BIOAVAILABILITY OF GENOTOXIC MIXTURES IN SOIL

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ABSTRACT Contaminated media at Superfund sites typically consist of complex mixtures of organic and inorganic chemicals which are difficult to characterize, both analytically and toxicologically. The current EPA approach to risk assessment uses solvent extraction to remove chemicals from the soil as a basis for estimating risk to the human population. However, contaminants that can be recovered with a solvent extract may not represent the mixture of chemicals that are available for human exposure. A procedure using an aqueous extraction was investigated to provide a more realistic estimate of what chemicals are bioavailable. A study was conducted with two soil types: creosote-contaminated sandy soil and coal tar-contaminated clay soil spiked with benzo(a)pyrene [B(a)P], and trinitrotoluene (TNT). Samples were extracted with hexane:acetone and water titrated to pH2 and pH7. HPLC analysis demonstrated up to 35% and 29% recovery of contaminants using the aqueous extracts. The estimated cancer risk for the aqueous extract was one order of magnitude less than that for solvent extracts. Analysis using the *Salmonella*/microsome assay demonstrated that solvent extracts were genotoxic (133 revertants/mg) with metabolic activation while aqueous extracts of clay soil were not genotoxic. Sandy soil showed genotoxicity both with and without metabolic activation. These results suggest that solvent extraction techniques may overestimate the concentration of contaminants that are available for human exposure and, hence, the risk associated with the presence of the contaminants in soil.

KEY WORDS: risk assessment, bioavailability, aqueous extraction, genotoxicity

INTRODUCTION

Risk assessment calculations usually assume that the majority of contaminants in soil are available for human absorption. Current EPA [1] guidelines require Soxtec extraction with hexane:acetone to recover organic chemicals from the soil. In using an organic solvent to extract compounds, bonds are often ruptured between the particles in the soil and the chemical. The quantity of chemicals that are solvent-extracted from a matrix will depend on the ionic strength of the particular solvent and the characteristics of the matrix [2]. Hexane and acetone generally produce maximum recovery of the polar and non-polar organic compounds. These extracts are then analyzed for chemical concentration and the results used in a risk calculation to estimate the maximum chemical exposure associated with the contaminated soil.

Most hydrocarbons will bind strongly to organic matter in the soil. Leaching or adsorption of contaminants from the soil may be impeded due to binding of the chemical to the micropores in the soil particle. To be released from these micropores, the contaminant must break the bonds between it and the soil particle. As it passes through the particle, the formation of additional bonds further impedes removal of the chemical from the soil [3]. The literature suggests that aging of chemicals results in continuous diffusion from the more readily accessible solid:water interface to the more remote micropores. Hatzinger and Alexander [5] reported that the length of time a compound remains in the soil is directly

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related to its extractability and biodegradability. Phenanthrene and 4-nitrophenol were aged from 0, 13, 27, up to 84 days. On the designated day an inoculum of *Pseudomonas* strain R or isolate WS-5 was added. Mineralization rates for days 0, 13, 27, and 84 were measured at 17.6, 14.4, 13.3, and 11.2%, respectively. Recovery of unaged phenanthrene from butanol extraction was 94.5% and decreased to 67.0% when aged for 13 days; subsequent extraction of butanol-extracted soil with methylene chloride in a Soxhlet apparatus recovered up to 0.66% of the unrecovered phenanthrene from the butanol extraction.

Chemicals that are tightly bound to the soil are unlikely to be available for cellular uptake and metabolism or for leaching into ground water. Bonaccorsi, *et al.* [6], administered 2,3,7,8-tetrachlorodibenzo (p)dioxin (TCDD) aged in soil and TCDD freshly added to soil to rabbits through oral gavage. The concentrations in the liver were measured after seven days as an indicator of bioavailability. It was demonstrated that concentrations of TCDD in aged soil were not detected in the rabbit liver at soil concentrations of 20 and 40 ppm; however freshly amended soils yielded TCDD concentrations of 0.26 and 1.1 ppm from 20 and 40 ppm soil concentrations. Shu, *et al.* [7], compared concentrations of soil-bound TCDD and TCDD in corn oil and found that only 43% TCDD in contaminated soil was available to rats. Umbreit, *et al.* [8], used TCDD-contaminated soil and administered this to guinea pigs. TCDD was then extracted from the soil and the soil was recontaminated with the extract. The recontaminated soil was then administered to the guinea pigs through oral gavage. A reduction was seen in the bioavailability of TCDD when bound in soil and an increase in availability in soil recontaminated with the extracted TCDD.

