Manuscript:

In-situ phytoremediation of TNT-contaminated soil

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Summary

Parts of the area of the derelict world war II ordnance plant "Werk Tanne" (Clausthal-Zellerfeld, Harz, Germany) are heavily contaminated by chemicals of the TNT-production and particularly by TNT itself. High soil contaminations have to be treated with *ex-situ* methods, but for the extended contaminations of surface soil *in-situ* phytoremediation is appropriate. The TNT-degrading potential of the rhizosphere of the planted trees and shrubs themselves is augmented by highly active mycorrhiza and white-rot fungi.

A phytoremediation measure was established to scale with heavy machinery (soil grader), including incorporation of white-rot fungi into the soil and planting of mycorrhized trees and shrubs. The effects of site preparation, mycorrhized rhizosphere and white-rot fungi on the degradation of TNT were assessed over one year with a complex monitoring scheme, including a battery of five biotests and field investigations on selected indicators (soil mesofauna, decomposition).

The results of the monitoring show a great influence of the grading procedure for site preparation, a diversified sensitivity of the biotest-battery and complex reactions of the field indicators. The contamination is effectively reduced by the grading. The phytoremediation measure as a whole reduces hazards of transport of nitro-aromatics by dust or leachate, initiates a secondary succession of the soil ecosystem and may in the longer term transform remaining TNT and metabolites and thus proves as an effective decontamination measure, which is applicable in large scale technology.

Introduction

The world-war-II ordnance plant 'Werk Tanne' close to Clausthal-Zellerfeld (Harz, Germany) was one of the most productive explosives factories of the Third Reich. From 1939 to 1944, a total of 105.358 tons of 2,4,6-trinitrotoluene (TNT) was manufactured and processed [1]. The industrial activities have caused an extended, but not uniform contamination of the area (120 ha) with TNT, intermediate and by-products of the manufacturing and metabolites

(particularly amino-di-nitrotoluene), which have remained a considerable hazard since then, due to the toxicity and potential cancerogeneity of the substances [2]. Additionally to the industrial activities, the soil was heavily influenced by bombardment, destruction and deforestation, as well as by subsequent afforestation with spruce in the late forties. A considerable background of heavy metals (lead in particular) is typical for the soils of the Harz mountains.

On site, biological remediation techniques have been tested to scale since 1998 [3], which have focussed mainly on *ex-situ* technologies requiring excavation of soil [4,5]. These treatments are designed for rather localised high TNT-contamination. In contrast, the *in-situ* phytoremediation process described in this paper is appropriate for extended contamination of surface soils, which should not exceed 1000 mg TNT / kg dm soil. The procedure makes use of technological (grading) and biological operations (incorporation of biota). It is expected that processes in the rhizosphere of the planted shrubs and trees in combination with introduced fungi (white-rot fungi, mycorrhiza) efficiently metabolise the nitro-aromatics. Additionally, the uptake of metabolites in plants and the formation of bound residues will be encouraged [6,7,8]. A complex monitoring programme, including field observations and laboratory testing, allows to evaluate the process. The experimental design has been described in detail in [9]. The paper focuses on the description of the efficiency of the procedure for degradation of TNT, on the re-establishment of intact ecosystem properties by prevention of adverse side effects and on the technical practicability.

Materials and Methods

Site preparation

A site close to the so-called former tonsil-soil storage (building 110) was selected for its moderate contamination in the surface-soil. In times of operation, tonsil was used in a unique recycling procedure for spoilt TNT. Because of the peculiarities of the former ordnance plant, no replicates could be established.

Soil type is very loamy (brown soil from loessy loam on greywacke), with pH in the range of 4 and with considerable gravel from underlying shale and industrial activities. Climate is montan, cool and humid (alt. 560 m; precip. 1300 mm / yr.; annual mean temp. 6,2 °C).

