4 Pure substances

4.1 Abstract

The toxicity of the explosives TNT, Hexyl, Hexogen and Octogen as well as of TAT, the end product of the reductive microbial degradation of TNT, was evaluated in the standard soil material Lufa 2.2 for two terrestrial invertebrates: the collembola *F. candida* and the enchytraeid *E. crypticus*. For that three different tests were used: a mortality, a reproduction and a choice test with the corresponding test parameters mortality, reproduction and choice of uncontaminated or contaminated soil material.

A comparison between the mortality and the reproduction test showed the reproduction test to be the more sensitive test for both animals. In these tests *F. candida* was more sensitive to TNT than *E. crypticus*. In the case of TAT only the enchytraeid showed a significant reduction of the reproduction, but on neither of the test species the TNT-metabolite had a lethal effect. Hexyl, too, was not lethal to *F. candida*, but it was to *E. crypticus.* It reduced the reproduction of both species, even more for the collembola than the enchytraeid, which is thought to be the effect of a high mortality of the juveniles. The choice test was the most sensitive test for *E. crypticus.* However, only for Hexyl a significant choice behaviour could be detected with *F. candida*, indicating that the animals have no perception of the other compounds.

Since the enchytraeid seemed to be affected in the mortality tests with TNT and Hexyl, the fertility of these animals was further investigated on agar-agar. For both substances the cocoon placement and the hatching rates were reduced at high concentrations.

TNT was much more toxic than any of the other substances. As it was also the most widely used explosive during the First and Second World War, it must be considered as the one causing the highest ecological risk. The following order of toxicity can be given for the collembola- and enchytraeid-biotest in the standard soil material Lufa 2.2:

F. candida: TNT > Hexyl > TAT/Hexogen/Octogen

E. crypticus: TNT > Hexyl > TAT > Hexogen/Octogen

4.2 Theoretical background

Explosives are compounds that undergo rapid burning or decomposition resulting in the generation of large amounts of gas and heat and the consequent production of sudden changes in pressure (F & W: 2001). They can be either pure compounds or mixtures. The pure compounds are usually subdivided into three groups according to their use (YINON, 1981: 1-3):

- Primary explosives: also called initiators are highly sensitive to mechanical shock, friction and heat. They are readily ignited by direct contact with flame or electrical sparks (eg lead azide).
- High explosives: explosions proceed with extreme rapidity and are transmitted instantaneously throughout the substance with the release of a great amount of heat. They are not readily detonated by heat, flame or shock, but rather under the influence of the shock of an exploding primary explosive. Examples are, apart from TNT, Hexyl, Hexogen, Octogen tested in this thesis, Picric acid (3,4,6-Trinitrophenol) or Nitroglycerin (Glycerol trinitrate).
- Non explosive ingredients: like stabilisers and plasticisers.

This thesis examines high explosives, which can be further subdivided into three groups according to their structure. Nitro compounds are characterised by the carbon-nitrogen bond (C-NO_2). Nitrate esters are substances in which the nitro group is attached to the carbon atom via an oxygen atom (C-O-NO_2). In nitramines a nitro group is bound to a nitrogen atom (N-NO_2) (YINON, 1981: 4-20). The ecotoxicity of two representatives of the aromatic nitro compounds, TNT and Hexyl, is investigated together with two representatives of the nitramines, Hexogen and Octogen. No nitrate esters were included in the investigation, since they are much more likely to explode under the influence of heat, friction or light. Consequently the handling of these compounds would have been too dangerous in an ordinary laboratory. The investigated explosives are all considered to be relevant in the evaluation of military waste.

4.2.1 TNT

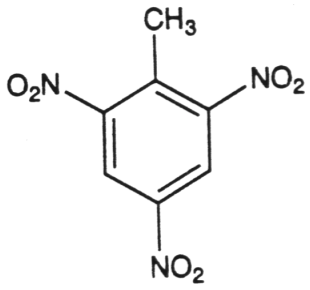


Fig. 4.2-1: Structural formula of TNT (MERCK-INDEX, 1996).

The high explosive 2,4,6-Trinitrotoluene, commonly known as TNT, is a yellow, odourless solid, which does not occur naturally in the environment. It has a high explosive power with a detonation velocity of 6900 m/s, which is the rate with which a detonation propagates in an explosive (KÖHLER & MEYER, 1995). TNT is used as an explosive in military shells, bombs and grenades as well as in industrial and underwater blastings (ATSDR, 1996b). A summary o the properties of the explosive is given in Table 4.3-1.

TNT was first synthesised in 1863 by J. Wilbrand and has been manufactured since 1900 in a very simple and relatively safe process. Due to its high chemical stability and low sensitivity to impact and friction, TNT is relatively safe to handle. Hence, it became the most important explosive during both World Wars and in Germany alone 300 000 t/a were manufactured during World War II (MARTINETZ & RIPPEN, 1996: 334):

> "The outstanding advantages of TNT like its low sensitiveness to impact and friction, its safe handling, its considerable safety in storage, its relative safety in manufacture and its relatively high explosive power, have made TNT the most widely used of all high explosives since the beginning of the twentieth century up to the present time" (URBANSKI, 1964: 321).

TNT may enter the environment through waste water and solid waste from the manufacturing stage and its processing up to the recycling through the destruction of bombs and grenades. During the production many by-products are formed, which are released into the environment with the waste water. So far up to 30 different nitro-aromatics have been identified (ROSENBLATT, 1991: 208). For TNT it is known that sorption into the soil occurs and that biological degradation is possible, but not easy. In surface waters it is rapidly broken down by sunlight. However, there is always the danger of TNT reaching and polluting the groundwater through soil or in surface water.

For humans, exposure can occur through eating, drinking, touching or inhaling of contaminated soil, water, food or air. After oral intake irritations of the gastrointestinal system like vomiting and diarrhoea have been observed. The following symptoms have been registered after intake through the skin: headaches, dizziness, sickness, weakness, restlessness, cyanoses, breathing difficulties, methaemoglobin-anaemia, dark brown urine, a drop in blood pressure, unconsciousness, coma and occasionally tremors. Long-term side effects include anaemia, hepatitis, jaundice, liver atrophy and damage of the kidneys. A chronic exposure to TNT gives rise to the same symptoms, but also depressions, rashes with lesions, pain in the muscles and cataracts (MARTINETZ & RIPPEN, 1996: 336).

Due to its similarity in structure to known carcinogens TNT is considered as one. In the USA ist is classed C, a possible human carcinogen, and in Germany III B, resulting from well-founded suspicion for carcinogen potential. No evidence for human carcinogenicity can be given, but in rats urinary bladder papilloma and carcinoma were detected (IRIS, 1998). In an Ames-test its mutagenic potential was found to be positive (MARTINETZ & RIPPEN, 1996: 337).

In the mammalian system TNT is transformed into the principal metabolites 2-amino-4,6-dinnitrotoluene (2A46DNT) and 4-amino-2,6-dinitrotoluene (4A26DNT), small amounts of 2,4-diamino-6-nitrotoluene (24DA6NT) and traces of 2,6-diamino-4-nitrotoluene (26DA4NT). These

metabolites were found in the urine of munitions workers, the blood of rabbits and the urine of rats (ROSENBLATT, 1991: 216).

The following threshold limits are recommended in Germany and the USA (MARTINETZ & RIPPEN,1996: 337, 621):

- Acceptable daily intake (ADI): 0.28 mg/kg (bw)[1] day
- Threshold limit value (TLV): Germany: 0.01 ppm; 0.1 mg/m^3
  USA: 0.5 mg/m^3
- Drinking water: Germany: 1.0 µg/L based on toxicological reasons
  USA: 0.020 mg/L drinking water equivalent level (DWEL).

4.2.2 Hexyl

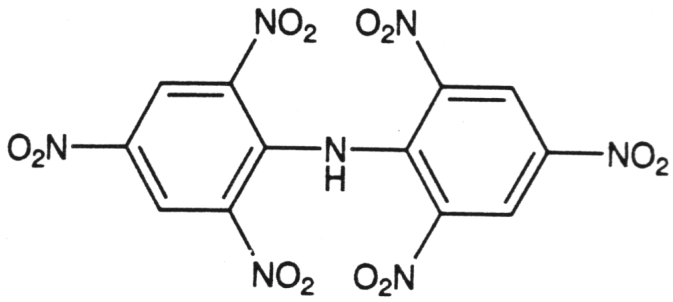


Fig. 4.2-2: Structural formula of Hexyl (MERCK-INDEX, 1996).

Hexyl is chemically named 2,2´,4,4´,6,6´-hexanitrodiphenylamine, but also known as dipicrylamine, hexamine, hexite or hexanitrodiphenylamine. In an often used mixture with TNT it is called hexanite. Hexyl is a brightly yellow solid, but turns brownish under the influence of sunlight. It was first discovered in 1874 by P.T. Austin and R. Gnehm and already used in World War I by the German military as an underwater-explosive. As an explosive it is superior to TNT (MARTINETZ & RIPPEN, 1996: 348) with a higher detonation velocity of 7200 m/s (KÖHLER & MEYER, 1995). The properties of the explosive are summarised in Table 4.3-1.

Hexyl can enter the environment in the same way as TNT. Long persistency in the soil and ground water are expected as well as a slow migration through soil due to strong sorption (MARTINETZ & RIPPEN, 1996: 350).

Upon contact it causes irritation of the eyes, the membranes of the respiratory organs, the lungs and the skin. The results of chronic exposure are an orange-colouring of the hair, rashes or even long-lasting eczemas, chronic headaches and weakness. It has been found to be carcinogenic leading to skin cancer in animal tests with rats and mutagenic for Salmonella in the Ames-test (MARTINETZ & RIPPEN, 1996: 349). No recommended threshold values have been found.

[1] (bw) body weight

4.2.3 Hexogen (RDX)

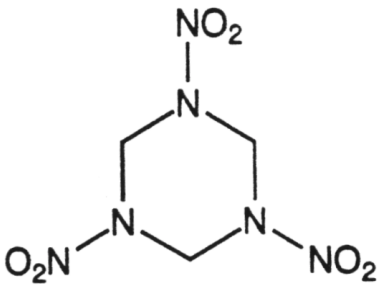


Fig. 4.2-3: Structural formula of Hexogen (MERCK-INDEX, 1996).

The chemical compound hexahydro-1,3,5-trinitro-1,3,5-triazine is also called cyclotrimethylenetrinitramine, but commonly known as Hexogen in Germany, cyclonite in the USA, T4 in Italy or RDX in Britain, an acronym for *R*esearch *D*epartment or *R*oyal *D*emolition *eX*plosive (MEGALOMANNIA: 2000c). It is a white powder with no known taste or odour (ATSDR: 1996a). Hexogen was first synthesised by Lenze in 1897, but industrial manufacturing was not developed until World War II (RÖMPP, 1995). Its explosive power surpasses that of TNT with a detonation velocity of 8750 m/s (KÖHLER & MEYER, 1995). In addition it has a high chemical stability, but lower than nitro compounds (URBANSKI, 1964: 77). More properties of the explosive are listed in Table 4.3-1.

Hexogen was widely used during World War II as a constituent of explosive mixtures with great explosive power, especially in mixtures with TNT called Hexolite or Composition B (URBANSKI, 1964: 77). In Germany the mean production per month during World War II thus reached 2800 t (MARTINETZ & RIPPEN, 1996: 351). Today Hexogen is the most important military high explosive in the USA and has thus replaced TNT. It is formulated into munitions either alone as Composition A or in combination with molten TNT as Composition B (ROSENBLATT, 1991: 198/199) or with plasticisers as C-4 or Semtex (MEGALOMANNIA: 2000b). As an impurity it contains Octogen, which is produced during the manufacturing process (ROSENBLATT, 1991: 198/199).

Hexogen can enter the environment similarly to TNT during its manufacture, processing or its destruction at open-burning or incineration sites. It migrates slowly in soils and strongly resists biodegradation (ROSENBLATT, 1991: 208), however in surface water it can easily be broken down photochemically (ROSENBLATT, 1991: 211).

In case of toxification the central nervous system is affected and produces convulsions and/or unconsciousness. Early symptoms include headache, dizziness, nausea and vomiting, weakness, thirst and sweet tasting (MARTINETZ & RIPPEN, 1996: 353). Recovery time for humans has been within days or at worst several months (ROSENBLATT, 1991: 217). Hexogen is metabolised in the liver into unidentified metabolites. The half life is about 24-30 hours

(ROSENBLATT, 1991: 215). Due to limited evidence Hexogen has been classified as a class C carcinogen – a possible human carcinogen in the USA (ROSENBLATT, 1991: 218).

The following threshold limit values have been recommended in the USA and in Germany (MARTINETZ & RIPPEN, 1996: 355, 658):

- ADI: 3 µg/kg bw d
- TLV: USA: 1.5 mg/m^3
- Drinking water: USA: 0.10 mg/L DWEL (ROSENBLATT, 1991: 224)
  2µg/L (ASTDRb: 2001).

4.2.4 Octogen (HMX)

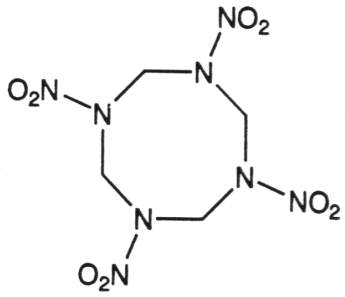


Fig. 4.2-4: Structural formula of Octogen (MERCK-INDEX, 1996).

Octogen is chemically named octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine and in English usually referred to as HMX, the short form of *H*igh *M*elting e*X*plosive or *H*er *M*ajesties e*X*plosive, depending on the country (MEGALOMANNIA, 2000b). It exists in four polymorphous forms, but is usually obtained in the ß-form, a colourless solid with a not known taste or smell, which is the form least sensitive to impact (URBANSKI, 1964: 117). Its chemical stability is superior to Hexogen, and it has also the superior properties as an explosive due to its higher ignition temperature (URBANSKI,1964: 118) and its higher detonation velocity of 9100 m/s (KÖHLER & MEYER, 1995). An overview of the properties of the explosive is given in Table 4.3-1.

It is manufactured by a modification of the Hexogen-synthesis and contains Hexogen as an acceptable impurity (ROSENBLATT, 1964: 199). Today, it is increasingly being used as a propellant and in maximum-performance explosives (ROSENBLATT, 1991: 199).

It does not occur naturally and may of course enter the environment in the same ways as TNT or Hexogen. Its microbial degradation is possible, but not easy. In surface water photochemical degradation takes place, but 2-3 times slower than for Hexogen (ROSENBLATT, 1991: 214).

No acute poisoning is known, but similar effects as for Hexogen have been expected. In animal tests chronic effects on the liver, spleen and kidney could be detected, but no evidence of poisoning in humans could be found (MARTINETZ & RIPPEN, 1996: 357). In higher doses it has similar effects on the central nervous system of mammals as Hexogen (ROSENBLATT, 1991:

218). It is classed as a carcinogen class D, thus not considered to be carcinogenic due to lack of animal evidence (ROSENBLATT, 1991: 224).

The following threshold values have been recommended (MARTINETZ & RIPPEN, 1996: 354, 359):

- ADI: 3 µg/kg bw day
- TLV: USA: 1.5 mg/m^3
- Drinking water: USA.: 1.8 mg/L DWEL (ROSENBLATT, 1991: 224)
  0.4 mg/L (ATSDRa: 1997).

