6 Ageing of TNT-contaminated soil materials

6.1 Abstract

The toxicity of TNT in the mortality and reproduction test with *F. candida* decreased if the soil material had been stored for some time before the tests were performed, which is considered to be the effect of microbial degradation as well as sorption to the soil matrix. The toxicity of the aged soil material was related with the ageing time and the original TNT-concentration. In the mortality tests the decrease in mortality manifested itself earlier than in the reproduction tests, since the mortality is the less sensitive parameter. It developed faster in soil materials stored at 20°C than in those stored at 4°C.

Some of the soil samples had been analysed and their TNT-content can be linked with their toxicity. Thus, in soil materials with a TNT-content of 18-19 mg TNT/kg (dw) the mortality was strongly reduced. The reproduction was no longer significantly reduced if the TNT-content was around 2 mg TNT/kg soil (dw), although an improvement was already observed at 19 mg TNT/kg soil (dw). If the TNT-concentration was above this value or the concentrations of the ADNTs were quite high (2A46DNT > 9 mg/kg soil (dw) and/or 4A26DNT > 13 mg/kg soil (dw)) the reproduction remained completely reduced.

During the duration of the reproduction test itself the TNT-content decreased and the concentration of TNT-degradation products increased, indicating microbial degradation.

6.2 Theoretical background

In Germany most contamination by ammunitions took place during both World Wars or immediately afterwards as a result of the dismanteling by the allied forces. Hence, the contamination is more than 50 years old. The toxicity of a substance, however, depends also on the time it has already been in the soil, as the pollutant can be adsorbed, absorbed and even bound by soil particles. In addition, degradation can take place in soil reducing but also increasing the toxicity of the soil, depending on the degradation products formed.

It was observed that Herbicides had become resistant to desorption as a result of ageing, thus limiting degradation and plant uptake (SCRIBNER et al, 1992). From polyaromatic hycrocarbons (PAK) it is known that they form non-extractable bound residues in aged soils (KÄSTNER et al, 1997). The presence of such persistent residual fractions of substances might cause a slow and continuous leaching to groundwater and limit the effectiveness of soil remediation (SCRIBNER et al, 1992). The effects of ageing on TNT have so far not been investigated.

6.2.1 Sorption

The sorption of TNT to the soil matrix has already been described in detail in the previous chapter (see p. 57-59, chapter 5.2). Until an equilibrium between the sorbed or bound and the dissolved toxicant is established, some time is necessary. In the case of TNT it was found that in the first week sorption takes place, but that much more time is needed for irreversible binding (COMFORT et al, 1995).

In a first set of experiments the influence of sorption versus microbial degradation was investigated. One batch of soil material was stored at 4°C to suppress microbial degradation, thus focusing on sorption as a result of ageing. Another batch was stored at 20°C to enhance microbial degradation during the ageing process. Some soil samples were analysed for their TNT-content at the beginning of the tests and at the end of the reproduction tests.

6.2.2 Microbial degradation of TNT

TNT is very resistant to electrophilic attack at the aromatic ring, as each of the three nitro groups withdraws electrons from the ring and thus renders the aromatic ring electron deficient (RIEGER & KNACKMUSS, 1995: 2). However, the nitro groups are easily susceptible to reductive attack, yielding to the reduction of the nitro groups as the mayor pathway for TNT degradation (PREUß & RIEGER, 1995: 72). As a consequence, TNT is converted in a stepwise process via the aminodinitrotoluenes (ADNT) and diaminonitrotoluenes (DANT) to triaminotoluene (TAT), which is outlined more detailed and with structural formulas later (see p. 116-117, chapter 8.2.2.1). Only the last step, the formation of TAT, is strictly anaerobe (PREUß & RIEGER, 1995: 76). Under aerobic conditions, however, many different intermediates and condensation products like azoxy compounds can be formed, some of them even more toxic than their precursors (RIEGER & KNACKMUß, 1995: 8-9). An overview of the various possible intermediates is given in Fig. 6.2-1.

