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Effects of pesticides on soil fauna: Development of ecotoxicological test methods for tropical regions

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ABSTRACT

Soil organisms play a crucial function in the ecosystem and are the main driving force responsible for organic matter breakdown, nutrient cycling and soil structural stability. Plant Protection Products (PPPs) (i.e., pesticides) have long been used in agriculture for control pests and diseases in plants. However, many PPPs are also toxic for non-target (beneficial) species and may have negative impacts on soil functions.

Little research has been done on the impact of PPPs on tropical ecosystems, considering the amount of studies already conducted in temperate regions. Often, most of the standardized data used in the risk assessment of chemicals in tropical countries are generated in temperate regions, whereas comparable data are relatively scarce for tropical regions. Based on this situation, mainly two questions were addressed in this study: (1) Do fate and effects of pesticides differ between tropical and temperate regions? (2) Can data generated under temperate conditions be used for the Environmental Risk Assessment (ERA) in tropical regions?

The effects of three pesticides (two fungicides (benomyl and carbendazim) and one insecticide (lambda-cyhalothrin)) were evaluated using structural (i.e., earthworms and arthropods) and functional (i.e., organic matter decomposition) endpoints of soil biota in Amazonia. Tests were performed on the laboratory, semi-field and field level. They were based on standard methodologies as described in international OECD and ISO guidelines. Laboratory tests performed under tropical conditions had to be modified accordingly (e.g., an increase in temperature from 20 °C to 28 ºC). Besides using some native species, the tests were done mainly with two (temperate and tropical) strains of earthworms (*Eisenia fetida*) and the peregrine isopod species *Porcellionides pruinosus*, using both temperatures. The chemicals were spiked in four (natural and artificial) soils. A tropical artificial soil (TAS), containing a fern product (Xaxim) as organic matter, was developed in this study. After the tests were finished, Xaxim had to be replaced with coir dust due to its scarcity in the market. The methodology of semi-field and field experiments, already used in temperate regions, was slightly adapted for use under tropical conditions. The results from laboratory as well as semi-field and field tests showed that the toxicity of the test chemicals was strongly influenced by the tropical factors (abiotic like temperature and biotic like species). Accordingly, in the case of the fungicides the toxicity was lower but in the case of the insecticide higher under tropical than under temperate conditions. Significant effects could also be detected in the Terrestrial Model Ecosystem (TME). In the field test, carbendazim as well as lambda-cyhalothrin negatively affected OM breakdown. With one exception, no effects on the soil macrofauna were observed one year after the first application of both substances: the abundance of the native earthworm *Andiorrhinus amazonius* decreased in all carbendazim treatments.

Coming back to the initial two questions, it can be stated that due to the experiences gained in this study the following preliminary answers can be given: (1) Yes, depending on the PPP assessed, its fate and effect can differ in the two regions. (2) No, in case a PPP is used in the tropics, existing toxicological data from temperate regions should be carefully evaluated and, if necessary, additional tests performed under tropical conditions. Further research is necessary: A standard tropical field soil should be identified and the suitability of coir dust as an OM source in TAS must be verified. The chronic laboratory test with isopods needs further development.

Auswirkungen von Pestiziden auf die Bodenfauna: Entwicklung ökotoxikologischer Testmethoden für die Tropen

KURZFASSUNG

Bodenorganismen sind ein zentraler Bestandteil terrestrischer Ökosysteme, insbeson-dere in Hinsicht auf Streuabbau, Nährstoffkreisläufe und Bodenstabilität. Pflanzen-schutzmittel (PPPs; d.h. Pestizide) werden seit langem in der Landwirtschaft zur Kontrolle von Schädlingen und Pflanzenkrankheiten eingesetzt. Allerdings sind viele PPPs zugleich toxisch für "nützliche" Arten und können zudem negative Wirkungen auf Bodenfunktionen haben. Die Auswirkungen von PPPs in tropischen Ökosystemen wurden bisher wenig erforscht, vor allem im Vergleich zur Situation in gemässigten Breiten. Daher stammen die für die Risikobeurteilung von PPPs in tropischen Ländern notwendigen standardisierten Daten meist aus Tests, die unter gemässigten Bedingungen erstellt wurden. In dieser Studie wurden primär zwei Fragen bearbeitet: (1) Unterscheidet sich das Verhalten und die Wirkung von Pestiziden unter tropischen und gemässigten Bedingungen? (2) Können Daten aus gemässigten Regionen für die ökologische Risikobeurteilung (ERA) von PPPs in den Tropen verwendet werden?

Die Auswirkungen von drei Pestiziden (zwei Fungizide (Benomyl, Carben-dazim) und ein Insektizid (lambda-cyhalothrin)) auf Bodenorganismen in Amazonien wurden unter Verwendung struktureller (Regenwürmer, Arthropoden) und funktionaler (Streuabbau) Endpunkte beurteilt. Tests wurden im Labor-, Halbfreiland (Terrestrischen Modellökosystemen (TME) und Freiland durchgeführt. Sie basierten auf international standardisierten Verfahren, d.h. OECD und ISO Richtlinien. In Hinsicht auf tropische Bedingungen mussten die Labortests modifiziert werden (z.B. bei 28 ºC statt 20°C). Die Tests wurden meist mit zwei Varianten der Regenwurmart *Eisenia fetida* (aus Deutschland bzw. Brasilien stammend) sowie einer peregrinen Asselart (*Porcellionides pruinosus*) durchgeführt. Die Chemikalien wurden in vier (natürliche und künstliche) Böden eingemischt. Ein tropischer Kunstboden (TAS), der als organisches Material ein Farnprodukt (Xaxim) enthält, wurde in dieser Studie entwickelt. Nach Durchführung der Tests musste Xaxim durch „Coir dust" ersetzt werden, da ersteres in Brasilien nicht mehr erhältlich war. Die ebenfalls aus gemässigten Breiten bekannten Halbfreiland- bzw. Freilandverfahren wurden nur wenig modifiziert. Die Ergebnisse der Labor-, Halbfreiland- und Freilandtests zeigen, dass alle drei PPPs negativ auf die Testorganismen wirkten und dass ihre Toxizität durch die tropischen Bedingungen (primär Temperatur und Spezies) stark beeinflusst wurde. Im Fall der beiden Fungizide war die Toxizität unter tropischen geringer als unter gemässigten Bedingungen, während es bei dem Insektizid genau umgekehrt war. Signifikante Wirkungen auf einzelne Arten (z.B. die einheimische Regenwurmspezies *Andiorrhinus amazonius*) oder den Streuabbau wurden sowohl in den TMEs als auch im Freiland festgestellt. In Hinsicht auf die beiden eingangs gestellten Fragen können die folgenden (vorläufigen) Antworten gegeben werden: (1) Ja, in Abhängigkeit von den Eigenschaften des zu beurteilenden PPP können sich dessen Verhalten und Auswirkungen in den beiden Regionen unterscheiden. (2) Nein. Wenn ein PPP in den Tropen eingesetzt wird müssen die aus gemässigten Breiten stammenden Daten sorgfältig überprüft werden und zusätzliche Tests sind gegebenenfalls durchzuführen. Weitere Forschung ist notwendig: Ein "tropischer" natürlicher Boden ist zu identifizieren und die Verwendbarkeit von "Coir dust" als Bestandteil der TAS ist zu verifizieren. Ein chronischer Labortest mit Asseln ist weiter zu entwickeln.

RESUMO

Os organismos de solo desempenham importante função no ecossistema e têm papel essencial na decomposição da matéria orgânica, ciclagem de nutrientes e estabilidade estrutural do solo. Os pesticidas têm sido, há muito tempo, usados na agricultura para o controle de pragas e doenças. Entretanto, muitos destes produtos são também tóxicos para organismos não-alvo (benéficos) e podem produzir impactos negativos nas funções do solo. Poucos estudos têm sido feitos sobre o impacto dos pesticidas em ecossistemas tropicais, considerando as diversas pesquisas já conduzidas neste sentido em regiões temperadas. Em geral, os dados usados para a avaliação de risco ambiental de produtos químicos em países tropicais são oriundos de regiões temperadas. Diante disto, duas importantes questões foram levantadas neste estudo: (1) O efeito e destino dos pesticidas em solos de regiões temperadas podem ser diferentes em solos tropicais? (2) Os dados produzidos nas regiões temperadas podem ser usados para a avaliação de risco ambiental nos trópicos?

Os efeitos de três pesticidas (dois fungicidas (benomyl e carbendazim) e um inseticida (lambda-cyhalothrin)) foram avaliados utilizando parâmetros estruturais (i.e., fauna de minhocas e artrópodos) e funcionais (i.e., decomposição da matéria orgânica) da biota do solo na Amazônia. Experimentos foram desenvolvidos em laboratório, em condições de semi-campo (microcosmos) e de campo. Ensaios foram feitos conforme metodologias padronizadas e descritas em protocolos internacionais OECD e ISO. Em laboratório, testes de toxicidade padronizados foram modificados conforme as condições tropicais (e.g., temperatura aumentada de 20 °C para 28 ºC). Estes foram feitos em duas populações de minhocas (*Eisenia fetida*) originárias das regiões temperadas (Alemanha) e tropical (Brazil) e do crustáceo *Porcellionides pruinosus* (Isopoda) de origem tropical, sob ambas temperaturas. Os pesticidas foram adicionados em quatro tipos de substratos (solos naturais e artificiais). Os testes de toxicidade foram feitos em solo tropical artificial, desenvolvido neste estudo, contendo xaxim como matéria orgânica. Posteriormente, devido à escassez do xaxim no mercado, foi usado pó de casca de coco na composição do solo. A metodologia dos experimentos em microcosmos e campo, já em uso nas regiões temperadas, foi adaptada para sua utilização sob condições tropicais. Os resultados de testes de laboratório, em microcosmos e campo mostraram que a toxicidade dos pesticidas foi fortemente influenciada pelos fatores tropicais, como temperatura e espécie. A toxicidade dos fungicidas aos organismos do solo, foi menor sob condições tropicais que temperada. Ao contrário, o inseticida apresentou maior toxicidade nas condições tropicais. Efeitos significantes foram também observados em microcosmos. No ensaio de campo, observou-se o efeito negativo de carbendazim e lambda-cyhalothrin sobre a decomposição da matéria orgânica. Após sucessivas aplicações destes pesticidas no campo, durante um ano, não foi observado efeito sobre a macrofauna do solo. Entretanto, a abundância de *Andiorrhinus amazonius*, uma espécie nativa de minhoca, diminuiu em todos os tratamentos com carbendazim. As experiências obtidas neste estudo permitem responder as questões formuladas acima: (1) Sim, conforme o pesticida, seu destino e efeito no solo podem ser diferentes nas duas regiões. (2) Nao, para os trópicos, os dados ecotoxicológicos de regiões temperadas devem ser avaliados com cautela e, se necessário, outros ensaios devem ser feitos sob condições tropicais. Entretanto, outras pesquisas são necessárias: identificação de um solo natural tropical padronizado, testes em diferentes laboratórios com o solo artificial contendo casca de coco e elaboração de um teste para toxicidade crônica em isopodos.

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List of abbreviations, acronyms and definitions:

C_{org}	Organic carbon content
C.V.	Coefficient of variation
dw	dry weight
DT_{50}	*Disappearance time* – Time within which the concentration of the test substance is reduced by 50 %.
EC_{50}	*Median effective concentration* – the concentration which affects 50% of the test population after a specified exposure time, compared with the control. The EC_{50} is based on effects like reproduction rate, immobilization or growth rate.
ECT	ECT Oekotoxikologie GmbH, Floersheim, Germany
ELISA	Enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency (USA)
Embrapa	Empresa Brasileira de Pesquisa Agropecuária (Brazilian Agricultural Research Corporation), Manaus, Brazil
fw	fresh weight
GLP	Good Laboratory Practice
ha	hectare
Half-life	The time required for 50 % of a chemical to degrade in the environment.
Henry's constant	The ratio of concentration of a volatile chemical in air to the concentration in an aqueous solution (at equilibrium). It can be used as a general indicator of the volatility of a chemical.
IRGA	Infrared Gas Analyzer
ISO	International Organization for Standardization
IUPAC	International Union of Pure and Applied Chemistry
K_{oc}	*Soil-water partition coefficient* – or adsorption coefficient normalized on organic carbon. It describes the sorption of a chemical to soil particles compared to remaining dissolved in water. A chemical with a high K_{oc} value is unlikely to be transported to groundwater; instead, it is bound to soil particles.
K_{ow}	*Octanol-water partition coefficient* – a measure that describes the equilibrium partitioning of a chemical between octanol and water phases. The parameter is used to estimate the bioaccumulation in body tissues. A chemical with a high K_{ow} value tends to be bioaccumulated in organisms and, thus, in the food chain.
LC_{50}	*Median lethal concentration* – the concentration of a substance that is estimated to be lethal to 50% of the test organisms within the test period.
LOEC	*Lowest-observed-effect concentration* – the lowest concentration of a substance that has a statistically adverse effect on the exposed organism compared with the controls.

LUFA	*Landwirtschaftliche Untersuchungs- und Forschungs - Anstalt Speyer* – LUFA Speyer. Governmental institution that provides standardized soils to be used for experiments concerning the effect of chemicals on soil organisms or investigating the fate of chemicals in soil.
lux	Unit of illumination based on units per square meter
n.a.	not applicable
n.d.	not determined or no data
n.s.	not statistically significant (at P = 0.05, if not specified)
NOEC	*No-observed-effect concentration* – the highest tested concentration that does not cause any observed and statistically significant adverse effects on the organism compared with the controls.
OECD	Organization for Economic Co-operation and Development
OM	Organic matter
PPPs	Plant Protection Products
PEC	Predicted Environmental Concentration
PNEC	Predicted No Effect Concentration
ppb	parts per billion
ppm	parts per million
RF test	Range Finding test – preliminary toxicity test to find the range of concentrations to be used in the definitive test
SD	Standard Deviation
SOP	Standard Operating Procedures
TAS	Tropical Artificial Soil – Artificial soil adapted for tropical regions, proposed in this paper.
TASx	Tropical Artificial Soil – Artificial soil with xaxim as organic matter component.
TASs	Tropical Artificial Soil – Artificial soil with sphagnum moss as organic matter component.
TASc	Tropical Artificial Soil – Artificial soil with coconut coir dust as organic matter component.
TNS	Tropical Natural Soil – Natural soil classified as Acrisol (Red Yellow Podzolic)
TME	Terrestrial Model Ecosystem – microcosm consisting of an intact block of soil
v/v	volume per volume
Vapor pressure	The pressure exerted by the vapor of pure substance at a given temperature in a closed system at equilibrium. The vapor pressure is used to estimate the rate of emission of volatile chemicals from soil and water sources into atmosphere.
w/v	weight per volume
WHO	World Health Organization
95%-CL	Confidence limits at 95%

1 INTRODUCTION

In nature, the soil is one of the key elements that enable the life on earth. It plays a central role in all terrestrial ecosystems, functions as habitat for many organisms and as a filter and buffer, allowing clean groundwater storage. Important parts of the natural cycle of carbon, nitrogen, phosphorus and sulphur take place in the soil. The main ecological soil functions are those related to organic matter breakdown and nutrient mineralization by soil invertebrates and microbes. The soil dwelling organisms play a crucial role in the ecosystem by mediating the geochemical cycling of elements and nutrient supplies to plants. They are also very benefitial to soil structure and structural stability.

The technological advances in agriculture have led to an increased production and emission of chemical substances, which end up in the soil. The soil constituents, like the clay and organic matter, have a great capacity to retain chemicals. Therefore, the soil is a net sink for all kinds of chemicals, and its concentrations are often considerably higher than in any other environmental compartment (Verhoef and Van Gestel 1995).

Intensified agriculture to meet demands for high crop production has led to a long-term emission of agrochemicals in soil. The impact of agrochemicals on soil fauna diversity and soil functions has become an issue of great concern. Agrochemicals are applied in the environment to fulfil a specific purpose, but at the same time may cause damage to the soil biota, decreasing its diversity, growth or reproduction, and consequently organic matter decomposition and soil fertility. Therefore, there is an increasing need for appropriate methods to assess the side effects of these chemicals on soil ecosystems.

1.1 Terrestrial (soil) ecotoxicology

1.1.1 Definition and objectives

Ecotoxicology is the science that deals with the ecological effects of substances potentially toxic in environment. The term “ecotoxicology” was first coined in 1969 by R. Truhaut (Butler 1984). The science of ecotoxicology is an outgrowth of the link between toxicology, ecology and chemistry (Römbke and Moltmann 1996). During the development of ecotoxicology, a great number of bioassays for aquatic fauna have

reached a considerable level of standardization, but soil toxicity tests still require substantial development. Up to 1995, the existing international guidelines for soil organisms were the acute tests for earthworm (OECD 1984a) and plants (OECD 1984b). During the last years, new methods for soil toxicity assessment have been proposed, but despite that, not all of them have been internationally standardized yet (Løkke and Van Gestel 1998). Over the last decade, most of the attention has been focused on the ecotoxicological effects of soil contaminants, mainly because of the numerous polluted sites discovered and the high clean-up costs (Van Leeuwen 1995). However, the number of standardized test procedures available for the terrestrial medium (soil compartment) is still significantly lower than those which exist for the aquatic medium (Van Straalen and Van Gestel 1993).

The aim of soil ecotoxicology is to provide a scientific basis which will be used to evaluate and assess the fate and effects of a chemical substance in the soil, taking its quantities and the structure (i.e., biodiversity) and function (i.e., biological processes) of the soil biota into consideration. Final aim of these activities is the protection of the soil ecosystem against anthropogenic stress.

1.1.2 Principles of Environmental Risk Assessment (ERA)

In order to avoid duplication of work and, at the same time, to facilitate the comparison between data from tropical and temperate regions, general and internationally widely accepted principles of the Environmental Risk Assessment (ERA) were taken as a starting point. The most stringent definition was given by Barnthouse (1992): ERA is simply a systematic means of developing a scientific basis for regulatory decision making in an iterative process. ERA was developed in the USA, firstly for evaluating the risk of chemicals to humans but was later adapted for the environment (Fava et al. 1987; OECD 1989). The European Union started using ERAs for the registration of pesticides. Later, ERAs were also required for the notification of new and existing chemicals and for the registration of biocides (EC 2003b). Until today, many modifications have been proposed in various countries and for different purposes (leading to some confusion in terms of names and definitions). However, basically an ERA consists of the following steps (Figure 1.1):

1. Hazard identification
2. Exposure (PEC) and effect (PNEC) analysis

3. Risk characterization (PEC/PNEC ratio)

4. Risk management

Depending on the data, steps 2 and 3 of the process can be performed several times before a conclusive result has been reached. In any case, data should come from internationally standardized tests performed according to GLP (Good Laboratory Practice; OECD 1998) rules. Since details of data interpretation and the PEC/PNEC comparison for pesticides are becoming more and more complex, guidance is available from international organizations (EPPO 2003).

When determining the PEC/PNEC ratio, initially two assessment classes are defined to categorize the outcome of the ERA:

PEC/PNEC > 1: Improvement of exposure and effect analysis necessary

PEC/PNEC < 1: No indication of environmental risk potential

If after a refinement of the data (e.g., using the results of ecologically more relevant results from field studies instead of those from simple laboratory tests) the PEC/PNEC is still > 1, risk management measures become necessary.

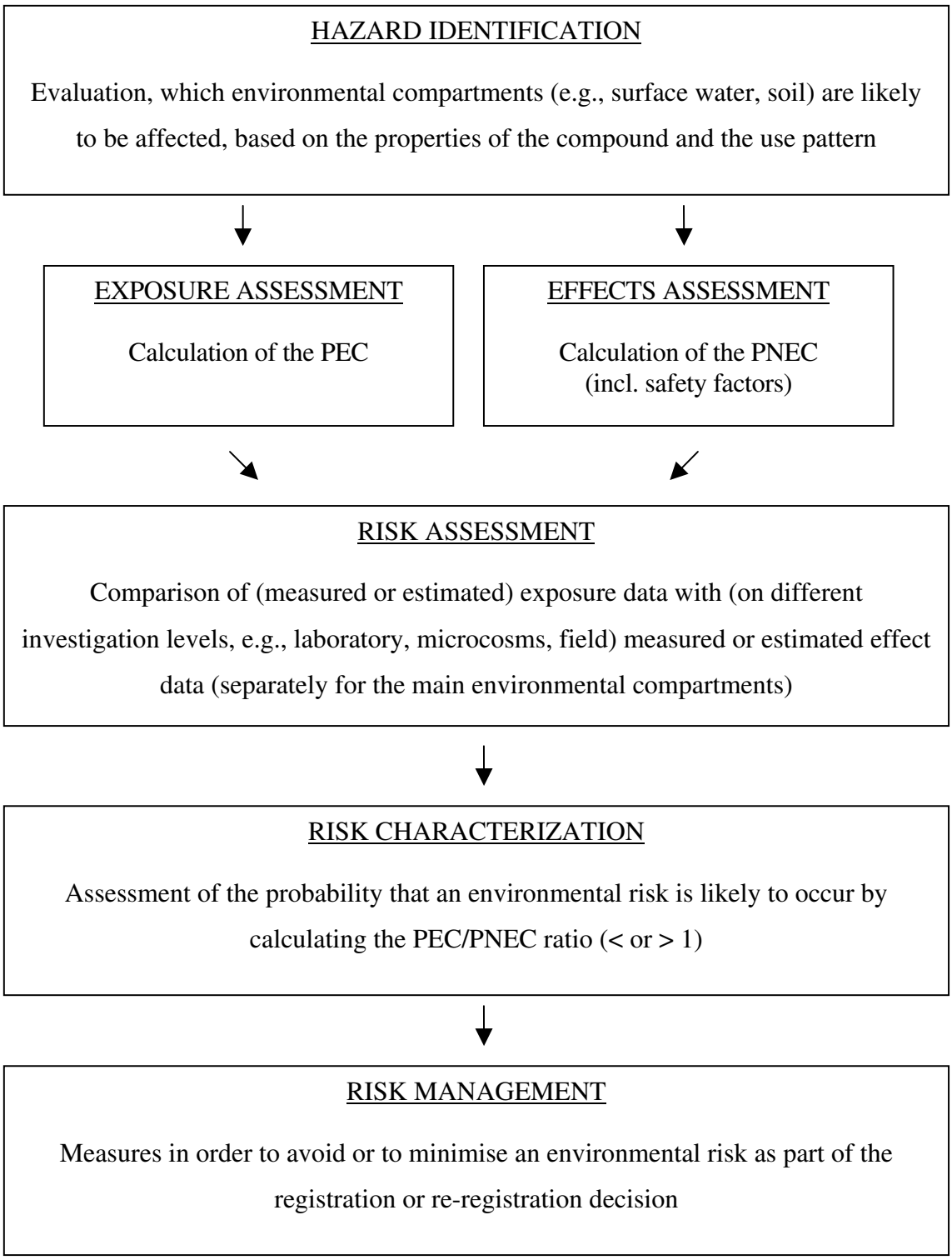


Figure 1.1: General principles of an Environmental Risk Assessment (ERA) process (modified according to various authors).

1.1.3 Different test levels

The objective of the environmental risk assessment is to determine whether the integrity of an ecosystem, rather than an individual organism, is disturbed when a chemical stressor is introduced into the system (Kupermann et al. 2002). Usually, the effects on soil organisms have been assessed based on the results of single species laboratory tests. The soil ecosystem is very complex in its physical, chemical and biological interactions. However, in laboratory tests, these interactions between organisms and their environment cannot be taken into account. For this reason, Römbke et al. (1996) suggested that chemicals in soil should be tested using a tiered test strategy with increasing ecological relevance and complexity: (1) basic laboratory tests (mainly acute); (2) extended laboratory tests (mainly chronic); (3) tests using microcosms (model ecosystem tests) or even field tests. In the first tier, basic laboratory tests, the most important functional groups in the soil ecosystem are covered (microorganisms, earthworms, arthropods and plants). In the second tier, an extension of the species spectrum of different trophic levels and a more detailed investigation of those organisms which were affected in the basic tests has to be performed. In the third tier, microcosms (e.g., Terrestrial Model Ecosystems - TME) are performed in soil cores taken in the field containing the native soil biota, which are treated with the contaminant under controlled conditions. Even field tests are possible in this highest tier. However, despite the fact that tests at the highest tier are most important for an ecotoxicological risk assessment, they have rarely been performed due to their high complexity, costs and time needed (Römbke and Notenboom 2002).

1.1.4 Differences between temperate and tropical ecosystems

Despite the great development of ecotoxicology in the last decades, only recently has concern arisen about environmental problems in tropical regions. Little research has been done on the impact of contaminants on tropical ecosystems, considering the amount of studies already conducted in temperate regions. In addition, and also in contrast to temperate ecosystems, the tropical environment is poorly studied. It is known that the physical and chemical variables affecting biotic processes as well as the fate of chemicals are different from those in temperate regions (Lacher and Goldstein 1997; Laabs et al. 2000; Paraiba et al. 2003), but details like the effects of higher temperatures on chemical metabolization in organisms or the soil are often unknown.

The question that should be addressed is how the fate and effects of pesticides differ between tropical and temperate climates. Regarding the fate of pesticides in the tropics, there are some general tendencies: the high temperature and humidity seem to favour degradation and volatilization of the chemical in the soil (Klein 1989). On the other hand, humid and warmer conditions may enhance the toxicity of some pesticides by increasing the penetration through skin of animals, and these may thus be taken up more quickly by tropical biota. In general, hot and humid conditions appear to potentiate the toxicity of most pesticides (Viswanathan and Krishna Murti 1989). These differences should be considered when assessing the potential risk of chemicals, in particular pesticides, in tropical ecosystems.

Nearly nothing is known about ecotoxicological effects in the terrestrial environment: most of the available work has been done with old pesticides in non-standardized tests (Bharathi and Subba Rao 1984; Cook et al. 1980; Hans et al. 1990; Senapati et al. 1991; Verma and Pillai 1991), while data on economically relevant chemicals are currently very scarce (e.g., Helling et al. 2000). Practically no tests have been performed in Latin America or Brazil. This is especially strange, since already very early Knäpper (1981) addressed the urgency of this problem.

Often, most of the data used in the risk assessment of chemicals in tropical countries are generated in North America or Europe (i.e., where temperate species were used), whereas comparable datasets are relatively scarce for the tropical and subtropical regions (Leung et al. 2003). Consequently, an extrapolation of temperate data to tropical conditions without a scientific basis can lead to erroneous results. This study was planned to contribute to the construction of such a scientific basis for tropical soil ecotoxicology.

1.2 Aims of this study

The main goal of this study was the assessment of the ecotoxicological effects of pesticides on the structure (i.e oligochaetes and arthropods) and the function (i.e., organic matter decomposition) of the soil biota under tropical (i.e., Amazonian) conditions. In order to reach this goal, the internationally accepted principles of Environmental Risk Assessment (ERA) were followed as far as possible.

Consequently, the following objectives can be formulated:

1. Selection of appropriate (e.g., representative for the study area and ecologically relevant) test chemicals (pesticides);
2. Modification of existing standardized test systems in terms of test species, substrate and conditions in order to make them suitable for tropical regions;
3. Adaptation of existing semi-field (TME - Terrestrial Model Ecosystem) and field (litterbag method) tests to tropical conditions;
4. Setting up of a data set with different tests (laboratory, semi-field and field level) and the selected pesticides, performed under tropical and temperate conditions (the latter to be used for comparison purposes);
5. Estimation of the exposure concentrations in soil;
6. Comparison of the results on three test levels for the selected pesticides with data gained under temperate conditions;
7. Performance of an ERA for these pesticides and comparison of the result with the one required by the European Commission for their registration in the European Union (i.e., temperate conditions);
8. Formulation of recommendations for the ecotoxicological assessment of pesticides in tropical soils, based on the experiences gained in this study and the discussion in the literature.

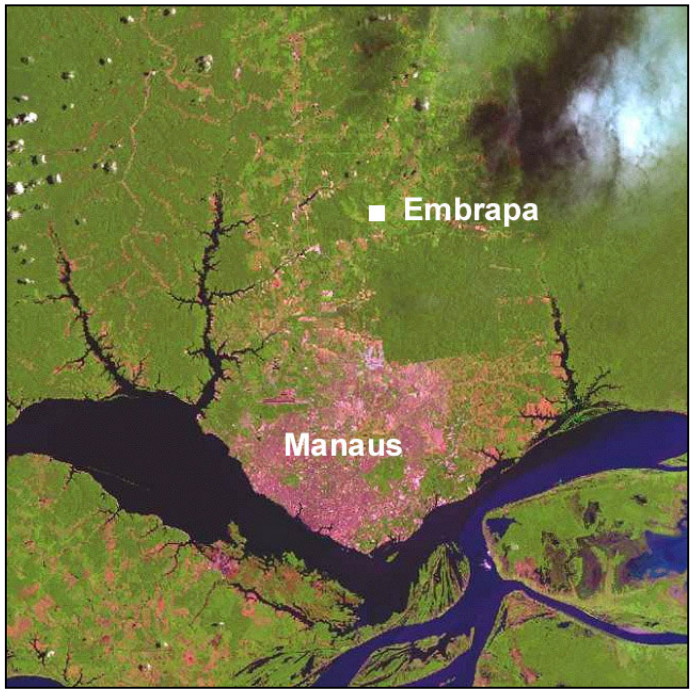
2 MATERIALS AND METHODS

2.1 Study site description

This study was carried out at Embrapa research station, located in central Amazonia, about 30 km north the city of Manaus, at 02°53'S, 59°59'W and 120 m a.s.l. (Figure 2.1).

The Embrapa station, comprising an area of 1800 ha, is covered by primary rainforest ("terra firme" forest), secondary forest originated from abandoned pastures, and some areas with annual crops and agroforestry systems.

The soils are acidic (pH 3.5 - 5.0), and very poor in nutrients. The most common soil type is the clayey Ferralsol (WRB[1] classification) (Yellow Latosol: Brazilian classification). In lower frequency than the Ferralsol, the Acrisol (WRB classification) (Red Yellow Podzolic: Brazilian classification) and other sandy soils occur near the streams (Rodrigues et al. 1972).



Scale: 1:500.000

Figure 2.1: Satellite image of study area (Source: Embrapa 2002).

[1] World Reference Base for Soil Resources

The climate is humid tropical, classified as Af-type (A = mean temperature of all months over 18°C; f = monthly precipitation over 60 mm), according to Köppen´s classification (Heyer 1984). The rainy season occurs from October to May with an average annual rainfall of 2672 ± 294 mm and a dry season (precipitation in 1-3 months below 100 mm) from July to September. The air temperature varies between 22 and 34 °C (mean = 28 ºC) and soil temperature ranges from 28 to 33 ºC (mean = 29 ºC) in the uppermost 20 cm layer. The relative air humidity ranges from 80 to 92% (mean = 89%) (Embrapa, unpublished data, see Appendices 1 and 2).

Laboratory, semi-field and field tests were done at the Embrapa station. In addition, laboratory tests were performed at ECT Oekotoxikologie GmbH located in Flörsheim (Germany).

2.2 Test substrates

In this study, different (artificial and natural) soils had to be identified for the performance of laboratory tests. In the following, the selection process and methods used for their characterization are described before details of the properties of the four selected soils are presented.

The preparation of the main test substrate for terrestrial studies, artificial soil, is based on an international guideline published by OECD (1984a). Originally, this guideline was written focusing on the situation in countries of temperate regions. In these countries, the components of artificial soil are easily to obtain. However, in order to allow its use in other regions of the world where the components are not readily available, some modifications are necessary. In particular, the original organic component peat moss proposed in the standard OECD guideline, had to be replaced by a material easier to find in tropical regions. In this study, the materials to be tested as a source of organic matter (OM) in the artificial substrate were Xaxim fiber extracted from a tree fern (*Dicksonia sellowiana* (Presl.) Hook), coir dust from coconut peel (*Cocos nucifera* L.) and Sphagnum moss (*Sphagnum sp*.). The final aim of this work was the development of a tropical artificial soil (TAS). In order to facilitate the extrapolation of test results to field conditions, two natural field soils were selected as test substrates in addition to the OECD artificial soil and the tropical artificial soil:

- The standard field soil LUFA 2.2 due to its wide acceptance in Europe;
- A natural field soil of the Amazon region.

The soil substrates will be herein referred to as OECD soil (the artificial soil standardized by OECD), TAS soil (the modified tropical artificial soil), LUFA soil (the standard natural soil LUFA 2.2 coming from Germany) and TNS soil (the tropical natural soil selected for this study).

The artificial and natural soil substrates were characterized by the following parameters:

- The soil water content was determined based on dry matter according to the guideline ISO-11465 (ISO 1993b). Samples of 10 g were dried at 105 ºC in a drying oven for at least three hours. The water content was calculated by the difference in mass before and after the drying process.
- The soil acidity (pH value) was measured by the addition of 25 ml of 0.01mol/L $CaCl_2$ solution to 10 g of air dried soil, according to the guideline ISO-10390 (ISO 1994). Samples were shaken for five minutes and after at least one hour were measured using a pH-meter.
- The maximum water holding capacity (WHC_{max}), i.e., the amount of water kept by a soil against the gravity force, was determined according to Annex C of the guideline ISO-11268-2 (ISO 1998a). Its determination consists in the saturation of the soil sample with water during three hours before the water is allowed to flow out for two hours. After drying the sample at 105 ºC the WHC_{max} is calculated based on the percentage of water held by the soil.
- The particle size distribution was determined according to the guideline ISO-11277 (ISO 1998b). It was determined by a combination of the sieving and sedimentation method. The particles not passing a 2 mm aperture sieve were separated by dry sieving; particles passing such sieve but retained by a 0.063 mm aperture sieve were separated by wet sieving; while the particles passing the latter sieve were determined by sedimentation.

2.2.1 Temperate artificial soil

The artificial standard soil was prepared according to OECD guideline 207 (OECD 1984a). It has been widely used in ecotoxicological experiments, mainly for earthworms but also for other soil fauna species. It consists of the following components: fine sand, white clay and a source of organic matter (Table 2.1).

Table 2.1: Components of artificial soil (OECD).

Soil component	Content % dry mass
Peat moss (air dried), finely ground	10
Kaolinite clay (air dried, containing not less than 30 % of kaolinite)	20
Industrial quartz sand (air dried), predominantly fine sand with more than 50 % by mass of particle size 0.05-0.2 mm (amount dependent on calcium carbonate required)	70
Calcium carbonate ($CaCO_3$, pulverized, analytical grade) to obtain an initial pH of 6.0 ± 0.5	0.3 -1.0

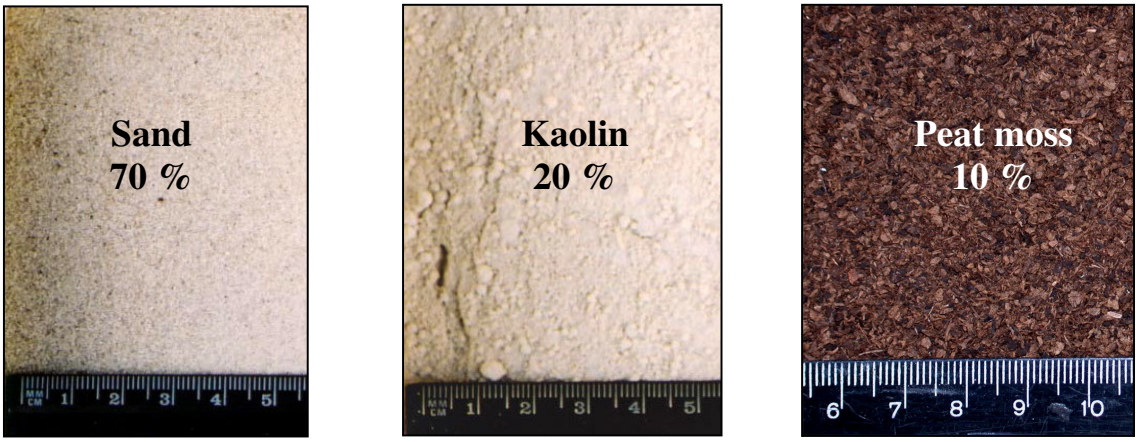


Figure 2.2: Components of standard artificial soil (OECD).

Figure 2.3: Standard artificial soil (OECD), mixed and wetted.

The dry components of artificial soil (Figure 2.2), weighed in the correct proportions, were mixed thoroughly in an electric cement mixer (capacity: 30 kg). After mixing (Figure 2.3), samples were taken for pH measurement and more calcium carbonate was added if the pH value was below 5.5. In a two-step procedure, water was added to achieve a moisture content of about 35% dry weight.

2.2.2 Temperate natural soil

The natural field soil LUFA 2.2 was selected by a working group consisting of governmental institutions and industry in order to find a soil being representative for Central European crop sites already in 1973 (Schinkel 1985). Such a soil was needed for testing the environmental fate of pesticides (e.g., degradation or mobility; Brodsky et al. 1997), as part of the registration process in Germany and the European Union. Later, this soil became popular also for the performance of ecotoxicological tests with invertebrates (most notably in the EU project SECOFASE; Løkke and Van Gestel 1998). In this project, both earthworms and isopods were tested with this soil. It is also recommended in the guideline ISO-15799 (ISO 1999a) as a control soil for testing the quality of soils. For these reasons, it was used in this project.

The standard soil LUFA 2.2 is commercially provided by the German governmental institution Landwirtschaftliche Untersuchungs- und Forschungsanstalt in Speyer. It is an uncontaminated soil, classified as “loamy sand” according to its texture (Table 2.4). Its chemical properties and some physical characteristics were determined (Table 2.2).

2.2.3 Tropical artificial soil

The “tropical artificial soil” (TAS) developed and used here is based closely on the OECD artificial soil (i.e., it consists of the same five components), but has been modified as follows: The original peat moss of the standard soil substrate (OECD) was replaced by triturated tree fern (Xaxim). The Xaxim is a material obtained from the “trunk,” (in fact a root) of a tree fern (*Dicksonia sellowiana* (Presl.) Hook), occurring in the South of Brazil and usually found in local garden stores (Figure 2.4). Before use, it was dried at room temperature and finely ground (Figure 2.5). For the sand component, the OECD guideline recommends a grain size composition of 50% of particles having a size between 0.05-0.2 mm. Due to the difficulty in obtaining this standard size grain, the

sand used in TAS soil was sieved in the laboratory and a maximum of 25% of particles between 0.05-0.2 mm was obtained.

Besides "Xaxim" two other materials feasible as a substitute for peat moss were tested:

"Coir dust": Coir is the name given to the fibrous material that constitutes the thick mesocarp (middle layer) of the coconut fruit (Meerow 1994) (Figure 2.4). Coconut peels extracted from green fruits were air dried and finely ground (Figure 2.5). Before use in soil substrates, the resulting coconut powder was wetted and stored for a complete composting process for at least 30 days. After the fermentation activity ceased, the material was air dried and sieved.

"Sphagnum": A non-decomposed and pure Sphagnum moss (Figure 2.4), usually found in garden stores of Brazil and used for growing orchids. The material was ground after drying it at room temperature (Figure 2.5).

Figure 2.4: Sources of organic matter for tropical artificial soil. Left to right: Coir, Sphagnum and Xaxim.

The artificial soil substrates which contained different organic matter sources will be herein referred to as TASx (with xaxim), TASc (with coir dust) and TASs (with sphagnum).

The usual peat moss (as recommended by OECD), and also the alternative organic matter sources, Xaxim, Coir dust and Sphagnum were chemically analyzed (Table 2.3). The new artificial substrates were analyzed with regard to their chemical and physical properties (Table 2.2).

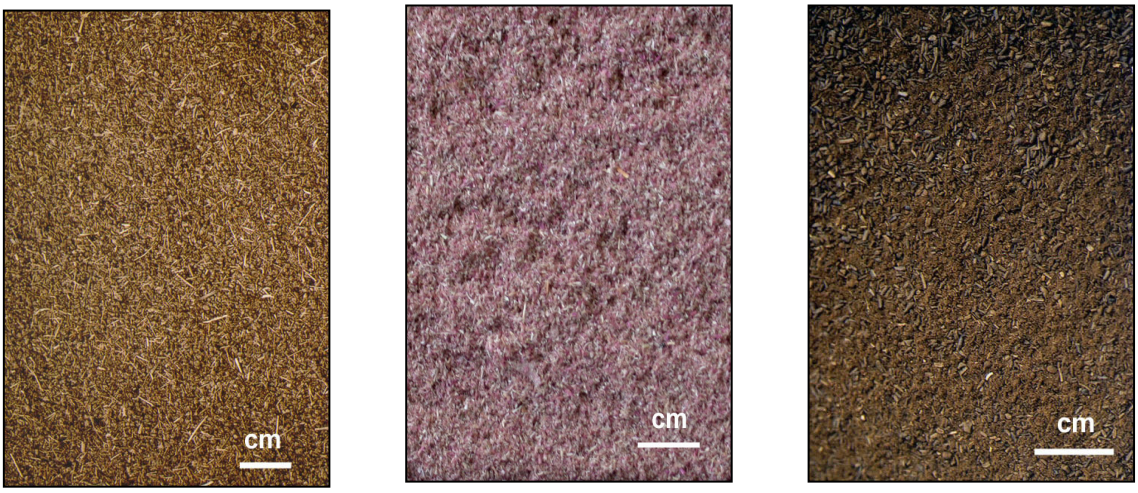


Figure 2.5: Source of organic matter for tropical artificial soil (after milling). Left to right: Coir, Sphagnum and Xaxim.

2.2.4 Tropical natural soil

In order to facilitate extrapolation of laboratory test results to field conditions, tests were performed in a natural field soil of the Amazon region. Two major soils are common in central Amazonia: the Ferralsol (Yellow Latosol) and the Acrisol (Red Yellow Podzolic). The Ferralsol has a clayey texture, whereas the Acrisol has a sandy-loam to sand texture. The Acrisol soil was selected as standard natural substrate for toxicity tests, because it is very widespread locally and its mineral composition - lower in clay in comparison to the Ferralsol - makes it easier to handle in the laboratory. Besides, this soil is often found in Manaus on the market for seedling production and garden construction.

The natural soil was collected from a site near Manaus, located near the Tarumã River and covered by natural secondary vegetation (Figure 2.6). The uppermost 25 cm of the "A" horizon were taken, while the litter layer, consisting only of organic material, was discarded. Before using the soil in the tests, it was sieved (5 mm) and air dried at room temperature. Free from any kind of contamination, it is classified as "sandy clay loam" according to its particle composition (Table 2.4). Chemical and physical analyses for natural soils are presented in Table 2.2

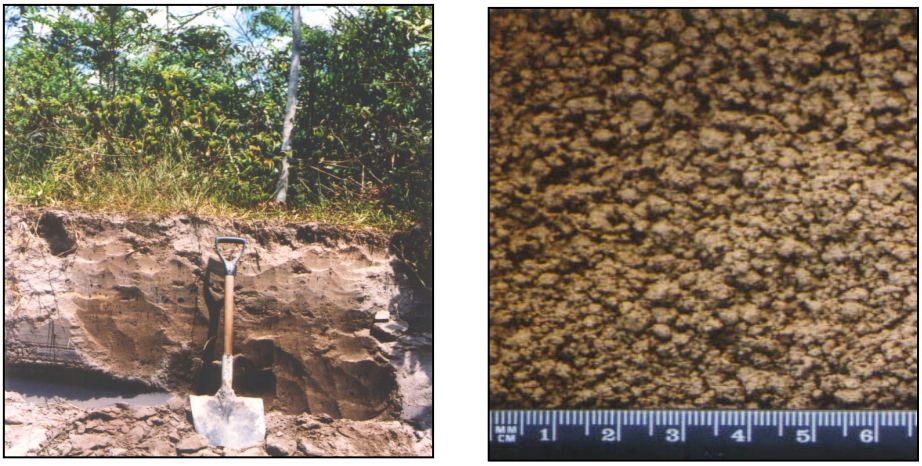


Figure 2.6: Tropical natural soil: In the field (left), wetted and homogenized in laboratory (right).

Table 2.2: Physico-chemical characterization of artificial and natural soils.

Characteristic	**Unit**	**TASc**	**TASx**	**TASs**	**OECD**	**European Natural Soil**	**Tropical Natural Soil**
pH ($CaCl_2$)		6.3	6.6	6.4	6.1	6.1	3.9
P	mg/dm^3	35	12	11	1	324	6
K		660	340	106	10	49	4
Na		160	90	67	64	22	4
Ca	c.molc /dm^3	2.46	8.40	4.11	6.29	10.54	0.06
Mg		0.56	0.0	0.0	0.55	0.05	0.04
N total	%	0.07	0.15	0.06	0.11	0.19	0.13
C org		3.59	3.48	1.49	3.59	2.70	2.49
Organic Matter		6.17	5.99	2.56	6.17	4.64	4.28
CN Ratio		51.3	23.2	24.8	32.6	14.2	19.2
Fe	mg/dm^3	8	24	13	9	288	179
Zn		2.54	0.98	2.07	1.30	31.9	1.00
Mn		0.58	2.49	2.38	0.41	84	1.68
Cu		0.74	0.41	0.50	0.02	8.52	0.45
Density	g/cm^3	1.0	1.1	0.9	1.1	1.2	13
WHCmax	%	48.5	47.7	74.5	56.1	50.0	40.1

Table 2.3: Chemical characterization of organic matter sources used in artificial soils.

Characteristic	Unit	Xaxim	Coir Dust	Sphagnum	Peat Moss
pH ($CaCl_2$)	g/kg	3.43	6.21	3.26	2.77
P		0.69	1.05	0.51	0.19
K		3.5	8.5	2.0	0.1
Ca		0.85	1.95	1.87	1.13
Mg		0.25	1.08	0.49	0.94
S		1.21	0.68	0.61	1.97
B	mg/kg	4.50	15.5	3.15	6.6
Cu		10.0	9.0	2.00	2.0
Fe		1908	189	437	514
Mn		85.0	10.0	141	9.0
Zn		7.0	14.0	12.0	34.0
Ash content	%	2.82	2.95	23.35	1.92
C_{total}		44.4	43.6	36.4	47.2
N_{total}		1.14	0.8	0.63	0.72
CN Ratio		39.0	54.3	57.5	65.7

Table 2.4: Textural characterization of natural soils.

	Clay (%)	Silt (%)	Sand (%)	Soil type
	Ø < 0.002 mm	Ø 0.002 - 0.02 mm	Ø 0.02 - 2.0 mm	
European Natural Soil (LUFA 2.2)	8.1	7.4	84.5	Loamy Sand
Tropical Natural Soil (Acrisol)	29.1	0.73	70.2	Sandy Clay Loam

2.3 Test organisms

2.3.1 Criteria and general selection

According to Edwards (1989), Edwards et al. (1996a) and Römbke et al. (1996), some criteria should be considered when organisms are selected for ecotoxicological tests. For a tropical species, the most important of these criteria are:

- It should play an important ecological role (here: in the soil compartment);
- It should have a wide range of distribution in tropical areas;
- It should live in intensive contact with the substrate to ensure exposition to chemicals;

- It should have a high reproduction rate in a short period of time, to allow keeping the animals in mass cultures.

Applying these criteria, in a first step the following species, common in the Amazon region, were selected:

- *Trigoniulus corallinus* (Gervais, 1847) (Diplopoda, Pachybolidae);
- *Circoniscus ornatus* (Verhoeff, 1941) (Isopoda, Scleropactidae);
- *Porcellionides pruinosus* (Brandt, 1833) (Isopoda, Porcellionidae);
- *Pontoscolex corethrurus* (Müller, 1857) (Oligochaeta, Glossoscolecidae);
- *Eisenia fetida* (Savigny, 1826) (Oligochaeta, Lumbricidae).

In addition, the pantropical earthworm *Eudrilus eugeniae* (Kinberg, 1866) (Oligochaeta, Eudrilidae), coming originally from West Africa, was used. Except for the earthworm *E. fetida* and the isopod *P. pruinosus*, the other species did not completely fulfill the selection criteria (in particular they are very difficult to breed in laboratory mass cultures). Nevertheless, in this project they were used for non-standard ecotoxicity tests and as soil fauna components in microcosms (TME) experiments. Only the earthworm *E. eugeniae* was skipped altogether, since the experience with this species in some pre-standard tests led to the conclusion that it is unsuitable for single-species testing as well as for being kept in model ecosystems due to its tendency to escape most kind of vessels (Knieriemen 1985). In addition, *E. eugeniae* is relatively sensitive against low oxygen levels and high population densities. These experiences are in accordance with the results of studies on the suitability of various earthworm species for vermicomposting performed mainly in South Africa (Reinecke et al. 1992): The authors concluded that *E. fetida* is preferable to *E. eugeniae*, since the handling of the latter species is more difficult.

2.3.2 Earthworms (Oligochaeta)

Eisenia fetida* and *E. andrei

The compost earthworm *Eisenia fetida*, probably originating from the Caucasus, is nowadays separated into two distinct species, *E. fetida* and *E. andrei* Bouché, 1972 (Bouché 1992). Both species (*E. fetida* or *E. andrei*) are listed as potential test organisms for temperate (OECD 1984a; 2003) as well as for tropical conditions (IBAMA 1990). It must be highlighted that the compost worm, most notably known as

a species from temperate regions, has “invaded” many tropical soils following European settlements (Barois et al. 1999).

These *Eisenia* species are dispersed over many tropical and temperate countries, where they are commercially used as organic waste decomposer for the production of compost (Mitchell 1997). Both species are often found growing together in mixed cultures. Ecologically, both species are classified as epigeics, i.e., the worms live on the soil surface as litter-dwelling species and they are restricted to habitats rich in organic matter (Bouché 1972).

Growth rates in both species are similar, but there are some differences regarding their reproductive strategy: *E. andrei* has a higher cocoon production, more hatchlings per cocoons and attains maturity earlier than *E. fetida*. However, these differences do not indicate any significant ecological distinctions (Reinecke and Viljoen 1991).

In this work, mass cultures of a “tropical variant” of *E. fetida* (Figure 2.7) were established in both laboratories (Embrapa in Brazil and ECT GmbH in Germany) from samples provided by local earthworm breeders in the area of Manaus. In the Embrapa laboratory, the cultures were kept in plastic boxes (25 x 36 cm area, 12 cm height) (Figure 2.7) filled with a mixture of tropical artificial soil (TASx) with cattle manure (70:30, v/v) at room temperature (23 to 30 ºC; mean = 26 ºC), relative air humidity near 90%. The animals were maintained in a shaded place with a natural light cycle (12h light /12h dark). In the ECT laboratory the cultures of tropical *Eisenia* were kept in a chamber at 28 ± 2 ºC in the dark. The breeding substrate was artificial soil with a pH-value set to 6 ± 0.5. The moisture was adjusted to 50 ± 10% dry weight using deionized water. The earthworms were fed according to demand, usually once a week, with finely ground cattle manure free of any chemical contamination.

In addition, mass cultures of the species *E. fetida* (now called the European variant”), originally delivered by a local worm breeder (Co. Landenberger, D-72355 Schömberg), have been kept at the ECT laboratory since 1994. The earthworms were bred in a mixture of OECD artificial soil and food (1 - 30 vol. %) at room temperature of 15 - 30 °C (mean: 22 °C) and in permanent darkness. The food was mainly dried cow dung free of contamination, but from time to time finely ground corn meal, stinging nettle and bran powder were added. Feeding was done on demand, usually once a week.

Due to its properties (easy to breed, well accepted as a test organism), the tropical variant of *E. fetida* was used as a standard test organism for the ecotoxicological tests in this project.

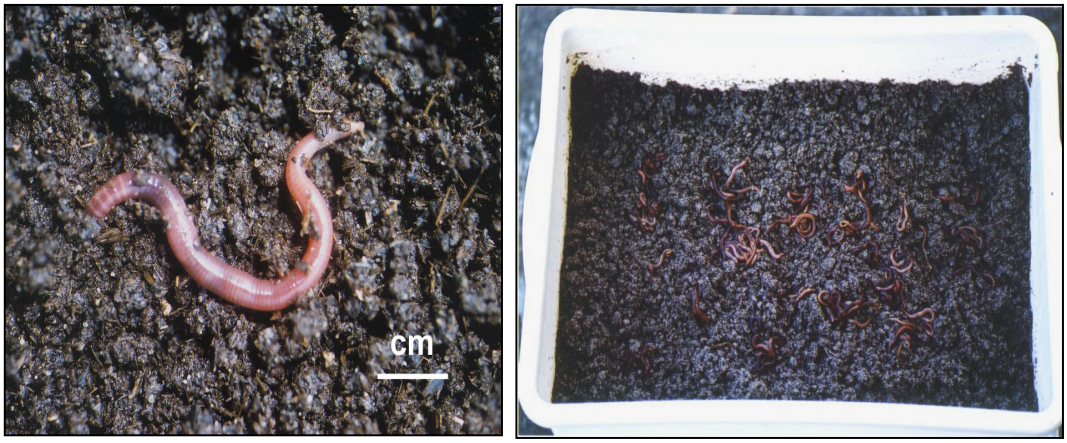


Figure 2.7: *Eisenia fetida*: Individual (left) and a culture box (right)

The tropical earthworm *Pontoscolex corethrurus*

The peregrine species *Pontoscolex corethrurus* (Figure 2.8) is widely distributed in tropical regions, where it is very common in anthropogenic soils (Römbke and Verhaagh 1992). Probably it originates from the Guyana plateau (Righi 1984). *P. corethrurus* is abundant in the Amazon region; it is mainly found in fallows, pastures and cultivated areas but rarely in secondary forests, while it is literally absent in primary forests. It has an endogeic ecological strategy, i.e., lives in the soil, usually in the upper 20 cm layer. *P. corethrurus* is very tolerant to different types of soil and changes of their physico-chemical as well as climatic factors like temperature and moisture. Adults collected in the field can easily produce cocoons in the laboratory when kept in natural soil mixed with cattle manure at a rate of 2 % (Bernardes and Kiehl 1997). According to Hamoui (1991), at a temperature between 20 and 23 ºC, the development period from hatching to sexual maturation seems to be long (14 - 19 months). However, higher temperatures and moisture rates can cause an increase in the growth rate of juveniles (Lavelle et al.1987). Although the culture techniques for *P. corethrurus* in the laboratory are poorly known, this species was considered a suitable tropical species for acute tests and microcosm studies, due to its ecological importance and dominance in tropical regions. For ecotoxicological experiments, the individuals were not reared in the laboratory but collected in their natural habitat and acclimatized in the test substrates

(TAS) at 28 ºC for at least 24 hours before the test. No mass culture could be established due to the slow development of these animals.

Figure 2.8: *Pontoscolex corethrurus* on tropical natural soil (TNS)

2.3.3 Woodlice (Isopoda)

The cosmopolite *Porcellionides pruinosus*

The terrestrial isopod *Porcellionides pruinosus*, originating from the Mediterranean region, has been carried unintentionally elsewhere by humans, colonizing temperate and tropical regions (Leistikow and Wägele 1999). Except in the Mediterranean region, this species is not found in natural biotopes, but as a synanthropic species it is frequently associated to human habitations, living in compost heaps and animal dung mounds. Due to large geographical variations, *P. pruinosus* is considered to be a complex of species. In some places, the presence of distinct species was observed (Garthwaite and Sassaman 1985); however, no detailed taxonomical review is available.

In Manaus, central Amazonia, *P. pruinosus* is often found under sheltered deposits of compost, cattle and chicken dung. Generally, the animals are found in dense populations in abandoned chicken houses, living on old chicken dung, probably because of the high amount of calcium available in this substrate. It seems that the distribution of the isopods depends on the availability of calcium in the environment. The cuticle of isopods contains large amounts of $CaCO_3$ as the main mineral component (Greenaway 1985). *P. pruinosus* (cited as *Methoponorthus*) stores calcium carbonate and resorbs it during the formation of the new cuticle (Ziegler and Miller 1997).

Cultures of *P. pruinosus* (Figure 2.9) were established in both laboratories taking into account the preference of these animals for a moist but not too wet substrate. Colonies grew quickly in plastic boxes (25 x 36 cm area, 12 cm height) (Figure 2.9) containing a layer of humus (from earthworm cultures), and were fed with leaves of Kudzu (*Pueraria phaseoloides* (Roxb.) Benth., in Manaus) or sycamore maple leaves (*Acer pseudoplatanus* L.; in Floersheim) and fish food (e.g., Tetramin). Besides the high practicability concerning the production of mass cultures, *P. pruinosus* has the habit of entering holes and crevices of the soil, thus having an intensive contact with the substrate. This habit makes it particularly suitable for toxicity tests in soil (Souza et al. 2000; Vink et al. 1995). Therefore, *P. pruinosus* was used as a standard organism for ecotoxicological tests in this project.

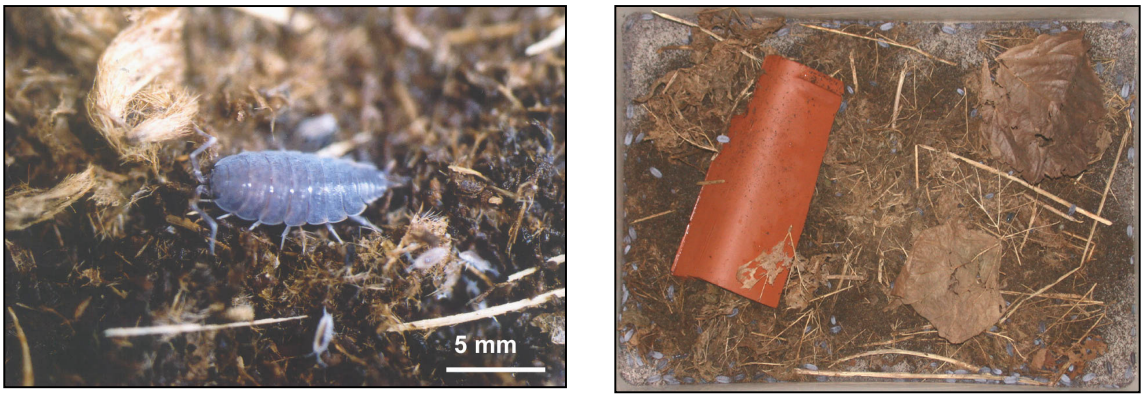


Figure 2.9: *Porcellionides pruinosus*: Individual (left) and a box culture (right).

The native isopod species *Circoniscus ornatus*

The species *Circoniscus ornatus* (Figure 2.10) is commonly found in anthropogenic areas near Manaus, often occurring associated with residues of Arecaceae species, such as in the litter of coconut (*Cocos nucifera* L.) and peach palm (*Bactris gasipaes* Kunth) plantations and in residues of fruit bunches of oil palm (*Elaeis guineensis* Jacq.). Attempts to start mass cultures of *C. ornatus* were made in the laboratory but despite the fact that the juveniles were apparently active, feeding intensively on leaves, their growth was very slow. For this reason, the species was not selected for mass cultures, but stock cultures were established in the Embrapa laboratory. Plastic boxes (25 x 36 cm area, 12 cm height) (Figure 2.11) containing a layer of humus were used and the animals were fed with leaves of Kudzu and fish food. For ecotoxicological experiments,

the individuals were taken from stock cultures and were acclimatized in the substrates (TAS) at 28 ºC for at least 24 hours before starting the test.

2.3.4 Millipedes (Diplopoda)

The pantropical millipede *Trigoniulus corallinus* (Figure 2.10), probably coming from Southeast Asia, has been introduced by man into Africa, Central and South America, and tropical islands throughout the world (Shelley and Lehtinen 1999). In the region of Manaus, it is often found in anthropogenic areas, dwelling in compost heaps and cow dung deposits on farms and in the gardens of urban areas. To establish cultures of this species, attempts were made at Embrapa, using as food source leaves of the leguminous Kudzu (*Pueraria phaseoloides*) and the peach palm (*Bactris gasipaes*). During four months, the animals survived eating both types of leaves but they did not reproduce. Considering that the life cycle of *T. corallinus* might be too long, this species was not selected for breeding in the laboratory as a test species. Stock cultures were established in the laboratory in plastic boxes (25 x 36 cm area, 12 cm height) (Figure 2.11) containing a layer of humus, and the animals were fed with leaves of Kudzu and fish food. For the ecotoxicological experiments, the individuals were taken from stock cultures and were acclimatized in the substrates (TAS) at 28 ºC for at least 24 hours before starting the test.

Figure 2.10: Individuals of *Circoniscus ornatus* (left) and *Trigoniulus corallinus* (right).

Figure 2.11: Box cultures of *Circoniscus ornatus* (left) and *Trigoniulus corallinus* (right).

2.4 Test performance: Single species tests in the laboratory

To assess the risk of pesticides to soil it is necessary to extrapolate laboratory toxicity data from single species tests (e.g., earthworm tests) to predict effects under field conditions. These data are usually obtained from acute and chronic toxicity tests. The acute test, in which the toxic endpoint is mortality, indicates the maximum response of an organism. In the chronic test, the chemical is applied in low concentrations (sublethal) and it is considered more sensitive to predict the environmental effects. The range of concentrations of these tests was based on the results of preliminary tests.

All laboratory experiments were carried out in close agreement with GLP (Good Laboratory Practice) requirements (OECD 1998).

2.4.1 Preliminary tests (Range Finding)

Considering that the tests were carried out for the first time with tropical species, and in different conditions (higher temperature) than those usually recommended, the toxicity of the test substances for the organisms was not known. Therefore, a preliminary or range-finding test (RF test) was performed for each combination of test species, test substrate and test chemical in order to identify the concentrations causing mortality. In this way, the range of concentrations for the definitive test was determined. The test design and performance did not vary according to the test organism.

In these range-finding tests, the organisms were exposed to the following concentrations: 1000; 100; 10; 1; 0.1 mg of the chemical substance per kilogram of test substrate (dry weight). Glass vessels (12.5 cm diameter, 16 cm height) with perforated plastic lids were used (Figure 2.12). One test vessel was used per concentration and one

for the untreated control. Usually, no replication is required in these preliminary tests. Ten individuals were put in each test vessel. After seven days, the live animals were counted and weighed, the dead ones removed. The final evaluation was done on day 14, when the live animals were again counted and the mortality in percent of the initial number was calculated. Except of the missing replication, the test performance and evaluation are the same as those described for the definitive tests (Section 2.4.2). The LC_{50} values were calculated for each test, thus allowing the definition of the concentrations to be used in the definitive acute tests. The final concentrations for acute tests were chosen in a way that two concentrations above and two concentrations below the LC_{50} as determined in the RF test were chosen. When the preliminary tests did not show mortality at all or the LC_{50} was equal or greater than 1000 mg /kg, the acute test was performed as a limit test, i.e., using an untreated control and a treatment with 1000 mg/kg (each with four replicates). According to existing guidelines (e.g., OECD 1984a), no substance must be tested at concentrations higher than 1000 mg/kg. For some cases, it was not necessary to perform the RF test, since the toxicity data were available in literature. For chronic tests, no specific range-finding test was performed but the appropriate range of concentrations was determined using the sublethal concentrations from the acute test.

2.4.2 Acute toxicity tests

Earthworms

The test system for the determination of the acute toxicity of chemicals to earthworms was based on the guidelines OECD n^{o}. 207 (OECD 1984a) and ISO-11268-1 (ISO 1993a). These international guidelines describe a method for determining the acute toxicity of substances to *Eisenia fetida* by dermal and alimentary uptake using an artificial substrate. Tests with the German variant of *E. fetida* were done directly according to these guidelines at ECT, while the tests performed with the “tropical” variant at Embrapa were modified by using different test substrates and a higher temperature. Usually, only adult earthworms (with clitellum) with a fresh weight between 300 and 600 mg were used (Figure 2.12). When necessary, some adult individuals with a slightly lower biomass (minimum of 250 mg) were taken. The selected test animals were

acclimatized in untreated soil substrate at least 24 hours prior to the start of the test. Tests were performed with benomyl, carbendazim and lambda-cyhalothrin.

The concentrations of the test substance were calculated using the dry weight of the artificial soil. The test substance was mixed in different concentrations in the soil substrate and was applied only once at the beginning of the test. The test period (exposure of earthworms to the soil substrate with test substance) was 14 days unless delayed effects were observed during this period of time. Test parameters (7 and 14 days after application) were mortality, biomass development and effects on the morphology and behavior of the earthworms. The relative loss in biomass, i.e., changes in weight of test animals between first and the fourteenth day, was calculated only in treatments where the mortality was lower than 15% (according to Kula 1998). For treatments where a mortality rate higher than 15% occurred, the biomass data at the fourteenth day are as not applicable (n.a.) in tables and graphs.

Test substrates and test conditions

Tests were performed with two artificial substrates (the standard OECD soil and the modified tropical artificial soil - TASx) and two natural soils (LUFA soil and the tropical natural soil - TNS). The test vessels were kept in a chamber at 28 ± 2 ºC or 20 ± 2 ºC in the dark and with relative humidity near 90%. (The OECD guideline recommends performing the test in the light; however this factor does not influence the test results).

Experimental procedure

The acute toxicity tests were done with five or six concentrations ranging from 0.2 to 1000 mg a.i./kg according to the chemical and the substrate used in the tests. The treatments, including one untreated control, were set in four replicates containing 10 earthworms per vessel.

The following procedures, described in chronological sequence, were applied in both laboratories:

- Earthworms were selected out of the breeding culture and acclimatized for at least 24 h in untreated soil substrate (wetted with deionized water to obtain a moisture of 35 ± 5% dw), at a controlled temperature (28 ± 2ºC or 20 ± 2 ºC), at a level which would be used later in the test.

- The substrate used in the test was pre-moistened with deionized water with 40-50% of the required moisture.
- Glass vessels (12.5 cm diameter, 16 cm height) were filled with 500g (dw) of the substrate (Figure 2.12).
- The preparation of the test solutions was done for each concentration by weighing and dissolving an amount of the chemical formulation in deionized water. In cases where the amount of the chemical to be weighed was very small, a stock solution was prepared and used to prepare the different test substance concentrations through a series of dilutions. Each concentration was dissolved in such a way that in each replicate an amount of 100 ml of aqueous solution could be mixed in the substrate of the test containers. When applying the 100 ml of the chemical solution, the final soil moisture was adjusted to 35 ± 5% dw.
- Using a small mixer, the substrate was completely mixed with the chemical solution until the test substance was homogeneously distributed.
- After the chemical solution was mixed in the substrate, samples for determination of pH and moisture were taken in one replicate per treatment according to the respective guidelines ISO-11465 (ISO 1993b) and ISO-10390 (ISO 1994).
- The acclimatized earthworms were individually weighed prior to the test and put into groups of ten. These groups were organized in weight order, i.e., from the heaviest group to the lightest. These were separated into sets of six, taking one of each set as a replicate. Consequently, within the replicate, the worm groups were assigned to the treatment randomly. Afterwards the animals were put onto the soil surface of each test container.
- On day 7, the earthworms were sampled out of the test containers. During this process, morphological and behavioral changes and the number of surviving earthworms in each test vessel were recorded. Individuals were classified as dead when they did not respond to a mechanical stimulus to the anterior part of body. Surviving earthworms were weighed (all earthworms of one vessel together). Afterwards the earthworms were put back onto the soil surface in the containers.

- On day 14, the earthworms were removed from the test containers. During this process morphological and behavioral changes and the number of surviving earthworms in each test vessel were recorded. Surviving earthworms were weighed separately.
- At the end of the test, samples for the determination of pH and moisture were taken in one replicate per treatment again.

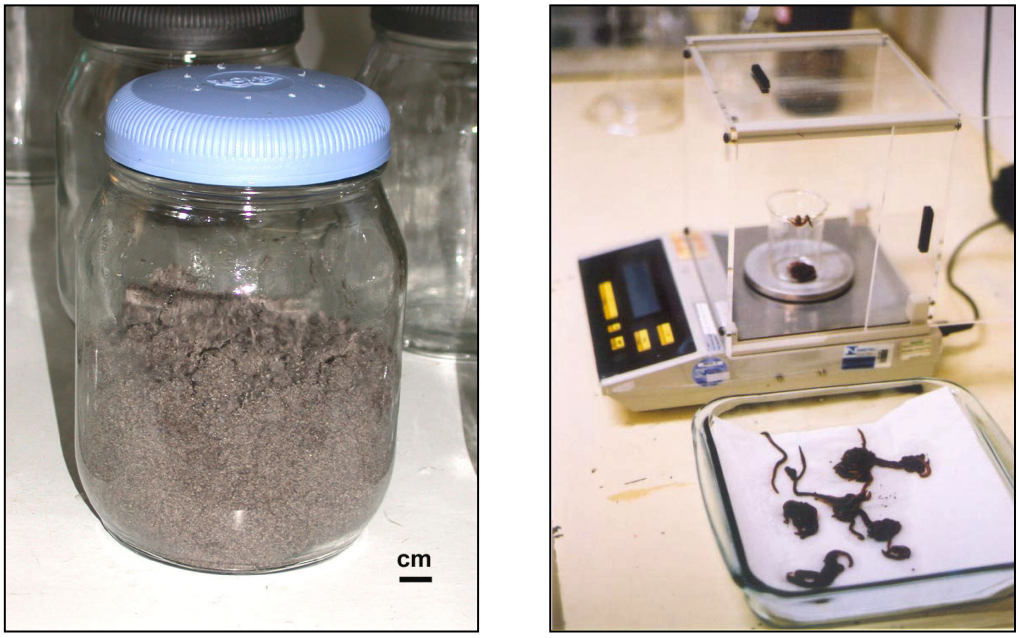


Figure 2.12: Test vessel (left) and earthworm weighing (right).

Test validity

According to the test protocol the results should be checked for some criteria in order to be considered valid. The OECD guideline n°. 207 (OECD 1984a) recommends that the mortality in the control should be ≤ 10%. In the guideline ISO-11268-1 (ISO 1993a), it is suggested that in addition the biomass loss should not exceed 20%. Currently, mortality is the most accepted criterion whereas the biomass is still under discussion (Kula 1998).

Isopods

Standard test protocols are not available for the toxicity of chemicals to isopods. Hornung et al. (1998a) developed draft test protocols and Standard Operating Procedures (SOP) for culturing the European isopod *Porcellio scaber*. In this study, the

procedures used for acute tests on the isopod *Porcellionides pruinosus* were based on the methods available for *P. scaber*. Tests were performed with benomyl, carbendazim and lambda-cyhalothrin.

Test substrates and test conditions

Tests were performed with two artificial substrates (standard OECD soil and modified tropical artificial soil - TASx) and two natural soils (LUFA soil and tropical natural soil - TNS). The test vessels were kept in a chamber at 28 ± 2 ºC in the dark and with a relative air humidity near 90%.

Experimental procedure

According to the results obtained in the RF tests, the acute toxicity tests were done in five or six concentrations ranging from 0.003 to 3.16 mg a.i./kg (1000 mg a.i./kg for limit tests) according to the chemical and the substrate used in the tests. The treatments, including one untreated control, were set in four replicates containing 10 individuals per vessel. Only adult isopods with a fresh weight between 20 and 30 mg were used and no difference was made between male and female or the reproductive stage of females. The exposure was via contact in soil and lasted 14 days. The final moisture of the test substrates was adjusted to 25 ± 5% dw for TASx, OECD and LUFA soil and 20 ± 5% dw for TNS soil.

The following procedures, described in chronological sequence, were applied in both laboratories:

- Isopods were selected from the breeding culture and acclimatized for at least 24 h in an untreated soil substrate (wetted with deionized water to achieve a moisture content of 25 ± 5% dw) at a controlled temperature (28 ± 2ºC), on the level to be used in the test.
- The substrate used in the test was pre-moistened with deionized water with 40-50 % of the required moisture, and the final moisture was reached after the application of the chemical solution.
- The preparation of the test solutions was done for each concentration by weighing and dissolving an amount of chemical formulation in deionized water. In cases where the amount of chemical to be weighed was very small, a stock

solution was prepared and used to prepare the different test substance concentrations.

- Using a small mixer, the substrate was completely mixed with the chemical solution until the test substance was homogeneously distributed.
- Plastic vessels (area 11 cm x 15.5 cm, height 6 cm) (Figure 2.13) were filled with 250g (dw) of the treated substrate.
- After the chemical solution was mixed in the substrate, samples for the determination of pH and moisture were taken in one replicate per treatment according to the respective guidelines ISO-10390 (ISO 1994) and ISO-11465 (ISO 1993b).
- Prior to the test, the acclimatized isopods were weighed together in groups of ten. Afterwards, the animals were put onto the soil surface of each test container. Test containers were closed using perforated and transparent lids.
- On day 7, the isopods were removed from the test containers. During this process, the number of surviving individuals in each test vessel was recorded. Individuals were classified as dead when they did not move or respond to a mechanical stimulus. Surviving isopods were weighed (all individuals of one vessel together). Afterwards, they were put back onto the soil surface in the containers.
- On day 14, the isopods were removed from the test containers. During this process the number of surviving individuals in each test vessel was recorded. Surviving isopods were weighed together for each test vessel.
- At the end of the test, samples for the determination of the pH-value and the moisture were taken again in one replicate per treatment.

Figure 2.13: Test vessel with isopods (*Porcellionides pruinosus*).

Validity of the test

There is no standard guideline for toxicological tests using *P. pruinosus*. Thus, the criterion used in the test proposed with *Porcellio scaber* (Hornung et al. 1998a) was adopted, i.e., less than 20% of mortality in the control.

Native species

Acute toxicity tests were performed with the following native species:

- Earthworm (*Pontoscolex corethrurus*)
- Isopod (*Circoniscus ornatus*)
- Millipede (*Trigoniulus corallinus*)

The tests using *P. corethrurus* and *T. corallinus* were done according to the experimental procedure described above for *E. fetida*, while the test with *C. ornatus* followed the procedures used for *P. pruinosus* (see above). The main differences were:

- The organisms did not come from standard cultures but were collected in the field and acclimatized in the laboratory for few days prior to the test.
- Only two chemicals (carbendazim and lambda-cyhalothrin) were used and one type of artificial soil (TASx), tested at 28 ºC.

The soil was dosed in five concentrations ranging from 0.032 to 1000 mg a.i./kg according to the chemical, species and substrate used in the tests. The treatments, including one untreated control, were set in four replicates containing 10 individuals per

vessel. The range of weight used for each species in the tests was previously defined based on the natural variation (standard deviation of mean) found in adults sampled in the field.

2.4.3 Chronic toxicity tests

Earthworms

The test system for the determination of the chronic toxicity of chemicals to earthworms was based on the guidelines OECD n°. 222 (OECD 2003) and ISO-11268-2 (ISO 1998a). These international guidelines describe a method for determining the chronic toxicity of substances to *Eisenia fetida* by dermal and alimentary uptake using an artificial substrate. Tests with the German variant of *E. fetida* were done according to these guidelines at ECT, while the tests performed with the "tropical" variant at Embrapa were modified by using different test substrates and a higher temperature. Usually, only adult earthworms (with clitellum) with a fresh weight between 300 and 600 mg were used. When necessary, some adult individuals with a slightly lower biomass (minimum of 250 mg) were taken. Usually, the earthworms for reproduction tests came from synchronized cultures, i.e., with individuals having a homogenous age and physiological stage (developed clitellum). The selected test animals were acclimatized in an untreated soil substrate at least 24 hours prior to the start of the test.

The test substance was mixed in different concentrations in the soil substrate and was applied only once at the beginning of the test. The test period (exposure of earthworms to the soil substrate with test substance) was 56 days. After 28 days, the adult worms, which had been exposed to the test chemical, were removed from the test vessels. During the following 28 days, juveniles hatched from cocoons laid by the adults. Test parameters were mortality and biomass development of the adults 28 days after application and the number of juveniles 56 days after application.

Test substrates and test conditions

Tests were performed with two artificial substrates (standard OECD soil and modified tropical artificial soil, TASx) and two field soils (European natural soil (LUFA) and tropical natural soil, TNS). The test vessels were kept in chambers at 28 ± 2 ºC or 20 ± 2 ºC in the dark and at a relative air humidity near 90%.

Experimental procedure

The concentrations of the test substance were calculated using the dry weight of the artificial soil. Five or six concentrations ranging from 0.1 to 100 mg a.i./kg were selected according to the chemical and the substrate used in the tests. The treatments, including one untreated control were set in four replicates containing 10 earthworms per vessel.

The following procedures, described in a chronological sequence, were applied in both laboratories (Embrapa and ECT GmbH):

- One week before the test, approximately 500 adult worms were selected out of the synchronized cultures. The worms were acclimatized for 7 days in an untreated test substrate mixed with air-dried cow manure as food source.
- One day before the test, the artificial soil (500 g per replicate) was moistened to a preliminary water content of 30 ± 10% dw. The dried food material (5 g per 500 g dry weight) was mixed into the artificial soil.
- Start of test: Preparation of the test item solutions. Sufficient test item for all vessels was dissolved in deionized water to prepare a stock solution. This stock solution was diluted with deionized water to obtain one test item solution (= dosage solution) for each test item concentration. Each concentration was dissolved in such a way that in each replicate vessel 100 ml aqueous solution could be mixed into the artificial soil.
- When applying the test item solution the final required soil moisture (50 ± 10% dry weight) was achieved; 100 ml test solution was added to each vessel. The artificial soil was mixed thoroughly until the solution was homogeneously distributed.
- Artificial soil was filled into the test containers (750 g wet weight per replicate (corresponding to nominal 500 g dry weight). The height of the soil column in

the containers was 5 - 6 cm. Moisture and pH of the artificial soil were determined once at each concentration.

