Journal of Hazardous Materials, 30 (1992) 71–81
Elsevier Science Publishers B.V., Amsterdam

Bacterial mutagenicity of soil extracts from a bioremediation facility treating wood-preserving waste

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(Received May 4, 1991; accepted September 7, 1991)

Abstract

Bioremediation is gaining acceptance as a viable alternative for remediating contaminated soils at Superfund sites. However, it has the potential for resulting in the production of intermediate breakdown products which may be more toxic and soluble than the parent compound. This study was designed to monitor the mutagenic activity of contaminated soils from an abandoned wood preserving facility undergoing bioremediation in an intensively managed land treatment unit (LTU). Sample collection included two waste samples, twelve soil samples from the LTU, one on-site control soil sample, and one off-site control sample. Samples were sequentially extracted with methylene chloride and methanol, and the residues from these extractions were dissolved in dimethyl sulfoxide for testing with the *Salmonella*/microsome assay (Ames test). Each extract was tested using strain TA98 with and without metabolic activation, and the results were used to measure the bacterial mutagenicity which is an indicator of the carcinogenic potential. Although chemical analysis had suggested that biodegradation had reduced the concentration of toxic organics to below method detection limits, the results of bioassay testing detected mutagenic chemicals still present in both lifts of soil in the LTU. The weighted activity (revertants per gram of soil) of the soil from the upper lift which had been in the LTU for only five weeks exceeded two times the background, whereas the weighted activities of soil samples from the lower lift which had been in the LTU for three months were within background values for this site. These data indicate that three months of remediation in an intensively managed LTU is sufficient to reduce the weighted activity of contaminated soil from this facility to near background levels.

1. Introduction

Bioremediation is designed to use the diverse metabolic capabilities of microorganisms to detoxify hazardous constituents. The ultimate goal of bioremediation is to completely mineralize the toxic constituents to carbon dioxide,

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water and residual elements or ions. However, the metabolic pathways used to biodegrade polynuclear aromatic hydrocarbons to intermediates which can be mineralized typically involve a series of different enzyme systems. Thus, the intermediates of biodegradation may persist in the environment or be further degraded. In addition, the intermediates of biodegradation may be both more water soluble and more toxic than the parent compounds.

Procedures for bioremediation of industrial waste include trickling filtration, land treatment, activated sludge digestion, oxidation lagoons and soil inoculation. Bioreactors may also be used to increase contact time and enhance biodegradation of excavated materials. At least seven sites in the United States are presently evaluating some form of bioremediation as a treatment technique for wastes from wood-preserving facilities [1]. The predominant compounds present in a wood-preserving waste include polynuclear aromatic hydrocarbons (PAHs) (including naphthalene, 2,6-dimethylnaphthalene, chrysene and benzo[a]pyrene), phenolics (including phenol, cresol and pentachlorophenol) and heterocyclics (including nitrogen, oxygen and sulfur heterocyclics) [2]. Although biodegradation of the majority of these compounds has been reported in the literature [3,4], little information is available to evaluate the effect of bioremediation on mutagenicity. Degradation under ambient conditions was found to increase the specific activity of a soil amended with a wood-preserving waste [5]. Thus, the present study was conducted to monitor the mutagenic potential of waste from a wood-preserving facility during bioremediation in an intensively managed land treatment unit (LTU) subjected to frequent tillage and periodic additions of nutrients and bacteria.

2. Materials and methods

2.1 Site

The site is an inactive wood-preserving facility located in the northwest of the United States. Historically, the facility had used creosote, pentachlorophenol, copper, chromium and arsenic as wood-treating chemicals. A site investigation revealed the presence of contaminated surface soils, buried waste pits and a plume of contaminated groundwater with a non-aqueous phase. It was estimated that more than 18 000 m^3 of contaminated soil are present at the site. Contaminant levels in soil range from 1 to 2700 mg kg^{-1} for pentachlorophenol, and 2 to 26 555 mg kg^{-1} for polynuclear aromatic hydrocarbons.