4.2.5 Triaminotoluene (TAT)

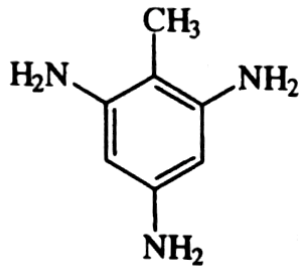


Fig. 4.2-5: Structural formula of TAT (MARTINETZ & RIPPEN, 1996: 768).

Triaminotoluene (TAT) is the end product of a strictly anaerobic reduction of TNT by microorganisms (RIEGER & KNACKMUSS, 1995: 2). Hardly anything is known about the substance itself and no use is known. It can only be obtained as an analytical standard and even the attached material safety data sheet does not provide any useful information. In addition, the substance is unstable under aerobic and under many anaerobic conditions. Thus, it undergoes acid hydrolysis in three hours at pH 3, but remains stable over the same time period at pH 6 (MARTINETZ & RIPPEN, 1996: 775).

So far TAT has not been detected in soil as a free substance, only in soil-free systems, due to its high ability to adsorb to the soil matrix. It can even be assumed that TAT is bonded by a nucleophilic addition to the carbonyl functions or the C-C double bonds of the quinoid structures of humic substances (DAUN et al, 1998). A study with radioactively labelled TNT proved that after aerobic degradation only 2% of the radioactivity could be extracted, whereas 98% was bound to the soil (ACHTNICH et al, 1999).

4.3 Materials and methods

4.3.1 Contaminants

The explosives TNT, Hexogen and Octogen (technical grades) used in the tests were obtained from the Bundesanstalt für Materialforschung (BAM, Germany). The purity of TNT was more than 99% according to BAM and no other compounds could be detected by high pressure liquid chromatography (HPLC). Hexogen contained 5% wax and Octogen was kept under water. 2,4,6-TAT in the form of 2,4,6-triaminotoluene hydrochloride (analytical reference) was purchased from Promochem (Wessel, Germany). At delivery it already had a red-brown colour, indicating a possible transformation process. An overview of the properties of the explosives is given in Table 4.3-1.

Table 4.3-1: Properties of explosives

	TNT	Hexyl	Hexogen	Octogen
chemical abstracts services registry number (CAS)[2)]	118-96-7	131-73-7	121-82-4	2691-41-0
gross formula[1)]	$C_7H_5N_3O_6$	$C_{12}H_5N_7O_{12}$	$C_3H_6N_6O_6$	$C_4H_8N_8O_8$
molecular weight[1)]	227.1	439.2	222.1	296.2
density [g/cm^3][1)]	1.64	1.64	1.82	1.96 (ß-form)
melting point [°C][1)]	80.8	240-241 under de-composition	204	282
energy of formation [kJ/kg][1)]	- 219.1	162.1	400.2	353.8
specific energy [kJ/kg][1)]	908	1098	1370	1366
heat of explosion H_20 fl [kJ/kg][1)]	3725	4042	5625	5601
detonation velocity [m/s][1)]	6900	7200	8750	9100
impact sensitivity [Nm][1)]	15	7.4	7.4	7.4
friction sensitivity [N pistil load][1)]	up to 353 no reaction	up to 353 no reaction	120	120
aqueous solubility [mg/L, 25°C][2)]	150	11	60	5
organic solvent[2)]	acetone	n.a.	acetone slightly	acetone slightly
log K_{OW}[2)*]	1.6 - 2	not researched	0.8 - 1.6	0.1 – 0.3
K_{OC}[2)]**	470-1600	not researched	60-100	(3.5)

1) Köhler & Meyer, 1995
2) Martinetz & Rippen, 1996
n.a. not applicable
* partitioning coefficient between water and 1-octanol
** partitioning coefficient between organic carbon and water

For the application TNT was dissolved in acetone and poured onto purified quartz sand (2% soil (dw)). The spiked sand was mixed into standard soil material Lufa 2.2 after the acetone had evaporated in a fume cupboard. To correct possible toxic effects of the solvent, the control

assays were treated in the same way. The other explosives and TAT were mixed into the soil material as dry solids. The necessary amount of water was then added to adjust the MWC to 60% and was mixed thoroughly with an electric mixer. Various concentrations were obtained by mixing uncontaminated control soil material with the highest contaminated one. Unless stated otherwise the contaminated soil materials were immediately used for the tests.

4.3.2 Tests

The mortality, reproduction and choice tests were carried out with all contaminants as already described (see p. 5-8, chapter 3.3.1). If the surviving animals of an mortality test seemed to be affected or if the EC50 was very much lower than the LC50, the influence of the toxicant on the reproduction was further investigated.

In the case of *F. candida* the surviving animals from the mortality test were placed on uncontaminated soil material and after three weeks the reproduction was determined. The shorter test period was chosen to ensure that the adult animals originally introduced in the test could still be distinguished from the juveniles. The reproduction rate was then calculated by the number of adults found at the end of the test and not by ten as usually (see p. 7, chapter 3.3.1.1). This was useful since the possibility of damage to the animals during the transfer from the contaminated soil material to the uncontaminated soil material could not be ruled out. The reproduction rates were statistically evaluated with a one way ANOVA (see p. 9, chapter 3.3.3) using the programme SigmaStat and graphically illustrated with SigmaPlot software.

In the case of *E. crypticus* the surviving animals were transferred onto agar-agar. Thus the cocoon placement could be observed directly. The freshly laid cocoons were placed on a fresh agar-agar plate in order to observe the hatching. The cocoon rate was calculated by dividing the number of cocoons laid in a day by the number of adults of the day before. For the hatching rate, cocoons found empty were considered as hatched. Their number on a certain is represented the percentage of the number of cocoons laid in this concentration over the whole observation period. For a better handling of the data and to minimise the possibility that cocoons had been overlooked the results from three consecutive days were combined for the calculation of the cocoon and the hatching rates.

The cocoon rate was statistically estimated with a one way ANOVA using SigmaStat software (see p. 9, chapter 3.3.3) looking for a difference in time and concentrations. Since no replicates were tested for the hatching, no statistical test could de applied. The graphs were drawn using SigmaPlot software.

Non-soil tests like the contact or water test could only be used with substances soluble in water or a suitable solvent. Thus they were only applicable for TAT and TNT (see p. 18, Table 4.3-1).

4.4 Results

4.4.1 TNT

4.4.1.1 Mortality and reproduction test

In order to assess the toxicity of the explosive TNT mortality and reproduction tests were carried out. TNT increased the mortality and reduced the reproduction rates in both test systems (Fig. 4.4-1). In the collembola-biotest with *F. candida* the LC50(7d) was 139.9 ± 9.4 mg TNT/kg (four tests) and the EC50 (28d) was 64.3 ± 22.4 mg TNT/kg (five tests). The LC50(7d) in the enchytraeid-biotest with *E. crypticus* was at 949,9 ± 617.8 mg TNT/kg (three tests) and the EC50 (28) was 501.2 ± 278.3 mg TNT/kg (three tests). The concentration-effect relationships show only one representative test (Fig. 4.4-1) of the various ones used for the calculation of the 50%-value. In case of the enchytraeid, however, the tests varied very much, which is demonstrated by the high standard deviation (SD).

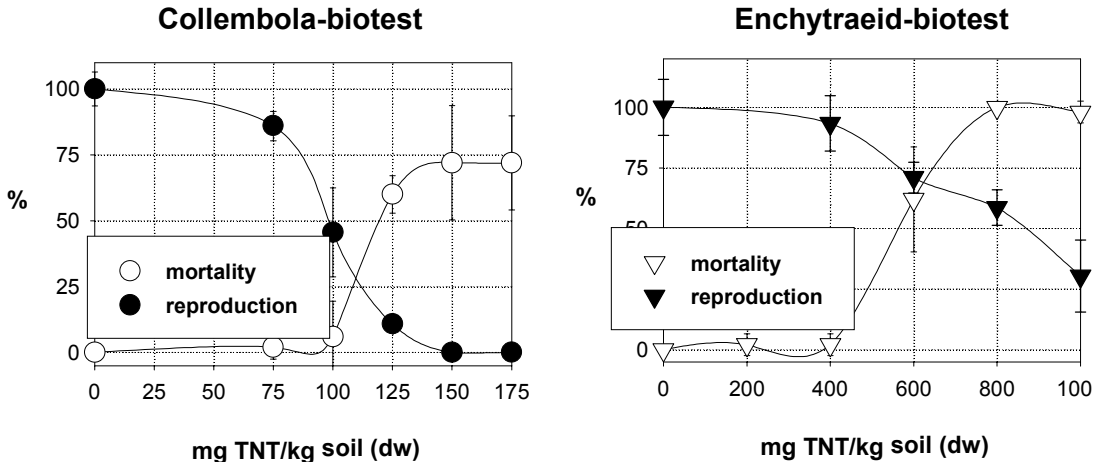


Fig. 4.4-1: Concentration-effect relationship for TNT in standard soil material Lufa 2.2. The data for mortality and reproduction are given in relation to the values in the standard soil material Lufa 2.2. Symbols: mean ± SD.

In the mortality tests with *E. crypticus* it was evident, that the worms became smaller with an increasing concentration of TNT. For the demonstration of this effect a mortality test was chosen, in which some animals had survived very high concentrations (Fig. 4.4-2).

0 mg/kg 100 mg/kg 200 mg/kg 800 mg/kg 1600 mg/kg

Fig. 4.4-2: Surviving *E. crypticus* of a mortality test with various concentrations of TNT-contaminated soil materials.

4.4.1.2 Reproduction of contaminated *E. crypticus*: cocoon and hatching rate

The observed decrease in size of the surviving enchytraeids of the mortality tests was an obvious effect of the toxicant, which might have an influence on their fertility. To analyse the effect on the reproduction the surviving animals from a mortality test were transferred onto agar-agar and the cocoon placement was directly observed. The freshly laid cocoons were placed on a new agar-agar plate in order to observe the hatching. The cocoon placement and the hatching of the worms transferred from the uncontaminated soil material were taken as control (see p. 19, chapter 4.3.2).

Cocoon rate

The cocoon rate was calculated by dividing the number of cocoons laid on a day by the number of adults from the day before (see p. 19, chapter 4.3.2). For a better handling of the data and to minimise the possibility that cocoons had been overlooked the results from three consecutive days were combined for the cocoon rates. Thus two different observation periods were distinguished: 1-3 days and 4-6 days after the transfer. The mortality of the adult worms was not significantly different neither in time nor concentration. For each concentration five replicates were tested.

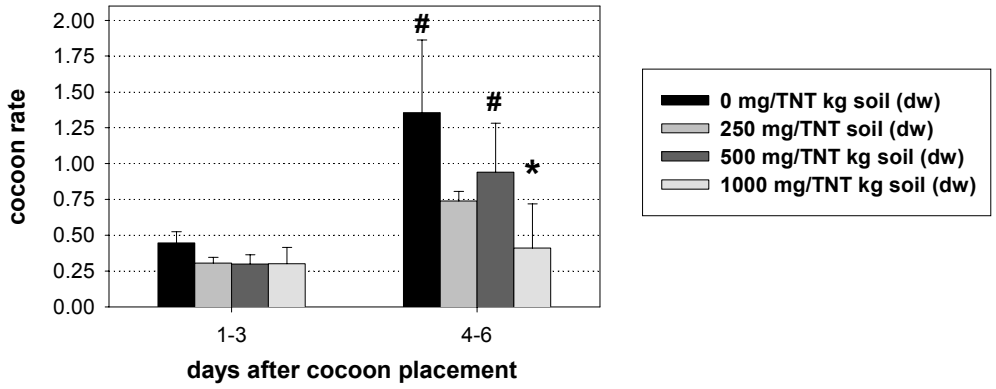
Table 4.4-1: Cocoon rates for *E. crypticus* transferred from different concentrations of TNT-contaminated soil materials onto agar-agar

mg TNT/kg soil (dw)	days after transfer			
	1-3		4-6	
	mean	SD	mean	SD
0	0.446	0.077	1.356#	0.508
250	0.305	0.040	0.740	0.065
500	0.298	0.065	0.941#	0.341
1000	0.300	0.114	0.410*	0.309

* significant difference to control
\# significant difference to previous period

In all the tested concentrations more cocoons were laid in the second period of 4-6 days than in the first period 1-3 days after the transfer. The difference however, was only statistically significant for the control and for 500 mg TNT/kg soil (dw). The cocoon rate of the control was in both periods higher than the rates of the worms from the contaminated soil materials.

At the first period the cocoon rate from all contaminated assays was about the same, whereas for the second period of 4-6 days after cocoon placement it became heterogeneous. The highest value was obtained for 500 mg TNT/kg, followed by the lowest tested concentration of 250 mg TNT/kg soil (dw). For the highest tested concentration of 1000 mg TNT/kg soil (dw) the cocoon rate was found to be the lowest and was then placement the significantly lower than in the control.



* significant difference to control from the same period of cocoon placement
\# significant difference to cocoons placed at 1-3 days after transfer

Fig. 4.4-3: Cocoon rates for *E. crypticus* transferred from different concentrations of TNT-contaminated soil materials onto agar-agar. Bars mean ± SD.

Hatching rate

For the hatching rate, cocoons found empty were considered as hatched. (see p. 19, chapter 4.3.2). Their number on a certain day was divided by the number of cocoons laid in this concentration over the whole period, which was set as 100%. For the cocoon rates the results from three consecutive days were combined for the calculation of the hatching rates in order to minimise the possibility that cocoons had been overlooked and to ensure a better handling of the data. The hatching rate was only observed for the cocoons of the first batch 1-3 days after the cocoon placement. Since no replicates were tested for the hatching, no statistical test could be applied. As control served the hatching rates of the animals transferred from the uncontaminated soil material.

Table 4.4-2: Hatching rates for the cocoons laid 1-3 days after the transfer of adult *E. crypticus* from different concentrations of TNT-contaminated soil materials

mg TNT/kg soil (dw)	days after cocoon placement			
	5-7	6-8	7-9	8-10
0	24.6%	52.5%	68.9%	73.8%
250	38.1%	66.7%	69.0%	73.8%
500	2.4%	41.5%	56.1%	65.9%
1000	4.8%	9.5%	23.8%	31.0%

Hatching started in all concentrations 5-7 days after the cocoons had been laid and a convergence to 100% was observed. However, even 8-10 days after cocoon placement 100% had not been reached in any of the samples including the control. At the first of observation period the hatching rate of the lowest tested concentration of 250 mg TNT/kg soil (dw) was remarkably higher than in the corresponding controls, which reached the same level just 7-9 days after the cocoons were laid. On the other hand the hatching rates for the higher concentrations were very much lower than those in the control, especially at 5-7 days after cocoon placement. For the highest concentration of 1000 mg TNT/kg soil (dw) hatching

remained relatively low compared to the other concentrations until the end of the observation period, whereas for 500 mg TNT/kg soil (dw) nearly level values with the control at 6-8 were observed.