The increasing evidence that bound contaminants may be unavailable for exposure needs to be more carefully considered in preparation of a risk assessment. In addition, as the compound concentration and composition changes in a mixture, the synergistic and antagonistic interactions between compounds will also change. A method utilizing aqueous extraction may provide a more reasonable estimate of the bioavailability of compounds resultant from leaching as well as ingestion of soil. Risk assessment based on bioavailability as well as chemical interactions could be employed with a microbial assay to determine genotoxicity.

MATERIALS AND METHODS

Chemicals

Chemicals purchased from Sigma were 99% purity, including: benzo(a)pyrene, *d*-glucose-6-phosphate, *l*-histidine, dimethyl sulfoxide (ACS reagent grade), *B*-nicotinamide adenine dinucleotide phosphate (NADP), and d-biotin. Trinitrotoluene (98% purity) with 10-20% water was purchased from Chem Service. All standards for HPLC analysis were purchased from AccuStandards and were 99.9% purity.

Samples

An aged coal tar-contaminated soil with a relatively high clay content was obtained from a manufactured gas plant site in the northeast United States. Samples were spiked with 200 ppm of B(a)P or 200 ppm each of B(a)P and TNT. A control with no spike was also prepared. Additional studies were conducted using a creosote-contaminated sandy soil obtained from a wood treatment facility. Samples were spiked in the same manner. Spikes for TNT included calculation to compensate for water content.

Extraction procedure

Solvent

Coal tar-contaminated soil was extracted using a Tecator Automatic Soxtec extraction unit (Tecator, Hoganus, Sweden). Approximately 10 g of sample was placed in a pre-weighed cellulose thimble with a glass wool plug on top of the contents in each extraction thimble. Fifty ml of a 1:1 (v/v) mixture of hexane:acetone was poured into pre-weighed extraction cups and heated to 140°C. Solvent was allowed to boil and then thimbles were immersed into boiling solvent for 60 min and then rinsed for an additional 60 min. The solvent was allowed to evaporate and collect in the condenser. Extraction cups containing the sample extracts were removed and allowed to air dry. Sample residue was transferred with a glass pipet and several volumes of solvent into a pre-weighed culture tube. The culture tube was placed under a stream of nitrogen gas, weighed, and then the extract was redissolved in DMSO. Samples of the creosote-contaminated soil were extracted with methylene chloride and methanol using current EPA guidelines [1].

Aqueous

Samples were also extracted with potassium phosphate buffer at pH 2 and distilled water (pH 7) and incorporated into the bottom agar for a genotoxicity assay. A 4:1 volume of water:soil was prepared then shaken in the dark for 24 hours according to methods of Donnelly, *et al.* [9]. The sample was then poured into centrifuge bottles and centrifuged at high speed for 15 minutes. Supernatant was then filtered through a Buchner funnel with Whatman glass microfibre filters (70 mm) to remove large particles and adjusted to pH 7. Supernatant was then passed through a ZapCap® CR disposable bottle-top filter with a pore size of .45 μm. Vogel Bonner minimal media was prepared with 10, 30, and 50% less the normal amount of water. The sterile filtrate was aseptically decanted into the prepared agar and brought to a volume of 250 ml. Agar was poured into sterile Petri dishes which were incubated upside down at 37°C for 24 hours to ensure sterility. Each dose of the water extract was tested in duplicate plates in two independent experiments.

Chemical analysis

The chromatographic set-up consisted of a gradient HPLC system incorporating a Waters (Milford, MA) 600 Controller, an injection valve (type 7725I, Rheodyne, Cotari CA) with an internal sample loop of 20 μl volume and a Waters 996 photodiode array (PDA) detector with a 200-800 nm resolution. The mobile phase consisted of 50% acetonitrile and 50% water which was sonicated in an ultrasonic bath until completely degassed. Separation was achieved on a Waters Novapak® C18 column (3.9 x 150 mm); a guard column (type Waters C18, 30 mm) was used to prevent clogging of the separation column.

Approximately 2 ml of both aqueous and solvent extracts were reserved for HPLC analysis. Aqueous extracts were prepared by extracting with toluene in a glass separatory funnel, while solvent extracts were dissolved in toluene. Identification, integration, and quantification was completed using a set of PAH standards provided by AccuStandards (New Haven, CT) and the Millenium software program. All concentrations were reported in part-per-million (mg/l) concentration.

