3 Materials and methods

To assess the toxicity of substances in soil with biotests, it is necessary to optimise the living conditions of the test organisms. For that, certain soil parameters have to be adjusted, which is described in the first part of this chapter. Then a brief description of the two test organisms is given before the performed tests are outlined and summarised in Table 3.3-2.

3.1 Determination of soil parameters

3.1.1 Water content

The water content was determined on dry matter bases by complete drying of the soil samples according to ISO 11465. The difference in mass before and after the drying procedure represents the water content. For that a double set of soil (20-50 g) was dried to constant mass at 105°C in a drying oven. After at least two hours the samples were placed in a desiccator for half an hour to cool down before they were weighed again. The water content can be calculated according to the following equation (ISO 11465, 1993; LEWANDOWSKI et al, 1997):

$$\text{water content } [\%] = \frac{m_1 - m_2}{m_2 - m_0} \times 100$$

m_0 mass of empty container in g

m_1: mass of container with moist soil in g

m_2: mass of container with dry soil in g.

3.1.2 Maximum water holding capacity (MWC)

The MWC is the amount of water a soil can keep against gravity. For its determination the soil sample must be first saturated with water. For that a double set of soil (50 g) was weighed into cylindrical sieves with a fine mesh (Ø inside 3 cm, mesh Ø 50 µm). Then the sieves were placed into a vessel filled with water. The water level had to be equal to that of the soil material in the sieves. After one hour the sieves were put on a wet sand bath (filling height 10 cm) and covered with a watch glass. The soil was saturated with water after three hours and its water content determined, which equals the maximum water holding capacity expressed in % or in g H_2O/100 g soil (dw) (ACHAZI et al, 2000a: 34; SCHINNER et al, 1993: 346).

3.1.3 Soil acidity

The acidity of the soil including the sorbed cations was determined after the addition of 0.01mol/L $CaCl_2$-solution according to ISO 10390. Usually, the amount of the solution is 2.5 fold of the soil weight, but for soils with high organic matter the amount of solution is 10 fold. Both samples were shaken for 15 minutes. After at least two hours at room temperature the pH

was measured with a calibrated pH-meter. Between measurements the glass electrode was rinsed with distilled water (ISO 10390, 1994; SCHINNER et al, 1993: 34).

3.1.4 Content of organic matter and organic carbon

An easy approach for the determination of the organic matter is by the glowing loss. For that dried soil was weighed in a porcelain crucible which was placed in a muffle furnace at 550°C. After 2.5 hours the glowed probes were put in a desiccator for half an hour to cool down before they were weighed again. The glowing loss is the weight difference before and after the glowing. It is calculated on basis of the following equation (LEWANDOWSKI et al, 1997: 94):

$$\text{glowing loss [\%]} = \frac{m_1 - m_2}{m_2 - m_0} \times 100$$

m_0: mass of empty container in g

m_1: mass of container with soil in g before glowing

m_2: mass of container with soil in g after glowing.

The glowing loss represents the organic matter. The amount of organic carbon (C_{org}) can be calculated by dividing the organic matter content by 1.72 or 2.0 for very peaty soils (DUNGER & FIEDLER, 1997: 54).

3.1.5 Grain size

The grain size was determined according to DIN 19683-2. For that 10 g of the air-dried sieving fraction < 2mm was weighed into plastic bottles and 25 ml of 0.4 N sodium pyrophosphate ($Na_4P_2O_7$) was added. The solution was left over night and then shaken in an inverted shaker for three hours. The solution was transferred into 1 L glass cylinders and filled with distilled water up to 1 L. In a pipette apparatus after Köhn the medium silt (63-20 µm), the fine silt (2-63 µm) and the clay fraction (< 2 µm) were taken after the time required at the given room temperature. Each fraction was transferred into a container and completely dried in a drying oven. The sand fraction was determined by sieving under running water with sieves of 63 µm for the fine sand, 200 µm for the middle sand and 630 µm for the coarse sand. The sand fractions were completely dried in small porcelain bowls in a drying oven. When the fractions were completely dried, they were transferred into a dessicator and later weighed. For the calculation of the percentages of each fraction the original soil sample represented 100% after the organic matter and the water content had been subtracted. In case of the pipette fractions the portion of sodium pyrophosphate was also subtracted (DIN 19683-2, 2000).

3.2 Test organisms

As test organisms two representatives of the soil mesoflora were chosen: the collembola *Folsomia candida* and the enchytraeid *Enchytraeus crypticus*. Both invertebrates are decomposers. *F. candida* as an insect is a representative of the animals living in the air-filled

soil pores, whereas *E. crypticus* as an annelid lives in the soil pore water. Thus the primary ways of exposure to pollutants are different for both species: via the air for the collembola and via the water for the enchytraeid. Both species live of bacteria and fungi. Exposure via the food is possible for both test organisms and an intake via water through the ventral tube is discussed for the collembola (ACHAZI et al, 2000).