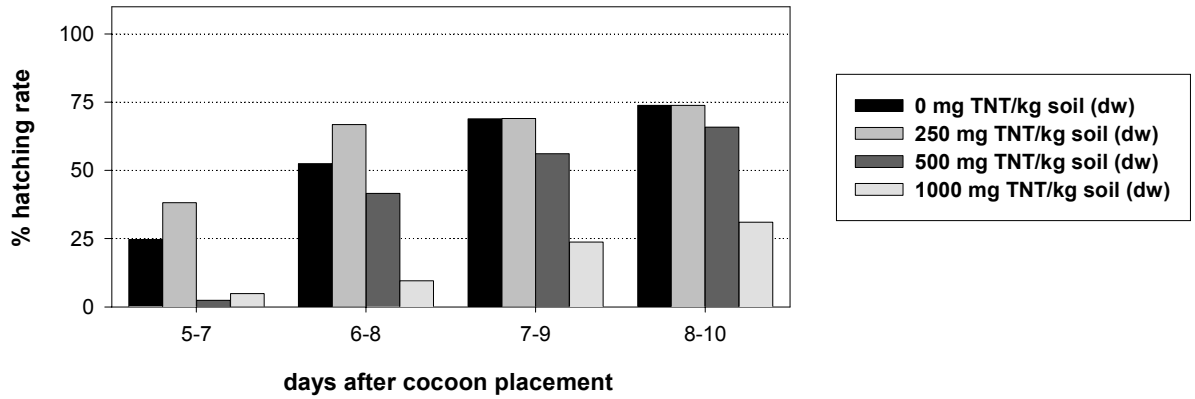


Fig. 4.4-4: Hatching rates for the cocoons laid 1-3 days after the transfer of the adult *E crypticus* from TNT-contaminated soil materials. Bars: mean.

4.4.1.3 Non-soil tests

To evaluate the toxicity of TNT without the influence of soil for *F. candida* a contact test (see p. 8, chapter 3.3.2.1. and for *E. crypticus* a water test with artificial fresh water was performed (see p. 8, chapter 3.3.2.2). The mortality was determined after 24, 48 and 72 hours, respectively.

The highest tested concentration in the water test was restricted by the low water solubility of TNT of 150 mg TNT/L (see p. 18, Table 4.3-1). Thus the mortality was too low determine a LC50-value after 24 hours.

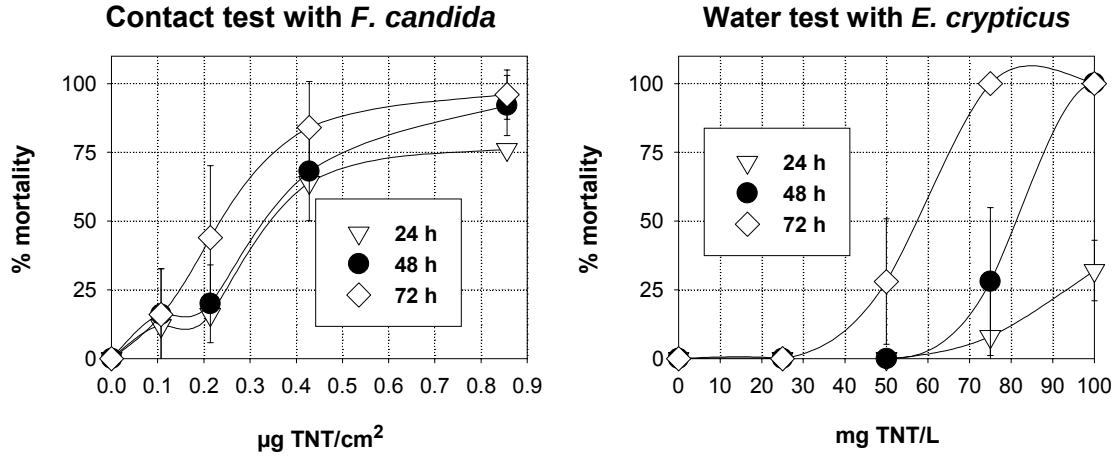


Fig. 4.4-5: Concentration-effect relationship for TNT in the contact test with *F. candida* and in the water test with *E. crypticus.* Symbols: mean ± SD.

From the graphs the LC50-values were determined for the contact test with *F. candida* and the water test with *E. crypticus*.

Table 4.4-3: LC 50 values of TNT for *F. candida* in the contact test and *E. crypticus* in the water test after 24, 48 and 72 hours

F. candida			***E. crypticus***		
LC 50 contact test in µg/cm²			**LC 50 water test in mg/L**		
24 h	**48 h**	**72 h**	**24 h**	**48 h**	**72 h**
0.735	0.700	0.500	> 100	83	58

4.4.1.4 Choice test

In this test system the animals were given the choice between contaminated and uncontaminated soil material during a test period of two days. Either the animals would migrate into the contaminated soil material or escape from in, thus indicating an attractant or repellent effect of the contaminant. Two different test sets were used:

- combined test set: 10 animals on each side
- separate test set: 10 animals on side with uncontaminated soil material of five test vessels (attraction or avoidance)
  10 animals on side with contaminated soil material of five different test vessels (escape)

The combined test set gives an overview about the possible reactions, whereas a certain choice behaviour can only be distinguished with the separated test set (see p. 7, chapter 3.3.1.2).

For the statistical evaluation with a Chi-square test (see p. 9, chapter 3.3.3) the sum of all replicates of a tested concentration was evaluated to reduce the effect of outliers. Graphs are only given if a significant choice behaviour was observed. The percentages of all the animals were calculated for each replicate. In the graphs the mean and the SD of the percentages are represented only for the uncontaminated sides.

With TNT separate tests were carried out with both test species as results obtained from the combined tests were not very clear.

Table 4.4-4: Distribution of *F. candida* in a separated test set with TNT; the side on which the animals were placed is indicated by shading; n 5

		% animals found on		
	mg TNT/kg soil (dw)	**uncontaminated side**	**contaminated side**	**result**
animals placed on unconta-minated side	**0**	61	39	**no significance**
	100	76	28	
	150	80	20	
	200	66	34	
	250	61	39	
animals placed on conta-minated side	**0**	28	72	**no significance**
	100	33	68	
	150	43	57	
	200	52	48	
	250	31	69	

In the separated test set the collembola showed up to 250 mg TNT/kg soil (dw) no significant choice behaviour, neither indicating an attraction for nor a repellence of the TNT-contaminated soil material.

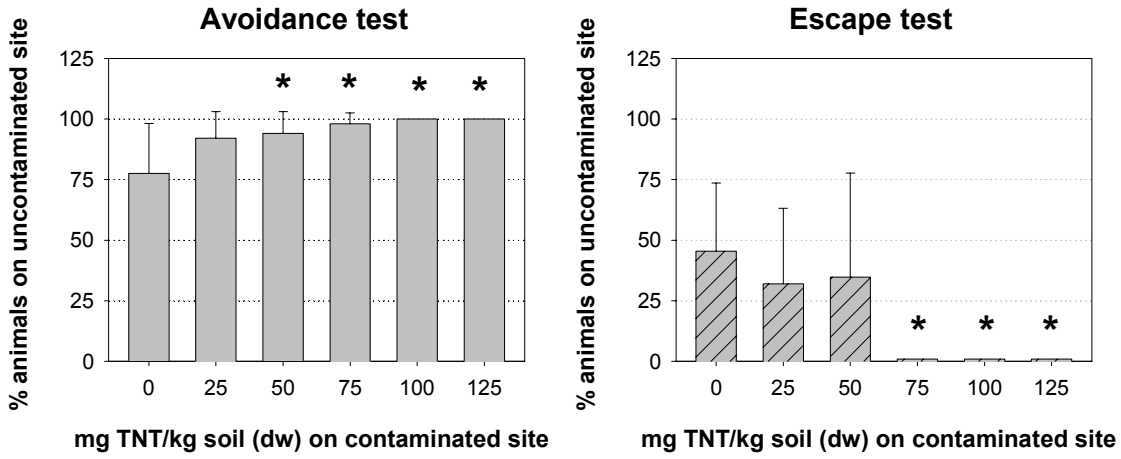
In the case of *E. crypticus* the combined test indicated that the animals avoided high TNT-concentrations. On the other hand animals, which had been placed on highly contaminated soil materials, were not able to leave. They were found in a bundle in the same manner as they had been introduced. Transferred into water they were again able to move, even though they had previously been unable to dig themselves into the soil material. Therefore a separated test set was performed.

Table 4.4-5: Distribution of E. crypticus in a separated test set with TNT; the side on which the animals were placed is indicated by shading; n 5

		% animals found on		
	mg TNT/kg soil (dw)	**uncontaminated side**	**contaminated side**	**result**
animals placed on unconta-minated side	**0**	77	23	
	25	92	8	
	50	94	6	**avoidance**
	75	100	0	**avoidance**
	100	100	0	**avoidance**
	125	100	0	**avoidance**
animals placed on conta-minated side	**0**	45	55	
	25	32	68	
	50	34	66	
	75	0	100	**no escape**
	100	0	100	**no escape**
	125	0	100	**no escape**

In a separated test set it became obvious that the animals significantly avoided concentrations ≥ 50 mg TNT/kg soil (dw). However, no significant escape behaviour could be detected. In

concentrations ≥ 75 mg TNT soil (dw) the animals were unable to leave the contaminated soil material.



* significant differene in distribution to control

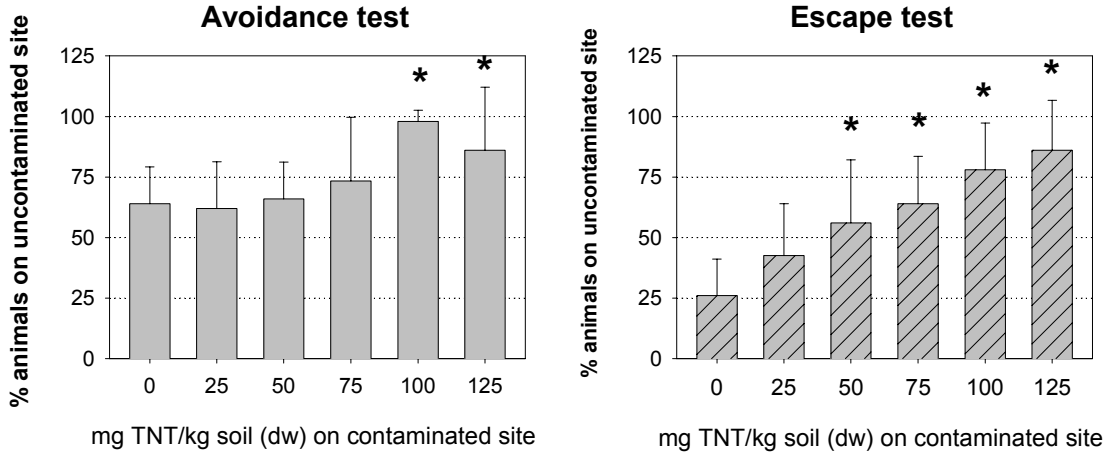
Fig. 4.4-6: Distribution of *E. crypticus* in a separated test set of a choice test with TNT. Bars mean ± SD.

Since the animals were not able to escape in the higher concentrations the same test was performed after the soil material had been stored for 7 days. Substances are adsorbed by the soil matrix, leading the toxicity of substances in soil to decrease with time.

Table 4.4-6: Distribution of *E. crypticus* in a separated test set with TNT after an ageing period of 7 days; the side on which the animals were placed is indicated by shading, n 5

		% animals found on		
	mg TNT/kg soil (dw)	**uncontaminated side**	**contaminated side**	**result**
animals placed on unconta-minated side	**0**	64	36	
	25	62	38	
	50	66	34	
	75	73	27	
	100	98	2	**avoidance**
	125	85	15	**avoidance**
animals placed on conta-minated side	**0**	26	74	
	25	39	61	
	50	56	44	**escape**
	75	64	36	**escape**
	100	78	22	**escape**
	125	86	14	**escape**

After the ageing period the animals significantly avoided concentrations ≥ 100 mg TNT/kg soil (dw) and significantly fled from concentrations ≥ 50 mg TNT/kg soil (dw).



* significant differene in distribution to control

Fig. 4.4-7: Distribution of *E. crypticus* in a separated test set of a choice test with TNT after 7 days of ageing. Bars mean ± SD.

4.4.1.5 Summary of results with TNT

TNT influenced the mortality and the reproduction of the collembola. In a contact test the mortality was affected at much lower concentrations than in soil. No choice behaviour was observed for the collembola.

The mortality and the reproduction of the enchytraeid, too was affected by TNT. Again the mortality was affected at much lower concentrations in the water test than in soil. The surviving worms from contaminated soil materials in the mortality tests, however were much smaller than the ones from the uncontaminated soil material, indicating an effect on the animals below mortality. It was shown, that the cocoon placement of the worms from the contaminated soil material was lower than for worms transferred from the uncontaminated soil material. The hatching of these cocoons also remained lower. In the choice test the enchytraeid avoided contaminated soil material at concentrations much below the LC(50)- or EC(50)-values and was not able to escape from these soil materials. This changed after the soil material had been stored for 7 days, indicating a repellent effect of the contaminated soil material.

4.4.2 Hexyl

4.4.2.1 Mortality and reproduction test

The toxicity of Hexyl was assessed in a mortality and reproduction test with *F. candida* and *E. crypticus.* For the collembola no mortality was detected up to the highest tested concentration of 4000 mg Hexyl/kg soil (dw). The observed mortalities in the different Hexyl-contaminated soil materials are given in Table 4.4-7. In contrast, the EC50(28d) was relatively low with 175.6 ± 39.6 mg Hexyl/kg soil (dw) (4 tests), but the adults survived all tested concentrations.

Table 4.4-7: Mortality for *F. candida* with Hexyl; mean ± SD; n 5

mg Hexyl/kg soil (dw)	% mortality
0	13.3 ± 12.5
250	16.7 ± 17.0
500	10.0 ± 0.0
1000	13.3 ± 12.5
2000	10.0 ± 8.2
4000	6.7 ± 9.4

Because of the difference in toxicity for *F. candida* between the mortality and the reproduction test an additional test was carried out in order to check, whether the reproduction itself was affected.

In the enchytraeid-biotest the LC50(7d) was 2402.8 ± 56.2 mg Hexyl/kg soil (dw) (two tests) and the EC50(28d) was 530.4 mg Hexyl/kg soil (dw) (Fig. 4.4-8). The surviving animals of the mortality test seemed to be affected by the toxicant (Fig. 4.4-10). Thus additional tests for the cocoon placement and hatching were performed with contaminated enchytraeids as already described for TNT.

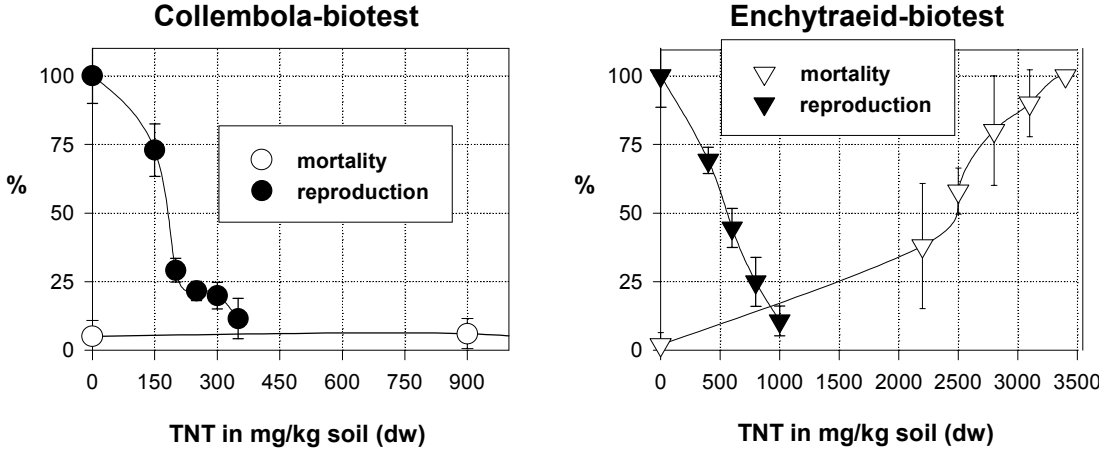


Fig. 4.4-8: Concentration-effect relationship with Hexyl for *F. candida* and *E. crypticus.* The data for mortality and reproduction are given in relation to the values in the standard soil material Lufa 2.2. Symbols: mean ± SD.

