A Battery of Toxicity Tests as Indicators of Decontamination in Composting Oily Waste

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Heterogenous oily waste from an old dumping site was composted in three windrows constructed from different proportions of waste, sewage sludge, and bark. The objectives of this pilot study were to examine the usefulness of composting as a treatment method for this particular waste and to study decontamination in the composting process by using a battery of toxicity tests. Five samples from the windrow having intermediate oil concentrations were tested with toxicity tests based on microbes (*Pseudomonas putida* growth inhibition test, ToxiChromotest, MetPLATE, and three different modifications of a luminescent bacterial test), enzyme inhibition (reverse electron transport), plants (duckweed growth inhibition and red clover seed germination), and soil animals (*Folsomia candida*, *Enchytraeus albidus*, and *Enchytraeus* sp.). The luminescent bacterial tests were used as prescreening tests. Chemical analyses of samples were carried out simultaneously. Both toxicity and oil concentration, including those of polyaromatic hydrocarbons (PAHs), were reduced during composting and soil quality improved significantly. The total oil hydrocarbon concentration decreased from 90,000 to 19,000 mg/kg, measured with the IR method, in 4 months, and from 86,000 to 1400 mg/kg, measured with GC method. The concentration of PAHs decreased from 135 to 23.5 mg/kg. During the fourth month of composting (stabilization stage), the proportion of the heaviest oil fractions (asphaltenes) became dominant. Toxicity varied between different samples and between different bioassays; however, the first sample was significantly more toxic than the others, and most of the tests revealed a decrease in toxicity during the composting process. © 2000 Academic Press

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INTRODUCTION

Cleanup of oil-contaminated soil by land farming or by composting has become popular in the last couple of decades. The intention is not usually to deposit composted soil in a special landfill, but to reuse it, e.g., for the construction of green areas. Therefore, the success of the composting process depends not only on decreasing chemical concentrations, but also on the soil's suitability to support the normal soil fauna and flora.

The assessment of the environmental hazard of solid wastes or contaminated soil is, in many countries, still performed by clinical analysis. However, the chemical data are not sufficient to evaluate the toxic effects, because it is impossible to analyze all the chemical compounds contributing to toxicity. Assessment of synergistic and antagonistic effects is not possible using chemical data. For proper assessment of polluted soils and monitoring of cleanup processes, toxicity data, chemical analysis, and ecological information should be combined (Plotkin and Ram, 1984; Linder *et al.*, 1992; Schrab *et al.*, 1993; Jean and Fruget, 1994; Lambolez *et al.*, 1994; Keddy *et al.*, 1995; Bernand *et al.*, 1996; Dutka *et al.*, 1996; Debus and Hund, 1997; Meier *et al.*, 1997).

In many studies on solid wastes in which ecotoxicological tests have been used, little attention has been paid to such aspects as the selection of test species, the sensitivity of tests, and the simplicity and cost of the assays. Very few serious endeavors have been made to determine which is the minimum battery of tests needed (Bernand *et al.*, 1996). A battery of toxicity tests should be used to evaluate the effects on different trophic levels, as well as acute, chronic, and genotoxic effects. Various single-species screening tests are often employed to detect possible harmful effects of chemicals on biotic systems. In the environment, however, different species and a complex food web are exposed.

Various bacterial bioassays have been developed for the screening of chemicals, effluents, sediments, and soil toxicity. Microbial tests (e.g., MetPLATE, ToxiChromotest, Microtox, Mutatox, BioTox) are simple, rapid, sensitive, and inexpensive toxicity tests (Arfsten *et al.*, 1994; Bitton *et al.*, 1994; Keddy *et al.*, 1995; Bernand *et al.*, 1996). These

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tests are based on the measurement of enzyme activity or metabolism of bacterial cells.

Mitochondrial enzymes and enzyme reactions, including mitochondrial electron transport (reverse electron transport, RET), are well-understood processes in living organisms. Being fast an inexpensive, mitochondrial enzyme reactions have the potential to be sensitive and efficient screening tests for general toxicity (Read *et al.*, 1998). The RET assay has been suggested as an alternative to Microtox for screening water-soluble chemicals (Weideborg *et al.*, 1997). Inhibition of RET has been found to correlate well with whole-animal toxicity in aquatic organisms (Blondin *et al.*, 1989; Betterman *et al.*, 1996) and man (Knobeloch *et al.*, 1990).

Soil animals are relatively vulnerable to the adverse effects of oil contamination. For example Pirhonen and Huhta (1984) studied the soil animal community response to light fuel oil and hydraulic oil. Soil arthropods and enchytraeid worms were especially sensitive to oil spillage. Neuhauser *et al.* (1989) concluded that arthropods, enchytraeids, and earthworms all suffered from oily waste application onto field soil irrespective of the amount applied. The greater the amount applied, the longer the time required for recovery. Soil animals have been used as bioassays in several studies. Earthworms are the most common species (e.g., Callahan *et al.*, 1991; Crop and Morgan 1991; Menzie *et al.* 1992; van Gestel *et al.*, 1993), but collembolans have also been used (van Gestel and van Diepen, 1997).

Several species of higher plants have been used to test phytotoxicity. Terrestrial plant tests, seed germination and root elongation tests, may reflect both direct and indirect (water-mediated) effects of the soil (Linder *et al.*, 1990). In general, plant tests are cost effective, relatively easy to perform, and are suitable for turbid sample testing (Wang, 1991).

The aims of this study were: (i) to characterize samples of composted oily wastes using ecotoxicological analyses; and (ii) to compare the sensitivity and applicability of commercial microbiotests and other bioassays representing different trophic levels, to select a representative and cost-effective battery of tests for assessing further cleanup processes.

