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Environmental Protection Technology Series

EVALUATION OF SELECTED METHODS FOR CHEMICAL AND BIOLOGICAL TESTING OF INDUSTRIAL PARTICULATE EMISSIONS

Industrial Environmental Research Laboratory
Office of Research and Development
U.S. Environmental Protection Agency
Research Triangle Park, North Carolina 27711

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EVALUATION OF SELECTED METHODS FOR CHEMICAL AND BIOLOGICAL TESTING OF INDUSTRIAL PARTICULATE EMISSIONS

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ABSTRACT

The report gives results of chemical analyses and cellular biological assays performed on size-classified particulate material collected at nine industrial sites using a new series cyclone sampling train. The excercise was formulated to determine the performance of the train and whether the chemical analyses or the bioassays, alone or in combination, were sufficient to characterize the hazards associated with particulate emissions. This program lends support to the view that size-classified particulate matter is needed for the various chemical or biological tests. Elemental analysis and partial organic characterization of the particulate samples have been performed. A cellular bioassay, utilizing rabbit alveolar macrophages, has been used to estimate the toxic potential of particulate samples in terms of their observed acute cytotoxic activity. A bacterial screening technique, utilizing several histidine deficient Salmonella typhimurium strains, has been used to study the mutagenic potential of the particulate samples. No strong correlation was observed between the chemicals analysis and biological activity of the samples.

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The MITRE Corporation was assigned the role of coordinator in this project, and has been responsible for the collation, analysis, and interpretation of the results. The author thanks Mr. G. Erskine and Dr. N. Zimmerman for their assistance in this endeavor.

This work has been performed under Contract Number 68-02-1859 for the Process Measurements Branch, Industrial Environmental Research Laboratory, Environmental Protection Agency, Research Triangle Park, North Carolina 27711.

1.0 CONCLUSIONS

In order to determine a rapid, effective, and inexpensive means for evaluating the potential hazards associated with particulate emissions, two methods, chemical analysis and cellular bioassay, were carried out on size-classified particulate material collected at nine industrial sites. The experiment was designed to test whether such methods, alone or in combination, were capable of indicating potential hazards associated with particulate emissions.

The rabbit alveolar macrophage (RAM) cytotoxicity bioassay and a mutagenicity screening test, using three Salmonella typhimurium bacterial tester strains, were utilized to predict the acute toxicity and mutagenic behavior of the particulate samples. Partial organic characterization, with emphasis on polycyclic hydrocarbons of known carcinogenic potential, and inorganic elemental analysis were performed on the same size-classified particulate samples. Several conclusions can be drawn from the research as conducted:

(1) the series cyclone sampling train developed for this study has been shown to be a useful tool in collecting, within a one-to-five hour sampling period, sufficient quantities of size classified particulate material from a variety of industrial sources to permit further chemical and biological testing. Furthermore, the need for size-classified particulate material has been demonstrated, since different size particles collected at the same industrial source do not necessarily possess similar chemical and biological characteristics.

(2) the rabbit alveolar macrophage (RAM) cytotoxicity bioassay can provide a consistent, ordinal ranking of particulate samples, based on their acute cellular toxicity.

(3) the mutagenic screening test is capable of indicating that some of the industrial particulate samples possess positive mutagenic activity.

(4) no strong correlation has been established between the chemical characteristics of the particulate samples (elemental and partial organic characterization) and their observed biological activities (acute cytotoxic and mutagenic behavior).

The chemical analyses have provided comparable results, both from independent laboratory analysis as well as independent testing methods. The expense and sophistication of each technique reflects the intent of the screening program. The RAM cytotoxicity bioassay is capable of providing a consistent, ordinal ranking of particulate samples based on their acute cellular toxicity. If the desired ranking should reflect proportional differences in observed cytotoxicities, then the current RAM testing protocol must be enlarged to include more definitive concentration--response information. For more intensively studied priority streams, additional RAM response parameters (e.g., functional impairment, membrane integrity) will supplement the currently used index of cell viability (dye exclusion).

The mutagenic screening test has indicated that several particulate samples possess mutagenic activity, under the test conditions. It should be recognized that a positive mutagenic screening test using S. typhimurium is the first step in a battery of tests to evaluate the mutagenic hazard of the particular sample. Additional solvent vehicles and microbial tester strains can be added to the experimental protocol, as warranted.

The sensitivity and specificity of a testing program must be compatible with the test program's intent. If a large number of pollutant emissions are to be screened, so that the more hazardous ones can be identified and intensely studied, then the initial screening sequence need not be extremely sensitive or specific. Both bioassay procedures have indicated their potential utility in assessing the specific biological activities of industrial praticulate emissions.

2.0 INTRODUCTION

The Process Measurements Branch of the Industrial Environmental Research Laboratory (EPA/RTP)* is developing a phased sampling and analysis strategy that provides for the environmental source assessment of industrial and energy processes. In this phased approach, initial survey testing (Level 1) is used to evaluate the potential environmental hazards of pollutant or process streams through examination of their physical and chemical characteristics as well as their biological activity. Those streams identified as potentially hazardous will be subjected to more intensive testing procedures (Level 2 and 3) on a priority basis.

At all phases of the analytical program, the sophistication of each measurement technique is compatible with its companion techniques and with the quality of the sample to be assayed. The ultimate goal of an environmental source assessment is to insure that the waste streams from a given process are environmentally acceptable or that adequate technology exists for control.

This report presents several methods which have been used to characterize the hazards associated with particulate material emitted from industrial sources. Although there are alternative approaches and techniques which could be used to define environmentally hazardous streams, these selected methods were chosen to be both complimentary and cost-effective.

2.1 APPROACH

The development of a rapid, effective, and inexpensive means for evaluating the potential hazards associated with particulate emissions

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is an essential part of PMB's environmental source assessment program. To this end, two methods, chemical analysis and cellular bioassay, were carried out on size-classified particulate material collected at nine industrial sites. The experiment was designed to determine whether the chemical analyses or bioassay procedures, alone or in combination, could assess potential hazards associated with particulate material.

Chemical analysis, alone, cannot provide sufficient data for complete evaluation of pollutant emissions, because the biological activity of the samples cannot be consistently predicted. Interactive effects (e.g., synergism, antagonism) between chemical constituents are difficult to assess. The biological availability of a given constituent is likewise not easily predicted from chemical data alone. The presence or absence of given toxic components neither precludes nor indicates a relationship of the effluent to a suspected toxic effect. Chemical analysis should not be restricted to an *a priori* determination of known hazardous or toxic compounds. The possibility of overlooking unanticipated, biologically active materials must be avoided.

