare species increased with the number of replicates. However, by combining both treatment levels

(i.e. calculation of the treatment mean), the number of replicates is identical to those of the control and the quality of species, i.e. presence of species and number of species can be compared. When comparing combined treatment level with the control, the above mentioned reduction can be clearly seen by the loss of species. Within the control TMEs all in all 25 species were recorded, while only 13 species were found in the treatment TMEs (13 at 7.5 kg/ha, 9 at 20 kg/ha). The four most dominant species were still present in the treatments, whereas their abundance was highly reduced. Those species with very small numbers in the control were not found in the treatments (*Folsomia fimetaria, Leopidocyrtus lignorum, Pseudosinella alba, Sminthurinus viridis, Stenaphorura denisi*). The same was true for some subdominant or recendent species like *Spaeridia pumilis, Entomobrya multifaciata, Mesaphorura macrochaeta, Folsomides parvulus*.

The species *Parisotoma notabilis* belongs to the hemiedaphic life-form type (Figure 39). Within the study most of the individuals in the control were captured in layer A (0-2.5 cm, 303 ind.), followed by layer B (2.5 cm, 108 ind.). Only few animals were recorded by pitfall trapping on the soil surface and in deeper soil depth (Figure 39). The abundance of the species was reduced for both application rates maximally by 100 % in comparison to the control. Significant effects were found for layer A on all sampling dates except of day 140 and for layer B on day 14 at the highest application rate (Table 39).

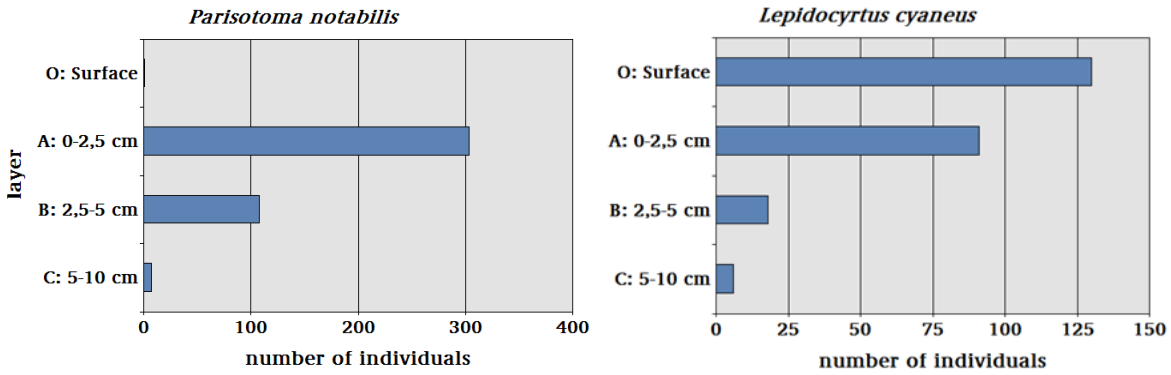

Figure 39 Vertical distribution of the collembolan species *Parisotoma notabilis* and *Lepidocyrtus cyaneus* in the control TMEs of study [1]. Shown is the total number of individuals captured in all soil cores and pitfall traps on all sampling dates.

The species *Lepidocyrtus cyaneus* can be classified to the epedaphic life-form type (Figure 39). Most of the individuals (130 ind.) were recorded by pitfall trapping i.e. capturing individuals that actively moving on the soil surface. In soil, the uppermost soil layer A was preferred (91 ind.), followed by layer B (18 ind.). In layer C, only six individuals were recorded. The species was strongly affected by Lindane in both application rates and on every sampling date (Table 39). According to the statistical power that is based on a sufficient number of individuals and a low variance within the data set, significant effects were mainly observed in the uppermost soil layer A. Nevertheless additional significant effects were observed at day 14 when considering all layers, whereas no significance was noticed for the single layers. This observation gives a strong hint to sample the whole soil profile and not just the uppermost layers.

Lepidocyrtus lanuginosus is an epedaphic species living on the surface and within the uppermost soil layer (Figure 40). Most of the individuals were captured on the surface (100 ind.) and in layer A (66 ind.), whereas only small amounts were recorded in deeper soil depth of the control TMEs. Effects on *Lepidocyrtus lanuginosus* were detected for all layers on any sampling date (Table 39). Until day 140, both application rates resulted in an extinction of this species in the soil cores and a clear marked reduction on the surface. On day 189 and 364, the effects in the soil cores were less pronounced. On day 364 no significant effects were observed within the soil (layer A-C) any more, whereas a significant reduction for the surface layer still remained.

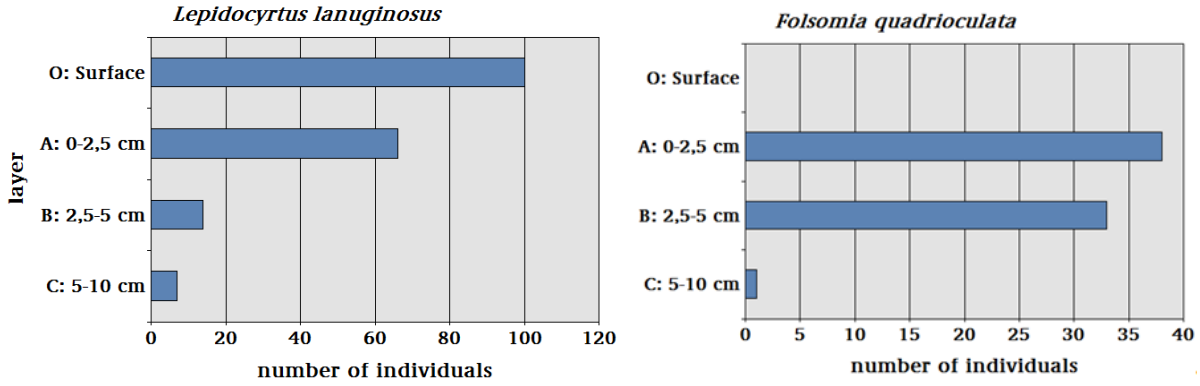

Figure 40 Vertical distribution of the collembolan species *Lepidocyrtus lanuginosus* and *Folsomia quadrioculata* in the control. Shown is the total number of individuals captured in all soil cores and pitfall traps on all sampling dates.

The species *Folsomia quadrioculata*, recorded only in smaller numbers than the species discussed so far, is classified as hemiedaphic (Figure 40). The vertical distribution in the control showed the preference of this species for the uppermost soil layers A (38 ind.) and B (33 ind.). Only a few individuals were recorded for layer C and no individuals were captured on the surface. Except of the first sampling after 14 days, the population was reduced by 100 % in comparison to the control (Table 39). Regarding the total abundance in all layers (A-C), significant effects were observed on all sampling dates for both application rates. However, due to the overall low numbers of individuals in the single layers the findings there have to be interpreted with caution (see chapter 4.4.1).

Table 39 Summary of the statistical analysis of the effect of Lindane on four different collembolan species in TMEs of study [1]. Results are given for the different soil layers (O-C) and different sampling dates (14 days to364 days after application). See text for details. Red: decrease of abundance ≥ 50%; Grey: decrease of abundance < 50%; X: no data available; Blank fields: data not sufficient for statistical calculation. Bold: significant difference (p-value Williams-test < 0.05); (see also appendix 1).

Lindane 7.5 kg a.s./ha		*Parisotoma notabilis* days after application				
	layer	14	42	140	189	364
O	Surface	-100			X	
A	0 - 2.5 cm	**96**	**100**	100	**100**	**96**
B	2.5 - 5 cm	85	100	100	100	100
C	5 - 10 cm	100	100		X	100
	all layers	**96**	100	100	**100**	**98**

Lindane 20 kg a.s./ha		*Parisotoma notabilis* days after application				
	layer	14	42	140	189	364
O	Surface	100			X	
A	0 - 2.5 cm	**98**	**100**	100	**100**	**100**
B	2.5 - 5 cm	**100**	-400	100	100	100
C	5 - 10 cm	60	100		X	100
	all layers	**97**	89	100	**100**	**100**

Lindane 7.5 kg a.s./ha		*Lepidocyrtus cyaneus* days after application				
	layer	14	42	140	189	364
O	Surface		**100**	100	X	100
A	0 - 2.5 cm	100	**100**	**100**	**100**	33
B	2.5 - 5 cm			100	100	
C	5 - 10 cm	100	33		X	100
	all layers	**100**	**83**	**100**	100	0

Lindane 20 kg a.s./ha		*Lepidocyrtus cyaneus* days after application				
	layer	14	42	140	189	364
O	Surface		**100**	96	X	80
A	0 - 2.5 cm	100	**100**	**100**	**100**	100
B	2.5 - 5 cm			100	88	
C	5 - 10 cm	100	100		X	100
	all layers	**100**	**100**	**100**	97	100

Lindane 7.5 kg a.s./ha		*Lepidocyrtus lanuginosus* days after application				
	layer	14	42	140	189	364
O	Surface		100	**80**	X	**52**
A	0 - 2.5 cm	100	100	**100**	**89**	33
B	2.5 - 5 cm	100	100	100	100	
C	5 - 10 cm		100	100	X	-100
	all layers	100	**100**	**100**	**92**	23

Lindane 20 kg a.s./ha		*Lepidocyrtus lanuginosus* days after application				
	layer	14	42	140	189	364
O	Surface		96	**93**	X	**84**
A	0 - 2.5 cm	100	100	**100**	**95**	83
B	2.5 - 5 cm	100	100	100	80	
C	5 - 10 cm		100	100	X	100
	all layers	100	**100**	**100**	**92**	85

Lindane 7.5 kg a.s./ha		*Folsomia quadrioculata* days after application				
	layer	14	42	140	189	364
O	Surface				X	
A	0 - 2.5 cm	100	**100**	100	100	100
B	2.5 - 5 cm	**100**		100	**100**	**100**
C	5 - 10 cm	-100			X	
	all layers	**82**	**100**	**100**	**100**	**100**

Lindane 20 kg a.s./ha		*Folsomia quadrioculata* days after application				
	layer	14	42	140	189	364
O	Surface				X	
A	0 - 2.5 cm	33	**100**	100	100	100
B	2.5 - 5 cm	**100**		100	**100**	100
C	5 - 10 cm	100			X	
	all layers	**82**	**100**	**100**	**100**	**100**

In addition to the above, the results from the different treatments, calculated for all soil layers except of the surface captures, were compared using the number of taxa and two diversity indices (Shannon index and Evenness). The resulting pattern is presented in Table 40. The main outcome of this comparison is that even more significant effects were found for the community endpoints than for the single taxa. All endpoints were significantly affected at least on three consecutive sampling dates. At the highest treatment level, the community effects on all endpoints were still present after one year, whereas, at the lower Lindane treatment, the Shannon diversity and Evenness showed a recovery after one year. However, the PRC and the number of taxa indicated long lasting effects on the collembolan community at both treatment levels.

Table 40 Summary of the results for the statistical diversity analyses, Principal Response Courve PRC (p-value t-test < 0.05 of PCA sample scores) , number of taxa, Shannon and Eveness, *: significance (p-value Williams-test < 0.05) of collembolans summed up over all TME layers (0-2.5 cm, 2.5-5 cm, 5-10 cm) treated with Lindane (left) rates of 7.5 kg a.s./ha (right) or 20 kg a.s./ha (study [1]). Data based on 10 replicates of control TMEs and 5 replicates for each treatment.

Lindane 7.5 kg a.s./ha						Lindane 20 kg a.s./ha					
	days after application						days after application				
all layers	14	42	140	189	364	all layers	14	42	140	189	364
PRC	*	*	*	*	*	PRC	*	*	*	*	*
Number of taxa	*		*	*	*	Number of taxa	*		*	*	*
Shannon	*	*	*	*		Shannon	*	*	*	*	*
Evenness		*	*	*		Evenness		*	*	*	*

The similarity of the collembolan communities measured with Steinhaus´ and Stander´s indices is presented for all layers (A-C) in Figure 41. Both indices showed a clear effect of the Lindane applications on the collembolan community. The lower treatment level showed the tendency of a delayed effect in comparison to the higher treatment level and a tendency of recovery after one year, whereas the effect was long lasting without recovery at the highest Lindane treatment level.

The results of the multivariate statistical analysis are given in Figure 42. The analyses via Principal Response Couve PRC indicated a negative effect of the applied Lindane rates on mostly all collembolan species with an overall significance of the first canonical axis of p = 0.005. The performed Redundancy Analysis (RDA) showed a significant difference for all layers (A-C) and all sampling dates. Furthermore the sample scores of the corresponding Principal Componant Analysis (PCA) were significantly different for all sampling dates and both Lindane treatment levels in comparison to the control. The dominant species *Parisotoma notabilis*, *Lepidocyrtus cyaneus*, *L. lanuginosus* and *Folsomia quadrioculata* (Figure 32) contributed most to the difference in community response. Most species showed a decrease in abundance except for the species *Brachystomella parvula* which showed increasing abundances after Lindane treatment.

In conclusion, Lindane showed consistent effects on the collembolan community when summing up the individuals of the soil column from 0-10 cm (layer A-C). The total abundances of collembolans were found to be significantly reduced on all sampling dates, at day 140 only at the highest application rate. The effects lasted long right up to the end of the study. The multivariate analyses of the community as well as the diversity endpoints confirm these findings. The similarity indices show a tendency for recovery for the lower application rate on day 364. The method of summing up the abundances in all soil layers led to stronger detectable effects when comparing with the results for single layers only. This is due to the increasing statistical power that is based on higher numbers of individuals and lower variability. Because of the different life-form types with respect to the preferences of species to a specific soil depth, the sum for single species led not necessarily to the best results. For one of the most abundant single species *Parisotoma notabilis*, effects occur for single layers but not when assessing the sum of layer A-C. The pattern of effects for the single species is diverse and species-specific. Effects on one species do not imply

effects for others. Only *Folsomia quadrioculata* was significantly affected by Lindane on all sampling dates in all soil layers albeit, according to the study design with lower number of replicates for the treatments than for the control, statistical bias of this findings cannot be excluded (chapter 4.4.1).

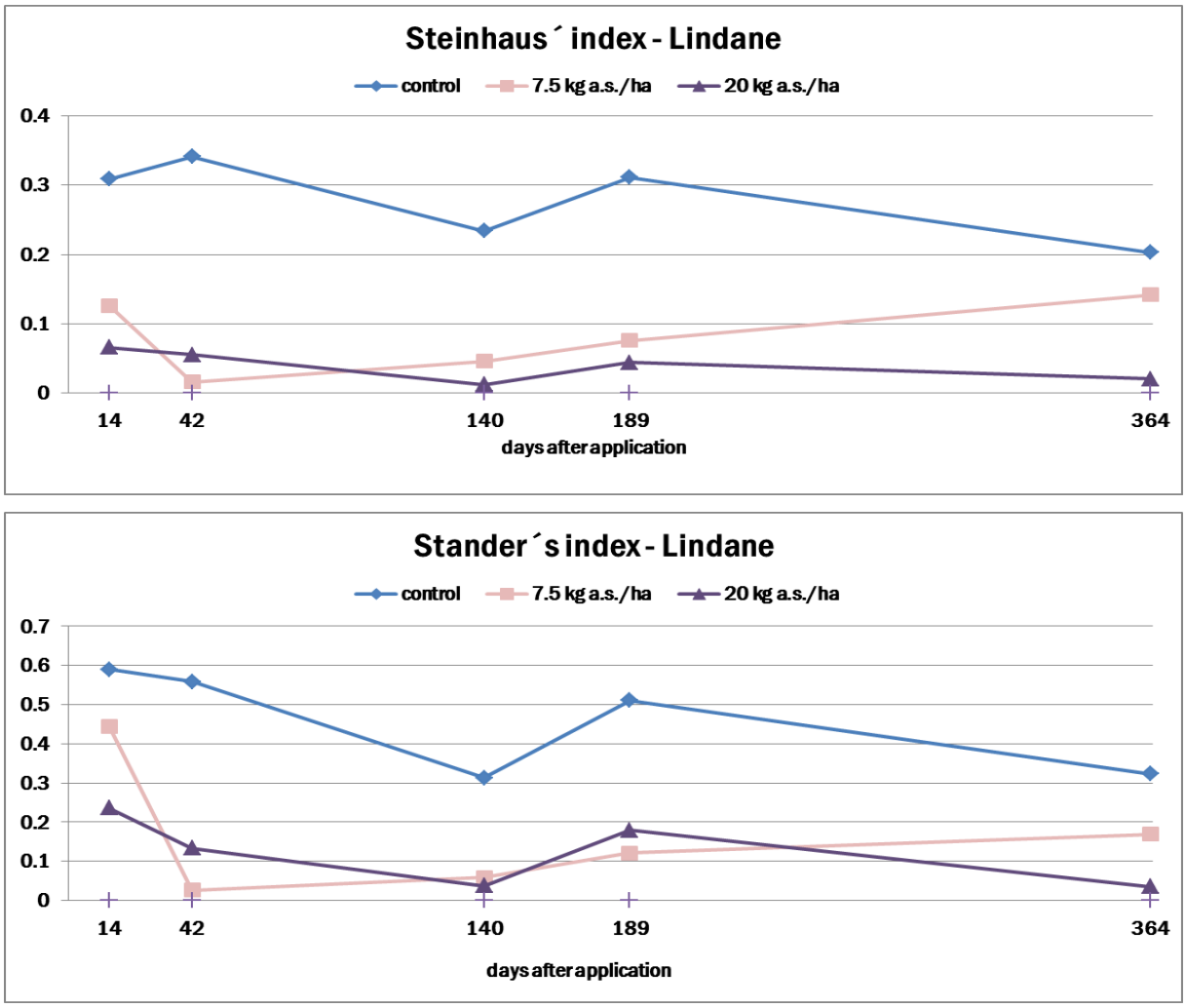

Figure 41 Similarity of Collembolan diversity summed up over soil layers A-C (above) in the different treatment of TMEs in study [1]. Steinhaus index (below) Standers index. Calculation is based on 10 replicates for the control and 5 replicates for each treatment.

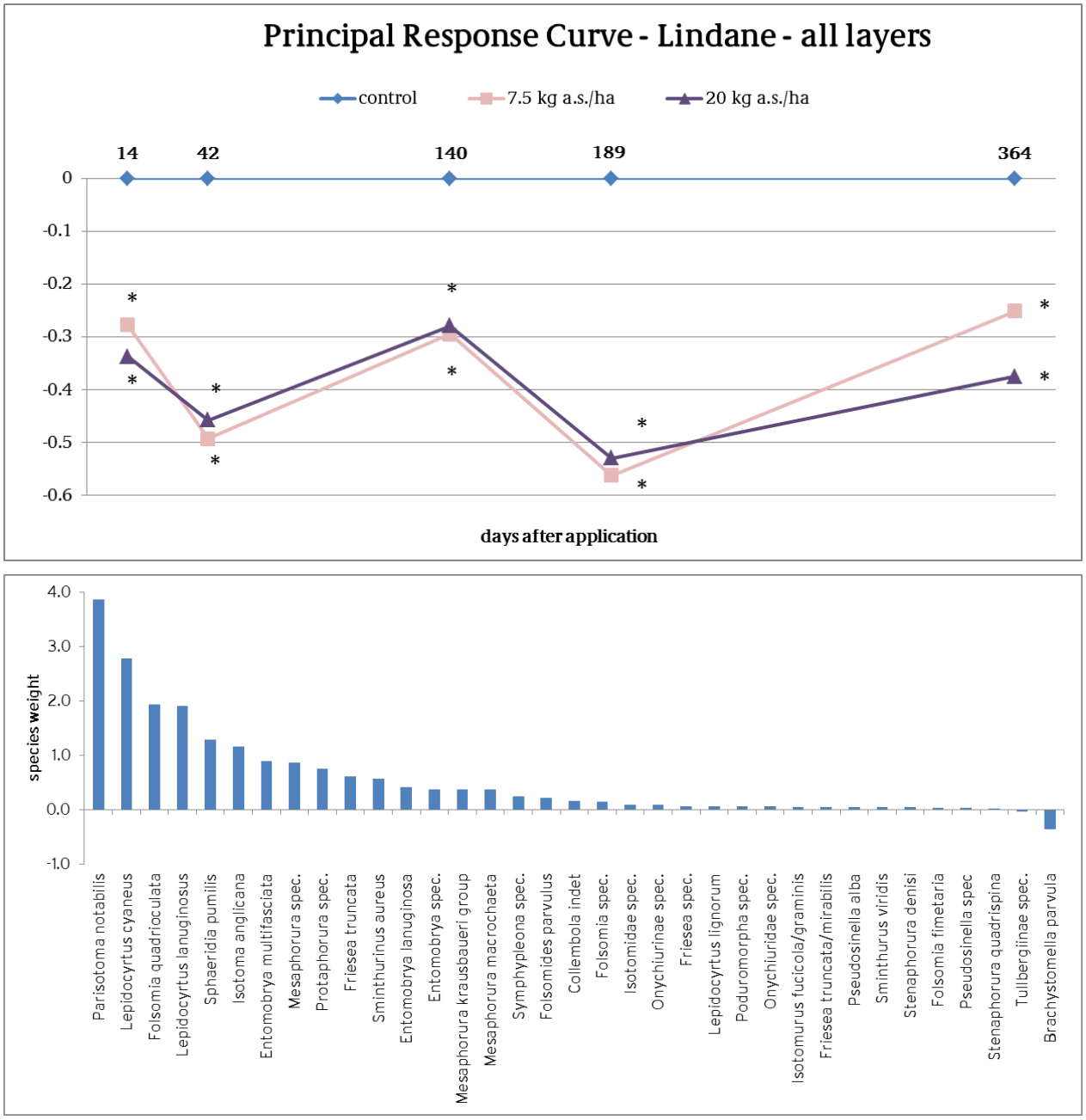

Figure 42 Principal response curves (PRC) of the collembolan community after application of Lindane calculated for the sum of all layers (layer A, B & C) in the TMEs of study [1]. Results of all collembolan species, mean of 10 replicates for controls, 5 replicates for the treatments; *: significant effects measured by sample scores of the PCA for the single sampling date. Species weights indicating the share of difference contributed by the different species.

4.4.3.2 Effects on oribatid mites

Oribatid mites were investigated in three different layers A (0-2.5 cm), B (2.5-5 cm) and C (5-10 cm). Because of the reduced mobility of oribatid mites, the captures by means of pitfall traps were not sufficient to analyse those species. Oribatid mites were recorded mainly in the upper layer A (91 %, 7 % in layer B, 2 % in layer C). In the controls, the mean number of individuals in layer A increased over time from 6 ind. at day 14 to 10 ind. (day 42) up to more than 20 ind. at day 189. The abundance of oribatid mites was affected by the treatment of Lindane at both application rates (Figure 43, Table 41). On the first sampling, the abundance of oribatid mites decreased by 67 % for both application rates regarding all layers. On this sampling date, the decrease was statistically significant for all layers and for layer A for both application rates. The reason for the lack of statistically significant effects at the two lower layers was presumably the high variance of the control TMEs for this sampling date. The reduction of abundance of oribatid mites increased over time (day 42, day 140) to more than 95 % in the higher application rate (only one individual could be recorded at day 189 here), so the reduction increased close to 100 %. After 1 yr., the reduction of the population size was still about 90 % (95 % layer A; 100 % layer C). The arising effects reflect a dose related dependency. Because of the low abundances of oribatid mites in layer B and C even in the controls, no statistically significant effects were detected on any sampling date - except at day 42 for the higher application rate in layer B. At days 42 and 140, no oribatid mite individuals were recorded in the layer C of the controls.

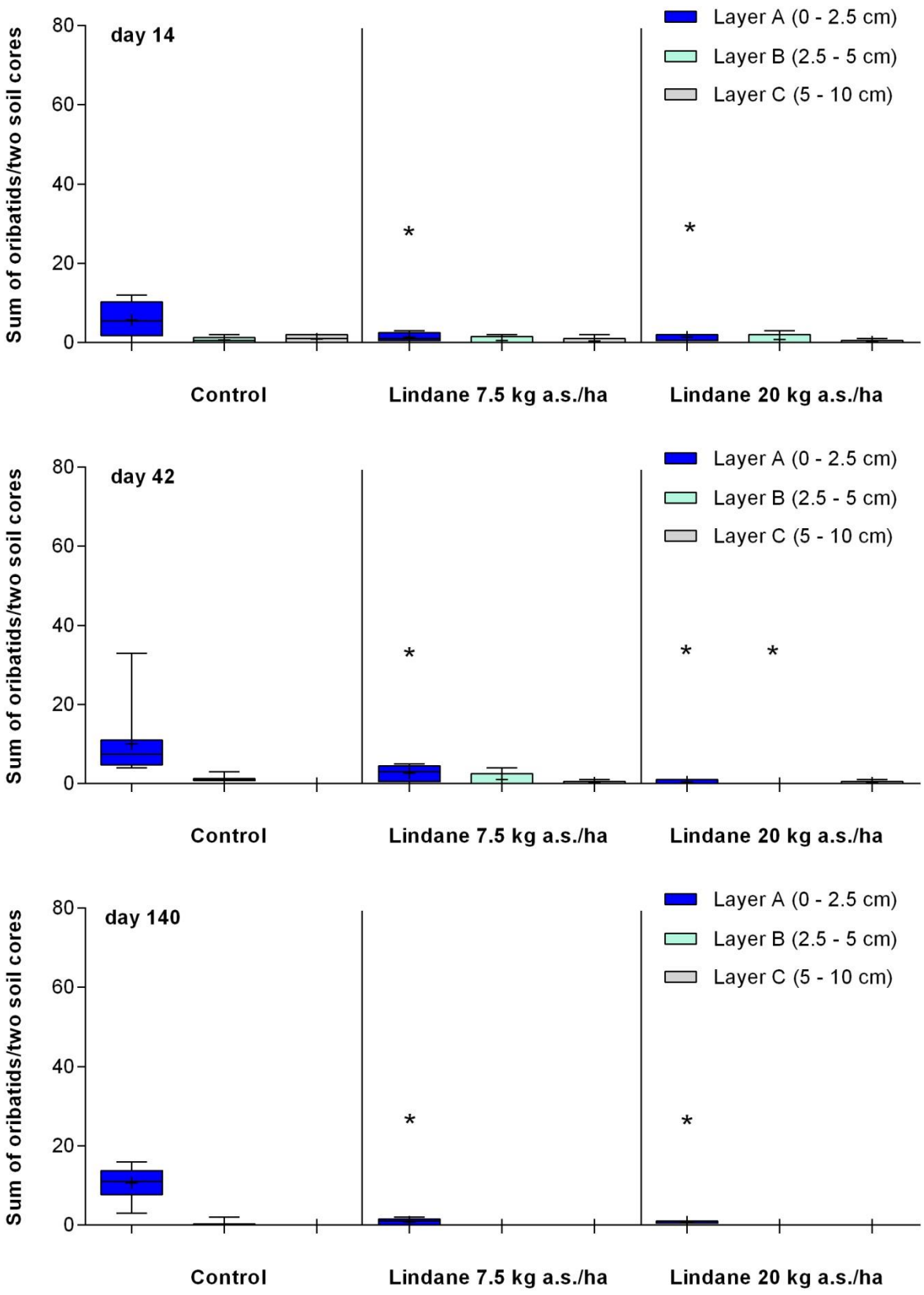

Figure 43 caption see below

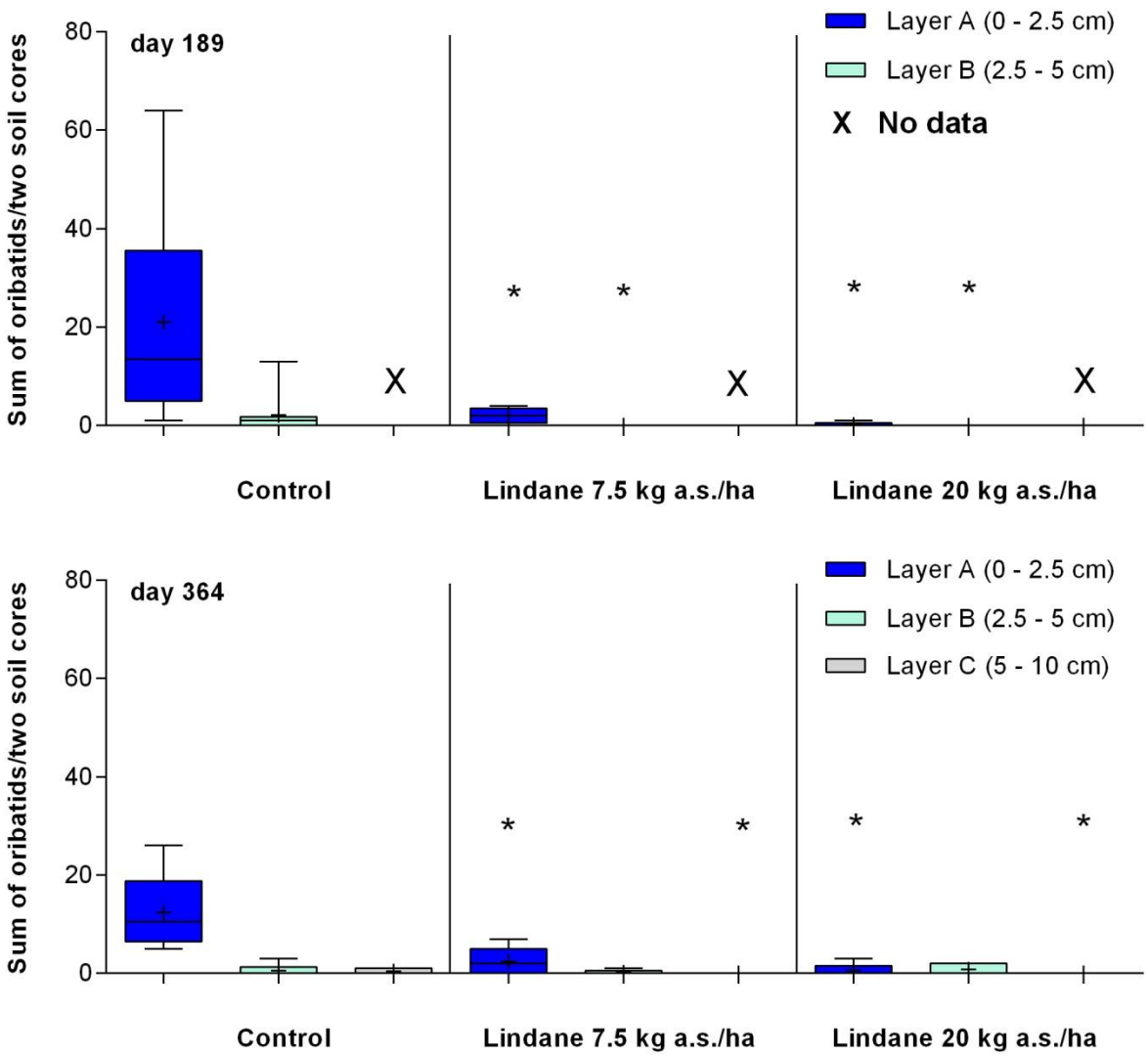

Figure 43 Total abundance of oribatid mites in the soil layers of the TMEs in study [1] (10 replicates for control; 5 replicates for lindane treatments). X: no data; cross: mean; *: significance (p-value Williams-test < 0.05). Sampling dates at day 14-364 from top to bottom

Table 41 Decrease of total abundance [%] of oribatid mite species in the different soil layers on different sampling dates of TME study [1], 14-364 days after application of Lindane. Red: decrease more than 50% in comparison to the control; grey: less than 50% decrease in comparison to the control; X: data not available; Blank fields: data not sufficient for statistical calculation; Bold: significant effects (p-value <0.05, Mann-Whitney Rank Sum); calculation is based on 10 replicates for the control and 5 replicates for the respective treatment.

Lindane 7.5 kg a.s./ha							Lindane 20 kg a.s./ha						
		days after application							days after application				
	layer	14	42	140	189	364			14	42	140	189	364
A	0 - 2.5 cm	**75**	**74**	**93**	**90**	**81**	A	0 - 2.5 cm	**75**	**96**	**93**	**99**	**95**
B	2.5 - 5 cm	14	9	100	**100**	67	B	2.5 - 5 cm	-14	**100**	100	**100**	-33
C	5 - 10 cm	56			X	**100**	C	5 - 10 cm	78			X	**100**
	all layers	**67**	**66**	**93**	**91**	**81**		all layers	**67**	**95**	**93**	**99**	**90**

The structure of the oribatid mite community in the TME soil from a meadow site is highly dominated by one species, *Liebstadia similis*, while others are only present in low numbers. Nevertheless, the community is clearly affected by the Lindane treatments (Table 42). 15 species were recorded within the control, while only 7 (5 for each application rate) were captured within both treatments. Two species could only be recorded within the treatments (*Oppiella falcata* in soil cores, *Scutovertex minutus* in pitfall traps). Another two species, *Scheloribates laevigatus* and *Ramusella clavipectinata* were recorded with higher numbers in the treatments than in the control.

Table 42 Presence and mean abundance of captured oribatid mite species in the control TMEs (10 replicates) and the two different application rates of the treatment TMEs (5 replicates). The Treatment mean is calculated as the mean abundance of oribatid mite species in all treatment TMEs (10 replicates), 5 with higher and 5 with lower pesticide rate.

	Control		**Lindane**	
		Treatment mean	*7.5 kg a.s./ha*	*20 kg a.s./ha*
Number of replicates	**10**	**10**	**5**	**5**
Soil cores				
Liebstadia similis	47.5	4.2	5.4	3.0
Juveniles	12.1	1.4	2.2	0.6
Eupelops occultus	2.6	-	-	-
Banksinoma lanceolata	0.7	-	-	-
Mnunthozetes semirufus	0.5	0.1	0.2	-
Ramusella clavipectinata	0.5	0.8	1.0	0.6
Scheloribates laevigatus	0.5	1.8	2.6	1.0
Galumna obvia	0.4	-	-	-
Pantelozetes paolii	0.2	-	-	-
Achipteria coleoptrata	0.1	-	-	-
Gustavia microcephala	0.1	-	-	-
Oppiella (Oppiella) nova	0.1	-	-	-
Punctoribates punctum	0.1	0.1	-	0.2
Scheloribates latipes	0.1	-	-	-
Suctobelba spec.	0.1	-	-	-
Tectocepheus velatus	0.1	-	-	-
Oppiella (Oppiella) falcata	-	0.1	0.2	-
Pitfall traps				
Scutovertex minutus	-	x	-	x
Number of all taxa	**15**	**7**	**5**	**5**

In the statistical analysis on the population level, for 3 out of 19 species (cp. appendix 1) significant effects were detected for at least one single date and soil layer. As already mentioned above, the statistical power for the data on oribatid mites in deeper soil layers was mostly low due to the naturally low numbers in those layers. According to that, it can be assumed that significant reductions of numbers of individuals for single species in deeper single layers are hard to detect.

When focussing on single species, it must be considered that the relation of dominance for the different species is strongly askew (cp. Figure 34). The community was strongly dominated by one species, *Liebstadia similis,* with more than 75 % of all individuals. Additionally, a main part of the juveniles representing more than 12 % of the individuals belong probably to this species. *Liebstadia similis* is according to the findings a hemiedaphic/epigeic species (Figure 44) which was significantly affected in layer A at day 42 right up to 1 year post application. Because of very low numbers of this species in deeper soil layers (especially in layer C), the decrease of abundance in these layers - even when being significant - must be interpreted with care (chapter 4.4.1). The rate of false positive effects could be increased. However the strong decrease in layer B on all sampling dates except of day 364 of the higher application rate in comparison to the findings for Imidacloprid (see below) leads to the conclusion that effects especially for layer B can be assumed. According to Table 43 below, effects of Lindane on Liebstadia similis were above 80 % in the treatment with 7.5 kg a.s./ha, with exception of 42 d after treatment, where the effects were 60 %. In the treatment with 20 kg as/ha, effects at day 14 after treatment were approx 70 % and increased over time.

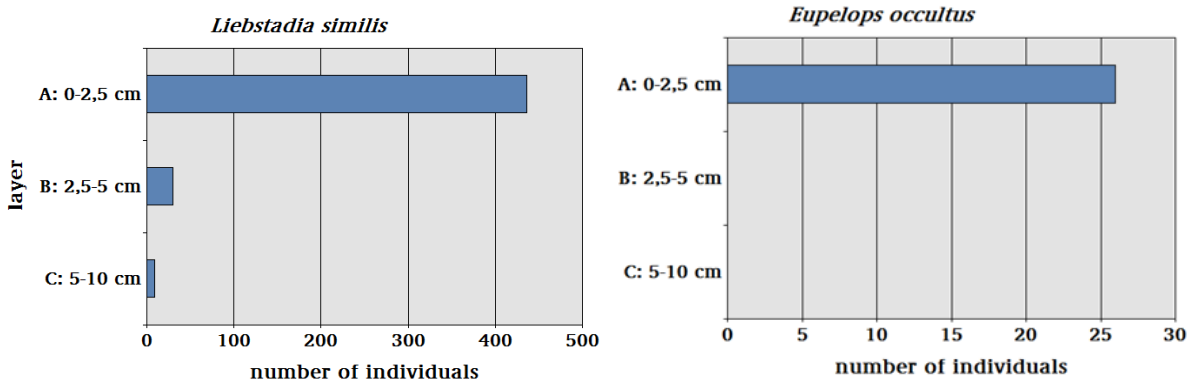

Figure 44 Vertical distribution of the oribatid mite species *Liebstadia similis and Eupelops occultus* in the control TMEs of study [1]. Shown is the total number of individuals captured in all soil cores on all sampling dates.

The second typical species for grassland communities among oribatid mites is *Eupelops occultus* that can be classified according to our findings as hemiedaphic/epigeic species (Figure 44). This species was recorded only from day 42 to day 364 in the upper layer A. During this time significant effects occur for both application rates, i.e. the species was absent from both treatment soils and in all layers (see Table 42, Table 43).

Ramusella clavipectinata was a species that occurred only at a few sampling dates during the study in very few numbers (Figure 45). It was only recorded once in the controls at day 42 with a sum of four individuals and then was detected as significantly reduced in both application rates.

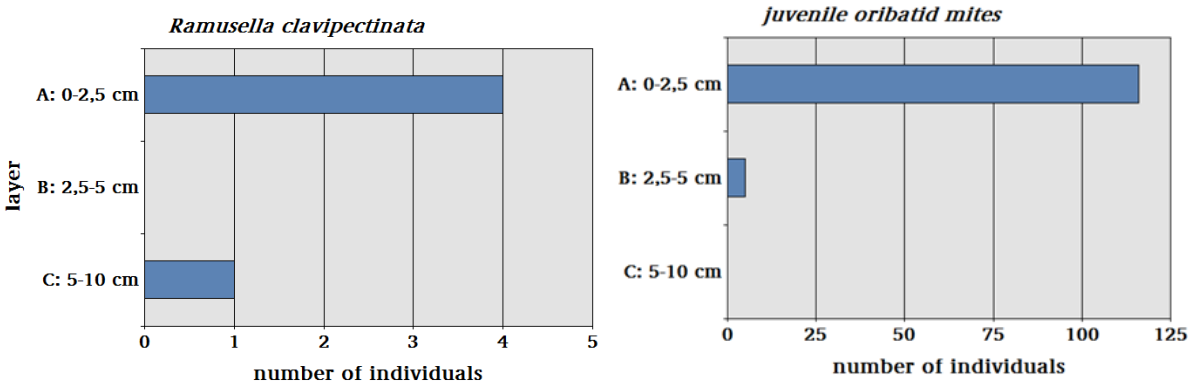

Figure 45 Vertical distribution of the oribatid mite species *Ramusella clavipectinata* and juvenile oribatid mites in the control. Shown is the total number of individuals captured in all soil cores on all sampling dates.

Juvenile oribatid mites were recorded mainly in layer A (Figure 45). Only few individuals were recorded in layer B and there were no records in layer C. The juvenile oribatid mites were statistically significantly reduced in layer A and when considering all layers together. Significant differences could be measured in layer A at day 42, 189 and 364 and for all layers at day 189 and day 364 for both application rate (Table 43). Numbers of juvenile oribatid mites were reduced by approx 75 % at first sampling date in the treatment with 7.5 kg Lindane/ha. Effects increased in strength during the experiment. This was also observed in the treatment with 20 kg Lindane /ha, whereby the initial effects already reached 88% reduction compared to the control in layer A.

When comparing the results of different endpoints of diversity, the multivariate community approach is most significant (Table 44). Except for day 42 for the lower application rate, all results show significant reductions due to the application of Lindane. The number of taxa is significantly different for day 42, day 189 and day 364 for both application rates. Shannon and Evenness were indicating effects for the higher application rate from day 42 to day 364. The picture for the lower application rate shows no consistent pattern.

The similarity of the oribatid mite communities for all layers measured with Steinhaus´ and Stander´s indices is presented in Figure 46. The curves are reflecting dose-related dependencies of effects except of sampling at day 14. The response of both indices showed a similar pattern for both Lindane application rates. The similarity of the treated communities was on all sampling dates, except of day 42 for the lower application rate, clearly different to control.

The PRC summarizing all sampling dates derived significant treatment effects when summing up all layers (p-value 0.005, Monte Carlo test). The subsequent PCA analysis indicated a significant effect on the community on all sampling dates and both application rates except of day 42 (p-value 0.052, t-test) for the lower application rate (Figure 47). Except of day 14 the curves were dose related with the strongest effects at day 189. According to the dominance distribution within the oribatid mite community, the highest share of these effects is referred to *Liebstadia similis*, juveniles oribatid mites and the species *Eupelops occultus*. The population of *Scheloribates laevigatus* showed an opposite pattern, i.e. no reductive effect of Lindane treatment on the community.

In summary, the effect of Lindane on oribatid mites was significant for all sampling dates when considering all layers. The sum of all layers is very similar to the findings for layer A, because by

far most of the oribatid mites did occur in the uppermost soil layer. Mainly affected were *Liebstadia similis*, *Eupelops occultus* and the juvenile oribatid mites which represented more than 90 % of the total number of oribatid mites.

Table 43 Summary of statistical analysis of four different oribatid mite species in the soil layers of TME study [1]. Results are given for the different soil layers (A-C) and different sampling dates (14 days-364 days after application). Red: decrease of abundance ≥ 50% Grey: decrease of abundance < 50% X: no data available. Blank fields: data not sufficient for statistical calculation. Bold: significant difference (p-value Williams-test < 0.05); (see also appendix 1).

Lindane 7.5 kg a.s./ha		*Liebstadia similis* days after application				
layer		14	42	140	189	364
A	0 - 2.5 cm	85	**60**	**92**	**98**	**84**
B	2.5 - 5 cm	100	60	100	100	100
C	5 - 10 cm	100			X	**100**
all layers		87	**60**	**92**	**98**	**86**

Lindane 20 kg a.s./ha		*Liebstadia similis* days after application				
layer		14	42	140	189	364
A	0 - 2.5 cm	69	**96**	**94**	**99**	**100**
B	2.5 - 5 cm	100	**100**	100	100	0
C	5 - 10 cm	60			X	**100**
all layers		70	**93**	**94**	**99**	**96**

Lindane 7.5 kg a.s./ha		*Eupelops occultus* days after application				
layer		14	42	140	189	364
A	0 - 2.5 cm		100	**100**	**100**	**100**
B	2.5 - 5 cm					
C	5 - 10 cm				X	
all layers			100	**100**	**100**	**100**

Lindane 20 kg a.s./ha		*Eupelops occultus* days after application				
layer		14	42	140	189	364
A	0 - 2.5 cm		100	**100**	**100**	**100**
B	2.5 - 5 cm					
C	5 - 10 cm				X	
all layers			100	**100**	**100**	**100**

Lindane 7.5 kg a.s./ha		*Ramusella clavipectinata* days after application				
layer		14	42	140	189	364
A	0 - 2.5 cm		**100**			
B	2.5 - 5 cm					
C	5 - 10 cm	-300			X	
all layers		-700	50			

Lindane 20 kg a.s./ha		*Ramusella clavipectinata* days after application				
layer		14	42	140	189	364
A	0 - 2.5 cm		**100**			
B	2.5 - 5 cm					
C	5 - 10 cm	100			X	
all layers		-500	100			

Lindane 7.5 kg a.s./ha		*Juveniles* days after application				
layer		14	42	140	189	364
A	0 - 2.5 cm	75	**89**	100	**100**	**85**
B	2.5 - 5 cm		-100	100		
C	5 - 10 cm				X	
all layers		63	71	100	**100**	**85**

Lindane 20 kg a.s./ha		*Juveniles* days after application				
layer		14	42	140	189	364
A	0 - 2.5 cm	88	**95**	100	**100**	**100**
B	2.5 - 5 cm		100	100		
C	5 - 10 cm				X	
all layers		88	**95**	100	**100**	**93**

Table 44 Summary of the results for the statistical diversity analyses, PRC (p-value t-test < 0.05 of PCA sample scores), number of taxa, Shannon and Eveness, *: significance (p-value Williams-test < 0.05) of oribatid mites treated with Lindane (left) application rate of 7.5 kg a.s./ha (right) 20 kg a.s./ha. Database: 10 replicates of control TMEs and 5 replicates for each treatment in study [1].

Lindane 7.5 kg a.s./ha	days after application				
all layers	14	42	140	189	364
PRC	*		*	*	*
Number of taxa		*		*	*
Shannon			*	*	
Evenness			*		

Lindane 20 kg a.s./ha	days after application				
all layers	14	42	140	189	364
PRC	*	*	*	*	*
Number of taxa		*		*	*
Shannon		*	*	*	*
Evenness		*	*	*	*

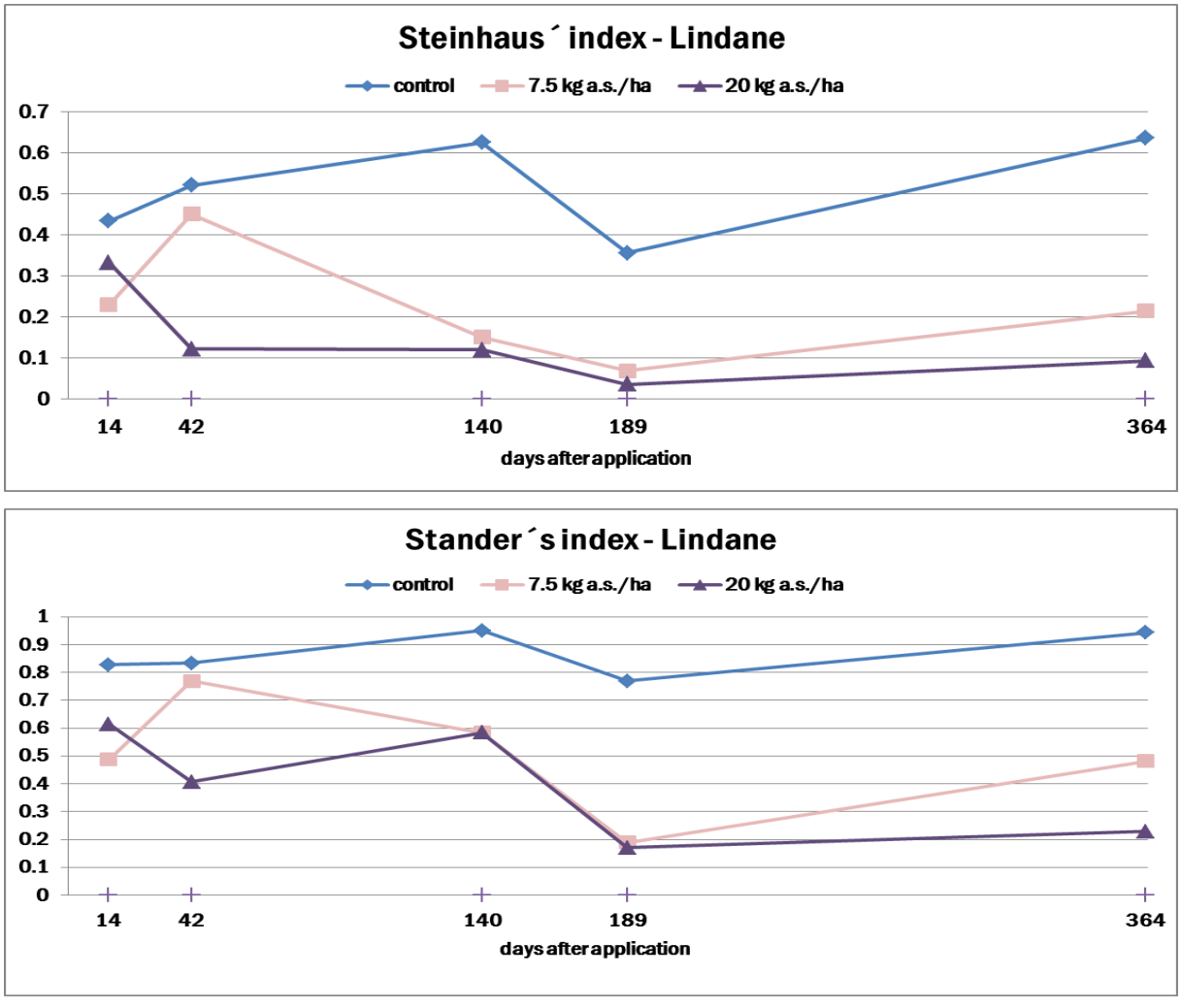

Figure 46 Similarity of oribatid mite diversity summed up over soil layers A-C (above) Steinhaus index (below) Standers index. Calculation is based on 10 replicates for the control and 5 replicates for each treatment.

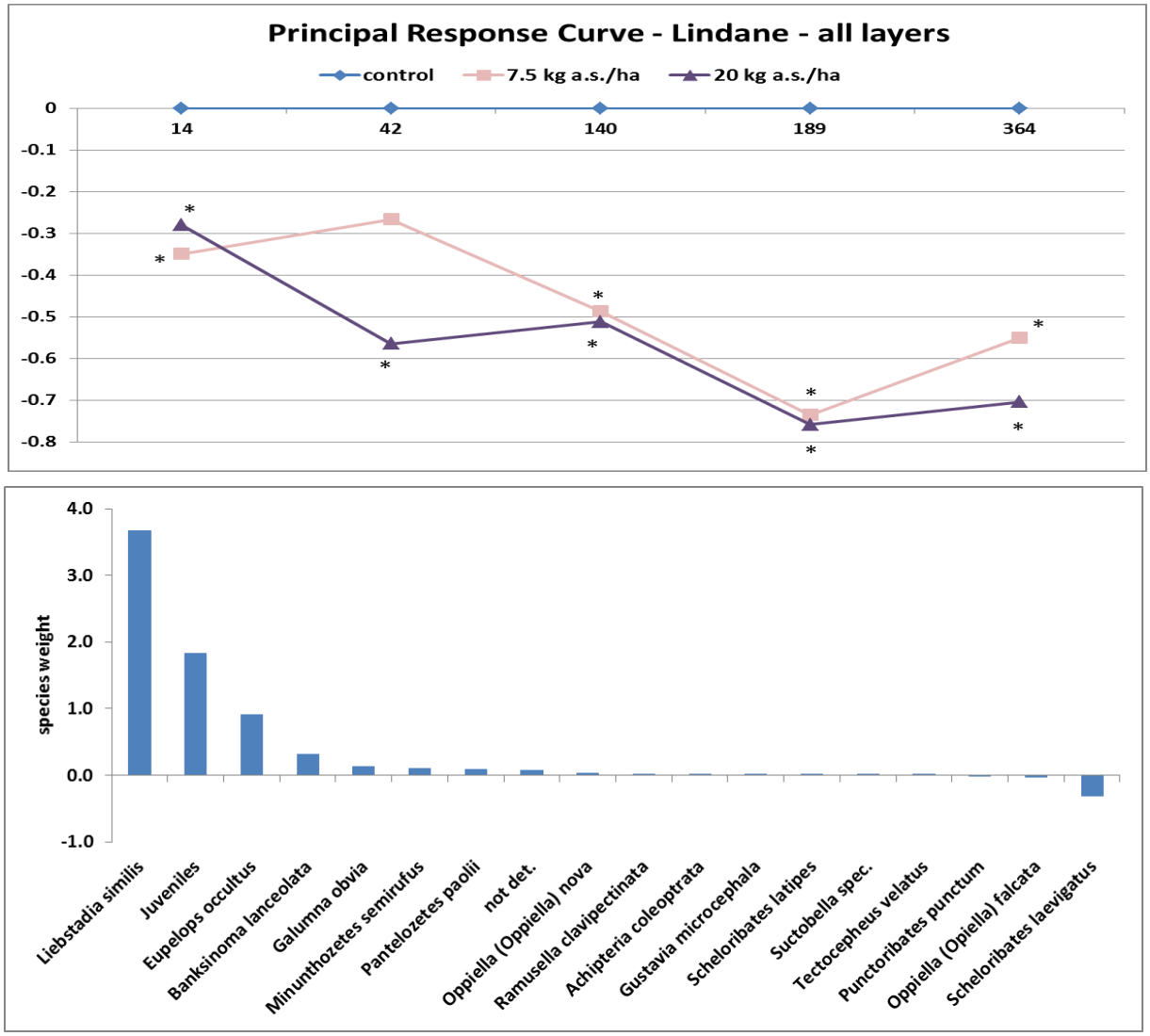

Figure 47 Principal Response Curves of the oribatid community in the different TME treatments after the application of Lindane calculated for all layers (layer A, B & C) Results for all oribatid mite species, mean of 10 replicates for controls, 5 replicates for treatments; *: significant effects measured by sample scores of the PCA for the single sampling date. Species weights indicating the share of difference for the different species

4.4.3.3 Effects on enchytraeidae

The enchytraeid abundance was significantly affected by Lindane (Figure 48, Table 45), although not in an expected manner. On the first sampling date, their number increased in both Lindane treatments, especially in layer A – something which happened again on the next sampling date at day 42, this time also in layer B and in layer C for the higher application rate. On the third sampling date at day 140, such an increase was observed too, but only in the higher Lindane treatment. The effect occurred in the two upper soil layers. On the fourth sampling date, control numbers were higher than those in the two Lindane treatments. On the last sampling date, there was no difference in all three layers. No effects could be observed in layer C on all sampling dates. Variability was high on some dates and treatments but not in general.

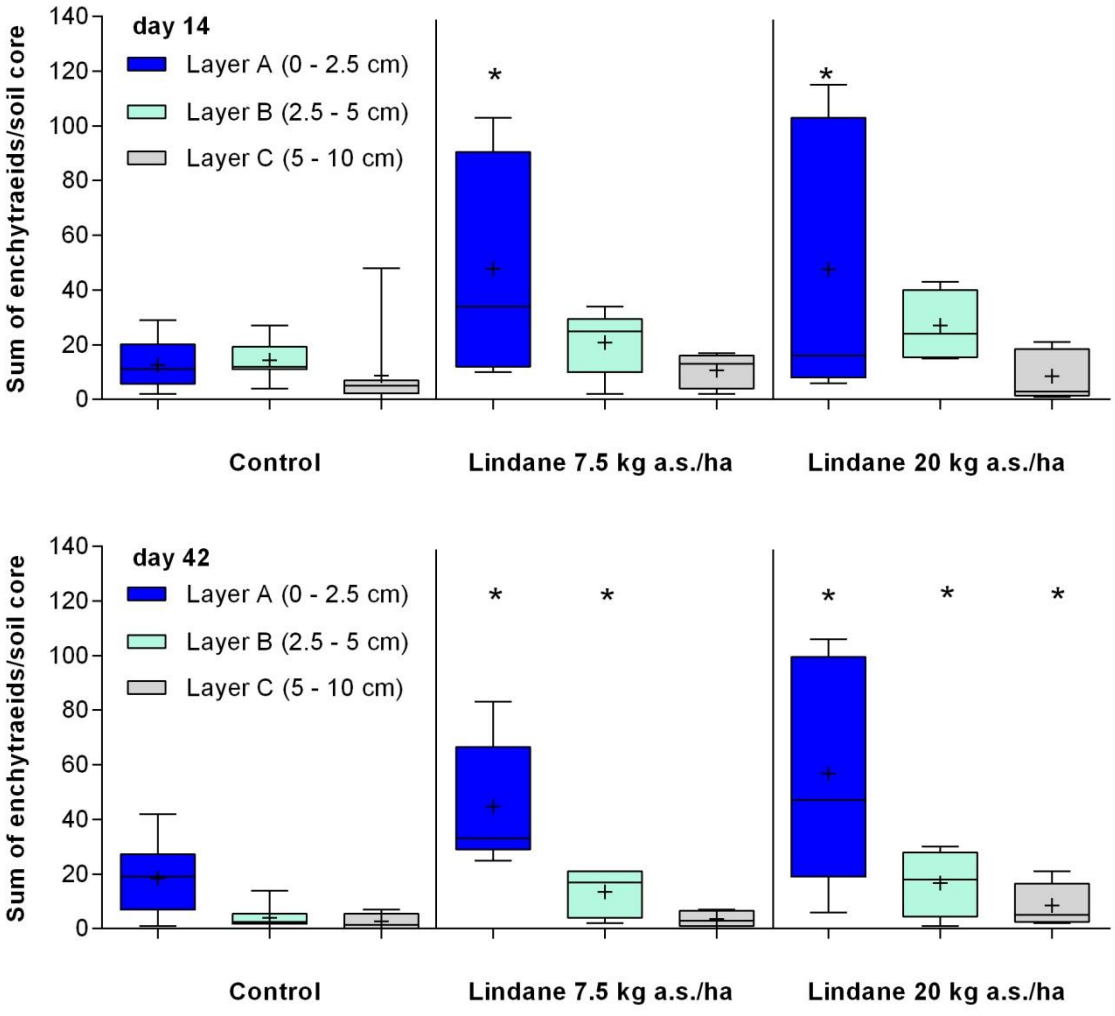

Figure 48 caption see below

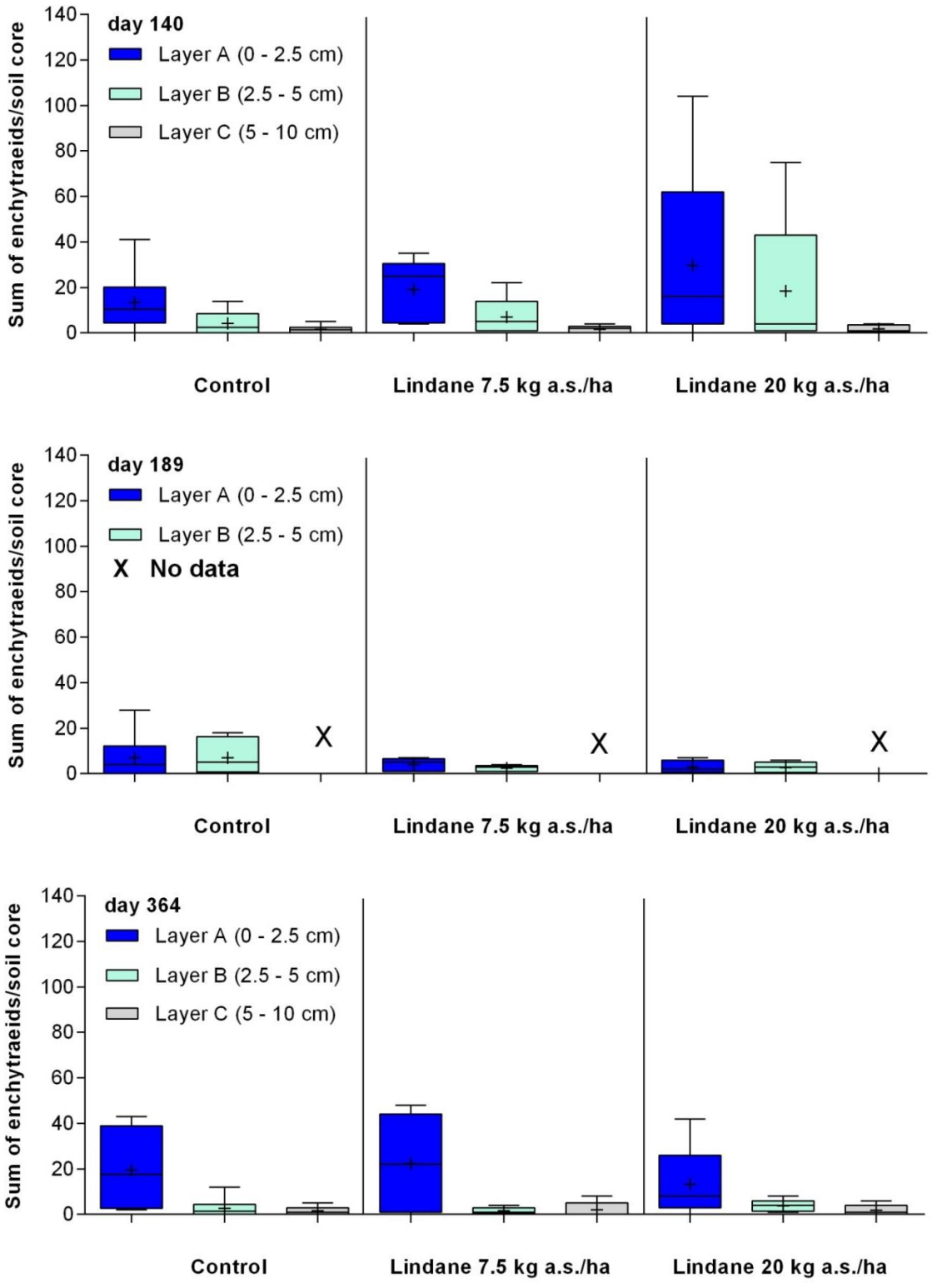

Figure 48 Total abundance of enchytraeids in the soil layers of the TMEs in study [1] (10 replicates for control; 5 replicates for lindane treatments). X: no data; cross: mean; *: significance (p-value Williams-test < 0.05). Sampling dates on day 14-364 from top to bottom

Table 45 Decrease of total abundance [%] of enchytraeid species in the different soil layers on different sampling dates of study [1], 14-364 days after application of Lindane in the TMEs. Red: decrease in abundance of more than 50% in comparison to the control; grey: less than 50% decrease in comparison to the control; X: no data available; Bold: significant effects (p-value <0.05, Mann-Whitney Rank Sum); calculation is based on 10 replicates for the control and 5 replicates for the respective treatment.

Lindane 7.5 kg a.s./ha

		days after application				
	layer	14	42	140	189	364
A	0 - 2.5 cm	**-276**	**-142**	-42	42	-14
B	2.5 - 5 cm	-45	**-235**	-71	66	43
C	5 - 10 cm	-22	-33	6	X	-25
	all layers	-122	**-145**	-44	54	-8

Lindane 20 kg a.s./ha

		days after application				
	layer	14	42	140	189	364
A	0 - 2.5 cm	**-275**	**-207**	-121	57	33
B	2.5 - 5 cm	-89	**-315**	-349	60	-36
C	5 - 10 cm	1	**-219**	-6	X	-13
	all layers	**-133**	**-225**	-159	58	22

Table 46 Presence and mean abundance of captured enchytraeid species in the control TMEs (10 replicates) and the two different application rates of the treatment TMEs (5 replicates) in study [1]. The Treatment mean is calculated as the mean abundance of enchytraeid species in all treatment TMEs (10 replicates), 5 with higher and 5 with lower pesticide rate.

	Control		**Lindane**	
		Treatment mean	*7.5 kg a.s./ha*	*20 kg a.s./ha*
Number of replicates	**10**	**10**	**5**	**5**
Soil cores				
Archaeta "dzwilloi"	26.2	10.10	88.2	113.8
Fridericia connata	22.7	22.1	25.4	18.8
Fridericia ulrikae	8.5	8.2	8.6	7.8
Enchytronia parva s.l.	8.4	2.10	18.4	23.6
Enchytraeus sp. PALE	7.7	12.1	8.0	16.2
Fridericia galba	7.5	8.7	8.2	9.2
Fridericia bulboides	5.8	6.0	5.4	6.6
Fridericia paroniana	4.8	3.2	1.8	4.6
Fridericia bisetosa	4.3	5.3	6.0	4.6
Enchytraeus sp. GRAN	4.1	8.8	8.4	9.2
Buchholzia appendiculata	3.5	5.8	4.0	7.6
Fridericia sylvatica	2.5	1.5	1.4	1.6
Henlea perpusilla	2.0	3.4	3.6	3.2
Marionina communis	1.7	1.6	1.8	1.4
Fridericia benti	1.5	0.7	0.6	0.8
Fridericia dura cf.	0.9	2.0	2.4	1.6
Enchytraeus norvegicus	0.1	0.9	0.6	1.2
Cognettia glandulosa	-	0.2	0.2	0.2
Number of taxa	17	18	18	18

Regarding the presence of species and enchytraeid community, no difference can be observed (Table 46), except of *Cognettia glandulosa* which only could be captured within the treatments

with low numbers, the species are evenly distributed. However, abundance were different between control TMEs and TMEs treated with Lindane (see figures above)

In the statistical analysis on the population level for 4 out of 18 species (appendix 1), significant effects could be detected in a few cases. Actually, effects of both application rates were observed mostly in layer B, less often when combining all soil layers. Regarding sampling dates, in five out of six cases such effects were observed on the first sampling date (day 14). An impact only of the higher application rate was detected at day 42, 140 and 364 in individual or all soil layers. When trying to identify a similar pattern of significant effects on different species it seems there is no such pattern. However, species that could be analysed further due to sufficient abundances showed an increase in individual densities, as was seen when analyzing the total enchytraeids abundance.

The species *Achaeta "dzwilloi"* has not been classified into one of the three ecological groups, but it is quite likely that it is a mineral dweller (most of the species of this genus prefer deeper layers). In this study it was predominantly found in layer A with 51 % (layer B 28 %; layer C 21 %, Figure 49). It has been affected significantly twice (day 14 and 189) at both application rates and once (day 42) only at the higher application rate (Table 47). The first two impacts occurred in soil layer B, the latter in in soil layer C at day 42. Interestingly, at day 14 there was also a significant difference when putting the enchytraeids of all three layers together, since this species were most abundant in the TMEs.