- The worms were taken out of the acclimatization substrate and weighed individually. Sets of 10 worms were placed into different vessels. The vessels with worms were then arranged in weight order, i.e., from the heaviest to the lightest group. These were separated into sets of 6 (= treatments), using one set as a replicate. Within the replicate, the worm groups were assigned to the treatment levels randomly. Afterwards, the animals were put onto the soil surface of each test container. The test containers were closed using perforated lids.
- One day after the start of the test. Feeding: 5 g dry food material was spread on the soil surface of each test container. The food was moistened with 5 ml deionized water per test container. The amount of food was adjusted in order to minimize the risk of moulding.
- One week after the start of the tests. Moistening: determination of the weight of the test containers. Water loss was compensated with deionized water. Feeding: the feeding activity was recorded for each test container.
- Two and three weeks after the start of the tests: Moistening and feeding as described above.
- Four weeks after the start of the tests: The adult worms were taken out of the test containers. During this process, morphological and behavioral changes and the number of surviving worms in each test vessel were recorded. Individual weighing of the surviving worms. Mixing in of dry food material (5g per test vessel separately) into the artificial soil. Afterwards the artificial soil (including cocoons) was returned into the test containers. The test containers were closed again. For another 4 weeks they were kept under the same test conditions as described above.
- Five, six and seven weeks after the start of the tests: Moistening as described above.
- Eight weeks after the start of the tests: The juvenile worms were sampled out of the test containers. Extraction was done using the water bath method (50 - 60 °C water temperature) (Figure 2.14), followed by hand-sorting in all replicates per

treatment to control the extraction efficiency. During this process morphological and behavioral changes and the number of juveniles in each test vessel were recorded.

- Determination of pH and soil moisture for each treatment separately at the end of the test.

Figure 2.14: Extraction of juveniles of *E. fetida* in a water bath

Test validity

According to the guideline n°. 222 (OECD 2003), the following criteria should be satisfied in the controls for a test result to be considered valid:

- Each replicate (containing 10 adults) should have at least 30 juveniles by the end of the test.
- Coefficient of variation (CV) of reproduction should be ≤ 30 %.
- Adult mortality over the initial 4 weeks of the test should be ≤ 10 %.

Isopods

Twenty-four hours prior to performing chronic toxicity, tests truly gravid females of the species *Porcellionides pruinosus* were selected (Figure 2.15). This was partly done for practicability reasons: The number of non-gravid females and males in the cultures is usually much smaller than the number of true gravid females. Thus a sufficient number of non-gravid females and males was not available for testing. Another reason for this approach was that apparently females of *P. pruinosus* are able to store male sperm for at

least four broods (Vink and Kurniawati 1996). Thus, the fertilization phase of the reproduction cycle does not usually occur during the test period.

Testing was carried out in 750 ml Bellaplast vessels with pierced transparent plastic lids for ventilation, containing 150 g dw soil. The amount of soil was reduced compared to acute testing in order to facilitate the very labour-consuming hand-sorting procedure for counting the juveniles. Four replicates were used for each concentration. Moisture was adjusted to about 30% of soil dw for OECD artificial soil and about 28% for LUFA 2.2 standard soil. Ten individuals were used per test vessel, and animals had an average weight of 20 - 30 mg per individual. Test duration was 14 days under continuous dark conditions at 28°C. Moisture was checked and adjusted to the initial amount at day 7. The animals were fed with 250 mg TetraMin at day 0 and 7 as the only food source. Based on the experience from preliminary feeding tests, TetraMin proved to be essential for reproduction success, including the provision of food for the hatchlings. The sum-weight of the adults was recorded at the beginning of the test. Sets of 10 adults each were grouped by weight and thus evenly assigned to the individual replicates.

The sum-weight of adults, and mortality and number of juveniles was determined at day 14, the latter by hand-sorting.

Concentrations tested were:

- 0; 0.01; 0.032; 0.1; 0.32; 1.0 mg a.i./kg for lambda-cyhalothrin
- 0; 10; 31.6; 100; 316; 1000 mg a.i./kg for benomyl and carbendazim

Chemicals were dissolved in deionized water and mixed into the soil. The soil was afterwards distributed to the four replicate vessels.

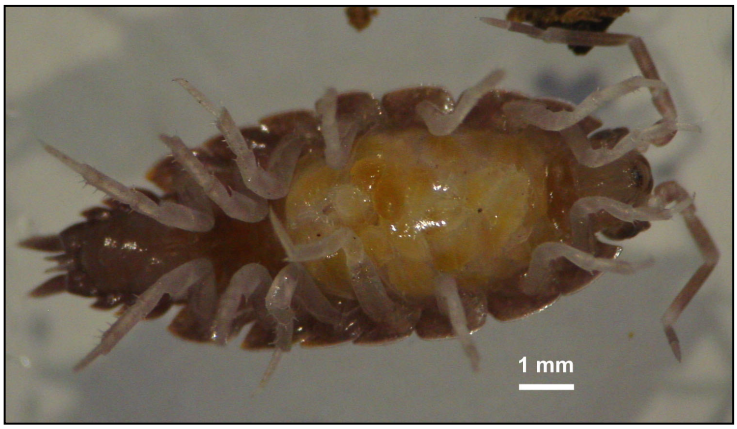


Figure 2.15: Female (gravid) of *Porcellionides pruinosus*.

2.4.4 Avoidance test with earthworms

The habitat function of soils is often assessed using different earthworm tests with either acute or chronic endpoints in order to obtain information on environmental effects. However, the disadvantage of these tests is that they are labour-intensive and time-consuming. For example, for the determination of the number of juveniles in the ecologically relevant and often very sensitive earthworm reproduction test, a long incubation period of 56 days is needed. Even the acute earthworm test using the mortality parameter requires 14 days - but this relatively short duration is countered by an often low sensitivity. The test duration of both tests is long enough to delay the risk assessment decisions and the corrective actions to address an environmental contamination. Therefore, there is a high need for a short and easy-to-perform screening test.

Since earthworms have many chemoreceptors in their body wall (especially in the anterior segments of the body) they show a high sensitivity to chemicals in their environment (Edwards and Bohlen 1996). This sensitivity, coupled with their locomotory abilities, enables them to avoid contaminated areas (Stephenson, et al. 1998). Therefore, the avoidance test, an alternative for the rapid toxicity assessment based on the behavioral responses of earthworms, has been proposed by Yeardley et al. (1996), Slimak (1997), Stephenson et al. (1998), Hund-Rinke and Wiechering (2001) and Hund-Rinke et al. (2003). The principle of this test is that the earthworms in one test vial are simultaneously exposed to the soil sample to be evaluated and to a control soil. After a short test period of a few days the location of the animals is determined.

In the present study, avoidance tests were performed with *Eisenia fetida* using the pesticides benomyl, carbendazim and lambda-cyhalothrin at different concentrations in artificial soils (OECD soil and TASx) and one natural soil (LUFA soil). The tropical natural soil (TNS) was not suitable for the avoidance test due to the high mortality rate of earthworms. Tests were performed in 7 concentrations (ranging from 1 to 1000 mg a.i./kg) for benomyl and carbendazim and in 6 concentrations (ranging from 0.32 to 100 mg a.i./kg) for lambda-cyhalothrin. Each treatment consisted of 4 replicates. Plastic vessels (11 x 15.5 cm area, 6 cm height) were filled with soil up to a height of about 4 to 5 cm (about 500g soil, dry weight). Using a piece of plastic fitted transversally in the vessel, one half of the vessel was filled with contaminated soil, the other filled with control soil, without chemical. Then the plastic separator was removed and 10 adults of *Eisenia fetida* (weight: 250 - 600 mg) were placed on the separating line of each test vessel (Figure 2.16). Afterwards, the vessels were closed with transparent and perforated lids. A scheme of the avoidance test procedure is presented in Figure 2.17.

Before the test vessels were transferred to the test chambers, they were exposed to the light in the laboratory until all earthworms had entered in the substrate. The tests were carried out at 20 ± 2 ºC for OECD and LUFA soils, and at 28 ± 2 ºC for tropical artificial soil (TASx). The vessels were kept in the dark in order to avoid lateral effects of light in the vessels. The animals were not fed during the test. Soil moisture and pH were determined according the ISO guidelines described in Section 2.2. The test lasted 48 h. At the end of the test period, the control and the contaminated soil sections were carefully separated and the number of earthworms was determined for both sections of the vessels. Individuals found between the sections (on the separating line) were counted according to the direction they were moving, i.e., considered in the section where the anterior part of body was. Very toxic substances or soils containing highly available chemicals might cause mortality. This kind of effect goes beyond the scope of an avoidance test. To consider this effect in the assessment, dead earthworms were classified as escaped animals. A random distribution of the earthworms in the test containers (i.e., individuals did not display any congregation behavior) is a prerequisite for a valid avoidance test. To determine if worms were distributing themselves at random, dual control tests were performed using uncontaminated soil in both sections of the test container in 4 replicates. Thus, in such control tests, the avoidance behavior was

considered absent when the proportion of earthworms between the two sections of the test vessel was not significantly different from 0.50.

Computation of avoidance response

For each replicate, the net response (NR) (expressed as percentage) was calculated as following:

$$NR = ((C - T) / 10) \times 100$$

where: C = sum of earthworms observed in control soil
T = sum of earthworms observed in treated soil
10 = total number of earthworms per replicate

A positive (+) net response indicates avoidance of, and a negative net response (-) indicates a non-response (or attraction) to the chemical tested in given concentration.

For the test results, the value of EC_{50} (including 95%-confidence limits) was calculated and graphics created showing the values of the mean net avoidance response.

Figure 2.16: Avoidance test: Vessel filled with control and treated soil (left) and earthworms distributed on the soil surface (right).

2.4.5 Non-standardized tests

Acute test in factorial design

Aiming to verify a possible difference in sensitivity to chemicals between European and tropical *Eisenia* under different soils and temperatures, tests following a factorial design were applied. For these experiments, a fractional factorial test design was used, which allows for the determination of the effects of single factors as well as synergistic and

antagonistic effects (Morgan 1991). In preliminary tests, a factorial design, without replicates, was used. The factors, species, temperature and soil types, were selected. Each factor was assessed at two levels. Evaluation was carried out with an ANOVA ($P < 0.01$). Based on the preliminary results, a second experiment using a full factorial design (with replicates) was performed as follows:

- For each chemical, carbendazim and lambda-cyhalothrin was applied in a 2 x 2 factorial design (two factors at 2 levels each), in three replicates and TASx as substrate (Table 2.5).
- The factors were: earthworm species (tropical and European *Eisenia*) and temperature (20 ºC and 28 ºC).
- Controls and treatments with carbendazim (10 and 1000 mg a.i./kg) and lambda-cyhalothrin (10 and 50 mg a.i./kg).
- Mortality and the weight development (% of the initial weight) were measured.
- With exception of the number of replicates, the experimental procedures were the same applied in the standard acute test with earthworms (Section 2.4.2).

Table 2.5: Description of factors (two levels each) used in the experiment.

Factors		**Chemical**					
Origin	**Temperature**	**Carbendazim**			**Lambda-cyhalothrin**		
European *Eisenia*	20 ºC	Control	10 mg a.i./kg	1000 mg a.i./kg	Control	10 mg a.i./kg	50 mg a.i./kg
	28 ºC						
Tropical *Eisenia*	20 ºC						
	28 ºC						

Tests using TASc (coir dust) and TASs (sphagnum) artificial soil

In order to test the feasibility to use the TASc or TASs soil as a tropical artificial substrate for toxicity tests, acute tests with tropical *E. fetida* were performed for carbendazim and lambda-cyhalothrin, in both substrates. With exception of the number of replicates (3 instead of 4), the experimental procedures were the same applied in the standard acute test with earthworms (Section 2.4.2).

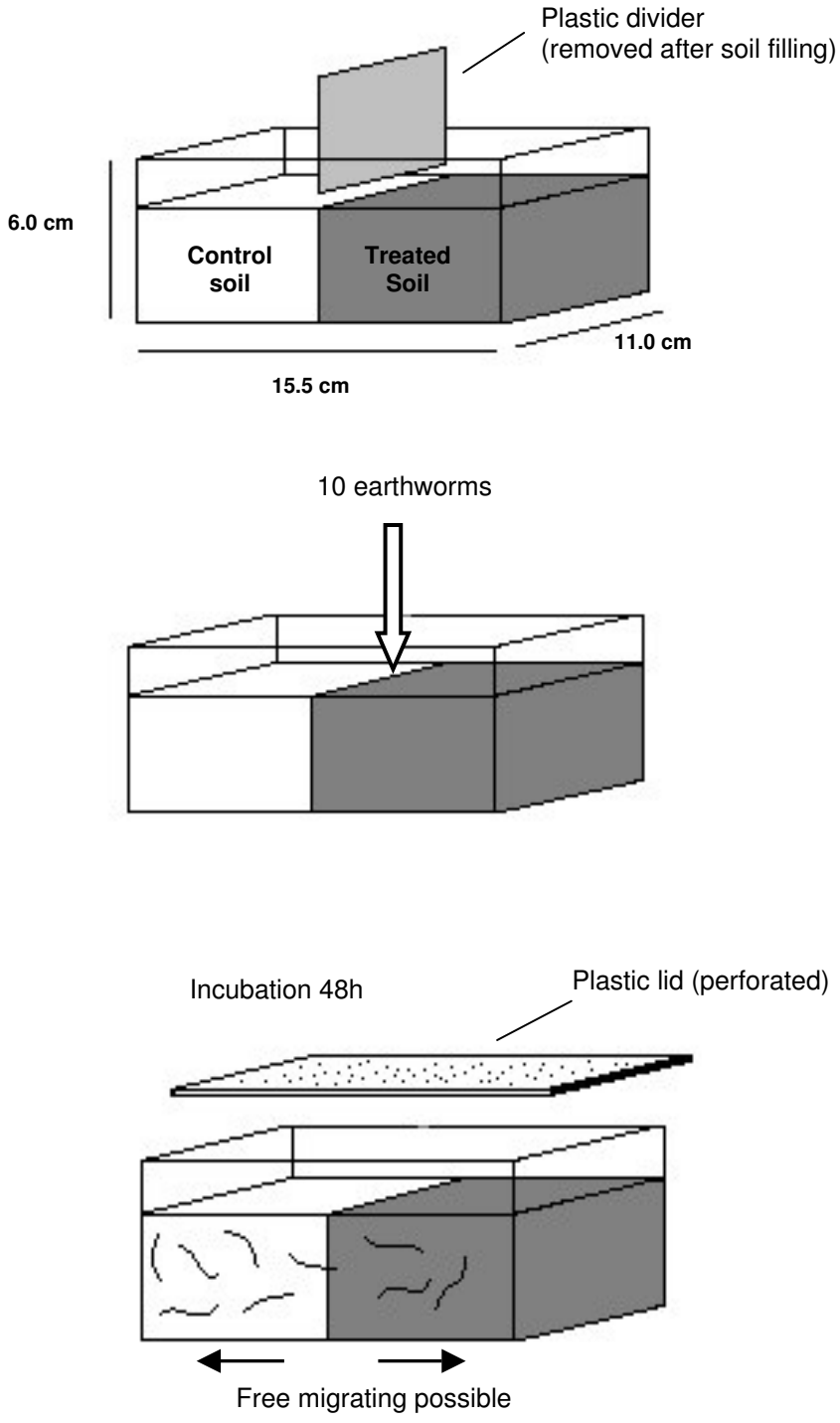


Figure 2.17: Experimental procedure for avoidance test (after Hund-Rinke and Wiechering 2001, slightly modified).

2.5 Test performance: Higher-tier tests

2.5.1 Functional tests on different levels

Bait-lamina test

One relatively new approach for the use of functional methods in soil ecology is the bait-lamina test in which the feeding activity of a variety of soil animals is measured (Von Törne 1990; Kratz 1998). In principle, the loss of artificial or natural organic material (bait) exposed to a variety of soil animals (e.g., earthworms, Collembola, Enchytraeidae) in the holes of small plastic strips that are inserted into the soil is measured (Pfeif et al. 1996). The number of empty holes (i.e., from which the bait material was removed) as well as their vertical distribution is evaluated, thus allowing the use of the feeding activity of soil animals as an additional functional parameter for the assessment of the biological status of soils. The important advantages of the method are simplicity and short exposure periods (some days to a few weeks). Additionally, nearly no training, special skills or equipment are necessary. In comparison to the measurement of other functional parameters like litter decomposition, the bait-lamina method practically does not disturb the soil structure. Since environmental conditions like climate or soil moisture can strongly influence the results (Kratz and Pieper 1999), the method should only be applied for comparing the biological activity between closely related plots.

Bait-lamina consist of plastic strips 120 mm*6 mm*1 mm, which have a pointed tip at the lower end. In the lower part (85 mm) of each strip, 16 holes of 1.5 mm diameter are drilled, which are 5 mm apart from each other (Figure 2.18). They are filled with bait material (a mixture of cellulose (70 %) and bran (25 %) powder together with 5% of activated carbon). This mixture has been used successfully in many studies performed in temperate regions (e.g., Kratz 1998). The strips were exposed in a way that the uppermost hole was just beneath the soil surface (Figure 2.19). Measurement parameters were the total amount of fed holes and the vertical distribution of the feeding activity. At the end of the exposure period (in temperate regions between 10 and 20 days; in Amazonia 4 days as determined in a pre-test), bait-lamina were retrieved from the soil and visually assessed (the strips were held against the light and holes fed upon were counted in a yes/no manner). The feeding rate is measured as number of “fed” holes (i.e., holes from which the organic bait material was removed).

Despite regular use in temperate regions, so far bait-lamina tests have been performed in the tropics only twice (Geissen et al. 2001 and Römbke et al. 2004a). In the present study, this technique was used in the semi-field experiments (TME study; Section 2.5.2, Figure 2.23F) and in the field experimental plots of the litterbag study (Section 2.5.3).

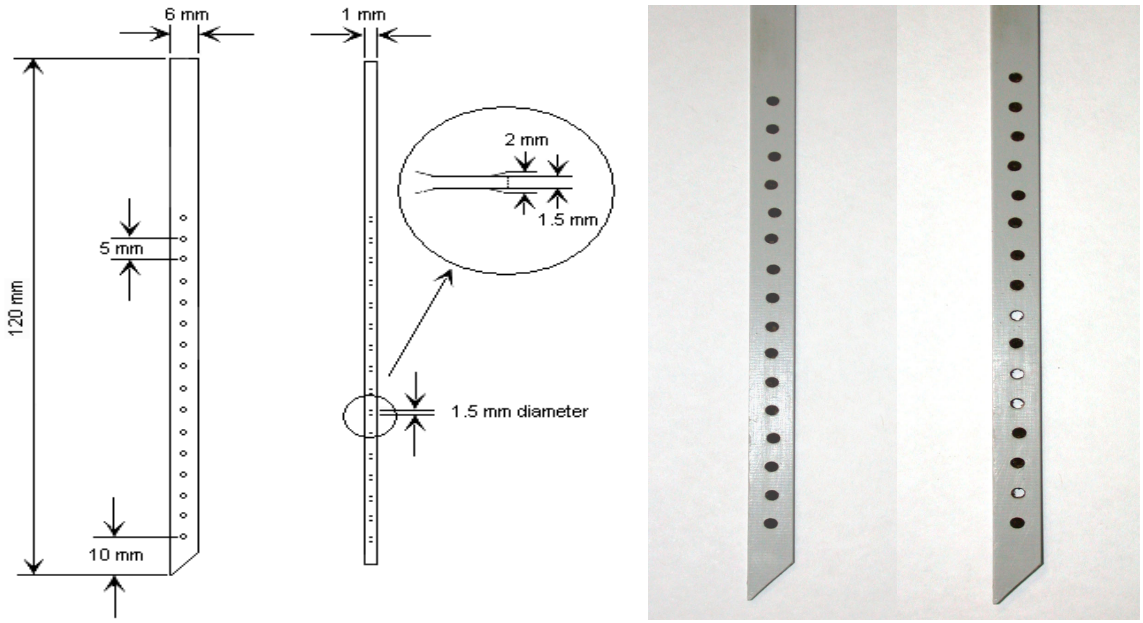


Figure 2.18: Bait-lamina test system (according to Kratz 1998).

Figure 2.19: Bait-lamina sticks exposed in the field.

Soil respiration

Soil microorganisms play an important role in the decomposition of organic matter and in the cycling of nutrients (Swift et al. 1979). In the evaluation of toxicity of crop protection products to the environment, determination of effects on soil microbial activity is required when exposure of soil microorganisms to chemicals is expected (OECD 2000a,b).

The potential effects of the pesticides carbendazim and lambda-cyhalothrin on soil microbial activity were measured in semi-field (microcosms, TME) and field (litterbags) experiments.

The method for estimating soil respiration consisted of measuring carbon dioxide production over time in soil samples in the laboratory under controlled conditions. The ECT Respiration Device (Förster and Farias 2000), a soil respiration system based on an Infra-Red Gas Analyzer - IRGA, was used. The respiration of soil samples was determined by measuring the carbon dioxide production over time in a continuous flow through the system at a constant flow rate of 300 ml fresh air per minute.

A portable computerized photosynthesis measuring system HCM-1000 (Heinz Walz GmbH, Effeltrich, Germany) was used. The central unit of the system consists of an infra-red gas analyzer (IRGA), a peristaltic air pump and a mass flow meter, this is connected to a measuring chamber (cuvette). It works in an open flow mode (differential mode) measuring the difference between the CO_2 concentration of the ambient air before and after passing the cuvette. The system is controlled via a computer. To measure soil respiration, the central unit was connected to a specially designed rag containing 17 cuvettes. Each cuvette was connected via tubing and solenoid valves to the central unit. A special software allowed switching the soil or litter containing cuvettes alternately to the IRGA. One blank chamber was left without sample and with difference between the CO_2 passed in this chamber (CO_2-containing air) and the CO_2 in the chamber containing the sample, the soil respiration was calculated.

The CO_2 production was measured in two stages:

- Basal respiration, during 6 hours, conducted at natural soil moisture and without adding glucose.

- Substrate-induced respiration (SIR), during at least 12 hours, at optimal soil moisture, adding glucose.

The basal and SIR were sequentially measured in the same soil sample. After the basal respiration measurements were completed (6 hours), the soil sample was taken from the chamber and a mixture of glucose and talcum (24 : 50, w/w) was added. If the sample had lost water (checked by weight difference), deionized water was added to achieve optimal soil moisture. The sample was put back into the measuring chamber for another 12 hours to determine the substrate-induced respiration.

Microbial respiration was calculated according to:

$$Respiration\ [nl\ CO_2\ min^{-1}\ g^{-1}\ soil] = (C*F) / S$$

where: C = IRGA measured CO_2 - value [ppm]
F = Flow rate through cuvette [ml/min]
S = Soil dry weight [g]

2.5.2 Semi-field tests: Terrestrial Model Ecosystems (TME)

Terrestrial Model Ecosystems (TMEs) are defined as undisturbed soil columns taken from the field and managed in the laboratory in order to assess the impacts of chemicals on biological structures and processes in the terrestrial environment (Morgan and Knacker 1994; Sheppard 1997). Since the data obtained in TMEs are in good agreement with field data from corresponding sites, they have been suggested as a reliable test system to predict effects of chemicals on soil ecosystems (Weyers et al. 2004). In recent years, the number of ecotoxicological studies using TMEs has increased, mainly under temperate conditions (Morgan and Knacker 1994; Förster et al. 2004).

Considering that no experience was available for the use of TMEs for toxicity tests in tropical soils, in the present study the experiments were performed in three sets:

- A preliminary test without pesticides, aiming to gain some experience with regard to the sampling of soil cores in the field and their management in the laboratory, the selection of soil organisms and litter to be used in the test.
- An experiment to assess the effects of the chemicals carbendazim and lambda-cyhalothrin using intact soil cores.

- An experiment to assess the effects of the chemicals carbendazim and lambda-cyhalothrin using homogenized soil.

Experimental design

The preliminary experiment consisted of a 2 x 3 factorial design with 8 replicates, comprising 48 TME units in total. The TMEs were collected in the field and cleared of its native litter layer, leaving them only with soil. Thereafter, in the laboratory, the soil cores were placed on moveable carts and the soil surface was covered with 10 g of leaf litter of either *Hevea pauciflora* (Spruce ex Benth.) Müll. Arg., *Flemingia macrophylla* (Willd.) Merr. or *Brachiaria decumbens* Stapf. During the first month, the TMEs were acclimatized, without adding the soil fauna, and kept wetted by applying 20 ml of water every two days. At the second month, in each TME, soil organisms were inserted, i.e., either *E. fetida* and *P. pruinosus* ("laboratory fauna") or *P. corethrurus* and *C. ornatus* ("field fauna"), with 5 individuals of each species. In addition, *T. corallinus* was added (also 5 individuals per TME), because it is considered to belong to laboratory as well as field fauna, since only one species of diplopod was available. The moisture was maintained by applying 20 ml water per TME three times a week. The experiment lasted 4 months. Every 30 days, the survival of animals in the TME units was checked and dead ones were replaced. In cases where the litter had been highly consumed, new material (10 g) was added. The air temperature in the room was recorded every 30 minutes with automatic temperature logger. Luminosity ranged from 150 to 300 lux in a light / dark cycle of 12h /12h. About 10 days before the end of the test, 4 bait-lamina sticks were put into each TME. The material used in the bait-laminas consisted of cellulose (60%), agar (20%) and powder of *Pueraria* leaves (20%). After 7 to 10 days, the sticks were taken out for the evaluation of the soil fauna feeding activity. At the end of the test period, the remaining litter was carefully removed from the soil surface and weighted for mass loss calculation. Each soil core was then sorted for soil macrofauna, first in the upper 5 cm and later in the remaining deeper soil layers.

The second experiment was performed using two chemicals (carbendazim and lambda-cyhalothrin). For each chemical, a 2 x 2 factorial design (two factors at two levels each) with 4 replicates was applied, consisting of 24 TME units, including the control. The TMEs were sampled in the field, managed as in the preliminary

experiment, and treated with 10 g (fw) of two litter species (*Flemingia* and *Hevea*) and two concentrations (high and low) of each chemical. The high / low concentrations were 4 mg / 0.4 mg a.i. of lambda-cyhalothrin and 100 mg / 10 mg a.i. of carbendazim per TME, which means 180 mg / 18 mg a.i. and 4510 mg / 451 mg a.i. per square meter, respectively. The application of chemicals was as follows:

- Application of both chemicals (high and low treatments) at the start of the test
- Application of both chemicals (low treatments) every month.

The chemicals were sprayed over the litter layer. After 24 h, five individuals of native soil fauna (*Trigoniulus*, *Circoniscus* and *Pontoscolex*) were added per TME. One day before the monthly application, the TMEs were checked for survival of animals and the dead ones replaced. In cases where the litter had been highly consumed, new material (10 g) was added. The experiment lasted for 5 months. The TMEs were kept moist and the temperature in the room was recorded as in the preliminary experiment. At the end of the test period, the experiment was evaluated regarding litter decomposition rate, soil respiration and microbial biomass in the upper 5 cm layer and for the mortality of macrofauna.

The aim of the third experiment was to test the chemicals carbendazim and lambda-cyhalothrin in TME with homogenized soil. For each chemical, a 2^4 factorial design (4 factors at 2 levels each) was used, i.e., the chemical in two concentrations, two application methods (on the soil surface and by mixing it in to the soil), two soil types (Ferralsol and Acrisol) and two litter species (*Pueraria* and *Flemingia*), comprising 48 TME units (including controls). The soils were collected in the field, sieved and sampled for pH, moisture and texture analyses. The Ferralsol was clayey in texture, whereas the Acrisol was sandy. The chemicals were applied only once at the beginning of the test. The TMEs were treated with two concentrations (high and low) of each chemical. The high / low concentrations were 0.8 mg / 0.08 mg a.i. of lambda-cyhalothrin and 200 mg / 20 mg a.i. of carbendazim per kg of soil (dw). After the soil was treated with chemicals, 10 g (fw) of each litter species (*Flemingia* and *Pueraria*) were added, according to the respective treatment. One day later, five individuals of native soil fauna (*Trigoniulus*, *Circoniscus* and *Pontoscolex*) were added per TME. The moisture was maintained by applying 20 ml water per TME, three times a week. The temperature in the room was recorded as in the preliminary experiment. The experiment

lasted for ten weeks and during the test period no animals or litter were added. About 10 days before the end of the test, four bait-lamina sticks were put into each TME. The material used in bait-laminas consisted of cellulose (60%), agar (20%) and powder of *Pueraria* leaves (20%). After 7 to 10 days, the sticks were taken out for evaluation of soil fauna feeding activity. At the end of the test period, the experiment was evaluated regarding litter decomposition rate, soil respiration and microbial biomass in the upper 5 cm layer and for the mortality of macrofauna.

Site for soil core sampling

The field site selected for soil core sampling was a rubber (*Hevea pauciflora*) plantation established 20 years ago. Before the rubber trees had been planted, this area had been a pasture since the primary forest was cut and burned in 1960. During the sampling, the soil surface between the tree lines was covered with a litter layer of rubber tree leaves. At least during the previous 10 years, this area had not received any chemical contamination or soil management.

Equipment for TMEs

The extraction of soil cores and the equipment used for TME experiments were performed according to Förster et al. (2004). A single model ecosystem consisted of: a 30-cm long high density polyethylene tube (17.5 cm diameter), a thin layer of inert gauze to fit between the drilled holes of the Buchner funnel and the bottom of the soil core, polyvinylchloride tubing to connect the funnel to a 1000 ml wide neck polyethylene bottle which acted as a collection vessel for the leachate (Figure 2.20).

Extraction of soil cores

Soil cores were extracted such as to avoid soil compaction or any other disturbance of the natural soil layering. A specially designed steel extraction tube (Figure 2.22) and a hydraulic excavator was used for extraction (Figure 2.21). The hydraulic excavator arm and steel extraction tube were positioned vertically and the arm pushed the tube slowly down into the ground (Figure 2.21). Then, the excavator was used to pull the soil core slowly out of the ground using a wire fixed on the handles of the extraction tube (Figure 2.21). Finally, the steel cap was removed from the steel extraction tube and the soil core

lifted out. The steel extraction tube with a new soil core tube was then moved on to an adjacent area. Any extracted soil core which showed compaction was discarded. At least 20% more soil cores were extracted than were strictly necessary according to the experimental design. This excess meant that soil cores that had problems could be discarded. The soil cores were extracted approximately one week before application of the two chemicals.

Installation of soil cores in the laboratory

Extracted soil cores were transported to the laboratory in an upright position while trying to avoid jarring as much as possible. In the laboratory, the soil cores were placed into moveable carts and the Buchner funnel was connected to the leachate collection bottle to which a small amount of concentrated HCl was added (Figure 2.20). During installation, each TME that appeared to have holes or cracks in the soil surface was discarded immediately. TMEs were moistened with distilled water three times a week after they had been collected and assembled. Details of the TME at the laboratory are shown in Figures 2.23, A-F.

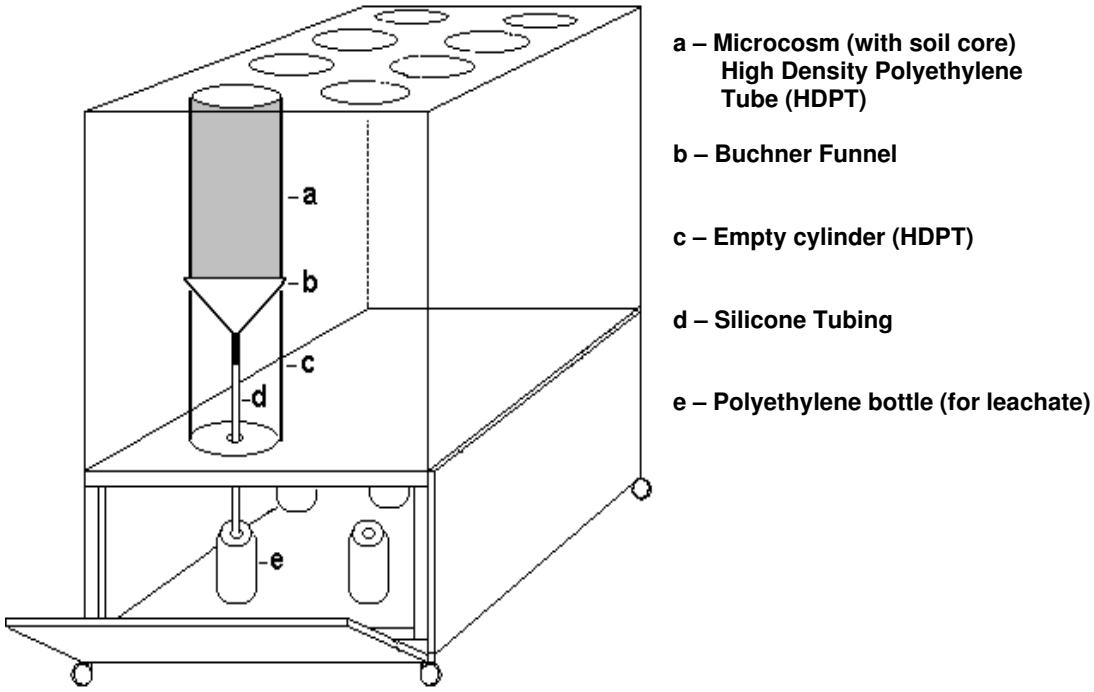


Figure 2.20: Terrestrial Model Ecosystem (TME) - Mounted in movable cart (after Knacker and Römbke 1997, slightly modified).

Figure 2.21: Terrestrial Model Ecosystem (TME) - Extraction of soil cores.

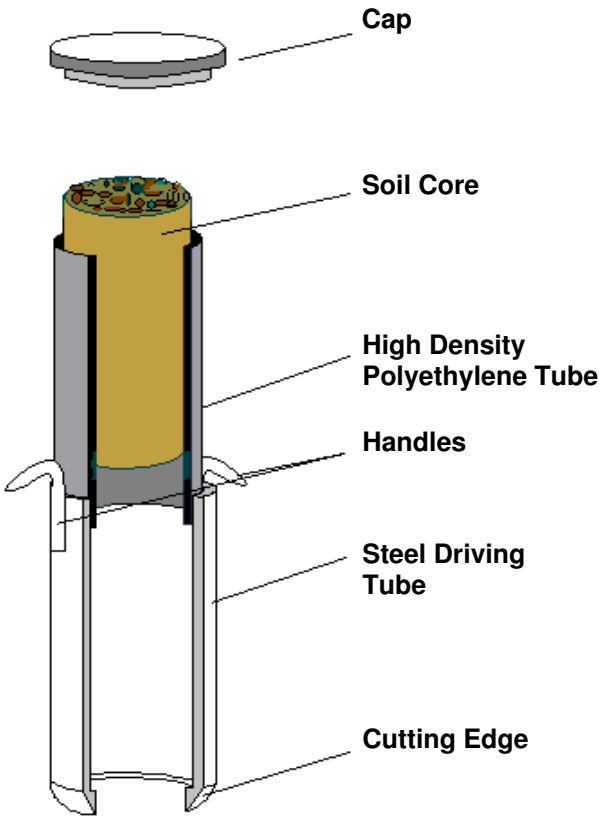


Figure 2.22: Terrestrial Model Ecosystem (TME) - Soil core extractor (after Knacker and Römbke 1997, slightly modified).

Figure 2.23: Terrestrial Model Ecosystem (TME): Sampled in field (A); set on movable carts in laboratory (B and C); front view of microcosm with litter and fauna (D); chemical application in microcosm (E); microcosm with bait-lamina sticks (F).

2.5.3 Field tests

Organic matter (OM) decomposition is a critical process in terrestrial ecosystems, and is responsible for the maintenance of soil fertility (Swift et al. 1979). Different methods to assess OM breakdown are available (Kula and Römbke 1998) but the litterbag method is usually the most appropriate (Crossley and Hoglund 1962; Heath et al. 1964; Paulus et al. 1999; Knacker et al. 2003). This test, designed to determine the effects of chemicals on the decomposition of organic matter in the field, can also be used in terrestrial model ecosystems.

The test consists of enclosed natural (e.g., dried leaves) or artificial (cellulose) organic material in litterbags, which are exposed to normal climatic conditions in the field. Depending on the palatability of the material, the activity of soil organisms (especially the macrofauna) and the influence of the climatic conditions, the enclosed organic material is decomposed within a few months or years. During the test, some samples are retrieved periodically for the evaluation of weight loss and the OM degradation rate.

Up to now, there is no standardized guideline for the litterbag test. However, an international working group (EPFES) has prepared a draft method (Römbke et al. 2003).

In this study, a litterbag experiment was conducted in the field to assess the effects of the fungicide carbendazim and the insecticide lambda-cyhalothrin on OM breakdown.

The study area was situated in the experimental Embrapa station (see description in Section 2.1) in a rubber tree (*Hevea pauciflora*) plantation. The soil is classified as Ferralsol (Yellow Latosol) with a texture composition of 81 % clay, 9 % sand; 10 % silt and pH 4.0; C_{org}: 1.0 - 2.0 %; N_{total} 0.1 - 0.2 %; organic matter 1.9 - 3.5 % (Rodrigues et al. 1972). The rubber plantation was established 15 years ago after the primary forest had been cut and burned (Figure 2.24 A). The soil surface between the tree lines was covered with the leguminous Kudzu plant (*Pueraria phaseoloides*) in order to avoid erosion.

During the first two years the plantation received fungicide application (mainly benomyl) for the control of fungal leaf diseases. Chemical fertilizers were also used during the five years after the trees were planted. After that, all agricultural

practices were suspended. Years later, the leguminous vegetation was extinguished due to the shadow produced by the rubber trees (6 to 7 years old) and consequently a litter layer was formed by the rubber tree leaves. Thus, the study site is a system that had not suffered from any chemical contamination for at least the eight preceding years. Previous evaluation of the main soil fauna groups (e.g., earthworms, millipedes and woodlice) showed that there was an active and functional soil biocenosis in the area (Hanagarth, personal comm.). Climatic conditions, including air temperature and precipitation, were recorded during the experimental period.

The organic material used in this test consisted of leaves of *Vismia guiannesis* (Aubl.) Choisy, which is a common shrub in secondary forests of this region. The *Vismia* leaves were chosen instead of the autochthonous litter (*Hevea pauciflora*) due to the availability of data about its decomposition rates at in the same study site (Höfer et al. 2000 and 2001). The leaves were obtained from a site close to the one in which the study was conducted. The litterbags were filled with dried leaves of *Vismia*, laid on the surface of the treatment plots and then exposed directly to spray applications of the chemicals. Leaves of *Vismia* trees were air dried indoors for at least 4 weeks until a constant weight was achieved. Each litterbag (10 cm x 15 cm) was filled with 10 g air-dried leaves and sealed using a sewing machine. After preparing each group of 30 weighed samples, three sub-samples were taken at random to determine the air-dry and oven-dry weight (72 h at 65°C). In addition, these leaves were used to determine the ash-free dry weight (heated at 550 °C in a muffle furnace for 4 - 5 hours).

The experiment for each chemical consisted of 4 treatments (3 concentrations and a control) with 4 replicates each. The same control plots were used for both chemicals. Two extra control plots and 4 extra litterbags per plot were used in case some of the other bags were lost (e.g., due to the activity of termites) during the study. Litterbags containing dried leaves of *Vismia* were exposed on the surface of the test plots three days before application. In total, 36 (32 + 4 reserve) litterbags were distributed randomly over each of the 5m x 5m plots (Figure 2.24 C). Therefore, the total number of litterbags was: 4 x 4 x 36 = 576 for each tested substance. The experiment lasted about one year, and every three months 8 litterbags were randomly selected in each plot, removed from the soil and transported under ambient conditions to the laboratory for analysis.

The chemical treatments were based on the recommended application rates for carbendazim (1000 g per ha) and for lambda-cyhalothrin (40 g per ha) as follows: low (recommended) concentration applied every 3 months (T1); low (recommended) concentration applied every month (T2), and 10-fold the low concentration applied once (T3). During the experimental period of one year, in the treatments T1, T2 and T3 four, eleven and one application were made, respectively. Chemical treatments were applied using a movable application device, which was equipped with the same type of nozzles as used in agricultural practice (Figure 2.24 B). During the test period, the litterbags were progressively covered by the *Hevea* leaves falling from the surrounding trees (Figure 2.24 D), becoming completely covered by the litter layer at the end of the experiment. The experimental plots were however left in natural conditions, without any kind of management.

The weight change of the organic matter in the litterbags was calculated as percent of initial ash-free dry weight at each sampling date. The decomposition rates in the plots treated with chemicals were compared to the control plots. Additionally, the time was estimated when 50 % ($T_{0.50}$) or 90 % ($T_{0.90}$) of the initial ash-free dry weight was lost. The carbon nitrogen ratio (C/N) in the remaining litter was determined in four litterbags sampled from each plot after 6 months (second sampling) and after 12 month (last sampling) of exposure.

One month after the last chemical application, the last sampling of the litterbags was performed. Thereafter, the litter layer and the soil (0-5 cm depth) of an area of 25 x 25 cm was sampled in each plot and transported to the laboratory where each sample was put on Berlese-type extractor funnels for the evaluation of soil macrofauna and mesofauna. An additional soil sample (area 25 x 25 cm, 5 cm depth) was collected in each plot for evaluation of the number and biomass of earthworms (mainly juveniles) using the hand sorting method. In order to evaluate the deep burrowing earthworm species, on every plot an area of 1 m^2 was treated with 20 L of a 0.25% formalin solution (i.e., 25 ml formalin (= 37% formaldehyde solution) in 10 L water). The earthworms that escaped from the soil within a time period of 20 min after adding the formalin solution were fixed in 70% alcohol for further determination of species and biomass.

Soil respiration was evaluated taking a mixed sample (from 3 sub-samples) collected up to 5 cm deep from each plot. Basal and induced soil respiration were measured according to the method described in Section 2.5.1. For the evaluation of the feeding activity of the soil organisms, the bait-lamina method (see description in Section 2.5.1) was used. The bait-lamina sticks were filled with a bait material consisting of 60% cellulose, 20% finely ground litter of Kudzu (*P. phaseoloides*) and 20 % agar-agar. Five sticks were distributed randomly in each plot and exposed for 8 days.

Figure 2.24: Field experiment (litterbags): A - View of experimental plots; B - Chemical application on litterbags; C and D - Litterbags at the start and at the end of the test, respectively.

2.6 Statistics

Routine statistical analyses were applied according to Zar (1984). Prior to analysis, data were tested for homogeneity of variance (Levene's test) and for normal distribution (Kolmogorov-Smirnov test). Data with non-homogeneous variances were transformed using arc-sin for surviving rates, and square root for biomass changes. If the data were still inhomogeneous after transformation, an equivalent non-parametric test was used. Significant differences were considered at $p < 0.05$ probability level. For comparison between tests, the confidence limits values of two LC_{50} (acute tests) or EC_{50} (avoidance and chronic tests) were used. Two LC_{50} or EC_{50} values were considered significantly different if their 95 % confidence limits did not overlap. The statistical methods used here are detailed in Meister and Van den Brink (2000) and Chapman et al. (1996).

2.6.1 Standard tests

Acute tests

In acute toxicity tests, the endpoint survival rate was analyzed according to the parameters: Median Lethal Concentration (LC_{50}), Lowest Observed Effect Concentration (LOEC) and Highest No Observed Effect Concentration (NOEC). For the relative loss of biomass, the parameters LOEC and NOEC were calculated taking only the treatments where the mortality was < 15% as proposed by Kula (1998).

LC_{50} estimation

Values of the lethal median concentration (LC_{50}) were determined by the Probit Analysis Method (Finney 1971). In cases where the number of intermediate responses were > 3, and the chi-square test was not significant, the Trimmed Spearman-Kärber (Hamilton et al. 1977) method was used to estimate LC_{50}. The 95%-confidence limit associated with an LC_{50} was calculated. The computer program TOXRAT® was used for Probit Analysis and to build the dose response curves. The Trimmed Spearman-Kärber program is available on the U.S. EPA Internet site: http://www.epa.gov/nerleerd/stat2.htm. In both methods, the 95%-confidence limits associated with an LC_{50} was calculated, if the data were appropriate. In case mortality occurred in the control, before the LC_{50} calculation, the Abbott's correction was applied according to the formula:

$$(\%)Mortality\ (corrected) = \frac{(\%\ of\ treated\ \ mortality)\ -\ (\%\ of\ control\ \ mortality) \times 100}{100\ -\ (\%\ of\ control\ \ mortality)}$$

LOEC and NOEC estimation

The means of survival rates in each treatment were compared using One-way Analysis of Variance (ANOVA). A multiple comparison method (Dunnett's test) was used to determine which concentrations had mean responses that were significantly different from the control. When data was not appropriate (variances not homogenous), a transformation was performed prior to ANOVA. If data were still inhomogeneous after transformation, an equivalent non-parametric test (Kruskal-Wallis) was used.

Thus, the NOEC was defined as the highest concentration that has no statistically significant effect when compared to the control and the LOEC was defined as the next higher concentrations (usually the lowest concentration that produces statistically significant effect on organisms when compared to the control).

Chronic tests

In chronic toxicity tests, the endpoint reproduction (number of juveniles) was analysed according to the parameters: Median Effective Concentration (EC_{50}), Lowest-Observed-Effect-Concentration (LOEC) and Highest-No-Observed-Effect-Concentration (NOEC).

EC_{50} estimation

A non-linear regression model was used to verify a quantitative relation between concentration and the measured response (i.e., mean number of juveniles). The model for logistic response described by Haanstra et al. (1985) was used for calculation of EC_{50} and its 95% confidence limits.

This logistic regression model was fitted through the following equation:

$$Y = \frac{c}{1 + e^{(b*(x-a))}}$$

where:
Y = number of juveniles
a = natural logarithm (Ln) of EC_{50} (mg/kg)
b = slope
c = mean number of juveniles in control
x = Ln of concentration (mg/kg).

LOEC and NOEC estimation

The mean of the number of juveniles in each concentration was compared with control using ANOVA and Dunnett's test to determine which concentrations were significantly different from the control. Thus, the NOEC was defined as the highest concentration that has no statistically significant effect when compared to the control and the LOEC was defined as the next higher concentrations (usually the lowest concentration that produces statistically significant effect on organisms when compared to the control).

Avoidance tests

EC_{50} estimation

The EC_{50} values and its 95%-confidence limits were determined using Trimmed Spearman-Kärber method (Hamilton et al. 1977).

LOEC and NOEC estimation

The Student *t* test (P = 0.05) was used for comparing the means of proportions of individuals in the sections (control and treated) of each test vessel, in the chemical treatments (5 concentrations) as well as in the dual control tests (4 replicates). The LOEC and NOEC values were determined using ANOVA and Dunnett's test.

2.6.2 Factorial design

Experiments using factorial designs were evaluated by means of multi-factor analysis of variance. Three-factor analysis of variance was used to test for differences between samples according to the levels of each factor, and for interactions between the factors. Multiple comparison procedure was done using Tukey's test. For a detailed description of this approach see Morgan (1991).

2.6.3 TME and field tests evaluation

Results from the TME and field tests were analyzed using different statistical procedures in the three experiments. Prior to the use of the statistical procedures, the data were checked for normality (Kolmogorov-Smirnov's test) and homogeneity of variances (Levene's test). When these assumptions were not met, the data were transformed. If the transformation had not conferred normality and homogeneity of

variances, a non-parametric test was used. The effects of chemicals were compared between treatments and control. For single comparisons of means between two variables, the Student t-test (at P = 0.05) was used. In cases where, after transformation the data did not meet a normal distribution and homogeneity of variances, the Wilcoxon-Mann-Whitney test was used. For multiple comparisons between the treatments and the control, an ANOVA followed by the Dunnett's test was used. Otherwise, the Kruskal Wallis rank test was applied for data with no normality and homogeneity of variances. In case of differences, a multiple comparison test was used (e.g., Dunnett's test).

2.7 Test chemicals

2.7.1 Selection of appropriate substances

Since it was not possible to test a wide range of chemicals in this study, three substances were selected as model substances. Their selection was based on the following criteria:

- No more than two, at the highest three chemicals could be tested, for practical reasons
- One chemical should cause effects on oligochaete worms while the other one should be effective against arthropods in order to cover the two main groups of soil organisms
- The chemicals should represent two different classes of pesticides (e.g., concerning the use or the mode of action)
- In order to facilitate the assessment of the test results the physico-chemical and toxicological properties of the model chemicals should be well known
- In addition, the number of ecotoxicological information should be high for these substances
- Finally, the model chemicals should be ecotoxicologically relevant in tropical countries, in particular in Brazil.

For these reasons, the fungicide carbendazim and the insecticide lambda-cyhalothrin were selected. While the former is known to be highly toxic to oligochaete worms, the latter has strong insecticidal properties. Both represent different use classes having clearly different modes of action. Since they have been available on the market for many years, enough data on their various properties as well as on their

ecotoxicological fate and effects are available in the open literature. Finally, they are sold regularly both in Europe and Brazil (especially in the State of Amazonas; Waichman et al. 2002). Despite the fact that no detailed data are available, it is known that they are widely used in many regions of the world.

Within this study it was also tested whether the closely related fungicides carbendazim and benomyl show different ecotoxicological effects. Such results could be important in order to decide whether data from one compound could be extrapolated to other substances. The answer to this question might influence the amount of work (i.e., testing effort) which is required in order to assess the environmental risk of pesticides (e.g., must any individual substance be tested or would it be sufficient to test only representatives of various (use) classes?). Benomyl was an economically important pesticide for about two decades. Therefore, and despite the fact that its strong ecotoxicological side effects (as well as some toxicological problems) have been known for some time, it was surprising that the producing company decided only recently to withdraw this compound from the market (EU 2002). Independent of this development, most of the laboratory tests done with carbendazim and lambda-cyhalothrin were also performed with benomyl.

Benomyl

Benomyl is a broad-spectrum fungicide of the benzimidazole chemical group, which is registered for use in more than 50 countries, and represents about 50 % of the benzimidazole fungicides market worldwide, followed by carbendazim (20%) and thiophanate-methyl (20%) (WHO 1993a). It is available as a wettable powder formulation (500 g /kg of active ingredient) and used for the control of a wide range of diseases on more than 70 crops, including fruit trees, vegetables and cereals. The recommended application rates range from 0.1 to 1.0 kg a.i./ha and applications from once per year to spray intervals ranging from 7 to 14 days. As a systemic fungicide, it is absorbed through the leaves and roots, having a protective and curative action. Benomyl is rapidly converted to carbendazim in the environment with half-lives of 2 and 19 h in water and in soil, respectively. Carbendazim is the primary metabolite of thiophanate methyl, another fungicide, and is also registered as an active ingredient (WHO 1993a). Benomyl is classified by the World Health Organization (WHO) as an “unlikely

hazardous" (U) substance when used at recommended application rates. A general description, including some physical, chemical and toxicological properties, is presented in Table 2.6.

The chemical manufacturer that first registered Benomyl as a systemic fungicide in 1969, and by the end of 2002, decided to phase out the sale of all benomyl products (US EPA 2002 and EU 2002).

Carbendazim

Carbendazim is a systemic benzimidazole fungicide, produced commercially since 1970. It is used to control a broad range of diseases on cereals, fruits and vegetables, as well as in post-harvest food storage, and as a seed pre-planting treatment. It is formulated as an aqueous dispersion, aqueous suspension, and a wettable powder containing 500 g /kg of active ingredient. The recommended application rates range from 0.18 to 0.6 kg a.i./ha and spray intervals vary from 8 to 15 days (WHO 1993b). According to WHO, carbendazim is classified as an "unlikely hazardous" (U) substance when in normal use. A general description, including some physical, chemical and toxicological properties, is presented in Table 2.7. The decision whether carbendazim will be registered in the EU has been postponed until the end of 2003 (EC 2003a).

Lambda-cyhalothrin

Lambda-cyhalothrin is a pyrethroid insecticide and acaricide, developed in 1977. It is highly active against a broad spectrum of pests in public and animal health, but is also employed in agriculture against pests in many crops. It is a non-systemic pesticide formulated as 2.5%, 5.0%, and 12% emulsifiable concentrates (WHO 1990). The recommended application rates range from 5 to 20 g a.i./ha and spray intervals vary from 7 to 15 days. According to WHO, lambda-cyhalothrin is classified as a "moderately hazardous" (Group II) substance. A general description, including some physical, chemical and toxicological properties, is presented in Table 2.8. Recently, this compound has been included on Annex I of EU Directive 91/414, meaning that lambda-cyhalothrin is classified as causing no concern to humans or the environment (EC 2003a).

Table 2.6: Physical, chemical and toxicological characterization of benomyl.

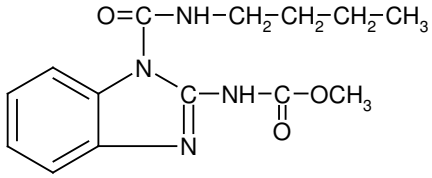
Characteristic	Information	Reference
Common name	Benomyl	Kidd and James (1991)
Chemical name	Methyl 1-(butylcarbamoyl) benzimidazol-2-ylcarbamate (IUPAC)	Kidd and James (1991)
Chemical family	Benzimidazole; MBC	Kidd and James (1991)
Molecular formula	$C_{14}H_8N_4O_3$	Kidd and James (1991)
Molecular structure		WHO (1993a)
Molecular weight	290.62	Kidd and James (1991)
Physical form	Crystalline solid	WHO (1993a)
Melting point	Decomposes just after melting at 140 °C	WHO (1993a)
Vapor pressure	< 1 mPa at 20 ºC	Kidd and James (1991)
Henry's constant	< 4.2 x 10-9 atm m3/mol. at pH 5 and 25 °C	WHO (1993a)
Water solubility	2 mg/l at 25 ºC	Kidd and James (1991)
Solubility in selected organic solvents	Acetone: 18 g/kg at 25 ºC Ethanol: 4 g/kg at 25 ºC	Kidd and James (1991)
Adsorption coefficient (Koc)	≅ 250	Aharonson and Kafkafi (1975)
Partition coefficient (Kow)	Log Kow =1.36	WHO (1993a)
Biodegradation half-life	Quick degradation to carbendazim (DT_{50}: 10 - 20 d)	Domsch (1992)
Stability	Decomposed by strong acids and strong alkalis. Decomposes slowly in the presence of moisture.	Kidd and James (1991)
Toxicology		
Earthworm LC_{50}	0.4 - 27 mg/kg soil	Van Gestel (1992)
Earthworm $NOEC_{reproduction}$	0.25 - 1.0 mg/kg soil	Van Gestel (1992)
Enchytraeid LC_{50}	22.0 mg/kg soil	Römbke and Knacker (1989)
Enchytraeid $NOEC_{reproduction}$	4.04 mg/kg soil	Römbke et al. (1998)
Bees (*Apis mellifera*)	Not toxic to bees. LD50 > 10µg/bee	Kidd and James (1991)
Acute Rat Oral LD50	> 10000 mg /kg	Kidd and James (1991)

Table 2.7: Physical, chemical and toxicological characterization of carbendazim.

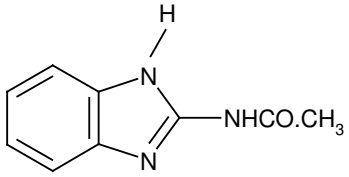
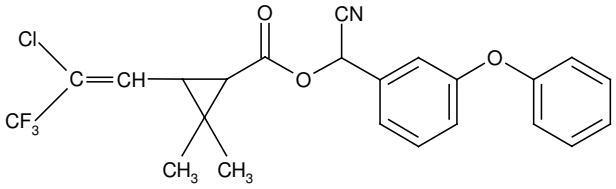
Characteristic	Information	Reference
Common name	Carbendazim	Kidd and James (1991)
Chemical name	Methyl benzimidazole-2-ylcarbamate (IUPAC)	Kidd and James (1991)
Chemical family	Benzimidazole	Kidd and James (1991)
Molecular formula	$C_9H_9N_3O_2$	Kidd and James (1991)
Molecular structure		WHO (1993b)
Molecular weight	191.19	Kidd and James (1991)
Physical form	White, crystalline solid	WHO (1993b)
Melting point	250 ºC (melts), 302-307°C (decomposes)	WHO (1993b) ; Kidd and James (1991)
Vapor pressure	65 nPa at 20°C	Kidd and James (1991)
Henry's constant	1.02 x 10-9 atm m3/mol at 20 °C	WHO (1993b)
Water solubility	28 mg/l (pH 4) and 8 mg/l (pH 7) at 24 °C	Kidd and James (1991)
Solubility in selected organic solvents	Acetone: 300 mg /l at 24 ºC Hexane: Insoluble Ethanol: 300 mg /l at 24 ºC	Kidd and James (1991)
Adsorption coefficient (Koc)	≅ 250	Aharonson and Kafkafi (1975)
Partition coefficient (Kow)	Log Kow = 1.49	WHO (1993b)
Biodegradation half-life	DT_{50} in sandy soils (pH 4.6 - 4.9): 50 - 230 d DT_{50} in clay soils (pH 6.1 - 6.3): 10 - 50 d	Domsch (1992)
Stability	Stable for at least two years below 50ºC. Decomposes slowly in alkaline solution. Stable in acid, forming water-soluble salts.	Kidd and James (1991)
Toxicology		
Earthworm LC_{50}	1 - 10 mg/kg soil	Van Gestel (1992)
Earthworm $NOEC_{reproduction}$	0.6 mg/kg soil	Van Gestel (1992)
Enchytraeid LC_{50}	5 - > 32 mg/kg soil	Römbke and Moser (2002)
Enchytraeid $NOEC_{reproduction}$	0.8 - 1.4 mg/kg soil	Römbke and Moser (2002)
Bees (*Apis mellifera*)	relatively non-toxic	WHO (1993b)
Acute Rat Oral LD50	> 15000 mg /kg	WHO (1993b)

Table 2.8: Physical, chemical and toxicological characterization of lambda-cyhalothrin.

Characteristic	Information	Reference
Common name	lambda-cyhalothrin	Kidd and James (1991)
Chemical name	α-cyano-3-phenoxybenzyl 3-(2-chloro-3,3,3-trifluoroprope-nyl)-2,2-dimethylcyclopropanecarboxylate	Kidd and James (1991)
Chemical family	Pyrethroid	Kidd and James (1991)
Molecular formula	$C_{23}H_{19}ClF_3NO_3$	Kidd and James (1991)
Molecular structure		WHO (1990)
Molecular weight	449.9	Kidd and James (1991)
Physical form	Colorless solid	Kidd and James (1991)
Melting point	49.2 ºC (melts), >275°C (decomposes)	WHO (1990) ; Kidd and James (1991)
Vapour pressure	200 nPa at 20°C	Kidd and James (1991)
Henry's constant	0.02 Pa-m3/mol at 20 °C	EC (2001)
Water solubility	0.005mg/l (pH 6.5) at 20 °C	Kidd and James (1991)
Solubility in selected organic solvents	Soluble in hexane, toluene, dichloromethane, methanol, acetone and ethyl acetate: > 500 g/l	EC (2001)
Adsorption coefficient (Koc)	157000 (mean, n=4)	EC (2001)
Partition coefficient (Kow)	Log Kow = 7.0	EC (2001)
Biodegradation half-life	4 - 12 weeks (in soil)	Kidd and James (1991)
Stability	Stable for more than 6 months at 15 - 25°C	Kidd and James (1991)
Toxicology		
Earthworm LC_{50}	> 1000 mg/kg soil	Maroni et al. (2002)
Earthworm $NOEC_{reproduction}$	100 mg/kg soil (biomass)	Maroni et al. (2002)
Enchytraeid LC_{50}	Not available	-
Enchytraeid $NOEC_{reproduction}$	Not available	-
Bees (*Apis mellifera*)	Oral LD50 = 0.91µg/bee	EC (2001)
Acute Rat Oral LD50	56-79 mg/kg	WHO (1990)

2.7.2 Screening of test concentrations (Rapid-Kits)

An ELISA-Plate Test Kit was used to determine the concentration of carbendazim in two test substrates (OECD artificial soil and LUFA standard soil), in the soil from the litterbag field trial and in hydrous leachates and soil from a terrestrial microcosm study. The ELISA-Plate Test Kit is originally designed to quantify the concentration of carbendazim in water and foodstuff. This method has rarely been used in ecotoxicological tests (Römbke and Moser 2002) and is therefore described in detail here. The principle of the method is based on the ability of polyclonal antibodies to selectively bind carbendazim and cross-reacting substances. The Test kit, produced by Beacon Analytical Systems, Inc., Portland ME, USA, was received from Coring System Diagnostix GmbH, Gernsheim, Germany. One kit consists of a microtiterplate (12 x 8 cavities) with antibodies fixed at the bottom of each cavity, MBC-enzyme conjugate, substrate, stopping solution, negative control and three standards (0.05, 0.2 and 1.0 ppb carbendazim) (Figure 2.25).

Sample preparation

Soil samples were homogenized by thoroughly mixing. Any stones, roots or other plant materials or animals were sorted out. A sub-sample of 10 g dry weight equivalent was taken for the extraction procedure.

Test kit preparation

The test kit was stored at 4-8° C. It was not frozen or exposed to temperatures above 37° C. Prior to the start of the test the kit should be adapted to room temperature.

Extraction procedure

Moist soil of 10 g dry weight equivalent was filled into a 30 ml plastic bottle, amended with a small portion of sodium bicarbonate to achieve an alkaline medium, and with 15 ml of methanol. Five steel balls (diameter 5 mm) were added and the flask was shaken for two minutes, left to settle for five minutes, and shaken again for two minutes. Thereafter, the solution was filtered through folded paper filter (Schleicher & Schuell 595½). The filtrate was diluted with PBS buffer to achieve nominal concentrations of carbendazim. The PBS buffer consisting of the following salts solved in 800 ml water:

2.9 g Na_2HPO_4 x 12 H_2O, 0.20 g KH_2PO_4 x H_2O, 8.00 g NaCL, 0.20 g KCL; pH adjusted to 7.2 ± 0.2 with NaOH or HCL; filled up to 1000 ml with distilled water.

Analysis

One hundred µL each of the negative control, standards and samples were added into the cavities. Thereafter, 100 µL MBC enzyme conjugate was added to each cavity, which was then covered by a adhering foil and the plate incubated at room temperature in the dark for one hour. After incubation, the cavities were emptied and washed with water 5 times. Then 100 µL substrate was added to each cavity, the cavities were covered by a foil and incubated for another 30 minutes in the dark. To stop the color reaction, 100 µL stopping solution was added to each cavity (Figure 2.26). The optical density was then measured at 460 nm.

Calculation of concentrations

The concentration of carbendazim in the extracts was calculated via software provided by the company that produces the ELISA kit. The optical density values of the control and the calibration standards and of the respective soil extracts were given into a defined matrix and the concentration of carbendazim calculated according to the calibration curve based on the standards. The concentration of the test substance in the dilution was given as ppb (= µg/L). To calculate the carbendazim concentration in the soil extract, the results were multiplied by the dilution factor. The total recovery of carbendazim was calculated as percentage of the initial nominal concentration in the soil. The latter was calculated from the applied amount of the test substance assuming no degradation and a 100% extraction efficiency of carbendazim. For the field and the TME, an even distribution of the applied amount of the test substance in the top 5 cm soil and an average soil density of 1 kg/dm^3 were assumed. In a second step, the degradation of carbendazim was calculated assuming a DT_{50} value for clay soils of 50 days (Kidd and James 1991).

Figure 2.25: Equipment of the RAPID kit test (samples, extracts, standards etc.).

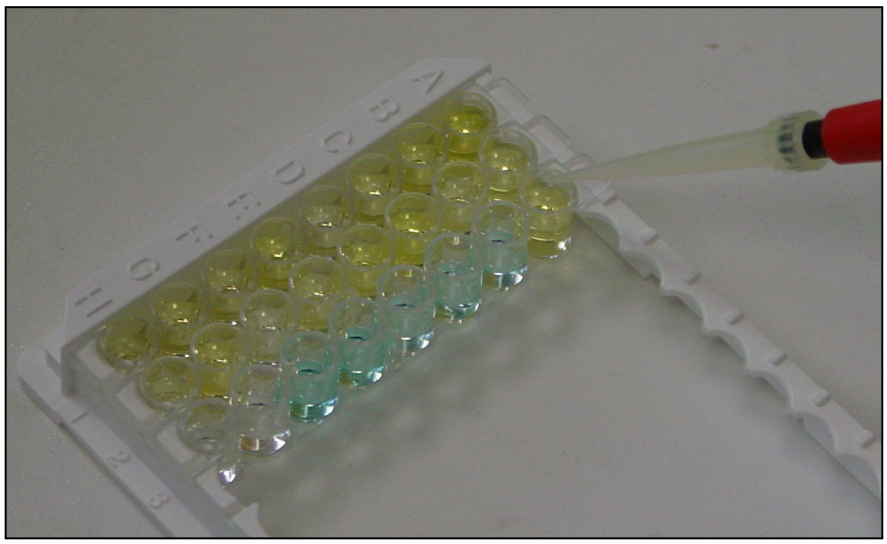


Figure 2.26: Addition of standard and sample extracts to the microtiterplate.

3 RESULTS

3.1 Validity of laboratory tests

3.1.1 Earthworms

The test system for the determination of the acute toxicity of chemicals to earthworms was based on the guidelines OECD n°. 207 (OECD 1984a) and ISO-11268-1 (ISO 1993a). According to these guidelines, the mortality of adult worms in the controls must be ≤ 10 % in order to consider a test as valid. In addition, the ISO guideline requires that the control worms should not loose more than 20 % of their initial biomass.

The mortality criterion was met for all tests with both standard European soils and tropical soils. Concerning biomass loss, in all tests except those running in LUFA 2.2 soils, the criterion of 20% was not met. In the tests with benomyl in natural soil (TNS) and with lambda-cyhalothrin in tropical artificial soil (TASx), the loss of biomass was even higher than 30%. However, taking into account that in acute tests mortality is the most important criterion and that the biology of the tropical variant of *Eisenia fetida* is not well known, the results can be considered as valid.

No specific reason for these effects on biomass can be given except in the case of TNS: The low pH (approx. 4.1) is probably responsible for the very poor performance of the worms in this soil (the other three soils had pH-values between 6.4 and 6.8). The moisture was always in a range suitable for the worms (around 30%). It should be noted that the lower moisture values in TNS do not mean that it was too dry for the worms in this soil: Depending on soil properties like the grain size distribution, the amount of available water is quite different; so, it has to be assumed that the worms had an optimum moisture regime. Except from the (intended) effects of these soil properties no specific observations were made during the tests. With the exception of benomyl in OECD soil where no data (n.d.) were available, the acute tests performed in OECD and LUFA soil with European *Eisenia fetida* were valid (Table 3.1).

Table 3.1: Validity of acute tests with European and tropical *Eisenia fetida* (values in %).

Substrate	Benomyl		Carbendazim		Lambda-cyhalothrin	
	Mortality in the control	Biomass loss	Mortality in the control	Biomass loss	Mortality in the control	Biomass loss
TASx	0.0	27.9	0.0	30.0	5.0	33.7
TNS	0.0	31.9	0.0	28.7	0.0	28.5
OECD ^{Eur}	n.d.	n.d.	0.0	0.0 [9.9]	0.0	8.1
LUFA ^{Eur}	0.0	0.0 [8.2]	0.0	0.0 [16.1]	0.0	0.0 [9.9]
OECD ^{Trop}	0.0	24.6	0.0	25.4	7.5	21.2
LUFA ^{Trop}	0.0	11.4	0.0	0.0 [7.1]	0.0	0.0 [1.9]

Trop - Tropical *Eisenia* at 28 ºC; Eur - European *Eisenia* at 20 ºC; Values in square brackets - increase of biomass.

In chronic tests, the following criteria must be met in the controls: (1) adult mortality ≤ 10%; (2) number of juveniles per replicate ≥ 30; and (3) coefficient of variation (CV) of reproduction to be ≤ 30 %. All test results for the three chemicals were considered valid for standard European soils. With TASx, only the CV criterion was not met in the tests with benomyl and lambda-cyhalothrin, while in the tests with tropical natural soil (TNS) too few juveniles were found, and also the CV values were clearly higher than 30%. Despite the fact that in the test with European *Eisenia* in LUFA soil the mean number of juveniles slightly was lower than the accepted limit (≥30), the test was considered to be valid due to its relatively low variance (CV). So, all tests except those done in TNS were considered to be valid (Table 3.2).

At present, no validity criteria established by guidelines can be attributed for the tropical earthworm species. Therefore, basically the same criteria as used in the standard acute tests were applied, i.e., in the case of native earthworm *Pontoscolex corethrurus* the validity criteria mortality and loss of biomass were used. Taking this approach, the results of the tests with the tropical earthworm *P. corethrurus* were considered to be valid - despite the fact that the biomass criterion was slightly exceeded (Table 3.3).

Table 3.2: Validity of chronic tests with European and tropical *Eisenia fetida* (mortality and CV values in %).

Substrate	Benomyl			Carbendazim			Lambda-cyhalothrin		
	Mortality in the control	No. of juveniles	CV	Mortality in the control	No. of juveniles	CV	Mortality in the control	No. of juveniles	CV
TASx	0.0	128.3	38.2	0.0	72.8	28.7	0.0	46.8	37.8
TNS	0.0	< 30	21.9	2.5	< 30	72.4	2.5	< 30	148.6
OECD^{Eur}	n.d.	n.d.	n.d.	0.0	158.5	8.9	0.0	355.3	18.0
LUFA^{Eur}	2.5	207.0	15.1	0.0	28.3	18.0	0.0	205.8	14.8
OECD^{Trop}	0.0	154.8	9.3	5.0	100.3	9.9	2.5	56.8	10.4
LUFA^{Trop}	0.0	199.3	25.4	0.0	139.5	13.7	2.5	174.0	14.6

Trop - Tropical *Eisenia* at 28 ºC; Eur - European *Eisenia* at 20 ºC; n.d. = not determined

Table 3.3: Validity of acute tests with tropical earthworm *Pontoscolex corethrurus* (values in %).

Carbendazim		Lambda-cyhalothrin	
Mortality	**Biomass loss**	**Mortality**	**Biomass loss**
5.0	18.9	0.0	23.8

Up to now, no validity criteria for avoidance tests have been formally adopted. Therefore, an equal distribution of the worms between the two halves of a test vessel containing the same type of soil was defined as a validity criterion. As can be seen in Table 3.4, all tests performed with OECD and LUFA soil as well as those done with tropical artificial soil (TASx) are considered to be valid, since the earthworms showed an equal distribution in the test containers. Tests in the tropical natural soil (TNS) were not valid due to the high mortality of earthworms in this substrate (data not shown).

Table 3.4: Dual control tests for European *Eisenia fetida* in OECD and LUFA soils and for tropical *E. fetida* in TAS soil.

Soil type	Frequency of distribution of earthworms (%)		Mean net response (A-B) (%)	Standard Error	Significance (*t*-test, P= 0.05)	Moisture (%)	pH
	Section A	Section B					
OECD	50.0	50.0	0	10.8	n.s.	30.1	6.1
LUFA	50.0	50.0	0	9.1	n.s.	29.5	6.1
TASx	45.0	55.0	-10	8.7	n.s.	31.3	6.4

n.s. = not significant

3.1.2 Arthropods

Since no validity criterion has been defined for tests using *P. pruinosus*, the criterion suggested in the literature for *P. scaber* (Hornung et al. 1998a), i.e., ≤ 20% mortality in the control, was adopted for *P. pruinosus*. According to this criterion, all tests with OECD and TASx soils were valid, but nearly all tests done with the two field soils (TNS and LUFA, except the test with lambda-cyhalothrin with LUFA) did not meet this criterion (Table 3.5). However, taking into consideration that in most cases the mortality was only slightly higher than 20% and that the test performance of *P. pruinosus* is not well known, it is proposed to consider these tests as valid, too. Further research will show which validity criterion for this test is appropriate.

Table 3.5: Validity of acute tests with *Porcellionides pruinosus* (mortality values in %).

Substrate	Benomyl	Carbendazim	Lambda-cyhalothrin
TASx	20.0	17.5	17.5
TNS	27.5	30.0	22.5
OECD	7.5	7.5	10.0
LUFA	25.0	25.0	10.0

At present, no validity criteria established by guidelines can be attributed for the tropical species tested in this study. Therefore, basically the same criteria as used in the standard acute tests were applied: in the case of native earthworms the validity criteria mortality and loss of biomass were used, whereas for millipedes and isopods the criterion mortality was sufficient.

Likewise, the tests with *C. ornatus* are also valid according to the criteria used for *P. pruinosus*. The mortality in control was relatively high (> 20%) in both tests with the diplopod *T. corallinus*, but since no experience is available for the normal behavior of this species, the test results will be considered as “valid with restriction” for the time being. However, it is perfectly clear that further research is necessary to verify this assumption (Table 3.6).

Table 3.6: Validity of acute tests with tropical species (mortality values in %).

Species	Carbendazim		Lambda-cyhalothrin	
	Mortality	**Biomass loss**	**Mortality**	**Biomass loss**
Trigoniulus corallinus	27.5	n.a.	22.5	n.a.
Circoniscus ornatus	15.0	n.a.	12.5	n.a.

n.a. = not applicable

3.2 Toxicity tests with earthworms

3.2.1 *Eisenia fetida*: standard tests

Effect of fungicide benomyl on tropical *Eisenia fetida*

Acute tests

The toxicity of benomyl to tropical *E. fetida* was tested in preliminary assays (range-finding (RF) tests). The RF tests provided LC_{50} values as 373 and 25 mg a.i./kg for OECD and LUFA soils, respectively, and 316 mg a.i./kg for tropical TASx and TNS soils. The concentrations used for the acute tests were derived from the RF test results. The acute effects of benomyl on the tropical variant of *E. fetida* were tested in all four test soils. Significant effects on mortality and biomass development occurred in the tested range of concentrations (10 - 1000 mg a.i./kg for artificial soils and 3.16 - 316 mg a.i./kg for field soils). The LC_{50} values in artificial soils were 7 to 10 times higher than in natural soils (458 - 633 versus 61.4 - 66.8 mg a.i./kg). Since the data of TASx soil were not appropriate for the probit analysis, no dose response curve was fit and the LC_{50} value was calculated with the Trimmed Spearman-Kärber method. The LC_{50} values of the two artificial soils (OECD and TASx) were considered to be different (no overlap of the confidence limits) while between the two natural soils (LUFA and TNS) such a difference could not be observed (Table 3.7). Accordingly, the estimated LOEC and NOEC values for mortality were also higher in artificial than in natural soils (Table 3.8). The shape of the dose response curves of benomyl was quite similar in all four tested soils (Figures 3.1-3.4). The loss of biomass in earthworms was statistically different from the control only in the test with LUFA soil (Appendix 7).

In the case of the TNS, the low pH might have negatively influenced the worms in all treatments (including the controls), but for the OECD and TASx soils there is no obvious reason why a relatively high loss of biomass occurred even in the

controls. Neither the abiotic parameters nor the biological effect data show a high variability (see Appendix 7).

Table 3.7: Acute toxicity of tropical *Eisenia fetida* in benomyl-dosed substrates: median lethal concentrations - LC_{50} values (mg/kg).

Soil		LC_{50}	95 % Confidence Limits (CL)		CL - overlapping			
			Lower	Upper	1	2	3	4
1	**OECD**	458.3	383.7	547.5	*			
2	**TASx**	633.0	571.1	701.6		*		
3	**LUFA**	66.8	55.1	81.0			*	*
4	**TNS**	61.4	51.1	72.1			*	*

Table 3.8: Acute toxicity of *Eisenia fetida* in benomyl-dosed substrates: LOEC and NOEC values (mg/kg).

	OECD		TASx		LUFA		TNS	
	Mortality	Biomass	Mortality	Biomass	Mortality	Biomass	Mortality	Biomass
LOEC	316	> 100	1000	> 316	100	31.6	100	> 31.6
NOEC	100	≥ 100	316	≥ 316	31.6	10	31.6	≥ 31.6

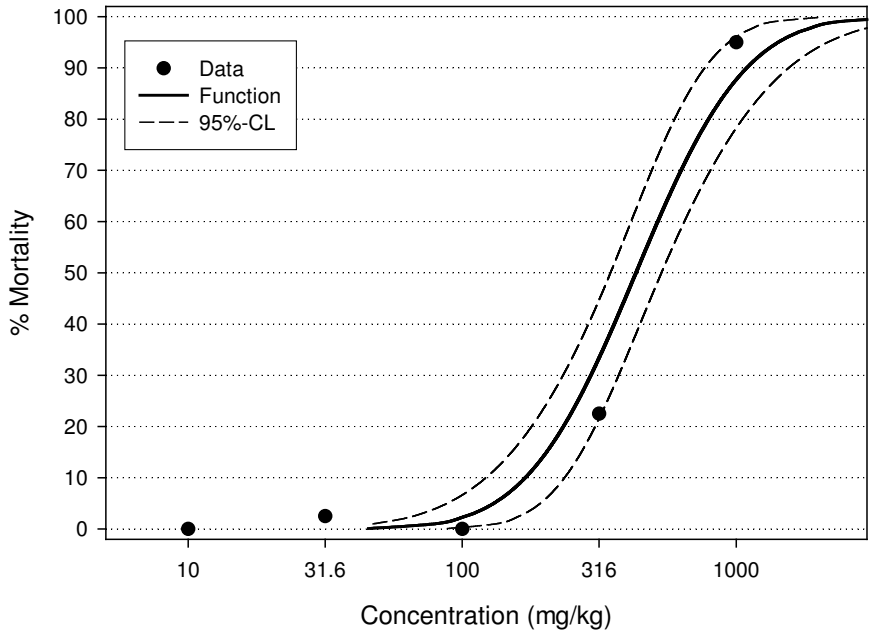


Figure 3.1: Dose response curve for the acute toxicity test with tropical *Eisenia fetida* in benomyl-dosed OECD soil.