Bioassay techniques can be used effectively to assess the biological activity of particulate samples. Classical whole animal, *in vivo* bioassay methods, as well as cellular *in vitro* tests, have been developed to monitor the potential effect of pollutants on living systems. The advantages of cellular bioassay include its relatively low cost, small sample requirement, and short experimentation time; hence, its appeal for rapid evaluation of numerous, potentially hazardous compounds. Criticism of cellular bioassay suggests that unsuspected effects may be missed since the whole animal with potential target organs is not being considered. Aspects of this

problem can be overcome by judicious choice of cell types and critical selection of cytological and biochemical test parameters.

Whereas cellular bioassay takes into account the biological activity of a given sample, it cannot specify the compounds in a crude sample responsible for the observed effects. A cost-effective screening method could involve the use of cellular bioassay to determine which effluent samples are biologically active, together with the use of chemical fractionation and analysis to ascertain which agents are responsible for the observed effects.

The rabbit alveolar macrophage (RAM) cytotoxicity bioassay and mutagenicity screening (using three Salmonella typhimurium bacterial strains) were utilized to predict the acute toxicity and mutagenic behavior of size-classified particulate samples collected at nine industrial sites. Partial organic characterization, with emphasis on polycyclic hydrocarbons of known carcinogenic potential (or structurally similar compounds), and inorganic elemental analysis were performed on the same size-classified particulate samples. These techniques should not be construed to represent a Level 1 sampling and analysis strategy, but rather a composite of selected protocols from both Levels 1 and 2.

2.2 OBJECTIVES

The purpose of this research effort is to evaluate the effectiveness of selected testing methods in accomplishing the following objectives:

(1) To determine whether the sequential cyclone sampling train can provide sufficient size-classified samples for chemical and biological tests, and whether this classification is useful;

(2) To determine whether the RAM bioassay can provide a reliable estimate of the acute cellular toxicity of particulate samples;

(3) To determine whether the mutagenic screening test is capable of indicating a positive mutagenic response to the particulate samples;

(4) To determine whether the chemical analyses can be correlated to the observed biological activity of the samples.

3.0 SAMPLE COLLECTION

3.1 FABRICATION AND CALIBRATION OF SAMPLING TRAIN

A series cyclone sampling train was developed to provide the capability to collect sufficient quantities of size-classified particulate material ($\geq$300mg per size range) from a variety of industrial sources so that subsequent chemical and biological characterization of the sample could be achieved. The cyclone train design called for collection of samples according to particle aerodynamic diameter, in ranges of >10μ, 3-10μ, 1-3μ, and <1μ (by filter). The sampling train was intended to operate continuously or intermittently over a period of five hours to acquire a one-day integrated sample representative of the process.

TRW Systems Group* fabricated the sampling train according to drawings provided by Southern Research Institute.** Since particulate samples were to be collected for biological testing, construction materials were selected for their nontoxic qualities. Assessment of toxicity was based upon information generated by TRW on the NASA-sponsored Viking Biology Lander Instrument Program (VBLI).[1] Stainless steel CRES 316 was used exclusively for the cyclones, tubing, and fittings; Viton "O" rings were used as seals. The filter material used was a Teflon needle felt material pretested and found acceptable for cytotoxicological purposes. The field sampling configuration of the series cyclone train is presented in Figure 1.

*One Space Park, Redondo Beach, CA 90278.
**2000 Ninth Avenue, South Birmingham, AL 35205.

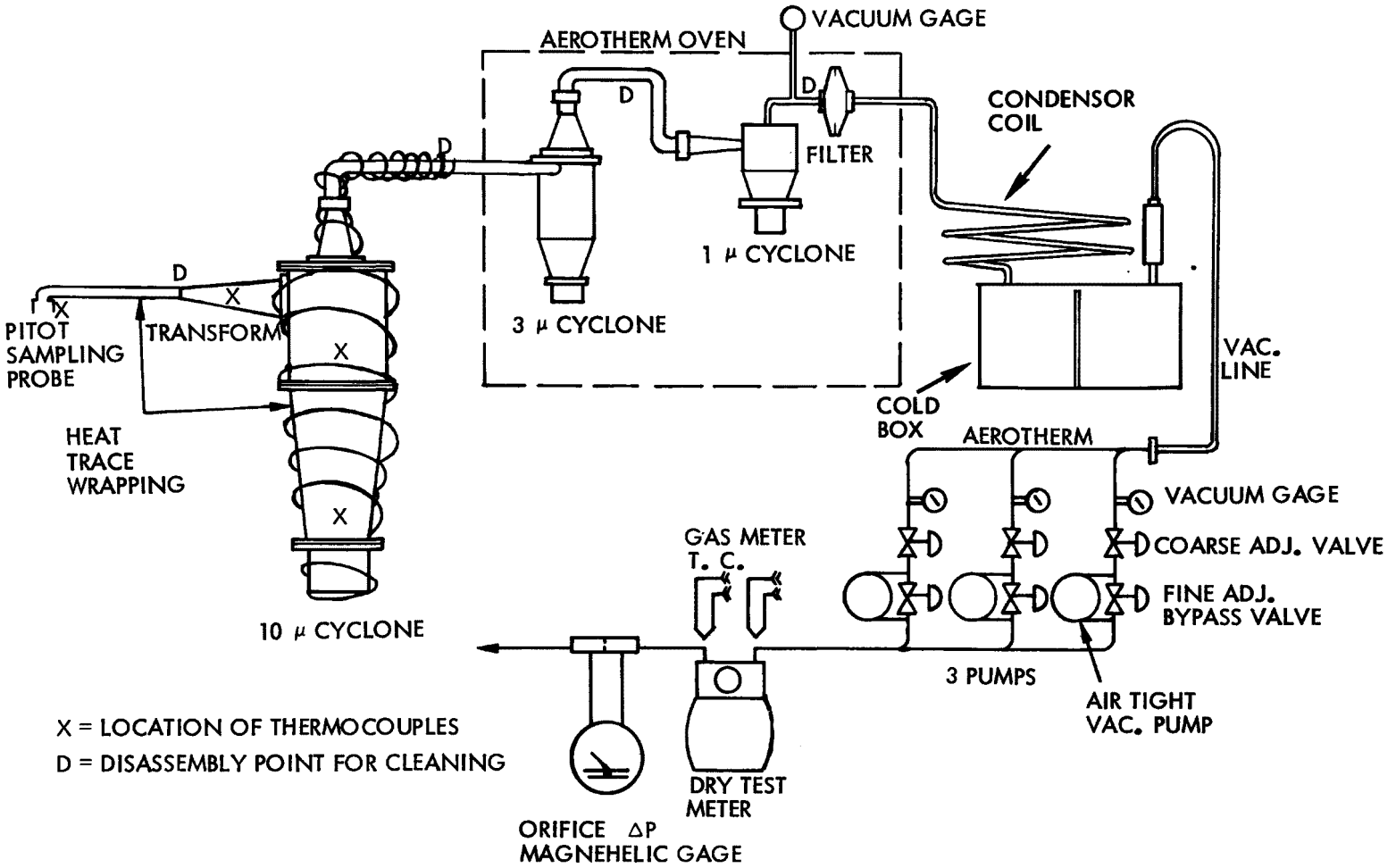

FIGURE 1
SERIES CYCLONE TRAIN, FIELD SAMPLING CONFIGURATION

Due to time constaints, TRW Systems Group was only able to calibrate the sampling train as follows:

1) generate a nebularized quantity of polydispersed talc and pull it through the train;
2) measure the amount of talc collected in each cyclone;
3) assume an "S"-shaped efficiency curve for each cyclone with corresponding unspecified constants; and
4) determine the efficiency curve constants which produce the cyclone residues from the original samples of known size distribution.

