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Bioassay-based risk assessment of complex mixtures

K.C. Donnelly[a,*], S.H. Safe[b], K. Randerath[c], E. Randerath[c]

[a] *Veterinary Anatomy and Public Health,* [b] *Veterinary Physiology and Pharmacology, Texas A&M University, College Station, TX 77843-4458, USA,* [c] *Baylor College of Medicine, Houston, TX, USA*

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Abstract

In order to compare a standard chemical-based risk assessment with in vitro genotoxicity assays, two complex environmental mixtures from a wood-preserving site were analyzed in the *Salmonella*/microsome and *E. coli* prophage induction assays. Using GC/MS, sample 003 was found to contain relatively low levels of polycyclic aromatic hydrocarbons (PAHs) and elevated levels of polychlorinated dibenzo-*p*-dioxins (PCDDs), while sample 005 had higher levels of PAHs and relatively low levels of PCDDs. The complex mixtures were sequentially extracted with methylene chloride and methanol for analysis in *Salmonella*, or extracted with a 1:1 hexane:acetone mixture for analysis in the *E. coli* prophage induction assay. At a dose of 1.0 mg/plate in *Salmonella* strain TA98 with metabolic activation, the methanol extract of sample 003 induced 197 net revertants, while sample 005 induced 436 net revertants. In the prophage induction assay, with activation, the hexane:acetone extract of sample 003 induced a genotoxic response that was slightly lower than that observed with sample 005. The estimated incremental carcinogenic risk for ingestion of PAHs was 1.5E − 3 for sample 003, while for sample 005 the estimated risk was 1.5E − 2. Thus, the sample which induced the maximum response in both bioassays also had the highest estimated cancer risk. However, the frequency of PAH-DNA adducts in both skin and liver tissues was appreciably higher with sample 003 than with sample 005. A combined testing protocol, using both biological and chemical analysis, therefore provides more accurate information from which to assess risk than the use of either method alone.

1. Introduction

The baseline risk assessment is a critical factor in the ranking and remediation of contaminated waste sites. Standard methodology for risk assessment [1] requires the selection of principal hazardous constituents, an exposure assessment, and a toxicity assessment. This information is then combined to form the basis of a risk

* Corresponding author. Tel.: 1-409-845-7956. Fax: 1-409-847-8981.

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characterization. The standard chemical-based risk assessment procedure calculates an incremental carcinogenic risk or hazard index (for systemic effects) for specific principal hazardous constituents at reasonable maximum exposures [1]. Although the existing methodology does provide a standardized procedure for estimating risk, both the public and the scientific community have been critical of the uncertainties associated with these methods.

The accuracy of a standard risk assessment methodology is affected by several parameters. In the collection and analysis of data, a concentration term is defined which must characterize contaminant levels which often exhibit a tremendous variability. Default factors are often used to characterize exposure levels in a population. These factors assume a standard weight, age and diet in a heterogeneous population. Toxicity factors are derived from animal studies and, for a large number of chemicals, are simply unavailable. Biological analysis of contaminated media offers a tool for overcoming several limitations of the standard risk assessment procedure. Bioassays can be used to detect the toxic or genotoxic potential of unknown and even unidentified compounds. In addition, bioassays can be used to characterize the toxicity of complex mixtures. This allows the biological test to measure the interactions of the components of a complex mixture.

Limited information is available in the literature to describe potential synergistic, antagonistic or additive interactions of complex mixtures. Chemicals may interact in a mixture to alter metabolism or transport characteristics with a significant effect on toxicity. In animal studies, DiGiovanni and Slaga [2] observed that benzo(e)pyrene enhanced the carcinogenic activity of benzo(a)pyrene. Rao et al. [3] observed similar interactions in a bacterial mutagenicity assay. Kawalek and Andrews [4] found that benzene and phenanthrene inhibited the mutagenicity of 2-aminoanthracene when using uninduced rat liver. Catechol, a major intermediate in the degradation of most aromatic hydrocarbons, was shown to both increase and inhibit the mutagenicity of benzo(a)pyrene [5]. In order to address these interactions, microbial mutagenicity assays have been utilized to characterize the genotoxic potential of a variety of complex mixtures including air particulate [6–8] and hazardous wastes [9–11]. These studies, therefore, were conducted to compare the results of a risk characterization based on chemical analyses and biological testing.