An experimental site of 25m x 20m was prepared in May 1999 (Fig. 1); an uncontaminated area (U) was included. A contaminated control of approx. 7 m x 7 m is located nearby in the spruce forest (F). Preparation of the site with a heavy-duty soil-grader (*MAK Systemgesellschaft Kiel* and UMWELTSCHUTZ NORD) loosened, aerated and homogenised the surface soil (0-30 cm); deeper layers may be compacted. Straw with white rot fungi (*Trametes versicolor, Pleurotus ostreatus*) was amended and incorporated into the soil. Bark mulch was applied for the reduction of dust emission, suppression of herb growth and improvement of microclimate, particularly soil moisture.

Spruce (*Picea abies*), poplar (*Populus tremula*) and elder (*Sambucus nigra*) were infected with mycorrhiza (*Pisolithus tinctorius, Paxillus involutus*) during nursery. In a factorial design, five variants (plots) were established (Fig. 1). On each of U, C and P, saplings of spruce, poplar and elder (n= 82, 17, 18, respectively) were planted in May 1999 in a regular 0,5 m grid.

Monitoring

The effects of site preparation, mycorrhized rhizosphere and white-rot fungi on the degradation of TNT were assessed over one year. The processes initiated by the applied procedures were followed with chemical and biological monitoring.

Sampling

Sampling schemes were integrated in space and time. For analytics of nitro-aromatics, 24 samples were taken from each plot in 0-10 and 10-30 cm depth (spacing 0,5 m; May 1999, August 1999, October 1999, May 2000). Suction lysimeters were driven 60 cm into the soil to evaluate the leachate (1 on U, 2 on the contaminated plots each). The uptake of nitro-aromatics by the plants was analysed from samples of root, shoot and leaves.

Samples for the biotest-battery and for the field monitoring were composed from 8 spots in each plot (not August 1999). Depths of 0 – 5 cm and 5 –10 cm are separated, some analyses include the depth of 10-30 cm. Also for these samples, chemical analyses were performed.

Nematodes were only analysed from two plots, from U and C.

Nitro-aromatics

Chemical analyses of contamination with TNT and its A-DNT metabolites use advanced methods (HPLC, thermal desorption plus GC / MS ?10?). High resolution was achieved with excellent selectivity and a positive identification (MS). Volatile components as well as PAHs may be detected simultaneously (TDS-GC/MS). Detection limit and limit of determination for nitro-aromatics are at 1 mg/ kg and 5 mg/kg dm soil, respectively, for HPLC and even lower for TDS-GC/MS. Polar compounds were searched after with HPLC/DAD [11].

Biotest-battery

The biotest-battery includes a set of five established bioassays, described in detail in [9, 12]. Based on the results from homogenised soil-samples, soil toxicity was assessed for all experimental plots (Tab. 1).

Soil fauna

The taxa for the soil zoological monitoring were selected on the grounds of the trophic systems, described by Heal and Dighton [13] to represent two main potential expositions: Nematodes from the micro-trophic system of the water-filled pores and Gamasina from the mesotrophic system of the air-filled pores. Representatives of the macrotrophic system (e.g. earthworms) do not occur in sufficient quantities in the soil of the site.

Nematode densities were assessed with Baermann extraction from 3 subsamples from the composite samples of the full treatment plots U and C (May 1999, Oct. 1999, May 2000). Families and feeding groups were determined (mainly after ?14?) and the maturity index was calculated ?15, 16?.

Microarthropods were driven out of the soil in a Tullgren-type extractor (three replicates of each composite sample). Estimates of abundances (ind. / 100 g dm) were calculated; species of predatory mites (Gamasina: Acari, Parasitiformes) were determined mainly after Karg ?17?.

Decomposition

The mini-container system after Eisenbeis et al. ?18? was used to estimate decomposition. A PVC-bar (length approx. 40 cm) holds 12 containers (diameter 16 mm, height 17 mm), 6 of which were filled with straw and the remaining 6 with spruce needles from uncontaminated areas of the site. The material was sterilised by freeze-heating cycles. The access of decomposers was influenced with mesh sizes of 20 µm and 500 µm, resulting in n=3 per variant per bar. In July 1999, 15 bars were exposed horizontally in each plot (5 cm below surface), 5 of which were removed from each plot in October 1999. Ash-free mass loss as a measure for decomposition was evaluated as box-plots (medians with quartiles).