The design criteria of the sampling train were best met when the flow rate through the train was 3 scfm. The computed cyclone cut-off diameters (D_{50}s) were 9.5μ, 2.0μ, and 0.5μ for the three cyclones, respectively.

The sampling train, as prepared by TRW Systems Group, should not be considered optimal since the intent was to provide a fail-safe sampling system capable of collecting a variety of particulate samples under a variety of operating conditions. In its reports,[2,3] TRW Systems Group urges that the train be redesigned and recalibrated to minimize weight, to reduce the number of components, to reduce the amount of sample wall-loss, and to identify more precisely the minimum critical flow that gives acceptable size classifications. In addition, the use of a more suitable filter material was advised, since the type used in this study was subject to thermal degradation during sampling train operation.

3.2 SAMPLING SUPPORT PROCEDURES

Since particulate material collected would undergo biological testing and chemical analysis, an intensive effort was expended during field

operations to insure sample integrity. All equipment that would come into contact with the particulate samples was cleaned, inspected, and packaged according to procedures demonstrated under the VBLI Program.[1] To avoid unnecessary field cleaning, appropriate components of the sampling train, storage containers, and transfer equipment were pre-cleaned and packaged in the laboratory clean room which met Class 10,000 requirements of Federal Standard No. 209.[4] Nylon drapes were used to isolate the sample containers (pre-weighed Nalgene) from the rest of the environment during field transfer of the sample material.

Upon return to the TRW facilities, the samples were transferred to pre-cleaned, tared, high density polyethylene storage bottles. All transfers were performed in a Class 1000 laminar flow bench[4] to prevent particulate contamination from the surrounding environment. This transfer technique was also followed for sample disbursement. When necessary, only polyethylene utensils were allowed to touch the sample. All samples were stored in a limited access safe situated in a suitably controlled environment.

3.3 FIELD SAMPLING

Industrial sites sampled were selected to provide particulate material possessing a variety of physical and chemical characteristics, as well as an anticipated range of cellular toxicity. In order to evaluate the cyclone train, a wide range of sampling conditions under which the cyclone train would operate was chosen. Table 1 provides the sampling logistics at the ten industrial sites.* The sampling

*Sample collected at one industrial site was insufficient for further analysis.

TABLE 1

LOGISTICS OF SAMPLE COLLECTION

Source	Open Hearth Furnace	Coke Oven Heater	Basic Oxygen Furnace	Iron Sintering Plant	Oil Fired Power Plant	Clay Aggregate Plant	Copper Smelter	Aluminum Smelter	Minicipal Sludge Incinerator	Kraft Mill Process
Sampling location	Electrostatic precipitator (4 duct diameters downstream from ESP, 4 duct diameters upstream of stack)	Base of stack	Downstream of ESP, downstream of induction fan, upstream of stack	Inlet to baghouse	Wet scrubber inlet	Between primary and secondary cyclones	Outlet of roaster reverberator inlet to baghouse	Inlet to baghouse	Duct between furnace and water quench	Stack effluent from ESP
T^{o} stack*	350^{o}-425^{o}	400^{o}	150-225^{o}	400^{o}	170^{o}	510^{o}	250^{o}	210^{o}	1100^{o}	335^{o}
T^{o} 10μ cyclone*	350^{o}	380^{o}	270^{o}	350^{o}	195^{o}	400^{o}	275^{o}	300^{o}	410^{o}	335^{o}
T^{o} oven*	350^{o}-400^{o}	380^{o}-400^{o}	270^{o}	390^{o}	250^{o}	400^{o}	300^{o}	300^{o}	380^{o}	350^{o}
Flow rate through sampling train	4.8 scfm	3.5 scfm	4.6 scfm	5 scfm	4.5 scfm	3.8 scfm	4.7 scfm	5 scfm	4.7 scfm	4.8 scfm
Total sampling time	5 hours continuous	5 hours continuous	5 hours continuous	2 hours intermittent over 5 hour period	2 hours continuous	1.25 hours intermittent over 5 hour period	1 hour continuous	2 hours intermittent over 5 hour period	5 hours continuous	5 hours continuous

*Degrees fahrenheit

locations at the industrial sites were not consistent with respect to control devices and the particulate material collected should not be construed to represent the actual emissions of that particular site.

The specific sampling location at each industrial facility was selected to provide a representative sample of the effluent stream. Total sampling time was limited to a maximum of five hours; in those instances where particulate grain loading was high, intermittent sampling periods over the five-hour interval were utilized. Site characteristics, as well as more specific sampling locations are presented in the following sections.

3.3.1 Steel Mill, Open Hearth Furnace

The open hearth furnace system sampled in this study was of the oxygen-lanced variety with a production capability of 225 tons per heat. The sampling port was located approximately four duct diameters downstream from the electrostatic precipitators and about four duct diameters upstream from the entrance to the base of the stack. The sampling port itself was located sixty feet above ground level. Sampling time was five hours, continuous.

3.3.2 Steel Mill, Basic Oxygen Furnace

The basic oxygen furnace sampled in this study has a production capability of 109 tons per heat. The sampling port was located downstream from the electrostatic precipitator and induction fan, upstream from the stack, and about six feet above the site floor. Total elapsed sampling time was five hours.

3.3.3 Steel Mill, Coke Oven Heater

The coke oven operation sampled in this study consisted of a number of coke oven batteries, each battery containing forty-five ovens. The particulate sample was collected at the base of a stack serving one of the batteries. Effluent from this stack is representative of the combustion of the by-product coking gas used to heat the coke ovens. The by-product coking gases, having been passed through electrostatic precipitators, cooled and compressed, are burned in the fire boxes to heat the ovens. The sampled stream is representative of only a minor portion of the coking process. The particulate samples were obtained over a five-hour period.

3.3.4 Steel Mill, Iron Sintering Plant

The sintering process sampled in this study employed two Dwight Lloyd sintering machines with combined capacity of 850 tons per day. The sampling site was located upstream from the baghouse entrance. The gas stream itself was generated by the sintering process. The particulate samples were obtained during two hours of intermittent sampling over a five-hour period.