Biological analysis for solvent-based extracts

The *Salmonella/*microsome assay [10] with procedural modification described by Maron

and Ames [11] was used to evaluate the mutagenicity of solvent extracts. *Salmonella* strain TA98, kindly provided by Dr. B.N. Ames (University of California, Berkeley, CA), was tested, with and without metabolic activation, using S9 mixture. The S9 mixture (9000 x *g* supernatant from homogenized liver) contained 0.2 ml rat liver S9 and 0.7 ml cofactor supplement (11.4 mM $MgCl_2$, 47 mM KCL, 7.1 mM glucose-6-phosphate, 5.7 mM NADP, and 140 mM potassium phosphate buffer at pH 7.4). Aroclor-induced Sprague-Dawley rat liver was obtained from Molecular Toxicology (Annapolis, MD).

The solvent extracts were tested at 20, 10, 5, 2, and 1 mg residue/ml DMSO. The plates were incubated upside down for 72 h and cells reverting to wild-type appeared as large colonies on the plate. Colonies were counted using an Artek automatic colony counter (Dynaktek Laboratories, Chantilly, VA). Strain TA98 was tested monthly for nutritional markers (histidine and biotin), sensitivity to crystal violet, ampicillin, and UV light. On each test date, TA98 was calibrated with positive and negative controls.

Biological analysis for aqueous extracts

Aqueous extracts were run in the standard plate incorporation assay with procedural modifications suggested by Donnelly, *et al.* [9]. Top agar additions included only bacteria TA98 and cofactor; aqueous extracts were added to the bottom agar at a rate of 10, 30, and 50% extract per 25 ml bottom agar. The data were analyzed using the modified two-fold rule [12]. A positive response is considered if the average response for at least two consecutive dose levels is greater than twice the average response for the concurrent negative/solvent control [12].

Risk calculations

Risk calculations were performed to rank soils using calculations for ingestion of soil based on both solvent and aqueous extraction for childhood exposure.

$$\text{Intake (mg/kg-day)} = \frac{\text{CS x IR x CF x FI x EF x ED}}{\text{BW x AT}}$$

where: CS = chemical concentration in soil (mg/kg); IR = ingestion rate (mg soil/day), 200 mg/kg (children 1 through 6 yrs. old); CF = conversion factor (10^{-6} kg/mg); FI = fraction ingested (0.7); EF = exposure frequency (days/year), 350 days/year; ED = exposure duration (years), 6 years; AT = averaging time (70 year lifetime for carcinogenic effects * 365 days); and BW = body weight (16 kg)

RESULTS AND DISCUSSION

The risk associated with exposure to the two contaminated soils was estimated using chemical and biological analysis of solvent and aqueous extracts. The results of the chemical analysis of aqueous extracts of both soil types are presented in Table 1; the extraction efficiencies assume a 100% extraction rate from the solvent extracts. In general, the creosote-contaminated sandy soil showed a greater aqueous extractability of contaminants than the coal tar-contaminated clay soil. The aqueous extract of the sandy soil at pH7 resulted in a 43% recovery of acenaphthene compared to 34% recovery in the clay soil. Extraction of the sandy soil at pH2 yielded 36% recovery of acenaphthene verses 27% recovery from the clay soil. Samples spiked with B(a)P showed a recovery of 24 and 13% for pH7 and pH2, respectively. Soils spiked with B(a)P and TNT resulted in a recovery of 29% TNT for both soil types at pH2, and 23 and 17% for

clay soil and sandy soil at pH7, respectively. Thus, the recovery of the various chemicals appeared to be influenced greatly by the physical characteristics of the soil.

A comparison of the genotoxicity of the solvent and aqueous extracts was conducted using a microbial mutagenicity assay. The mutagenic potential of hexane/acetone and aqueous extracts were measured using *S. typhimurium* strain TA98 with and without metabolic activation. The data presented in Table 2 provides the mutagenicity of the solvent extracts of the various soils. None of the solvent extracts induced a positive mutagenic response in the absence of metabolic activation. All of the solvent extracts induced a doubling of revertants at two consecutive dose levels (or a positive mutagenic response) with metabolic activation. With metabolic activation, the hexane/acetone extracts of the clay samples induced a maximum response of 133 revertants at a dose of 0.5 mg/plate, while the solvent extract of sandy soils induced a maximum response of 87 revertants/plate.

The results of the aqueous extracts at pH7 and pH2 are provided in Tables 3 and 4. The pH7 extract of the B(a)P:TNT-spiked sandy soil induced a positive mutagenic response with and without metabolic activation. This result reflects the presence of the direct-acting TNT in the extract. None of the aqueous extracts of the clay soils induced a doubling of revertants at two consecutive dose levels. The B(a)P-amended sand induced 114 revertants at a dose of 12.5 ml of extract (50%) per plate with S9, while the sand spiked with both B(a)P and TNT induced 183 revertants without S9 and 141 revertants with S9. The data in Table 4 indicate that extracts collected at pH2 failed to induce a doubling of revertants at two consecutive doses. These data suggest that a reduced level of genotoxic material was recovered in the extraction performed at the lower pH.