Results

TNT in soil

The soil contamination with TNT ranges up to some 10 000 mg/kg dm soil in the forest (Fig. 1, Fig. 2). With values generally well under 500 mg/kg dm soil (May 1999, after site preparation) it is in the range suitable for phytoremediation, although rather low compared to the site exploration. In spite of grading, the distribution of TNT remained extremely heterogeneous in space (horizontally and vertically) and in type (fine clay-like material to course bits). Arithmetic means in mg TNT/kg dm soil for May 1999, 0-30cm, are (medians are given in parentheses): C= 46 (20), P=190 (53) , M=59 (9), F=2369 (22). Distribution of A-DNTs is more uniform, with means (mg A-DNT/kg dm soil) in the range of 15-40 on the experimental plots and 170 (61) on F. Concomitant with high nitro-aromatic contamination, values for lead are elevated spotwise by more than factor 3 (ca. 1800 mg/kg dm, site F).

The patterns of change of the nitro-aromatic concentrations in the soil are summarised in the trajectories of Fig. 2. Compared to the treatment plots, the forest site remains in a different domain. Initially, the drastic decrease of TNT is accompanied only by slight changes of the A-DNT-content. With time, TNT-concentrations stabilise at a low level with continuing decrease of A-DNT. On the treated plots, nitro-aromatic decrease is highly significant (Wilcoxon-Mann-Whitney Test P<0,01).

Polar components were not detected.

TNT in leachate

Nitro-aromatics in the leachate reflect quantitatively the soil contamination (F > P > C > M), but show interesting qualitative differentiation. Highest concentrations of nitro-aromatics in the leachate are measured in F (13 mg/l TNT, 1,8 mg/l A-DNT). On P, TNT reaches values of 2,8 mg/l with A-DNTs well below 1 mg/l, on M all concentrations in the leachate are well below 1, mostly even below 0,5 mg/l. Noticeably on plot C, A-DNTs occurs in rather high concentrations in the leachate (up to 2,3 mg/l) with only small loads of TNT (max. 0,5 mg/l). Drastic differences between the two suction lysimeters in each plot may reflect a southerly draining effect from an adjacent train aisle.

TNT in plants

Nitro-aromatics are detected in spruce on all contaminated plots, in poplar only on P. For spruce, there is no clear correlation between the soil contamination and the uptake of TNT or A-DNTs. However, highest concentrations are found in stem and needles from C, followed by P. The A-DNT values in stem/needles are by approx. 60% higher than those for TNT, reflecting the specific situation of the leachate from C. It is noteworthy, that roots do not show the highest nitro-aromatic concentrations.

Biotest-battery

The results of the biotest-battery reflect the influence of site characteristics, particularly low pH (U, Fig. 3a), and offer a diversified toxicity-classification for the soil samples of the experimental plots (C, Fig. 3b; only two examples are selected for Fig 3). Especially the bioluminescence-test reflects the TNT-concentrations in the soil.

Soil biological monitoring

Soil fauna

Whereas TNT-transformation occurs within the first months after the treatment, soil biological parameters undergo a succession at a very different time-scale. The analysis of the Nematode community in a c-p-triangle [19] clearly reflects the initial disturbance with

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subsequent regeneration (only plot U, C; Fig. 4). It is stronger on C, where the subsequent regeneration process is slower. This is even more pronounced in terms of abundances, that increase from May 1999 to May 2000 from 10 to almost 50 Ind./g dm on U and only to less than 15 Ind./g dm on C. Persistent high dominance of bacterial feeders on C points at a long-lasting effect of the TNT-contamination.

The Gamasina show a transitory inoculation with species from the fungus cultures, followed by a diverse colonisation in the direction of a forest community (Tab. 2). No definite indications can be derived concerning the TNT-contamination.