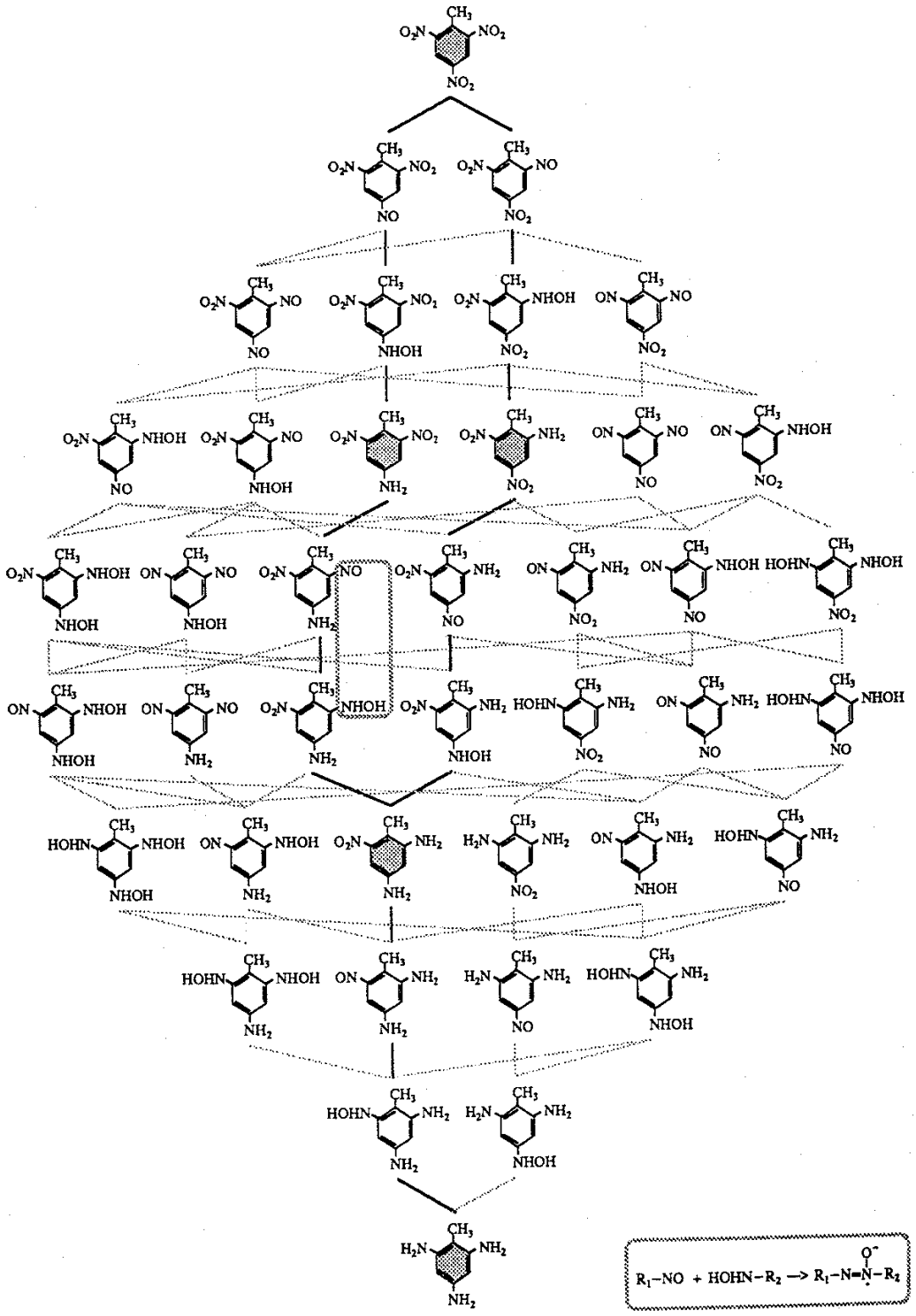


Fig. 6.2-1: Possible intermediates and condensation products of TNT originating from gratuitous reduction. Identified metabolites are indicated by shading of the aromatic rings. The heavy lines connecting these compounds indicate the most likely reaction sequence between TNT and TAT (RIEGER & KNACKMUSS 1995: 9).

In a second set of experiments the influence of the ageing process at 20°C was examined in more detail at lower TNT-concentrations.

6.3 Materials and methods

The effect of ageing on the toxicity of TNT was assessed with mortality and reproduction tests (see p. 6, chapter **3.3.1.1**), but only for the collembola as the more sensitive organism for this toxicant. For the tests the usual standard soil material Lufa 2.2 was used (see p. 5, chapter 3.3.1).

6.3.1 Ageing at different temperatures

A first set of experiments was performed with the concentrations 0, 150, 300, 450, 600 and 700 mg TNT/kg soil (dw) after ageing periods of 15 days, one, two, four and six months. Each concentration was mixed individually. However, due to the huge amount of soil material, it became lumpy, although the WHC was correctly adjusted to 60%.

The contaminated soil material was stored in bottling jars in the dark to prevent photochemical degradation. One set of jars was stored at 20°C to enhance microbial activity and another one at 4°C to suppress it. At the beginning of the ageing as well as before each test and at the end of each reproduction test a sample of each contaminated soil material was stored at -18°C and later analysed for its TNT-content.

The toxicity for the test organisms in soil materials of both ageing temperatures was determined at the same time. Six samples per concentration were used.

The mortality and the reproduction were statistically evaluated as usual with a one way ANOVA (see p. 9, chapter 3.3.3). A mortality of 100% and a reproduction rate of 0% were considered to be significant without testing. If thus only one concentration remained to be compared with the control, an unpaired t-test or a U-test was performed (see p. 9, chapter 3.3.3). In addition, the influence of the ageing on each TNT-concentration at different ageing periods was tested in the same way for the mortality and the reproduction tests. The mortality and the reproduction rate at the two temperatures were compared with an unpaired t-test or a U-test.

6.3.1.1 Analysis of soil samples

The analysis was performed by the Technische Akademie Wuppertal e.V, Institut für Umweltanalytik und Altlastenerkundung at Wildau, Germany. TNT and its degradation products were extracted with diethyl ether. For the detection high pressure liquid chromatography (HPLC) and a dioden array detector (DAD) were used. Only TNT and the ADNTs 2-amino-4,6-dinintrotoluene (2A46DNT) and 4-amino-2,6-dinitrotoluene (4A26DNT) were detected at a detection limit between 0.1 and 0.2 mg/kg soil (dw). In the unspiked control with Lufa 2.2 no nitroaromates were found. A full test report is given in Appendix B.

6.3.1.2 More detailed assessment of the ageing at 20°C

Since the differences between the concentrations were too high a second set of experiments was performed with 150, 200, 250 and 300 mg TNT/kg soil (dw) after 7 days, 14 days, one month, two and four months of ageing. This time the concentrations were obtained by mixing the highest contaminated soil material with the uncontaminated one. The water was added in several portions to prevent the soil material from becoming lumpy. The bottling jars were only stored at 20°C in the dark and no samples were taken for analysis. Five samples of each concentration were tested.

Unfortunately, no synchronised test populations were available after 7 and 14 days of ageing, thus these two ageing experiments were repeated with a new set of soil material prepared in the same way.

6.4 Results

6.4.1 Ageing at different temperatures

6.4.1.1 Effect on the TNT-content

Those soil samples in which at either temperature an effect on the mortality or the reproduction had been observed, were analysed for their content of TNT and its degradation products.

Effect of the ageing process

Table 6.4-1: Concentrations of TNT and the ADNTs detected in the spiked soil samples after different ageing periods

soil sample			contamination					
nominal TNT-concentration	period of ageing	ageing tempe-rature	mg/kg soil (dw)			% nominal TNT		
			TNT	2A46DNT	4A26DNT	TNT	2A46DNT	4A26DNT
150 mg TNT/kg soil (dw)	0 d		43.1	4.77	4.44	28.7	3.2	3.0
	2 mo	20°C	n.d.	6.60	4.10	0.0	4.4	2.7
	2 mo	4°C	18.5	3.66	3.49	12.3	2.4	2.4
	6 mo	4°C	1.94	6.07	4.38	1.3	4.0	2.9
300 mg TNT/kg soil (dw)	0 d		161	9.15	6.18	53.7	3.1	2.1
	2 mo	20°C	18.1	9.07	11.20	6.0	3.0	3.7
	2 mo	4°C	102	5.57	9.08	34.0	1.8	3.0
450 mg TNT/kg soil (dw)	0 d		288	3.88	5.43	64.0	0.9	1.2
	4 mo	20°C	28.7	15.50	15.80	6.4	3.4	3.5
	4 mo	4°C	235	4.79	9.38	52.0	1.1	2.1

n.d. not detected

The analysis of the spiked soil materials revealed that not all TNT could be extracted, even in the soil materials which had been immediately frozen after the spiking at day zero of the ageing. Hence, in the soil material spiked with 150 mg TNT/kg soil (dw) 106.9 mg TNT could not be

extracted. Hence, only 29% of the original amount of TNT were detected, whereas 3.2% of the originally added TNT was transformed into 2A46DNT and 3.0% into 4A26DNT. In the soil material spiked with 300 mg TNT/kg soil (dw) 139.0 mg TNT could not be recovered. Overall 54% of the original TNT were found as well as 3.1% 2A46DNT and 2.1% 4A26DNT. In the soil material spiked with 450 mg TNT/kg 162.0 mg TNT could not be extracted. Thus, only 64% of the original TNT were discovered together with 0.9% 2A46DNT and 1.2% 4A26DNT.