4.4.2.2 Reproduction of contaminated *F. candida*

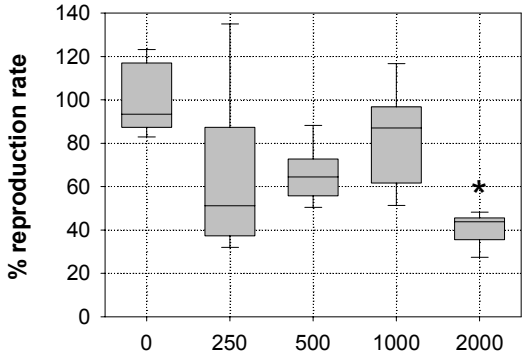
Since only the reproduction, but not the mortality of the collembola was affected by Hexyl, its effect on the reproduction was further investigated. For that the surviving animals of a mortality test were transferred onto uncontaminated soil materials and the reproduction determined after three weeks (see p. 19, chapter 4.3.2). The number of adults introduced or surviving the test as well as the reproduction rates were statistically evaluated with an one way ANOVA (see p. 9, chapter 3.3.3).

Table 4.4-8: Number of adults transferred from the Hexyl-contaminated soil materials onto the uncontaminated soil material, number of adults found after a test period of three weeks and the reproduction rate after 3 weeks; mean ± SD; n 5

mg Hexyl/kg soil (dw) in mortality test	number of adults introduced into reproduction test	number of adults surviving reproduction test	% reproduction rate after 3 weeks per surviving adults
0	8.6 ± 0.5	6.4 ± 0.8	100.6 ± 15.2
250	8.8 ± 0.4	6.2 ± 1.9	65.7 ± 37.2
500	9.0 ± 0.6	6.0 ± 1.1	65.6 ±12.7
1000	8.6 ± 0.5	4.4 ± 0.5	82.0 ± 22.5
2000	8.6 ± 0.8	6.4 ± 1.0	40.5 ± 7.3*

* significant difference to control

The mortality of the adults in the contaminated soil material did not differ significantly from each other. Neither was any significant difference in the number of adults found after three weeks in the reproduction test. The reproduction rate differed only significantly from the control for the highest tested concentration of 2000 mg Hexyl/kg soil (dw).

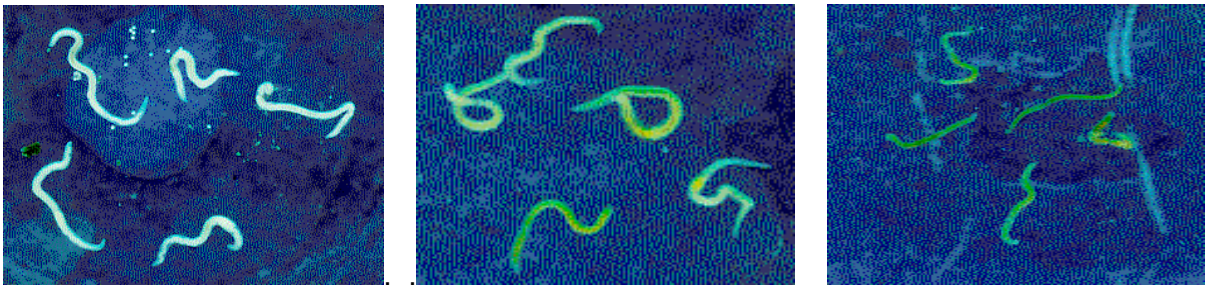


* siginificant difference to control

Fig. 4.4-9: Reproduction rates of *F. candida* transferred from a mortality test in various Hexyl-contaminated soil materials into uncontaminated soil material. Boxes represent median as a line with 25th and 75th percentile; error bars are 10th and 90th percentile.

4.4.2.3 Reproduction of contaminated *E. crypticus*: cocoon and hatching rate

In the mortality test with *E. crypticus* the animals were affected by Hexyl. In the contaminated soil materials the animals were smaller in size than in the uncontaminated control. In addition the clitella became yellow, indicating that the animals had taken up the brightly yellow toxicant. At higher concentrations even the whole animal turned yellow and became rigid (Fig. 4.4-10). In order to determine whether this uptake of Hexyl affected the reproduction, the cocoon and hatching rates of animals transferred from Hexyl-contaminated soil materials onto agar-agar were determined (see p. 19, chapter 4.3.2). As control served the animals transferred from the uncontaminated soil material.



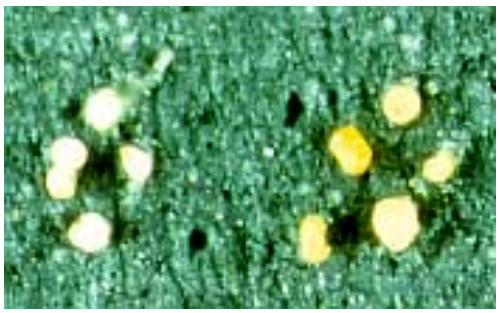
0 mg/kg 1800 mg/kg 2400 mg/kg

Fig. 4.4-10: Photos of surviving *E. crypticus* from a mortality test in various concentrations of Hexyl-contaminated soil materials.

For a better handling of the data and to minimise the possibility that cocoons had been overlooked the results from three consecutive days were combined for the cocoon as well as for the hatching rates. Three different observation periods were established: 1-3, 4-6 and 7-9 days after the transfer. The mortality of the adult worms differed not significant in time and concentration with the exception of 1000 mg Hexyl/kg soil (dw). In this concentration the mortality after six days differed significantly from the control and also from the mortality at the previous days. However, the worms from this concentration had been selected according to their appearance: white worms in the first replicate, those with a yellow clitella in the second and third and the completely yellow ones in the fourth. Although no significance in the mortality between the replicates was found, the possibility cannot be excluded, that the different treatment might have caused the significant differences, as they did not occur in the other concentrations.

Cocoon rate

For each concentration five replicates were tested, but only four for 1000 mg Hexyl/kg soil (dw) due to the lesser number of animals recovered from the contaminated soil material. The first cocoons laid by the yellow-coloured worms were yellow (see Fig. 4.4-10). After the yellow cocoons were laid, the worms with the yellow clitella became normally white again. The completely yellow worms lost their yellow colour slowly over the following two weeks.



control Hexyl

Fig. 4.4-11: Cocoons laid by worms out of the control and cocoons laid by worms out of Hexyl-contaminated soil material immediately after the transfer.

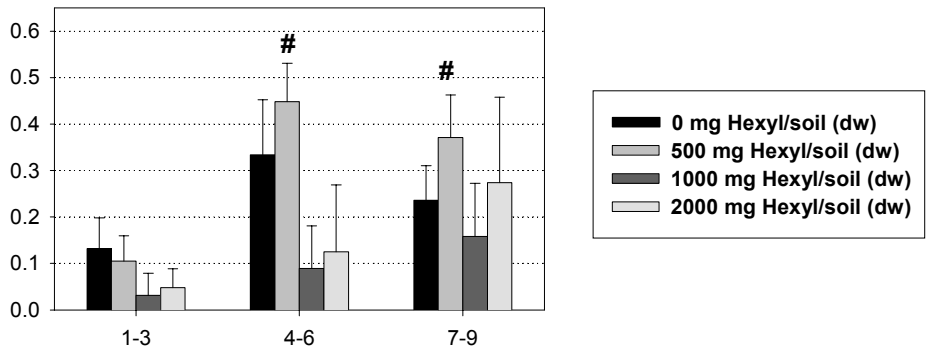
The cocoon rates were determined as being the number of cocoons laid by the number of worms found alive the previous day (see p. 19, chapter 4.3.2). They were tested for significance with an one way ANOVA (see p. 9, chapter 3.3.3).

Table 4.4-9: Cocoon rate for *E. crypticus* transferred from different concentrations of Hexyl-contaminated soil materials onto agar-agar

mg Hexyl/kg soil (dw)	days after transfer					
	1-3		4-6		7-9	
	mean	SD	mean	SD	mean	SD
0	0.132	0.666	0.334	0.118	0.236	0.074
500	0.105	0.054	0.448#	0.083	0.371#	0.092
1000	0.031	0.048	0.089	0.092	0.158	0.114
2000	0.048	0.040	0.125	0.144	0.274	0.184

significant difference to previous periods

In all tested concentrations more cocoons were laid 4-6 and 7-9 days after the transfer than immediately after the transfer. At the third observation period the cocoon rates of the two highest concentrations of 1000 and 2000 mg Hexyl/kg soil (dw) even exceed those of the second period. A significant difference to the cocoon rate 1-3 days after the transfer, however, was only determined for the lowest tested concentration of 500 mg Hexyl/kg soil (dw) either 4-6 days and 7-9 days after the transfer. Both times the cocoon rate was even higher than in the corresponding control. The cocoon rate of the two highest concentrations was always lower than in the control, with the cocoon rate of the highest concentration being even higher than the other. However, no significance was detected in relation to the control of the corresponding time period.



* sifnificant difference to cocoons placed 1-3 days after transfer

Fig. 4.4-12: Cocoon rates for *E. crypticus* transferred from different concentrations of Hexyl-contaminated soil materials onto agar-agar. Bars: mean ± SD.

Hatching rate

All empty cocoons were considered as hatched. Their number on a certain day was divided by the number of cocoons laid in this concentration over the whole period, which was set to 100%. For the cocoon rates the results from three consecutive days were combined for the calculation

of the hatching rates in order to minimise the possibility that cocoons had been overlooked and to ensure a better handling of the data (see p. 19, chapter 4.3.2).

The hatching rates were observed for the cocoons of the first, second and third batch taken 1-3, 4-6 and 7-9 days after the transfer of the adult worms from the Hexyl-contaminated soil materials onto the agar-agar. No statistical test could be applied, since no replicates had been tested.

Table 4.4-10: Hatching rates for the cocoons laid 1-3 days after the transfer of adult *E. crypticus* from different concentrations of Hexyl-contaminated soil materials

mg Hexyl/kg soil (dw)	days after cocoon placement						
	5-7	6-8	7-9	10-12	11-13	19-21	21-23
0	15.8%	52.6%	63.2%	78.9%	78.9%	84.2%	84.2%
500	0.0%	66.7%	73.3%	100.0%	100.0%	100.0%	100.0%
1000	0.0%	50.0%	75.0%	100.0%	100.0%	100.0%	100.0%
2000	0.0%	0.0%	50.0%	75.0%	75.0%	100.0%	100.0%

5-7 days after the cocoons had been laid the hatching had only begun in the control. In the lowest tested concentration of 500 mg Hexyl/kg soil (dw) and in the medium concentration of 1000 mg Hexyl/kg soil (dw), the hatching started after 6-8 days. In the lowest concentration the hatching rate even exceeded the one of the control and in the medium concentration it was at about the same level.

After 7-9 days the cocoons from the highest concentration of 2000 mg Hexyl/kg soil (dw) began hatching, the rate remained below the level of the control. But after 10-12 days it reached the same level and exceeded the hatching rate of the control after 19-21 days by reaching 100%. In the control a hatching rate of 100% was never reached, but in the other concentrations already after 10-12 days.

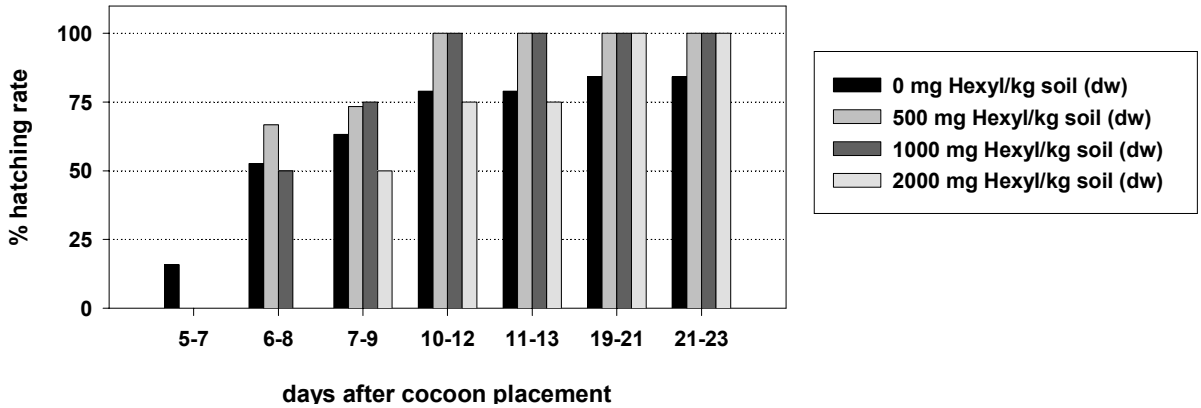


Fig. 4.4-13: Hatching rates of cocoons laid 1-3 days after the transfer of adult *E. crypticus* from various Hexyl-contaminated soil materials onto agar-agar. Bars: mean.

The cocoons laid four to six days after the transfer onto the agar-agar were combined in the second batch.

Table 4.4-11: Hatching rates for the cocoons laid 4-6 days after the transfer of adult *E. crypticus* from different concentrations of Hexyl-contaminated soil materials

mg Hexyl/kg soil (dw)	days after cocoon placement			
	7-9	8–10	16-18	18-20
0	0.0%	75.0%	85.7%	89.3%
500	47.5%	47.5%	100.0%	100.0%
1000	71.4%	85.7%	100.0%	100.0%
2000	100.0%	100.0%	100.0%	100.0%

The hatching began 7-9 days after the cocoon placement in all tested concentrations apart from the control, in which the hatching started after 8-10 days. In the control a hatching rate of 100% was never reached, but in all other concentrations. Already at the first day of hatching after 7-9 days for the highest concentration of 2000 mg Hexyl/kg soil (dw) and for the others after 16-18 days. The hatching rate of the highest concentration was always the highest, the one of the control always the lowest, except after 8-10 days when the hatching rate of the lowest tested concentration was lower.

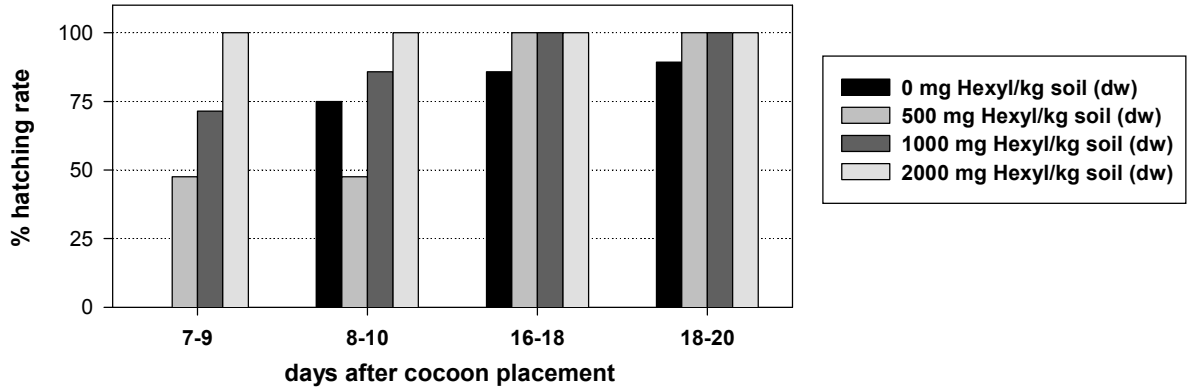


Fig. 4.4-14: Hatching rate of cocoons laid 4-6 days after the transfer of adult *E. crypticus* from various Hexyl-contaminated soil materials onto agar-agar. Bars: mean.