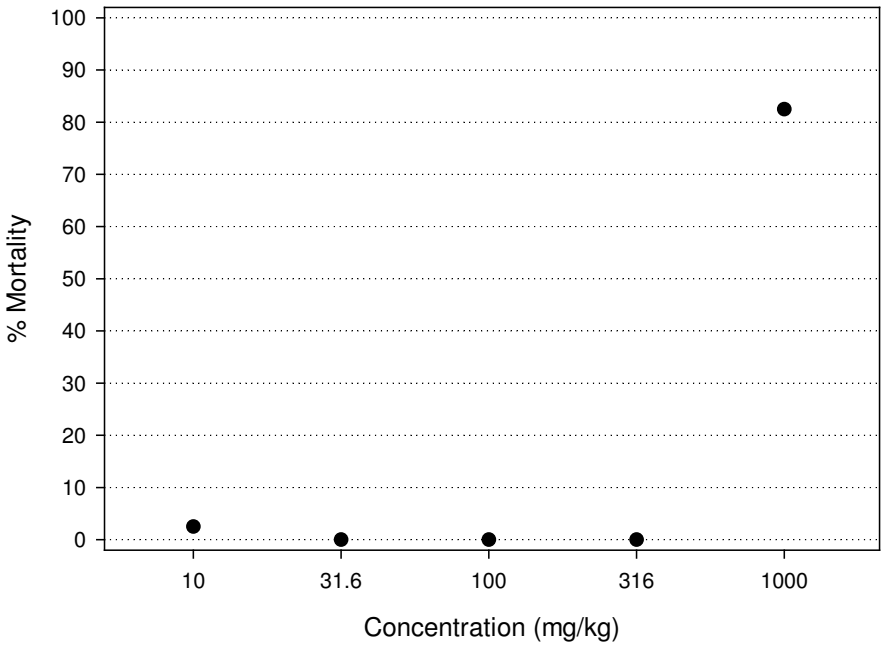


Figure 3.2: Mortality of tropical *Eisenia fetida* in benomyl-dosed TASx soil (data not appropriate for probit analysis).

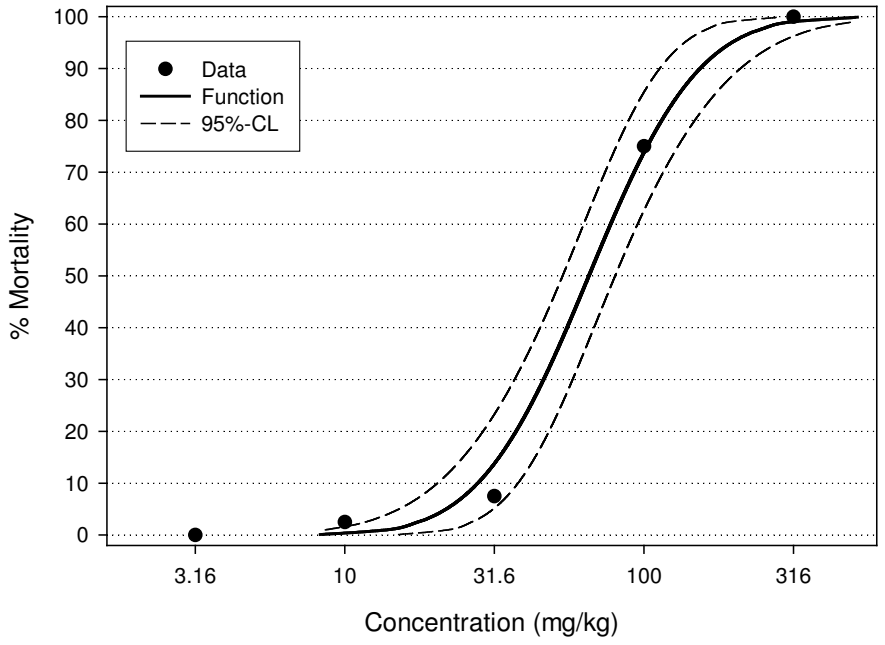


Figure 3.3: Dose response curve for the acute toxicity test with tropical *Eisenia fetida* in benomyl-dosed LUFA soil.

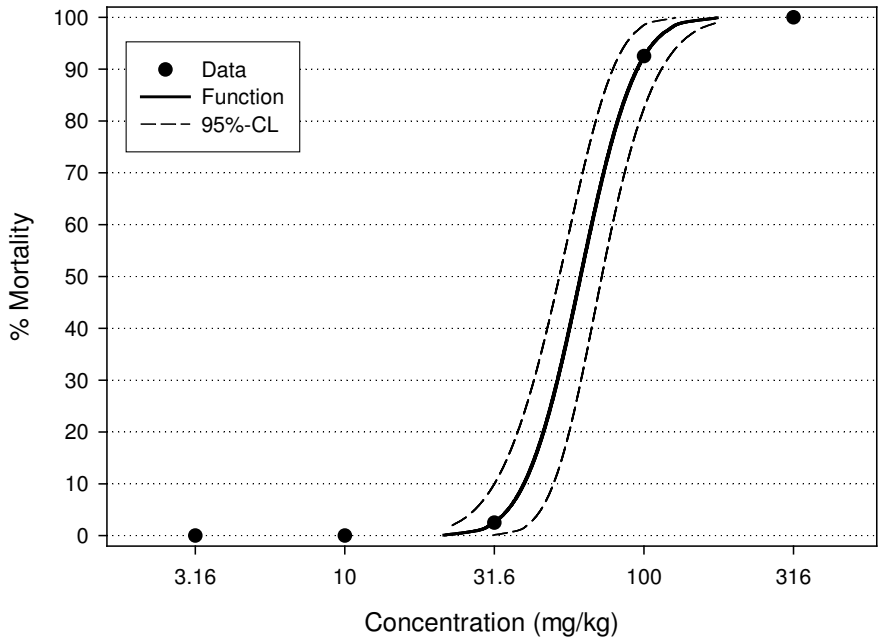


Figure 3.4: Dose response curve for the acute toxicity test with tropical *Eisenia fetida* in benomyl-dosed TNS soil.

Chronic tests

In the chronic tests, covering a concentration range between 0.1 and 100 mg a.i./kg, the effect on the number of juveniles differed from the picture seen in the acute tests (Appendix 8). The estimated EC_{50} in natural LUFA soil was about 5 and 16 times lower than in artificial TASx and OECD soils, respectively, covering a range between 0.8 to 12.9 mg a.i./kg. The EC_{50} values in OECD and TASx soils were statistically different (Table 3.9). No data are available from the tests with tropical natural soil (TNS) due to the insufficient number of juveniles in the control (Appendix 8). The dose response curve was relatively similar in the two artificial soils, showing a steep decrease (but not at the same concentration), while the curve in the LUFA soil was very shallow without any steep step (the curves are partly influenced by the fact that the tested concentration range differed in the individual tests) (Figures 3.5 - 3.7). The differences in the dose response curves are not reflected by the NOEC values: In OECD and LUFA soil it was determined as 0.32 mg a.i./kg, but in the test with TASx it was nearly as high as the EC_{50} (3.16 versus 3.8 mg a.i./kg). Less than 10% mortality of adults occurred in all three tests. Neither in the test with OECD nor with LUFA soil was the biomass of adults

affected, while in the test with TASx a decrease of about 10 and 20 % was found at the two highest concentrations (31.6 and 100 mg a.i./kg), respectively. During the 56 days of test, the moisture was kept near the initial value and the pH varied in 0.3 to 0.5 units up or down (Appendix 8).

Table 3.9: Chronic toxicity of *Eisenia fetida* in benomyl-dosed substrates: EC_{50} and its 95%-confidence limits, LOEC and NOEC (values in mg/kg).

	Effect on reproduction			
	OECD	**TASx**	**LUFA**	**TNS***
EC_{50}	12.9 [3.3 - 51.1]	3.8 [1.6 - 9.0]	0.8 [n.d.]	n.d.
LOEC	1	10	1	n.d.
NOEC	0.32	3.16	0.32	n.d.

* tests were not valid

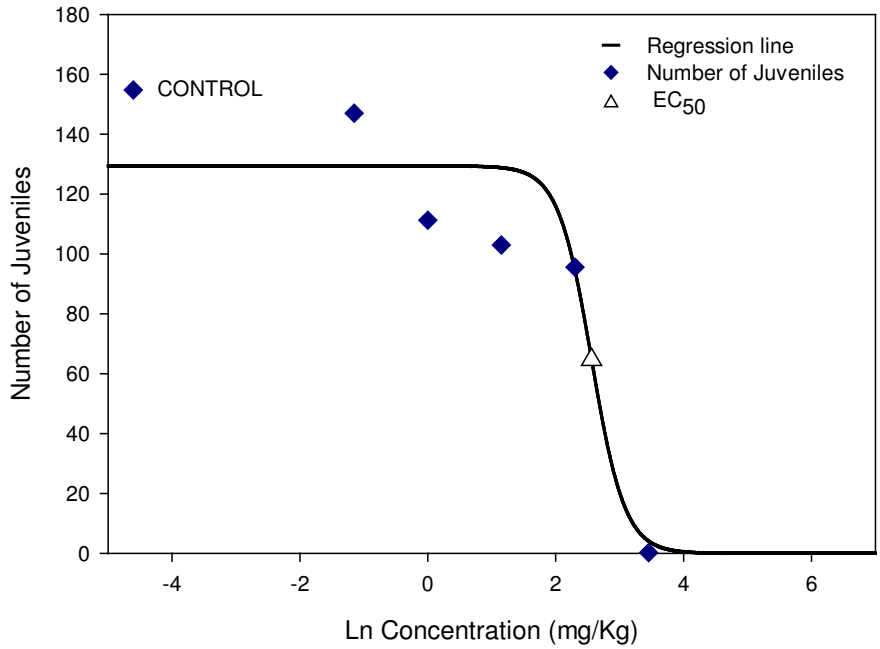


Figure 3.5: Dose response curve in the chronic toxicity test with tropical *Eisenia fetida* in benomyl-dosed OECD soil.

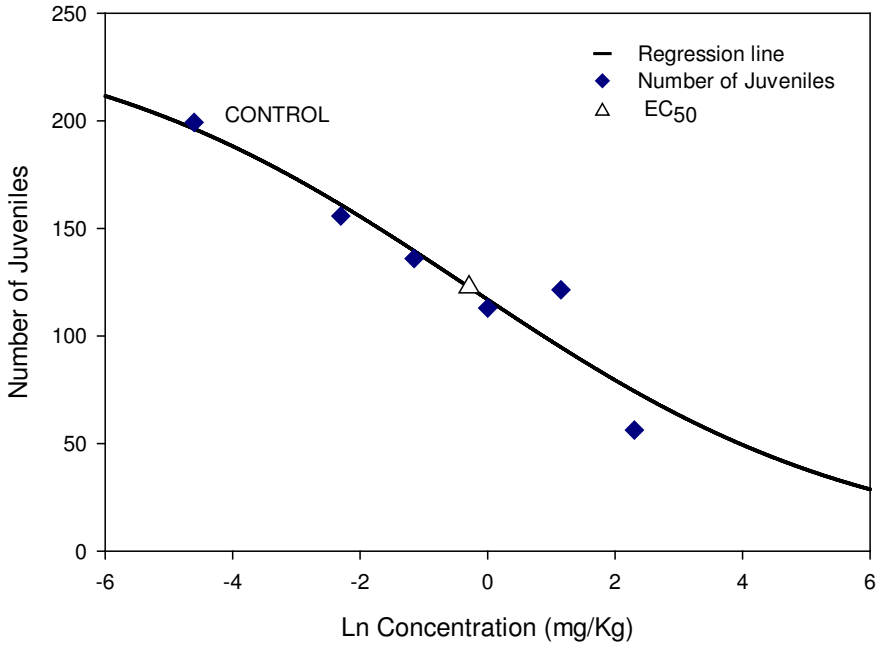


Figure 3.6: Dose response curve in the chronic toxicity test with tropical *Eisenia fetida* in benomyl-dosed LUFA soil.

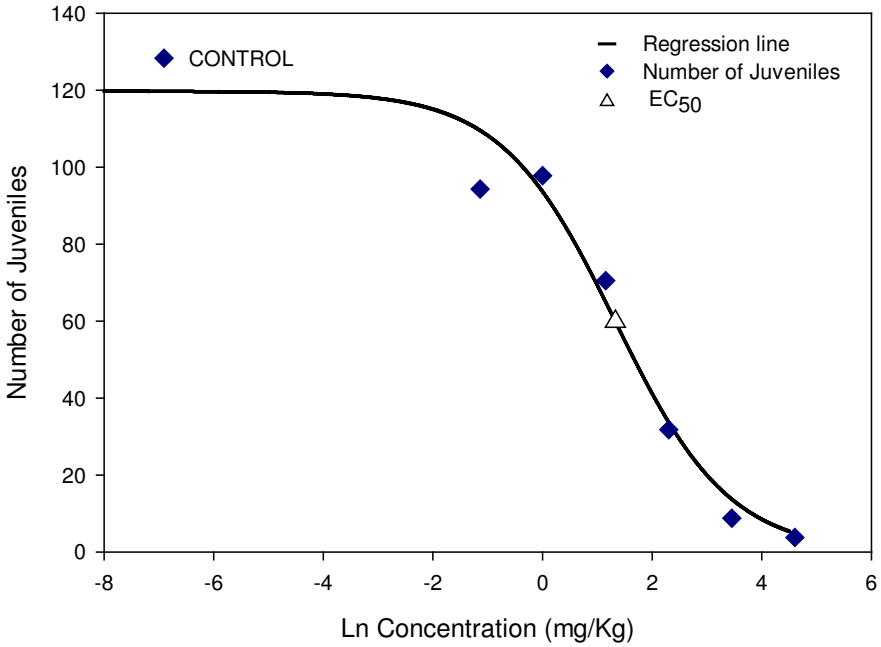


Figure 3.7: Dose response curve in the chronic toxicity test with tropical *Eisenia fetida* in benomyl-dosed TASx soil.

Avoidance tests

The avoidance behavior of earthworms regarding benomyl was tested in TASx soil in a range of concentrations from 1.0 to 1000 mg a.i./kg. The results indicate a significant reaction of earthworms to concentrations ≥ 31.6 mg a.i./kg (Figure 3.8). The EC_{50} value was determined as 54.9 mg a.i./kg [43.1 - 69.9, 95%-CL] and LOEC and NOEC values as 31.6 and 10 mg a.i./kg, respectively. Avoidance tests were not valid in natural TNS soil due to the aggregating behavior or the high mortality rate of earthworms when exposed in this substrate. Values of mean net response are presented in Appendix 9.

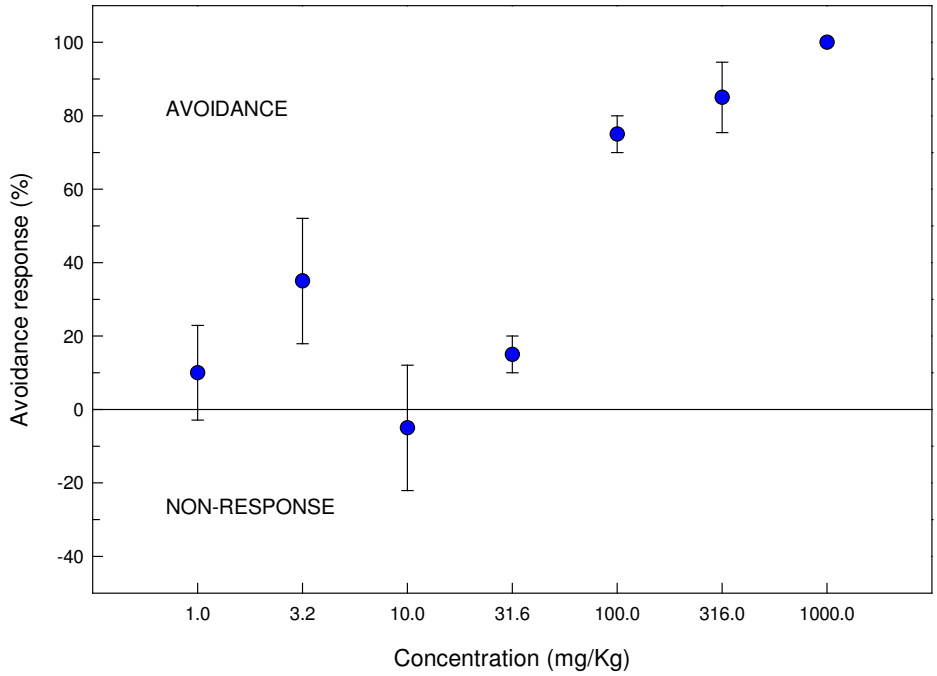


Figure 3.8: Avoidance response or non-response (negative) during the exposure of tropical *Eisenia fetida* to benomyl concentrations in TASx soil (mean net response and standard error bars).

Effect of fungicide benomyl on European *Eisenia fetida*

Acute tests

Preliminary tests (RF tests) with benomyl and European *E. fetida* in OECD soil provided an LC_{50} value of 250 mg a.i./kg. The respective result from the definitive acute test was taken from the literature (22 mg a.i./kg; Heimbach 1984). For LUFA soil, the test concentration range was also taken from the literature. In the acute test with benomyl and the European *E. fetida* in LUFA soil, significant effects on the mortality were observed in the tested concentrations ranging from 2 to 200 mg a.i./kg. The LC_{50} value was determined as 14.6 mg a.i./kg [11.3 - 18.7, 95%-CL] and LOEC and NOEC values as 6.3 and 2.0 mg a.i./kg, respectively. The dose response curve of benomyl in LUFA soil did not fit properly to the data and the 95% confidence limit could not be estimated (Figure 3.9). The computation for biomass change was not applicable (n.a.), since high mortality occurred after the second concentration (6.3 mg a.i./kg). Neither the abiotic parameters nor the biological effect data show a high variability (Appendix 10).

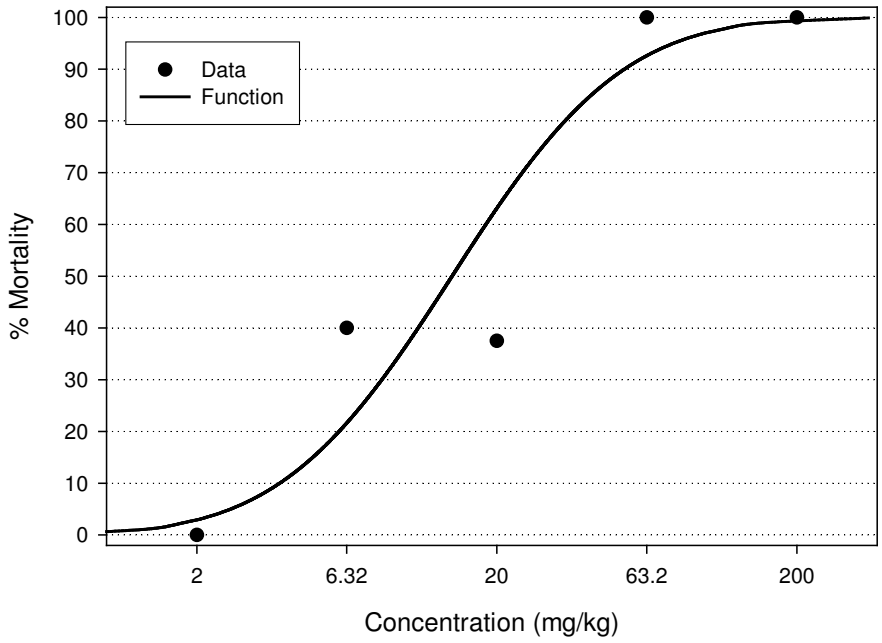


Figure 3.9: Dose response curve for the acute toxicity test with European *Eisenia fetida* in benomyl-dosed LUFA soil (95%-CL not determined).

Chronic tests

The chronic effects of benomyl on the European *E. fetida* were tested only in the LUFA soil covering a concentration range between 0.1 and 10 mg a.i./kg. For the artificial OECD soil, the data were obtained from literature: the EC_{50} value was 1.6 mg a.i./kg [1.2 - 2.3, 95%-CL] and the LOEC and NOEC values were 3.2 and 1.0 mg a.i./kg, respectively (Van Gestel et al. 1992). The estimated EC_{50} in natural LUFA soil was determined as 1.0 mg a.i./kg [95%-CL n.d.] and the LOEC and NOEC values as ≥ 1.0 and 0.32 mg a.i./kg, respectively (Figure 3.10). Less than 10% mortality of adults occurred in the control. During the 56 days of test, the moisture was kept near the initial value and the pH varied in 0.3 to 0.5 units up or down (Appendix 11).

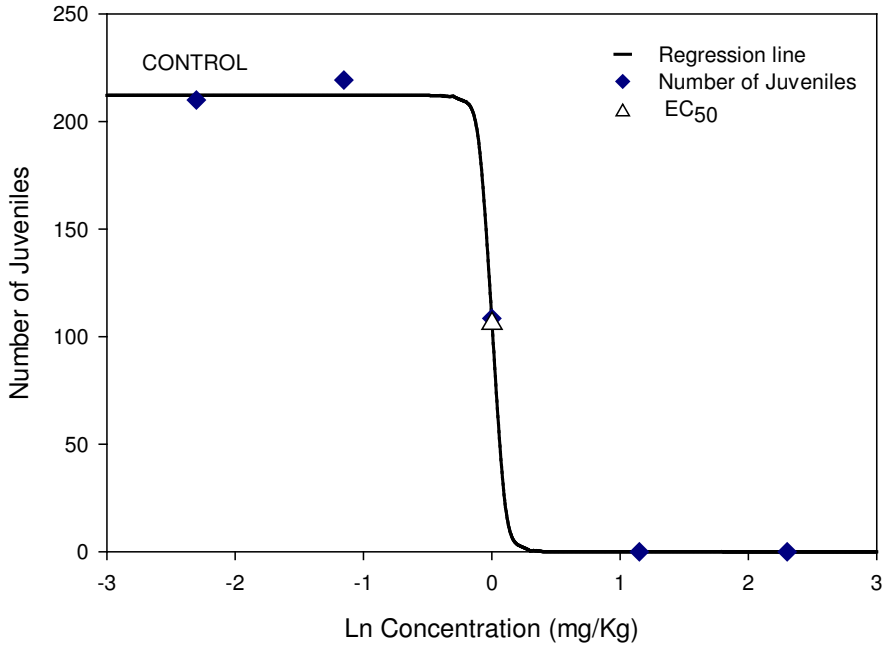


Figure 3.10: Dose response curve in the chronic toxicity test with European *Eisenia fetida* in benomyl-dosed LUFA soil.

Avoidance tests

The avoidance behavior of the European *E. fetida* to benomyl was tested in OECD and LUFA soil, at seven concentrations ranging from 1.0 to 1000 mg a.i./kg. The results indicate a similar tendency as in acute and chronic tests, i.e., the chemical was more toxic (available) in natural than in artificial soils. In natural LUFA soil, the earthworms showed significant avoidance behavior in the lowest concentration (1.0 mg a.i./kg), while in artificial OECD soil they reacted at concentrations of ≥ 10 mg a.i./kg (Figures 3.11 and 3.12). The EC_{50} value for LUFA soil was determined as 1.6 mg a.i./kg [1.2 - 2.2, 95%-CL] and the LOEC and NOEC values as < 1.0 mg a.i./kg. In OECD soil the EC_{50} value was 28.2 mg a.i./kg [24.5 - 32.4, 95%-CL] and the LOEC and NOEC value was determined as 10 and 3.16 mg a.i./kg, respectively. The EC_{50} values were considered to be different among the tested soils (no overlap of confidence limits). Values of mean net response are presented in Appendix 12.

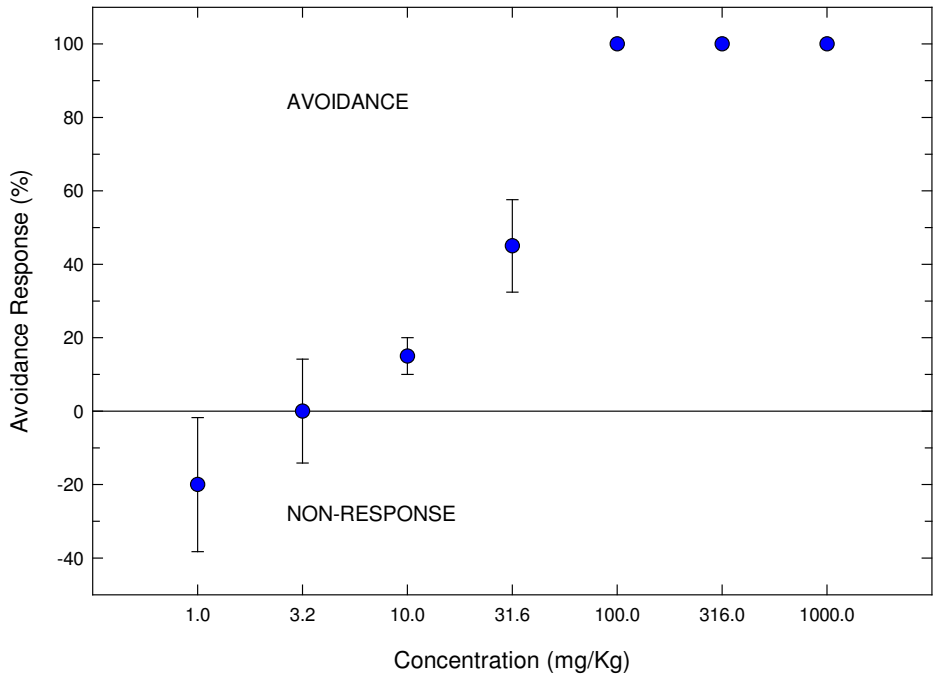


Figure 3.11: Avoidance response or non-response (negative) during the exposure of European *Eisenia fetida* to benomyl concentrations in OECD soil (mean net response and standard error bars).

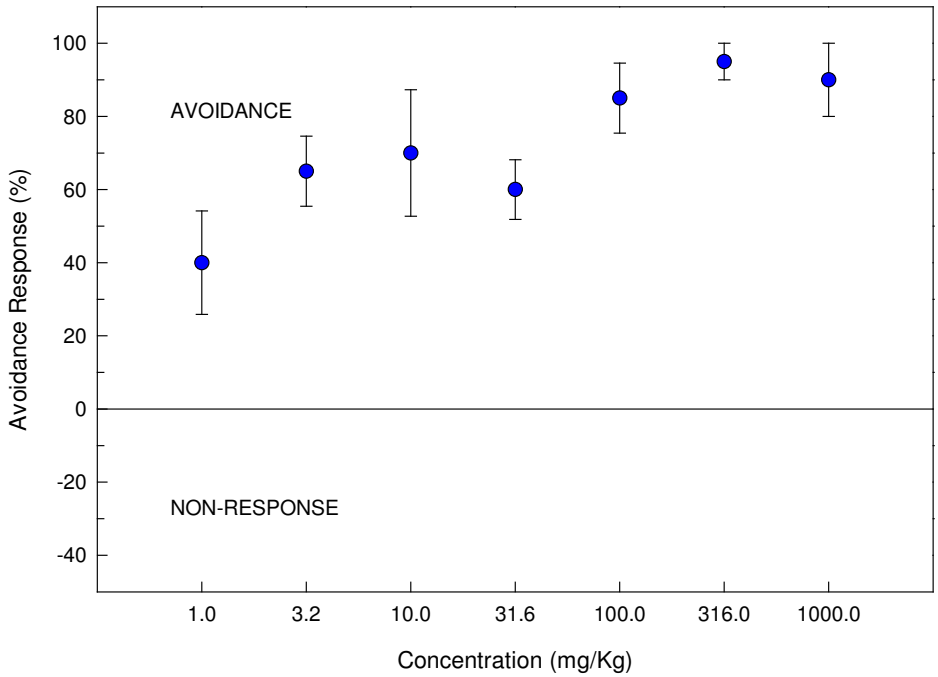


Figure 3.12: Avoidance response or non-response (negative) during the exposure of European *Eisenia fetida* to benomyl concentrations in LUFA soil (mean net response and standard error bars).

Effect of fungicide carbendazim on tropical *Eisenia fetida*

Acute tests

The fungicide carbendazim was previously tested with tropical *E. fetida* in RF tests. The RF tests provided LC_{50} values as ≥ 1000 mg a.i./kg for artificial soils (OECD and TASx) and 100 mg a.i./kg for natural soils (LUFA and TNS). The concentrations defined for the acute tests were derived from the RF tests results. The acute effects of carbendazim on the tropical variant of *E. fetida* were tested in all four test soils (Appendix 13). Significant effects on mortality and biomass development were observed in the tested range of concentrations (10 - 1000 mg a.i./kg for the OECD, LUFA and TASx soils and 1.0 - 100 mg a.i./kg for the TNS soil). The LC_{50} values in all soils except the TNS soil were higher than 1000 mg a.i./kg, while the LC_{50} value in the TNS soil was about 20 times lower (57.1 mg a.i./kg). Clearly, the difference between the LC_{50} values of the two groups (OECD, LUFA and TASx versus TNS) was statistically highly significant (Table 3.10). Accordingly, the estimated LOEC and NOEC values for mortality were also lower in TNS compared to the other three soils,

but the difference was much smaller: just one concentration level (Table 3.11). The shape of the dose response curves of carbendazim differed strongly between the individual tests: while in the first three soils no mortality higher than 25% up to the highest concentration of 1000 mg a.i./kg occurred, a very steep increase was found in the test with TNS soil (no mortality up to 316 mg a.i./kg but 95% at 1000 mg a.i./kg) (Figures 3.13 – 3.16). The loss of biomass in earthworms was statistically different from the control only in the test with LUFA soil (Appendix 13), which is partly caused by the fact that in the LUFA soil the worms did not show any biomass loss in the control (in fact, they gained weight). This sensitive reaction is mirrored in the respective NOEC and LOEC values for biomass: while in all other tests this endpoint did not differ from the mortality results in the case of the LUFA test a NOEC of < 10 mg a.i./kg was determined - the lowest value of any endpoint in all acute tests. Taking mortality and biomass results together, it seems that the worms clearly reacted more in the two field soils compared to the two artificial soils.

In the case of the TNS, the low pH might have negatively influenced the worms in all treatments (including the controls), but for the OECD and TASx soils there is no obvious reason why a relatively high loss of biomass occurred even in the controls. Neither the abiotic parameters nor the biological effect data showed a high variability (Appendix 13).

Table 3.10: Acute toxicity of tropical *Eisenia fetida* in carbendazim-dosed substrates: median lethal concentrations - LC_{50} values (mg/kg).

Soil		LC_{50}	95 % Confidence Limits (CL)		CL - overlapping			
			Lower	Upper	1	2	3	4
1	**OECD**	2649	1274	18155	*	*	*	
2	**TASx**	3280	858	12541	*	*	*	
3	**LUFA**	24195	2829	> 10000 (n.d.)	*	*	*	
4	**TNS**	57.1	54.1	60.2				*

Table 3.11: Acute toxicity of tropical *Eisenia fetida* in carbendazim-dosed substrates: LOEC and NOEC values (mg/kg).

	OECD		TASx		LUFA		TNS	
	Mortality	Biomass	Mortality	Biomass	Mortality	Biomass	Mortality	Biomass
LOEC	316	> 100	316	> 316	316	10	100	> 31.6
NOEC	100	≥ 100	100	≥ 316	100	< 10	31.6	≥ 31.6

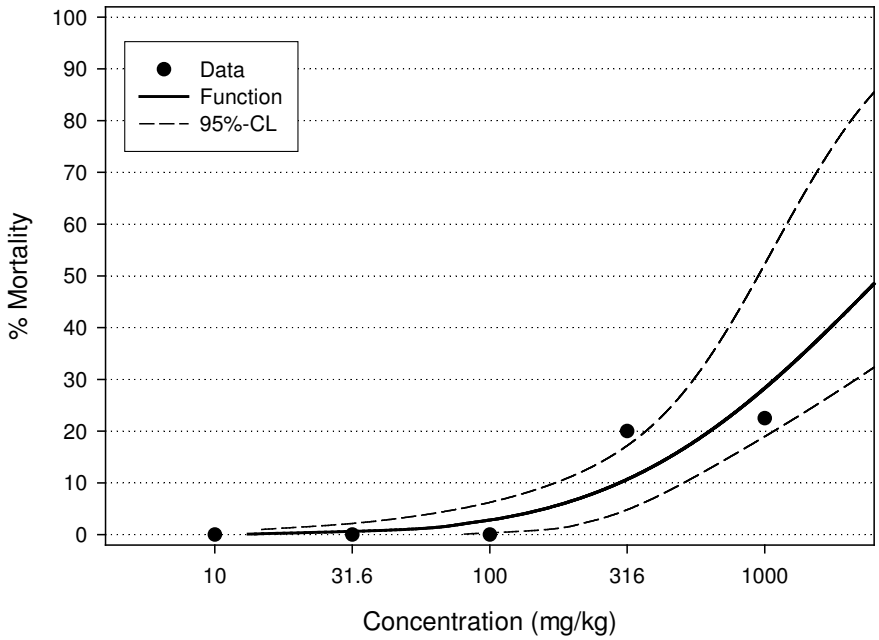


Figure 3.13: Dose response curve for the acute toxicity test with tropical *Eisenia fetida* in carbendazim-dosed OECD soil.

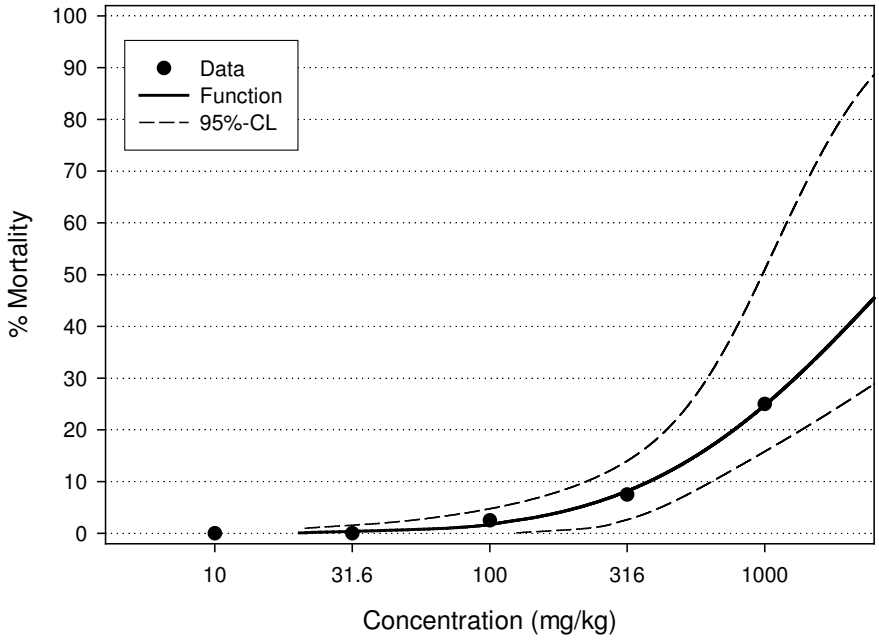


Figure 3.14: Dose response curve for the acute toxicity test with tropical *Eisenia fetida* in carbendazim-dosed TASx soil.

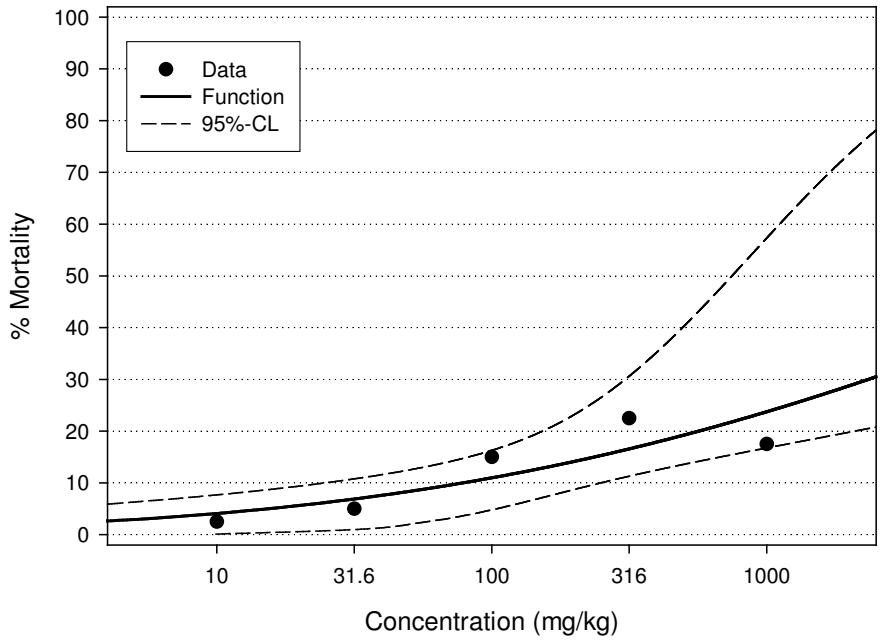


Figure 3.15: Dose response curve for the acute toxicity test with tropical *Eisenia fetida* in carbendazim-dosed LUFA soil.

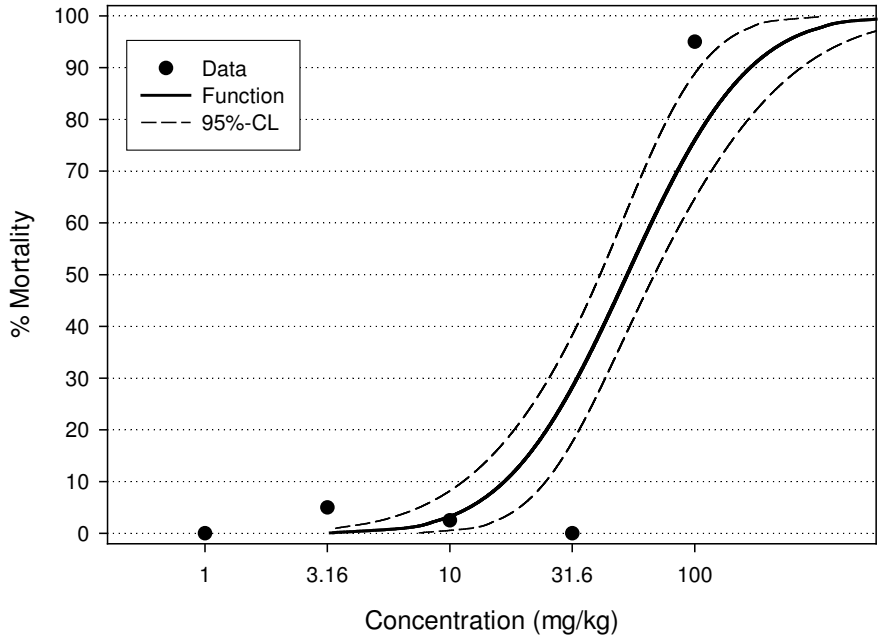


Figure 3.16: Dose response curve for the acute toxicity test with tropical *Eisenia fetida* in carbendazim-dosed TNS soil.

Chronic tests

In the chronic tests, covering a concentration range between 0.1 and 100 mg a.i./kg, the effect on the number of juveniles differed from the picture seen in the acute tests. The EC_{50} values in the soils of the first group (i.e., OECD, LUFA and TASx) were not statistically different, covering a range between 4.6 to 14.2 mg a.i./kg (Table 3.12). No data are available from the tests with tropical natural soil (TNS) due to the insufficient number of juveniles in the control (see Appendix 14). The dose response curve was relatively similar in the three soils, showing a steep decrease (Figures 3.17 - 3.19). No differences were found concerning the NOEC values: in all three soils it was the same (3.16 mg a.i./kg). With the exception of the highest concentration in the test with TASx (20 % at 100 mg a.i./kg), less than 10 % mortality of adults occurred in all three tests. This value is higher than in the respective acute test with the same concentration (there it was 2.5%), indicating that an increase in test duration may have lead to lower LC_{50} values. No effect on the biomass of adults was found in any of the three tests. During the 56 days of test, the moisture was kept near the initial value and the pH varied in 0.3 to 0.5 units up or down (Appendix 14).

Table 3.12: Chronic toxicity of tropical *Eisenia fetida* in carbendazim-dosed substrates: EC_{50} and its 95%-confidence limits, LOEC and NOEC (values in mg/kg).

	Effect on reproduction			
	OECD	**TASx**	**LUFA**	**TNS***
EC_{50}	14.2 [5.5 - 36.2]	4.6 [1.9 - 11.1]	9.6 [6.4 - 14.3]	n.d.
LOEC	10	10	10	n.d.
NOEC	3.16	3.16	3.16	n.d.

* tests were not valid

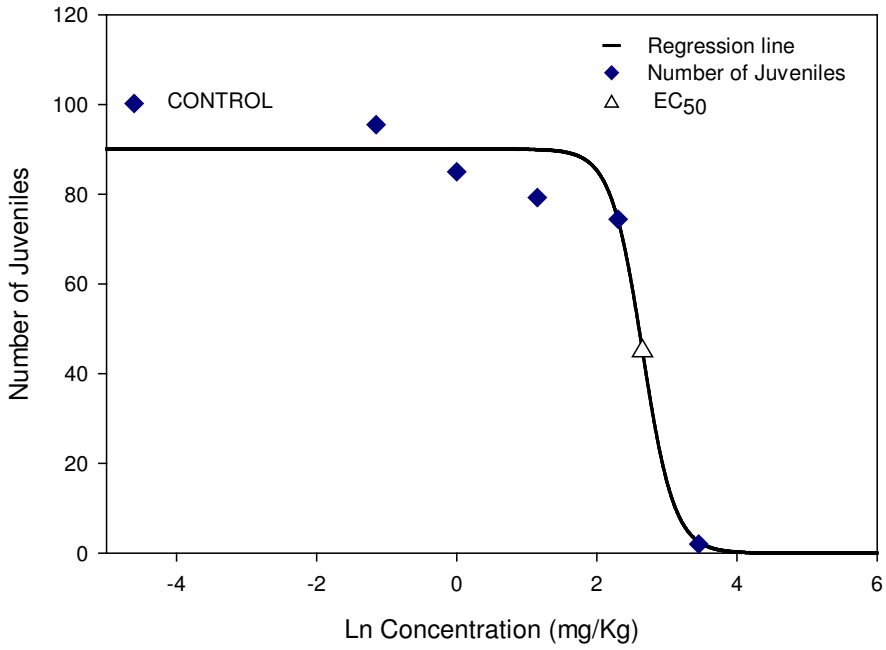


Figure 3.17: Dose response curve in the chronic toxicity test with tropical *Eisenia fetida* in carbendazim-dosed OECD soil.

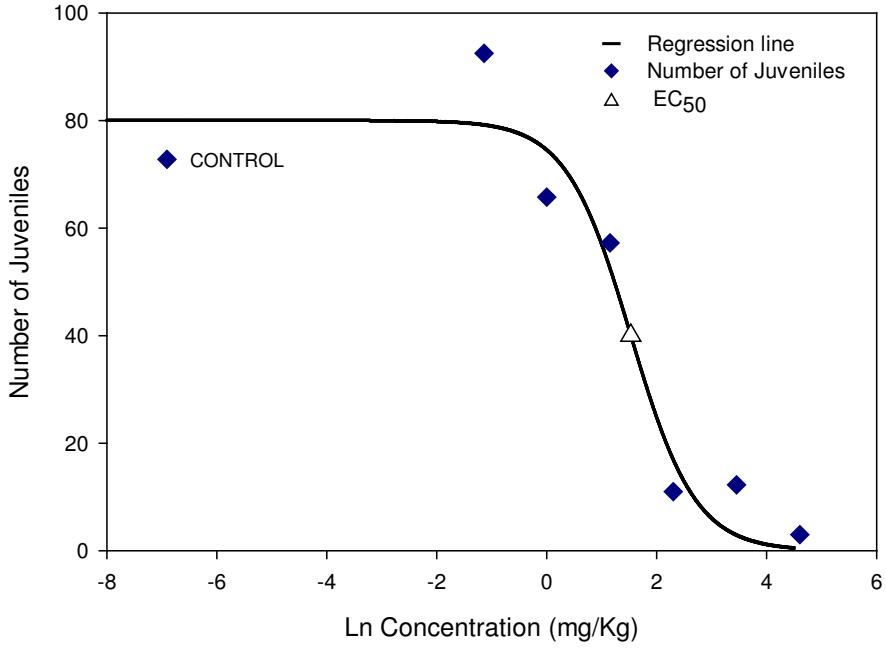


Figure 3.18: Dose response curve in the chronic toxicity test with tropical *Eisenia fetida* in carbendazim-dosed TASx soil.

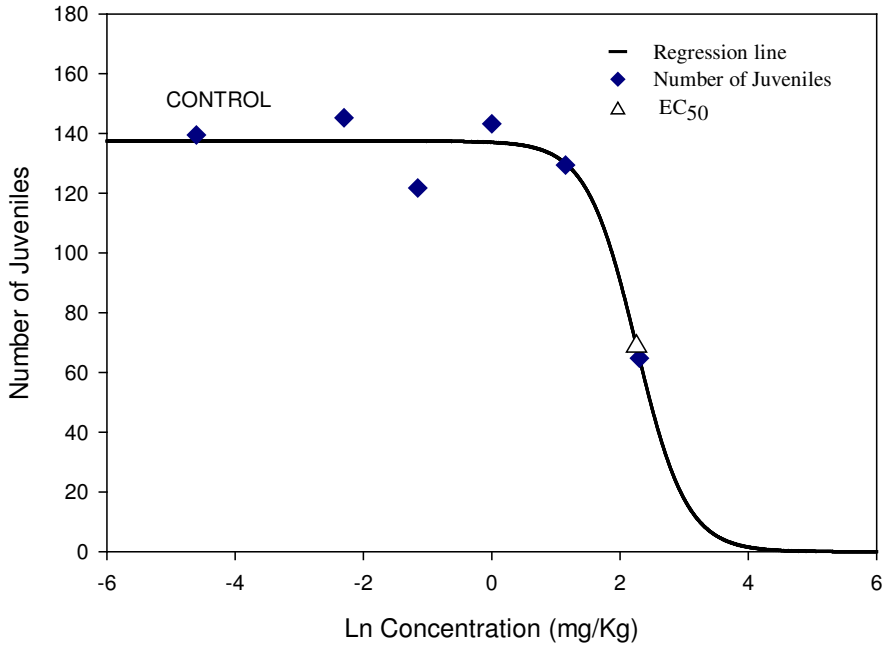


Figure 3.19: Dose response curve in the chronic toxicity test with tropical *Eisenia fetida* in carbendazim-dosed LUFA soil.

Avoidance tests

The avoidance behavior of the tropical *E. fetida* regarding carbendazim was tested in TASx soil at seven concentrations ranging from 1.0 to 1000 mg a.i./kg. The results indicate a significant avoidance behavior of earthworms to concentrations ≥ 1.0 mg a.i./kg (Figure 3.20). The EC_{50} values were determined as 33.3 mg a.i./kg [21.7 - 51.2, 95%-CL] and LOEC and NOEC values as < 1.0 mg a.i./kg. Tests with carbendazim were not valid in natural TNS soil due to the aggregating behavior or the high mortality rate of earthworms when exposed to this substrate. Values of mean net response are presented in Appendix 15.

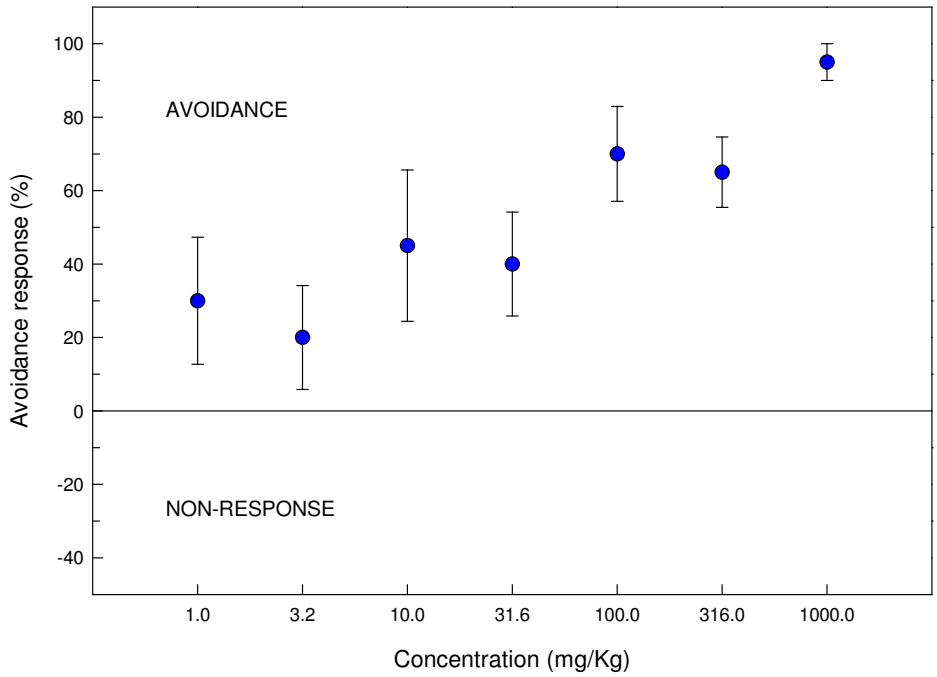


Figure 3.20: Avoidance response or non-response (negative) during the exposure of tropical *Eisenia fetida* to carbendazim concentrations in TASx soil (mean net response and standard error bars).

Effect of fungicide carbendazim on European *Eisenia fetida*

Acute tests

Preliminary tests (RF tests) of carbendazim with European *E. fetida* in OECD soil provided an LC_{50} value of 100 mg a.i./kg. With LUFA soil, no RF test was performed, but the data necessary to define the concentration range for the acute test came from the literature (Hund-Rinke et al. 2002). The acute effects of carbendazim on the European *E. fetida* were tested only in the LUFA soil. The original data of the acute test with OECD soil were provided by Dr. C.A.M. Van Gestel (a summary is published in Van Gestel et al. 1992). Significant mortality occurred in LUFA soil in the tested concentrations ranging from 0.2 to 10 mg a.i./kg. The LC_{50} value was determined as 4.1 mg a.i./kg [3.6 - 4.7, 95%-CL] and the LOEC and NOEC values as 3.6 and 1.3 mg a.i./kg, respectively. Likewise, a significant effect of carbendazim on mortality was observed in the OECD soil in the tested concentrations ranging from 0.6 to 60 mg a.i./kg. The LC_{50} value was determined as 5.8 mg a.i./kg [4.9 - 6.9, 95%-CL] and the LOEC and NOEC values as 6.0 and 1.9 mg a.i/kg. The dose response curves of

carbendazim in both soils fitted properly to the data (Figures 3.21 and 3.22). Despite the small difference between the LC_{50} values for both soils, they are considered to be different (no overlap of the confidence limits). In the LUFA soil test, the biomass loss of earthworms did not statistically differ from the control. In the test with OECD soil, the biomass of earthworms increased, i.e., it did not decrease as would have been expected. This was due to the special conditions used by the author (Van Gestel) in this experiment, i.e., the earthworms were fed in order to evaluate the effect on reproduction simultaneously. Neither the abiotic parameters nor the biological effect data show high variability (see Appendix 16).

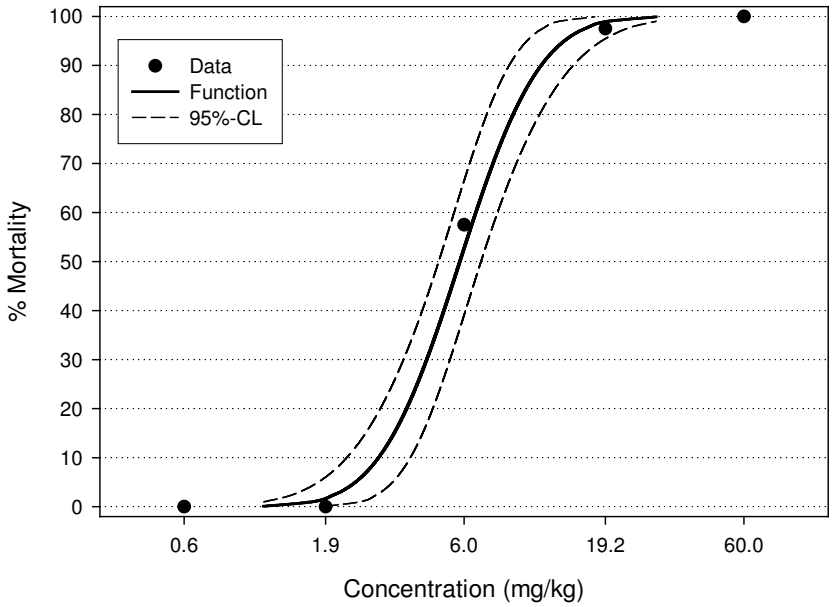


Figure 3.21: Dose response curve for the acute toxicity test with European *Eisenia fetida* in carbendazim-dosed OECD soil. (Data from Van Gestel et al. 1992).

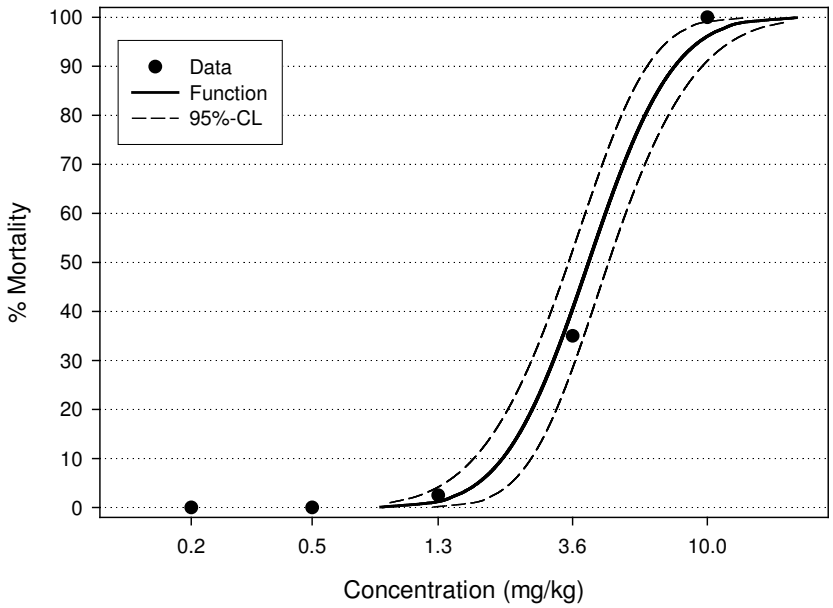


Figure 3.22: Dose response curve for the acute toxicity test with European *Eisenia fetida* in carbendazim-dosed LUFA soil.

Chronic tests

The chronic effects of carbendazim on the European *E. fetida* were tested in the OECD and LUFA soils. Significant reduction of the number of juveniles was observed for both tested soils (Figures 3.23 and 3.24). For the artificial OECD soil, the test was performed only with three concentrations, 1.0, 3.0 and 5.0 mg a.i./kg. The EC_{50} value was determined as 2.7 mg a.i./kg [95%-CL n.d.] and LOEC and NOEC values as 1.0 and 0.1 mg a.i./kg, respectively. The test in LUFA soil was done using a dose response design with concentrations ranging from 0.2 to 10 mg a.i./kg. The EC_{50} value was determined as 0.6 mg a.i./kg [0.1 - 4.1, 95%-CL] and LOEC and NOEC values as 1.3 and 0.5 mg a.i./kg, respectively. Despite the fact that the mean number of juveniles was slightly lower than the accepted limit (≥30), the test was considered to be valid due to its relatively low variance (CV). During the 56 days of test, the moisture was kept near the initial value and the pH varied in 0.3 to 0.5 units up or down (see Appendix 17).

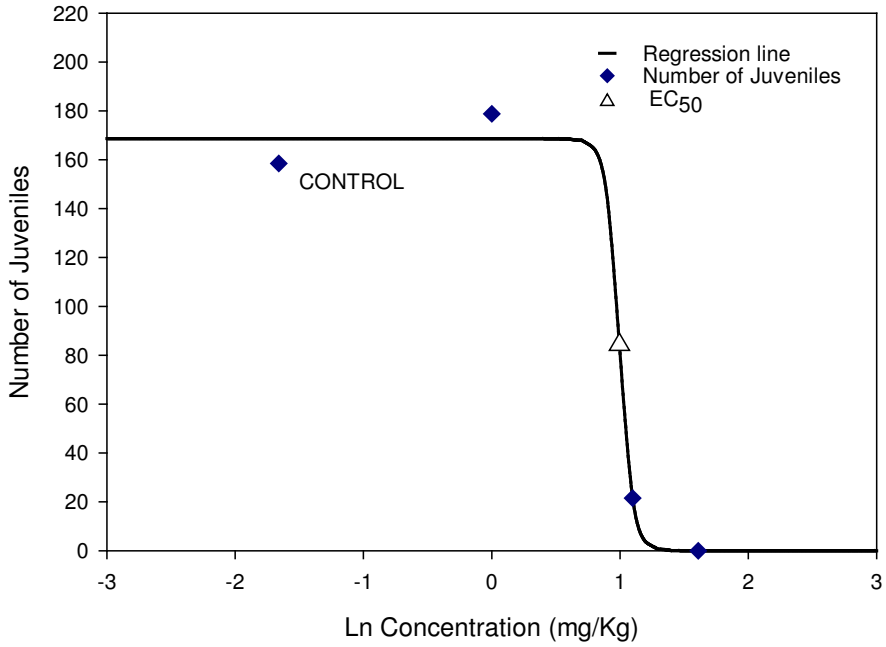


Figure 3.23: Dose response curve in the chronic toxicity test with European *Eisenia fetida* in carbendazim-dosed OECD soil.

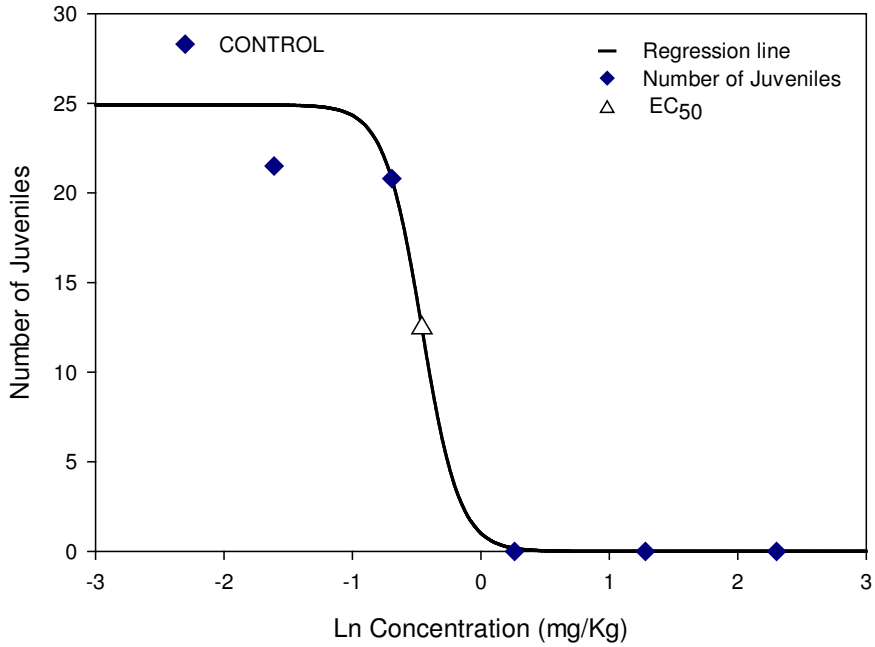


Figure 3.24: Dose response curve in the chronic toxicity test with European *Eisenia fetida* in carbendazim-dosed LUFA soil.

Avoidance tests

The effect on the avoidance behavior of the European *E. fetida* regarding carbendazim was tested in OECD and LUFA soils, at seven concentrations ranging from 1.0 to 1000 mg a.i./kg. The results indicate a similar tendency as in acute and chronic tests, i.e., the chemical was more toxic (available) in natural than in artificial soils. In natural LUFA soil the earthworms showed significant avoidance behavior at the lowest concentration (1.0 mg a.i./kg), while in artificial OECD soil they reacted strongly only at the concentration of 100 mg a.i./kg (Figures 3.25 to 3.26). The EC_{50} value for LUFA soil was determined as 7.1 mg a.i./kg [0.7 - 69.2, 95%-CL] and the LOEC and NOEC values as < 1.0 mg a.i./kg. In OECD soil the EC_{50} value was 127.4 mg a.i./kg [90.1 - 179.6, 95%-CL] and the LOEC and NOEC values were determined as < 1.0 mg a.i./kg. The EC_{50} values were considered to be different among the tested soils (no overlap of the confidence limits). Values of mean net response are presented in Appendix 18.

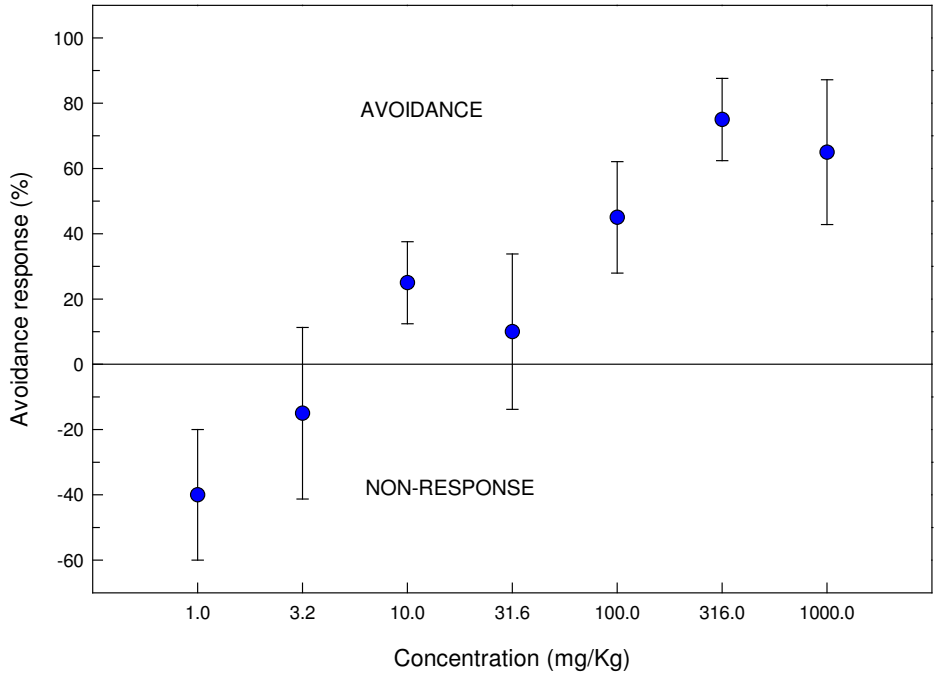


Figure 3.25: Avoidance response or non-response (negative) during the exposure of European *Eisenia fetida* to carbendazim concentrations in OECD soil (mean net response and standard error bars).

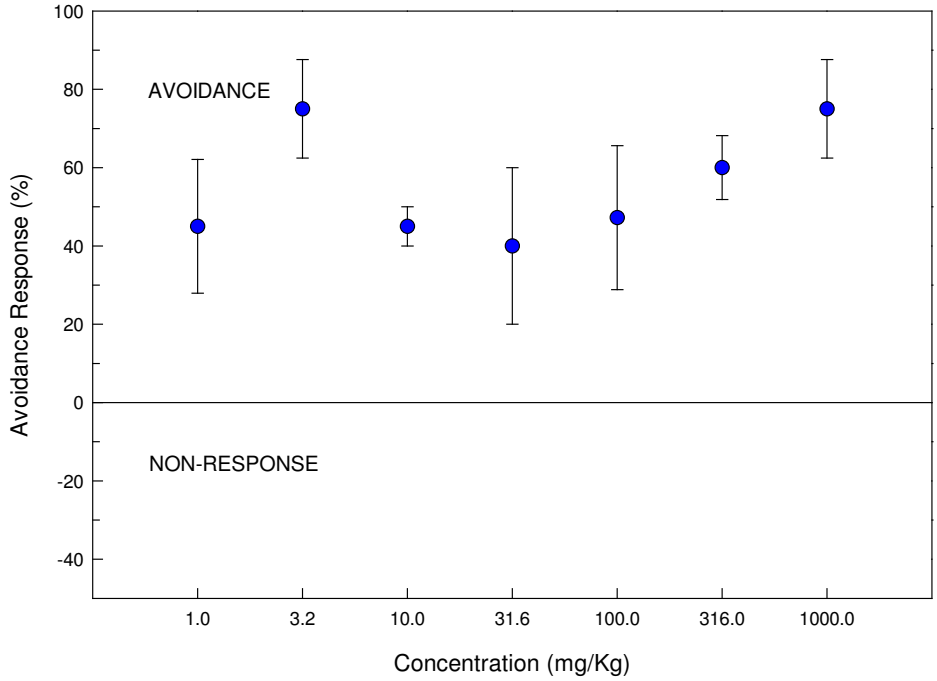


Figure 3.26: Avoidance response or non-response (negative) during the exposure of European *Eisenia fetida* to carbendazim concentrations in LUFA soil (mean net response and standard error bars).

Effect of insecticide lambda-cyhalothrin on tropical *Eisenia fetida*

Acute tests

The toxicity of lambda-cyhalothrin to tropical *E. fetida* was tested in preliminary assays (range-finding (RF) tests). The RF tests provided LC_{50} values of 100 mg a.i./kg for LUFA soil and 31.6 mg a.i./kg for tropical TASx and TNS soils. For OECD soil, the RF test was not performed, but the value is based on literature data. The concentrations used for the acute tests were derived from the RF test results. The acute effects of lambda-cyhalothrin on the tropical variant of *E. fetida* were tested in all four test soils. The dose response curves did not fit properly to the data of all tests (Figures 3.27 - 3.30). For the test with LUFA soil, no confidence limits could be estimated. In the test with OECD soil, the number of intermediate responses was not enough to fit a curve, despite the fact that LC_{50} was estimated. Significant effects on mortality and biomass development were observed in the tested range of concentrations (1 - 100 mg a.i./kg for the artificial soils and 3.16 - 316 and 6.25 - 100 mg a.i./kg for the field soils, respectively). The LC_{50} value in OECD artificial soil was 9.6 times higher than in the

TASx (228.7 versus 23.9 mg a.i./kg) and about 3.5 times higher than in the two field soils (228.7 versus 68.5 and 65.0 mg a.i./kg, respectively). LC_{50} values of the OECD soil and the three other soils were assumed to be different, but there was no such difference between TASx and the two natural soils (LUFA and TNS) (Table 3.13). Accordingly, the estimated LOEC and NOEC values for mortality were also higher in OECD soil than in TASx and TNS, but not in LUFA soil (Table 3.14). The results concerning the loss of biomass are more complex: in three out of four tests (with OECD, LUFA, and TNS soil), statistically significant effects were found at lower concentrations than for mortality. Consequently, the NOEC values were low (3.16 (TASx), 10 (OECD, LUFA) and 25 (TNS) mg a.i./kg), but quite similar considering the great differences in LC_{50} values (Table 3.14). However, these results on biomass have to be viewed with caution, since already in the controls of the OECD soil (slightly) but mainly of the two tropical soils, a loss of biomass (up to 33.7% in TASx) occurred. In the case of the TNS, the low pH might have negatively influenced the worms in all treatments (including the controls), but for the OECD and TASx soils there is no obvious reason why a relatively high loss of biomass occurred even in the controls. Neither the abiotic parameters nor the biological effect data show a high variability (see Appendix 19). Taking mortality and biomass results together, it seems that the worms clearly reacted most in the TASx soil, while the effects of the two field soils and, in particular, OECD soil were less pronounced.

Table 3.13: Acute toxicity of tropical *Eisenia fetida* in lambda-cyhalothrin-dosed substrates: median lethal concentrations - LC_{50} values (mg/kg).

Soil		LC_{50}	95 % Confidence Limits (CL)		CL - overlapping			
			Lower	Upper	1	2	3	4
1	**OECD**	228.7	131.4	> 1000	*			
2	**TASx**	23.8	19.3	29.5		*		
3	**LUFA**	68.5	55.8	84.2			*	*
4	**TNS**	65.0	60.3	70.2			*	*

Table 3.14: Acute toxicity of tropical *Eisenia fetida* in lambda-cyhalothrin-dosed substrates: LOEC and NOEC values (mg/kg).

	OECD		TASx		LUFA		TNS	
	Mortality	Biomass	Mortality	Biomass	Mortality	Biomass	Mortality	Biomass
LOEC	100	31.6	31.6	10	100	31.6	50	50
NOEC	31.6	10	10	3.16	31.6	10	25	25

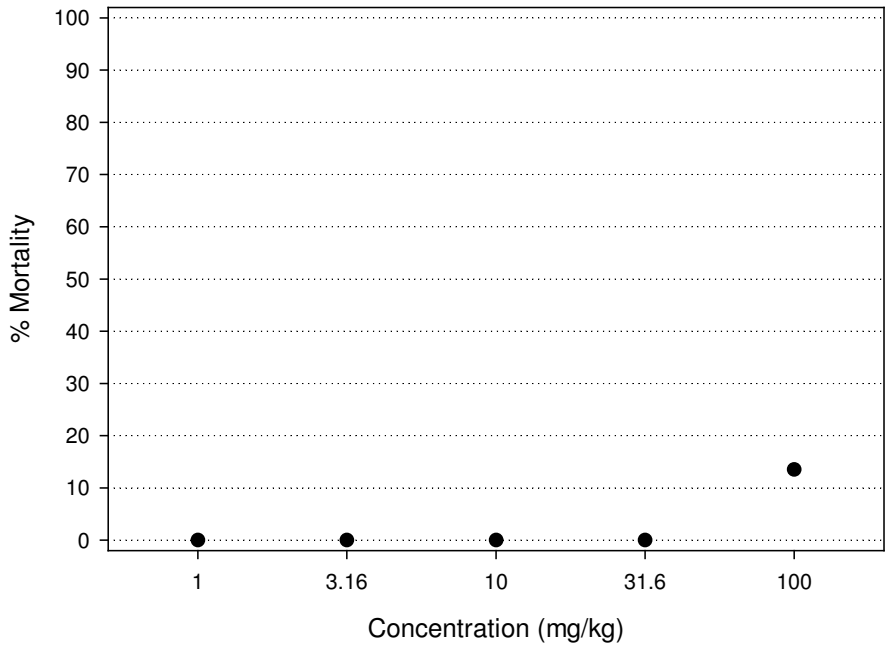


Figure 3.27: Mortality of tropical *Eisenia fetida* in lambda-cyhalothrin-dosed OECD soil (after Abbott's correction) (data not appropriate for probit analysis).

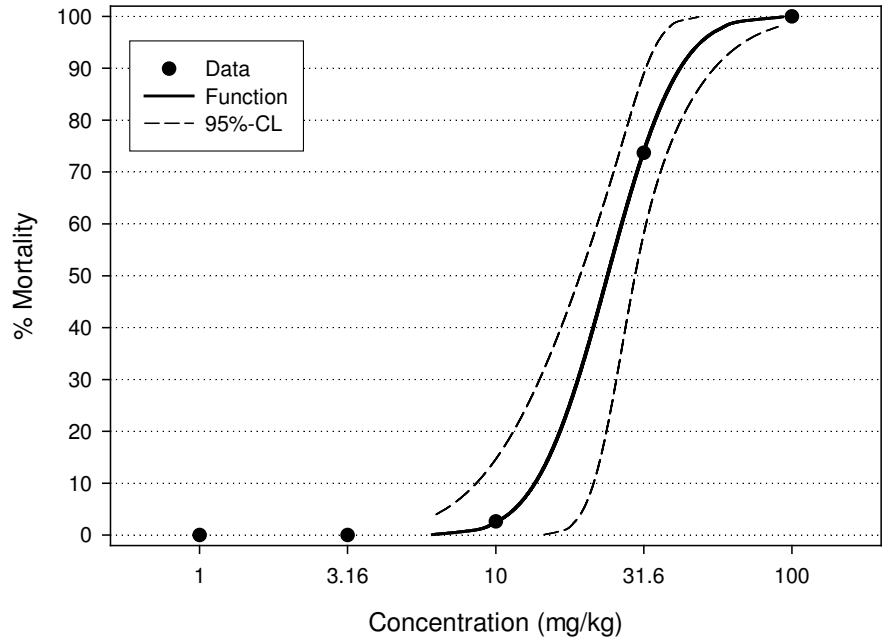


Figure 3.28: Dose response curve for the acute toxicity with tropical *Eisenia fetida* in lambda-cyhalothrin-dosed TASx soil (after Abbott's correction).

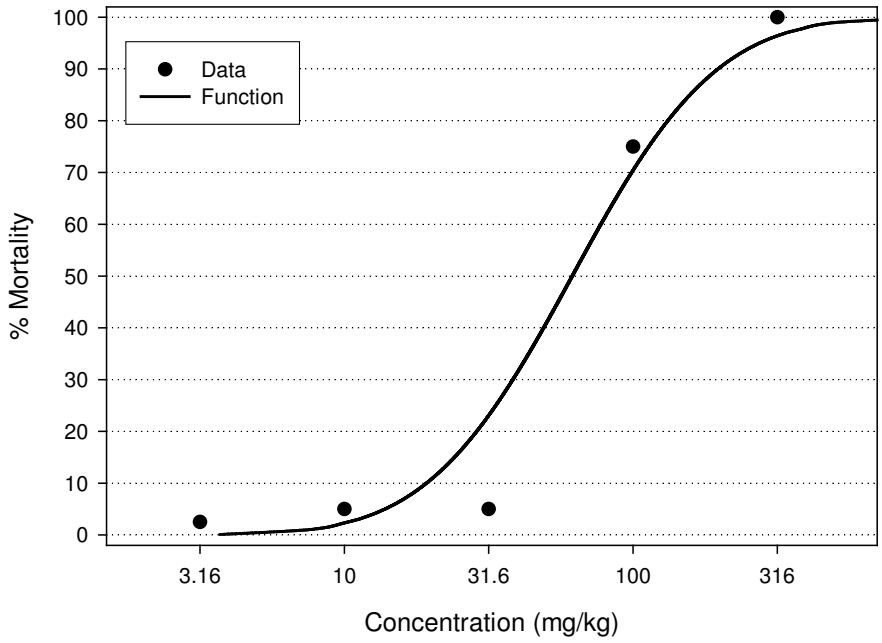


Figure 3.29: Dose response curve for the acute toxicity with tropical *Eisenia fetida* in lambda-cyhalothrin-dosed LUFA soil (95%-CL not determined).

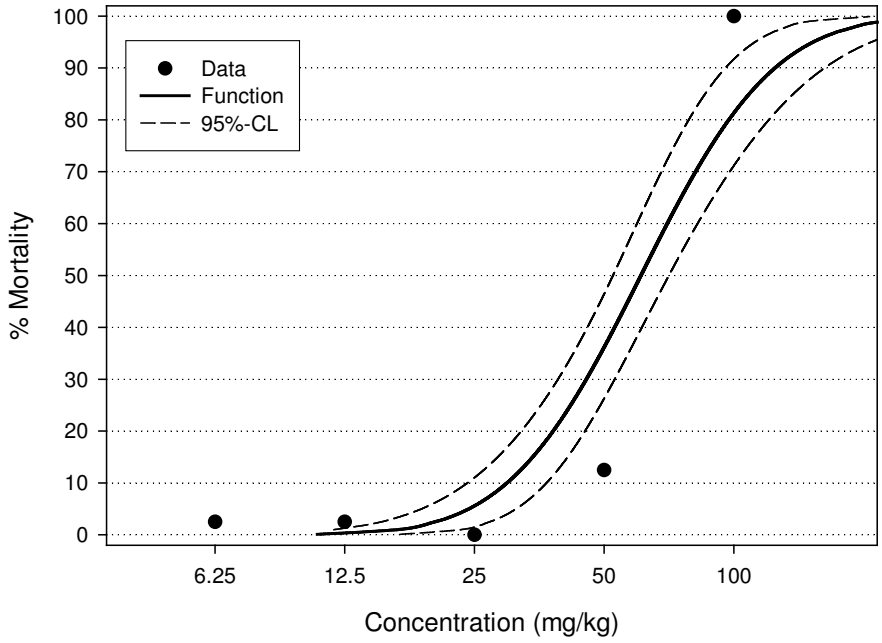


Figure 3.30: Dose response curve for the acute toxicity with tropical *Eisenia fetida* in lambda-cyhalothrin-dosed TNS soil.

Chronic tests

In the chronic tests with lambda-cyhalothrin and the tropical variant of *E. fetida* covering a range of 1 - 316 mg a.i./kg, the effect on the number of juveniles did not differ strongly from the picture seen in the acute tests. The EC_{50} values in OECD soil and in natural LUFA soil were about the same (60.2 and 54.1 mg a.i./kg, respectively), while the EC_{50} value in the test with TASx was about 7.5 times lower than the other two. Accordingly, no statistical difference was found between OECD and LUFA, but both differed from TASx (Table 3.15). No data are available from the tests with tropical natural soil (TNS) due to the insufficient number of juveniles in the control (see Appendix 20). The dose response curve was relatively similar in all three soils tested, showing a steep decrease (Figures 3.31 - 3.33). On the other hand, the NOEC values are more closely together: in TASx 6.25 mg a.i./kg, in LUFA 10 mg a.i./kg and in OECD 31.6 mg a.i./kg. Less than 10% mortality of adults occurred in all three tests except for the highest concentration in the test with OECD soil, where 80% of the worms died at 316 mg a.i./kg (which is in accordance with the acute test). Starting at 100 mg a.i./kg, a decrease in biomass was observed in the tests with OECD and LUFA soils, while in TASx even an increase at the highest concentration occurred. During the 56 days of test, the moisture was kept near the initial value and the pH varied in 0.3 to 0.5 units up or down (Appendix 20).

Table 3.15: Chronic toxicity of tropical *Eisenia fetida* in lambda-cyhalothrin-dosed substrates: EC_{50} and its 95%-confidence limits, LOEC and NOEC (values in mg/kg).