In general, the TNT-concentration was lower after the same ageing period, if the soil material had been stored at 20°C than at 4°C. For instance after two months no TNT could be detected in the soil material spiked with 150 mg TNT/kg soil (dw), if it had been stored at 20°C, whereas still 12.3% of the original TNT could be found in the soil material stored at 4°C. Even after six months 1.3% of the spiked TNT could still be detected in this soil material. The difference is even more striking in soil materials with higher TNT-concentrations. Hence, after two months only 6% of the spiked TNT were detected in the soil material with 300 mg TNT/kg soil (dw) aged at 20°C, whereas 34% could still be found in the soil material aged at 4°C. In the soil material originally contaminated with 450 mg TNT/kg soil (dw) 6.4 % of the spiked TNT could still be detected after four months if it had been stored at 20°C, but 52% if stored at 4°C.

Effect of the reproduction test

The test duration of 28 days for the reproduction test might have an effect on the toxicity, especially since microbial degradation is likely at the test temperature of 20°C. Hence, if the reproduction of the collembola had improved at either ageing temperature, samples of the soil material at the beginning and the end of the test were analysed.

Table 6.4-2: Concentrations of TNT and the ADNTs detected in the spiked soil samples after different ageing periods at the beginning and the end of the reproduction test

soil sample				contamination					
nominal TNT-con-centration	period of ageing	ageing tempe-rature	test	mg/kg soil (dw)			% nominal TNT		
				TNT	2A46DNT	4A26DNT	TNT	2A46DNT	4A26DNT
150 mg TNT/kg soil (dw)	2 mo	20°C		n. d.	6.60	4.10	0.0	4.4	2.7
	2 mo	20°C	repro	n. d.	6.43	3.52	0.0	4.3	2.4
	2 mo	4°C		18.5	3.66	3.49	12.3	2.4	2.3
	2 mo	4°C	repro	n. d.	3.91	3.96	0.0	2.6	2.6
	6 mo	4°C		1.98	6.07	4.38	1.3	4.0	2.9
	6 mo	4°C	repro	1.48	8.33	5.65	1.0	5.6	3.8
300 mg TNT/kg soil (dw)	2 mo	20°C		18.1	9.07	11.20	6.0	3.0	3.7
	2 mo	20°C	repro	4.16	12.8	15.9	1.4	4.3	5.3
	2 mo	4°C		102	5.57	9.08	34.0	1.8	3.0
	2 mo	4°C	repro	24.4	7.62	11.2	8.1	2.5	3.7
	4 mo	20°C		1.76	8.63	7.28	0.6	2.9	2.4
	4 mo	20°C	repro	1.36	8.89	9.10	0.5	3.0	3.0

n.d. not detected repro reproduction test

The TNT-concentration found after the reproduction test was always lower than before. Thus in the soil material spiked with 150 mg TNT/kg soil (dw) after two months of ageing at 4°C the TNT-content of 18.5 mg TNT/kg soil (dw) has completely disappeared during the reproduction test. In the soil material spiked with 300 mg TNT/kg soil (dw) the reduction of the TNT-content during the reproduction tests after two months of ageing at 20°C was not complete, but the concentration decreased from 18.1 to 4.16 mg TNT/kg soil (dw). However, after four months of ageing the TNT-content only decreased from 1.76 to 1.36 mg TNT/kg soil (dw) during the reproduction test. If the same soil material had been stored for two months at 4°C the TNT was reduced during the reproduction test from 102 to 24.4 mg TNT/kg soil (dw). On the other hand the percentages of ADNTs produced during the reproduction tests rose, with the exception of the soil material spiked with 150 mg TNT/kg tested after two months of ageing at 20°C.

6.4.1.2 Effect on the mortality

Ageing at 4°C

Table 6.4-3: Mortality for *F. candida* with TNT-contaminated soil material stored at 4°C after different periods of ageing; mean ± SD; n 6

	% mortality after different ageing periods at 4°C				
mg TNT/kg soil (dw)	**15 days**	**1 month**	**2 months**	**4 months**	**6 months**
0	6.0 ± 4.9	0.0 ± 0.0	14.0 ± 8.0	12.0 ± 7.5	1.7 ± 3.7
150	23.3 ± 23.6	41.7 ± 18.6#	23.3 ± 13.7	11.7 ± 10.7	1.7 ± 3.7
300	61.7 ± 31.3*	90.0 ± 11.5*	86.7 ± 13.7*	70.0 ± 21.6*	73.3 ± 11.1*
450	95.0 ± 5.0*	100.0 ± 0.0*	98.3 ± 3.7*	88.3 ± 13.4*	96.7 ± 4.7*
600	100.0 ± 0.0*	96.7 ± 4.7*	100.0 ± 0.0*	73.3 ± 26.2*	91.7 ± 6.9*
750	100.0 ± 0.0*	91.7 ± 10.7*	100.0 ± 0.0*	66.7 ± 22.9*	86.7 ± 11.1*

* significant difference to control at the same ageing period
\# significant difference to other ageing periods of the same concentration

The mortality only clearly decreased in the lowest tested concentration of 150 mg TNT/kg soil (dw), as it was no longer significantly higher than in the control. In all other concentrations the mortality remained significantly higher than in the control of the same ageing period.

Ageing at 20°C

Table 6.4-4: Mortality for *F. candida* with TNT-contaminated soil materials stored at 20°C after different periods of ageing; mean ± SD; n 6

	% mortality after different ageing periods at 20°C				
mg TNT/kg soil (dw)	**15 days**	**1 month**	**2 months**	**4 months**	**6 months**
0	4.0 ± 4.9	3.3 ± 4.7	5.0 ± 7.6	6.0 ± 4.9	1.7 ± 3.7
150	15.0 ± 15.0	16.7 ± 12.5	16.7 ± 7.5	6.0 ± 4.9	14.0 ± 13.6
300	61.7 ± 15.7*	78.3 ± 15.7*	23.3 ± 18.0*#	18.3 ± 6.9#	16.7 ± 11.1#
450	93.3 ± 7.5*	88.3 ± 10.7*	80.0 ± 11.3*	38.3 ± 10.7#	25.0 ± 17.1#
600	100.0 ± 0.0*	91.7 ± 10.7*#	90.0 ± 11.5*#	78.3 ± 12.1*#	91.7 ± 6.9*#
750	98.3 ± 3.7*	93.3 ± 4.7*	81.7 ± 13.4*	85.0 ± 16.1*	96.7 ± 4.7*

* significant difference to control at the same ageing period
\# significant difference to earlier ageing periods of the same concentration