The remedial alternative that was selected for contaminated soil was excavation and treatment in a 0.4 ha (approximately 1 acre) lagoon lined with a flexible membrane liner. The first (lower) lift (layer of soil) was composed of soils from which all rocks had been removed, and was applied to the LTU on June 21, 1989. The carcinogenic polynuclear aromatic hydrocarbon (PAH) concentration in the soil was approximately 279 mg kg^{-1} and the pentachlorophenol (PCP) concentration in the first lift was 176 mg kg^{-1}. This was

reportedly reduced to below the Record of Decision (ROD)-mandated concentrations (88 mg kg^{-1} carcinogenic polynuclear aromatic hydrocarbons) after 40 days of treatment. Treatment conditions included daily rototilling and periodic additions of nutrients and freeze-dried bacteria. The second (upper) lift was applied on August 8, 1989 and contained 126 mg kg^{-1} carcinogenic PAH and 31 mg kg^{-1} PCP.

2.2 Soil samples

Thirteen soil and two waste samples were collected on September 27, 1989 from the facility. The first waste sample (No. 001) was a dry tar which had been exposed to air and sunlight, and was collected from the soil surface adjacent to a pond which was being excavated. The second waste sample (No. 004) was a hardened tar sample collected from a seam of waste approximately 4 feet beneath the soil surface.

Twelve soil samples were collected from the LTU; nine samples from the upper lift (0–6 in.; Samples 005–013) and three samples from the lower lift (8–12 in.; Samples 014–016). In addition, one on-site background soil sample was collected from an area approximately 10 feet south of the LTU (Sample No. 017). A second (off-site) background soil sample was collected from a remote area of a nearby national park. A minimum of 250 g soil was collected from each sampling location using precleaned stainless steel trowels.

2.3 Soil extraction procedure

Each soil and waste sample was sequentially extracted using the Tecator* Soxtec HT 12 apparatus (Tecator, Hoganas, Sweden) [6] first with methylene chloride and secondly with methanol. Samples of 15–20 g (dry weight basis) were weighed and added to cellulose extraction thimbles. Glass cups were filled with 25 ml extraction solvent and clamped into the condensers. Each sample was extracted for 5 min in the boiling position and 55 min in the rinsing position. After the rinsing time was completed, the residual solvent was evaporated. The residue from each extraction was then weighed and redissolved in dimethyl sulfoxide (DMSO Grade I, Sigma Chemical Co., St Louis, MO). All extractions were conducted in triplicate and the residue (dissolved in DMSO) was composited.

2.4 Bioassay

The *Salmonella*/microsome assay of Ames et al. [7] was used to evaluate the mutagenicity of the solvent extractable organic compounds in the soil and waste extracts. The *Salmonella* strains were kindly supplied by Dr B.N. Ames (University of California, Berkeley, CA). The procedural methods were those of Ames et al. [7] with modifications suggested by Maron and Ames [8].

*Mention of a trade name does not constitute an endorsement.

The *Salmonella* tester strain TA98 was used with and without metabolic activation because preliminary testing of sample extracts indicated that this strain was the most sensitive strain for the types of chemicals to be evaluated. The S9 mixture (9000 *g* supernatant from homogenized liver) contained 0.3 ml rat liver S9 and 0.7 ml cofactor supplement (11.4 m*M* $MgCl_2$, 47 m*M* KCl, 7.1 m*M* glucose-6-phosphate, 5.7 m*M* NADP and 140 m*M* potassium phosphate buffer at pH 7.4). This high level (30%) of metabolic activation was employed because preliminary testing of a waste extract indicated that this was the optimum level for the types of samples anticipated (data not shown). Aroclor induced Sprague–Dawley rat liver S9 homogenate was obtained from Organon Teknika (West Chester, PA).

Each of the two extracts obtained from the soil or waste was tested in the standard plate-incorporation assay at a minimum of five dose levels (mg $plate^{-1}$): 0.05, 0.1, 0.25, 0.5 and 1.0. The data were analyzed using the modified two-fold rule [9]. Using this procedure, a response is considered positive if the average number of revertants for at least two consecutive dose levels was greater than twice the average number of revertants for the concurrent solvent control [9].