For biotests both organisms are suitable according to the criteria defined by WESTHEIDE and BETHKE-BEILFUSS (1991: 506) for enchytraeids, as they have a short reproduction time and high reproduction rates, are easy to cultivate in the laboratory and the toxicity endpoints mortality or reproduction are easy to determine. Another advantage of the test species for reproduction tests is that *F. candida* is parthenogenetic and *E. crypticus,* like all enchytraeids, is a hermaphrodite.

The permanent culture of *F. candida* was kept on a moist Plaster of Paris prepared with charcoal (57% Plaster of Paris, 5.1% activated charcoal) in polyethylene boxes (15 x 10 x 6 cm) at 20-22°C and fed with dry yeast twice a week. In order to get a synchronised test population, adults were transferred into a new container to induce oviposition. The freshly laid eggs were removed with a brush and collected on a small plate of Plaster of Paris. The juveniles hatched after 7–10 days and were used at approximately 25 days (ACHAZI et al, 1997).

E. crypticus (WESTHEIDE & GRAEFE, 1992) was kept as a permanent culture on agar-agar (1.7% high strength agar-agar in artificial fresh water) in petri dishes (Ø 8.5 cm) at 20–23°C and fed with grained oats. Synchronised populations were obtained by transferring adult worms from the permanent culture onto fresh agar-agar twice a week. One or two days later the new cocoons were transferred onto another petri dish. The juveniles hatched after 7-10 days and were used for the tests about 25 days after the transfer of the cocoons so at the age of about 15-18 days (ACHAZI et al, 1997).

3.3 Tests

Usually the tests were performed in soil, but occasionally non-soil tests were used to further investigate the toxicity of a substance, without the influence of soil. A summary of the tests is given in Table 3.3-2.

3.3.1 Soil tests

As standard soil material Lufa 2.2 from the Landwirtschaftliche Untersuchungsanstalt in Speyer (Rhineland-Palatinate, Germany) was used. A loamy sand with hardly any contamination, an organic carbon content of 2.2% and a pH of 5.9. Further properties of the soils are given in Appendix A. Before it was used in the tests it was defaunated by freezing it twice at -18°C.

During an intermediate phase at room temperature the hatching of cocoons, eggs or survival stages was allowed.

3.3.1.1 Mortality test and reproduction test

For the reproduction test with *F. candida* the International Organization for Standardization (ISO) has developed a guideline (ISO 11267) and a draft guideline for enchytraeids (ISO/WD 16378 for *Enchytraeus sp.).* The tests were conducted on the basis of these guidelines (ISO 11267, 1999 and ISO 16378, 2000), with slight modifications. In contrast to the ISO-Norms, mortality tests with a duration of one week were performed to determine the effect on the mortality and not only as a range finding test as described in the ISO-norms. The alterations for both test species are described by ACHAZI et al (2001: tables 5-7) for *F. candida* and by RÖMBKE et al (2001: tables 5-6) for *E. crypticus.* An overview of these tests is given in Table 3.3-2.

The tests were conducted in glass containers with Teflon-lid screw caps, which were closed tightly during the test period. For the mortality test 15 g soil (fw) and 30 g soil (fw) for the reproduction test were added after its water content had been adjusted to 60% of the MWC. In each container ten adult, synchronised animals were placed and five replicas of each concentration were tested. For each test the mortality or the reproduction of the test organisms was determined in a control with uncontaminated soil material, which had been treated in the same way as the contaminated soil materials.

F. candida was fed at the beginning of the test with about 40 mg dry yeast and again after two weeks in the reproduction test. *E. crypticus* was fed with ground oats which were mixed thoroughly into the soil. For the mortality test 0.5% of the soil matter (fw) were added and 2% for the reproduction test.

The test containers were kept in a climatic enclosure at 20 ± 1°C, either at a light regime of natural daylight for *F. candida* or in permanent darkness for *E. crypticus*. The containers were aired once a week and the water content was checked every two weeks.

After a test period of 7 days in the mortality test (short-term test) or 28 days in the reproduction test (long-term test), the animals were counted. For that the containers were filled with water and the soil with the animals transferred into a counting container. In the mortality test for *E. crypticus* petri dishes (Ø 15 cm) were used and for *F. candida* glass bowls (Ø 11.5 cm). In the reproduction test for *E. crypticus* the same containers were used, but the soil was transferred into at least three different petri dishes. In addition the test containers were filled with water one day in advance and kept at 8°C overnight to force the worms out of the soil. For the reproduction test with *F. candida* beakers (250 mL) were used. The water was dyed with blue ink and the vessels with the animals floating on the surface photographed. The slides were later counted by using a screen and a colony counter.