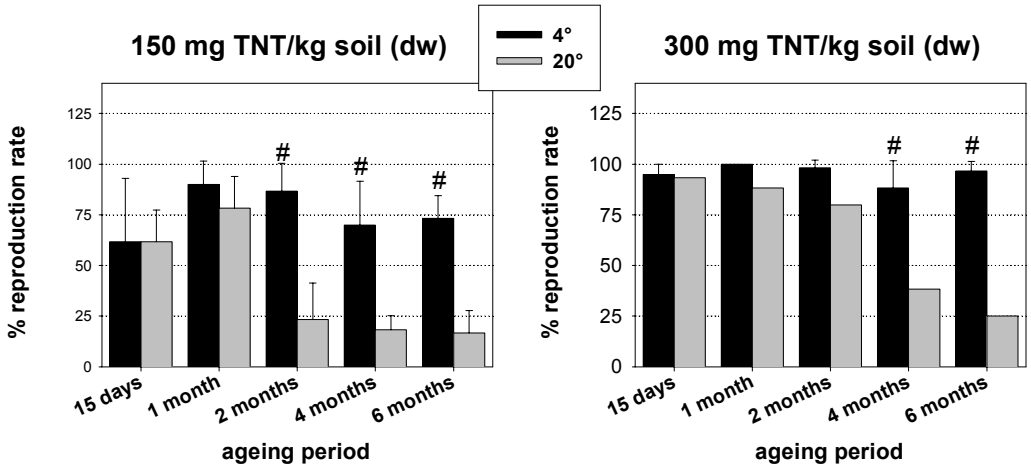
In the lowest tested concentration of 150 mg TNT/kg soil (dw) the mortality was already low 15 days after the ageing started. It was neither significantly different to the control nor was there any significant difference between the different ageing periods for this concentration. The mortality in the medium concentrations of 300 and 450 mg TNT/kg soil (dw) decreased with the period of ageing and after four months the mortality was no longer significantly different from the control. In the second lowest concentration of 300 mg TNT/kg soil (dw) the mortality decreased so much that after two months it was significantly different to the one at the beginning of the ageing. In the soil material with 450 mg TNT/kg soil (dw) the mortality was after four months significantly lower than before. Over the whole period of ageing the mortality remained significantly higher in the two highest tested concentrations of 600 and 750 mg TNT/kg soil (dw) than in the controls at the same ageing period. In the soil material with 600 mg TNT/kg soil (dw) the mortality decreased slightly from 100% 15 days after the ageing started and was significantly lower at the later ageing periods. In the highest tested concentration of 750 mg TNT/kg soil (dw) the different ageing periods did not differ from each other significantly, not indicating any improvement.

Comparison of the effect of ageing at different ageing periods on the mortality

In the lowest tested concentration of 150 mg TNT/kg soil (dw) the mortality was at both ageing temperatures right from the beginning not significantly higher than in the control of the same ageing period. Only after one month of ageing differed the two ageing temperatures significantly from each other.

In contrast, in the soil materials with higher TNT-concentrations stored at 4°C the mortality remained significantly higher than in the corresponding controls over the whole ageing period, whereas an improvement was observed in the soil materials stored at 20°C. Thus, after two months of ageing the mortality was already significantly lower in the soil material with 300 mg

TNT/kg soil (dw) stored at 20°C than in the soil material with the same TNT-content stored at 4°C and this remained so until the end of the experiment (see Fig. 6.4-1). In the soil material with 450 mg TNT/kg soil (dw) this difference became significant after four and six months of ageing (see Fig. 6.4-1).



significant difference to other ageing temperature

Fig. 6.4-1: Reproduction rates for *F. candida* in soil materials aged at different temperatures for the two lowest tested concentrations. Bars: mean ± SD.

Another possibility for a comparison between the soil materials aged at different temperatures is by their LC50(7d) values.

Table 6.4-5: LC50(7d)-values for *F. candida* in TNT-contaminated soil materials aged at 4°C and 20°C in comparison to the one without ageing indicated by shading

	LC50(7d) in mg TNT/kg soil (dw)	
ageing period	**ageing at 4°C**	**ageing at 20°C**
0 days	139.9 ± 8.2	
15 days	246.7	259.9
1 month	153.8	241.3
2 months	246.0	362.9
4 months	284.5	491.1
6 months	274.8	433.9

The LC50(7d)-values of all aged soil materials were higher than the one determined for the freshly spiked soil material with 139.9 ± 8.2 mg/TNT/kg soil (dw). They increased with the duration of the ageing process for both ageing temperatures. Exceptions from this general increase in the LC50(7d)-values were the soil materials tested after one months and six months of ageing. Overall, the increase in the LC50(7d)-values is more striking in the soil materials stored at 20°C, as they triple until the end of the ageing period in comparison to the one without ageing.

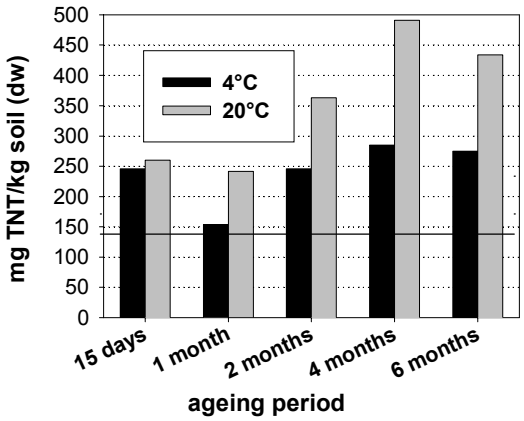


Fig. 6.4-2: LC50(7d)-values for *F. candida* in TNT-contaminated soil materials aged at 4°C and 20°C in comparison to unaged soil material indicated by the line.

6.4.1.3 Effect on the reproduction

Ageing at 4°C

Table 6.4-6: Reproduction rates for *F. candida* with TNT-contaminated soil materials stored at 4°C after different periods of ageing; mean ± SD; n 6

	% reproduction rate after different ageing periods at 4°C				
mg TNT/kg soil (dw)	**15 days**	**1 month**	**2 months**	**4 months**	**6 months**
0	100.0 ± 14.0	100.0 ± 14.0	99.8 ± 24.8	99.8 ± 26.2	100.1 ± 8.3
150	5.7 ± 5.2*	14.4 ± 13.0*	50.6 ± 15.1*#	45.7 ± 30.8*#	103.7 ± 9.9#
300	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*
450	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*
600	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*
750	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*

* significant difference to control at the same ageing period
\# significant difference to earlier ageing periods of the same concentration

Only in the lowest tested concentration the reproduction increased during the ageing process. After two months of ageing the reproduction rate had already improved so much that it was significantly higher than at the beginning of the ageing. After six months of ageing it was even no longer significantly lower than in the control of the same ageing period. In all other concentrations the reproduction remained completely reduced over the whole ageing period.