2. Materials and methods

2.1. Site

The site used for the collection of samples was an abandoned wood-preserving facility which included approximately 26 acres and was used for railroad tie trimming, treating and storage. Residential areas are on two sides of the facility, and other industries are located on two sides. Creosote had been used since operations began, while pentachlorophenol was used from the early 1970s until 1984, and zinc naphthenate was used from 1984 until closing. As asphalt driveway sealer composed of coal tar derivatives and montmorillonitic clay was also manufactured at this facility.

The site includes several buried surface impoundments, abandoned processing areas contaminated with treatment materials, and a large drip track area covered with a thick layer of tarry material. Visual inspection revealed several areas outside of the facility fenceline which appear to have been impacted by waste materials. An off-site background sample was collected from a schoolyard located approximately one mile from the facility.

2.2. Samples

Two samples collected from the abandoned wood-preserving facility are described in this report. The first sample was collected from a mound of hardened tar. This material had been recycled from waste products for use as a road sealant. This sample (003) was a black viscous tar collected from the surface to a depth of approximately 10 cm using a precleaned stainless steel trowel. The second sample (005) was a black material with the texture of sediment collected with a precleaned stainless steel auger from a depth of 10–25 cm. This sample was collected from a depressed area adjacent to an above ground storage tank. The area from which the sample was collected had previously been the location of a lagoon used for storage of wood-preserving waste. All samples were placed in a precleaned 16 oz glass jar and stored on ice (approximately 4 °C) prior to shipment and analysis in the laboratory.

2.3. Soil and solid waste extraction procedure

Samples were extracted using a Tecator Automatic Soxtec extraction unit (Tecator, Hoganus, Sweden). Approximately 15 g of sample and 7 g^{-} anhydrous sodium sulfate were mixed in a soil moisture can (samples were prepared in triplicate). The sample was then placed in a cellulose thimble and extracted using 25 ml of solvent. The temperature of the extraction unit was set at 130 °C for methylene chloride and 200 °C for methanol. Each sample was extracted for 5 min in the boiling position and 55 min in the rinsing position. Following the methylene chloride extraction, the extraction was repeated using methanol. At the end of each extraction cycle, the extraction cup was removed and the sample transferred to a tarred screw-capped culture tube. The sample was then dried under a stream of nitrogen and redissolved in DMSO for bioassay analysis.

2.4. Chemical analysis

Quantitative identifications were carried out using a GC-MS Library Search. The GC-MS used was an HP 5890 with HP 5971A Mass Selective Detector. Approximately 40 peaks were identified. The GC conditions were: initial oven temperature, 35 °C for 4 min; final temperature, 310 °C for 20 min; temperature program rate, 8 °C/min; injector temperature 260 °C; detector temperature 310 °C. The column used was a PTE-5, 0.25 mm i.d., 30 m length, and 0.25 μm film thickness. Quantitative results were obtained mainly using GC-FID analysis with an HP 5880 GC.

2.5. Bioassays

The *Salmonella*/microsome assay of Ames et al. [12] was used to evaluate the mutagenicity of the solvent extractable organic compounds recovered from the soil samples from both sites. *Salmonella* strains were kindly supplied by Dr. B.N. Ames (University of California, Berkeley, CA). The procedural methods were the same as Ames et al. [12] with the modifications suggested by Maron and Ames [13]. The *Salmonella* tester strain TA98 was utilized with and without metabolic activation. 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). Prior research indicated that this high level of S9 yielded the optimum response for these samples [14]. Aroclor-induced Sprague–Dawley rat liver was obtained from Molecular Toxicology (Annapolis, MD).

The extracts of the various environmental samples were tested in the standard plate incorporation assay at a minimum of four dose levels (mg/plate): 0.1, 0.25, 0.5, and 1.0. The plates were incubated for 72 h and cells reverting to the wild-type appeared as obvious large colonies on the plate. The number of colonies appearing on each plate was determined using an Artek automatic colony counter (Dynatek Laboratories, Chantilly, VA). All strains were checked monthly for nutritional markers (histidine and biotin) and other genetic characteristics, i.e., sensitivity of crystal violet, ampicillin, and UV light. On each test date, all tester strains were calibrated with positive and negative controls.

The data were analyzed using the modified twofold rule [15]. Using this procedure, a response is considered positive only if the average response for at least two consecutive dose levels was greater than twice the average response for the concurrent negative/solvent control [15]. The data have also been evaluated based on specific activity values and weighted activity values. The specific activity provides a qualitative measurements of the mutagenic potential of a specific sample. The weighted activity was obtained by multiplying the specific activity at a dose of 1 mg/plate times the weight of organic residue in the original soil or solid waste sample. The weighted activity provides a more quantitative measure of the mutagenic potential and accounts for both the degree of contamination in a specific sample, as well as the potency of contaminants in the sample.