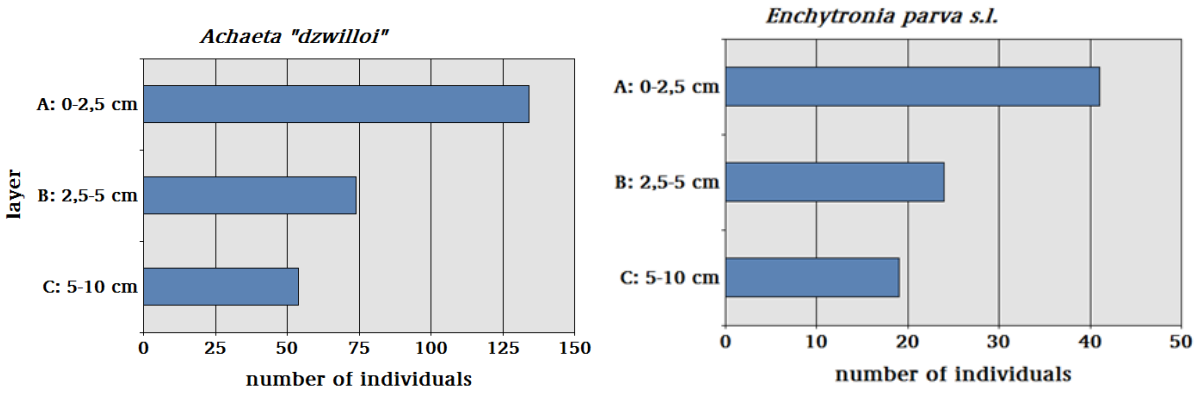

Figure 49 Vertical distribution of the enchytraeid species *Achaeta "dzwilloi"* and *Enchytronia parva s.l.* in the control. Shown is the total number of individuals captured in all soil cores on all sampling dates.

The other mineral dweller selected for this evaluation, *Enchytronia parva* s.l., is probably a mixture of several closely related species. Within this study it was found with 49 % of individuals in layer A (layer B 29 %; layer C 23 %, Figure 49). Besides significant effects in soil layer B at day 14 such differences were also observed in layers A and B at day 140 and in soil layer C at day 364 (Table 47).

The intermediate species *Enchytraeus* sp. GRAN is also a mixture of several small species, some for them already described with an own name, others not. Morphologically they are (almost) not distinguishable, but genetic characterizations are not (yet) done in a way that individual species could be clearly separated. In this study it was also predominantly found in layer A with 61 % (layer B 27 %; layer C 12 %, Figure 50). *Enchytraeus* sp. GRAN was statistically significantly affected at day 42 and 140 in soil layer A and when taking all layers together (Table 47). This pattern does

not give a hint whether the different species belonging to this “bunch” do have different sensitivities towards Lindane.

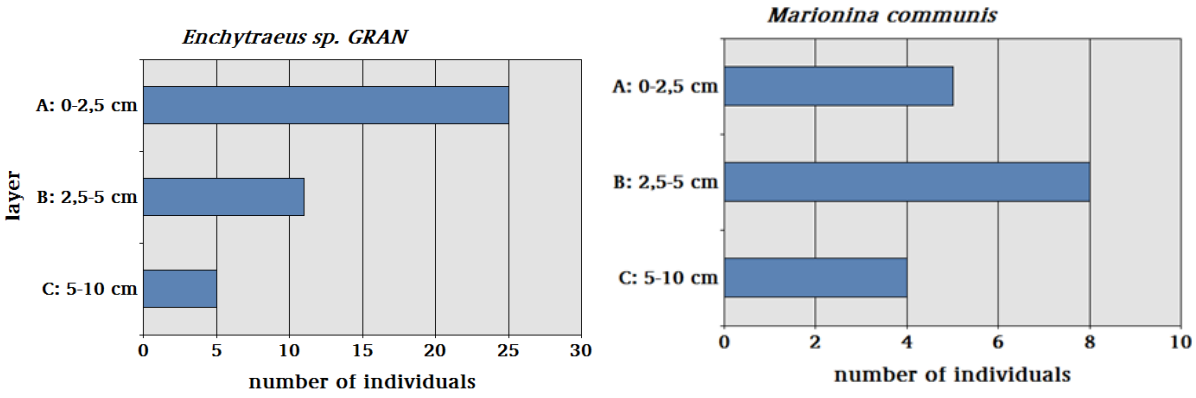

Figure 50 Vertical distribution of the enchytraeid species *Enchytraeus sp. GRAN* and *Marionina communis* in the control. Shown is the total number of individuals captured in all soil cores on all sampling dates.

Finally, effects on the litter dweller *Marionina communis* were studied in the same way. Despite its preference for the soil surface described by literature, within this study the species occurs in the whole upper soil column from 0-10 cm (layer A 29 % layer B 47 %; layer C 23 %, Figure 50) This small species has often been overlooked in enchytraeid sampling programs, but it seems that it is not really rare in Central and Northern Europe. The population was significantly affected in soil layer C at day 14 for both application rates (Table 47). In the lower application rate, the population decreased by 100 % afterwards. However, the numbers were to small in this case to draw further conclusions from the effect pattern.

The analyses of endpoints related to the diversity of the enchytraeids community like PRC, number of species. evenness and Shannon index showed no significant effect of Lindane for any sampling date.

The similarity of the enchytraeid communities measured with Steinhaus´ and Stander´s indices is presented for all layers in Figure 51 and shows no differences between control and one of the two treatments. The control curves and those of the treatments were at the same level on all sampling dates beside of very small differences (e.g. Stander´s at day 42).

Table 47 Summary of statistical analysis of four different enchytraeid species in the TMEs of study [1]. Results are given for the different soil layers (A-C) and different sampling dates (14 days-364 days after application). Red: decrease of abundance ≥ 50% Grey: decrease of abundance < 50% X: no data available. Blank fields: data not sufficient for statistical calculation. Bold: significant difference (p-value Williams-test < 0.05); (see also appendix 1).

Lindane 7.5 kg a.s./ha		*Achaeta "dzwilloi"* days after application				
layer		14	42	140	189	364
A	0 - 2.5 cm	-474	-332	-411	-33	-143
B	2.5 - 5 cm	**-384**	-700	-267	**100**	100
C	5 - 10 cm	64	-100	-50	X	50
all layers		**-256**	-372	-318	67	-16

Lindane 20 kg a.s./ha		*Achaeta "dzwilloi"* days after application				
layer		14	42	140	189	364
A	0 - 2.5 cm	-478	-400	-622	33	-329
B	2.5 - 5 cm	**-500**	-650	-1067	**100**	-8
C	5 - 10 cm	3	**-271**	-50	X	100
all layers		**-302**	-430	-712	83	-139

Lindane 7.5 kg a.s./ha		*Enchytronia parva s.l.* days after application				
layer		14	42	140	189	364
A	0 - 2.5 cm	-1233	-50	-500	100	37
B	2.5 - 5 cm	**-243**	-33		0	60
C	5 - 10 cm	-8	100	33	X	-300
all layers		**-354**	-30	-220	25	28

Lindane 20 kg a.s./ha		*Enchytronia parva s.l.* days after application				
layer		14	42	140	189	364
A	0 - 2.5 cm	-1000	-167	**-900**	100	100
B	2.5 - 5 cm	**-586**	-67	↑	33	20
C	5 - 10 cm	23	-300	100	X	**-1500**
all layers		**-377**	-150	**-660**	50	20

Lindane 7.5 kg a.s./ha		*Enchytraeus sp. GRAN* days after application				
layer		14	42	140	189	364
A	0 - 2.5 cm		-167	-700	-300	-26
B	2.5 - 5 cm	-300		-500	43	
C	5 - 10 cm	100		-300	X	100
all layers		-140	-367	-550	0	-14

Lindane 20 kg a.s./ha		*Enchytraeus sp. GRAN* days after application				
layer		14	42	140	189	364
A	0 - 2.5 cm		**-700**	**-2100**	-300	16
B	2.5 - 5 cm	100		-100	71	
C	5 - 10 cm	100		100	X	100
all layers		100	**-700**	**-1050**	25	24

Lindane 7.5 kg a.s./ha		*Marionina communis* days after application				
layer		14	42	140	189	364
A	0 - 2.5 cm	-700	100		100	
B	2.5 - 5 cm	-33			100	
C	5 - 10 cm	**100**			X	
all layers		-50	100		100	

Lindane 20 kg a.s./ha		*Marionina communis* days after application				
layer		14	42	140	189	364
A	0 - 2.5 cm	-700	100		100	
B	2.5 - 5 cm	100			-20	
C	5 - 10 cm	**100**			X	
all layers		0	100		0	

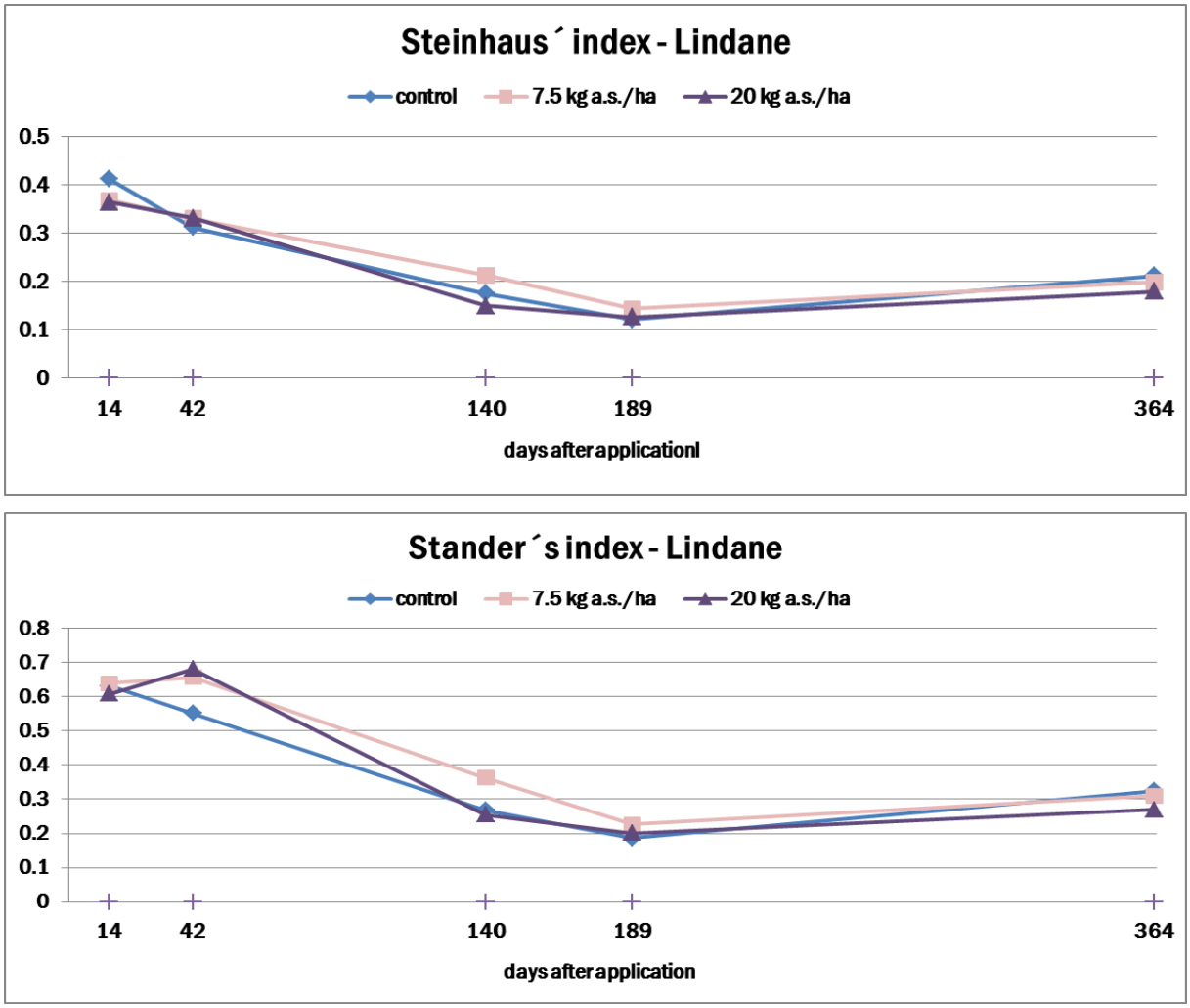

Figure 51 Similarity of enchytraeid diversity in TMEs of study [1] summed up over soil layers A-C (above) Steinhaus index (below) Standers index. Calculation is based on 10 replicates for the control and 5 replicates for each treatment.

In sum, the enchytraeid abundance was not significantly negatively affected by Lindane when considering all layers together. However, on the first sampling date their number increased in both treatments. This increase was observed also at the next sampling dates (day 42 and day 140, at the latter only in the higher treatment). The increase in enchytraeid abundance is possibly caused by a higher amount of available food, since competition by Collembola decreased due to the strong impact of Lindane on these arthropods. Another possibility is that important predators of enchytraeids, e.g. gamasid mites or small beetles, might have been also stronger affected by the insecticide Lindane than the worms. However, both hypotheses cannot be confirmed since neither feeding rates nor the number of predators were assessed in this study. When assessing the effects of Lindane on individual species, significant differences between both treatments and the control could be detected in a few cases, mainly on the first sampling date and for the dominant species *A. "dzwilloi"* and *E. parva* s.l. Despite the fact that significant effects did not occur in all soil layers, the overall difference was significant. Similar patterns could also be observed when assessing the effects of the high Lindane application rate.

4.4.3.4 Effects on earthworms

Lindane did not affect earthworms significantly when analysing them as one group: in one case a significant difference was found in treated TMEs compared to controls (Figure 52, Table 48). On the first sampling date, day 14, their mean numbers were almost similar both in the control and the two application rates. Also the vertical distribution was similar; i.e. the highest numbers were not found close to the surface but in deeper layers: 5 to 10 cm (control) and 2.5 – 5 cm (treatment). Numbers in the control and in the deepest layer (E) were again on the same level. On the second earthworm sampling date at day 140, this pattern changed, i.e. on average more or less similar numbers were found in the three uppermost soil layers and decreased in the deeper layers. On the third earthworm sampling date, there was one significant difference in abundance between control and the high Lindane treatment (t-test; p = 0.03 for layer E), but most worms were found in soil layer A. This pattern seems to indicate a seasonal influence: in May 2011 the uppermost soil was dry due to low precipitation at the coring site, while later on the TMEs were irrigated when dryness occurred. High variability (including the controls) did impact the identification of statistically significant changes in individual numbers.

No differences between the two treatments and the control could be found with regards to the species presence (Table 49). The effect of Lindane on single species is discusses below. The mean abundances for the lower application rate - calculated based on five replicates - were in some cases higher than in the control (e.g. juveniles, *Aporrectodea caliginosa*, *A. rosea*, *Lumbricus castaneus*). The mean abundance for the higher application rate of Lindane was for all species lower than in the control.

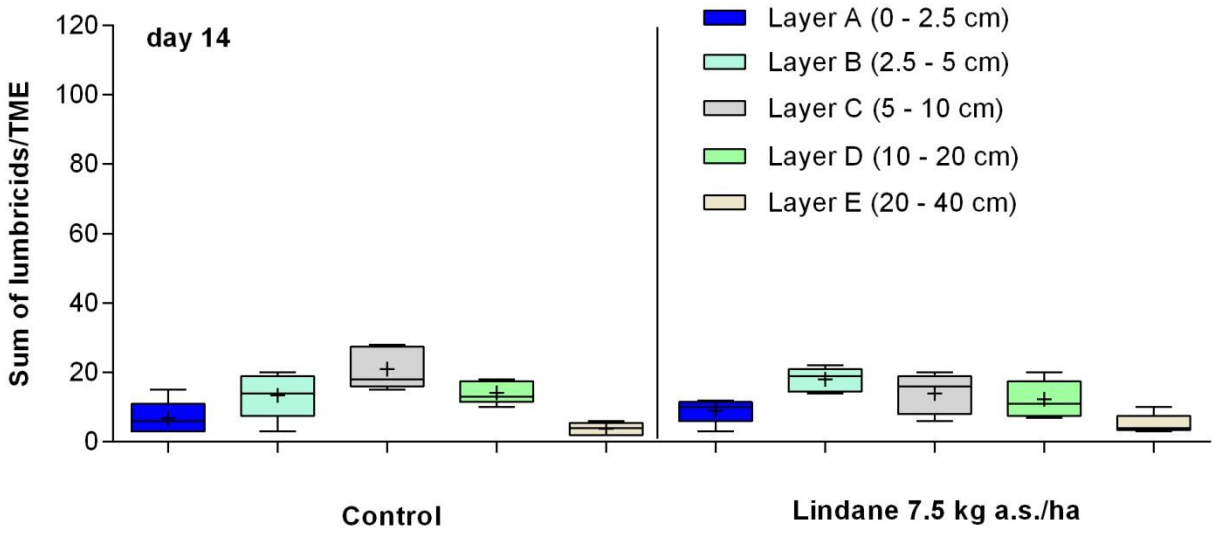

Figure 52 caption see below

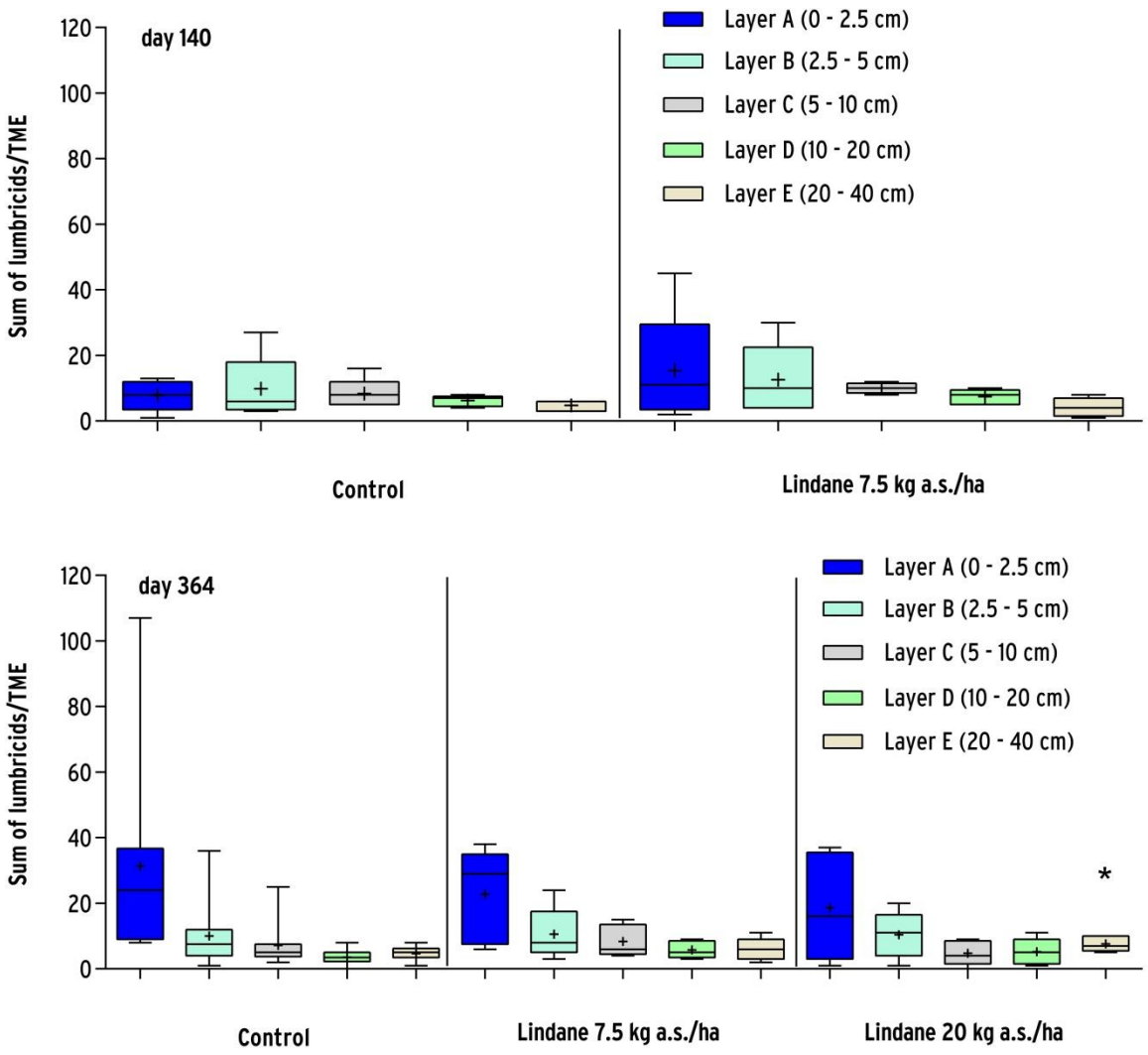

Figure 52 Total abundance of lumbricids in the soil layers of the TMEs in study [1] (5 replicates for control at day 14 and 140; 10 replicates for control at day 364; 5 replicates for lindane treatments). cross: mean; *: significance (p-value Williams-test < 0.05). Sampling dates at day 14, 189, 364 from top to bottom

Table 48 Decrease of total abundance [%] of lumbricid species in the different soil layers on different sampling dates in TMEs of study [1], 14-364 days after application. Red: decrease more than 50% in comparison to the control; Grey: less than 50% decrease in comparison to the control; X: no data available; Bold: significant effects (p-value <0.05, Mann-Whitney Rank Sum); calculation is based on 10 replicates for the control and 5 replicates for the respective treatment.

Lindane 7.5 kg a.s./ha

		days after application				
	layer	14	42	140	189	364
A	0 - 2.5 cm	-15	X	-97	X	27
B	2.5 - 5 cm	-15	X	-29	X	-6
C	5 - 10 cm	39	X	-19	X	-17
D	10 - 20 cm	7	X	-19	X	-61
E	20 - 40 cm	-41	X	13	X	-28
	all layers	6	X	-34	X	6

Lindane 20 kg a.s./ha

		days after application				
	layer	14	42	140	189	364
A	0 - 2.5 cm	X	X	X	X	41
B	2.5 - 5 cm	X	X	X	X	-4
C	5 - 10 cm	X	X	X	X	31
D	10 - 20 cm	X	X	X	X	-44
E	20 - 40 cm	X	X	X	X	**-62**
	all layers	X	X	X	X	18

Table 49 Presence and mean abundance of captured lumbricid species in the control TMEs of study [1] (10 replicates) and the two different application rates of Lindane of the treatment TMEs (5 replicates). For Treatment mean is calculated the mean abundance of lumbricid species in all treatment TMEs (10 replicates), 5 with higher and 5 with lower pesticide rate.

	Control	**Lindane**		
		Treatment mean	*7.5 kg a.s./ha*	*20 kg a.s./ha*
Number of replicates	**10**	**10**	**5**	**5**
TME				
Juveniles	59.8	39.4	62.0	16.8
Aporrectodea caliginosa	28.8	23.3	36.2	10.4
Lumbricus terrestris	6.0	3.2	4.4	2.0
Octolasion cyaneum	5.5	1.9	2.6	1.2
Aporrectodea rosea	4.1	2.5	4.4	0.6
Lumbricus rubellus	2.9	1.5	2.8	0.2
Lumbricus castaneus	2.0	1.4	2.4	0.4
Number of taxa	6	6	6	6

The statistical analysis on the population level for 4 out of 6 species (cp. appendix 1) resulted that significant effects could be detected not constantly but on single sampling dates and in single soil layer. Actually, an effect of both application rates was observed once for *Lumbricus castaneus* in soil layer A (at day 364), and twice in soil layer D (day 14 and 140) for *Lumbricus terrestris* and *Aporrectodea caliginosa*. Looking at the species under study from an ecological point of view, representatives from all three ecological groups were found (Table 50). Conspicuously, statistically significant effects were detected mostly in the layer that was determinied to be the preferred one for the species in this study – and also partly confirmed by literature records. Possibly, in the preferred layer the statistical power of the assay was higher, since numbers were high enough for statistical detection of effects.

L. terrestris, well-known as an ecosystem engineer and thus highly important for soil functions such as organic matter decomposition, or regulation of water infiltration (Lavelle et al. 1997), was mainly recorded in the deeper layers D and E (10-20 cm and 20-40 cm, Figure 53). The species was

actually statistically significantly affected in soil layer D at day 14 (Table 50). These worms live in deep burrows but are feeding and mating on the soil surface. Thus, this finding might be an indication of a quick vertical transport of Lindane within these burrows, which is however not supported by the findings of Lindane in the TME leachates, see chapter 4.2.3. Possibly, the vertical movement of *L. terrestris* –as described before - for feeding at the soil surface brought the animals in contact with contaminated soil layers.

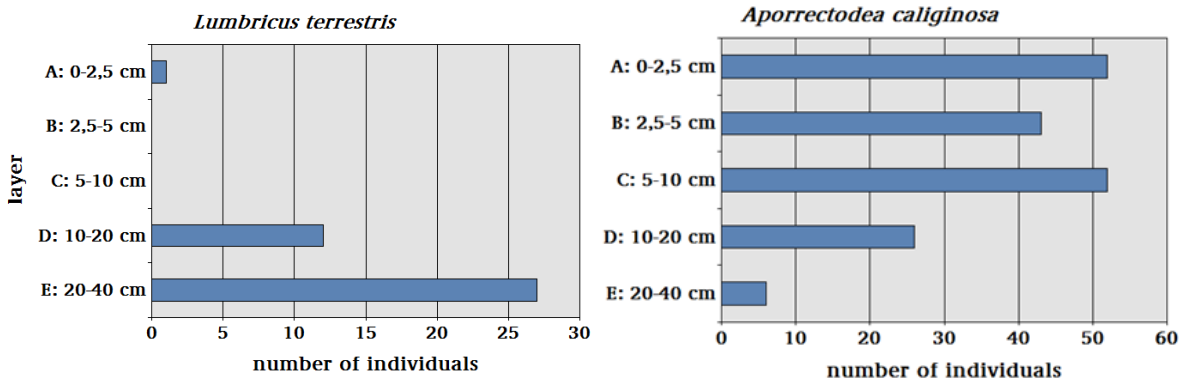

Figure 53 Vertical distribution of the lumbricid species *Lumbricus terrestris* and *Aporrectodea caliginosa* in the control. Shown is the total number of individuals captured in all TMEs on all sampling dates in the study [1].

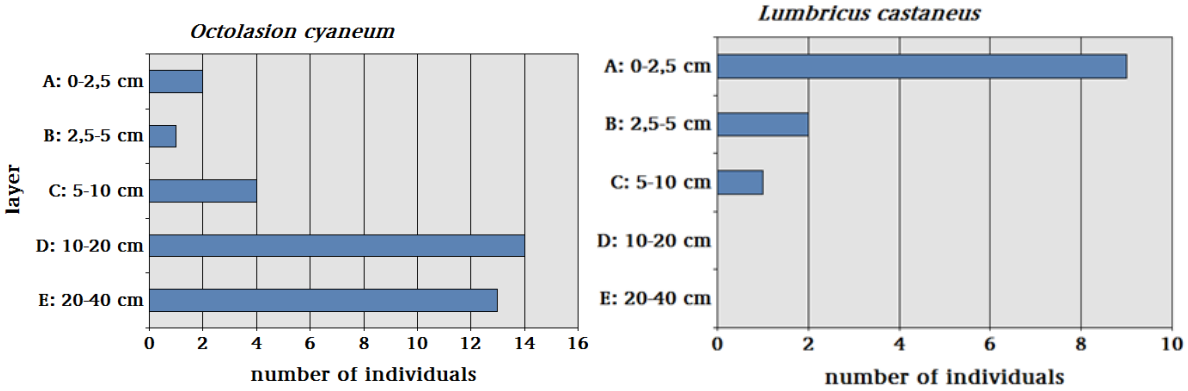

Figure 54 Vertical distribution of the lumbricid species *Octolasion cyaneum* and *Lumbricus castaneus* in the control. Shown is the total number of individuals captured in all TMEs on all sampling dates in the study [1].

The endogeic species *A. caliginosa* (probably the most wide-spread earthworm in Central Europe; Römbke et al. 2013) was mainly recorded in the soil layers A-D (0-20 cm) and only few numbers were found in layer E (20-40 cm, Figure 53). Another endogeic species, *O. cyaneum*, was distributed with higher numbers mainly in the deeper soil layers D and E, only few individuals were recorded for the upper soil layers A-C (Figure 54). Both species *A. caliginosa* and *O. cyaneum* were statistically significantly affected at day 140 when combining all soil layers, or in soil layer D, respectively (Table 50). Finally, the epigeic species *L. castaneus* (Figure 54) was statistically significantly affected at day 364 in soil layer A. As the classification indicates, this species lives always on or closely to the soil surface.

Table 50 Summary of statistical analysis of four different lumbricid species. Results are given for the different soil layers (A-E) and different sampling dates (14 days-364 days after application). Red: decrease of abundance ≥ 50% Grey: decrease of abundance < 50% X: no data available. Blank fields: data not sufficient for statistical calculation. Bold: significant difference (p-value Williams-test < 0.05); (see also appendix 1).

Lindane 7.5 kg a.s./ha		*Lumbricus terrestris* days after application		
	layer	14	140	364
A	0 - 2.5 cm			100
B	2.5 - 5 cm			
C	5 - 10 cm			
D	10 - 20 cm	**100**	-33	-33
E	20 - 40 cm	60	25	14
	all layers	78	9	0

Lindane 20 kg a.s./ha		*Lumbricus terrestris* days after application		
	layer	14	140	364
A	0 - 2.5 cm	X	X	100
B	2.5 - 5 cm	X	X	
C	5 - 10 cm	X	X	
D	10 - 20 cm	X	X	0
E	20 - 40 cm	X	X	0
	all layers	X	X	0

Lindane 7.5 kg a.s./ha		*Octolasion cyaneum* days after application		
	layer	14	140	364
A	0 - 2.5 cm			100
B	2.5 - 5 cm			100
C	5 - 10 cm			100
D	10 - 20 cm	50	56	-300
E	20 - 40 cm	50	50	43
	all layers	50	**62**	38

Lindane 20 kg a.s./ha		*Octolasion cyaneum* days after application		
	layer	14	140	364
A	0 - 2.5 cm	X	X	100
B	2.5 - 5 cm	X	X	100
C	5 - 10 cm	X	X	100
D	10 - 20 cm	X	X	-100
E	20 - 40 cm	X	X	-14
	all layers	X	X	8

Lindane 7.5 kg a.s./ha		*Lumbricus castaneus* days after application		
	layer	14	140	364
A	0 - 2.5 cm		60	**100**
B	2.5 - 5 cm		50	
C	5 - 10 cm		50	
D	10 - 20 cm			
E	20 - 40 cm			
	all layers		50	**100**

Lindane 20 kg a.s./ha		*Lumbricus castaneus* days after application		
	layer	14	140	364
A	0 - 2.5 cm	X	X	67
B	2.5 - 5 cm	X	X	
C	5 - 10 cm	X	X	
D	10 - 20 cm	X	X	
E	20 - 40 cm	X	X	
	all layers	X	X	0

Lindane 7.5 kg a.s./ha		*Aporrectodea caliginosa* days after application		
	layer	14	140	364
A	0 - 2.5 cm	0	-110	36
B	2.5 - 5 cm	-80	-18	0
C	5 - 10 cm	-80	-18	24
D	10 - 20 cm	-5	**-267**	-400
E	20 - 40 cm	0	100	-167
	all layers	-17	-70	-34

Lindane 20 kg a.s./ha		*Aporrectodea caliginosa* days after application		
	layer	14	140	364
A	0 - 2.5 cm	X	X	22
B	2.5 - 5 cm	X	X	-100
C	5 - 10 cm	X	X	-52
D	10 - 20 cm	X	X	-200
E	20 - 40 cm	X	X	-167
	all layers	X	X	-49

The multivariate statistical analysis with PRC showed no significant effects on the community (Table 51, PRC not shown). The number of species, the Shannon index and Evenness were significantly affected at day 140 for the lower Lindane application rate. At day 364, the number of taxa and the Evenness was significantly changed, but no statistically significant effects could be detected for the parameter Shannon index. Both indices (Steinhaus, Stander) did not show any differences between control and treatments on any sampling date (Figure 55).

Table 51 Summary of the results for the statistical diversity analyses, PRC (p-value t-test < 0.05 of PCA sample scores), number of taxa, Shannon and Eveness, *: significance (p-value Williams-test < 0.05) of lumbricids treated with Lindane (left) application rate of 7.5 kg a.s./ha (right) 20 kg a.s./ha. X: no data available. Database 10 replicates of control TMEs and 5 replicates for each treatment.

Lindane 7.5 kg a.s./ha						Lindane 20 kg a.s./ha					
	days after application						days after application				
all layers	14	42	140	189	364	all layers	14	42	140	189	364
PRC		X		X		PRC	X	X	X	X	
Number of taxa		X	*	X		Number of taxa	X	X	X	X	*
Shannon		X	*	X		Shannon	X	X	X	X	
Evenness		X	*	X		Evenness	X	X	X	X	*

Regarding the effects of Lindane on earthworms there is no consistent effect pattern for total abundance visible. Lindane did not affect earthworms strongly when looking at all soil layers together. In detail, the number of species was reduced at sampling day 140 and day 364, and both Shannon index and Evenness indicated an effect of Lindane on the earthworm community at day 140. Regarding the species level, no strong negative effects were found, but at day 140 the endogeic species *O. cyaneum* and at day 364 the epigeic species *L. castaneus* were affected negatively. Earthworm species showed apparently different distribution patterns between the layers in the treatment compared to the control distribution. However, even if changes affected 100 % of the individual numbers, the differences were not statistically significant, due most likely to a low statistical power of the assay.

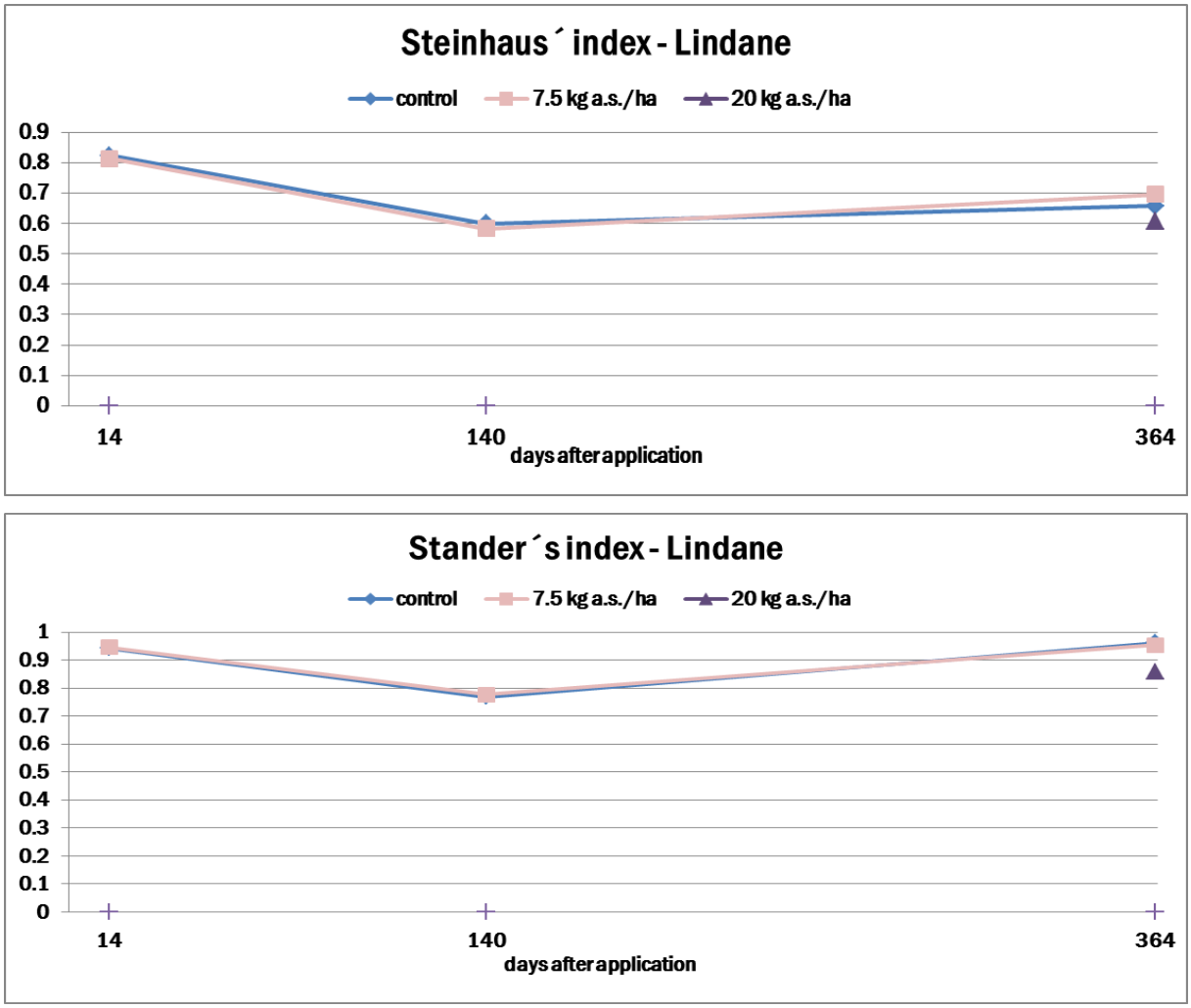

Figure 55 Similarity of Lumbricids diversity summed up over all soil layers (above) Steinhaus index (below) Standers index. Calculation is based on 5 replicates for the control and 5 replicates for each treatment at day 14 and day 140. Calculation is based on 10 replicates for the control and 5 replicates for each treatment at day 364.

4.4.4 Effects of Imidacloprid

4.4.4.1 Effects on collembola

The abundance of the collembolans was significantly affected by the Imidacloprid treatment (Figure 56, Table 52). For the lower application rate, the highest impact was measured at day 42, when in every layer statistically significant effects were observed. The effect rate increased with the depth i.e. lowest decrease for the surface layer (60 %) and highest decrease for layer C (100 %, 5-10 cm, 87 % layer A, 90 %, layer B). For the higher application rate, the strongest effects were also measured at day 42, albeit also strong effects were already visible at day 14. The effects were in most of the cases dose related i.e. the higher dose of Imidacloprid has induced the highest effect. At day 140, no statistically significant change in abundance could be observed in the deeper soil layers, however a significant decrease in abundance was measured for the surface layer. This is not due to missing numbers of collembolans in the soil at this time (cp. Table 36). At day 189, only layer A and B were sampled while significantly reduced individual numbers could be detected for layer B in both application rates. After 364 days, effects still occurred for both treatment rates, while the effect size decreased. The population of all layers except of layer B were statistically significantly affected in the higher application rate. The abundance of collembolans treated by the lower application rate of Imidacloprid was statistically significantly affected in layer O and layer C, but not in layer A.

In the statistical analysis on population level for 13 species of 25 species (cp. appendix 1; 52 % of the recorded species in the study) statistically significant effects could be detected for at least a single date and soil layer. The patterns are species specific.

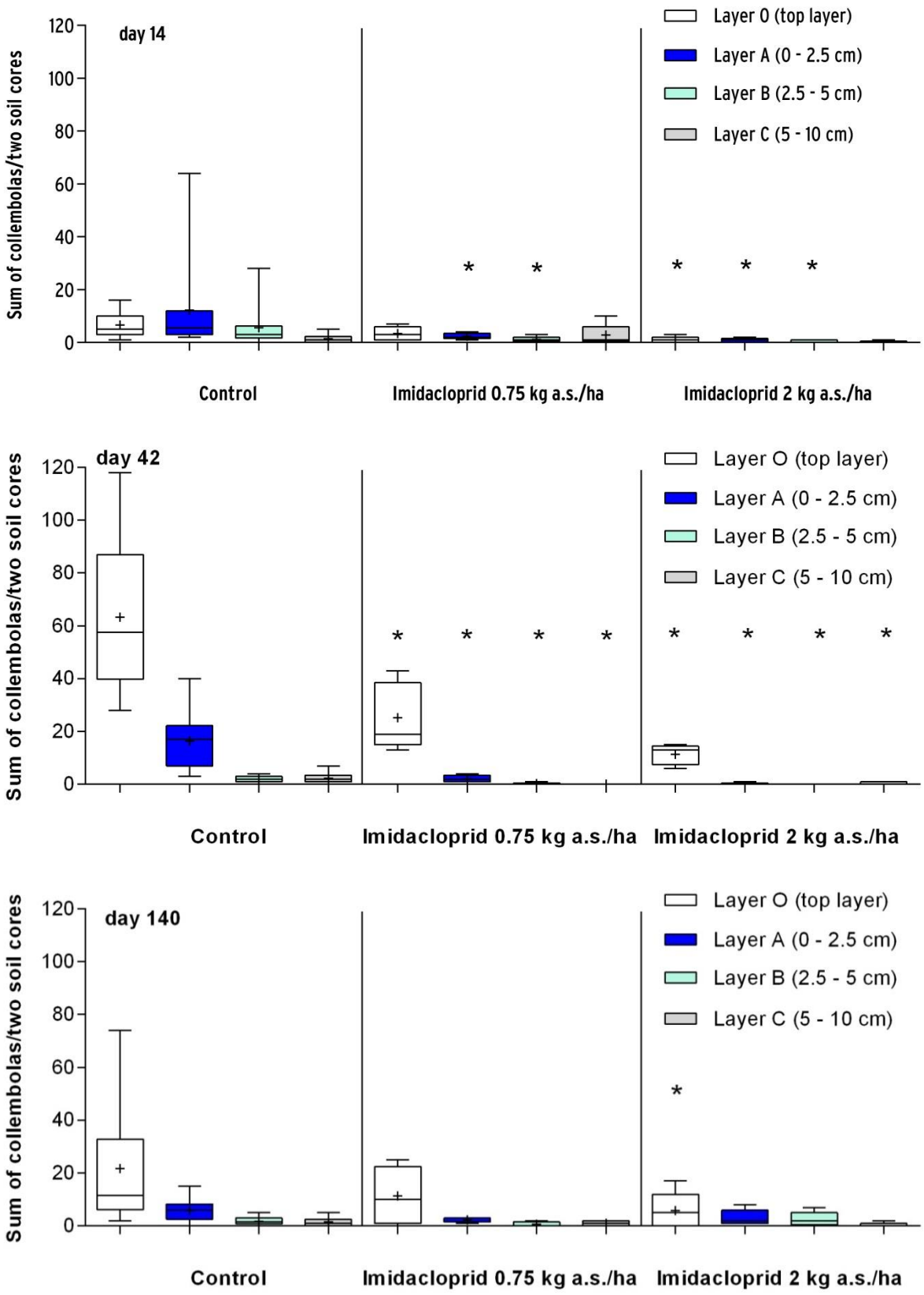

Figure 56 caption see below

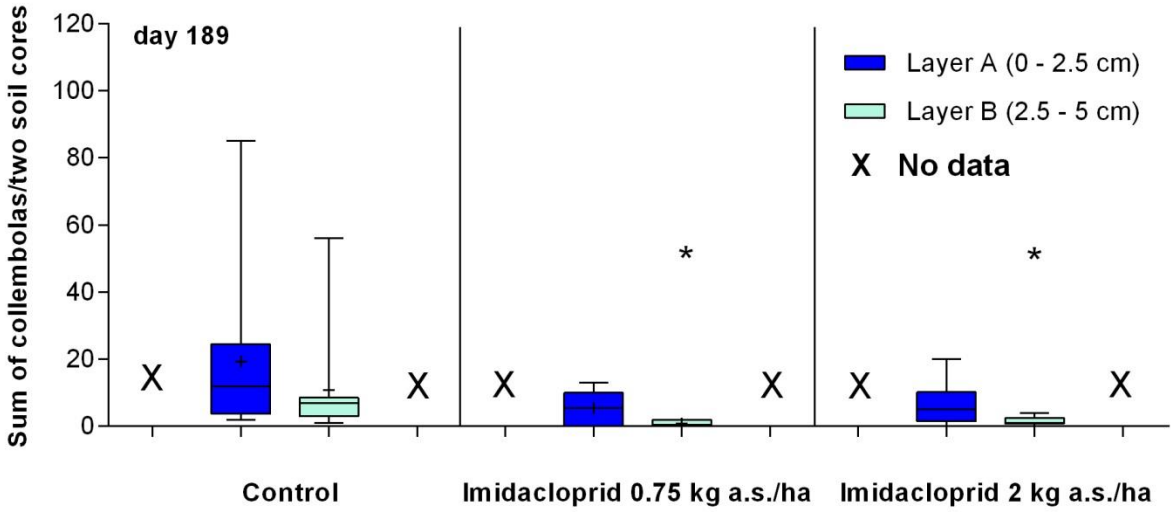

Figure 56 Total abundance of collembolans in the soil layers of the TMEs in study [1] (10 replicates for control; 5 replicates for imidacloprid treatments). X: no data; cross: mean; *: significance (p-value Williams-test < 0.05). Sampling dates at day 14 - 364 from top to bottom

Table 52 Decrease of total abundance [%] of collembolan species in the different soil layers on different sampling dates, 14-364 days after application. Red: decrease more than 50% in comparison to the control; Grey: less than 50% decrease in comparison to the control; X: no data available; Bold: significant effects (p-value <0.05, Mann-Whitney Rank Sum); calculation is based on 10 replicates for the control and 5 replicates for the respective treatment.

Imidacloprid 0.75 kg a.s./ha

		days after application				
	layer	14	42	140	189	364
O	Surface	49	**60**	48	X	**67**
A	0 - 2.5 cm	**80**	**87**	59	66	55
B	2.5 - 5 cm	**79**	**90**	67	**92**	52
C	5 - 10 cm	-100	**100**	38	X	**73**
	all layers	**67**	**88**	57	**75**	**57**

Imidacloprid 2 kg a.s./ha

		days after application				
	layer	14	42	140	189	364
O	Surface	**85**	**82**	**73**	X	**56**
A	0 - 2.5 cm	**93**	**99**	45	60	**74**
B	2.5 - 5 cm	**93**	**100**	-44	**86**	72
C	5 - 10 cm	86	**75**	75	X	**91**
	all layers	**93**	**96**	33	**68**	**76**

Table 53 Presence and mean abundance of captured collembolan species in the control TMEs (10 replicates) and the two different application rates of the treatment TMEs (5 replicates). The Treatment mean is calculated as the mean abundance of collembolan species in all treatment TMEs (10 replicates), 5 with higher and 5 with lower pesticide rate.

	Control	Imidacloprid		
		Treatment mean	*0.75 kg a.s./ha*	*2 kg a.s./ha*
Number of replicates	**10**	**10**	**5**	**5**
Soil cores				
Parisotoma notabilis	41,8	1,7	3,2	0,2
Lepidocyrtus cyaneus	11,5	5,8	6,6	5,0
Lepidocyrtus lanuginosus	8,7	5,0	4,6	5,4
Folsomia quadrioculata	7,2	0,1	-	0,2
Isotoma anglicana	4,1	0,1	0,2	-
Sphaeridia pumilis	3,3	1,3	1,0	1,6
Entomobrya multifasciata	2,1	0,9	1,2	0,6
Entomobrya lanuginosa	1,9	1,5	2,2	0,8
Brachystomella parvula	1,8	0,1	0,2	-
Friesea truncata	1,3	0,1	0,2	-
Sminthurinus aureus	1,2	0,3	0,2	0,4
Mesaphorura macrochaeta	0,8	0,1	0,2	-
Folsomides parvulus	0,7	-	-	-
Isotomurus fucicola/graminis	0,3	0,1	0,2	-
Stenaphorura quadrispina	0,3	-	-	-
Folsomia fimetaria	0,1	0,1	0,2	-
Lepidocyrtus lignorum	0,1	0,1	0,2	-
Pseudosinella alba	0,1	-	-	-
Sminthurus viridis	0,1	-	-	-
Stenaphorura denisi	0,1	-	-	-
Isotoma viridis	-	0,3	0,6	-
Pitfall traps				
Bourletiella hortensis	x	x	x	x
Deuterosminthurus pallipes	x	x	x	x
Heterosminthurus bilineatus	x	-	-	-
Isotoma viridis	x	-	-	-
Isotomurus graminis	x	x	-	x
Tomocerus vulgaris	-	x	x	-
Number of taxa	**25**	**20**	**18**	**11**

The community structure of collembolans was affected by Imidacloprid following a dose response relationship (Table 53). When considering both treatments at a time, only a moderate decrease of species presence (20) can be noticed in comparison to the control (25). This is mainly caused by the relatively small decrease in species presence for the lower application rate (18), especially when taking into account that these species numbers were based on only 5 replicates for the treatment (10 replicates for the control, cp. Chapter 4.4.1). In contrast to these results, a strong decrease of species numbers (11) was measured for the higher application rate. There, even

dominant species like *Parisotoma notabilis* and *Folsomia quadrioculata* showed low abundances close to extinction.

The hemiedaphic species *Parisotoma notabilis* (see Figure 39, distribution in the control < 1 % layer O, 72 % layer A, 26 % layer B, 2 % layer C) was affected in all layers while significant effects occurred mainly for layer A (Table 54). However, the species was completely reduced after day 42 with only one exception for the lower application rate at day 189 (85 %).

The epedaphic species *Lepidocyrtus cyaneus* (Figure 39) was significantly affected by Imidacloprid at day 42 (Table 54). The numbers of individuals of this species was according to its phenology highest at day 42 and day 140. This effect, occurring for both application rates for the surface layer at day 42 and for layer A for the higher application rate, was not visible after 140 days anymore.

The hemiedaphic species *Isotoma anglicana* (90 % layer O, 8 % layer A, 1 % layer B, 1 % layer C) was recorded in this study predominantly in the surface layer O in the pitfalls (Figure 57). In this layer significant effects of Imidacloprid could be found for both application rates on all sampling dates with high rates of reduction (> 96 %, Table 54). In all soil layers the population was reduced by 100 %, except layer A at day 364 (80 %). At day 42 the population in layer A was also significantly affected in both application rates and at day 364 in the higher application rate.

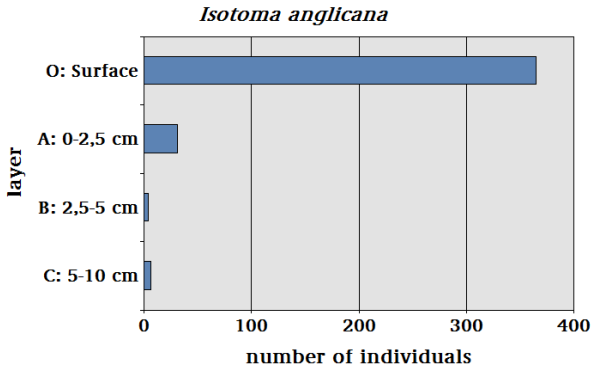

Figure 57 Vertical distribution of the collembolan species *Isotoma anglicana* in the control. Shown is the total number of individuals captured in all soil cores and pitfall traps on all sampling dates.

Effects on the hemiedaphic species *Folsomia quadrioculata* (no observations in layer O, 38 % layer A, 33 % layer B and 1 % layer C, Figure 40) could be observed for every layer (except of layer O) at any time. Significant effects were mainly found for the sum of all layers (day 42 to 364). It should be noted that all effects detected but one (high Lindane rate, day 14, layer B = 71 % effect) were 100 % reduction of abundance of this species. However, statistically significant effects could be detected for the single layer A at day 42 and for layer B at day 189 and day 364 for both application rates.

In sum, considering the effects on the different species, statistically significant effects could be observed predominantly in the layer were the species occurred with highest abundances.

The comparison of the treated populations using diversity indices is shown in Table 55. The overall result for those endpoints (Evenness, Shannon and species number) is that most of the effects occur at day 42 and day 364. The most affected endpoint is the number of taxa, based on the sum of all layers on all sampling dates except day 189.

Table 54 Summary of statistical analysis of four different collembolan species. Results are given for the different soil layers (O-C) and different sampling dates (14 days-364 days after application). Red: decrease of abundance ≥ 50% Grey: decrease of abundance < 50%. X: no data available. Blank fields: data not sufficient for statistical calculation. Bold: significant difference (p-value Williams-test < 0.05); (see also appendix 1).

Imidacloprid 0.75 kg a.s./ha		*Parisotoma notabilis* days after application				
layer		14	42	140	189	364
O	Surface	100			X	
A	0 - 2.5 cm	**96**	**100**	100	**97**	**100**
B	2.5 - 5 cm	85	100	100	100	100
C	5 - 10 cm	-300	100		X	100
all layers		**78**	**100**	100	**98**	**100**

Imidacloprid 2 kg a.s./ha		*Parisotoma notabilis* days after application				
layer		14	42	140	189	364
O	Surface	100			X	
A	0 - 2.5 cm	**98**	**100**	100	**100**	**100**
B	2.5 - 5 cm	**100**	100	100	100	100
C	5 - 10 cm	100	100		X	100
all layers		**99**	**100**	100	**100**	**100**

Imidacloprid 0.75 kg a.s./ha		*Lepidocyrtus cyaneus* days after application				
layer		14	42	140	189	364
O	Surface		**69**	42	X	80
A	0 - 2.5 cm	-33	-11	71	38	-100
B	2.5 - 5 cm			100	**100**	
C	5 - 10 cm	100	100		X	-100
all layers		20	17	64	53	-150

Imidacloprid 2 kg a.s./ha		*Lepidocyrtus cyaneus* days after application				
layer		14	42	140	189	364
O	Surface		**93**	69	X	60
A	0 - 2.5 cm	100	**100**	43	49	-100
B	2.5 - 5 cm			-300	**100**	
C	5 - 10 cm	100	100		X	100
all layers		**100**	**100**	27	61	-50

Imidacloprid 0.75 kg a.s./ha		*Isotoma anglicana* days after application				
layer		14	42	140	189	364
O	Surface	**100**	**100**	**96**	X	**97**
A	0 - 2.5 cm		**100**	100		80
B	2.5 - 5 cm		100	100	100	
C	5 - 10 cm		100	100	X	
all layers			**100**	100	100	80

Imidacloprid 2 kg a.s./ha		*Isotoma anglicana* days after application				
layer		14	42	140	189	364
O	Surface	**100**	**100**	**100**	x	**98**
A	0 - 2.5 cm		**100**	100		**100**
B	2.5 - 5 cm		100	100	100	
C	5 - 10 cm		100	100	X	
all layers			**100**	100	100	**100**

Imidacloprid 0.75 kg a.s./ha		*Folsomia quadrioculata* days after application				
layer		14	42	140	189	364
O	Surface				X	
A	0 - 2.5 cm	100	**100**	100	100	100
B	2.5 - 5 cm	100		100	**100**	**100**
C	5 - 10 cm	100			X	
all layers		100	**100**	**100**	**100**	**100**

Imidacloprid 2 kg a.s./ha		*Folsomia quadrioculata* days after application				
layer		14	42	140	189	364
O	Surface				X	
A	0 - 2.5 cm	100	**100**	100	100	100
B	2.5 - 5 cm	71		100	**100**	**100**
C	5 - 10 cm	100			X	
all layers		82	**100**	**100**	**100**	**100**

Table 55 Summary of the results for the statistical diversity analyses, PRC (p-value t-test < 0.05 of PCA sample scores), number of taxa, Shannon and Eveness, *: significance (p-value Williams-test < 0.05) of collembolas treated with Imidacloprid (left) application rate of 0.75 kg a.s./ha (right) 2 kg a.s./ha. Database: 10 replicates of control TMEs and 5 replicates for each treatment.

Imidacloprid 0.75 kg a.s./ha	days after application				
all layers	14	42	140	189	364
PRC		*			*
Number of taxa	*	*	*		*
Shannon		*			*
Evenness					

Imidacloprid 2 kg a.s./ha	days after application				
all layers	14	42	140	189	364
PRC	*	*		*	*
Number of taxa	*	*	*		*
Shannon	*	*			*
Evenness	*	*			

The similarity of the collembolan communities measured with Steinhaus´ and Stander´s indices is presented for all layers in Figure 58. Both diagrams are very similar. The curves of both application

rates of Imidacloprid were visibly different to the control at day 14 and day 42. Afterwards, at day 140 and day 189 the curves were at the same level while at day 364 they get apart again showing a – small - difference again.

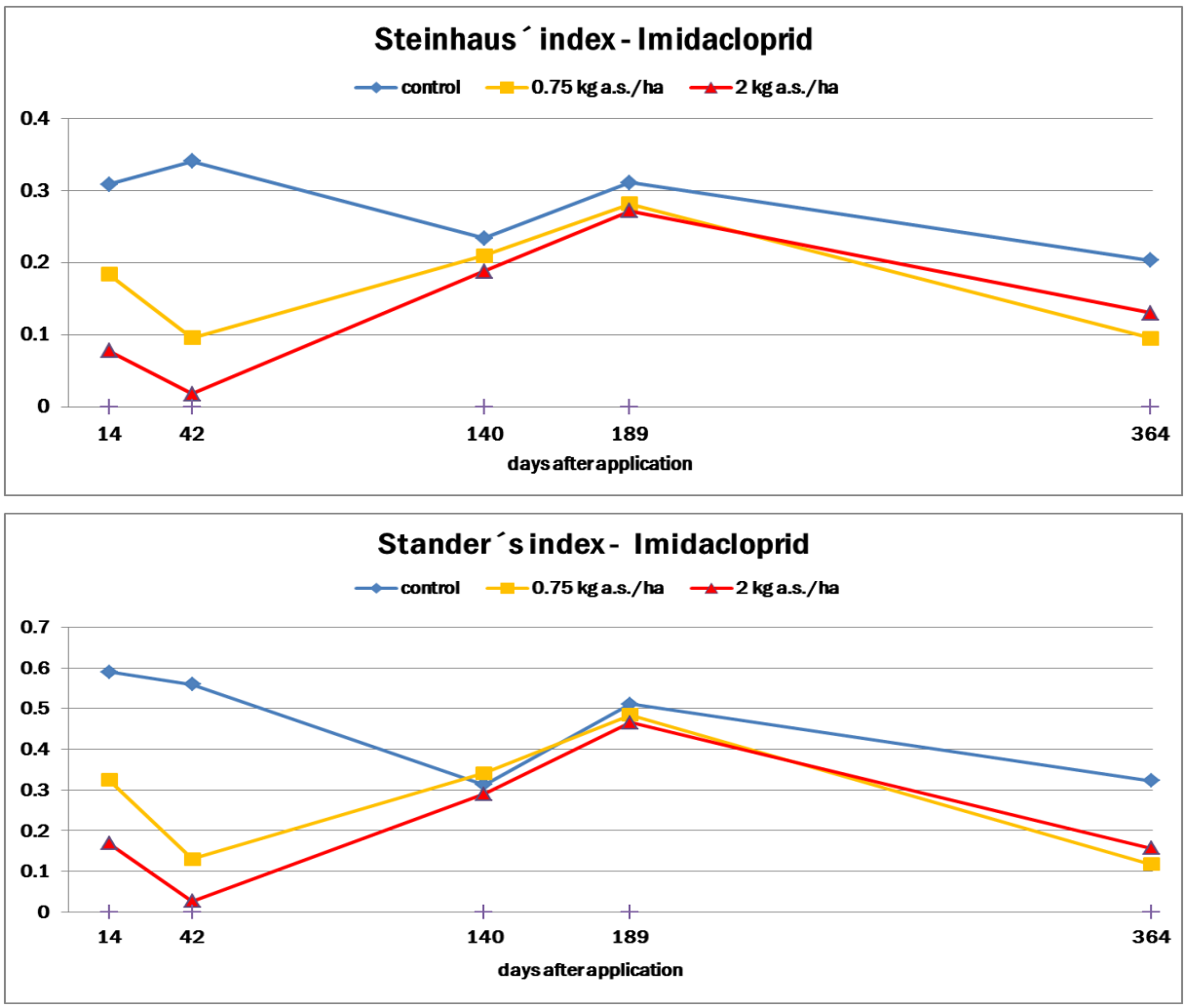

Figure 58 Similarity of Collembola diversity summed up over soil layers A-C (above) Steinhaus index (below) Standers index. Calculation is based on 10 replicates for the control and 5 replicates for each treatment.

The results of the multivariate statistical analysis are given in Figure 59. The RDA summarizing all sampling dates was significant for all layers in sum (p=0.001). Considering all layers in sum, effects occurred on any sampling date except of day 140. At day 14 and day 189, these effects were significant for the higher application rate. For the high application rate at day 140 and for the low application rate at day 14 and 189, treatments were different from the control at a significance level of alpha ≤ 10 %.

The dominant species *Parisotoma notabilis*, *Lepidocyrtus cyaneus*, *L. lanuginosus* and *Folsomia quadrioculata* (Figure 32) contributed most to the difference in community response. Most of all species showed a decrease in abundance except for the species *Brachystomella parvula* which showed increasing abundances after Imidacloprid treatment.

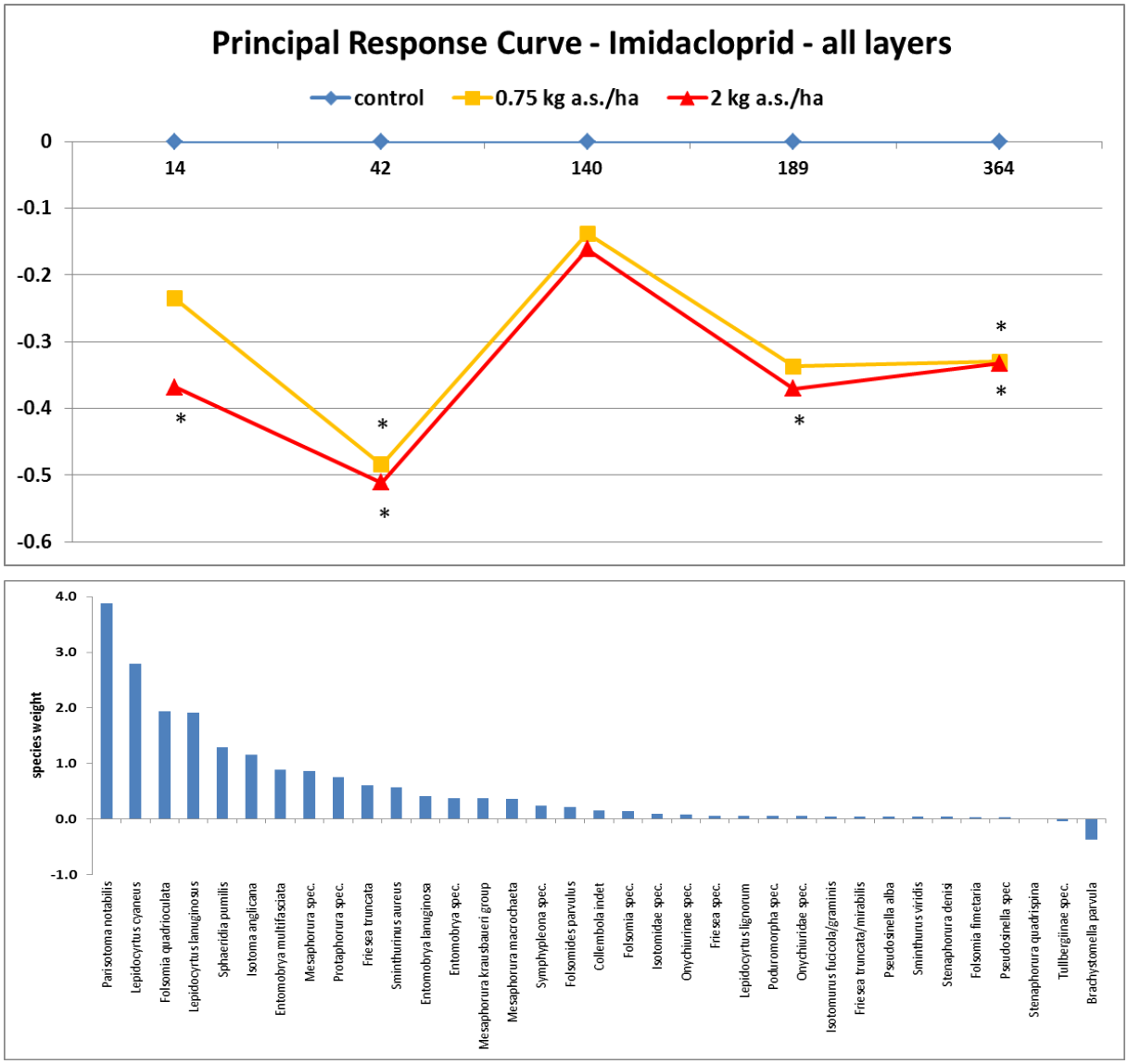

Figure 59 Principal Response Curves to the application of Imidacloprid of the collembolan community calculated for the sum of all layers (layer O A, B & C). Results of all Collembolan species, mean of 10 replicates for controls, 5 replicates for treatments; *: significant effects measured by sample scores of the PCA for the single sampling date. Species weights indicating the share of difference for the different species

In sum, Imidacloprid showed consistent effects on collembolans when summing up the individuals of the soil column from 0-10 cm (layer A-C). The strongest effect on their number was found at days 14 and 42 after application, but effects rose up again at day 189 at the lower application rate and at day 364 for both application rates. These findings were confirmed by the similarity indices as well as by the Shannon index that showed differences for the first two samplings, but also small differences for day 364. The community composition via the endpoint PRC indicate effects of the Imidacloprid treatments for all samplings. These findings were similar to those for the species *Parisotoma notabilis* and *Folsomia quadrioculata*. Both species were always affected except of one sampling date.

4.4.4.2 Effects on oribatid mites

The population of oribatid mites was not consistently affected by Imidacloprid (Figure 60, Table 56). During the study time with five samplings in different layers, their abundance seemed to increase in comparison to the control. This was the case e.g. at day 14 in the low application rate, at day 140 in the low and high application rates and at day 364 in high application rate. This could be due to higher food availability, since potential competitors were more strongly affected (i.e. Collembola). It is also possible that due to an increasing number of dead invertebrates the bacterial or/and fungal biomass increased, meaning that more food resources were available for the mites. Since neither the bacterial nor the fungal biomass was measured during the study, these assumptions could not be confirmed.