In the third batch the cocoons laid 7-9 days after the transfer from the Hexyl-contaminated soil materials onto the agar-agar were combined.

Table 4.4-12: Hatching rates for the cocoons laid 7-9 days after the transfer of adult *E. crypticus* from different concentrations of Hexyl-contaminated soil materials

mg Hexyl/kg soil (dw)	days after cocoon placement			
	4-6	5-7	13-15	13-17
0	0.0%	51.7%	96.6%	96.6%
500	2.1%	46.8%	97.9%	97.9%
1000	35.7%	42.9%	85.7%	85.7%
2000	26.3%	26.3%	100.0%	100.0%

In the third batch of observation as in the second batch, the cocoons from the contaminated worms began hatching 4-6 days after they were laid. Only in the control the hatching started after 5-7 days, but already with a rate of 51.7%. The hatching rate in the highest concentration

of 2000 mg Hexyl/kg soil (dw) was always the highest apart from 5-7 days after the cocoon placement and it was the only one to reach 100%.

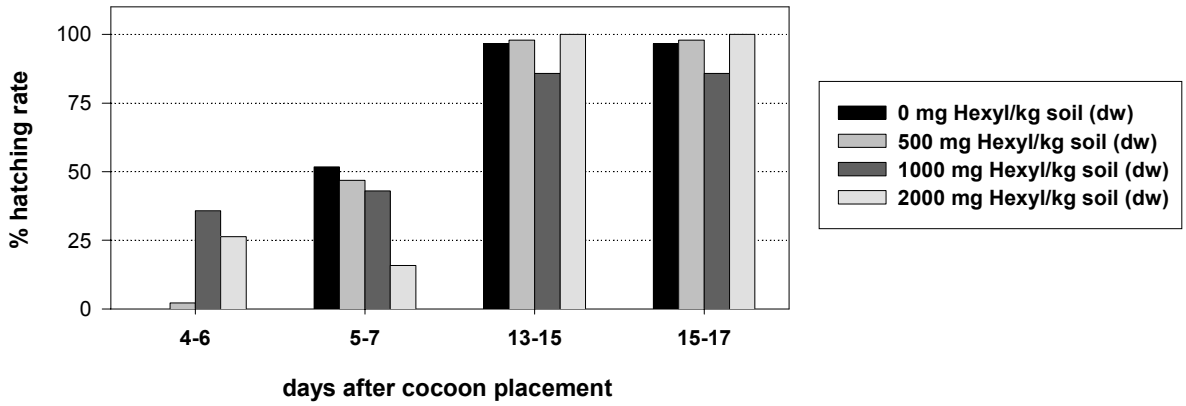


Fig. 4.4-15: Hatching rates of cocoons laid 7-9 days after the transfer of adult *E. crypticus* from various Hexyl-contaminated soil materials onto agar-agar. Bars: mean.

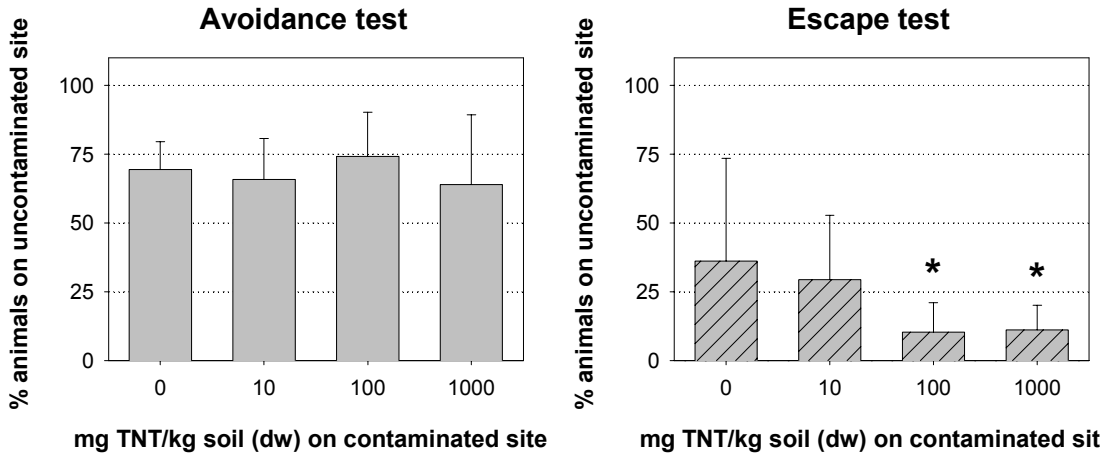
4.4.2.4 Choice test

A choice test can determine whether the test organisms perceive a substance or not. This is important for the evaluation of the toxicity of a pollutant in an ecosystem as the animals might avoid contaminated areas or be attracted by them. For both test organisms separated test sets were performed. The percentages of the animals found for each side are given and the results were evaluated with a Chi-square test.

Table 4.4-13: Distribution of *F. candida* in a separated test set with Hexyl; the side on which the animals were placed is indicated by shading, n 5

		% animals found on		
	mg Hexyl/kg soil (dw)	**uncontaminated side**	**contaminated side**	**result**
animals placed on unconta-minated side	**0**	69	31	
	10	66	34	
	100	74	26	
	1000	63	37	
animals placed on conta-minated side	**0**	37	63	
	10	30	70	
	100	10	90	**no escape**
	1000	12	88	**no escape**

F. candida was not significantly attracted or repelled up to 1000 mg Hexyl/kg soil (dw). At a concentration of ≥ 100 mg Hexyl/kg soil (dw) the collembola showed a significant preference to stay on the contaminated site if placed there.



* significant differene in distribution to control

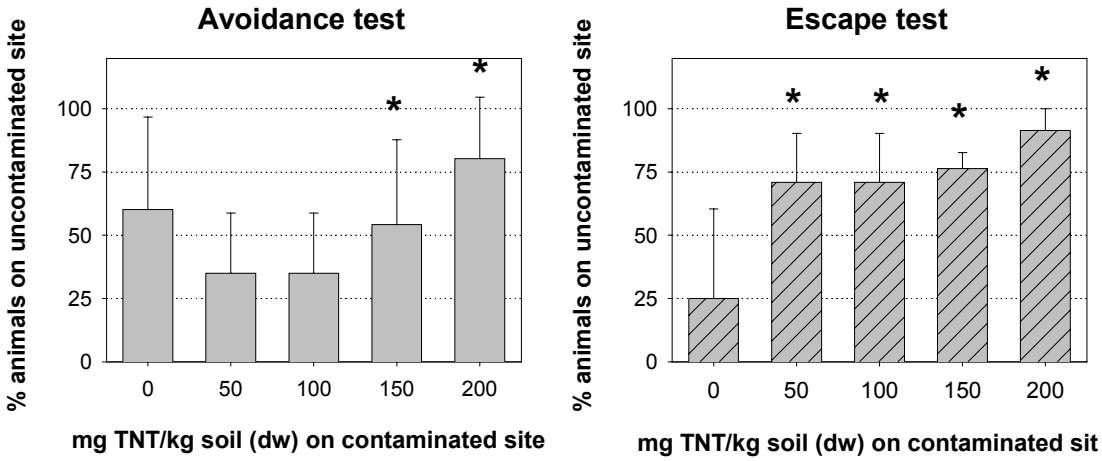
Fig. 4.4-16: Distribution of *F. candida* in a separated test set of a choice test with Hexyl. Bars: mean ± SD.

The behaviour of the enchytraeid was completely different from the one of the collembola.

Table 4.4-14: Distribution of *E. crypticus* in a separated test set with Hexyl; the side on which the animals were placed is indicated by shading, n 5

		% animals found on		
	mg Hexyl/kg soil (dw)	**uncontaminated side**	**contaminated side**	**result**
animals placed on unconta-minated side	**0**	59	41	
	50	38	63	
	100	45	55	
	150	87	13	**avoidance**
	200	73	27	**avoidance**
animals placed on conta-minated side	**0**	27	73	
	50	70	30	**escape**
	100	76	24	**escape**
	150	91	9	**escape**
	200	92	8	**escape**

E. crypticus showed a significant avoidance behaviour at 150 mg Hexyl/kg soil (dw), but not at the next higher concentration. The Chi-square test, however, stated that this test is not reliable enough and that negative findings should be interpreted cautiously. Considering that the lower concentration already showed a significant avoidance behaviour, this can also be attributed to this concentration. The escape behaviour is much clearer and significantly manifested itself at concentrations ≥ 50 mg Hexyl/kg soil (dw).



* significant differene in distribution to control

Fig. 4.4-17: Distribution of *E. crypticus* in a separated test set of a choice test with Hexyl.

4.4.2.5 Summary of results with Hexyl

For the collembola Hexyl was not mortal, but strongly reduced its reproduction. However, the reproduction of animals transferred from contaminated onto uncontaminated soil materials was not affected. In the choice tests the animals did not escape from concentrations below the EC50-values.

The enchytraeid had its mortality and reproduction affected. In the mortality test the surviving animals seemed to be influenced by the explosive, as they became yellow and smaller than the animals from the uncontaminated soil materials. It was shown that the cocoon and the hatching rates of these animals were indeed affected, but not as strongly as might have been expected by their change in colour and size. In a choice test the enchytraeid avoided and escaped from concentrations below the LC50- and EC50-values.

4.4.3 Hexogen (RDX)

4.4.3.1 Mortality and reproduction test

The toxicity of Hexogen was assessed with the mortality and the reproduction test. Up to a concentration of 8000 mg Hexogen/kg soil (dw) it did not show toxic effects on the two species with respect to mortality and reproduction. The observed mortalities and reproduction rates in the Hexogen-contaminated soil materials are given in Table 4.4-15. This concentration was chosen as the highest one, because up to the present knowledge only one soil with 8500 mg Hexogen/kg is known from the Umatilla Munitions Depot Activity, Umatilla, Oregon, USA (PENNINGTON et al, 1995).

Table 4.4-15: Mortalitly and reproduction rates for *F. candida* and *E. crypticus* with Hexogen; mean ± SD; n 5

mg Hexogen/kg soil (dw)	Collembola-biotest		Enchytraeid-biotest	
	% mortality	% reproduction rate	% mortality	% reproduction rate
0	4.0 ± 4.9	100.0 ± 17.5	0.0 ± 0.0	100.0 ± 7.1
1000	6.0 ± 4.9	87.4 ± 6.3	6.0 ± 4.7	98.9 ± 9.2
2000	4.0 ± 4.9	70.1 ± 5.4	2.0 ± 4.0	71.8 ± 34.5
4000	8.0 ± 11.7	85.1 ± 18.7	4.0 ± 4.9	58.5 ± 22.3
8000	2.0 ± 4.0	84.9 ± 13.4	0.6 ± 1.2	90.1 ± 42.0

4.4.3.2 Choice test

The perception of the explosive and a possible choice behaviour were tested. Neither *F. candida* nor *E crypticus* showed a significant choice behaviour for concentrations up to 10 000 mg Hexogen/kg soil (dw) in a combined test set. Therefore no separate tests were carried out. The table gives the percentages of all the animals found on each side for both test organisms. The results were statistically evaluated with a Chi-square test.

Table 4.4-16: Distribution of *F. candida* and *E. crypticus* in a combined test set with Hexogen; n 5

		% animals found on		
	mg Hexogen/kg soil (dw))	uncontaminated side	contaminated side	result
F. candida	**0**	48	52	**no significance**
	10	41	59	
	100	41	59	
	1000	56	44	
	10000	46	54	
E. crypticus	**0**	53	47	**no significance**
	10	52	48	
	100	54	46	
	1000	40	60	
	10000	47	53	

4.4.3.3 Summary of results with Hexogen

The explosive Hexogen is neither toxic for the collembola nor for the enchytraeid in relation to mortality and reproduction up to 8000 mg Hexogen/kg soil (dw). Additionally no choice behaviour was observed for both species up to 10 000 mg Hexogen/kg soil (dw).

4.4.4 Octogen (HMX)

4.4.4.1 Mortality and reproduction test

The toxicity of a substance can be determined with mortality and reproduction tests. Ooctogen was non-toxic for *F. candida* and *E. crypticus* in either test up to 8000 mg Octogen/kg. This concentration was chosen in relation to Hexogen. Table 4.4-17 lists the observed mortalities and reproduction rates in the Octogen-contaminated soil materials.

Table 4.4-17: Mortality and reproduction rates for *F. candida* and *E. crypticus* with Octogen; mean ± SD; n 5

mg Octogen/kg soil (dw)	Collembola-biotest		Enchytraeid-biotest	
	% mortality	% reproduction rate	% mortality	% reproduction rate
0	4.0 ± 4.9	100.0 ± 17.5	0.0 ± 0.0	100.0 ± 7.1
1000	2.0 ± 4.0	106.4 ± 12.8	2.0 ± 4.0	85.8 ± 11.6
2000	2.0 ± 4.0	83.7 ± 5.6	2.0 ± 4.0	65.9 ± 27.3
4000	0.0 ± 0.0	81.0 ± 10.4	0.0 ± 0.0	46.5 ± 28.1
8000	0.0 ± 0.0	78.0 ±23.0	2.0 ± 4.0	60.5± 22.9

4.4.4.2 Choice test

With choice tests the perception of a test species for a substance and a the thereof resulting reaction can be observed. Neither for *F. candida* nor for *E. crypticus* a significant choice behaviour was detected for concentrations up to 10 000 mg Hexogen/kg soil (dw) in a combined test set. Thus, no separate tests were performed. The table gives the percentages of all animals found for each side for both test species. The results were evaluated with a Chi-square test.

Table 4.4-18: Distribution of *F. candida* and *E. crypticus* in a combined test set with Octogen; n 5

		% animals found on		
	mg Octogen/kg soil (dw))	uncontaminated side	contaminated side	result
F. candida	**0**	49	51	**no significance**
	10	53	47	
	100	45	55	
	1000	40	60	
	10000	46	54	
E. crypticus	**0**	53	47	**no significance**
	10	41	59	
	100	49	51	
	1000	54	46	
	10000	47	53	

4.4.4.3 Summary of results with Octogen

Octogen is not toxic either for the collembola or the enchytraeid in the mortality or the reproduction test up to 8000 mg Octogen/kg soil (dw). In addition, no choice behaviour was observed for either species up to 10 000 mg Octogen/kg soil (dw).

4.4.5 Triaminotoluene (TAT)

4.4.5.1 Mortality and reproduction tests

In order to asses the toxicity of TAT mortality and reproduction tests were carried out. For the collembola the end product of microbial reduction of TNT under anaerobic conditions (RIPPEN & KNACKMUSS, 1995) was not toxic either in the mortality or in the reproduction test in the concentrations used. No effect on the mortality of the enchytraeid was observed, but its reproduction was reduced. The observed mortalities and reproduction rates for both species are given in Table 4.4-19.