The bioassay data have been interpreted using specific activities and weighted activities. The specific activity is the total number of induced revertants (at a dose of 1 mg residue per plate) less the solvent control value. The specific activity provides a qualitative evaluation of the mutagenic potential of the extract. This value is not influenced by the quantity of residue extracted from the soil. The weighted activity was obtained by multiplying the specific activity (net revertants per mg) by the weight of residue extracted (per gram of soil). This value provides a more quantitative representation of the mutagenic potential of the soil as it defines both the mutagenic response and the total organic concentration of the soil extract. Since previous studies have shown that agricultural soil may have a weighted activity as high as 99 revertants per gram [10], a 50% margin of error has been added, and a soil will be considered to be contaminated if the mutagenic potential is greater than 150 revertants per gram.

All strains were checked monthly for nutritional markers (histidine and biotin) and other genetic characteristics, i.e. sensitivity to crystal violet, ampicillin and UV light. On each test date, all tester strains were calibrated with positive and negative controls. Negative controls included only dimethyl sulfoxide (DMSO, Grade I, Sigma) in the top agar. Positive controls including 25 μg $plate^{-1}$ 2-nitrofluorene (Aldrich, Milwaukee, WI) and benzo[a]pyrene (Sigma, St Louis, MO) at 10 μg $plate^{-1}$ were used to verify the functioning of the metabolic activation system. All reagents and extracts were tested for sterility.

3. Results and discussion

Of the total organic residues extracted from the wastes, approximately 30% was extracted with methylene chloride in the surface tar sample, while over 90% of the subsurface tar sample was extracted with methylene chloride (Table 1). For the LTU soil samples (005–016), the total quantity of solvent extractable organics ranged from 1.5 to 9.7 mg g^{-1} in the upper lift (Samples 005–013); and, from 1.1 to 6.0 in the lower lift (Samples 014–016) (Table 1). In the two background soils, the total solvent extractable organics was 0.7 and 1.7 mg g^{-1} for Samples 017 and 023, respectively (Table 1). Thus, the majority of the soil samples collected from the LTU contained greater quantities of organic chemicals than did the background soils.

None of the extracts induced a positive mutagenic response in the absence of metabolic activation (Table 2). In addition, methylene chloride fractions extracted from the tar samples failed to induce mutagenic responses in the presence of metabolic activation (Table 2). The methanol extracts of both tar samples did induce a mutagenic response with metabolic activation. Of the waste tar samples, No. 004 induced the maximum response of 114 total revertants. Of the two background soil samples, the methylene chloride extract of

TABLE 1

Sample characteristics and solvent extractable organic residues for soil and waste samples from a bioremediation facility

Sample identification	Texture	% water	Residue weight (mg g^{-1})		
			CH_2Cl_2	CH_3OH	Total
001	Tar-like (surface waste)	20.4	34.9	96.9	131.8
004	Hardened tar (subsurface waste)	NT	385.8	10.1	395.9
005	Sandy loam (upper lift)	7.3	2.3	0.5	2.8
006	Sandy loam (upper lift)	10.3	2.8	6.9	9.7
007	Sandy loam (upper lift)	8.8	1.8	0.9	2.7
008	Sandy loam (upper lift)	8.8	2.1	0.5	2.6
009	Sandy loam (upper lift)	7.6	2.7	0.6	3.3
010	Sandy loam (upper lift)	10.9	1.2	0.3	1.5
011	Sandy loam (upper lift)	7.6	0.9	0.7	1.6
012	Sandy loam (upper lift)	10.5	1.2	1.1	2.3
013	Sandy loam (upper lift)	9.2	0.9	1.3	2.2
014	Loam (lower lift)	11.7	2.1	1.5	3.6
015	Loam (lower lift)	11.9	3.0	3.0	6.0
016	Loam (lower lift)	17.3	0.6	0.5	1.1
017	Gravelly sand (on-site bkg)	12.0	0.4	0.3	0.7
023	Gravelly loam (off-site bkg)	11.0	0.7	1.0	1.7

NT = not tested.