	Effect on reproduction			
	OECD	**TASx**	**LUFA**	**TNS***
EC_{50}	60.2 [54.3 - 66.8]	7.7 [3.2 - 18.3]	54.1[26.5 - 110.4]	n.d.
LOEC	100	12.5	31.6	n.d.
NOEC	31.6	6.25	10	n.d.

*tests were not valid

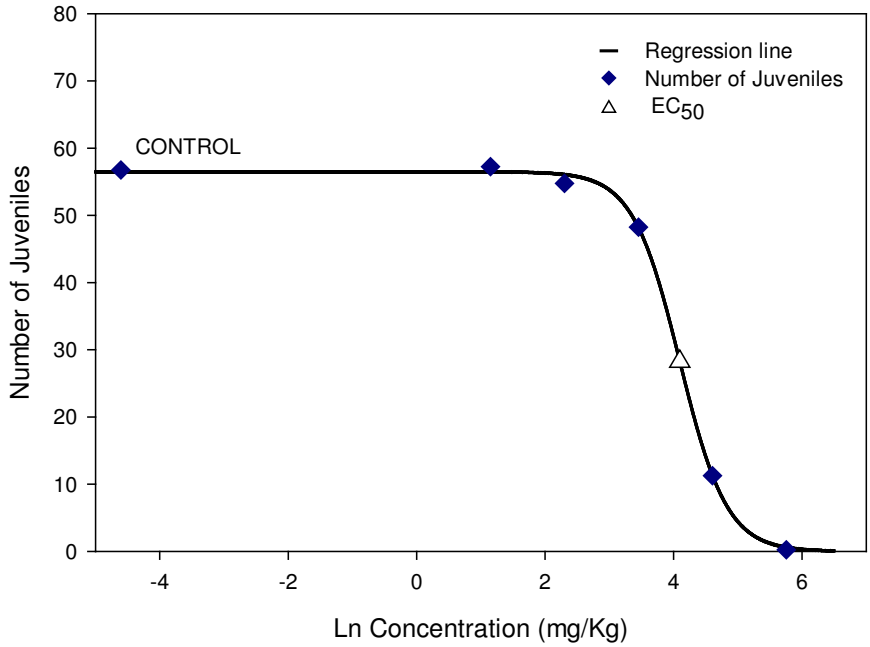


Figure 3.31: Dose response curve for the chronic toxicity test with tropical *Eisenia fetida* in lambda-cyhalothrin-dosed OECD soil.

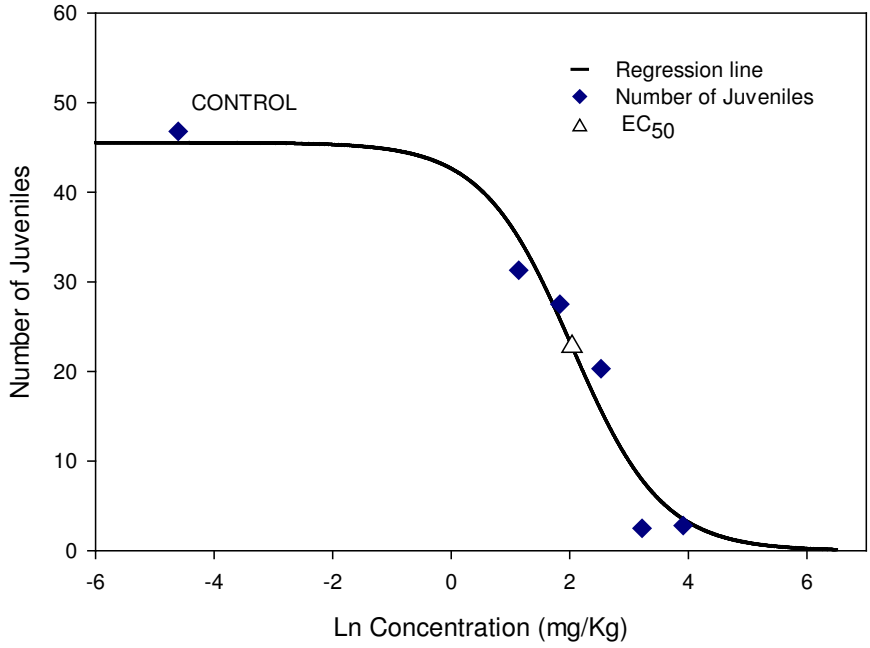


Figure 3.32: Dose response curve for the chronic toxicity test with tropical *Eisenia fetida* in lambda-cyhalothrin-dosed TASx soil.

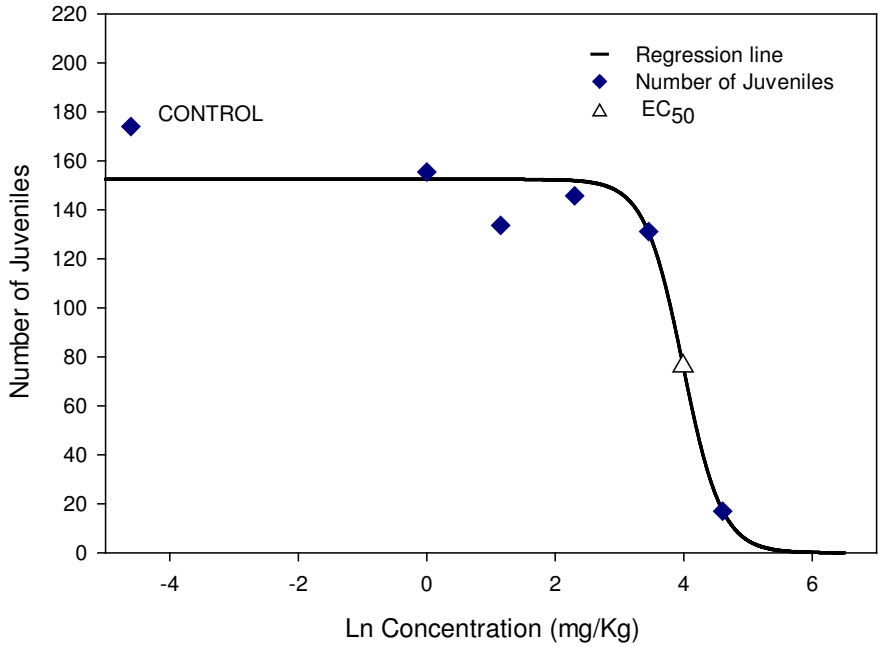


Figure 3.33: Dose response curve for the chronic toxicity test with tropical *Eisenia fetida* in lambda-cyhalothrin-dosed LUFA soil.

Avoidance tests

The avoidance behavior of the tropical *E. fetida* regarding lambda-cyhalothrin was tested in TASx soil at six concentrations ranging from 0.32 to 100 mg a.i./kg. The earthworms showed significant avoidance behavior in the lowest concentration (0.32 mg/kg) (Figure 3.34). The EC_{50} value was determined as 0.2 mg a.i./kg [0.0 - 0.8, 95%-CL] and the LOEC and NOEC values as ≤ 0.32 mg a.i./kg. Tests were not valid in natural TNS soil due to the aggregating behavior or the high mortality rate of earthworms when exposed to this substrate. Values of mean net response are presented in Appendix 21.

Effect of insecticide lambda-cyhalothrin on European *Eisenia fetida*

Acute tests

Preliminary tests (RF tests) of lambda-cyhalothrin with European *E. fetida* provided LC_{50} values of 316 mg a.i./kg for OECD and LUFA soils. The concentrations used for the acute tests were derived from the RF test results. The acute effects of lambda-cyhalothrin on the European *E. fetida* were tested in OECD and LUFA soil. In the OECD soil a dose response test design with a range of 2 to 632 mg a.i./kg was used.

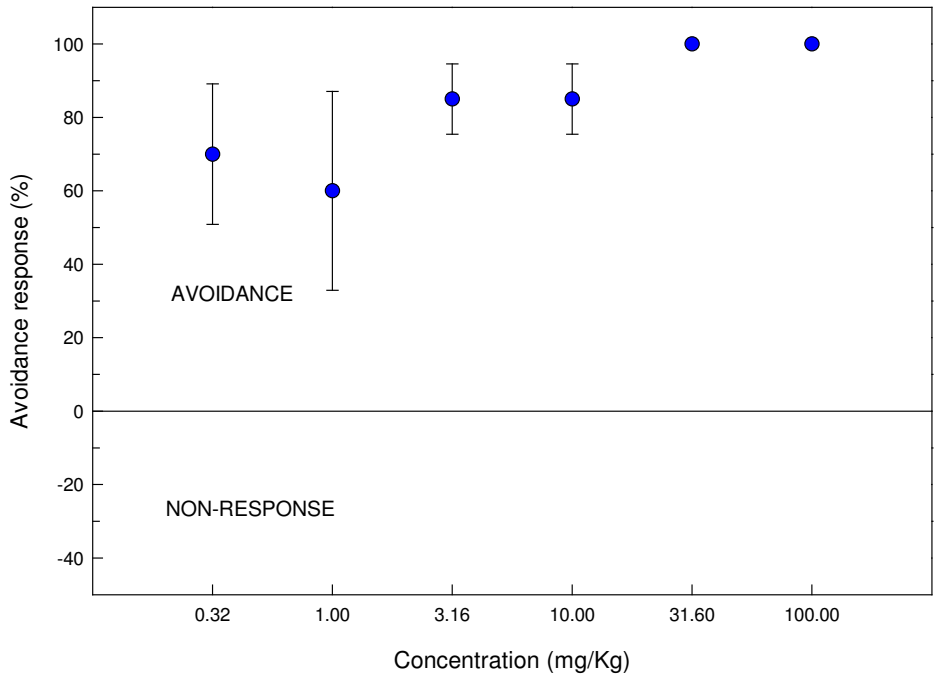


Figure 3.34: Avoidance response or non-response (negative) during the exposure of tropical *Eisenia fetida* to lambda-cyhalothrin concentrations in TASx soil (mean net response and standard error bars).

The LC_{50} value was determined as 99.8 mg a.i/kg [95%-CL, n.d.] and LOEC and NOEC values as 200 and 63.2 mg a.i./kg, respectively. Significant effects on mortality occurred only at the two highest concentrations. The dose response curve did not fit properly to the data and the confidence limits could not be estimated (Figure 3.35). The biomass loss was statistically different from the control at the concentrations of 20 and 63.2 mg a.i./kg (Figure 3.36). In the LUFA soil, tested in a range of concentrations from 10 to 1000 mg a.i./kg, the LC_{50} value was determined as 139.9 mg a.i./kg [113.1 - 173.0, 95%-CL] and LOEC and NOEC values as 100 and 31.6 mg a.i./kg, respectively. The LC_{50} values from both soils did not differ statistically. The biomass loss was statistically different from the control at 31.6 mg a.i./kg. In both soils, the computation for biomass change was not applicable (n.a.) in the highest concentrations, due to the excessive mortality of earthworms (see Appendix 22).

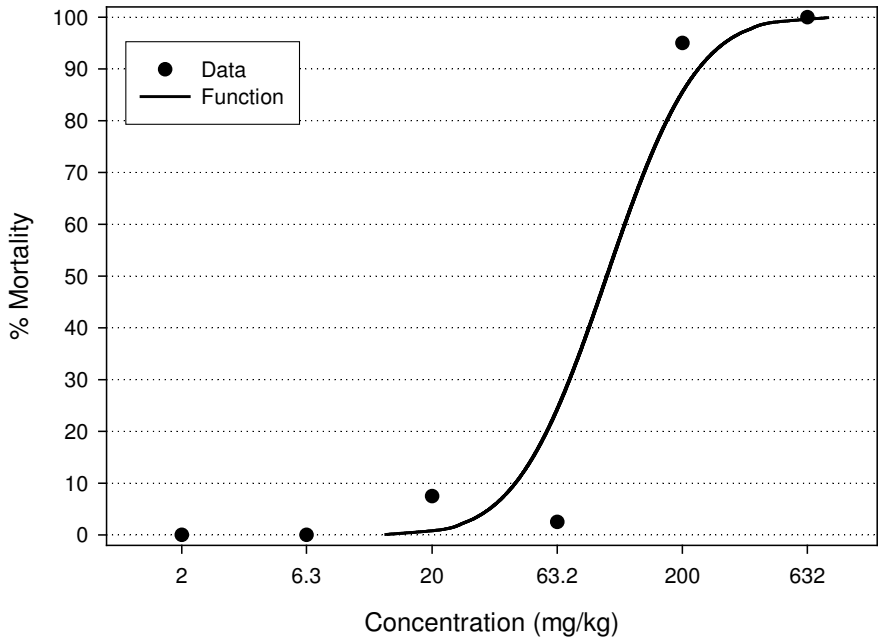


Figure 3.35: Dose response curve for the acute toxicity test with European *Eisenia fetida* in lambda-cyhalothrin-dosed OECD soil (95%-CL not determined).

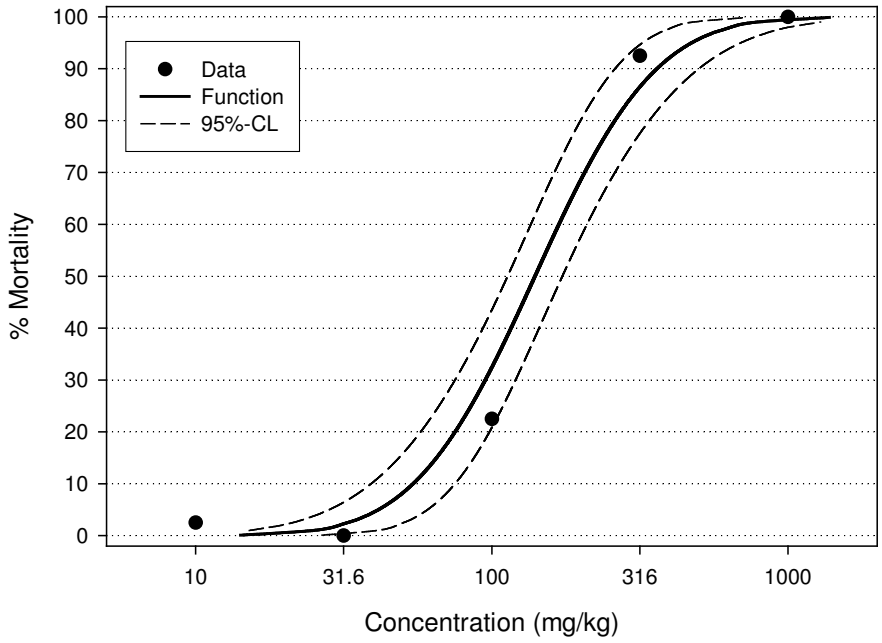


Figure 3.36: Dose response curve for the acute toxicity test with European *Eisenia fetida* in lambda-cyhalothrin-dosed LUFA soil.

Chronic tests

The chronic effects of lambda-cyhalothrin on the European *E. fetida* were tested in OECD and LUFA soils in a dose response design with concentrations ranging from 1 to 100 mg a.i./kg. A significant reduction in the number of juveniles was observed for both tested soils (Figures 3.37 - 3.38). In the OECD soil, the EC_{50} was determined as 37.4 mg a.i./kg [21.4 - 65.2, 95%-CL] and the LOEC and NOEC values as 31.6 and 10 mg a.i./kg, respectively. In the LUFA soil, the EC_{50} was determined as 44.5 mg a.i./kg [1.9 - 1004, 95%-CL] and a LOEC and NOEC values as 10 and 3.16 mg a.i./kg, respectively. The EC_{50} values in both soils were not statistically different. During the 56 days of test, the moisture was kept near the initial value and the pH varied in 0.3 to 0.5 units up or down (see Appendix 23).

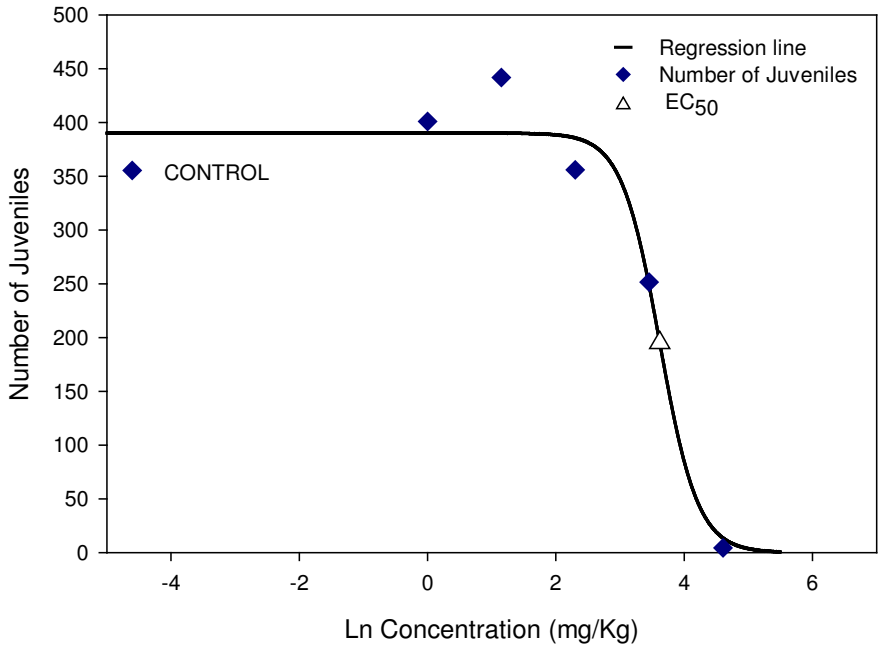


Figure 3.37: Dose response curve in the chronic toxicity test with European *Eisenia fetida* in lambda-cyhalothrin-dosed OECD soil.

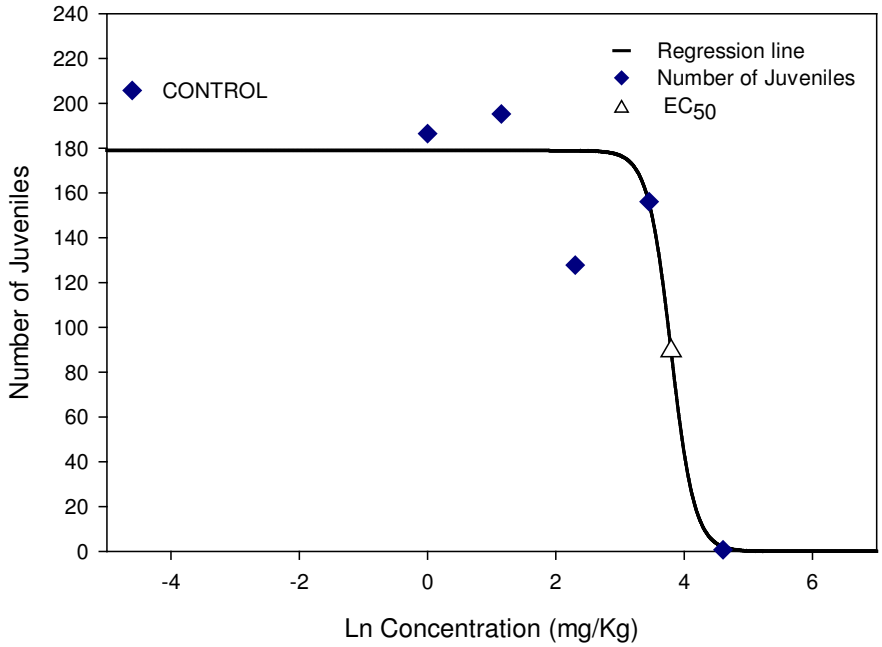


Figure 3.38: Dose response curve in the chronic toxicity test with European *Eisenia fetida* in lambda-cyhalothrin-dosed LUFA soil.

Avoidance tests

The avoidance behavior of European *E. fetida* regarding lambda-cyhalothrin was tested in OECD and LUFA soils, at six concentrations ranging from 0.32 to 100 mg a.i./kg. The results indicate a similar tendency as in acute and chronic tests, i.e., the chemical was more toxic (available) in natural than in artificial soils. In natural LUFA soil, the earthworms showed significant avoidance behavior in the lowest concentration (0.32 mg a.i./kg) while in artificial OECD soil they strongly reacted at the concentration of 3.16 mg a.i./kg (Figures 3.39 - 3.40). The EC_{50} value for LUFA soil was determined as 0.5 mg a.i./kg [0.4 - 0.6, 95%-CL] and the LOEC and NOEC values as $\leq$ 0.32 mg a.i./kg. In OECD soil the EC_{50} value was 2.3 mg a.i./kg [2.9 - 3.7, 95%-CL] and the LOEC and NOEC was determined as 3.16 and 1.0 mg a.i./kg, respectively. The EC_{50} values were considered to be different among the tested soils (no overlap of the confidence limits). Values of mean net response are presented in Appendix 24.

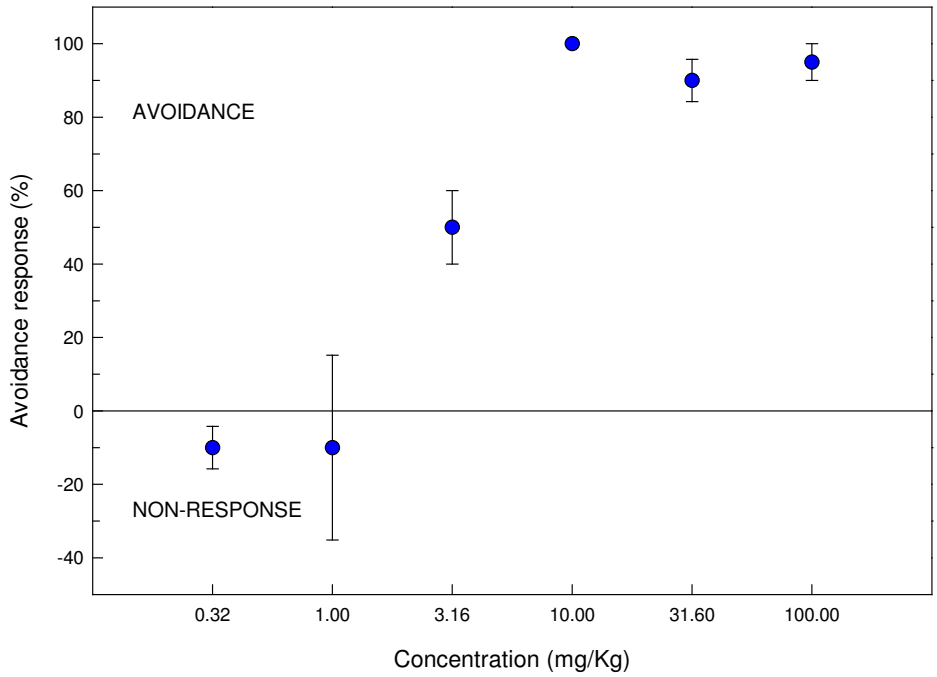


Figure 3.39: Avoidance response or non-response (negative) during the exposure of European *Eisenia fetida* to lambda-cyhalothrin concentrations in OECD soil (mean net response and standard error bars).

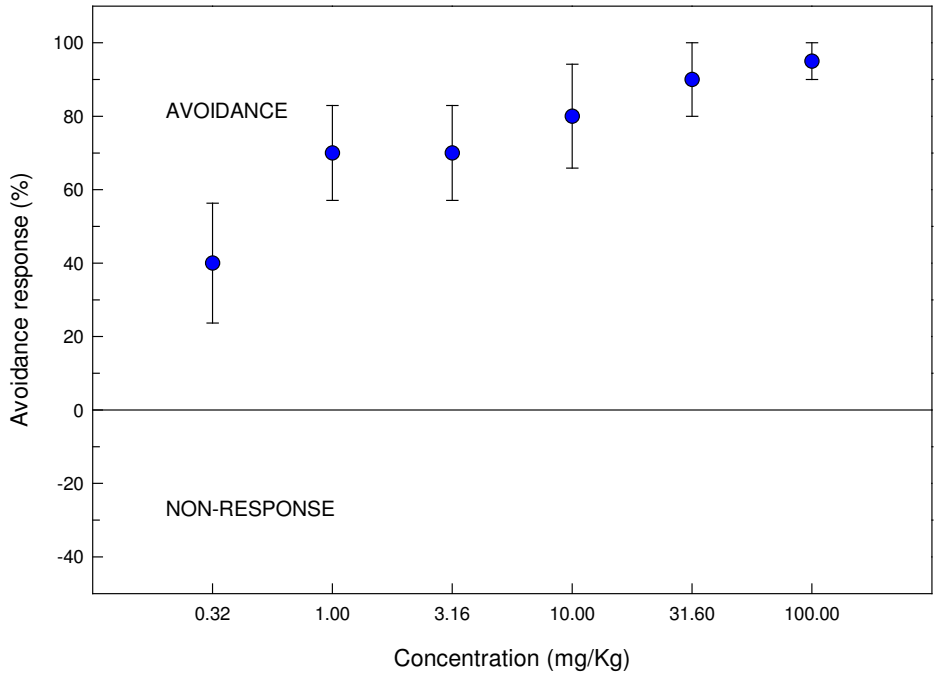


Figure 3.40: Avoidance response or non-response (negative) during the exposure of European *Eisenia fetida* to lambda-cyhalothrin concentrations in LUFA soil (mean net response and standard error bars).

3.2.2 *Eisenia fetida*: special tests

Acute tests in factorial design

Results of preliminary tests (no replicates) performed according to a factorial design with European standard soils showed that the toxicity (mortality) of carbendazim depends on temperature, type of soil and the origin of *E. fetida*, whereas in lambda-cyhalothrin tests the soil type was the main factor affecting both mortality and biomass (Table 3.16). Results of preliminary factorial design tests performed with tropical soils indicated a significant influence of the three factors but only for biomass in the carbendazim test (Table 3.17). Since the factor "soil" is completely covered (all combinations of species and chemicals were tested in all soils in laboratory tests) it was not considered in the definitive factorial test.

The results of the definitive factorial design test (with three replicates) indicate that:

- The effect of carbendazim on mortality of *E. fetida* depends on the temperature, i.e., higher mortality at 20 ºC than at 28 ºC, independent of the European or tropical origin of the earthworms. When testing the high concentration, the effect on biomass loss of the earthworms at day 7 also depends on the temperature, i.e., a higher loss of biomass occurred at 20 ºC than at 28 ºC. In the control and at the low concentration, mortality and biomass loss were not influenced by the tested factors (Tables 3.18 to 3.20);
- The effect of lambda-cyhalothrin on the mortality of *E. fetida* depends on its origin, i.e., higher mortality occurs in tropical than in European *Eisenia*, independent of temperature. The biomass loss of earthworms at the low concentration at day 7 depends also on their origin. A significant interaction between temperature and origin was found for biomass loss when testing the low concentration. At the high concentration, the computation for biomass change was not applicable (n.a.), due to the mortality of tropical *Eisenia* at day 7 (Tables 3.21 to 3.23).

Table 3.16: Acute toxicity test (preliminary) in factorial design for European and tropical *Eisenia fetida* in two soils (OECD and LUFA) at two temperatures (20 ºC and 28 ºC).

Factors	**Carbendazim**		**Lambda-cyhalothrin**	
	Mortality	Biomass loss	Mortality	Biomass loss
Origin	*	n.s.	n.s.	n.s.
Soil	*	n.s.	*	*
Temperature	**	n.s.	n.s.	n.s.

* (P = 0.05) and ** (P = 0.01)

Table 3.17: Acute toxicity test (preliminary) in factorial design for European and tropical *Eisenia fetida* in two soils (TASx and TNS) at two temperatures (20 ºC and 28 ºC).

Factors	**Carbendazim**		**Lambda-cyhalothrin**	
	Mortality	Biomass loss	Mortality	Biomass loss
Origin	n.s.	*	n.s.	n.s.
Soil	n.s.	*	n.s.	n.s.
Temperature	n.s.	*	n.s.	n.s.

* (P = 0.05)

Table 3.18: Acute toxicity test in factorial design for European *Eisenia fetida* in carbendazim-dosed TASx soil.

Treatment	**Temperature (ºC)**	**Mortality (%)**	**Biomass (mg) Day 0**	**Biomass (mg) Day 7**	**Percent of the initial weight**	**Moisture (%)**	**pH**
Control	20	13.3	407.6 ± 9.7	353.8 ± 48.9	86.8	34.6	6.8
10 mg/kg	20	3.3	365.5 ± 46.9	306.2 ± 54.0	83.8	34.0	6.8
1000 mg/kg	20	80.0	363.8 ± 30.4	231.0 ± 23.8	63.5	32.8	6.8
Control	28	13.3	386.6 ± 24.4	316.0 ± 18.7	81.7	32.7	6.8
10 mg/kg	28	0.0	356.3 ± 27.5	312.6 ± 14.6	87.7	34.0	6.8
1000 mg/kg	28	13.3	356.3 ± 21.6	267.2 ± 16.2	75.0	31.4	6.8

Table 3.19: Acute toxicity test in factorial design for tropical *Eisenia fetida* in carbendazim-dosed TASx soil.

Treatment	Temperature (ºC)	Mortality (%)	Biomass (mg) Day 0	Biomass (mg) Day 7	Percent of the initial weight	Moisture (%)	pH
Control	20	16.7	410.9 ± 17.6	339.7 ± 18.4	82.7	32.2	6.9
10 mg/kg	20	3.3	411.2 ± 33.1	336.1 ± 26.8	81.7	33.0	6.6
1000 mg/kg	20	96.7	395.0 ± 28.7	216.0 ± 18.6	54.7	32.2	6.2
Control	28	13.3	418.0 ± 16.4	323.0 ± 15.3	77.3	32.1	6.8
10 mg/kg	28	0.0	421.5 ± 50.8	349.8 ± 33.8	83.0	32.7	6.8
1000 mg/kg	28	3.3	415.8 ± 39.7	317.0 ± 24.6	76.2	31.7	6.7

Table 3.20: Acute toxicity test in factorial design for *Eisenia fetida* in carbendazim-dosed TASx soil - effects influenced by test temperature or its origin.

Factors	Survival			Biomass Change 7d		
	Control	10mg/kg	1000 mg/kg	Control	10 mg/kg	1000 mg/kg
Temperature	n.s.	n.s.	** (P < 0.001)	n.s.	n.s.	** (P = 0.002)
Origin	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Temperature x Origin	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

* (P = 0.05) and ** (P = 0.01) by Tukey's multiple comparison procedure

Table 3.21: Acute toxicity test in factorial design for European *Eisenia fetida* in lambda-cyhalothrin-dosed TASx soil.

Treatment	Temperature (ºC)	Mortality (%)	Biomass (mg) Day 0	Biomass (mg) Day 7	Percent of the initial weight	Moisture (%)	pH
Control	20	0.0	506.0 ± 18.0	409.2 ± 8.3	80.9	34.5	6.8
10 mg/kg	20	0.0	439.2 ± 31.4	314.8 ± 21.9	71.7	34.2	6.9
50 mg/kg	20	6.7	443.0 ± 58.9	285.3 ± 32.8	64.4	31.7	6.7
Control	28	3.3	471.7 ± 24.3	373.1 ± 22.8	79.1	33.6	6.6
10 mg/kg	28	3.3	462.8 ± 32.2	324.2 ± 23.0	70.1	33.7	6.7
50 mg/kg	28	10.0	449.1 ± 25.1	279.3 ± 11.5	62.2	33.0	6.8

Table 3.22: Acute toxicity test in factorial design for tropical *Eisenia fetida* in lambda-cyhalothrin-dosed TASx soil.

Treatment	Temperature (ºC)	Mortality (%)	Biomass (mg) Day 0	Biomass (mg) Day 7	Percent of the initial weight	Moisture (%)	pH
Control	20	0.0	454.3 ± 31.8	362.6 ± 14.2	79.8	34.6	6.8
10 mg/kg	20	20.0	443.0 ± 13.5	276.7 ± 14.1	62.5	34.0	6.8
50 mg/kg	20	90.0	431.8 ± 18.2	n.a.	n.a.	32.4	6.9
Control	28	0.0	443.2 ± 21.0	341.7 ± 19.7	77.1	34.9	6.9
10 mg/kg	28	10.0	452.3 ± 41.6	296.3 ± 39.8	65.5	34.3	6.8
50 mg/kg	28	96.7	423.2 ± 7.1	n.a.	n.a.	33.1	6.7

Table 3.23: Acute toxicity test in factorial design for *Eisenia fetida* in lambda-cyhalothrin-dosed TASx soil - effects influenced by test temperature or its origin.

Factors	Survival			Biomass Change 7d		
	Control	10 mg/kg	50 mg/kg	Control	10 mg/kg	50 mg/kg
Temperature	n.s.	n.s.	n.s.	n.s.	n.s.	n.a.
Origin	n.s.	* (P = 0.012)	** (P < 0.001)	n.s.	** (P < 0.001)	n.a.
Temperature x Origin	n.s.	n.s.	n.s.	n.s.	* (P = 0.027)	n.a.

* (P = 0.05) and ** (P = 0.01) by Tukey's multiple comparison test

Tests using TASc (coir dust) artificial soil

The results of the avoidance experiments (Table 3.24) using the tropical artificial soil (TASc) show that:

- There is no statistical significant behavioral difference visible in the avoidance tests using OECD and TASc soil;
- The earthworms did not react (e.g., congregating behavior) when exposed only in TASc soil;
- The earthworms showed avoidance behavior, moving from the non-composted to the composted substrate.

Table 3.24: Avoidance behavior of tropical *Eisenia fetida* in TASc soil.

Frequency distribution of earthworms (%)		**Net Response (%)**	**Standard Error**	**Significance (*t*-test)**
OECD	**TASc (composted)**			
62.0	38.0	24.0	13.4	n.s. (P = 0.195), n = 5
TASc (composted)	**TASc (composted)**			
57.5	42.5	15.0	4.8	n.s. (P = 0.069), n = 4
TASc (composted)	**TASc (non-composted)**			
92.9	7.1	86.0	4.8	** (P = < 0.01), n = 7

The acute effects of lambda-cyhalothrin and carbendazim on the tropical variant of *E. fetida* were tested in TASc. Both tests were valid concerning the mortality, since only few worms died in the two controls (2.5 and 0%, respectively). However, the biomass criterion was missed slightly (about 23% loss in both tests). Neither the abiotic parameters nor the biological effect data showed a high variability (see Appendix 25).

In the test with lambda-cyhalothrin, significant effects on the mortality and the biomass development occurred in the tested range of concentrations (1 - 100 mg a.i./kg) (Figure 3.41). The LC_{50} value was determined as 11.0 mg a.i./kg [9.1 - 13.3, 95% CL] and the LOEC and NOEC values as 10 and 3.16 mg a.i./kg, respectively. In comparison to the results gained with the other tropical artificial soil (TASx, see Table 3.13), this value is considered to be different (no overlap of the confidence limits). Concerning biomass, the NOEC determined with TASc was lower than the lowest tested concentration (<1 mg a.i./kg), which is lower than the result for TASx (3.16 mg a.i./kg).

In the test with carbendazim, effects on the mortality and the biomass development in the tested range of concentrations (10 - 1000 mg a.i./kg) (Figure 3.42) were almost non-significant. The LC_{50} value was determined as > 1000 mg a.i./kg, which is in agreement with the result gained with TASx soil. Concerning biomass, the NOEC values also did not differ (316 mg a.i./kg here and ≥ 316 mg a.i./kg for TASx) (see Appendix 25).

Summarizing these results it seems that coir dust is an alternative to xaxim when preparing artificial soil in the tropics.

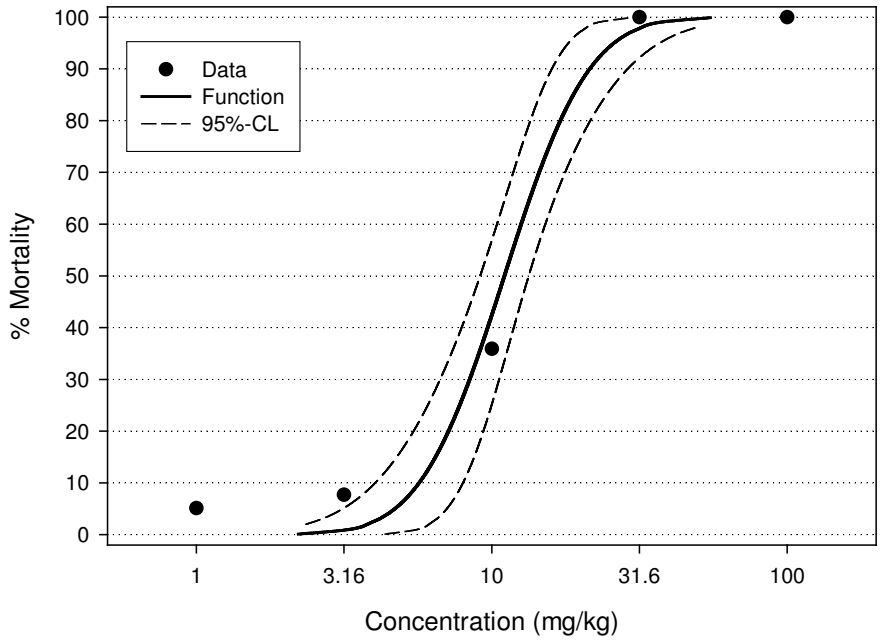


Figure 3.41: Dose response curve for the acute toxicity with tropical *Eisenia fetida* in lambda-cyhalothrin dosed TASc soil (after Abbott's correction).

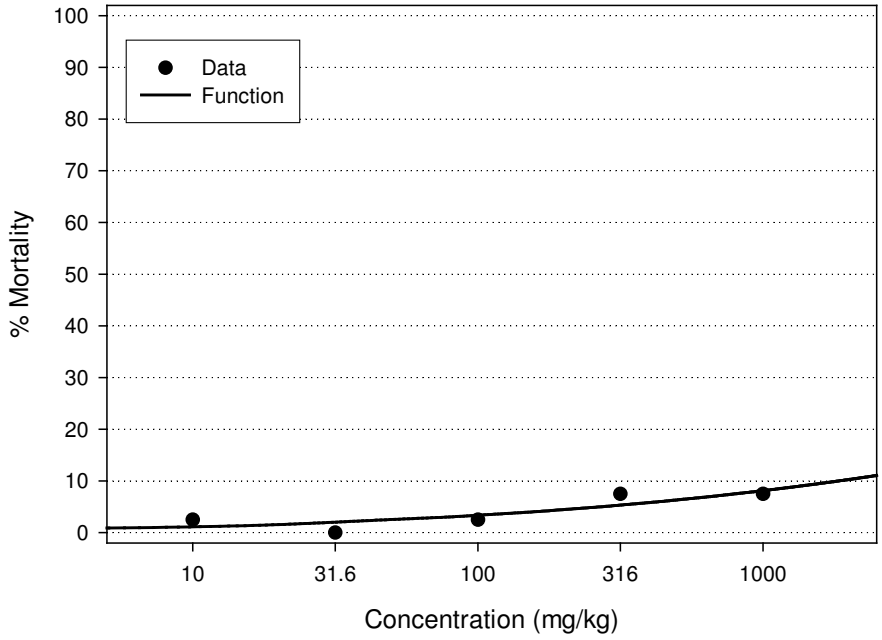


Figure 3.42: Dose response curve for the acute toxicity with tropical *Eisenia fetida* in carbendazim-dosed TASc soil.

Tests using TASs (sphagnum) artificial soil

The results of the avoidance experiments show that TASs soil is not a feasible substrate for toxicological tests. In these experiments *E. fetida* showed a significant congregating behavior when exposed in uncontaminated TASs soil (mean net response = 20%, P = 0.013, n = 4, *t* - test), which indicates that the substrate is not suitable for earthworms.

The acute effects of lambda-cyhalothrin and carbendazim on the tropical variant of *E. fetida* were tested in TASs (Table 3.25). Both tests were valid concerning mortality, since no worms died in the two controls. Due to the high mortality at all tested concentrations, no biomass data were available.

In the test with lambda-cyhalothrin, significant effects on the mortality were observed in the tested range of concentrations (25 - 100 mg a.i./kg). The LC_{50} value was determined as 1.58 mg a.i./kg. This value is clearly lower than those determined for TASx (23.9 mg a.i./kg) and TASc (12.9 mg a.i./kg).

In the test with carbendazim, highly significant effects on the mortality were observed in the tested range of concentrations (31.6 - 1000 mg a.i./kg). The LC_{50} value was determined as < 31.6 mg a.i./kg, which is clearly lower than the result gained with TASx and TASc (both > 1000 mg a.i./kg).

Table 3.25: Acute toxicity test with tropical *Eisenia fetida* in carbendazim and lambda-cyhalothrin-dosed TASs soil.

Carbendazim			**Lambda-cyhalothrin**		
Treatment	**Mortality (%)**	**LC_{50} / LOEC / NOEC**	**Treatment**	**Mortality (%)**	**LC_{50} / LOEC / NOEC**
Control	0.0	LC_{50} < 31.6 mg/kg	Control	0.0	LC_{50} = 1.58 mg/kg
31.6 mg/kg	63.3	LOEC < 31.6 mg/kg	25 mg/kg	100	LOEC = n.d.
316 mg/kg	93.3	NOEC < 31.6 mg/kg	50 mg/kg	100	NOEC = n.d.
1000 mg/kg	70.0		100 mg/kg	100	

3.2.3 *Pontoscolex corethrurus*

Acute toxicity of fungicide carbendazim

The acute effects of carbendazim on the earthworm *Pontoscolex corethrurus* were tested in TASx soil. Significant effects on mortality and biomass development of this species were observed in the tested range of concentrations (1 - 100 mg a.i./kg). The LC_{50} value was determined as 45.6 mg a.i./kg [37.2 - 56.0, 95% CL] and the LOEC and

NOEC values as 100 and 31.6 mg a.i./kg, respectively (Figure 3.43). The biomass of earthworms measured in the vessels treated with carbendazim were not statistically different from the control, but it seems that the biomass is affected at about 10 mg a.i./kg (due to the high loss in the control the NOEC could not be calculated). Since this species is adapted to acid soils it might be that the pH of TASx (6.4 - 6.8) caused this loss. Neither the abiotic parameters nor the biological effect data show a high variability (see Appendix 26).

Acute toxicity of insecticide lambda-cyhalothrin

Significant effects on the mortality and biomass development of *Pontoscolex corethrurus* were observed in the tested range of concentrations (1 - 100 mg a.i./kg). The LC_{50} value was determined as 40.2 mg a.i./kg [31.2 - 51.7, 95% CL] and the LOEC and NOEC as 31.6 and 10.0 mg a.i./kg, respectively (Figure 3.44). The effects of lambda-cyhalothrin on the biomass of earthworms were not statistically different from the control. This result is strongly influenced by the high loss of biomass in all treatments including the control. Since this species is adapted to acid soils it might be that the pH of TASx (6.3 - 6.8) caused this loss. Neither the abiotic parameters nor the biological effect data show a high variability (see Appendix 26).

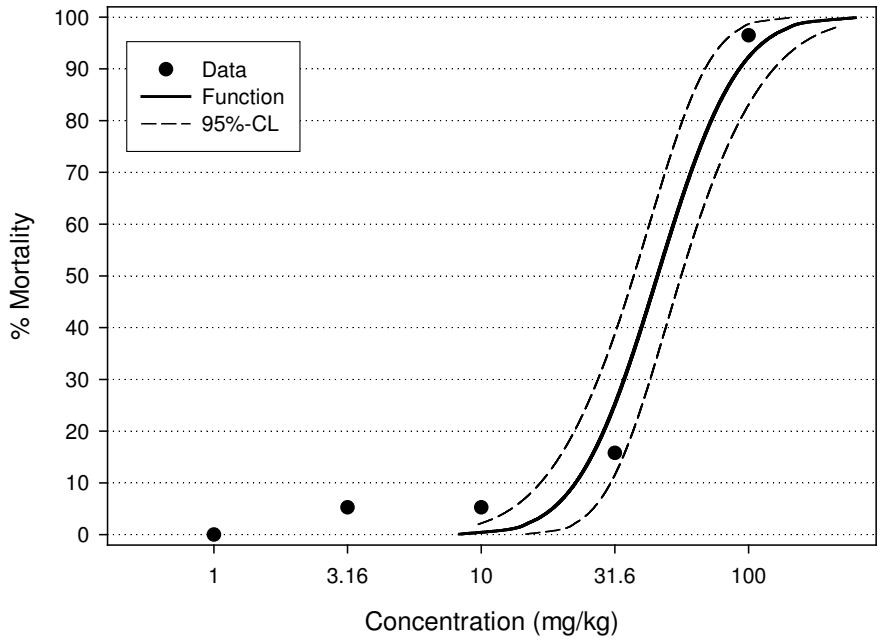


Figure 3.43: Dose response curve for the acute toxicity test with *Pontoscolex corethrurus* in carbendazim-dosed TASx soil (after Abbott's correction).

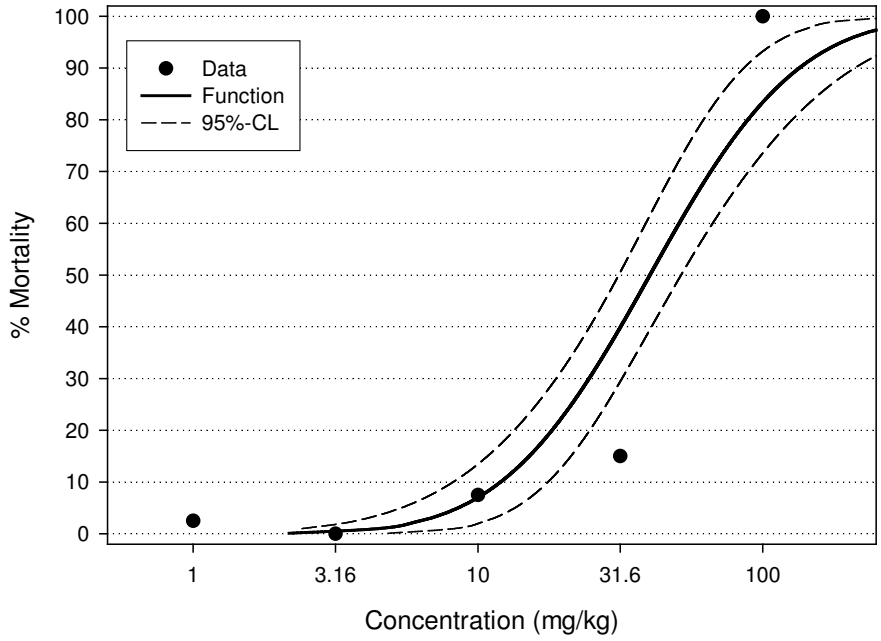


Figure 3.44: Dose response curve for the acute toxicity test with *Pontoscolex corethrurus* in lambda-cyhalothrin-dosed TASx soil.

3.3 Tests with arthropods

3.3.1 Isopods: *Porcellionides pruinosus*

Acute toxicity of fungicides benomyl and carbendazim

The acute effects of benomyl and carbendazim on *Porcellionides pruinosus* were tested in all four soils using a limit test design. Despite the fact that in the tests with the two natural soils a relatively high mortality was observed, these results will be discussed too. In the two artificial soils OECD and TASx, LC_{50} values greater than 1000 mg/kg were found. In natural soils the LC_{50} values can also be estimated as being higher than 1000 mg/kg (see Tables 3.26 - 3.29).

Table 3.26: Acute toxicity (limit test) of benomyl for *Porcellionides pruinosus* in OECD soil and TASx soil.

Treatment	OECD			TASx		
	Mortality (%)	Moisture (%)	pH	Mortality (%)	Moisture (%)	pH
Control	7.5	32.1	6.7	20	30.5	6.7
1000 mg/kg	15	30.8	5.7	25	31	6.7

Table 3.27: Acute toxicity (limit test) of benomyl for *Porcellionides pruinosus* in LUFA soil and TNS soil.

Treatment	LUFA			TNS		
	Mortality (%)	Moisture (%)	pH	Mortality (%)	Moisture (%)	pH
Control	25	27.5	6.0	27.5	17.9	3.9
1000 mg/kg	10	27.2	6.0	42.5	17.4	4.1

The results indicate that benomyl and carbendazim are not toxic to *P. pruinosus* in artificial soils, since the LC_{50} values are greater than 1000 mg/kg. In natural soils, the test results were not valid due to the high mortality in control. However, the relatively low mortality found in the treated vessels leads to the conclusion that the two chemicals are not toxic to *P. pruinosus* when tested in natural soils.

Table 3.28: Acute toxicity (limit test) of carbendazim for *Porcellionides pruinosus* in OECD and TASx soils.

Treatment	OECD			TASx		
	Mortality (%)	**Moisture (%)**	**pH**	**Mortality (%)**	**Moisture (%)**	**pH**
Control	7.5	32.1	6.0	17.5	30	6.7
1000 mg/kg	20	30.9	5.7	17.5	30.3	6.6

Table 3.29: Acute toxicity (limit test) of carbendazim for *Porcellionides pruinosus* in LUFA and TNS soils.

Treatment	LUFA			TNS		
	Mortality (%)	**Moisture (%)**	**pH**	**Mortality (%)**	**Moisture (%)**	**pH**
Control	25	27.5	6.0	30	17.9	4.0
1000 mg/kg	20	27.8	6.0	32.5	17.4	3.9

Chronic toxicity (limit test) of fungicides benomyl and carbendazim

In preliminary tests (RF tests) with carbendazim and benomyl in OECD and LUFA soils, no effect was found on reproduction (juveniles) of *Porcellionides pruinosus.* Based on these results, definitive tests were carried out using a control and the highest concentration following a limit design. No effects of carbendazim and benomyl were observed in the OECD soil (Table 3.30), while in LUFA soil the number of juveniles was significant lower than in the control (Table 3.31).

Table 3.30: Chronic toxicity test with *Porcellionides pruinosus* in benomyl and carbendazim-dosed OECD soil.

Chemical	Treatment	Number of juveniles	Juveniles % of the control	Adult Mortality (%)	Moisture (%)	pH
	Control	145.0 ± 46.2	100.0	7.5	27.5	5.8
Benomyl	1000 mg/kg	102.3 ± 54.6	70.5	17.5	26.4	6.2
Carbendazim	1000 mg/kg	97.5 ± 27.9	67.2	10.0	23.9	5.8

Table 3.31: Chronic toxicity test with *Porcellionides pruinosus* in benomyl and carbendazim-dosed LUFA soil.

Chemical	Treatment	Number of juveniles	Juveniles % of the control	Adult Mortality (%)	Moisture (%)	pH
	Control	158.3 ± 36.2	100.0	10.0	22.6	6.2
Benomyl	1000 mg/kg	56.5 ± 11.9 **	37.5	5	25.5	6.3
Carbendazim	1000 mg/kg	62.0 ± 26.4 **	39.2	10	28.9	5.9

** Statistically different from control ($P < 0.01$) (Mann-Whitney test)

Acute toxicity of insecticide lambda-cyhalothrin

The acute effects of lambda-cyhalothrin on *Porcellionides pruinosus* were tested in all four test soils using a dose response design. Despite the fact that in the tests with the tropical field soil TNS a relatively high control mortality was observed, these results will also be discussed here. In all four soils, a high toxicity was observed (LC_{50} values between 0.1 (TNS), 0.2 (TASx), 0.5 (OECD) and 1.4 (LUFA) mg a.i./kg). All four values are statistically significantly different (Table 3.32). Accordingly, the NOEC value for the two artificial soils was determined as 0.1 mg a.i./kg, while it was higher (1.0 mg a.i./kg) in the LUFA soil and lower (< 0.032 mg a.i./kg) in TNS soil. A clear dose-dependent effect can be found in all four soils (Figures 3.45 - 3.48). Neither the abiotic parameters nor the biological effect data show a high variability (Appendix 27).

Table 3.32: Acute toxicity of *Porcellionides pruinosus* in lambda-cyhalothrin-dosed substrates: LC_{50} and its 95%-confidence limits, LOEC and NOEC (values in mg/kg).

	OECD	TASx	LUFA	TNS
LC_{50}	0.5 [0.4 - 0.6]	0.2 [0.1 - 0.3]	1.4 [1.2 - 1.8]	0.1[0.03 - 0.1]
LOEC	0.32	0.32	3.16	≤ 0.032
NOEC	0.1	0.1	1.0	< 0.032

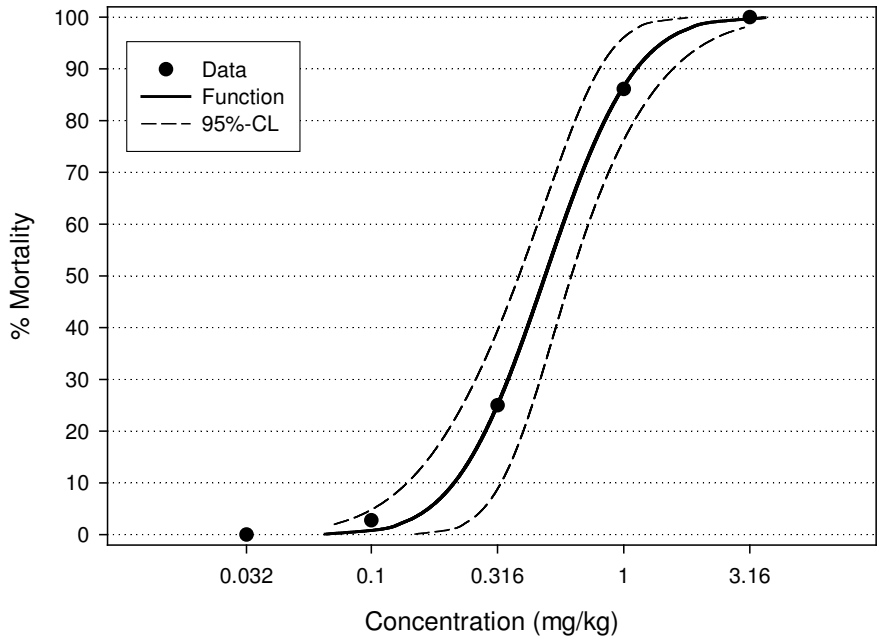


Figure 3.45: Dose response curve for the acute toxicity test with *Porcellionides pruinosus* in lambda-cyhalothrin-dosed OECD soil.

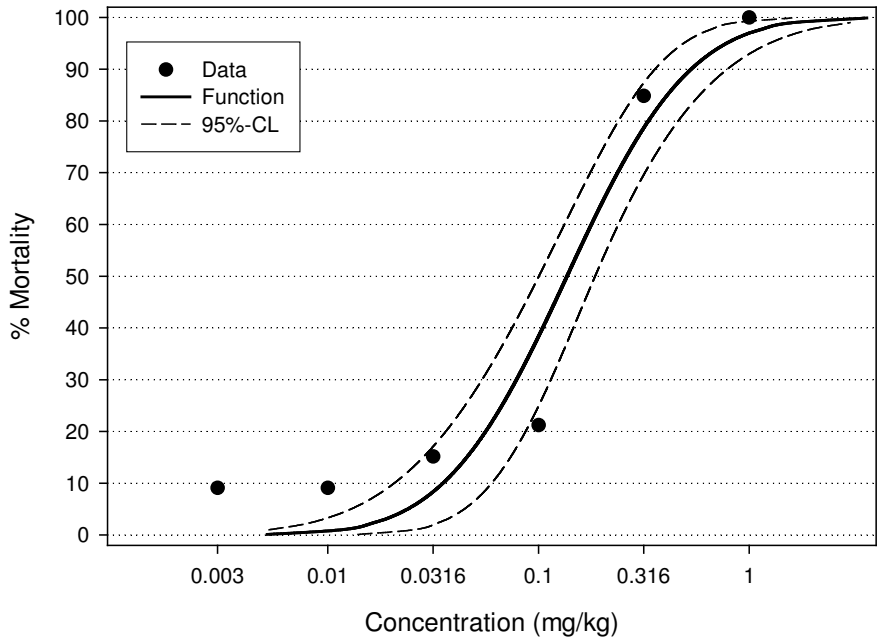


Figure 3.46: Dose response curve for the acute toxicity test with *Porcellionides pruinosus* in lambda-cyhalothrin-dosed TASx soil (after Abbott's correction).

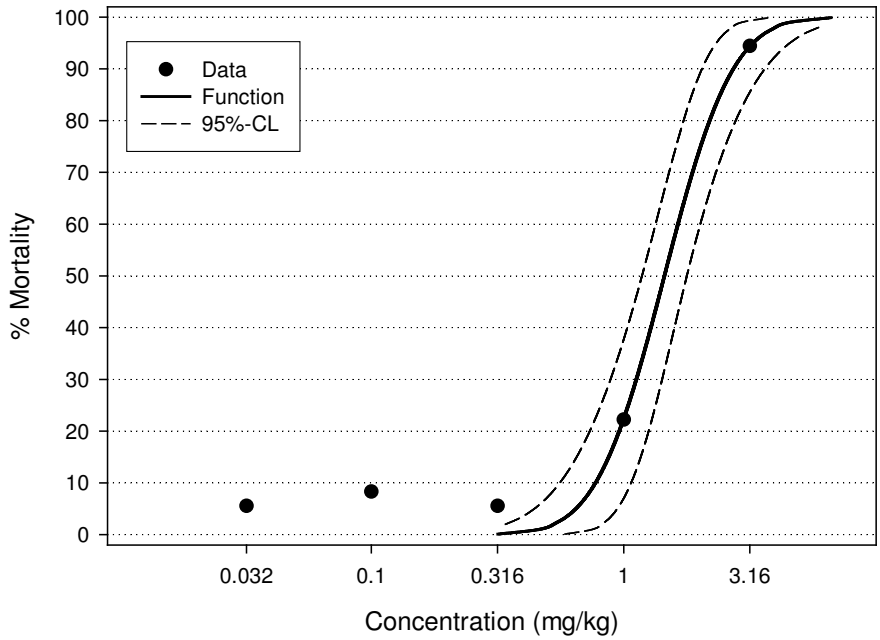


Figure 3.47: Dose response curve for the acute toxicity test with *Porcellionides pruinosus* in lambda-cyhalothrin-dosed LUFA soil (after Abbott's correction).

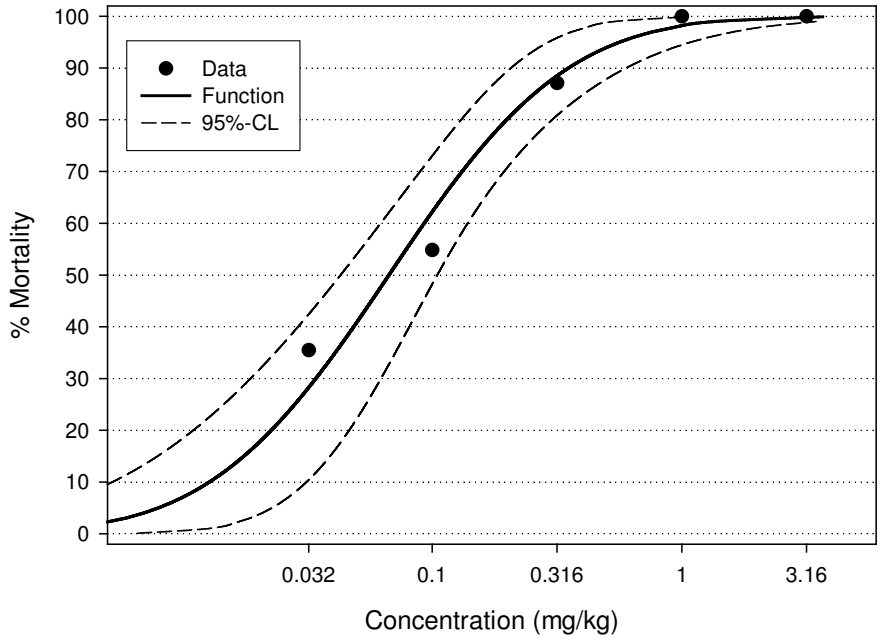


Figure 3.48: Dose response curve for the acute toxicity test with *Porcellionides pruinosus* in lambda-cyhalothrin-dosed TNS soil (after Abbott's correction).

Chronic toxicity of insecticide lambda-cyhalothrin

Chronic effects of lambda-cyhalothrin on the reproduction of *Porcellionides pruinosus* were tested in OECD and LUFA soils. Significant effects on mortality were observed in both soils at concentrations of 1 mg a.i./kg in OECD soil and 0.1 - 1.0 mg a.i./kg in LUFA soil. The EC_{50} for reproduction was lower in LUFA (0.13 mg a.i./kg) than in OECD soil (0.4 mg a.i./kg), while LOEC and NOEC values were similar (0.32 and 0.1 mg a.i./kg, respectively) (Figures 3.49 and 3.50). Neither the abiotic parameters nor the biological effect data showed a high variability. The absolute number of juveniles in the untreated vessels was roughly twice as high in OECD compared to LUFA soil (see Appendix 28).

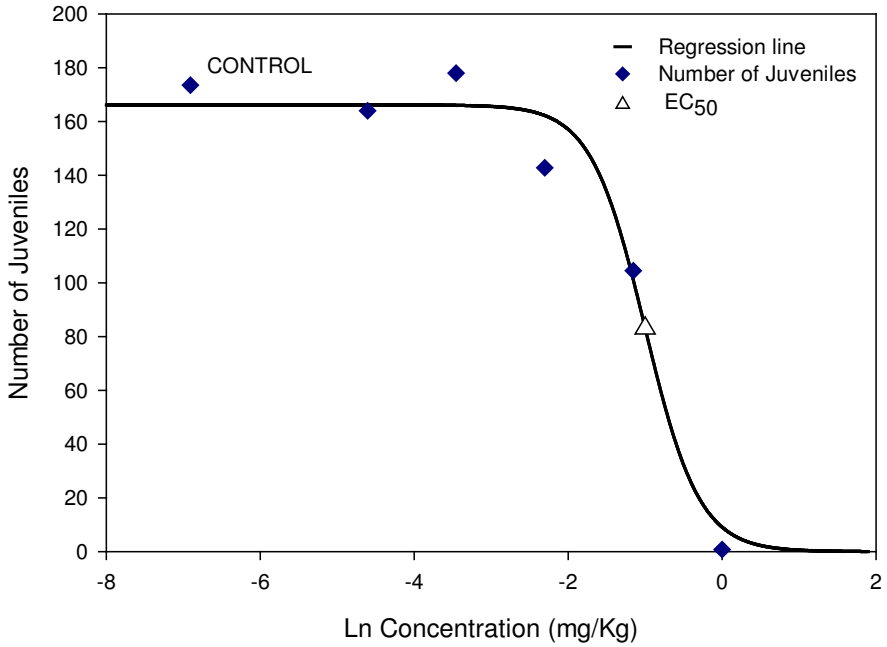


Figure 3.49: Dose response curve in the chronic toxicity test with *Porcellionides pruinosus* in lambda-cyhalothrin-dosed OECD soil.

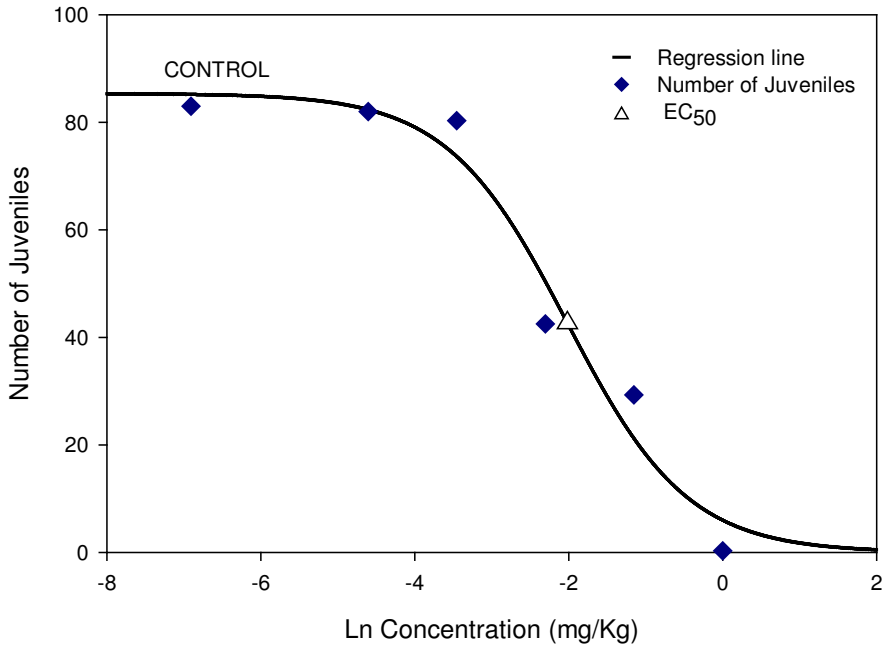


Figure 3.50: Dose response curve in the chronic toxicity test with *Porcellionides pruinosus* in lambda-cyhalothrin-dosed LUFA soil.

3.3.2 Isopods: *Circoniscus ornatus*

Acute toxicity of fungicide carbendazim

The toxicity of carbendazim to *C. ornatus* was tested in range-finding (RF) tests. These RF tests provided an LC_{50} value > 1000 mg a.i./kg for TASx soil. Based on this result, the definitive test was carried out following a limit design (i.e., using a control and the concentration of 1000 mg a.i./kg). The LC_{50} value for *Circoniscus ornatus* was determined as > 1000 mg a.i./kg in TASx soil. Carbendazim is clearly not toxic to this arthropod species. Neither the abiotic parameters nor the biological effect data show a high variability (Table 3.33).

Table 3.33: Acute toxicity (limit) test with *Circoniscus ornatus* in carbendazim-dosed TASx soil.

	Mortality (%)	**Moisture (%)**	**pH**
Control	15.0	29.8	6.6
1000 mg/kg	2.5	29.5	6.6

Acute toxicity of insecticide lambda-cyhalothrin

Based on the RF test result (LC_{50} = 6.25 mg a.i./kg), the acute effect of lambda-cyhalothrin on *C. ornatus* was tested in TASx soil using a dose response design ranging from 0.32 to 31.6 mg a.i./kg (Figure 3.51). The LC_{50} was determined as 2.3 mg a.i./kg [1.4 - 3.7, 95%-CL] and the LOEC and NOEC values as 1.0 and 0.32 mg a.i./kg, respectively. Neither the abiotic parameters nor the biological effect data showed a high variability (Appendix 29).

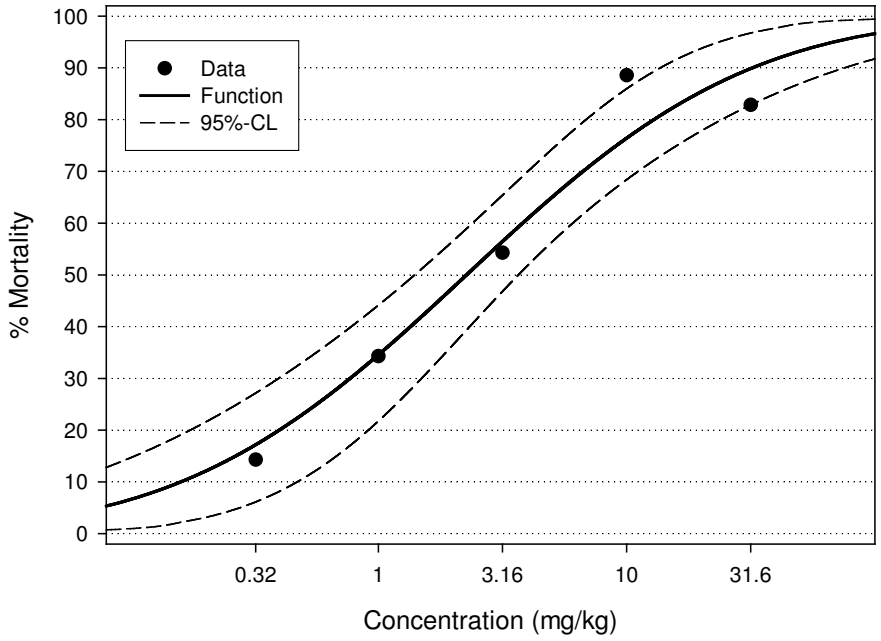


Figure 3.51: Dose response curve for the acute toxicity test with *Circoniscus ornatus* in lambda-cyhalothrin-dosed TASx soil (after Abbott's correction).

3.3.3 Diplopods: *Trigoniulus corallinus*

Acute toxicity of fungicide carbendazim

Acute toxicity effects on the tropical millipede *Trigoniulus corallinus* were tested in a dose response design ranging from 10 to 1000 mg a.i./kg in TASx soil. Significant effects on mortality were observed at the highest concentration (1000 mg a.i./kg) of carbendazim (Figure 3.52). The data were not appropriate for the probit analysis, no dose response curve was observed and, therefore, the LC_{50} value was calculated with the Trimmed Spearman-Kärber method. The LC_{50} value was determined as 503.5 mg a.i./kg

[363.5 - 697.5, 95% CL] and the LOEC and NOEC values as 1000 and 316 mg a.i./kg, respectively. Neither the abiotic parameters nor the biological effect data showed a high variability (see Appendix 30).

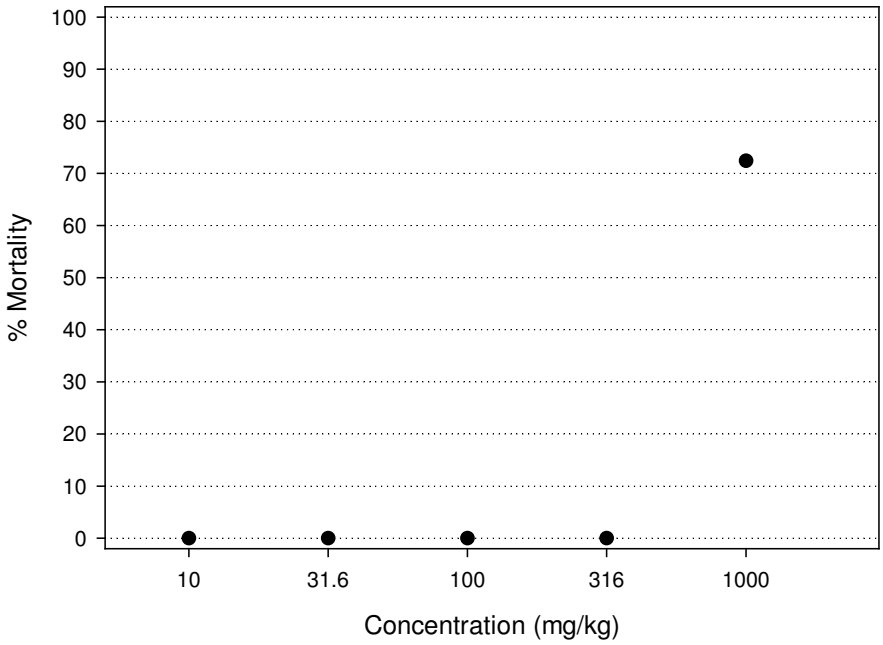


Figure 3.52: Mortality of *Trigoniulus corallinus* in carbendazim-dosed TASx soil (after Abbott's correction).

Acute toxicity of insecticide lambda-cyhalothrin

The acute effects of lambda-cyhalothrin on the tropical diplopod *Trigoniulus corallinus* were tested in TASx soil in a dose response design ranging from 0.39 to 6.25 mg a.i./kg. A clear dose response curve was observed (Figure 3.53). The LC_{50} value was determined as 1.2 mg a.i./kg [0.8 - 1.7, 95%-CL]. Despite a slightly elevated mortality in the controls, the values are considered as valid. Mortality increased steadily over the whole concentration range tested. Neither the abiotic parameters nor the biological effect data show a high variability (Appendix 30).

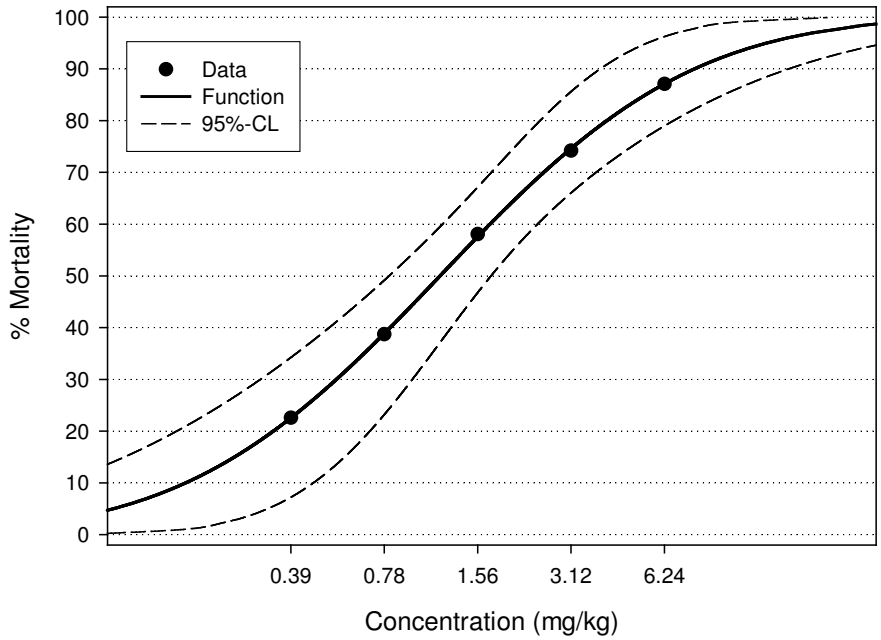


Figure 3.53: Dose response curve for the acute toxicity test with *Trigoniulus corallinus* in lambda-cyhalothrin-dosed TASx soil (after Abbott's correction).

3.4 Results of semi-field tests with tropical soils

3.4.1 Test with organisms in untreated TME

The soil fauna species studied in the microcosms (intact soil cores; TMEs) were active and acted as litter decomposers, despite their high mortality during the test (25.0 - 86.7%). No obvious reason could be identified for those high mortality rates. The mean mortality of the five species did not differ among them (but it was lowest with the "field species" *Pontoscolex corethrurus*). Also, no differences between the species from laboratory mass cultures (Lab group = *Eisenia fetida* and *Porcellionides pruinosus*) and those collected in the field (Field group: *Trigoniulus corallinus, Circoniscus ornatus and Pontoscolex corethrurus*) could be observed. No correlation between animal mortality and the three litter types was found (Table 3.34).

The decomposition rates were statistically different between the litter species but were not correlated with one of the two soil fauna groups (Table 3.35). Feeding activity in microcosms, evaluated with bait-lamina tests, was significantly ($P>0.01$) higher in microcosms treated with the 'Field' fauna group (15%) than with the 'Lab'

fauna group (8%), whereas no statistical differences between the three litter types were found.

During the experimental period, the abiotic conditions did not vary: the room temperature ranged from 26 to 31 ºC (mean = 28.0 ± 1.0 ºC) and the soil moisture in the TMEs ranged from 30 to 35 %.

Table 3.34: Mortality of five species of the soil fauna, added to untreated microcosms (intact soil core) with three litter types for three months (Lab. group shown in bold).

	Flemingia		*Hevea*		*Brachiaria*	
	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD
Trigoniulus corallinus	86.7	6.9	83.0	13.1	77.5	14.0
Circoniscus ornatus	67.8	24.6	73.0	21.4	42.3	24.9
Porcellionides pruinosus	72.9	20.9	74.5	16.5	63.9	10.5
Eisenia fetida	67.5	45.3	65.0	35.1	45.0	38.2
Pontoscolex corethrurus	25.0	20.7	35.0	33.4	52.5	33.7

Table 3.35: Litter biomass loss from three species exposed in untreated microcosms (intact soil core) for four months.

	Flemingia		*Hevea*		*Brachiaria*	
	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD
Lab Fauna group	73.3a	7.6	33.8b	4.1	41.4c	7.3
Field Fauna group	78.3a	6.1	30.4b	10.2	43.9c	11.2

Means followed by a common letter in a row are not significantly different at P = 0.01 (Tukey's test)

3.4.2 Effect of carbendazim and lambda-cyhalothrin

Microcosms with intact soil cores

The three soil organisms reacted differently in the microcosms treated with carbendazim and lambda-cyhalothrin, respectively. The millipede *T. corallinus* showed a high susceptibility to both chemicals in all concentrations. The isopod *C. ornatus* was significantly affected only at the higher concentration of lambda-cyhalothrin (Table 3.36), while the earthworm *P. corethrurus* was very susceptible to carbendazim in both concentrations. All species showed a high mortality in the controls (Table 3.37).

The decomposition rate of litter was influenced by the application of the chemicals. The litter mass loss of *Flemingia* was significantly lower when treated with

carbendazim and lambda-cyhalothrin compared to the control. In the case of *Hevea*, the reduction of litter mass was significant only at the high concentration of carbendazim (Table 3.38). The microbial respiration was not influenced by the treatments (Table 3.39). It must be kept in mind that the litter of *Flemingia* was much better decomposed (90.1% within 5 months) than the litter of *Hevea* (34.7% in the same period). Due to the slow breakdown of *Hevea* leaves it was difficult to detect significant differences despite the fact that in all treatments the difference to the control was clearly higher than 10% of mass loss.

During the experimental period, the room temperature ranged from 26 to 28 ºC (mean = 27.0 ± 0.5 ºC) and the soil moisture in the TMEs ranged from 30 to 35 %.

Table 3.36: Mortality of *T. corallinus* and *C. ornatus*, in microcosms (intact soil core) treated with one application of carbendazim and lambda-cyhalothrin exposed during one month.

	Trigoniulus corallinus		*Circoniscus ornatus*	
	Mean (%)	SD	Mean (%)	SD
Control	36.3	25.5	31.5	14.5
Carbendazim (451 mg a.i. / m^2)	67.5 **	18.3	30.0	26.2
Carbendazim (4510 mg a.i. / m^2)	85.0 **	14.1	42.5	19.8
L-cyhalothrin (18 mg a.i. / m^2)	80.0 **	10.7	40.0	26.2
L-cyhalothrin (180 mg a.i. / m^2)	100 **	0.0	92.5 **	14.9

** Statistically different from control at P = 0.01 (Dunnett's test)

Table 3.37: Mortality of *P. corethrurus* in microcosms (intact soil core) treated with carbendazim and lambda-cyhalothrin, exposed during five months. Applied (1)five times (monthly) and (2)once at beginning.