Decomposition

Compared to U, the mass loss of the substrates introduced with the minicontainers is not reduced on the contaminated treatment plots C and P (Fig. 5). On M and F, decomposition seems to be slightly retarded, which may be attributed to a tendency towards water-logging on M and the high contamination on F. Minor influences of mesofauna can be derived for the plots with introduced plants (U, C, P), however, statistically insignificant.

Discussion

The phytoremediation measure described here, combines

- a mechanical encroachment by grading,

- the import of selected biota with saplings, mycorrhiza and white-rot fungi,

- the input of organic matter (fungus substrate) and

- the control of microclimate (mulch).

The remediation process is an integrated function of all these components, each of which proved to fulfil various tasks in the treatment of the contaminated site. The plants provide optimal protection against side-contamination by dust and leachate control. The remediation measure was conducted to scale and proved to be applicable in large scale technology. The experimental set-up allows a step-wise discussion.

The grading is a severe, short term disturbance of autochthonous soil biota, initiating a vivid secondary succession. A stimulation of microbial activity in the aerobic zone (< 30cm depth) is most probable, as was observed by soil-respiration measurements of the biotest-battery and by a slight increase of the C/N-ratio. Such a stimulation of autochthonous microflora is regarded by Thomas et al. [20] as being mainly responsible for TNT-transformation Depending on the soil conditions, compaction beyond grading-depth (> 30cm) may occur, resulting in water-logging and anoxic conditions.

Lower TNT-concentrations (as in M and C; Fig. 2) are brought beyond detection limit within less than six months after grading. The activation of soil microflora, but also abiotic, chemical reactions may contribute. Higher TNT-loads (as in P; Fig. 2) are not transformed to such low endpoints, since further transformation is retarded or even stopped. This observation is in accordance with the persistence of the contamination in the forest soil for more than 50 yrs. , but has to be proven in longer-term investigations.

The imported biota are of paramount relevance in the initial phase immediately after grading to establish at least some biotic activity in the disturbed soil. However, the survival of many groups is limited in time, as could be shown for the Gamasina or as is expected due to substrate limitation for the white-rot fungi. But since planted shrubs and trees as well as fungus-inoculated shredder-substrate provide organic matter to accelerate secondary succession, a site specific community soon replaces the functions of the introduced biota. The plants not only activate biotic soil activity in their rhizosphere, including that of mycorrhiza,

but also protect the open soil from erosion, reduce transport of toxic dust, provide a favourable microclimate and control water infiltration, which is important for limiting the leaching of contaminants. The mulch layer supports these functions.

As described by [21], uptake of nitro-aromatics in plants depend on the plant species. Only spruce saplings may contribute with their rhizosphere and mycorrhiza to the reduction of the contamination, hypothetically with interactions with white-rot fungi (plot C) and by taking up mobile A-DNTs from the soil water. In contrast to [22], only diffuse relations to the level of soil contamination were found. Two vegetation periods are too short to assess quantitatively the effect of the plants on decontamination. In combination with their fungal partners, they may be important particularly after the initial transformation phase has levelled off.

Summarising, the plants not only stabilise the remediation plots and control water fluxes, but also reduce the hazard of an export of the contamination by dust and leachate. Compared to F, the concentrations of nitro-aromatics in the leachate from the experimental sites are rather low, however, with high percentages of A-DNTs on the full treatment site C. This observation stresses the importance of leachate control by plantation.

The biological monitoring system allows an evaluation of the remediation process on different scales. With the biotest-battery, aqueous and solid exposure-pathways are assessed. From the bioluminescence-assay it can be concluded that almost all of the mobilisable toxicity is caused by TNT [23]. The other bioassays were less sensitive, but in combination provided useful information for soil toxicity classification. Regarding soil respiration, adaptation to the site-specific conditions have to be taken into consideration, including 50 years of contamination.