TABLE 1. EXTRACTION EFFICIENCY OF AQUEOUS EXTRACTS, IN PERCENT, BASED ON 100% RECOVERY OF PAHS FROM SOLVENT EXTRACT ON HPLC (ACENAPH = ACENAPHTHENE, B(b)F = BENZO(b)FLUORANTHENE, D(a)A = DIBENZO(a)ANTHRACENE, PHEN = PHENANTHRENE; N/A = NOT DETECTED ON HPLC.

	Clay soil			Sandy soil		
Chemical	pH7+ B(a)P	pH2 + B(a)P	pH7 + B(a)P+ TNT	pH2 + B(a)P+ TNT	pH7 B(a)P+ TNT	pH2 B(a)P+ TNT
Acenaph	34	29	30	32	43	36
Anthracene	29	27	28	27	32	32
B(b)F	25	21	20	21	22	21
B(a)P	24	13	21	13	25	13
Chrysene	21	17	21	17	23	19
D(a)A	3	3	3	3	n/a	n/a
Fluoranthene	3	2	3	3	3	3
Fluorene	0	0	0	0	3	2
Phen	26	27	26	25	24	25
Pyrene	11	13	11	10	n/a	n/a
TNT	n/a	n/a	23	29	17	29

A weighted activity was also calculated for the solvent and aqueous extracts of the soils. The weighted activity accounts for the amount of organic material extracted from a sample. The solvent extract of sample B induced a weighted activity of 8,339 revertants/0.5 g soil, although the aqueous extract induced a weighted activity of only 31 revertants/0.5 g soil (Figure 1). The data based on biological testing suggest an increased risk associated with the solvent extracts as compared to aqueous extracts.

TABLE 2. TOTAL HIS+ REVERTANTS FOR SOLVENT EXTRACTS.

Sample	Dose mg/ml	-s9	+s9
B(a)P-clay	1	28 ± 4	112 ± 11
	2	27 ± 8	115± 19
	5	36 ± 2	124± 17
	10	31 ± 11	130± 16
	20	51 ± 12	113± 11
B(a)P:TNT clay	1	27 ± 4	71 ± 17
	2	32 ± 6	98 ± 18
	5	30 ± 5	129 ± 10
	10	30 ± 5	133 ± 22
	20	40 ± 8	123 ± 6
No-spike clay	1	29 ± 5	63 ± 8
	2	30 ± 7	82 ± 13
	5	25 ± 2	107 ± 24
	10	30 ± 6	125 ± 13
	20	33 ± 8	115 ± 21
B(a)P-sand	1	19 ± 4	57 ± 14
	2	23 ± 7	71 ± 5
	5	18 ± 8	73 ± 8
	10	18 ± 5	78 ± 6
	20	18 ± 4	70 ± 12
B(a)P:TNT sand	1	36 ± 8	48 ± 12
	2	38 ±15	63 ± 14
	5	35 ± 10	71 ± 12
	10	29 ± 13	87 ± 18
	20	28 ± 6	81 ± 11
No-spike sand	1	21 ± 6	56 ± 1
	2	23 ± 5	67 ± 14
	5	25 ± 7	71 ± 11
	10	20 ± 3	67 ± 12
	20	21 ± 7	67 ± 9
DMSO	control	26	39

*All spikes are 200 ppm.

TABLE 3. TOTAL HIS+ REVERTANTS FOR pH7 AQUEOUS EXTRACTS.

Sample	Dose %	-s9	+s9
B(a)P-clay	10	23 ± 4	39 ± 7
	30	30 ± 5	43 ± 12
	50	28 ± 6	39 ± 6
B(a)P:TNT clay	10	34 ± 4	38 ± 7
	30	28 ± 4	41 ± 5
	50	39 ± 10	48 ± 10
No-spike clay	10	26 ± 7	38 ± 6
	30	28 ± 5	40 ± 10
	50	33 ± 9	44 ± 16
B(a)P- sand	10	40 ± 4	76 ± 1
	30	51 ± 6	90 ± 4
	50	47 ± 0	114 ± 1
B(a)P: TNT sand	10	45 ± 11	85 ± 0
	30	71 ± 6	126 ± 1
	50	183 ± 49	141 ± 20
DMSO	control	28	38

*All spikes are 200 ppm.