Ageing at 20°C

Table 6.4-7: Reproduction rates for *F. candida* with TNT-contaminated soil materials stored at 20°C after different periods of ageing; mean ± SD; n 6

	% reproduction rate after different ageing periods at 20°C				
mg TNT/kg soil (dw)	**15 days**	**1 month**	**2 months**	**4 months**	**6 months**
0	100.0 ± 14.0	99.9 ± 20.9	100.0 ± 13.7	100.0 ± 8.6	100.0 ± 8.4
150	48.2 ± 7.2*	24.5 ± 10.5*#	107.1 ± 19.2	82.8 ± 13.3	97.2 ± 5.6
300	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*	28.8 ± 30.4*	96.9 ± 9.8#
450	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*
600	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*
750	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*

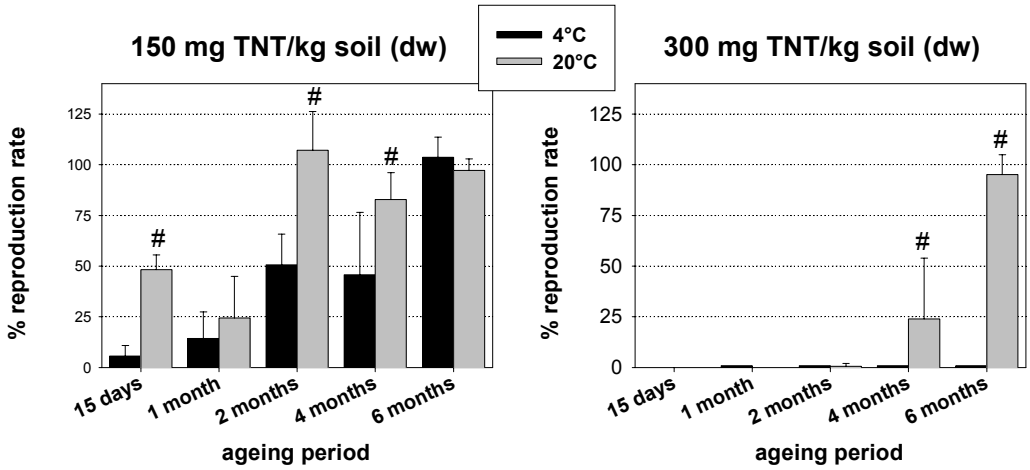
* significant difference to control at the same ageing period
\# significant difference to other ageing periods of the same concentration

The reproduction in the higher concentrations of 450, 600 and 750 mg TNT/kg soil (dw) remained completely reduced until the end of the ageing period. For the TNT-concentration of 300 mg TNT/kg soil (dw) the reproduction rate was after four months of ageing still significantly reduced in comparison to the control of the same ageing period, but not completely as before. After six months it was not even anymore significantly lower than the control and significantly higher than at earlier ageing periods. The reproduction rate in the lowest tested concentration of 150 mg TNT/kg soil (dw) was only 15 days and one month after the ageing started significantly lower than in the corresponding control. One month after the beginning of the ageing the reproduction rate was so low that it was even significantly lower than at the later ageing periods.

Comparison of the effect of ageing at different temperatures on the reproduction

The reproduction increased much more in the soil materials stored at 20°C than in those stored at 4°C. In the lowest tested concentration the reproduction rates increased in both cases, but for the soil material stored at 20°C it was already after two months no longer significantly lower than in the control. If it had been stored at 4°C this was only achieved after six months. After 15 days, two and four months the difference between the two ageing period was so big, that they differed significantly from each other (see Fig. 6.4-3).

In the next tested concentration of 300 mg TNT/kg soil (dw) only in the soil material aged at 20°C an increase of the reproduction rate could be detected after four months. After six months the difference was no longer significant to the control. The same soil material aged at 4°C, however, showed no improvement at all and the reproduction remained completely reduced. Hence, the reproduction rates in these two soil materials were significantly different from each other four and six month after the ageing started (see Fig. 6.4-3). In all other soil materials at both temperatures the reproduction remained completely reduced.



significant difference to other ageing temperature

Fig. 6.4-3: Reproduction rates for *F. candida* in TNT-contaminated soil materials aged at different temperatures for the two lowest tested concentrations. Bars: mean ± SD.

Unfortunately, no EC50(28d)-values could be determined as the reproduction in the higher concentrations remained completely reduced until the end of the ageing period.

6.4.2 More detailed assessment of ageing at 20°C

The effect of the ageing, especially for the reproduction, could not be clearly determined in the previous test, as the difference between the concentrations was very high. Thus, a second set of experiments was performed with lower concentrations, but this time the soil materials were only stored at 20°C during the ageing process.

6.4.2.1 Mortality

Table 6.4-8: Mortality for *F. candida* with TNT-contaminated soil materials stored at 20°C after different periods of ageing; mean ± SD; n 5

	% mortality after different ageing periods at 20°C				
mg TNT/kg soil (dw)	**7 days**	**15 days**	**1 month**	**2 months**	**4 months**
0	6.0 ± 8.0	6.0 ± 4.9	20.0 ± 11.0	10.0 ± 6.3	8.0 ±7.5
150	12.5 ± 10.9	6.0 ± 4.9	14.0 ± 8.0#	10.0 ± 8.9	10.0 ± 8.9
200	66.0 ± 12.0*#	16.0 ± 10.2	32.0 ± 21.4	14.0 ± 4.9	16.0 ± 10.2
250	96.0 ± 4.9*#	44.0 ± 10.2*#	50.0 ± 17.9#	6.0 ± 4.9	2.0 ± 4.0
300	98.0 ± 4.0*#	92.0 ± 7.5*#	80.0 ± 16.7*#	2.0 ± 4.0*	8.0 ± 9.8

* significant difference to control at the same ageing period
\# significant difference to other ageing periods of the same concentration

The mortality in the lowest tested concentration was never significantly higher than in the control of the corresponding ageing period. After one month of ageing, however, the mortality was significantly higher than at the other ageing periods. For the other concentrations a steady decrease in the mortality was observed. In all of them the mortality was no longer significantly lower than the control after two months of ageing. The highest tested concentration of 300 mg

TNT/kg soil (dw) was still significantly different after two months, but lower than the control, whereas before it had been significantly higher. The second lowest concentration of 200 mg TNT/kg soil (dw) did only differ from the control after 7 days and the soil material with 250 mg TNT/kg soil (dw) after 7 and 15 days of ageing.

As a result of the decrease in mortality the tests performed at earlier ageing periods differed significantly from the later ones. The mortality in the soil material with 200 mg TNT/kg soil (dw) was only significantly higher at 7 days of ageing, whereas the higher concentrations of 250 and 300 mg TNT/kg soil (dw) were still higher until one month of ageing.

6.4.2.2 Reproduction

Table 6.4-9: Reproduction rates for *F. candida* with TNT-contaminated soil materials stored at 20°C after different periods of ageing; mean ± SD; n 5

	% reproduction rate after different ageing periods at 20°C				
mg TNT/kg soil (dw)	**7 days**	**15 days**	**1 month**	**2 months**	**4 months**
0	100.0 ± 14.3	100.0 ± 15.8	100.0 ± 15.7	100 ± 16.7	99.9 ± 12.4
100	76.5 ± 19.7	105.2 ± 22.1	104.7 ± 24.0	94.1 ± 4.6	112.8 ± 17.1
150	18.1 ± 15.4*	102.7 ± 10.3	90.1 ± 23.8#	44.2 ± 14.4*#	81.3 ± 20.6#
200	0.0 ± 0.0*	26.9 ± 12.3*#	27.1 ± 15.6*#	59.9 ± 8.4*#	120.7 ± 13.4#
250	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*	63.0 ± 15.6*#	96.9 ± 27.4#
300	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*	32.6 ± 40.6*	119.7 ± 16.9#

* significant difference to control at the same ageing period
\# significant difference to earlier ageing periods of the same concentration

In all concentrations the reproduction rates increased over the ageing period of four months and was then even not significantly different from the control as at earlier ageing periods. An exception was the lowest concentration of 100 mg/TNT/kg soil (dw), which never differed significantly from the control. The increase was steady apart from the soil material with 150 mg TNT/kg soil (dw), in which the reproduction dropped to 44.2 % after two months. Then it was significantly lower than at 15 days and one month and also lower than in the control of the same ageing period. Afterwards, the reproduction rate rose and became again significantly higher than at 7 days.