MATERIALS AND METHODS

Composting

Heterogeneous oily waste from an old landfill of Neste Oil Refinery at Naantali, Finland, was composted in windrows. Ten to twenty percent of the total amount of the waste was heavy oil fractions. The rest of the waste consisted of fly ash, metals, solid sulfur, catalysts used in the oil refining process, and different types of soil.

The experiment was started in June 1997 and continued for 4 months (123 days), until the cold and wet weather forced completion of the process. The waste was excavated from four different locations in the area, and then it was combined and sieved to obtain a homogenous matrix. Three compost windrows (50 m^3) with different proportions of oily waste were constructed from the waste, sewage sludge, and coniferous bark. The windrows were aerated by effective turning with a screw-type mixer, one to six times per month, and irrigated when necessary. The composting process was monitored by measuring the temperatures twice a week and by taking samples at intervals of 1 or 2 weeks. The samples from one windrow (intermediate oil concentration) were used for the ecotoxicological studies.

SAMPLING AND PRETREATMENT OF THE SAMPLES

The samples for the ecotoxicological tests were taken at the beginning of the process (Day 0) and on Days 32, 60, 74, and 123. Bioluminescent bacterial tests were used as screening tests and performed at intervals of 1 or 2 weeks. In addition, a subsample of the last compost sample (Day 123) was extracted with hexane to remove the hydrocarbons still present in the compost. Immediately after the turning, samples were taken from 10 to 15 different locations of the windrow at 5 to 20 cm depth. The samples were pooled, air-dried, sieved, and stored at $-18°C$ until testing. Bioluminescent bacterial tests were carried out immediately. Toxicity tests and chemical analyses were done on the same samples.

CHEMICAL ANALYSES

The samples were analyzed for physical and chemical parameters; pH, moisture, ATP, loss on ignition, nutrients, oil concentration. Total oil hydrocarbon concentration, hydrocarbon distribution (C: $\leq$ 10, 11–20, 21–30, 31–44), and concentrations of benzene, toluene, polyaromatic hydrocarbons (PAHs), and heavy metals (As, Cd, Cr, Cu, Pb, Ni, Zn, and V) were analyzed at Neste Oyj Research Centre, Porvoo, Finland. Total oil hydrocarbon concentration was measured by IR spectroscopy and the GC–MS method. Hydrocarbon distribution and concentrations of benzene and toluene were analyzed with the GC–MS method. PAHs were analyzed using GC–MS SIM technique. Arsenic and cadmium were analyzed with the AAS method, and the other heavy metals, with the ICP method.

SAMPLE EXTRACTION

One hundred grams of solid sample was suspended in 100 ml of deionized water, and the mixture was shaken on a rotary shaker (280 rpm/min, 1 h, room temperature). The mixture was centrifuged at 4000 rpm at $+4°C$ for 15 min. The extraction was repeated with another 100 ml of water, and the supernatants were combined. The precipitate was extracted again with 150 ml of 10% DMSO solution and

treated as before. The water and DMSO–water supernatants were used separately for toxicity analyses. Elutriates were neutralized if the pH was outside the range of 5–8. The extracts were stored in plastic bottles (250 ml) at 4°C until testing. Samples were filtered (0.2 μm) before ecotoxicological tests.

A slightly different extraction procedure was used to obtain water extracts for the BioTox screening test: 10 g of solid sample was extracted with 90 ml of deionized water by shaking in a shaker (250 strokes/min, 24 h, room temperature). The suspensions were neutralized if the pH was outside the range of 6–8.5. The concentration of NaCl was adjusted to 2%. A 1.5-ml aliquot of sample extract was centrifuged at 7000 rpm in an Eppendorf centrifuge for 3 min at room temperature and the supernatant was used for the test.

ECOTOXICOLOGICAL TESTS

Composting of the toxic oil compounds was followed by measuring the ecotoxicity with 13 tests. A brief description of the tests is given in Table 1. The results are calculated as percentages of inhibition at the given concentration or as EC_{50}/EC_{20} or IC_{50} values, except for soil animal tests, the results of which are expressed as number of individuals in sample/number of individuals in control.

Cell organelles. The RET assay is an enzymatic *in vitro* screening test based on the effects of toxic compounds on NAD reduction by submitochondrial particles. The reaction conditions are arranged so that the enzyme NADH–coenzyme Q reductase is operating in reverse direction. The procedure and a kinetic modification of it have been described earlier in detail (Blondin *et al.*, 1989; Knobeloch *et al.*, 1994, Argese *et al.*, 1995). Submitochondrial particles were prepared from beef heart mitochondria. Before the test, samples were diluted with a concentrated assay buffer to achieve final concentrations of 50%. The reaction was followed spectrophotometrically at 340 nm for ca. 10 min. A linear portion of the reaction was used to calculate the increase in absorbance units per minute. The inhibition of the reaction was calculated by comparing the reaction rate of the samples to the controls. Deionized water or DMSO–water was used for the controls.