In contrast to the laboratory tests, an uncontaminated standard does not exist for the field monitoring. A screening of the "Werk Tanne" area did not prove strict relations of Gamasina abundance or species number with TNT contamination as was reported for Oribatida [24], but the species composition did [25]. The results from the phytoremediation show a strong impact of the grading, followed by a vivid secondary succession which may lead in reasonable time to soil development and intact nutrient cycles. The successional dynamics as well as the maturity index for Nematoda give evidence of another disturbing factor, probably the nitro-aromatics. The complex monitoring scheme is necessary to document the safe progression of the procedure and to counteract possible hazards.

Tab. 1: Bioassay-based soil-toxicity classification - scheme

BIOASSAY	ORGANISM	PARAMETER	SOIL-TOXICITY CLASSES			
			1	2	3	4
Plant growth	*Lepidium sativum*	Biomass-production (14 d): mg dry wt/pot	> 200	101 - 200	11 - 100	0 - 10
Collembola reproduction	*Folsomia candida*	Reproduction-rate (28 d): number of offspring/adults	> 100	51 - 100	11 - 50	0 - 10
Soil respiration	Autochthonous microorganisms	Respiratory quotient (unamended respiration/SIR)	< 0,1	0,1 - 0,2	0,2 - 0,3	> 0,3
Eluate-toxicity	*Vibrio fischeri*	Inhibition of luminescence (30 min, EC_{20}): µL leachate /mL incubation-medium	>500	101 - 499	10 - 100	< 10
Eluate-mutagenicity	*Vibrio fischeri*	Reversion measured as increased luminescence (16-24 h): positive / negative	negative	not defined	not defined	positive
		Interpretation	nontoxic	slightly toxic	clearly toxic	highly toxic

Tab. 2: The Gamasina community in the soil of the experimental plots (for plot code see Fig. 1, bm= bark mulch).

	date	bm	May99					Oct99					May00				
	plot		U	C	P	M	F	U	C	P	M	F	U	C	P	M	F
	Abundances (ind/100g dm)	5,8	0,6	1,6	0,5	0,1	10,0	0,8	1,1	0,4	0,1	1,6	0,8	0,8	1,2	0,3	2,4
1	**Parasitus eta**	43															
2	Ameroseius spec.	14															
3	Leptogamasus alstoni	14															
4	Macrocheles merdarius	14															
5	**Glyphtholaspis americana**		30														
6	**Digamasellus punctum**		26	16													
7	**Dendrolaelaps stammeri**		29	68													
8	Parasitus consanguineus			6													
9	Dendrolaelaps arvicola			5													
10	Pachylaelaps fuscinuliger				49												
11	*Gamasellus montanus*				17												
12	Leptogamasus suecicus					100											
13	Pachylaelaps vexilliger						7										
14	*Pergamasus crassipes*						6										
15	Arctoseius magnanalis						4										
16	*Veigaia cerva*						2										
17	*Lysigamasus conus*						2	20									
18	Hypoaspis angusta			5				10	7								
19	*Prozercon kochi*							11									
20	Dendrolaelaps sellnicki								35					10			
21	*Lysigamasus runcatellus*							30	22	60				12		34	
22	*Veigaia planicola*						2		7								
23	*Hypoaspis aculeifer*									40							
24	Rhodacarellus silesiacus										100						
25	*Veigaia nemorensis*						42					47			7		45
26	Holoparasitus stramenti											22					
27	*Lysigamasus vagabundus*						28	10	14			9	51	11		34	31
28	Parholaspulus alstoni												13				
29	Dendrolaelaps spec.													23			
30	*Pergamasus quisquiliarum*														22		
31	*Veigaia kochi*						1								7		9
32	*Parasitus kraepelini*								7						7		
	Juvenile	14	15		34		7	20	8			22	36	44	57	32	15

italics: known from previous studies in the area; **bold**: species from the straw/white-rot fungus cultures

Fig. 1: Experimental design with five variants.