Genotoxicity of chemicals in the extracts of samples 003 and 005 were also measured in the *Escherichia coli* prophage induction assay [16, 17]. Tester strains *E. coli* lysogen strain $WP2_s(\lambda)$ (lon_{11}, $sulA_1$, $trpE_{65}$, $uvrA_{155}$, $lamB^+$), and indicator strain TH008 (streptomycin^r) were provided by D.M. DeMarini (US EPA, Research Triangle Park, NC). The assay was conducted in two parts: the microsuspension assay and the plaque formation assay. The microsuspension assay consisted of overnight exposure at 37 °C of mid-log phase $WP2_s(\lambda)$ ($\simeq 2 \times 10^6$ cells) to residues resuspended in acetone. The residues were tested with and without metabolic activation (2.5% S9 mix) in 96-well microtiter plates. The S9 mix components were the same as that described for the *Salmonella* assay; a 1 ml volume of 2.5% S9 mix contained 0.025 ml S9 fraction and 0.975 ml cofactor supplement. The S9 fraction-to-extract mass ratio

for S9 mix of 30% in the *Salmonella* test and 2.5% in the *E. coli* prophage induction assay was the same. Positive direct-action (2-nitrofluorene (2NF): 0.0065–0.21 g/l), positive indirect-action (benzo(a)-pyrene (BAP): 0.0039–0.13 g/l), and negative solvent (acetone) controls were tested with the extracts to ensure consistency in *E. coli* DNA damage induced and S9 mix activity.

The plaque formation assay monitored *E. coli* TH008 plaque formation by phage induction. From the incubated mictotiter plates of the microsuspension assay, five dose levels for testing were determined as the concentrations in the first five turbid wells (indicating cell growth) adjacent to a clear well (indicating cytotoxicity). Phage solution in each of the five turbid wells was diluted 1:10 in 5 ml supplemented minimal medium. 100 μl of the diluted phage was vortexed with approximately 4×10^7 TH008 cells in soft agar, which was poured onto streptomycin/tryptone agar plates. Dose levels ranged from 0.026 to 0.83 g/ml. The extract residues were tested in duplicate in at least two independent experiments.

Plaque forming units (PFUs) were counted manually. The criterion for a positive response was at least a threefold increase of induced PFU/plate caused by the test compound over that induced by the acetone control at two or more doses. If a test compound exhibited a threefold increase over that induced by the acetone control at only one dose, the result for that compound in the experiment was scored as a weak positive. A summary response was given to each tested extract based on the reproducibility of the results from two independent experiments. If a given response was not reproducible, the response summary was scored as questionable. Routine strain maintenance included monthly testing of tryptophan requirements, sensitivity to ultraviolet light ($WP2_s(\lambda)$), and streptomycin resistance (TH008).

3. Results and discussion

The risk associated with exposure to the two contaminated soils was estimated using chemical- and bioassay-based procedures. The results of the chemical analysis of the hexane:acetone extracts of samples 003 and 005 are presented in Table 1. In general, the polycyclic aromatic hydrocarbon (PAH) concentration in sample 005 was from 5 to 10 times greater than that detected in sample 003. The benzo(a)pyrene (BAP) content of sample 005 was 1233 mg/kg, while 125 mg/kg of BAP was detected in sample 003. The concentrations of polychlorinated dibenzofurans (PCDFs) and dibenzo-*p*-dioxins (PCDDs) were generally higher in sample 003 than in sample 005 (Table 1). The total tetrachlorodibenzo-*p*-dioxin (TCDD) content in sample 003 was 1418 pg/g, while TCDD levels in sample 005 were below analytical detection limits. Total octachlorodibenzo-*p*-dioxin (OCDD) contents in samples 003 and 005 were 198016 pg/g and 1696 pg/g, respectively. Thus, the sample of tar (003) had generally higher levels of PCDDs and lower levels of PAHs than did the sample of contaminated sediment (005).