Microbes. The BioTox test was performed according to the standard procedure for aqueous samples (DIN 38 412, 1991; ISO/CD 11348, 1994). Freeze-dried bacteria (naturally luminescent *Vibrio fischeri* NRRL B-11177) were first reconstituted to obtain a stable suspension. The inhibition of the light production of the bacteria, caused by water extracts of the samples, was measured with the Bio-Orbit 1257-luminometer using 5- and 15-min contact times. The

TABLE 1
Battery of Biotests Used to Assess the Toxicity of Composted Oily Waste[a]

Biotest	Type of test	Endpoint	Duration
i. Cell organelles			
Reverse electron transport assay (RET), beef heart submitochondrial particles	*In vitro* test, enzyme complex	Enzyme inhibition	20 min
ii. Microbes			
BioTox, bacteria *Vibrio fischeri*	Microbiotest	Luminescence	5–30 min
Bioluminescent direct contact test, *Vibrio fischeri*	Microbiotest	Luminescence	5–30 min
Bioluminescent direct contact flash test, *V. fischeri*	Microbiotest	Luminescence	30 s
ToxiChromotest, *E. coli*	Microbiotest	Enzyme inhibition	6 h
MetPLATE, *E. coli*	Microbiotest	Enzyme inhibition	4–5 h
Bacterium growth inhibition test, *Pseudomonas putida*	Microbiotest	Growth inhibition	16 h
iii. Plants			
Seed germination test, red clover	Plant test	Growth inhibition	4 or 7 days
Duckweed, *Lemna minor*	Plant test	Growth inhibition	3 or 5 days
iv. Soil animals			
Soil arthropod, *Folsomia candida*	Soil animal test	Survival and reproduction	14 and 32 days
Enchytraeid worm, *Enchytraeus albidus*	Soil animal test	Survival and reproduction	14 and 32 days
Enchytraeid worm, *Enchytraeus* sp.	Soil animal test	Survival and reproduction	14 and 32 days
v. Genotoxicity			
Mutatox, genotoxicity assay, *V. fischeri*	Microbiotest	Luminescence	24 h

[a] The tests are divided into five groups: (i) cell organelles, (ii) microbes, (iii) plants, (iv) soil animals, and (v) genotoxicity.

extracts were tested as duplicates and the inhibitions obtained were corrected for the inhibitions caused by 2% NaCl.

An automated modification of the standard *Pseudomonas putida* test (ISO 10712, 1995) with a Bioscreen C analyzer (Labsystems Oy, Helsinki, Finland) was used to detect bacterium growth inhibition. The bacterium *P. putida* represents a common aquatic heterotrophic microorganism. When *P. putida* cells are cultured under specified conditions, in a defined medium with different concentrations of wastewater over several generations, toxic substances present in the sample can inhibit the cell multiplication of the bacteria (Brinkmann and Kühn 1977). In this test *P. putida* MIGULA (DSM 50026) were grown for 16 h in a liquid medium in special cuvettes, and turbidity due to bacterial growth was measured by vertical photometry. The pH of the samples were checked and the samples were centrifuged 10 min/10,000 rpm and then sterile-filtered (0.2 mm) to eliminate other bacterial growth. In this test, the nutrients in the tested extract can also stimulate the bacterial growth and mask possible toxic effects. 3,5-Dichlorophenol was used as a reference toxicant.

ToxiChromotest is a rapid microbial colorimetric assay in kit form that can be used to test toxicants in liquids. It is based on the ability of toxicants in inhibit *de novo* synthesis of β-galactosidase from a mutant strain of *Escherichia coli*. Bacterial response to a toxic sample was observed on the test pad where the intensity of the blue color is inversely proportional to sample toxicity. The test is suitable for rapid screening. The bioassay was carried out according to the manufacturer's (Group 206 Technologies, Gainesville, FL) procedure.

MetPLATE assay is a rapid quantitative microbial assay. The test is specific for heavy metal toxicity. MetPLATE is in a 96-well microtitration plate format and is suitable for determining toxicity characteristics, such as median inhibitory concentrations (Bitton *et al.*, 1992, 1994). MetPLATE is based on the activity of β-galactosidase from a mutant strain of *E. coli* and uses chlorophenol red galactopyranoside as the enzyme substrate. The bacterial suspension was incubated with sample dilutions in a 96-well microplate. The bioassay was carried out according to manufacturer's (Group 206 Technologies) procedure and the absorbance was measured at 570 nm.

The bioluminescent direct contact test was modified from the direct contact method (Brouwer *et al.*, 1990) using naturally luminescent *V. fischeri*. In the current procedure, 5 g of the solid sample was suspended with 45 ml of 2% NaCl by mixing vigorously for 5 min. Dilutions of this stock suspension were measured in duplicate to determine the EC_{50} and EC_{20} at 5- and 15-min contact times, using the BioTox toxicity screening system according to the standard procedure for aqueous samples (DIN 38412, 1991; ISO/CD 11348, 1994). Effect of color and turbidity was not taken into account in the calculations.

The bioluminescent direct contact flash test is a new modification of the direct contact luminescent bacterial test (Brouwer *et al.*, 1990). The suitability of the flash test for monitoring oil compost toxicity was evaluated. The method has been published recently (Lappalainen *et al.*, 1999). In this new method, kinetic measurement of luminescence was started at the same time the *V. fischeri* suspension was added to the sample (the same stock suspension as in the direct contact test described earlier). Luminescence signal was measured 20 times per second throughout the 30-s exposure period. Peak height at 0–5 s (I_0) and signal at 30 s (I_t) were measured. The result was expressed as the ratio of I_t to I_0, and converted to inhibition percent (INH%). The results were compared with the inhibition caused by 2% NaCl, which was used as a clean control sample. The procedure was carried out with the Bio-Orbit 1251 luminometer (Bio-Orbit Oy, Turku, Finland) at 20°C and with the BioTox kit containing freeze-dried *V. fischeri*.