	Mean (%)	SD
Control	58.8	36.9
(1) Carbendazim (451 mg a.i. / m^2)	85.0 **	14.1
(2) Carbendazim (4510 mg a.i. / m^2)	95.0 **	9.3
(1) L-cyhalothrin (18 mg a.i. / m^2)	30.0	28.3
(2) L-cyhalothrin (180 mg a.i. / m^2)	42.5	12.8

** Statistically different from control at P = 0.01 (Dunnett's test)

Table 3.38: Litter biomass loss in microcosms (intact soil core) treated with carbendazim and lambda-cyhalothrin and a control, exposed during five months. Applied [(1)]five times (monthly) and [(2)]once at beginning.

	Flemingia macrophylla		*Hevea pauciflora*	
	Mean (%)	SD	Mean (%)	SD
Control	90.1	5.5	34.7	16.9
[(1)] Carbendazim (451 mg a.i. / m^2)	54.3 **	7.9	18.0	7.3
[(2)] Carbendazim (4510 mg a.i. / m^2)	59.0 **	7.3	12.0 *	6.9
[(1)] L-cyhalothrin (18 mg a.i. / m^2)	32.0 **	10.1	20.0	6.5
[(2)] L-cyhalothrin (180 mg a.i. / m^2)	26.1 **	11.2	19.0	8.3

**, * Statistically different from control at P = 0.01 and P = 0.05 (Dunnett's test), respectively

Table 3.39: Microbial respiration in upper soil layer in microcosms (intact soil core) treated with carbendazim and lambda-cyhalothrin and a control, exposed during five months. Applied [(1)]five times (monthly) and [(2)]once at beginning (values in µL CO_2 h^{-1} g^{-1}).

	Flemingia macrophylla		*Hevea pauciflora*	
	Mean (%)	SD	Mean (%)	SD
Control	8.2	2.4	6.8	0.7
[(1)] Carbendazim (451 mg a.i. / m^2)	6.8	1.3	7.7	0.7
[(2)] Carbendazim (4510 mg a.i. / m^2)	5.4	0.9	5.8	0.2
[(1)] L-cyhalothrin (18 mg a.i. / m^2)	6.3	1.1	7.1	1.4
[(2)] L-cyhalothrin (180 mg a.i. / m^2)	7.0	0.2	6.4	3.7

Microcosms with homogenized soil

In this experiment, the mortality of the soil fauna caused by carbendazim and lambda-cyhalothrin (applied to the soil in field-relevant – i.e., low - concentrations) was not significantly different from the control. The natural mortality rate was high *for T. corallinus,* followed by *C. ornatus* and *P. corethrurus*. No statistically significant differences in mortality were found between the tested soils, the mode of application of the pesticides, their concentrations or the litter type (Table 3.40). The mean decomposition rate in litter of *Pueraria* was slightly higher than in *Flemingia* but not statistically significantly different. Also, in the treatments with chemicals, the decomposition rate was not distinct from the control (Table 3.41).

The microbial respiration in the upper soil layer was not negatively influenced by the treatments. However, the clayey soil had a higher respiration rate than the sandy soil (Table 3.42).

The feeding activity in the microcosms, evaluated with bait-lamina tests, was not statistically different between the treatments. However, a significant ($P<0.01$) difference was observed between the clayey soil (15%) and the sandy soil (1%).

During the experimental period, the room temperature ranged from 26 to 31 ºC (mean = 29.0 ± 1.0 ºC). The moisture in the TMEs ranged from 29.0 to 37.9 % (mean = 33.8 ± 2.6 %) and 6.5 to 13.1% (mean = 9.8 ± 1.7%) in the clayey and sandy soils, respectively.

Table 3.40: Mortality of soil fauna exposed during two months in microcosms (homogenized soil) treated with carbendazim and lambda-cyhalothrin.

	Trigoniulus corallinus		*Pontoscolex corethrurus*		*Circoniscus ornatus*	
	Mean (%)	SD	Mean (%)	SD	Mean (%)	SD
Control	93.8	9.6	22.5	21.8	46.3	21.6
Carbendazim (20 mg a.i. / kg)	97.5	7.1	12.5	10.4	35.0	23.3
Carbendazim (200 mg a.i. / kg)	100	0	32.5	26.0	40.0	18.5
L-cyhalothrin (0.08 mg a.i. / kg)	92.5	10.4	32.5	28.2	35.0	17.7
L-cyhalothrin (0.8 mg a.i. / kg)	97.5	7.1	32.5	23.8	30.0	21.4

Table 3.41: Litter biomass loss in microcosms (homogenized soil) treated with carbendazim and lambda-cyhalothrin, exposed during two months.

	Flemingia macrophylla		*Pueraria phaseoloides*	
	Mean (%)	SD	Mean (%)	SD
Control	18.9	11.5	37.2	21.1
Carbendazim (20 mg a.i. / kg)	14.2	10.8	33.2	12.6
Carbendazim (200 mg a.i. / kg)	21.6	10.5	24.8	10.0
L-cyhalothrin (0.08 mg a.i. / kg)	22.7	6.3	45.8	19.2
L-cyhalothrin (0.8 mg a.i. / kg)	24.2	8.0	39.7	9.0

Table 3.42: Microbial respiration in upper soil layer in microcosms (homogenized soil) with two types of soil treated with carbendazim and lambda-cyhalothrin, exposed during two months (mean and standard deviation; values in µL CO_2 h^{-1} g^{-1}).

	Clayey Ferralsol		Sandy Acrisol	
	Mixed	Surface	Mixed	Surface
Control	14.6 ± 0.6	8.2 ± 1.7	2.8 ± 0.4	2.1 ± 0.6
Carbendazim (20 mg a.i. / kg)	10.6 ± 3.2	11.0 ± 0.5	2.4 ± 0.0	2.4 ± 0.3
Carbendazim (200 mg a.i. / kg)	11.2 ± 3.7	10.1 ± 2.4	4.0 ± 0.5	3.2 ± 0.2
L-cyhalothrin (0.08 mg a.i. / kg)	13.1 ± 1.0	12.1 ± 0.9	2.7 ± 0.1	2.3 ± 0.2
L-cyhalothrin (0.8 mg a.i. / kg)	13.1 ± 0.1	9.0 ± 2.8	2.4 ± 0.0	2.3 ± 0.1

Mixed and Surface = mode of application of the pesticide

3.5 Results of field tests in tropical soil

3.5.1 Effect of fungicide carbendazim

Carbendazim treatments did not have a statistically significant effect on litter decomposition. High variability in the litter mass loss was observed among the treated plots (i.e., standard deviations in the treatments as high or higher than in the control). However, the decomposition rate in the three chemically treated plots was smaller than in the control plot during most of the experimental period (Table 3.43 and Figures 3.54 - 3.56). These differences varied according to the treatments and the duration of the exposure (Table 3.43). At the plots treated monthly with 1 kg/ha of carbendazim decomposition was slightly faster than in the control for the first six months of exposure before it became slower. In both periods, the deviation between control and treated plots was smaller than 10%. At plots treated with the same amount of carbendazim in 3-month intervals, the remaining weight differed by less than 10% at 3 and 12 months of exposure while being about 12% smaller at the two samplings at 6 and 9 months of exposure. The data gained from the plot treated once with 10 kg a.i./ha carbendazim showed nearly the same picture as the 3-month plot. Despite the differences in remaining weight at some intermediate dates, the difference between treated plot and control was always smaller than 10% after one year. At that point of time and in absolute numbers, the remaining weight at all plots was 34.7 to 40.4% of the initial weight.

Changes in the carbon and nitrogen content of the litter, evaluated after six and twelve months in the treated plots, were not statistically different from the control

(Tables 3.44 and 3.45). However, the carbon content (and consequently the C/N ratio) showed a slight and dose-dependent increase at the treated plots compared to the control (in particular at the plots treated once with 10 kg a.i./ha carbendazim).

The results of the arthropod soil fauna survey performed at the end of the study (after 12 months of exposure) indicate a high variability among the replicates, showing standard deviations between 15 and 95% of the mean. This variability is neither correlated with litter or soil fauna nor with mesofauna and macrofauna. The abundance of arthropods in litter and in soil was not influenced by carbendazim (Tables 3.46 and 3.47). In addition, no specific group of litter and soil fauna showed significant differences in abundance between control and the treatments. Individual data on the abundance of arthropod litter and soil fauna (showing the different animal groups) are provided in the Appendices 3 and 4.

Due to the known sensitivity of Oligochaeta to carbendazim (Van Gestel 1992), earthworms were assessed separately. Considering all earthworm species together, no significant differences in abundance and biomass between control and treatments were found. Despite that, the average values of both variables were higher in the control than in the treatments (Table 3.48). However, in the analyses according to earthworm species, significant differences in abundance were found between control and all carbendazim treatments for *Andiorrhinus amazonius*, the most abundant species in the study site (Table 3.49).

Concerning the effect on feeding activity, with bait-lamina tests, no data was obtained due to intense rainfall during the test in the field.

Table 3.43: Remaining weight of organic matter in litterbags exposed to three treatments with carbendazim in comparison to the control (means and standard deviations, values in %).

Time (months)	**Control**	**1 kg/ha (monthly)**		**1 kg/ha (3-monthly)**		**10 kg/ha (once)**	
Start	100	100	[1]Effect	100	[1]Effect	100	[1]Effect
3	65.5 ± 6.9	57.4 ± 11.8	8.1	68.2 ± 4.4	-2.7	71.3 ± 1.2	-5.8
6	49.4 ± 13.9	46.5 ± 19.2	2.9	61.8 ± 10.4	-12.4	61.2 ± 5.0	-11.8
9	37.1 ± 6.7	43.3 ± 14.2	-6.2	49.2 ± 6.7	-12.1	45.7 ± 8.5	-8.6
12	34.7 ± 7.0	35.7 ± 13.7	-1.0	40.4 ± 11.0	-5.7	38.2 ± 11.3	-3.5

[1] Difference to the control: the values (negative) mean the % of reduction of the decomposition.

Table 3.44: Carbon and nitrogen composition of organic matter in litterbags exposed to three treatments with carbendazim and a control evaluated after 6 months.

	N_{total} (%)		**C_{total} (%)**		**CN ratio**	
	Initial content	After 6 months	Initial content	After 6 months	Initial content	After 6 months
Control	0.9 ± 0.01	1.2 ± 0.1	51.0 ± 0.1	35.4 ± 5.2	56.7 ± 0.3	30.4 ± 4.3
1 kg/ha (monthly)		1.2 ± 0.1		35.8 ± 4.4		30.0 ± 3.1
1 kg/ha (3-monthly)		1.1 ± 0.1		36.9 ± 5.9		32.0 ± 3.6
10 kg/ha (once)		1.2 ± 0.1		39.8 ± 2.0		33.9 ± 3.2

Table 3.45: Carbon and nitrogen composition of organic matter in litterbags exposed to three treatments with carbendazim and a control evaluated after 12 months.

	N_{total} (%)		**C_{total} (%)**		**CN ratio**	
	Initial content	After 12 months	Initial content	After 12 months	Initial content	After 12 months
Control	0.9 ± 0.01	1.1 ± 0.2	51.0 ± 0.1	29.0 ± 4.6	56.7 ± 0.3	26.0 ± 1.6
1 kg/ha (monthly)		1.1 ± 0.2		33.5 ± 9.0		29.5 ± 3.3
1 kg/ha (3-monthly)		1.2 ± 0.2		33.7 ± 2.6		29.2 ± 1.2
10 kg/ha (once)		1.2 ± 0.2		35.3 ± 6.1		29.8 ± 3.4

Table 3.46: Litter fauna abundance (individual/m^2) exposed to three treatments with carbendazim.

	Control		**1 kg/ha (monthly)**		**1 kg/ha (3-monthly)**		**10 kg/ha (once)**	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Arthropods (Mesofauna)	1173	722	1316	900	1684	950	652	332
Arthropods (Macrofauna)	605	405	784	120	1220	262	1232	1211

Table 3.47: Soil fauna abundance (individual/m^2) exposed to three treatments with carbendazim.

	Control		**1 kg/ha (monthly)**		**1 kg/ha (3-monthly)**		**10 kg/ha (once)**	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Arthropods (Mesofauna)	1157	659	700	362	1624	616	1716	1141
Arthropods (Macrofauna)	741	274	756	378	772	394	544	425

Table 3.48: Earthworm abundance (individual/m^2) and biomass (g fw/m^2) exposed to three treatments with carbendazim and a control.

	Control		**1 kg/ha (monthly)**		**1 kg/ha (3-monthly)**		**10 kg/ha (once)**	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Earthworms (abundance)	27.7	24.0	12.5	3.8	9.3	7.4	2.8	1.7
Earthworms (biomass)	6.1	6.4	4.0	4.1	2.4	1.2	1.1	1.3

Table 3.49: Earthworm species abundance (individual/m^2) exposed to three treatments with carbendazim and a control.

Species	**Control**		**1 kg/ha (monthly)**		**1 kg/ha (3-monthly)**		**10 kg/ha (once)**	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Andiorrhinus amazonius	14.3	9.1	4.8 *	2.9	5.0 *	5.6	0.3 *	0.5
Tuiba dianaea	1.5	2.3	1.3	1.3	1.3	0.5	0.5	0.6
Urobenus brasiliensis	6.3	11.0	5.3	3.1	3.3	2.5	1.5	1.3
Rhinodrilus sp.	0.5	0.8	0	0	0	0	0	0
Pontoscolex corethrurus	2.5	6.1	0.5	1.0	0	0	0	0
Rhinodrilus contortus	0	0	0	0	0	0	0	0
Tuiba sp.	0	0	0	0	0	0	0.5	1.0

* Statistically different from control at P = 0.05 (Dunnett's test).

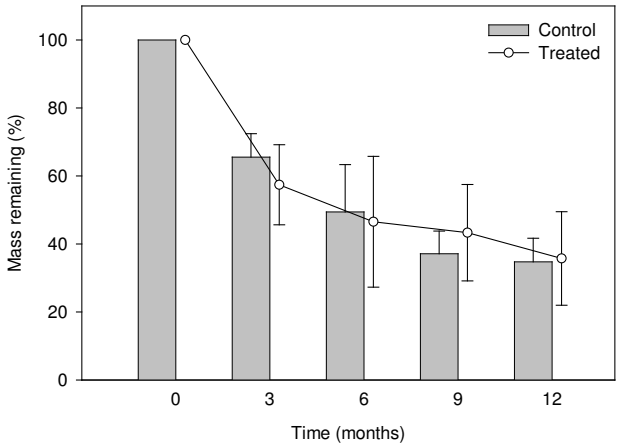


Figure 3.54: Remaining weight in litterbags treated monthly with 1kg/ha of carbendazim and a control (means and standard deviation bars).

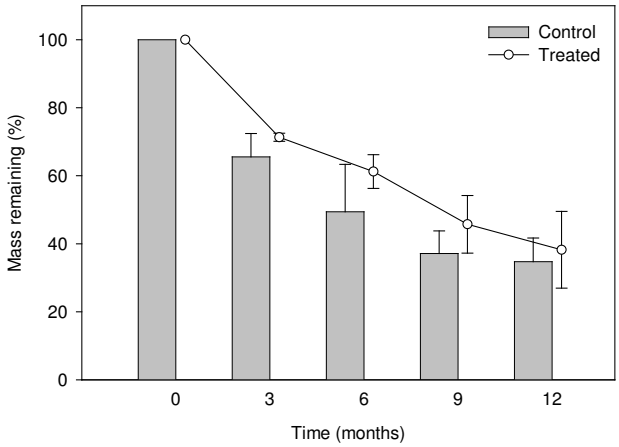


Figure 3.55: Remaining weight in litterbags treated 3-monthly with 1 kg/ha of carbendazim and a control (means and standard deviation bars).

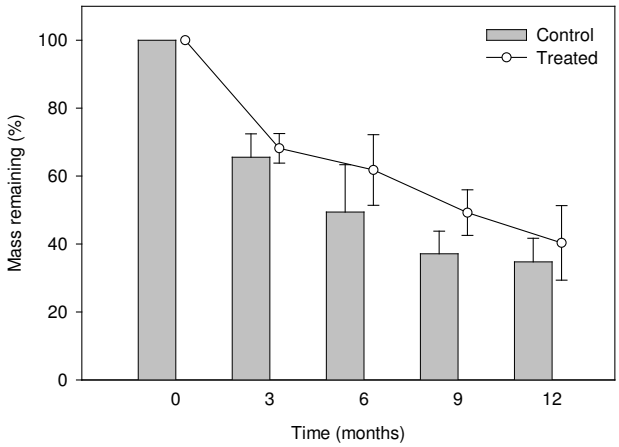


Figure 3.56: Remaining weight in litterbags treated once with 10 kg/ha of carbendazim and a control (means and standard deviation bars).

3.5.2 Effect of insecticide lambda-cyhalothrin

The decomposition of litter was negatively influenced by lambda-cyhalothrin applications. In comparison with the carbendazim treatment, a lower variability of the litter mass loss was observed among the treated plots (i.e., standard deviations in the treatments always lower than in the control). After nine months, statistically significant effects were found at the low (monthly application of 40 g a.i./ha) and the highest concentration (one application of 400 g a.i./ha lambda-cyhalothrin). At some dates, differences between the decomposition in the control and those in the chemical treatments were also in the range of 10 %, but did not show statistical significance (Table 3.50 and Figures 3.57 - 3.59). At all dates, the remaining weight was lower at the treated plots than in the control. After monthly application of 40 g a.i./ha lambda-cyhalothrin, the difference to the control increased over time, reaching 10.3% (statistically significant) at 9 months of exposure. At the end of the study, these differences vanished. At the plots treated 3-monthly with the same concentration, the difference in remaining weight compared to the control increased during the whole study period, reaching a maximum of 10.3% after 12 months. The application of 400 g a.i./ha lambda-cyhalothrin once led also to an increase of the remaining weight when compared to the control, reaching 10.2% after 6 months and 11.6% after 9 months (only the latter value being statistically significantly different to the control). However, in this case the difference decreased to 6.4% at 12 months of exposure. Therefore, only at the plots treated 3-monthly with 40 g a.i./ha lambda-cyhalothrin a difference higher than 10% remained at the end of the study. At that point of time and in absolute numbers, the remaining weight at all plots was 33.8 to 45.6% of the initial weight.

Changes in the carbon and nitrogen content of the litter, evaluated after 6 months in the treated plots, were not statistically different from the control (Table 3.51). However, with one exception (in the plots treated every 3-months with 40 g a.i./ha lambda-cyhalothrin after 12 months) the carbon content (but less the C/N ratio) showed a slight and dose-dependent increase at the treated plots compared to the control (in particular at the plots treated once with 400g a.i./ha lambda-cyhalothrin). In addition, the C/N ratio was significantly different compared to the control at the highest concentration at 12 months of exposure (Table 3.52).

The results of the arthropod soil fauna survey performed at the end of the study (after 12 months of exposure) indicate a high variability among the replicates, showing standard deviations between 20 and 80% of the mean. This variability is neither correlated with litter or soil fauna nor with mesofauna and macrofauna. The abundance of litter and soil arthropods was not negatively influenced by the application of lambda-cyhalothrin (Tables 3.53 and 3.54). In fact, the number of mesofauna in the litter and the soil was at least as high as in the control plots and often considerably higher. In addition, no specific group of litter and soil fauna show significant differences in abundance between control and the treatments. Individual data on the abundance of arthropod litter and soil fauna (showing the different animal groups) are provided in the Appendices 5 and 6.

In addition, the earthworm fauna was separately assessed in lambda-cyhalothrin treatments. Considering all earthworm species together, no significant differences in abundance and biomass between control and treatments were found. The mean number of individuals was lower on all treated plots, while the biomass was lower at the 3-monthly and the highest treatment but considerably higher in the monthly treatment (Table 3.55). The latter result was caused by the occurrence of two adult individuals of the giant glossoscolecid species *Rhinodrilus contortus*. Without these two, the average would have been in the same range as the control. However, in the analyses according to earthworm species, a significant differences in abundance were found between control and the low (3-monthly application of 40 g a.i./ha) treatment for *Andiorrhinus amazonius*, the most abundant species in the study site (Table 3.56).

Table 3.50: Remaining weight in litterbags treated monthly with three treatments of lambda-cyhalothrin and a control (means and standard deviations, values in %).

Time (months)	**Control**	**40 g/ha (monthly)**		**40 g/ha (3-monthly)**		**400 g/ha (once)**	
Start	100	100	Effect[1]	100	Effect[1]	100	Effect[1]
3	65.5 ± 6.9	70.8 ± 5.3	-5.3	69.3 ± 3.2	-3.8	70.3 ± 3.9	-4.8
6	49.4 ± 13.9	56.1 ± 2.8	-6.7	57.0 ± 3.9	-7.6	59.6 ± 4.4	-10.2
9	37.1 ± 6.7	47.4 ± 5.2 (**)	-10.3	43.6 ± 4.6	-6.5	48.7 ± 7.3 (**)	-11.6
12	34.7 ± 7.0	33.8 ± 4.1	-0.9	45.6 ± 8.4	-10.9	41.1 ± 4.5	-6.4

** Statistically different from control at P=0.01 (Dunnett's test). [1] Difference to the control: the values mean the % of reduction of the decomposition.

Table 3.51: Carbon and nitrogen content in organic matter from litterbags exposed to three treatments with lambda-cyhalothrin and a control evaluated after 6 months.

	N total (%)		**C total (%)**		**CN ratio**	
	Initial content	After 6 months	Initial content	After 6 months	Initial content	After 6 months
Control	0.9 ± 0.01	1.2 ± 0.1	51.0 ± 0.1	35.4 ± 5.2	56.7 ± 0.3	30.4 ± 4.3
40 g/ha (monthly)		1.3 ± 0.1		39.0 ± 1.4		32.9 ± 2.5
40 g/ha (3-monthly)		1.3 ± 0.1		39.8 ± 2.7		31.7 ± 2.3
400 g/ha (once)		1.3 ± 0.1		41.6 ± 1.4		32.8 ± 2.4

Table 3.52: Carbon and nitrogen content in organic matter from litterbags exposed to three treatments with lambda-cyhalothrin and a control evaluated after 12 months.

	N total (%)		**C total (%)**		**CN ratio**	
	Initial content	After 12 months	Initial content	After 12 months	Initial content	After 12 months
Control	0.9 ± 0.01	1.1 ± 0.2	51.0 ± 0.1	29.0 ± 4.6	56.7 ± 0.3	26.0 ± 1.6
40 g/ha (monthly)		1.2 ± 0.1		34.9 ± 1.2		28.0 ± 0.5
40 g/ha (3-monthly)		1.2 ± 0.1		32.5 ± 5.7		27.3 ± 2.4
400 g/ha (once)		1.4 ± 0.1		43.1 ± 1.9		31.6 ± 0.5 (**)

Table 3.53: Litter fauna abundance (individual/m^2) exposed to three treatments with lambda-cyhalothrin and a control.

	Control		**40 g/ha (monthly)**		**40 g/ha (3-monthly)**		**400 g/ha (once)**	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Arthropods (Mesofauna)	1173	722	1520	705	2144	1733	3292	2274
Arthropods (Macrofauna)	605	405	472	208	552	176	944	470

Table 3.54: Soil fauna abundance (individual/m^2) under exposed to three treatments with lambda-cyhalothrin and a control.

	Control		**40 g/ha (monthly)**		**40 g/ha (3-monthly)**		**400 g/ha (once)**	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Arthropods (Mesofauna)	1157	659	2036	758	2064	595	1136	119
Arthropods (Macrofauna)	741	274	712	359	572	200	596	165

Table 3.55: Earthworm abundance (individual/m^2) and biomass (g fw/m^2) exposed to three treatments with lambda-cyhalothrin and a control.

	Control		**40 g/ha (monthly)**		**40 g/ha (3-monthly)**		**400 g/ha (once)**	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Earthworms (abundance)	27.7	24.0	15.5	14.1	8.5	7.0	11.3	4.8
Earthworms (biomass)	6.1	6.4	18.8	20.9	3.8	4.1	3.5	3.2

Table 3.56: Earthworm species abundance (individual/m^2) exposed to three treatments with lambda-cyhalothrin and a control.

Species	**Control**		**40 g/ha (monthly)**		**40 g/ha (3-monthly)**		**400 g/ha (once)**	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Andiorrhinus amazonius	14.3	9.1	5.3	4.1	4.5 *	4.7	7.0	3.6
Tuiba dianaea	1.5	2.3	0	0	1.0	1.4	1.5	1.3
Urobenus brasiliensis	6.3	11.0	1.0	0.8	1.3	1.9	1.0	1.2
Rhinodrilus sp.	0.5	0.8	0.8	1.5	0.3	0.5	1.0	2.0
Pontoscolex corethrurus	2.5	6.1	0	0	0.8	1.5	0	0
Rhinodrilus contortus	0	0	0.5	1.0	0	0	0	0
Tuiba sp.	0	0	0.5	1.0	0	0	0	0

* Statistically different from control at P = 0.05 (Dunnett's test).

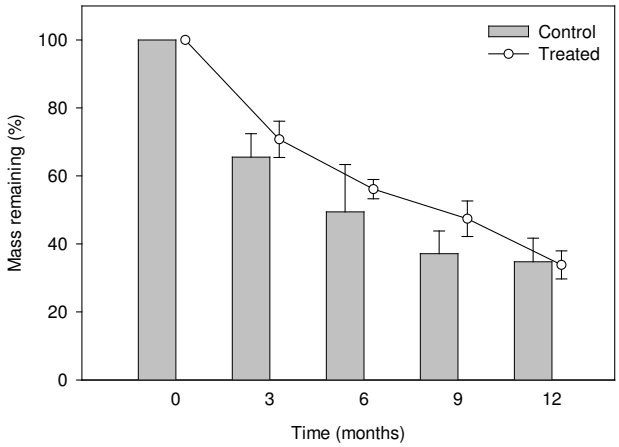


Figure 3.57: Remaining weight in litterbags treated monthly with 40 g/ha of lambda-cyhalothrin and a control (means and standard deviation bars).

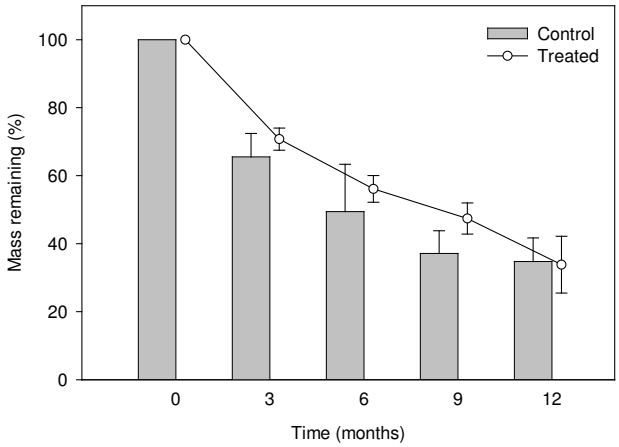


Figure 3.58: Remaining weight in litterbags treated 3-monthly with of 40 g/ha of lambda-cyhalothrin and a control (means and standard deviation bars).

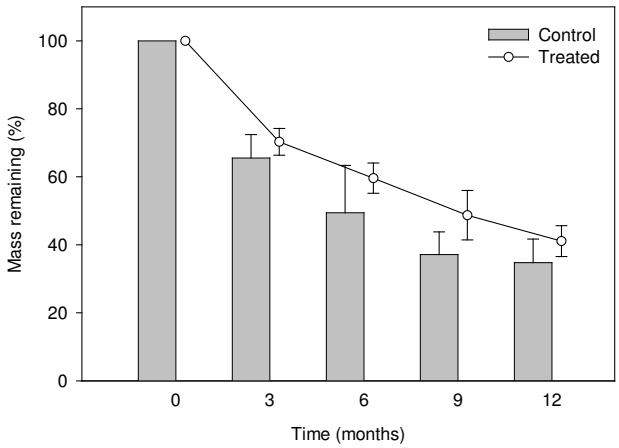


Figure 3.59: Remaining weight in litterbags treated once with of 400 g/ha of lambda-cyhalothrin and a control (means and standard deviation bars).

3.6 Screening of carbendazim concentrations

Using the method described in Section 2.7.2, the concentrations of carbendazim were determined in two laboratory tests (one with OECD artificial soil, the other with LUFA soil), in one TME test (both in leachate and mineral soil) and in the field test (mineral soil). Due to the restricted number of test kits, these measurements were intended to give an idea about exposure concentrations or, in a more general sense, to confirm the exposure (i.e., using this method as some kind of quality measure). The results of the five tests are given in Appendices 31 to 35.

According to these data, only 1 - 15 % of the original amount of carbendazim applied to OECD artificial soil could be determined in samples taken eight weeks after application. Due to storage at relatively high temperature (4 °C) for several months, further degradation during this time cannot be ruled out. Immediately after application, the concentrations detected in LUFA soil varied between 20 and 50 %. Two weeks later, the concentrations differed between 17 and 42 % of the applied amount. While in the test with OECD artificial soil the measured amount decreased in correlation with the initial nominal concentration, such a correlation could not be detected in the test with LUFA soil.

In the leachate of the TME test, very small concentrations of carbendazim were detected in nearly each sample, independent of the application scenario. Despite a high variability between the individual soil cores, the average concentration in both sample series was nearly identical (38.5 versus 40.8 µg a.i./L). In relation to the applied amount, only a tiny fraction leached through the soil column.

In the TME soil samples, carbendazim could be detected in all samples five months after application of the test substance. The detected concentration in the soil was higher in the second (monthly applications of 4.51 a.i. kg/ha) than in the first (application of 45.1 kg a.i./ha once) scenario: 0.97 versus 2.71 mg a.i./kg soil. In relation to the initial nominal soil concentration (i.e., calculated without degradation), 1.6 % (one high application) and 45.13 % (several small applications) of the expected amount were measured. However, a more realistic relationship can only be attained when taking into consideration the degradation of carbendazim during the five months of exposure (for details see Section 4.2.1).

In the litterbag field test, carbendazim could be detected in (nearly) all samples 12 months after application of the test substance. The mean measured concentration differed clearly depending on the application scenario: in the first scenario (3-monthly application of 1.00 kg a.i./ha) and in the second scenario (monthly applications of 1.00 kg a.i./ha for eleven months) it was 0.02 mg ai./kg, while in the third scenario (one application of 10 kg a.i./ha) it was 0.11 mg a.i./kg (i.e., about 5 times higher). In relation to the initial nominal soil concentrations (i.e., calculated without degradation), the detected amounts differed less: here the values are 1.1 %, 1.5 % and 0.87 %. However, a more realistic relationship can only be attained when taking into consideration the degradation of carbendazim during the 12 months of exposure (for details see Section 4.3.1).

4 DISCUSSION

4.1 Laboratory tests

4.1.1 Earthworm test methodology

Acute toxicity tests with *E. fetida* were done using the standard methodology as described in the guidelines OECD nº. 207 (OECD 1984a) and ISO-11268-1 (ISO 1993a) as well as according to the modified "tropical" version developed in this work. No specific methodological problem was observed during the performance of these tests. All acute tests were considered valid taking into account that the criterion mortality (< 10%) is the most important one. The second validity criterion, change of earthworm body weight (≤ 20% during the test), which was not met in some tests performed here, is specified only in ISO guideline 11268-1 (ISO 1993a). According to Kula (1998), in only 5% of tests (OECD soil, at 20 ºC) presented to the German Pesticide Registration Agency, a weight decrease of higher than 20% was observed in control boxes. Therefore, the author (op. cit.) suggested the inclusion of this criterion for the next revision of this OECD guideline. In the tests performed here, the biomass change varied according to the test conditions. Higher losses of biomass were found in the tests done using tropical (TAS soil and 28 ºC) compared to those using the standard temperate (OECD soil and 20 ºC) conditions. Since both soils differ only slightly in their composition (in particular in the source of organic matter), it is assumed that the temperature is the main factor responsible for differences in biomass loss. At higher temperatures the earthworms show a higher burrowing activity and might also be more exposed to desiccation. This assumption is backed up by the fact that the loss of biomass observed in the tests with TNS (a soil with a low pH; ca. 4.1) and with tropical *E. fetida* in OECD soil but at 28 °C is in the same range of magnitude. The same distribution regarding species, soils, and temperatures was found concerning the tests with LUFA soil: European *E. fetida* at 20 °C showed a clear increase in biomass while the tropical variant partly lost little weight, partly gained some. Independently from the type of soil, the biomass of the worms was mainly influenced by temperature – assuming that the basic metabolism of the two variants is the same.

Chronic tests were done following the guidelines OECD nº. 222 (OECD 2003) and ISO-11268-2 (ISO 1998a). No methodological difficulties occurred during the

performance of these tests. As in the acute tests, no problem occurred concerning the validity criterion mortality. In addition, with two exceptions, the number of juveniles was in all cases higher than the required number of 30. One exception occurred in LUFA soil but as the criterion was only slightly missed, the test was considered to be valid. The other exception occurred with TNS, which was probably caused by the low pH of this soil. Tests with European soils gave similar results: pH mainly affects reproduction but not mortality (Scheffczyk, personal comm.). Concerning the CV of the number of juveniles, again nearly all tests (except those with TNS) fulfilled the validity criterion of 30% indicated in the OECD and ISO guidelines. In the tests with TASx, the CV was higher than in the tests with OECD and LUFA (the 30% value was slightly missed twice), but this result is probably due to a lack of experience with this modified test.

So far, no standard protocol has been published for avoidance tests, but a draft version is under review (ISO 2003a). The methodology for this test including the validity criteria was based on the specific literature (e.g., Stephenson et al. 1998 and Hund-Rinke et al. 2003). As long as the worms did not show a significant different distribution in the two halves of a test vessel filled with the same soil, the test was considered to be valid. In addition, the mortality in a test should not be higher than 10%. These criteria will be included in the next version of the draft ISO protocol (Römbke, personal comm.). No problems occurred concerning the validity of the avoidance test, showing that the use of TAS or a higher temperature do not influence the performance of this method.

Summarizing the situation, it has to be questioned whether for tests performed under tropical conditions (e.g., 28 ºC) the validity criteria and their acceptable limits as listed in guidelines with conditions can be accepted. While this seems to be easily possible in the case of the parameters mortality, number and juveniles and avoidance behavior, the biomass has to be treated separately. Here the higher temperature as used in tests under tropical conditions causes higher metabolic rates, which can lead to a quick decease in earthworm weight even within 14 days. For this reason, it was decided not to use the biomass change as a cut-off criterion. Further research is needed whether this criterion should be re-set (e.g., to 30%). Other possibilities include feeding of the worms or the shortening of the test duration.

Species selection

As already listed in Section 2.3, specific criteria must be taken into account when selecting a suitable test species. In addition, the number of the selected test species should be kept low in order to maintain practicability and to increase the acceptance of the idea to introduce new tests especially for tropical regions. In accordance with the international discussion about terrestrial test strategies (e.g., Samsøe-Petersen and Pedersen 1994), an oligochaete species should be selected in any case due to its high ecological relevance.

Among terrestrial oligochaetes, earthworms of the family Lumbricidae are the most widely used test organisms. The acute toxicity test with the compost worm *Eisenia fetida* in artificial soil (OECD 1984a) is the oldest and to date the most important test with soil organisms. In the meantime, a large number of slightly modified variations of this method have been published (e.g., NEN 1988; ISO 1993a; ASTM 1995). A first overview shows that the earthworm subacute test (FDA 1988) is also very similar, but two changes are remarkable: (i) explicitly the measurement endpoint behavior is roughly quantified and (ii) very rarely in terrestrial ecotoxicological tests is the measurement of the actual concentration of the test chemical required. In Brazil, the environmental agency (IBAMA) did not adopt the OECD earthworm acute test for the assessment of chemicals but requires instead the ARTISOL test (AFNOR 1989), which was standardized in France (see IBAMA 1990). The earthworm reproduction test (ISO 1998a; OECD 2003) is based largely (e.g., with regard to test substrate and test species) on the acute test.

It has been tried several times to use ecological more important species as a test organism, especially the lumbricid mineral soil dweller *Aporrectodea caliginosa*, today distributed worldwide due to European colonization. Despite the fact that a proposal in standardized form is available (Kula and Larink 1997), it seems unlikely that this will be widely used due to the fact that the reproduction of *A. caliginosa* in the laboratory is difficult and time consuming. In addition, there are a number of proposals for tests with the vertical burrowing species *Lumbricus terrestris*, which is probably the most important "key species" (or "ecosystem engineer" according to Jones et al. 1994) of soils in temperate regions of the world. Due to its long generation period and large body size, these

methods (e.g., the Daniel-funnel test (Bieri 1992)) have not become established for lack of practicability and efficiency.

Summarizing the experiences from the Holarctic, the compost worm *Eisenia fetida* (nowadays separated into two distinct species, *E. fetida* and *E. andrei* (Bouché 1992)) is still the most often used terrestrial test species due to several reasons. *E. fetida* is widely available in temperate and tropical areas, can be reared at high densities in laboratory cultures and has, in comparison to other Lumbricidae, a short life-cycle. Secondly, it is an organism that lives in intensive contact with the test substrate soil. The species is a typical representative of epigeic worms (i.e., those living close to the soil surface, mainly in the litter layer), which are likely to be affected by chemicals reaching the soil via spraying (which is the case for most pesticides). In addition, due to the fact that tests with these species are required, for about 20 years, as part of the registration process of pesticides in Europe and North America, extensive ecotoxicological information is now available (e.g., Edwards and Bohlen 1992). On the other hand, the choice of this test species has been a major source of criticism of the OECD protocol (Spurgeon and Weeks 1998). Actually, *Eisenia fetida* does not occur in agricultural or natural soils, but inhabits sites rich in organic matter such as compost and manure heaps.

In general, earthworm species do not differ strongly in sensitivity to many PPPs. Heimbach (1984) has already shown that the sensitivity of various lumbricid earthworms towards chemicals varies, but no species is always the most sensitive one. Of course, certain species can react differently to selected compounds (Bauer and Römbke 1997), e.g., some species are more exposed to chemicals due to their behavior (Edwards 1983). When comparing results of laboratory tests with *Eisenia fetida* with those from other species, differences in LC_{50} values were nearly exclusively within a factor of 10. One noteworthy exception is Propoxur, where the LC_{50} of *Eisenia fetida* was 72 times higher than that for the endogeic species *Aporrectodea longa* (Jones and Hart 1998). Another example is the higher sensitivity of the species *Lumbricus rubellus* and *Aporrectodea rosea* compared to *E. fetida* with respect to metal contamination like Zinc (Spurgeon and Weeks 1998).

So, for the foreseeable future, the two *Eisenia* species will be the standard earthworm species for terrestrial ecotoxicological tests under temperate conditions.

Based on the experience from the Holarctic (e.g., the suitability of litter-inhabiting (epigeic), fast growing worms) and adding a preference for high temperatures, the following three (in fact four: the older literature does not differentiate between *E. fetida* and *E. andrei*) earthworm species are considered for ecotoxicological tests under tropical conditions:

- *Perionyx excavatus* (Perrier 1872), Megascolecidae (originally from South-East Asia);
- *Eudrilus eugeniae* (Kinberg 1866), Eudrilidae (originally from West Africa);
- *Eisenia fetida* (Savigny 1826), Lumbricidae (originally from the Caucasus?).

It might be surprising that the same species (*E. fetida* or *E. andrei*) is listed as a potential test organism for temperate as well as for tropical conditions. As already shown by Graff (1978) and Raquet (1983), several populations (or races?) of this species from different parts of the world differ in their physiology and reproduction. Among peregrine exotic species, Barois et al. (1999) could only identify few epigeic worms: besides *E. andrei* and *E. fetida, Perionyx excavatus, Drawida willsi* (both originally from Asia), *Hyperiodrilus africanus, Dichogaster bolaui* (both originally from Africa). Actually, only *E. fetida/andrei, P. excavatus* and *E. eugeniae* are classified by these authors as well culturable – out of a list of about 25 potential candidates.

Maybe their long history as an inhabitant of habitats like compost heaps with their often fluctuating temperature, and their world-wide distribution by man in often small "starter" groups has facilitated this differentiation. In any case, it is known that *E. fetida* and *E. andrei* tolerate wider temperature conditions than the two other species (e.g., Reinecke et al. 1992).

Nearly nothing is known about the use of these species in ecotoxicological testing under tropical conditions. In fact these species have originally been investigated due to their potential suitability for waste management, i.e., vermicomposting, or as a protein source (Edwards et al. 1985). One of the most important criteria for this role – as well as for the use as test organisms – is the practicability of breeding: It must easily be possible to produce a mass culture of these worms without great effort and within a short period of time. The dissertation of Knieriemen (1984, 1985) and several articles from South Africa (Reinecke and Viljoen 1988; Viljoen and Reinecke 1988, 1989;

Reinecke et al. 1992) are still the most important sources when comparing these three species. The information listed by Knieriemen (1984, 1985) refers explicitly to *E. andrei* (the situation in South Africa is less clear). Due to the fact that the Asian species *P. excavatus* is not available commercially and has also not been found in the vicinity of the Brazilian study area so far, the comparison of the culture requirements consequently focussed on *E. eugeniae* and *E. andrei*. However, quickly it became clear when performing own studies with these two species that *E. eudrilus* is relatively sensitive to low oxygen levels and high population densities. In addition, it needs food of high quality and likes to escape from the culture boxes. In other words, it is much more difficult to handle than the *Eisenia* species. Therefore, Knieriemen (1984) and Reinecke et al. (1992) concluded that *E. fetida* is the preferred species for vermicomposting.

In addition to the earthworms discussed so far, the peregrine species *Pontoscolex corethrurus* could be a candidate for ecotoxicological testing. It is the only member of the primarily South American oligochaete family Glossoscolecidae which is distributed circumtropically today (Lee 1985). *P. corethrurus*, a mesohumic inhabitant of deeper layers of the mineral soil according to the classification of Lavelle (1984) and Barois et al. (1999), is very common in nearly all anthropogenically influenced tropical soils (Römbke and Verhaagh 1992). It is used intensively in ecological studies (in the laboratory as well as in the field (e.g., Lavelle et al. 1987; Hamoui 1991; Fragoso et al. 1999). At the Embrapa sites it is rare or even absent in the secondary and primary forest, but very abundant and dominant in many of the polyculture plantation cultures (Römbke et al. 1999). However, in all studies reported in the literature reviewed so far (e.g., Barois et al. 1999; Brown et al. 1999) these worms have been collected in the field; i.e., at least a mass rearing is problematic. Own experiences support this conclusion. However, due to its ecological relevance and dominance in the field sites it was included in the trials to identify the most suitable test species.

Based on the above discussion, a tropical strain of *E. fetida* was selected as the most suitable organism for soil ecotoxicological tests done under tropical conditions.

Substrate selection

Artisol, consisting of an amorphous silica gel mixed between glass balls, was the first standard substrate recommended to expose earthworms in toxicity assays (AFNOR

1989). Adults of *Eisenia fetida* were exposed to chemicals which had been mixed into the Artisol (Ferrière et al. 1981). However, the silica gel does not present any similarity with natural soils and consequently, for reasons of ecological realism, the test was not recommended as a tool for the Environmental Risk Assessment of chemicals (Verhoef and Van Gestel 1995). Nowadays, the substrate to be used in ecotoxicological soil tests recommended by international agencies is artificial soil (OECD 1984a; ISO 1993a), since the test conditions are closer to those in the natural environment of earthworms. However, in most tropical countries, international guidelines for the environmental assessment of chemicals in the soil are officially not in use. In case they are, these guidelines are neither adapted for their specific conditions nor modified according to the international discussion. As an example, Artisol is still recommended in local guidelines in Brazil (IBAMA 1990).

However, there are also technical reasons why OECD artificial soil is difficult to use in tropical countries. The main limitation is the unavailability of the type of organic matter (i.e., *Sphagnum* peat moss) described in the guidelines. Originally, these guidelines were written focussing on the situation of countries in temperate regions, where the peat moss is easy to obtain. In order to allow its use in other regions of the world where this component is not readily available, a new source of organic matter had to be identified in order to get a modified OECD artificial soil especially modified for tropical regions: the Tropical Artificial Soil – TAS. In this study, three sources of organic matter potentially suited as a replacement for peat moss were investigated.

The first material tested as a source of organic matter (OM) in the new artificial substrate (Tropical Artificial Soil – TASx) was xaxim. The artificial soil (TASx), with xaxim as source of organic matter, is a feasible substrate for the earthworms, i.e., no mortality was observed. The loss of biomass is more correlated with the temperature at which the test was done and the species which was used than with the type of substrate. Thus, this material was used in the majority of the toxicity tests done in this study. However, the commercial use of Xaxim is restricted by Brazilian law, since this species was included in the list of endangered species due to its overuse.

Some commercial laboratories in Brazil have used the pure *Sphagnum* plant (instead of *Sphagnum* peat) as a source of organic matter for artificial soil, but

unfortunately, no data are available due to confidentiality reasons. In this study, *Sphagnum* was tested in artificial soil, but was found to be not suitable as a substrate in earthworm tests. In addition, the chemicals mixed into were found to be more available, thus resulting in a higher toxicity than in the tests with other organic matter, probably because *Sphagnum* is not a decomposed organic material.

In parallel, coir dust was used as a third alternative for the composition of tropical artificial soil. It consists of the short fibers and dust that remain after the coconut husks are processed for the extraction of the long fibers. Also called "coconut peat", with similar physical properties to sphagnum peat, it has been suggested as a substitute for growing media for plants (Meerow 1994; Abad et al. 2002). Artificial soil prepared with coir dust (TASc) showed similar characteristics (e.g., water retention capacity) to TASx and OECD soil. The earthworms did not show any adverse response to TASc soil when compared to OECD soil in control vessels of the avoidance test. However, when coir dust is obtained directly from fresh coconut peels, a fermentation process occurs in the substrate causing avoidance behavior to earthworms. Thus, before its use in artificial soil, composting the coir dust until the fermentation process has ceased is recommended. Finally, the validity criteria were fulfilled in two toxicity tests using TASc performed with carbendazim and lambda-cyhalothrin. While the results of the tests performed in TASx and TASc were nearly identical in the case of carbendazim, the LC_{50} as well as the NOEC and LOEC values were lower in TASc than in TASx. Of course, these tests should be repeated with other chemicals in order to be sure that the use of coir dust in TAS does not strongly influence the results of toxicity tests.

Coir dust is common in many tropical countries and is also commercially available in temperate regions. In conclusion, coir dust is a suitable alternative to *Sphagnum* peat for the composition of artificial soil modified for tropical regions (TAS).

Besides the tests with artificial soils, two natural soils were used in this study. The aim was to obtain an idea about the toxicity of the test chemicals in ecologically relevant substrates. In temperate regions, the standard soil LUFA 2.2 is recommended as a control for testing the quality of soils (ISO 1999a), because it is considered to be representative for agricultural soils of Central Europe. It is a loamy sand with a pH ca. 6.0 (Schinkel 1985), thus being relatively similar to OECD artificial soil except its

lower amount of organic matter (4.6 and 6.2 %, respectively). This soil is often used for the performance of ecotoxicological research projects with invertebrates, including *E. fetida* (Løkke and Van Gestel 1998). For this reason, it was also used in this study.

In parallel, it was attempted to identify a natural soil (TNS) suitable for the tests performed under tropical conditions. In the region around Manaus, no natural soil with similar properties of those of artificial soils or LUFA soil could be found. Usually these tropical soils are very acid with pH values ranging from 3.5 to 5.0. Therefore, it was decided to use a sandy clay loam soil (TNS) typical for Central Amazonia despite its low pH of 3.9. Due to its low pH, the attempts to test *E. fetida* were not successful. The earthworms were able to survive for a short period (14 d) in TNS soil but showed an aggregating behavior, which indicates adverse conditions in the substrate. In chronic tests (reproduction test, 56 d) the pH increased from 3.9 to 5.5, due to the food mixed in the substrate. In this case the earthworms survived better but produced a low number of juveniles. In the short-term avoidance tests the earthworms did not enter the TNS and died on the surface. Summarizing these results TNS is considered not to be feasible for toxicity tests using *E. fetida*. Further research is needed in order to determine whether a more suitable tropical field soil can be identified or whether another oligochaete species has to be selected for testing such soils. Finally, the question remains how the results of artificial soils can be extrapolated to the real field situation when the soil properties are different.

Test conditions

In comparison to the test conditions required by the "temperate" guidelines (OECD 1984a and ISO 1993a), nearly no changes are proposed when performing tests under tropical conditions. The one big exception is temperature, because this is the main factor that differs between temperate and tropical regions. It can influence the toxicity of pesticides to soil invertebrates in two ways, chemically and biologically:

- Higher temperature may enhance the degradation as well as alter the metabolism (i.e., the degradation pathway) of chemicals. However, few data are available on how pesticides react under tropical conditions (e.g., Andrea and Wiendl 1995; Sahoo et al. 1990; Sattar 1990). A common assumption is that the degradation

will be quicker (thus the exposure shorter) due to a higher activity of soil micro-organisms.

- At higher temperature, the toxicity (e.g., of zinc) can increase (Spurgeon et al. 1997), since the organisms are more active and consequently may be more exposed to the chemicals. On the other hand, due to a higher physiological rate, organisms might be better able to metabolize potentially toxic chemicals.

The same observation (i.e., the impossibility to differentiate between the biological and chemical effects of temperature) was made in tests with collembolans (Martikainen and Krogh 1999).

In any case, there is no general rule for the effect of temperature on the toxicity of chemicals with respect to soil invertebrates and in particular to earthworms.

For practical reasons (e.g., room temperature is very suitable for *E. fetida*, a species that naturally lives in - often warm - compost and that does not reproduce at low temperatures) and in order to be able to compare the results of different tests, a temperature of 20 ºC was fixed as a standard for ecotoxicological tests performed in temperate regions. Probably this value could also be used for subtropical climates. For tropical humid areas no recommendation concerning the optimum test temperature exists so far. Therefore, a standard tropical temperature of 28 ºC was used in this study. This value was selected for two reasons:

- The annual average of the soil temperature at the study site close to Manaus is about 28 °C (see Appendix 1).
- The optimum reproduction temperature of several oligochaetes including *E. fetida* is lower than 30 °C; in other words both the number and the health of juveniles declines when the soil temperature is constantly higher (Graff 1983; Knieriemen 1984; Reinecke and Kriel 1981).

Chemicals

The model test chemicals used in this study were selected due to the fact that they fulfilled criteria, e.g., have effects on oligochaetes and arthropods, are well-known (in temperate regions), and are used in tropical areas, in particular in the State of Amazonas, Brazil. In this respect, no problems occurred during the work. In addition, their handling (e.g., mixing into the soil substrate) was also easy. In particular it is

worth mentioning that, as expected, effects on very different organisms could be observed. Unfortunately, nearly nothing is known about their environmental fate under tropical conditions, but this is true for nearly all pesticides with the exception of some very old ones like DDT (Kaushik 1991).

With respect to the further use of the data gained here it was not foreseeable that one of the test chemicals (benomyl) was withdrawn from the producing company by the end of 2002. However, the insight between the toxicity of a parent (benomyl) and a metabolite (carbendazim) as well as the information about the influence of different substrates, endpoints, species, etc. on the toxicity of a pesticide will be useful in any case.

Since the basic idea of an Environmental Risk Assessment (ERA) is the comparison of the exposure and the effects of a chemical, it is necessary to quantify the concentration of the test chemical in the soil. The determination of chemical concentrations in environmental substrates (here mainly soil) usually requires the use of very elaborated chemical analytical methods like HPLC, GC-MS and so on. In case the necessary resources are not available, the RAPID-kit method is considered to be a quick and simple alternative. However, this method has two disadvantages:

- Since the method is usually used in the area of food quality assurance, kits are available for very few environmentally relevant pesticides. In addition, the method was primarily developed for food stuff like fruits and had to be adapted to the complex substrate soil. Only the concentrations of carbendazim could be checked in this study, since for lambda-cyhalothrin no kit is available on the market (its development, while possible, is very expensive and time-consuming).
- Due to the measurement principle (and, in particular, the simplified extraction process), the RAPID-kit is (only) a screening method; i.e., it is not possible to determine – the often very low - concentrations with the same level of accuracy as in “normal” chemical residue analyses. In food chemistry, only samples that contain the test substance according to the RAPID-kit results are measured by “normal” residue analysis.

However, taking these restrictions into consideration, the RAPID-kit should be able to indicate reliable concentration ranges and tendencies concerning the fate of carbendazim in soil (i.e., it can be used to verify the exposure of the test organisms). In

addition, the results of these measurements can be used to estimate whether degradation of carbendazim in the TME and field studies took place as calculated. So, it can be used as a quick check to determine whether the initial nominal concentrations were (more or less) reached or whether there is still any exposure. In any case it is more suitable for laboratory test soils due to their higher homogeneity than for field soils.

However, before recommending this method, it must be remembered that the quality assurance must be improved. In this case, the available resources (i.e., the number of kits available) did not allow performing all the additional measurements usually done in residue analysis. For example, the quality of the extraction process is checked by measuring the recovery of the test substance: After mixing a known amount of the chemical into the soil, the soil sample is worked up immediately. Depending on the extraction process used, up to 100 % of the applied amount is measured. Also control samples should be measured in order to clarify whether substances normally contained in the soil can be mixed up with the test substance. Finally, all measurements should be done at least in duplicate. In the present study, only the last step was done in the LUFA test. The few data gained in this test show some but still acceptable variability (e.g., 47.8 to 46.9, 32.6 to 43.0 or 61.9 to 39.0; all data in percent of the initial nominal concentrations).

Summarizing these experiences, chemical residue analysis is still the most appropriate method for determining the concentration of a test substance in soil. There are, however, certain circumstances where RAPID-kits may be an appropriate alternative (test kit available, screening approach).

Practicability

The procedures recommended in OECD guidelines for acute and chronic ecotoxicological tests with soil invertebrates do not require a complex laboratory structure or high-tech equipment. The same is true for the tests modified for the tropical conditions proposed and used in this study. Thus, with simple and accurate instruments (e.g., breeding chambers, balances) and following the GLP rules (e.g., the adoption of Standard Operation Procedures - SOPs), these test were implemented without difficulty in the Embrapa laboratory of Manaus (there, ecotoxicological work had never been

done before). The slight adaptations needed (e.g., a new source of organic matter) does not make the procedures more complicated.

However, as is the case for all new test systems (and as laid down in the OECD rules for the standardization of tests), the reproducibility of the modified tests proposed here (i.e., adapted for tropical conditions), should be proven in a ring test. In such a ring test, the same substances are tested in different facilities according to the new or modified guideline (Römbke and Moser, 2002). Since in this case the differences compared to the existing guideline are not overwhelming, a relatively small group of about five to six laboratories would be sufficient.

Endpoints

The following endpoints are standardized and described in the guidelines for acute and chronic ecotoxicological tests with earthworms: mortality, reproduction and body weight change. A behavioral endpoint was used in avoidance tests, but has not yet been standardized.

Mortality (main endpoint in acute tests) and reproduction (endpoint for chronic tests) were considered practicable and reproducible in both laboratories. However, the mean number of juveniles in the controls was lower in most tests performed at the Embrapa laboratory compared to the tests at the ECT laboratory. These differences were probably caused by the quality of the cow manure used as food for the earthworms. The manure used in the Embrapa laboratory was obtained from extensive cattle-raising, where the animals have a diet poor in protein. On the other hand, the manure used at ECT was got from a non-conventional farmer who used high quality food. According to Kula (1998), such differences may also occur between European laboratories when the manure is heated, sterilized or has a high ammonia content.

The body weight change is used in the acute and reproduction tests, although it is not the main endpoint in either of them. It is not clearly defined and its use needs to be more discussed. According to the experiences in a ring test with contaminated soils, it seems that the sensitivity of this endpoint is nearly always somewhere between mortality and reproduction (Hund-Rinke et al. 2002). So, it can be a useful indicator for chronic effects when measured in acute tests, but in chronic tests the reproduction is clearly more sensitive and, moreover, ecologically relevant. In addition, its use in the

former case is restricted due to the fact that the biomass data can only be assessed at concentrations where no or nearly no mortality occurred.

As mentioned earlier, the behavioral endpoint avoidance has not been used for long. There are indications that its sensitivity is comparable to the one determined in reproduction tests (Hund-Rinke et al. 2003). However, it seems that some chemicals cannot be detected by the earthworms, meaning that they will die in the test soil without trying to escape (e.g., for organophosphates; Hodge et al. 2000). Despite this problem, the endpoint should be investigated further since it is potentially a quick and easy alternative to the acute mortality tests currently used.

Assessment

The statistical procedures used to determine toxicity parameters such as $LC_{50,}$ $EC_{50,}$ LOEC and NOEC were based on the recommendations given in the respective guidelines. Furthermore, for the behavioral endpoint (avoidance), the parameters $EC_{50,}$ and NOEC were calculated according to the draft ISO guideline (ISO 2003a). The appropriate methods for each test are well discussed in the literature and were applied without difficulty. However, some problems occurred regarding the estimation of the NOEC parameter:

- In some cases it was not obtained when the lowest test concentration produced a statistically significant effect
- As the confidence intervals cannot be calculated, it was not possible to compare the NOEC values from different tests.

Due to these statistical problems with NOECs, the importance of this endpoint has been criticized (see e.g., Chapman et al. 1996). Despite this criticism, the NOEC estimation is still required by the existing guidelines (e.g., for the registration of a new pesticide, NOECs have to be reported to the authorities). On the other hand, regression analysis (i.e., determination of ECx values) is becoming more and more accepted. Some guidelines already allow or require both values (e.g., the new OECD Earthworm Reproduction Test (OECD 2003)). Thus, in this study the existing recommendation for the estimation of NOEC and the calculation of EC_{50} was used.

Methodological summary

The methodological experiences gained in this study can briefly be summarized as follows:

- The existing earthworm test methods standardized by OECD and ISO for temperate regions were successfully modified for tropical conditions.
- In particular, a Tropical Artificial Soil (TAS) has been developed and a tropical variant of the well-known compost worm *Eisenia fetida* was found to be suitable as tropical test species.
- The modified tests are easy to perform and do not require specific equipment.
- No tropical field soil comparable to the LUFA standard soil could be identified due to the differences between the properties of Amazonian soils and the requirements of *E. fetida*.
- Most of the tests performed were valid. In those cases where these criteria were not met, the reasons could usually be identified (e.g., poor food quality).

However, open areas for further research were also identified:

- A species suitable for tests with acidic tropical field soils is needed.
- More tests with coir dust as source of organic matter have to be performed.

4.1.2 Tests results with *Eisenia fetida*

Before discussing the effects of the three chemicals on earthworms, the results of the RAPID-kit measurements have to be taken into consideration (only for the test substance carbendazim). Referring to literature data gained with the RAPID-kit method and OECD artificial soil (Römbke and Moser 2002), it is highly probable that carbendazim in a range of 0.32 to 3.2 mg a.i./kg at 20 °C is stable for about six weeks. However, at concentrations > 2 mg a.i./kg a decrease was observed (at 3.2 mg a.i./kg about 60 % were recovered), indicating that microorganisms start to use this chemical when available at a minimum amount. The data gained in this study in OECD artificial soil at 28 °C after eight weeks are much lower (1.5 to 2 % of the nominal value), but show the same kind of concentration dependency. This means that the extraction process was not completely successful. When looking at the results of the LUFA test, the data show that bioavailability is clearly higher than that of the OECD soil (this statement is surely also true for the tests performed at 20° C). A degradation of

carbendazim during the test period is unlikely due to the short period of time, in particular in the case of the first LUFA samples.

Therefore, the RAPID-kits confirm that the test organisms were exposed to carbendazim during most of or even the whole test period in the test performed at 20° C. Under tropical conditions, exposure was probably less but due to the small amount of data it was not possible to determine exactly how much. However, it is obvious that exposure was clearly higher in LUFA than in OECD (or TAS) artificial soil. As it was not possible to use detailed exposure concentrations, it was decided to use nominal values for the discussion of laboratory test results.

Acute effects

Benomyl

The fungicide benomyl is reported in the literature as being very toxic to Oligochaeta. For example, LC_{50} values for *E. fetida* and *E. andrei* in artificial soil at 20 °C vary between 3.2 and 27 mg a.i./kg (one outlier is at 82.6 mg a.i./kg), while in some field clayey soils (with about 12 % organic matter) clearly higher values (up to 360 mg a.i./kg) were found (Table 4.1). On average, *L. terrestris* was more sensitive (LC_{50} values < 10 mg a.i./kg; even in a clay soil the value was only 34 mg a.i./kg) than *E. fetida*.

However, in this study, benomyl was much less toxic when tested with tropical *E. fetida* at 28 ºC. In OECD soil, the toxicity was 20.8 times lower (LC_{50} value: 458.3 mg a.i./kg) with tropical than with European *E. fetida*, and in TAS soil benomyl was even less toxic (LC_{50} value: 633 mg a.i./kg; i.e., 28.8 times lower). The same tendency was observed in the tests with the field soils LUFA and TNS: the toxicity was lower by a factor of about 4 when using tropical worms compared to European *E. fetida* (Tables 4.3 and 4.4).

Table 4.1: Results of oligochaete acute tests with benomyl performed in various soils; all data are given in mg a.i./kg.

Species	Soil	% OM	LC_{50}	Reference
Lumbricidae				
Not specified	OECD	10	3.2	Maroni et al. (2002)
E. fetida	OECD	10	27	Haque and Ebing (1983)
E. fetida	OECD	10	82.6	ESG (1999)
E. fetida	Field	n.d.	< 200	ESG (1999)
E. andrei	OECD	10	22	Heimbach (1984)
E. andrei	OECD	10	19	Heimbach (1985)
E. andrei	OECD	10	5.7	Van Gestel (1992)
E. andrei	Field	2.9	13.5	ESG (2002)
E. andrei	Field	3.5	24.5	ESG (2002)
E. andrei	Field	12.2	360	ESG (2002)
L. terrestris	Field	11	3.5	Haque and Ebing (1983)
L. terrestris	OECD	10	< 50	ESG (1999)
L. terrestris	Field	2.9	1.6	ESG (2002)
L. terrestris	Field	3.5	2.2	ESG (2002)
L. terrestris	Field	12.2	34.0	ESG (2002)

When benomyl is exposed to soil, it is rapidly (i.e., within some hours to a few days) converted to carbendazim. This step is so quick that an effect of temperature would be difficult to determine. The half-lives of carbendazim at 15, 20 and 25°C were determined as 28, 25 and 21 days, respectively, indicating a more rapid degradation of this substance with increasing temperature (WHO/FAO 1994). Therefore, the low toxicity of benomyl observed in the tests performed under tropical conditions (i.e., 28 ºC) is probably caused by a higher degradation rate.

In addition, it cannot be ruled out that the tropical *E. fetida* is, due to some unknown reason, less sensitive to benomyl (and carbendazim) than the European variant. Independent of the reason, it is noteworthy that the difference in toxicity is much higher in the artificial soils than in the field soils: While at 20 °C the LC_{50} values are very similar in OECD artificial soil and LUFA soil, there is a factor of seven between artificial and field soils under tropical conditions. In other words, the temperature effect is much smaller in the field soils. This may be caused by the different microorganism communities: In artificial soils with (usually) a low number of micro-

organisms, a higher temperature may increase the growth of these communities more rapidly than in field soils with their already more active communities. However, this idea has not been investigated so far.

Carbendazim

This fungicide, the first metabolite of benomyl, is also known to be highly toxic to a wide range of oligochaetes (Table 4.2). LC_{50} values below 10 mg a.i./kg have often been reported from laboratory tests with lumbricid and enchytraeid species. Due to this high toxicity and stability in soil (depending on the soil type and temperature, DT values are reported between 10 and 230 days in temperate climates; in the tropics probably shorter on average), carbendazim was selected as reference compound in the enchytraeid reproduction test (ISO 2003b). Currently, it is not clear why in one test with the enchytraeid species, *E. coronatus* is obviously much less sensitive than all other oligochaete species tested so far. No difference in sensitivity was found between the various lumbricid test species.

Table 4.2: Results of oligochaete acute tests with carbendazim performed in various soils; all data are given in mg a.i./kg

Species	Soil	% OM	LC_{50}	Reference
Enchytraeidae				
E. albidus	OECD	10	4.9	Römbke and Federschmidt (1995)
E. albidus	Field	n.d.	2.5	Römbke and Federschmidt (1995)
E. albidus	OECD	10	> 3.6	Römbke and Moser (2002)
F. ratzeli	Field	n.d.	15.2	Federschmidt (1994)
E. coronatus	OECD	10	321.8	Arrate et al. (2002)
Lumbricidae				
Not specified	OECD	10	3.9	Maroni et al. (2002)
E. fetida	OECD	10	6.0 - 9.3	Adema et al. (1985)
E. fetida	OECD	10	5.5	Federschmidt (1994)
E. andrei	OECD	10	5.7	Van Gestel (1992)
L. terrestris	Field	n.d.	0.9 - 2.6	Cluzeau et al. (1992)
L. rubellus	Field	n.d.	6.2	Burrows and Edwards (2002)

As in the case of benomyl, carbendazim was highly toxic to the European *E. fetida* at 20 ºC and practically non-toxic when tested with tropical *E. fetida* at 28 ºC

(LC_{50} values of 5.8 mg a.i./kg versus > 1000 mg a.i./kg in both OECD and TAS). The difference in toxicity (factor > 200) was even higher than with benomyl (Tables 4.3 and 4.4). However, in this case a similar difference was also determined in the test with LUFA soil: the LC_{50} values of the tests under temperate and tropical conditions differed by a factor of > 244 (LC_{50} values of 4.1 mg a.i./kg versus > 1000 mg a.i./kg). Despite the fact that the toxicity of carbendazim to *E. fetida* was much higher in TNS, even here the factor was relatively high (13.9; with LC_{50} values of 4.1 mg a.i./kg and 57.1 mg a.i./kg, respectively).

This situation is difficult to explain; in particular, when being compared to the results of the tests with benomyl. In factorial design tests it could be shown that the temperature was the main factor influencing the toxicity of carbendazim in TAS soil, i.e., both the tropical and European *E. fetida* were more affected when exposed at 20 ºC compared to 28 °C (both variants have the same sensitivity towards carbendazim). Neither the origin of the worms (i.e., whether they came from Europe or the tropics) nor the interaction between origin and temperature influenced the test results significantly. The higher toxicity of carbendazim in TNS under tropical conditions compared to the tests with the three other soils could be explained by its low pH value (ca. 3.9). An influence of the content of organic matter (one of the most important factors affecting bioavailability of chemical substances in soil) can be ruled out, since this amount does not differ from that in LUFA – but the toxicity differs by about a factor of more than 70. One could argue that either a combination of relatively low organic matter content and low pH or other soil properties like the cation exchange capacity cause a higher availability of carbendazim in soil and thus a higher toxicity. But if this is true: why did the LC_{50} values of benomyl not differ between LUFA and TNS soil? At present, these differences cannot be explained. However, in any case it must be pointed out that under tropical conditions the toxicity of carbendazim to earthworms (tropical variant) is clearly lower than under temperate conditions using European *E. fetida*.

Lambda-cyhalothrin

In contrast to the two fungicides, little information exists about the toxicity of this insecticide to earthworms. However, it is known that all pyrethroids (chemical class of lambda-cyhalothrin) due to their arthropod-specific mode-of-action have a low acute

toxicity to oligochaetes (Inglesfield 1989). LC_{50} values of > 1000 mg a.i./kg and effects on the biomass at 100 mg a.i./kg have also been mentioned from standard laboratory tests with OECD soil and *E. fetida* (Maroni et al. 2002).

In the tests reported here, the insecticide lambda-cyhalothrin was more toxic to tropical than to European *E. fetida* in all but the OECD artificial soil. The toxicity towards the tropical earthworm variant was 2-fold (LUFA and TNS) to 5-fold (TAS) higher than when testing European *E. fetida* (Tables 4.3 and 4.4). Results of factorial design tests showed that the origin of *E. fetida* (European or tropical) was the main factor influencing the toxicity in TAS soil, i.e., the tropical earthworms were more sensitive than the European ones, independent of the tested temperature and also of the soil used. However, a small but significant influence of the interaction between origin and temperature was found when looking at the biomass development. Since this effect was not dose-dependent (i.e., it occurred at low but not at high concentrations in the factorial test), it is not further considered here.

However, as mentioned above, one exception was observed: the toxicity of lambda-cyhalothrin was lower for the tropical species tested in OECD soil at 28 ºC compared to the European variant tested at 20 °C. This result could be explained by the influence of the quality of the organic matter used in OECD artificial soil (sphagnum peat). This peat is naturally decomposed and probably has a greater microbial activity than the Xaxim material used in TAS soil. In soil, lambda-cyhalothrin is mainly degraded biologically, while photochemical reactions can sometimes contribute to the degradation of residues on exposed surfaces. All the isomers of cyhalothrin, including those that constitute lambda-cyhalothrin, are readily degraded in soil under a range of conditions. Half-lives for lambda-cyhalothrin at 20 °C in soils ranged from 22 to 82 days (WHO 1990). Under tropical conditions, the biodegradation of lambda-cyhalothrin in soil would even be faster. Therefore, it is probable that lambda-cyhalothrin was more quickly (or differently) degraded in OECD soil and consequently was less available as a toxicant.

However, independent of the differences in toxicity observed and the potential reasons for these differences, it can be stated that:

- The acute toxicity of lambda-cyhalothrin was clearly higher than reported in the literature.

- The factor between the results gained under temperate and tropical conditions was relatively small in all four soils (independent of the direction, between 2 and 5). There is a clear tendency (3 out of 4 cases) that tropical *E. fetida* are more sensitive to lambda-cyhalothrin than temperate species.

Table 4.3: Acute toxicity of three pesticides to European (at 20 ºC) and tropical (at 28 ºC) *E. fetida* in artificial soils (LC_{50} values in mg a.i./kg).

	European *Eisenia fetida* (20 ºC)	**Tropical *Eisenia fetida* (28 ºC)**			
	OECD	**OECD**	**Toxicity ratio**	**TAS**	**Toxicity ratio**
Benomyl	22.0 *	458.3	20.8	633	28.8
Carbendazim	5.8	> 1000	> 172.4	> 1000	> 172.4
Lambda-cyhalothrin	99.8	228.7	3.3	23.8	0.2

*Heimbach (1984)

Table 4.4: Acute toxicity of three pesticides to European (at 20 ºC) and tropical (at 28 ºC) *E. fetida* in natural soils (LC_{50} values in mg a.i./kg).

	European *Eisenia fetida* (20 ºC)	**Tropical *Eisenia fetida* (28 ºC)**			
	LUFA	**LUFA**	**Toxicity ratio**	**TNS**	**Toxicity ratio**
Benomyl	14.6	66.8	4.6	61.4	4.2
Carbendazim	4.1	> 1000	> 243.9	57.1	13.9
Lambda-cyhalothrin	139.9	68.5	0.5	65	0.5

Chronic effects

Benomyl

The effects of benomyl on the reproduction of oligochaete worms are well studied (but rarely published), because this compound is recommended as a reference substance in chronic laboratory tests according to OECD (2003) and ISO (1998a) guidelines. NOEC values of about 1 mg a.i./kg were determined for *E. andrei* in artificial soil, while the same species in field soil as well as enchytraeids react less sensitively (Table 4.5). In any case, this fungicide is one of the most toxic chemicals known for oligochaetes to date.

Table 4.5: Results of oligochaete chronic tests with benomyl performed in various soils; all data are given in mg a.i./kg.

Species	Soil	$EC_{50Repro}$	$NOEC_{Repro}$	Reference
Enchytraeidae				
Enchytraeus sp.	Field	-	16	Puurtinen and Martikainen (1997)
Lumbricidae				
Not specified	OECD	-	1.0	Maroni et al. (2002)
E. andrei	OECD	3.5	0.3 - 7.7	Van Gestel et al. (1995)
E. andrei	Field	11.8	9.4	ESG (2002)

As in the acute tests, the toxicity of benomyl was lower when tested with tropical *E. fetida* compared to European *E. fetida* (Tables 4.7 and 4.8). However, this difference was clearly smaller in the chronic tests (factor varied between 2 (OECD) and 8 (TAS) in artificial soil, while in LUFA no difference was found at all (in TNS no reproduction was possible at all; probably due to the low pH of this soil). Therefore, the same trend as in the acute test was observed in the chronic test. The lower factors observed can partly be explained by the low absolute level of the effects: EC_{50} values of about 1 mg a.i./kg or even lower belong to the lowest soil toxicity values reported in terrestrial ecotoxicology in general.

In comparison to other pesticides tested so far, benomyl has a high acute-to-chronic ratio (ACR) up to 167 (TAS). Under temperate conditions this ACR is about 10. This is a striking difference between the test results performed under tropical and temperate conditions, easily explicable by the low acute toxicity, while chronic effects are much less influenced by temperature.

Carbendazim

The chronic toxicity of carbendazim to oligochaetes is also well documented (Van Gestel 1991). Since it is the first metabolite of benomyl it is not astonishing that the test results reported from literature do not differ significantly from those reported for the mother compound (Table 4.6). In fact there seems to be a slight tendency that carbendazim is even more toxic to oligochaetes than benomyl. Again, with the exception of an enchytraeid (*E. coronatus*), all tests revealed NOEC values between 0.6 and 2.9 mg a.i./kg. In addition, in all tests the EC_{50} values were very similar as the

NOEC values, indicating again the extremely high toxicity of this chemical. Interesting are the differences in sensitivity between the various enchytraeid species.

Table 4.6: Results of oligochaete chronic tests with carbendazim performed in various soils; all data are given in mg a.i./kg

Species	Soil	$EC_{50Repro}$	$NOEC_{Repro}$	Reference
Enchytraeidae				
E. albidus	OECD	-	1.2	Collado et al. (1999)
E. albidus	OECD	1.0 - 1.3	0.8 - 1.4	Römbke and Moser (2002)
E. buchholzi	OECD	-	2.7	Collado et al. (1999)
E. coronatus	OECD	14.1	10.2	Arrate et al. (2002)
E. crypticus	LUFA	10	-	Achazi et al. (1997)
Lumbricidae				
Not specified	OECD	-	0.6	Maroni et al. (2002)
E. andrei	OECD	0.6	2.9	Van Gestel (1992)
E. fetida	OECD	1.4	1.0	Hayward (2000)
E. fetida	OECD	2.2	1.0	Hayward (2001)
E. fetida	OECD	-	1.0	Hayward (2002)

In the tests reported here (Tables 4.7 and 4.8), the difference between tropical and European *E. fetida* was highest in LUFA soil (factor 16 in EC_{50} values of 0.6 and 9.6, respectively), but this ratio should not be over-interpreted, since the absolute values are on a very low level (e.g., in LUFA 0.6 mg a.i./kg). As discussed for the acute tests, temperature is probably the single key factor responsible for the lowered toxicity of this compound under tropical conditions. In any case carbendazim is a chemical highly toxic to earthworms, especially when looking at chronic effects (acute-to-chronic ratio (ACR) can be as high as about 200 (TAS)). Under temperate conditions, it is smaller than 10. This is a striking difference between the test results performed under tropical and temperate conditions, easily explicable by the lowered acute toxicity, while chronic effects are much less influenced by temperature.

Lambda-cyhalothrin

Nothing is known about the chronic effects of this insecticide on oligochaete worms. Due to its insecticidal mode-of-action, it might be expected that there is only a low

toxicity. However, as could be shown in this study (Tables 4.7 and 4.8), the overall level of toxicity is relatively high: the EC_{50} values vary between 7.7 and 60.2 mg a.i./kg under tropical compared to 37 and 45 mg a.i./kg under temperate conditions. There was no striking difference between the results of the tests done in the three soils (the toxicity ratio differed between 0.2 and 1.2). Only in TAS soil the tropical earthworms were slightly more sensitive towards lambda-cyhalothrin, but again this difference should not be over-interpreted. The ACR was very low; with the exception of the test with tropical *E. fetida* and OECD artificial soil (where it was nearly 6) in all other cases it varied between 1.1 and 3.1. These results can be summarized as follows: Due to the results of the factorial design tests and the fact that in 3 out of 4 acute tests the tropical *E. fetida* was more sensitive than the European one (up to a factor of 5), a tendency towards a difference in sensitivity between the two variants of *E. fetida* was observed. However, when looking at the results of the chronic tests this indication becomes smaller (despite the fact that in one out of three) tests again a factor of 5 was found). Further research is needed to determine whether a difference in sensitivity towards certain chemicals exists between the two variants.

Table 4.7: Chronic toxicity of three pesticides to European (at 20 ºC) and tropical (at 28 ºC) *E. fetida* in artificial soils (EC_{50} values in mg a.i./kg).

	European *Eisenia fetida* (20 ºC)	**Tropical *Eisenia fetida* (28 ºC)**			
	OECD	**OECD**	**Toxicity ratio**	**TAS**	**Toxicity ratio**
Benomyl	1.6	12.9	8.1	3.8 (n.s.)	2.4
Carbendazim	2.7	14.2	5.3	4.6 (n.s.)	1.7
Lambda-cyhalothrin	37.4	60.2 (n.s.)	1.6	7.7	0.2

n.s. = not significant (compared with European *E. fetida*)

Table 4.8: Chronic toxicity of three pesticides to European (at 20 ºC) and tropical (at 28 ºC) *E. fetida* in natural soil (EC_{50} values in mg a.i./kg).