Table 4.4-19: Mortality and reproduction rates for *F. candida* and *E. crypticus* with TAT; mean ± SD; n 4/5

mg TAT/kg soil (dw)	Collembola-biotest		Enchytraeid-biotest	
	% mortality	% reproduction rate	% mortality	% reproduction rate
0	0.0 ± 0.0	100 ± 27.1	1.3 ± 3.3	100.0 ± 3.3
500	n. t.	111.2 ±14.2	0.0 ± 0.0	85.5 ± 20.7
1000	10.0 ± 8.2	103.6 ±14.9	2.5 ± 4.3	68.2 ± 24.5
1500	15.0 ± 5.0	88.2 ± 19.0	n.t.	n.t.
2000	10.0 ± 8	133.4 ± 18.1	0.0 ± 0.0	29.1 ± 23.8*

n.t. not tested
* significant difference to control

For the reproduction of *E. crypticus* an EC50(28d) of 1324.6 mg TAT/kg soil (dw) was determined.

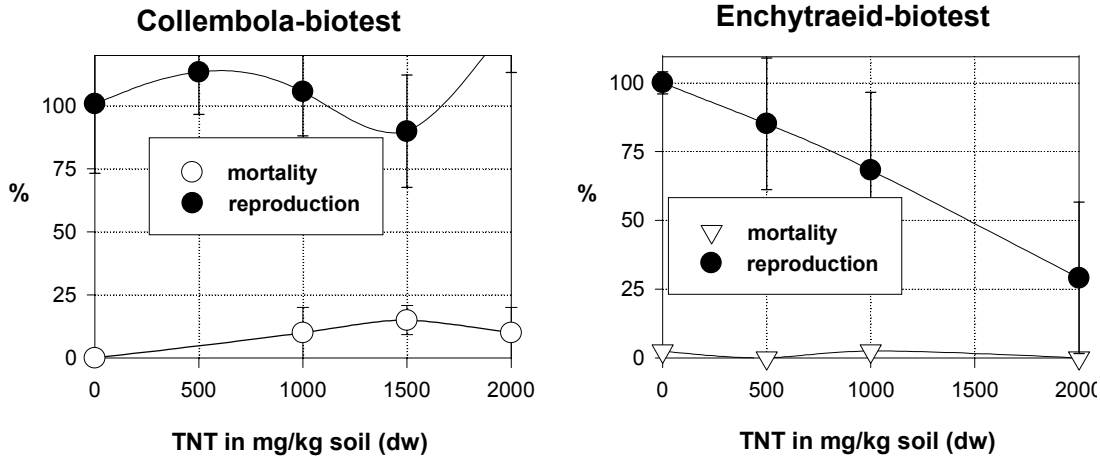


Fig. 4.4-18: Concentration-effect relationship for TAT with *F. candida* and *E. crypticus.* The data for mortality and reproduction are given in relation to the values in the standard soil material Lufa 2.2. Symbols: mean ± SD.

4.4.5.2 Non-soil tests

Since TAT did not show a very strong toxicity in the soil tests, its toxicity was tested without the interference of soil, to which it is known to adsorb easily. Thus, for *F. candida* a contact test on filter paper (see p. 8, chapter 3.3.2.1) and for *E. crypticus* a water test with artificial fresh water (see p. 9, chapter 3.1.2) were performed. The mortality was determined after 24, 48 and 72 hours, respectively. As no mortality was found in the contact test up to 28.5 µg TAT/cm^2 for *F. candida* an additional water test was carried out to exclude even the possibility of an adsorption of the compound to the filter paper.

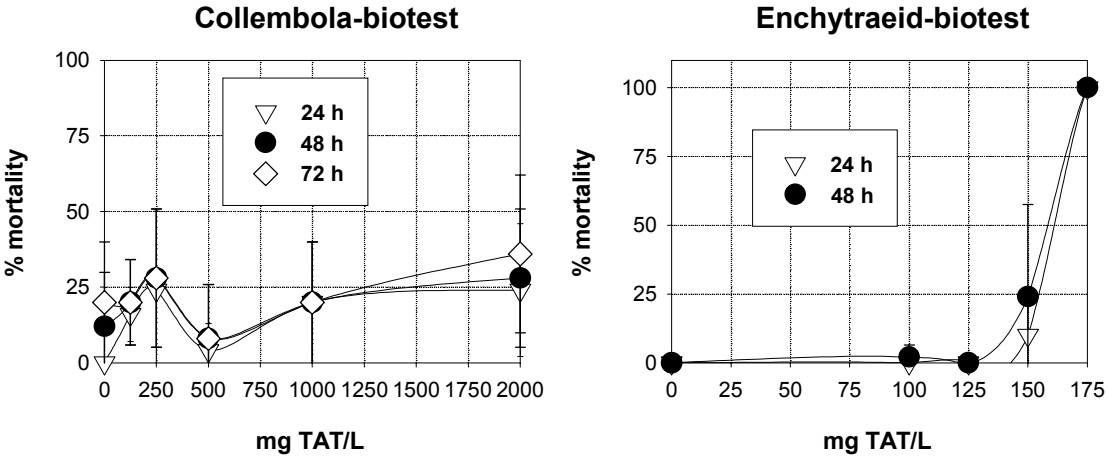


Fig. 4.4-19: Concentration-effect relationship for TAT in the water tests with *F. candida* and *E. crypticus*. Symbols: mean ± SD.

Not even in a water test TAT was toxic for the collembola, as the mortality of the highest tested concentration of 2000 mg/L was not significantly different from the control. For the enchytraeid LC50-values were determined after 24, 48 and 72 hours.

Table 4.4-20: LC 50 values of TAT for *F. candida* and *E. crypticus* in the water test after 24, 48 and 72 hours

LC50 water test in mg/L					
F. candida			***E. crypticus***		
24 h	**48 h**	**72 h**	**24 h**	**48 h**	**72 h**
> 2000	> 2000	> 2000	162.5	159	159

4.4.5.3 Choice test

The choice behaviour is a sensitive parameter for the perception of a substance by organisms. For both test organisms separated test sets were performed with TAT. The results were evaluated with a Chi-square test and in the tables the percentages of all animals found are given for each side.

Table 4.4-21: Distribution of *F. candida* in a separated test set with TAT; the side on which the animals were placed is indicated by shading, n 5

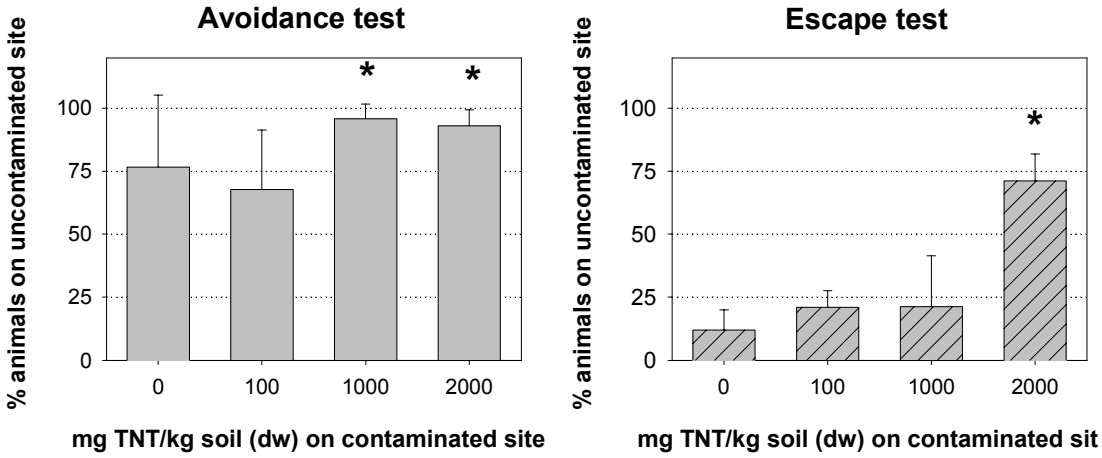
		% animals found on		
	mg TAT/kg soil (dw)	**uncontaminated side**	**contaminated side**	**result**
animals placed on uncontaminated side	**0**	71	29	**no significance**
	100	63	38	
	1000	51	49	
	2000	52	48	
animals placed on contaminated side	**0**	39	61	**no significance**
	100	46	54	
	1000	32	68	
	2000	30	70	

F. candida showed no significant choice behaviour. The animals were neither attracted nor repelled by the TAT-contaminated soil material. The behaviour of the enchytraeid differed from the collembola.

Table 4.4-22: Distribution of *E. crypticus* in a separated test set with TAT; the side on which the animals were placed is indicated by shading; n 5

		% animals found on		
	mg TAT/kg soil (dw)	**uncontaminated side**	**contaminated side**	**result**
animals placed on uncontaminated side	**0**	74	26	
	100	69	31	
	1000	95	5	**avoidance**
	2000	93	7	**avoidance**
animals placed on contaminated side	**0**	12	88	
	100	20	80	
	1000	20	80	
	2000	71	29	**escape**

E. crypticus significantly avoided concentrations of ≥ 1000 mg TAT/kg soil (dw). If placed on the contaminated side they significantly fled from the highest tested concentration of 2000 mg TAT/kg soil (dw). In the graph only the distribution of the side with the uncontaminated soil material is represented.



* significant difference in distribution from control

Fig. 4.4-20: Distribution of *E. crypticus* in a separated test set of a choice test with TAT.

4.4.5.4 Summary of results with TAT

The end product of microbial reduction was non-toxic for the collembola in the mortality and the reproduction test up to 2000 mg TAT/kg soil (dw). In the non-soil tests, too, it was not toxic, not even in a water test up to 2000 mg TAT/L. In the choice test no choice behaviour was detected.

In case of the enchytraeid TAT reduced the reproduction, but had no influence on the mortality in soil materials up to 2000 mg TAT/kg soil (dw). In a water test, however, the mortality was affected at much lower concentrations.

4.4.6 Summary of Results

With the explosives TNT, Hexyl, Hexogen and Octogen, as well as with TAT mortality, reproduction and choice tests in the standard soil material Lufa 2.2 were carried out. Since the surviving enchytraeids of the mortality tests with TNT and Hexyl seemed to be affected by the toxicants additional cocoon and hatching tests on agar-agar were performed. As Hexyl was not lethal for the collembola, but strongly reduced the reproduction, the fertility of animals transferred from contaminated soil material onto uncontaminated soil material was investigated.

Non-soil tests could only be performed with TNT and TAT, due to the low solubility of the other substances. The results for the soil and the non-soil tests are given in Table 4.4-23. The tests with animals transferred from contaminated soil materials are not included in the summary, as no definite toxicity end point can be given.

Table 4.4-23: Summary of the results with the soil and non-soil tests

	F. candida			*E. crypticus*		
	mortality and reproduction tests					
	LC50(7d) in mg/kg soil (dw)	**EC50(28d) in mg/kg soil (dw)**		**LC50(7d) in mg/kg soil (dw)**	**EC50(28d) in mg/kg soil (dw)**	
TNT	139.9 ± 9.4	64.3 ± 22.4		949.9 ± 617.8	501.2 ± 278.3	
Hexyl	> 4000	175.6 ± 39.6		2402.8 ± 56.2	530.4	
Octogen	> 8000	> 8000		> 8000	> 8000	
TAT	> 2000	> 2000		> 2000	1324.6	
	water tests					
	LC50(24h) in mg/L	**LC50(48h) in mg/L**	**LC50(72h) in mg/L**	**LC50(24h) in mg/L**	**LC50(48h) in mg/L]**	**LC50(72h) in mg/L**
TNT	n.t.	n.t.	n.t.	>100	83	58
TAT	> 2000	> 2000	> 2000	162.5	159	159
	contact tests					
	LC50(24h) [µg/cm²]	**LC50(48h) [µg/cm²]**	**LC50(72h) [µg/cm²]**	not applicable		
TNT	0.735	0.700	0.500			
TAT	>28.5	>28.5	>28.5			
	choice tests					
	avoidance	**escape**		**avoidance**	**escape**	
TNT	no significance			50 mg/kg soil (dw)	no escape	
TNT 7d	n.t.	n.t.		100 mg/kg soil (dw)	50 mg/kg soil (dw)	
Hexyl	no significance	no escape ≥ 100 mg/kg soil (dw)		150 mg/kg soil (dw)	50 mg/kg soil (dw)	
Hexogen	no significance			no significance		
Octogen	no significance			no significance		
TAT	no significance			1000 mg/kg soil (dw)	2000 mg/kg soil (dw)	

n.t. not tested

4.5 Discussion

A comparison of the toxicity of the various ammunition like substances showed that TNT was the most toxic one. Hexyl, an aromatic nitro compound like TNT, was much less toxic, whereas TAT, the end product of microbial degradation of TNT, was not toxic for *F. candida*, but for *E. crypticus*. The heterocyclic nitramines Hexogen and Octogen were neither toxic for *F. candida* nor *E. crypticus* in any of the performed tests.

4.5.1 TNT

4.5.1.1 Comparison of results

In the mortality and reproduction tests *F. candida* was much more sensitive to TNT (LC50(7d) 139.9 ± 9.4 mg TNT/kg soil (dw) and EC50(28d) 64.3 ± 22.4 mg TNT/kg soil (dw)) than

E. crypticus (LC50(7d) 949.9 ± 617.8 mg TNT/kg soil (dw) and EC50(28d) 501.2 ± 278.3 mg TNT/kg soil (dw)). As the variance between the replicates of a test was also much lower for the collembola, *F. candida* is the more suitable organism to test for the toxicity of TNT in soil.

In the case of *E. crypticus* the variance between replicates of a test was very high, as indicated by the high standard deviation for the LC50- and the EC50-value. Some of the mortality tests could not be considered for the LC50, as concentrations which were absolutely lethal in one test had no effect on the mortality of *E. crypticus* in another test. This difference in toxicity cannot be explained by faults of the experimental design. Rather a periodicity of the enchytraeid has to be assumed, which alters its sensibility for certain toxicants (ACHAZI et al, 1997).

In the choice test *F. candida* did not show any significant reaction to TNT. Thus, the collembola has probably no perception for TNT. *E. crypticus* on the other hand showed a very clear avoidance behaviour at concentrations as low as 50 mg TNT/kg soil (dw). This is more than 10% lower than the EC 50(28d) or the LC50(7d), indicating that the worm is able to detect the toxicant. Although, if placed on the contaminated soil materials, the animals did not escape, but stayed there. A closer look revealed that the animals had remained on the soil surface and did not dig themselves into the soil material – a completely untypical behaviour for enchytraeids. This indicates that the animals perceived the toxicant so strongly that they completely avoided any contact, as they were not unable to move if transferred into water. After the TNT had been given the possibility of sorption to the soil matrix by an ageing period of 7 days, the animals were able to escape and were significantly repelled at 50 mg TNT/kg soil (dw). The threshold level for avoidance on the other hand had risen to 100 mg TNT/kg soil (dw).