3.3.5 Oil-Fired Power Plant

The oil-fired power plant sampled for this study was a horizontally fired unit, fired at right angles to the walls of the rectangular firebox. The sampling port was located on the inlet duct to the low-energy wet scrubber. High-sulfur fuel oil was being combusted during sampling. The sample was collected continuously over a two-hour period. A power plant shutdown precluded a longer sampling period.

3.3.6 Copper Smelter

Particulate samples were collected from the side of a tear-shaped horizontal duct issuing from the roaster/reverberator process upstream from the baghouse. The sampling effort experienced extremely heavy grain loading; the sampling probe became clogged after one hour of operation, at which time the sampling effort was terminated. Sampling at the copper smelter was the only instance of a high grain load process that did not utilize an intermittent sampling schedule to obtain an integrated sample over a five-hour period.

3.3.7 Aluminum Smelter

The duct sampled at the aluminum smelting complex contained effluent resulting from the electrolytic process. The sampling probe was inserted into the horizontal effluent duct upstream from the baghouse. Since high grain loading conditions existed, fifteen-minute sampling intervals, once every hour for five hours, provided the representative sample.

3.3.8 Paper Mill, Kraft Pulping

Sample collection occurred in the recovery furnace effluent stream, downstream from the electrostatic precipitator. Accumulated sample mass after five hours of operation was negligible (0.008g), with approximately ninety percent being deposited in the 1-3 micron cyclone.

3.3.9 Ceramics Plant, Clay Aggregate Production

Particulate effluent from a ceramic aggregate production plant rotary kiln furnace was collected. The sampling probe was situated between the primary and secondary cyclones approximately fifteen feet above ground level in the side wall of a vertical circular duct. Since heavy grain loading conditions existed, fifteen minute sampling intervals, once every hour for five hours, provided the sample.

3.3.10 Municipal Waste Water Sludge Incinerator

The sludge incineration system sampled in this study utilized a three-stage spiral design, where sludge is injected at the top and is directed downward through successive stages of incineration, until the final ash product is removed at the bottom. Due to extreme temperatures, direct sampling of the incinerator was not feasible. A water-cooled sampling probe was inserted on the pre-cooler portion of the incinerator outlet. Sampling time was five hours, continuous.

4.0 SAMPLE ANALYSIS

The size-classified particulate material collected at the industrial sites was subjected to three chemical analyses as well as two cellular bioassays. Particulate samples collected in the largest cyclone (>10μ) were not analyzed because it was felt that these particles would settle out of the atmosphere in a short period of time and thus, represented a minor air pollution hazard. Table 2 provides the disbursement schedule for the samples collected. All chemical and biological tests were not run on every sample collected, and Table 2 must be consulted for the actual tests conducted on a particular sample. Analytical methodologies for those tests follow.

4.1 CHEMICAL ANALYSIS

Three types of chemical analysis were performed on the size-classified particulate material. Elemental composition of the samples was determined by spark source mass spectrometry. Partial organic characterization, emphasizing polycyclic hydrocarbons, was obtained by gas chromatography-mass spectrometry and high resolution mass spectrometry.

4.1.1 Spark Source Mass Spectrometry

Spark source mass spectrometry (SSMS) was performed on the industrial particulate samples by Accu-Labs Research.* Assays were performed

*11485 W. 85th Avenue, Wheat Ridge, CO 80033

TABLE 2
SAMPLE DISBURSEMENT

SOURCE	STEEL PLANT Open Hearth Furnace			STEEL PLANT Coke Oven Heater			STEEL PLANT Basic Oxygen Furnace			STEEL PLANT Iron Sintering			OIL FIRED POWER PLANT		
Sample Size (μ)	1-3	3-10	F[a]	1-3	3-10	F	1-3	3-10	F	1-3	3-10	F	1-3	3-10	F
TOTAL SAMPLE COLLECTED (mg)	1,718.6	243.2		297.9	14.5		1,254.5	116.2		1,054.4	1,774.7		90.6	0.4	
Spark Source Mass Spectrometry	X	X		X			X	X		X	X		X		
Gas Chromatography-Mass Spectrometry	X	X	X	X		X	X	X	X	X	X	X			X
High Resolution Mass Spectrometry	X	X		X			X			X	X				
Cytotoxicity Bioassay	X	X	X	X	X	X	X	X	X	X	X	X	X		X
Mutagenicity Bioassay	X						X				X				

SOURCE	CERAMICS PLANT Clay Aggregate Rotating Kiln			COPPER SMELTER			ALUMINUM SMELTER			WASTE WATER TREATMENT PLANT Sludge Incinerator		
Sample Size (μ)	1-3	3-10	F	1-3	3-10	F	1-3	3-10	F	1-3	3-10	F
TOTAL SAMPLE COLLECTED (mg)	4,221.4	12,788.4		598.9	2,193.6		983.3	1,613.6		1,613.5	13,357.3	
Spark Source Mass Spectrometry	X	X		X	X		X	X		X	X	
Gas Chromatography-Mass Spectrometry	X	X	X	X	X	X	X	X	X	X	X	X
High Resolution Mass Spectrometry	X	X		X	X		X	X		X	X	
Cytotoxicity Bioassay	X	X	X	X	X	X	X	X	X	X	X	X
Mutagenicity Bioassay	X	X		X	X		X	X		X	X	

[a] F - filter (>1μ)

Note: In addition, a pulp and paper mill was sampled; unfortunately, the sample mass collected was to small for any subsequent analysis to be performed.

for seventy-five different elements. The SSMS technique provided a lower detection limit of 0.1 ppmw* for each element. Additional semi-quantitative scans (geo-scans) were run to identify concentrations of those elements present in quantities greater than one percent by weight. No repetitive analyses of individual particulate samples were performed. The quantitative SSMS results are estimated to be accurate within two hundred percent while the geo-scan's accuracy is within approximately five hundred percent.

4.1.2 Gas Chromatography-Mass Spectrometry

Gas chromatography-mass spectrometry (GC-MS) analysis of twenty-four particulate samples was performed by Battelle Columbus Laboratories.** The analysis focused on polycyclic hydrocarbons of known carcinogenic potential or structurally similar compounds. Each sample was subjected to ultrasonic extraction with methylene chloride at 50° C, and the resulting solution was recovered by centrifugation. Internal standards were added before the volume of the solution was reduced to 200 µl; the added standards were 9-methylanthracene, 9-phenylanthracene, and 9,10-diphenylanthracene. All samples were analyzed using GC-MS with quantification by specific absolute ion current integration. The detection limit for individual polycyclic organic species was slightly less than 10 ng. No repetitive analyses of individual particulate samples were performed.

4.1.3 High Resolution Mass Spectrometry

High resolution mass spectrometry was performed by a United States Energy Research and Development Administration facility (ERDA/PERC)

*parts per million by weight
**505 King Avenue, Columbus, OH 43201

in Pittsburgh, Pennsylvania*. High resolution mass spectrometry has the capability of determining the precise mass of various polynuclear hydrocarbons from which the chemical formula can be derived, but isomeric identification cannot be performed by HRMS alone. High resolution mass spectrometry can be used for the preliminary screening of complex mixtures for the possible presence of several hundred hazardous and/or toxic compounds.