The second procedure utilized to estimate risk associated with the two samples was a microbial mutagenicity assay. The mutagenic potential of the methylene chloride and methanol extract of the two samples was measured using *S. typhimurium* strain

Table 1
Chemical characteristics of samples from a wood-preserving facility

Chemical	Sample 003	Sample 005
	Sample concentration (mg/kg)[a]	
Benzanthracene	318	3631
Chrysene	290	3435
Benzo(b)fluoranthene	132	1533
Beno(k)fluoranthene	119	1383
Benzo(a)pyrene	125	1233
Benzo(e)pyrene	100	1026
Dibenz(a,h)anthracene	4	26
2,3,7,8-TCDF	113	BD
2,3,7,8-TCDD	0	0
Total TCDF	216	2
Total TCDD	1418	BD
Total HxCDF	2236	645
Total HxCDD	BD	74
OCDF	1101	17
OCDD	198016	1696

[a] Sample concentration in mg/kg for PAHs and pg/g for PCDDs and PCDFs; BD = below analytical detection limits.

TA98 with and without metabolic activation. PAHs generally require some form of mammalian metabolism in order to reach their ultimate carcinogenic form. In the absence of metabolic activation, neither sample induced a positive mutagenic response. With metabolic activation, the methylene chloride extract of sample 003 induced 117 net revertants at a dose of 1 mg/plate, while the methylene chloride extract of sample 005 induced 248 net revertants per mg (Table 2). The methanol extracts typically represent the more polar and water soluble components of the sample. The methanol extract of sample 003 induced 197 net revertants at a dose of 1 mg/plate. The maximum mutagenic response observed for the two samples was induced by the methanol extract of sample 005 which induced 437 net revertants per mg (Table 2). Using *Salmonella*, higher levels of mutagenicity were detected in the extracts of sample 005 than were detected in the extracts of sample 003. The results obtained using the *E. coli* assay were less conclusive. Responses at all doses were comparable, with sample 003 inducing a 3.3-fold increase at a dose of 0.78 mg/plate, and sample 005 inducing a 3.7-fold increase at the same dose.

A summary of the risk characterizations prepared using chemical analysis, two microbial mutagenicity assays, and a mouse skin painting study is presented in Table 3. The data for the skin painting study were generated in another laboratory [18] and is presented in this manuscript for comparative purposes only. Using the *Salmonella* assay, risk was estimated qualitatively and quantitatively. The specific activity provides a qualitative indication of the mutagenic potential of a complex mixture.

Table 2
Mutagenic potential of various extracts of wood-preserving waste samples as measured in *S. typhimurium* strain TA98 with and without metabolic activation

Sample number	Dose (mg/ml)	Net TA98 His^+ revertants			
		$MeCl_2$		MeOH	
		− S9	+ S9	− S9	+ S9
003	1	33 ± 4	84 ± 5	31 ± 13	141 ± 18
	2	19 ± 6	106 ± 20	31 ± 4	179 ± 36
	5	31 ± 12	108 ± 20	28 ± 10	155 ± 24
	10	24 ± 8	140 ± 7	29 ± 10	235 ± 9
	20	32 ± 6	150 ± 22	15 ± 7	230 ± 41
005					
	1	32 ± 6	134 ± 21	23 ± 4	202 ± 52
	2	37 ± 14	170 ± 48	20 ± 5	258 ± 22
	5	36 ± 10	229 ± 35	18 ± 8	151 ± 33
	10	42 ± 14	288 ± 15	25 ± 13	380 ± 112
	20	46 ± 7	281 ± 45	10 ± 5	470 ± 125
	0	30 ± 6	33 ± 10	30 ± 16	33 ± 10
Controls[a]					
2NF		1278 ± 290	ND	1278 ± 290	ND
BAP		ND	420 ± 116	ND	420 ± 116
DMSO		29 ± 6	34 ± 7	29 ± 6	34 ± 7

[a] Controls: 2NF = 25 μg 2-nitrofluorene; BAP = 10 μg benzo(a)pyrene; DMSO = dimethylsulfoxide; ND = not determined.

The specific activity of both samples was lower than that for the benzo(a)pyrene control, with sample 005 inducing a maximum specific activity that was approximately twice that observed for sample 003. The weighted activity accounts for the amount of organic material extracted from a sample and provides a more quantitative estimate of mutagenic potential. Sample 003 induced a weighted activity of 14 223 revertants per gram and sample 005 induced a weighted activity of 23 518 revertants per gram. The maximum weighted activity detected in a background soil from this facility was 229 revertants per gram. These data indicate that the weighted activities of the two on-site samples were more than 60 times greater than the maximum weighted activity observed in a background soil.