Plants. For the red clover seed germination test 30 g of sample was layered on plastic Petri dishes ($\phi = 14$ cm) and moistened with 20 ml of deionized water. The dishes were covered and incubated at room temperature for 4 h before the seeding. The area of the dishes was divided by plastic strips into four sectors. Two replicates per sample were used, except the sample for Day 74 was not replicated. Visibly healthy red clover seeds (*Trifolium pratense*) were placed on the soil substrate, 20 seeds in every sector. Artificial soil (OECD, 1984) was used as the reference soil substrate. The test vessels were incubated in the dark at 25°C and the germinated seeds were counted on Days 4 and 7. Seeds having a primary root 5 mm or longer was defined as germinated (Wang, 1991). The percentages of germinated seeds were calculated and compared with those of controls.

The extracts were tested with a *Lemna minor* growth inhibition test (OECD, 1996). A local strain of *L. minor* was used. If the beginning of the test, 10 young plants with two leaves (fronds) were transferred into vessels containing 15 ml of the test solution. The vessels were placed under a light source (4000–5000 lx, light dark period of 16/8). The temperature was held at 20 ± 2°C and the relative humidity at 40–60%. The test solutions were mixed with a buffered nutrient medium in 50 and 10% final concentrations. Three replicates of the controls, the test solutions, and the positive controls with potassium dichomate (5 mg Cr/liter) were tested. The number of fronds was calculated on Day 3 and at the end of the test on Day 5. Fresh weight and chlorophyll a content were also measured. Chlorophyll was extracted by homogenizing the plant tissue in ethanol. The homogenate was stored at -20°C for at least 24 h and centrifuged to remove the tissue. The clear supernatant was used for spectrophotometric determination of chlorophyll a (APHA/AWWA/WPCF, 1985). Inhibition of *L. minor*

growth was calculated using number of fronds, fresh weight, and chlorophyll a concentrations.

Soil animals. Three different soil animal species (*Folsomia candida*, *Enchytraeus albidus*, and *Enchytraeus* sp.) were used for testing. The test procedures were modified from the standard proposals (ISO/FDIS 1998; ISO/WD, 1999). Samples were moistened with deionized water and mixed carefully until a moist and crumpy structure was achieved. Twenty grams (dry wt) of each soil was weighed into 10 glass beakers (50 ml) and into five glass jars (250 ml), except hexane-washed soil, which weighed only 15 g due to a shortage of the soil. Ten *F. candida* specimens were transferred into five beakers, 10 *Enchytraeus* sp. specimens into another five beakers, and 10 *E. albidus* specimens into five glass jars. Hexane-washed soils and Day 60 samples were prepared only for the collembolan and *E. albidus* tests and replicated three and five times, respectively. Small amounts of baker's yeast for the collembolans or grinded oat flakes for the enchytraeids were given as food. Finally, the beakers were closed with a plastic film (Parafilm) that was pierced for gas exchange, and the jars were closed with a glass lid.

The vessels were incubated in a climate chamber ($+20^\circ$C, constant light 400–800 lx) for 32 days. Evaporated water was compensated and food was added once a week. After 14 days *E. albidus* adults were removed from the soil and counted. After 32 days the soils in the jars were wetted with ethanol (99.5%) and ca. 10 drops of Bengal red (1% ethanol solution) were added into each jar. The juveniles were hand sorted and counted after dying. Collembolans were extracted from the soil samples with a modified high-gradient extractor (see Martikainen and Rantalainen, 1999). Enchytraeids were extracted with the wet funnel method (O'Connor, 1955) where temperature was raised with glow bulbs for 4 h. Worms were stained in ethanol and then dyed Bengal red before counting. For all species, adults and juveniles were counted separately.

Genotoxicity. Extracts were tested with the Mutatox genotoxicity assay using a dark mutant strain of the luminescent bacterium *V. fischeri*. Toxicity assays were conducted using the Mutatox procedure according to the manufacturer's (Microbics Corp., Carlsbad, CA) instructions. DNA-damaging substances are recognized by measuring the ability of a test sample to restore the luminescent state in the bacterial cells. The amount of light increase indicates the genotoxicity of the sample.

RESULTS

Results of the Chemical Analyses

The concentrations of the oil hydrocarbons decreased substantially during the entire composting period (Fig. 1). The 60-day sample, containing more than 1000 mg/kg PAHs, differed significantly from the others. This extremely
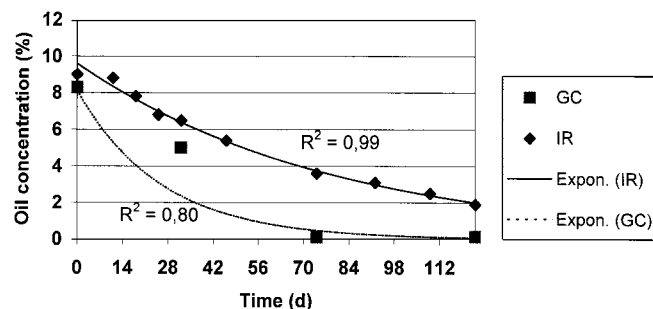

FIG. 1. Mineral oil concentrations in compost samples, measured with IR and GC methods. Exponential correlations (R^2) with composting time are represented.

high concentration of PAHs was mainly phenantrene, while the other polyaromatics were on the same level as the earlier sampling (32 days), which is why the 60-day sample was considered to be nonrepresentatve. Table 2 summarizes the results of the chemical analyses.