	European *Eisenia fetida* (20 ºC)	**Tropical *Eisenia fetida* (28 ºC)**	
	LUFA	**LUFA**	**Toxicity ratio**
Benomyl	1.0	0.8 (n.s.)	0.8
Carbendazim	0.6	9.6	16.0
Lambda-cyhalothrin	44.5	54.1 (n.s.)	1.2

n.s. = not significant (compared with European *E. fetida*)

Avoidance tests

Benomyl, carbendazim and lambda-cyhalothrin

In the literature, it is stated that the avoidance behavior of *E. fetida* is a sensitive parameter for the detection of low concentrations of pesticides and other chemicals. Different methods have been suggested as a potential tool to assess the toxicity of contaminants in soil (Yeardley et al. 1996; Slimak 1997; Stephenson et al. 1998; Hund-Rinke and Wiechering 2001; Hund-Rinke et al. 2003). However, there are also examples where earthworms were not able to avoid a toxic chemical in soil (e.g., the endogeic lumbricid species *Aporrectodea caliginosa* did not avoid toxic concentrations of organophosphate insecticides (Hodge et al. 2000)). According to Reinecke et al. (2002), *E. fetida* was able to detect and avoid low concentrations of the fungicide Mancozeb, but on the contrary showed even a preference for a soil contaminated with lead nitrate. Clearly, the extent of the avoidance behavior depends on the respective chemical to be tested.

Out of the three pesticides tested in this study, only benomyl has been used in avoidance tests so far (ESG 2002). Both *E. andrei* and *L. terrestris* were exposed in three field soils with different organic matter content (2.9, 3.5 and 12.2 %, respectively). No correlation with this soil property was found: with *E. fetida*, EC_{50} values between 2.7 and 19.4 mg a.i./kg were determined, while the NOEC values of *L. terrestris* varied between 50 and 100 mg a.i./kg. Interestingly, in the case of *E. fetida* the sensitivity did not differ between avoidance and reproduction, while in the case of *L. terrestris* the avoidance behavior was even less sensitive than mortality.

In this study, it could be demonstrated that the sensitivity of *E. fetida* in avoidance tests is extremely high, but – as to be expected – depends on the soil type and the test chemical. For example, in all tests performed with OECD artificial soil and the two fungicides benomyl and carbendazim, the earthworms had difficulties to detect and avoid the chemical-contaminated soil, especially in the case of carbendazim (LC_{50}/EC_{50} as well as NOEC values were used for the assessment of the results and for the comparison with the results of acute and chronic tests). In the same soil, even low concentrations of lambda-cyhalothrin were detected. On the contrary, in TAS and, in particular, in LUFA the chemicals were avoided at much lower concentrations, indicating a clearly better availability to the earthworm sense organs. In the latter soil,

often even the lowest test concentrations (1.0 mg a.i./kg for the fungicides and 0.32. mg a.i./kg for the insecticide) were still avoided. No avoidance tests could be done with TNS due to the high mortality (probably caused by the low pH). Since an avoidance test can only be assessed when sublethal effects are not confounded with mortality, these data have been omitted from this discussion. The fact that the tests with OECD and LUFA soil were done under temperate (i.e., with European *E. fetida* at 20 °C) and the tests with TAS under tropical (i.e., with tropical *E. fetida* at 28 °C) conditions did not have an obvious effect on the results.

The question about the relationship between the quick and easy-to-perform avoidance test and the established, much longer lasting acute and chronic earthworm tests has been intensively discussed in the literature. The central issue in this discussion is the sensitivity of the three tests. For example, some studies, mainly done with contaminated soil (not individual chemicals) have been shown that the avoidance tests are at least as sensitive as the reproduction tests (Hund-Rinke and Wiechering, 2001 and Hund-Rinke et al. 2003). Stephenson et al. (1998), assessing condensate-contaminated soil, concluded that the avoidance tests can predict the results of acute and chronic tests. On the contrary, in tests with crude oil, a lower sensitivity of the avoidance test was determined in relation to chronic tests (Schaefer, 2001). In this study, it was observed that the earthworms responded clearly more sensitively in avoidance tests than in acute tests, except for carbendazim (and maybe benomyl) in OECD soil (Table 4.9). In addition, avoidance tests were as sensitive as the reproduction tests for the fungicides benomyl (in OECD and LUFA soil) and carbendazim (in LUFA and TAS soil). In all other cases (i.e., all tests with the insecticide lambda-cyhalothrin and the one with benomyl in TAS) the avoidance tests were more sensitive than the chronic tests (Table 4.9).

The practical importance of the significant relationship is that the avoidance test can be used as a quick and economical substitute for acute tests in the assessment of contaminated soils. However, one limitation is that a database about the avoidance-response to different types of chemicals is necessary in order to minimize erroneous results. However, this chance is small since in those cases where the worms do not avoid a contaminated soil very often mortality occurs, thus indicating that this soil is of

concern. Only in those cases where the test duration of 48 h is too short to show such mortality an incorrect assessment might be possible.

Table 4.9: Comparison of the sensitivity of *E. fetida* in acute chronic and avoidance tests (values in mg a.i./kg).

		Avoidance test		**Acute test**		**Avoid. test sensitivity**	**Chronic test**		**Avoid. test sensitivity**
Soil	**Chemical**	**EC_{50}**	**NOEC**	**LC_{50}**	**NOEC**		**EC_{50}**	**NOEC**	
OECD[1]	Benomyl	28.2	1.0	22.0	n.d.	- (?)	1.6	1.0	=
	Carbendazim	127.4	100	5.8	1.9	-	2.7	0.1	-
	L-cyhalothrin	3.3	1.0	99.8	63.2	+	37.4	10.0	+
LUFA[1]	Benomyl	1.6	< 1.0	14.6	2.0	+	1.0	0.32	=
	Carbendazim	7.1	< 1.0	4.1	1.3	+	0.6	0.5	=
	L-cyhalothrin	0.5	< 0.32	139.9	31.6	+	44.5	3.16	+
TAS[2]	Benomyl	54.9	1.0	633	316	+	3.8	3.16	+
	Carbendazim	33.3	3.16	>1000	100	+	4.6	3.16	=
	L-cyhalothrin	0.2	< 0.32	23.9	10	+	7.7	6.2	+

(1) Test done with European *E. fetida* at 20 ºC; (2) Test done with tropical *E. fetida* at 28 ºC

4.1.3 Test comparison

For the ecological risk assessment of chemicals in soil, data from toxicity tests with earthworms are required. According to various national and international guidelines, the risk of pesticides to soil organisms is assessed in a tiered approach, in which firstly the results of acute and chronic laboratory tests lasting 14 and 56 days, respectively, are used. Later on, these data are considered for the prediction of effects under field conditions. In case of concern, the potential effects have to be tested in semi-field or field experiments. The acute test with the endpoint mortality indicates the maximum response of an organism, and has been considered not to be sensitive, since high concentrations of the test chemical are needed to cause such a drastic effect. The chronic test, aiming at sublethal effects, is considered to be more sensitive. Therefore, it is a more realistic approach for the prediction of environmental effects, since in the field the exposure concentrations of pesticides are usually quite low. In particular, the chronic test is very time-consuming (56 days) so that more simple and short but also sensitive

alternatives are urgently needed. However, in order to be on the safe side when predicting the effect of a chemical in the field, the ecological relevance of a test result must be taken into account (for details of the environmental risk assessment see Section 4.4).

In the following it is discussed whether the results of the tests performed with the three chemicals confirm their characteristics as described above. First, the results of the mortality tests show effects at high concentrations – probably much higher than those possible under realistic field conditions. Secondly, as expected, the results of the chronic tests indicate effects at lower concentrations as shown by the ACR ratio. While the two fungicides benomyl and carbendazim have very high ACR values, meaning that there is a strong increase in sensitivity when performing a chronic test, the insecticide lambda-cyhalothrin has only low ACR values. These differences can be explained by the different modes-of-action of the various pesticides. The avoidance test is proposed as an easy and quick alternative to the two other tests. In fact, it is clearly more sensitive than the acute test and often at least as sensitive as the chronic one (for lambda-cyhalothrin even more). However, considering the importance of the chronic test (on this level it is decided whether a pesticide is of concern or not (in the former case it has to be tested in the field) and, therefore, the need for high ecological relevance of the test results, it would be very premature to recommend the avoidance tests as a substitute for the chronic test. However, the results obtained in the present study support the idea of more research (in particular broadening the range of test substances with very different modes-of-action and properties) in this area before the avoidance test can replace one of the standard toxicity tests currently adopted by international guidelines.

4.1.4 Tests results with *P. corethrurus*

As discussed earlier in Section 4.1.1 (species selection) for *Eisenia fetida*, this peregrine species naturally occurring in the (anthropogenically influenced) soils of Amazonia and many other tropical regions of the world, was tested in order to determine the acute effects of the two pesticide carbendazim and lambda-cyhalothrin. For the Environmental Risk Assessment of these chemicals it is important to evaluate whether such a tropical native earthworm would have a different sensitivity compared to *E. fetida* when tested in TASx soil. The results indicate that *P. corethrurus* is very much

more sensitive to the fungicide carbendazim (LC_{50} = 45.6 mg a.i./kg) than the tropical *E. fetida* (LC_{50} > 1000 mg a.i./kg). On the other hand, the sensitivity of *P. corethrurus* to the insecticide lambda-cyhalothrin was comparable to that of *E. fetida* under tropical conditions (40.2 and 23.8 mg a.i./kg, respectively). In part, this difference can be explained by the distinct ecological strategy of *P. corethrurus*, which lives in close contact with the soil and feeds on soil particles. Since the species is well adapted to acid soils (pH 3.5 - 4.5), the elevated pH value (6.5) in TAS could also have influenced the toxicity. However, this effect cannot be overwhelming since in that case it would have been visible in both tests.

In fact, more research is necessary regarding the effect of pesticides on tropical earthworm species like *P. corethrurus*. Up to now, this species has only very rarely been used in ecotoxicology. In a non-standard laboratory test with Sevin (a carbamate insecticide), an estimated LC_{50} of about 400 mg a.i./kg was found (Kale and Krishnamoorthy 1979). Other carbamate compounds revealed LC_{50} values for *Eisenia fetida* in a range between 66 and >1000 mg a.i./kg (Maroni et al. 2002). In sugarcane regions of Southern Brazil, the insecticides lindane and heptachlorepoxide were detected in the tissue of field-collected *P. corethrurus*, but no biological effects of these compounds were studied (Sparovek et al. 2001). Based on the information of just two tests it cannot be decided whether this species is more sensitive than the standard test species *E. fetida.* In addition to testing more chemicals, basic biological information (e.g., how to optimise breeding) is needed.

4.1.5 Tests results with arthropods

In general, the same conclusions drawn already for the earthworm tests (Section 4.1.2) are true here: a lower exposure of carbendazim under tropical conditions and higher availability in LUFA than in artificial soils. However, due to the short duration of the isopod tests (14 days), the probability of exposure was even higher in these tests compared to those with earthworms. In any case, the conclusion remains the same: The discussion of the laboratory results is based on nominal test concentrations.

Methodology

Until about 1990, among soil invertebrates only earthworms have seriously been considered as standard test organisms. Only recently have tests been developed with other species, often arthropods. The most often used test with arthropods is the collembolan test using the springtail *Folsomia candida* (ISO 1999b). However, due to the complete lack of knowledge about potential micro-arthropod test species from the tropics, it was not possible to develop a respective tropical springtail test.

Many macro-arthropod species, like millipedes or isopods living mainly on the soil surface or in the litter layer, are also promising test animals for laboratory tests due to their important ecological role in many soil ecosystems (Verhoef and Van Gestel 1995), in particular in those where earthworms are scarce (e.g., due to soil acidity or low moisture level like in many tropical soils (Höfer et al. 2000)). However, due to problems in breeding and keeping macro-arthropods (e.g., for many millipedes basic biological data concerning food preferences or life-cycles are not available), most of them are not likely to be standardized in the foreseeable future (Løkke and Van Gestel 1998). A remarkable exception is the isopods group, since some species can be bred in the laboratory (in Europe: the wide-spread *Porcellio scaber* and also *Trachelipus ratzeburgii* (Dunger et al. 1997)).

Despite the fact that isopods are widely used in ecotoxicological research, no official guideline is available for the trophic level they represent (e.g., Drobne and Hopkin 1994; Drobne 1997; Santos et al. 2003). Protocols for temperate regions have been proposed by Hornung et al. (1998a) to evaluate effects on growth and reproduction, but further standardization and more data (especially with organic chemicals and pesticides) is needed. Further progress has been made by improving culturing conditions (Caseiro et al. 2000), considering other exposure routes as well as the use of isopods in bioaccumulation (Sousa et al. 2000). The millipedes (Diplopoda), another important group of soil fauna, have been considered even less for toxicity tests. Tajovsky (1998) performed toxicity tests with the millipede *Brachydesmus superus* in temperate conditions, and suggested procedures for testing and to maintain this species in the laboratory. However, none of the tests proposed with these species have been standardized so far or are likely to be standardized in the foreseeable future.

According to the literature, the isopod species *Porcellionides pruinosus* (Brandt 1833) seems to be the most likely test organism for tropical regions (Vink et al. 1995; Vink and Van Straalen 1999). The main reasons for this proposal are the cosmopolitan distribution of this species, which is not only very often found in tropical countries but can also occur in regions with Mediterranean climatic conditions like Southern Portugal (Loureiro, personal comm.). Following the World List of Terrestrial Isopoda, at least eight subspecies have been described (mainly from North Africa), but their taxonomical validity seems to be doubtful (Spelda, personal comm.).

In addition to the "standard" arthropod test species *P. pruinosus* used in this study, two species occurring at the Embrapa study site were selected for testing purposes: the millipede *Trigoniulus corallinus*, widely distributed in tropical regions, and the tropical isopod *Circoniscus ornatus*, native in Amazonia (Hoefer et al. 2000). Due to the difficulties in the culture of *T. corallinus* and *C. ornatus* in the laboratory, it was not possible to perform long-term tests with sublethal endpoints. Therefore, only acute tests (14 days) were performed with these species, through repeated collections of adults from field populations. According to Tajovsky (1998), the only other millipede species proposed for testing purposes (*B. superus*) cannot be cultured either, and collection of adults in field are necessary. However, *C. ornatus* and *T. corallinus* were considered to be useful for toxicity tests in microcosms in tropical regions (see Section 4.2).

Since it was not the aim of this study to propose a fully developed and standardized macro-arthropod test, details of the test performance will not be discussed here. However, some issues should be highlighted:

- It is probably not a problem to standardize an acute mortality test with *P. pruinosus* since the species is easy to breed and handle.
- Much more difficult will be the development of a chronic reproduction test due to problems concerning the determination of the right age and sex of the test organisms, and the small size of the juveniles to be counted at the end of the test.
- Other test ideas proposed for isopods, focusing, for example, on the uptake of chemicals via food, are not very useful for the development of a soil ecotoxicity test.

- Other tropical macro-arthropod species, which could serve as an alternative to *P. pruinosus* (in particular non-isopods), have not yet been identified (mass breeding will probably be a big issue in any potential test species).
- In all practical aspects, an isopod test system should follow as much as possible existing OECD and / or ISO guidelines (in particular the use of artificial soil).
- Referring to own experiences, OECD artificial soil, as well as TAS, are very suitable for testing purposes (the same is true for a test temperature of 28 °C).

Regarding the other issues discussed in Section 4.1.1 (e.g., suitability of the test chemicals) no differences between the experiences gained with earthworms and isopods could be found. Due to the experiences gained with *P. pruinosus* (i.e., the fact that standardized cultures could be established) this species can be considered a suitable test species for inclusion in standard protocols and use in tropical regions (including Mediterranean areas, e.g., Santos et al. 2003).

Acute tests

Benomyl and carbendazim

Nearly no data on the toxicity of the two pesticides to macro-arthropods like isopods or millipedes exist. In a pure sand, an LC_{50} of 1.22 mg a.i./kg in a test with adult *P. pruinosus* was determined after two weeks (Vink et al. 1995). In a paper from Libya, Mohamed and Nair (1989) state that *P. pruinosus* is tolerant (i.e., is not sensitive) towards benomyl. The results of three acute tests with OECD, LUFA and TAS show that the isopod *P. pruinosus* was not affected by the fungicides benomyl and carbendazim (i.e., no mortality occurred up to 1000 mg a.i./kg). In the case of TNS, a high mortality was found for the two chemicals (42.5% and 32.5%), but since at the same time mortality in the control was 27.5% and 30.0%, only the respective difference between control and treatment values is considered to be due to the test chemical. In addition, carbendazim did not cause mortality at 1000 mg a.i./kg for the native isopod species *C. ornatus*. Due to the mode-of-action of these fungicides, these results were expected.

Surprisingly, in comparison to the other arthropods, the millipede *T. corallinus* was relatively sensitive to carbendazim, showing an LC_{50} value of 503.5 mg a.i./kg. At present it is not clear why there was such a difference, since there has been little

research on the ecotoxicology of millipedes in general (e.g., Gromysz-Kalkowska and Szubartowska 1994; Tajovsk 1998).

However, it is clear that both fungicides have no acute effects at environmentally realistic concentrations on the isopods and millipedes tested in this study.

Lambda-cyhalothrin

Again, nothing is known from the literature concerning the ecotoxicity of this insecticide on isopods or millipedes. All arthropods tested here were very sensitive to lambda-cyhalothrin. The LC_{50} values for *P. pruinosus* were extremely low in the two artificial soils (0.5 and 0.2 mg a.i./kg (OECD and TAS, respectively) and in TNS soil (0.1 mg a.i./kg)) while higher in LUFA (1.4 mg a.i./kg). This result is different from those gained in earthworm tests, where the toxicity of lambda-cyhalothrin was usually higher in the field soils. The difference may possibly be due to the different way of exposure in both organisms.

In TAS soil, the native isopod *C. ornatus* was less sensitive towards lambda-cyhalothrin, showing an LC_{50} value of 2.3 mg a.i./kg, while the millipede *T. corallinus* reacted in the same range as *P. pruinosus* (LC_{50} value of 1.2 mg a.i./kg). All tested macro-arthropods are highly sensitive towards this insecticide at low concentrations, which can be considered to be field-relevant. Differences between species and soils are not very large but could be significant in some cases. However, no further discussion of these results is possible since the amount of data available is too small (both from this study and from the literature).

Chronic tests

As can be expected from the information presented in the previous sections, nothing is known about the chronic effects of lambda-cyhalothrin on macro-arthropods. Methodological problems also exist. Sublethal effects of chemicals on isopods have been studied using different approaches in order to find an easy and inexpensive test method (Drobne and Hopkin 1995; Drobne 1997; Hornung et al. 1998b), but no agreed standard method for such tests has yet been determined.

Consequently, in this study the toxicity of the three test chemicals on the reproduction of *P. pruinosus* was difficult to assess, because, after mating, females may retain sperms for a long period of time and produce eggs for at least four broods (Vink and Kurniawati 1996). For this reason, the test approach presented here covers only partially the reproduction process, since the tests began with the exposure of gravid females.

However, the results gained seem to be valid: In both the OECD and LUFA soils, the reproduction rate of *P. pruinosus* was not affected by the fungicides benomyl and carbendazim at relevant concentration levels. Based on the results of the acute tests, a limit test design was used, testing only a concentration of 1000 mg a.i./kg. No effects at all were found at this concentration in OECD soil, but in LUFA soil the number of juveniles was significantly reduced. Unfortunately, due to time restrictions it was not possible to determine the exact EC_{50} value. However, it will most likely be somewhere in the range between 100 and 1000 mg a.i./kg, which is far from field relevant concentrations.

In contrast, the insecticide lambda-cyhalothrin showed a high toxicity. EC_{50} values of 0.4 and 0.13 mg a.i./kg, respectively, were found for the two soils, indicating that:

- the reproductive endpoint is (slightly) more sensitive than the acute endpoint;
- exposure and thus effects are higher in the field than in the artificial soil.

In any case, this insecticide is highly toxic to this isopod species. Being already on a toxicity level of less than 1 mg a.i./kg, it is not easy to determine reliable effect values on even lower levels.

Due to the methodological difficulties of the chronic test with *P. pruinosus*, the results obtained here are not fully conclusive (e.g., is the difference between the two soils reliable?). Therefore, more investigations will be necessary in order to improve the test design.

4.2 Semi-field tests

The decomposition of organic matter under field conditions and the influence of chemicals on this process are discussed in detail in Section 4.3.

4.2.1 Methodology

The environmental effects of chemicals have rarely been assessed directly in the field due to the complexity and variability of the ecosystem and the difficulties in interpreting the results. Thus, the environmental risk assessment of chemicals is based on the results obtained from toxicity tests performed under standard laboratory conditions with single species (Van Leeuwen and Hermens 1995; Gillett and Witt 1980). It has long been recognized that the effects of chemicals on the structural and functional level of the ecosystem may not be predicted from single species toxicity tests (Cairns 1984). Therefore, it has been suggested that microcosm studies could help to bridge the gap between laboratory tests and field studies (Gillett and Witt 1980). Terrestrial Model Ecosystems (TMEs) were developed as a higher-tier level tool to assess the effects at structural and functional level in the ecosystem (Morgan and Knacker 1994; Sheppard 1997). Studies using TMEs were performed in a project at four European field sites in The Netherlands, United Kingdom, Portugal and Germany (Knacker et al. 2004). These studies showed that TMEs can be an useful instrument for environmental risk assessment when concerns about the potential risk of a chemical remain after the performance of single species laboratory tests (Weyers et al. 2004).

In this study, toxicity tests using the TME method were applied for the first time in the tropics. With regard to the sampling of soil cores in the field and their management in the laboratory, it could be demonstrated that the TME method is suitable for tropical conditions. No problems specifically caused by the study area concerning extraction of soil cores, installation in the laboratory or maintenance of the soil cores there were observed. Like in TME studies in temperate regions, care must be taken when selecting the extraction site, since some (e.g., very sandy or very clayey soils) can be difficult to extract.

However, already in a preliminary test without pesticide application, a high mortality (25 - 87%) of the soil invertebrates introduced in the TMEs was observed. This high mortality also occurred in the main tests with intact soil cores (31 - 59%) and with homogenized soil (22 - 94%). All introduced species showed this high mortality, independent of their very different life-style, behavior, taxonomic and physiological conditions. In addition, there were no differences between the animals coming from laboratory cultures and those collected in the field. Interestingly, the oligochaetes and

arthropods obviously were very active before their death – as can be proven by the decomposition of the litter material. The microclimate (i.e., decreasing and/or variable moisture conditions) in the TMEs during the three months of testing may have caused the high mortality rate for some species (see Section 4.25). Thus, an improvement of the microclimatic conditions in the TMEs, in particular a regularly suitable moisture content in litter and soil, has to be achieved (e.g., by installation of an automatic moistener for each soil core or by checking the actual moisture more often).

When comparing the performance of the test with intact soil cores (as proposed in the original guidance for TMEs) with that gained in the test using homogenized soil (i.e., like in gnotobiotic microcosms; Edwards et al. 1996b), no differences concerning handling were observed. The setting-up of the latter test was easier since no heavy equipment was necessary for the extraction of the cores in the field; however, the data from the respective controls show that there was no obvious difference concerning microbial respiration, mortality of introduced animals or decomposition of *Flemingia* litter (of course, only when comparing the same soil and litter type and also taking the different test periods into consideration). However, a significant difference in feeding rate was determined between the two soils used in the test with homogenized soil, indicating that the activity of the soil fauna is much higher in the clayey Ferralsol compared to the sandy Acrisol – a difference which was also found concerning microbial activity. Both results are easily explained by the respective soil properties (in particular their moisture regime).

In Section 3.6, the results of the RAPID-kit measurements of the second TME series were presented. Due to the fact that no DT_{50} value for the degradation of carbendazim in tropical soils is available, the actual data were given as percentage of the nominal concentrations without taking into consideration any degradation of the test substance. This leads to very low values (0.3 - 11.0 %), indicating a bad recovery rate. However, it seems to be more realistic to assume a DT value of 50 days (i.e., using the lowest known value for acidic sandy soils and the highest for basic clayey soils under temperate conditions; Domsch 1992) and to re-calculate the actual soil concentrations in the TMEs after 5 months (Table 4.10). For tropical conditions, this DT_{50} value can be seen as a worst-case approach, because carbendazim is probably degraded more quickly at higher temperatures. The usual approach recommended by the European Union for

the calculation of such values in the context of pesticide registration was used (e.g., soil layer = 0 - 5 cm, bulk density of soil 1.5 kg/dm^3, no plant interception).

Table 4.10: Calculated concentrations of carbendazim in the soil of the TMEs five months after application of two different rates and using a DT_{50} value of 50 d in order to incorporate degradation (all data given as mg a.i./kg).

Date after Application	**Scenario 1: 45.1 kg a.i./ha (one application)**	**Scenario 2: 4.51 kg a.i./ha (monthly application)**
Day 0	60.13	6.01
Day 28	40.79	4.08
Day 35	n.d.	9.31
Day 42	n.d.	8.45
Day 49	30.07	7.67
Day 100	n.d.	12.47
Day 120	n.d.	15.46
Day 150	7.52	10.20

When comparing the two concentrations calculated for day 150 (i.e., 5 months after application) with the mean measured values (0.97 and 2.71 mg a.i./kg), recovery rates of 12.9 % and 26.6 % were found. Taking into consideration the assumptions used for this calculation (e.g., concerning the effect of temperature, the DT values or soil density), this result can be considered as being very good. In any case, the data confirm that the organisms living in the TMEs were exposed to carbendazim over the whole test period.

Summarizing the experiences with this method in this study it is clear that there are no specific problems which can be traced back to tropical conditions in general. Those difficulties observed (e.g., problems with the moisture content) are the same as those under temperate conditions. They did not greatly influence the test results.

4.2.2 Comparison of test litter types

In the preliminary TME experiment, significant differences in mass loss were observed among the three litter species used. The litter of *Flemingia* (leguminous) was much more decomposed than *Brachiaria* (grass) and in particular *Hevea* (rubber tree). In the TME experiment with intact soil cores, these differences could be confirmed: *Flemingia*

was again decomposed quicker than the other two litter types. In the TME test with homogenized soil, the litter of another leguminous species, *Pueraria*, was even more attractive for the soil organisms than *Flemingia*. Such a quick decomposition of leguminous litter has also been found in other tropical ecosystems (e.g., crop sites in India; Arunachalam et al. 2003). In the same study from India it was pointed out that the initial lignin content is probably responsible for differences in the decomposition of leguminous litter.

The millipede *T. corallinus*, representing the major part of the soil fauna biomass in the TMEs, was the most active litter consumer. The feeding activity of *T. corallinus*, higher in litter of *Flemingia* than *Brachiaria* and *Hevea,* explains the difference in the decomposition rate observed among these litter types. Consequently, the effect of pesticides on litter decomposition was more evident in *Flemingia* due to the high mortality of *T. corallinus* when exposed to the chemicals. Pobozsny et al. (1992) also observed that *T. corallinus* (cited as *Trigonoiulus lumbricinus*) is differently attracted by different litter types: In their experiments a higher feeding activity in *Coffea* and *Hibiscus* than in *Panicum* (grass) leaves was observed. Comparable differences in feeding preferences are also well known for earthworms (Barley 1959), but nothing is known in this respect for the epigeic species living at the Embrapa site. The feeding biology of *P. corethrurus* has not been studied in detail, but since this species lives in the mineral soil, a direct effect of the litter type is unlikely. In any case, earthworms prefer nitrogen-rich food material, which can easily be seen by their quicker growth and higher cocoon production (Evans and Guild 1948).

Right now it can only be speculated about the reasons for these different preferences, but two issues are highly probable:

- The animals are attracted by the higher N-content of leguminous tissue (as in the case of *Flemingia* and *Pueraria*).
- The animals avoid non-palatable litter (e.g., leaves which are difficult to break up mechanically; usually the leaves have to be exposed to weather influences for some time before they can be eaten by soil invertebrates).

Hevea is one example of non-palatable litter. Chaudhuri et al. (2003), observed different feeding preferences in leaves of *Hevea* by three earthworm species and related such differences to toxic substances present in the leaves.

Summarizing it can be stated that in TME experiments, which include functional endpoints like decomposition of organic matter, the selection of the best litter is essential for the test performance and interpretation of the results. The quality of the litter directly influences the well-being of the organisms and determines also the duration of test (i.e., tests with non-palatable litter have to run longer). In addition, the palatability and, therefore, the feeding activity, is crucial for the effects of chemical on the endpoint mass loss. In this study, the selection of several litter types with different properties allowed clarification of these issues. However, when performing TME tests routinely, it is recommended to use only one litter type, i.e., one that is most appropriate for the identification of potential chemical effects like *Flemingia* or *Pueraria*.

4.2.3 Comparison of test organisms

In the preliminary experiment without pesticide application, all tested species showed a high mortality (only *P. corethrurus* had slightly lower rates). This effect might partly be related to the loss of body water when arthropods were exposed on the soil surface. The highest mortality (78 - 87%) was found for the millipede *Trigoniulus corallinus*. Shukla and Tripathi (1985) observed that *T. corallinus* (cited as *Trigoniulus lumbricinus*) is more sensitive to body water losses in comparison to other arthropod groups like insects. Therefore, they bury themselves in soil or litter in order to avoid dry conditions. The mortality was also high for the isopods species *Circoniscus ornatus* (42 - 73%) and *Porcellionides pruinosus* (64 - 75%) in this experiment. Likewise, it is well known from the literature that terrestrial isopod species are also sensitive to water loss. Similar to the millipedes, isopods look for humid and sheltered habitats to avoid desiccation. In the TME test with intact soil cores, the water regime was better controlled and the mortality rates of the two arthropod species tested (*C. ornatus, T. corallinus*) were lower (31 and 36%).

In addition, it was speculated whether the use of field-collected animals compared to those coming from laboratory cultures might have influenced the mortality rate. Background for this assumption is the difference in age, physiological stage and individual history of the individuals of *C. ornatus* and *T. corallinus.* However, this factor can be neglected due to the fact that the individuals of *P. pruinosus,* coming from

a laboratory culture, showed the same high mortality rates as the other arthropods (64 - 75%).

With regard to the earthworm species, the native species *Pontoscolex corethrurus* showed a lower mortality than *Eisenia fetida* (25 - 53% versus 45 - 68%). While *Pontoscolex corethrurus* is an endogeic species, well adapted to the acid soils in the tropics used in the preliminary experiment, *E. fetida* is an epigeic species, restricted to sites rich in organic matter and, probably more important, prefers neutral to slightly acidic soils. Consequently, it was observed in the TME experiment that *E. fetida* did not enter the soil but remained on the soil surface. The same behavior had already been observed in the avoidance laboratory tests with this species. In this context it must be remembered that in the acute (i.e., just 14 days) laboratory tests both species did not show mortality but high biomass losses in TNS (*E. fetida* about 30% and *P. corethrurus* about 20%). In the preliminary TME test, lasting much longer (3 months), this biomass loss ended up as mortality. In the even longer running test with intact soil cores, the mortality of *P. corethrurus* was even higher (59%). However, this can be explained by a behavior often seen in long-term experiments with this species: After starting a culture, for a period of a few months the population size decreases before it rises again reaching a maximum after about 6 months if enough food is provided (Pashanasi, personal comm.). In this study, however, the size of the TMEs and the food supply were probably not high enough in order to allow such a population increase.

In the bait-lamina tests, an evident difference in feeding activity in favour of the field fauna group was observed, being mainly caused by the presence of the earthworm *P. corethrurus*. Thus, it can be concluded that in tropical regions, native species like *P. corethrurus* should be used in TME experiments instead of *E. fetida*.

4.2.4 Effect of test substances

Test with carbendazim in TMEs

After exposure to carbendazim, the soil fauna species introduced to the TMEs reacted differently. The millipede *T. corallinus* and the native earthworm *P. corethrurus* showed a high sensitivity to the two carbendazim applications (6.0 and 61.1 mg a.i./kg initially; i.e., their LC_{50} values were about 61.1 mg a.i./kg, taking the high control mortality into consideration), whereas the isopod *C. ornatus* was not affected. Partly,

the same results had already been observed in the acute laboratory tests, in which *C. ornatus* was not affected up to 1000 mg a.i./kg while for *P. corethrurus* the LC_{50} value was determined as 45.6 mg a.i./kg. Only for the millipede was the toxicity lower in the laboratory (LC_{50} = 503.5 mg a.i./kg) than in the TME test. A more detailed comparison is not possible due to the different design and duration of the two tests.

In TME studies with carbendazim performed in temperate regions, the native earthworm species were highly affected (Römbke et al. 2004b) at concentrations starting at 1.08 mg a.i./kg ($NOEC_{Biomass}$) at one out of four sites. At the three other tested sites, partly much weaker effects ($NOECs_{Biomass}$ between 3.24 and >87.5 mg a.i./kg) were measured. The EC_{50}-values for the effect of carbendazim on earthworm abundance ranged between 2.0 and 48.8 kg carbendazim/ha (2.71 to 65.2 mg carbendazim/kg soil) and on earthworm biomass from 1.0 to 34.6 kg carbendazim/ha (1.36 to 46.0 mg carbendazim/kg soil). In general, a high variability of the data derived from replicate samples reduced the probability of determining significant differences. Carbendazim induced similar effects on the abundance and the biomass of earthworms. Effects were most pronounced 16 weeks after application of the model chemical. The effects did not differ between the TME tests and the respective field-validation studies. Effects on earthworm diversity were difficult to assess since the numbers for individual species were low.

Using a gnotobiotic microcosm (i.e., filled with homogenized soil), Burrows and Edwards (2002) determined an LC_{50} value of 6.2 mg a.i./kg and an EC_{50} (growth) of 1.9 mg a.i./kg for *Lumbricus rubellus* in a silty clay loam. A quite similar LC_{50} value was found by Rodriguez et al. (2003) for *Eisenia fetida*: 6.12 mg a.i./kg. In TME studies with carbendazim the effect on soil microarthropod communities was not consistent and varied according to the species (Koolhaas et al. 2004). Since macro-arthropods like isopods and diplopods were not tested, a comparison between the two arthropod groups used in the two studies is difficult, but it seems that the tendency (species-specific effects of carbendazim) is the same.

In the literature, a strong inhibiting effect of carbendazim on litter decomposition is reported (Knacker et al. 2003; Sousa et al. 2004; Van Gestel et al. 2004). For that reason, this fungicide was discussed for some time as a reference substance in litterbag experiments, to be used in concentrations of up to 5000 mg a.i./ha.

However, due to the high variability of the test results (depending probably on composition, size and activity of the oligochaete fauna at the respective study site), this proposal was finally cancelled (Römbke et al. 2003).

The leaf litter decomposition in TMEs, measured as weight loss, was also affected by carbendazim. The effect was more evident (i.e being significant at both concentrations) with *Flemingia* than with *Hevea* litter (where only the higher concentration caused a significant decrease). From the pre-tests it is known that the fauna prefer the *Flemingia* leaves in comparison to *Hevea.* Since diplopods and earthworms were strongly affected, a significant difference to the control appeared in the treatments with the more palatable litter. In a case where even in the control little faunal influence was observed, and consequently effects on the fauna did not materialise in a decrease of the decomposition rate. In temperate regions, Förster et al. (2004) found a clear dose response relationship of carbendazim on organic matter (cellulose paper) decomposition in TME experiments with different soils after eight weeks of exposure. The carbendazim-induced effects on organic matter decomposition in TMEs and in the field were comparable, but in TMEs the cellulose paper was decomposed faster than under field conditions. The calculated EC_{50} values after 8 weeks of incubation of the filter paper were 9.5, 7.1 and 2.1 kg carbendazim/ha for grassland TMEs, grassland field and arable TMEs, respectively. Effects on decomposition were correlated with effects on earthworms and enchytraeids but not with effects on bait-lamina consumption. In general, the lack of earthworm influence (probably less feeding than indirect effects on micro-organisms) after application of carbendazim cannot be compensated by the microflora or mesofauna (Förster 2001).

No influence of the test substances in the bait-lamina tests was observed (only performed in the TME test with homogenized soil), but this result might be influenced by the strong difference in feeding activity in the cores filled with clayey (15 % feeding rate) and sandy soil (1 % feeding rate). This difference could have masked an effect of the chemical. This assumption is backed up by the fact that in a TME test with a silty clay loam at 20 °C lasting 7 days, an EC_{50} of 8.2 mg a.i./kg carbendazim was found in a bait-lamina test performed in a TME test with homogenized soil (Burrows and Edwards, 2002). In a TME test with intact soil cores (different soil types), the EC_{50} values ranged between 2.0 and 56 kg carbendazim/ha (Förster et al. 2004). In this case, bait-lamina

consumption was influenced by the soil moisture content, and correlated significantly with earthworm abundance. The contribution of enchytraeids and, in particular, of micro-arthropods to bait-lamina consumption was less pronounced. The low number of earthworms found in one of the tested soils (from an arable site) is thought to be the reason for the fact that an effect of carbendazim on the feeding activity was not detected at that site.

The microbial respiration in soil indicated by CO_2 production is usually considered as an indicator of microbiological activity. However, a great variability of the effects of pesticides on microbial respiration has been reported in the literature (e.g., Domsch 1992). In the TME experiments reported here, microbial respiration was not influenced by the carbendazim applications. Likewise, in temperate regions, no significant reduction in soil respiration and microbial biomass was found in microcosms treated with carbendazim (Förster et al. 1996; Vink and Van Straalen 1999; Sousa et al. 2004). In addition, no effects on nutrient cycling were observed in TME studies after application of carbendazim, which is in accordance with the very low concentrations of this substance in TME leachates (Van Gestel et al. 2004).

Summarizing these results it can be concluded that the decrease in (mainly) *Flemingia* leaf litter decomposition was a consequence of the direct toxic effect of carbendazim on the soil fauna (in particular, but not only, earthworms) rather than on microbial activity.

Test with lambda-cyhalothrin

The arthropods *C. ornatus* (at the higher concentration) and in particular *T. corallinus* (at both concentrations) were highly affected by lambda-cyhalothrin applications in the TMEs, while no influence was observed on the earthworm *P. corethrurus*. In the TMEs, the arthropods, living on the soil surface and in the litter layer, were more exposed to the chemical than the earthworms in the soil. In addition, the biodegradation of lambda-cyhalothrin in the moist soil could be faster compared to the degradation in the litter that was not always moist. Again, these results are in general comparable to the information gained from the acute laboratory tests in which the toxicity was much higher for the arthropod (LC_{50} values of 2.3 and 1.2 mg a.i./kg, respectively) than for the earthworm

species (40.2 mg a.i./kg). Unfortunately, no comparison is possible with literature data, since this insecticide has not been tested in terrestrial microcosms so far.

As a consequence of the effects on saprophagous arthropods, the applications of lambda-cyhalothrin highly reduced the decomposition of *Flemingia* litter. Due to the slow breakdown of *Hevea* litter no such inhibitory effect was observed. However, in both treatments the average decomposition rate was only half of that in the control, but due to the high variability, this difference was not significant. Again, no effect of the test substance was found concerning the microbial respiration in the TMEs or the feeding activity. As discussed above, the latter result might be influenced by the overall low feeding activity and the strong difference between the soil cores filled with clayey (15 % feeding rate) and sandy (1 % feeding rate) soil. However, in a 21-day laboratory test with collembolans and OECD artificial soil, a significant inhibition of consumption was found already at 0.05 mg a.i./kg. This was caused by the lethal effect of this pyrethroid on the springtails (Kampmann 1994).

Summarizing these results it is highly probable that the decrease in *Flemingia* leaf litter decomposition was a consequence of the direct toxic effect of lambda-cyhalothrin on the arthropod soil fauna rather than on earthworms or microbial activity. Despite the fact that this conclusion is backed up by the specific mode-of-action of this insecticide, it has to be considered as preliminary, since data on the effects of this substance on microbes are missing.

4.2.5 Evaluation of results of semi-field tests

Summarizing the results of the TME semi-field tests, it is clear that methodologically – with the exception of an improvement of handling the soil moisture regime - no problems occurred. In addition, both structural (composition of soil fauna) and functional (litter decomposition) endpoints could succesfully be manipulated in these studies (due to the low and unknown number and the often difficult taxonomy it was decided not to rely on the natural fauna but to introduce test animals into the TMEs). From an ecological point of view, at least three soil fauna groups seem to be necessary for experiments in tropical regions:

- Millipede species (e.g., *T. corallinus*), acting mainly as a litter comminutor;

- Isopod species (e.g., *P. pruinosus* or a native species), feeding on litter and debris on the soil surface;
- Earthworm species (e.g., *P. corethrurus*) as a soil dwelling organism.

These species (or related ones) can be found in most tropical regions.

However, keeping added organisms alive and active is not easy. Therefore, a better control of the microclimate conditions should be obtained, e.g., by irrigating the soil cores regularly via special rain-heads (see Knacker et al. 2004) or by keeping the natural litter layer or vegetation on the surface of the soil core. But despite high mortality rates even in the controls, the effects of the chemicals could be determined.

Regarding the soil and litter type used, no general recommendations can be given except that the choice should be ecologically realistic for the chemical and ecosystem to be assessed. In addition, the requirements of the introduced test species should be fulfilled in a way that potential effects of a chemical are clearly distinguishable from other effects (e.g., those of the soil properties themselves). In addition, a well palatable litter type is recommended, since an indirect effect of the test chemical acting via the soil fauna then becomes more clearly visible.

In the following, the results of the tests performed in this study are compared to those done under temperate conditions. For this purpose, the discussion will focus on the work with carbendazim, since no data are available for tests with lambda-cyhalothrin. However, it can be expected that the main conclusions do not differ concerning the test chemical discussed.

Generally speaking, the main difference between the tests performed under temperate and tropical conditions is temperature (20 °C versus 28 °C), because this factor directly influences the fate of the test chemical (other factors like soil properties, duration, vegetation cannot directly be compared) as well as the physiology and behavior of the test species. As determined already in the laboratory tests with earthworms, the higher temperature causes an increased (and different?) metabolization of carbendazim, leading potentially to a decrease in toxicity. It is a potential, because this effect can only be seen directly when comparing the same endpoint and test species. For example, the effect of lowered or shorter exposure can be counteracted by an enhanced natural sensitivity towards carbendazim in one or more of the tropical test species. Interestingly, in the case of the millipede *T. corallinus* the test conditions

(longer duration?) in the TME supported a higher toxicity than in the short-term laboratory test.

Under temperate conditions (and including 4 different soils), the two most sensitive endpoints were the abundance of earthworms and the feeding activity (NOECs 1.08 and <0.36 mg a.i./kg), respectively (Weyers et al. 2004). A direct comparison with the results of this study is not easy, since here the TME tests could not be set up using a dose response design. However, it is clear that there was no effect on the bait-lamina test at all up to 60.1 mg a.i./kg. Effects on tropical earthworms and macro fauna occur at concentrations in the same order of magnitude, since significant effects occurred at < 6 mg a.i/kg, indicating that the NOEC values are probably similar for the most sensitive species. However, this can only be proven by performing a TME test under tropical conditions using a dose response design. The lack of clear effects in the TME test with homogenized soil might be masked by another factor also causing a high mortality in the controls, supporting the need for an additional TME test.

In any case, the results presented from the TME tests seem to support the proposal from Versteeg et al. (1999), that the results of chronic laboratory tests (here: < 10 mg a.i./kg carbendazim) are sufficient for the risk assessment of chemicals on higher tiers. However, it is also clear that a sensitive endpoint is chosen and that acute data are only useful if safety assessment factors are applied (see Section 4.4 for a more detailed discussion).

4.3 Field tests

4.3.1 Methodology

Organic matter (OM) breakdown is one of the most important ecological functions in soil ecosystems. This complex process integrates several interacting physical, chemical, climatic and biological factors (Römbke et al. 2003):

- Physico-chemical environment (location, soil properties, climate);
- Quality of the resource (nutrient quality, chemical and physical defences towards feeding);
- Decomposer community (microflora, microfauna, mesofauna, macrofauna, megafauna).

If dead plant material OM (e.g., litter) were not decomposed and mineralized, living plants would not get the nutrients necessary for their growth (Odum 1971). Finally, in the long run, soil fertility would be influenced (Eijsackers and Zehnder 1990). Therefore, it is of extreme importance that this process is not disturbed by anthropogenic activities (e.g., the application of chemicals).

In temperate regions as well as parts of the tropics OM breakdown has often been studied (Crossley and Hoglund 1962, Heath et al. 1964; Swift et al. 1979; Cadisch and Giller 1997). However, field experiments using the litterbag method have only rarely been performed in the Amazon region. In most of them, no species differentiation of the litter was done (e.g., Dantas and Phillipson 1989; Höfer et al. 1996; Cornu et al. 1997). In those studies in which the decomposition of the litter of one species was addressed (e.g., Luizao and Schubert 1987; Heneghan et al. 1998; Mesquita et al. 1998; Höfer et al. 2001), with the exception of the last paper (*Vismia* sp.) other species than used in this study were investigated. Actually, due to methodological differences the results of most of the old studies are difficult to compare – a problem, which was already addressed by Proctor (1983). In addition, with the exception of one working group in East Africa (e.g., Tingle and Grant 1995) no study is known in which the effect of PPPs on OM breakdown was tested under tropical conditions.

The lack of an internationally accepted and standardized test method for evaluating the effects of PPPs on OM breakdown is probably due to the complexity of this soil ecological process (Kula and Römbke 1998; Scheu and Setälä 2001). While a historical overview of potential test methods was done by Heal et al. (1997), their relevance for the environmental risk assessment of pesticides was reviewed by Knacker et al. (2003). Despite the fact that the litterbag method as an ecotoxicological tool has been described in numerous papers, no standardized guideline has been established for this technique. According to Knacker et al. (2003), the litterbag method, with mass loss of OM as the measured endpoint, is the most appropriate technique available. Based on these recommendations a draft protocol on testing the effects of PPPs on OM breakdown has recently been prepared by an international working group (Römbke et al. 2003).

In this study, a litterbag experiment was conducted in the field to assess the effects of the fungicide carbendazim and the insecticide lambda-cyhalothrin on OM

breakdown. The methodology was based on existing experience from temperate regions (e.g., Paulus et al. 1999; early versions of the draft guideline proposed by Römbke et al. (2003)). No specific technical problems were observed during the performance of the experiment except that the tropical climatic conditions must be taken into consideration (Aerts 1997), e.g., the amount of precipitation occurring within short periods of time. So, the selection of an appropriate site can be more difficult than under temperate conditions. As a general rule, one can expect that the more complex, i.e., the closer a test method is to natural conditions, the higher the variability of the results will be.

In accordance with the draft guideline, an ecotoxicological litterbag test is considered to be valid if at least 60 % of mass loss occurred at the end of the study in the control plots. In the present study, the averaged biomass loss reached ca. 65% in the control; therefore the test was valid. Depending on specific crops or agricultural practices, the litterbags can be exposed on the soil surface (e.g., in orchards) or buried into the soil (e.g., arable fields). As this study was performed in a rubber tree plantation where no agricultural practice was used, the litterbags were exposed on the surface as part of the litter layer. However, during the test period the bags were progressively covered by *Hevea* leaves falling from the trees, and at the end of the test, were completely covered by a litter layer. It is known from temperate studies that the variability of results on OM breakdown is minimized when litterbags are inserted into the soil, because the climatic fluctuations affecting the degradation process are less pronounced. Therefore, it can be assumed that the variability of the results was buffered due to this coverage. On the other hand, in a rubber tree plantation a high percentage of the study area is not shaded completely, thus being exposed to rapidly changing climatic extremes (temperature).

Sampling of the soil organisms (mainly macrofauna: earthworms, isopods, millipedes and other arthropods) was done using methods widely recommended in the ecological literature (e.g., Dunger and Fiedler 1997; Höfer et al. 2000). Actually, for earthworm sampling a method recently standardized by ISO was used (ISO 2003c). No problems specific for tropical sites were observed. The same calculations as described at the end of Section 4.2.1 were also done for the litterbag test in the field, using exactly the same assumptions concerning DT values, soil properties, etc. The results of these calculations are summarized in Table 4.11.

Table 4.11: Calculated concentrations of carbendazim in soil of litterbag site in the field 12 months after application of three different rates and using a DT_{50} value of 50 d in order to incorporate degradation (all data given as mg a.i./kg).

Date after Application	**Scenario D1: 1.0 kg a.i./ha (four applications)**	**Scenario D2: 1.0 kg a.i./ha (eleven (monthly) applications)**	**Scenario D3: 10 kg a.i./ha (one application)**
Day 0	1.33	1.33	13.33
Day 30	0.88	2.21	9.04
Day 90	1.72	3.18	n.d.
Day 150	0.75	3.60	n.d.
Day 180	1.83	3.71	n.d.
Day 240	0.79	3.83	n.d.
Day 270	1.86	3.86	n.d.
Day 365	0.50	1.58	0.08

When comparing the three concentrations calculated for day 365 (i.e., 12 months after application) with the mean measured values (0.02, 0.02 and 0.11 mg a.i./kg), recovery rates of 4.0 %, 1.3 % and 137.5 % were found. It is difficult to understand why in the case of the repeated applications the measured concentrations were in the range of a few percent of the calculated values, while in the third scenario a better compliance was found. To a certain extent, the field conditions (e.g., high temperature fluctuations) might have played a role, but maybe the repetition of low concentrations caused an increase of microbiological activity, thus leading to a higher degradation rate (i.e., lower DT_{50} value) as assumed in this calculation. In general, the calculations as well as the measurement show that the exposure was relatively low but lasted for a long time (except in D3, where the concentration decreased relatively quickly to very low levels). When comparing these results with those from the TME study, it should be taken into consideration that the application rate was considerably lower in the field study and that the environmental conditions were much more stable in the TME study. In any case, the calculated as well as the via RAPID-kit measured concentrations should be handled with care.

Summarizing the experiences with the litterbag test in this study, it is clear that there are – with one exception – no specific problems which can be traced back to tropical conditions in general. When selecting the study site, the much stronger

influence of climatic factors (e.g., surface temperature depending on whether a site is shaded or not or on amount of precipitation per event) has to be taken into consideration. It is recommended to include these issues in the currently proposed draft guideline (Römbke et al. 2003).

4.3.2 Effect of test substances

Organic matter breakdown

The effect on litter mass loss is the most important measurable parameter in ecotoxicological litterbag experiments. In older studies performed in temperate regions, a high variability among litterbags occurred, but the average mass loss values of plots are less variable (Römbke et al. 2003). Likewise, the same situation concerning variability was observed in litterbag studies performed in tropical regions (Höfer et al. 2000 and this study). Depending on climatic factors causing differences in abiotic conditions on an often small scale, the litter may degrade at different rates at different spots in the same area.

In addition to the micro-environmental factors, biological factors can also be an important source of variability (according to Lavelle et al. (1993), in the humid tropics these factors are even more important than abiotic ones). While in temperate ecosystems earthworms are often the most essential organisms governing OM breakdown (Edwards and Heath 1963; Edwards and Bohlen 1996), the macrofauna in general seems to be the main driving force for the decomposition process in the tropics (Anderson et al. 1983; Tian et al. 1992; Yamashita and Takeda 1998; Gonzalez and Seastedt 2001). At the same site close to Manaus as in the study presented here, Höfer et al. (2001) could show in a litterbag study that in primary as well as secondary forests and plantations macrofauna biomass is positively correlated with decomposition rates and negatively with litter stocks. However, in the different systems studied, different organism groups were dominant. As a general rule, the importance of earthworms is highest in the primary forest and decreases with anthropogenic influence (also the species spectrum changes from native to peregrine species; see also Gonzalez et al. 1996), while arthropods like isopods and millipedes are most active in plantations. In addition, the specific feeding behavior of some soil fauna groups, in particular termites

(e.g., *Syntermes* sp. (Martius 1994; Martius 1998), in very restricted spots might be one reason for the high variability in litter decomposition results in tropical regions.

Due to this substantial variability in decomposition, there is some concern about the statistical data analysis in litterbag experiments. However, it is necessary to point out that the biological importance and statistical significance are logically distinct. Thus, in field tests, effects of great biological relevance may not be statistically significant when the test results are extremely variable. According to Römbke et al. (2003), the participants of an international workshop (EPFES) agreed on how to assess the results of a litterbag test as follows: if the difference in mass loss between the treated area and the control is ≤ 10% at the end of the test (usually 12 months after application), then the risk of this PPP for OM breakdown is considered to indicate a low concern. If the difference is > 10% or statistically significant differences in the breakdown rate between control and the treatment plots occur at one year, then a refined risk assessment has to be performed.

In temperate regions, the effects of carbendazim on litter decomposition have been investigated in various studies (e.g., Eder et al. 1992; Förster et al. 2004). According to Förster (2001), in various test designs (including laboratory, microcosm and field studies) the OM breakdown of hay, beech litter and cellulose paper was significantly affected at concentrations between 0.24 and 2.4 mg a.i./kg (= 180 and 1800 kg a.i/ha). However, such effects occurred when the litterbags were exposed on the soil surface during the chemical application. In addition, they were usually connected with high abundance and biomass of earthworms (in particular the ecosystem engineer *Lumbricus terrestris*). When carbendazim (or the closely related benomyl) was studied under different conditions, effects were not found (e.g., Martikainen et al. 1998). Carbendazim and benomyl were used as a reference substance in litterbag experiments in concentrations of up to 5.0 kg a.i./ha (= 6.65 mg a.i./kg) until 2002. However, due to the high variability of the test results (depending probably on composition, size and activity of the earthworm fauna at the respective study site), this proposal was cancelled in the draft guideline (Römbke et al. 2003).

In the present study, the carbendazim treatments did not show significant effects on litter decomposition. High variability of litter mass loss occurred in all treatments but were highest in the plots treated monthly with 1 kg a.i./ha. The

differences in litter mass loss between the treatments and the control plots were higher than (or nearly) 10 % after 6 and 9 months of exposure on the plots treated 3-monthly or once, but did not exceed 10% at the end of the test. The effects were observed at concentrations roughly similar to those reported from studies performed in temperate regions.

This result does not fit with the calculated concentrations of the test chemical in the soil. According to these values (see Table 4.11), the concentration in the plots treated 3-monthly was never higher than 1.86 mg a.i./kg, while in the monthly treatments it could reach 3.86 mg a.i./kg and in the plots with one high application, concentrations as high as 13.3 mg a.i./kg could be expected. The RAPID-kit data do not help here a lot because these measurements were done only at the end of the tests where the concentrations in all three treatments were quite low (0.01 - 0.14 mg a.i./kg). One explanation could be that for the soil organisms (and therefore, indirectly, for OM breakdown) the concentration in the litter was more important than that in the soil.

In the literature, no data concerning the effects of lambda-cyhalothrin or another pyrethroid on organic matter breakdown were found. In this study, the treatments with this insecticide clearly affected OM breakdown negatively. After 6 months of exposure, the effects on mass loss were statistically different from the control in the plots treated once with lambda-cyhalothrin. Three months later (i.e., 9 months after application), this difference was also significant at the plots treated monthly with the test substance. Interestingly, at the end of the experiment an effect of higher than 10 % (but not statistically significant) was only found in the plots treated 3-monthly. No RAPID-kit values are available for this insecticide, and the concentrations of lambda-cyhalothrin could also not be calculated since no reliable DT values are known. Thus, this insecticide applied in roughly field-relevant concentrations can be a risk for OM breakdown.

In comparison to carbendazim treatments, a lower variability of litter mass loss was observed for lambda-cyhalothrin. As discussed earlier, the activity of macroarthropods, the main force in litter comminution, might be responsible for the high variability in litter mass loss between replicated plots. Consequently, the lower variability observed in the plots treated with lambda-cyhalothrin can be explained by the reduction of soil fauna populations due to the high toxicity of this insecticide.

With the exception of the 3-monthly treatment with lambda-cyhalothrin, a reduction of effects was observed for both chemicals at the end of the experiment, i.e., 12 months after application. Given that the litterbags were progressively covered by leaves fallen from the rubber trees, their exposure to the pesticides, changed during the experimental period. Thus, the reduction of effects can be explained by the decrease in exposure to the chemicals. This can be seen by the differences between the individual sampling dates at the same plots: For example, during the first three months at each plot the mass loss is about 30 % due to leaching processes and macrofauna feeding. During the last three months, where only badly palatable litter remained in the bags, 0 - 14 % mass loss occurred.

Soil organisms

The effects of carbendazim and lambda-cyhalothrin on the arthropods and earthworms were evaluated at the end of the experiment after 12 months of exposure. The abundance of the soil fauna varied considerably between the individual plots. Such a variability has also been reported by authors for tropical ecosystems (e.g., Höfer et al. 2001) and may be explained by different factors. Some fauna groups such as Collembola, Acari or Oligochaeta have a clustered distribution. Sometimes they are restricted to microhabitats, which usually leads to a large variation between the samples. Besides, due to time constraints concerning the extraction and determination of the high number of animals, the sampling was limited to one sample per field plot.

The abundance of litter- and soil-dwelling arthropods was not influenced by the treatments with carbendazim or lambda-cyhalothrin. Due to the high variability between the individual plots, no significant differences between the treatments and the control could be detected by the statistical analyses of the data. However, a negative effect on soil arthropods after application of lambda-cyhalothrin seems to be evident, since a significant reduction of mass loss was observed in the litterbag test. Probably, such effects occurred only for short periods of time and were followed by a quick recovery of (at least some) arthropod populations. This recovery could be due to different sensitivity to the chemicals of the individual species (see Koolhaas et al. 2004; Inglesfield 1989), often short generation time of some soil fauna groups (see Prinzing et

al. 2002) or by re-colonization (the plots were relatively small compared to the migration ability of many macroarthropod species).

The earthworms found at the study site were very inhomogeneously distributed and the different species varied considerably in body size – between 2 cm and more than 1.2 m in length (Römbke et al. 1999). Due to this high variability in abundance and biomass, it is often not possible to detect significant differences between treatments and control in field experiments. For example, in the standardized earthworm field test according to the ISO guideline 11268-3 (ISO 1999c), often only the analysis (i.e., all species together) reveals significant differences between treated plots and the control, while the numbers and biomass of the individual species are most variable (Kula, personal comm.).

In the present study, an assessment of the effects of carbendazim on abundance and biomass of all oligochaetes showed no significant difference between treatments and the control. However, when the data were analysed on the species level, a statistically significant effect on the most abundant species *Andiorrhinus amazonius* was found in all carbendazim treatments. This result is not very surprising, remembering that, according to the calculations, the concentration of all carbendazim-treated plots was (often clearly) higher than 1 mg a.i./kg. Such concentrations have been shown to affect earthworms negatively in chronic laboratory tests under tropical as well as under temperate conditions. In a very rough estimation, assuming that NOEC values are often lower by a factor of 10 compared to LC_{50} values, even an effect on the tropical species *P. corethrurus* can be considered under field conditions. Realizing that the fauna samples were taken at the end of the litterbag test, i.e., 12 months after the first application of the chemicals, also avoidance reactions might have occurred in the carbendazim-treated plots.

In temperate regions, the effect of carbendazim on earthworms is well known from laboratory, microcosms and field tests. Since this chemical (and the closely related fungicide benomyl) have been used for many years in the standard earthworm field test (ISO 1999c), its toxicity to earthworms is very well documented. For example, NOEC values (for abundance) as low as 3.24 kg a.i./ha (= 4.31 mg a.i./kg) and EC_{50} values (for biomass) as low as 8.69 kg a.i./ha (= 11.55 mg a.i./kg) were found at European meadow and agricultural sites (Römbke et al. 2004b). According to the ISO guideline (ISO

1999c), application rates of 2 - 4 kg a.i./ha (= 2.66 - 5.32 mg a.i./kg) are considered to cause a decrease in abundance of 40 - 80 %. Van Gestel (1992) found effects on earthworms independent of site use and soil characteristics at concentrations ranging from 0.4 to 1.6 mg a.i./kg. In addition, different earthworm species show a different sensitivity to this fungicide. So, no clear differences between the sensitivity of earthworms towards carbendazim at the temperate and the tropical sites investigated here are obvious.

Nearly no information concerning the effects of lambda-cyhalothrin on native earthworms in field tests is available in the literature. Three annual applications of lambda-cyhalothrin at rates of up to 250 g ai/ha (= 0.33 mg a.i./kg) to field plots had no adverse effect on populations of individual species of earthworms or total earthworm numbers or weight (Coulson et al. 1986). In addition, Inglesfield (1989) stated that pyrethroids in general do not effect earthworms at concentrations up to 5 kg a.i./ha (= 6.65 mg a.i./kg). In this study, the low concentration (3-monthly) of lambda-cyhalothrin negatively influenced the abundance of the native earthworm *Andiorrhinus amazonius* in the field experiment. However, the toxicity of lambda-cyhalothrin to this species was not consistent, since no significant effects were found in the other treatment with monthly applications. It is difficult to explain this result. Maybe an avoidance reaction took place, since in a test with *Eisenia fetida* in LUFA soil such a reaction occurred already at 0.32 mg a.i./kg (see Figure 3.40).

Summarizing the results of the field study, it seems that carbendazim had strong (i.e., > 10 % mass loss difference) but only short-term effects on OM breakdown at concentrations roughly similar to those reported from studies performed in temperate regions. Lambda-cyhalothrin caused higher differences in mass loss at the treated plots compared to the control 6, 9 and 12 months after application (according to the application scenario). This has to be considered as being a risk for OM breakdown.

No effects on the soil macrofauna were found one year after the first application of both test substances. The only exception was the native earthworm *Andiorrhinus amazonius,* which was negatively affected by all carbendazim treatments as well as by the 3-monthly application of lambda-cyhalothrin. While in the former case chronic, maybe even acute, effects of the fungicide are likely, it is difficult to identify the reason for the effects of the insecticide (avoidance behavior?). Differences in

sensitivity between the reactions found here and those reported from temperate sites seem to be small.

4.4 Environmental Risk Assessment

4.4.1 Introduction

One aim of this work was to perform an Environmental Risk Assessment of three test substances. Mainly two questions have to be addressed in this section:

1. Is the outcome of an ERA different when using either data generated under temperate or under tropical conditions?
2. Does the inclusion of higher-tier data (semi-field and field) have an influence on the outcome of the ERA?

Due to the limitations of a Ph.D. thesis and the data availability in general, no complete ERA for these substances was possible (see as an example: Schmuck and Keppler 2003). Instead, the ERA focuses on the soil as part of the terrestrial compartment. According to the guidelines for the registration of pesticides in Europe, soil data on the fate (mainly degradation) and the effects on micro-organisms and earthworms are required for a PPP (EC 2003b). Only under specific conditions (in particular substances with high persistence in soil), which are not fulfilled for the substances considered here, more effect data have to be presented to the authorities:

- If the DT90 (field) value > 100 days, collembolan and predatory soil mite tests are necessary.
- If these additional effect tests indicate a risk or if the DT90 (field) value > 365 days, a litterbag test in the field is required.

According to the Brazilian rules for pesticide registration, only the fate in the soil, the acute test for earthworm and the effect on soil microorganisms are considered for the soil compartment (IBAMA 1996). However, all requirements for registration are under revision (IBAMA 2003). Therefore, in the following, fate data from the literature plus own estimations (based e.g., on the Rapid-Kit measurements) and effect data from the literature as well as the results presented so far will be used to discuss the two questions presented above.

Legally speaking, the situation of the three substances is as follows:

Benomyl:

- European Union: Due to serious problems in the area of human toxicology and ecotoxicology (in particular the effects on earthworm populations), the producing company decided in December 2002 to withdraw the substance from the market (EU 2002).
- Brazil: Due to concerns about toxic effects on human health, the substance was canceled after a re-evaluation (ANVISA 2001) and excluded from the list of pesticides registred in Brazil (ANVISA 2003).

Carbendazim:

- European Union: Partly due to the same problems (e.g., the ecotoxicological side-effects on earthworms), a decision on the re-registration of this substance is pending (EU 2002). Originally announced for 2001, the EU will not make a decision until the end of 2004, since the results of further studies have to be incorporated in the dossier.
- Brazil: Concerns about effects on human health led to a re-evaluation (together with benomyl; ANVISA 2001) which resulted in the decision to allow its use in the country (ANVISA 2003).

Lambda-cyhalothrin:

- European Union: This insecticide has been successfully re-registered in 2000 (EU 2002). Its use is allowed in all EU countries except in Ireland and Spain, where further, regionally specific tests have to be done.
- Brazil: To date, its use is allowed (ANVISA 2003).

In the following, the situation for the three substances is discussed separately.

4.4.2 Benomyl

Benomyl is only addressed briefly since nothing is known about the exposure and fate of benomyl under tropical conditions and since nearly no higher-tier data (except Vink and Van Straalen 1999) are available. It is assumed that due to the quick metabolization of benomyl to carbendazim, the risks of this compound are covered by an ERA for carbendazim.

Due to the reasons mentioned above, for this substance only the first question (this section) with a clear focus on the effect data can be addressed. No formal ERA is possible, since no reliable exposure and fate data, in particular for tropical conditions, are available. For the discussion here, application rates of 125 g a.i./ha (= 0.17 mg a.i./kg) as recommended for German crop sites are considered. According to the results of the acute, chronic and avoidance tests with earthworms (*E. fetida*) and those of acute and chronic tests with isopods (*P. pruinosus*) in artificial soils (Table 4.12), the following statements can be made:

- Values gained from the earthworm tests under tropical conditions are always higher by about a factor of at least 3 (acute tests: 30, chronic tests: 3).
- Values gained from the acute isopod tests are the same under both conditions, while the chronic value from the former cannot be compared with data from tropical regions. However, data from Vink et al. (1995) indicate a low toxicity (NOEC = 1221 mg a.i./kg) for this species for different exposure routes in a non-standardized test.

For temperate regions the results of the earthworm tests as well as information from literature (see Section 4.1.2) indicate concern for the soil ecosystem (in any case the NOECs gained in isopod tests are so much higher than any possible exposure that they will not be considered any further here). This evaluation is backed up by the results of earthworm tests with field soils, where lower acute NOECs (in LUFA and TNS = 31.6 mg a.i./kg) and the same chronic $NOEC_{repro}$ for LUFA as in OECD artificial soil (0.32 mg a.i./kg) were found. In addition, data from behavioral tests performed under temperate conditions revealed NOECs of 1.0 mg a.i./kg or lower in the different soils.

Assuming that benomyl would be used in the tropics in similar application rates as in Germany, these (few) results gained under tropical conditions indicate that the concern identified for temperate regions is valid for tropical regions too. Despite the fact that the toxicity to earthworms (isopods are not affected) is lower by a factor of 30 (acute) or 3 (chronic) than under temperate conditions, this difference is not large enough to change this (still preliminary) assessment. Therefore, the classification of benomyl as a compound of concern is still valid for tropical conditions.

However, since the data availability is poor (e.g., no reliable exposure or DT values, no NOECs from an earthworm test with a tropical field soil, no semi-field (e.g.,

TME) or field data at all), it is recommended to improve this situation before making a final assessment. Despite the fact that the production of benomyl has been stopped by the main producing company, this recommendation is still valid as long as it is not really clear that the use of this compound is banned world-wide.

Table 4.12: LC_{50} and NOEC values for benomyl from acute, chronic and avoidance tests with earthworms (*E. fetida*) and isopods (*P. pruinosus*) in artificial soils (OECD, TAS) under temperate and tropical conditions (20 °C, 28 °C); always the lowest value known was used (all data in mg a.i./kg)

Test system	Temperate conditions (OECD soil)	Tropical conditions (TAS soil)
Earthworm acute test (LC_{50})	22.0	633
Earthworm chronic test (NOEC)	1.0	3.16
Earthworm avoidance test (NOEC)	1.0	1.0
Isopod acute test (LC_{50})	≥ 1000	≥ 1000
Isopod chronic test (NOEC)	≥ 1000	n.d.

4.4.3 Carbendazim

An ERA for carbendazim is much more complex than the one for benomyl due to the larger amount of data available. The situation is improved by the fact that recently an EU project was finished in which an ERA based on semi-field and field data in addition to laboratory data was performed (Weyers et al. 2004). Therefore, in the following both questions formulated above will be addressed.

Fate

Under temperate conditions, carbendazim is moderately persistent under field conditions (DT_{50} values between 10 and 50 days in acid sandy soils, and between 50 and 230 days in neutral clayey soils (Domsch 1992) have been reported). Own data from laboratory tests gained with Rapid-kits indicate that the substance is stable in OECD artificial soil for the usual test duration of 2-8 weeks, but no reliable DT values can be derived from these data. Since vapour pressure and photo degradation are low, a constant exposure in the laboratory and semi-field tests can be assumed. In long-term field tests, a decrease over time (strongly depending on site-specific soil and climatic conditions) was determined in the EU project mentioned earlier (Jones et al. 2004).

For the temperate scenario mentioned earlier (Weyers et al. 2004), the application pattern for tomatoes, with the highest application rates, was used. Four applications per year at 600 g a.i./ha each, 14 d interval, with a DT_{50} soil of 100 d (thus rate constant k = - ln0.5/100) and 50% a.i. reaching soil (50% plant cover) were assumed. For the degradation of carbendazim, a first order kinetic, soil density of 1.7, soil depth of 5 cm and plant cover of 50% were assumed. As a result, a long-term PEC immediately after the last application of long term PECsoil of 1.228 mg a.i./kg was determined as a worst-case approach (for details of the calculation, all done according to the respective EU guidelines, see Weyers et al. 2004).

No fate data on carbendazim are available for tropical conditions. Own data gained with the Rapid-kit method indicate that the degradation of this substance is quicker at higher temperatures. The same tendency can be assumed when looking at the results of the earthworm factorial design tests, in which a significant relationship between lower toxicity and higher temperature was found. Summarizing these hints, it seems certain that the respective DT values are lower than those under temperate conditions, but how much lower these values are can only be estimated. For the calculations presented in Sections 4.2.1 and 4.3.1, a DT value of 50 days, application rates of 1.0 kg a.i./ha, applied monthly or 3-monthly, respectively, as well as a 10-fold higher rate (10 kg a.i./applied once) were used. Therefore, a range of initial PEC values between 1.33 and 13.3 mg a.i./kg are considered as a worst-case approach for the ERA presented here (a long-term PEC is difficult to define, since no details of the application pattern under tropical conditions is known).

Summarizing this discussion, PEC values of 1.228 and 1.33 mg a.i./kg for temperate and tropical conditions, respectively, will be used for the ERA. In addition, a PEC of 13.33 mg a.i./kg is considered as a worst-case scenario in order to cover all application scenarios under tropical conditions.

Effects

In this section, the results of the standard laboratory tests, tests with field soils and/or native species and tests described in the literature are discussed. According to the results of the acute, chronic and avoidance tests with earthworms (*E. fetida*) and those of acute and chronic tests with isopods (*P. pruinosus*) in artificial soils (Table 4.13), the

following differences between the results from temperate and tropical conditions can be made:

- The values gained from the earthworm tests under tropical conditions are clearly higher than those from temperate conditions by a factor of > 170 (acute tests) and 30 (chronic tests).
- The values gained from the acute isopod tests are the same under both conditions while the chronic value from the former cannot be compared with data from tropical regions.

The data gained in isopod tests are so much higher than any possible exposure that they will not be considered any further here. For the same reason, even an effect in the chronic limit test with LUFA soil at 1000 mg a.i./kg does not indicate a risk for these organisms. In addition, the two tested native arthropod species did not show a high sensitivity (the LC_{50} values were >1000 mg a.i./kg for the isopod *C. ornatus* and 503.5 mg a.i./kg for the milliped *T. corallinus*).

For temperate regions the results of the earthworm tests as well as information from literature (see Section 4.1.2) indicate concern. This evaluation is backed up by the results of earthworm tests with LUFA field soil, where a lower LC_{50} value (4.1 mg a.i./kg) and a more or less similar chronic $NOEC_{repro}$ (0.5 mg a.i./kg) were found. Avoidance results are in the same area of magnitude (< 1.0 mg a.i./kg). The values used in the ERA performed by Weyers et al. (2004) (see also Table 4.14) are very comparable: For the earthworm acute test, an LC_{50} value of 5.03 mg a.i./kg and for the earthworm chronic test a NOEC of 0.53 mg a.i./kg was determined. Data gained in enchytraeid tests are even lower ($EC10_{Repro}$: 0.15 mg a.i./kg). In the TME studies at four European sites, the lowest NOEC value for earthworms was 1.08 mg a.i./kg (in Table 4.14 given as 1.27 mg a.i./kg due to a re-calculation to wet weight). A functional endpoint (the feeding rate) revealed a NOEC in the same order of magnitude.

Under tropical conditions, the same tendency is visible as for temperate regions: A very low acute toxicity (> 1000 mg a.i./kg) for earthworms is combined with a quite high chronic toxicity (3.16 mg a.i./kg). However, it is interesting that the difference between artificial and field soil is much higher (LC_{50} for *E. fetida* in tropical field soil: 57.1 mg a.i./kg) than under temperate conditions. The inclusion of data from acute tests with the native earthworm species *P. corethrurus* does not change the overall

picture (LC_{50} 45.6 mg a.i./kg). In the TMEs, only LC_{50} values could be determined. However, assuming a ratio of 10 between LC_{50} values and NOECs (often found in ecotoxicology), the lowest effect values seem to be in the same order of magnitude. Moreover, looking at the functional endpoint OM breakdown, significant effects were found at 6.01 mg a.i./kg, meaning that the NOEC must be even lower. In the field, a decrease in the abundance of the native earthworm species *Andiorrhinus amazonius* was found at concentrations as low as 1.33 mg a.i./kg, while no effect on decomposition was found at the three application scenarios covering a range of initial PECs of up to 13.3 mg a.i./kg. Summarizing these higher-tier data, the results of these tests confirm effects of carbendazim in the range of a few mg a.i./kg, either on structural (earthworm abundance in the field) or functional (OM breakdown in TMEs) endpoints. In addition, it seems that the decrease in toxicity under tropical conditions compared to test results gained under temperate conditions as seen in the laboratory (i.e., being even higher than in the case of benomyl) cannot be found under semi-field or field conditions.

Table 4.13: LC_{50} and NOEC values for carbendazim from acute, chronic and avoidance tests with earthworms (*E. fetida*) in artificial soils (OECD, TAS) under temperate and tropical conditions (20 °C, 28 °C); always the lowest value known was used (all data in mg a.i./kg)

Test system	Temperate conditions (OECD soil)	Tropical conditions (TAS soil)
Earthworm acute test	5.8	> 1000
Earthworm chronic test	0.1	3.16
Earthworm avoidance test	< 1.0	3.16
Isopod acute test	≥ 1000	≥ 1000
Isopod chronic test	n.d.	≥ 1000

Risk assessment

In the most recent attempt to assess the risk of carbendazim to soil invertebrates (including the use of TME and field data), Weyers et al. (2004) compiled the information available from the literature (Table 4.14), in particular the data gained in a project sponsored by the European Union between 1998 and 2002 (Knacker et al. 2004, Moser et al. 2004 and Römbke et al. 2004b). In a quite broad approach, different data sources and their respective assessment factors are listed, showing clearly how the risk assessment process is refined from level to level. The different outcome of the ERA,

i.e., the lowering of the PEC/PNEC ratio, is mainly caused by a smaller assessment factor. This decrease of the assessment factor is based on the idea that data from higher test level (e.g., semi-field tests) are more reliable than those from lower (e.g., laboratory tests) level. In other words: The same effect data determined on the laboratory or on the TME level would lead to a lower PEC/PNEC ratio for the latter due to a lower assessment factor.

For example, if only aquatic effect data were available for a chemical, the Equilibrium Partitioning Method (Di Toro et al. 1991) has to be used in order to extrapolate these data to soil organisms. Due to the high insecurity of this data source, the comparison of these values with the PECsoil would lead to unreliable high PEC/PNEC ratios, thus indicating strong concern (i.e., the PEC/PNEC ratio is much higher than 1). On the other hand, when TME data (field data would be in a similar range) are used, the PEC/PNEC ratio drops considerably. According to the latter data, carbendazim can cause concern when used at the assumed application rates of 600 g a.i./ha. However, it can easily be seen that at lower application rates and/or fewer applications it would be possible to gain a PEC/PNEC ratio < 1, thus indicating no concern. In such a situation it is up to the producing company, to decide whether the PPP is still effective against the target organism (in the case of carbendazim: certain fungi) at such changed application rates and frequencies.

For tropical conditions, a similar detailed ERA is not possible. However, using the data from the study presented here, a preliminary ERA was performed (Table 4.15). Note that always the most sensitive data are considered, i.e., here only earthworm and OM breakdown values. According to this compilation, an application rate of 1 kg a.i./ha carbendazim when applied several times (3-monthly) lead to a concern for the soil ecosystem, since the PEC/PNEC values are always > 1.

However, this ERA for the use of carbendazim under tropical conditions can only be seen as preliminary for the following reasons:

- While a very large amount of valid laboratory data is available, the number of higher-tier tests is low. Furthermore, the latter were not performed according to a dose response design (i.e., no detailed value could be calculated).
- The number of assumptions is, naturally, high: this statement is true for the application rates and in particular for the exposure values used.

- Finally, the PEC/PNEC ratios estimated are relatively close to 1.

Table 4.14: Synopsis of PEC/PNEC ratios based on data typically available for the different substance groups. Data are given in mg a.i./kg (soil, wet weight) for terrestrial organisms. Values reported on a dry weight basis were corrected by a factor of 0.882, due to assumptions set soil water content to 12% by weight (20% by volume).

Test level	Data used	Value [mg/kg]	Assessment factor	PNEC [mg/kg]	PEC [mg/kg]	PEC/PNEC Ratio
Theory: no test data available	EPM[1]	-	Part of EPM	0.000626	1.228	1962
Laboratory: Single-species tests	LC_{50} earthworm	5.03	1000	0.005	1.228	246
	NOEC earthworm	0.53	10	0.053	1.228	23
	EC_{10} enchytraeids	0.15	10	0.015	1.228	82
Semi-field: TME data	NOEC earthworm	1.27	5	0.254	1.228	5
	NOEC bait lamina	0.42	5	0.084	1.228	15

[1]EPM = Equilibrium partitioning method (Di Toro et al. 1991).