Further sample analysis was conducted utilizing the *E. coli* prophage induction assay. DeMarini et al. [17] observed this test to be more sensitive to chlorinated phenols than the *Salmonella* assay. Although both samples induced a positive response (as indicated by a doubling of induced prophage) in the assay, there was not an appreciable difference in the response induced by the hexane: acetone extracts of the two samples (Table 3). The third bioassay used to characterize risk was a mouse skin painting study. Data reported by Randerath et al. [18] indicate that the normalized relative DNA adduct level observed for sample 003 was 316E + 9 per 10 mg of

Table 3
Summary of risk characterization for extracts of samples from a wood-preserving facility

Characteristic[a]	Sample number	
	003	005
BAP (mg/g)	125	1233
2,3,7,8-TCDD eq. (pg/g)	0.44	0.003
Specific activity	197	436
Weighted activity	14223	23518
Normalized RAL (skin)	316	129
Prophage induction (FI)	3.3	3.7
ICR (PAHs)	1.5E − 3	1.4E − 2
ICR (PCDFs + PCDDs)	3.8E − 2	2.6E − 4

[a] Characteristics: BAP = benzo(a)pyrene concentration in mg/g; 2,3,7,8-TCDD eq. = equivalent weight of all PCDD and PCDF congeners based on toxic equivalency factors described by Safe [19]; specific activity = net TA98 revertants/mg; weighted activity (revertants/g) = specific activity (revertants/g) times residue weight (mg/g); normalized RAL = relative adduct levels ($\times 10^9$) normalized for 10 mg of sample (hexane:acetone extract); prophage induction (FI) = fold increase in *E. coli* prophage induction assay (values greater than 3.0 indicate positive response); ICR (PAHs) = total incremental carcinogenic risk (estimated for PAHs using EPA (1989) methods); ICR (PCDFs + PCDDs) = incremental carcinogenic risk estimated for PCDDs and PCDFs. Risk estimation assumed the ingestion of 100 mg of soil/d for 350 d/yr for 30 yr by a 70 kg worker.

sample, while a normalized RAL of 129E + 9 was observed for sample 005. Based solely on chemical analysis, elevated risk levels were estimated for both samples. Based on PAH content, sample 003 incurred an incremental carcinogenic risk (ICR) of 1.5E − 3 (or 1500 excess cancers in a population of one million), while sample 005 had an estimated risk of 1.4E − 2 (or 14000 excess cancers in a population of one million). Risk estimates based on PCDD and PCDF content indicate that sample 003 had an ICR of 3.8E − 2, while sample 005 had an ICR of 2.6E − 4.

The *Salmonella* assay is sensitive of PAH carcinogens and relatively insensitive to halogenated chemicals. The data obtained in the present study indicate that *Salmonella* appears to be a good indicator of potential carcinogenic risk associated with complex mixtures of PAHs, whereas it exhibited an inverse correlation between weighted activity and the risk estimated for PCDDs and PCDFs. The most accurate estimate of risk should have been obtained from the mouse skin painting study. The normalized adduct levels induced by sample 003 were approximately three times greater than levels induced by sample 005, while the estimated risk for chlorinated compounds suggested a difference of more than two orders of magnitude. A review of the data suggests that the chlorinated chemicals in sample 003 induced metabolism in the mouse with a resultant increase in PAH-DNA adduct formation. The risk characterization based on PCDD and PCDF content may have overestimated risk because of interactions in the complex mixture. The results of a risk assessment may be used to rank sites or to establish acceptable levels for site remediation. The results

of the present study indicate that both of the areas sampled require remediation. The *Salmonella* bioassay and PAH risk characterization rank sample 005 as the greatest hazard, whereas the DNA-adduct assay and PCDD risk characterization rank sample 003 as the greatest hazard. The results of both chemical-based and biological risk characterizations are in agreement that contaminant levels must be reduced by several orders of magnitude to be within acceptable levels. Based on the anticipated uncertainty associated with any risk assessment, it is unlikely that there is a significant difference in the risk associated with these two samples.

4. Conclusions

These results do indicate that there are limitations to risk assessment based on either biological or chemical test methods. Chemical analysis cannot define the toxicity of a contaminant, nor can it interpret the interactions of the components of a complex mixture. In vitro biological testing is unable to provide a model to measure all possible toxicological endpoints, nor can bioassays account for metabolism, distribution and excretion of a toxic chemical. Chemical analysis is an important tool for identifying specific contaminants and for a preliminary site assessment. Biological analysis however, provides a tool to rank site toxicity and to compare the effectiveness of remedial techniques. In addition, bioassays measure the toxicity and interactions of all the components of a complex mixture. The data suggest that a more accurate risk characterization can be obtained when using a combined testing protocol with both chemical and biological test methods.

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