TABLE 4. TOTAL HIS+ REVERTANTS FOR pH2 AQUEOUS EXTRACTS.

Sample	Dose %	-s9	+s9
No-spike clay	10	32 ± 5	41 ± 5
	30	22 ± 3	31 ± 4
	50	33 ± 4	28 ± 11
B(a)P- clay	10	30 ± 11	37 ± 9
	30	27 ± 5	24 ± 5
	50	35 ± 2	27 ± 4
B(a)P:TNT clay	10	27 ± 4	31 ± 5
	30	37 ± 6	30 ± 4
	50	35 ± 9	22 ± 3
B(a)P:TNT sand	10	27 ± 0	51 ± 8
	30	37 ± 0	32 ± 7
	50	17 ± 0	17 ± 0
DMSO	control	28	38

*All spikes are 200 ppm.

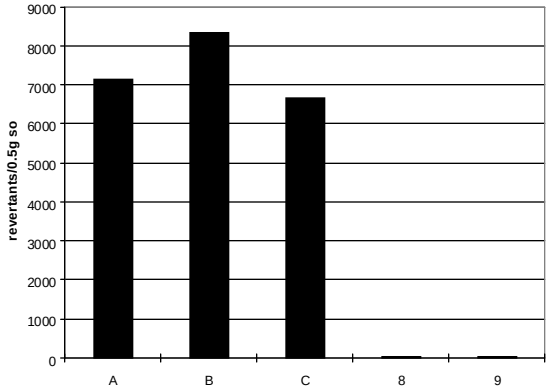

FIGURE 1. WEIGHTED ACTIVITY OF SOLVENT AND AQUEOUS EXTRACTS: WITH METABOLIC ACTIVATION. A = B(a)P-SPIKED CLAY SOIL, SOLVENT EXTRACT; B = B(a)P:TNT-SPIKED CLAY SOIL, SOLVENT EXTRACT; C = CLAY SOIL WITH NO SPIKE, SOLVENT EXTRACT; 8 = B(a)P-SPIKED SANDY SOIL, AQUEOUS EXTRACT pH7; 9 = B(a)P:TNT-SPIKED SANDY SOIL, AQUEOUS EXTRACT pH7.

The estimated excess lifetime cancer risk was calculated for the aqueous and methylene chloride extracts based on HPLC analysis (Table 5). The highest estimated risk was obtained from the methylene chloride extract spiked with B(a)P and TNT as compared to the lowest risk associated with pH7. In general, the solvent-extracted samples were at least one order of magnitude greater risk than that of the aqueous extracts.

CONCLUSION

As expected, appreciable differences were observed in both the chemical composition and genotoxicity of the solvent and aqueous extracts of contaminated soils. Although a positive mutagenic response was more consistently observed in the solvent extracts, the maximum mutagenic response was observed in the aqueous extract of a spiked soil. In addition, none of the extracts collected at a pH2 induced a positive response. In most cases, chemical analysis suggested that the estimated risk associated with the solvent extracts was approximately one order of magnitude greater than the risk associated with the aqueous extract. These differences most likely reflect both the compound concentration and composition of solvent versus aqueous extracts. These data verify that the solutions recovered by an

TABLE 5. ESTIMATED CANCER RISK ASSOCIATED WITH EXPOSURE TO AQUEOUS AND SOLVENT EXTRACTS OF CONTAMINATED SOILS (* DENOTES SANDY SOIL).

Extraction technique	Lifetime cancer risk
*$MeCl_2$	5.3e-3
*$MeCl_2$ [B(a)P + TNT]	1.58e-2
*Aqueous pH 7 [B(a)P + TNT]	3.72e-3
*Aqueous pH 2 [B(a)P + TNT]	4.9e-3
Hexane:Acetone	1.4e-2
Hexane:Acetone [B(a)P]	2.2e-2
Hexane:Acetone [B(a)P + TNT]	2.2e-2
Aqueous extract pH7	1.5e-3
Aqueous extract pH7 [B(a)P]	3.9e-3
Aqueous extract pH7 [B(a)P + TNT]	4.2e-3
Aqueous extract pH2	1.6e-3
Aqueous extract pH2 [B(a)P]	2.9e-3
Aqueous extract pH2 [B(a)P + TNT]	2.9e-3

aqueous or solvent extract will differ appreciably. Further research is needed to identify the system or systems that most closely emulate the processes of leaching or bioavailability. These studies should provide information that can be used to reduce the uncertainty associated with estimating the risk associated with contaminated soil.

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