Summarizing the results of this ERA, it is strongly recommended to improve the data quality by performing more higher-tier tests (including accompanying chemical residue analyses) in order to minimise the number of assumptions. However, even this preliminary ERA indicates that despite the (probably) quicker degradation of carbendazim under tropical compared to temperate conditions, a risk of this PPP to the soil ecosystem when applied in practice-relevant concentrations cannot be excluded. Referring to the second question raised above, it is clear that the inclusion of higher-tier data improves the ERA considerably.

Table 4.15: Synopsis of PEC/PNEC ratios based on data gained under tropical conditions in this study for carbendazim. Data are given in mg a.i./kg for terrestrial organisms.

Test level	Data used	Value [mg/kg]	Assessment factor	PNEC [mg/kg]	PEC [mg/kg]	PEC/PNEC Ratio
Laboratory: Single-species tests	LC_{50} earthworm	> 1000	1000	> 1.00	1.33	<1.33
	NOEC earthworm	3.16	10	0.316	1.33	4.21
	LC_{50} native earthworm	45.6	1000	0.05	1.33	26.6
Semi-field: TME data	NOEC OM breakdown	<6.01	5	<1.20	1.33	>1.30
Field:	NOEC native earthworm	<6.01	5	<1.20	1.33	>1.30

4.4.4 Lambda-cyhalothrin

Considering the performance of an ERA, this compound is situated somewhere between benomyl and carbendazim for the following reasons:

- For temperate regions, no data compilation or even a full ERA has been published. However, due to the fact this PPP was re-registered in the European Union quite recently, it is clear that the ERA performed by the authorities did not reveal a risk for the soil compartment.
- Due to the work performed in the study presented here, a relatively high amount of data was gained for the effect side. On the other hand, no reliable exposure data are available.

Fate

Under temperate conditions, lambda-cyhalothrin is non- to moderately persistent under field conditions (DT_{50} values between 4 and 12 weeks (WHO 1990; Tomlin 1997). On soil surfaces and in aqueous solutions at pH 5, lambda-cyhalothrin degrades in sunlight with a half-life of approximately 30 days (WHO 1990). Although no additional environmental biodegradation data specific to lambda-cyhalothrin were found, pyrethroid insecticides in general are often readily biologically degraded (WHO 1990). Fate data from tropical conditions in general are not available, but Domsch (1992) mentions that in India other pyrethroids like deltamethrin are degraded more rapidly than under European conditions. Own data were not gained, but the results of the

earthworm factorial design tests indicate that temperature is not a factor influencing the toxicity and, therefore, probably the exposure (i.e., the persistence) was more or less the same under both conditions.

For the purpose of the ERA performed here, no reliable DT values for lambda-cyhalothrin were found. Therefore, following a worst-case approach (i.e., simple addition of all applied amounts), the highest concentration expected to occur in the soil under realistic field conditions is 0.21 mg a.i./kg (sum of four 3-monthly applications). This value is based on an application rate of 40 g a.i./ha, which is twice as high as recommended for German crop sites. However, taking the higher pest pressure in tropical regions into consideration, it was decided to use this higher value of 40 g a.i./ha, which is used as a still realistic worst-case scenario when calculating the respective PEC values. The underlying assumptions for these calculations follow EU guidelines and do not differ from those used in the case of carbendazim (see Section 5.3.1).

Effects

The results of the standard laboratory tests, tests with field soils and/or native species and tests described in the literature are discussed in the following. According to the results of the acute, chronic and avoidance tests with earthworms (*E. fetida*) and those of acute and chronic tests with isopods (*P. pruinosus*) in artificial soils (Table 4.16), some differences between the results from temperate and tropical conditions have been identified:

- The values gained from the earthworm tests under tropical conditions are lower than those from temperate conditions by a factor of 2-3 (acute, chronic and avoidance tests) - a difference which was tracked down in the factorial design tests as being caused by the worms themselves (i.e., the origin was a decisive factor). The same difference was found in tests with temperate (LUFA) and tropical (TNS) field soils with LC_{50} of 139.9 and 65.5 mg a.i./kg.
- The values gained from the acute isopod tests are also lower under tropical conditions, while the chronic value from the former cannot be compared with data from tropical regions.

The data gained in earthworm tests are higher than the estimated exposure values. However, the difference is relatively small. Isopod data are usually not available during the registration process of a PPP, since no tests with macroarthropods are required in the respective EU guidelines. However, data from tests with beneficial (= non-target) arthropods (e.g., leaf-inhabiting predatory mites or parasitoids; rarely also staphylinid beetles like *Aleochara bilineata*) are required – but this information is usually not used to assess the risk for soil organisms (Candolfi et al. 2001). In this section, risk assessment is performed with both organism groups.

In tests from temperate regions, no acute toxicity of lambda-cyhalothrin to earthworms has been reported so far. Chronic tests have not yet been performed, indicating that a $NOEC_{biomass}$ of 100 mg a.i./kg is possibly the lowest effect value for oligochaetes found in the literature (Maroni et al. 2002). While no ecotoxicological studies with isopods are known, it is clear - as expected - that this insecticide is highly toxic to macroarthropods like carabid beetles or spiders (Inglesfield 1989).

From tropical regions, no earthworm or macroarthropod data are available except those gained here. In addition to the unexpectedly low acute and chronic effect values from the standard laboratory earthworm tests (Table 4.16), even lower effect values were found in tests with field soils (e.g., a NOEC of 3.16 in LUFA) and in avoidance tests (≤1.0 mg a.i./kg). The native earthworm species *P. corethrurus* seems to be even more sensitive (LC_{50} of 40.2 mg a.i./kg).

The high toxicity of an insecticide to arthropods could be confirmed in the test with isopods, where all LC_{50} values (including the tropical artificial soil TNS) were clearly lower than 1 mg a.i./kg. Interestingly, only in LUFA soil was a higher LC_{50} found (1.4 mg a.i./kg) – the only case in all tests where the sensitivity in a field soil was lower than in artificial soils. However, this difference should not be overestimated, since the results of the chronic tests show the “normal” relationship (NOEC values of 0.4 versus 0.13 mg a.i./kg, respectively). The sensitivity of the native macroarthropod *T. corallinus* was slightly lower than that of the isopods (LC_{50} 1.2 mg a.i./kg).

In the TME tests performed in this study, at 0.24 and 2.39 mg a.i./kg (initial concentrations) no effects on earthworms but significant mortality of native millipedes and isopods (the latter only at the higher concentration) were found. In addition, OM breakdown was significantly affected at both concentrations. The latter result was partly

confirmed in the field, where an effect > 10% on mass loss was found at concentrations of about 0.21 mg a.i./kg. Structural effects on macroarthropods were not observed in the field, but there are indications that a native earthworm species is affected (*Andiorrhinus amazonius*) at the same concentration.

Summarizing the research on the effect of lambda-cyhalothrin, it is clear that soil organisms react more sensitively under tropical than under temperate conditions. Unexpectedly high sensitivity of earthworms was found, while the efficiency of this insecticide towards macroarthropods could be confirmed. TME and field data indicate effects on structural and functional endpoints under realistic conditions and relevant concentrations (i.e., 0.21 mg a.i./kg).

Table 4.16: LC_{50} and NOEC values for lambda-cyhalothrin from acute, chronic and avoidance tests with earthworms (*E. fetida*) in artificial soils (OECD, TAS) under temperate and tropical conditions (20 °C, 28 °C); always the lowest value known was used (all data in mg a.i./kg)

Test system	Temperate conditions (OECD soil)	Tropical conditions (TAS soil)
Earthworm acute test	99.8	23.9
Earthworm chronic test	10.0	6.2
Earthworm avoidance test	1.0	< 0.32
Isopod acute test	0.5	0.2
Isopod chronic test	0.4	n.d.

Risk assessment

As already mentioned, for temperate regions no ERA has been published. Based on the data from standardized laboratory tests gained in this study (mainly on earthworm toxicity) concern is not indicated (PEC/PNEC value based on chronic toxicity (NOEC = 10 mg a.i./kg; assessment factor: 10): 0.21 mg < 1). However, concern arise if earthworm avoidance or macroarthropod acute and chronic test data were included; however, these values are usually not part of an ERA in the European Union. Obviously, the European authorities responsible for the registration of PPPs followed the standard approach, which leads to the conclusion, that lambda-cyhalothrin poses no risk to the soil ecosystem.

For tropical regions, a very preliminary ERA for the use of lambda-cyhalothrin in the soil was performed. This ERA is based on an assumed application

rate of 40 g a.i./ha (4 times per year), leading to a realistic worst-case exposure of 0.21 mg a.i./kg (no degradation of the compound included) and of the effect data gained in this study.

Table 4.17: Synopsis of PEC/PNEC ratios based on data gained under tropical conditions in this study with lambda-cyhalothrin. Data are given in mg a.i./kg for terrestrial organisms.

Test level	Data used	Value [mg/kg]	Assessment factor	PNEC [mg/kg]	PEC [mg/kg]	PEC/PNEC Ratio
Laboratory: Single-species tests	LC_{50} earthworm	23.9	1000	0.024	0.21	8.75
	NOEC earthworm	6.20	10	0.62	0.21	0.34
	LC_{50} isopod	0.20	1000	0.0002	0.21	1050
	LC_{50} native millipede	1.20	1000	0.0012	0.21	175
Semi-field: TME data	NOEC native millipede + OM breakdown	<0.24	5	<0.048	0.21	>4.38
Field:	NOEC OM breakdown	<0.21	5	<0.042	0.21	>5.0

According to the data given in Table 4.17, the use of earthworm data would lead to the conclusion “no concern”, since the PEC/PNEC value based on the NOEC is clearly < 1.The inclusion of field observations, in particular the decrease in abundance of the native earthworm species *Andiorrhinus amazonius*, would slightly change this picture, but since this result is not conclusive (no such effects at the other application scenarios with comparable exposure), it would not change the outcome of the ERA. However, this fact in combination with the structural and functional effects obtained in the TMEs at an initial concentration of 0.24 mg a.i./kg, would indicate some concern.

The situation becomes much more clear if the results of the macroarthropod tests (isopods and millipedes) are included, since they clearly indicate concern (PEC/PNEC values of 175 and 1050!). This result is confirmed in the TME, where at comparable initial concentrations effects on these organisms as well as on OM breakdown were found (NOECs < 0.24 mg a.i./kg). As already mentioned, OM breakdown is affected in the field up to one year after the first application, while there are no detectable effects on the abundance of the macroarthropods. The latter observation can be explained either by a recovery of the arthropod populations or, more probably, by recolonization of the relative small study plots.

Summarizing the results of this ERA, it becomes clear that lambda-cyhalothrin can be a risk for the soil ecosystem. However, due to the lack of detailed exposure data, this result is based on a worst-case approach. It is strongly recommended to check this result by performing a field study under realistic use conditions, i.e., with larger plots, application rates according to "Good Agricultural Practice" as used in Brasil, and an accompanying chemical residue analysis. Both structural (abundance of macroarthropods) as well as functional (OM breakdown) endpoints should be measured. The different outcome of this ERA for tropical regions compared to the ones done for temperate regions is due to the fact that at tropical plantation sites macroarthropods play a more important ecological role than earthworms (Höfer et al. 2001), while just the opposite is true for temperate regions (Edwards and Bohlen 1996).

4.4.5 Summary of experiences and comparison with ERA approaches in the tropics

Since up to now no specific ERAs for tropical conditions have been performed, this issue can only be briefly discussed. As has been shown above, the ERA for temperate conditions can also be used other ecosystems like the tropics (under the assumption, that specific data are available); i.e., there is no need for some kind of special "Tropical ERA". However, it has become clear that, due to changes in exposure and effect values, a "normal" ERA is necessary using data gained under tropical conditions. Whether this process leads to another result and thus to other consequences regarding potential risk management measures, depends on the individual test substance. Theoretically (and partly already shown by the few examples tested in this study), an increase as well as a decrease in concern are possible.

Regarding the two questions raised in the introduction of this chapter, the following outcome of the preliminary ERAs for the three PPPs assessed here can be summarized as follows:

- Benomyl

Its toxicity is lower (earthworms) or equal (isopods) under tropical compared to temperate conditions. However, the differences are relatively small (factor of 3 - 30). Therefore, the ERA would lead to concern for both regions. Since no higher-tier data are available for tropical regions, their relevance cannot be discussed.

- Carbendazim

The differences in earthworm toxicity values between those gained under tropical and temperate conditions are even higher in the case of benomyl (factors 30 - 170). For isopods, no difference could be observable due to a lack of toxicity. Again, this difference is not large enough to reject the concern identified under temperate conditions. The inclusion of higher-tier results (TME as well as field) strongly supports the identification of concern due to their ecological relevance.

- Lambda-cyhalothrin

The toxicity to earthworms and, probably, isopods is higher under tropical conditions compared to temperate ones. Again, the difference is relatively small (factor of about 2 - 3). However, the outcome of the ERA is clearly different (concern for tropical but not for temperate regions), since not earthworm but macroarthropod data have been used as a basis for the ERA. This change was made beacuse these organisms are ecologically more important at tropical plantation sites than earthworms. The concern identified could be supported by results from the TME and field level. The last statement confirms the importance of higher-tier data.

In this context, it must be mentioned that in aquatic ecotoxicology, proposals have been made to define a "general factor" between the effect level under temperate and tropical conditions (e.g., based on the LC_{50} values of several pesticides which have been tested under both conditions as compiled from the literature; e.g., Leung et al. 2003). Such use of a general factor is considered to be inappropriate, because it does not take into account the considerable differences between the effect levels in individual cases and the very small database for the derivation of such a factor. Finally, for theoretical reasons (in particular the high number of factors which can influence this ratio – in both directions) it is highly unlikely that such a factor can be defined without creating a high number of over- and underprotection cases. Also based on data compilations done in aquatic ecotoxicology, it has been proposed to protect the

ecosystem level by using results from chronic laboratory tests (Versteeg et al. 1999). However, even if this is true just by looking at the numbers, the ecological relevance of higher-tier data is much higher and thus more useful for the decision on the registration of a PPP.

The performance of a full ERA is not possible within a Ph.D. study. However, the preliminary ERAs performed here can be considered as a first step towards a "full-scale" environmental risk assessment of these PPPs. The greatest obstacle is the lack of reliable exposure and fate data gained under tropical conditions. However, the experiences gained so far can be summarized as follows:

- If PPPs are used in tropical regions, their environmental risk has to be assessed using the generally accepted ERA principles, which have to be based on tests performed under tropical conditions.
- "Tropical conditions" require modification of the abiotic test properties (e.g., higher temperature) as well as – potentially – inclusion of other test species (e.g., isopods).
- Test methods for the compartment soil are now available (earthworms) or are under development (isopods). However, in agreement with international standards (e.g., OECD), these methods must be formally validated.
- In addition to single species tests, higher-tier methods should be included in the ERA.
- The availability of realistic exposure and fate data is of utmost importance.

5 CONCLUSIONS

The present study aimed to improve the scientific basis for tropical soil ecotoxicology. The effect of pesticides on soil organisms and organic matter decomposition was studied in a comparative approach between temperate and tropical regions.

The results from laboratory as well as semi-field and field tests show that the toxicity of the test chemicals was strongly influenced by the tropical abiotic factors. Depending on the chemical, its fate and effect in tropical soil may be very different compared with temperate regions. Thus, it can be concluded that, to assess the environmental risk of pesticides in tropical regions, the existing ecotoxicological data from temperate regions should be carefully evaluated and, if necessary, additional tests should be performed under tropical conditions. Consequently, for an environmental risk assessment (ERA) for pesticides in the tropics, the simple extrapolation of temperate data to tropical conditions can lead to erroneous results.

Despite the fact that earthworms are considered the standard test organism in (temperate) soil ecotoxicology, additional tests using ecologically relevant species like soil arthropods as in this study (e.g., isopods) should be included in a tropical ERA. While such changes of existing test methods and strategies are necessary on the laboratory level, existing approaches and methods on the semi-field (i.e., TMEs) and field (i.e., OM breakdown tests) level with only slight technical modifications are useful under tropical conditions. Based on this extended set of methods but relying on the same tiered approach, an ERA of pesticides (and, probably, chemicals in general) for moist tropical regions is possible.

The basic methodology in soil ecotoxicology is now available for tropical regions. However, there are some areas open for further research: In this study, a new formulation is proposed for tropical artificial soil, but the suitability of the OM source (coir dust) in this substrate must be verified, since most test were done with a fern product (Xaxim). A standard field soil should be identified for tropical regions. A standardized sublethal isopod test for chemicals needs further development. New tests using microcosms should be performed using a dose response design. The amount of data generated under tropical conditions should be extended. For example, is it possible to extrapolate the experience gained in this study to savannah sites with a dry climate?

6 SUMMARY

The soil plays a central role in all terrestrial ecosystems, functioning as habitat for many organisms and as a medium for decomposition, balance and restoration as a result of its filter, buffer, storage and substance-converting properties including groundwater protection. The soil dwelling organisms play a crucial function in the ecosystem and are the main driving force responsible for organic matter breakdown, nutrient cycle and soil structural stability. Plant Protection Products (PPPs) (i.e., pesticides) have long been used in agriculture to control pests and diseases in plants. It is recognized that their use has increased crop yields and food production. However, most of them are also toxic to non-target species and may have negative impacts on beneficial organisms. Thus, in the past decades, the impact of chemicals on soil fauna diversity and soil functions has become a matter of special concern.

Therefore, there is an increasing need for the development of appropriate methods to assess the side effects of these chemicals on soil ecosystems. Little research has been done on the impact of contaminants on tropical ecosystems, considering the amount of studies already conducted in temperate regions. Besides, the physical and chemical variables affecting biotic processes as well as the fate of chemicals differ in both regions. Such differences should be considered when assessing the potential risk of chemicals, in particular pesticides, in tropical ecosystems. Often, most of the standardized data used in the risk assessment of chemicals in tropical countries are generated in North America or Europe (i.e., where temperate boundary conditions were used), whereas comparable data are relatively scarce for the tropical regions. Consequently, an extrapolation of temperate data to tropical conditions without a scientific basis can lead to erroneous results.

Aiming to improve the scientific basis for tropical soil ecotoxicology, this study was planned to assess the effects of selected pesticides on the structure (i.e., earthworms and arthropods) and the function (i.e., organic matter (OM) breakdown) of soil biota in Amazonia. With this purpose, two main questions were addressed here:

- Do fate and effects of pesticides differ between tropical and temperate regions?
- Can data generated under temperate conditions be used for the Environmental Risk Assessment (ERA) in tropical regions?

For this purpose, the chemicals benomyl and carbendazim (fungicides) and lambda-cyhalothrin (insecticide) were selected as model substances to be investigated on three levels of ecological relevance:

- Basic laboratory tests – mainly acute and chronic toxicity tests with selected soil fauna species (plus a recently developed screening test using behavior as an endpoint);
- Semi-field tests – microcosm tests performed under controlled conditions (e.g., Terrestrial Model Ecosystems-TME) using soil cores taken in the field containing the native soil biota and treated with the test chemical;
- Field tests – studying the effects of PPPs on OM breakdown and the native soil fauna.

All tests were based on standard methodologies as described in international OECD and ISO guidelines. Laboratory tests performed under tropical conditions had to be modified accordingly (e.g., a temperature increase from 20 °C to 28 ºC). The tests were done mainly with two strains (from Germany and Brazil) of earthworms (*Eisenia fetida*). The chemicals were spiked in two natural soils and in OECD artificial soil (already developed in 1984). In addition, a tropical artificial soil (TAS) was developed in this study. Originally, TAS contained a fern product (Xaxim) as organic matter, but after the tests had been completed, this had to be replaced due to its scarcity in the market. In additional test runs, it was proven that coir dust (a coconut waste product) is a very good replacement for Xaxim. The methodology of semi-field and field experiments, already tested in temperate regions, was slightly adapted for tropical conditions. The main results of this study can be summarized as follows:

- Standardized laboratory tests with the earthworm *Eisenia fetida* could easily be performed under tropical conditions. For acute endpoints, the suitability of the isopod *Porcellionides pruinosus* could be shown. In addition, the newly developed Tropical Artificial Soil can be recommended for general use in tropical ecotoxicology.
- Results from laboratory tests show that the toxicity of the test chemicals was strongly influenced by the tropical abiotic factors (artificial soil: higher temperature; field soil: soil properties). For example, carbendazim was clearly less toxic under tropical than under temperate conditions, while the situation was

the opposite (less clearly) for lambda-cyhalothrin. As expected, the two fungicides were highly toxic to earthworms (but showed no effect on arthropods), while the insecticide strongly affected the arthropods and surprisingly, moderately toxic to earthworms. Nearly always, the lowest NOEC and EC50 values were found in the avoidance tests.

- Tests in microcosms (TME) were done for the first time in the tropics and were performed successfully, but the moisture regime (i.e., life conditions for introduced animals) should be improved. The toxicity values found do not differ strongly between tropical and temperate conditions, but obvious differences (e.g., functional endpoint bait-lamina) were also identified.
- In the field, carbendazim as well as lambda-cyhalothrin negatively affected OM breakdown. No effects on the soil macrofauna were found one year after the first application of both substances with one exception: the abundance of the native earthworm *Andiorrhinus amazonius* decreased in all carbendazim treatments.
- Laboratory as well as semi-field tests with native earthworm and arthropod species show valuable results, but due to a lack of basic biological knowledge their mortality in long-term tests was high. Thus, these tests need further improvement.
- Despite a major drawback (the exposure concentrations could only be estimated using RAPID-kit data and model calculations) a preliminary Environmental Risk Assessment was performed for the three PPPs, being the first (terrestrial) ERA for a tropical region. Using data from three test levels, the concern identified for benomyl and carbendazim under temperate conditions could be confirmed. Due to the effects on macroarthropods, the risk of lambda-cyhalothrin is higher for tropical than for temperate regions.

In addition, the following areas open for further research were identified: A standard tropical field soil should be identified and the suitability of coir dust as OM source in TAS must be verified. The chronic laboratory test with isopods needs further development. TME tests should be repeated using a dose response design. In order to assess the importance of faunal re-colonization processes, the plot size of field tests should be increased.

Coming back to the initial two questions, it can be stated that due to the experiences gained in this study the following preliminary answers can be given:

- Yes, depending on the PPP assessed, its fate and effect can differ in the two regions;
- No, in case a PPP is used in the tropics, new ecotoxicological data should be generated and an additional ERA has to be performed.

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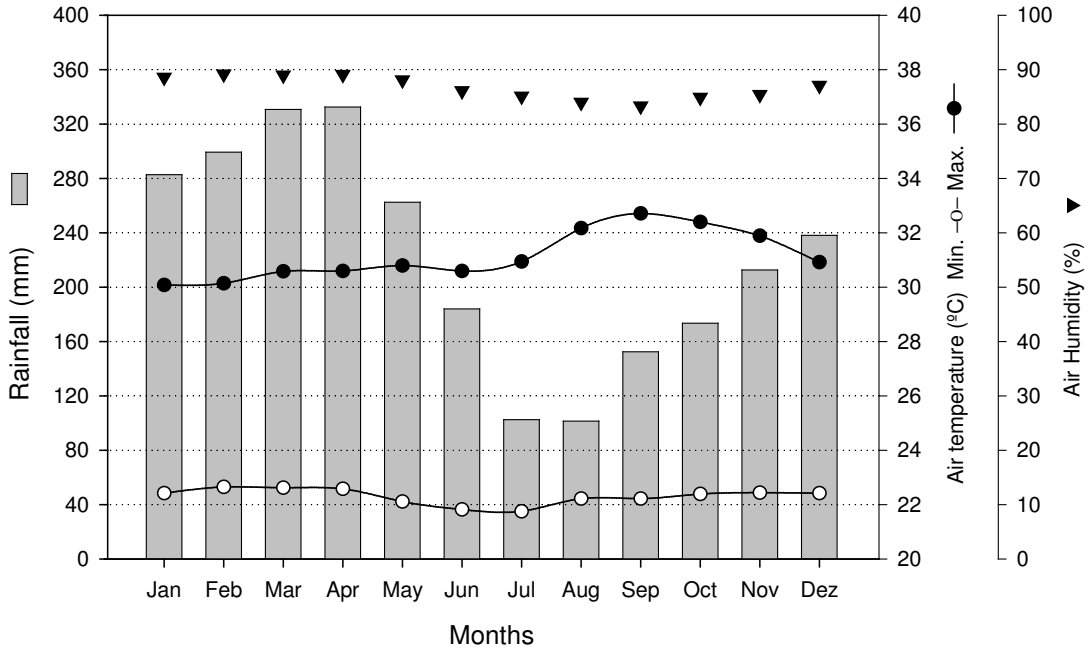
8 APPENDICES

Appendix 1: Climate data from the study area during 2002. Source: Embrapa, Manaus (unpublished data).

Month	Air temperature (ºC)			Soil temperature (ºC) at different depths (cm)				Air Humidity (%)	Total Rainfall (mm)
	Max	Min	Mean	2	5	10	20		
Jan	31.5	23.0	27.4	29.9	29.0	28.8	28.8	90.3	241.4
Feb	31.0	22.9	27.3	29.4	28.5	28.3	28.2	91.6	367.9
Mar	30.9	22.7	26.9	29.2	28.4	28.4	28.5	92.3	473.1
Apr	31.9	22.4	27.5	29.6	28.5	28.2	28.4	92.4	359.8
May	31.6	22.8	27.7	29.6	28.5	28.2	28.6	91.9	351.2
Jun	30.8	22.1	27.4	29.6	28.7	28.2	28.7	89.4	161.7
Jul	32.2	22.0	27.9	29.9	28.8	28.6	28.8	90.0	167.8
Aug	33.1	21.9	28.4	31.0	29.6	29.0	29.3	88.4	148.0
Sep	34.3	22.1	29.2	33.1	31.2	30.4	30.9	81.6	55.8
Oct	33.2	22.2	28.5	31.5	30.2	29.3	30.0	82.7	187.8
Nov	32.8	22.5	28.6	31.1	29.7	28.9	29.3	86.4	155.4
Dec	31.4	22.4	27.1	29.3	28.3	28.0	28.5	91.5	267.3
Mean	**32.1**	**22.4**	**27.8**	**30.3**	**29.1**	**28.7**	**29.0**	**89.0**	**Sum (2937.2)**

Appendix 2: Climate data from the study area: monthly means of ten years (1987 to 1996). Source: Embrapa, Manaus (unpublished data).



Appendix 3: Abundance of litter fauna groups (individual/m^2) under three treatments with carbendazim.

	Control		**1 kg/ha (monthly)**		**1 kg/ha (3-monthly)**		**10 kg/ha (once)**	
Mesofauna	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Acari	867	528	836	565	1224	768	688	686
Collembola	251	167	408	355	392	227	488	484
Symphyla	56	84	72	62	68	53	56	66
Macrofauna								
Araneae	5	13	4	8	20	20	8	9
Chilopoda	3	7	0	0	0	0	4	8
Coleoptera (ad.)	83	66	108	67	192	139	56	56
Coleoptera (la.)	32	46	40	38	28	24	16	13
Diplopoda	40	41	0	0	24	38	44	78
Diptera (la.)	19	12	8	9	16	13	12	15
Diptera (ad.)	72	36	92	48	104	59	80	82
Formicidae	144	180	208	152	356	157	132	117
Gryllidae	3	7	12	24	8	9	8	9
Heteroptera	3	7	4	8	0	0	4	8
Homoptera	43	37	52	33	48	23	44	33
Isoptera	0	0	8	9	24	28	8	16
Hymenoptera	91	105	52	60	228	114	72	50
Pseudoscorpionida	0	0	60	100	8	16	12	15
Psocoptera	29	24	24	38	32	29	56	33
Trichoptera	3	7	12	15	4	8	0	0
Thysanura	0	0	0	0	0	0	96	152
Thysanoptera	37	37	100	70	128	141	0	0
Number of groups	18		18		18		19	

Appendix 4: Abundance of soil fauna groups (individual/m2) under three treatments with carbendazim.

	Control		**1 kg/ha (monthly)**		**1 kg/ha (3-monthly)**		**10 kg/ha (once)**	
Mesofauna	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Acari	605	130	332	209	880	539	648	294
Collembola	315	397	136	70	552	523	264	102
Symphyla	187	154	208	142	172	77	760	1266
Diplura	51	68	24	48	20	20	44	54
Macrofauna								
Araneae	16	18	36	35	24	21	52	27
Chilopoda	0	0	12	8	0	0	0	0
Coleoptera (ad.)	99	64	164	197	96	57	36	40
Coleoptera (la.)	3	7	12	15	8	9	12	15
Diplopoda	27	31	16	18	12	15	0	0
Diptera (la.)	0	0	0	0	0	0	0	0
Diptera (ad.)	96	30	88	38	244	332	100	91
Formicidae	237	115	208	176	292	127	148	140
Gryllidae	0	0	0	0	0	0	0	0
Heteroptera	0	0	4	8	0	0	4	8
Homoptera	32	36	44	35	24	31	20	20
Isoptera	67	106	12	24	4	8	16	23
Hymenoptera	56	86	16	32	0	0	0	0
Pseudoscorpionida	107	200	100	89	68	70	156	195
Ricinulei	3	7	44	88	0	0	0	0
Psocoptera	0	0	0	0	0	0	0	0
Trichoptera	0	0	0	0	0	0	0	0
Thysanura	0	0	0	0	0	0	0	0
Thysanoptera	0	0	0	0	0	0	0	0
Number of groups	15		17		13		13	

Appendix 5: Abundance of litter fauna groups (individual/m^2) under three treatments with lambda-cyhalothrin.

	Control		**40 g/ha (monthly)**		**40 g/ha (3-monthly)**		**400 g/ha (once)**	
Mesofauna	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Acari	867	528	608	352	1036	807	2416	1868
Collembola	251	167	904	437	1100	926	624	263
Symphyla	56	84	8	9	8	16	248	245
Diplura	0	0	0	0	0	0	4	8
Macrofauna								
Araneae	5	13	0	0	12	15	64	75
Blattodea	0	0	0	0	4	8	0	0
Chilopoda	3	7	0	0	16	23	4	8
Coleoptera (ad.)	83	66	44	27	36	53	96	32
Coleoptera (la.)	32	46	4	8	0	0	28	27
Dermaptera	0	0	0	0	4	8	4	8
Diplopoda	40	41	8	9	8	16	52	33
Diptera (la.)	19	12	8	9	0	0	0	0
Diptera (ad.)	72	36	56	21	60	46	240	228
Formicidae	144	180	120	61	132	161	192	88
Gryllidae	3	7	0	0	0	0	28	46
Heteroptera	3	7	4	8	0	0	8	9
Homoptera	43	37	28	38	52	62	36	20
Isoptera	0	0	4	8	20	40	8	16
Gastropoda	0	0	0	0	4	8	4	8
Hymenoptera	91	105	104	166	176	159	52	35
Opilionida	0	0	0	0	4	8	0	0
Palpigradi	0	0	4	8	0	0	0	0
Pseudoscorpionida	0	0	24	31	0	0	12	8
Ricinulei	0	0	0	0	0	0	0	0
Psocoptera	29	24	60	33	24	21	64	32
Trichoptera	3	7	4	8	0	0	0	0
Thysanura	0	0	0	0	0	0	0	0
Thysanoptera	37	37	0	0	0	0	0	0
Number of groups	18		17		17		20	

Appendix 6: Abundance of soil fauna groups (individual/m^2) under three treatments with lambda-cyhalothrin.

	Control		**40 g/ha (monthly)**		**40 g/ha (3-monthly)**		**400 g/ha (once)**	
Mesofauna	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Acari	605	130	560	284	856	659	516	216
Collembola	315	397	1236	590	872	302	352	257
Symphyla	187	154	204	110	280	263	224	70
Diplura	51	68	36	42	56	81	44	78
Macrofauna								
Araneae	16	18	4	8	20	40	76	74
Chilopoda	0	0	16	23	16	13	12	8
Coleoptera (ad.)	99	64	44	24	36	24	28	38
Coleoptera (la.)	3	7	4	8	12	15	16	32
Diplopoda	27	31	20	24	4	8	32	35
Diptera (la.)	0	0	0	0	0	0	0	0
Diptera (ad.)	96	30	108	142	156	119	80	35
Formicidae	237	115	272	95	184	134	172	66
(Gryllidae)	0	0	4	8	0	0	4	8
Heteroptera	0	0	8	16	4	8	0	0
Homoptera	32	36	24	28	28	38	4	8
Isoptera	67	106	28	56	4	8	48	76
Hymenoptera	56	86	64	108	92	116	0	0
Pseudoscorpionida	107	200	116	109	16	32	108	116
Psocoptera	3	7	0	0	0	0	0	0
Ricinulei	0	0	0	0	0	0	0	0
Trichoptera	0	0	0	0	0	0	0	0
Thysanura	0	0	0	0	0	0	0	0
Thysanoptera	0	0	0	0	0	0	0	0
Number of groups	15		17		16		15	

Appendix 7: Acute toxicity of benomyl in tropical *Eisenia fetida*

I) Acute toxicity test with tropical *Eisenia fetida* in benomyl-dosed OECD soil.

Treatment	**Mortality (%)**	**Biomass (mg) Day 0**	**Biomass (mg) *Day 14***	**Percent of the initial weight**	**Moisture (%)**	**pH**
Control	0.0	428.6 ± 23.1	323.2 ± 7.9	75.4	36.3	6.2
10 mg/kg	0.0	402.7 ± 40.0	316.2 ± 31.5	78.5	35.0	6.1
31.6 mg/kg	2.5	403.3 ± 27.5	315.1 ± 24.7	78.1	32.5	6.0
100 mg/kg	0.0	398.7 ± 26.7	306.8 ± 19.5	76.9	35.4	6.1
316 mg/kg	22.5	401.2 ± 26.7	n.a.	n.a.	34.7	6.0
1000 mg/kg	95.0	398.2 ± 22.4	n.a.	n.a.	32.5	6.1

II) Acute toxicity test with tropical *Eisenia fetida* in benomyl-dosed TASx soil.

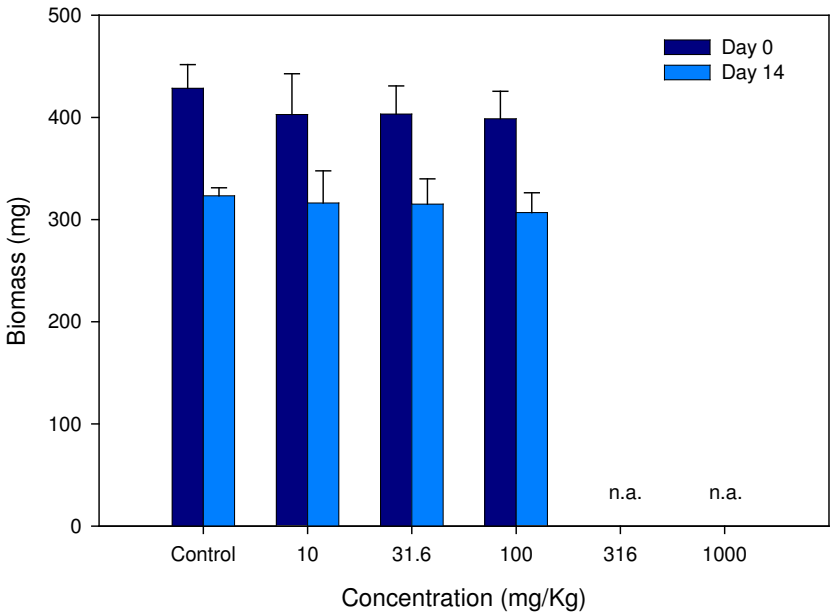
Treatment	**Mortality (%)**	**Biomass (mg) Day 0**	**Biomass (mg) Day 14**	**Percent of the initial weight**	**Moisture (%)**	**pH**
Control	0.0	318.2 ± 12.2	229.5 ± 8.7	72.1	31.8	6.8
10 mg/kg	2.5	295.3 ± 4.3	220.8 ± 5.0	74.8	33.4	6.7
31.6 mg/kg	0.0	294.6 ± 18.3	218.8 ± 10.6	74.3	30.9	6.7
100 mg/kg	0.0	295.2 ± 6.3	225.1 ± 13.4	76.2	27.4	6.8
316 mg/kg	0.0	298.7± 8.9	224.0 ± 16.0	75.0	29.8	6.8
1000 mg/kg	82.5	298.2 ± 8.6	n.a.	n.a.	32.6	6.8

III) Acute toxicity test with tropical *Eisenia fetida* in benomyl-dosed LUFA soil.

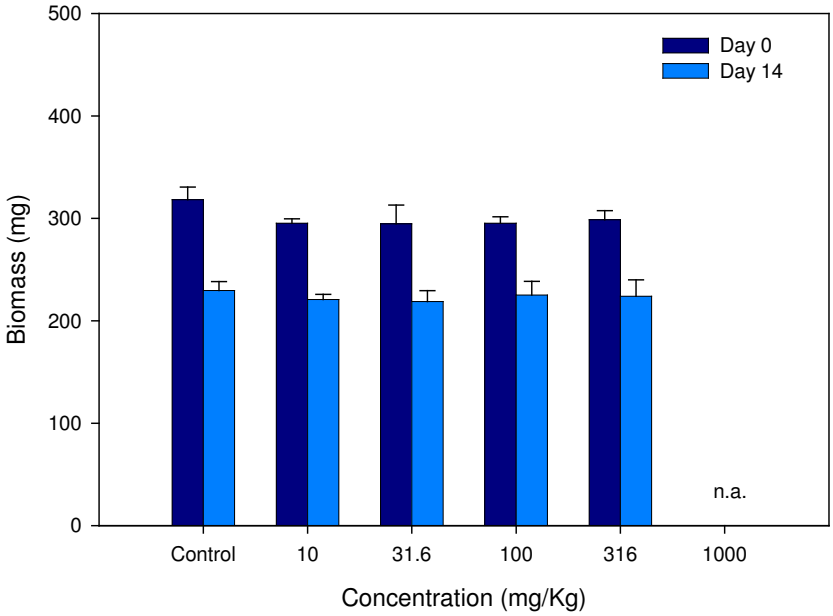
Treatment	**Mortality (%)**	**Biomass (mg) Day 0**	**Biomass (mg) Day 14**	**Percent of the initial weight**	**Moisture (%)**	**pH**
Control	0.0	454.0 ± 35.8	402.2 ± 36.5	88.6	32.2	6.4
3.16 mg/kg	0.0	452.6 ± 31.4	417.9 ± 9.5	92.3	31.9	6.4
10 mg/kg	2.5	454.1 ± 34.6	391.4 ± 32.8	86.2	31.4	6.5
31.6 mg/kg	7.5	451.2 ± 19.1	323.9 ± 23.0	71.8	31.3	6.5
100 mg/kg	75.0	447.7 ± 19.1	n.a.	n.a.	31.9	6.4
316 mg/kg	100.0	442.3 ± 24.4	n.a.	n.a.	31.6	6.5

IV) Acute toxicity test with tropical *Eisenia fetida* in benomyl-dosed TNS soil.

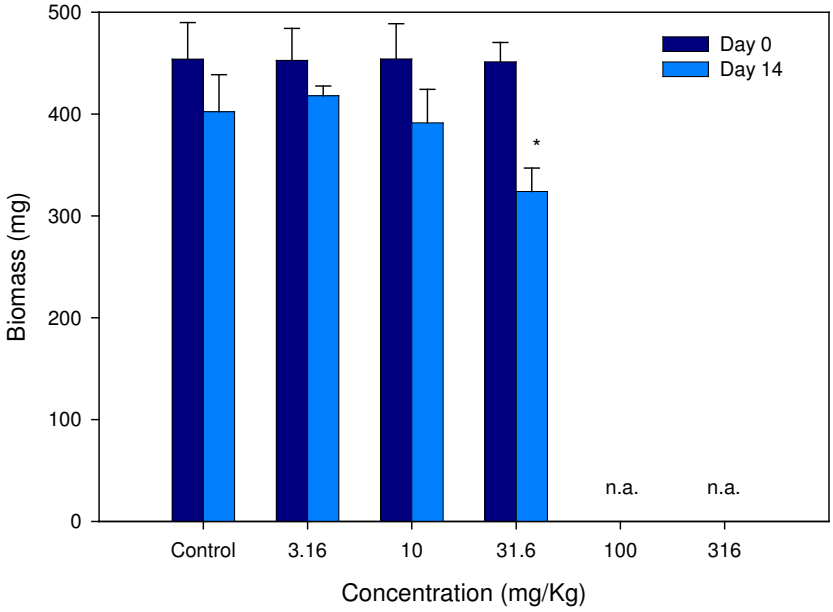
Treatment	**Mortality (%)**	**Biomass (mg) Day 0**	**Biomass (mg) Day 14**	**Percent of the initial weight**	**Moisture (%)**	**pH**
Control	0.0	380.8 ± 26.8	259.4 ± 30.0	68.1	20.9	4.1
3.16 mg/kg	0.0	380.8 ± 31.9	274.2 ± 11.2	72.0	20.8	4.1
10 mg/kg	0.0	378.9 ± 24.9	281.4 ± 24.2	74.3	20.4	4.0
31.6 mg/kg	2.5	381.2 ± 22.7	293.5 ± 10.8	77.0	20.6	4.0
100 mg/kg	92.5	380.3 ± 23.5	n.a.	n.a.	20.7	4.1
316 mg/kg	100.0	379.0 ± 22.7	n.a.	n.a.	20.6	4.1



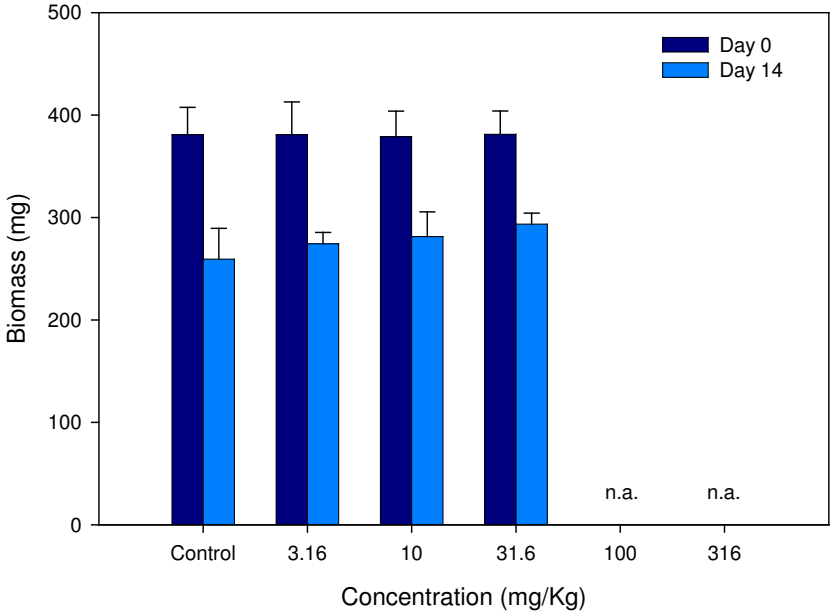
V) Biomass change of tropical *Eisenia fetida* in benomyl-dosed OECD soil (means and standard deviations; treatments do not differ from control).



VI) Biomass change of tropical *Eisenia fetida* in benomyl-dosed TASx soil (means and standard deviations; treatments do not differ from control).



VII) Biomass change of tropical *Eisenia fetida* in benomyl-dosed LUFA soil (means and standard deviations; * statistically different from control at P = 0.01).



VIII) Biomass change of tropical *Eisenia fetida* in benomyl-dosed TNS soil (means and standard deviations; treatments do not differ from control).

Appendix 8: Chronic toxicity of benomyl in tropical *Eisenia fetida*.

I) Chronic toxicity test with tropical *Eisenia fetida* in benomyl-dosed OECD soil.

Treatment	Number of juveniles	Juveniles % of the control	Adult Mortality %	Biomass (mg) Day 0	Biomass (mg) Day 28	Moisture (%)	pH
Control	154.8 ± 14.4	100.0	0.0	377.1 ± 35.6	420.5 ± 75.7	43.0	6.6
0.316 mg/kg	147.0 ± 25.1	95.0	0.0	369.1 ± 39.5	439.7 ± 51.7	41.0	6.7
1 mg/kg	111.3 ± 16.3	71.9	0.0	370.4 ± 38.1	429.8 ± 30.3	40.8	6.6
3.16 mg/kg	103.0 ± 13.6	66.6	0.0	376.9 ± 35.1	411.5 ± 20.0	39.5	6.7
10 mg/kg	95.6 ± 21.5	30.7	0.0	388.3 ± 50.8	463.2 ± 61.8	40.0	6.6
31.6 mg/kg	0.3 ± 0.5	0.2	0.0	379.8 ± 47.8	411.5 ± 49.2	40.4	6.7

II) Chronic toxicity test with tropical *Eisenia fetida* in benomyl-dosed LUFA soil.

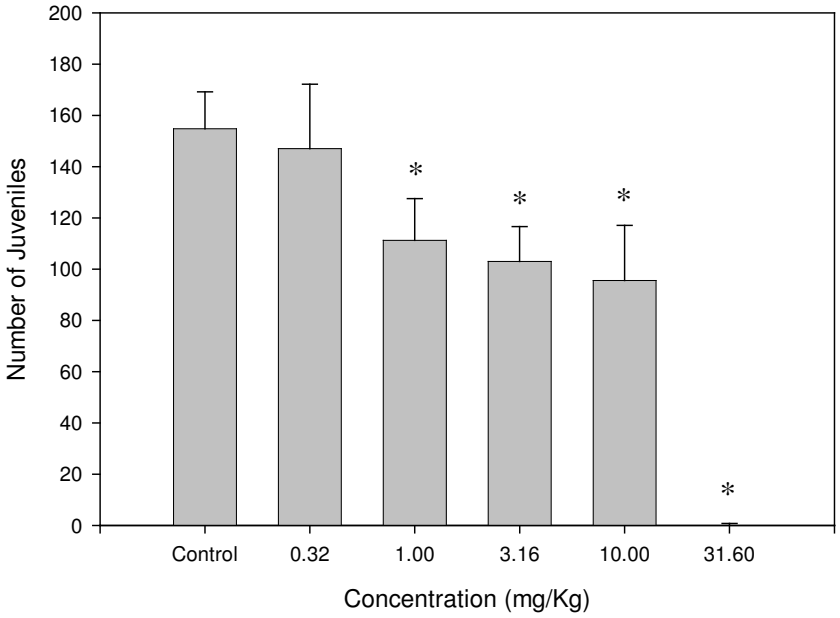
Treatment	Number of juveniles	Juveniles % of the control	Adult Mortality (%)	Biomass (mg) Day 0	Biomass (mg) Day 28	Moisture (%)	pH
Control	199.3 ± 50.6	100.0	0.0	437.9 ± 28.9	627.3 ± 32.4	35.2	6.4
0.1 mg/kg	155.8 ± 44.8	78.2	0.0	443.3 ± 33.8	625.1 ± 64.1	33.3	6.4
0.316 mg/kg	136.0 ± 17.1	68.3	2.5	439.6 ± 23.0	660.3 ± 49.4	31.8	6.3
1 mg/kg	113.0 ± 60.0	56.7	7.5	435.7 ± 35.4	752.7 ± 65.9	37.3	6.3
3.16 mg/kg	121.5 ± 40.6	48.3	7.5	449.3 ± 33.6	722.9 ± 63.6	33.8	6.3
10 mg/kg	56.3 ± 34.6	28.2	0.0	436.8 ± 27.2	628.4 ± 64.1	34.2	6.3

III) Chronic toxicity test with tropical *Eisenia fetida* in benomyl-dosed TASx soil.

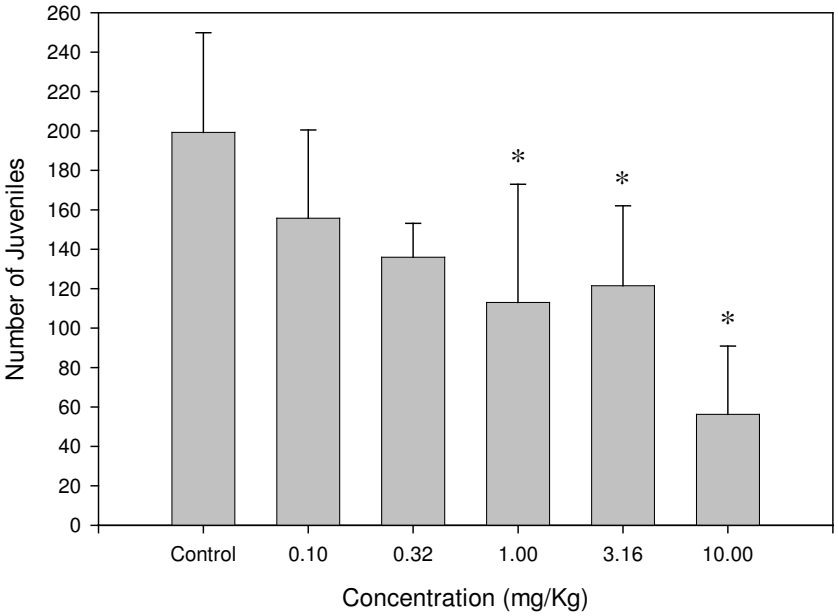
Treatment	Number of juveniles	Juveniles % of the control	Adult Mortality (%)	Biomass (mg) Day 0	Biomass (mg) Day 28	Moisture (%)	pH
Control	128.3 ± 49.0	100.0	0.0	363.7 ± 67.6	332.5 ± 36.8	39.2	6.5
0.316 mg/kg	94.3 ± 11.6	73.5	0.0	353.0 ± 38.8	356.5 ± 45.6	36.9	6.6
1 mg/kg	97.8 ± 23.2	76.2	0.0	359.0 ± 43.5	353.6 ± 24.3	37.9	6.6
3.16 mg/kg	70.5 ± 16.7	55.0	7.5	347.0 ± 44.3	349.5 ± 45.6	38.4	6.6
10 mg/kg	31.8 ± 18.9	24.8	0.0	346.4 ± 32.1	366.0 ± 29.7	39.5	6.5
31.6 mg/kg	8.8 ± 9.6	6.8	2.5	341.4 ± 37.0	321.5 ± 55.2	37.8	6.6
100 mg/kg	3.8 ± 4.8	2.9	7.5	355.4 ± 51.5	301.2 ± 40.2	40.2	6.5

IV) Chronic toxicity test with tropical *Eisenia fetida* in benomyl-dosed TNS soil.

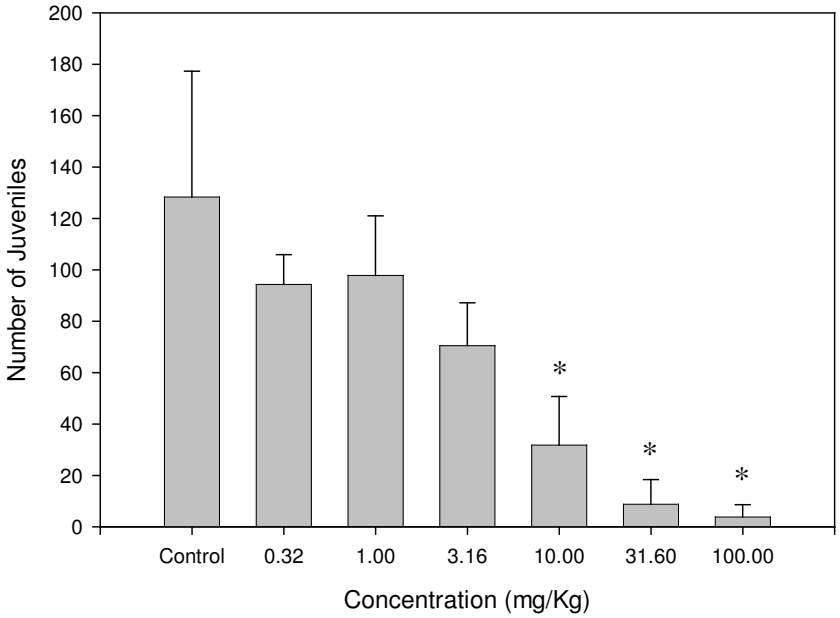
Treatment	Number of juveniles	Juveniles % of the control	Adult Mortality (%)	Biomass (mg) Day 0	Biomass (mg) Day 28	Moisture (%)	pH
Control	18.8 ± 4.1	100.0	0.0	336.0 ± 31.1	323.7 ± 23.6	16.9	4.1
0.316 mg/kg	28.8 ± 6.8	153.3	2.5	342.4 ± 51.5	323.6 ± 26.6	15.9	4.1
1 mg/kg	36.0 ± 9.9	192.0	5.0	334.5 ± 42.5	336.5 ± 20.0	17.3	4.1
3.16 mg/kg	23.3 ± 4.1	124.0	7.5	335.1 ± 30.8	325.3 ± 26.5	16.1	4.0
10 mg/kg	11.0 ± 10.6	58.7	0.0	341.1 ± 50.1	313.7 ± 31.0	13.6	4.2
31.6 mg/kg	0.5 ± 1.0	2.7	7.5	327.4 ± 34.4	281.0 ± 51.1	17.9	4.1



V) Reproduction rate of tropical *Eisenia fetida* in benomyl-dosed OECD soil (number of juveniles per test vessel, means and standard deviations; * statistically different from control at P = 0.01).



VI) Reproduction rate of tropical *Eisenia fetida* in benomyl-dosed LUFA soil (number of juveniles per test vessel, means and standard deviations; * statistically different from control at P = 0.01).



VII) Reproduction rate of tropical *Eisenia fetida* in benomyl-dosed TASx soil (number of juveniles per test vessel, means and standard deviations; * statistically different from control at P = 0.01).

Appendix 9: Avoidance test with tropical *Eisenia fetida* in benomyl-dosed TASx soil.

Treatment	Frequency distribution of earthworms (%)		Net Response (%)	Standard Error	Significance (*t*-test)	Moisture (%)	pH
	Control	Treated					
1 mg/kg	55.00	45.00	10	12.9	n.s.	32.7	6.3
3.16 mg/kg	67.50	32.50	35	17.1	*	32.3	6.5
10 mg/kg	47.50	52.50	-5	17.1	n.s.	32.8	6.3
31.6 mg/kg	57.50	42.50	15	5.0	**	35.4	6.4
100 mg/kg	87.50	12.50	75	5.0	**	33.0	6.4
316 mg/kg	92.50	7.50	85	9.6	**	33.2	6.3
1000 mg/kg	100	0	100	0.0	**	33.3	6.3
Untreated soil						32.4	6.4

* P=0.05 and ** P=0.01

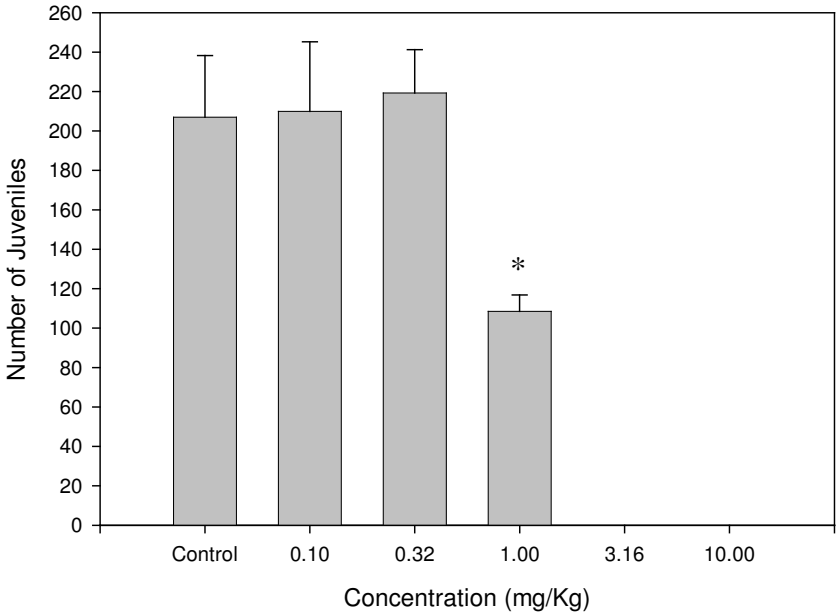
Appendix 10: Acute toxicity test with European *Eisenia fetida* in benomyl-dosed LUFA soil.

Treatment	Mortality (%)	Biomass (mg) Day 0	Biomass (mg) Day 14	Percent of the initial weight	Moisture (%)	pH
Control	0.0	408.6± 21.2	442.1± 18.9	108.2	31.4	5.5
2.0 mg/kg	0.0	401.3 ± 14.1	404.1 ± 4.7	100.7	32.1	5.5
6.3 mg/kg	40.0	402.5 ± 11.7	n.a.	n.a.	32.5	5.5
20 mg/kg	37.5	399.6 ± 22.2	n.a.	n.a.	30.1	5.5
63.2 mg/kg	100.0	396.7 ± 17.3	n.a.	n.a.	32.1	5.5
200 mg/kg	100.0	434.7 ± 33.9	n.a.	n.a.	32.6	5.5

Appendix 11: Chronic toxicity of benomyl in European *Eisenia fetida*.

I) Chronic toxicity test with European *Eisenia fetida* in benomyl-dosed LUFA soil.

Treatment	Number of juveniles	Juveniles % of the control	Adult Mortality (%)	Biomass (mg) Day 0	Biomass (mg) Day 28	Moisture (%)	pH
Control	207.0 ± 31.3	100.0	2.5	448.8 ± 45.7	614.2 ± 51.5	28.6	6.0
0.1 mg/kg	210.0 ± 35.3	101.4	0.0	436.3 ± 38.7	564.9 ± 27.6	29.8	6.0
0.316 mg/kg	219.3 ± 22.0	105.9	0.0	432.8 ± 24.2	599.4 ± 27.3	28.5	6.0
1 mg/kg	108.5 ± 8.3	52.4	0.0	436.3 ± 31.3	533.6 ± 33.0	26.9	5.8
3.16 mg/kg	0.0	0.0	95.0	425.3 ± 43.0	289.5 ± 38.9	31.8	5.9
10 mg/kg	0.0	0.0	97.5	419.7 ± 32.4	337.0 ± 0.0	30.4	6.0



II) Reproduction rate of European *Eisenia fetida* in benomyl-dosed LUFA soil (number of juveniles per test vessel, means and standard deviations; * statistically different from control at P = 0.01).

Appendix 12: Avoidance test with European *Eisenia fetida* in benomyl.

I) Avoidance test with European *Eisenia fetida* in benomyl-dosed OECD soil.

Treatment	**Frequency distribution of earthworms (%)**		**Net Response (%)**	**Standard Error**	**Significance (*t*-test)**	**Moisture (%)**	**pH**
	Control	**Treated**					
1 mg/kg	40.00	60.00	-20	18.3	n.s.	31.3	5.5
3.16 mg/kg	50.00	50.00	0	14.1	n.s.	31.6	5.9
10 mg/kg	57.50	42.50	15	5.0	**	32.3	5.7
31.6 mg/kg	72.50	27.50	45	12.6	**	31.3	5.7
100 mg/kg	100	0	100	0.0	**	30.6	5.7
316 mg/kg	100	0	100	0.0	**	31.2	5.7
1000 mg/kg	100	0	100	0.0	**	32.5	5.8
Untreated soil						31.5	5.7

** P=0.01

II) Avoidance test with European *Eisenia fetida* in benomyl-dosed LUFA soil.

Treatment	Frequency distribution of earthworms (%)		Net Response (%)	Standard Error	Significance (*t*-test)	Moisture (%)	pH
	Control	Treated					
1 mg/kg	70.00	30.00	40	14.1	**	31.1	6.4
3.16 mg/kg	82.50	17.50	65	9.6	**	32.5	-
10 mg/kg	85.00	15.00	70	17.3	**	32.2	6.3
31.6 mg/kg	80.00	20.00	60	8.2	**	32.6	-
100 mg/kg	92.50	7.50	85	9.6	**	32.2	6.4
316 mg/kg	97.50	2.50	95	5.0	**	32.4	6.5
1000 mg/kg	95.00	5.00	90	10.0	**	35.6	6.5
Untreated soil						31.1	6.2

** P=0.01

Appendix 13: Acute toxicity of carbendazim in tropical *Eisenia fetida*.

I) Acute toxicity of with tropical *Eisenia fetida* in carbendazim-dosed OECD soil.

Treatment	Mortality (%)	Biomass (mg) Day 0	Biomass(mg) Day 14	Percent of the initial weight	Moisture (%)	pH
Control	0.0	433.6 ± 16.7	323.5 ± 7.6	74.6	36.3	6.2
10 mg/kg	0.0	423.3 ± 28.1	321.6 ± 10.3	76.0	35.4	6.2
31.6 mg/kg	0.0	407.9 ± 17.7	322.1 ± 20.4	79.0	33.1	6.2
100 mg/kg	0.0	433.5 ± 29.4	309.3 ± 13.5	71.4	33.3	6.2
316 mg/kg	20.0	423.6 ± 27.6	n.a.	n.a.	35.0	6.2
1000 mg/kg	22.5	427.9 ± 43.2	n.a.	n.a.	32.9	6.1

II) Acute toxicity test with tropical *Eisenia fetida* in carbendazim-dosed TASx soil.

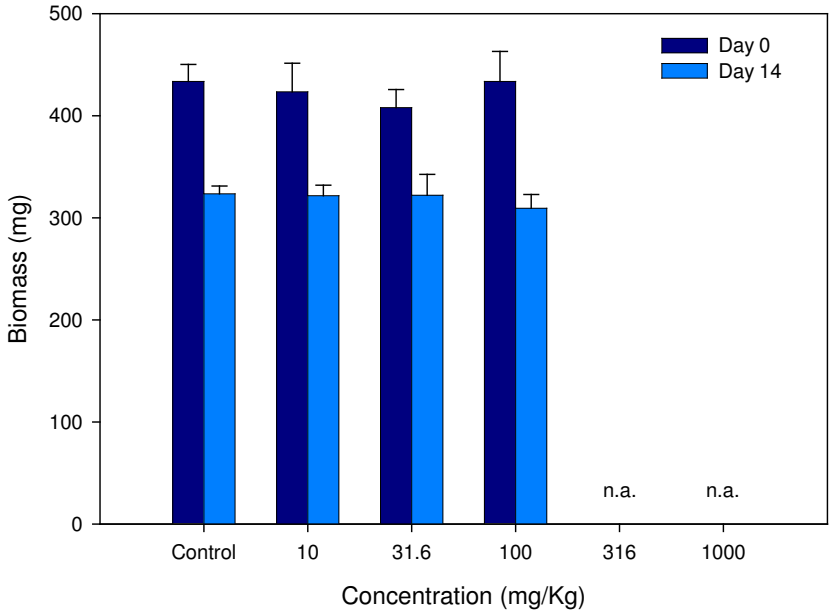
Treatment	Mortality (%)	Biomass (mg) Day 0	Biomass (mg) Day 14	Percent of the initial weight	Moisture (%)	pH
Control	0.0	307.3 ± 13.2	215.2 ± 12.3	70.0	32.9	6.5
10 mg/kg	0.0	316.3 ± 21.9	246.0 ± 10.3	77.8	32.1	6.7
31.6 mg/kg	0.0	297.1± 12.5	228.3 ± 4.9	76.8	29.3	6.7
100 mg/kg	2.5	291.2 ± 3.7	218.2 ± 12.5	74.9	32.3	6.7
316 mg/kg	7.5	293.6 ± 9.1	192.9 ± 10.7	65.7	31.9	6.7
1000 mg/kg	25.0	294.0 ± 8.3	n.a.	n.a.	33.4	6.7

III) Acute toxicity test with tropical *Eisenia fetida* in carbendazim-dosed LUFA soil.

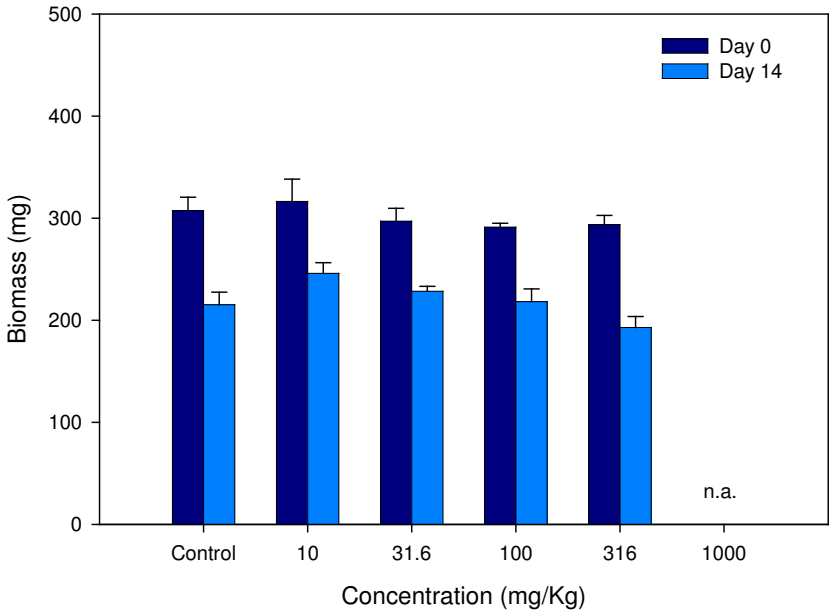
Treatment	Mortality (%)	Biomass (mg) Day 0	Biomass (mg) Day 14	Percent of the initial weight	Moisture (%)	pH
Control	0.0	319.6 ± 20.1	342.2 ± 28.1	107.1	32.3	6.2
10 mg/kg	2.5	312.3 ± 11.0	248.7 ± 14.3	79.6	32.1	6.3
31.6 mg/kg	5.0	315.3 ± 10.7	182.6 ± 17.8	57.9	32.1	6.2
100 mg/kg	15.0	317.0 ± 18.2	183.3 ± 14.9	57.8	33.1	6.2
316 mg/kg	22.5	315.3 ± 15.9	n.a.	n.a.	36.3	6.3
1000 mg/kg	17.5	315.1 ± 11.9	n.a.	n.a.	33.3	6.2

IV) Acute toxicity test with tropical *Eisenia fetida* in carbendazim-dosed TNS soil.

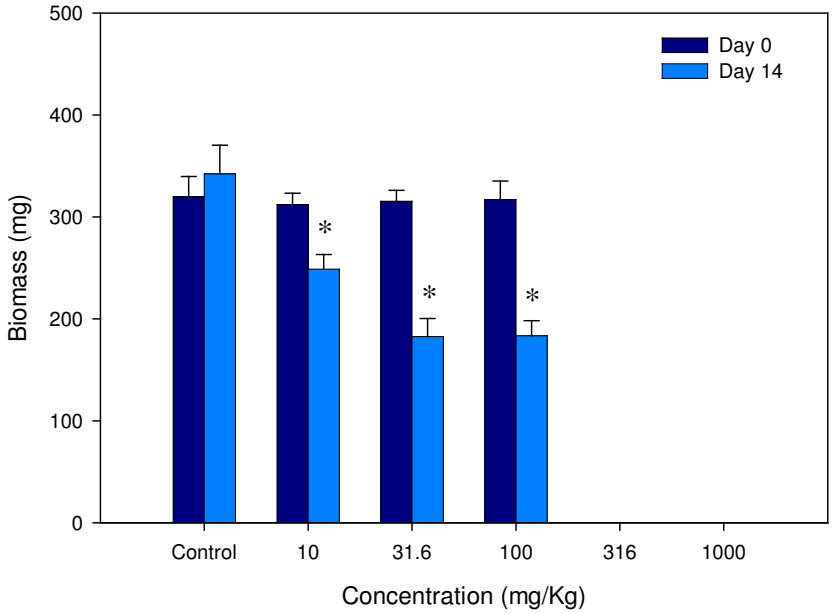
Treatment	Mortality (%)	Biomass (mg) Day 0	Biomass (mg) Day 14	Percent of the initial weight	Moisture (%)	pH
Control	0.0	301.2 ± 5.0	214.7 ± 13.6	71.3	18.5	3.9
1 mg/kg	0.0	298.8 ± 22.1	232.2 ± 21.2	77.7	18.7	3.9
3.16 mg/kg	5.0	279.1± 5.9	202.5 ± 6.6	72.5	20.0	3.9
10 mg/kg	2.5	292.2 ± 8.9	233.7 ± 3.6	80.0	18.1	4.0
31.6 mg/kg	0.0	291.8 ± 3.4	218.5 ± 17.3	74.9	19.4	3.9
100 mg/kg	95.0	295.2 ± 18.6	n.a.	n.a.	17.8	3.9



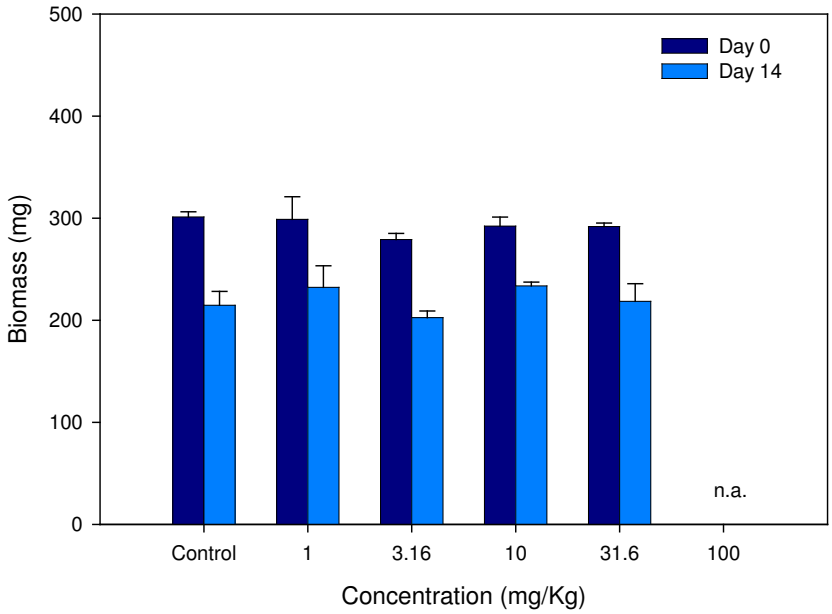
V) Biomass change in the acute toxicity test with tropical *Eisenia fetida* in carbendazim dosed OECD soil (means and standard deviations; treatments do not differ from control).



VI) Biomass change in the acute toxicity test with tropical *Eisenia fetida* in carbendazim-dosed TASx soil (means and standard deviations; treatments do not differ from control).



VII) Biomass change in the acute toxicity test with tropical *Eisenia fetida* in carbendazim-dosed LUFA soil (means and standard deviations; * statistically different from control at P = 0.01).



VIII) Biomass change in the acute toxicity test with tropical *Eisenia fetida* in carbendazim-dosed TNS soil (means and standard deviations; treatments do not differ from control).

Appendix 14: Chronic toxicity of carbendazim in tropical *Eisenia fetida*.

I) Chronic toxicity test with tropical *Eisenia fetida* in carbendazim-dosed OECD soil.

Treatment	**Number of juveniles**	**Juveniles % of the control**	**Adult Mortality (%)**	**Biomass (mg) Day 0**	**Biomass (mg) Day 28**	**Moisture (%)**	**pH**
Control	100.25 ± 9.9	100.0	5.0	409.5 ± 54.0	450.4 ± 53.3	27.8	6.2
0.316 mg/kg	95.50 ± 11.6	95.3	0.0	398.0 ± 33.5	441.5 ± 34.3	30.8	6.2
1 mg/kg	85.00 ± 13.8	84.8	0.0	405.1 ± 25.0	467.9 ± 35.5	34.4	6.2
3.16 mg/kg	79.25 ± 4.8	79.1	2.5	395.2 ± 31.7	424.8 ± 45.2	30.9	6.3
10 mg/kg	74.43 ± 20.2	51.4	0.0	401.1 ± 26.2	451.5 ± 37.1	30.2	6.2
31.6 mg/kg	2.00 ± 2.4	2.0	2.5	404.2 ± 39.3	386.6 ± 30.1	29.2	6.2

II) Chronic toxicity test with tropical *Eisenia fetida* in carbendazim-dosed TASx soil.

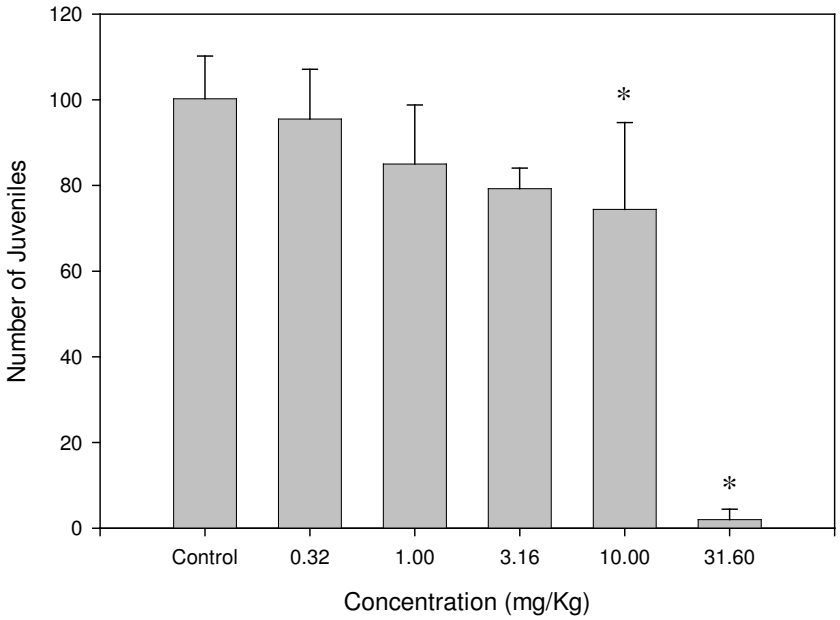
Treatment	Number of juveniles	Juveniles % of the control	Adult Mortality (%)	Biomass (mg) Day 0	Biomass (mg) Day 28	Moisture (%)	pH
Control	72.8 ± 20.9	100.0	0.0	371.5 ± 22.0	336.5 ± 28.4	35.4	6.3
0.316 mg/kg	92.5 ± 27.5	127.1	2.5	388.2 ± 32.4	343.8 ± 12.2	34.9	6.4
1 mg/kg	65.8 ± 13.8	90.4	2.5	377.7 ± 26.3	330.7 ± 16.1	36.7	6.4
3.16 mg/kg	57.3 ± 19.5	78.7	0.0	383.1 ± 29.7	347.4 ± 20.8	37.6	6.4
10 mg/kg	11.0 ± 8.0	15.1	2.5	367.9 ± 21.8	353.2 ± 11.1	36.7	6.5
31.6 mg/kg	12.3 ± 8.2	16.8	5.0	379.0 ± 25.9	351.8 ± 21.8	36.1	6.6
100 mg/kg	3.0 ± 2.6	4.1	20.0	363.1 ±24.8	341.1 ± 34.7	36.5	6.5

III) Chronic toxicity test with tropical *Eisenia fetida* in carbendazim-dosed LUFA soil.

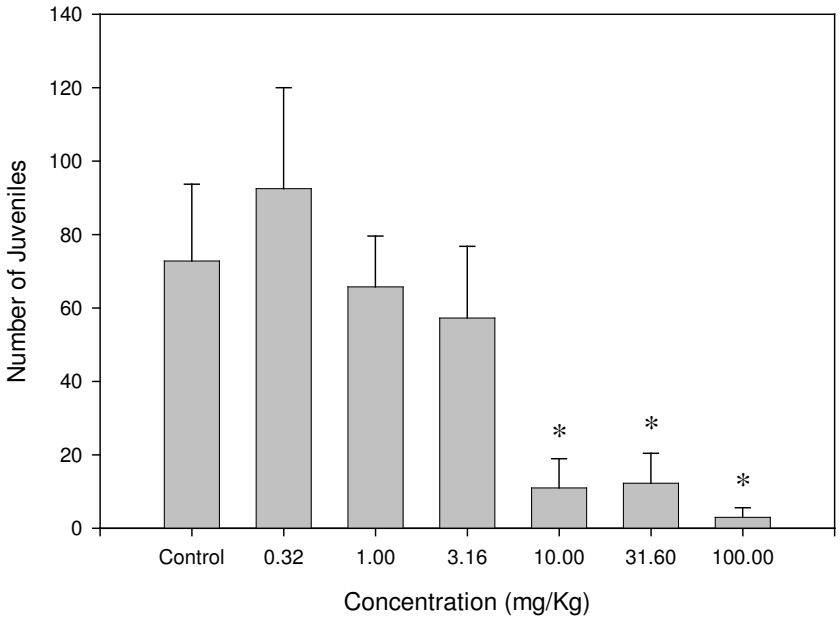
Treatment	Number of juveniles	Juveniles % of the control	Adult Mortality (%)	Biomass (mg) Day 0	Biomass (mg) Day 28	Moisture (%)	pH
Control	139.5 ± 19.1	100.0	0.0	447.2 ± 22.3	463.0 ±18.7	30.5	6.2
0.1 mg/kg	145.3 ± 29.2	104.1	0.0	448.4 ± 24.1	447.7 ± 38.3	31.1	6.2
0.316 mg/kg	121.8 ± 24.6	87.3	0.0	442.1 ± 36.0	482.3 ± 45.6	30.5	6.2
1 mg/kg	143.3 ± 45.7	102.7	0.0	432.0 ± 29.8	463.2 ± 21.3	30.2	6.2
3.16 mg/kg	129.4 ± 35.6	87.3	0.0	448.7 ± 39.6	500.9 ± 47.2	33.3	6.2
10 mg/kg	64.8 ± 21.8	46.4	7.5	444.5 ± 18.9	431.1 ± 43.2	31.7	6.2

IV) Chronic toxicity test with tropical *Eisenia fetida* in carbendazim-dosed TNS soil .