The oil concentration decreased by 79% according to IR analysis and by 98% according to GC analysis. The hydrocarbon distribution demonstrated that 55% of the hydrocarbons on the last sample had carbon chains longer than 31 atoms, whereas on the first sample the percentage was only 24%. The decrease in polyaromatics (PAHs) was 83%

TABLE 2
Results of the Chemical Analyses[a]

Contaminant	Days from the start				
	0	32	60	74	123
Total oil (%) (IR spectroscopy)	9.0	6.5	3.8	3.6	1.9
Total oil (%) (GC spectroscopy)	8.3	5.0	4.7	0.11	0.14
Hydrocarbons (mg/kg)					
< C11	495	44	47	6	8
C11–C20	37,200	22,911	14,400	290	260
C21–C30	25,400	16,300	18,000	340	370
C31–C44	20,300	10,700	14,600	450	790
Benzene	0.407	0.008	0.02	0.005	0.005
Toluene	0.46	0.24	2.30	0.20	0.09
PAHs	135	53	>1,000	31.7	23.5
Metals (mg/kg)					
AS	8.5				11
Cd	0.7				0.9
Cr	44				52
Cu	210				190
Pb	72				100
Ni	45				55
Zn	230				280
V	65				77

[a] Samples were taken on Days 0, 32, 60, 74, and 123 from the start. Analyses were carried out from pooled samples, which were air-dried and sieved to yield a particle size <2 mm.

and the residue (23.5 mg/kg) consisted of PAHs that had four or more rings. Although the decrease in oil hydrocarbons continued throughout the whole composting process, the most dramatic changes in hydrocarbon distribution was seen during the first and fourth months of composting. The most volatile hydrocarbons (carbon chains shorter than 11 C atoms, BTEX compounds, and PAHs having fewer than four rings) disappeared mainly during the first month. During the fourth month of composting the profile of the GC chromatogram (diagrams not provided) shifted strongly to the right, meaning that the proportion of the heaviest oil fractions, e.g., asphaltenes and waxes, increased, compared with the short-chained fractions.

The concentrations of heavy metals increased by 18–39%, except for the concentration of copper which decreased slightly (Table 2). The increase in heavy metal concentra-

tions is approximately of the same magnitude as the decrease in organic matter during the process.

Results of Ecotoxicological Tests

The toxicity of the composted soil decreased during composting, judging by the ecotoxicological test results (Table 3 and Figs. 1–6). Water and DMSO extracts inhibited RET (at 50% final concentration of the extract); the first three samples almost totally (73–98%) inhibited RET. The inhibition gradually decreased with composting time, but after 123 days inhibition was still about 40% (Fig. 2).

According to the bioluminescent direct contact test (Fig. 3a) toxicity varied considerably during the process but an overall decrease in toxicity can clearly be seen. The same kind of decrease in toxicity can be seen at the end of the

TABLE 3
Results of Toxicity Tests[a]

Biotest		0	32	60	74	123	Hx
		Days from the start				
i. Cell organelles							
RET							
Water extract (INH %)		81	86	82	39	35	ND
DMSO–water extract (INH %)		96	94	73	56	42	ND
ii. Microbes							
BioTox	Water extract (INH %)	65	41	40	37	16	ND
Bioluminescence direct contact test	Sample–water suspension EC_{50}, 5 min (mg/liter)	1.3	4.7	8.1	12.3	30.2	ND
Bioluminescence direct contact flash	Sample–water suspension INH %	47	28	19	16	−40	0
ToxiChorotest	INH %	60	NT	NT	NT	NT	NT
MetPLATE	INH %	NT	ND	ND	ND	ND	ND
P. putida growth inhibition	INH % at 1% dilution	−80	80	70	70	55	ND
	INH % at 2% dilution	−110	60	75	80	55	ND
	INH % at 5% dilution	−140	60	80	75	40	ND
	INH % at 10% dilution	−65	75	75	70	50	ND
	INH % at 25% dilution	75	70	75	60	70	ND
iii. Plants							
Seed germination	4-day INH %	99	81	75	69	47	ND
	7-day INH %	96	79	65	64	43	ND
Lemna minor growth inhibition	Frond number, IC_{50}	31	NT	NT	NT	NT	ND
	Fresh weight, IC_{50}	32	NT	NT	NT	NT	ND
	Chlorophyll a concentration, IC_{50}	31	NT	NT	NT	NT	ND
iv. Soil animals							
Soil arthropod,	Adults (sample/control)	0.0	0.0	0.0	0.0	1.0	1.9
F. candida	Juveniles (sample/control)	0.0	0.0	0.0	0.0	0.8	5.6
Enchytr. worm,	Adults (sample/control)	0.0	0.0	1.0	1.0	1.1	1.0
E. albidus	Juveniles (sample/control)	0.0	0.0	0.2	0.8	3.0	27
Enchytraeus sp.	Adults (sample/control)	0.0	0.2	0.5	ND	0.9	ND
	Juveniles (sample/control)	0.0	0.0	1.0	ND	1.5	ND
v. Genotoxicity							
Mutatox	Relative genotoxicity (%)	100	25	NT	NT	NT	ND

[a] Results are expressed as percentage inhibition (Inh %) at the given percentage concentration of the extract or as the EC_{50}/IC_{50} value (mg/liter or %) except for the results of the Mutatox genotoxicity assay (expressed as relative genotoxicity) and the results of soil animal tests (expressed as number of animals in sample compared with number of animals in OECD standard soil). The samples were taken at the beginning of the process (Day 0) and on Days 32, 60, 74, and 123. Hx is the 123-day sample that was washed with hexane. NT, not toxic. ND, not determined.
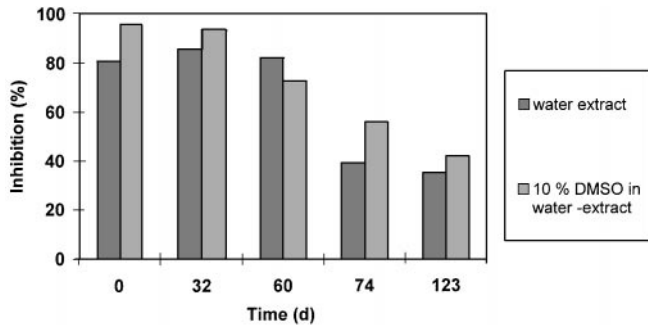
FIG. 2. Inhibition of water and 10% DMSO–water extracts of the compost samples of RET assay.