The mortality was calculated by subtracting the number of living animals at the end of the test from the ten animals introduced at the beginning. A mortality of ten represented 100%. For the reproduction rate first, the number of juveniles was calculated by subtracting the ten adults introduced at the beginning from the number of animals in the container. Secondly, the number of juveniles was divided by ten in order to get the reproduction rate per adult animal introduced in the test. The reproduction rate of the control with the uncontaminated soil was taken as 100%. The mean and the standard deviation (SD) were calculated and statistically evaluated with a one way *a*nalysis of *v*ariance (ANOVA) (see p. 9, chapter 3.3.3) using the programme SigmaStat. A mortality of 100% and a reproduction of 0% were considered to be significant without testing. If only one concentration was compared with the control an unpaired t-test or an U-test were performed (see p. 9, chapter 3.3.3).

The concentrations with a 50%-effect, the LC50 for lethality in the mortality test and the EC50 for the reduction of the reproduction, were calculated via a progression analysis according to FINNEY (1971). For the calculation all data from various tests were used, whereas only one exemplary set of data was used to illustrate the concentration-effect relationships. The mortality of the uncontaminated control is taken into account for the calculation of the LC50, which might lead to an apparent contradiction with the concentration-effect relationships if the mortality was high. The plots were drawn using the programme SigmaPlot.

If the animals were damaged, *E. crypticus* was considered as dead, but *F. candida* was counted as living, as rapid recovery had been observed in animals thought to be dying. Tests were valid if the mortality did not exceed 20% or the reproduction rate was above 40 juveniles/adult for *F. candida* and higher than 60 juveniles/adult for *E. crypticus* (WARNECKE et al, 2001).

3.3.1.2 Choice test

In this test system the animals were given the choice between contaminated and uncontaminated soil material. Either the animal would migrate into the contaminated soil material or escape from it, thus indicating an attractant or repellent effect of the contaminant. The test system is based on investigations with lumbricides (YEARDLEY et al, 1996) and enchytraeids (ACHAZI et al, 1999), but was developed in the course of this thesis yielding to two different test systems:

- combined test set: 10 animals on each side
- separated test set: 10 animals on uncontaminated side of five test vessels
  10 animals on contaminated side of five different test vessels.

First the combined test set was used, but the distribution of the animals at the end of the tests could not clearly be attributed to a certain choice behaviour. Thus, the separated test set was

developed, in which attraction and repellence could be observed for animals either placed on uncontaminated or contaminated soil material.

Overall, plastic sputum beakers with a screw top served as test containers. The two test sides were divided by a marked gauze, with holes (Ø 1mm) big enough for both test species to get through. As standard soil material Lufa 2.2 was used. The water content was adjusted to 60% of the MWC. Each side was filled with 10 g soil (fw). The test vessels were kept in a climatic enclosure at 20 ± 1°C in permanent darkness. For each test the distribution in a control with uncontaminated soil material on both sides was tested.

After two days the animals on each side were counted, by transferring the soil material from each side into a counting container. For the collembola glass bowls were used, for the enchytraeid petri dishes, as in the mortality tests. To prevent the soil material from mixing, a plastic division was put in the test vessel.

The sum of the animals found in the different replicas on each side was tested for significance with a Chi-square test (see p. 9, chapter 3.3.3) in relation to the distribution in the control vessels. If less than twelve animals were found at the end of the test in a replicate of the combined test set or less than six in a replicate of the separate test set, this replicate was not counted. In the tables the sum of the animals found on a side is represented as the percentage of all the animals found in this soil sample.

For the graphs the percentages of all the animals found were calculated for each replicate. The mean and the SD of these percentages are represented in the graphs only for the uncontaminated sides. They were drawn with SigmaPlot software. An overview of the test is given in Table 3.3-2.

3.3.2 Non-soil tests

The contact test for the earthworm *E. fetida* can be adapted for animals living in the air-filled soil pores, like *F. candida*. For animals living in the soil pore water, like *E. crypticus*, water tests can be used. In contrast to contact tests they offer the advantage that the test animals cannot dry out if the test containers are not tightly closed due to water evaporating.

3.3.2.1 Contact test

The contact test with *F. candida* was carried out on filter paper (Ø 2.5 cm) placed in scintillation vessels. If a test substance was not water soluble, a suitable solvent was used. After the solvent had evaporated, the filter paper was moistened with 70 µL of tab water before the animals were introduced. Each concentration was tested in five replicates, the control was the solvent or tab water. Five or ten animals were placed in each container and checked for mortality after 24, 48 and 72 hours. The LC50-values for each test period were graphically

determined by the help of a concentration-effect relationship. A short description of the test is given in Table 3.3-2.