The particulate samples were vaporized** and the components observed in the mass spectra. High resolution mass spectra were recorded on photographic plates, and the data processed by computer.

Semi-quantitative mass spectral analyses of two particulate samples were obtained by successive scans over the period of time during which the sample yielded vaporization products. Mass spectra from all fourteen particulate samples analyzed were screened by computer for nine precise masses that would indicate the possible presence of carcinogenic polynuclear aromatic hydrocarbons.

4.2 BIOLOGICAL CHARACTERIZATION

In this study, two <u>in vitro</u> bioassays were utilized to determine the acute toxicity and the mutagenic potential of the 3-10μ, 1-3μ and >1μ particulate samples from industrial sources. The rabbit alveolar macrophage (RAM) has been used to determine the potential acute cytotoxicity of the samples. The alveolar macrophage exists as a pulmonary free cell and provides an early line of defense against

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**@300°C, 10^{-6} torr

inhaled foreign bodies. Because of its phagocytic activity, it is particularly useful in the toxicologic evaluation of airborne particulate matter. The mutagenic bioassay utilizes several bacterial indicator strains (histidine deficient Salmonella typhimurium strains TA-1535, TA-1537, and TA-1538), with reversion to prototrophy indicative of mutation. Both bioassays have indicated their utility in studying the effects of certain pure compounds, but neither has been used extensively on complex mixtures.

4.2.1 Cytotoxicity Evaluation

Northrop Services, Inc.,* under contract with the Experimental Biology Laboratory** (EBL/RTP), performed the rabbit alveolar macrophage (RAM) cytotoxicity test, according to a modification of the procedure developed by Waters et al.[5,6] The RAM culture medium was added to pre-weighed particulate samples to achieve a desired final particulate concentration in the medium. The complete culture medium consisted of Medium 199 in Hanks' salts supplemented with 20 percent heat-inactivated fetal bovine serum, 100 units/ml penicillin-G, 100 µg/ml streptomycin sulfate and 100 µg/ml kanamycin. The particulate samples were incubated with continuous agitation on a rocking platform (12 oscillations per minute) for 20 hours in the culture medium (less serum but including antibiotics) to allow for dissolution of any soluble components of the particulate matter. Macrophage cells were then added (along with the bovine serum) to achieve a final concentration of approximately 5×10^5 cells/ml. The cultures were returned to the roller platform and incubated for 20 hours (@ 37° C in a humidified 5 percent CO_2 atmosphere). The culture medium containing

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**EBL, Environmental Protection Agency, Research Triangle Park, NC 27711

unattached cells was then poured off and retained. Cells remaining attached to the culture vessel were removed using trypsin and recombined with the original culture medium. Cell number was determined by direct count using a hemocytometer. Cell viability was estimated by light microscopy on the basis of trypan blue dye exclusion.

An initial cytotoxicity screening of the particulate samples was performed at a final particulate concentration of 1000 µg/ml of culture medium. Samples found in the initial screening to produce net cell death of greater than 15 percent, as compared with controls, were retested in a preliminary concentration-response test using particulate concentrations of 1000 µg/ml, 300 µg/ml, and 100 µg/ml of culture medium. The pH values of the cultures were monitored throughout, and if shifts below 6.8 or above 7.6 occurred, the sample was tested under both unadjusted and adjusted conditions.

In addition, an attempt was made to ascertain whether the toxicity of a given particulate sample was due to the particles themselves and/or soluble component(s) released into the medium. The particulate matter was incubated in the culture medium (less serum but including antibiotics) for 20 hours and then removed from the medium via centrifugation and filtration through a 0.22µ Millipore filter. The filtered supernatant and centrifuged particles (resuspended in fresh medium) were then independently tested for cytotoxicity. Figure 2 indicates the testing sequence used in this study.

The filters used to collect the sub-micron particles were cut into quarters, desiccated, weighed, and pre-incubated with sterile deionized water plus antibiotics for twenty hours. RAM cells were added to half of the pre-incubated filter samples (final concentrations: 5×10^5 cells/ml) after addition of the Medium 199 concentrate plus