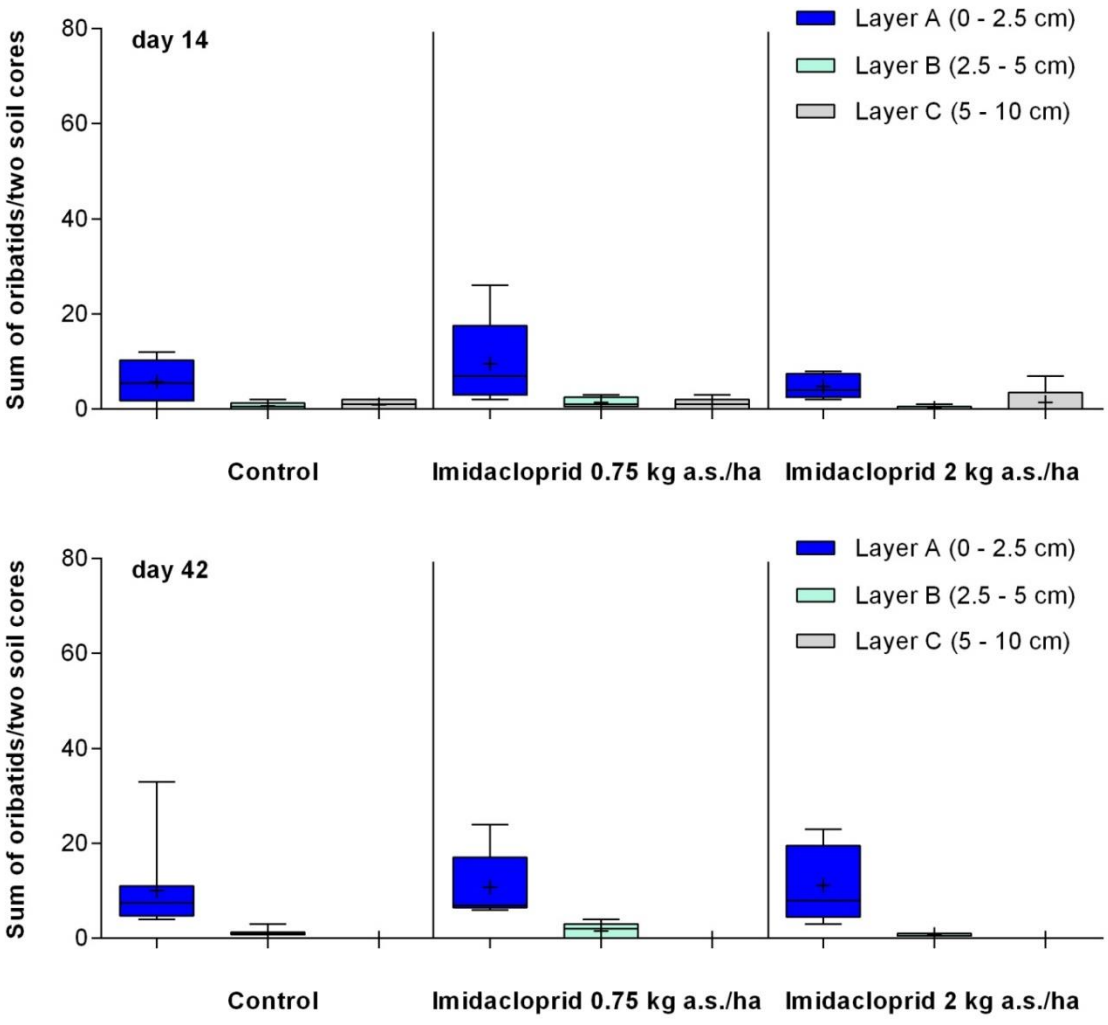

Figure 60 caption see below

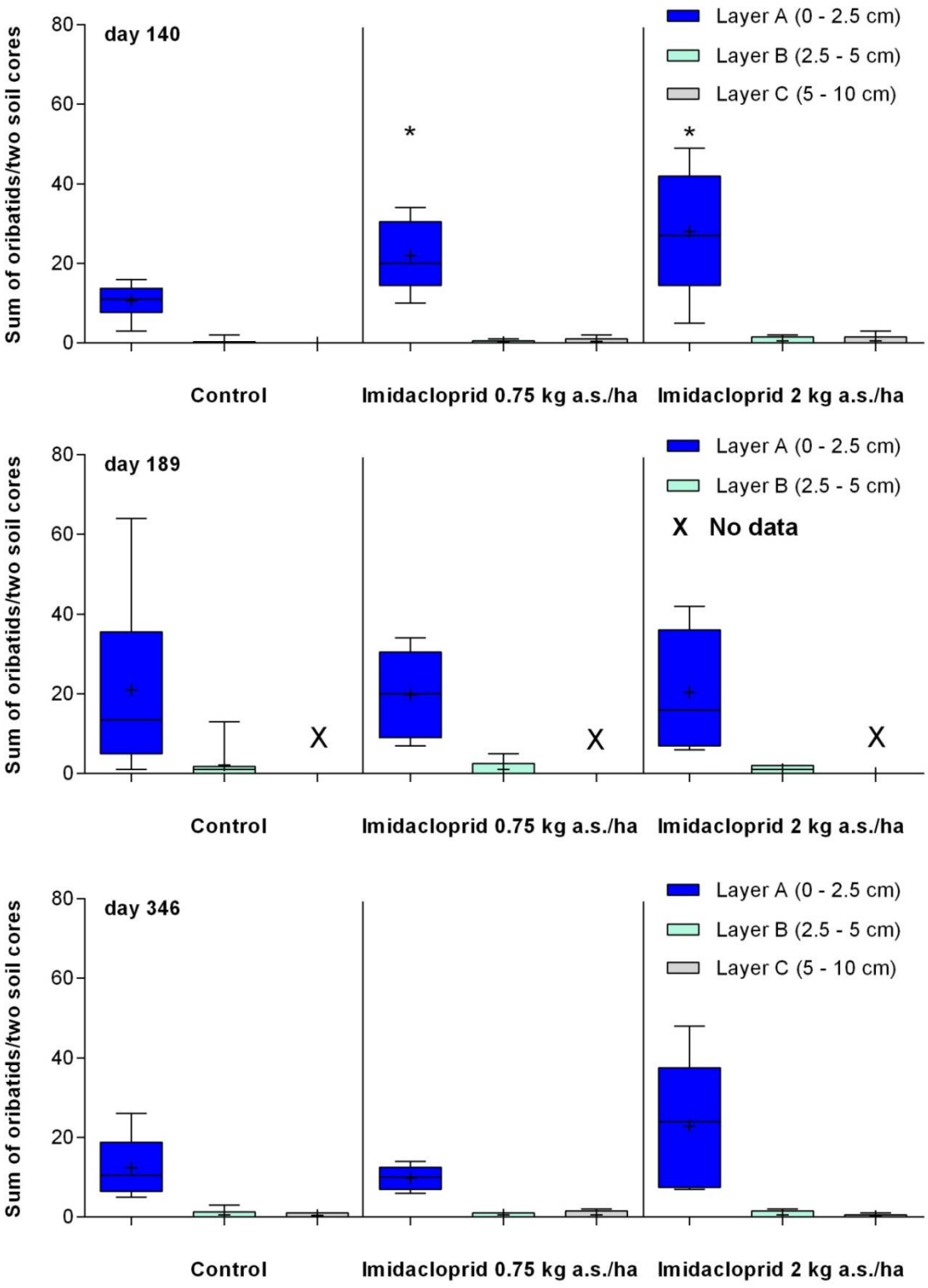

Figure 60 Total abundance of oribatid mites in the soil layers of the TMEs in study [1] (10 replicates for control; 5 replicates for imidacloprid treatments). X: no data, cross: mean; *: significance (p-value Williams-test < 0.05). Sampling dates at day 14 - 364 from top to bottom

Table 56 Decrease of total abundance [%] of oribatid mite species in the different soil layers on different sampling dates, 14-364 days after application. Red: decrease more than 50% in comparison to the control; grey: less than 50% decrease in comparison to the control; X: no data available; Blank fields: data not sufficient for statistical calculation; Bold: significant effects (p-value <0.05, Mann-Whitney Rank Sum); calculation is based on 10 replicates for the control and 5 replicates for the respective treatment.

Imidacloprid 0.75 kg a.s./ha

		days after application				
	layer	14	42	140	189	364
A	0 - 2.5 cm	-68	-7	**-106**	6	21
B	2.5 - 5 cm	-100	-45	50	50	0
C	5 - 10 cm	-11			X	-50
	all layers	-64	-11	**-105**	10	18

Imidacloprid 2 kg a.s./ha

		days after application				
	layer	14	42	140	189	364
A	0 - 2.5 cm	16	-11	**-162**	3	-84
B	2.5 - 5 cm	71	27	-50	50	0
C	5 - 10 cm	-56			X	50
	all layers	12	-7	**-165**	7	-76

Table 57 Presence and mean abundance of captured oribatid mite species in the control TMEs (10 replicates) and the two different application rates of Imidacloprid treatment TMEs (5 replicates). The Treatment mean is calculated as the mean abundance of oribatid mite species in all treatment TMEs (10 replicates), 5 with higher and 5 with lower pesticide rate.

	Control		**Imidacloprid**	
		Treatment mean	*0.75 kg a.s./ha*	*2 kg a.s./ha*
Number of replicates	**10**	**10**	**5**	**5**
Soil cores				
Liebstadia similis	47.5	66.6	60.6	72.6
Juveniles	12.1	12.1	10.4	13.8
Eupelops occultus	2.6	11	10	12
Banksinoma lanceolata	0.7	11	12	10
Mnunthozetes semirufus	0.5	0.7	0.8	0.6
Ramusella clavipectinata	0.5	0.3	0.2	0.4
Scheloribates laevigatus	0.5	2.0	2.6	14
Galumna obvia	0.4	0.7	0.6	0.8
Pantelozetes paolii	0.2	0.1	0.2	-
Achipteria coleoptrata	0.1	0.1	-	0.2
Gustavia microcephala	0.1	-	-	-
Oppiella (Oppiella) nova	0.1	0.1	0.2	-
Punctoribates punctum	0.1	0.4	0.2	0.6
Scheloribates latipes	0.1	-	-	-
Suctobelba spec.	0.1	-	-	-
Tectocepheus velatus	0.1	-	-	-
Ctenobelba pectinigera	-	0.1	0.2	-
Galumna alata	-	0.2	0.4	-
Pitfall traps				
Scutovertex minutus	-	X	X	-
Number of all taxa	**15**	**14**	**13**	**9**

The number of species in the Imidacloprid treatment especially in the higher application rate was lower than in the control (Table 57), but not statistically significant different. When considering both treatments together, there was no difference to the control numbers. The results for single species show no remarkable effects, except for *Liebstadia similis* (cp. Table 58). The epigeic species *L. similis* (Figure 44) shows a significant increase of individuals compared to the control at sampling day 140, which might be due to above mentioned possible increase of food resources (Table 58). Other species e.g. *Minunthozetes semirufus* and *Scheloribates laevigatus*, which are typical representatives for natural grassland communities, occurred only in low numbers (Figure 61), therefore limiting interpretation of any effects.

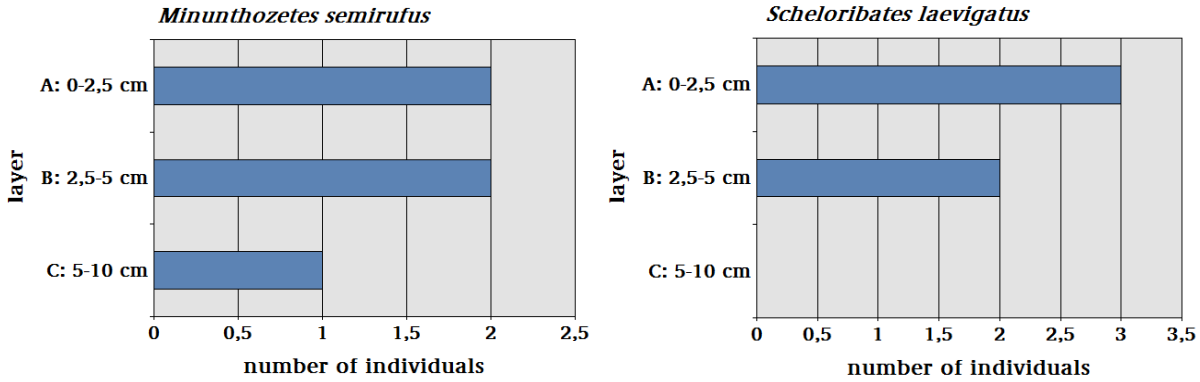

Figure 61 Vertical distribution of the oribatid mite species *Minunthozetes semirufus* and *Scheloribates laevigatus* in the control. Shown is the total number of individuals captured in all soil cores on all sampling dates.

Table 58 Summary of statistical analysis of four different oribatid mite taxa. Results are given for the different soil layers (A-C) and different sampling dates (14 days-364 days after application). Red: decrease of abundance ≥ 50% Grey: decrease of abundance < 50% X: no data available. Blank fields: data not sufficient for statistical calculation. Bold: significant difference (p-value Williams-test < 0.05); (see also appendix 1); ↑: significant increase of treatment while the number of individuals in the control is 0.

Imidacloprid 0.75 kg a.s./ha		*Liebstadia similis* days after application				
	layer	14	42	140	189	364
A	0 - 2.5 cm	-54	-40	**-108**	-11	6
B	2.5 - 5 cm	-33	100	33	87	100
C	5 - 10 cm	100			X	50
	all layers	-36	-27	**-106**	-2	11

Imidacloprid 2 kg a.s./ha		*Liebstadia similis* days after application				
	layer	14	42	140	189	364
A	0 - 2.5 cm	59	-28	**-176**	-11	-89
B	2.5 - 5 cm	100	60	-100	47	0
C	5 - 10 cm	-140			X	100
	all layers	40	-20	**-174**	-6	-77

Imidacloprid 0.75 kg a.s./ha		*Minuthozetes semirufus* days after application				
	layer	14	42	140	189	364
A	0 - 2.5 cm		-100			
B	2.5 - 5 cm	100	100			
C	5 - 10 cm	100			X	
	all layers	100	-33			

Imidacloprid 2 kg a.s./ha		*Minuthozetes semirufus* days after application				
	layer	14	42	140	189	364
A	0 - 2.5 cm	↑	100			
B	2.5 - 5 cm	100	100			
C	5 - 10 cm	100			X	
	all layers	-200	100			

Imidacloprid 0.75 kg a.s./ha		*Scheloribates laevigatus* days after application				
	layer	14	42	140	189	364
A	0 - 2.5 cm				-100	
B	2.5 - 5 cm	-100				-100
C	5 - 10 cm				X	
	all layers	-500			-100	-500

Imidacloprid 2 kg a.s./ha		*Scheloribates laevigatus* days after application				
	layer	14	42	140	189	364
A	0 - 2.5 cm				-100	
B	2.5 - 5 cm	100				100
C	5 - 10 cm				X	
	all layers	100			-167	-100

Imidacloprid 0.75 kg a.s./ha		*Juveniles* days after application				
	layer	14	42	140	189	364
A	0 - 2.5 cm	-100	26	100	64	63
B	2.5 - 5 cm		-200	100		
C	5 - 10 cm				X	
	all layers	-138	5	100	65	48

Imidacloprid 2 kg a.s./ha		*Juveniles* days after application				
	layer	14	42	140	189	364
A	0 - 2.5 cm	-50	-21	-100	64	-63
B	2.5 - 5 cm		50	100		
C	5 - 10 cm				X	
	all layers	-63	-14	-200	65	-70

The multivariate statistical analysis with PRC and RDA as well as the diversity endpoints showed no significant effects of both Imidacloprid treatments on the community of oribatid mites (PRC and results of diversity endpoints not presented). The similarity of these communities measured with Steinhaus´ and Stander´s indices is presented for all layers in Figure 62 and show no differences between control and one of the two application rates on any sampling date.

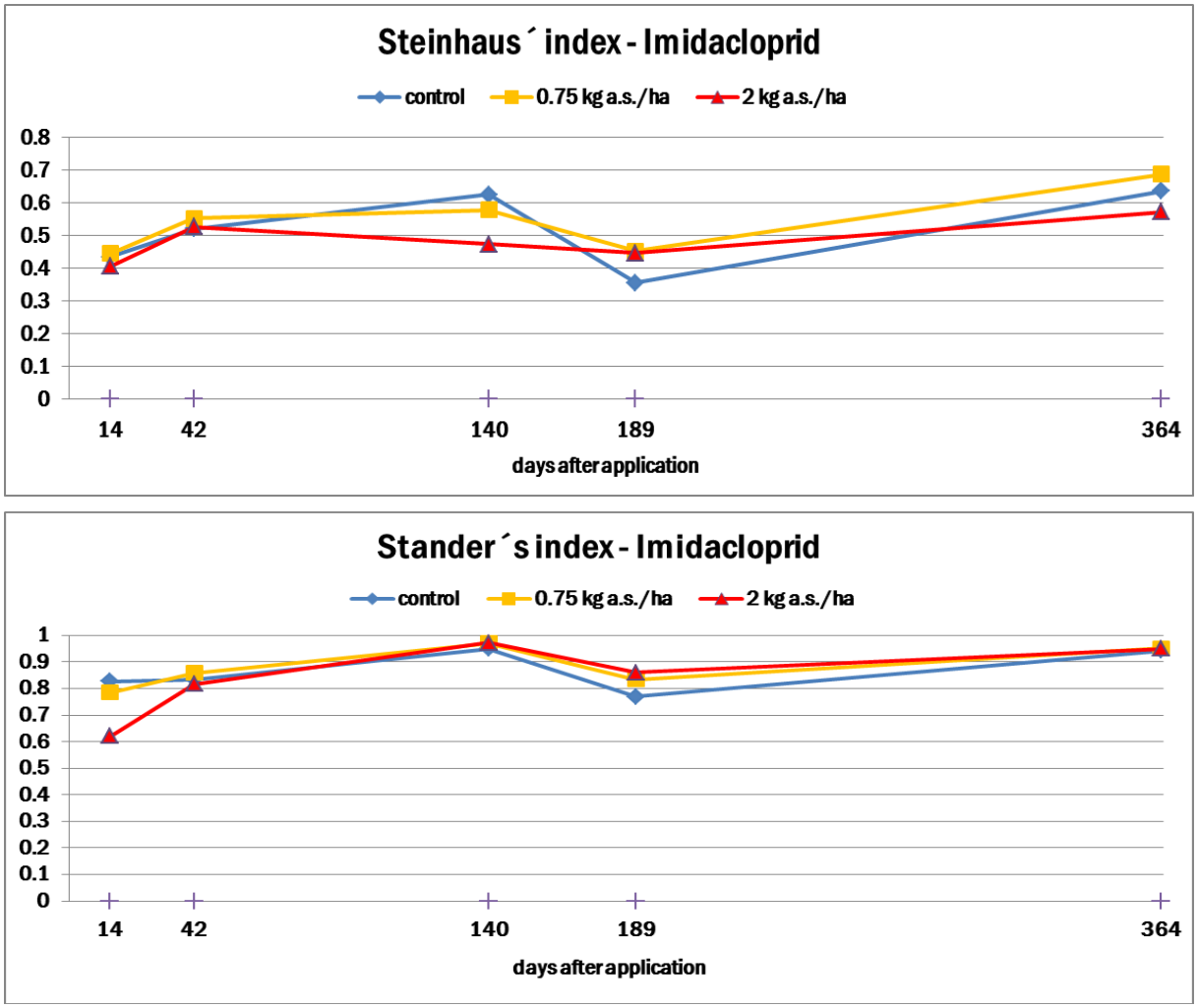

Figure 62 Similarity of Oribatid mite diversity summed up over all soil layers (above) Steinhaus index (below) Standers index. Calculation is based on 10 replicates for the control and 5 replicates for each treatment.

In sum, no consistent effects of Imidacloprid on the total abundance and community of oribatid mites could be detected in the TMEs of study [1].

4.4.4.3 Effects on enchytraeidae

The population of Enchytraeidae was not significantly affected by Imidacloprid (Figure 63, Table 59), except of day 42, when a significant increase in abundance in layer B was observed. On all sampling dates and in all soil layers their numbers in the treatments were in the same range as those of the control.

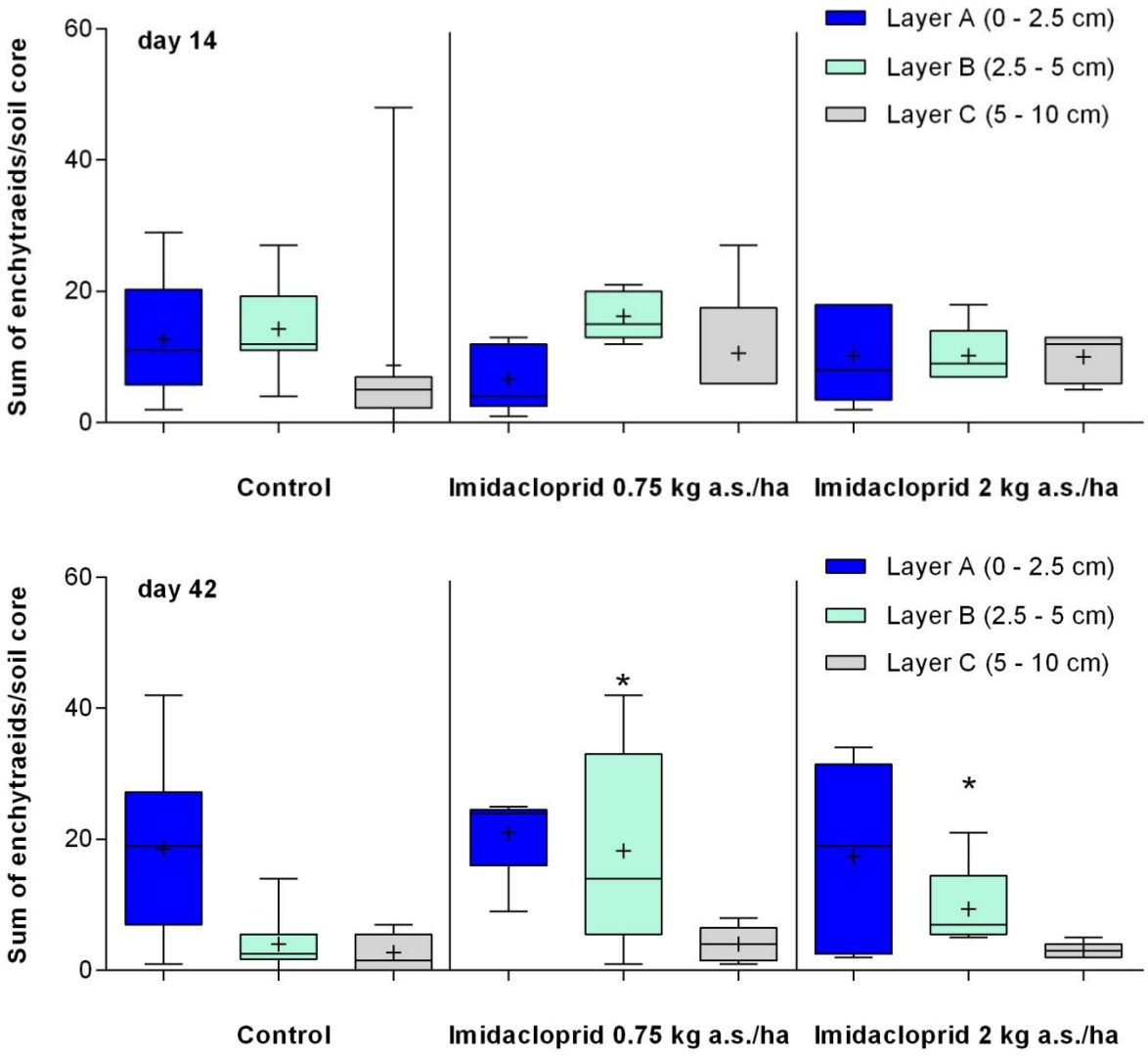

Figure 63 caption see below

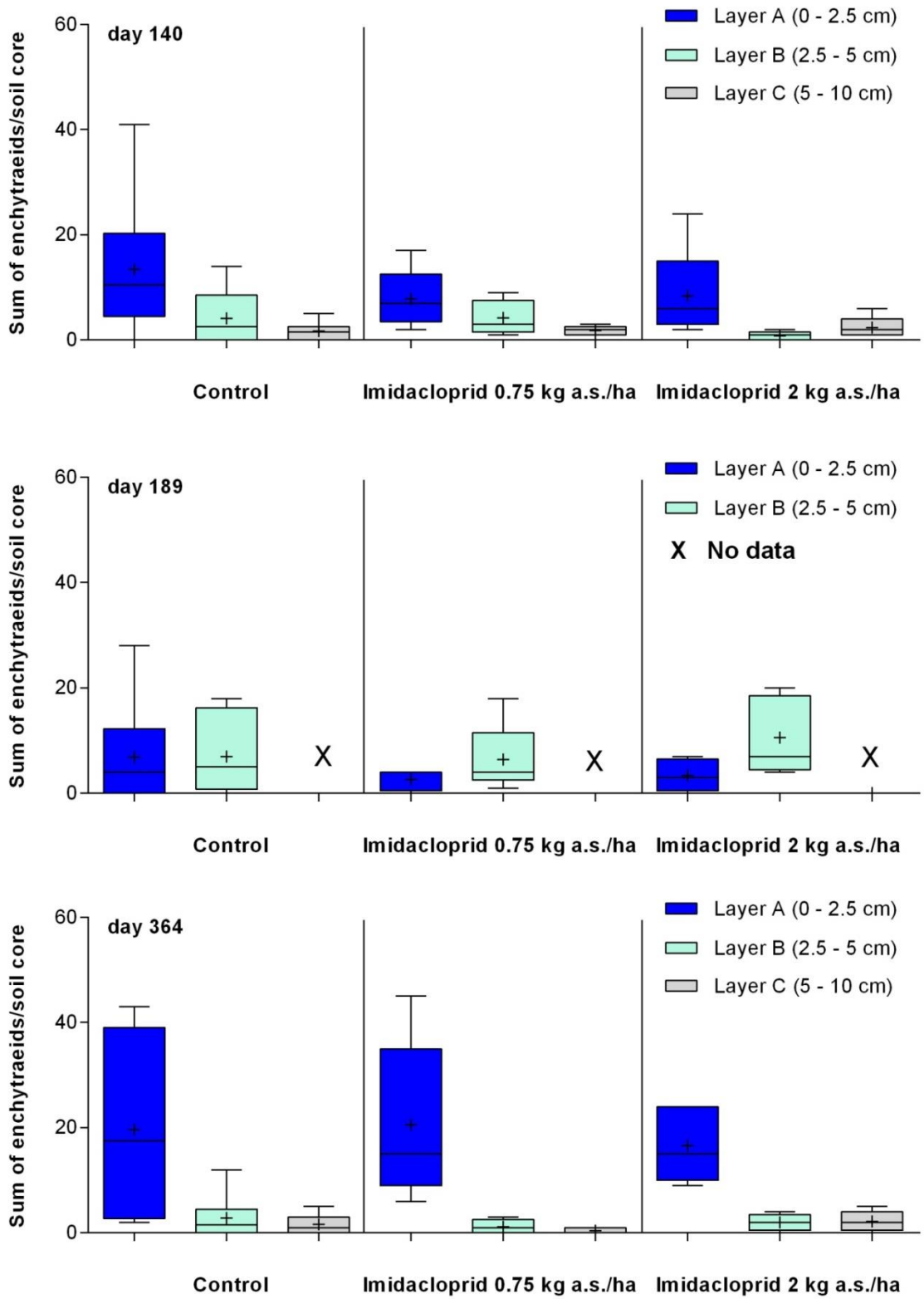

Figure 63 Total abundance of enchytraeids in the soil layers of the TMEs in study [1] (10 replicates for control; 5 replicates for imidacloprid treatments). X: no data, cross: mean; *: significance (p-value Williams-test < 0.05). Sampling dates at day 14 - 364 from top to bottom

Table 59 Decrease of total abundance [%] of enchytraeid species in the different soil layers on different sampling dates, 14-364 days after application of Imidacloprid. Red: decrease more than 50% in comparison to the control; grey: less than 50% decrease in comparison to the control; X: no data available; Bold: significant effects (p-value <0.05, Mann-Whitney Rank Sum); calculation is based on 10 replicates for the control and 5 replicates for the respective treatment.

Imidacloprid 0.75 kg a.s./ha

		days after application				
	layer	14	42	140	189	364
A	0 - 2.5 cm	48	-14	42	62	5
B	2.5 - 5 cm	-13	**-355**	-2	9	57
C	5 - 10 cm	-22	-48	-6	X	75
	all layers	6	-71	28	35	8

Imidacloprid 2 kg a.s./ha

		days after application				
	layer	14	42	140	189	364
A	0 - 2.5 cm	20	6	37	51	24
B	2.5 - 5 cm	29	**-135**	80	-51	29
C	5 - 10 cm	-15	-11	-41	X	-38
	all layers	15	-18	40	-1	13

Table 60 Presence and mean abundance of captured enchytraeid species in the control TMEs (10 replicates) and the two different application rates of the treatment TMEs (5 replicates). The Treatment mean is calculated as the mean abundance of enchytraeid species in all treatment TMEs (10 replicates), 5 with higher and 5 with lower pesticide rate.

	Control		**Imidacloprid**	
		Treatment mean	*0.75 kg a.s./ha*	*2 kg a.s./ha*
Number of replicates	**10**	**10**	**5**	**5**
Soil cores				
Archaeta "dzwilloi"	26.2	30.8	26.4	35.2
Fridericia connata	22.7	18.2	25.2	11.2
Fridericia ulrikae	8.5	7.9	11.2	4.6
Enchytronia parva s.l.	8.4	7.8	9.2	6.4
Enchytraeus sp. PALE	7.7	11.4	13.8	9.0
Fridericia galba	7.5	6.6	6.2	7.0
Fridericia bulboides	5.8	4.2	4.0	4.4
Fridericia paroniana	4.8	1.4	1.2	1.6
Fridericia bisetosa	4.3	3.3	4.0	2.6
Enchytraeus sp. GRAN	4.1	10.0	7.6	12.4
Buchholzia appendiculata	3.5	2.0	2.4	1.6
Fridericia sylvatica	2.5	1.9	2.2	1.6
Henlea perpusilla	2.0	1.1	0.8	1.4
Marionina communis	1.7	1.8	1.8	1.8
Fridericia benti	1.5	0.7	0.8	0.6
Fridericia dura cf.	0.9	1.0	0.2	1.8
Enchytraeus norvegicus	0.1	0.3	0.4	0.2
Cognettia glandulosa	-	0.3	0.6	-
Number of taxa	**17**	**18**	**18**	**17**

The community structure of enchytraeids in the TMEs treated with Imidacloprid was very similar to that in the control (Table 60). The species *Cognettia glandulosa* was only recorded in the lower treatment rate in low numbers

However, looking at single species, in the TMEs treated with Imidacloprid significant effects were found for 6 out of 18 enchytraeid species in at least one single layer on one sampling date (cp. appendix 1). Both increases and decreases of enchytraeid numbers were found, meaning that no consistent trend could be identified.

Those four enchytraeid species which reacted significantly towards Imidacloprid showed different preferences concerning their vertical distribution in the soil (Figure 49, Figure 50, Figure 64). The mineral dweller *A. dzwilloi* was affected in soil layers B and C (in the latter only at the highest application rate), which is surprising since most of these worms do occur in layer A (51.1 % in the control, Table 61). However, in the two lower layers there are still enough worms (28.1 and 20.6 %, referring to 74 and 54 individuals) in order to allow the identification of effects. The other two mineral dwellers, *F. connata* and *F. dura*, were affected either in soil layer A at sampling days 140 and 189, or in soil layers A, B, and C and different soil layers (day 14 or day 189, Table 61). Looking at the absolute numbers of *F. dura* collected (9 individuals) it becomes clear that the detection of robust statistical differences is difficult, even if all effects detected were 100 % decrease in individual numbers.

In contrast, the effect pattern found for *F. connata* seems to be more reliable: This mineral dweller was mainly and in high numbers found in soil layer A, which fits to the effect pattern found. The species *Enchytraeus* GRAN, classified as being an intermediate, occurs in high numbers in soil layer A, where also significant effects were found (Table 61). No explanation can be given why on the very late sampling date on day 364 effects were found in soil layer C, but the low numbers found in this layer in the control (see Figure 50) do not allow a robust interpretation of data. No comparison with literature data is possible here, partly because the number of observations in other studies is low to very low - mainly for *A. dzwilloi* and for *F. dura* which are not well known at all. In addition, often a group of closely related species with unknown ecology (mainly E. GRAN) might have been studied (Schmelz & Collado 2010).

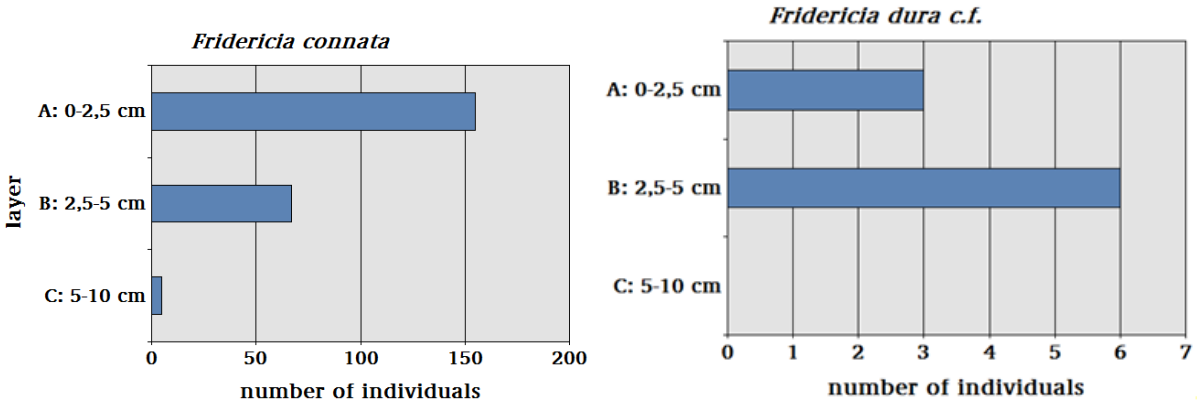

Figure 64 Vertical distribution of the enchytraeid species *Fridericia connata* and *Fridericia dura c.f.* in the control. Shown is the total number of individuals captured in all soil cores on all sampling dates.

Table 61 Summary of statistical analysis of four different enchytraeid taxa. Results are given for the different soil layers (A-C) and different sampling dates (14 days-364 days after application). Red: decrease of abundance ≥ 50% Grey: decrease of abundance < 50% X: no data available. Blank fields: data not sufficient for statistical calculation. Bold: significant difference (p-value Williams-test < 0.05); (see also appendix 1).

Imidacloprid 0.75 kg a.s./ha		*Achaeta "dzwilloi"* days after application				
layer		14	42	140	189	364
A	0 - 2.5 cm	70	28	89	33	71
B	2.5 - 5 cm	-111	**-733**	-33	33	100
C	5 - 10 cm	33	14	50	X	50
all layers		23	-106	41	33	81

Imidacloprid 2 kg a.s./ha		*Achaeta "dzwilloi"* days after application				
layer		14	42	140	189	364
A	0 - 2.5 cm	0	-112	-67	100	57
B	2.5 - 5 cm	-100	**-417**	67	33	54
C	5 - 10 cm	38	**-100**	100	X	0
all layers		-4	-164	0	50	48

Imidacloprid 0.75 kg a.s./ha		*Enchytraeus sp. GRAN* days after application				
layer		14	42	140	189	364
A	0 - 2.5 cm		-633	-300	-100	-26
B	2.5 - 5 cm	33		-100	100	
C	5 - 10 cm	100		-100	X	100
all layers		20	-900	-200	75	-33

Imidacloprid 2 kg a.s./ha		*Enchytraeus sp. GRAN* days after application				
layer		14	42	140	189	364
A	0 - 2.5 cm		-100	**-600**	**-900**	**-195**
B	2.5 - 5 cm	33		100	-43	
C	5 - 10 cm	100		-100	X	**-400**
all layers		-20	-300	**-300**	-150	**-233**

Imidacloprid 0.75 kg a.s./ha		*Fridericia connata* days after application				
layer		14	42	140	189	364
A	0 - 2.5 cm	44	-107	47	**100**	4
B	2.5 - 5 cm	-5	-100		-100	
C	5 - 10 cm	-300			X	
all layers		-2	-117	29	78	4

Imidacloprid 2 kg a.s./ha		*Fridericia connata* days after application				
layer		14	42	140	189	364
A	0 - 2.5 cm	83	-3	**100**	**100**	92
B	2.5 - 5 cm	29	14		-300	
C	5 - 10 cm	-260			X	
all layers		34	0	**100**	56	88

Imidacloprid 0.75 kg a.s./ha		*Fridericia dura cf.* days after application				
layer		14	42	140	189	364
A	0 - 2.5 cm		100			
B	2.5 - 5 cm	**100**	100		100	
C	5 - 10 cm				X	
all layers		100	100		100	

Imidacloprid 2 kg a.s./ha		*Fridericia dura cf.* days after application				
layer		14	42	140	189	364
A	0 - 2.5 cm		100		↑	
B	2.5 - 5 cm	**100**	100		100	
C	5 - 10 cm	↑			X	
all layers		0	50		-500	

The multivariate statistical analysis with PRC and RDA as well as the diversity endpoints showed no significant effects on the community of enchytraeids except of one, the evenness at day 189 for the higher Imidacloprid concentration (PRC and results of diversity endpoints not presented). Both indices (Steinhaus, Stander) did not show any differences on any sampling date or treatment (Figure 65).

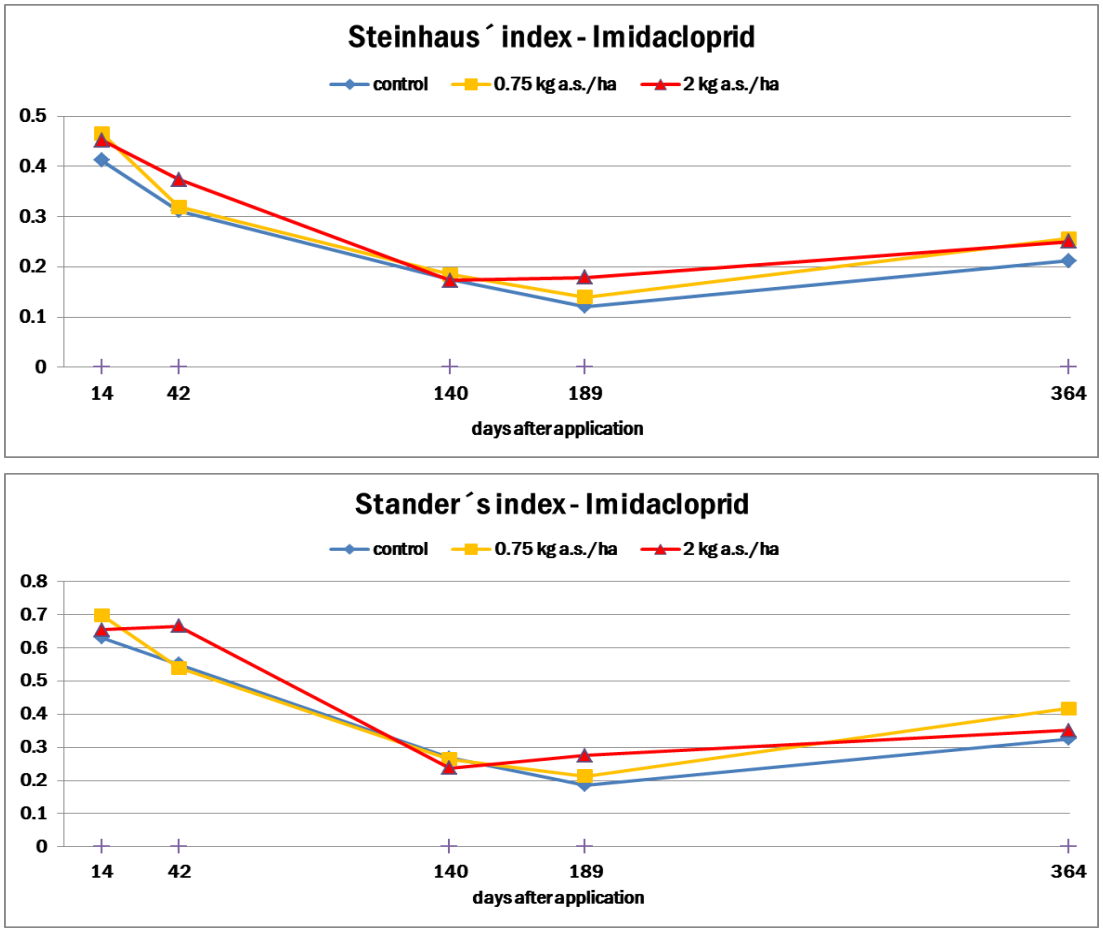

Figure 65 Similarity of Enchytraeid diversity summed up over soil layers A-C (above) Steinhaus index (below) Standers index. Calculation is based on 10 replicates for the control and 5 replicates for each treatment.

Summarising, the population of enchytraeids was not significantly affected by the treatment of Imidacloprid. Analyzed as total abundances, on all sampling dates in all soil layers their numbers in the treatments were in same range as those of the control.

A lack of overall effects was observed for the community parameters PRC and species diversity. In fact only at sampling day 140 the endpoint Evenness indicates an effect at the high application rate of this compound on the enchytraeid community (data not shown).

4.4.4.4 Effects on earthworms

Imidacloprid did not affect earthworms consistently through the experiment, but significant differences between control and treatments were found, especially on the last sampling date (Figure 66, Table 62). The earthworm numbers were almost similar both in the control and the lower application rate at day 14 and day 140. Also their vertical distribution was similar; i.e. at sampling day 14 the highest numbers were not found close to the surface but in 5 to 10 cm depth. Numbers in the layer A and in the deepest layer (E) were again on the same level. On the second sampling date for earthworms at day 140, this pattern changed, i.e. on average more or less similar and low numbers were found in all soil layers. However, on the third sampling date for earthworms at day 364, the earthworm numbers were significantly higher in the uppermost soil layer A of the control compared to both treatments, but variability was also high. In the soil layer B and C, the high application rate of Imidacloprid caused a significant decrease of earthworm numbers. Again, these differences between sampling dates indicate seasonal influences, at least at the start of the study.

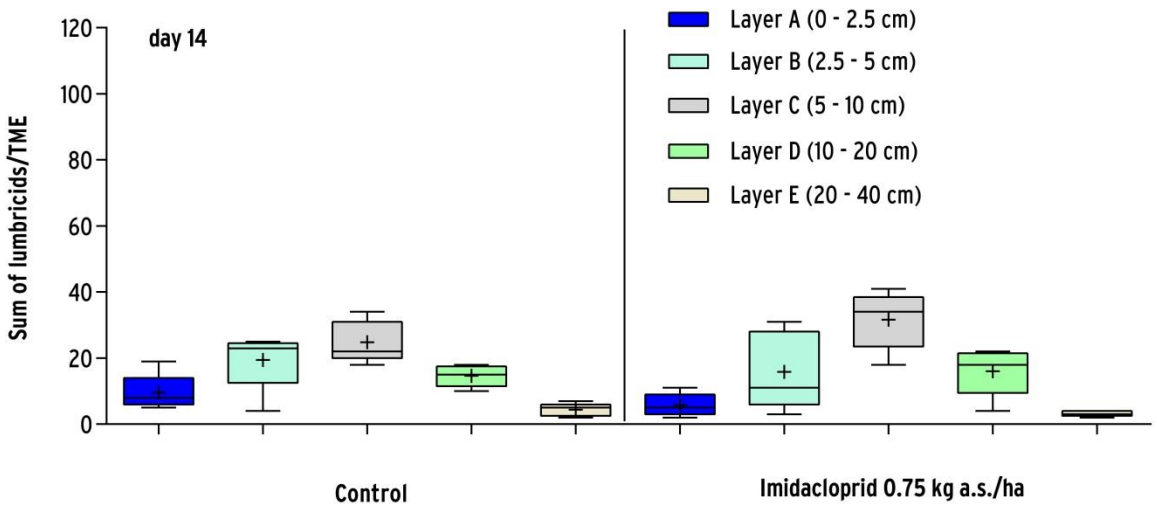

Figure 66 caption see below

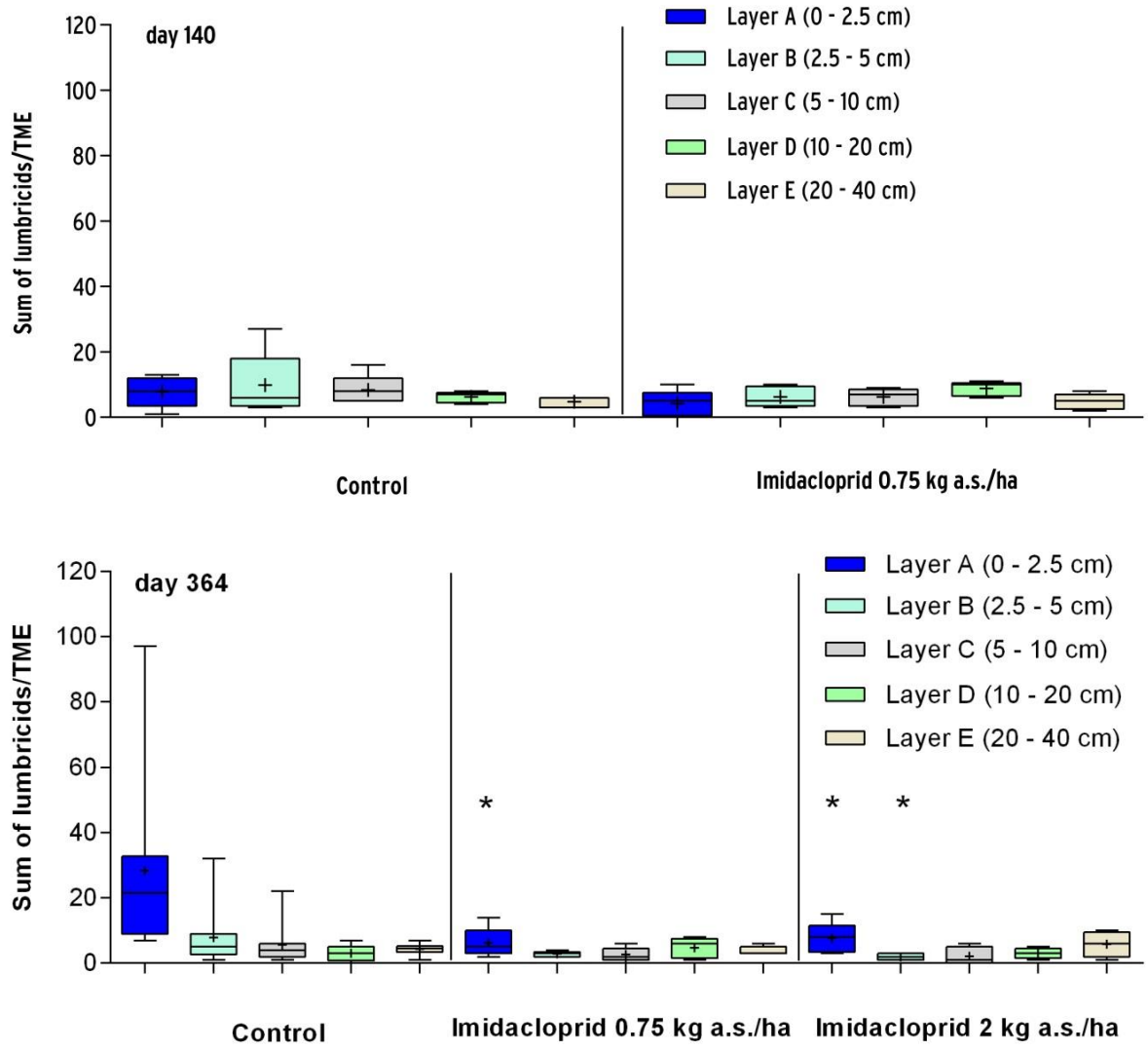

Figure 66 Total abundance of lumbricids in the soil layers of the TMEs in study [1] (5 replicates for control at day 14 and 140; 10 replicates for control at day 364; 5 replicates for imidacloprid treatments). cross: mean; *: significance (p-value Williams-test < 0.05). Sampling dates at day 14, 189, 364 from top to bottom

Table 62 Decrease of total abundance [%] of lumbricid species in the different soil layers on different sampling dates, 14, 140 and-364 days after application. Red: decrease more than 50% in comparison to the control; grey: less than 50% decrease in comparison to the control; Bold: significant effects (p-value <0.05, Mann-Whitney Rank Sum); calculation is based on 10 replicates for the control and 5 replicates for the respective treatment.

Imidacloprid 0.75 kg a.s./ha

		days after application				
	layer	14	42	140	189	364
A	0 - 2.5 cm	40	X	46	X	**75**
B	2.5 - 5 cm	19	X	37	X	54
C	5 - 10 cm	-27	X	26	X	42
D	10 - 20 cm	-10	X	**-42**	X	-50
E	20 - 40 cm	27	X	0	X	2
	all layers	1	X	28	X	**35**

Imidacloprid 2 kg a.s./ha

		days after application				
	layer	14	42	140	189	364
A	0 - 2.5 cm	X	X	X	X	**71**
B	2.5 - 5 cm	X	X	X	X	**72**
C	5 - 10 cm	X	X	X	X	42
D	10 - 20 cm	X	X	X	X	0
E	20 - 40 cm	X	X	X	X	-32
	all layers	X	X	X	X	**55**

Table 63 Presence and mean abundance of captured lumbricid species in the control TMEs (10 replicates) and the two different application rates of the treatment TMEs (5 replicates). The Treatment mean is calculated as the mean abundance of lumbricid species in all treatment TMEs (10 replicates), 5 with higher and 5 with lower pesticide rate.

	Control	**Imidacloprid**		
		Treatment mean	*0.75 kg a.s./ha*	*2 kg a.s./ha*
Number of replicates	**10**	**10**	**5**	**5**
TME				
Juveniles	59.8	23.4	41.0	5.8
Aporrectodea caliginosa	28.8	13.1	22.8	3.4
Lumbricus terrestris	6.0	5.2	7.0	3.4
Octolasion cyaneum	5.5	1.7	2.8	0.6
Aporrectodea rosea	4.1	2.5	4.2	0.8
Lumbricus rubellus	2.9	2.4	4.4	0.4
Lumbricus castaneus	2.0	0.6	1.2	-
Number of taxa	**6**	**6**	**6**	**5**

The structure of the earthworm community was not changed with regards to presence of species but the numbers of species decreased slightly at the higher application rate (Table 63). There, the epigeic species *Lumbricus castaneus* could not be recorded. However, it occurred rarely in the control, too.

In the TMEs treated with Imidacloprid, significant effects were found for 3 out of 6 species in at least one single layer on one sampling date (cp. appendix 1). Additionally, one group of species was effected, i.e. juveniles of the genus *Aporrectodea*. Actually, these animals are morphologically very difficult to distinguish from juveniles of the genus *Allolobophora*, but since no adult from this genus had ever been found in this study, it will not be considered likely. The significant differences on the latest sampling date are mainly caused by these juveniles (Table 64). These usually very small worms belong to the ecological group of endogeics. They are living in the uppermost soil layer while adult endogeics more often occur in deeper layers (Figure 67, Figure 53). Regarding the phenology of juvenile individuals of this group, the number decreased in the control until day 140 and increased up to nearly 30 individuals per TME at day 364. This increase did not happen in any of the treatments. The high sensitivity of the juveniles is also indicated by the significant effects observed in soil layer A at day 140. The assumption that

Imidacloprid is causing these differences at day 364 is further supported by the fact that adult endogeics, here *A. caliginosa* and *A. rosea*, are only affected on the last sampling date. *A. rosea* did occur mainly in deeper soil layers (Figure 67), mainly in layer C, where significant effects could be detected. Even the epigeic species *L. castaneus* (Figure 54) is only affected in soil layer A at day 364 and in layer C at day 14 (Table 64). Since the numbers of this last species are rather low, the interpretation of these results is difficult.

Summarizing these findings, it seems that the test chemical Imidacloprid has a significant effect on earthworms of different species and age classes – but almost always on those in the uppermost three soil layers (0 – 10 cm). However, the lack of significant effects in deeper soil layers may partly be caused by a methodological problem: in these layers the number of earthworms is almost always quite low, even if they are the preferred depth for species like *Lumbricus terrestris* and *Octolasion cyaneum*. Hence, the statistical power of the data for the deeper soil layer is low.

The different diversity endpoints analyzed were showing an inhomogeneous pattern of effects (Table 65). No effects on the community could be measured by the multivariate statistical method of PRC and RDA for any sampling date (PRC not shown). The number of taxa were significantly changed for day 140 in the lower application rate and day 364 for both application rates. Only the Shannon Index and the Evenness showed a significant effect 14 days after application. They also showed a significant change at day 364 for the higher application rate. Both similarity-indices (Steinhaus, Stander) did not show any statistically significant differences between control and treatments on any sampling date (Figure 68).

Summarising, the earthworm diversity was negatively affected almost exclusively on the last sampling date 364 days after application. Statistically significant effects were seen for total abundances in both Imidacloprid application rates in the upper soil layers. (A and B till 5 cm depth). In layer C (5-10 cm depth) effects were above 40 % but not statistically significant. In deeper soil layers, (D and E from 10 to 40 cm depth) total abundances decrease minimally or increased. This pattern was observed also at species level, and was consistent for the uppermost soil layer. Diverstity endpoint like the Shannon Index and Eveness confirmed this for the higher application rate.

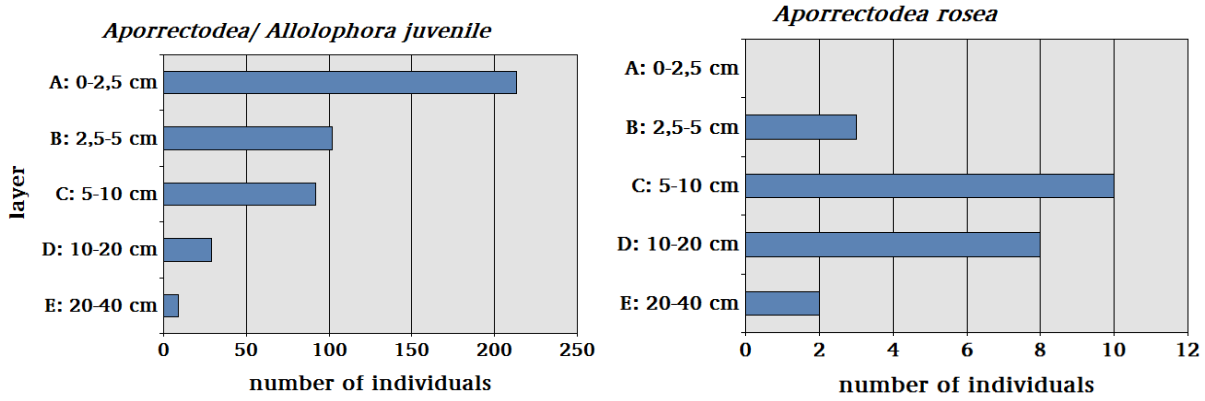

Figure 67 Vertical distribution of the juvenile individuals of the lumbricid group Aporrectodea/ Allolophora and the species *Aporrectodea caliginosa* in the control. Shown is the total number of individuals captured in all TMEs at all sampling dates in the study [1].

Table 64 Summary of statistical analysis of four different lumbricid taxa. Results are given for the different soil layers (A-E) and different sampling dates (14 days-364 days after application). Red: decrease of abundance ≥ 50% Grey: decrease of abundance < 50% X: no data available. Blank fields: data not sufficient for statistical calculation. Bold: significant difference (p-value Williams-test < 0.05); (see also appendix 1).

Imidacloprid 0.75 kg a.s./ha		*Aporrectodea / Allolobophora spec.* days after application		
layer		14	140	364
A	0 - 2.5 cm	60	**88**	**91**
B	2.5 - 5 cm	-5	53	**75**
C	5 - 10 cm	-55	-50	**88**
D	10 - 20 cm	-69	71	-33
E	20 - 40 cm	↑	-150	-71
all layers		**-34**	42	**80**

Imidacloprid 2 kg a.s./ha		*Aporrectodea / Allolobophora spec.* days after application		
layer		14	140	364
A	0 - 2.5 cm	X	X	**86**
B	2.5 - 5 cm	X	X	**75**
C	5 - 10 cm	X	X	**82**
D	10 - 20 cm	X	X	56
E	20 - 40 cm	X	X	-14
all layers		X	X	**80**

Imidacloprid 0.75 kg a.s./ha		*Lumbricus castaneus* days after application		
layer		14	140	364
A	0 - 2.5 cm		40	**100**
B	2.5 - 5 cm		50	
C	5 - 10 cm	↑	100	
D	10 - 20 cm			
E	20 - 40 cm			
all layers			50	**100**

Imidacloprid 2 kg a.s./ha		*Lumbricus castaneus* days after application		
layer		14	140	364
A	0 - 2.5 cm	X	X	**100**
B	2.5 - 5 cm	X	X	
C	5 - 10 cm	X	X	
D	10 - 20 cm	X	X	
E	20 - 40 cm	X	X	
all layers		X	X	**100**

Imidacloprid 0.75 kg a.s./ha		*Aporrectodea caliginosa* days after application		
layer		14	140	364
A	0 - 2.5 cm	100	70	**55**
B	2.5 - 5 cm	0	59	63
C	5 - 10 cm	-3	23	-100
D	10 - 20 cm	5	-100	-300
E	20 - 40 cm	100	50	-100
all layers		5	42	17

Imidacloprid 2 kg a.s./ha		*Aporrectodea caliginosa* days after application		
layer		14	140	364
A	0 - 2.5 cm	X	X	**70**
B	2.5 - 5 cm	X	X	75
C	5 - 10 cm	X	X	50
D	10 - 20 cm	X	X	33
E	20 - 40 cm	X	X	-300
all layers		X	X	**51**

Imidacloprid 0.75 kg a.s./ha		*Aporrectodea rosea* days after application		
layer		14	140	364
A	0 - 2.5 cm			
B	2.5 - 5 cm	100	100	100
C	5 - 10 cm	0	40	**100**
D	10 - 20 cm	-17	-150	
E	20 - 40 cm	0		
all layers		0	-13	20

Imidacloprid 2 kg a.s./ha		*Aporrectodea rosea* days after application		
layer		14	140	364
A	0 - 2.5 cm	X	X	
B	2.5 - 5 cm	X	X	-100
C	5 - 10 cm	X	X	50
D	10 - 20 cm	X	X	
E	20 - 40 cm	X	X	
all layers		X	X	-60

Table 65 Summary of the results for the statistical diversity analyses, PRC (p-value t-test < 0.05 of PCA sample scores), number of taxa, Shannon and Eveness, *: significance (p-value Williams-test < 0.05) of lumbricids treated with Imidacloprid (left) application rate 0.75 kg a.s./ha (right) 2 kg a.s./ha. Database: 10 replicates of control TMEs and 5 replicates for each treatment.

Imidacloprid 0.75 kg a.s./ha		days after application			
all layers	14	42	140	189	364
PRC		X		X	
Number of taxa		X	*	X	*
Shannon	*	X		X	
Eveness	*	X		X	*

Imidacloprid 2 kg a.s./ha		days after application			
all layers	14	42	140	189	364
PRC	X	X	X	X	
Number of taxa	X	X	X	X	*
Shannon	X	X	X	X	*
Eveness	X	X	x	X	*

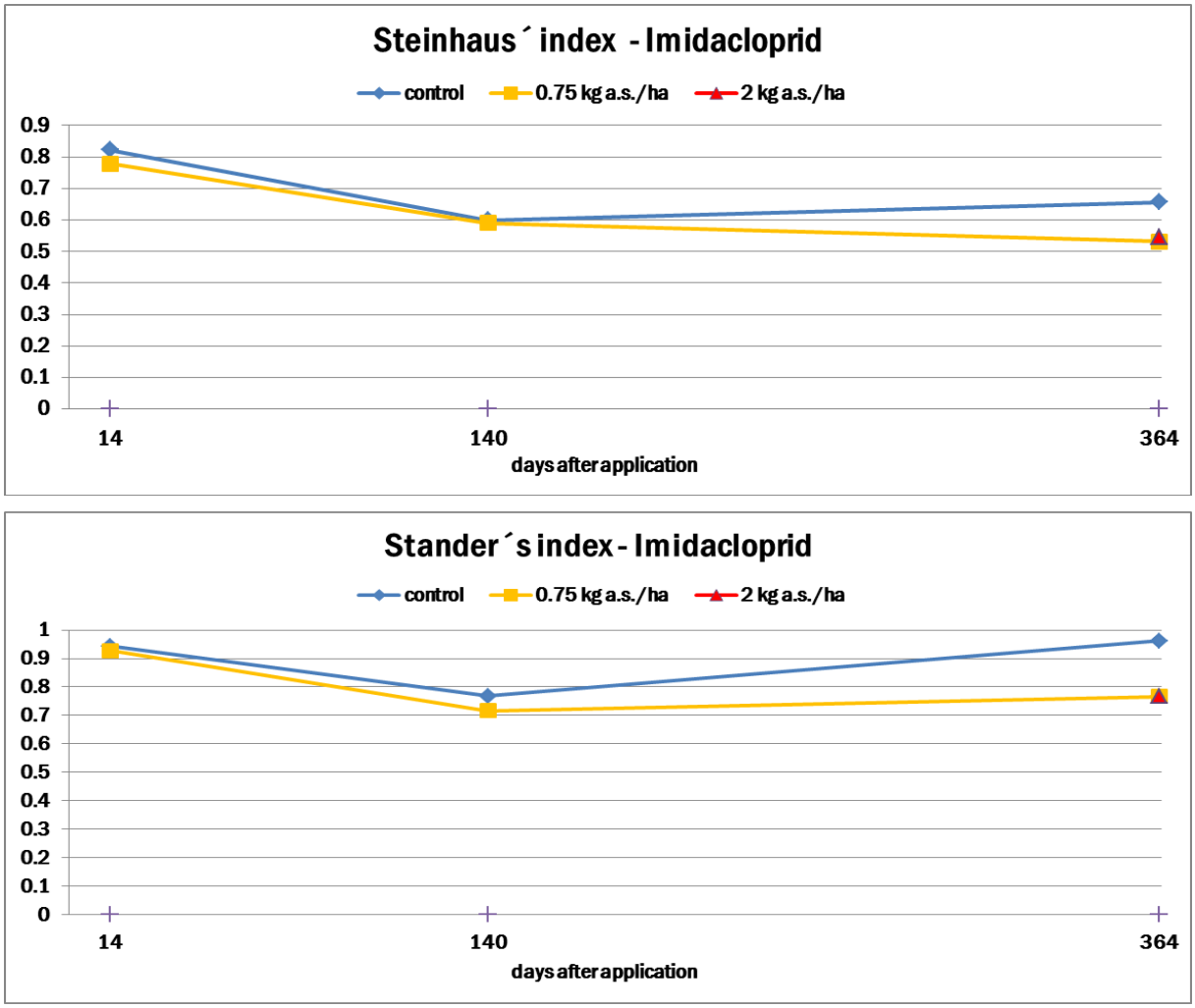

Figure 68 Similarity of Lumbricids diversity summed up over all soil layers (above) Steinhaus index (below) Standers index. Calculation is based on 5 replicates for the control and 5 replicates for each treatment at day 14 and day 140. Calculation is based on 10 replicates for the control and 5 replicates for each treatment at day 364.

5 Results of the experimental indoor study [2] testing the pesticides Lindane and Imidacloprid

5.1 Analyses of the leachate

5.1.1 Analyses of Lindane

During the experiment, only small amounts of radioactivity were found in the leachate. Altogether 420 ng of Lindane equivalents were detected in the total volume of 300 ml. The volume of leachate fitted very well to the volume occurring in the outdoor study. (cp. Chapter 4.2.3) But the amount of Lindane found in the water corresponded to less than 0.001 % of the initially applied amount of Lindane; and it was much lower than 0.05 %, which was found in the outdoor study. Also, the average concentration of Lindane equivalents of 1.15 ± 0.84 µg/L (average of the measured concentrations) was lower than 3.8 ± 4.2 µg/L, comparing to its contents detected outdoor. All results are summarised in Table 66.

Table 66 Results of the analysis of the leachate of the indoor Lindane study. Not detectable = n.d., the limit of quantification on 26th July was 1.1 µg/L.

Date	Volume ml of the sample	Leachate Conc in µg/L	Total ng lindane-equivalents
15.6.	74	n.d.	n.d.
21.7.	5.4	0.78	4.24
26.7.	15	<1.1	<17
10.8.	17	n.d.	n.d.
20.8.	81	1.76	142.55
21.8.	48	1.92	91.92
9.10.	8.2	1.4	11.47
15.10.	59	2.27	134.07
27.10.	21.5	1.68	36.08
SUM May-Nov	300.3		420.32
Average		1.15 ± 0.84	

5.1.2 Analyses of Imidacloprid

The obtained results for Imidacloprid were similar to those ones for Lindane. Calculated as Imidacloprid equivalents 442.84 ng were determined in the combined volume of leachate (247.3 ml). The amounts of leachate correlate well to its volume in the outdoor study. However, also a smaller amount of this substance was found in the leachate (0.03 % of the applied amount compared to 0.36 %) than those recovered in the outdoor study. The average concentration of Imidacloprid equivalents of 1.83 ± 1.33 µg/L (average of the measured concentrations) was also lower than 13.5 ± 34.8 µg/L detected in outdoor experiment. All results are presented in Table 67.

Table 67 Shown are the results of the analysis regarding the leachate of the indoor Imidacloprid study.

Date	Volume ml of the sample	Leachate Conc. in µg/L	Total ng imidacloprid-equivalents
15.6.	38	1.97	74.94
21.7.	7	0.31	2.16
26.7.	13	3.59	4.66
10.8.	11	3.89	4.28
20.8.	85	2.33	198.06
21.8.	49	2.32	113.46
9.10.	6.4	0.67	4.29
15.10.	45	0.7	31.31
27.10.	14.5	0.67	9.69
SUM May-Nov	247.3		442.84
Average ± SD		1.83 ± 1.33	

5.2 Results of the Lindane laboratory study – soil samples

In samples of the indoor study, using radiolabelled test substances, concentrations were determined based on the quantification of radioactivity.

Since the radiolabel after metabolisation will be present in the active ingredient, but also in primary (xenobiotic) and secondary (biogenic) metabolites, and therefore a distinction of the nature of the residues based on the amount of radioactivity is not possible, all results are given in mg “Lindane-equivalents”/kg dry matter. Three different concentrations were determined.

- Aliquots of the homogenised soil samples and grass were combusted, the radioactivity were trapped in a scintillation cocktail and measured. The calculated Lindane equivalents are the total residues determined in the sample.
- Soil samples were extracted similar to samples of the field study. The radioactivity of the extracts were measured and calculated as Lindane equivalents (= extractable residues).
- Extracted soil samples were combusted and the measured radioactivity calculated as Lindane equivalents (= non extractable residues, NER).

The results of the combustion of the soil and the grass before extraction combined with the weights of the samples are used to calculate the total amount of Lindane equivalents in the soil.

5.2.1 Results of the combustion of unextracted soil and grass

At each sampling time (except T0) the vegetation of all TMEs was cut to about 2 cm of its height (similar to the outdoor study). Radioactivity which was bound to the grass layer was therefore removed periodically to a large extent. Small amounts of radioactivity were detected in the leachate. Volatile residues, as parent compound, metabolite or as carbon dioxide, could not be trapped in the column experiment, and, therefore, are not included in the radioactivity balance of the experiment. The results of the combustion of unextracted samples at separate sampling dates are shown in Table 68- Table 72. The concentration of Lindane equivalents in µg/g (mg/kg) of dry matter, the total amount of Lindane equivalents of a soil core (sum of the layers) and the percentages with respect to the total applied amount are shown. Figure 69 shows the percentage distribution of all dates.