3.3.2.2 Water test

For water tests with *E. crypticus* artificial fresh water was used (294 mg $CaCl_2$ x H_20, 123 mg $MgSO_4$ x 7 H_20; 65 mg $NaHCO_3$ and 5.8 mg KCl in 1 L deionised water with a conductivity of less than 10 µS/cm and pH 7.8) (RÖMBKE & KNACKER, 1989). The test vessels had to be tightly closed, to prevent evaporating leading to scintillation vessels being used. In each container 5 ml of liquid were filled and five or ten animals placed. Each concentration was tested in five replicates, the control was plain artificial fresh water. After 24, 48 and 72 hours the vessels were checked for mortality. Damaged animals were also considered as dead. The LC50-values for each test period was graphically determined by the help of a concentration-effect relationship. The test is summarised in Table 3.3-2.

3.3.3 Statistics

In order to evaluate biological data statistical tests are necessary to exclude that variations between the data are overestimated. In the mortality and reproduction tests either in soil or not, the differences between groups were assessed. The groups represented the different concentrations of the tested compounds. Depending on the number of compared groups and whether the samples were drawn from normally distributed populations or not, different tests had to be applied. An overview is given in Table 3.3-1.

In the choice test the distribution in the control had to be compared with the distribution in the samples with contaminated soil material on one side. Hence, two different groups and two different categories exist. The distribution of the groups is compared whether it falls in the same category as the control group. Depending on the number of observations different tests are applied, which are briefly outlined in Table 3.3-1.

Table 3.3-1: Overview of the used statistical tests

	conditions	**two groups**	**more than two groups**
comparison of groups	normal distribution and equal variance	unpaired t-test	ANOVA with Bonferroni t-test
	non-normal distribution and/or unequal variance	U-test	ANOVA on ranks with Dunn´s test
comparison of groups in different categories	five or more observations	Chi-square test	not applicable
	five or less observations	Fisher exact test	not applicable

Table 3.3-2: Overview of the biotest-systems with *E.* crypticus and *F. candida*

species	*Folsomia candida*				*Enchytraeus crypticus*			
test	**contact test**	**choice test**	**mortality test**	**reproduction test**	**water test**	**choice test**	**mortality test**	**reproduction test**
purpose of test	mortality	choice behaviour	mortality	reproduction	mortality	choice behaviour	mortality	reproduction
test length	24, 48, 72 h	2 d	7 d	28 d	24, 48, 72 h	2 d	7 d	28 d
validity	mortality: > 20%	distribution between sides	mortality: > 20%	repro rate: < 50%	mortality: > 20%	distribution between sides	mortality: > 20%	repro rate: < 50%
age of test animals	30 - 40 d after hatching		24 -27d after hatching		30 - 40 d (20-33 d after cocoon placement		24 - 27d (14-21 d after cocoon placement)	
number of test animals	5 per sample	10/20 per sample	10 per sample		5 per sample	10/20 per sample	10 per sample	
number of samples	5 per concentration				5 per concentration			
test vessels	scintillation	sputum	100 mL Duran flasks, closed with Teflon-lined screw caps		scintillation	sputum	100 mL Duran flasks, closed with Teflon-lined screw caps	
airing	none	none	weekly		none	none	weekly	
soil per sample (fw)	n.a.	10 g per side	15 g	30 g	n.a.	10 g per side	15 g	30 g
soil material	n.a.	Lufa 2.2			n.a.	Lufa 2.2		
soil humidity	n.a.	60 % MWC			n.a.	60 % MWC		
feeding	none	none	40 mg dry yeast at the beginning	40 mg dry yeast at the beginning and after 14 days	none	none	0.5% ground oats mixed into the soil	2% ground oats mixed into the soil
test temperature	15 ± 2 °C	20 ± 2 °C			15 ± 2 °C	20 ± 2 °C		
light	permanent darkness	natural daylight			permanent darkness			
positive control [mg Betanal/kg soil (dw)]	none	avoidance: ≥ 22 escape: ≥ 108[2)]	LC50(7d): 95.5 ± 31.8 EC50(28d): 57.3 ± 19.1[1)]		none	avoidance: ≥ 127[3)]	LC50(7d): 573.3 ± 114.6 EC50(28d): 222.9 ± 95.5[1)]	

1) (WARNECKE et al, 2001) values converted from active ingredient Phenmedipham to substance Betanal
2) (Achazi et al, 2001) values converted from active ingredient Phenmedipham to substance Betanal
3) (WAGENR-VASKE, 2000) values converted from active ingredient Phenmedipham to substance Betanal
n.a. not applicable