composting period as the flash test (Fig. 3b). The contact time did not seem to have any major effect on the results because the results with 5- and 15-min contact times are of the same order of magnitude. Color and turbidity interfered with the assay when the EC_{50} concentrations exceeded 5 mg/liter. During the first month of composting the samples exhibited strong inhibition in the direct contact flash test (Fig. 3b), even though the contact time was only 30 s. The inhibitory effect gradually decreased during composting and the last two samples caused a strong inductive effect, indicating a dramatic change in the compost matrix. The inhibitions, caused by the water extracts on the BioTox test, (Fig. 3b), decreased gradually from 65 to 15% during the composting period and no change could be seen at the end of the composting process. Color and turbidity did not disturb the BioTox test because the extracts used for the tests had only a faint yellow or brown color and formed a near-colorless mixture with the bacterial suspension.

Inhibition and stimulation by the 1% sample extracts of the growth of *Pseudomonas putida* are illustrated in Fig. 2c. The other concentrations did not have any relation to the oil concentrations during the composting process. The extracts of the first samples at the start of composting caused strong stimulation of growth in dilutions up to 10%. But the dilution of 25% was clearly inhibitive. During the composting period the toxicity of the 1% extract decreased slightly but not significantly. On later sampling occasions no stimulation of growth was observed.

The ToxiChromotest demonstrated that, of the five oily samples tested, only the first (Day 0) sample was toxic and displayed enzyme inhibition 60% (Table 3). None of these oily samples displayed toxicity by MetPLATE (Table 3).

The *L. minor* assay results indicated that the water extract from the first oily sample inhibited growth, measured as the number of fronds, weight, and chlorophyll a content (Fig. 4a). The later samples did not have inhibitory effects. DMSO–water extracts could not be used because DMSO was toxic to the plants. The first compost sample inhibited red clover germination almost completely (98.7%) (Fig. 4b).

The inhibition decreased gradually during composting, but after 123 days it was still greater than 40%. A slightly smaller effect was seen after 7 days, than after 4 days, of counting the germinated seeds. More than 90% of the seeds of the controls were already germinated by the first counting on Day 4.

E. albidus adults survived well in the soils taken on Day 60 and afterward (Fig. 5a). The first two compost samples (Days 0 and 32) were highly toxic for this enchytraeid species. In these soils there were no juveniles. Reproduction was rather weak and only a few juveniles, if any, were found in all but the hexane-washed soil. The number of juveniles increased with time, but the variation between the replicates was rather high. *Enchytraeus* sp. appeared to be somewhat more resistant to oil contamination than *E. albidus* (Fig. 5b). Only in the first sample there were no adults. *Enchytraeus* sp. demonstrated increasing survival and reproduction
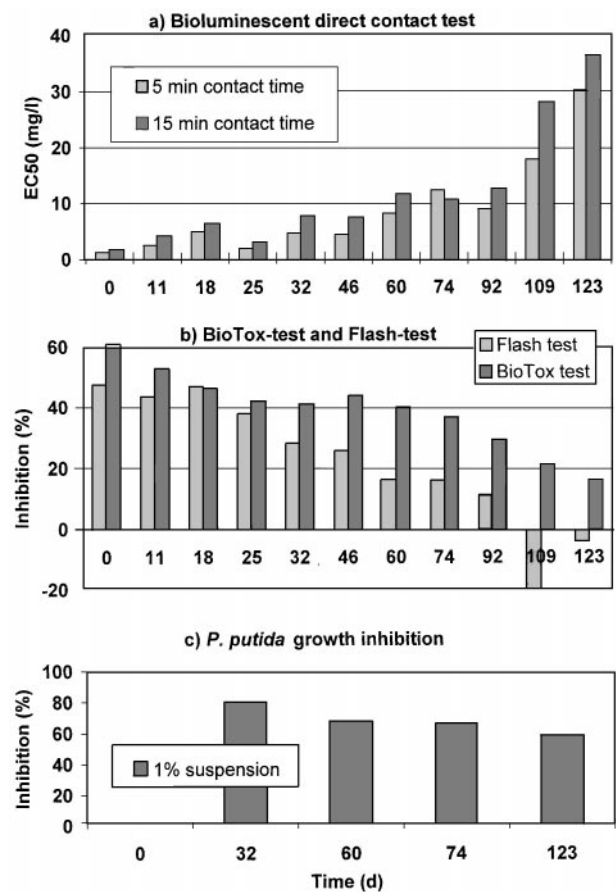