On day one 92 % of the applied amount could be recovered. Nearly 60 % of the residues were found in the upper centimetre (0-1 cm) and more than 30 % were retained in the grass cover. The content of Lindane in the grass layer rapidly decreased during the course of the experiment to about 2.5 %. The radioactivity of the top soil layer (0-1 cm) decreased more slowly to nearly 20 % of the applied amount. In the second soil layer (1 – 2.5 cm) radioactivity increased from 0.6 % at day 1 to 27 % at day 140 and decreased until the end of the experiment (189 days) slightly to 17 % of the applied amount. The result of T2 (42 days) is regarded as outlier (3.7 %). In the deeper layers radioactivity is detectable starting at T2, day 42, and increased during the incubation to a maximum of 1.5 %. In the leachates at day 42 and later sampling times only traces of Lindane are detectable (less than 10-3 % of the applied amount (cp. Chapter 5.1.1).

Table 68- Table 72 The concentration of Lindane equivalents in soil dry matter (DM), the total amount of Lindane equivalents in each layer and the proportion of Lindane equivalents of the applied amount of Lindane is given. * = µg Lindane / g fresh weight. DM = dry matter

Table 68 Concentration of Lindane T0, Day 1

	µg Lindane-eq./g DM	µg / total sample	% of the applied amount
Grass	710.39 ± 186.19*	5140.42 ± 1347.29	32.72 ± 8.58
0-1 cm	179.46 ± 46.28	9204.71 ± 2373.57	58.60 ± 15.11
1-2.5 cm	0.82 ± 0.26	90.01 ± 28.79	0.57 ± 0.18
2.5-5 cm	0.04 ± 0.01	8.23 ± 2.81	0.05 ± 0.02
5-10 cm	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
10-20 cm	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Leachate	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Sum [%] of the applied amount:		14443 ± 3752	*91.9 ± 23.9*

Table 69 Concentration of Lindane T1, Day 14

	µg Lindane-eq./g DM	µg / total sample	% of the applied amount
Grass	381.67 ± 88.02*	2557.18 ± 589.73	16.28 ± 0.00
0-1 cm	157.25 ± 24.25	6795.40 ± 1048.07	43.26 ± 6.67
1-2.5 cm	18.27 ± 2.51	2166.17 ± 298.07	13.79 ± 1.90
2.5-5 cm	0.14 ± 0.01	34.75 ± 2.84	0.22 ± 0.02
5-10 cm	0.10 ± 0.00	47.32 ± 0.00	0.30 ± 0.00
10-20 cm	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Leachate	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Sum [%] of the applied amount:		11601 ± 1349	73.8 ± 12.3

Table 70 Concentration of Lindane T2, Day 42

	µg Lindane-eq./g DM	µg / total sample	% of the applied amount
Grass	109.76 ± 10.68*	869.05 ± 84.56	5.53 ± 0.54
0-1 cm	135.29 ± 23.81	7683.62 ± 1352.41	48.92 ± 8.61
1-2.5 cm	3.90 ± 0.38	581.57 ± 56.86	3.70 ± 0.36
2.5-5 cm	0.23 ± 0.08	43.38 ± 15.43	0.28 ± 0.10
5-10 cm	0.11 ± 0.02	47.82 ± 6.70	0.30 ± 0.04
10-20 cm	0.02 ± 0.00	17.02 ± 0.00	0.11 ± 0.00
Leachate	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Sum [%] of the applied amount:		9242 ± 1516	58.8 ± 9.6

Table 71 Concentration of Lindane T3, Day 140

	µg Lindane-eq./g DM	µg / total sample	% of the applied amount
Grass	13.42 ± 5.23*	397.00 ± 154.61	2.53 ± 0.98
0-1 cm	137.72 ± 23.52	3512.49 ± 599.75	22.36 ± 3.82
1-2.5 cm	53.39 ± 4.34	4299.05 ± 349.73	27.37 ± 2.23
2.5-5 cm	3.26 ± 0.21	675.54 ± 44.51	4.3 ± 0.28
5-10 cm	0.26 ± 0.06	132.52 ± 30.85	0.84 ± 0.20
10-20 cm	0.12 ± 0.03	151.71 ± 34.81	0.97 ± 0.22
Leachate		0.24 ± 0.00	0.00 ± 0.00
Sum [%] of the applied amount:		9168 ± 1214	58,4 ± 7.7

Table 72 Concentration of Lindane T4, Day 189

	µg Lindane-eq./g DM	µg / total sample	% of the applied amount
Grass	15.61 ± 1.09*	368.74 ± 25.65	2.35 ± 0.16
0-1 cm	86.63 ± 16.39	2881.48 ± 545.07	18.34 ± 3.47
1-2.5 cm	33.61 ± 4.84	2666.76 ± 384.35	16.98 ± 2.45
2.5-5 cm	2.73 ± 0.63	489.46 ± 112.33	3.12 ± 0.72
5-10 cm	0.45 ± 0.09	236.56 ± 48.14	1.51 ± 0.31
10-20 cm	0.08 ± 0.02	91.64 ± 19.03	0.58 ± 0.12
Leachate		0.18 ± 0.00	0.00 ± 0.00
Sum [%] of the applied amount:		6735 ± 1135	42.9 ± 7.2

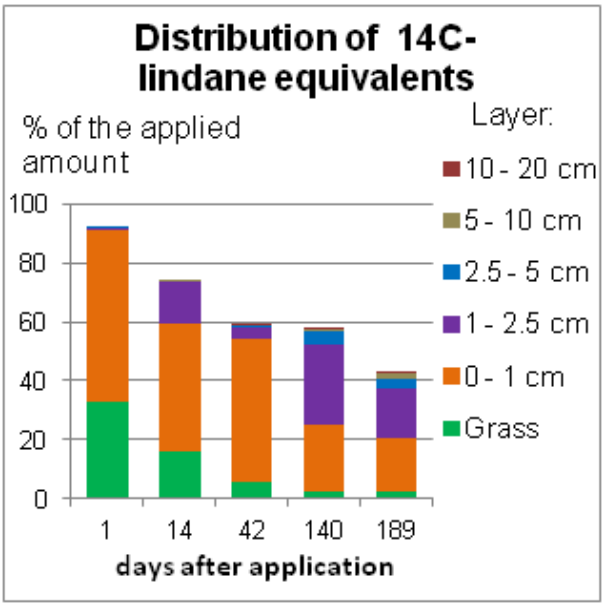

Figure 69 Distribution of ^{14}C -Lindane equivalents in the indoor study. Given is the total amount analysed (combustion of not extracted soil) in percent of the applied amount of Lindane of 15708 µg per TME.

5.2.2 Results of the extractable and non-extractable residues - time course within sample cores

In this section the course of concentrations of extractable and non-extractable residues within a sample core during the experiment is shown. The percentage distribution of Lindane-equivalents on a single day in the different layers can be found in Table 73 (100 % = Sum of the absolute amount in µg in the soil core found in the extractable and in the non-extractable fraction of the different layers).

Figure 70 summarises in the left charts the concentrations of Lindane equivalents in mg/kg dry matter in the different soil layers and in the right charts the relative distribution of the substance

in the extractable and the non-extractable fraction of one sample (100 % = concentration of Lindane-equivalents measured through combustion of the not extracted soil sample. The calculations were made with following equations:

$$\frac{\text{Conc}_{\text{Ext}}}{\text{Conc}_{\text{Tot}}} * 100 = \%\text{Ext} \qquad \text{Equation 12}$$

$$\frac{\text{Conc}_{\text{NER}}}{\text{Conc}_{\text{Tot}}} * 100 = \%\text{NER} \qquad \text{Equation 13}$$

Conc_{Ext} = Concentration of extractable Lindane equivalents in mg/kg dry soil matter

Conc_{Tot} = Concentration of total Lindane equivalents in mg/kg dry soil matter (combustion of soil)

Conc_{NER}= Concentration of not extractable Lindane equivalents in mg/kg dry soil matter (combustion of the extracted soil)

Table 73 Distribution of the amount of Lindane equivalents in µg/soil core in percent of the sum of the Lindane equivalents on a day, measured in the extractable and the non-extractable fraction.

		Percent of the sum of extractable and NER				
Soil layers		Day 1	Day 14	Day 42	Day 140	Day 189
0 - 1 cm	Extract	98.28	71.98	87.45	36.82	34.15
	NER	0.94	1.27	3.36	4.70	4.59
1 - 2.5 cm	Extract	0.73	24.54	6.57	40.12	41.89
	NER	0.05	1.61	0.71	7.41	6.89
2.5 - 5 cm	Extract	0.00	0.25	0.44	5.74	6.09
	NER	0.00	0.06	0.10	1.97	1.92
5 - 10 cm	Extract	0.00	0.29	0.56	1.20	2.41
	NER	0.00	0.00	0.16	0.48	1.11
10 - 20 cm	Extract	0.00	0.00	0.33	0.82	0.45
	NER	0.00	0.00	0.33	0.75	0.50
	Sum	100	100	100	100	100

One day after application radioactivity, recalculated as Lindane equivalents, was extracted mainly from the upper layer and only traces were detected in deeper layers (Table 73). On the next sampling dates, some transport of the residues is observed. After 189 days 48.8 % of the substance equivalent were found in the second layer A2 (1 – 2.5 cm) and traces moved to layers below 2.5 cm. The left bar charts of Figure 70 also show most of the substance remaining in the first soil centimetre (0-1 cm), but with increasing incubation time small amounts of the substance are

transported to deeper layers. After 42 days, only little radioactivity is detectable in the deepest layer D. The results of the samples of day 42, especially of layer A2, do not fit well to the concentrations of the other samples (Figure 71 and Figure 72). The analyses of the total, extractable and non-extractable residues, however, match well and the deviation between replicates did not exceed that of the other samples. The possible cause for this outlier is probably an application mistake.

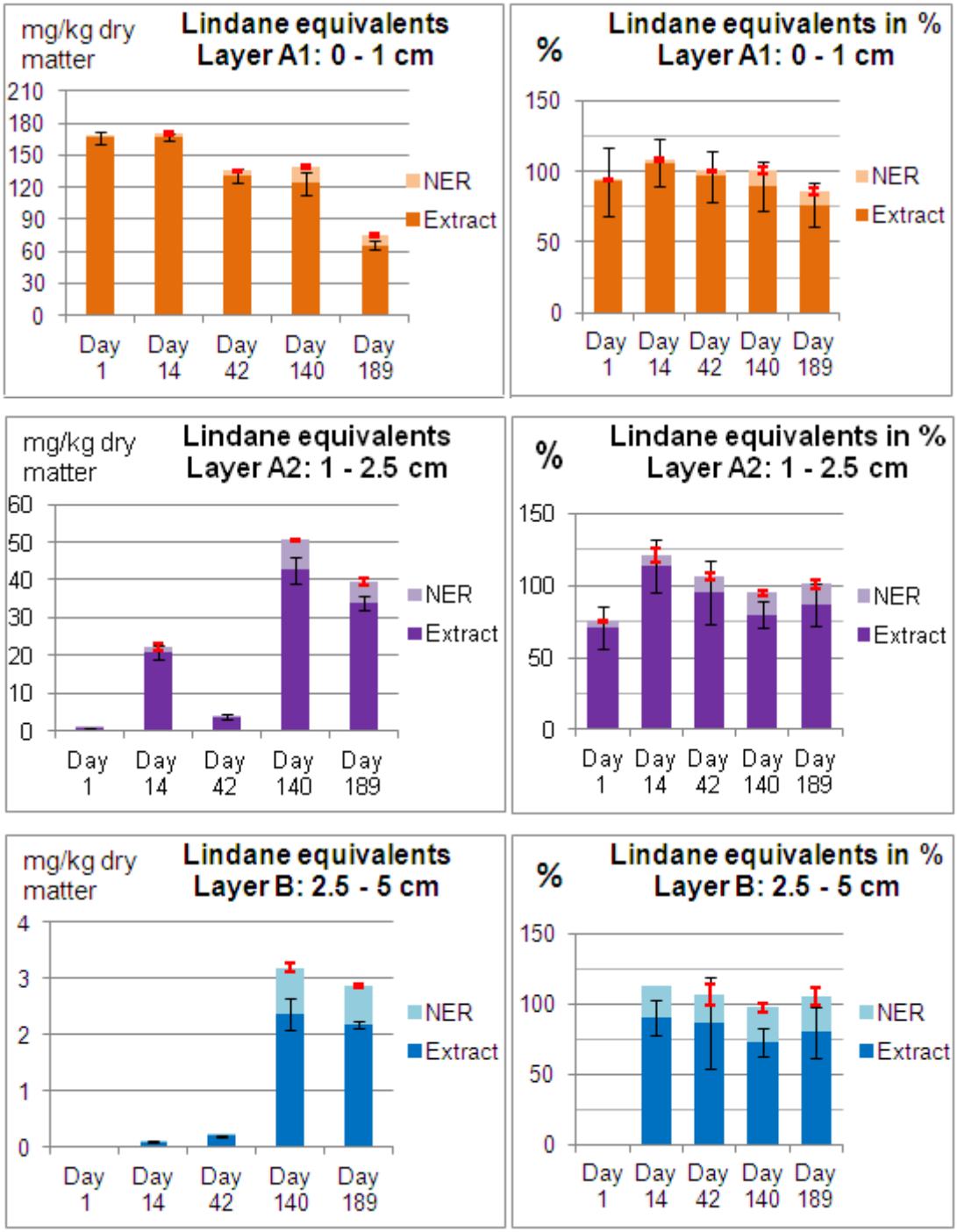

Figure 70 caption see below

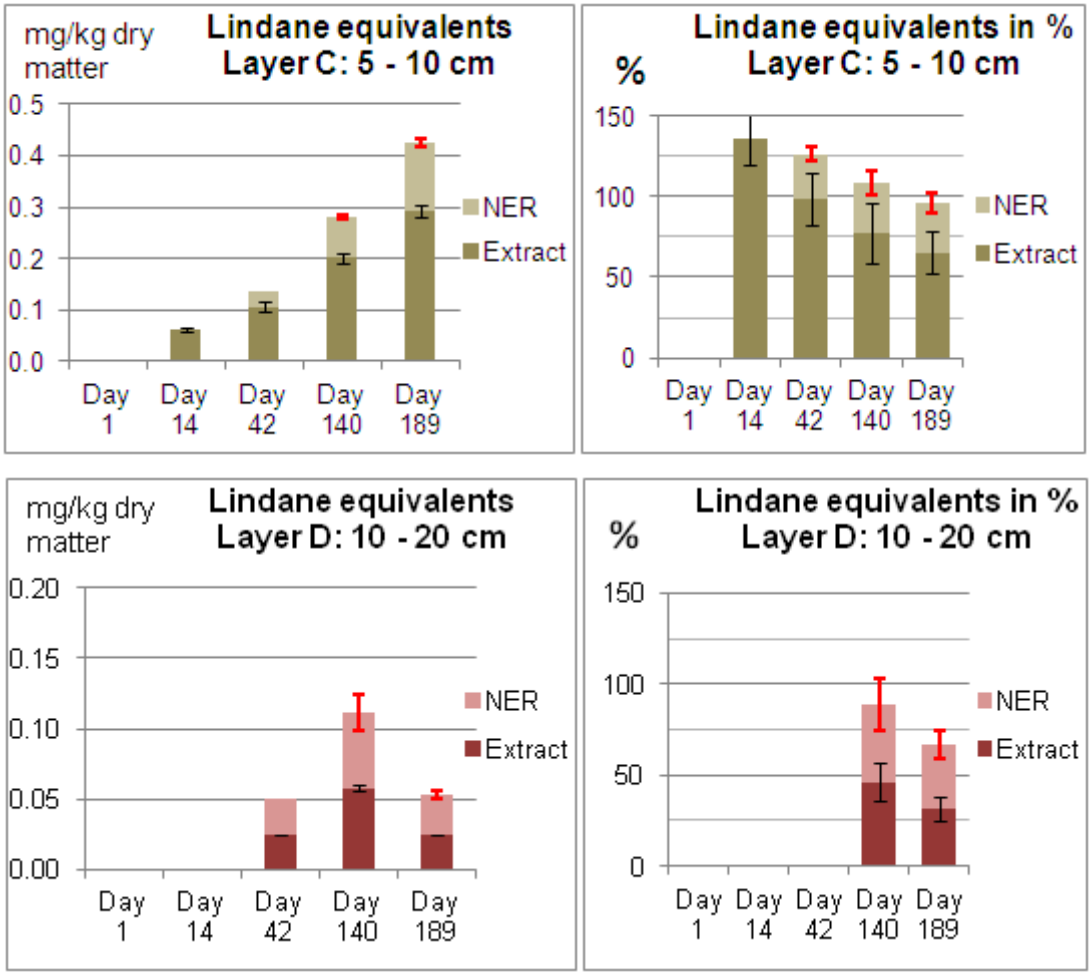

Figure 70 In the left figure the concentration of Lindane equivalents in soil dry matter are given in mg/kg for the respective layer. The right figure show the percentage of the Lindane equivalents of the extractable and the non-extractable residues referring to the total amount of Lindane. This total amount was measured after the combustion of the non-extracted soil sample.

5.2.3 Overview of the Lindane results of the indoor study

All data of the laboratory Lindane study are summarised in this section. The results of the two combustions and of the extraction are given in Table 74. In Figure 71 concentrations of extractable and non-extractable residues in mg/kg dry matter are given.

Figure 72 show separately the time course of the concentrations of the extractable and of the non-extractable residues, respectively. Table 74 confirms the pattern of the distribution of Lindane-equivalents in soil over the trial period shown in Table 73. The sum of the concentration of extractable and of non-extractable residues fits very well to the results of the combustion of the unextracted soil. Therefore, no losses during the extraction occurred. This is also evident in the right column of Figure 70. Greater deviations are due to results near the LOQ.

Figure 71 and Figure 72 visualise the data of Table 74. Also in these figures the results of day 42 appear as outliners. While the concentrations of the extractable residues in the soil layers decrease over time, the content of the non-extractable residues reached a maximum at day 140

which thereafter decreased. Thus, non-extractable residues are subject of remobilisation or (most probably further) degradation.

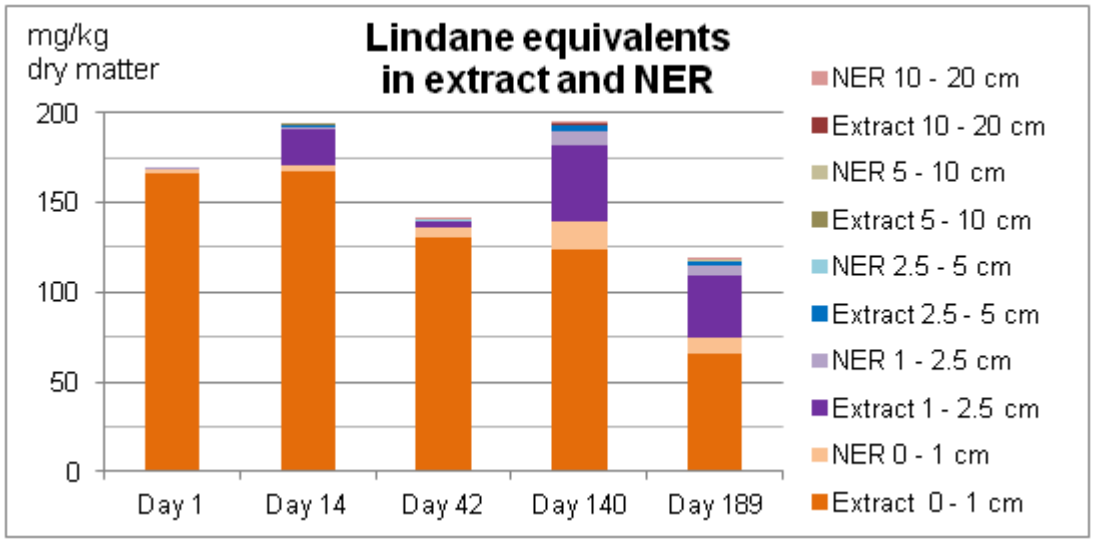

Figure 71 Lindane equivalents concentration of the extractable and the non-extractable residues (Extract and NER, respectively) in the respective soil layers. Summing up of the concentrations in the bars has been chosen for pragmatic reasons, each concentration stands for itself.

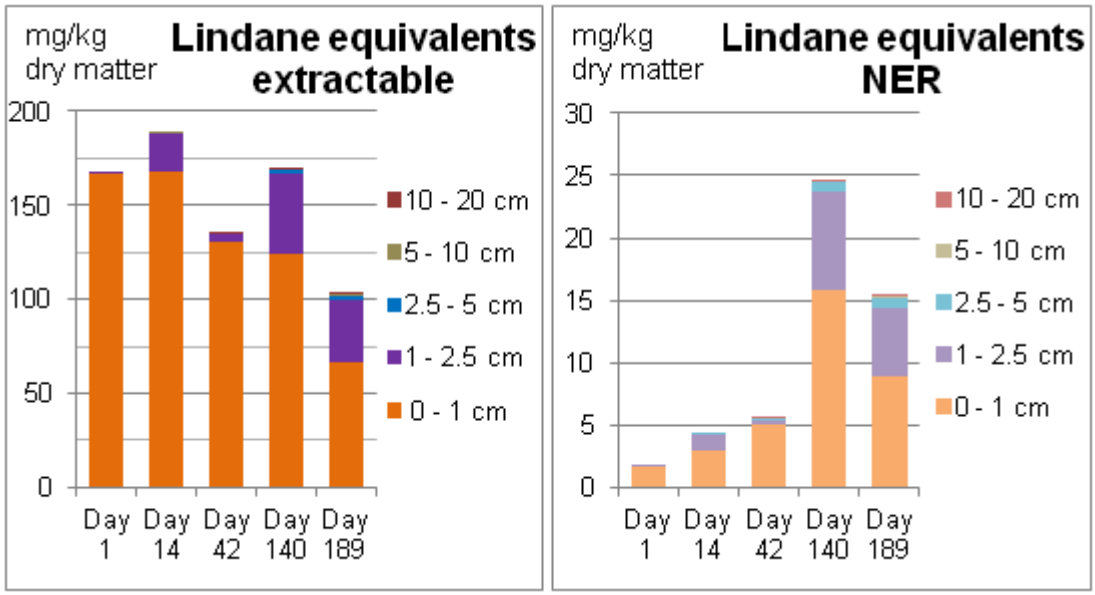

Figure 72 Presented are the calculated Lindane equivalents concentration of the extractable (left figure) and the non-extractable residues (NER, right figure). Summing up of the concentrations in the bars has been chosen for pragmatic reasons, each concentration stands for itself.

Table 74 Results of the radioactive analyses. Means are given in mg/kg dry matter (DM) ± standard derivation. n.d. = not detectable. Concentrations below the limit of quantification are given as <"value of the LOQ". Total = combustion of soil (not extracted), NER = Non-Extractable Residues, Extr + NER = Sum of the results in the extract and in the NER.

Lindane equivalents (mg/kg DM)					
Layer A1 (0 - 1 cm)	Day 1	Day 14	Day 42	Day 140	Day 189
Total	179.5 ± 46.3	157.3 ± 24.3	135.3 ± 23.8	137.7 ± 23.5	86.6 ± 16.4
Extract	166.6 ± 5.7	167.2 ± 3.1	130.5 ± 6.3	123.7 ± 11.0	66.0 ± 4.5
NER	1.60 ± 0.10	2.94 ± 1.35	5.01 ± 0.73	15.77 ± 1.87	8.88 ± 1.39
Extr + NER	168.2 ± 5.7	170.1 ± 3.3	135.6 ± 6.3	139.4 ± 11.1	74.9 ± 4.7
Layer A2 (1 - 2.5 cm)	Day 1	Day 14	Day 42	Day 140	Day 189
Total	0.82 ± 0.14	18.27 ± 2.51	3.90 ± 0.38	53.39 ± 4.34	39.05 ± 6.18
Extract	0.58 ± 0.07	20.78 ± 1.94	3.74 ± 0.78	42.68 ± 3.44	33.94 ± 1.72
NER	<0.08	1.37 ± 0.93	0.40 ± 0.09	7.89 ± 0.32	5.58 ± 0.75
Extr + NER	0.62 ± 0.07	22.14 ± 2.15	4.14 ± 0.79	50.57 ± 3.45	39.52 ± 1.88
Layer B (2.5 - 5 cm)	Day 1	Day 14	Day 42	Day 140	Day 189
Total	<0.08	0.11 ± 0.01	0.23 ± 0.08	3.26 ± 0.21	2.73 ± 0.63
Extract	n.d.	0.10 ± 0.01	0.20 ± 0.02	2.37 ± 0.28	2.18 ± 0.05
NER	n.d.	< 0.05	0.05 ± 0.00	0.81 ± 0.08	0.69 ± 0.03
Extr + NER	n.d.	0.13 ± 0.00	0.25 ± 0.02	3.19 ± 0.29	2.87 ± 0.06
Layer C (5 - 10 cm)	Day 1	Day 14	Day 42	Day 140	Day 189
Total	n.d.	0.05 ± 0.00	0.11 ± 0.02	3.26 ± 0.21	2.73 ± 0.63
Extract	n.d.	0.06 ± 0.00	0.11 ± 0.01	2.37 ± 0.28	2.18 ± 0.05
NER	n.d.	n.d.	< 0.06	0.81 ± 0.08	0.69 ± 0.03
Extr + NER	n.d.	0.06 ± 0.00	0.12 ± 0.01	3.19 ± 0.29	2.87 ± 0.06
Layer D (10 - 20 cm)	Day 1	Day 14	Day 42	Day 140	Day 189
Total	n.d.	n.d.	0.02 ± 0.00	0.12 ± 0.03	0.08 ± 0.02
Extract	n.d.	n.d.	< 0.05	0.06 ± 0.00	< 0.05
NER	n.d.	n.d.	< 0.05	0.05 ± 0.01	0.03 ± 0.00
Extr + NER	n.d.	n.d.	0.04 ± 0.00	0.11 ± 0.01	0.05 ± 0.00

5.3 Results of the Imidacloprid laboratory study – soil samples

The samples with radiolabelled Imidacloprid of the laboratory study were analysed similarly to the samples of Lindane. The radioactivity was measured a) in the unextracted soil, b) in the extracts and c) in extracted soil. The results were calculated as Imidacloprid equivalents. The concentrations were given in mg/kg dry matter and the total amount in a soil layer was given in µg/layer. The grass (unextracted) was also combusted and Imidacloprid equivalents determined.

5.3.1 Results of the combustion of unextracted soil and grass

The results of the combustion of unextracted samples at separate sampling dates are shown in Table 75- Table 79. The concentration of Imidacloprid equivalents in µg/g (mg/kg) of dry matter, the total amount of Imidacloprid equivalents of a total soil core (sum of the layers) and the percentages with respect to the total applied amount are shown. Figure 73 shows the percentage distribution at all sampling dates.

One day after application all of of the applied amount of radioactivity has been recovered (101 ± 26 %). Residues were found mainly in the grass layer (45 %) and in the top centimetre (0-1 cm, 55 %). About 1 % of the applied amount was analysed on day 1 in of the soil between 1 – 5 cm and no radioactivity (< LOD) moved into deeper layers. During the experiment the amount of substance decreased in the grass layer from 45 % at day 1 to 4 % of the applied amount on day 189. In the first soil layer (0 – 1 cm) the content decreased from 55 % at the beginning to 12 % after 189 days. In layer 1 – 2.5 cm the concentration of Imidacloprid equivalents increased from 13.3 µg (0.9 %) to 316 µg (20 %) on day 140 and decreased until day 189 to 147 µg (9 %). A similar pattern can be seen in layer B, 2.5 – 5 cm. Here, the analysed amounts increased from 2.2 µg on day 1 to 162 µg (10 %) on day 140 and decreased until day 189 slightly to 148 µg of Imidacloprid equivalents. In deeper soil layers only small amounts (between 3 and 8 %) are detectable starting at day 14. In total at the end of the experiment on day 189 only 43 % of the applied radioactivity is detectable in soil and vegetation, the rest most probably volatilised and/or mineralised (the experimental setup did not allow to trap gaseous metabolites.)

Table 75 The concentration of Imidacloprid equivalents in soil dry matter (DM), the total amount of Imidacloprid equivalents in each layer and the proportion of Imidacloprid equivalents of the applied amount of Imidacloprid (1571 µg/TME) is given. *) = µg Imidacloprid equivalents / g fresh weight. Imi-eq. = Imidacloprid equivalents.

Table 75 Concentration of Imidacloprid T0, Day 1

	µg Imi-eq. /g DM	µg / total sample	% of the applied amount
Grass	*329.68 ± 111.13	705.19 ± 237.71	44.89 ± 15.13
0-1 cm	17.15 ± 3.34	868.63 ± 169.21	55.29 ± 10.77
1-2.5 cm	0.10 ± 0.02	13.31 ± 3.31	0.85 ± 0.21
2.5-5 cm	0.01 ± 0.01	2.15 ± 0.94	0.14 ± 0.06
5-10 cm	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
10-20 cm	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Leachate		0.00	0.00
Sum [%] of the applied amount:		1589.3 ± 411.2	101.2 ± 26.2

Table 76 Concentration of Imidacloprid T1, Day 14

	µg Imi-eq. /g DM	µg / total sample	% of the applied amount
Grass	*112.35 ± 35.08	606.93 ± 189.52	38.63 ± 12.06
0-1 cm	18.45 ± 2.36	541.59 ± 69.36	34.47 ± 4.41
1-2.5 cm	1.36 ± 0.24	154.96 ± 27.2	9.86 ± 1.73
2.5-5 cm	0.08 ± 0.02	17.38 ± 3.38	1.11 ± 0.22
5-10 cm	0.08 ± 0.02	45.42 ± 10.91	2.89 ± 0.69
10-20 cm	0.12 ± 0.01	129.06 ± 8.9	8.22 ± 0.57
Leachate		0.01	0.00
Sum [%] of the applied amount:		1495.3 ± 309.3	95.2 ± 19.7

Table 77 Concentration of Imidacloprid T2, Day 42

			% of the

	µg Imi-eq. /g DM	µg / total sample	applied amount
Grass	*95.27 ± 15.43	158.92 ± 25.75	10.12 ± 1.64
0-1 cm	13.53 ± 1.28	852.93 ± 80.56	54.29 ± 5.13
1-2.5 cm	3.45 ± 0.58	283.70 ± 47.77	18.06 ± 3.04
2.5-5 cm	0.49 ± 0.11	75.77 ± 17.14	4.82 ± 1.09
5-10 cm	0.14 ± 0.01	74.48 ± 4.97	4.74 ± 0.32
10-20 cm	0.07 ± 0.01	68.28 ± 10.05	4.35 ± 0.64
Leachate		0.00	0.00
Sum [%] of the applied amount:		1514.1 ± 186.2	96.4 ± 11.9

Table 78 Concentration of Imidacloprid T3, Day 140

	µg Imi-eq. /g DM	µg / total sample	% of the applied amount
Grass	*14.46 ± 2.50	150.83 ± 26.12	9.60 ± 1.66
0-1 cm	6.57 ± 0.45	283.45 ± 19.27	18.04 ± 1.23
1-2.5 cm	4.11 ± 0.25	316.38 ± 19.54	20.14 ± 1.24
2.5-5 cm	0.66 ± 0.15	161.91 ± 36.58	10.31 ± 2.33
5-10 cm	0.11 ± 0.01	59.81 ± 5.72	3.81 ± 0.36
10-20 cm	0.04 ± 0.01	47.19 ± 7.26	3.00 ± 0.46
Leachate		0.31	0.02
Sum [%] of the applied amount:		1019.9 ± 114.5	64.9 ± 7.3

Table 79 Concentration of Imidacloprid T4, Day 189

	µg Imi-eq. /g DM	µg / total sample	% of the applied amount

Grass	*5.30 ± 1.13	65.31 ± 13.96	4.16 ± 0.89
0-1 cm	4.95 ± 1.32	185.17 ± 49.31	11.79 ± 3.14
1-2.5 cm	1.95 ± 0.33	147.09 ± 24.62	9.36 ± 1.57
2.5-5 cm	0.73 ± 0.03	147.67 ± 5.43	9.40 ± 0.35
5-10 cm	0.10 ± 0.01	51.38 ± 7.72	3.27 ± 0.49
10-20 cm	0.08 ± 0.03	85.80 ± 29.53	5.46 ± 1.88
Leachate		0.05	0.00
Sum [%] of the applied amount:		682.41 ± 130.57	43.44 ± 8.31

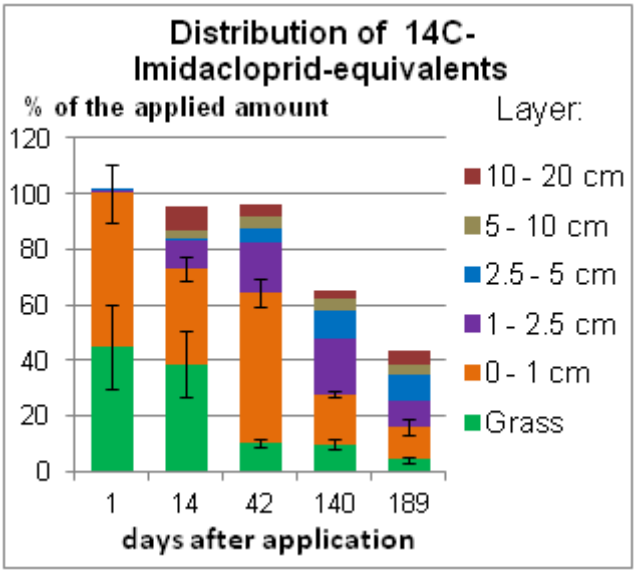

Figure 73 Distribution of ^{14}C -Imidacloprid equivalents in the indoor study. The total amount of Imidacloprid (combustion of not extracted soil) is presented as a percentage of the applied amount of Imidacloprid of 1571 µg per TME. For the clarity only the error bars for the grass layer and the upper soil layer (0–2.5 cm) are given.

5.3.2 Results of the extractable and non-extractable residues - time course within sample cores

This section shows the course of the concentrations of the extractable and the non-extractable residues within a sample core during the experiment. The relative distribution of Imidacloprid equivalents at one sampling date in the different soil layers (100 % = Sum of the absolute amount in µg in the soil core found in the extractable and in the non-extractable fraction of the different layers) is summarized in Table 80 Table 75 - Table 79 show the concentrations of Imidacloprid-equivalents in mg/kg dry matter for the different soil layers (left charts) and the relative distribution of the substance in the extractable and the non-extractable fraction of single sample (100 % = concentration of Imidacloprid-equivalents was measured after the combustion of the not extracted soil sample, formulas were given previously in Chapter 5.2.2.

Table 80 Distribution of the amount of Imidacloprid equivalents in µg/soil core in percent of the sum of the Imidacloprid equivalents at the various sampling times, measured in the extractable and the non-extractable (NER) fractions.

		Percent of the sum of extractable and NER				
Days after application		Day 1	Day 14	Day 42	Day 140	Day 189
0 - 1 cm	Extract	97.46	82.19	53.34	24.52	22.88
	NER	1.84	9.77	22.49	33.35	35.83
1 - 2.5 cm	Extract	0.51	5.63	14.79	14.51	15.39
	NER	0.10	0.91	5.05	20.10	13.14
2.5 - 5 cm	Extract	0.08	0.37	2.49	3.79	4.84
	NER	0.01	0.07	0.55	2.48	5.60
5 - 10 cm	Extract	0.00	0.38	0.60	0.69	0.80
	NER	0.00	0.06	0.28	0.26	0.63
10 - 20 cm	Extract	0.00	0.50	0.31	0.18	0.51
	NER	0.01	0.12	0.09	0.11	0.38
	Sum	100	100	100	100	100

Comparable to the Lindane study, nearly all of the Imidacloprid after one day of its application was found in the upper layer and was extractable (see Table 80). During the incubation time, up to 40 % of the substance moved into the deeper layers of soil. The content of the non-extractable residues increased from 1.9 % at day 1 to 56 % of the Imidacloprid-equivalents analysed at day 140 and at day 189.

The left bar charts of the Figure 74 show that the highest concentrations were measured in the upper soil layers (0 – 1 cm). While the concentration in the upper layer decreased during the course of the experiment, in the layer 1 - 2.5 cm its maximum content occurred at day 140. In layer B (2.5 – 5 cm) the concentration of Imidacloprid-equivalents increased during the experiment. Only traces were found in Layer C and D (5 – 10 and 10 – 20 cm, respectively).

The right bar charts show the percentage of extractable and non-extractable Imidacloprid residues for each sample. The proportion of the non-extractable increased in all soil layers in time course and reached about 40 – 60 % at the end (day 189). The recoveries of all analyses were between 90 and 110 % (sum of extractable and non-extractable as percentage of the total amount in a sample). This indicates that no losses of radioactivity during the extraction of the soil occurred.

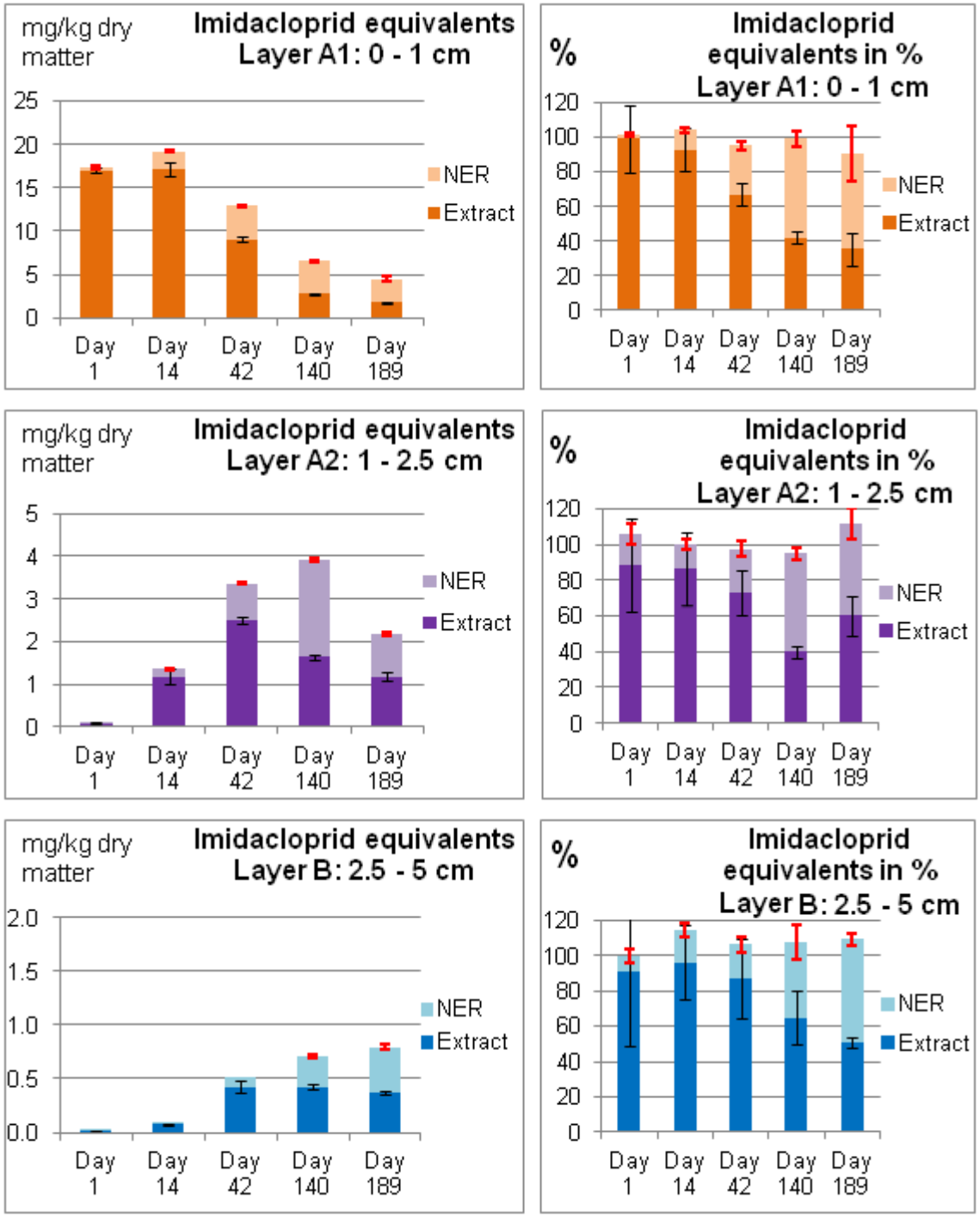

Figure 74 caption see below

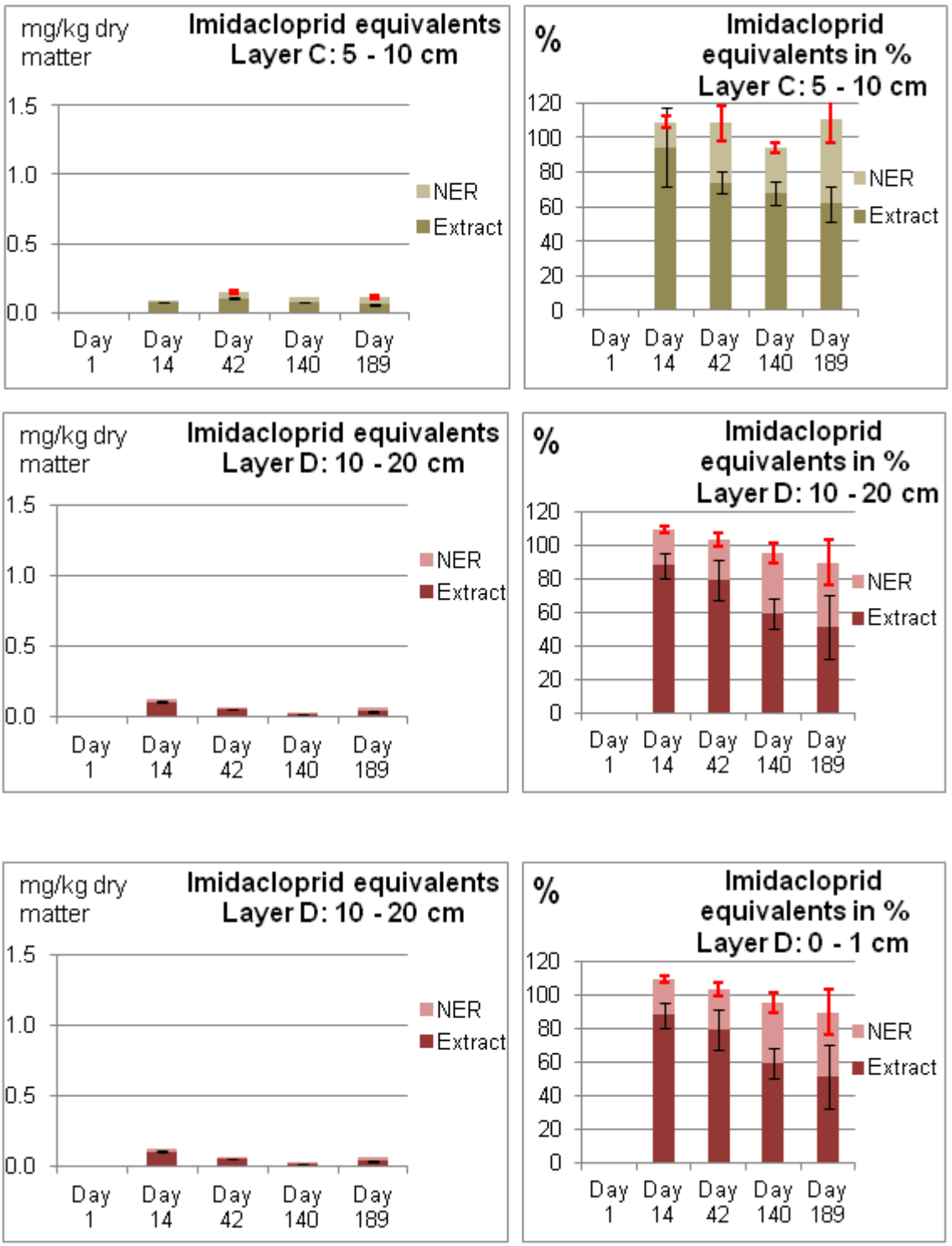

Figure 74 In the left figure the concentration of Imidacloprid equivalents in soil dry matter are given in mg/kg for the respective layer. Error bars indicating the standard deviation. The right figure show the percentage of the Imidacloprid equivalents of the extractable and the non-extractable residues referring to the total amount of Imidacloprid. This total amount was measured after the combustion of the non-extracted soil sample.

5.3.3 Overview of the Imidacloprid results of the indoor study

In this chapter all the data from the Imidacloprid indoor study are combined. Table 81 summarises all concentrations of Imidacloprid equivalents for the different fractions. Figure 75 sketches the concentrations of the insecticide in the respective soil fraction in the time course. Figure 76 show separately the time course of the concentrations of the extractable and of the non-extractable residues, respectively. A decrease in the concentration of the non-extractable and of the extractable residues was observed, which is in a good agreement with the results obtained in the corresponding radioactive Lindane study. The content of the non-extractable residues reached a maximum at day 140 which thereafter decreased.

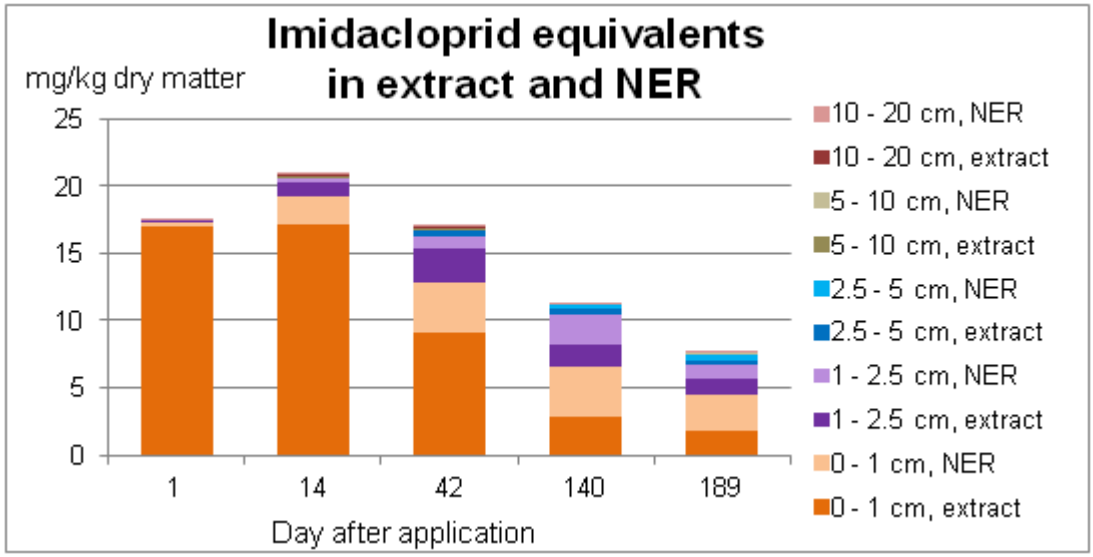

Figure 75 Imidacloprid equivalents concentration of the extractable and the non-extractable residues (Extract and NER, respectively) in the respective soil layers. Summing up the concentrations in the bars has been chosen for pragmatic reasons and for illustration.

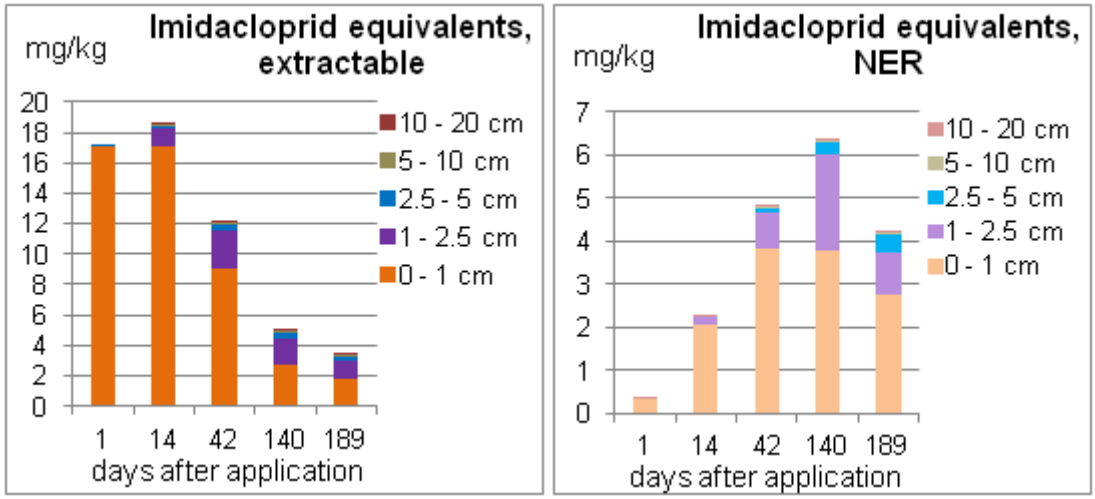

Figure 76 Imidacloprid equivalents concentration of the extractable (left figure) and the non-extractable residues (NER, right figure). Summing up the concentrations in the bars has been chosen for pragmatic reasons and for illustration.

Table 81 Results of the radioactive analyses. Means are given in mg/kg dry matter (DM) ± standard derivation. n.d. = not detectable. Concentrations below the limit of quantification are given as < "value of the LOQ". Total = combustion of soil (not extracted), NER = Non-Extractable Residues, Extr + NER = Sum of the results in the extract and in the NER

Imidacloprid equivalents (mg/kg DM)					
Layer A1 (0-1 cm)	**Day 1**	**Day 14**	**Day 42**	**Day 140**	**Day 189**
Total	17.15 ± 3.34	18.45 ± 2.36	13.53 ± 1.28	6.57 ± 0.45	4.95 ± 1.32
Extract	17.03 ± 0.23	17.14 ± 0.85	9.06 ± 0.35	2.76 ± 0.09	1.75 ± 0.03
NER	0.32 ± 0.13	2.04 ± 0.13	3.82 ± 0.08	3.76 ± 0.12	2.74 ± 0.32
Extr + NER	17.35 ± 0.37	19.18 ± 0.98	12.88 ± 0.43	6.52 ± 0.21	4.48 ± 0.35
Layer A2 (1-2.5 cm)	**Day 1**	**Day 14**	**Day 42**	**Day 140**	**Day 189**
Total	0.10 ± 0.02	1.36 ± 0.24	3.45 ± 0.58	4.11 ± 0.25	1.67 ± 0.28
Extract	0.09 ± 0.01	1.18 ± 0.18	2.51 ± 0.07	1.64 ± 0.07	1.01 ± 0.08
NER	0.02 ± 0.00	0.19 ± 0.02	0.86 ± 0.02	2.27 ± 0.04	0.86 ± 0.02
Extr + NER	0.11 ± 0.01	1.37 ± 0.19	3.37 ± 0.08	3.90 ± 0.08	1.86 ± 0.09
Layer B (2.5-5 cm)	**Day 1**	**Day 14**	**Day 42**	**Day 140**	**Day 189**
Total	0.01 ± 0.01	0.08 ± 0.02	0.49 ± 0.11	0.66 ± 0.15	0.73 ± 0.03
Extract	0.01 ± 0.00	0.08 ± 0.01	0.42 ± 0.05	0.43 ± 0.02	0.37 ± 0.02
NER	< 0.002	0.01 ± 0.00	0.09 ± 0.00	0.28 ± 0.02	0.43 ± 0.02
Extr + NER	0.01 ± 0.00	0.09 ± 0.01	0.52 ± 0.05	0.71 ± 0.03	0.80 ± 0.03
Layer C (5-10 cm)	**Day 1**	**Day 14**	**Day 42**	**Day 140**	**Day 189**
Total	n.d.	0.08 ± 0.02	0.14 ± 0.01	0.11 ± 0.01	0.10 ± 0.02
Extract	n.d.	0.08 ± 0.00	0.10 ± 0.01	0.08 ± 0.00	0.06 ± 0.01
NER	n.d.	0.01 ± 0.00	0.05 ± 0.01	0.03 ± 0.00	0.05 ± 0.01
Extr + NER	n.d.	0.09 ± 0.01	0.15 ± 0.01	0.11 ± 0.00	0.11 ± 0.01
Layer D (10-20 cm)	**Day 1**	**Day 14**	**Day 42**	**Day 140**	**Day 189**
Total	n.d.	0.11 ± 0.01	0.07 ± 0.01	0.04 ± 0.01	0.08 ± 0.03
Extract	n.d.	0.10 ± 0.01	0.05 ± 0.00	0.02 ± 0.00	0.04 ± 0.01
NER	< 0.002	0.03 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.03 ± 0.00
Extr + NER	< 0.002	0.13 ± 0.01	0.07 ± 0.00	0.03 ± 0.00	0.07 ± 0.01

6 Results of the experimental outdoor study [3] testing the pesticide Carbendazim

6.1 Leachate and environmental conditions

In Figure 77 the data from continuous measurement of soil moisture and temperature is shown. The highest soil temperature was measured in July 2011 (24.07.2013) with 28.8°C in the layer 0-6 cm, the lowest in October 2013 (12.10.2013) with 6.8 °C also in the layer 0-6 cm.

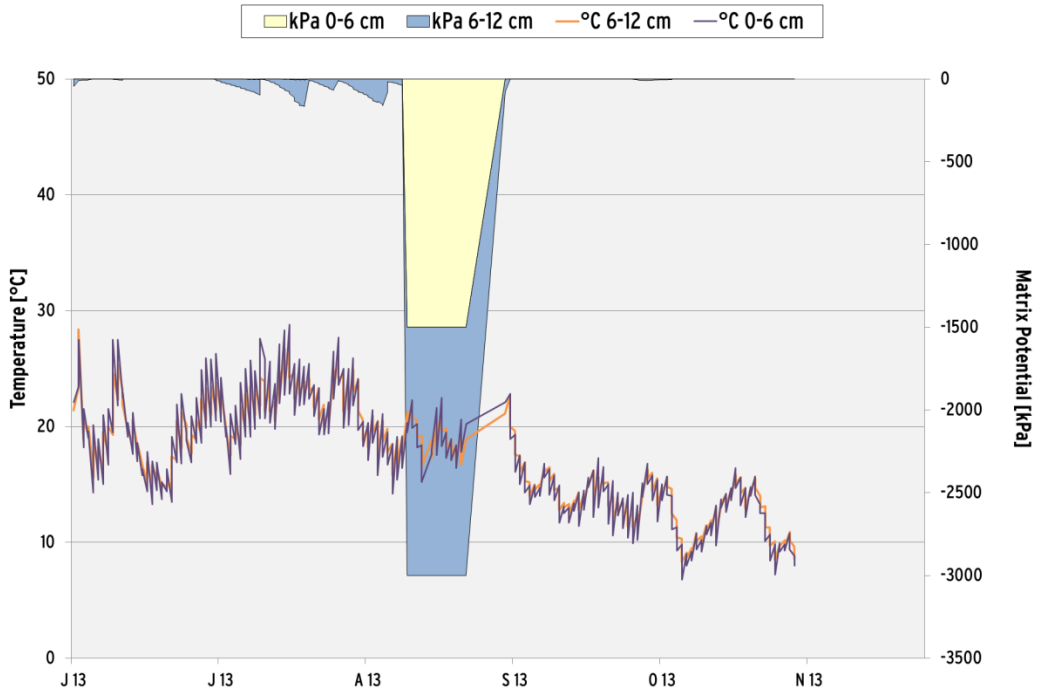

Figure 77 Data from continuous measurement of equitensiometer and temperature sensors in the study [3] from June 2013 to November 2013.

The data for precipitation and irrigation as well as the leachate measurements are given in Table 82. The TMEs were used in the months June, July and August. Due to an exceptional dryness in July and August 2013 they were additionally irrigated. Except of these two months, the difference was in a range that could be adjusted by irrigation or natural precipitation in the other months.

The highest precipitation was measured for September 2013 (128 mm), followed by October (79.5 mm). The total precipitation for the whole study period, i.e. from June 2013 to November 2013, was 447.5, including irrigation. When considering only natural precipitation, the total sum was 392.5 mm. The amount of leachate water was the same for the two measured TMEs except of September 2013 the month with the highest precipitation. The highest amount of leachate was recorded in TME 1 in September, followed by both TMEs in October (Figure 78)

Table 82 Precipitation, Irrigation and the amount of leached water in the period of study [3] from June 2013 to November 2013. Differences (diff.) between measured precipitation and desired values from Wetter.com (2011) fo Monschau/Höfen were given.

	Precipitation [mm]	Irrigation [mm]	Sum [mm]	Leachate water [ml]	
				TME 1	TME 2
June	77	16	16	1495	1085
	set value		81		
	diff.		-65		
July	41,5	19	19	2395	2475
	set value		132		
	diff.		-113		
August	41	20	61	6	0
	set value		113		
	diff.		-52		
September	128	0	128	5422	2296
	set value		106		
	diff.		22		
October	79,5	0	79,5	4420	4360
	set value		64		
	diff.		15,5		
November	25,5	0	25,5	2920	2710
	set value		124		
	diff.		not det.		
		Sum of the study	**329**	**16657**	**12926**

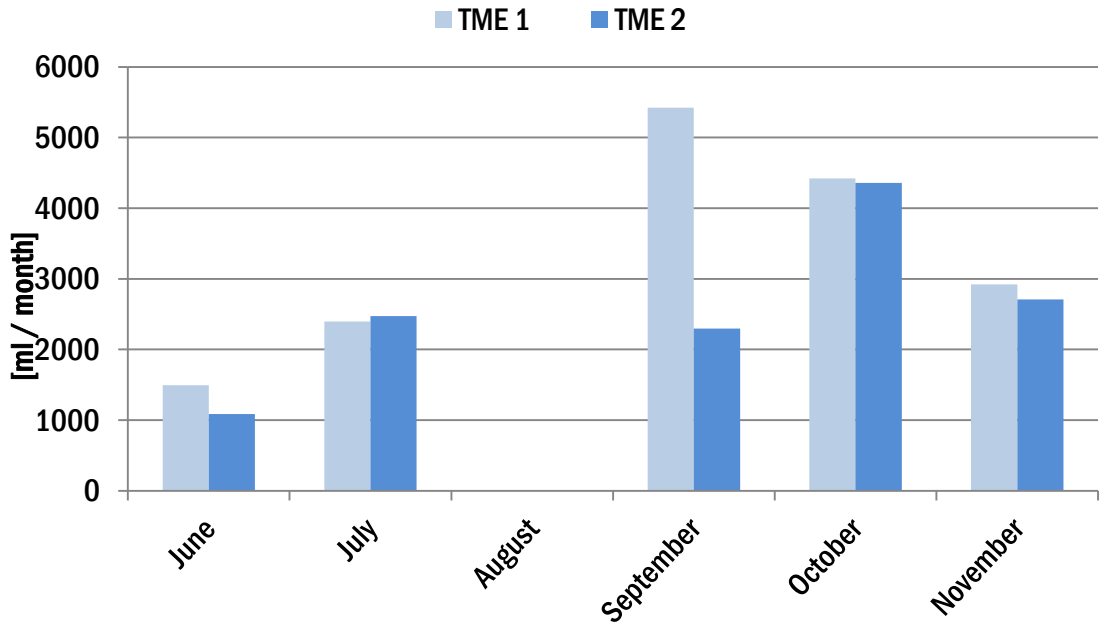

Figure 78 Measurement of leached water for the months June `13 to November `13 during the study.

6.2 Chemical analyses in soil layers

The results of the chemical analysis of Carbendazim for the three different sampling dates are given in Table 83 and Table 84 (see also Table 1-3 Appendix). Their standard deviation is high for some layers (e.g. std. 17.25; 15kg/ha layer 0-1 cm). However, the mean values calculated for the different soil layers show a clear trend regarding the concentrations of Carbendazim. Nearly all (> 95 % of the total recovery) of the chemical could be measured in the uppermost first centimetre of soil (cp. also Figure 79 and Figure 80) on all sampling dates (one exception: the highest concentration was found in the second level (0-2.5 cm) on the third sampling date – but at this time the absolute level of concentrations was already quite low.

The Carbendazim concentration at day 16 after application was 6.49 mg/kg dry weight (DW) of soil for the low application rate and 23.18 mg/kg DW for the high application rate in the uppermost centimetre of soil. One very high concentration (57.0 mg/kg) on this sampling date is considered to be an outlier. No explanation can be given for this value, but the mean value without this outlier is 14.7 mg/kg and thus much more reasonable. The concentration for the layer A (0-2.5 cm) was 4.36 mg/kg for the low application rate and 7.30 mg/kg for the high application rate. Less than 5 % were measured for layer B (2.5-5 cm). The concentrations for the layers below were often beneath the detection limit (cp. Table 83, Table 84).

For the second sampling, i.e. at day 114 after application the total recovery for the low application rate was 10.2 % of the amount measured at day 16 and for the high application rate it was 12.6 %, respectively. The half-life of Carbendazim is 28-36 days at 15°C (derived from laboratory studies), and approx. 18 days (n=4) derived from a field study (EU 2007)). The measured decrease within the present study is in the range of the assumed decrease that could be derived from literature.

The total recovery at day 148 after application for the low application rate was 1.7 % at day 16 and 1.5 % for the higher concentration. Only the surface layers (0-1 cm or 0-2.5 cm) were containing measurable amounts of Carbendazim at this time.

Table 83 Carbendazim concentrations given as [mg/kg] dry soil for the different replicates on the sampling dates at day 1-148 days after application of study [3] for the application rate 7.5 kg/ha, (loq 0.004 mg/kg)

	mg/kg dry matter			
Layer (cm)	Day 1	16	114	148
O: 0-1cm	**8.87**	**6.49**	**0.51**	**0.03**
A: 0 - 2.5	4.50	4.36	0.46	0.00
B: 2.5 – 5	0.18	0.21	0.03	0.00
C: 5 – 10	-	0.14	0.01	0.00
D: 10 – 20	-	0.01	0.01	0.00

Table 84 Carbendazim concentrations given as [mg/kg] dry soil for the different replicates on the sampling dates at day 1-148 days after application of study [3] for the application rate 15 kg/ha, (loq 0.004 mg/kg).

	mg/kg dry matter			
Layer (cm)	Day 1	16	114	148
O: 0-1cm	19.33	23.18	2.89	0.11
A: 0 - 2.5	9.40	7.30	0.89	0.11
B: 2.5 – 5	0.79	0.35	0.05	0.01
C: 5 – 10	-	0.06	0.04	0.00
D: 10 – 20	-	0.01	0.00	0.00

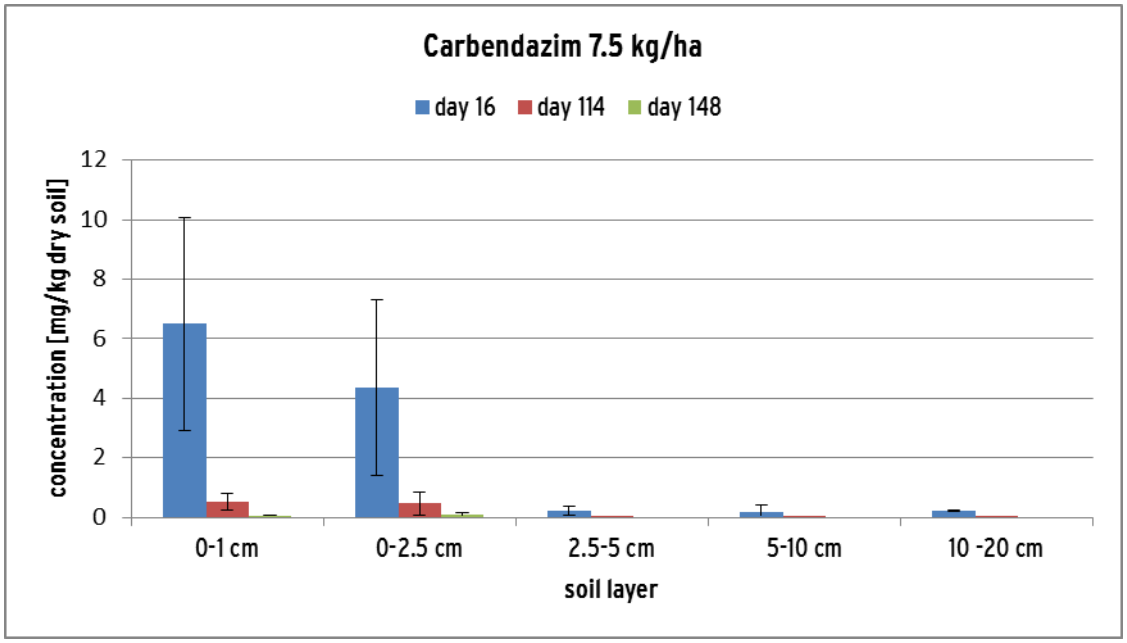

Figure 79 Concentration of Carbendazim (7.5 kg/ha) in the different soil layers for the three sampling dates day 16, day 114 and day 148

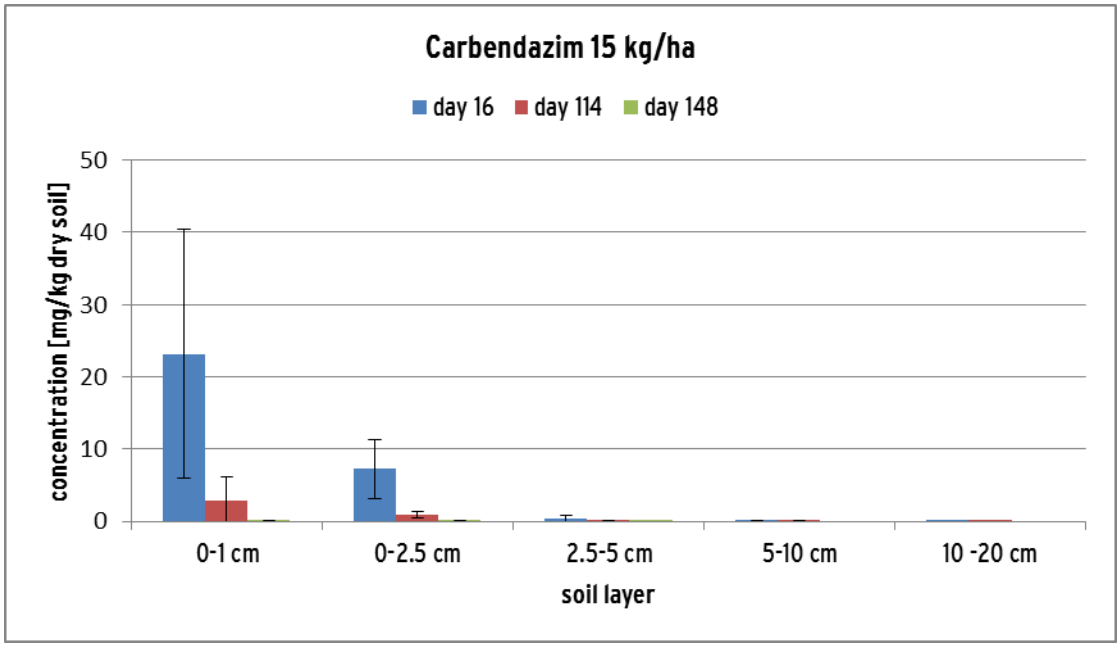

Figure 80 Concentration of Carbendazim (15 kg/ha) in the different soil layers for the three sampling dates day 16, day 114 and day 148

6.3 Distribution of the earthworms in different soil layers

During this study, earthworms were collected on three dates, i.e. 16 days, 114 days and 148 days after application of the test substance Carbendazim. Both abundance and biomass of the individual species as well as the total abundance and biomass of tanylobic species, epilobic species, adults, juveniles, endogeic adults, epigeic adults, anecic adults and the total number were recorded separately. Detailed results are given in the Appendix 1.