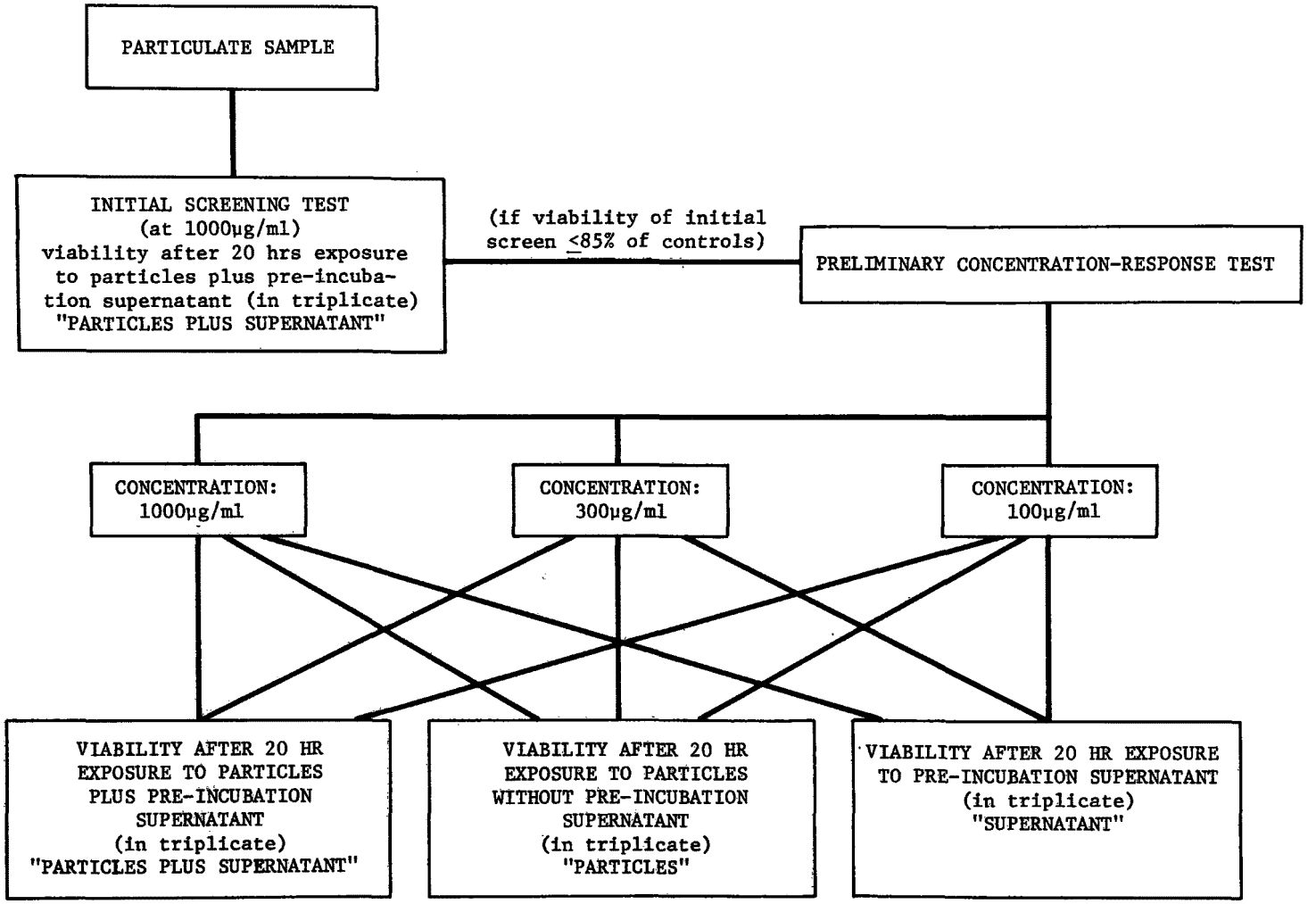

FIGURE 2
RABBIT ALVEOLAR MACROPHAGE CYTOTOXICITY SCREENING TEST PROCEDURE

serum to reconstitute the complete incubation medium described previously. The cultures were incubated with agitation for twenty hours, and the cell number and viability noted ("filter plus supernatant" fraction). The remainder of the pre-incubated filter samples were removed from the solution and RAM cells were added to the supernatant (final concentration: 5×10^5 cells/ml), incubated for twenty hours, and cell number and viability determined ("supernatant" fraction). The filters that were removed were dried, weighed, and pre-incubated with sterile deionized water plus antibiotics a second time. After addition of medium concentrate and serum, RAM cells were added (final concentration: 5×10^5 cells/ml), incubated for twenty hours, and cell number and viability noted ("dried filter" fraction).

4.2.2 Mutagenicity Evaluation

Litton-Bionetics, Inc.,* under sub-contract to Research Triangle Institute** performed the mutagenic screening tests according to a modification of the procedure developed by Ames *et al.*[7,8,9] Three histidine deficient *Salmonella typhimurium* strains (TA-1535, TA-1537, and TA-1538) were used, with reversion to prototrophy indicative of mutation. The TA-1535 strain is most likely to undergo base pair substitutions, and the TA-1537 and TA-1538 frameshift reverse mutations. All three strains have defective DNA excision repair systems as well as defective lipopolysaccharide coats, thereby increasing the sensitivity of the strains to observable mutational events.

All particulate samples were dissolved in a single solvent, dimethylsulfoxide (DMSO). Exposure of the bacterial populations to the particulate material, using DMSO as the vehicle, occurred on plates by the

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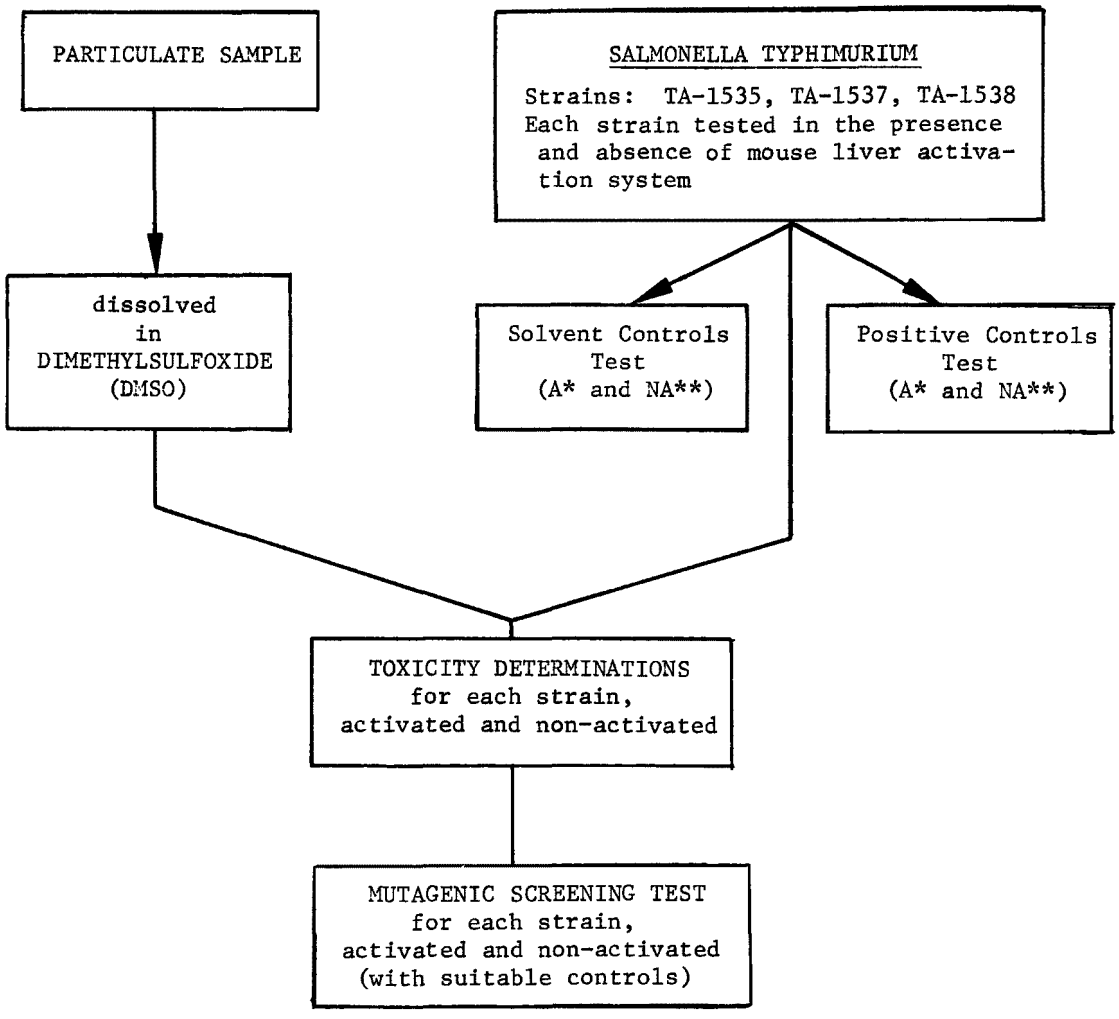
agar overlay method. In the event that the compound(s) might require metabolic activation in order to exhibit mutagenic activity, all bacterial tests were run in the presence and absence of a mouse liver activation system.[10] Positive control tests were run on the bacterial systems by exposing each to known active mutagens. Since DMSO was the solvent vehicle for each test, appropriate solvent controls were run. The toxicity of each particulate sample to the bacterial populations over a range of exposures was determined prior to the mutagenicity testing. The highest doses used in the mutagenic tests were restricted to those levels lethal to not more than twenty-five percent of the exposed population in the toxicity screening. This restriction minimized the potential for growth of non-mutant cells (phenocopies) utilizing histidine released from dead cells, while it allowed for a reasonable exposure level in order to detect only moderately mutagenic samples. The lowest mutagenic test dose was at least two orders of magnitude lower than a concentration which produced detectable toxicity. The experimental procedure used in the mutagenicity screening test is summarized in Figure 3.