Due to the increase in the reproduction rates the tests performed at later periods of ageing differed significantly from the earlier ones. Thus, the reproduction rate at all later ageing periods differed from the one after 7 days in the soil material with 200 mg TNT/kg soil (dw). For the soil material with 250 mg TNT/kg soil (dw) only the reproduction rate after two and four months differed. At the highest concentration of 300 mg TNT/kg soil (dw) only the reproduction rate of the last ageing period of four months was significantly higher than at the beginning of the ageing.

6.4.2.3 Comparison of the effect on mortality and reproduction

The reproduction needed much longer to show an improvement as a result of the ageing. Thus, in the soil material with 150 mg TNT/kg soil (dw) the reproduction rate was still significantly lower after 7 days of ageing, whereas the mortality showed no significant difference to the control for this TNT-concentration. In the soil material with 200 mg TNT/kg soil (dw) the reproduction rate was completely reduced 7 days after the ageing had started and also in the soil materials with 250 and 300 mg TNT/kg soil (dw) still after 15 days and one month of ageing. The mortalities in these soil materials had already improved after the same ageing periods, although they were still significantly lower than in the corresponding controls. After the next ageing period the mortality in all three soil materials was no longer significantly different. The reproduction on the other hand was no longer completely reduced. The reproduction rate was just after four months of ageing no longer significantly lower in the three highest tested soil materials than in the control.

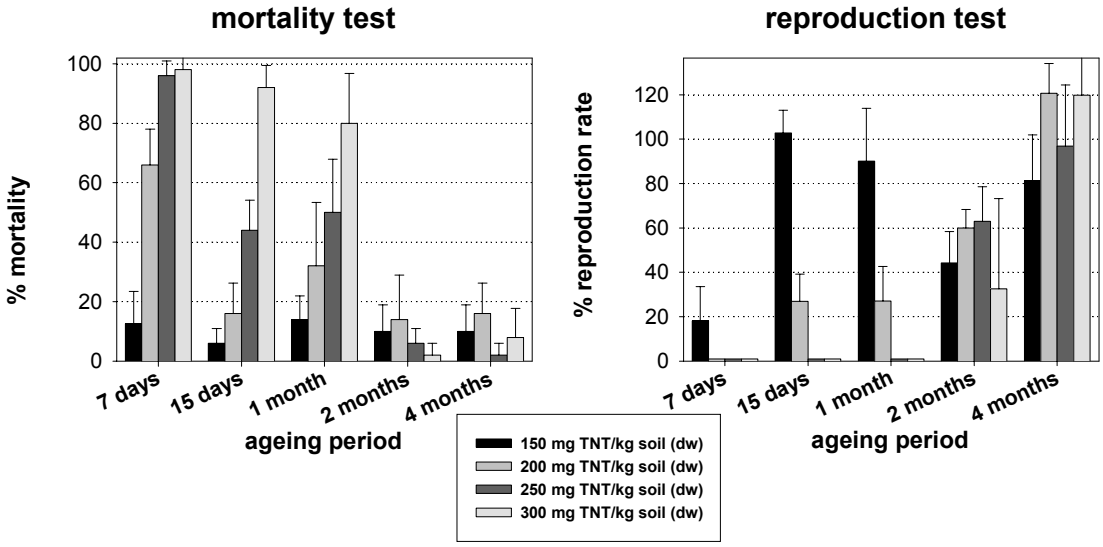


Fig. 6.4-4: Mortality and reproduction rates for *F. candida* in TNT-contaminated soil materials aged at 20°C.

In the graph it becomes obvious that the decrease in the mortality between one month and two months of ageing is enormous for the two highest tested concentrations, as the soil materials were no longer lethal after two months of ageing. For the reproduction the drop in the lowest tested concentration of 150 mg TNT/kg soil (dw) after two months of ageing manifests itself very clearly. In all other concentrations the reproduction rates increased steadily.

For this test set it was possible to determine LC50(7d)- as well as EC50(28d)-values for a comparison of the toxicity influenced by ageing.

Table 6.4-10: LC50(7d)-and EC50(28d)-values for *F. candida* in TNT-contaminated soil materials aged at 20°C in comparison to the one without ageing indicated by shading

ageing period	LC50(7d) in mg TNT/kg soil (dw)	EC50(28d) in mg TNT/kg soil (dw
0 days	139.9 ± 8.2	64.3 ± 20.0
7 days	191.3	119.6
15 days	251.9	155.3
1 month	261.5	186.1
2 months	> 300	230.2
4 months	> 300	> 300

The LC50(7d) and EC50(28d)-values increased steadily with the ageing. The most striking difference for the EC50-value is the one between the unaged soil material and the one after 7 days, as it nearly doubled. After two months the LC50(7d)-value could no longer be determined as it was above the highest tested concentration of 300 mg TNT/kg soil (dw). For the reproduction rate this was the case after four months.

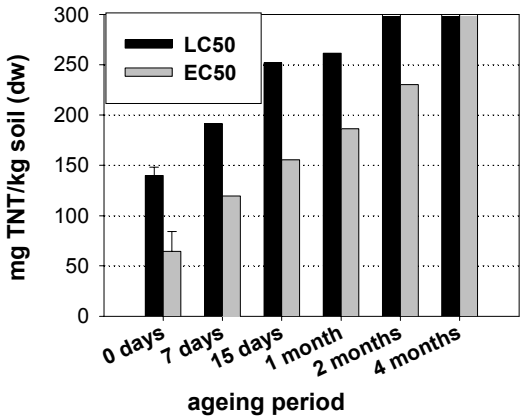


Fig. 6.4-5: LC50(7d)- and EC50(28d)-values for *F. candida* in TNT-contaminated soil materials aged at 20°C.

6.4.3 Summary of results

For an overview of the results of the two different tests sets the evaluated LC50(7d)- and EC59(28d)-values are given in Table 6.4-11.