In order to evaluate the non-soil tests a comparison with the reference substance, the herbicide Betanal with the active ingredient Phenmedipham is useful. In the standard soil material Lufa 2.2 the LC50(7d)-values for the collembola- and enchytraeid-biotest were 95.5 ± 31.8 and 573.2 ± 114.6 mg Betanal/kg soil (dw), respectively the EC 50(28d)-values were 57.3 ± 19.1 and 222.9 ± 95.5 mg Betanal/kg soil (dw) (WARNECKE et al, 2001). In the water test for *E. crypticus* the LC50 values for 24, 48 and 72 hours were 82.5, 72.0 and 67.5 µg Betanal/L, in the contact test with *F. candida* they were 13.0, 12.0 and 12.0 µg Betanal/cm^2 after 24, 48 and 72 hours (see Appendix D.1). For a comparison the LC50(48h) can be compared to the LC50(7d) in soil. For *F. candida* the difference is described by the factor 8 x 10^3 and for *E. crypticus* by the factor 8 for Betanal. In the case of TNT the equivalent factors are 200 x 10^3 for the collembola and 11 for the enchytraeid. Thus TNT is much more toxic for the collembola in the contact test than Betanal, indicating a stronger sorption of TNT to the soil. However, for *E. crypticus* the bioavailability of TNT in soil is comparable to the one of Betanal and not affected by different sorption properties of the substances in soil. This difference in the bioavailability of TNT for the two species can be explained by the different mode of exposure: the collembola is exposed to

the soil and the air, whereas the enchytraeid is mainly exposed to the soil water. The concentration of TNT in the soil pore water, however, is not affected by the sorption to the soil matrix, due to the low water solubility of TNT. Betanal on the other hand does easily emulsify in water. Thus, its sorption properties do not influence its toxicity so much as it is the case for TNT.

As the surviving individuals of *E. crypticus* in the mortality test were remarkably smaller in the contaminated soil materials than in the control, a special test was developed to check their ability to reproduce after an exposure to the toxicant: a cocoon and a hatching test. The number of cocoons laid, increased from the first observation period of 1-3 days to the second period of 4-6 days after the transfer from the contaminated soil materials in all concentrations. This might be the result of a recovery from the transfer itself, which must be considered as a disturbance for the animals. In the control, however, the cocoon rate was always higher than for the animals from the contaminated soil materials, but only significantly in comparison to the highest tested concentration of 1000 mg TNT/kg soil (dw) 4-6 days after the transfer. Hence it has to be concluded that the cocoon rate of the animals is reduced by their exposure to the contaminated soil material.

The hatching rate, too, was reduced for cocoons from animals out of the two highest concentrations of 500 and 1000 mg TNT/kg soil (dw). The hatching rates of these cocoons never reached the rates of the control, not even after 8-10 days. Since an improvement was evident in the control and the lowest tested concentration of 500 mg TNT/kg soil (dw), it has to be assumed that the hatching of cocoons from the animals out of the highly contaminated soil materials was delayed. On the other hand the hatching rate of the cocoons from animals transferred from the soil material contaminated with 250 mg TNT/kg soil (dw) was 5-7 and 6-8 days after the cocoon placement higher than in the control. Thus the hatching was accelerated in the lowest tested concentration, which might be the result of an overcompensation due to the exposure to the toxicant. This effect was, however, diminished after 9 days, when the hatching rates became level with the control. In none of the concentrations did the hatching rate reach 100% until the end of the test period. In general, it can be stated, that the reproduction of the enchytraeids transferred from TNT-contaminated soil material is affected.

4.5.1.2 Comparison with literature

In a Microtox assay with *Vibrio fischeri* all the explosives investigated in this thesis as well as all degradation products of TNT have been tested in aqueous solutions or in 1% acetone (DRZYZGA et al, 1995). According to the guideline “Deutsche Einheitsverfahren zur Wasser-, Abwasser-, und Schlammuntersuchung – Testverfahren mit Wasserorganismen; Gruppe L; DIN 38412, Teil 34; DEV 1991) TNT was classified as very toxic to aqueous organisms, with an EC50(30min)-

value of 3.59 mg/L (DRZYZGA et al, 1995). In a different study the EC50(15min)-value for *V. fischeri* in solutions of water and dimethylsulfoxide (DMSO) is given with 0.95 mg/L, which is within the range but slightly lower than in the previous study. The EC50(96h)-value for the unicellular freshwater algae *Selenastrum capricornutum* was 73 mg/L (SUNAHARA et al, 1998).

For a better evaluation of the environmental risk caused by TNT, the toxicity of TNT bound to sediments has to be taken into account, as it might be transported into streams by leaching and runoff or erosion from contaminated sites. Thus, the toxicity for the marine polychaete *Neanthes arenaceodentata* and the estuarine amphipod *Leptocheirus plumulosus* was determined (GREEN et al, 1999). For the polychaete an LC50(28d) of 320 mg TNT/kg sediment (dw) was found, but no 50%-value for growth could be given. In relation to the TNT tissue concentration the animals became larger with an increased tissue concentration. This effect cannot be attributed to an affluence in food for surviving animals due to a high mortality, as the mortality was only 10 and 15%. Thus as an explanation hormesis is given, the enhancement of growth at low toxicant concentration, where homeostatic regulatory mechanisms overcompensate for stressors such as toxicants. The amphipod showed significant effects with TNT on its survival, growth and reproduction. The LC50(28d) is 202.7 mg TNT/kg sediment (dw) and thus lower than the one for the polychaete. The growth was significantly reduced at higher concentration after having shown an insignificant hormesis effect for the lower ones. The reproduction was significantly reduced, but no EC50-value could be determined. Both test species are more sensitive to TNT than *E. crypticus*, but less sensitive than *F. candida*.

The acute toxicity of TNT on the earthworm *Eisenia andrei* has been investigated on filter paper and in two different soil materials (ROBIDOUX et al, 1999). In the filter paper contact test no toxicity was observed, when the TNT was applied in aqueous solution. However, if acetonitrile was used as a transfer solvent, toxicity was easily detected at 14 µg/cm^2 (849 mg TNT/L), when the filter paper was saturated with TNT and thus an unknown aqueous TNT-concentration became available to the earthworms. In the soil materials TNT was significantly toxic to the earthworms at 260 mg TNT/kg soil (dw) in a forest soil material and at 420 mg TNT/kg soil (dw) in OECD-soil, a artificial soil material consisting of 70% sand, 20% Kaolin clay and 10% sphagnum peat. The difference in toxicity was not related to the organic matter content, which was 2.24% in the OECD-soil and thus lower than in the forest soil material with 4.24%. Neither the water content nor the pH values were related. However, it is suggested that a difference in the microbial activity of the soil materials could yield to a difference in toxicity. In this study the TNT-concentration in the soil water with the lowest significant effect, the LOEC (forest soil material 2.4 mg TNT/L; OECD-soil 1.3 mg TNT/L), was compared with the TNT-concentrations applied on the filter paper (> 100 mg TNT/L). On the basis of this comparison the soil tests are two orders of magnitude more sensitive.

In the same workgroup the chronic toxicity for the earthworm was tested in OECD-soil with respect to adult growth and various reproduction parameters (ROBIDOUX et al, 2000). Adult growth was only significantly affected at the highest tested concentration of 881 mg TNT/kg soil (dw), some of the reproduction parameters, however, were already reduced at 110 mg TNT/kg soil (dw). Hence, the LOEC for reproduction was 110 mgTNT/kg soil (dw); the lowest concentration with no significant effect, the NOEC was 55 mg TNT/kg soil (dw). No significant mortality was observed at the tested concentrations ≥ 881 mg TNT/kg soil (dw). This result differed from the study on acute toxicity mentioned above and this difference is attributed to variations in the test conditions. Hence, in the mortality test no food had been added, which might reduce the toxicity of TNT by sorption. In addition the evaporation time of acetonitrile as transfer solvent was shorter, which might cause mortality, too. Thus the earthworm is less sensitive in the mortality test than *E. crypticus*, but more sensitive in the reproduction test. The collembola was more sensitive in both tests.

In a microcosm study with the nematode *Rhabditis maupasi* and the mite *Hypoaspis aculeifer* the effect of TNT on a food web was investigated on high gel strength agar (FRISCHE, 1998). No lethal effects on either test species were detected, but the population development was delayed as well as modified for the nematode. For the mites the population development was delayed and divergent with a significantly lower abundance at the end of the test period. This effect on the food web is attributed to a prolonged lag phase of bacterial growth as a short-term toxic effect of TNT. In a microcosm study with indigenous arthropods and nematodes in soil material the abundances were not significantly affected up to 200 mg TNT/kg soil (dw) as TNT was bound to the soil and thus not biologically available (PARMELEE et al, 1999).

Higher plants have been found to be affected by TNT. The growth of the dicotyledons cress *(Lepidum sativa)* and turnip (*Brassica rapa*) was significantly inhibited at 50 mg/kg, but at lower concentration a stimulation of growth could be observed in a BBA soil (a soil material from the Biologische Bundesanstalt (BBA)). Two tested monocotyledons oat (*Acena sativa*) and wheat *(Triticum aestivum)* were less sensitive to TNT (GONG et al, 1999). Tall fescue (either *Festuca elatior* or *Festuca gigantea*) and bromegrass (*Bromus inermis*) were found to tolerate 31 and 24 mg TNT/L, since they were still able to germinate and grow at these concentrations. However, the seed germination and the initial growth were affected at lower concentration than just growth (KRISHNAN et al, 2000). In an earlier study germination and the growth of the root as well as the shoot were delayed and reduced (PETERSON et al, 1999). A significant reduction or inhibition of germination and seedling development occurred at concentrations of 30 mg TNT/L (100 mg TNT/kg soil (dw)). However, for soils below 100 mg TNT/kg soil (dw) a remediation with tall fescue (*F. elatior* or *F. gigantea*) is suggested by a two stage remediation with phytoremediaton as the second step (PETERSON et al, 1996). For smooth bromegrass

(*B. inermis*) the germination was significantly reduced at 30 mg TNT/L, but switchgrass (*Panicum virgatum*) showed no effect. The seedlings of bromegrass (*B. inermis*) were able to tolerate 17.5 mg TNT/L and the ones of switchgrass (*P. virgatum*) 15 mg TNT/kg soil (dw). Thus smooth bromegrass (*B. inermis*) and switchgrass (*P. virgatum*) could be used for the bioremediation of soils below 15 mg TNT/L (50 mg extractable TNT/kg soil (dw) (PETERSON et al, 1999). In comparison to the two invertebrates examined in this thesis, plants are more sensitive to TNT.

4.5.2 Hexyl

4.5.2.1 Comparison of results

For *F. candida* no mortality was found up to 4000 mg Hexyl/kg soil (dw). The reproduction was, however, already significantly reduced at 150 mg Hexyl/kg soil (dw) and the EC50 (28d)-value was 175.6 ± 39.6 mg Hexyl/kg soil (dw). To investigate the difference between the mortality and the reproduction for the collembola an altered reproduction test was performed with adults transferred from contaminated soil materials onto uncontaminated soil material. After three weeks the reproduction rates were determined. Only in the highest tested concentration of 2000 mg Hexyl/kg soil (dw) the reproduction rate was significantly reduced to 40.5 ± 7.3% of the control. The lowest tested concentration of 250 mg Hexyl/kg soil (dw), which is already above the EC50(28d) showed no significant effect. Hence, the reduced reproduction in the standard reproduction test cannot be attributed to a reduced fertility of the adults. Since the eggs are known to be more resistant towards toxicants than any other life stage (GEIST, 2000), a higher mortality of the juveniles due to a higher sensitivity must be considered as the cause of the reduced reproduction.

In the choice test the collembola showed a significant tendency to remain in the Hexyl-contaminated soil material at a concentration of 100 mg Hexyl/kg soil (dw) and above, if they had been placed there However, no avoidance or attraction of Hexyl at these concentrations was observed. Hence, the tendency to remain in the contaminated soil materials might not be the result of an attraction, but of a toxic effect hindering the collembola from escape. Overall, it must be concluded that the reproduction of a population would be seriously endangered, as the EC50(28d)-value is with 175.6 ± 39.6 mg Hexyl/kg soil (dw) much lower.

In the enchytraeid-biotest the mortality as well as the reproduction were affected with a LC50(7d) of 2402.8 ± 56.2 mg Hexyl/kg soil (dw) and an EC50(28d) of 530.4 mg Hexyl/kg soil (dw). In the mortality test, however, the animals already showed an effect before the mortality was significantly increased at 2100 mg Hexyl/kg soil (dw): first their clitella became yellow and later the whole animal turned yellow and rigid (see p. 12, Fig. 4.4-9). Since the EC50(28d) was 4.5 times lower than the LC50(7d), an additional reproduction test was performed in order to

find out, whether these sublethal effects influence the fertility. For that animals from Hexyl-contaminated soil materials were transferred onto agar-agar and the cocoon placement and the hatching were observed. More cocoons were laid 4-6 days than 1-3 days after the transfer in all concentrations indicating a disturbing effect on the cocoon placement due to the transfer (see p. 31, Fig. 4.4-11). This effect continued for the observation period of 7-9 days with the exception of the control and 500 mg Hexyl/kg soil (dw), where the cocoon rates were lower than at 4-6 days. Thus, animals from the lower concentrations recovered faster from the additional disturbance of the transfer than animals from the higher concentrations. The cocoon rate of the lowest tested concentration of 500 mg Hexyl/kg soil (dw) was in both later observation periods significantly higher than after 1-3 days and even higher than in the corresponding control. Thus the worms seem to overcompensate the toxic influence of the explosive, after they had laid the yellow cocoons in the first observation period. Since the cocoon rates from worms of the two higher concentrations still increased from 4-6 to 7-9 days after the transfer, these worms seem to recover more slowly from the toxicant.

The hatching of the cocoons laid by the worms from the control 1-3 days after the transfer was faster than of the contaminated ones, as only cocoons in the control had hatched 5-7 days after they had been laid (see p. 32, Fig. 4.4-12). However, later the hatching rate of the cocoons from the animals out of the contaminated soil materials soon exceeded the rate of the control even reaching 100%, a level the control never reached. Thus, the hatching of cocoons laid by worms from contaminated soil materials was only slower at the beginning, but after a short delay it became faster than in the control, indicating a recovery. The cocoons of the highest concentration of 2000 mg Hexyl/kg soil (dw), however, recovered more slowly than the others. It has to be remarked that the yellow cocoons, too, hatched, releasing normally white worms. Thus the yellow colour of the cocoons, most likely to be caused by the explosive, does not cause any harm to the juveniles.

The hatching of the cocoons laid 4-6 days and 7-9 days after the transfer from the contaminated soil material was faster at the beginning than in the control (see p. 33, Fig. 4.4-13 and p. 34, Fig. 4.4-14). This remained so until the end of the observation period with the exception of the lowest tested concentration of 500 mg Hexyl/kg soil (dw) 4-6 days after the transfer. Remarkable is that the hatching rate of the cocoons laid 4-6 days after the transfer from the worms taken out of the soil material contaminated with the highest concentration of 2000 mg Hexyl/kg soil (dw) reached 100% as soon as the hatching began. Also, the cocoons laid from the same worms 7-9 days after the transfer reached a hatching rate of 100% sooner than the cocoons from animals transferred from the other concentrations. This indicates that the delayed hatching of the cocoons laid immediately after the transfer is overcompensated by the cocoons laid later.

In general, it can be concluded, that the reproduction of the enchytraeids transferred from the Hexyl-contaminated soil materials was affected, but not as strongly as might have been suggested by their change in colour and size. However, the recovery of the worms was very rapid.