The application of Carbendazim did affect the abundance of earthworms significantly on almost every sampling date (Figure 81, Table 85). A dose-response-relationship was observed. The highest reduction was recorded for the high application rate at day 114 and day 148 (98 % and 96 % of the control).

At the first sampling date, (day 16), no reduction in total abundance was measured for the lower concentration in layer C-E (5-40 cm) and for the high application rate in layer E. At this lowest layer an increase of abundance could be detected. This increase could be caused by avoidance movements of earthworms from the upper soil layers down to layers with lower Carbendazim concentrations. For all layers significant effects could be recorded through the study.

For the low application rate, significant reductions could be recorded for the uppermost layer A on every sampling date. At day 114 a significant reduction of earthworms was found in layer D (50 % in comparison to the control) and E (65 % in comparison to the control). After 148 days a significant reduction did occur in all layers except of layer D.

For the high application rate, significant changes in abundance were measured in layers A and C for every sampling date. At day 114 the abundance decreased in all layers by 85 % or more. This pattern was also visible for day 148 with the exception of layer D at this date (decrease of 50 % in comparison to the control). When considering all layers together the effect was higher than 90 % (high application rate) at days 114 and 148 after application (Figure 81, Table 85).

One month after the start of the study (day 16 after application) about 50 % of all worms were found in the uppermost 2.5 cm, but only about 1 % in a depth of 20–40 cm. In the TMEs treated with the low application rate of Carbendazim only 24 % are still in the uppermost soil layer, and similar percentages were found in the following layers. Almost 9 % were living in the lowest soil layer. The same vertical shift with similar percentages was also observed in the TMEs treated with the high application rate of Carbendazim.

On the second sampling date (T2; day 114 after application of the test substance), the vertical distribution of earthworms in the control TMEs did not change considerably. While their percentage in the uppermost soil layer increased slightly, there was a small decrease in the three middle layers and a considerable increase (up to 7 %) in the lowest layer. However, there is still a clear difference to the treated TMEs: Only 31 % of all worms were found in the highest soil layer of the TMEs treated with the low application rate of Carbendazim and even less (20 %) in the TMEs treated with the high concentration of Carbendazim. Actually, the remaining earthworms (which had possibly already moved downwards, depending on the chemical stress coming from above), stayed there.

Basically, the same situation could be observed also on the last sampling date (T3), but the percentage of worms in the uppermost soil layer of the controls increased further, up to 65 %, and decreased in all other layers with increasing depth. In the lower Carbendazim treatment, a further

decrease of lumbricid abundance was observed in all layers. No similar development could be recorded for the TMEs with the high application rate, probably because the worm numbers were already very low. No explanation can be given why this decrease is clearly less pronounced in Layer D (50 % decrease but not significantly different from the control), but the absolute low numbers of lumbricids may play a role here.

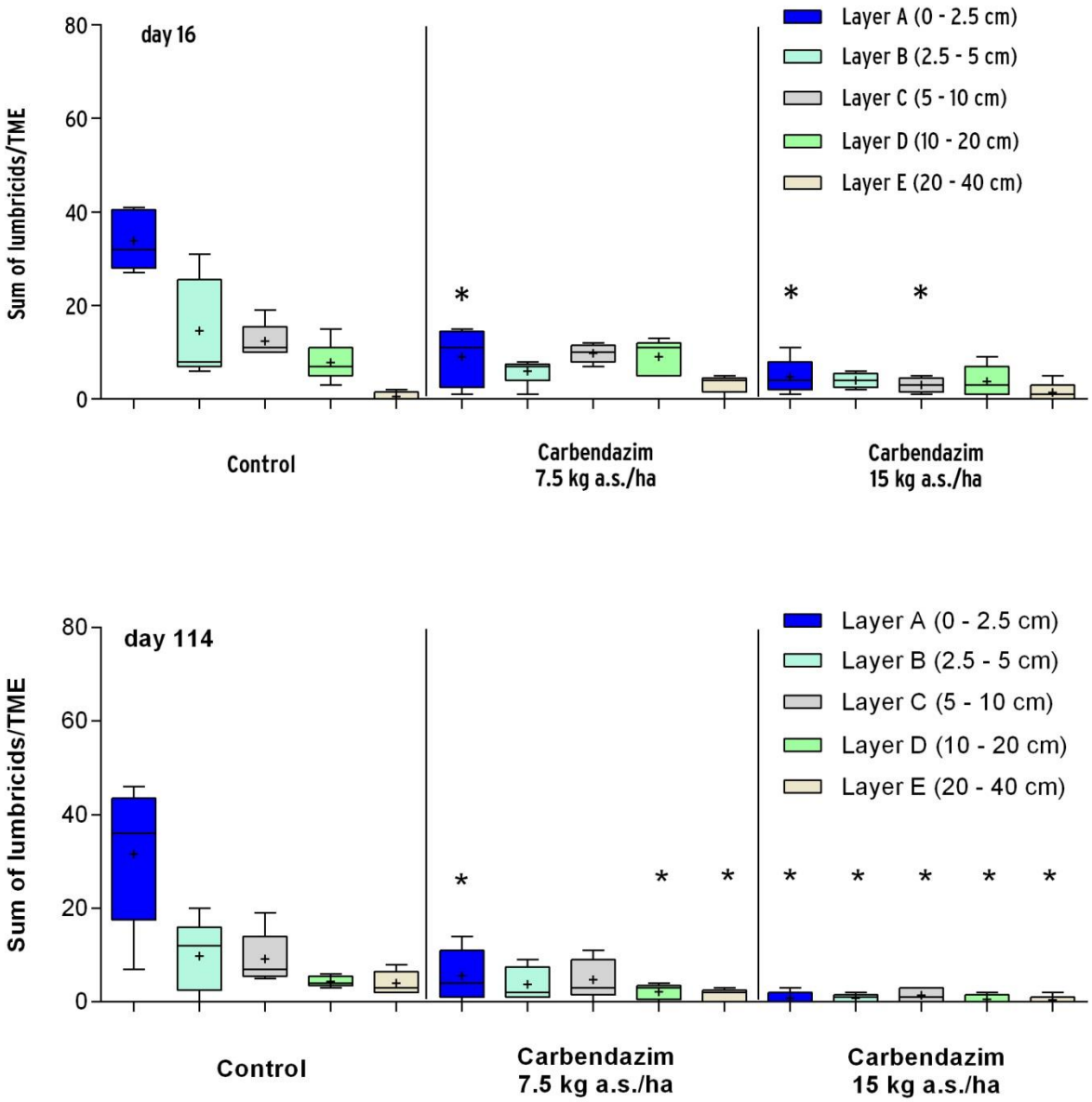

Figure 81 caption see below

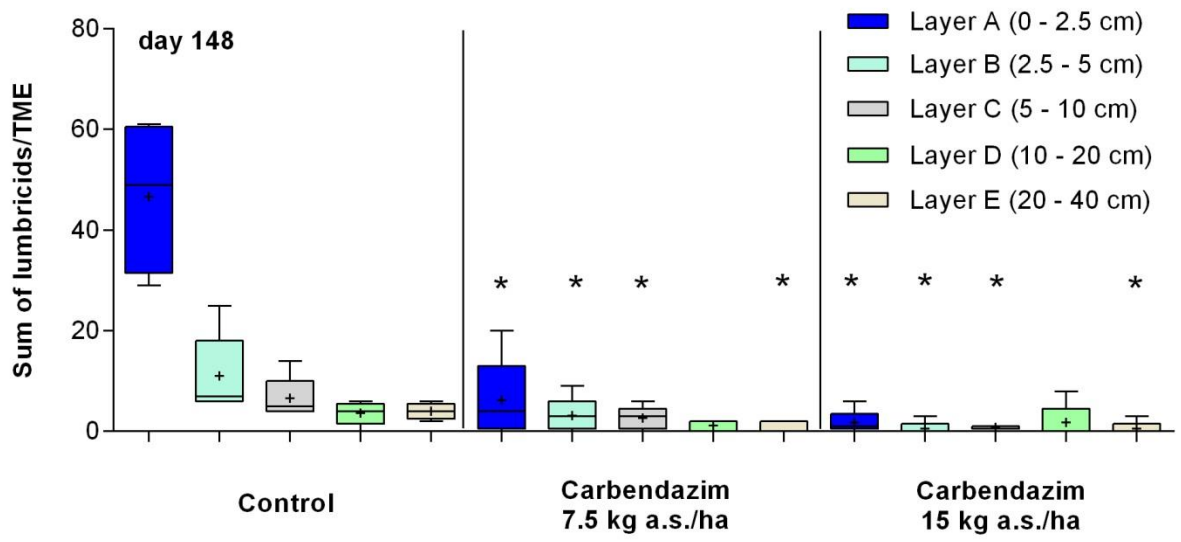

Figure 81 Total abundance of lumbricids in the soil layers of the TMEs in study [3] (5 replicates for control; 5 replicates for carbendazim treatments). cross: mean; *: significance (p-value Williams-t-test < 0.05). Sampling dates 16 days, 114 days and 148 days after application from top to bottom

Table 85 Decrease of total abundance [%] of lumbricid species in the different soil layers on the three sampling dates (16, 114, and 148 days after application). Red: decrease more than 50% in comparison to the control; Grey: less than 50% decrease in comparison to the control; Bold: significant effects (Williams test, p-value <0.05); calculation is based on 5 replicates for the control and 5 replicates for the respective treatment.

Carbendazim 7.5 kg a.s./ha				
		days after application		
	layer	16	114	148
A	0 - 2.5 cm	**74**	**81**	**85**
B	2.5 - 5 cm	55	47	**68**
C	5 - 10 cm	32	47	**56**
D	10 - 20 cm	-14	**50**	68
E	20 - 40 cm	-467	**65**	**80**
	all layers	**48**	**61**	**78**

Carbendazim 15 kg a.s./ha				
		days after application		
	layer	16	114	148
A	0 - 2.5 cm	**85**	**98**	**96**
B	2.5 - 5 cm	66	**86**	**93**
C	5 - 10 cm	**82**	**85**	**88**
D	10 - 20 cm	49	**82**	47
E	20 - 40 cm	-133	**90**	**85**
	all layers	**75**	**91**	**92**

Additionally to the above mentioned decrease in the total abundance of earthworms, the structure of the lumbricid community, especially the number of species present, was also affected in the TMEs treated with Carbendazim (Table 86). Out of nine species found in the control (three of them with less than 1 ind./m^2), only five species could be recorded in the TMEs of each treatment (with additional two and four species with less than 1 ind./m^2). In the lower Carbendazim application rate, the species *Lumbricus rubellus*, *Aporrectodea limicola*, *Octolasion lacteum* and *Allolophora chlorotica* were missing. In the higher concentration *Lumbricus castaneus*, *Lumbricus rubellus*, *Octolasion lacteum* and *Allolophora chlorotica* were not found.

Table 86 Presence and mean abundance of captured lumbricid species in the control TMEs (5 replicates) and the two different application rates of the treated TMEs (5 replicates).

	Control	Carbendazim	
		7.5 kg a.s./ha	*15 kg a.s./ha*
Number of replicates	**5**	**5**	**5**
TME			
Juveniles	103.2	47.4	18.0
Aporrectodea caliginosa	36.0	11.0	4.0
Lumbricus spec.	25.8	3.0	0.4
Aporrectodea rosea	7.6	1.6	0.4
Octolasion cyaneum	5.0	1.6	1.2
Lumbricus castaneus	3.4	0.2	-
Lumbricus terrestris	2.2	0.2	0.2
Lumbricus rubellus	2.0	-	-
Aporrectodea limicola	0.8	-	0.4
Octolasion lacteum	0.6	-	-
Allolobophora chlorotica	0.2	-	-
Number of taxa	9	5	5

After statistical analysis on the population level for 8 out of 9 earthworm species (cp. appendix 1) significant effects were found on one date for at least one single soil layer. Only the abundance of *Aporrectodea limicola* was statistically significantly affected (but this species was rare anyway).

The earthworm species were distributed within the soil core in the soil depth typical for their respective life-form types and were affected right in these soil layers (Table 87).

Lumbricus terrestris was found only in the two deepest layers within this study (Figure 82). In both layers, the abundance decreased to extinction at day 114 and day 148. Because of the variance within the dataset, these findings were only significant for layer E - where most of the individuals occurred. *Octolasion cyaneum* was found mainly in layer C and D (Figure 82). The species was reduced by Carbendazim by 100 % in layer C at day 114 and day 148 and more than 80 % in comparison to the control in layer D.

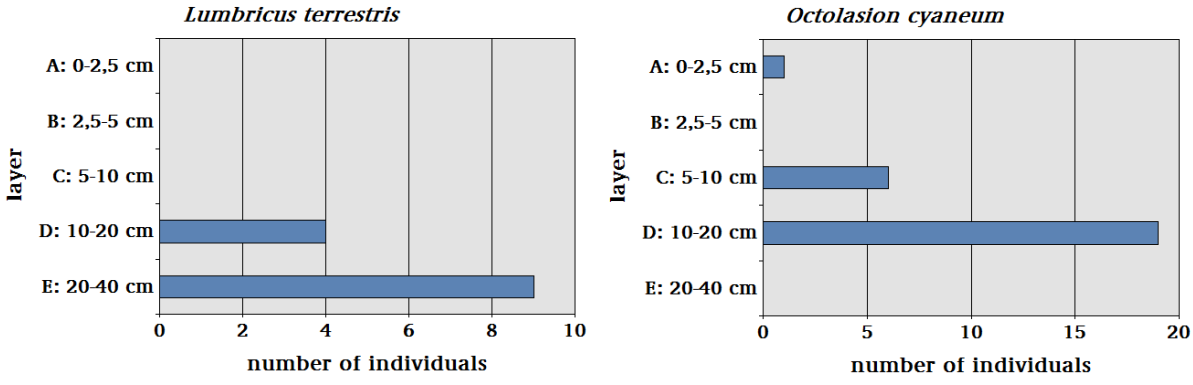

Figure 82 Vertical distribution of the lumbricid species *Lumbricus terrestris* and *Octolasion cyaneum* in the control. Shown is the total number of individuals captured in all TMEs on all sampling dates in the study [3].

Lumbricus castaneus was mainly distributed in the uppermost soil layer A (Figure 83). The abundance of this species decreased in all layers except of layer B at day 114 (low concentration) by 100 %. However, statistically significant changes were only detected in the uppermost layer A for day 114 and day 148.

The endogeic species *Aporrectodea caliginosa* was distributed in this study [3] mainly in the uppermost soil layer and showed in this layer higher numbers of individuals in comparison to study [1].

The species numbers decreased significantly in layer A and B at day 114 and 148 and in layer C at day 114 for the higher concentration. A reduction at day 16 can be only recognized for layer A, however, no decrease of species number occurred in the deeper soil layer at this time. Later on medium effects were recorded for both concentrations at day 114 in layer D and E and in layer D at day 148. A decrease of numbers in layer C was only recorded for the higher concentration on all sampling dates.

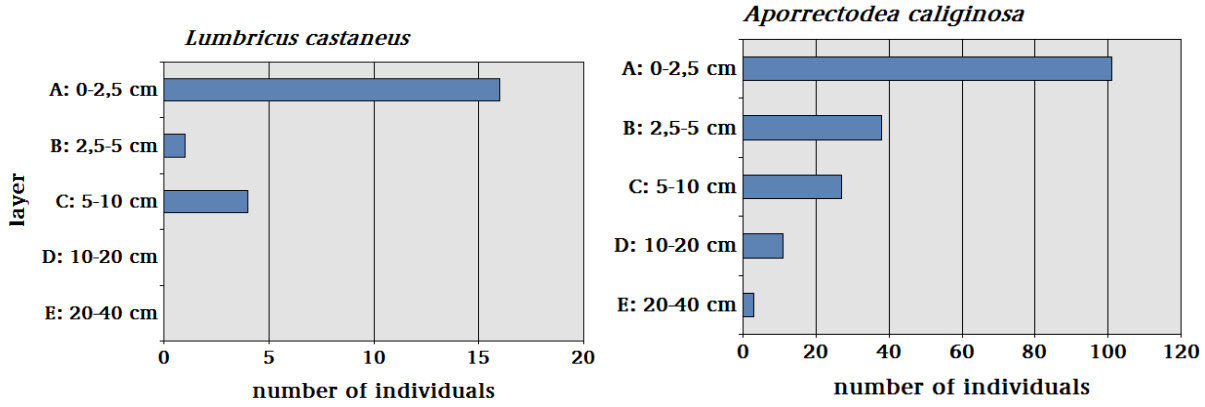

Figure 83 Vertical distribution of the lumbricid species *Lumbricus castaneus* and *Aporrectodea caliginosa* in the control. Shown is the total number of individuals captured in all TMEs on all sampling dates in the study [3].

The statistical community analyses showed significant effects on the community for every sampling date and for both application rates (Table 88). The number of taxa was significantly reduced at day 148 for the lower concentration and on all days for the higher concentration. The Shannon index was significantly different only for the last sampling for both concentrations. No effects were measured for the Evenness on any date. The similarity of the lumbricid community measured with Steinhaus´ and Stander´s indices is presented for all layers in Figure 84. Both indices show significant changes of diversity for the last sampling date, 148 days after application. The Steinhaus´ index, which considers the absolute densities of the species, showed a significant change of diversity also for day 16 and day 114. However, this change can´t be seen with Standers index, which is not as sensitive for changes of rare species because it considers the relative abundances (see chapter 3.10).

The multivariate statistical analysis with the Principal Response Curve showed a clear dose response correlation, with increasing effect size over time (Figure 85). The lumbricid community was significantly affected on all sampling dates for both concentrations. The largest share on this effect was related to juvenile individuals of the genus *Lumbricus*, followed by *Aporrectodea caliginosa* and juveniles of the *Aporrectodea/Allolobophora* complex.

The experiences made in this study can be summarised as follows:

- Carbendazim decreased the number and biomass (data not shown) of earthworms in a dose-dependent manner in a way which could be expected from literature data. These very strong impacts lasted until the end of the study.
- The vertical distribution of earthworms strongly changed after treatment with Carbendazim. Surviving worms moved downwards.
- Further assessments, especially on the level of ecological groups and individual species, are necessary in order to evaluate the influence of Carbendazim on the earthworm community in the long run.
- Knowing the reproduction times of the species found in the TMEs, recovery will take several months at least (species with annual reproduction cycles, especially *Lumbricus terrestris,* do occur only in small numbers in all TMEs).

Table 87 Summary of statistical analysis of four different lumbricid species. Results are given for the different soil layers (A-E) and different sampling dates (16 days, day 114 and day 148 after application). Red: decrease of abundance ≥ 50% Grey: decrease of abundance < 50% Blank fields: data not sufficient for statistical calculation. Bold: significant difference (p-value Williams-test < 0.05); (see also appendix 1).

Lumbricus terrestris
days after application

	layer	16	114	148
A	0 - 2.5 cm			
B	2.5 - 5 cm			
C	5 - 10 cm			
D	10 - 20 cm	100	100	100
E	20 - 40 cm		**100**	**100**
	all layers	50	**100**	**100**

Lumbricus terrestris
days after application

	layer	16	114	148
A	0 - 2.5 cm			
B	2.5 - 5 cm			
C	5 - 10 cm			
D	10 - 20 cm	50	100	100
E	20 - 40 cm		**100**	**100**
	all layers	50	**100**	**100**

Octolasion cyaneum
days after application

	layer	16	114	148
A	0 - 2.5 cm	100		
B	2.5 - 5 cm			
C	5 - 10 cm		**100**	**100**
D	10 - 20 cm	**71**	**83**	**83**
E	20 - 40 cm			
	all layers	25	**88**	**89**

Octolasion cyaneum
days after application

	layer	16	114	148
A	0 - 2.5 cm	100		
B	2.5 - 5 cm			
C	5 - 10 cm		**100**	**100**
D	10 - 20 cm	**86**	**83**	**100**
E	20 - 40 cm			
	all layers	50	**88**	**89**

Lumbricus castaneus
days after application

	layer	16	114	148
A	0 - 2.5 cm	100	**100**	**100**
B	2.5 - 5 cm		0	
C	5 - 10 cm		100	100
D	10 - 20 cm			
E	20 - 40 cm			
	all layers	100	67	**100**

Lumbricus castaneus
days after application

	layer	16	114	148
A	0 - 2.5 cm	100	**100**	**100**
B	2.5 - 5 cm		100	
C	5 - 10 cm		100	100
D	10 - 20 cm			
E	20 - 40 cm			
	all layers	100	100	**100**

Aporrectodea caliginosa
days after application

	layer	16	114	148
A	0 - 2.5 cm	100	**89**	**86**
B	2.5 - 5 cm	33	**84**	**75**
C	5 - 10 cm	0	14	29
D	10 - 20 cm	-67	50	75
E	20 - 40 cm		50	-200
	all layers	50	**73**	**73**

Aporrectodea caliginosa
days after application

	layer	16	114	148
A	0 - 2.5 cm	71	**98**	**100**
B	2.5 - 5 cm	33	**100**	**100**
C	5 - 10 cm	67	**86**	71
D	10 - 20 cm	0	75	75
E	20 - 40 cm		50	0
	all layers	58	**94**	**94**

Table 88 Summary of the results for the statistical diversity analyses, PRC (p-value Williams-test < 0.05 of PCA sample scores) , number of taxa, Shannon and Eveness, (p-value Williams-test < 0.05) of lumbricids summed up over all layers (0-2.5 cm, 2.5-5 cm, 5-10 cm, 10-20 cm, 20–40 cm) treated with Carbendazim (left) concentration 7,5 kg a.s./ha (right) 20 kg a.s./ha. Database 10 replicates of control TMEs and 5 replicates for each treatment.

Carbendazim 7.5 kg a.s./ha	days after application		
all layers	16	114	148
PRC	*	*	*
Number of taxa			*
Shannon			*
Evenness			

Carbendazim 15 kg a.s./ha	days after application		
all layers	16	114	148
PRC	*	*	*
Number of taxa	*	*	*
Shannon			*
Evenness			

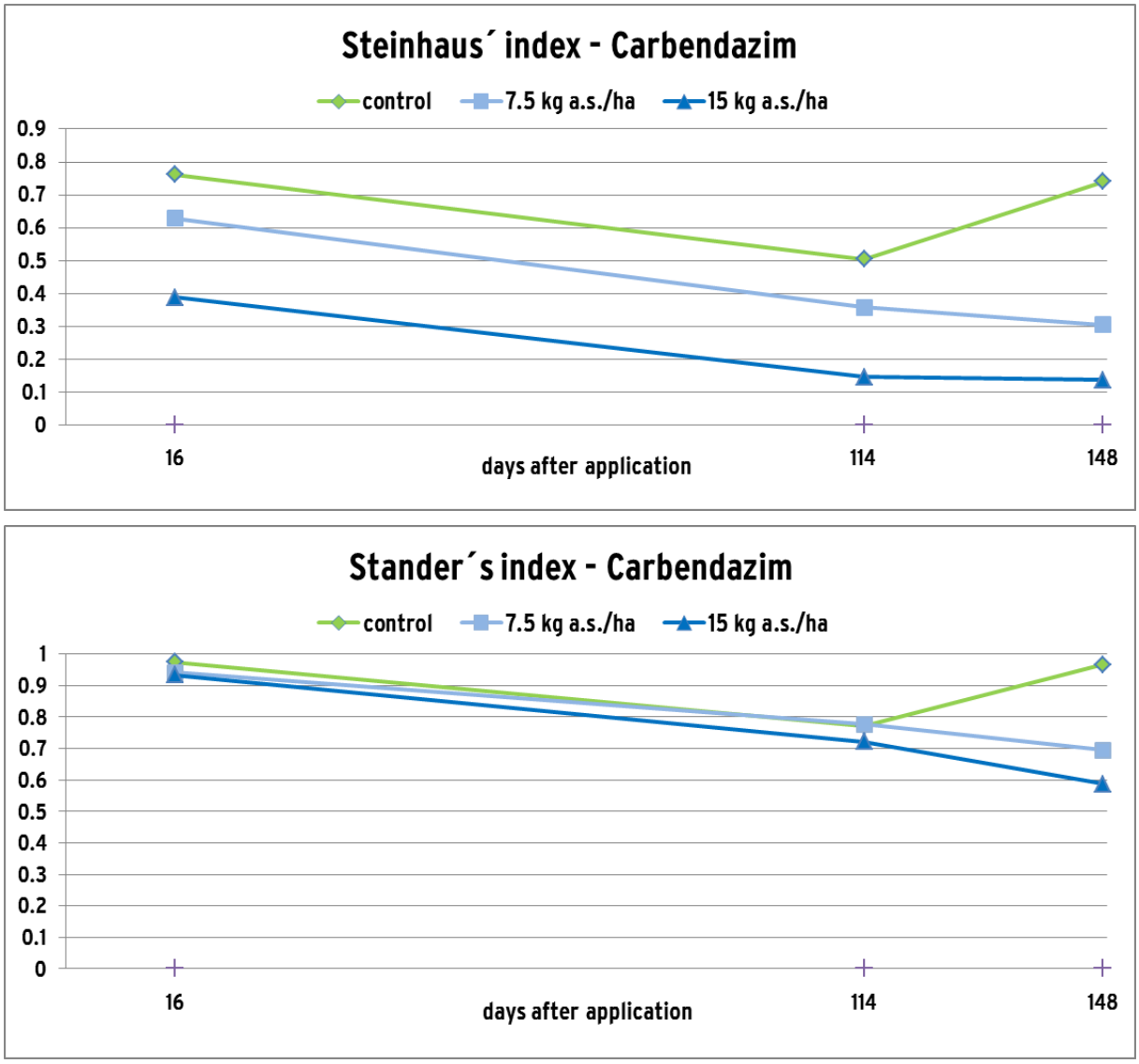

Figure 84 Similarity of Lumbricid diversity summed up over soil layers A-E (above) Steinhaus index (below) Standers index

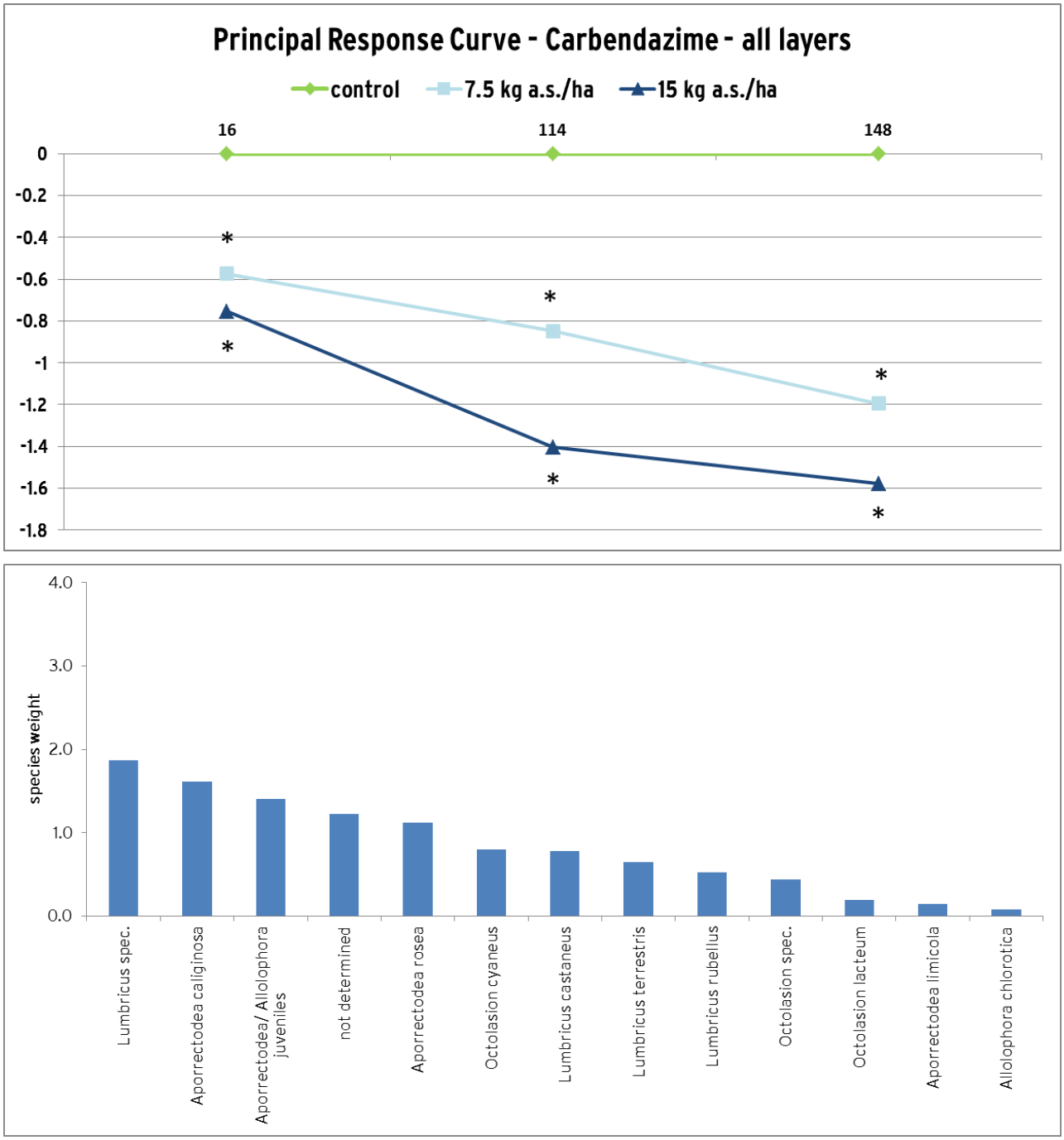

Figure 85 Principal Response Curves to the application of Carbendazim of the lumbricid community calculated for the sum of all layers (layer A, B & C) Results of all Lumbricid species,mean of 5 replicates for controls, 5 replicates for treatments; *: significant effects measured by sample scores of the PCA for the single sampling date. Species weights indicating the share of difference for the different species

7 Modelling the pesticide exposure of organisms in soil layers

7.1 Evaluation of the degradation of Lindane and Imidacloprid in soil using KinGUI version 1.1

The first step of the pesticide exposure modelling calculates degradation rates for Lindane and Imidacloprid applied at low and high dose. Input data are obtained from the outdoor studies which are presented in chapter 4. The experiments were carried out from May 2011 to April 2012. Soil cores (5 cm diameter) were sampled with a height of 2.5 cm, 2.5 cm, 5 cm and 10 cm, named layer A, B, C and D respectively. Usually seven parallel sample cores have been examined. For layer D (10-20 cm) and on day 140 for layer C (5-10 cm) only two sample cores and on day 1 (T0) six replicates were used. A summary of the results of the analysis is presented in Table 30 and Table 31 (Lindane) and Table 33 and Table 34 (Imidacloprid).

7.1.1 Model definitions

The data were analysed using the program KinGUI version 1.1. The kinetics considered were "single first order" (SFO) and "Hockey Stick (HS) for Lindane. Both kinetics were recommended by FOCUS degradation kinetics (FOCUS 2014).

Single First Order kinetics (SFO)

The SFO model is based on an exponential decline as shown in the following equation. The models estimates two fitting parameters the concentration at the beginning (C0) and the rate constant (k_{deg}).

(Equation 14)

$$C = C_0 * \exp(-k_{deg} * t)$$

C: substance concentration at time t (mg/kg)

C_0 substance concentration at time $t=0$ (mg/kg)

k_{deg}: rate contant(1/d)

Hockey-Stick kinetics (HS)

In contrast to the SFO model the hockey stick kinetics consist of two sequential first order declines. At the beginning the substances degrades according to the first leaching rate. At a defined time point the leaching rate switches completely to a second degradation rate. To describe the HS-model three parameters are needed namely two different first order rate constants and the time when the kinetics switches to the second rate constant..

(Equation 15)

$$C = C_0 * \exp(-k_{deg1} * t) \quad \text{for } t \leq t_b$$

$$C = C_0 * \exp(-k_{deg1} * t_b) * \exp[-k_{deg2} * (t-t_b)] \quad \text{for } t > t_b$$

C: substance concentration at time t (mg/kg)

C_0 substance concentration at time $t=0$ (mg/kg)

t_b breakpoint (when rate constant changes)

k_{deg1}: rate constant before $t = t_b$ (1/d)

k_{deg2}: rate constant after $t = t_b$ (1/d)

The structure of the model as considered in KinGUI is presented in Figure 86.

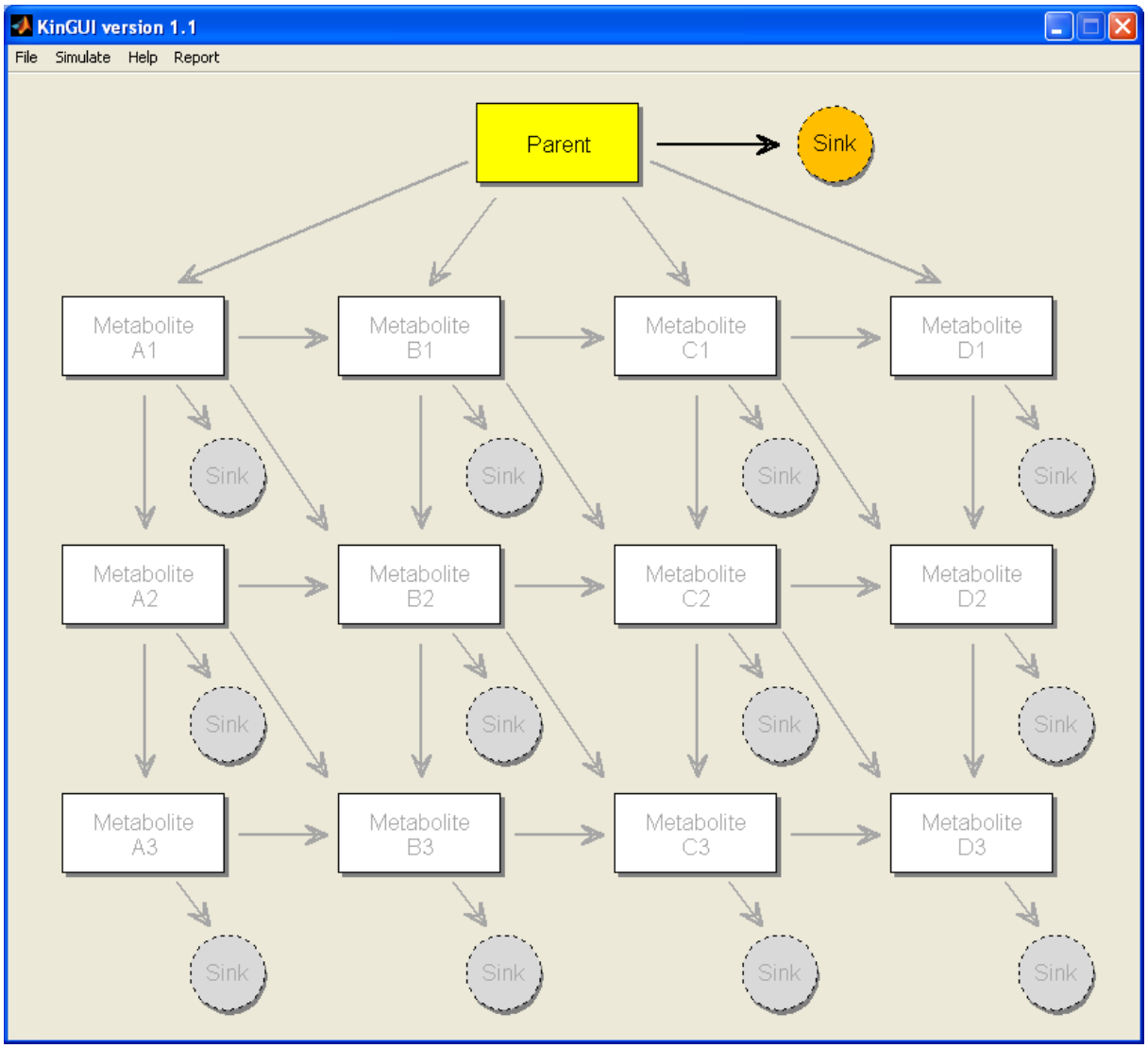

Figure 86 Structure of the models in KinGUI as used for the evaluation

7.1.2 Normalisation

In addition to fitting of the original data the experimental data were also normalised.

This was done based on the 12 hour temperature data in the top soil horizon using the time-step procedure as recommended by FOCUS (2014). The transformation is basically using the following equation.

$$NST_{TEMP}(i) = Q10^{((Temp(i-1)-20\,°C)/10)} + NST_{TEMP}(i-1) \quad \text{(Equation 16)}$$

NST_{TEMP}: Normalised study time for day i considering temperature

Temp(i): Soil temperature on day i

Q10: Q10-factor used for the normalisation (Q10=2.58 were used as recommended by EFSA (2010).

If the temperature of the previous day was 20 °C the NST was calculated to be exact 24 h. If the temperature was above 20 °C the NST was prolonged, if it was below 20 °C the NST was shortened. The effect of the normalisation is shown in the following table for the first month of the experiment.

Normalisation with regard to soil moisture was done using the results of PELMO simulations considering the on-site weather conditions during the study as no experimental data of soil moisture in the TMEs was available. The procedure followed the recommenddationof FOCUS (2014). The transformation is basically using the following equation.

$$NST_{SM}(i) = (SM(i-1)/SM0)^{0.7} + NST_{SM}(i-1) \quad \text{(Equation 17)}$$

NST_{SM}: Normalised study time for day i considering soil moisture

SM(i): Soil moisutre on day i

SM0: Reference Soil moisture (pF 2)

The final study time was found by multiplication of NST_{TEMP} and NST_{SM}

Table 89 Normalisation of the residues using the time-step approach

Date	Real Study time (d)	Temperature (°C)	Soil moisture	Normalised day duration Temperature (d)	Normalised day duration Soil moisture (d)	Total Normalised day duration (d)	Normalised study time (d)
10/05/2011	0	19.75	0.3165	0.98	0.88	0.86	0
11/05/2011	1	19.45	0.307375	0.95	0.86	0.82	0.86
12/05/2011	2	18.25	0.29925	0.85	0.85	0.72	1.68
13/05/2011	3	16.7	0.292625	0.73	0.83	0.61	2.39
14/05/2011	4	16.7	0.288	0.73	0.82	0.60	3.00
15/05/2011	5	14.95	0.284	0.62	0.82	0.51	3.61
16/05/2011	6	14.15	0.2805	0.57	0.81	0.46	4.11
17/05/2011	7	14.75	0.277375	0.61	0.80	0.49	4.58
18/05/2011	8	16.25	0.295875	0.70	0.84	0.59	5.06
19/05/2011	9	18.5	0.337625	0.87	0.92	0.80	5.65
20/05/2011	10	18.7	0.32375	0.88	0.89	0.79	6.45
21/05/2011	11	19.4	0.312125	0.94	0.87	0.82	7.24
22/05/2011	12	18	0.326375	0.83	0.90	0.74	8.06
23/05/2011	13	16.2	0.362875	0.70	0.97	0.68	8.81
24/05/2011	14	19	0.37875	0.91	1.00	0.91	9.48
25/05/2011	15	16.95	0.3655	0.75	0.97	0.73	10.39
26/05/2011	16	19.15	0.35125	0.92	0.95	0.87	11.12
27/05/2011	17	18.2	0.33975	0.84	0.92	0.78	11.99
28/05/2011	18	15.05	0.325625	0.63	0.90	0.56	12.77
29/05/2011	19	17.85	0.37875	0.82	1.00	0.81	13.33
30/05/2011	20	19.2	0.36225	0.93	0.97	0.90	14.15
31/05/2011	21	22.25	0.3495	1.24	0.94	1.17	15.04
01/06/2011	22	15.1	0.335125	0.63	0.92	0.58	16.21
02/06/2011	23	16.8	0.378	0.74	1.00	0.74	16.79
03/06/2011	24	18.95	0.361875	0.91	0.97	0.87	17.52
04/06/2011	25	20.45	0.340625	1.04	0.93	0.97	18.40
05/06/2011	26	23	0.32025	1.33	0.89	1.18	19.36
06/06/2011	27	22.55	0.353125	1.27	0.95	1.21	20.54
07/06/2011	28	21.35	0.34175	1.14	0.93	1.06	21.75
08/06/2011	29	20.65	0.37875	1.06	1.00	1.06	22.81
09/06/2011	30	18.6	0.36525	0.88	0.97	0.85	23.87
10/06/2011	31	18.5	0.35	0.87	0.94	0.82	24.72

7.1.3 Input data

The input data were based on the average concentration over the top 20 cm in all soil profiles considering the measured residues in all soil sampled soil layers. The actual concentrations are summarised in the following tables.

Table 90 Input data used for the fitting (Lindane, low dose)

Sample Code	Day	Month	Year	Study time (d)	Normalised study time (d)	Concentration in soil (mg/kg)
L10Aa	10	5	1	0	0	2.9144
L10Ac	10	5	1	0	0	3.4910
L10Ad	10	5	1	0	0	9.3698
L10Ae	10	5	1	0	0	1.6188
L10Af	10	5	1	0	0	2.4300
L10Ag	10	5	1	0	0	1.7103
L11Aa	24	5	1	14	9.48	5.0175
L11Ab	24	5	1	14	9.48	1.8975
L11Ac	24	5	1	14	9.48	2.1200
L11Ad	24	5	1	14	9.48	4.1613
L11Ae	24	5	1	14	9.48	2.2913
L11Af	24	5	1	14	9.48	2.6360
L11Ag	24	5	1	14	9.48	0.1141
L12Aa	21	6	1	42	33.20	0.4928
L12Ab	21	6	1	42	33.20	1.3425
L12Ac	21	6	1	42	33.20	0.2533
L12Ad	21	6	1	42	33.20	0.5561
L12Ae	21	6	1	42	33.20	2.0943
L12Af	21	6	1	42	33.20	1.7094
L12Ag	21	6	1	42	33.20	0.4741
L13Aa	27	9	1	140	104.76	0.9208
L13Ab	27	9	1	140	104.76	1.9443
L13Ac	27	9	1	140	104.76	1.2980
L13Ad	27	9	1	140	104.76	0.8314
L13Ae	27	9	1	140	104.76	0.5615
L13Af	27	9	1	140	104.76	0.8091
L13Ag	27	9	1	140	104.76	2.1174
L14Aa	15	11	1	189	126.03	1.0703
L14Ab	15	11	1	189	126.03	0.7779
L14Ac	15	11	1	189	126.03	0.9388
L14Ad	15	11	1	189	126.03	1.0160
L14Ae	15	11	1	189	126.03	1.0815
L14Af	15	11	1	189	126.03	0.7344
L14Ag	15	11	1	189	126.03	0.5061
L15Aa	8	5	2	363	172.09	0.6373
L15Ab	8	5	2	363	172.09	0.8139
L15Ac	8	5	2	363	172.09	0.6976
L15Ad	8	5	2	363	172.09	0.7396
L15Ae	8	5	2	363	172.09	0.7268
L15Af	8	5	2	363	172.09	0.1078
L15Ag	8	5	2	363	172.09	0.0505

Table 91 Input data used for the fitting (Lindane, high dose)

Sample Code	Day	Month	Year	Study time (d)	Normalised study time (d) (d)	Concentration in soil (mg/kg)
L20Aa	10	5	1	0	0	10.4738
L20Ac	10	5	1	0	0	7.4275
L20Ad	10	5	1	0	0	7.1525
L20Ae	10	5	1	0	0	5.7975
L20Af	10	5	1	0	0	3.4925
L20Ag	10	5	1	0	0	11.9175
L21Aa	24	5	1	14	9.48	8.4434
L21Ab	24	5	1	14	9.48	10.4210
L21Ac	24	5	1	14	9.48	6.2614
L21Ad	24	5	1	14	9.48	4.1730
L21Ae	24	5	1	14	9.48	2.9303
L21Af	24	5	1	14	9.48	4.2065
L21Ag	24	5	1	14	9.48	0.2970
L22Aa	21	6	1	42	33.20	1.1859
L22Ab	21	6	1	42	33.20	3.4030
L22Ac	21	6	1	42	33.20	1.7334
L22Ad	21	6	1	42	33.20	0.6898
L22Ae	21	6	1	42	33.20	5.6603
L22Af	21	6	1	42	33.20	1.9326
L22Ag	21	6	1	42	33.20	3.5608
L23Aa	27	9	1	140	104.76	6.0358
L23Ab	27	9	1	140	104.76	3.1284
L23Ac	27	9	1	140	104.76	4.8195
L23Ad	27	9	1	140	104.76	2.0566
L23Ae	27	9	1	140	104.76	2.6611
L23Af	27	9	1	140	104.76	1.3795
L23Ag	27	9	1	140	104.76	2.0256
L24Aa	15	11	1	189	126.03	4.6093
L24Ab	15	11	1	189	126.03	2.0194
L24Ac	15	11	1	189	126.03	4.0473
L24Ad	15	11	1	189	126.03	1.1665
L24Ae	15	11	1	189	126.03	3.7183
L24Af	15	11	1	189	126.03	1.4904
L24Ag	15	11	1	189	126.03	2.6114
L25Aa	8	5	2	363	172.09	3.0354
L25Ab	8	5	2	363	172.09	2.4378
L25Ac	8	5	2	363	172.09	1.7436
L25Ad	8	5	2	363	172.09	1.1999
L25Ae	8	5	2	363	172.09	0.9638
L25Af	8	5	2	363	172.09	2.5591
L25Ag	8	5	2	363	172.09	2.8049

Table 92 Input data used for the fitting (Imidacloprid, low dose)

Day	Month	Year	Study time (d)	Normalised study time (d)	Concentration (mg/kg)
10	5	1	0	0	0.3187
10	5	1	0	0	0.3047
10	5	1	0	0	0.2546
10	5	1	0	0	0.3251
10	5	1	0	0	0.2630
24	5	1	14	9.48	0.5693
24	5	1	14	9.48	0.3861
24	5	1	14	9.48	0.4316
24	5	1	14	9.48	0.3357
24	5	1	14	9.48	0.5652
21	6	1	42	33.20	0.3019
21	6	1	42	33.20	0.2609
21	6	1	42	33.20	0.1590
21	6	1	42	33.20	0.2387
21	6	1	42	33.20	0.3017
27	9	1	140	104.76	0.1061
27	9	1	140	104.76	0.0372
27	9	1	140	104.76	0.0442
27	9	1	140	104.76	0.1007
27	9	1	140	104.76	0.0363
15	11	1	189	126.03	0.0829
15	11	1	189	126.03	0.0916
15	11	1	189	126.03	0.1163
15	11	1	189	126.03	0.0755
15	11	1	189	126.03	0.0450
8	5	2	363	172.09	0.0225
8	5	2	363	172.09	0.0271
8	5	2	363	172.09	0.0222
8	5	2	363	172.09	0.0728
8	5	2	363	172.09	0.0712

Table 93 Input data used for the fitting (Imidacloprid, high dose)

Day	Month	Year	Study time (d)	Normalised study time	Concentration
10	5	1	0	0	0.6890
10	5	1	0	0	1.4682
10	5	1	0	0	1.2788
10	5	1	0	0	0.8476
10	5	1	0	0	1.5528
24	5	1	14	9.48	0.8702
24	5	1	14	9.48	0.4098
24	5	1	14	9.48	1.1338
24	5	1	14	9.48	0.5556
24	5	1	14	9.48	0.9888
21	6	1	42	33.20	0.9067
21	6	1	42	33.20	0.7729
21	6	1	42	33.20	0.2319
21	6	1	42	33.20	0.8474
24	5	1	42	33.20	0.6432
27	9	1	140	104.76	0.1865
27	9	1	140	104.76	0.1040
27	9	1	140	104.76	0.0793
27	9	1	140	104.76	0.0769
27	9	1	140	104.76	0.0692
15	11	1	189	126.03	0.2405
15	11	1	189	126.03	0.1971
15	11	1	189	126.03	0.2203
15	11	1	189	126.03	0.1652
15	11	1	189	126.03	0.1683
8	5	2	363	172.09	0.1729
8	5	2	363	172.09	0.2411
8	5	2	363	172.09	0.1748
8	5	2	363	172.09	0.1318
8	5	2	363	172.09	0.1052

7.1.4 Results of the fitting

The analyses were based on the models shown in Figure 86 assuming SFO (Single First Order) and HS (Hockey Stick) kinetics. The results of the optimisation are presented in the following two tables. Results are presented for the normalised as well as the non-normalised results. However, when comparing the DT50 values with the inverse modelling study only the normalised values should be considered as they reflect the same conditions as the PELMO simulations.

Table 94 Statistical Results (SFO)

Data set	chi² (%)	C0 (mg/kg)	Sd(C0) (mg/kg)	rate (1/d)	DT50 (d)	sd(rate) (1/d)
Results obtained from normalised study time used as input data						
Lindane low dose	25.9	2.99	0.4095	0.0126	55	0.0041
Lindane high dose	23.9	5.96	0.6505	0.0072	96	0.0020
Imidacloprid low dose	24.9	0.3858	0.0276	0.0127	55	0.0023
Imidacloprid high dose	14.1	1.0774	0.0815	0.0161	43	0.0032
Results obtained from study time used as input data (not normalised)						
Lindane low dose	28.3	2.93	0.4131	0.0085	81	0.0032
Lindane high dose	26.2	5.69	0.6368	0.0041	170	0.0013
Imidacloprid low dose	26.0	0.3812	0.0284	0.0088	79	0.0018
Imidacloprid high dose	15.7	1.0889	0.0858	0.0127	55	0.0028

Table 95 Statistical Results (HS)

Data set	chi²	M0 (%)	Tb (d)	rate 1 (1/d)	DT50 (1) (d)	sd(rate 1) (1/d)	rate 2 (1/d)	DT50 (2) (d)	sd(rate 2) (1/d)
Results obtained from normalised study time used as input data									
Lindane low dose	22.7	3.64	10.5	0.0554	12.5	0.0277	0.0080	86.6	0.0038
Lindane high dose	16.4	7.74	10.2	0.0594	11.7	0.0209	0.0041	169.1	0.0021
Imidacloprid low dose	24.8	0.3727	10.0	$7.6\ 10^{-11}$	>1000	0.0155	0.0155	44.7	0.0029
Imidacloprid high dose	13.5	1.1630	0.1041	1.1172	0.6	71.0064	0.0136	51.0	0.0033
Results obtained from study time used as input data (not normalised)									
Lindane low dose	35.6	2.93	2.2e-014	0.8208	0.8	>1000	0.0085	81.5	0.0036
Lindane high dose	18.2	7.70	0.2669	2.2137	0.3	>1000	0.0022	315	0.0012
Imidacloprid low dose	32.7	0.38	2.2e-014	5.5e-009	>1000	>1000	0.0088	79	0.0021
Imidacloprid high dose	19.7	1.09	2.2e-014	0.1906	3.5	>1000	0.0127	55	0.0035

The non-normalised optimisations always led to higher chi² values than the respective normalised results. Obviously, taking into account more information from the study conditions (temperature, soil moisture) for the fitting improves the quality of the results. The following discussion is therefore focusing to the normalised DT50 values.

The normalised DT50 and DT90 values, which are reliable to describe the the dissipation of Lindane and Imidacloprid during the experiment are summarised in Table 96.

According to the results there is evidence that the degradation follows biphasic kinetics for Lindane. This is also supported by the visual representations in the following figures. The DT50 values based on the HS kinetics (bold characters) are recommended as appropriate normalised values to describe the dissipation of Lindane in the TMEs during the experiment. The slow rate DT50s of 87 and 169 days of the HS kinetics (rate 2) probable better describe the degradation of the active substance in the TMEs.

According to the results there is no evidence that the degradation follows biphasic kinetics for Imidacloprid. This is also supported by the visual representations in the following figures. The DT50 values of 55 and 43 days based on the SFO kinetics (bold characters) seem to be as appropriate normalised values to describe the dissipation of Imidacloprid in the TMEs during the experiment. For the low dose variation the resulting DT50 in the first phase was calculated to be >>1000 d. That could be interpretated as a lag phase. However, this was not further evaluated since the SFO fits for Imidacloprid were anyway considered as more suitable. A summary of the results considering only the relevant kinetics is presented in the following table.

Table 96 Calculated DT50 and DT90

Data set	SFO kinetic		HS kinetic			
	DT50 (d)	DT90(d)	First phase (fast)		Second phase (slow)	
			DT50 (d)	DT90(d)	DT50 (d)	DT90(d)
Results obtained from normalised study time used as input data						
Lindane low dose	-	-	**13**	41	**87**	286
Lindane high dose	-	-	**12**	39	**169**	558
Imidacloprid low dose	**55**	182	-	-	-	-
Imidacloprid high dose	**43**	143	-	-	-	-
Results obtained from study time used as input data						
Lindane low dose	-	-	**0.8**	2.6	82	269
Lindane high dose	-	-	**0.3**	1.0	315	1040
Imidacloprid low dose	79	263	-	-	-	-
Imidacloprid high dose	55	182	-	-	-	-

Graphs of the fittings both for normalized and not normalized input data are presented in Annex 4.

7.2 Inverse modelling with PELMO

Inverse modelling studies are usually performed within the process of PPP authorisation in order to obtain key sorption and degradation parameters for risk assessment, such as Kfoc (Freundlich sorption constant related to organic carbon) and DegT50 (degradation time to 50%) from higher tier studies (e.g. outdoor lysimeter studies or field dissipation studies), instead of directly deriving such compound properties from standard laboratory studies. Hence, inverse modelling can be used to improve the standard kinetic modelling by considering additional information such as weather information and residue distributions from field studies over a longer time period. Using this methodology within the project context may help to better understand both dissipation and leaching processes that led to certain residue concentrations in the different soil layers of the terrestric model ecosystems over time.

The same input data were used as for the KinGui fitting in the previous section (outdoor studies presented in chapter 4). The experiments were carried out from May 2011 to April 2012. Soil cores (5 cm diameter) were sampled with a height of 2.5 cm, 2.5 cm, 5 cm and 10 cm, named layer A, B, C and D respectively. Usually seven parallel sample cores have been examined. For layer D (10-20 cm) and on day 140 for layer C (5-10 cm) only two sample cores and on day 1 (T0) six replicates were used. A summary of the results of the analysis is presented in Table 30 and Table 31 (Lindane) and Table 33 and Table 34 (Imidacloprid).

7.2.1 Methodology

The inverse modelling study was done using the software tool “inversePELMO” which combines the simulation model PELMO with the optimisation tool “PEST” (Klein 2011b).

Generally, two steps have to be conducted when performing inverse modelling studies:

First, the hydrology in soil is optimised, followed by the optimisation of pesticide fate as shown in Figure 87.

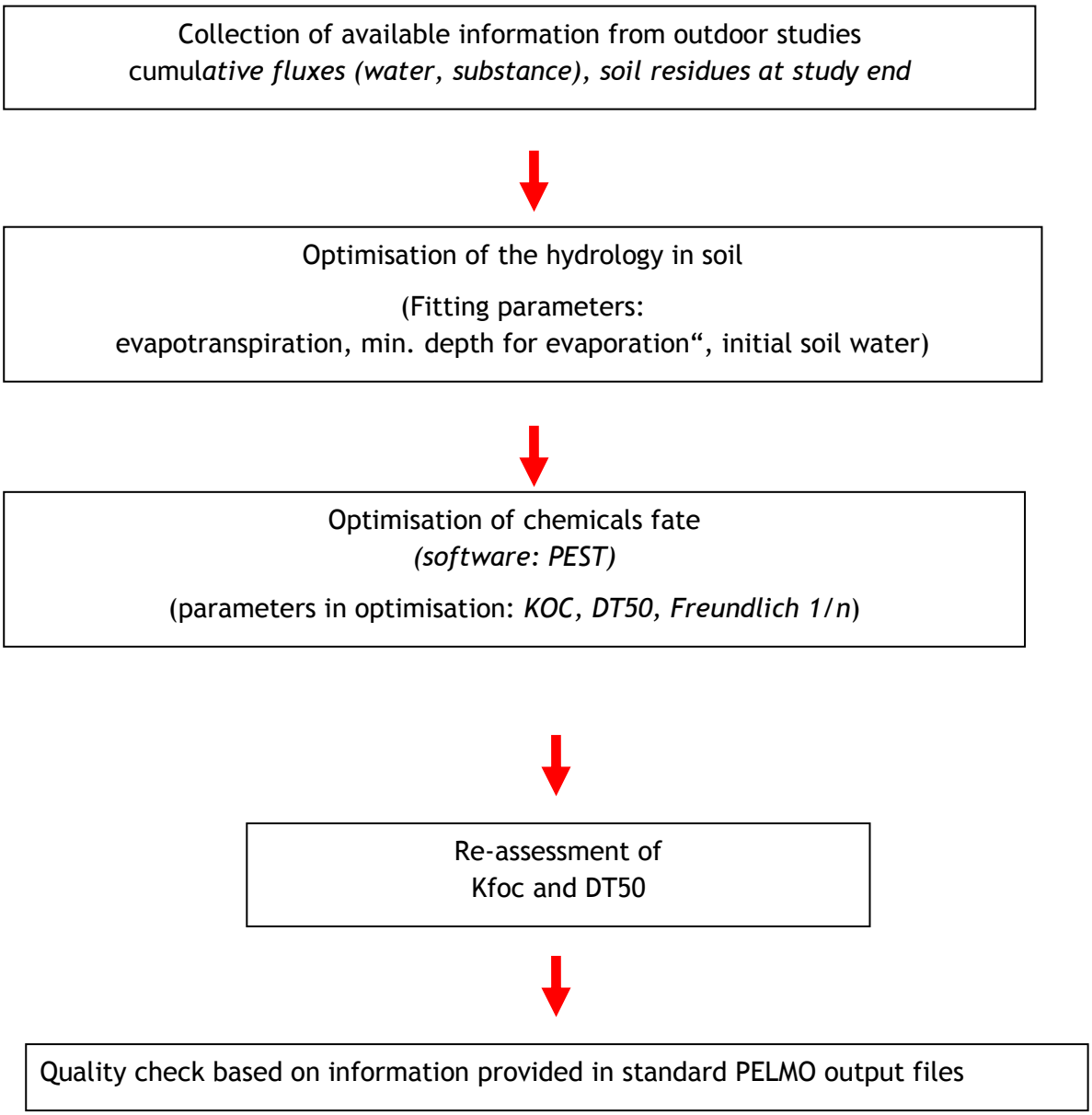

Figure 87 Flow chart: File handling of a flux optimisation with InversePELMO

PELMO (Klein 1995, Jene 1998, Klein 2011a) is the standard model for doing leaching simulations for registration purposes in Germany (Holdt et al. 2011) and in Europe FOCUS (2009). However, PELMO with its normal shell is not designed to perform inverse modelling studies because these studies require several model runs including automatic modification of input files based on the comparison with experimental results.

A scheme that shows the file handling is presented in Figure 88 for an optimisation of pesticide properties based on cumulative fluxes in the leachate. All pesticide and application parameters are gathered in text files with extension “psm”. The scenario input data can be found in files with extension „sze”. Before starting the inverse modelling calculation a first simulation (with initial conditions for either the soil hydrology or pesticide properties) should be prepared using the normal shell (which can be called directly from *InversePELMO*).

The optimisation itself is done automatically by *InversePELMO*.

As shown in Figure 88 *InversePELMO* calls PEST which then reads the control file pest_pesticide.pst with all information about the parameters considered for the optimisation including their initial values and their allowed ranges. Also the experimental data (e.g. cumulative fluxes) can be found in pest_pesticide.pst.

According to the information in pesticide.tpl *PEST.exe* is able to create pesticide input files (pesticide.psm) for PELMO including the correct position for the input parameters used in the optimisation. After this file has been written PEST calls PELMO for a simulation. To make the interface between PELMO and PEST more stable a second program is always executed after PELMO (in the example presented in Figure 88: PELMO_results_pesticide.exe) which gathers the important simulation results (e.g. calculated cumulative pesticide fluxes) and writes them into the file pest.plm. After both programs (PELMO and PELMO_results_pesticide.exe) are finished PEST gets control again and will read the important simulation results listed in pest.plm (instructions for PEST to read pest.plm is given in pest.ins). According to the simulation results a new iteration is initiated with new DT50 and Kfoc data for the optimisation until the optimisation is finalised.

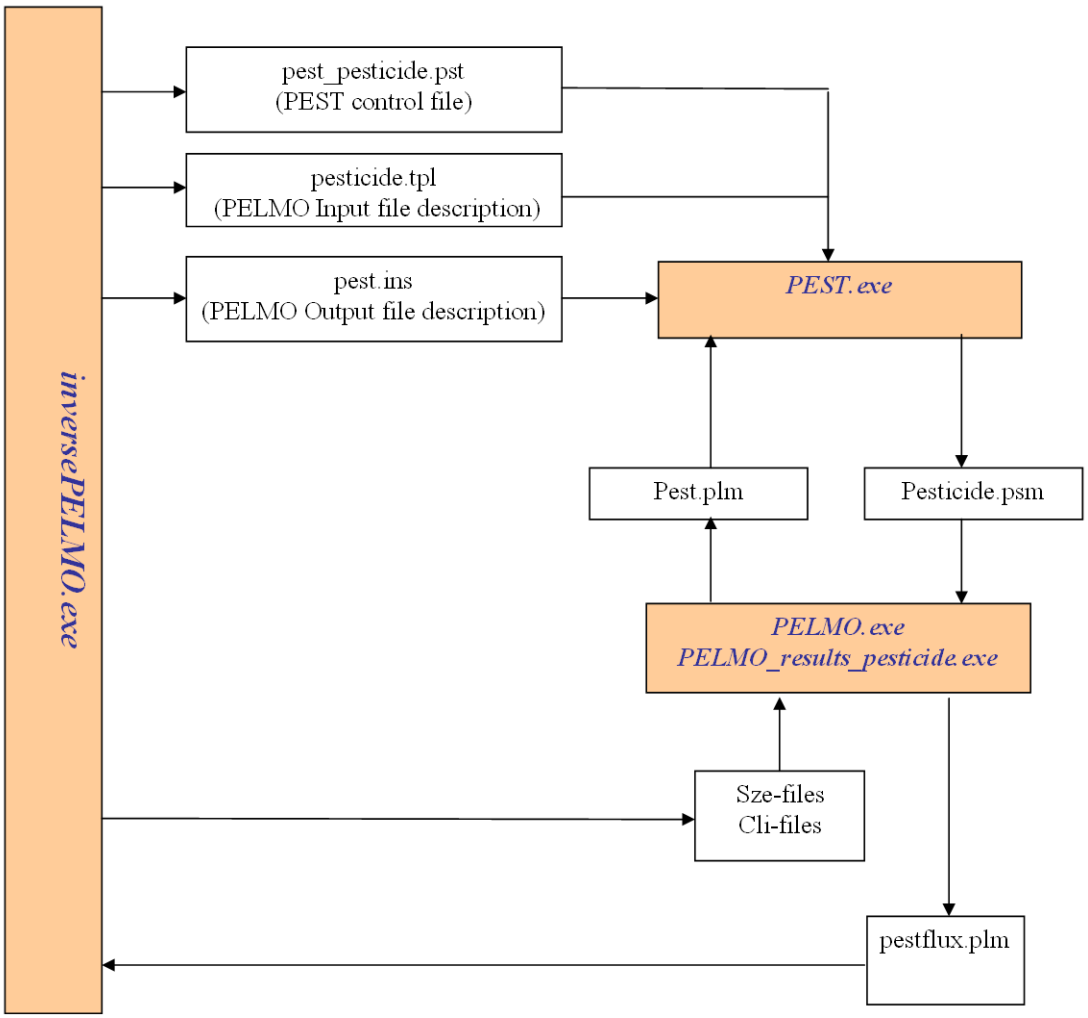

Figure 88 Flow chart: File handling of a flux optimisation with InversePELMO

7.2.2 Environmental and agricultural data

For the calculation soil information was based on the actual soil description in the experiments. The soil profile information is summarised in Table 97.

Table 97 Soil profile information used for the inverse modelling study

Layer	Corg (%)	pH	Sand content (%)	Silt content (%)	Clay content (%)	Disp. length (cm)
0-5 cm	4.7	6.5	20.8	73.1	6.1	2.5
5 – 10 cm	3.1	6.1	34.2	60.5	5.4	2.5
10 – 25 cm	2.7	6.2	26.3	68.3	5.4	2.5
25 – 40 cm	2.7	6.2	26.3	68.3	5.4	2.5

The Hamon equation was used to estimate potential evapotranspiration. The depth to which evaporation was computed year-round was 10 cm.

The monthly and annual precipitation and temperature data during the study is given in Table 98. Begin of the study was the 10th May 2011.

The nominal application rates for Lindane were 7.5 kg/ha (low dose) and 22 kg/ha (high dose). However, according to the kinetic analysis in the previous section the actual rate for the high dose variation was only 16.3 kg/ha which was therefore used in the optimisation.

According to the kinetic analysis there were significant losses (about 70 %) due to a fast process presumably volatilisation. The application depth in PELMO was set to 0.125 cm (instead of surface application) in order to simulate similar volatilisation losses as estimated from the previous data fitting.

The nominal application rates for Imidacloprid were 0.75 kg/ha (low dose) and 2.0 kg/ha (high dose). However, according to the kinetic analysis in the previous section the actual rate for the high dose variation was 2.38 kg/ha which was therefore used in the optimisation.

Table 98 Climate data during the study (TME study 1) considered for the inverse modelling

Month	Monthly Precipitation [mm]**	Annual Precipitation [mm]	Monthly Temperature [°C]	Annual [°C]	Temperature
May*	83		22.9		
June	77.5		24.0		
July	78		23.6		
August	133.5		24.0		
September	59		21.5		
October	52		15.2		
November	49		12.0		
December	151		9.7		
January	137		4.7		
February	39		-0.2		
March	14.5		8.9		
April	14.5		10.5		
May***	0	888	15.9	14.8	

* after application ** including precipitation *** only until study end

The crop “grass” was considered for the study (see Table 99 for further information). Standard parameters were considered for all other crop parameters.