Table 6.4-11: LC50(7d)- and EC50(28d)-values for *F. candida* determined in both test sets

	LC50(7d) in mg TNT/kg soil (dw)					
ageing period/ temperature	**7 days**	**15 days**	**1 month**	**2 months**	**4 months**	**6 months**
4°C	n. t.	246.7	153.8	246.0	284.5	274.8
20°C 1st set	n. t.	259.9	241.3	362.9	491.1	433.9
20°C 2nd set	194.3	251.9	261.5	--	--	--
	EC50 (28d) in mg TNT/kg soil (dw)					
4°C	n. t.	--	--	--	--	--
20°C 1st set	n. t.	--	--	--	--	--
20°C 2nd set	119.6	185.8	186.1	230.2	--	--

n.t. not tested -- not assessable

6.5 Discussion

In both test sets the toxicity decreased with the ageing period. However, in the first test set in which the ageing at different temperatures was observed no EC50(28d) could be determined. Hence, for the reproduction the decrease in toxicity can be better evaluated in the second test set.

For the mortality, on the other hand, more LC50(7d)-values could be determined in the first test set, and thus the decrease in toxicity can be better observed in this test set. For the ageing period of one month it was possible to determine the LC50(7d)-value for both test sets. In the first set of experiments it was lower than in the second, but it was also lower than at the ageing period before, indicating that the test itself might not have been correct. After six months of ageing the value decreased again, however, as the differences in the tested concentrations were very big, this should not be overestimated.

6.5.1 Ageing at different temperatures

On the basis of the soil analysis the influence of the ageing process and of the duration of the reproduction test on the TNT-content can be evaluated. In addition the TNT-concentration of the soil samples can be linked with their toxicity in the mortality and reproduction tests.

6.5.1.1 Effect on the TNT-content

Effect of the ageing process

Even in soil samples, which were immediately frozen after the spiking at day zero of the ageing process, not all of the added TNT could be detected (see p. 75, Table 6.4-1). On the one hand this has to be attributed to the discovery rate of 90% for TNT with the used method and also to the sorption of TNT and its subsequent binding to the soil matrix. On the other hand the existence of ADNTs as degradation products indicates that degradation by microorganisms indigenous for the standard soil material Lufa 2.2 has taken place, although the soil samples were frozen. Either the degradation had already taken place before the soil materials were frozen, or it took place while the samples were frozen. However, in soils, which had been contaminated by use a long time ago, no change in the content of nitroaromates was detected during a storage of 18 months (DOTT et al, 2001). Thus a degradation prior to the freezing seems more likely.

In the unaged soil materials with higher TNT-concentrations obviously more of the originally introduced TNT could be discovered, as the rate of degradation is limited by the number of bacteria present in the soil material and the rate of sorption is limited by the number of sorption sides. The undetectable amount of TNT, however, was fairly similar with 106.9 mg TNT in the

soil material spiked with 150 mg TNT/kg soil (dw) and 139 mg TNT or 162 mg TNT in the soil materials to which 300 or 450 mg TNT/kg soil (dw) had been added. Hence, the rate of sorption or degradation was even higher in the higher contaminated soil materials.

The general indication of microbial degradation even before the ageing process started, renders the planned differentiation between sorption at 4°C and degradation at 20°C useless. Hence, the effect of sorption is not further discussed, but has to be kept in mind.

In the soil materials stored at 20°C the TNT-content was much lower after the same ageing period, than in the soil materials which had been stored at 4°C, suggesting at faster degradation at the higher ageing temperature. At the lower temperature, however, the degradation was slower, but not completely inhibited, which is indicated by the increasing percentages of ADNT´s detected in these soil materials. A clear preference whether more 2A46DNT or 4A26DNT were formed cannot be made. Unfortunately, the further degradation cannot be reported, as the soil materials were not analysed for diaminonitrotoluenes (DANTs).

In general, in soil materials spiked with a higher TNT-concentration, the percentages of recovered TNT were higher after the same period of ageing than in soil materials with a lower original TNT-content, since the rate of degradation is limited by the number of bacteria present in the soil material. However, even in the high contaminated soil material, no inhibition of the degradation was observed.

The degradation of TNT was not only related with the concentration and the temperature, but also with the duration of the ageing process. The longer the ageing was allowed to proceed, the lower the detected TNT-concentrations. Thus the soil material spiked with 150 mg TNT/kg soil (dw) contained 18.5 mg TNT/kg soil (dw) after four months of ageing at 4°C, but only 1.94 mg TNT/kg soil (dw**)** after six months of ageing. In the soil material originally spiked with 300 mg TNT/kg soil (dw) the TNT-content fell from 18.1 mg TNT/kg soil (dw) after two months of ageing at 20°C to 1.7 mg TNT/kg soil (dw) after four months of ageing at the same temperature.

Effect of the reproduction test

Due to the time relation of the degradation, it is not surprising that during the four weeks duration of the reproduction tests further degradation took place (see p. 76, Table 6.4-2). This was especially the case, if the TNT-concentration was still very high at the beginning of the test. On the other hand hardly any of the remaining TNT was transformed if its concentration had already been very low at the beginning of the reproduction test, suggesting a strong sorption or binding of the TNT. In soil materials, in which no TNT had been detected at the beginning and/or at the end of reproduction test, the concentration of both ADNTs decreased, indicating a further transformation.

An exception thereof was the soil material spiked with 150 mg TNT/kg soil (dw) after two months of ageing at 20°C. In this soil material no TNT could be detected at the beginning and at the end of the reproduction test. However, during the test the percentage of 2A46DNT fell slightly from 4.4% to 4.3% (6.60 to 6.43 mg 2A46DNT/kg soil (dw)) and the percentage of 4A26DNT from 2.7% to 2.4% (4.10 to 3.52 mg 4A26DNT/kg soil (dw)). This diminution of the ADNT-content suggests a further transformation.

In the soil material spiked with 150 mg TNT/kg soil (dw) after two months of ageing at 4°C the concentrations of the ADNTs did hardly rise from 2.4% to 2.6% for 2A46DNT (from 3.66 to 3.91 mg/kg soil (dw)) and for 4A26DNT from 2.3% to 2.6% (from 3.49 to 3.96 mg/kg soil (dw)). On the other hand the TNT-content of 18.5 mg/kg soil (dw) disappeared completely during the reproduction test. Again, the relatively small rise in the ADNT-concentration in comparison to the complete disappearance of the TNT indicates a further transformation.

Unfortunately, former transformation processes cannot be followed, as neither the subsequent degradation products of the reductive pathway, the DANTs, were analysed, nor the possible condensation products, the azoxy compounds.

6.5.1.2 Mortality

The mortality decreased much more rapidly in the soil materials stored at 20°C than in those stored at 4°C during the ageing process as is demonstrated by the increase in the LC507d)-values (see p. 80, Fig. 6.4-2). This is consistent with the faster decrease of the TNT-content in the soil materials stored at the higher ageing temperature. In the lowest tested concentration this difference in toxicity was not visible, since the mortality in the soil materials of both ageing temperatures was not significantly different from the control (see p. 77, Table 6.4-3 and p. 78 Table 6.4-4). At the higher TNT-concentrations, however, the difference in toxicity between the soil samples stored at different temperature was even significantly different (see p. 79, Fig. 6.4-1).