*Activated
**Non-Activated

FIGURE 3
SALMONELLA TYPHIMURIUM MUTAGENICITY SCREENING TEST PROCEDURE

5.0 RESULTS

5.1 CHEMICAL ANALYSIS

The elemental composition of the industrial particulate samples, as determined by spark source mass spectrometry, is provided in Table 3. In addition to the Accu-Labs Research analyses, two particulate samples, the coke oven heater 1-3μ sample and the copper smelter 1-3μ sample, were analyzed for major and minor species by the Analytical Chemistry Branch of the Environmental Monitoring and Support Laboratory (EMSL/RTP).* All results are reported as parts per million by weight, except as noted. As evidenced in Table 3, the elemental concentrations of both particulate samples collected from the same site are not always similar (e.g., sodium in the aluminum smelter samples, beryllium and aluminum in the copper smelter samples, silver and uranium in the basic oxygen furnace samples.

Results of gas chromatographic-mass spectrometric analyses of certain samples are provided in Table 4. Polycyclic hydrocarbons are reported as parts per million by weight, except for filter analyses. Due to thermal degradation of the filter, the amount of particulate material deposited on any filter was undetermined, so filter analyses are reported as total nanograms detected. Three samples were not analyzed because of insufficient sample (i.e., coke oven heater 1-3μ sample, and the oil-fired power plant 1-3μ and 3-10μ samples). The GC-MS analysis focused on hydrocarbons of known carcinogenic potential, or of structurally similar compounds. When examining GC-MS and HRMS

*Environmental Protection Agency, Research Triangle Park, NC 27711

TABLE 3
ELEMENTAL ANALYSIS OF PARTICULATE SAMPLES AS DETERMINED BY SPARK SOURCE MASS SPECTROMETRY [a]

Element	Steel Plant Open Hearth Furnace, 3-10 Micron	Steel Plant Open Hearth Furnace, 1-3 Micron	Steel Plant Coke Oven Heater, 1-3 Micron	Steel Plant, Basic Oxygen Furnace 3-10 Micron	Steel Plant, Basic Oxygen Furnace 1-3 Micron	Steel Plant, Iron Sintering, 3-10 Micron	Steel Plant, Iron Sintering, 1-3 Micron	Oil Fired Power Plant, 1-3 Micron	Copper Smelting 1-3 Micron
Aluminum	68	140	5.3 (17)[†]	73	21	≈1300	890	340	250 (940)[†]
Antimony	27	70	3.7	17	9.7	2.5	3.5	7.8	8.0% (5.8%)
Arsenic	190	480	3.9 (200)	67	52	35	120	9.3	3.0% (21%)
Barium	6.3	12	1.0 (15)	68	40	60	140	280	170 (31)
Beryllium	<0.24	0.34	0.25	<0.26	<0.15	0.57	0.39	8.1	5.9
Bismuth	11	21	48 (135)	5.0	2.9	5.7	100	1.1	2.0% (5700)
Boron	47	31	3.3 (15)	130	50	61	76	16	11
Bromine	10	43	1.1	110	65	150	270	10	750 (52)
Cadmium	5.4	44	9.4 (131)	9.7	12	14	22	0.97	0.49% (3800)
Calcium	2.0%	0.83%	≈1100 (220)	4.0%	2.0%	40.0%	30.0%	1.0%	0.75% (800)
**Carbon	NR**	NR**	NR**	NR**	NR**	NR**	NR**	NR**	NR**
Cerium	1.2	1.1	0.10	5.9	0.73	38	46	14	3.9
Cesium	0.26	2.5	0.53	2.8	1.2	18	25	4.6	1.9 (60)
Chlorine	270	260	17	3.0%	3.0%	≈3300	1.0%	33	610 (135)
Chromium	880	60	94 (170)	220	170	76	52	≈1000	190 (153)
Cobalt	120	110	2.6 (11)	130	57	86	59	220	180 (64)
Copper	≈3600	≈2900	170 (190)	1100	800	≈1000	≈2200	300	5.0% (2.9%)
Dysprosium						1.8	1.3	0.31	
Erbium						0.53	0.37		
Europium						0.98	0.67	0.33	
Fluorine	790	760	220 (100)	5.0%	6.0%	1.0%	0.7%	31	210
Gadolinium						0.41	0.28	0.10	
Gallium	90	87	4.0	97	57	6.3	4.4	190	6.5 (18)
Germanium	7.1	18	0.98	10	6.0	2.9	9.2	4.7	140 (120)
Gold									25
Hafnium						0.63	0.44		1.3
Holmium						0.13	0.09		
*Indium	IS*	IS*	IS*	IS*	IS*	IS*	IS*	IS*	IS*
Iodine	1.7	3.9	1.8 (5.8)	19	2.6	29	69	0.61	160 (25)
Iridium									
Iron	40.0%	40.0%	>1% (4500)	>80%	40.0%	6.0%	2.0%	1.0%	3.0% (1%)
Lanthanum	0.21	0.20	0.03	2.2	0.28	7.3	10	0.52	4.1
Lead	900	≈1900	940 (1500)	170	210	≈2100	0.82%	450	35.0% (11.2%)
Lithium	59	110	6.1	27	16	18	28	13	43
Lutecium						0.06	0.24		
Magnesium	1.0%	1.0%	170	2.0%	≈2500	6.0%	4.0%	0.59%	2.0% (1.1%)
Manganese	0.52%	≈4400	250 (82)	1.0%	0.77%	640	321	220	310 (73)
Mercury	NR	NR	NR	NR	NR	NR	NR	NR	***
Molybdenum	35	79	17 (12)	38	10	11	17	814	0.51% (570)
Neodymium	0.68	0.65		0.73	0.18	9.5	6.5	0.68	9.8

[a] Reported as parts per million by weight, except as noted

*IS: Internal Standard

**Not Reported

***Mercury Observed

[†] ( ) Reported by independent laboratory (EMSL/RTP)

TABLE 3
ELEMENTAL ANALYSIS OF PARTICULATE SAMPLES AS DETERMINED BY SPARK SOURCE MASS SPECTROMETRY [a]
(CONTINUED)