FIG. 3. EC_{50} values of sample suspensions with 5- and 15-min contact times on the luminescent bacterial direct contact test (a), inhibitions caused by water extracts on the BioTox luminescent bacterial test and 5% sample suspensions on the direct contact flash test (b), and inhibition of 1% water extracts on growth of the bacterium *P. putida* (c). The logarithmic correlations (R^2) with mineral oil concentration are 0.84 for bioluminescent direct contact test, 0.76 for BioTox, 0.88 for the flash test, and 0.26 for *P. putida* ($P < 0.01$).
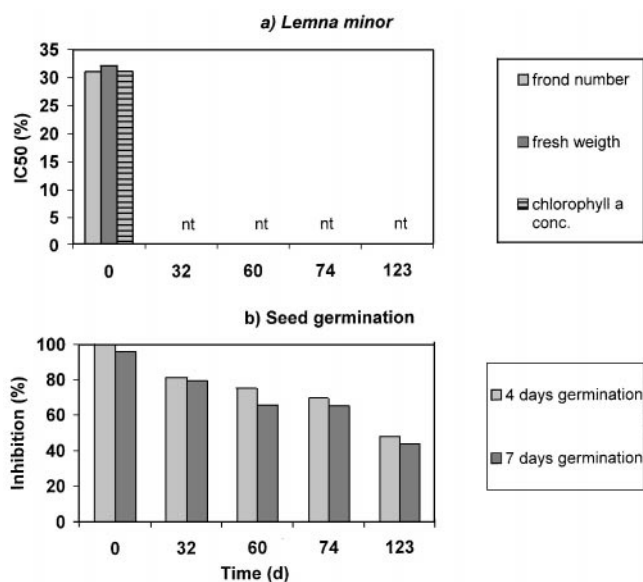
FIG. 4. Inhibitions by water extracts of growth of fonds, weight, and chlorophyll content in the *Lemna minor* assay (a) and the inhibitory effect of moistened solid samples on germination of red clover (b). Linear correlation (R^2) with oil concentration measured with IR method for seed germination test is 0.87 ($P < 0.01$). nt, not toxic.

success with time in the compost soils. *F. candida* proved to be extremely sensitive to oil contamination (Fig. 5c). Neither adults nor juveniles were found until Day 123. Their numbers were still higher in the hexane-washed soil.

According to the Mutatox genotoxicity assay, only two (Days 0 and 32) of the five samples were mutagenic (Fig. 6).

DISCUSSION

The Success of the Composting Process

The results reveal that both the toxicity and concentration, including those of PAHs, can be reduced by composting and a clear improvement in soil quality can be achieved. The results of the toxicity tests indicated a clear decrease in toxicity in the latter stages of composting, so that even seed germination of red clover and reproduction of soil arthropods and worms could be observed. The reasons for the reduced toxicity are evidently the degradation of the different oil fractions by microorganisms, which reduces the concentrations of oil hydrocarbons, and also the stabilization of the compost, which reduces the bioavailability of the oil residues.

Chemical analyses revealed three different stages in the 4-month process: (1) the first month of composting, when the most volatile substances in the compost disappeared; (2) the second and third months of composting, when degradation continued in all oil hydrocarbon groups; and (3) the fourth month of composting, when the proportion of ex-

tremely heavy oil fractions became dominant and stabilization of the compost took place.

The Sensitivity of the Biotests

The ecotoxicological tests seemed to have a different sensitivity to the degradation of hydrocarbons in the three stages. The toxicity to *E. coli* in ToxiChromotest and the toxicity to *L. minor* disappeared during the first month. High nutrient concentrations in the compost may have affected the growth of *L. minor* or the intake of toxicants by *E. coli* so that the toxicity may have been overlooked. Genotoxicity, according to the Mutatox assay, disappeared during the first 2 months of composting. This indicates either that genotoxic compounds were not extracted into the water or that only the volatile components present in the first two samples were genotoxic. The MetPLATE assay did
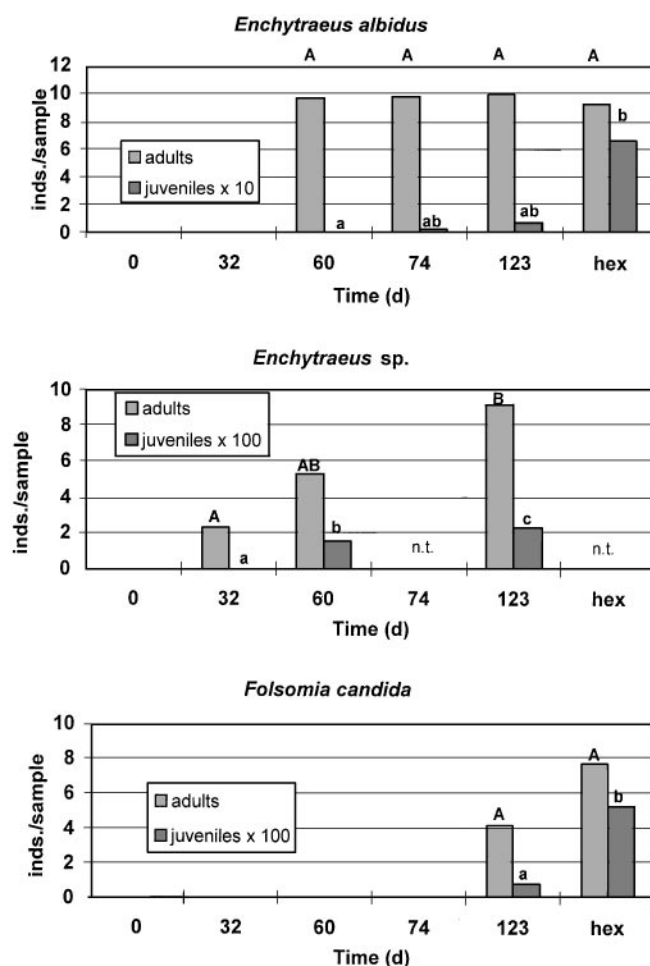