It is very difficult to link the mortality in the soil materials with the concentrations of TNT or the ADNTs found in the soil material. Thus, the soil material spiked with 150 mg TNT/kg soil (dw) contained after two months of ageing at 4°C with 18.5 mg TNT/kg soil (dw) a fairly similar content of TNT as the soil material spiked with 300 mg TNT/kg soil (dw) after two months of ageing at 20°C with 18.1 mg TNT/kg soil (dw). However, in the first the mortality was not significantly higher than in the control, but in the second. Hence, the higher toxicity of the soil material with the higher original TNT-content might be attributed to the higher concentrations of the ADNTs found in this soil material. However, the insignificant difference to the control of the soil material with the lower TNT-concentration could also be the result of the high mortality in the control of the ageing period.

In another soil material spiked with 450 mg TNT/kg soil (dw) after four months of ageing at 20°C the mortality was not significantly higher than in the corresponding control, although the detected TNT-concentration was with 28.7 mg TNT/kg soil (dw) higher than in the above mentioned soil materials. The concentrations of the ADNTs, too were higher. However, the significance had been evaluated with an ANOVA on ranks, which is not as exact as an ANOVA due to the formation of ranks. Therefore this test was not considered for a link between mortality and TNT-concentration.

Hence, a concentration at which TNT is no longer toxic for the collembola cannot be exactly determined, but at it can be stated that at concentrations of 18-19 mg TNT/kg soil (dw) the mortality is strongly reduced. Extractable concentrations of > 100 mg TNT/kg soil (dw) have to be considered as still toxic, since the soil material spiked with 300 mg TNT/kg soil (dw) after two months of ageing at 4°C had still a higher mortality than the corresponding control.

6.5.1.3 Reproduction

The effect of the different ageing temperature on the reproduction in TNT-spiked soil materials is even more distinct (see p. 82, Fig. 6.4-3) and in consistence with the slower degradation of TNT at the lower ageing temperature.

Only in two of the analysed soil materials the reproduction was not significantly lower than in the corresponding control, indicating that the TNT-content was too low to affect the reproduction of the collembola. In one of these soil materials, the one spiked with 150 mg TNT/kg soil (dw) after two months of ageing at 20°C, no TNT at all could be detected. In the other soil material, also originally contaminated with 150 mg TNT/kg soil (dw), but aged at 4°C for six months, 1.94 mg TNT/kg soil (dw) were detected at the beginning of the test.

On the other hand, in a soil material with an even lower TNT-concentration of 1.76 mg TNT/kg soil (dw) at the beginning of the reproduction test, the reproduction rate was significantly higher than at earlier ageing periods, but still significantly lower than in the control. However, this soil material, which had been spiked with 300 mg TNT/kg soil (dw) and aged for four months at 20°C, had higher concentrations of the ADNTs than the previously described soil materials. Hence, the content of 8.63 mg 2A46DNT/kg soil (dw) and/or 7.28 mg 4A26DNT/kg soil (dw) at the beginning of the test, might cause the higher toxicity of this soil material, especially as the concentration of both increased during the duration of the test.

In another soil material with a higher TNT-content of 18.5 mg TNT/kg soil (dw) at the beginning of the test the reproduction did also improve, but was still significantly lower than in the control. Hence, the toxicity of this soil material originally contaminated with 150 mg TNT/kg soil (dw) and stored for two months at 4°C should be attributed to its TNT-content. Especially since the concentrations of the ADNTs were quite low with 3.66 mg 2A46DNT/kg soil (dw) and 3.49 mg

4A26DNT/kg soil (dw). These concentrations increased only slightly during the reproduction test to 3.91 mg 2A46DNT/kg soil (dw) and 3.96 mg 4A26DNT/kg soil (dw), whereas TNT did disappear completely.

The soil material spiked with 300 mg TNT/kg soil (dw) and aged for two months at 20°C contained a similar TNT-concentration at the beginning of the test with 18.1 mg TNT/kg soil (dw). However, the reproduction in this soil material was much stronger affected than in the previously described soil. This is considered to be the effect of the high concentrations of ADNTs found in this soil material at the beginning of the test with 9.07 mg 2A46DNT/kg soil (dw) and 11.2 mg 4A26DNT/kg soil (dw). During the reproduction test the concentration of the ADNTs did further increase to 12.8 mg 2A46DNT/kg soil (dw) and 15.9 mg 4A26DNT/kg soil (dw), while the TNT-content decreased to 4.16 mg TNT/kg soil (dw). Hence, the concentration of the ADNTs was even higher than in the soil material spiked with 300 mg TNT/kg soil (dw) and stored at 20°C for four months for which the ADNTs were already considered to be responsible for its toxicity.

In the soil material spiked with 300 mg TNT/kg and aged for two months at 4°C the reproduction remained completely reduced, which must be attributed to its still high content of TNT with 102 mg TNT/kg soil (dw). Even at the end of the test, the TNT-concentration was still quite high with 24.4 mg TNT/kg soil (dw), as were the concentrations of the ADNTs with 7.62 mg 2A46DNT/kg soil (dw) and 11.2 mg 4A26DNT/kg soil (dw).

Hence, the reproduction of the collembola is not affected at TNT-concentrations around 2 mg TNT/kg soil (dw). An improvement can be already expected at concentrations of about 19 mg TNT/kg soil (dw). If the TNT-concentration is above this value or the concentrations of the ADNTs are quite high (2A46DNT > 9 mg/kg soil (dw) and/or 4A26DNT > 13 mg/kg soil (dw)) the reproduction is completely reduced.

6.5.2 More detailed assessment of ageing at 20°C

In the second set of experiments the effect of the ageing period on the toxicity of TNT in the soil materials is even more evident. The mortality as well as the reproduction showed a steady improvement with the ageing process. The decrease in the toxicity manifested itself earlier in the mortality than in the reproduction tests (see p. 84, Fig. 6.4-4).

An exception of this steady improvement was the mortality test after one month of ageing. However, at this ageing period the mortality in the control was also much higher than at the other periods, suggesting a general unfitness of the test animals at the time of the test (see p. 82, Table 6.4-8). Also in the reproduction tests of the soil material spiked with 150 mg TNT/kg soil (dw) after two months and four months of ageing the reproduction was much lower than before and as in the higher TNT-concentrations of the same ageing period (see p. 84,

Fig. 6.4-4). The reproduction rate in the control at this ageing period does not suggest an unfitness of the test animals. Thus the reduced reproduction might be the result of degradation products, although this effect was not observed in the first set of experiments.

The steady improvement of the soil materials is even better indicated by the steady increase of the LC50(7d)- and EC50(28d)-values (see p. 85, Table 6.4-10 and Fig. 6.4-5). Already after 7 days of ageing the 50%-values were above the ones in Lufa 2.2 without ageing. Unfortunately, the values could no longer be determined when the soil materials did no longer differ from the control - after two months in the mortality test and after four months in the reproduction tests.