In the choice test with *E. crypticus* a reaction to the presence of Hexyl could be observed at much lower concentrations. The animals significantly avoided concentrations of 150 mg Hexyl/kg soil (dw) and above and they fled significantly from concentrations of 50 mg Hexyl/kg soil (dw) and higher. Thus, in the field no animals would be detected in Hexyl-concentrations effecting the mortality or the reproduction.

4.5.2.2 Comparison with literature

Hexyl has not very often been investigated in the literature. Again the study of Drzyzga is the most futile one. It states Hexyl as very toxic to aquatic organisms with an EC50(30min)-value of 6.32 mg/L for the luminescent bacteria *V. fischeri* in aqueous solutions (DRZYZGA et al, 1995). Thus Hexyl is nearly half as toxic as TNT for this organism.

4.5.3 Hexogen (RDX) and Octogen (HMX)

4.5.3.1 Comparison of results

In none of the performed soil tests, either for *F. candida* or *E. crypticus* was a significant effect of the two explosives found up to a concentration of 8000 mg/kg soil (dw). Neither the mortality was increased nor the reproduction reduced nor did the contaminated soil materials induce a certain choice behaviour. No higher concentrations were tested, since up to the present no higher field contamination with either explosive is known. Thus Hexogen and Octogen have to be assumed as non-toxic for *F. candida* and *E. crypticus* .

4.5.3.2 Comparison with literature

On the basis of the Microtox assay with *V. fischeri* Hexogen as well as Octogen have been classed as toxic to aqueous organisms, with EC50(30min)-values of 74.56 mg/L for Hexogen and > 25 mg/L for Octogen. These concentrations, however, were not considered to be relevant for the environment, since they are above the water saturation point of these compounds (DRZYZGA et al, 1995). In the similar study of Sunahara the values for the concentrations with a 50% inhibition, the IC50(15min)-values, are given with > 40.20 mg/L for Hexogen and > 6.43 mg/L for Octogen. The toxicity assessment of the heterocyclic nitramines was restricted by their low solubility in water, but for Hexogen a 20% inhibition could be detected at this concentration. In the test with the freshwater unicellular green algae *S capricornutum* The EC50(96h)-values were > 40.20 mg/L for Hexogen and > 6.52 mg/L for

Octogen, with the water solubility again limiting the determination of toxicity (SUNAHARA et al, 1998).

Only one study in soil is known either for Hexogen or Octogen on the chronic toxicity of the earthworm *E. andrei* (ROBIDOUX et al, 2000; PHILLIPS et al, 1993). Both were performed in the same workgroup and adult growth as well as various parameters related to reproduction were investigated. For Hexogen the adult growth was not significantly affected up to the highest tested concentration of 756 mg/kg soil (dw). An effect on some of the parameters for reproduction could already be observed at the lowest tested concentration of 95 mg Hexogen/kg soil (dw), whereas no mortality was observed at the tested concentrations ≤ 756 mg Hexogen/kg soil (dw) (ROBIDOUX et al, 2000). In a different study on the acute toxicity of Hexogen for the earthworm *E. fetida*, no mortality but a weight loss at concentrations ≥ 100 mg Hexogen/kg soil (dw) was observed, which can be considered as an indicator for sublethal effects (PHILLIPS et al, 1993).

In the case of Octogen no mortality was observed for *E. andrei* up to the highest tested concentration of 3013 mg/kg, but most of the investigated sublethal parameters were significantly reduced at the lowest tested concentration of 296 mg Octogen/kg soil (dw) and above. Hence the NOEC-value on earthworm growth and reproduction is ≥ 296 mg Octogen/kg soil (dw) and it was considered possible that major reproduction effects may occur at lower concentrations (ROBIDOUX et al, 2001). In another study on the acute toxicity of Octogen no mortality for the earthworm *E. fetida*, but an increased weight loss in concentrations ≥ 200 mg Octogen/kg soil (dw) was observed, a possible indicator for sublethal effects (PHILLIPS et al, 1993).

4.5.4 TAT

4.5.4.1 Comparison of results

The end product of the microbial reduction of TNT, TAT, was non-toxic for *F. candida* up to a concentration of 2000 mg TAT/kg soil (dw) in the soil tests. Neither the mortality increased nor was the reproduction reduced nor did the animals show a certain choice behaviour. Since TAT is known to be easily adsorbed and even to be incorporated in the soil matrix (DAUN et al, 1998; ACHTNICH et al, 1999), additional non-soil tests were performed. In the contact test no toxicity could be detected, even if 2000 mg TAT/L were applied on the filter paper, leading to a concentration of 28.5 µg TAT/cm^2. As it cannot be excluded that TAT does also easily adsorb to the cellulose of the filter paper, a water test was performed, although water is not the typical media for the collembola. Even then no significant mortality was observed up to 2000 mg

TAT/L. Thus TAT has to be considered non-toxic up to a concentration of 2000 mg TAT/L or 2000 mg TAT/kg soil (dw) for the collembola *F. candida*.

In the case of the enchytraeid *E. crypticus* no significant mortality was detected up to 2000 mg TAT/kg soil (dw), but the reproduction was reduced with an EC50(28d) of 1324.6 mg TAT/kg soil (dw). For this test species, too, a water test was performed resulting in LC50-values of 162.5 mg TAT/L after 24 hours and 159.0 mg TAT/L after 48 and 72 hours. Hence, the water test is more than 12.5 times more toxic than the soil test. The difference in toxicity between the soil and the non-soil-test is much bigger for TAT than for TNT or the reference substance Betanal, which are 11 respectively 8 times more toxic (see p. 44, chapter 4.5.1.1). Thus a strong sorption of TAT to the soil matrix has to be assumed resulting in a lower bioavailability of the substance.

In the choice test *E. crypticus* significantly avoided concentrations of 1000 mg TAT/kg soil (dw) and above. A repellence, however, was only evident at 2000 mg TAT/kg soil (dw). This concentration is higher than the EC50(28d). Thus, the animals would stay in the soil material, even though their reproduction would be significantly reduced, but animals from outside would not immigrate into a soil material of this concentration. Hence, for TAT the avoidance-test is the most sensitive test.

4.5.4.2 Comparison with literature

In the literature the toxicity of TAT has not been widely evaluated. In the study of Drzyzga an EC50(30min)-value of 101.3 mg/L for *V. fischeri* in aqueous solutions is given. TAT was thus classed as less toxic to aquatic organisms. In comparison to the prior degradation products and the parent compound TNT, TAT was much less toxic (DRZYZGA et al, 1995). Another study found an IC50(15min) for TAT of > 25.81 mg/L for *V. fischeri*, which was again lower than that of its predecessors. Tests with the green algae *S. capricornutum* gave similar results for the other degradation products, but TAT was not tested (SUNAHARA et al, 1998).

4.5.5 Comparison of the toxicity to different organisms

Overall TNT is much more toxic than any of the other substances. As it was also the most widely used explosive during the First and Second World War, it must be considered as the one causing the highest ecological risk. The following order of toxicity can be given for the enchytraeid- and collembola-biotest:

F. candida: TNT > Hexyl > TAT/Hexogen/Octogen

E. crypticus: TNT > Hexyl > TAT > Hexogen/Octogen

From the studies on earthworm toxicity no order of toxicity for the three tested explosives can be concluded, since for two of them only LOECs have been observed, but TNT is the most toxic

one. The earthworm *E. andrei* is more sensitive than *E. crypticus* in respect to reproduction, but less sensitive in regard to acute toxicity. The collembola is in both cases more sensitive (Table 4.5-1).

For the luminescent bacteria *V. fischeri* the order was the same as for the enchytraeid- and collembola-biotest, except that Hexogen is even more toxic for the bacteria than TAT. The toxicity of Octogen cannot be exactly classified, as no toxicity was observed due to its low water solubility.

V. fischeri: TNT > Hexyl >Hexogen/Octogen? > TAT/Octogen?

A direct comparison with the EC50-values for the species investigated in this thesis is not possible, since the concentrations in solution would have to be compared with the concentrations in soil (Table 4.5-1).

TNT is more toxic to the marine polychaete *N. arenaceodentata* and the estuarine amphipod *L. plumulosus* than to either *F. candida* or *E. crypticus* (Table 4.5-1).

The up to now investigated higher plants cress (*L. sativa)*, turnip (*B. rapa)*, oat (*A. sativa)*, wheat (*T. aestivum)*, tall fescue (*F. elatior* or *F. gigantea)*, bromegrass (*B. inermis*) and smooth switchgrass (*P. virgatum*) are all more sensitive to TNT than the terrestrial invertebrates (Table 4.5-1). Since these plants take up TNT, they offer the advantage of phytoremediation of soils with low TNT-contamination.

Table 4.5-1: Overview of the toxicities of TNT, Hexyl, Hexogen, Octogen and TAT to different test organisms in respect to mortality and reproduction

		TNT			Hexyl		Hexogen		Octogen		TAT
		LC50	EC50	LOEC	LC50	EC50	LC50	LOEC	LC50	LOEC	EC50
water risk	*V. fischeri* (mg/L)		3.59[1] 0.95[2]			16.32[1]	74.56[1] >40.2[2]		>25[1] >6.43[2]		101.3[1] >25.81[2]
	S. capricornutm (mg/L)		3.2[2]				>40.2[2]		>6.52[2]		
sediments	*N. arenaceodentata* (mg/kg)	320[3]									
	L. plumulosus (mg/kg)	202.7[3]									
terrestrial inver-tebrates	*F. candida* (mg/kg)	139.9	64.3			176.3					
	E. crypticus (mg/kg)	949.4	501.2		2402.8	530.4					1324.6
	E. andrei (mg/kg)	> 881[4]		110[4]			>756[4]	95[4]	>3013[5]	296[5]	
phyto-toxicity	*F. elatior/F. gigantea* (mg/kg)			100[6]							
	B. inermis (mg/kg)			50[7]							
	P. virgatum (mg/kg)			50[7]							
	L. sativa (mg/kg)			50[8]							
	B. rapa (mg/kg)			50[8]							

1) DRZYZGA et al, 1995
2) SUNAHARA et al, 1998
3) GREEN et al, 1999
4) ROBIDOUX et al, 2000
5) ROBIDOUX et al, 2001
6) PETERSON et al, 1996
7) PETERSON et al, 1999
8) GONG et al, 1999

4.5.6 Comparison of tests

A comparison between the mortality and the reproduction test proved the reproduction test to be the more sensitive one for both animals. In these tests *F. candida* was more sensitive to TNT than *E. crypticus*. In the case of TAT only the enchytraeid showed a significant reduction of the reproduction, but on neither of the test species the TNT-metabolite had a lethal effect. Hexyl, too, was not lethal for *F. candida*, but for *E. crypticus* and it reduced the reproduction of both species, even more for the collembola than for the enchytraeid.

For the collembola, however, only for Hexyl a significant choice behaviour was detected. For the other tested substances no choice behaviour was observed, indicating that the animals have no perception of the compounds. In other investigations it was already established that *F. candida* can perceive heavy metals like cadmium (Cd) and copper (Cu) as well as organic compounds like the reference substance Betanal. The collembola was attracted by copper in concentrations ≥ 20 mg Cu/kg soil (dw), even in concentrations above the LC50(7d) of 778 mg Cu/kg soil (dw) or the EC50(28d) of 283 mg Cu/kg soil (dw) (PANNECK, 2000). Cadmium was avoided at low concentrations (20 and 40 mg Cd/kg soil (dw)), but the collembola was attracted by higher concentrations (≥ 400 mg Cd/kg soil (dw), even if they were above the EC50(28d) of 640 mg Cd/kg soil (dw) (PANNECK, 2000). The herbicide Betanal was avoided at concentrations of ≥ 22.2 mg Betanal/kg soil (dw), which is below the LC50(7d) of 95.5 ≥ 9.4 mg Betanal/kg soil (dw). However, the animals showed an escape behaviour only at concentrations of 108.3 mg Betanal/kg soil (dw). Other species of collembola like *Isotoma anglica, Heteromurus nitidus, Lepidocyrtus violaceus* or *Onychiurus armatus* were even more sensitive to the herbicide (ACHAZI et al, 2000: 99-100). For *Folsomia fimetaria* (Isotomidae) it was proven that the collembola avoided and escaped the insecticide dimethoate, even at the recommended dose (in Denmark 1L per ha containing 280 mg active ingredient) (FÁBIAN & PETERSEN, 1994). For the herbicide AAnetos L with the active ingredient 2,4,5-T-trichlorophenoxyacetic acid a repellency leading to avoidance was shown for *Onychiurus quadriocellatus* on a soil/agar substrate. The tested concentrations of 0.0131 mL/dm^2 and higher correspond to the quantity of herbicide ending up in the ground if the usual dose is applied (500 to 800 L/ha of a 1.5 or 1% solution) (EIJSACKERS, 1978).

For the evaluation of the toxicity of contaminated soil materials with an avoidance test system with the earthworm *Eisenia fetida* it was suggested, that the habitat function of the soil material is affected if 80% or more of the worms escaped from the contaminated side (HUND-RINKE et al, 2000: 69). This method is not suitable for the choice tests with *F. candida* and *E. crypticus*, as in some tests the animals hardly moved at all from the side on which they had been placed at the beginning of the test. Aggregation has been observed regularly for the collembola even

under laboratory conditions (USHER & HIDER, 1975). Therefore a Chi-square test was applied, which took into account the distribution of the control in the same test.

The choice test was the most sensitive test for *E. crypticus.* This was also the case for other substances tested with this test systems, like the heavy metals cadmium (Cd) and copper (Cu) as well as the reference substance Betanal. The enchytraeid avoided Cu at concentrations ≥ 200 mg Cu/kg soil (dw), which is below the LC50(7d) of 798 mg Cu/kg soil (dw) or the EC50(28d) of 301 mg Cu/kg soil (dw) (PANNECK, 2000). The choice behaviour towards Cd however, was contrary: the enchytraeids were attracted by the highest tested concentration of 100 mg Cd/kg soil (dw), which is above the EC50(28d) of 64 mg Cd/kg soil (dw) and below the LC50(7d) of 210.5 mg Cd/kg soil (dw) (PANNECK, 2000). The herbicide Betanal was strongly avoided by *E. crypticus* at concentrations ≥ 127 mg Betanl/kg soil (dw) (WAGNER-VASKE, 2000), which is much lower than the LC50(7d)-value or the EC50(28d)-value of 573.2 ± 114.6 or 222.9 ± 95.5 mg Betanal/kg soil (dw), respectively.

Table 4.5-2: Comparison of the sensitivity of the tests for the two test organisms for the tested explosives and TAT

	F. candida					***E. crypticus***				
	TNT	**Hexyl**	**Hexo-gen**	**Octo-gen**	**TAT**	**TNT**	**Hexyl**	**Hexo-gen**	**Octo-gen**	**TAT**
mortality test	**+**	**--**	**--**	**--**	**--**	**+**	**+**	**--**	**--**	**--**
reproduc-tion test	**+**	**+**	**--**	**--**	**--**	**+**	**+**	**--**	**--**	**+**
contact test	**+**	n.t.	n.t.	n.t.	**--**	n.t.	n.t.	n.t.	n.t.	n.t.
water test	n.t.	n.t.	n.t.	n.t.	+	**+**	n.t.	n.t.	n.t.	**+**
choice test	**--**	**+**	**--**	**--**	**--**	**+**	**+**	**--**	**--**	**+**

\+ toxicity found
-- no toxicity found
n.t. not tested