Table 99 crop rotation dates (TME study 1) considered for the inverse modelling

Stage	Date
emergence	01-May-11
maturation	01-Sept-11
Harvest	31-Dec-12

7.2.3 Compound data

The compound Lindane was analysed in the inverse modelling study.

The input parameters KOC and DT50 were considered in the optimisation. An overview of the other substance specific data is given in Table 100.

Table 100 Pesticide input parameters used for Lindane

Parameter	Unit	Value
Molar mass	(g mol-1)	290.8
Solubility in water at 20 °C	(mg L-1)	7
Solubility in water at 30 °C (estimated)	(mg L-1)	14
Vapour pressure at 20° C	(Pa)	0.01
Vapour pressure at 30° C (estimated)	(Pa)	0.04
Reference temperature for degradation, vaporisation and dissolution	(°C)	20
Reference soil moisture for degradation	(-)	at 10 kPa (field capacity)
Q10-factor (increase of degradation rate with an increase of temperature of 10°C)	(-)	2.58
Exponent of degradation - moisture relationship according to Walker)	(-)	0.7
Exponent of the Freundlich-Isotherm	(-)	0.9
Non-equilibrium sorption	(-)	not considered
TSCF = transpiration stream concentration factor	(-)	not considered

The compound Imidacloprid was analysed in the inverse modelling study.

The input parameters KOC and DT50 were considered in the optimisation. An overview of the other substance specific data is given in Table 101.

Table 101 Pesticide input parameters used for Imidacloprid

Parameter	Unit	Value
Molar mass	(g mol-1)	255.66
Henry's law constant at 20° C*	(J/Kmol)	0
Henry's law constant at 30° C	(J/Kmol)	0
Reference temperature for degradation, vaporisation and dissolution	(°C)	20
Reference soil moisture for degradation	(-)	at 10 kPa (field capacity)
Q10-factor (increase of degradation rate with an increase of temperature of 10°C)	(-)	2.58
Exponent of degradation - moisture relationship according to Walker)	(-)	0.7
Exponent of the Freundlich-Isotherm	(-)	0.9
Non-equilibrium sorption	(-)	not considered
TSCF = transpiration stream concentration factor	(-)	not considered

* Volatilisation not considered

7.2.4 Experimental results

The total average leachate amount collected during the study time was 353 L/m². The amounts of leachate at different sampling times are provided in Table 102. Significant Lindane residue concentrations were not found in the leachate.

Table 102 Time dependent average Leachate amounts in the TMEs

Date	Month	Year	Leachate (L/m²)
30	5	11	0.06
6	6	11	1.49
8	6	11	0.06
21	6	11	0.12
29	6	11	0.17
14	7	11	0.05
25	7	11	0.81
28	7	11	0.22
9	8	11	0.29
15	8	11	0.08
18	8	11	0.03
19	8	11	21.64
22	8	11	0.06
12	9	11	0.10
22	9	11	0.04
7	10	11	1.05
12	10	11	5.21
26	10	11	3.26
2	12	11	41.22
31	12	11	105.09
4	1	12	32.69
5	1	12	22.77
6	1	12	7.01
9	1	12	15.18
20	1	12	27.44
23	1	12	25.54
27	1	12	7.30
17	2	12	11.15
24	2	12	12.84
27	2	12	1.08
9	3	12	9.05
12	3	12	0.06

A summary of the measured Lindane residues in the soil cores is given in Table 30 (low dose) and Table 31 (high dose). The respective summary for the Imidacloprid residues in the soil cores are shown in Table 33(low dose) and Table 34(high dose).

7.2.5 Results of the inverse modelling study

7.2.5.1 Optimisation of the leachate

For the optimisation of the leachate three input parameters were considered in the fitting. Their initial values and their possible range are shown in Figure 89.

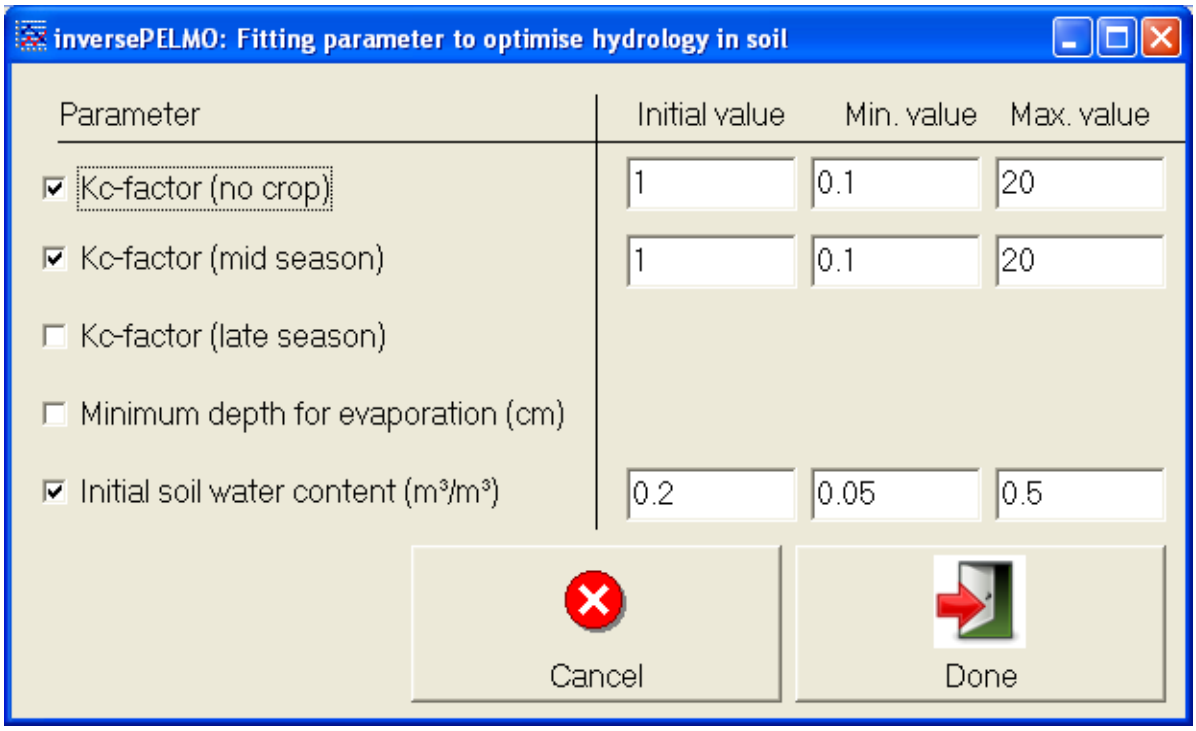

Figure 89 Parameters used in the optimisation of the leachate (TME study 1)

After the optimisation the results summarised in Figure 90 (taken from inversePELMO) were obtained.

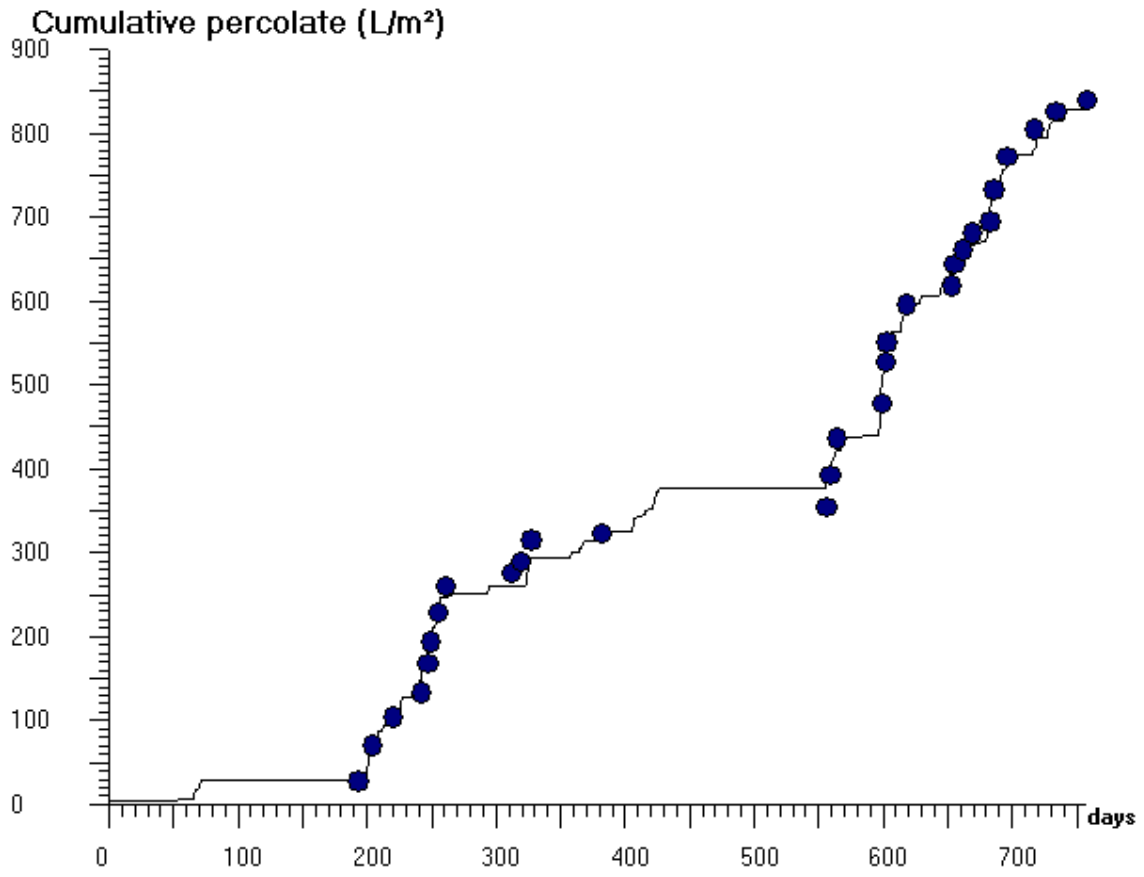

Figure 90 Results of the optimisation (cumulative leachate, TME)

The minimum error for which the Chi²-Test passes according to FOCUS was found to be 7.82 % which supports the excellent agreement shown in the figure.

Table 103 Optimised parameter for the leachate (TME study 1)

Parameter	Initial values	Estimated values
KC0	1	2.242
KC1	1	0.63233
MOI0	0.2	0.5

7.2.5.2 Optimisation of Lindane residues in soil

For the optimisation of the Lindane residues after the study the parameters “DT50” and “KOC” were considered in the fitting. Their initial values and their possible range are shown in Figure 91.

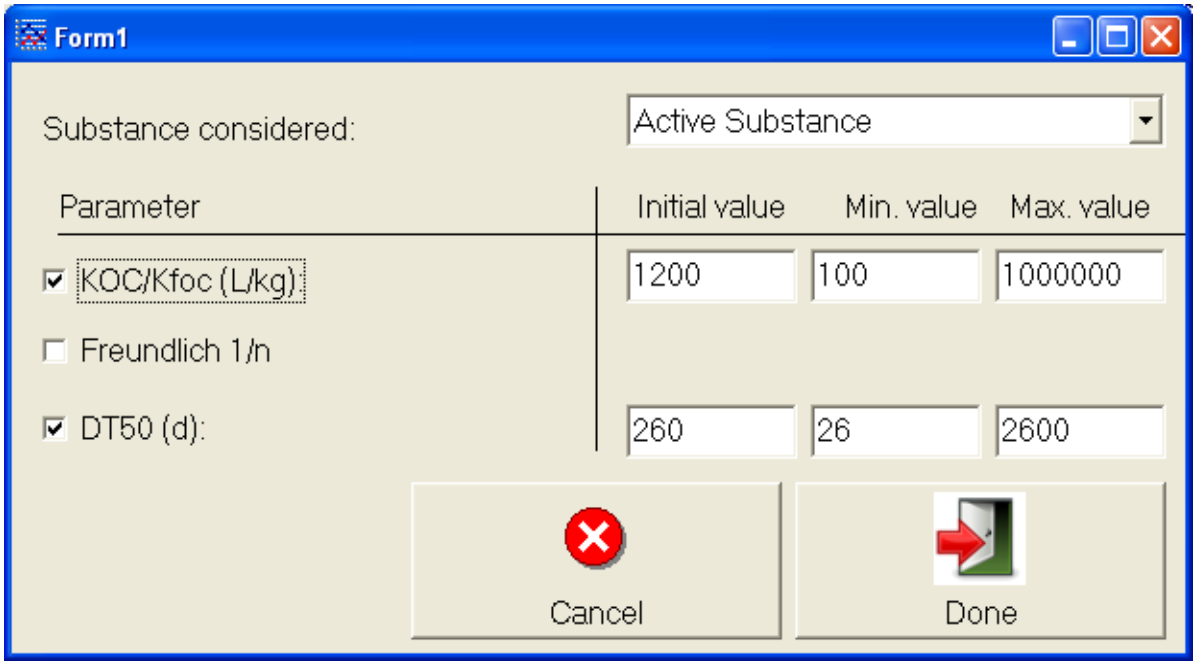

Figure 91 Parameters used in the optimisation of Lindane

TMEs with low dose

Two variations were considered with and without residue weighting.

After the optimisation the results summarised in Figure 92 (no residue weighting) and Figure 93 (with residue weighting) was obtained.

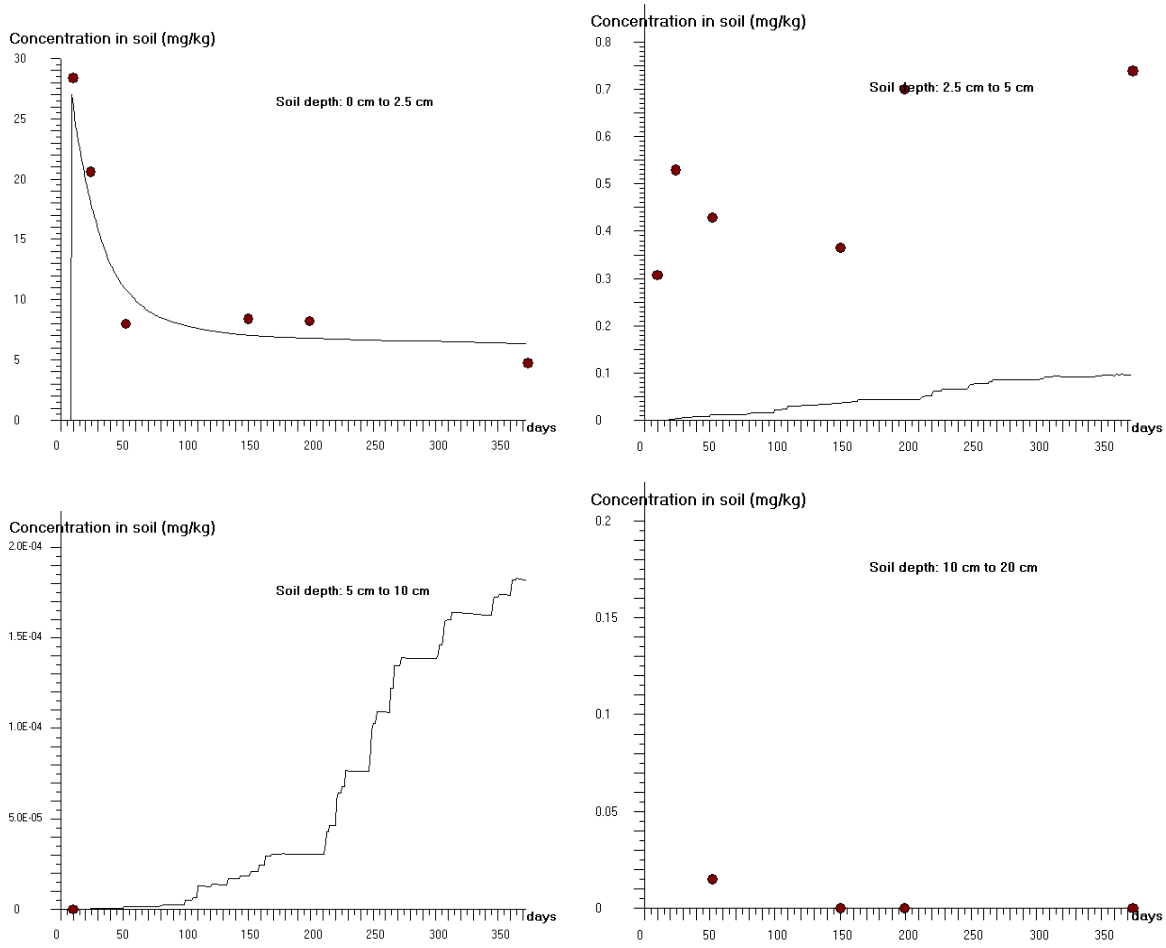

Figure 92 Results of the optimisation (soil residues, Lindane, low dose, no residue weighting)

The data weighting was done to focus on the transport of Lindane to the deeper soil layers. As the experimental soil concentrations below 2.5 cm were generally rather low they were hardly considered in the optimisation (see Figure 92).

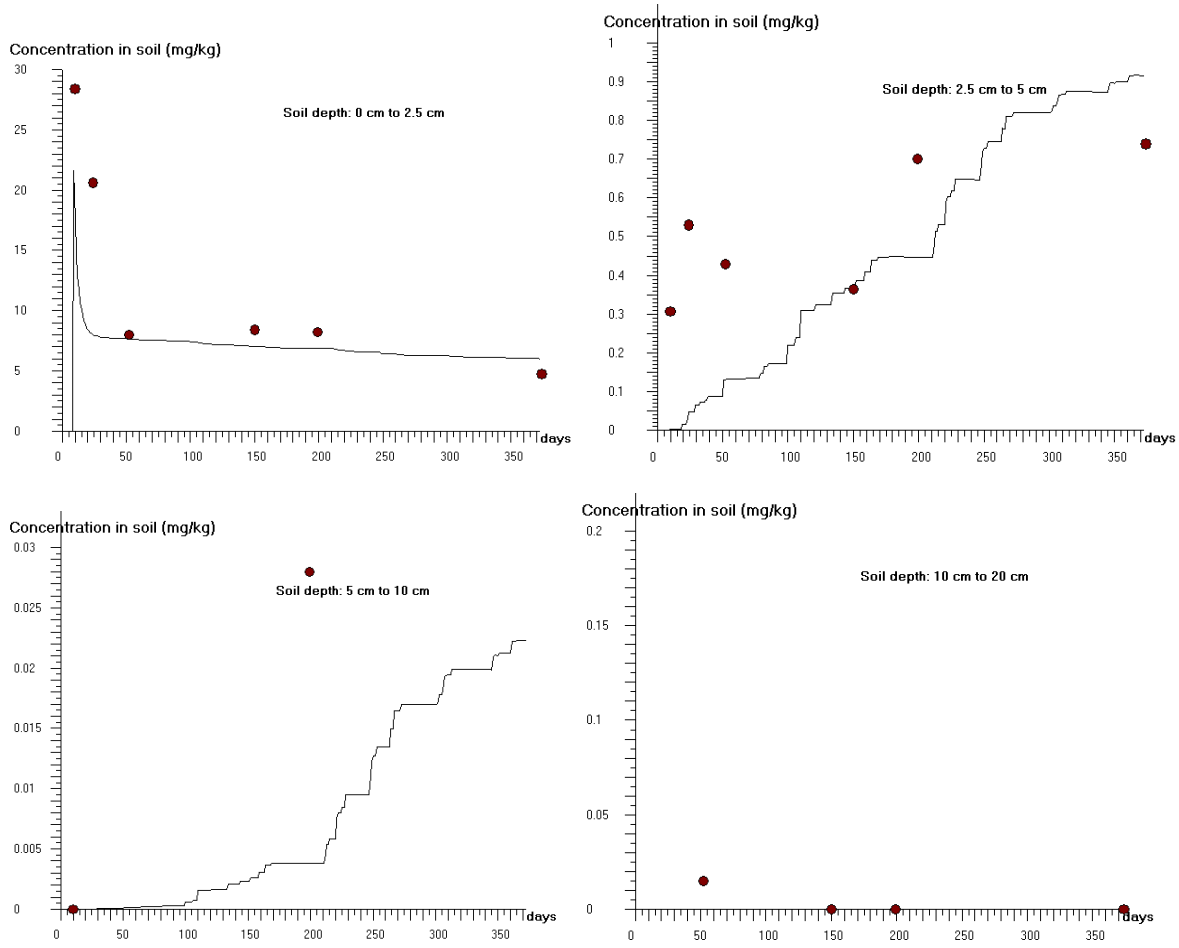

Figure 93 Results of the optimisation (soil residues, Lindane, low dose, residue weighting)

Figure 93 shows the result of the optimisation when more weight was put to the soil residues below 2.5 cm. The overall graphical agreement is better for the variation including data weighting. The optimised parameters are summarised in the following table. It shows that in both variations Lindane was found to be significant more persistent than expected based on the initial DT50 (260 days). This result is in line with the previous kinetic evaluation in the previous section.

Table 104 Optimised parameter for the soil concentrations (TMEs low dose)

Variation	Parameter	Estimated value	95% confidence limits lower limit	value	Initial parameter
	koc	28000	20998	35000	1200
no weighting	DT50	598	268	0	260
residue	koc	3676	2036	5315	1200
weighting	DT50	2475	365	0	260

The FOCUS chi² test was calculated to be 23.15 % (no weighting of residues). The volatilisation losses in the two simulations were 71 %.

TMEs with high dose

Two variations were considered with and without residue weighting.

After the optimisation the results summarised in Figure 94 (no residue weighting) and Figure 95 (with residue weighting) was obtained.

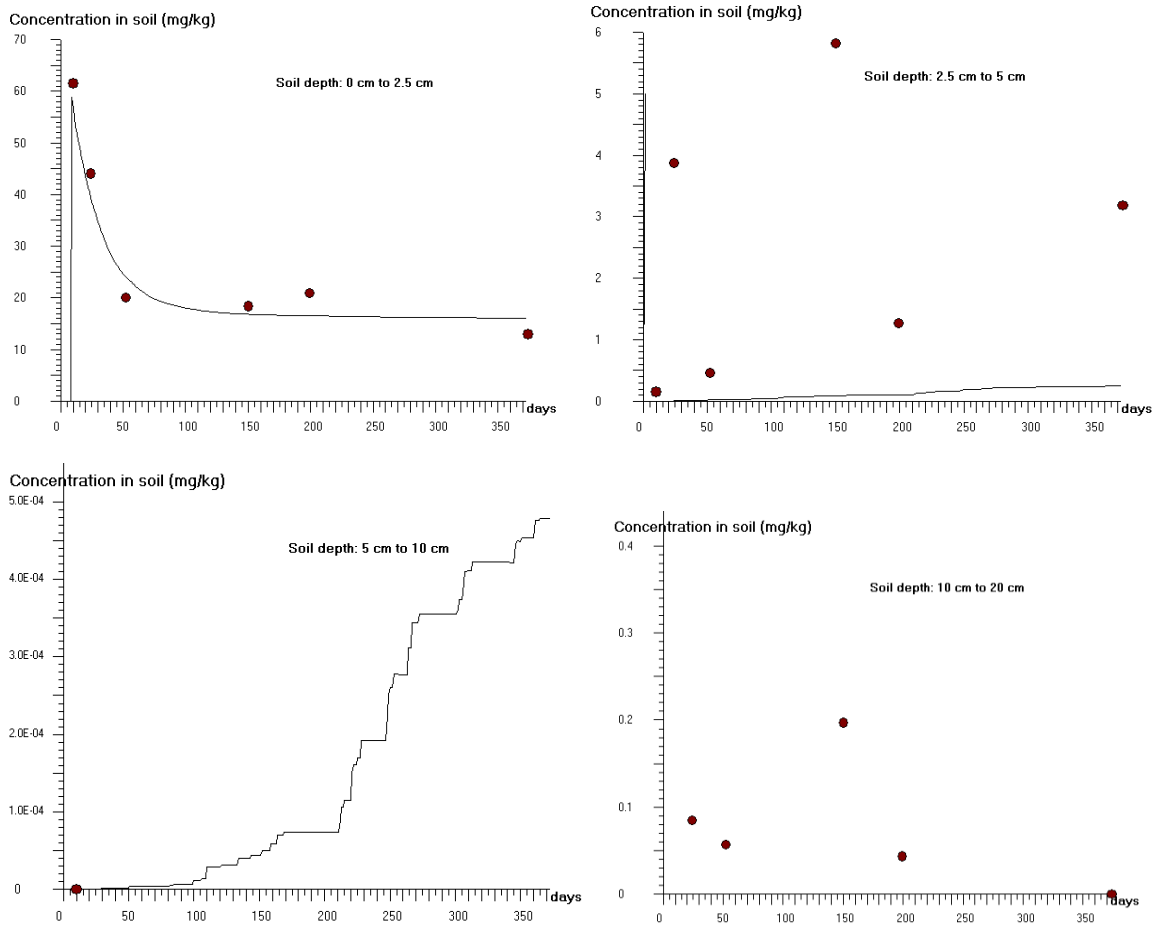

Figure 94 Results of the optimisation (soil residues, Lindane, high dose, no residue weighting)

The data weighting was done to focus on the transport of Lindane to the deeper soil layers. As the concentrations below 2.5 cm were generally rather they were hardly considered in the optimisation (see Figure 94).

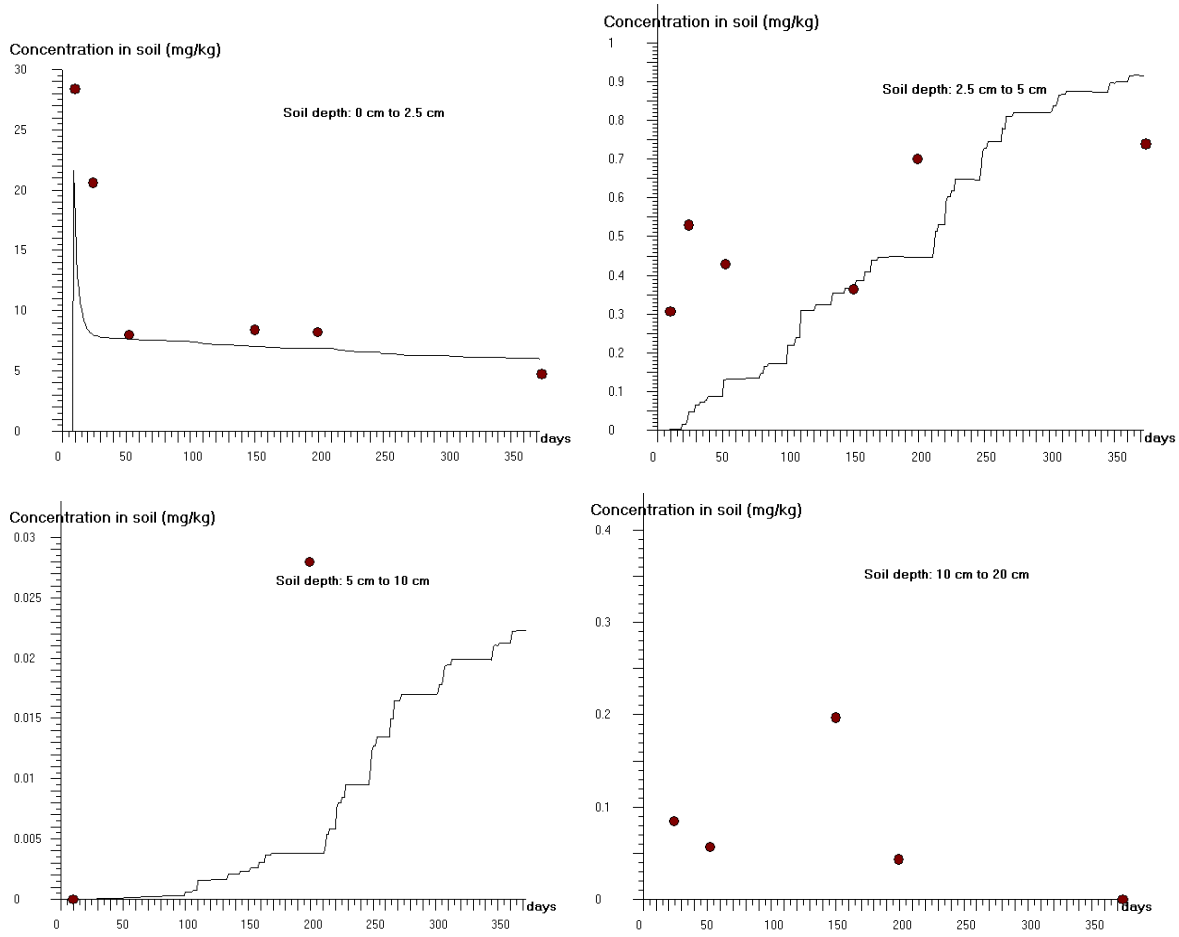

Figure 95 Results of the optimisation (substance flux, TMEs, high dose, residue weighting)

Figure 95 shows the result of the optimisation when more weight was put to the soil residues below 2.5 cm. The overall graphical agreement is better for the variation including data weighting. The optimised parameters are summarised in the following table. It shows that in both variations Lindane was found to be significant more persistent than expected based on the initial DT50 (260 days). This result is in line with the previous kinetic evaluation in the previous section.

Table 105 Optimised parameter for the soil concentrations (TMEs high dose)

Variation	Parameter	Estimated value	95% confidence limits lower limit	value	Initial parameter
	koc	30000	21944	38056	1200
no weighting	DT50	2567	444	0	260
residue	koc	1344	319	2369	1200
weighting	DT50	1308	166	0	260

The FOCUS chi² test was calculated to be 23.52 % (no weighting of residues).

The volatilisation losses in the two simulations were 72 %.

7.2.5.3 Optimisation of Imidacloprid residues in soil

For the optimisation of the Imidacloprid residues after the study the parameters “DT50” and “KOC” were considered in the fitting. Their initial values and their possible range are shown in Figure 96.

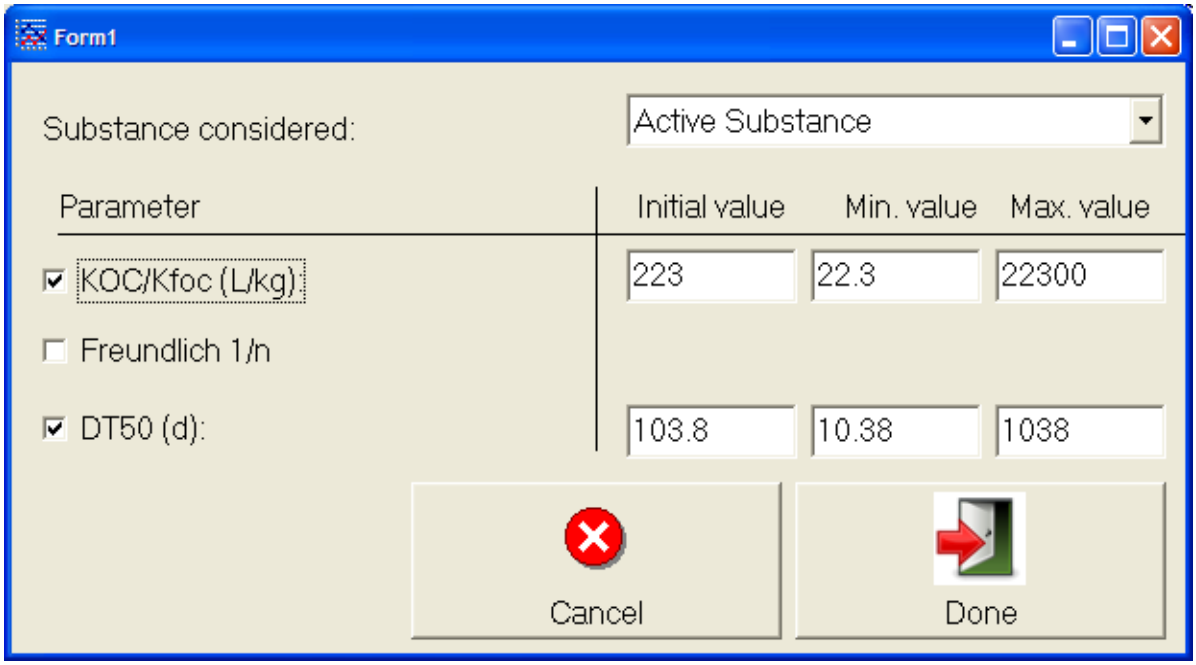

Figure 96 Parameters used in the optimisation of Imidacloprid

TMEs with low dose of Imidacloprid

Weighting of residues was not considered. After the optimisation the results summarised in Figure 97 (no residue weighting) were obtained.

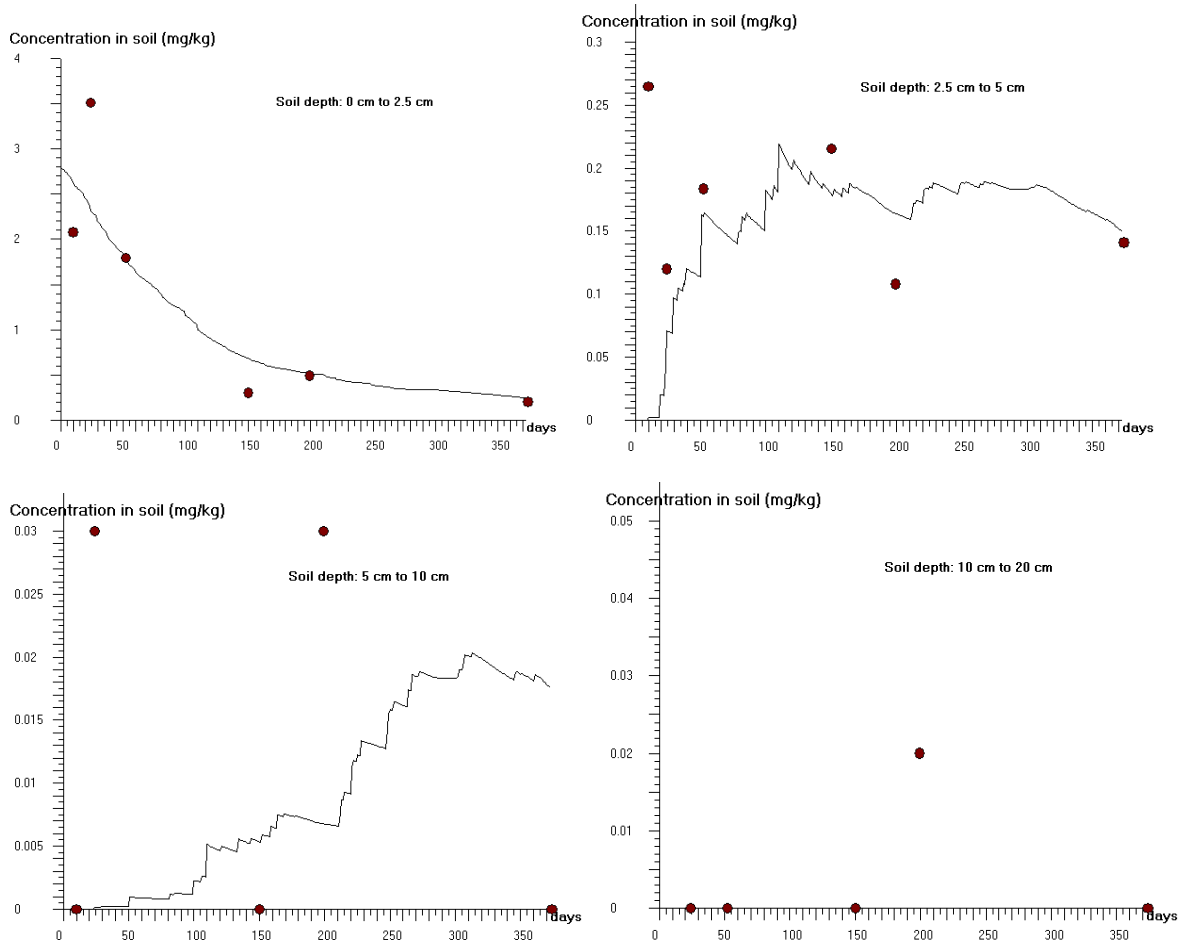

Figure 97 Results of the optimisation (soil residues, Imidacloprid, low dose, no residue weighting)

The optimised parameters are summarised in the following table. It shows that Imidacloprid was found to be slightly less mobile and less persistent than expected based on the initial DT50 (104 days) and KOC (223 L/kg). This result is in line with the previous kinetic evaluation in the previous section.

Table 106 Optimised parameter for the soil concentrations (Imidacloprid low dose)

Parameter	Estimated value	95% confidence limits lower limit	value	Initial parameter	KinGUI (normalised)
koc	497	1.5	993	223	-
DT50	65	43	132	104	55

The FOCUS chi² test was calculated to be 58 % (no weighting of residues). The volatilisation losses in the optimisation were not significant.

TMEs with high dose of Imidacloprid

Residue weighting was not considered for the optimisation. After the optimisation the results summarised in Figure 98 were obtained.

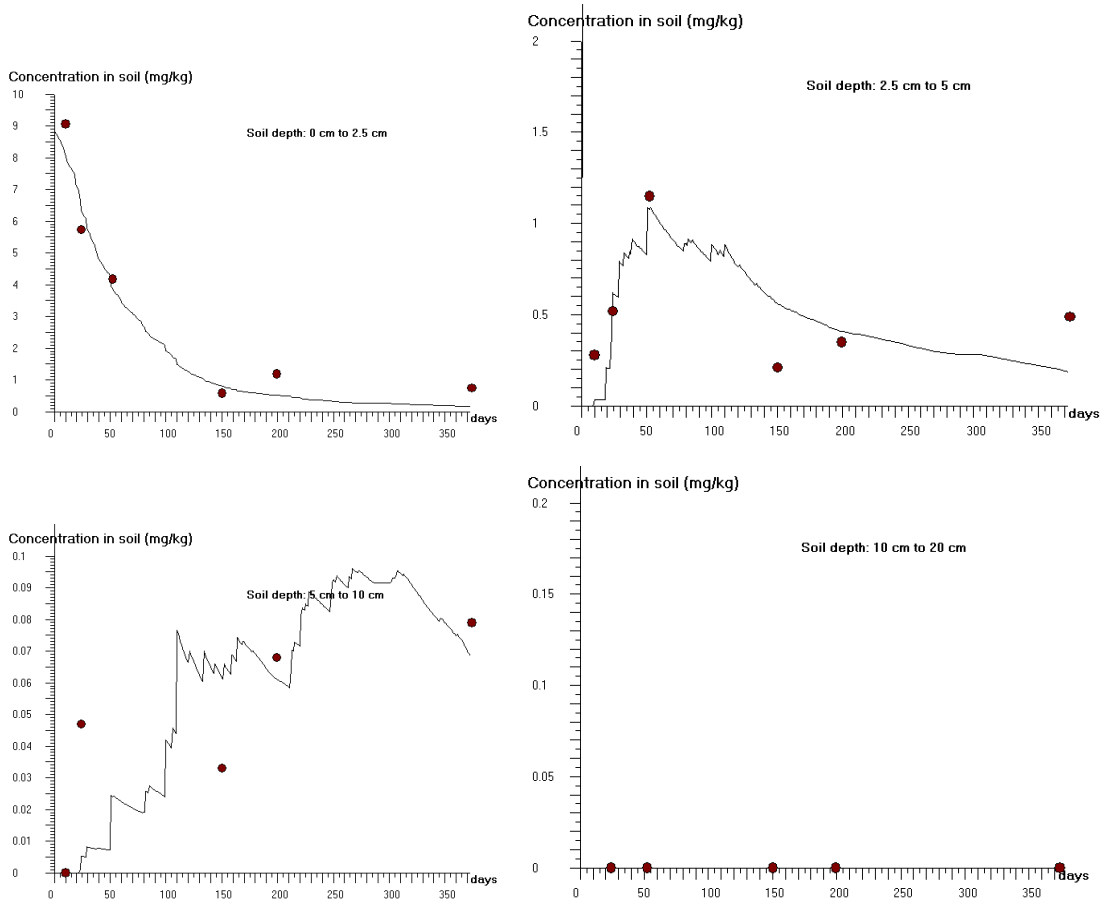

Figure 98 Results of the optimisation (soil residues, Imidacloprid, high dose, no residue weighting)

The overall graphical agreement is better than indicated by the result of the chi²-Test (27 %). The optimised parameters are summarised in the following table. It shows that in both variations Imidacloprid was found to be less persistent than expected based on the initial DT50 (104 days). This result is in line with the kinetic evaluation in the previous section (DT50 = 20 days) thought less significant. As transport to deeper soil layers cannot be neglected fit can be better explained as disappearance rather than degradation.

Table 107 Optimised parameter for the soil concentrations (TMEs high dose)

Parameter	Estimated value	95% confidence limits lower limit	value	Initial parameter	KinGUI (normalised)
koc	258	148	368	223	
DT50	42	34	95	55	43

The FOCUS chi² test was calculated to be 27 % (no weighting of residues).

The volatilisation losses in the two simulations were not significant.

7.2.6 Summary of results and conclusions

The comparison of the relatively simple fitting with KinGUI according to FOCUS (2014) and more advanced inverse modelling using inversePELMO showed nevertheless relatively similar results for Imidacloprid (DegT50 low dose: KinGUI: 55d , inverse PELMO: 65 d, high dose: KinGUI: 43 d , inverse PELMO: 42 d). That indicates that transport out of the 20 cm zone (mixing depth for KinGUI optimisation) was not a dominant process for this compound in the experimental study.

For Lindane a different outcome was observed: Whereas KinGUI estimated half lives in the rage of 87 d to 169 d the optimisation based on inversePELMO led to about 10 (low dose) to 25 (high dose) times higher DegT50 values (low dose KinGUI: 87 d , inverse PELMO: 598 d, high dose KinGUI: 196 d , inverse PELMO: 2567 d).

The differences between the two compounds can be explained when considering the volatilisation losses in PELMO: for Imidacloprid this process was not relevant, and both techniques came to similar results with regard to the decline in soil. However, for Lindane PELMO estimated volatilisation losses of about 70%. This a process which could be considered only partly by the KinGUI optimisation. Volatilisation is dominant at the soil surface but for the KinGUI fitting all concentrations were averaged over 20 cm. That may principally explain the differences between the two methodologies for Lindane. However, also the PELMO simulations are just an interpretation and no justification of the decline of the Lindane residues in the outdoor experiments as the amount of volatilsation could not be experimentally determined. Whether the decline of Lindane was really caused by volatilisation losses rather than degradation could only be answered based on additional standard degradation studies in the laboratory using the same soil and including full mass balance.

Nevertheless, though the conditions with regard to the special experimental design (outdoor study, heavy soil, averaging of results from different soil cores) were not optimal the residues of both compounds in soil could be reasonably explained with both modelling techniques.

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7.3 Transformation to other environmental conditions

In order to analyse the variability of soil concentrations for Lindane and Imidacloprid the optimised parameters were used to simulate the fate at other locations.

The soil and climate scenarios defined by FOCUS (2000) were considered for the transformation as this dataset is well documented and established in the EU. Daily weather data is available for the scenarios, covering a period of 26 years. The locations of the scenarios are shown in the following figure.

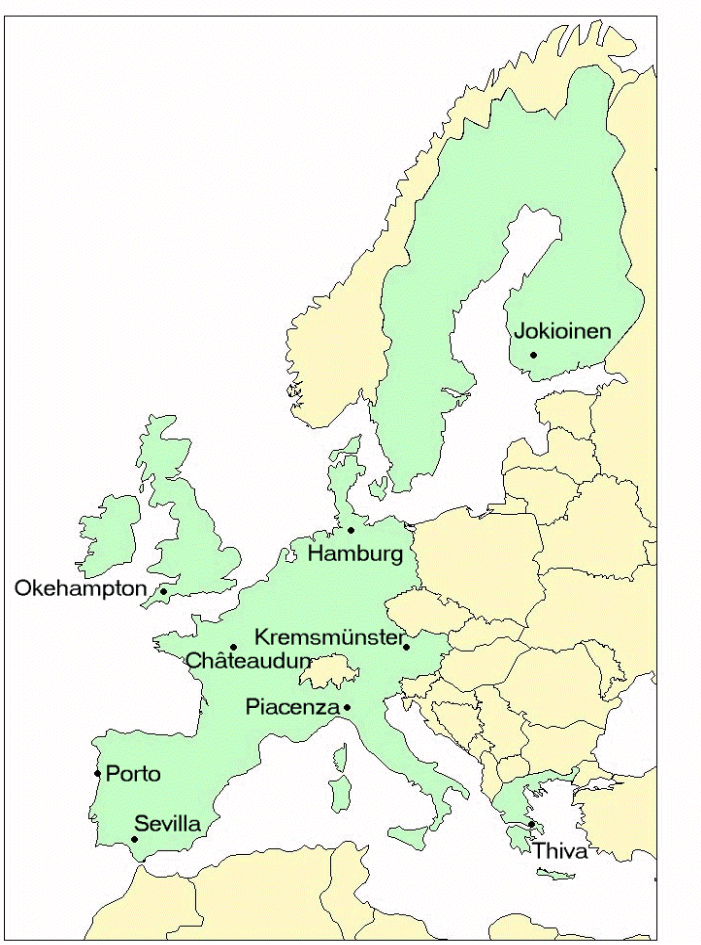

Figure 99 Locations of the FOCUS groundwater scenarios

Further information about the scenarios is summarised in Table 108.

Table 108 Characteristics of the nine weather and soil scenarios created by FOCUS

Location	Soil type (USDA)	Organic Matter [%]	Annual average air temperature [°C]	Annual sum of precipitation [mm]
Châteaudun	silty clay loam	2.4	11.3	648+ I*
Hamburg	sandy loam	2.6	9.0	786
Jokioinen	loamy sand	7.0	4.1	638
Kremsmünster	loam/silt loam	3.6	8.6	900
Okehampton	loam	3.8	10.2	1038
Piacenza	loam	2.2	13.2	857 + I*
Porto	loam	2.4	14.8	1150
Sevilla	silt loam	1.6	17.9	493 + I*
Thiva	loam	1.3	16.2	500 + I*

*irrigation relevant; grey letters = soil scenario not assumed for current calculations

However, only four standard FOCUS locations that are representing the central European zone have some representiveness for Germany (Châteaudun, Hamburg, Kremsmünster, Okehampton) and were therefore selected in for the additional simulations.

Furthermore, two scenarios were considered with regard to the crop: grass/alfalfa (close to the experimental design) and bare soil.

As a worst case simulation always the (optimised) experimental high dose was used in the simulations (Lindane: 16.3 kg/ha, Imidacloprid: 2.38 kg/ha).

Due to the persistence of the substance they could accumulate in the soil. In order to consider also the effect of accumulation annual applications were simulated for both substances. The figures showing the time dependent concentrations at different soil depth are also related to the final application.

7.3.1 Lindane grass cover

Figure 100 to Figure 103 describe the results for the time dependent Lindane concentrations at four different soil depths for the locations Châteaudun, Hamburg, Kremsmünster, Okehampton when the soil is cropped with grass/alfalfa. The annual application rate was 16.3 kg/ha

The shape of the daily concentrations is similar for all locations. However, the absolute concentrations are not. As Lindane is rather persistient after 26 annual applications it has accumulated at all locations at all soil depth of the upper 20 cm. Looking, e.g. at the top soil the background concentration at Châteaudun, Hamburg, Kremsmünster and Okehampton was simulated to be about 45 µg/cm³, 35 µg/cm³, 55 µg/cm³, and 50 µg/cm³, respectively. Also at deeper soil layers the maximum concentrations were simulated for Châteaudun. As shown in Table 108 Châteaudun is the location with the minimum rainfall of the four locations. There is no quick explanation what may have caused the differences. It seems to be a combination of the organic matter content together with annual rainfall. Higher concentrations were primarily found at the location with maximum organic matter. Compared to that the influence of annual precipitation was smaller (e.g. when comparing Kremsmünster and Okehampton).

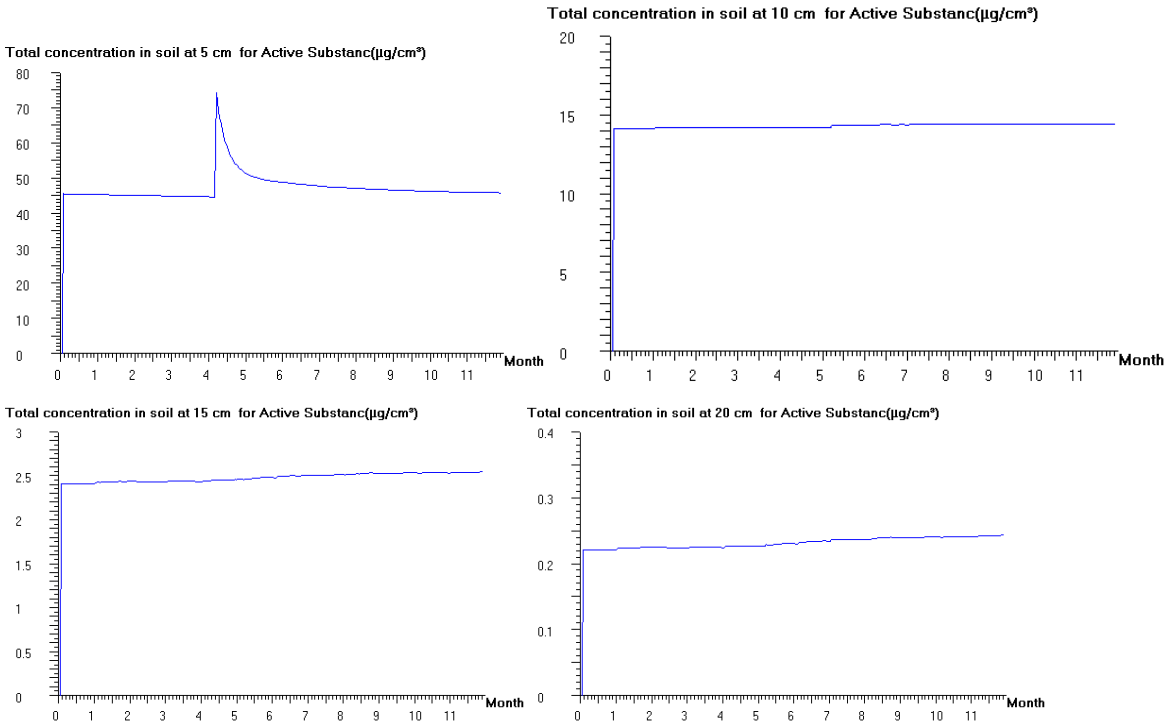

Figure 100 Lindane concentrations in soil at Châteaudun calculated with PELMO 553 based on parameters obtained by inverse modelling (grass/alfalfa, annual application of 16.3 kg/ha)

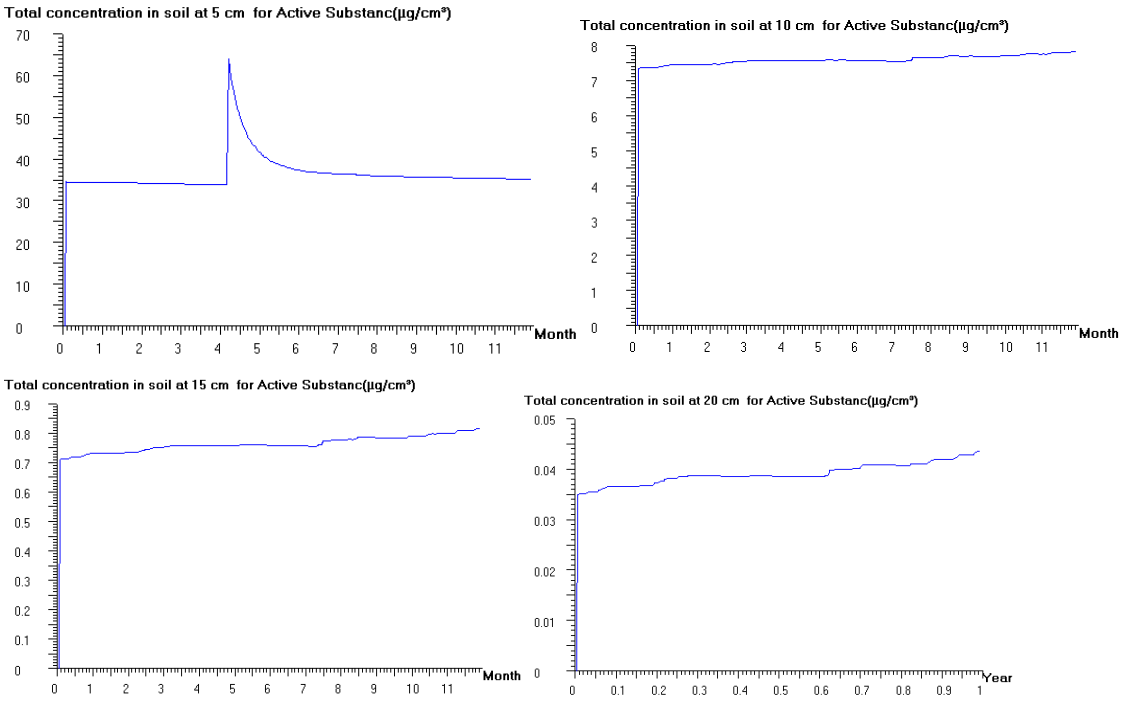

Figure 101 Lindane concentrations in soil at Hamburg calculated with PELMO 553 based on parameters obtained by inverse modelling (grass/alfalfa, annual application of 16.3 kg/ha)

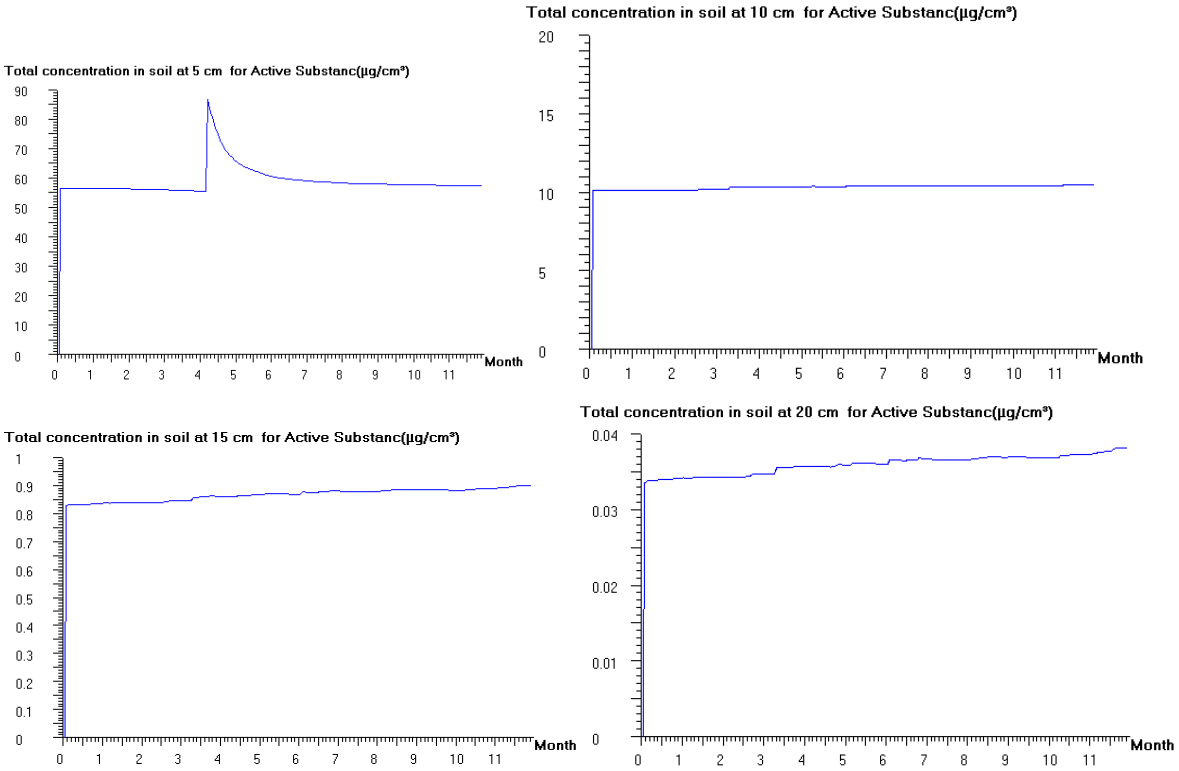

Figure 102 Lindane concentrations in soil at Kremsmünster calculated with PELMO 553 based on parameters obtained by inv. modelling (grass/alfalfa, annual application of 16.3 kg/ha)

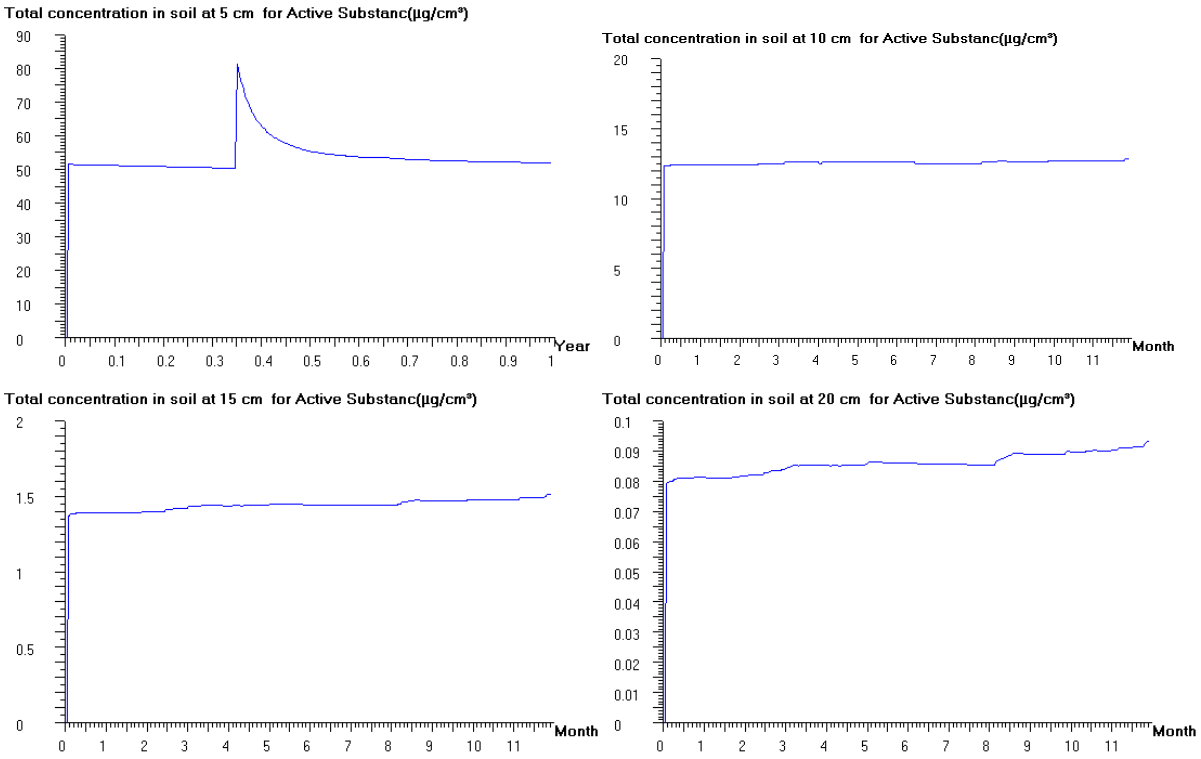

Figure 103 Lindane concentrations in soil at Okehampton calculated with PELMO 553 based on parameters obtained by inv. modelling (grass/alfalfa, annual application of 16.3 kg/ha)

Generally, the concentrations at deeper soil layers are decreasing very much, due to the fact that the leaching of the strongly sorbing substance Lindane is not transported to a high extend.

The range of concentrations simulated at a depth of 20 cm was between 0.03 g/cm³ (Kremsmünster) and 0.2 µg/cm³ (Châteaudun).

7.3.2 Lindane bare soil

Figure 104 to Figure 107 describe the results for the time dependent Lindane concentrations at four different soil depths for the locations Châteaudun, Hamburg, Kremsmünster, Okehampton. These simulations describe bare soil conditions. The annual application rate was the same as for the simulation covered with grass/alfalfa (16.3 kg/ha).

For the scenarios Hamburg, Kremsmünster and Okehampton the differences between bare soil and grass/alfalfa are not very pronounced. However, for Châteaudun the soil concentrations at bare soil conditions are generally lower than for the cropped variation. The major difference between the two variations is irrigation. Châteaudun is a so called "irrigated" scenario. Of course the bare-soil variation is not irrigated. Irrigation may transport Lindane faster to the next soil layer, whereas in the non-irrigated scenario more substance is volatised.

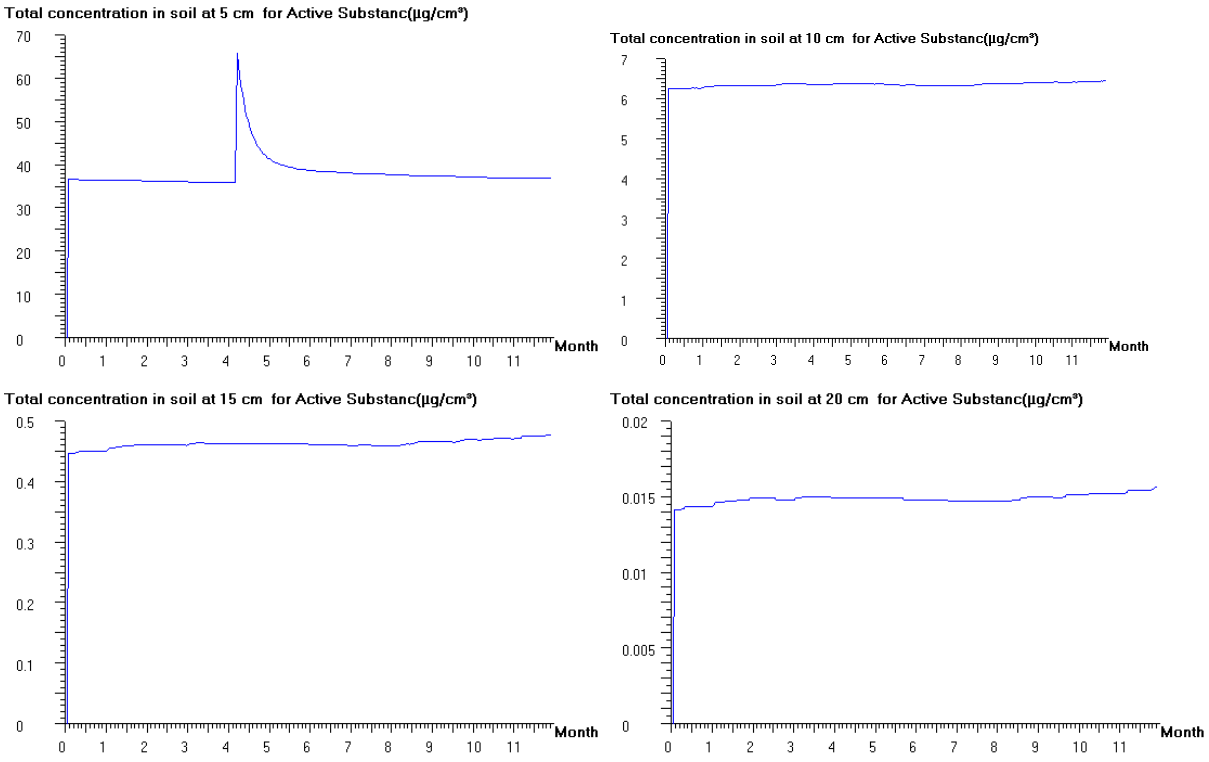

Figure 104 Lindane concentrations in soil at Châteadun calculated with PELMO 553 based on parameters obtained by inverse modelling (bare soil, annual application of 16.3 kg/ha)

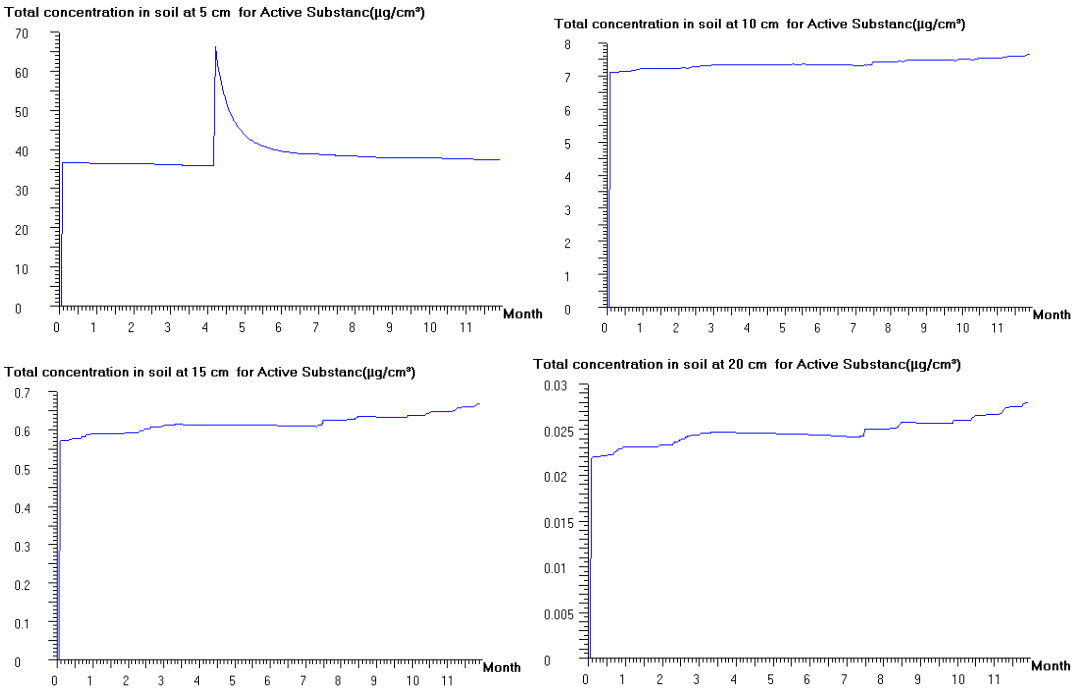

Figure 105 Lindane concentrations in soil at Hamburg calculated with PELMO 553 based on parameters obtained by inverse modelling (bare soil, annual application of 16.3 kg/ha)

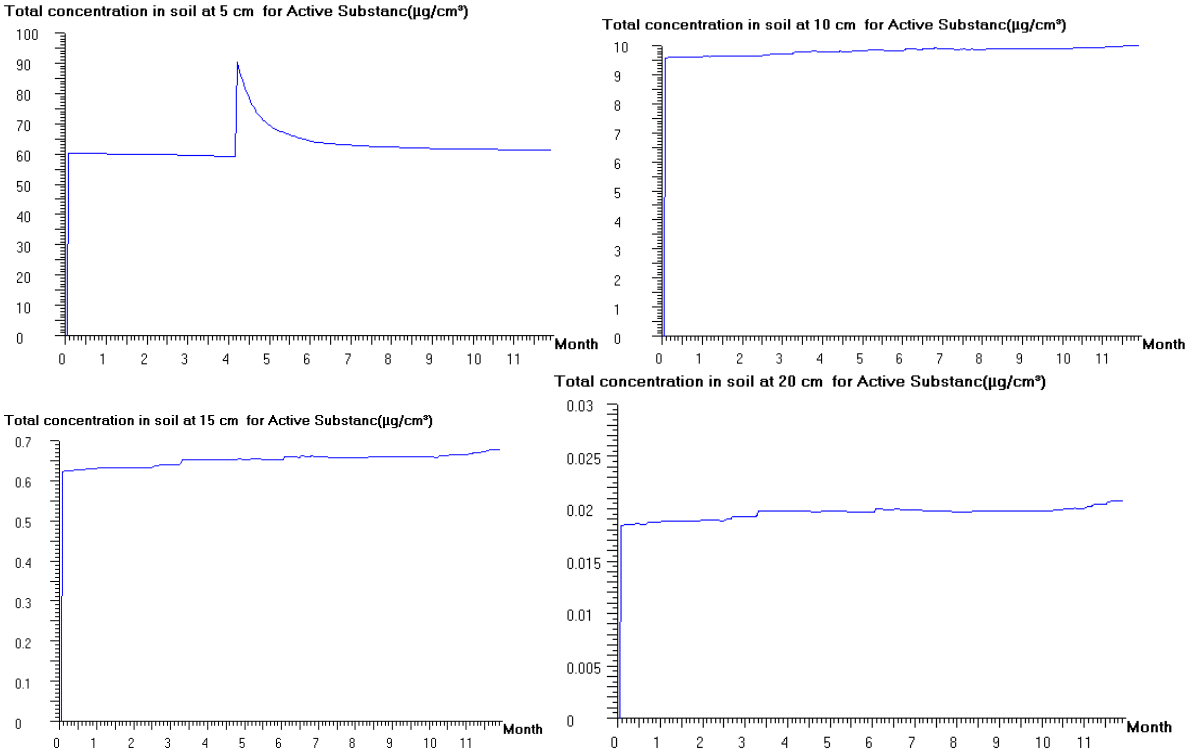

Figure 106 Lindane concentrations in soil at Kremsmünster calculated with PELMO 553 based on parameters obtained by inv. modelling (bare soil, annual application of 16.3 kg/ha)

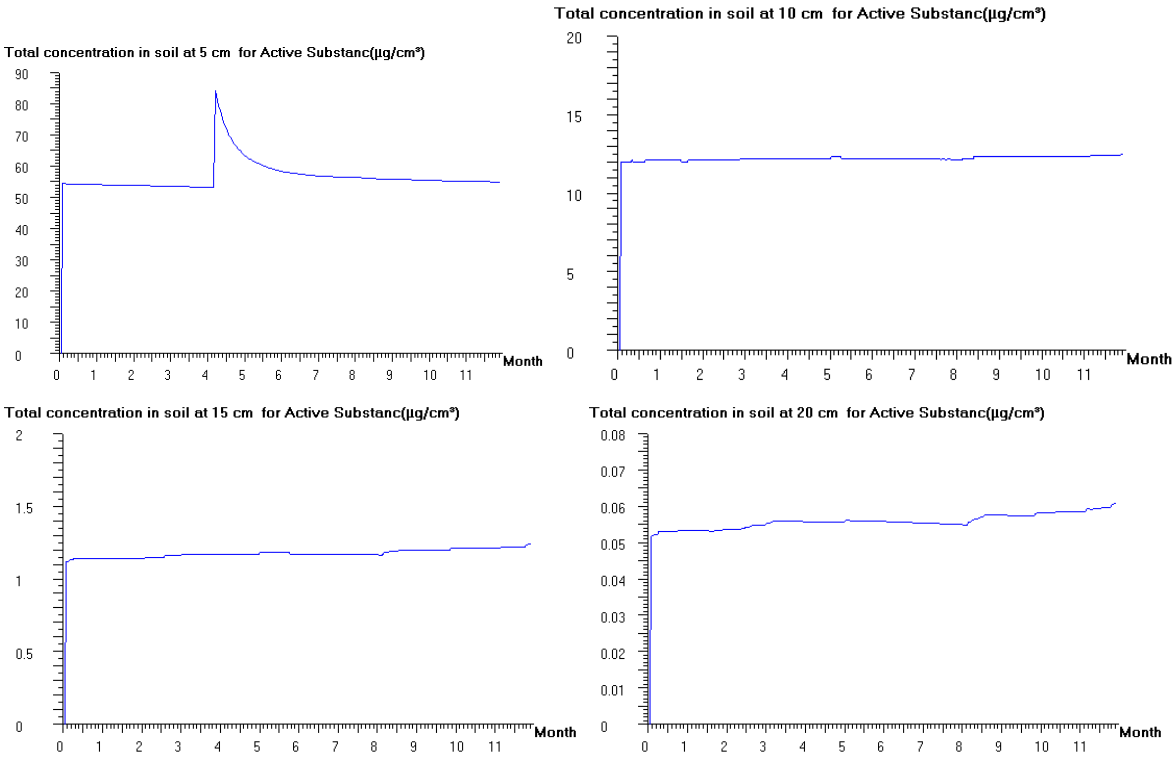

Figure 107 Lindane concentrations in soil at Okehampton calculated with PELMO 553 based on parameters obtained by inv. modelling (bare soil, annual application of 16.3 kg/ha)

7.3.3 Imidacloprid grass cover

Figure 108 to Figure 111 describe the results for the time dependent Imidacloprid concentrations at four different soil depths for the locations Châteaudun, Hamburg, Kremsmünster, Okehampton when the soil is cropped with grass/alfalfa. The annual application rate was 2.38 kg/ha.