Plots plotsize 6.5 x 4.5 m² each			*full ttt* uncontamin. U	*full ttt* contaminated C	*partial ttt* plants P	*partial ttt* mechnical M	forest control F
TNT-contamination (1)			▓	▓	▓	▓	▓
treatments (ttt)	mechanical	grader (0-30 cm), mulch	▓	▓	▓	▓	
	planted	with mycorrhized saplings (2)	▓	▓	▓		
	inocculation of soil with straw + white-rot fungi (3)		▓	▓			

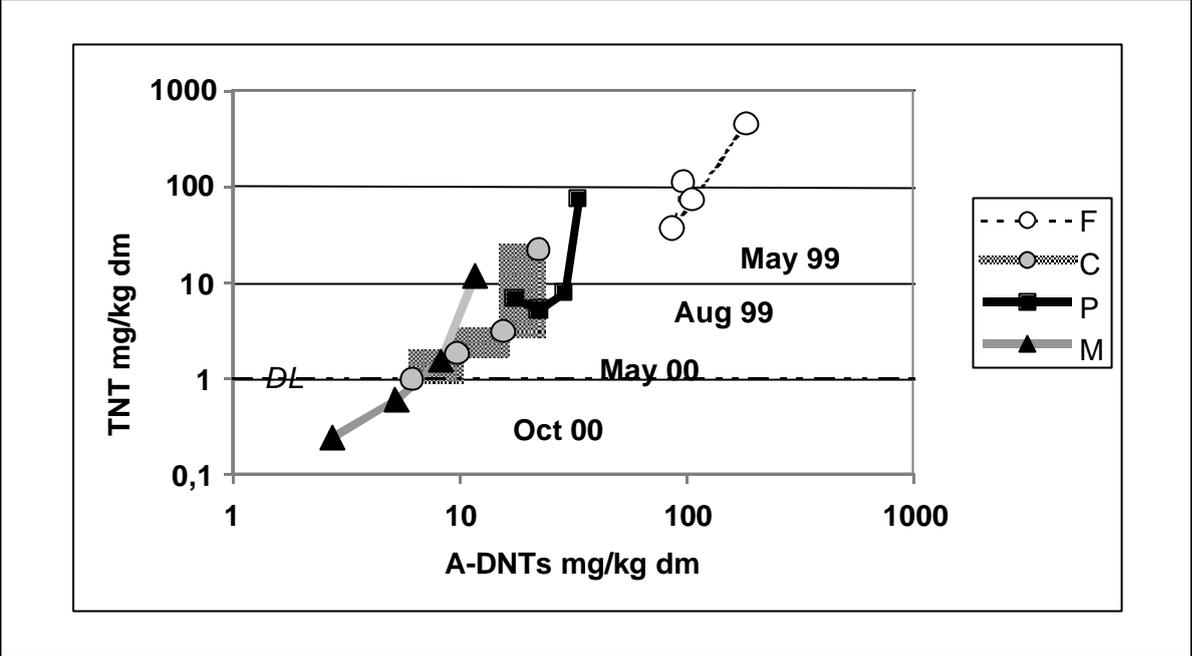(1) TNT in mg/kg dm soil (May 99, 0-30cm):
mean = 373, median = 14, max = 30300

Introduced organisms

(2) spruce, poplar, elder
(2) mycorrhiza: *Pisolithus tinctorius, Paxillus involutus*