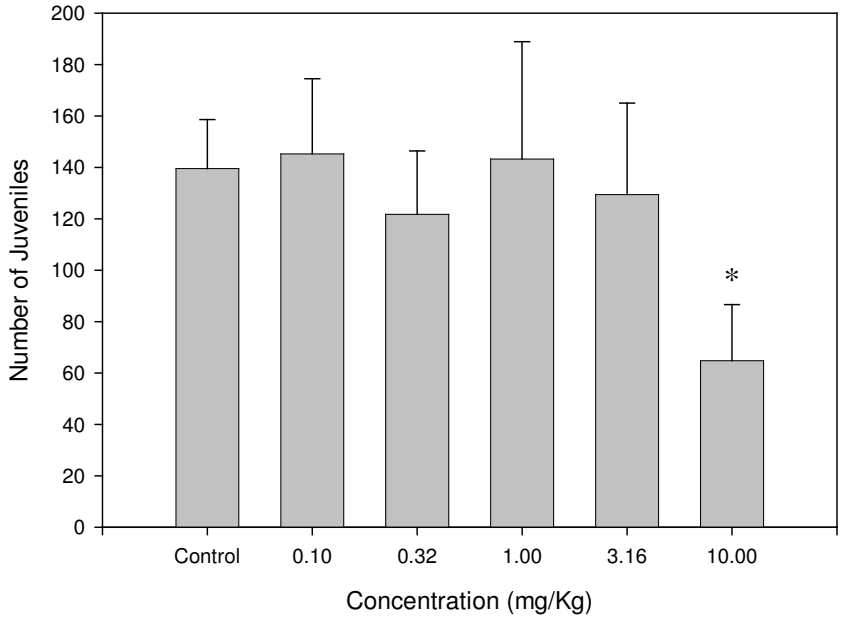
Treatment	Number of juveniles	Juveniles % of the control	Adult Mortality (%)	Biomass (mg) Day 0	Biomass (mg) Day 28	Moisture (%)	pH
Control	7.3 ± 5.3	100.0	2.5	320.0 ± 20.1	291.4 ± 10.8	18.9	3.91
0.316 mg/kg	12.0 ± 10.8	165.5	12.5	319.0 ± 17.5	317.3 ± 47.9	17.8	3.89
1 mg/kg	15.3 ± 6.8	210.3	0.0	319.2 ± 15.4	278.2 ± 28.7	16.2	3.83
3.16 mg/kg	4.3 ± 4.0	58.6	5.0	318.4 ± 14.1	265.1 ± 30.1	16.4	3.86
10 mg/kg	1.3 ± 1.9	17.2	2.5	318.9 ± 13.7	289.5 ± 42.1	16.3	4.11
31.6 mg/kg	0.0 ± 0.0	0.0	25.0	320.4 ± 14.3	189.6 ± 9.8	16.8	3.88



V) Reproduction rate of tropical *Eisenia fetida* in carbendazim-dosed OECD soil (number of juveniles per test vessel, means and standard deviations; * statistically different from control at P = 0.01).



VI) Reproduction rate of tropical *Eisenia fetida* in carbendazim-dosed TASx soil (number of juveniles per test vessel, means and standard deviations; * statistically different from control at P = 0.01).



VII) Reproduction rate of tropical *Eisenia fetida* in carbendazim-dosed LUFA soil (number of juveniles per test vessel, means and standard deviations; * statistically different from control at P = 0.01).

Appendix 15: Avoidance test with tropical *Eisenia fetida* in carbendazim-dosed TASx soil.

Treatment	**Frequency distribution of earthworms (%)**		**Net Response (%)**	**Standard Error**	**Significance (*t*-test)**	**Moisture (%)**	**pH**
	Control	**Treated**					
1 mg/kg	65.00	35.00	30	17.3	*	31.0	6.3
3.16 mg/kg	60.00	40.00	20	14.1	*	31.1	6.3
10 mg/kg	72.50	27.50	45	20.6	*	33.4	6.3
31.6 mg/kg	70.00	30.00	40	14.1	**	31.8	6.4
100 mg/kg	85.00	15.00	70	12.9	**	31.7	6.3
316 mg/kg	82.50	17.50	65	9.6	**	32.6	6.3
1000 mg/kg	97.50	2.50	95	5.0	**	33.3	6.4
Untreated soil						30.7	6.2

* P=0.05 and ** P=0.01

Appendix 16: Acute toxicity of carbendazim in European *Eisenia fetida*.

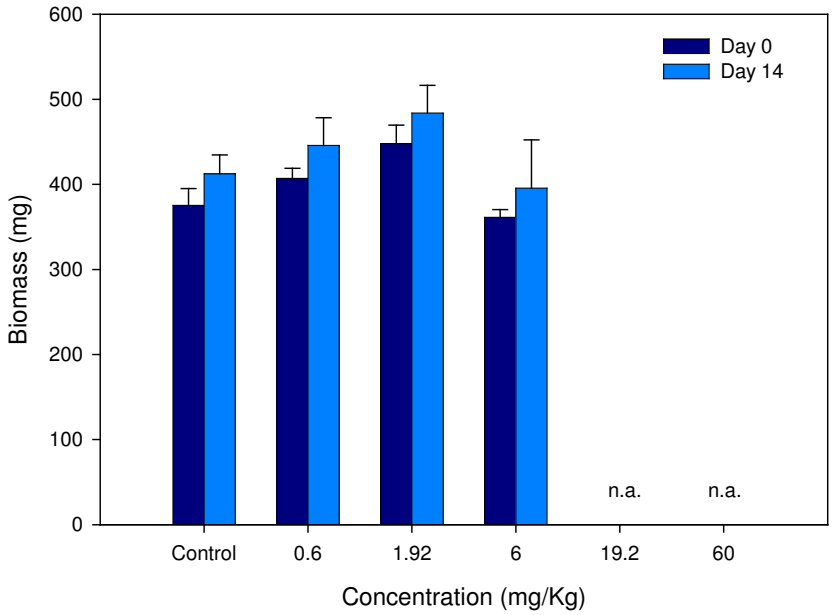
I) Acute toxicity test with European *Eisenia fetida* in carbendazim-dosed OECD soil.*

Treatment	Mortality (%)	Biomass (mg) Day 0	Biomass (mg) Day 21	Percent of the initial weight	Moisture (%)	pH
Control	0.0	375.3 ± 19.7	412.3 ± 22.4	109.9	35.0	6.5
0.6 mg/kg	0.0	407.0 ± 11.9	445.8 ± 32.6	109.5	35.0	6.5
1.92 mg/kg	0.0	447.8± 21.9	483.8 ± 32.6	108.0	35.0	6.5
6.0 mg/kg	57.5	361.3 ± 9.1	393.5 ± 56.9	108.9	35.0	6.5
19.2 mg/kg	97.5	391.5 ± 35.3	n.a.	n.a.	35.0	6.5
60 mg/kg	100.0	396.8 ± 44.8	n.a.	n.a.	35.0	6.5

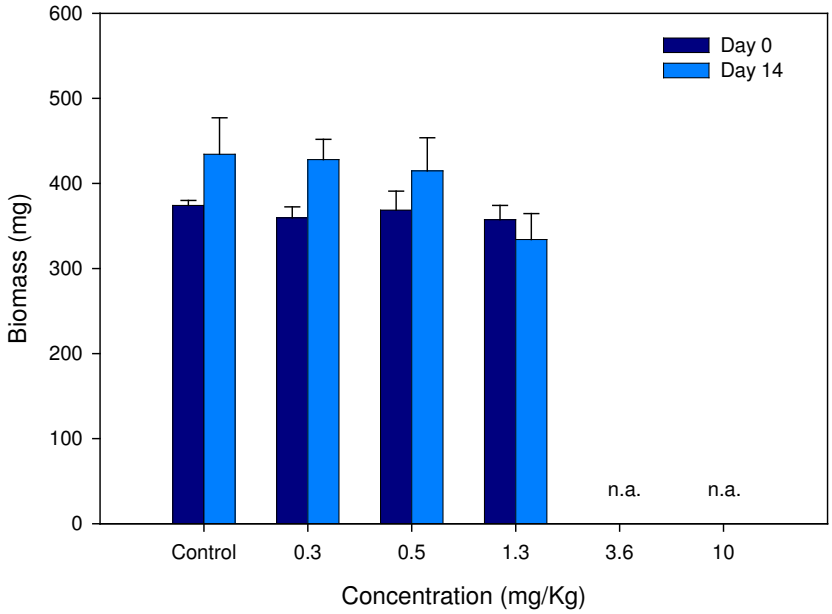
* Van Gestel et al. 1992

II) Acute toxicity test with European *Eisenia fetida* in carbendazim-dosed LUFA soil.

Treatment	Mortality (%)	Biomass (mg) Day 0	Biomass (mg) Day 14	Percent of the initial weight	Moisture (%)	pH
Control	0.0	374.2 ± 5.8	434.4 ± 42.7	116.1	32.1	5.3
0.2 mg/kg	0.0	359.6 ± 12.8	428.0 ± 23.8	119.0	33.6	5.3
0.5 mg/kg	0.0	368.7± 22.2	414.7 ± 39.0	112.5	32.9	5.3
1.3 mg/kg	2.5	357.3 ± 16.9	334.1 ± 30.4	93.5	33.2	5.4
3.6 mg/kg	35.0	369.3 ± 24.5	n.a.	n.a.	32.7	5.3
10 mg/kg	100.0	367.7 ± 7.7	n.a.	n.a.	35.5	5.3



III) Biomass change in the acute toxicity test with European *Eisenia fetida* in carbendazim-dosed OECD soil (means and standard deviations; biomass loss not observed). (Data from Van Gestel et al. 1992).



IV) Biomass change in the acute toxicity test with European *Eisenia fetida* in carbendazim-dosed LUFA soil (means and standard deviations; biomass loss observed only at the concentration of 1.3 mg a.i./kg).

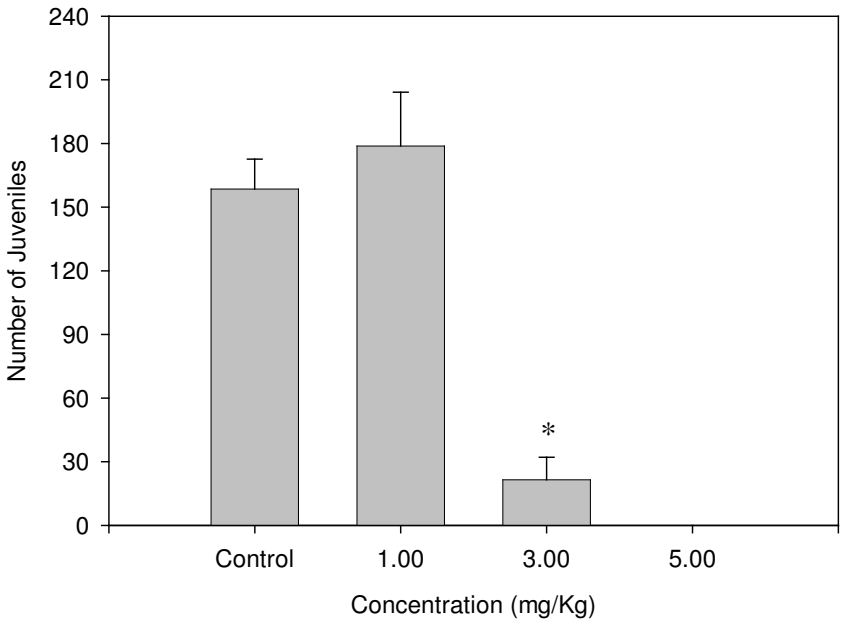
Appendix 17: Chronic toxicity test with European *Eisenia fetida*

I) Chronic toxicity test with European *Eisenia fetida* in carbendazim-dosed OECD soil.

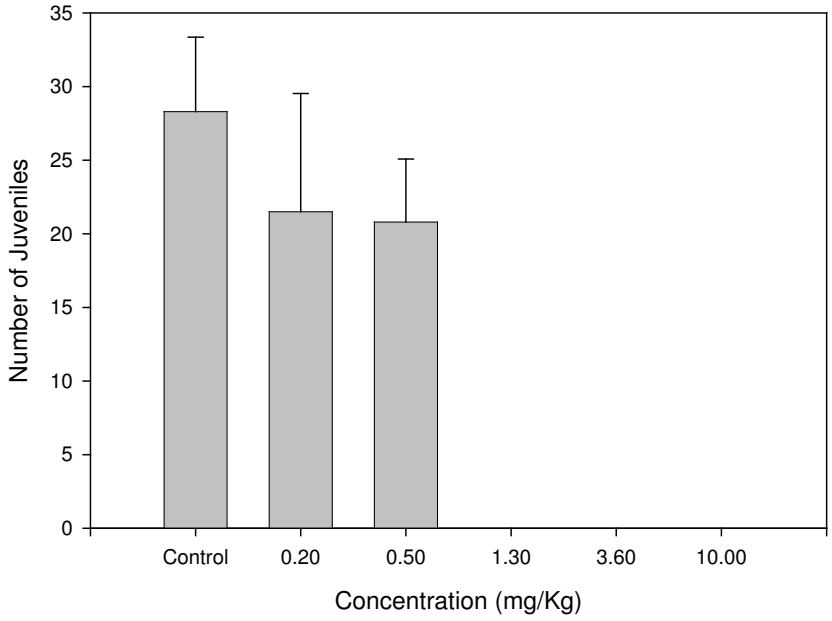
Treatment	Number of juveniles	Juveniles % of the control	Adult Mortality (%)	Biomass (mg) Day 0	Biomass (mg) Day 28	Moisture (%)	pH
Control	158.5 ± 14.2	100.0	0.0	382.3 ± 15.4	559.9 ± 18.3	41.7	6.3
1.0 mg/kg	178.8 ± 25.3	112.8	0.0	386.9 ± 13.9	533.4 ± 36.9	39.3	6.4
3.0 mg/kg	21.5 ± 10.6	13.6	0.0	374.3 ± 26.9	448.8 ± 27.7	39.1	6.3
5.0 mg/kg	0.0 ± 0.0	0.0	2.5	371.8 ± 26.5	365.4 ± 20.0	37.6	6.4

II) Chronic toxicity test with European *Eisenia fetida* in carbendazim-dosed LUFA soil.

Treatment	Number of juveniles	Juveniles % of the control	Adult Mortality (%)	Biomass (mg) Day 0	Biomass (mg) Day 28	Moisture (%)	pH
Control	28.3 ± 5.1	100.0	0.0	374.2 ± 5.8	408.2 ± 18.5	32.1	5.3
0.2 mg/kg	21.5 ± 8.0	76.1	2.5	359.6 ± 12.8	350.1 ± 27.6	33.6	5.3
0.5 mg/kg	20.8 ± 4.3	73.5	2.5	368.7 ± 22.2	365.1 ± 16.0	32.9	5.3
1..3 mg/kg	0.0 ± 0.0	0.0	2.5	357.3 ± 16.9	300.2 ± 15.7	33.2	5.4
3.6 mg/kg	0.0 ± 0.0	0.0	50	368.3 ± 24.5	98.7 ± 14.7	32.7	5.3
10 mg/kg	0.0 ± 0.0	0.0	100	367.7 ± 7.7	0.0 ± 0.0	33.5	5.3



III) Reproduction rate of European *Eisenia fetida* in carbendazim-dosed OECD soil (number of juveniles per test vessel, means and standard deviations; * statistically different from control at P = 0.01).



IV) Reproduction rate of European *Eisenia fetida* carbendazim-dosed LUFA soil (number of juveniles per test vessel, means and standard deviations).

Appendix 18: Avoidance test with European *Eisenia fetida* in carbendazim.

I) Avoidance test with European *Eisenia fetida* in carbendazim-dosed OECD soil.

Treatment	Frequency distribution of earthworms (%)		Net Response (%)	Standard Error	Significance (*t*-test)	Moisture (%)	pH
	Control	Treated					
1 mg/kg	30.00	70.00	-40	20.0	*	30.8	5.8
3.16 mg/kg	42.50	57.50	-15	26.3	n.s.	31.4	5.7
10 mg/kg	62.50	37.50	25	12.6	*	30.6	5.8
31.6 mg/kg	55.00	45.00	10	23.8	n.s.	30.2	5.5
100 mg/kg	72.50	27.50	45	17.1	*	30.3	5.5
316 mg/kg	87.50	12.50	75	12.6	**	29.9	6.1
1000 mg/kg	82.50	17.50	65	22.2	**	31.1	5.8
Untreated soil						30.6	5.4

n.s. (not significant); * (P = 0.05) and ** (P = 0.01)

II) Avoidance test with European *Eisenia fetida* in carbendazim-dosed LUFA soil.

Treatment	Frequency distribution of earthworms (%)		Net Response (%)	Standard Error	Significance (*t*-test)	Moisture (%)	pH
	Control	Treated					
1 mg/kg	72.50	27.50	45	17.1	*	30.8	6.3
3.16 mg/kg	87.50	12.50	75	12.6	**	31.2	6.4
10 mg/kg	72.50	27.50	45	5.0	**	30.2	6.3
31.6 mg/kg	70.00	30.00	40	20.0	*	30.6	6.3
100 mg/kg	72.50	27.50	45	18.4	*	31.8	6.3
316 mg/kg	80.00	20.00	60	8.2	**	31.0	6.4
1000 mg/kg	87.50	12.50	75	12.6	**	32.1	6.3
Untreated soil						30.5	6.4

* P=0.05 and ** P=0.01

Appendix 19: Acute toxicity of lambda-cyhalothrin in tropical *Eisenia fetida*.

I) Acute toxicity test with tropical *Eisenia fetida* in lambda-cyhalothrin-dosed OECD soil.

Treatment	Mortality (%)	Biomass (mg) Day 0	Biomass(mg) Day 14	Percent of the initial weight	Moisture (%)	pH
Control	7.5	288.4 ± 27.0	227.3 ± 37.0	78.8	39.5	6.2
1 mg/kg	0.0	291.7 ± 26.4	248.7 ± 15.1	85.3	43.7	6.2
3.16 mg/kg	0.0	287.6 ± 30.8	254.2 ± 26.7	88.4	41.4	6.2
10 mg/kg	0.0	287.1 ± 31.3	233.8 ± 28.8	81.4	39.7	6.2
31.6 mg/kg	2.5	281.8 ± 22.9	181.0 ± 36.6	64.3	42.3	6.2
100 mg/kg	20.0	282.3 ± 24.6	n.a.	n.a.	42.1	6.1

II) Acute toxicity test with tropical *Eisenia fetida* in lambda-cyhalothrin-dosed TASx soil.

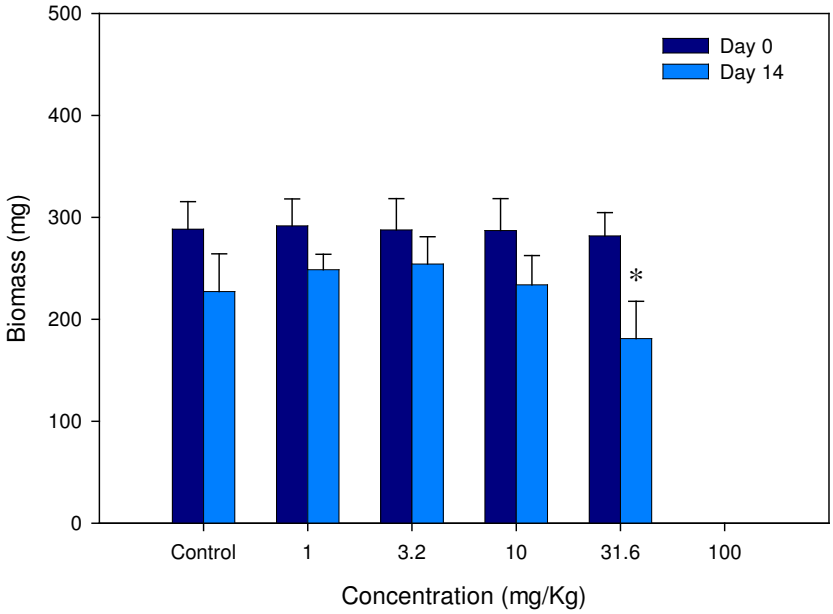
Treatment	Mortality (%)	Biomass (mg) Day 0	Biomass (mg) Day 14	Percent of the initial weight	Moisture (%)	pH
Control	5.0	449.5 ± 11.0	298.0 ± 20.9	66.3	39.8	6.7
1 mg/kg	5.0	448.7± 41.0	276.0 ± 42.7	61.5	35.1	6.8
3.16 mg/kg	5.0	451.4 ± 14.2	261.0 ± 10.9	57.8	37.7	6.6
10 mg/kg	7.5	438.7 ± 37.7	230.7 ± 26.9	52.6	35.2	6.7
31.6 mg/kg	75.0	450.7 ± 16.1	n.a.	n.a.	36.8	6.7
100 mg/kg	100.0	447.5 ± 26.3	n.a.	n.a.	36.9	6.8

III) Acute toxicity test with tropical *Eisenia fetida* in lambda-cyhalothrin-dosed LUFA soil.

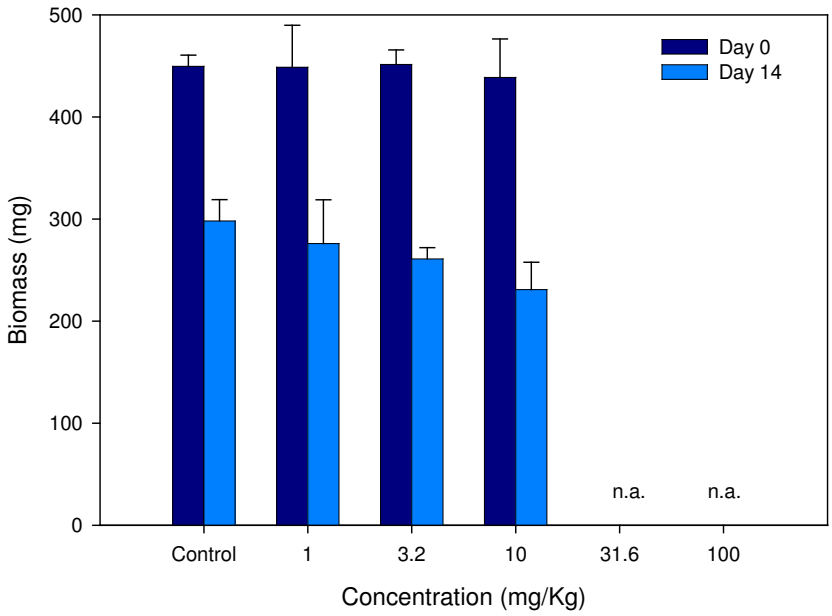
Treatment	Mortality (%)	Biomass (mg) Day 0	Biomass (mg) Day 14	Percent of the initial weight	Moisture (%)	pH
Control	0.0	354.7 ± 16.2	361.3 ± 24.5	101.9	28.6	6.3
3.16 mg/kg	2.5	333.2 ± 7.8	366.0 ± 14.5	109.9	31.9	6.3
10 mg/kg	5.0	351.1 ± 28.9	322.0 ± 7.8	91.7	29.1	6.3
31.6 mg/kg	5.0	330.4 ± 17.8	262.3 ± 31.1	79.4	33.3	6.3
100 mg/kg	75.0	351.2 ± 12.2	n.a.	n.a.	31.6	6.3
316 mg/kg	100.0	327.7 ± 6.2	n.a.	n.a.	30.9	6.4

IV) Acute toxicity test with tropical *Eisenia fetida* in lambda-cyhalothrin-dosed TNS soil.

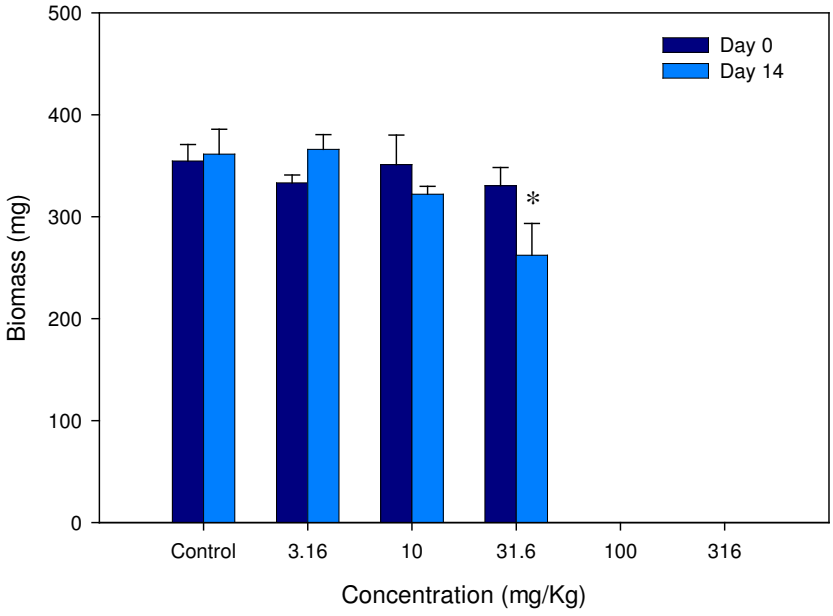
Treatment	Mortality (%)	Biomass (mg) Day 0	Biomass (mg) Day 14	Percent of the initial weight	Moisture (%)	pH
Control	0.0	295.0 ± 18.5	211.0 ± 16.3	71.5	18.6	3.8
6.25 mg/kg	2.5	323.6 ± 10.1	224.4 ± 19.1	69.4	18.5	3.8
12.5 mg/kg	2.5	307.9 ± 13.5	203.8 ± 20.3	66.2	18.6	3.8
25 mg/kg	0.0	313.5 ± 9.8	207.2 ± 20.7	66.1	18.4	3.8
50 mg/kg	12.5	304.1 ± 16.9	177.6 ± 10.1	58.4	18.4	3.8
100 mg/kg	100.0	306.9 ± 12.3	n.a.	n.a.	17.7	3.8



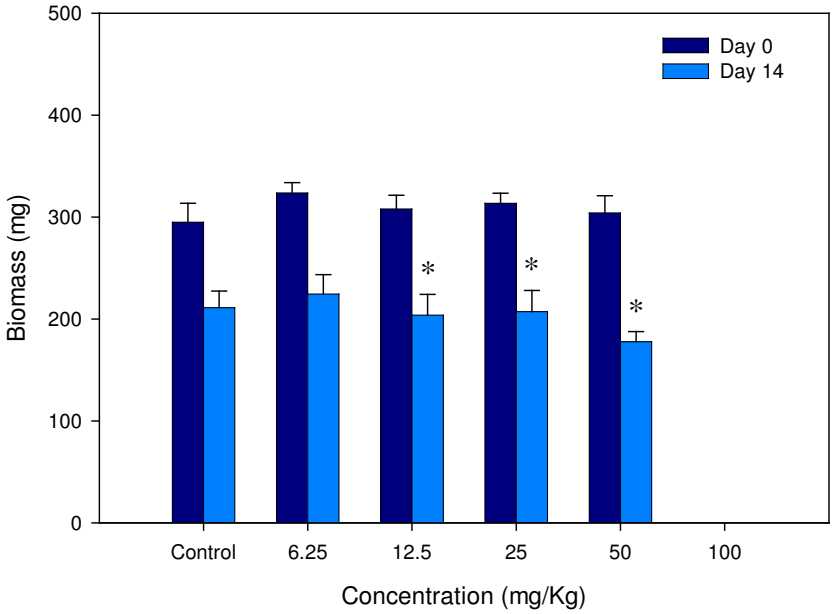
V) Biomass change in the acute toxicity test with tropical *Eisenia fetida* in lambda-cyhalothrin dosed OECD soil (means and standard deviations;
* statistically different from control at P = 0.01).



VI) Biomass change for the acute toxicity test with tropical *Eisenia fetida* in lambda-cyhalothrin-dosed TASx soil (means and standard deviations; treatments do not differ from control).



VII) Biomass change in acute toxicity test with tropical *Eisenia fetida* in lambda-cyhalothrin-dosed LUFA soil (means and standard deviations;
* statistically different from control at P = 0.01).



VIII) Biomass change for the acute toxicity test with tropical *Eisenia fetida* in lambda-cyhalothrin-dosed TNS soil (means and standard deviations; * statistically different from control at P = 0.01).

Appendix 20: Chronic toxicity of lambda-cyhalothrin in tropical *Eisenia fetida*.

I) Chronic toxicity test with tropical *Eisenia fetida* in lambda-cyhalothrin-dosed OECD soil.

Treatment	**Number of juveniles**	**Juveniles % of the control**	**Adult Mortality (%)**	**Biomass (mg) Day 0**	**Biomass (mg) Day 28**	**Moisture (%)**	**pH**
Control	56.8 ±5.9	100.0	2.5	386.9 ± 34.2	440.5 ± 38.4	30.5	6.2
3.16 mg/kg	57.3± 15.1	100.9	2.5	380.4 ± 18.3	449.2 ± 26.0	36.4	6.3
10 mg/kg	54.8 ± 19.6	96.5	2.5	381.9 ± 17.3	453.1 ± 29.9	32.3	6.3
31.6 mg/kg	48.3 ± 16.3	85.0	0.0	381.1 ± 23.1	445.5 ± 9.0	26.3	6.3
100 mg/kg	11.3 ± 4.6	19.8	0.0	371.9 ± 26.3	348.1 ± 31.1	32.1	6.3
316 mg/kg	0.3 ± 0.5	0.4	80.0	387.6 ± 34.7	280.6 ± 36.6	32.4	6.3

II) Chronic toxicity test with tropical *Eisenia fetida* in lambda-cyhalothrin-dosed TASx soil.

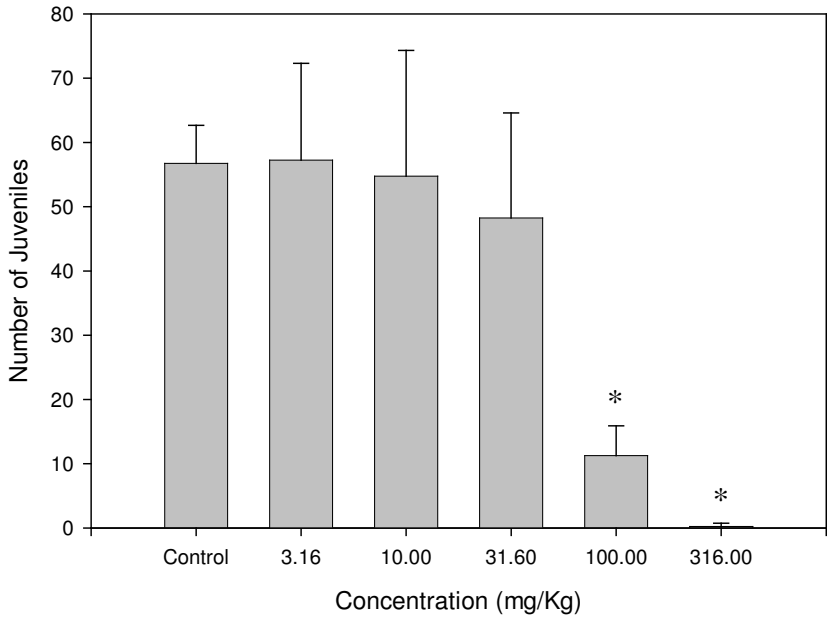
Treatment	Number of juveniles	Juveniles % of the control	Adult Mortality (%)	Biomass (mg) Day 0	Biomass (mg) Day 28	Moisture (%)	pH
Control	46.8 ± 17.7	100.0	0.0	309.0 ± 11.5	357.7 ± 16.3	39.7	6.4
3.12 mg/kg	31.3 ± 9.5	66.8	0.0	315.1 ± 20.5	384.8 ± 41.0	36.7	6.3
6.25 mg/kg	27.5 ± 8.7	58.8	0.0	314.5 ± 12.3	371.4 ± 8.4	36.0	6.3
12.5 mg/kg	20.3 ± 14.3	43.3	0.0	313.3 ± 9.2	334.7 ± 21.0	35.4	6.5
25 mg/kg	2.5 ± 1.9	5.3	0.0	313.4 ± 12.9	327.6 ± 10.4	39.0	6.4
50 mg/kg	2.8 ± 2.2	5.9	10.0	310.8 ± 8.7	366.4 ± 28.0	37.0	6.5

III) Chronic toxicity test with tropical *Eisenia fetida* in lambda-cyhalothrin-dosed LUFA soil.

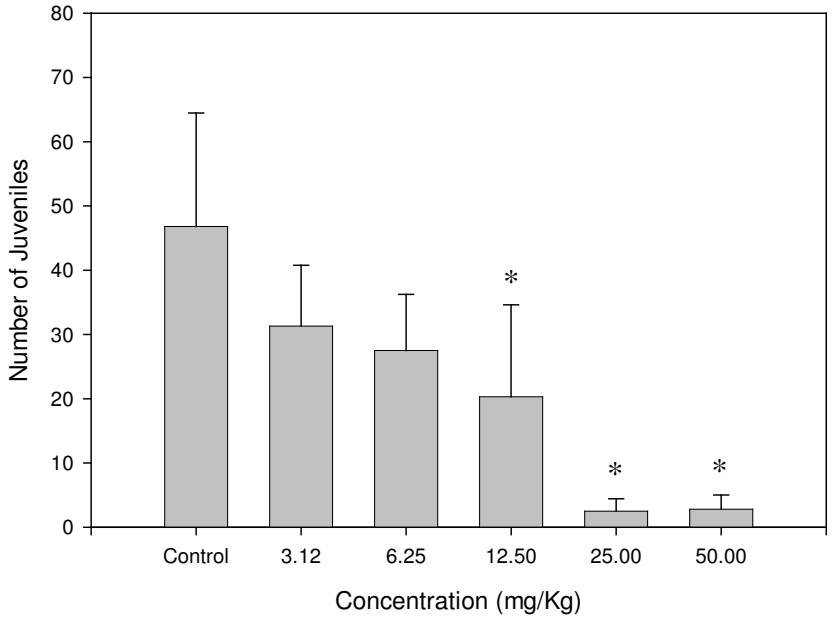
Treatment	Number of juveniles	Juveniles % of the control	Adult Mortality (%)	Biomass (mg) Day 0	Biomass (mg) Day 28	Moisture (%)	pH
Control	174.0 ± 25.4	100.0	2.5	379.5 ± 45.3	465.9 ± 21.5	28.6	6.3
1 mg/kg	155.5 ± 63.7	89.4	5.0	360.1 ± 39.5	424.0 ± 39.3	31.9	6.3
3.16 mg/kg	133.7 ± 31.8	76.8	2.5	362.3 ± 29.5	443.6 ± 34.3	29.1	6.3
10 mg/kg	145.8 ± 18.3	83.8	2.5	371.9 ± 47.3	473.3 ± 28.2	33.3	6.4
31.6 mg/kg	131.2 ± 33.0	49.7	7.5	362.5 ± 33.2	440.6 ± 54.3	31.6	6.4
100 mg/kg	17.0 ± 20.3	9.8	2.5	365.3 ± 27.2	315.4 ± 55.3	30.9	6.4

IV) Chronic toxicity test with tropical *Eisenia fetida* in lambda-cyhalothrin-dosed TNS soil.

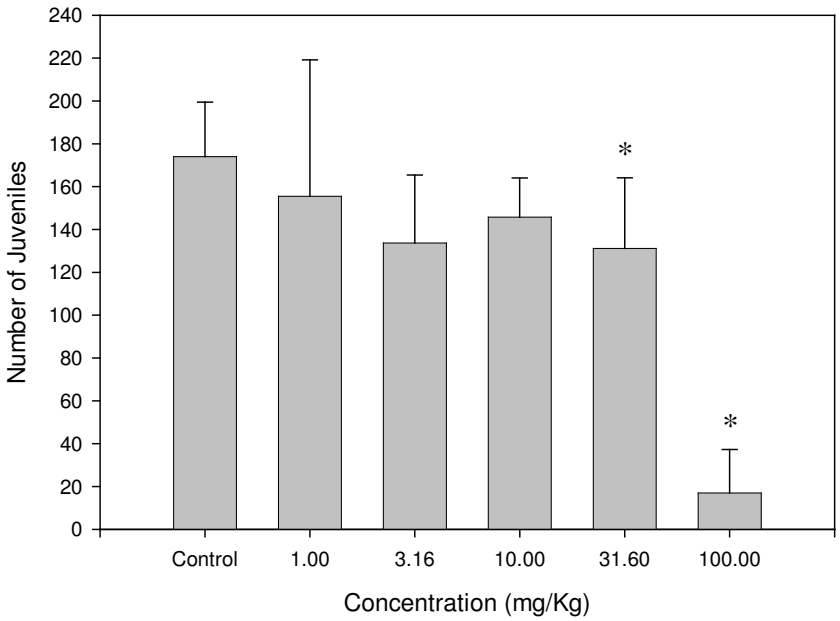
Treatment	Number of juveniles	Juveniles % of the control	Adult Mortality (%)	Biomass (mg) Day 0	Biomass (mg) Day 28	Moisture (%)	pH
Control	13.8 ± 20.4	100.0	2.5	305.5 ± 19.5	339.1 ± 36.1	15.5	3.97
1.56 mg/kg	17.5 ± 10.7	127.3	0.0	307.3 ± 15.6	340.0 ± 20.1	15.7	3.92
3.12 mg/kg	10.5 ± 11.6	76.4	0.0	304.6 ± 30.7	326.7 ± 28.6	14.8	3.95
6.25 mg/kg	26.3 ± 20.2	190.9	5.0	304.9 ± 17.7	350.7 ± 29.9	14.6	3.88
12.5 mg/kg	7.5 ± 7.5	54.5	5.0	303.0 ± 19.6	321.6 ± 21.0	15.3	3.92
25 mg/kg	2.5 ± 2.1	18.2	0.0	306.5 ± 28.9	315.5 ± 21.2	14.3	3.91
50 mg/kg	0.0 ± 0.0	0.0	12.5	304.2 ± 19.0	276.4 ± 45.4	15.0	4.19



V) Reproduction rate of tropical *Eisenia fetida* in lambda-cyhalothrin-dosed OECD soil (number of juveniles per test vessel, means and standard deviations; * statistically different from control at P = 0.01).



VI) Reproduction rate of tropical *Eisenia fetida* in lambda-cyhalothrin-dosed TASx soil (number of juveniles per test vessel, means and standard deviations; * statistically different from control at P = 0.01).



VII) Reproduction rate of tropical *Eisenia fetida* in lambda-cyhalothrin-dosed LUFA soil (number of juveniles per test vessel, means and standard deviations; * statistically different from control at P = 0.01).

Appendix 21: Avoidance test with tropical *Eisenia fetida* in lambda-cyhalothrin-dosed TASx soil.

Treatment	**Frequency distribution of earthworms (%)**		**Net Response (%)**	**Standard Error**	**Significance (*t*-test)**	**Moisture (%)**	**pH**
	Control	**Treated**					
0.316 mg/kg	85.00	15.00	70	19.1	**	32.7	6.3
1mg/kg	80.00	20.00	60	27.1	*	33.1	6.3
3.16 mg/kg	92.50	7.50	85	9.6	**	33.6	6.3
10 mg/kg	92.50	7.50	85	9.6	**	33.6	6.3
31.6 mg/kg	100	0	100	0.0	**	34.0	6.4
100 mg/kg	100	0	100	0.0	**	34.0	6.4
Untreated soil						34.1	6.4

*P=0.05 and ** P=0.01

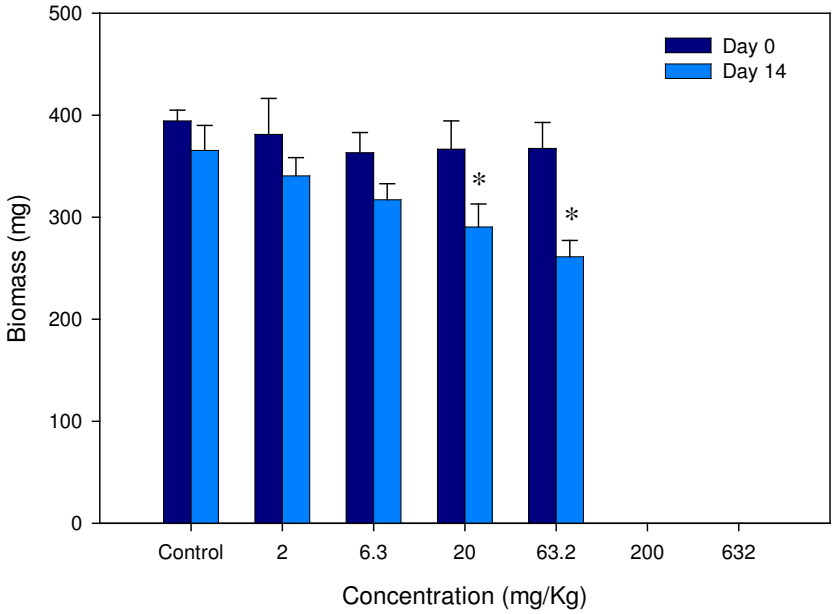
Appendix 22: Acute toxicity of lambda-cyhalothrin in European *Eisenia fetida*.

I) Acute toxicity test with European *Eisenia fetida* in lambda-cyhalothrin-dosed OECD soil.

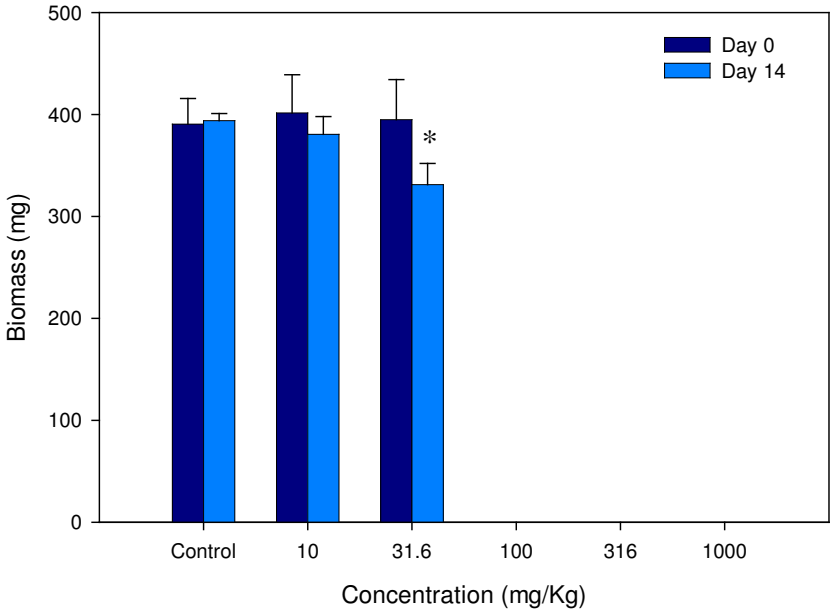
Treatment	Mortality (%)	Biomass (mg) Day 0	Biomass (mg) Day 14	Percent of the initial weight	Moisture (%)	pH
Control	0.0	394.4 ± 10.6	365.3 ± 24.6	91.9	45.8	6.5
2.0 mg/kg	0.0	381.1 ± 35.3	340.5 ± 17.8	89.3	46.6	6.3
6.3 mg/kg	0.0	363.2 ± 19.8	317.1 ± 15.7	87.3	47.6	6.3
20 mg/kg	7.5	366.7 ± 27.6	290.3 ± 22.7	79.2	46.1	6.7
63.2 mg/kg	2.5	367.3 ± 25.5	261.2 ± 16.0	71.1	45.3	6.8
200 mg/kg	95.0	337.1 ± 12.2	n.a.	n.a.	46.4	6.7
632 mg/kg	100.0	364.2 ± 24.4	n.a.	n.a.	45.0	6.5

II) Acute toxicity test with European *Eisenia fetida* in lambda-cyhalothrin-dosed LUFA soil.

Treatment	Mortality (%)	Biomass (mg) Day 0	Biomass (mg) Day 14	Percent of the initial weight	Moisture (%)	pH
Control	0.0	390.5 ± 25.1	393.9 ± 7.1	109.9	31.8	5.6
10 mg/kg	2.5	401.5 ± 37.5	380.5 ± 17.4	94.8	31.1	5.6
31.6 mg/kg	0.0	395.0 ± 39.2	331.2 ± 20.7	83.8	31.7	5.6
100 mg/kg	22.5	403.6 ± 30.7	n.a.	n.a.	30.7	5.6
316 mg/kg	92.5	393.9 ± 16.0	n.a.	n.a.	29.1	5.6
1000 mg/kg	100.0	394.6 ± 14.2	n.a.	n.a.	34.2	5.6



III) Biomass change in the acute toxicity test with European *Eisenia fetida* in lambda-cyhalothrin-dosed OECD soil (means and standard deviations;
* statistically different from control at P = 0.01).



IV) Biomass change in the acute toxicity test with European *Eisenia fetida* in lambda-cyhalothrin-dosed LUFA soil (means and standard deviations; * statistically different from control at P = 0.01).

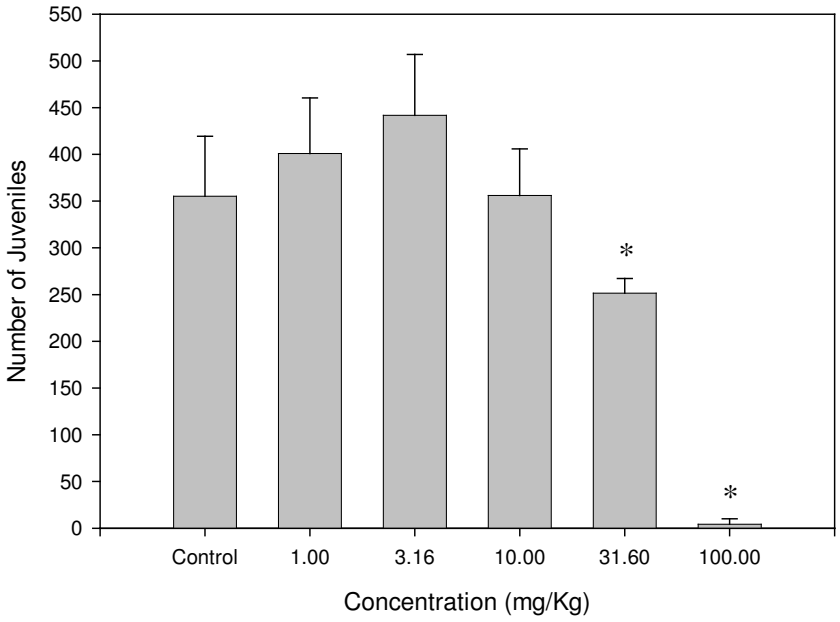
Appendix 23: Chronic toxicity of lambda-cyhalothrin in European *Eisenia fetida*

I) Chronic toxicity test with European *Eisenia fetida* in lambda-cyhalothrin-dosed OECD soil.

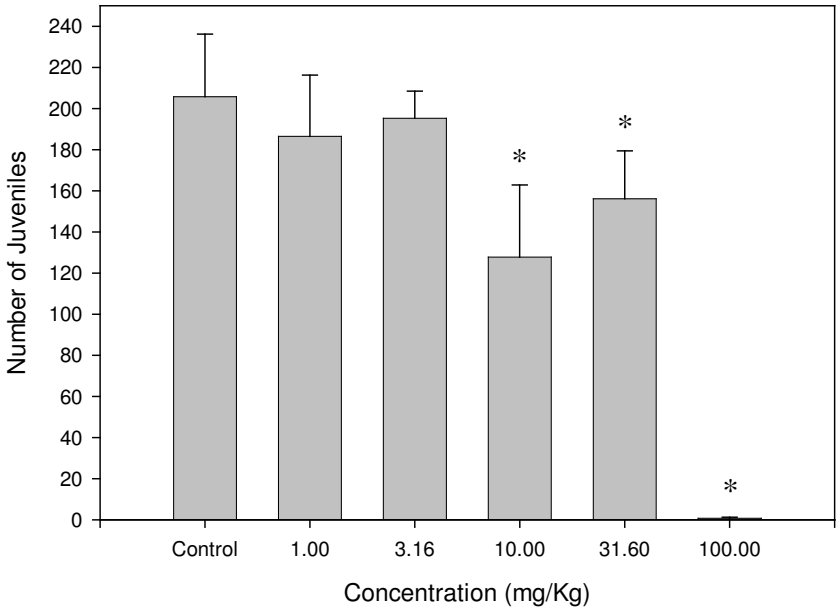
Treatment	**Number of juveniles**	**Juveniles % of the control**	**Adult Mortality (%)**	**Biomass (mg) Day 0**	**Biomass (mg) Day 28**	**Moisture (%)**	**pH**
Control	355.3 ± 64.0	100.0	0.0	361.6 ± 30.0	590.3 ± 20.4	36.9	6.3
1 mg/kg	401.0 ± 59.3	112.9	2.5	368.9 ± 26.5	567.2 ± 14.1	41.3	6.2
3.16 mg/kg	441.8 ± 65.1	124.3	0.0	374.2 ± 53.0	588.4 ± 48.5	42.5	6.2
10 mg/kg	356.0 ± 49.8	100.2	0.0	364.4 ± 55.7	584.6 ± 52.3	42.7	6.2
31.6 mg/kg	251.5 ± 15.7	70.8	0.0	357.4 ± 45.6	528.1 ± 38.4	40.8	6.2
100 mg/kg	4.3 ± 5.9	1.2	10.0	348.6 ± 44.8	316.9 ± 45.8	41.5	6.3

II) Chronic toxicity test with European *Eisenia fetida* in lambda-cyhalothrin-dosed LUFA soil.

Treatment	Number of juveniles	Juveniles % of the control	Adult Mortality (%)	Biomass (mg) Day 0	Biomass (mg) Day 28	Moisture (%)	pH
Control	205.8 ± 30.5	100.0	0.0	390.5 ± 17.8	544.8 ± 36.6	41.8	6.2
1 mg/kg	186.5 ± 29.7	90.6	0.0	394.4 ± 47.9	535.6 ± 18.9	36.8	6.2
3.16 mg/kg	195.3 ± 13.2	94.9	0.0	384.4 ± 28.9	563.2 ± 41.5	37.7	6.2
10 mg/kg	127.8 ± 35.1	62.1	0.0	381.7 ± 31.8	527.2 ± 21.5	37.2	6.3
31.6 mg/kg	156.1 ± 23.3	65.2	0.0	390.8 ± 23.6	567.6 ± 48.9	39.3	6.3
100 mg/kg	0.8 ± 0.5	0.4	15.0	394.2 ± 25.5	329.6 ± 33.1	35.4	6.2



III) Reproduction rate of European *Eisenia fetida* in lambda-cyhalothrin-dosed OECD soil (number of juveniles per test vessel, means and standard deviations; * statistically different from control at P = 0.01).



IV) Reproduction rate of European *Eisenia fetida* in lambda-cyhalothrin-dosed LUFA soil (number of juveniles per test vessel, means and standard deviations; * statistically different from control at P = 0.01).

Appendix 24: Avoidance test with European *Eisenia fetida* in lambda-cyhalothrin.

I) Avoidance test with European *Eisenia fetida* in lambda-cyhalothrin-dosed OECD soil.

Treatment	**Frequency distribution of earthworms (%)**		**Net Response (%)**	**Standard Error**	**Significance (*t*-test)**	**Moisture (%)**	**pH**
	Control	**Treated**					
0.316 mg/kg	45.00	55.00	-10	5.8	n.s.	31.7	5.9
1mg/kg	45.00	55.00	-10	25.2	n.s.	32.1	6.6
3.16 mg/kg	75.00	25.00	50	10.0	**	31.5	6.0
10 mg/kg	100.00	0.00	100	0.0	**	31.1	6.5
31.6 mg/kg	95.00	5.00	90	5.8	*	31.9	5.9
100 mg/kg	97.50	2.50	95	5.0	**	31.0	6.1
Untreated soil						31.5	5.8

* (P = 0.05) and ** (P = 0.01)

II) Avoidance test with European *Eisenia fetida* in lambda-cyhalothrin-dosed LUFA soil.

Treatment	Frequency distribution of earthworms (%)		Net Response (%)	Standard Error	Significance (*t*-test)	Moisture (%)	pH
	Control	Treated					
0.316 mg/kg	70.00	30.00	40	16.3	*	29.4	6.2
1mg/kg	85.00	15.00	70	12.9	**	28.9	6.1
3.16 mg/kg	85.00	15.00	70	12.9	**	28.5	6.2
10 mg/kg	90.00	10.00	80	14.1	**	28.2	6.3
31.6 mg/kg	95.00	5.00	90	10.0	**	28.2	6.2
100 mg/kg	97.50	2.50	95	5.0	**	28.7	6.3
Untreated soil						28.8	5.9

* (P = 0.05) and ** (P = 0.01)

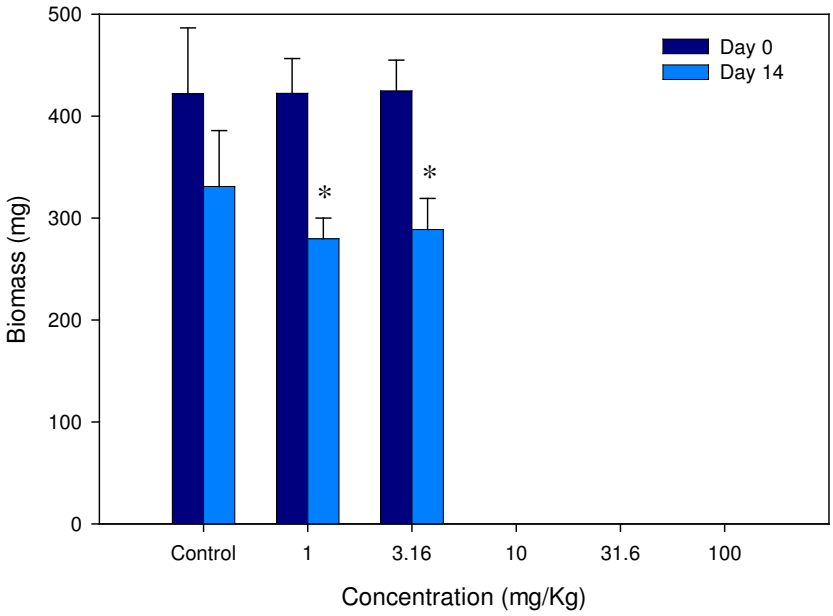
Appendix 25: Acute toxicity tests with tropical *Eisenia fetida* in TASc soil.

I) Acute toxicity of lambda-cyhalothrin.

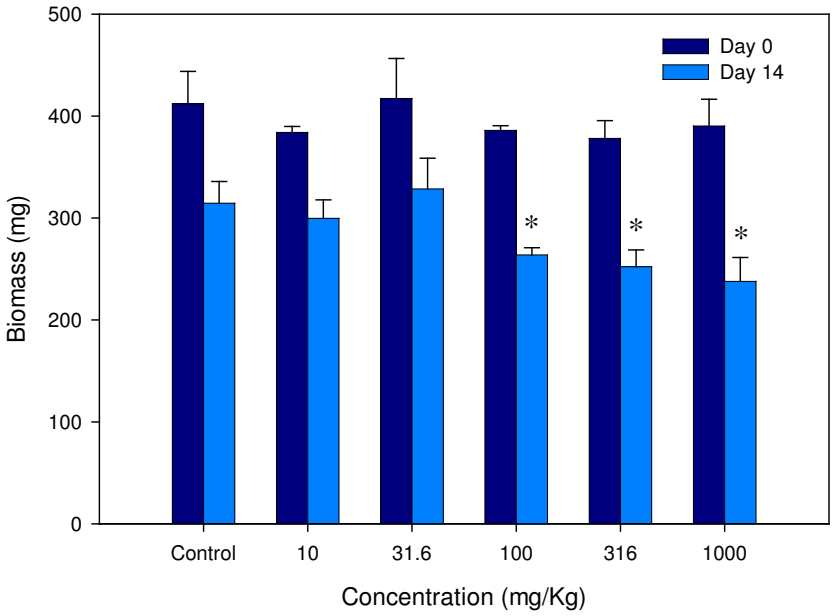
Treatment	Mortality (%)	Biomass (mg) Day 0	Biomass (mg) Day 14	Percent of the initial weight	Moisture (%)	pH
Control	2.5	422.0 ± 64.6	331.0 ± 54.8	78.4	41.2	6.7
1 mg/kg	7.5	422.4 ± 34.2	279.7 ± 20.3	66.2	42.1	6.6
3.16 mg/kg	10.0	424.7 ± 30.2	288.7 ± 30.4	68.0	42.5	6.7
10 mg/kg	37.5	411.4 ± 38.9	267.8 ± 39.8	65.1	42.2	6.7
31.6 mg/kg	100.0	427.9 ± 37.4	n.a.	n.a.	37.3	6.7
100 mg/kg	100.0	433.8 ± 39.8	n.a.	n.a.	39.2	6.6

II) Acute toxicity of carbendazim.

Treatment	Mortality (%)	Biomass (mg) Day 0	Biomass (mg) Day 14	Percent of the initial weight	Moisture (%)	pH
Control	0.0	412.0 ± 31.8	314.5 ± 21.3	76.3	39.4	5.9
10 mg/kg	2.5	384.0 ± 5.8	299.6 ± 18.2	78.0	34.0	6.0
31.6 mg/kg	0.0	417.3 ± 39.3	328.5 ± 30.2	78.7	39.2	6.0
100 mg/kg	2.5	385.9 ± 4.6	263.8 ± 7.0	68.3	36.7	6.0
316 mg/kg	7.5	378.1 ± 17.5	252.2 ± 16.4	66.7	36.3	6.0
1000 mg/kg	7.5	390.2 ± 26.3	237.7 ± 23.5	60.9	37.8	6.0



III) Biomass change for the acute toxicity test with tropical *Eisenia fetida* in lambda-cyhalothrin-dosed TASc soil (means and standard deviations; * statistically different from control at P = 0.01).



IV) Biomass change for the acute toxicity test with tropical *Eisenia fetida* in carbendazim-dosed TASc soil (means and standard deviations; * statistically different from control at P = 0.01).

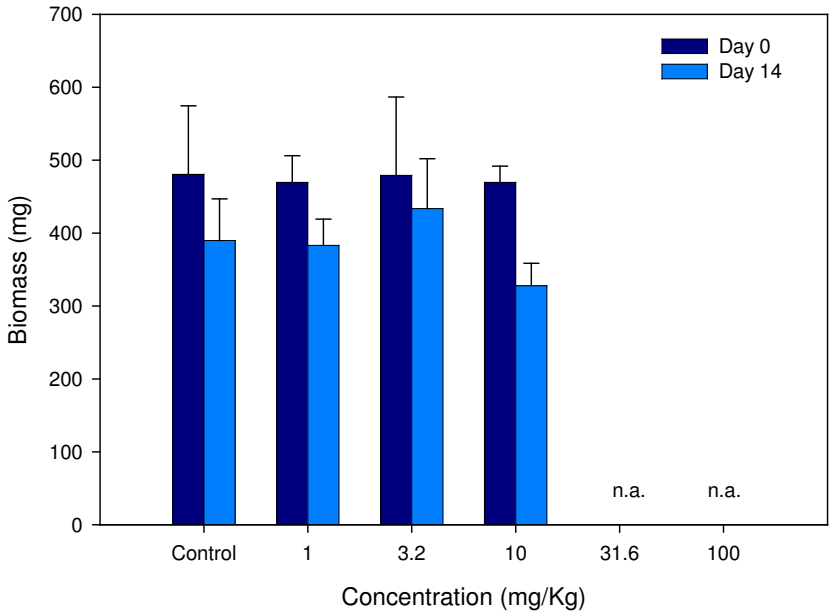
Appendix 26: Acute toxicity tests with *Pontoscolex corethrurus* in TASx soil.

I) Acute toxicity of lambda-cyhalothrin.

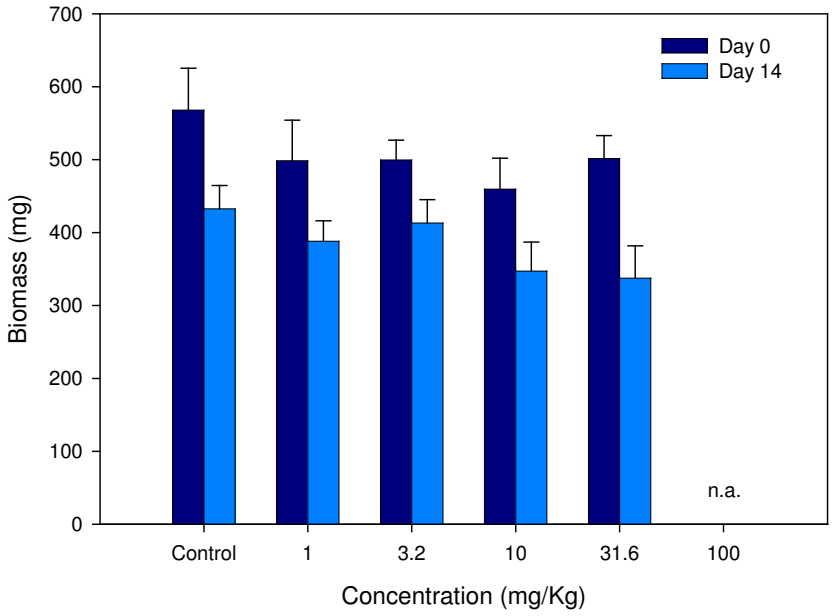
Treatment	Mortality (%)	Biomass (mg) Day 0	Biomass (mg) Day 14	Percent of the initial weight	Moisture (%)	pH
Control	0.0	567.9 ± 57.5	432.6 ± 31.9	76.2	34.9	6.3
1 mg/kg	2.5	498.5 ± 55.5	388.2 ± 27.9	77.9	35.5	6.4
3.16 mg/kg	0.0	499.6 ± 27.1	413.1 ± 32.0	82.7	35.0	6.5
10 mg/kg	7.5	459.5 ± 42.4	347.2 ± 39.8	75.6	35.6	6.7
31.6 mg/kg	15.0	501.5 ± 31.5	337.5 ± 44.2	67.3	37.3	6.8
100 mg/kg	100.0	477.1 ± 83.4	n.a.	n.a.	36.7	6.9

II) Acute toxicity of carbendazim.

Treatment	Mortality (%)	Biomass (mg) Day 0	Biomass (mg) Day 14	Percent of the initial weight	Moisture (%)	pH
Control	5.0	480.6 ± 94.0	389.8 ± 57.1	81.1	36.5	6.4
1 mg/kg	0.0	469.4 ± 36.6	383.1± 36.0	81.6	38.2	6.4
3.16 mg/kg	10.0	479.1 ± 107.4	433.5 ± 68.4	90.5	36.6	6.4
10 mg/kg	10.0	469.6 ± 22.1	327.6 ± 30.9	69.8	37.2	6.4
31.6 mg/kg	20.0	436.2 ± 45.3	n.a.	n.a.	38.2	6.5
100 mg/kg	96.7	438.2 ± 7.3	n.a.	n.a.	37.1	6.8



III) Biomass change in the acute toxicity test with *Pontoscolex corethrurus* in carbendazim-dosed TASx soil (means and standard deviations; treatments do not differ from control).



IV) Biomass change for the acute toxicity test with *Pontoscolex corethrurus* in lambda-cyhalothrin-dosed TASx soil (means and standard deviations; treatments do not differ from control).

Appendix 27: Acute toxicity of lambda-cyhalothrin in *Porcellionides pruinosus*.

I) Acute toxicity test with *P. pruinosus* in lambda-cyhalothrin-dosed OECD soil.

Treatment	Mortality (%)	Moisture (%)	pH
Control	10.0	30.1	5.0
0.0316 mg/kg	10.0	30.0	5.4
0.1 mg/kg	12.5	31.3	5.8
0.316 mg/kg	32.5	29.9	5.4
1 mg/kg	87.5	28.7	5.1
3.16 mg/kg	100.0	31.4	5.1

II) Acute toxicity test with *P. pruinosus* in lambda-cyhalothrin-dosed TASx soil.

Treatment	Mortality (%)	Moisture (%)	pH
Control	17.5	30.75	6.7
0.00316 mg/kg	25.0	31.32	6.7
0.01 mg/kg	25.0	30.83	6.7
0.0316 mg/kg	30.0	30.41	6.7
0.1 mg/kg	35.0	30.77	6.7
0.316 mg/kg	87.5	30.46	6.7
1 mg/kg	100.0	30.37	6.6

III) Acute toxicity test with *P. pruinosus* in lambda-cyhalothrin-dosed LUFA soil.

Treatment	Mortality (%)	Moisture (%)	pH
Control	10.0	28.8	6.0
0.0316 mg/kg	15.0	28.8	5.9
0.1 mg/kg	17.5	27.4	5.9
0.316 mg/kg	15.0	28.6	5.9
1 mg/kg	30.0	28.8	5.9
3.16 mg/kg	95.0	27.4	6.0

IV) Acute toxicity test with *P. pruinosus* in lambda-cyhalothrin-dosed TNS soil.

Treatment	Mortality (%)	Moisture (%)	pH
Control	22.5	17.4	4.0
0.0316 mg/kg	50.0	17.5	4.0
0.1 mg/kg	65.0	17.4	4.0
0.316 mg/kg	90.0	17.0	4.0
1 mg/kg	100.0	17.5	4.0
3.16 mg/kg	100.0	17.2	3.9

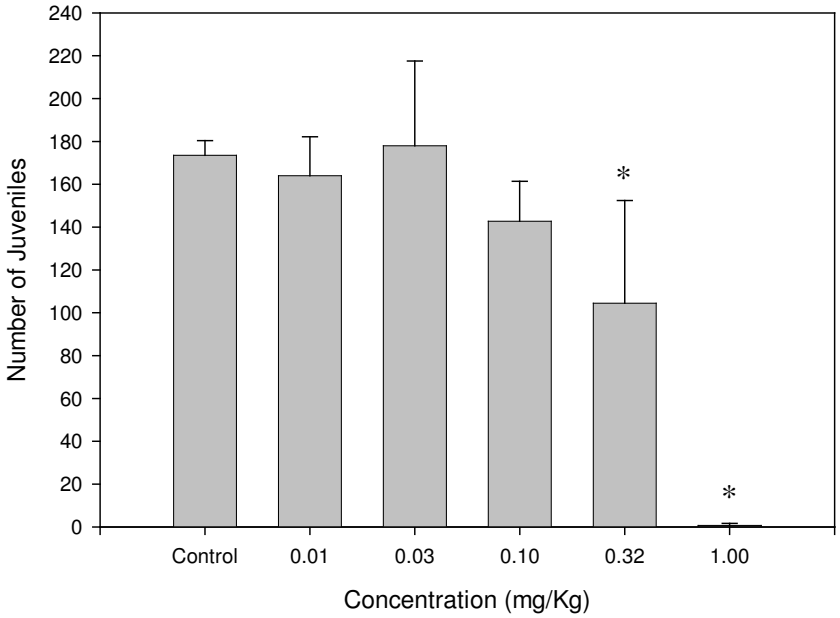
Appendix 28: Chronic toxicity of lambda-cyhalothrin in *Porcellionides pruinosus*.

I) Chronic toxicity test with *P. pruinosus* in lambda-cyhalothrin-dosed OECD soil.

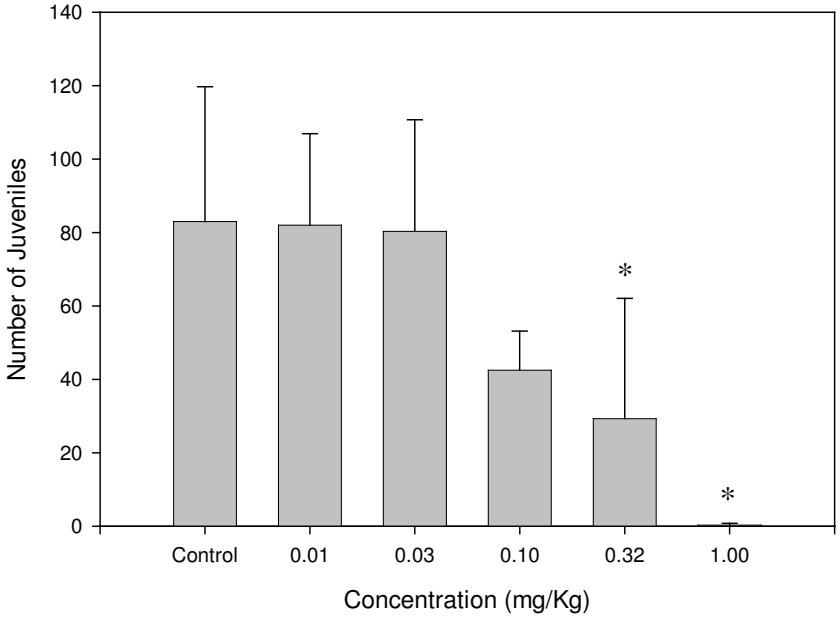
Treatment	Number of juveniles	Juveniles % of the control	Adult Mortality (%)	Moisture (%)	pH
Control	173.5 ± 6.9	100.0	12.5	34.4	6.2
0.001 mg/kg	164.0 ± 18.1	94.5	5	33.7	6.3
0.0316 mg/kg	178.0 ± 39.6	102.6	5	32.6	6.4
0.1 mg/kg	142.8 ± 18.6	82.3	7.5	34.6	6.4
0.316 mg/kg	104.5 ± 47.9	60.2	10	37.4	6.3
1 mg/kg	0.8 ± 1.0	0.4	52.5	32.6	6.4

II) Chronic toxicity test with *P. pruinosus* in lambda-cyhalothrin-dosed LUFA soil.

Treatment	Number of juveniles	Juveniles % of the control	Adult Mortality (%)	Moisture (%)	pH
Control	83.0 ± 36.7	100.0	10	30.9	5.7
0.001 mg/kg	82.0 ± 24.9	98.8	10	32.3	5.7
0.0316 mg/kg	80.3 ± 30.4	96.7	5	31.6	5.7
0.1 mg/kg	42.5 ± 10.6	51.2	25	30.6	5.7
0.316 mg/kg	29.3 ± 32.8	35.2	90	31.3	5.8
1 mg/kg	0.3 ± 0.5	0.3	100	30.4	5.7



III) Reproduction rate of *Porcellionides pruinosus* in lambda-cyhalothrin-dosed OECD soil (number of juveniles per test vessel, means and standard deviations; * statistically different from control at P = 0.01)



IV) Reproduction rate of *Porcellionides pruinosus* in lambda-cyhalothrin-dosed LUFA soil (number of juveniles per test vessel, means and standard deviations; * statistically different from control at P = 0.01).

Appendix 29: Acute toxicity test with *Circoniscus ornatus* in lambda-cyhalothrin-dosed TASx soil.

Treatment	**Mortality (%)**	**Moisture (%)**	**pH**
Control	12.5	28.8	6.6
0.316 mg/kg	25.0	29.1	6.5
1 mg/kg	42.5	29.4	6.5
3.16 mg/kg	60.0	29.4	6.5
10 mg/kg	90.0	29.4	6.3
31.6 mg/kg	85.0	30.2	6.6

Appendix 30: Acute toxicity with *Trigoniulus corallinus* in TASx soil.

I) Acute toxicity of carbendazim.

Treatment	**Mortality (%)**	**Moisture (%)**	**pH**
Control	27.5	33.8	6.7
10 mg/kg	17.5	34.0	6.3
31.6 mg/kg	10.0	33.5	6.9
100 mg/kg	12.5	34.1	6.6
316 mg/kg	27.5	35.2	6.1
1000 mg/kg	80.0	34.6	6.9

II) Acute toxicity of lambda-cyhalothrin.

Treatment	**Mortality (%)**	**Moisture (%)**	**pH**
Control	22.5	35.0	6.9
0.39 mg/kg	40.0	33.2	6.9
0.78 mg/kg	52.5	33.7	6.9
1.56 mg/kg	67.5	34.7	6.9
3.12 mg/kg	80.0	35.8	7.0
6.25 mg /kg	90.0	33.2	6.9

Appendix 31: Concentrations of carbendazim in OECD artificial soil determined with the RAPID-Kit method at the end of the test. All values are given in mg a.i. (either as mg/L water extract or mg/kg soil).

OECD-Soil (measured at the end of a test, i.e. 8 weeks after application)						
Sample	**Dilution factor**	**Concentration (measured)**		**Concentration (nominal)**		**Concentration (measured)**
		Sample	**Extract (after dilution)**	**Soil**	**Extract**	
		[µg/L]	[mg/L]	[mg/kg]	[mg/L]	[% nominal]
C1	50	0.626	0.03	0.316	0.21	14.9
C2	2000	0.050	0.10	1.00	0.67	15.0
C3	200	0.675	0.13	3.16	2.11	6.4
C4	1000	0.037	0.04	10.0	6.67	0.6
C5	5000	0.074	0.37	31.6	21.07	1.7

Appendix 32: Concentrations of carbendazim in LUFA standard soil determined with the RAPID-Kit method at the start and the end of the test, respectively. All values are given in mg a.i. (either as mg/L water extract or mg/kg soil).

I) LUFA-Soil: Measured at the start of the test, i.e. shortly after application.

Sample	**Dilution factor**	**Concentration (measured)**		**Concentration (nominal)**		**Concentration (measured)**
		Sample	**Extract (after dilution)**	**Soil**	**Extract**	
		[µg/L]	[mg/L]	[mg/kg]	[mg/L]	[% nominal]
C1	20000	0.076	1.52	10.0	6.7	22.8
C2	5000	1.294	6.47	31.6	21.1	30.7
C3	200000	0.159	31.85	100	66.7	47.8
C3	1000000	0.031	31.27	100	66.7	46.9
C4	400000	0.171	68.60	316	210.7	32.6
C4	2000000	0.045	90.54	316	210.7	43.0
C5	2000000	0.206	412.68	1000	666.7	61.9
C5	10000000	0.026	259.91	1000	666.7	39.0

II) LUFA-Soil: Measured at the end of the test, i.e. two weeks after application.

Sample	Dilution factor	Concentration (measured)		Concentration (nominal)		Concentration (measured)
		Sample	Extract (after dilution)	Soil	Extract	
		[µg/L]	[mg/L]	[mg/kg]	[mg/L]	[% nominal]
C1	20000	0.057	1.13	10.0	6.7	17.0
C2	5000	1.161	5.81	31.6	21.1	27.6
C3	200000	0.140	27.93	100	66.7	41.9
C4	400000	0.171	68.21	316	210.7	32.4
C5	2000000	0.079	158.53	1000	666.7	23.8

Appendix 33: Concentrations of carbendazim in the leachate of the second TME test determined with the RAPID-Kit method at the end of the test, i.e. five months after application (two application scenarios). All values are given in mg a.i. (either as µg/L water extract).

Sample Application rate	Sample	Sample Concentration [ppb]	Dilution factor	Extract concentration [µg/L]
TME (II) Leachate Application: 45.1 kg a.i./ha (once at start)	17	0.031	100	n.a.*
	18	0.329	100	32.93
	19	0.214	100	21.41
	20	0.243	100	24.28
	21	0.315	100	31.52
	22	0.671	100	67.05
	23	0.724	100	72.45
	24	0.583	100	58.27
TME (II) Leachate Application: 4.51 kg a.i./ha (monthly)	25	0.072	100	7.17
	26	0.275	100	27.53
	27	0.992	100	99.22
	28	0.344	100	34.39
	29	0.193	100	19.34
	30	0.969	100	96.85
	31	0.240	100	24.04
	32	0.181	100	18.07

Appendix 34: Concentrations of carbendazim in the soil of the second TME test determined with the RAPID-Kit method at the end of the test, i.e. five months after application (two application scenarios). All values are given in mg a.i. (either as mg/L water extract or mg/kg soil).

Sample application rate	Sample	Dilution factor	Concentration			
			Sample [ppb]	Extract [µg/L]	Soil [mg/kg]	Soil [% of nominal]
TME (II) Soil Initial nominal concentration: 60.1 mg/kg (once at start)	17	3000	0.062	187.30	0.28	0.47
	18	3000	0.621	1863.61	2.80	4.66
	19	3000	0.143	430.41	0.65	1.08
	20	3000	0.142	426.36	0.64	1.06
	21	3000	0.111	333.44	0.50	0.83
TME (II) Soil Initial nominal concentration: 6.0 mg/kg (monthly)	25	6000	0.115	692.58	1.04	17.33
	26	6000	0.132	790.60	1.19	19.83
	27	6000	0.481	2887.47	4.33	72.17
	28	6000	0.555	3327.44	4.99	83.17
	29	6000	0.222	1329.85	1.99	33.17

Appendix 35: Concentrations of carbendazim in the soil of the litterbag field test determined with the RAPID-Kit method at the end of the test, i.e. twelve months after application (three application scenarios). All values are given in mg a.i. (either as mg/L water extract or mg/kg soil).

Sample application rate	Sample	Dilution factor	Concentration			
			Sample [ppb]	Extract [µg/L]	Soil [mg/kg]	Soil [% of nominal]
Field D1(Soil) Initial nominal concentration: 1.33 mg/kg (3 x 1 kg/ha)	A	20	0.488	9.76	0.01	0.75
	B	20	0.926	18.52	0.03	2.26
	C	20	0.711	14.22	0.02	1.50
	D	20	0.012	n.a.*	n.a.	n.a.
Field D2 (Soil) Initial nominal concentration: 1.33 mg/kg (11 x 1 kg/ha)	A	20	0.752	15.04	0.02	1.50
	B	20	0.620	12.41	0.02	1.50
	C	20	0.841	16.82	0.03	2.26
	D	20	0.409	8.18	0.01	0.75
Field D3 (Soil) Intial nominal concentration: 13.3 mg/kg (1 x 10 kg/ha)	A	200	0.289	57.89	0.09	0.68
	B	n.a.	n.a.	n.a.	n.a.	n.a.
	C	n.a.	n.a.	n.a.	n.a.	n.a.
	D	200	0.462	92.39	0.14	1.05

* Sample concentration too low

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