Compared to the previous Lindane simulation the shape of the daily soil concentrations is different at all locations. Imidacloprid is less persistent than Lindane. Therefore, there is hardly any accumulation even after 26 years of annual applications. The consequence is that the top-soil concentrations at all four locations are rather similar maximal concentrations of about 5 µg/cm³ (which is close to the initial concentration after a single application of 4.76 µg/cm³).

On the other hand, the daily concentrations in deeper soil layers show a more indiviudal pattern at the four locations because the concentrations are more influenced by daily weather than Lindane.

Compared to Lindane the range of soil concentration between the top and the deeper soil layers is significantly smaller because higher fraction of Imidacloprid is transported through the soil because of its higher mobility.

The maximum concentrations at a depth of 20 cm are simulated for the location Hamburg, which is characterised by the combination of low organic carbon content and relatively high precipitation.

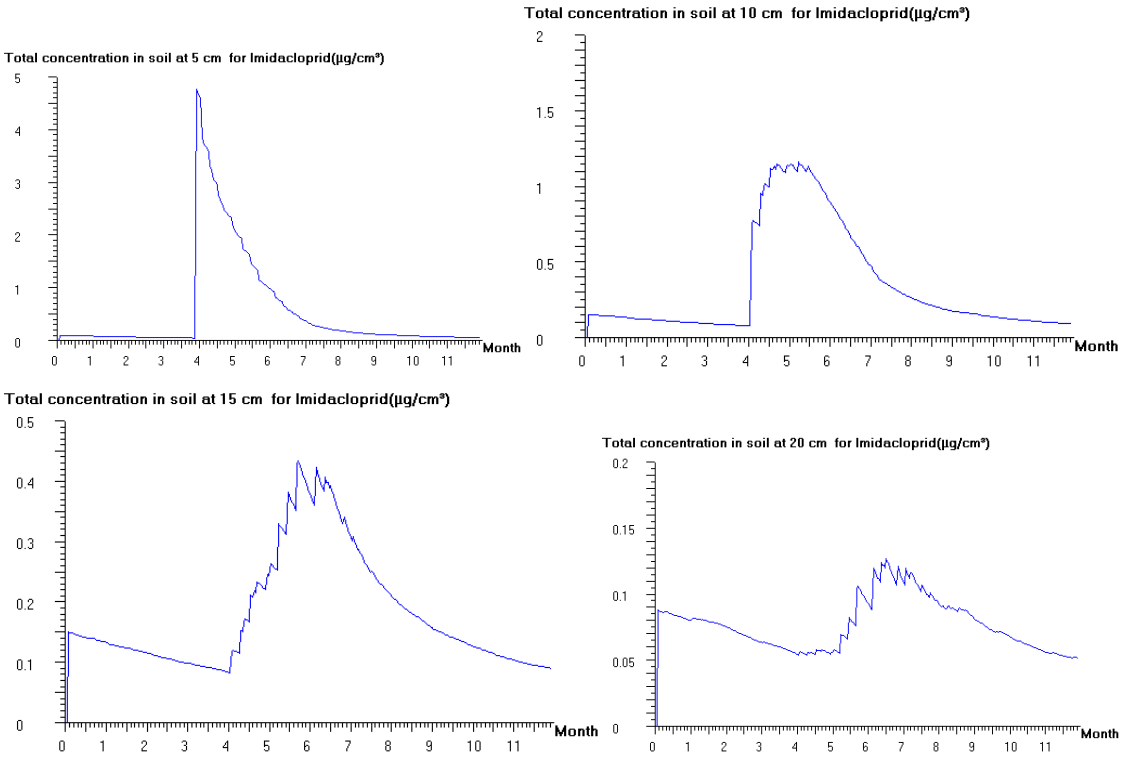

Figure 108 Imidacloprid concentrations in soil at Châteaudun calculated with PELMO 553 based on parameters obtained by inverse modelling (grass/alfalfa, annual application of 2.38 kg/ha)

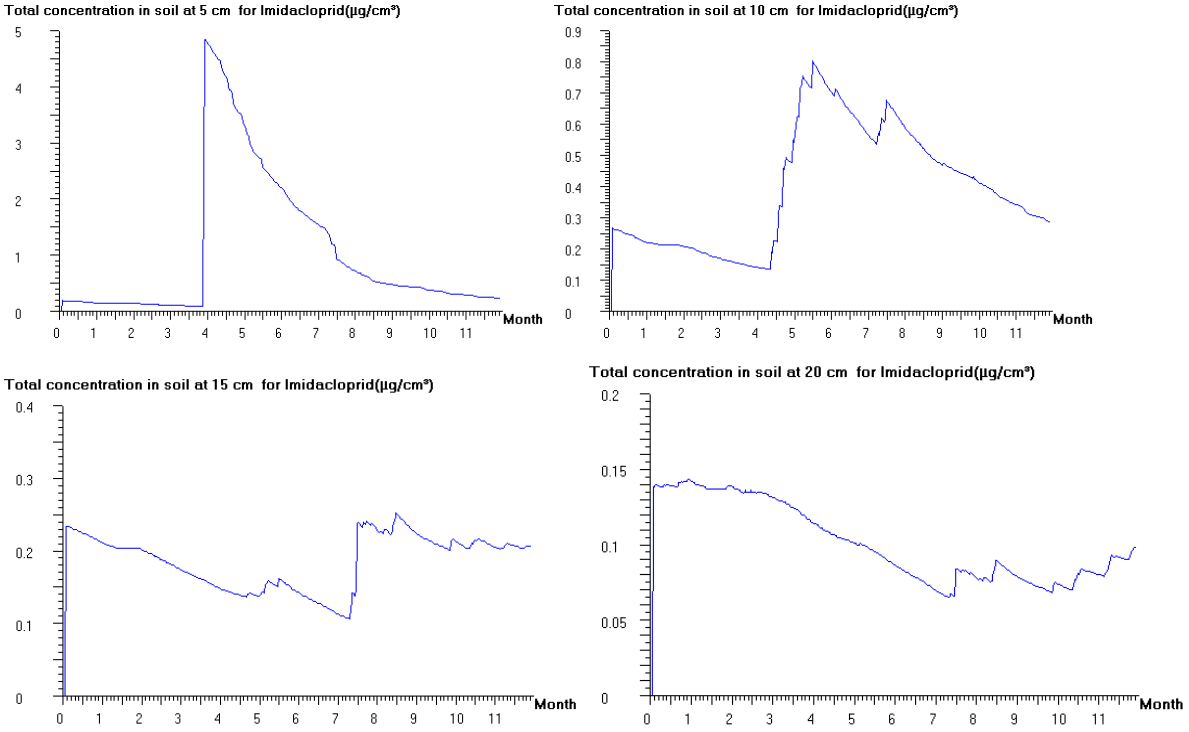

Figure 109 Imidacloprid concentrations in soil at Hamburg calculated with PELMO 553 based on parameters obtained by inverse modelling (grass/alfalfa, annual application of 2.38 kg/ha)

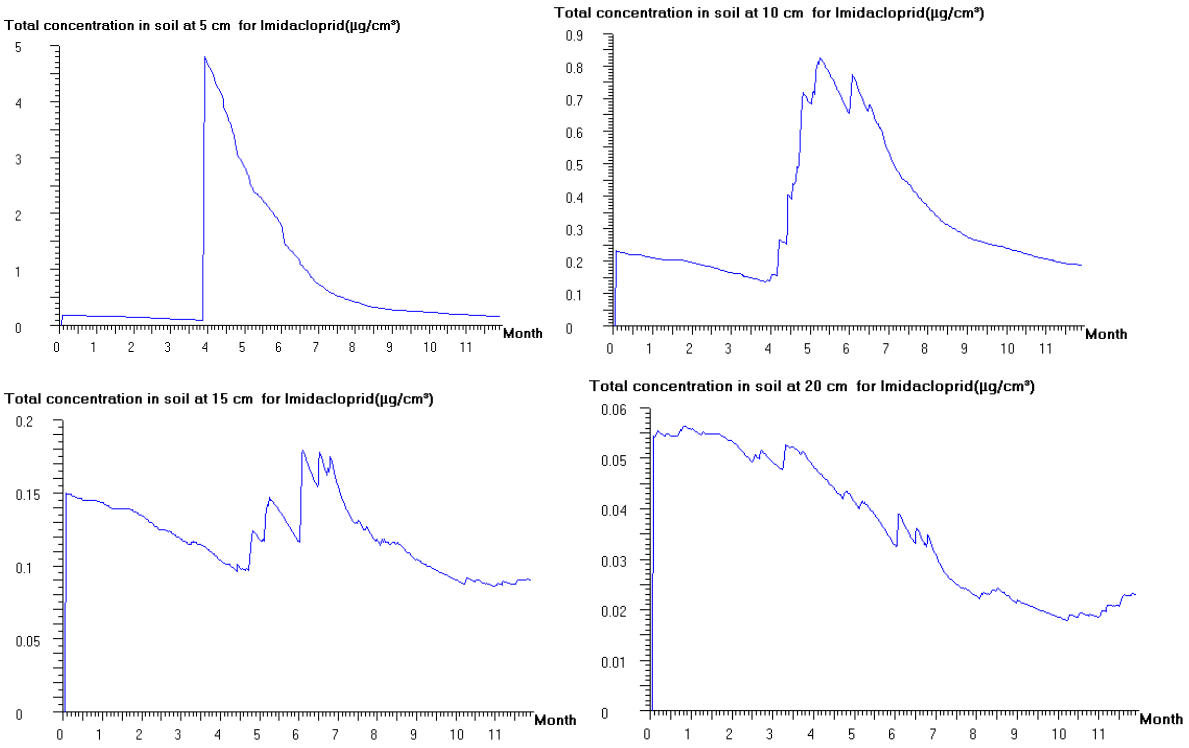

Figure 110 Imidacloprid concentrations in soil at Kremsmünster calculated with PELMO 553 based on parameters obtained by inv. modelling (grass/alfalfa, annual application of 2.38 kg/ha)

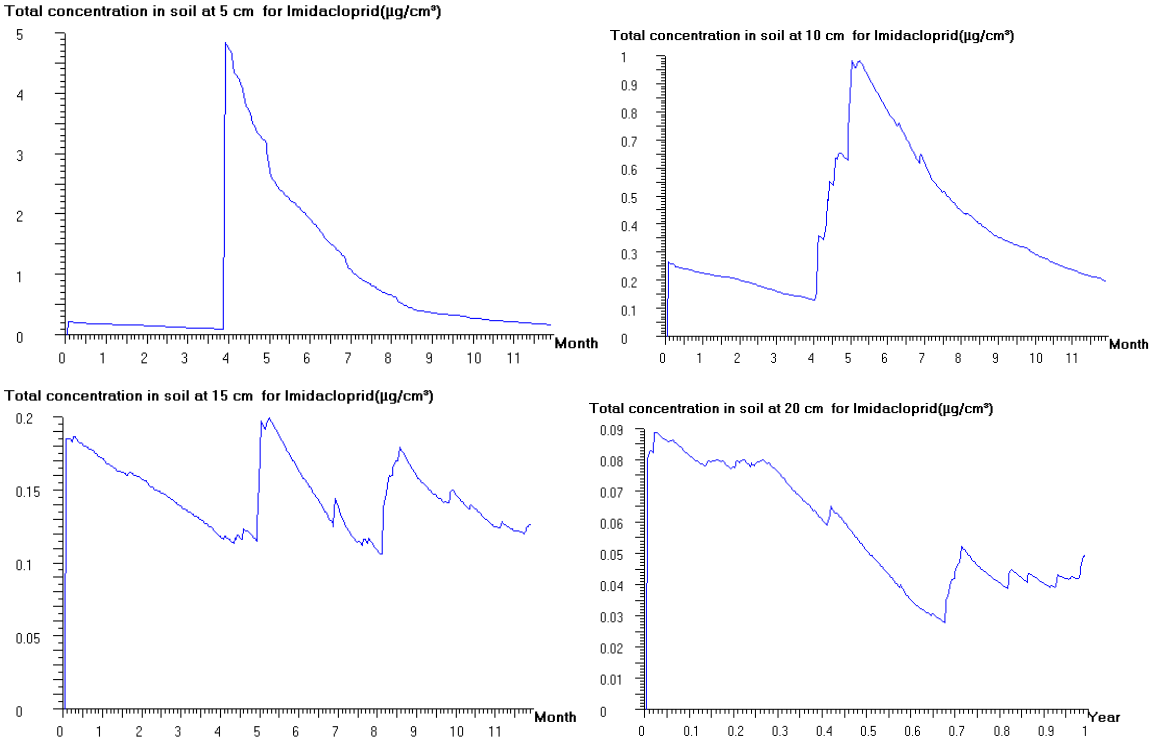

Figure 111 Imidacloprid concentrations in soil at Okehampton calculated with PELMO 553 based on parameters obtained by inv. modelling (grass/alfalfa, annual application of 2.38 kg/ha)

7.3.4 Imidacloprid bare soil

Figure 112 to Figure 115 describe the results for the time dependent Imidacloprid concentrations at four different soil depths for the locations Châteaudun, Hamburg, Kremsmünster, Okehampton. These simulations describe bare soil conditions. The annual application rate was the same as for the simulation covered with grass/alfalfa (2.38 kg/ha).

For the scenarios Hamburg, Kremsmünster and Okehampton the differences between bare soil and grass/alfalfa are not very pronounced. However, for Châteaudun the soil concentrations at bare soil conditions are generally lower than for the cropped variation. The major differences between the two variations is irrigation. Châteaudun is a so called "irrigated" scenario. Of course the bare-soil variation is not irrigated. Irrigation may transport Lindane faster to the deeper soil layers, which leads to higher soil concentrations in all soil layers below 5 cm compared to the non-irrigated bare-soil variation.

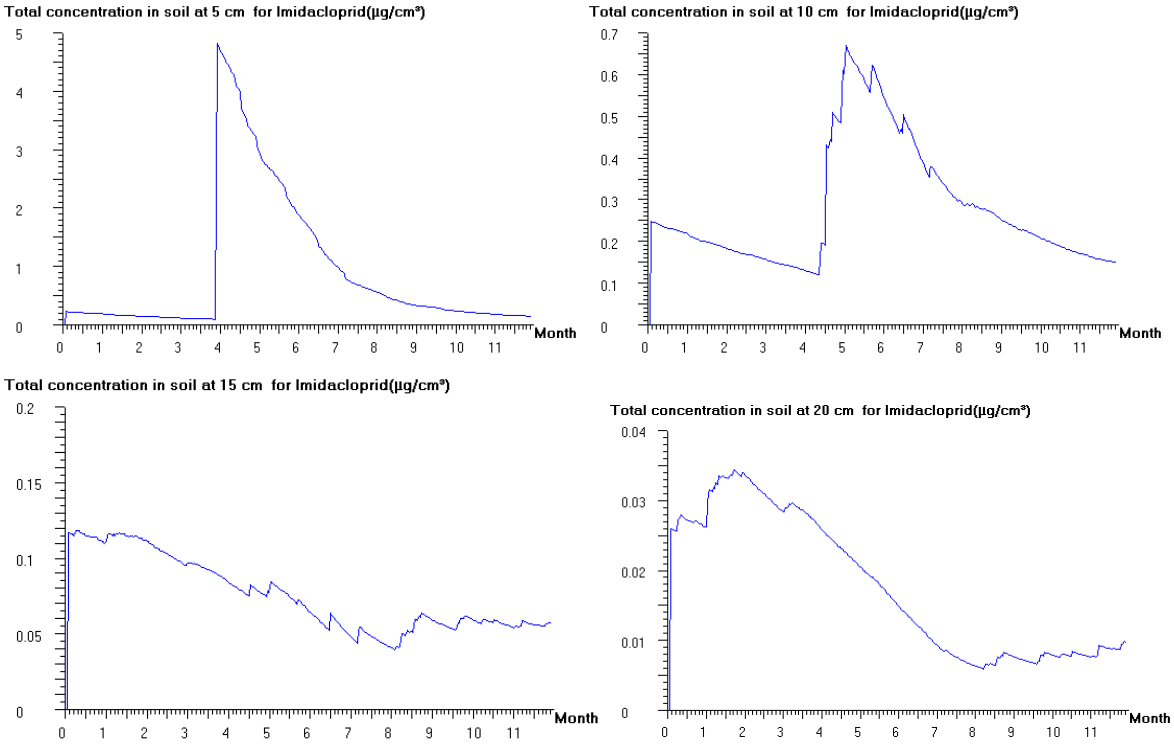

Figure 112 Imidacloprid concentrations in soil at Châteaudun calculated with PELMO 553 based on parameters obtained by inv. modelling (bare soil, annual application of 2.38 kg/ha)

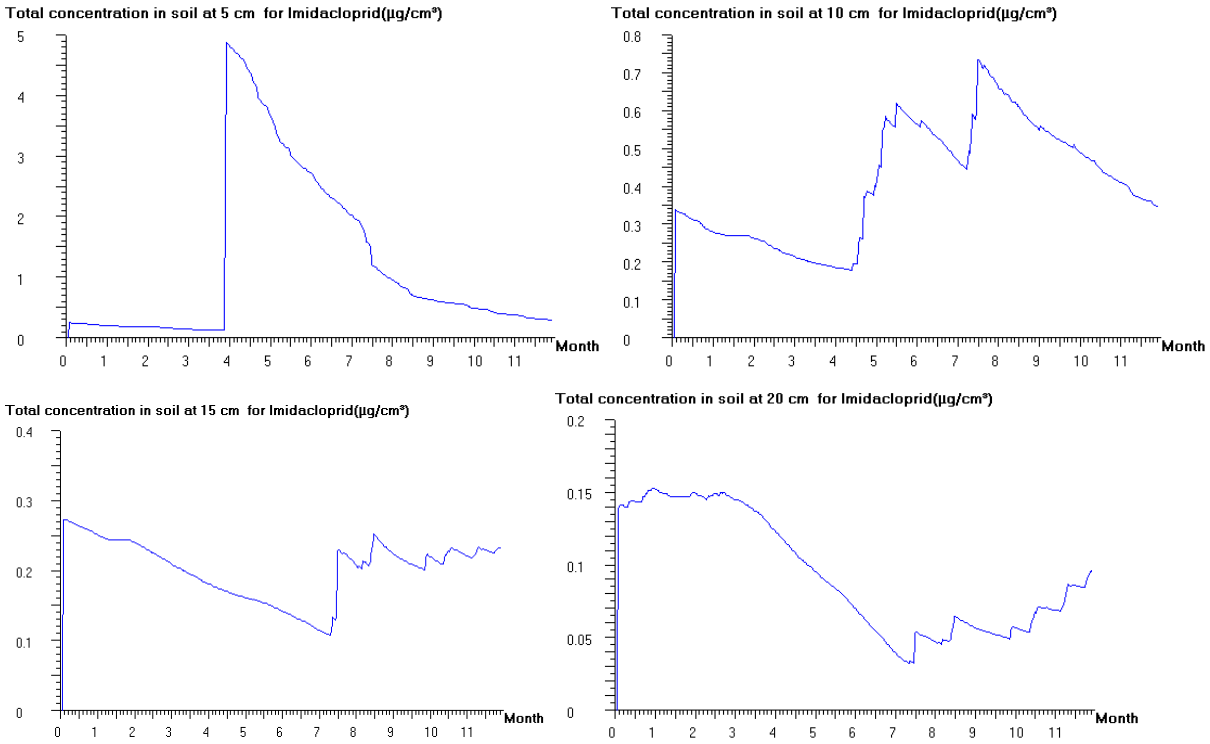

Figure 113 Imidacloprid concentrations in soil at Hamburg calculated with PELMO 553 based on parameters obtained by inverse modelling (bare soil, annual application of 2.38 kg/ha)

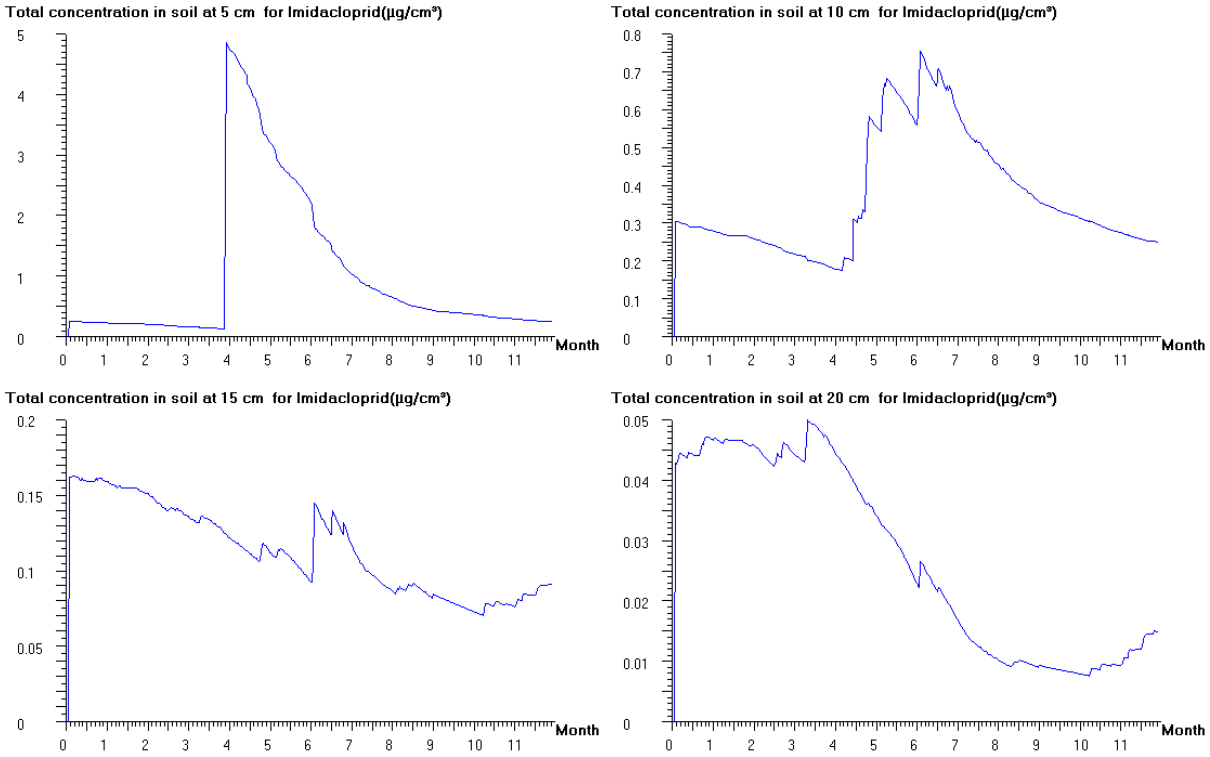

Figure 114 Imidacloprid concentrations in soil at Kremsmünster calculated with PELMO 553 based on parameters obtained by inv. modelling (bare soil, annual application of 2.38 kg/ha)

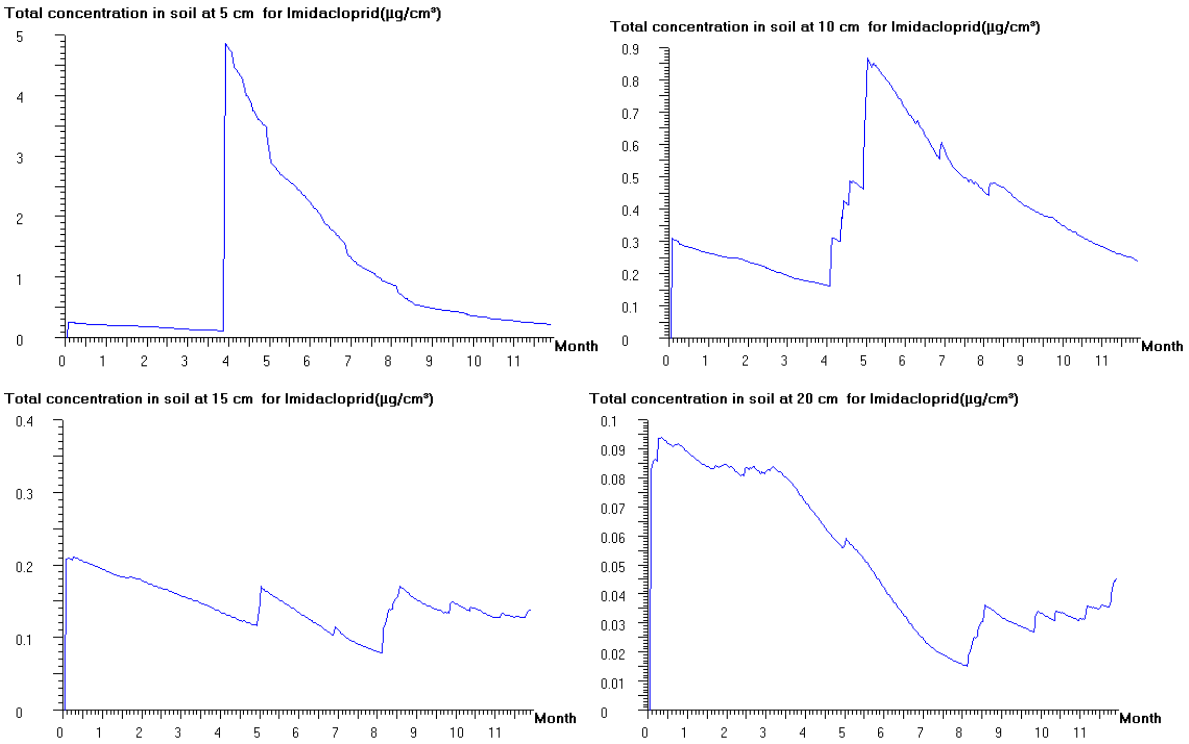

Figure 115 Imidacloprid concentrations in soil at Okehampton calculated with PELMO 553 based on parameters obtained by inv. modelling (bare soil, annual application of 2.38 kg/ha)

8 Consolidation and discussion of the results of the chemical analyses

8.1 Comparison of the analytical results of Lindane and Imidacloprid in study [1] and [2]

8.1.1 Lindane

Separate, additional studies were performed to investigate the influence of the air velocity, the water content, the temperature and vegetation cover on the volatility of Lindane [Hoen 2012, unpublished data]. In Schott bottles a 20 kg / ha corresponding amounts of radiolabelled Lindane were applied on soil with a water content of 40 or 60% of the water holding capacity. The systems were incubated for 7 days at 15 ° C or at 20 ° C and the bottles were vented with 30 or 60 gas exchange per hour. The air was either dry or saturated with water vapor. Volatile Lindane was collected with polyurethane foam and formed CO_2 with soda lime. In no case the mineralization after 7 days reached more than 0.3% of the applied radioactivity. After 2 days 5 - 20% of the radioactivity was found in the polyurethane and after 7 days 10 to 40%. By GC-ECD only Lindane could be detected in the extract of polyurethane. If the air velocity is reduced (from 60 to about 30 air changes / hour), the volatility decreased with otherwise identical parameters from 24 to 16.5% of the applied amount of radioactivity. Reducing the water content of the soil from 60 to 40 % of the water holding capacity leads to a decrease of volatile radioactivity from 24 to 11 %. Moistening the air, which was used to flush the system (by passage through a wash bottle), increased the proportion of volatiles from 24 to 43 % of the radioactivity. Decreasing the incubation temperature from 20 to 15 °C leads to a reduction from 43 to 21 % volatile radioactivity. In conclusion, the humidity of the air, the temperature and the air flow have a have a substantial influence on the volatility of Lindane. However, mineralisation of Lindane in one week can be neglected. It can be concluded, that the dissipation of Lindane is mostly due to votalisation and only small amounts were degraded. This was also found from Samuel & Pillai (1990). Only small amounts up to 1 % of the applied amount were mineralized during 28 days, but up to 20 % of Lindane volatised in this time (no air velocity, air replacement every 48 hours).

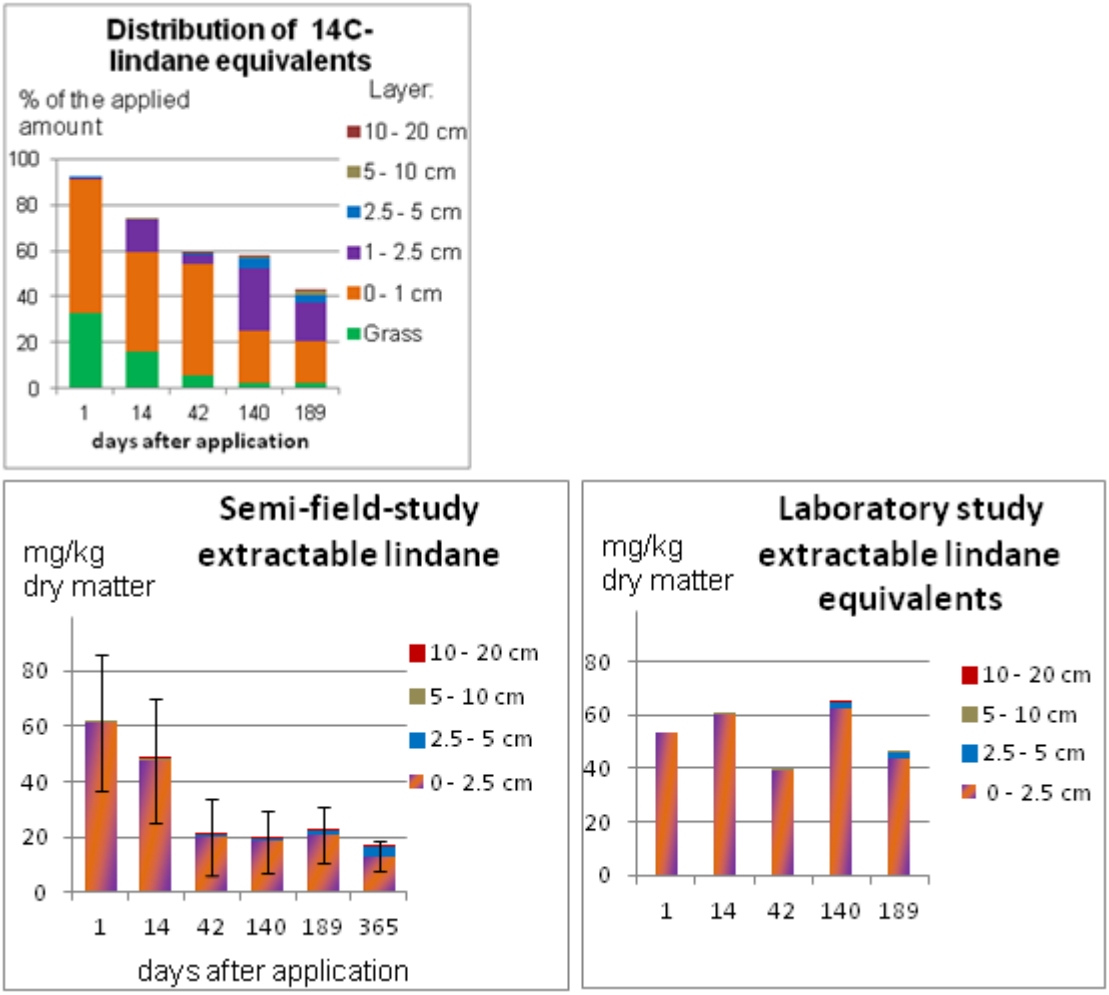

Figure 116 The concentrations of Lindane (application of 20 kg/ha) in the soil layers. The concentration in mg/kg dry matter are shown for each sampling date. The top graph pictures the distribution of radioactivity in the soil layers (Combustion of unextracted soil/grass). The bottom left graph shows results of the semi-field study [1] and the bottom right graph the results of the laboratory study. To get comparable graphs, the concentrations of Lindane of the lab study from the upper two layers (0-1 and 1-2.5 cm) are calculated into concentrations of the layer 0 – 2.5 cm. Summing up of the concentrations in the bars has been chosen for pragmatic reasons and for illustration.

This may explain some of the differences observed between the semi-field study [1] and the laboratory study [2]. Figure 116 shows the concentrations of extractable Lindane and Lindane-equivalents during the incubation time. The concentrations of the radiolabelled substance were calculated for the upper soil layer of 2.5 cm (although sampled in 0-1 and 1-2.5 cm), in order to simulate the conditions occurring in the outdoor study [1]. In both studies Lindane remained in the upper 2.5 cm layer of the soil. On the first day, the concentration of extractable pesticides residues was in both cases very similar (61.5 and 53.5 mg/kg in study [1] and [2], respectively). While the content in the semi-field study decreased rapidly, in the radioactive (indoor) study no clear decrease was observed. It has to be considered, that in study [2] also potential metabolites were calculated as Lindane-equivalents. However, during analyses of extracts from study [1] with GC-ECD no potential metabolite-peaks were found. The results from combustion of the unextracted soil and grass showed a good coherency (Figure 116). The total amount of Lindane

(based on radioactivity) was therefore indeed decreasing. During incubation, the structure of the soil was changing because the amounts of roots in the top soil centimetre increased considerably. At sampling date 140 and 189 days, the upper layer consisted mainly of roots which lead to a small weight of those samples (all weights and dry matter contents are listed in appendix 2). Due to the smaller surface in the lab TMEs, this did not occur to the same extent as in the outdoor study.

The dissipation rates found in our studies are comparable to those published elsewhere. Fuhremann et al. (1980) found that after 13 days 67 % of the applied radiolabel remained in the sample, 4.6 % was bound and 62.5 % was assigned to ^{14}C-extractable fractions in soil and plants (loamy soil with oats, 4 mg/kg of Lindane, 28 °C). This is in the same range of 74 ± 12 % of the applied amount which we found after 14 days, 2 % of them as detected as non-extractable.

8.1.1 Imidacloprid

The results of the semi-field-study [1] (application of 2 kg/ha) and the laboratory study [2] are compared in Figure 117. Shown are the extractable percentages of the initially applied amount. Similar to the Lindane study, higher amounts were also recovered in the lab study. Comparing this figure with the results of the combustion of unextracted soil and grass of study [2] (cp. Chapter 4.3.3), one can conclude that in study [2] a higher amount of Imidacloprid remained in the grass layer and was washed off during watering during the incubation to the soil. In the grass layer in the study [2] 45 % (day 0), 38 % (day 14) and 10 % (day 42) of the radioactivity was detected. This could lead to a significantly lower concentration found in the soil layer 0 – 2.5 cm. 9.0 mg/kg dry matter (application of 2 kg/ha) was found in the outdoor study, whereas in the indoor study this amount corresponded to 4.8 mg/kg (calculated value). In both studies the degradation rates of Imidacloprid were the highest during day 42 to 140 of the incubation. Matching to the higher amounts of radiolabel in the laboratory leachate (compared with Imidacloprid concentrations outdoor), in the lab study occurred a greater displacement of the substance in the lower layers 10 – 20 cm, although it has to be kept in mind, that the quantitative measurement of radioactivity does not correspond to Imidacloprid alone, but may also comprise it´s (bio)transformation products. In screening tests with thin layer chromatography up to 30 % of the radioactivity in extracts of layer 0 – 1 cm derived not from Imidacloprid, but to Imidacloprid metabolites, metabolite conjugates or microbial biomass compounds). Because Imidacloprid did not show any leaching into deeper soil layers (Krohn and Hellpointer, 2002), the proportion of metabolites (usually more polar) in the deeper soil layers is expected to be higher. Various studies showed that the DT50 (disappearance time of half of the original amount) are higher in laboratory studies than in field trials and they are higher in bare soil trials than in cropped soil (Krohn and Hellpointer, 2002 and Scholz and Spiteller, 1992). For example Baskaran et al. (1997) determined a DT50 of around 1000 days at high applied amounts (50 mg/kg) on bare soil. Rouchaud et al. (1996) found DT50s of about 42 to 130 days in six studies with different fertilizers (sugar beet crop, 7.2 mg/kg dry soil applied). The DT50 in our study are, due to few data points, difficult to estimate but they were approximately between 40 and 100 days. This could be explained by the fact that the grass roots (as other plants) increase the activity of soil microorganisms in the rhizosphere, thus affecting the higher degradation of a given compound in the soil environment.

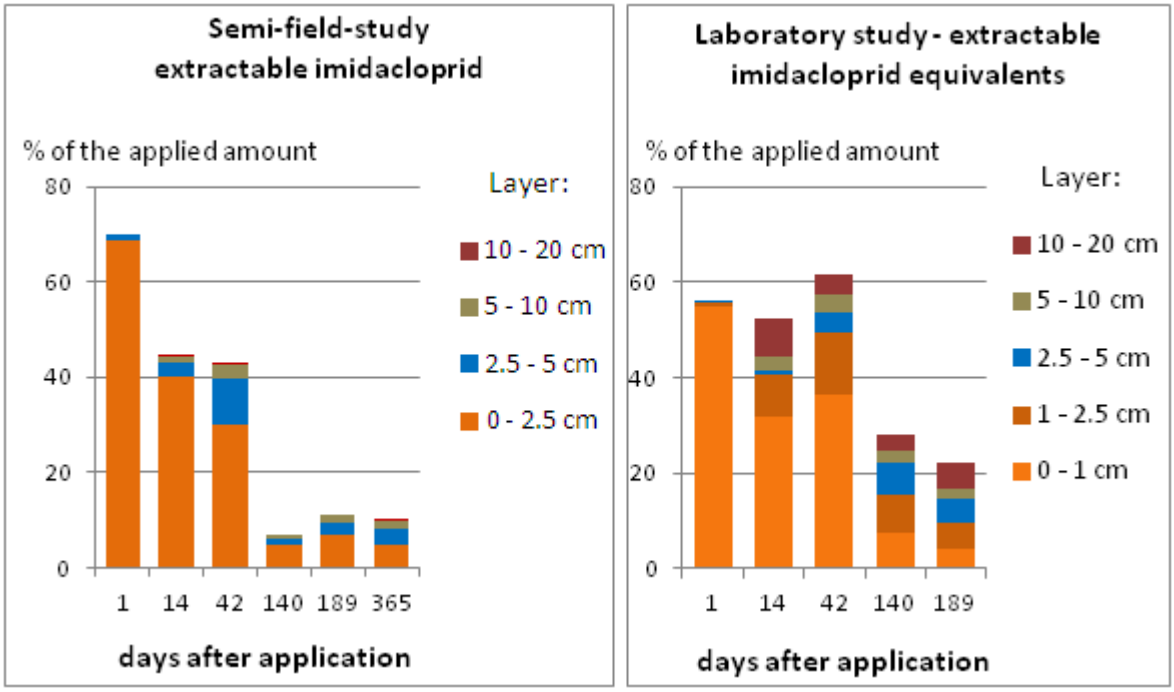

Figure 117 Shown is the percentage amount of Imidacloprid (application of 2 kg/ha) in the soil layers. The percentage of the applied amount is shown for each sampling date. The left graph shows results of the semi-field study [1] and the right graph the results of the laboratory study. To get comparable graphs, the concentrations of Imidacloprid of the lab study from the upper two layers (0-1 and 1-2.5 cm) are calculated into concentrations of the layer 0 – 2.5 cm.

8.2 Discussion of the analytical results of Carbendazim in study [3]

The total recovery of Carbendazim at day 1 after application was calculated to 20 % for the lower and 22 % for the higher application rate of Carbendazim. The recovery amount was far lower than it was expected for this pesticide. Due to this, a thorough error diagnostic was carried out in cooperation with the assigned analytical laboratory. Therefore the complete process from preparation of application solution up to the final calculation of concentration was cross-checked.

At first, some soil samples were independently analysed following the same method by a second laboratory. The second laboratory got raw soil samples, hence, the whole process i.e. pulverisation, extraction and measurement, was checked. The results were in the exact same range than the former measurements. As a second step all calculations i.e. dilution, unit calculation etc. were cross-checked by the responsible investigators and by independent collaborators with the result, that no error could be found. After cross-checking all errors that could have arose by measurement after soil sampling the process of sampling and application was cross-checked too. Therefore the retained sample (froze at -25 °C on day of application) of the application solution was analysed in the chemical laboratory with the result, that both concentrations of Carbendazim can be confirmed as right. Furthermore the standard protocol of application i.e. documentation of application process, loading the application sprayer etc. was checked with no error results.

The overall result was that the application and measurement can be assumed as correct. In the end the outage can be addressed in all probability to missing completeness of extraction of

Carbendazim from the soil. According to this assumption, the relative error-size of the resulted concentration values should be the same (approx. factor 4-5) for all measured concentrations, independent of soil layer, time and concentration itself.

8.3 Comparison of experimental and model assessment of exposure

Purpose of this chapter is to compare the distribution of the test substances in the soil profile and the leachates based on experimental and modelling activities in this project.

8.3.1 Results from field and laboratory studies

8.3.1.1 Experimental conditions

Rainfall and irrigation in the field experiment amounted to 3715 ml water during 189 days, i.e. during the incubation period of the laboratory TMEs. During this period the lab TMEs were watered with 3900 ml. 274 ml leachate was collected in the outdoor experiment, 306 ml in the lab study. In the outdoor study 0.001% and 0.03 % of the applied Lindane (application rate 20 kg/ha) and Imidacloprid (application rate 2 kg/ha) amounts, respectively, were found in the leachate, whereas 0.05 % (Lindane) and 0.4 % (Imidacloprid) pesticide equivalents were found in the indoor-leachate with the same application rates as in the field experiment.

We tested that humidity of the air, temperature and ventilation have a substantial influence on the volatility of Lindane amounting up to 40 % of the applied amount under certain conditions (worst case) whereas mineralisation of Lindane is minor. Volatilization has to be assumed for both set-ups, the field and the lab experiment although in the latter temperature was held constant compared to the fluctuating temperature profile in the field.

8.3.1.2 Field TME study: concentrations of test substances

Concentrations of *Lindane* (20 kg/ha) in the top soil layer (0-2,5 cm) were 61.5 mg/kg at day 1 and decreased to 13.0 mg/kg after one year; more than about 90 % of the extracted substance remained in this layer for half a year, and after one year still 71 % were present in this layer. The next layer (2.5-5 cm) contained 23 % of the extracted amount after one year (3.2 mg/kg), whereas the lower layers had concentrations way below 1% of the extracted amount. *Imidacloprid* (2 kg/ha) was only slightly more mobile with 9.1 mg/kg at day 1 and 0.75 mg/kg after one year in the top layer (97 % and 56 % of the extracted amount, respectively) and 0.28 mg/kg (day 1) and 0.49 mg/kg (day 365), equivalent to 3 % and 37 % of the extracted amount in the layer 2.5-5 cm. Concentrations in lower layers were below about 0.1 mg/kg.

8.3.1.3 Laboratory study: concentrations using ^{14}C-labelled pesticide

Lindane equivalent concentrations in the first cm layer were 168.2 mg/kg at day 1 and 74.9 mg/kg at day 180, and in the second layer (1-2.5 cm) 0.62 mg/kg and 39.52 mg/kg at day 1 and day 180, respectively. Concentrations in the next two layers (2.5-5 cm and 5-10 cm) were each 2.87 mg/kg after 180 days; the bottom layer (10-20 cm) contained below 0.1 mg/kg Lindane equivalents at day 180. Also *Imidacloprid* equivalents had the highest concentrations in the first cm of soil with 17.35 mg/kg (day 1) and 4.48 mg/kg (day 180), respectively. Corresponding values in the second layer (1-2.5 cm) were 0.11 mg/kg (day 1) and 1.86 mg/kg (day 180). The layer below contained less than 1 mg/kg (2.5-5 cm) and less than about 0.1 mg/kg in the bottom layer (10-20 cm) after 180 days.

8.3.2 Modelling results

When considering the standard PECsoil scenarios (soil bulk density = 1.5 kg/L) initial concentrations of *Lindane* (20 kg/ha) in the top soil layer were calculated in the range 27 mg/kg (0-5 cm) to 133 mg/kg (0-1 cm). For the 2.5 cm top soil layer the calculated initial concentration was 53 mg/kg. However, compared to the standard scenario the soil density of the experimental soil was only 1.07 kg/L which should have led to an underestimation of initial concentrations compared to the experimental data. In order to obtain reasonable input parameters an inverse modelling study was performed which resulted in initial concentrations of 55 mg/kg in the top 2.5 soil layer. After one year the concentrations were reduced to 15 mg/kg mainly due to volatilisation shortly after application and to some extent also due to transportation to deeper soil layers. However, the calculated concentrations in deeper soil layers remained very small (maximum values 2.5-5 cm: 0.9 mg/kg, 5 to 10 cm: 0.02 mg/kg).

When considering the standard PECsoil scenarios (soil bulk density = 1.5 kg/L) initial concentrations of *Imidacloprid* (2 kg/ha) in the top soil layer were calculated in the range 2.7 mg/kg (0-5 cm) to 13 mg/kg (0-1 cm). For the 2.5 cm top soil layer the calculated initial concentration was 5.3 mg/kg. However, compared to the standard scenario the soil density of the experimental soil was only 1.07 kg/L which should have led to an underestimation of initial concentrations compared to the experimental data. In order to obtain reasonable input parameters an inverse modelling study was performed which resulted in initial concentrations of 8.5 mg/kg in the top 2.5 soil layer corresponding to a value of 21 mg/kg for the 0-1 cm layer. After one year the calculated concentrations were significantly reduced to 0.1 mg/kg mainly due to degradation and to some extent also due to transportation to deeper soil layers. However, the calculated concentrations in deeper soil layers remained small compared to the top soil layer (maximum values 2.5-5 cm: 1.14 mg/kg, 5 to 10 cm: 0.06 mg/kg).

8.3.3 Comparison of experimental findings and modelling of exposure

8.3.3.1 Lindane, application rate 20 kg/ha

Initial concentrations (day 1) in the top 2.5 cm layer were 61.5 mg/kg (field study) and 55.1 mg/kg calculated by inverse modelling. In the lab study Lindane initial concentration in the first cm layer (0-1 cm) was 168.2 mg/kg and the modelled concentration in this layer at that time was 133.3 mg/kg.

After one year in the field experiment the concentration in the 0-2.5 cm decreased to 13.0 mg/kg (= 71 % of the applied amount); modelling resulted in a concentration of 14.8 mg/kg after that time. In the first cm layer after one year, experimental concentrations (obtained in the lab study) was 74.9 mg/kg, the modelled concentration was 50.7 mg/kg.

The next soil layer (2.5-5 cm) after one year contained 3.2 mg/kg in the field TMEs, whereas inverse modelling resulted in a concentration of 0.3 mg/kg. After one year 0.34 mg/kg were detected in the field TMEs (5-10 cm layer), but only 0.001 mg/kg were modelled. Only minor (experimental) or zero amounts (modelled) were detected and expected in the lower soil layers below 10 cm.

Thus, experimental findings and modelled concentrations were quite similar showing that Lindane remained mainly in the top soil layers even one year after application. However, modelling underestimated the concentration of Lindane in lower soil layers.

8.3.3.2 Imidacloprid, application rate 2 kg/ha

Initial concentrations (day 1) in the top 2.5 cm layer were 9.1 mg/kg (field study) and 8.5 mg/kg calculated by inverse modelling. In the lab study Imidacloprid initial concentration in the first cm layer (0-1 cm) was 17.4 mg/kg and the modelled concentration in this layer at that time was 21 mg/kg.

After one year in the field experiment the concentration in the 0-2.5 cm decreased to 0.75 mg/kg (= 56% of the applied amount); modelling resulted in a concentration of 0.1 mg/kg after that time. In the first cm layer after one year, experimental concentrations (obtained in the lab study) was 4.5 mg/kg, the modelled concentration was 1.2 mg/kg.

The next soil layer (2.5-5 cm) after one year contained 0.49 mg/kg in the field TMEs, whereas inverse modelling resulted in a concentration of 0.14 mg/kg. Only minor (experimental and modelled, < 0.1 mg/kg) amounts were detected and expected in the lower soil layers below 5 cm.

Thus, experimental findings and modelled concentrations were quite similar showing that Imidacloprid – despite a much lower Kow - remained mainly in the top soil layers even one year after application.

9 Consolidation and discussion of exposure and effects

The focus in this chapter is to link the fate and exposure of the three considered pesticides and the measured effects on the different species groups. The following strategy is to present on one hand the measured concentrations for the different soil layers at different times and on the other hand the effects on the soil organisms. The first type of results can be considered as a measure for exposure within the different soil layers and the second type of results related to connected effects. These values were compared to literature data for ecotoxicological values i.e. NOEC, EC10, EC50 and LC50 values as far as they are available.

9.1 Effects and exposure of Lindane

Most of the concentration of Lindane were found in the uppermost soil layer (95.9 %, 7.5 kg/ha, 95.5 % 20 kg/ha for layer 0-2.5 cm 14 days after application, chapter 4.3.2) and the concentration decreased over time. The highest concentration of Lindane was observed for both application rates on the first sampling date after 14 days in the uppermost soil layer (47.4 mg/kg, 20 kg/ha; 20.6 mg/kg, 7.5 kg/ha). The lowest concentration in the uppermost soil layer was found for both application rates after 364 days (4.7 mg/kg, 7.5 kg/ha, 13 mg/kg, 20 kg/ha). The concentrations of Lindane were found to decrease with deeper soil depth. In both treatments, the lowest concentrations could be found in the deepest soil layer D (10-20 cm), however, no significant change of concentration could be observed over time in this layer.

9.1.1 Exposure and effects on collembola

For the environmental risk assessment, the collembolan species *Folsomia candida* is used as test organism. According to Lock et al. (2002) several ecotoxicological values for effects of Lindane on different endpoints are available (see Table 109). For acute effects, the median lethal concentration in soils (LC50) is found to be 2.21 mg Lindane/kg soil dry weight. The no observed effect concentration (NOEC) based on a reproduction endpoint is 0.056 mg/kg. The effect concentration at which reproduction was reduced by 50 % (EC 50 value) is 0.189 mg/kg while the effect concentration at which 10 % reproduction decrease occurred (EC10) is 0.029 mg/kg and in this case lower than the NOEC value.

Table 109 Toxicity values of Lindane for the test species *Folsomia candida* (Collembola) for different endpoints and categories.

Species	Soil	Endpoint	Categorie	Value	Unit	Source
Folsomia candida	AS (OECD)	Mortality	LC50	2.21	mg/kg TG	Lock et al. 2002
Folsomia candida	AS (OECD)	Reproduction	NOEC	0.056	mg/kg TG	Lock et al. 2002
Folsomia candida	AS (OECD)	Reproduction	EC10	0.029	mg/kg TG	Lock et al. 2002
Folsomia candida	AS (OECD)	Reproduction	EC50	0.189	mg/kg TG	Lock et al. 2002

In the TMEs of study [1] at the lower Lindane application rate, the measured concentrations in the uppermost soil layers were at all times higher than the LC50 and EC50 values according to Lock et al. (2002). In this respect, and for the purpose of this experiments, the so-called ‘low’ application of Lindane was already quite high, since all effects were present straight from the first sampling date in the highest effect class.

However, the measured concentrations for layer C (5-10 cm) were in the range or lower than the NOEC value during the whole experiment. The measured concentration in layer B (2.5-5 cm) was lower than the LC50 value but higher than the NOEC.

For the higher Lindane application rate, all measured concentrations in layer A and B (0-2.5 cm, 2.5-5 cm) were higher than the LC50 value for *F. candida*. Only the measured concentration of layer D were in the range or lower than the NOEC value during the whole experiment. In layer B (5-10 cm), the measured concentration slightly increased right until 140 days after application (5.82 mg/kg) when the concentration was higher than the respective LC50 value for *F. candida*. Later on the measured concentration decreased to 1.27 mg/kg at day 189 (lower than the LC 50) and raised again to 3.19 mg/kg at day 364 (higher than the LC50).

As expected, effects on the collembolan population were measured during the whole study period from day 14 to day 364 after application (see chapter 4.4.3).

In some cases effects were not detected as statistically significant because of the missing dose response relationship that is required for the statistical method i.e. Williams t-test (e.g. day 42, 0-2.5 cm; day 189, 2.5-5 cm). This is likely because the effects of Lindane were in both treatments very high, making the detection of dose-dependency unlikely. In most of the cases, the effects could be explained by the high concentrations of Lindane. However, no effects for the lower application rate were observed at day 140 on the surface and in the uppermost soil layer, when the concentration of Lindane was still high enough to assume substantial effects.

By contrast, a decrease in abundance of collembola was visible at days 140 and 364 for the deepest analyzed layer C, even if the Lindane concentrations here were below the NOEC value known from literature.

As can be seen in Figure 118, effect elicitation and Lindane concentrations did not correlate in all layers. This is particularly true for the deeper sampling layers, where effects were detected already 14 day after Lindane application. At this time point, Lindane was still to be detected only in the uppermost layer.

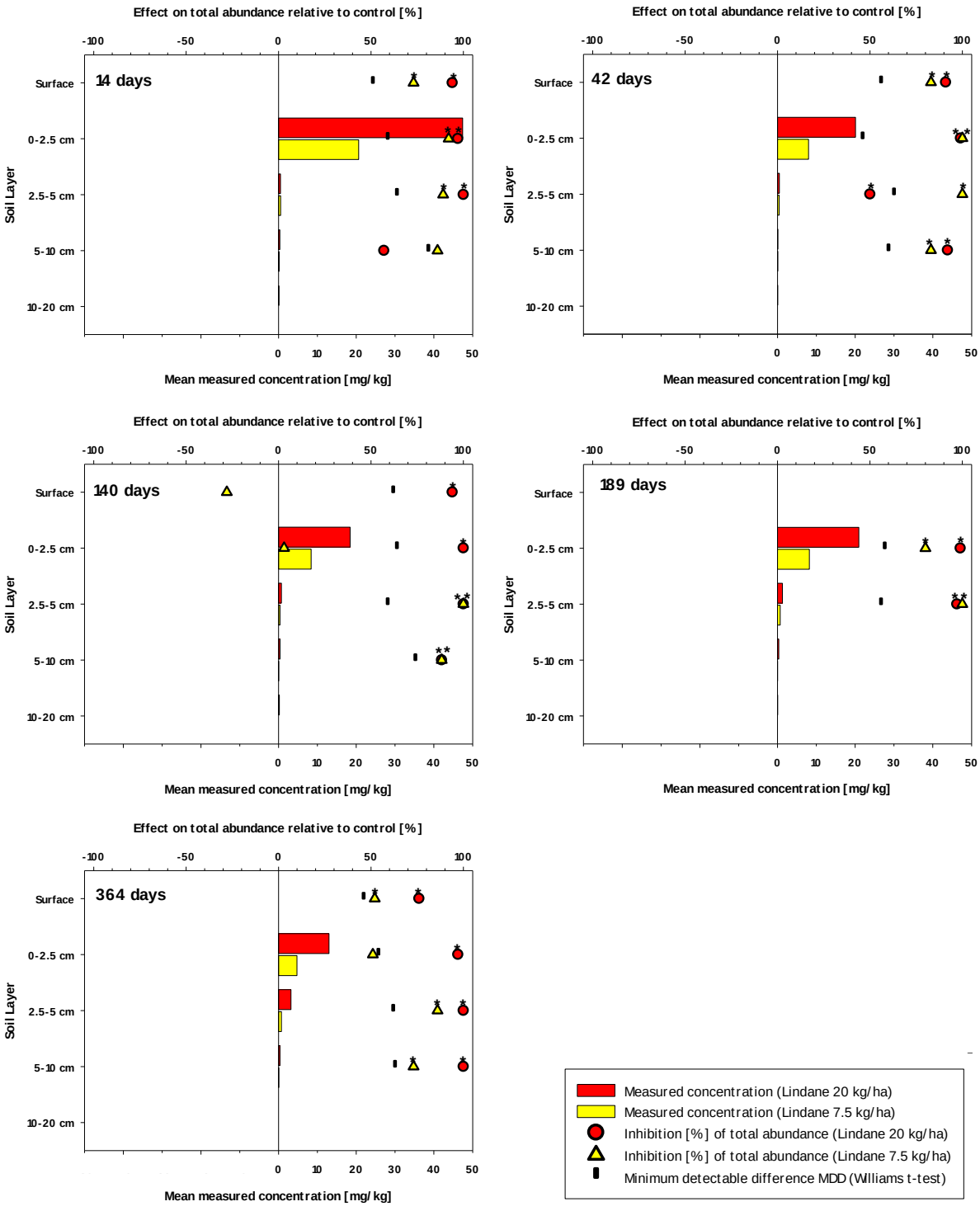

Figure 118 Decrease of total abundance of Collembolan species in the Lindane-treatments 7.5 kg a.s./ha and 20 kg a.s. /ha (5 replicates each) for the different soil layers in comparison to the control (10 replicates). Columns showing the measured concentration for the two application rates at the respective sampling date. *: significant difference according to Williams t-test, bars showing the minimum detectable difference (MDD) as value for the specific possible statistical resolution. MDD values higher than 100 % are not shown.

9.1.2 Exposure and effects on oribatid mites

For the first step of the environmental risk assessment of pesticides, laboratory tests with the gamasid species *Hypoaspis aculeifer* are performed. This species is used as surrogate test organism species for the group of Acari. No ecotoxicological values for any endpoints were found in literature for its sensitivity towards Lindane. There were also no further data for oribatid mites.

The concentrations of Lindane for the different soil layers were as described above in chapter 4.3, page 68. Due to missing data, it was not possible to compare the concentration level with the sensitivity level (ECx, NOEC etc.) of mite species. The Minimum Detectable Difference (MDD) was for layer A in the TMEs in a range between 44 % and 67 % on the different sampling dates, i.e. effects higher than these values could be detected as significant. However, at day 140 in layer B, the effect was relatively high but no significance was observed due to low individual numbers in the controls and high MDD (> 100 %). For the deeper soil layers of the TMEs, the MDD were higher - owed to the low abundances of species and only in few cases sufficient to detect effects at all (appendix 2). Effects of Lindane were as expected detectable in the uppermost soil layer, owing to the high application rates of Lindane and to the higher individual densities of oribatid mites (Figure 119).

As for collembola, effects were detected also in deeper soil layers where Lindane was not present all through the experiment. High effects were to be seen from first date of sampling on for layer B (2.5 to 5 cm) till the end of the study.

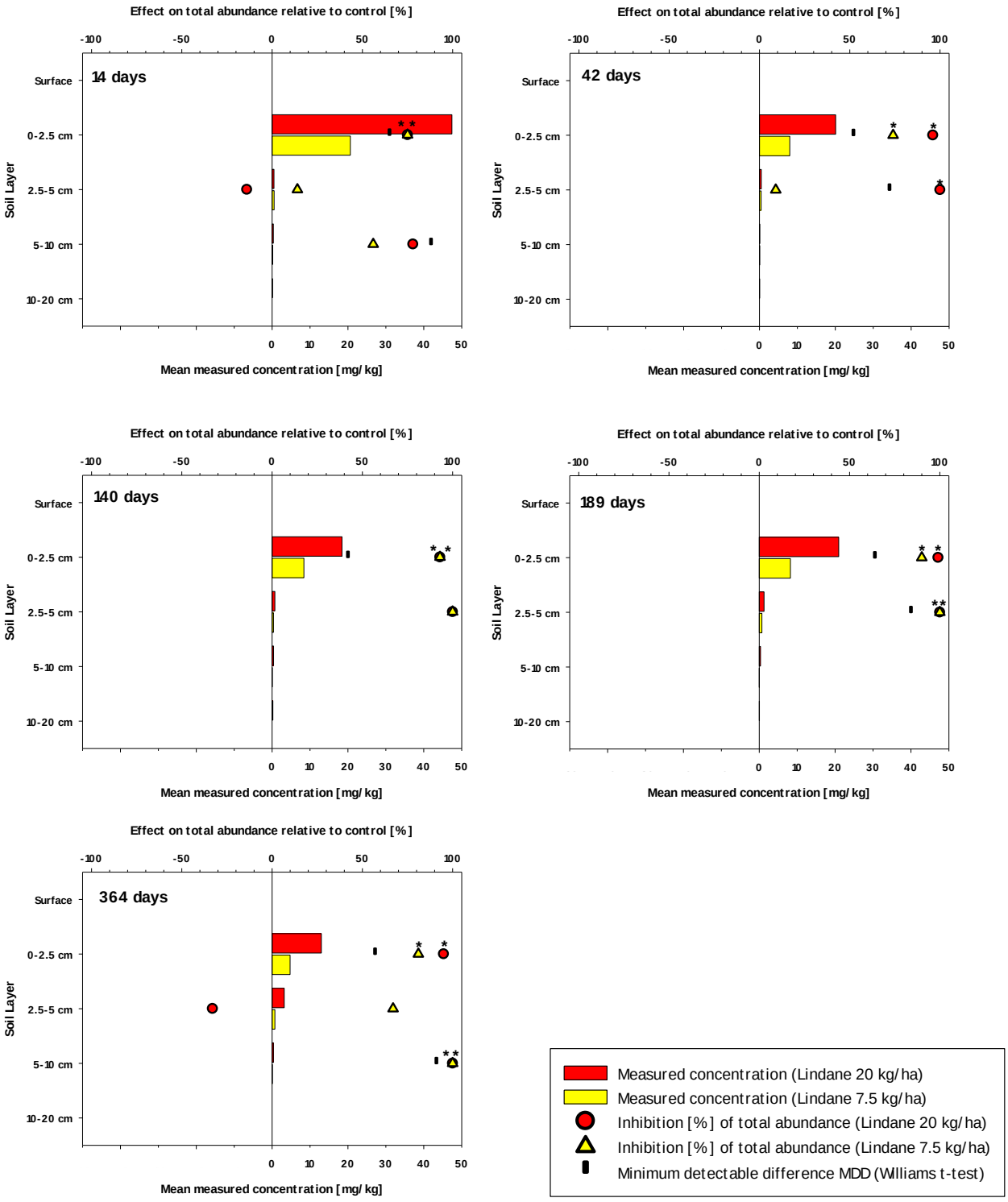

Figure 119 Decrease of total abundance of Oribatid species in the Lindane-treatments 7,5 kg a.s./ha and 20 kg a.s. /ha (5 replicates each) for the different soil layers in comparison to the control (10 replicates). Columns showing the measured concentration for the two application rates at the respective sampling date. *: significant difference according to Williams t-test; bars showing the minimum detectable difference (MDD) as value for the specific possible statistical resolution. MDD values higher than 100 % are not shown.