(3) WR-fungi: *Pleurotus ostreatus, Trametes versicolor*

Fig. 2. Trajectories in time of TNT and A-DNTs in soil (mg/kg dm soil, geometric means; DL detection limit)
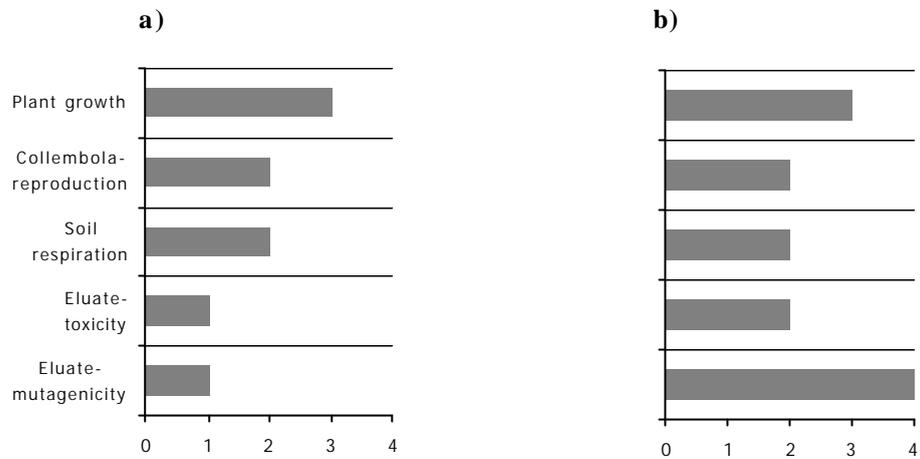
a) **b)**

Fig. 3: Bio-test based assessment of soil-toxicity (for uncontaminated reference [LUFA 2.2
soil] all values are 1, for heavily contaminated site F all values are 4):
a) uncontaminated site U: overall classification „slightly toxic" (2)
b) contaminated site B: overall classification „clearly toxic"(3)
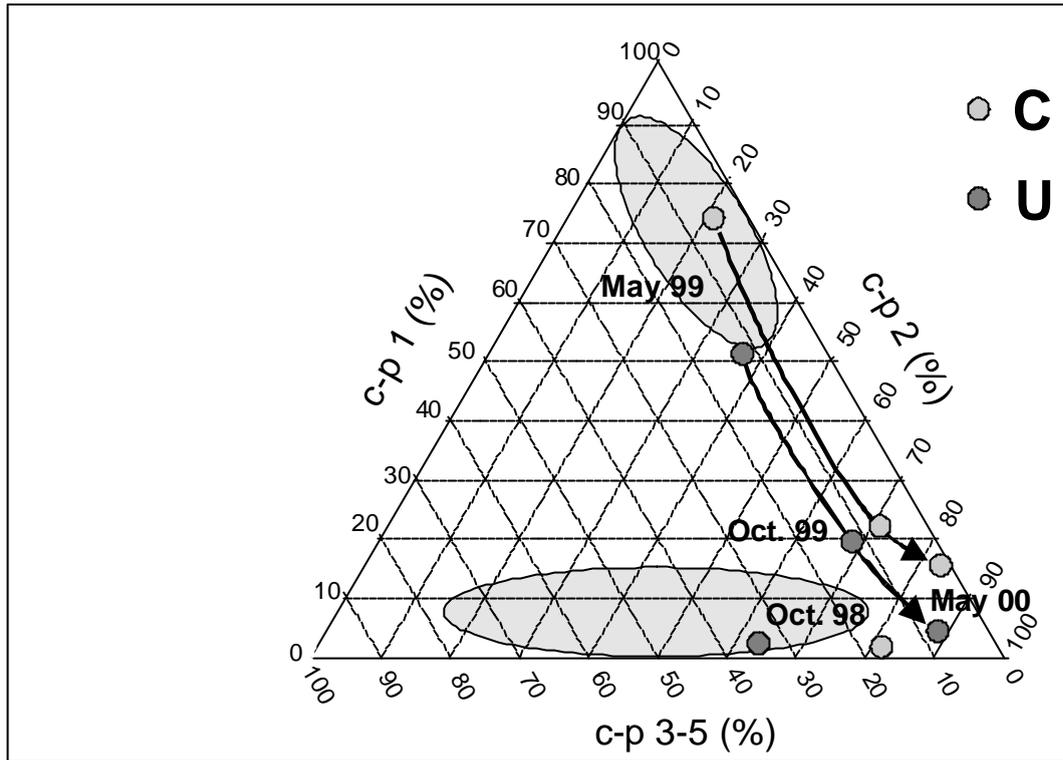
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Fig. 4: Trajectories for Nematoda within the C-P triangle; shaded top-area marks highly disturbed communities, that on the baseline undisturbed conditions. Data point Oct. 98 represents findings before the treatment.

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