TABLE 2

Mutagenic potential of CH_2Cl_2 and CH_3OH fractions extracted from soil samples from a bioremediation facility (values are given ± standard deviation)

Sample No.	Dose[a] (mg ml^{-1})	Fractions			
		CH_2Cl_2		CH_3OH	
		−S9	+30% S9	−S9	+30% S9
001	0	19± 3	27± 3	19± 3	27± 3
	1	16± 2	37±10	16± 1	41± 7
	2	18± 5	46±16	23± 8	55± 9
	5	17± 1	47±12	15± 2	65± 9
	10	16± 1	60±11	Toxic	70±11
	20	13± 4	52±22	Toxic	88±12
004	0	19± 3	27± 3	19± 3	27± 3
	1	22± 5	44± 9	16± 1	36±10
	2	16± 1	48± 4	19± 4	37± 6
	5	15± 1	40± 3	18± 4	77±17
	10	Toxic	65±10	20± 4	99±10
	20	Toxic	50± 9	27± 8	114± 9
005	0	19± 3	27± 3	19± 3	27± 3
	1	21± 4	31±10	17± 3	32± 8
	2	19± 3	42± 4	21± 4	33± 4
	5	21± 5	55±10	24± 5	45± 6
	10	29± 6	58±14	16± 1	68±13
	20	30±11	76± 6	25± 1	69± 9
006	0	19± 3	27± 3	19± 3	27± 3
	1	13± 4	46± 1	20± 5	39± 6
	2	18± 5	59± 8	18± 3	43± 8
	5	19± 4	57± 0	21± 4	48± 7
	10	19± 5	60± 5	25± 6	85±13
	20	20± 6	48± 4	19± 3	75±12
007	0	27± 7	30± 5	27± 7	30± 5
	1	21± 5	43± 7	19± 4	39±10
	2	28± 7	59±15	25±11	50±11
	5	28± 8	77± 7	28±10	68±18
	10	26±10	97±14	31±10	93±16
	20	30± 6	111±21	31± 6	115±11
008	0	27± 7	30± 5	27± 7	30± 5
	1	26± 4	57± 4	30± 4	38± 5
	2	25±11	77±12	23± 9	48± 9
	5	21± 5	87±16	27± 9	63± 4
	10	26± 6	119± 5	25± 7	103± 7
	20	29± 8	113±27	28± 8	115± 8

Sample No.	Dose[a] (mg ml^{-1})	Fractions			
		CH_2Cl_2		CH_3OH	
		−S9	+30% S9	−S9	+30% S9
009	0	27± 7	30± 5	27± 7	30± 5
	1	23± 8	34± 4	22± 4	45± 1
	2	21± 4	55±13	27±11	56± 4
	5	26±10	72±19	26±12	76±16
	10	26± 7	77±12	35±18	112±17
	20	19± 4	76± 6	25± 2	86±13
010	0	27± 7	30± 5	27± 7	30± 5
	1	27± 6	40±13	23±10	37± 3
	2	27±12	38± 7	24± 9	36± 6
	5	19± 5	59±15	21± 4	37± 0
	10	31± 6	72±15	25± 8	43± 3
	20	27± 4	86±11	21± 5	47±10
011	0	27± 7	30± 5	27± 7	30± 5
	1	25± 1	45±13	25± 9	38±11
	2	27± 4	51± 4	29± 5	55±16
	5	25± 7	74±18	26± 1	57±11
	10	26± 6	81±10	32± 4	75± 6
	20	30± 5	105±22	32± 9	96±11
012	0	32± 5	32± 6	32± 5	32± 6
	1	21± 4	40± 9	29± 5	36± 6
	2	28± 4	49±11	32± 4	40± 4
	5	33± 4	66±12	23± 5	32± 8
	10	33± 5	93± 8	33± 8	51± 8
	20	31± 5	88±16	39±10	75± 7
013	0	25± 7	30± 9	30± 5	31± 7
	1	23± 3	31±10	25± 6	36± 7
	2	26± 7	37± 7	26± 2	43± 7
	5	28± 8	49± 9	23± 8	42± 5
	10	32±12	92± 5	31± 5	61± 5
	20	NT	116±15	30± 5	85± 7
014	0	25± 7	30± 9	30± 5	30± 9
	1	26±10	33±10	21± 8	24± 8
	2	29± 8	41±11	30± 4	32±17
	5	25± 2	57± 9	29± 4	54± 8
	10	27± 6	76±11	27± 4	80±17
	20	27± 4	76±17	28± 8	81±14
015	0	25± 7	30± 9	30± 5	30± 9
	1	24± 8	46±12	29± 4	30±11
	2	28±12	54± 8	27± 4	42±19
	5	24± 9	69±16	29± 4	59±21
	10	26± 9	83±13	36±11	67±21
	20	25± 7	68±14	25± 2	64±27
016	0	25± 7	30± 9	30± 5	30± 9
	1	26± 6	29± 8	27[b]	36±12
	2	25± 6	42±11	21± 8	28±12
	5	34±16	47± 7	27± 1	25± 8
	10	39±13	101± 8	25± 2	35± 9
	20	37±12	148±22	28± 3	32±12