FIG. 5. Survival and reproduction of enchytraeid worms, *Enchytraeus albidus* and *Enchytraeus* sp., and a collembolan, *Folsomia candida*, in the compost samples and in the hexane-washed 123-day sample (hex). Significant differences between samples are indicated by uppercase (adults) or lowercase (juveniles) letters. n.t., toxicity not tested.
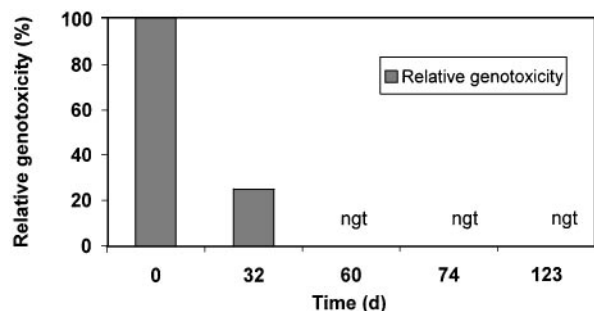
FIG. 6. Relative genotoxicity of water extracts in the Mutatox assay. ngt, not genotoxic.

not give any response, indicating that the heavy metals detected with chemical analysis were not in a bioavailable form, or the metals were not extracted, or the oil inhibited the test.

The *P. putida* bacterium growth inhibition test gave very poor correlation with the oil concentration. The stimulation of *P. putida* growth in all dilutions of the first samples was probably due to the high nutrient content in the compost at the start. The growth-inhibiting substances in the later samples seemed to be very persistent and no great improvement was observed. The reason for the inhibition is unknown.

According to the bacterial bioluminescent direct contact tests and the soil animal tests, there seemed to be a significant reduction or disappearance of toxicity during the fourth month of composting, when stabilization of the compost reduced the bioavailability of contaminants. At this stage the composted product can be left to mature. Maturation takes several months but there is no need for process treatment activities. It is important to note that none of the biotests, which assessed the effects of extracts, could detect the stabilization of the compost. The results also revealed differences between soil animal species (arthropods and annelid worms) in their sensitivity to oil contamination. This result confirms the earlier results of Pirhonen and Huhta (1984) who found that collembolans (and also other arthropods) were more sensitive than enchytraeids to hydraulic oil. Also juvenile collembolans, introduced into the soils at the age of 8–11 days, may have been more sensitive to toxic effects than the adult enchytraeids.

RET assay representing the cell organelles, the BioTox[TM] luminescent bacterial test, and the red clover seed germination test revealed a gradual decrease in toxicity during the process. According to these tests the toxicity did not completely disappear, indicating that on the last sample extract soluble toxicants were still present. A slightly smaller effect on red clover seeds was observed after 7 days than after 4 days of germination, indicating that the inhibition may be overcome with time. However, the seeds are vulnerable to microbial and fungal growth when germination is too slow.

The effects of the field situation may therefore differ from the laboratory tests. The toxic effects of the last sample were also seen in the soil animal tests where the reproduction (number of juveniles) on the last sample was less than in the sample that was washed with hexane.

Washing the soil with hexane improved the survival and reproduction of the soil animal species tested. This reveals that on Day 123 there were still bioavailable oil hydrocarbons, which hindered reproduction and which could be removed by hexane elution.

It is also noteworthy that the toxicity of water extracts on the BioTox luminescent bacterial test did not show the same kind of dramatic change in toxicity at the end of the composting process as could be seen in the results of the bioluminescent direct contact tests.

Minimum Battery of Ecotoxicological Tests and Tests Suitable for Process Monitoring

The most sensitive tests that also correlated with the oil hydrocarbon reduction were the RET assay, the BioTox test, the bioluminescent direct contact test, the bioluminescent flash test, the red clover seed germination test, the test with the soil arthropod *F. candida*, and the test with *Enchytraeus* sp. These tests represent different trophic levels and also assess the effects of solid samples and extracts. One test from each category should be used to assess the environmental impact of the composted product. The Mutatox assay can also be included in the battery to assess the disappearance of genotoxicity.

One biotest is adequate if only process monitoring is concerned. This screening test should be fast, user-friendly, and cost-effective to test a large quantity of samples. Sensitivity to oil hydrocarbon degradation during the entire composting process is also important. All commercial tests, as well as tests using lyophilized organisms, were fast and had no maintenance requirements, but only the luminescent bacterial tests were sensitive enough to assess the changes in toxicity during the entire composting process.

CONCLUSIONS

Both toxicity and oil concentration, including those of PAHs, can be reduced by composting and a clear improvement in soil quality can be achieved. The final oil fractions, measured by the GC method, were mainly heavy and stable hydrocarbons. The compost process treatment should be continued until the stabilization stage of composting. The final product should be assessed for its suitability to support normal soil fauna and flora with the aid of a battery ecotoxicity tests representing different trophic levels and several species. The most suitable tests for screening and monitoring during composting were the luminescent bacterial tests, especially the flash modification.

Stabilization of the compost made the waste suitable for the survival and reproduction of soil animals and for red clover germination. As these are the most relevant parameters to assess the soil suitability to support a more or less normal soil fauna and flora, the composting can be regarded as successful.

The most sensitive tests that also correlated with oil hydrocarbon reduction were the RET assay, BioTox test, bioluminescent direct contact test, bioluminescent flash test, red clover seed germination test, test with the soil arthropod *F. candida*, and test with *Enchytraeus* sp. The soil animal tests and the two direct contact luminescent bacterial tests proved to be the most sensitive to the stabilization of the compost.

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