9.1.3 Exposure and effects on enchytraeids

The different toxicity values determined for *Enchytraeus albidus* exposed to Lindane are presented in Table 110. *Enchytraeus albidus* is a test organism that can be assessed in the first step of environmental risk assessment of pesticides. The NOEC and EC50 values were detected in the same range (NOEC = 10 mg/kg; EC50 = 9.7 mg/kg), and the LC50 was much higher (107 mg Lindane/kg). Except of the value at day 14 in the uppermost soil layer A of the TMEs, the measured soil concentrations for the lower application rate was lower than the NOEC and EC50 value in all soil layers and on all sampling dates.

For the higher application rate, all measured soil concentrations in layer B and C were also lower than the NOEC and EC50 value during the whole experiment. The concentration in layer A was higher than the NOEC and EC50 value but lower than the LC50 value at all sampling dates. According to these results, effects were to be expected only for layer A and the higher application rate. No decrease in enchytraeid abundances could be detected in the TMEs of study [1] (Figure 120). Interestingly, enchytraeids displayed higher densities in the TMEs treated with Lindane, possibly as an indirect effect resulting from the decreased densities of arthropods. These increased densitites, especially at day 42 and 140 after treatment can be clearly seen in Figure 120. At the end of the experiment, the enchytraeid abundances in the TMEs treated with Lindane approached again the densities of the control TMEs.

Table 110 Toxicity values of Lindane for the test species *Enchytraeus albidus* (Enchytraeidae) for different endpoints and categories.

Species	Soil	Endpoint	Categorie	Value	Unit	Source
Enchytraeus albidus	AS (OECD)	Mortality	LC50	107	mg/kg TG	Lock et al. 2002
Enchytraeus albidus	AS (OECD)	Reproduction	NOEC	10	mg/kg TG	Lock et al. 2002
Enchytraeus albidus	AS (OECD)	Reproduction	EC10		mg/kg TG	
Enchytraeus albidus	AS (OECD)	Reproduction	EC50	9.7	mg/kg TG	Lock et al. 2002

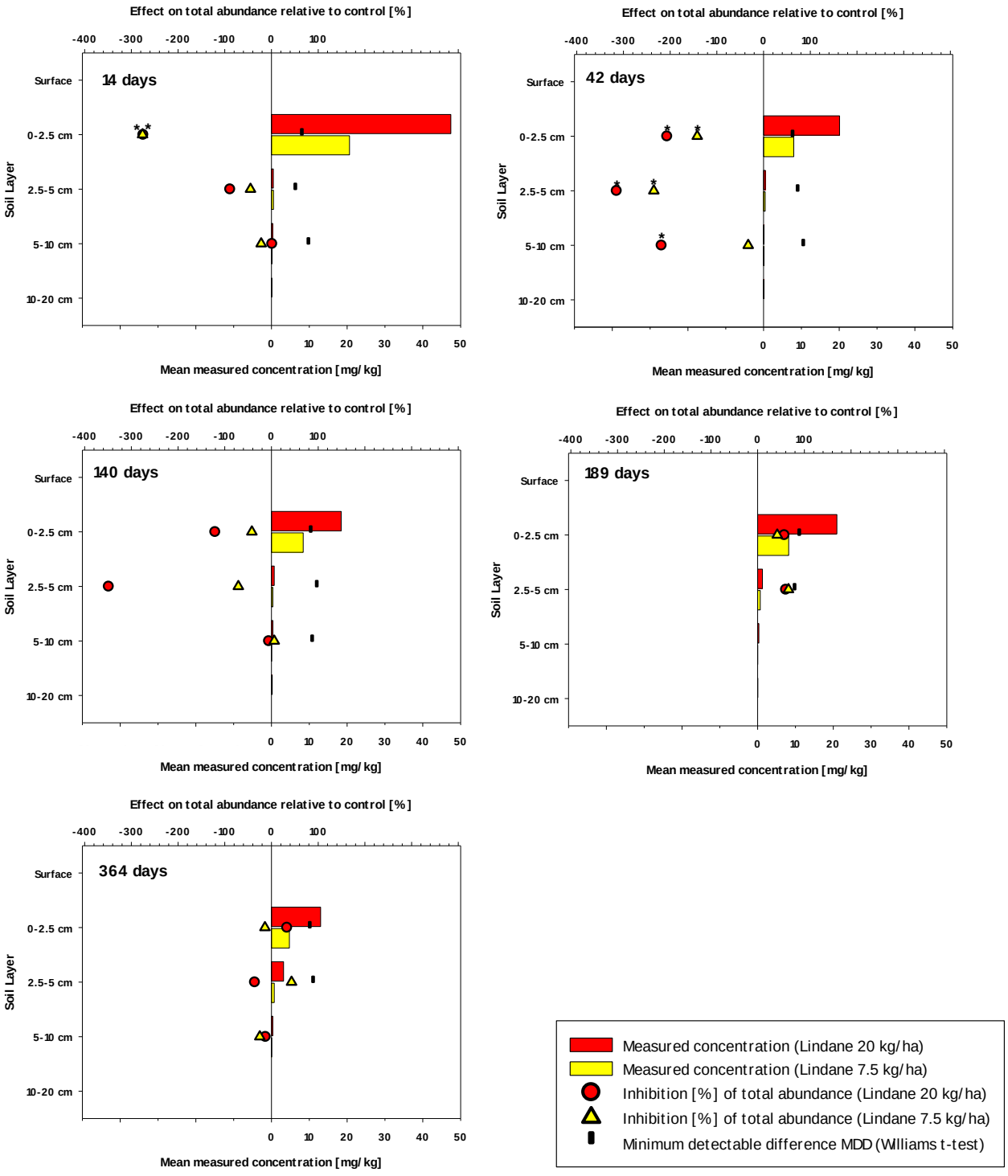

Figure 120 Decrease of total abundance of Enchytraeid species in the Lindane-treatments 7,5 kg a.s./ha and 20 kg a.s. /ha (5 replicates each) for the different soil layers in comparison to the control (10 replicates). Columns showing the measured concentration for the two application rates at the respective sampling date. *: significant difference according to Williams t-test; bars showing the minimum detectable difference (MDD) as value for the specific possible statistical resolution. MDD values higher than 100 % are not shown.

9.1.4 Exposure and effects on lumbricids

The assessment of the environmental risk of pesticides in the soil starts with standardized (OECD) laboratory tests with the lumbricid species *Eisenia fetida/andrei*. According to Lock et al. (2002), several ecotoxicological values from different endpoints are available for this standard test organisms (see Table 111). The LC50 for *E. fetida/ andrei* exposed to Lindane is 165 mg Lindane/kg soil, and the $NOEC_{Reproduction}$ is 14 mg/kg soil. The EC50 and the EC10 for the latter endpoint are 26.5 mg/kg and 14.4 mg/kg, respectively. All measured soil concentrations of Lindane for the lower application rate were lower than the NOEC value except of day 14 in the uppermost soil layer A (Figure 121). For the higher application rate, the measured soil concentrations were also lower than the NOEC value in layer B and C on all sampling dates and in layer A at day 364. The concentration in layer A in the time between day 14 and day 148 was higher than the NOEC but lower than the LC50 value.

Consistent effects on the total abundance of lumbricids were not measured at any time. However, the assessment of results at species level indicate that effects on single earthworm species did occur in the TMEs, usually in the soil depth which was preferred by the respective species. For example, *Lumbricus terrestris*, well-known as an ecosystem engineer and thus highly important as a provider of soil functions, was mainly recorded in the deeper layers D and E (10-20 cm and 20-40 cm, Figure 52). Statistically significant effects on this species were in the soil layers D (at day 14; Table 50). These anecic worms live in deep burrows but are feeding and mating on the soil surface. As described above, the concentrations of Lindane were always lower than the NOEC values in layer D. Thus, these earthworms might have been exposed to Lindane by its vertically transport via the earthworm burrows. This hypothesis is, however, not supported by the low concentrations of Lindane in the leachate of the TMEs (see chapter 4.2.3). Thus, the vertical movement of *L. terrestris* to the soil surface for feeding might have brought the worms in contact with soil layers contaminated by Lindane.

Table 111 Toxicity values of Lindane for the test species *Eisenia fetida/andrei* (Lumbricidae) for different endpoints and categories.

Species	Soil	Endpoint	Categorie	Value	Unit	Source
Eisenia fetida/andrei	AS (OECD)	Mortality	LC50	165	mg/kg TG	Lock et al. 2002
Eisenia fetida/andrei	AS (OECD)	Reproduction	NOEC	14	mg/kg TG	Lock et al. 2002
Eisenia fetida/andrei	AS (OECD)	Reproduction	EC10	14.4	mg/kg TG	Lock et al. 2002
Eisenia fetida/andrei	AS (OECD)	Reproduction	EC50	26.5	mg/kg TG	Lock et al. 2002

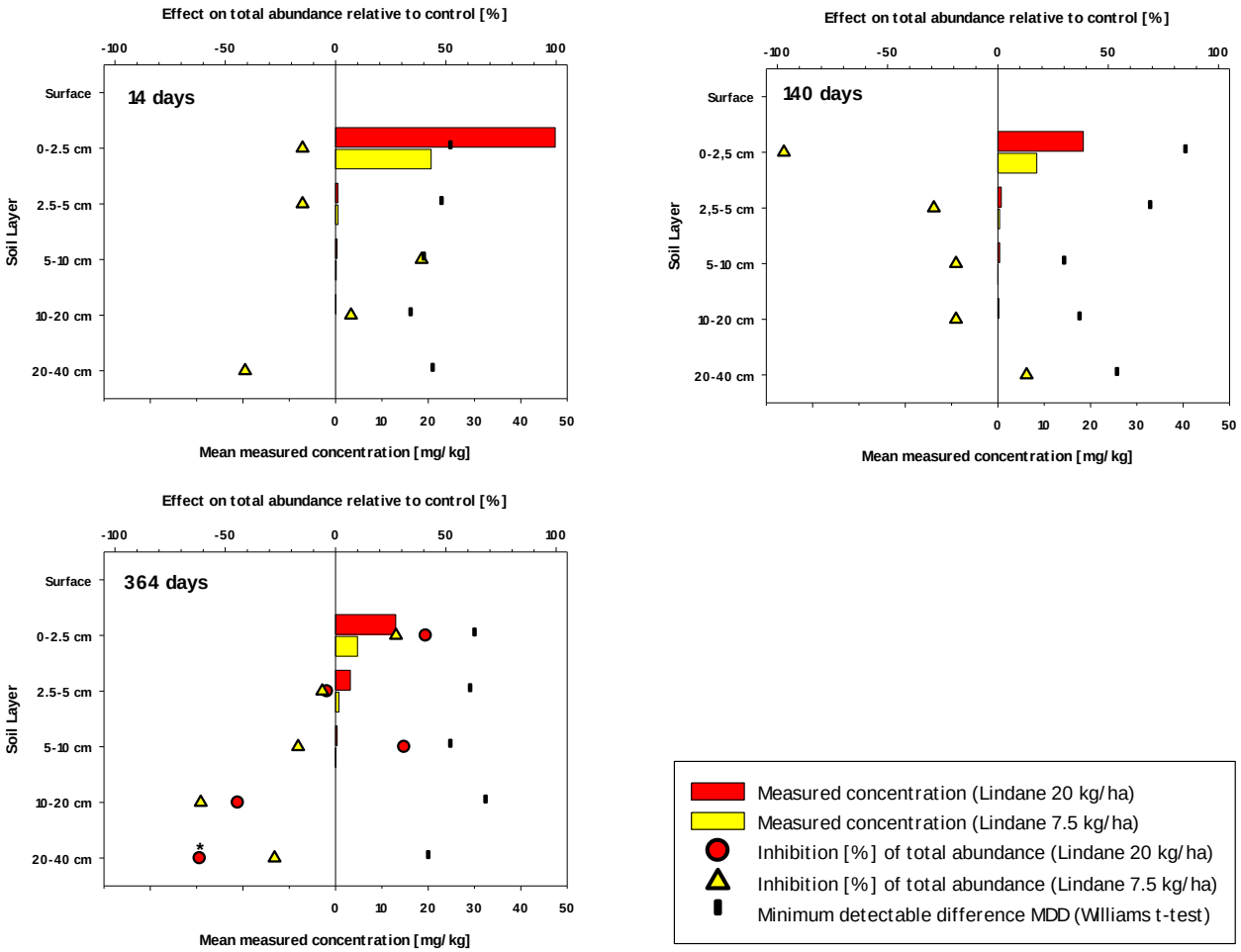

Figure 121 Decrease of total abundance of Lumbricid species in the Lindane-treatments 7,5 kg a.s./ha and 20 kg a.s. /ha (5 replicates each) for the different soil layers in comparison to the control (5 replicates at day 14 and 140; 10 replicates at day 364). Columns showing the measured concentration for the two application rates at the respective sampling date. *: significant difference according to Mann-Whitney Rank Sum; bars showing the minimum detectable difference (MDD) as value for the specific possible statistical resolution. MDD values higher than 100 % are not shown.

9.2 Effects and exposure of Imidacloprid

The highest concentrations of Imidacloprid in the TMEs of study [1] were detected in the uppermost soil layer (95.9 %, 0.75 kg/ha, 90.7 % 2.0 kg/ha for layer 0-2.5 cm 14 days after application, chapter 4.3.3). These concentrations decreased over time. The highest concentration of Imidacloprid was observed for both application rates on the first sampling date after 14 days in the uppermost soil layer (5.73 mg/kg, 2.0 kg/ha; 3.51 mg/kg, 0.75 kg/ha).

The lowest concentration in the uppermost soil layer was found for the lower application rate after 364 days (0.2 mg/kg, 0.75 kg/ha) and for the higher application rate at day 140 (0.58 mg/kg, 2.0 kg/ha).

The concentrations of Imidacloprid were found to decrease with soil depth. In both treatments, the lowest concentrations was found in the deepest soil layer D (10-20 cm), where no significant changes of concentration could be observed over time.

9.2.1 Exposure and effects on collembola

Only few toxicity data were available for the collembolan test species *Folsomia candida* exposed to Imidacloprid (Table 112). According to EFSA (2008), the NOEC value for this species based on the results of an OECD reproduction test is 1.25 mg/kg.

For both application rates, the measured concentration was higher than the NOEC value only at day 14 and day 42 in the uppermost soil layer of the TMEs. The concentration for all other soil layers at any time as well as for the latter sampling dates in the uppermost soil layer was below this NOEC value.

Effects of the Imidacloprid treatment on Collembola were measured on every sampling date (

Figure 122).

The strongest effects were observed as expected in the two uppermost soil layers (0-2.5 cm and 2.5-5 cm) on the first and second sampling date. At the sampling after 140 days and 189 days, the effects on total abundance were found to be reduced in comparison to the beginning of the study. Effects could sometimes not be validated as statistically significant, owed to the decreased individual numbers at these sampling dates and the high variability in the controls. The statistical power at these dates was lower than needed to detect effects lower than 60-70 % of total Collembola abundance.

Table 112 Toxicity values of Imidacloprid for the test species *Folsomia candida* (Collembola) for different endpoints and categories.

Species	Soil	Endpoint	Categorie	Value	Unit	Source
Folsomia candida	AS (OECD)	Mortality	LC50	-	mg/kg TG	-
Folsomia candida	AS (OECD)	Reproduction	NOEC	1.25	mg/kg TG	EFSA 2008
Folsomia candida	AS (OECD)	Reproduction	EC10	-	mg/kg TG	-
Folsomia candida	AS (OECD)	Reproduction	EC50	-	mg/kg TG	-

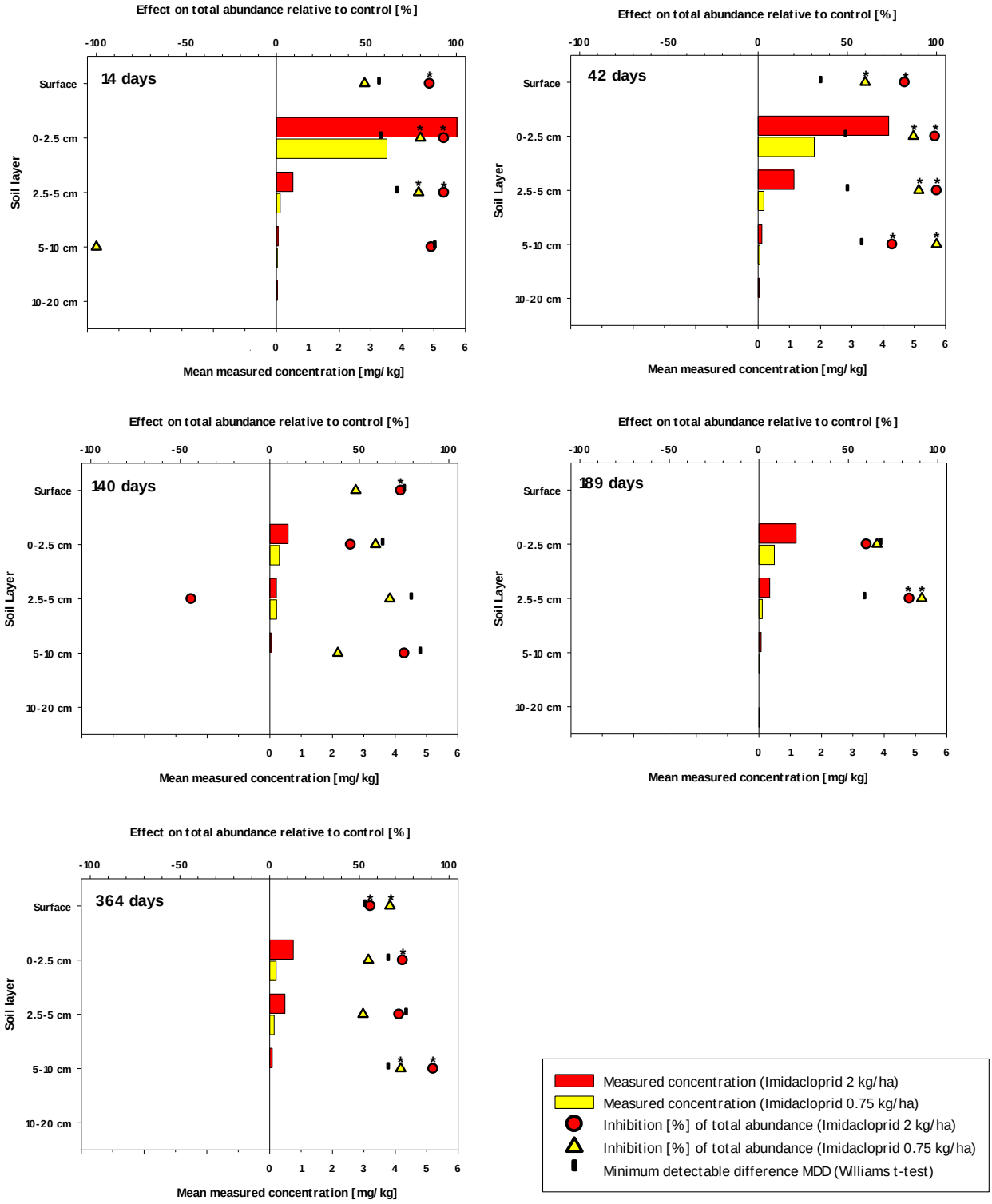

Figure 122 Decrease of total abundance of Collembolan species in the Imidacloprid-treatments 0.75 kg a.s./ha and 2.0 kg a.s. /ha (5 replicates each) for the different soil layers in comparison to the control (10 replicates). Columns showing the measured concentration for the two treatment concentrations at the respective sampling date. *: significant difference according to Williams t-test; bars showing the minimum detectable difference (MDD) as value for the specific possible statistical resolution. MDD values higher than 100 % are not shown.

After 364 days, the individual numbers recovered in the controls and the effects on collembolans in the TMEs treated with Imidacloprid showed effects between 50-90 %. At this time, due to the higher abundances of Collembola densities and the higher statistical power compared to the previous sampling dates, statistically significant effects were observed. Even in soil layer of 5-10 cm, their abundance was significantly decreased by the higher application rate of Imidacloprid. Statistically significant effects were detected in layer A but also in layer C, due to a minimum detectable difference of approx. 60 % at this last sampling date. For the lower Imidacloprid application rate, effects ranged between 50 and 70 %.

Again, in layer C, concentrations of Imidacloprid never reached levels approaching the NOEC determined in the laboratory tests with Collembola.

9.2.2 Exposure and effects on oribatid mites

According to EFSA (2008), the NOEC value for *Hypoaspis aculeifer* exposed to Imidacloprid is ≥2.67 mg/kg (Table 113). This concentration is higher than the NOEC determined for Collembola, reflecting that Imidacloprid is not as toxic for mites as it is for other arthropods. No specific data for oribatid mites were available from published literature. It should be noted that the given NOEC value is a value indicating that no effects were determined in the test and that no higher concentrations were tested (≥ value). Therefore, the NOEC determined in studies testing higher concentrations might be higher.

The concentration of the NOEC value for the mite *H. aculeifer* was reached in the TMEs on the first sampling date for both application rates and on the second sampling date for the higher application rate in the uppermost soil layer. However, no effects on total oribatid mite abundance were observed (Figure 123), except of significant increased abundances for both application rates at day 140.

The statistical power reflected by the MDD value (see Figure 123) indicates that an effects higher than 44-67 % in the uppermost soil layer could have been measured.

Table 113 Toxicity values of Imidacloprid for the test species *Hypoaspis aculeifer* (Acari: Gamasida) for different endpoints and categories.

Species	Soil	Endpoint	Categorie	Value	Unit	Source
H. aculeifer	AS (OECD)	Mortality	LC50		mg/kg TG	
H. aculeifer	AS (OECD)	Reproduction	NOEC	≥ 2.67	mg/kg TG	EFSA 2008
H. aculeifer	AS (OECD)	Reproduction	EC10		mg/kg TG	
H. aculeifer	AS (OECD)	Reproduction	EC50		mg/kg TG	

9.2.3 Exposure and effects on enchytraeids

No ecotoxicological test data were found for enchytraeids exposed to Imidacloprid in the literature. Due to missing data, it was not possible to compare the concentration level detected in the present TME study with the sensitivity level (ECx, NOEC etc.) of enchytraeid species. In the TMEs treated with Imidacloprid, no consistent effects on enchytraeid total abundance were observed (Figure 124). On the species level, an increase in enchytraeid abundance on day 42 after application of Imidacloprid was observed in the soil layer 2.5-5 cm depth. The same effect was similarly observed in the TMEs treated with Lindane. This observation might be attributable to an indirect effect of Imidacloprid on enchytraeids as a result of direct effects on arthropods (Collembola). The increase in enchytraeid abundance was consistent in both Imidacloprid treatment rates. The species being responsible for this pattern were *Achaeta "dzwilloi"*, Enchytraeus sp. GRAN and partly *Fridericia connata*.

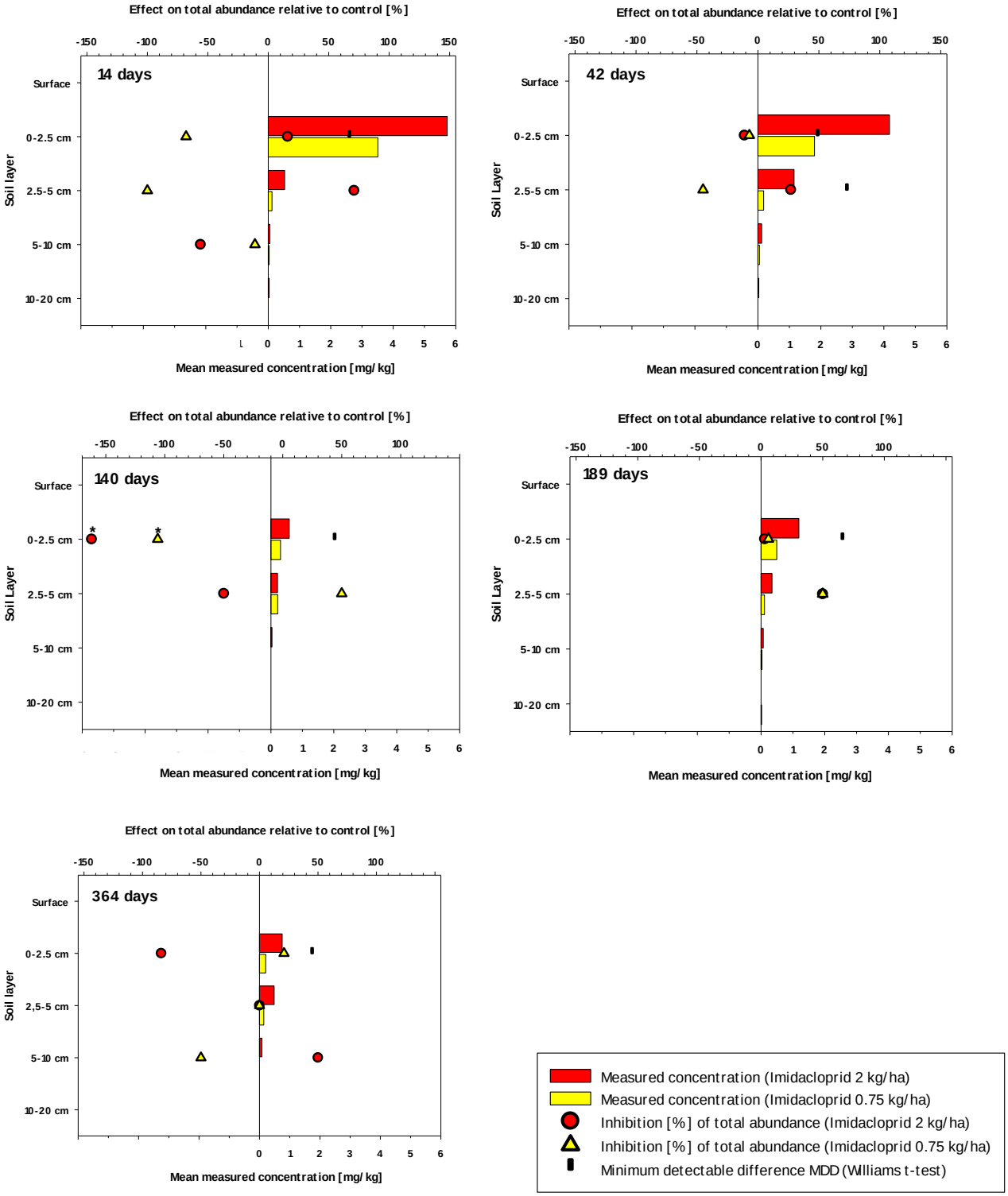

Figure 123 Decrease of total abundance of oribatid mite species in the Imidacloprid-treatments 0,75 kg a.s./ha and 2,0 kg a.s. /ha (5 replicates each) for the different soil layers in comparison to the control (10 replicates). Columns showing the measured concentration for the two application rates at the respective sampling date. *: significant difference according to Williams t-test; bars showing the minimum detectable difference (MDD) as value for the specific possible statistical resolution. MDD values higher than 100 % are not shown.

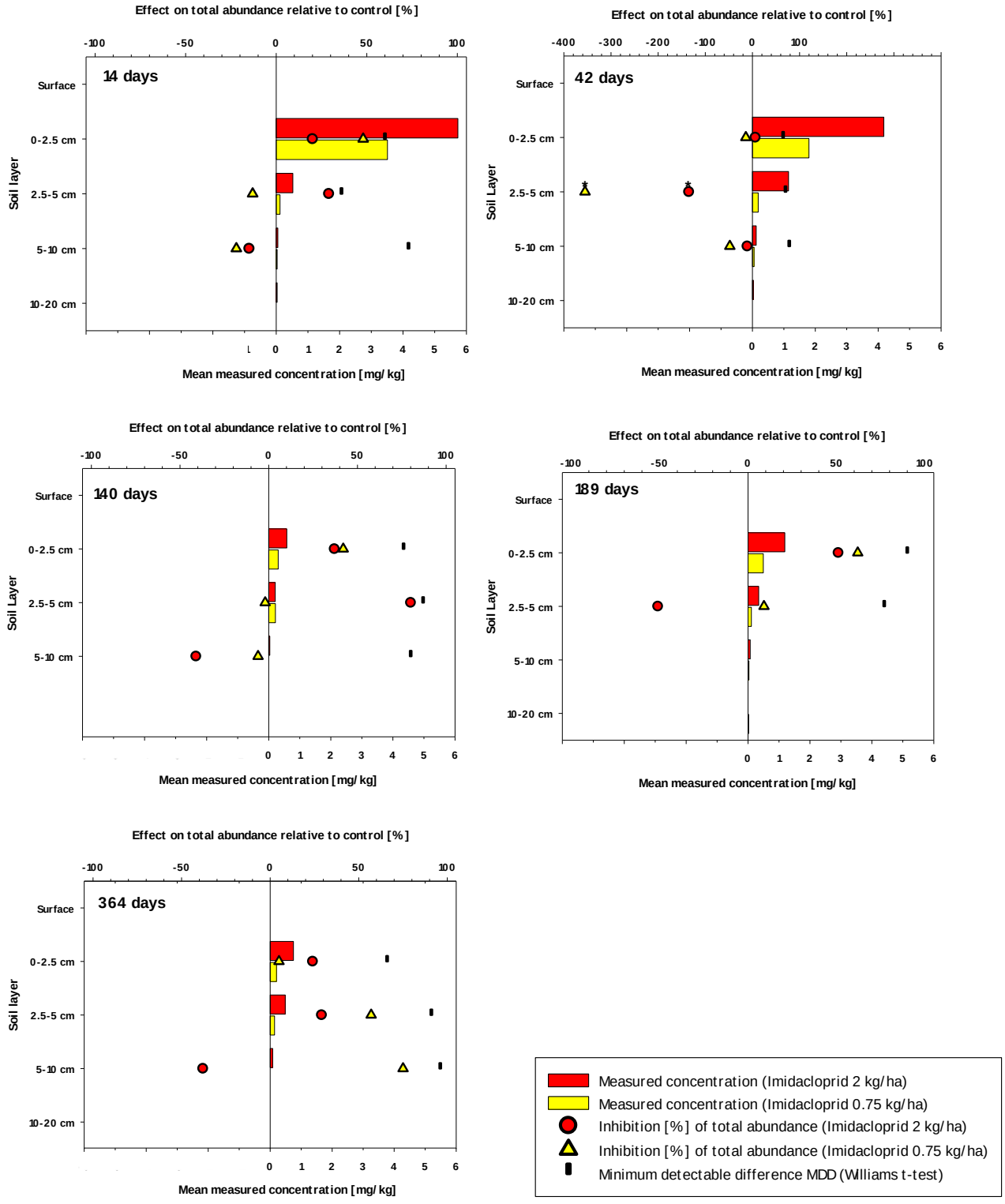

Figure 124 Decrease of total abundance of Enchytraeid species in the Imidacloprid-treatments 0,75 kg a.s./ha and 2,0 kg a.s. /ha (5 replicates each) for the different soil layers in comparison to the control (10 replicates). Columns showing the measured concentration for the two application rates at the respective sampling date. *: significant difference according to Williams t-test; bars showing the minimum detectable difference (MDD) as value for the specific possible statistical resolution. MDD values higher than 100 % are not shown.

9.2.4 Exposure and effects on lumbricides

According to the EU ecotox database (EFSA 2008), the LC50 value for effects of Imidacloprid on *Eisenia fetida/andrei* is 10.7 mg/kg soil. The NOEC value is ≥ 0.178 mg/kg (Table 114).

During the whole study period, the measured concentrations in the soil layers of the TMEs were below the median lethal concentration in the different soil layers of the treated TMEs. The highest concentration was 5.73 mg/kg, measured for the higher application rate at day 14 in layer A (0-2.5 cm).

In the uppermost soil layer A (0-2.5 cm), however, the measured concentration was for both application rates at every sampling date higher than the NOEC value for earthworm reproduction. For the higher application rate also the layer 2.5-5 cm shows always higher concentrations than 0.178 mg/kg. However, these concentartions were clearly lower than the lethal concentration.

Effects of Imidacloprid on total earthworm abundance were observed on the last sampling date (day 364; Figure 125). These effects occurred, as expected, in the uppermost soil layer for both application rates and for the higher application rate in layer B (2.5-5 cm). It can be hypothesized that the Imidacloprid concentrations higher than the determined NOEC for chronic effects from the literature have affected the reproduction of earthworms in the TMEs, as shown in the results of the sampling dates one year after application. This is supported by the fact that the significant differences on the latest sampling date are mainly caused by the decrease of the abundance of juveniles belonging to the genus *Aporrectodea*. In this context it was imoportant that adult earthworms of this genus, belonging to species classified as endogeics and which usually occurred in deeper layers, were affected by Imidacloprid (e.g. *A. caliginosa* and *A. rosea*).

Table 114 Toxicity values of Imidacloprid for the test species *Eisenia fetida/andrei* (Lumbricidae) for different endpoints and categories.

Species	Soil	Endpoint	Categorie	Value	Unit	Source
Eisenia fetida/andrei	AS (OECD)	Mortality	LC50	10.7	mg/kg TG	EU 2008
Eisenia fetida/andrei	AS (OECD)	Reproduction	NOEC	≥ 0.178	mg/kg TG	EU 2008
Eisenia fetida/andrei	AS (OECD)	Reproduction	EC10	-	mg/kg TG	-
Eisenia fetida/andrei	AS (OECD)	Reproduction	EC50	-	mg/kg TG	-

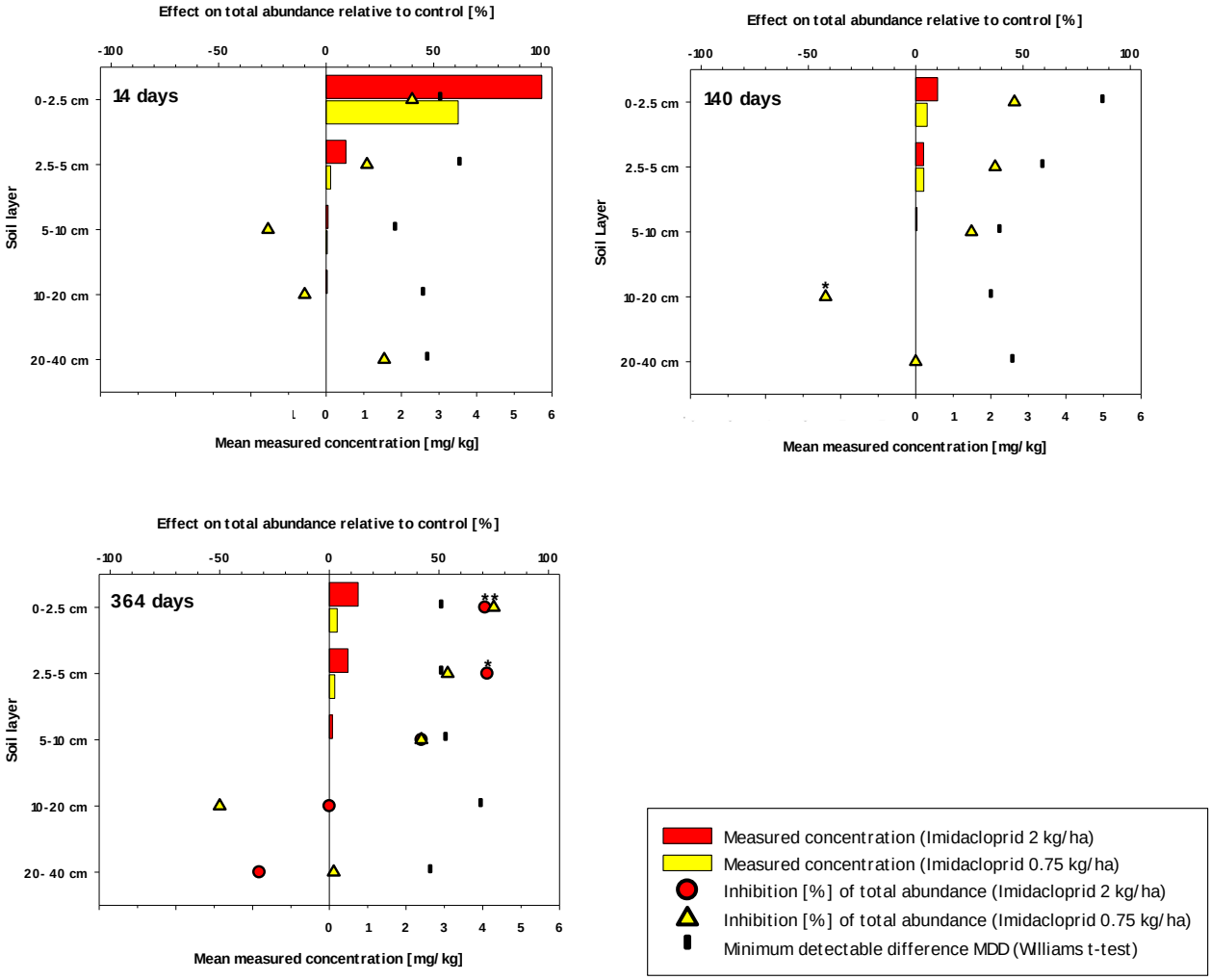

Figure 125 Decrease of total abundance of lumbricid species in the Imidacloprid-treatments 0,75 kg a.s./ha and 2,0 kg a.s. /ha (5 replicates each) for the different soil layers in comparison to the control (10 replicates). Columns showing the measured concentration for the two treatment concentrations at the respective sampling date. *: significant difference according to Mann-Whitney Rank Sum; bars showing the minimum detectable difference (MDD) as value for the specific possible statistical resolution. MDD values higher than 100 % are not shown.

9.3 Exposure and effects of Carbendazim

Most of the applied Carbendazim was found in the uppermost soil layer (87.6 %, 7.5 kg/ha, 94.2 %, 15 kg/ha for layer 0-2.5 cm 16 days after application, Chapter 6.2) and the concentrations decreased over time. The highest concentration of Carbendazim was observed for both application rates on the first sampling date after 16 days in the uppermost soil layer (0-1 cm, 23.8 mg/kg, 15 kg/ha; 6.49 mg/kg, 7.5 kg/ha).

The concentration in the layer 0-2.5 cm was lower than the concentration measured in the uppermost centimetre (0-1cm; 7.3 mg/kg, 15 kg/ha; 4.36 mg/kg, 7.5 kg/ha), which is probably due to a strong concentration gradient within the uppermost soil centimetres.

The lowest Carbendazim concentrations in the uppermost soil layer were found for both application rates after 148 days (0.03 mg/kg, 7.5 kg/ha, 0.11 mg/kg, 15 kg/ha). The concentrations of Carbendazim were found to decrease with soil depth. In both treatments, the lowest concentrations could be found in the deepest soil layer D (10-20 cm), where, however, no significant change of concentration could be observed over time.

9.3.1 Exposure and effects on lumbricides

The pesticide Carbendazim is known as highly toxic for earthworms and therefore it is used as a toxic reference in most of the field trials performed as part of the higher-tier earthworm risk assessment. Because of this widespread use of Carbendazim in earthworm environmental risk assessment, several specific toxicity values are known for this pesticide. According to Garcia (2004), the LC50 value for the standard earthworm test species is 5.8 mg Carbendazim/kg, the EC50 for reproduction is 2.7 mg/kg and the NOEC reproduction value is 0.1 mg/kg (Table 115).

Table 115 Toxicity values of Carbendazim for the test species *Eisenia fetida/andrei* (Lumbricidae) for different endpoints and categories.

Species	Soil	Endpoint	Categorie	Value	Unit	Source
E. fetida/andrei	AS (OECD)	Mortality	LC50	5.8	mg/kg TG	Garcia 2004
E. fetida/andrei	AS (OECD)	Reproduction	NOEC	0.1	mg/kg TG	Garcia 2004
E. fetida/andrei	AS (OECD)	Reproduction	EC10	-	mg/kg TG	-
E. fetida/andrei	AS (OECD)	Reproduction	EC50	2.7	mg/kg TG	Garcia 2004

Only on the first sampling date of the TMEs treated with Carbendazim (study [3]), the concentration in soil reached the LC50 value. The concentration in the uppermost centimetre of the soil after applying the lower application rate was 6.49 mg/kg, whereas the concentration related to the uppermost 2.5 centimetre was lower than the LC50 value (4.36 mg/kg).

For the higher application rate, the concentrations both upper layers were higher than the LC50 value (23.18 mg/kg, 0-1 cm; 7.3 mg/kg, 0-2.5 cm).

The EC50 value for reproduction was exceeded in the uppermost soil centimetre for the higher application Carbendazim rate at day 114 after application.

The NOEC value was exceeded at the first sampling date and the lower application rate at day 16 down to layer C (5-10 cm, 0.14 mg/kg) and in the higher application rate down to layer B (2.5-5 cm, 0.35 mg/kg). On later sampling dates, this value was reached in the uppermost soil layer (0-

1 cm and 0-2.5 cm) for the lower application rate only at day 114, whereas in the higher application rate this threshold was reached also after 148 days (chapter 6.2).

Additionally to the standard tests reported above, in a TME ring test performed at four European sites with different soils, the EC50-value for Carbendazim 16 weeks after starting the study was determined as 3 – 4 kg a.i./ha. This corresponds to a concentration of 4 – 5 mg a.i./kg soil DW following the formula used in EU risk assessment: application rate in kg/ha*1.33 = concentration in mg/kg soil at 0-5 cm (Römbke et al. 2004) in the upper lasoil layer. These numbers are in the same order of magnitude as the concentrations measured in our study in the first two soil layers A and B for the low application rate after 16 weeks (Table 84 , Figure 79) . Carbendazim is regularly used as a reference substance in earthworm field studies required for the registration of pesticides in Europe (EU 1991). Therefore, many such tests have been performed according to ISO Standard 11268-3 (1999), but their results have rarely been published in the open literature. For reasons still not completely understood, the toxicity of Carbendazim to earthworms decreased within the last twenty years: In the first version of the ISO guideline for earthworm field tests (ISO 1999) the application rate for Carbendazim which should cause an effect of 50% was given as four to eight kg a.i./ha. About ten years later, an international group of experts recommended to increase the application rate which should cause a reduction of about 50% of the number of earthworms at agricultural or grassland sites to 6 – 10 kg a.i./ha (Kula et al. 2006). Therefore, the allover effects on earthworm abundance observed in this study are exactly in the expected range and as foreseen for this experiment.

In the TME ring test cited above and similar studies (for a compilation see Schaeffer et al. 2008), it was not differentiated where the observed effects did occur, i.e. the number of worms was counted in the whole TMEs - depending on the individual study in a depth of 0 to 40 or 60 cm. In the present study, it was possible for the first time to exactly determine the reaction of the earthworm community in five different soil layers after application of Carbendazim on top of the TMEs.

On the first sampling date, Carbendazim reduced the total abundance of lumbricids by 81 % in the lower application rate and by 88 % in the higher application rate (Figure 126). The reduction of abundance in layer B (2.5-5 cm) was approximately 50 % for both application rates on this sampling date. The abundance was also reduced significantly in layer C (5-10 cm) but only for the higher application rate.

In comparison to the measured concentrations, the observed effects seem not to match for the different soil layers, since the NOEC was exceeded, but the lethal concentration could be detected only in the uppermost centimetre in the first sampling date (see figure below). On the later sampling dates, this relation was even less given. Strong effects, mostly statistically significant, occurred in all layers from the uppermost layer A down to layer E (20-40 cm). Except from effects in layer D (10-20 cm) at day 148, all effects were dose related i.e. the higher application rate caused higher effects than the lower.

At species level, it could be observed that *Octolasion cyaneum*, a species found mainly in deeper soil layers in the TMEs (C and D) was reduced by Carbendazim by 100 % in layer C at day 114 and day 148 and more than 80 % in comparison to the control in layer D. Already at day 16, effects detected in layer 10-20 cm were above 70 % and statistically significant. At this sampling date, concentrations of Carbendazim in layer D were lower than the NOEC value for Carbendazim.

Also for *Lumbricus terrestris*, a species that was found only in the two deepest layers within this study, the abundance decreased to extinction at day 114 and day 148 in both deeper soil layers. Effects on *L. terrestris* reached 100% already at day 16, but because of the variance within the dataset and the low statistical power of the assay, these findings were only significant for the deepest layer in the TMEs - where most of the individuals occurred and the concentration of Carbendazim was lowest.

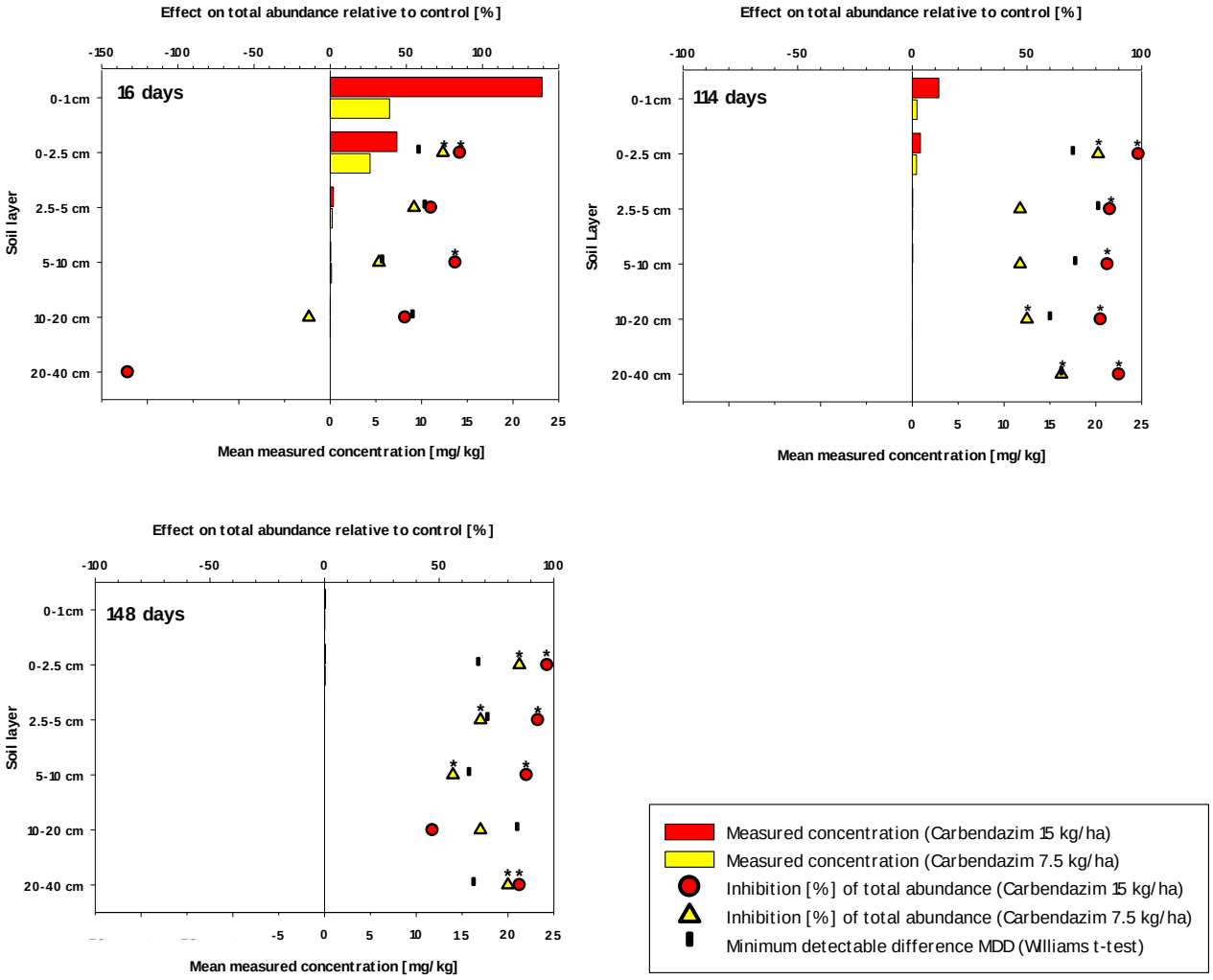

Figure 126 Decrease of total abundance of lumbricid species in the Carbendazim-treatments 7.5 kg a.s./ha and 15 kg a.s. /ha (5 replicates each) for the different soil layers in comparison to the control (5 replicates). Columns showing the measured concentration for the two application rates at the respective sampling date. *: significant difference according to Williams t-test; bars showing the minimum detectable difference (MDD) as value for the specific possible statistical resolution. MDD values higher than 100 % are not shown.

9.4 Conclusions

In the following, general conclusions regarding the above presented results of these TME studies are given by answering the questions asked in the beginning of the project (chapter 1.2).

- **Can the assumed functional relationships between spatial distribution of a PPP in the soil profile and the location of ecotoxicological effects be confirmed?**

In the most cases the applied amount of pesticides led to high concentrations in the different soil layers, as foreseen at the beginning of the experiment. Thus, the allover occurrence of measured effects was expected according to the applied amounts of the different pesticides. The main research questions of the experiment, though, regarded the distribution in the soil profile of the applied chemicals and the respective ecotoxicological effects on different soil organisms.
The results showed that effects of the applied chemicals were measured in the uppermost soil layers as to be expected, but that especially in deeper soil layers effects were detected that could not be explained or could not have been assumed by the related measured low concentration of the pesticide.

- **Is the exposure level and consequently the extent of ecotoxicological effects modulated by the preferred position and the behaviour of soil organisms in the soil profile?**

According to the high amounts of pesticides applied in our experiment, that was chosen in order to elicit effects on soil organisms deliberately, only in a few cases the concentration in the uppermost soil layer was lower than the assumed NOECs derived from standard laboratory tests. Thus, only in these cases a decoupling of exposure and effect was observed already in the first centimetres of the TMEs.
Regarding lower soil layers in the TMEs, often the concentrations were at all times lower than the NOEC reported in the literature, but effects could be nevertheless be observed already at the first sampling dates. This can be most plausibly explained by vertical movement of the soil organisms. The vertical movement is known for different soil organisms, e.g., earthworms that burrow deep in the soil but regularly move to the soil surface for feeding. Consequently it can be stated that soil organisms were affected, sometimes decoupled from the exposure derived from their preferred position in soil, especially in the case of species preferring deeper soil layers. In addition to the exposure measured in the preferred position in soil, the “real exposure” is triggered by the behaviour i.e. vertical movement of the soil organisms, hence both factors will influence the extent of ecotoxicological effects. This means, that the risk assessment approach has to be extended by inclusion of the migrational behaviour of organisms. This is particularly relevant for soils in which pesticides are distributed heterogeneously, displaying a concentration gradient.

- **Is the spatial transfer of the maximum concentration of a PPP into different soil layers over time accompanied by a sequence of effects in organism groups with different mode of exposure?**

For none of the three PPP a time-dependent shift of relevant proportions of the applied amounts in deeper soil layers was observed. Only small shares of the applied amounts were measured in

deeper soil layers for both the lipophilic lindane and the much less lipophilic imidacloprid. These amounts did probably not cause delayed effects in deeper soil layers, since these concentrations were below no observed effect concentrations reported in the literature. At the same time, in some cases a recovery could be observed later in the experiments in the uppermost soil layers.

- **Do active substances with different properties at a given time interfere with different groups of organisms, each representing a typical mode of exposure?**

In the soil type chosen for our experiments, the used pesticides did not behave very differently with regards to their movement in soil. Consequently, the active substances affected more or less the same organism groups at each individual point in time. This means that effects were detected in deeper soil layers - in organisms also preferring those layers - even if concentrations of pesticides with different properties were not high enough there to elicit the observed effects. Derived from the present study, it seems that the behaviour/mobility of the soil organisms plays a more important role in the overall effect pattern than the distribution of the substance in the different soil layers.

10 Recommendations

In the present studies, we investigated the effects of three different pesticides (lindane, imidacloprid and carbendazim) on different soil organisms of a grassland community in Terrestrial Model Ecosystems (TME). The three pesticides used have different physico-chemical properties (e.g. Kow) and are known to be differently toxic for various soil organism groups. The overall result of the chemical analysis was that the highest concentration for all three pesticides was always measured in the uppermost soil layers (study [1]: 0-2.5 cm; study [2]] and [3]: 0-1 cm). The concentrations of the pesticides increased in deeper soil depth over time, but at any time they were by far lower than in the uppermost layers.

All four observed organism groups (Collembola, Oribatida, Enchytraeidae, and Lumbricidae) showed group and species specific vertical distribution patterns in the soil. These distribution patterns were found to be also species-specific, but in each group the individual species could be classified accordingly into three groups (e.g. the well-known epigeic, endogeic and anecic earthworm groups). Consequently, different exposure patterns of species/ecological groups to the respective pesticide could be in principle assumed. The highest numbers of individuals were found in the uppermost soil layer (0-2.5 cm) for all organism groups (Collembola, 68 %; Oribatida, 91 %; Enchytraeidae, 60 %; Lumbricidae, 36 %). Except of lumbricids, more than 80 % of all individuals were observed in the uppermost 5 cm (Collembola, 92 %; Oribatida, 91 %; Enchytraeidae, 88 %). Effects of the three pesticides on soil organisms could be detected in every soil layer (0-10 cm soil depth for Collembola, Oribatida, and Enchytraeidae and 0-40 cm soil depth for Lumbricidae). They were found to be species and substance specific, (e.g. collembolans were affected especially by the insecticides). Both, acute effects (14 days after application) as well as long lasting effects (up to one year) were observed in the uppermost soil layer, as expected, due to the high application rates chosen, to affect all groups.

In the case of Lindane, the deliberately chosen high concentrations remained high during the experiment in the upper soil layers; the measured concentrations in the upper soil layers remained above the lethal values derived from laboratory tests with the corresponding standard test species (i.e. *Folsomia candida* for collembolans) over the course of the study. This was not the case for the other substances investigated.

For all substances investigated, effects were observed in deeper soil layers even if the measured concentrations were below the no observed effect concentrations known from literature. This was found for collembolans exposed to imidacloprid and lindane as well as for earthworms exposed to imidacloprid and carbendazim. These effects, e.g. on deep-digging earthworms, can be explained by the vertical movement of these organisms within the soil column reaching upper layers with higher pesticide concentrations.

Based on these results the following recommendations can be given:

Recommendation 1: Protection Goals

For the use of Terrestrial Model Ecosystems, field tests or ecological models e.g. as a higher tier options in risk assessment, it is mandatory to develop operational, spatially explicit protection goals.

Explanatory statement:

The current practice in the evaluation of field tests with soil organisms is based on a document by EPPO related to earthworm field tests, where recovery in-field within 1 year is considered acceptable (EPPO 2003). However, no official guidance is available yet for the evaluation of TME or field tests. EFSA is currently reviewing information in order to define specific protection goals (SPG) based on the ecosystem services approach (following e.g. EFSA PPR 2010, Nienstedt et al. 2012). SPGs should be operationally defined, including acceptable effect magnitudes and durations for soil organisms, possibly differentiated for e.g. in-field, edge-of-field (off-field) and other off-field areas. Such operational protection goals would also be needed if population models are used as additional higher tier tools, e.g. to extrapolate population level effects between different soils or climates. Elaboration of such protection goals should be based on a discussion between different stakeholders, e.g. regulators including risk managers, plant protection producers and soil ecologists.

Recommendation 2: Environmental chemisty

Regarding pesticide exposure, further research is needed to address the (partly not expected) behavior of the three PPPs of the present studies in the soil profiles.

Explanatory statement:

(1) Further PPPs, representative of certain chemical classes or intended uses and covering a range of physico-chemical properties, have to be investigated regarding their leaching behavior. The determined concentrations in the soil layers need to be compared with potential effects on soil organisms in the corresponding layers (as extension of the concept of the present studies).

(2) The degradation of individual pesticides in presence of further pesticides, for instance in pesticide spray series during the growing season, may be different compared to that of a pesticide alone. For instance, soil fungi –important degraders – may be inhibited by fungicides and degradation of chemicals (and natural organic matter) may be retarded. Thus, the rate and mechanism of pesticide degradation in soil should be studied in presence of further pesticides used in commercial product mixtures.

(3) In addition, the degradation of pesticides may be affected by additional (mainly natural) stress situations such as drought, water logging, or soil compaction. These scenarios should be further investigated to allow their consideration in the exposure estimations.

(4) Furthermore, a regular monitoring of pesticide residues in agricultural soils is lacking or is perfomed only in the frame of research programs. For instance, Chiaia-Hernández et al. (2017) showed that pesticides and major transformation products may persist for decades after application, even though dissipation half lives are reported to be much shorter.

(5) The formation of non-extractable residues (NER) leads to long-term persistence of the test substances and/or metabolites. Especially residues sequestered in the soil matrix (type I NER,

Kästner et al. 2014) are of environmental relevance as they are slowly released from the matrix. Pesticides with high potential to form type I NER should be tested by incubating exhaustively extracted soil with fresh soil and investigating the potential ecotoxicity for soil organisms.

Recommendation 3: Exposure Modelling

The reliability of mechanistic computer models like PELMO has to be improved for simulating loss processes at the soil surface such as photo- or microbial degradation and volatilisation.

Explanatory statement:

The current simulation models have been extensively tested to predict the fate of pesticides in the soil matrix. That includes transport as well as transformation processes. However, the validation status for processes at the soil surface is unsatisfying. To overcome this deficiency, more experiments should be performed to determine the soil moisture and temperature under these conditions. This is essential for the validation of the modules in the fate models that calculate moisture and temperature at the soil surface.

In the next step, it should be checked whether the modules in the fate models that extrapolate microbial degradation under standard conditions to the situation at the soil surface are really suitable.

With regard to soil photolysis, the experiments should reflect more realistic conditions when they are going to be used as input parameter for fate models; this is important since these standard laboratory studies are currently characterised by extreme high radiation which often leads to side effects (*e.g.,* increase of soil temperature during the study).

Also with regard to the process volatilisation from soil surface, more experiments should be performed which better reflect the special situation at the soil surface under various conditions; these data should be used as base for further validation of the fate models. That includes also the increase of adsorption at very dry soils surfaces which may reduce volatilisation significantly.

Recommendation 4: TME Performance

A TME or field study to determine the effects of pesticides on the soil community should mirror the (field) conditions of the target system and should be representative for the regional circumstances i.e. climatic conditions or soil properties. The organism groups to be monitored should be selected according to the special mode of action of the pesticide and the results of available laboratory tests.

Explanatory statement:

The community of soil organisms is adapted to the specific environmental conditions of the habitat they are living in. These conditions (mainly climate and soil properties) as well as the specific competition within the soil community are key factors for the presence and structure of the soil organism community. To assess the risk of pesticides used in specific regions and crops, e.g. olive trees in the Mediterranean region, soil communities of the respective region should be considered to claim realistic conditions. The organism groups to be tested should be selected based on a set of criteria, such as:

- **Exposure potential:** Are organisms potentially exposed considering the intended use of the pesticide (region, crop, season)?
- **Ecotoxicological sensitivity:** Are the species of the selected group(s) sensitive to the specific stress factor, e.g. as proven by the results of laboratory tests?
- **Ecological vulnerability**: are the organisms covering a range of different typical ecological traits (e.g. species with long reproduction cycles or slow spatial dispersal)?
- **Ecological relevance:** Are species of the respective group occurring in the targeted environment? Are the organisms dominant in terms of abundance or biomass and do they play a key role in food webs or do they act as ecosystem engineers?
- **Exposure pathway:** Do the organisms live in close contact with soil, pore water or plant residues?

Note that predators have been neglected in standard soil testing, while saprophagous groups are preferred due to their close contact with PPP adsorbed on organic material. However, it should be noted that predators as the gamasid mite *Hypoaspis aculeifer* are fed in the standard tests with uncontaminated food. As a rule-of-thumb, usually groups belonging to the macro- and mesofauna are preferred (i.e. in line with species selected for laboratory tests), covering at the same time a spectrum of hard- or softbodied organisms representing different exposure pathways (Peijnenburg et al. 2012). In the following a short overview on organisms potentially suitable for testing are listed.

Lumbricidae (earthworms):	Macrofauna	Softbodied
Isopoda (woodlice):	Macrofauna	Hardbodied
Enchytraeidae (potworms):	Mesofauna	Softbodied
Collembola (springtails):	Mesofauna	Hardbodied
Oribatida (oribatid mites)	Mesofauna	Hardbodied
Gamasida (gamasid mites)	Mesofauna	Hardbodied
Nematoda (nematodes)	Micro-/Mesofauna	Softbodied

When considering the respective ecological relevance, it is important to know which ecological function (and thus service) is covered by these organism groups (Nienstedt et al. 2012; Ockleford et al. 2017). Of course, all of them contribute due to their (especially for the mesofauna very high) biodiversity to the ecosystem service “genetic resource”. More or less the same is true for their contribution to the ecosystem service “nutrient cycling” because of their activity in organic matter decomposition. The ecosystem service “soil formation” is usually considered to be influenced mostly by macrofauna, i.e. mainly earthworms in temperate regions; in southern regions macroarthropods such as isopods, diplopods or termites take over, but in this case the important role of mesofauna in preparing and conserving soil structure on a smaller scale should not be neglected. Well-known is also the important contribution of deep-burrowing (= anecic) earthworms on water retention. Finally, and often not known, are the contributions of soil invertebrates to pest and disease control (e.g. earthworms or isopods destroying fungi spores when feeding on dead leaves).

Recommendation 5:

For the performance of TME or field studies in the context of risk assessment it is not necessary to differentiate between different soil layers. To assess effects of pesticides on soil organisms, a representative capture of abundance of the different species of the respective organism group must be guaranteed (and thus a certain soil depth has to be sampled). The recommended soil depth for sampling of the four soil organism groups studied here is at least 0-5 cm for Collembola, Oribatida, and Enchytraeidae as well as 0-40 cm for Lumbricidae and corresponds to a number of more than 80 % of their total abundance.

Explanatory statement:

To measure effects on soil organisms it is sufficient to capture the effect of the applied PPP on the total abundance and species individual abundance in the soil column per area. In the case of possible spatial decoupling of exposure to pesticides and effects on the soil organisms which are due to vertical movement of organisms in the soil core, no consequences for the risk assessment must be taken into account if the whole relevant soil column is analysed. However, data of the vertical distribution of soil organisms could be used for the parameterisation or the verification of population models.

For an extrapolation of the results of TME or field test to other conditions (soil type and/or climate) resulting in a different temporal and spatial distribution of the toxicant it would be useful to analyse the test item in different soil layers (e.g. 0-1, 1–2.5, 2.5–5, 5–10 and 10–20 cm). So, effect threshold could be expressed for different soil layers and compared to the PEC predicted for other soils and conditions.

Recommendation 6:

To evaluate the protection level of lower assessment tiers using calculated predicted environmental concentrations (PEC values) for different soil layers, it is recommended to compare the effect concentrations derived from lower tier studies with the effect concentrations derived from higher tiers, e.g. (semi-) field studies. A review on the comparison of the different assessment steps for different pesticides is desirable.

Explanatory statement:

The present studies were carried out with the premise to get information on the fate and effects of pesticides differing in their physico-chemical properties and thus behavior in soil and to affect organism groups in the soil. To reach this goal, effective concentrations of the respective pesticides were applied. Consequently, it was not the aim of the present study to detect the no-effect-threshold of the different pesticides for the different soil organisms like it would be required in present risk assessment. The applied rates led to a vertical profile of concentrations in the different soil layers. Concentrations in the uppermost soil layer elicited the expected effects on the organisms; in lower layers still effects were observed although the pesticide concentrations were lower than no effect concentrations reported in the literature. To be able to compare the equivalence of effects at lower and higher assessment steps, effect thresholds would be needed in both systems. The availability of effect values from (semi-) field studies is limited/not known. Therefore it is recommended to check whether such data are available for at least some pesticides. In case this is not possible, such (semi-) field studies should be performed in the future.

Recommendation 7:

For Environmental Risk Assessment schemes, the predicted environmental concentrations in soils ($PEC_{soil\text{-}initial}$) should be derived from the calculation of the concentration of the uppermost centimetre(s) and compared with the determined Effect Concentration.

Explanatory statement: For all perfomed studies here, the highest concentrations of all pesticides were found in the uppermost soil layer. This layer was 0-2.5 cm in study [1] but could be refined to 0-1 cm in study [2] and [3]. Due to the spray application of the pesticides onto the soil surface, a vertical gradient of concentrations occur in the soil column. Thus, at least for the first time after application, the PPP concentration is highest in the uppermost centimetre(s) of the soil. However, in the present studies this pattern was observed during the whole study period of 1 year for all three pesticides. The depth of the first layer in which soil organisms were separately analysed was in this experiment 0-2.5 cm. Due to practical limits (i.e. the uneven relief of the soil surface) soil organisms could not be sampled in smaller layers (e.g. the first soil centimetre) without limiting excessively the number of retrieved organisms. Some of the organisms (especially collembolans) were living and moving on the soil surface. The exact distribution pattern of the different organisms and species within the uppermost soil centimetres is not known. Hence, it is reasonable to consider the high pesticide concentrations of the upper soil layer together with the high amounts of exposed organisms in this layer.

Recommendation 8: basic ecology and Reference Values

Basic soil ecological questions have to be answered in order to improve the environmental risk assessment of plant protection products (PPP) for the soil compartment, especially for the evaluation of higher-tier tests such as TMEs or field studies.

Explanatory statement:

Relevant traits (e.g. regarding spatial behaviour) of the most important mesofauna groups (i.e. at least Collembola, Enchytraeidae, Oribatida and Gamasina) have to be identified and described. In this context, the definitions of existing proposals for the classification of ecological trait groups of meso- and macrofauna have to be critically investigated (e.g. “borderlines” of the three springtail ecological groups and their vertical movement in the soil profile have to be clarified). This work should cover Central Europe and should also form the basis for comparable investigations in Northern (e.g. boreal) and Southern (e.g. Mediterranean) Europe. This information can be collected in publicly available data bases (e.g. Edaphobase.org). Based on the compiled data, the structure and functions of soil organism communities at a specific site and soil can be classified by comparing the site-specific results with reference data.

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