TABLE 2 (Continued)

Sample No.	Dose[a] (mg ml^{-1})	Fractions			
		CH_2Cl_2		CH_3OH	
		−S9	+30% S9	−S9	+30% S9
017	0	25± 7	30± 9	30± 5	30± 9
	1	21± 4	40± 8	20± 5	28± 8
	2	27± 5	52± 8	24± 4	31±12
	5	25± 1	88±17	26± 1	33± 5
	10	26± 6	168±41	29± 3	27± 7
	20	NT	196±53	24± 1	29± 8
023	0	25± 7	30± 9	30± 5	31± 7
	1	14± 8	13±14	27± 1	31± 5
	2	31± 5	30±17	26± 6	31± 8
	5	28± 5	23±24	24± 6	29± 7
	10	33± 0	48±15	24± 4	30± 6
	20	35± 7	44±17	26± 7	39±14

[a]Doses of 0, 1, 2, 5, 10 and 20 mg ml^{-1} equal 0, 0.05, 0.1, 0.25, 0.5 and 1.0 mg plate^{-1}.
[b]Sample tested on duplicate plates in one independent experiment. Standard deviation not provided.
NT = not tested.

No. 017 induced the maximum mutagenic response of 196 revertants per mg of residue with activation. The elevated level of activity of this sample suggests that it may either contain natural mutagens (i.e. mycotoxins) or it may be contaminated by adjacent waste management activities.

The methylene chloride extracts of all soil samples from the LTU induced a mutagenic response with metabolic activation (Table 2). Of the upper lift samples (005–013), the methylene chloride extract of Sample No. 013 induced the maximum mutagenic response of 116 total revertants per mg with activation, while the lowest response was 48 total revertants per mg of residue induced by Sample No. 006 (Table 2). One methanol extract (Sample No. 010) from the upper lift failed to induce a mutagenic response. The mutagenic responses induced by the methanol extracts of the upper lift ranged from 69 total revertants per mg (Sample No. 005) to 115 total revertants per mg (Samples Nos. 007 and 008). The maximum response for all of the extracts of samples from the LTU was 148 total revertants per mg induced by the methanol extract of Sample No. 016 from the lower lift (Table 2). These data indicate that the mutagenic constituents of wood-preserving waste were persistent for at least 3 months under the conditions of the LTU.

The weighted activity provides a quantitative indication of the mutagenic potential of waste amended soils by defining the quantity of mutagens in a specified volume of soil. The weighted activity is calculated by multiplying the specific activity (determined by subtracting the solvent control value from the

total revertants obtained at a dose of 20 mg ml^{-1} in Table 2) by the residue weight of the organics solvent extracted from the soil (from Table 1). The weighted activities of the two tar samples collected in the present study were 873 revertants per gram for the methylene chloride fraction of tar sample No. 001 and 8873 revertants per gram for the methylene chloride fraction of tar sample No. 004 (Table 3). The methylene chloride fractions extracted from the upper lift of soil from the LTU-induced weighted activities ranged from 59 to 174 revertants per gram; while, the methanol extract-induced weighted activities ranged from 5 to 331 revertants per gram (Table 3). For the lower lift, the weighted activities ranged from 1 to 114 revertants per gram. These data suggest that bioremediation has slightly reduced the weighted activity of the wood-preserving waste contaminated soil.

In a previous study, the mutagenic potential of soils amended with a wood-preserving waste was observed to increase during the first 3 years following application [5]. The maximum response observed in the previous study was 1561 net revertants per mg induced by the base fraction extracted from the soil collected 540 days after waste application [5]. The application rates used in the previous study were greater than those in the present study. In addition, the previous study did not use any procedures for the enhancement of biodegradation. In the present study, mutagenic chemicals were detected in both the

TABLE 3

Weighted activity, as measured with metabolic activation, of CH_2Cl_2 and CH_3OH fractions extracted from soil samples from a bioremediation facility

Sample	Net TA98 His^+ revertants per gram		
	Description	CH_2Cl_2 +S9	CH_3OH +S9
001	Waste	873	5911
004	Waste	8873	879
005	Soil, upper lift	113	21
006	Soil, upper lift	59	331
007	Soil, upper lift	146	77
008	Soil, upper lift	174	43
009	Soil, upper lift	124	34
010	Soil, upper lift	67	5
011	Soil, upper lift	68	46
012	Soil, upper lift	67	47
013	Soil, upper lift	77	70
014	Soil, lower lift	97	77
015	Soil, lower lift	114	102
016	Soil, lower lift	71	1
017	Background soil	66	0
023	Background soil	10	8

upper and lower lifts of waste-contaminated soil. After 3 months of treatment, the specific activity of the extracts of surface soil from the LTU ranged from 17 net revertants to 86 net revertants at a dose of 1 mg per plate (Table 2). However, the specific activity detected in the soils from the LTU was appreciably lower than that observed for waste-amended soil in the previous study [5].

Previous studies have indicated that the weighted activity of an agricultural soil can range from 2 to 99 revertants per gram [10]. The weighted activity of background soils collected in a study of 18 Superfund sites ranged from below detection to 351 revertants per gram [11]. Following the bioassay of the background soil which yielded 351 revertants in the previous study, it was determined that this sample had been collected from a contaminated area at an inactive wood-preserving facility. In general, weighted activities of greater than 150 revertants per gram are assumed to be indicative of contaminated soil. In the present study, the maximum weighted activity of a contaminated soil from the LTU was 331 revertants per gram. Three samples from the upper lift induced weighted activities of greater than or equal to 150, while none of the samples from the lower lift induced a weighted activity greater than 150 revertants per gram (Table 3). Assuming that the initial weighted activity of the contaminated soil from this site is about 330 revertants per gram, these data indicate that bioremediation reduced the weighted activity of the lower lift of soil to background levels after approximately 3 months of treatment. Thus, although specific mutagens (possibly PAHs) could be detected in the extracts of the lower lift of soil, the quantitative mutagenic potential (or weighted activity) was less than 50% greater than that of an uncontaminated agricultural soil.

Although soil samples were not analyzed prior to placement in the LTU, the analysis of excavated waste material and some soil samples from the upper lift of the LTU indicate that mutagenic chemicals were present in the waste. The elevated specific activity observed with soil from the lower lift indicates that these chemicals may persist in the treatment system. However, the weighted activity of the majority of soil extracts was within background levels (0–150 revertants per gram), indicating that bioremediation reduced the overall mutagenic activity of waste-amended soil.

Significantly different results may be anticipated in other bioremediation systems. This may be due to differences in waste streams, microbial populations, soils, or other environmental or management parameters.

Acknowledgments

This study was made possible through funds from cooperative agreement No. CR-814386-01-0 from the United States Environmental Protection Agency

and grant No. P42-ES04917 from the National Institute of Environmental Health Services.

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