Element	Steel Plant Open Hearth Furnace, 3-10 Micron	Steel Plant Open Hearth Furnace, 1-3 Micron	Steel Plant Coke Oven Heater, 1-3 Micron	Steel Plant, Basic Oxygen Furnace 3-10 Micron	Steel Plant, Basic Oxygen Furnace 1-3 Micron	Steel Plant, Iron Sintering, 3-10 Micron	Steel Plant, Iron Sintering, 1-3 Micron	Oil Fired Power Plant, 1-3 Micron	Copper Smelting 1-3 Micron
Nickel	490	470	51	93	46	26	64	5.2	1300
Niobium	0.93	0.9		2.3	2.7	6.5	4.5	0.22	31 (5.3)[†]
**Nitrogen	NR**	NR**	NR**	NR**	NR**	NR**	NR**	NR**	NR**
Osmium									
**Oxygen	NR**	NR**	NR**	NR**	NR**	NR**	NR**	NR**	NR**
Palladium									
Phosphorus	0.5%	≈4900	41 (82)[†]	≈3500	≈1200	≈1300	890	≈1600	≈1300 (180)
Platinum									
Potassium	0.69%	3.0%	≈1700 (1500)	4.0%	2.0%	1.0%	3.0%	≈3500	10.% (1600)
Praseodymium	0.13	0.13		0.15	0.04	0.95	2.8	0.14	2.0
*Rhenium	IS*	IS*	IS*	IS*	IS*	IS*	IS*	IS*	IS*
Rhodium									
Rubidium	32	95	21 (340)	63	74	180	≈950	29	140 (105)
Ruthenium									
Samarium						1.1			
Scandium	<0.16	<0.16	>1%	<0.18	<0.10	1.1	1.1	<0.08	0.78 (<15)
Selenium	2.7	2.3	9.8	0.51	0.40	89	310	63	0.55% (865)
Silicon	0.89%	≈4300	200 (2500)	1.0%	0.56%	3.0%	2.0%	≈4500	1.0% (3000)
Silver	41	65	4.2 (35)	440	43	6.1	35	4.4	≈1100 (420)
Sodium	2.0%	8.0%	≈2000 (700)	9.0%	.077%	3.0%	5.0%	20.0%	3.0% (1700)
Strontium	4.8	11	1.2 (6.5)	43	14	160	110	57	29 (45)
Sulphur	2.0%	4.0%	>1% (7250)	≈3500	5.0%	0.31%	1.0%	7.0%	2.0%
Tantalum	0.82	0.79	0.18	0.44	0.52	0.29	0.20	0.41	1.3
Tellurium	0.15	0.40	0.85	0.15	<0.08	0.58	2.0	0.12	≈3600 (1700)
Terbium						0.10	0.15	0.04	
Thallium	0.53	1.5	7.5 (84)	0.78	0.97	11	35	0.78	≈3900 (875)
Thorium	<0.23	<0.22	0.24	<0.25	<0.14	2.3	3.7	1.3	0.56
Thullium						0.03	0.09		
Tin	340	610	66 (81)	140	40	4.4	6.1	14	4.0% (3700)
Titanium	33	15	8.0 (125)	0.44	9.6	290	370	210	560 (170)
Tungsten	11	10	0.87	12	6.8	3.6	2.4	13	180
Uranium	0.33	1.5	3.4 (16)	6.2	<0.14	2.3	3.7	1.3	5.6
Vanadium	290	330	38 (15.5)	40	23	48	33	2.0%	49 (128)
Ytterbium						0.57	0.21		
Yttrium	0.78	0.51	0.08	0.56	0.71	65	25	13	19 (16)
Zinc	2.0%	2.0%	≈1100 (850)	0.58%	≈4600	760	≈1100	900	1.0% (1.7%)
Zirconium	0.64	1.4	0.66	0.69	0.40	10	14	2.2	11 (9.8)

[a]Reported as parts per million by weight, except as noted

*IS: Internal Standard

**Not Reported

[†]( ) Reported by independent laboratory (EMSL/RTP)

TABLE 3
ELEMENTAL ANALYSIS OF PARTICULATE SAMPLES AS DETERMINED BY SPARK SOURCE MASS SPECTROMETRY [a]
(CONTINUED)

Element	Copper Smelting 3-10 Micron	Aluminum Smelter 1-3 Micron	Aluminum Smelter 3-10 Micron	Ceramics Plant 1-3 Micron	Ceramics Plant 3-10 Micron	Sludge Incinerator, 1-3 Micron	Sludge Incinerator, 3-10 Micron
Aluminum	≈2500	2.0%	≈3500	3.0%	3.0%	0.9%	0.69%
Antimony	3.0%	130	66	10	5.3	2.5	30
Arsenic	6.0%	≈4100	700	890	110	100	43
Barium	81	5.2	25	480	290	~1700	≈2000
Beryllium	0.59	2.9	1.8	2.3	1.2	2.4	14
Bismuth	1.0%	270	56	140	79	≈1500	≈1900
Boron	23	72	27	100	230	82	65
Bromine	750	86	23	14	7.6	720	420
Cadmium	≈1400	16	1.8	13	6.6	630	790
Calcium	4.0%	≈1800	2.0%	15.0%	15.0%	>1%	20.0%
**Carbon	NR**	NR**	NR**	NR**	NR**	NR**	NR**
Cerium	20	0.45	0.14	180	200	190	220
Cesium	3.7	7.5	0.27	6.0	6.6	18	11
Chlorine	≈1100	690	140	330	200	≈4600	≈2700
Chromium	79	90	56	150	120	~4000	≈2300
Cobalt	480	43	27	3.4	18	850	≈1000
Copper	20.0%	≈2000	≈4600	210	93	3.0%	3.0%
Dysprosium				1.7	4.5	1.8	2.5
Erbium				0.50	1.0	0.26	0.31
Europium	1.0			1.3	1.8	0.97	1.3
Fluorine	370	>50%	20.0%	9.0%	1.0%	~4300	≈2500
Gadolinium	0.31			0.89	0.99	1.5	0.55
Gallium	13	900	200	60	36	130	74
Germanium	24	3.4	2.1	6.3	1.4	2.8	1.7
Gold	58					84	120
Hafnium	0.65			0.99	1.3	2.7	3.7
Holmium				0.27	0.30	0.13	0.13
*Indium	IS*	IS*	IS*	IS*	IS*	IS*	IS*
Iodine	160	34	16	11	5.9	280	330
Iridium							
Iron	20.0%	4.0%	2.0%	6.0%	6.0%	7.0%	8.0%
Lanthanum	7.9	0.37	0.05	37	27	39	42
Lead	4.0%	330	73	60	33	≈1300	≈2200
Lithium	18	9.8	13	390	430	50	85
Lutecium				0.32	0.36	0.72	0.37
Magnesium	1.0%	330	870	10.0%	6.0%	6.0%	7.0%
Manganese	880	20	6.3	~1600	≈1300	850	≈1000
Mercury	***	NR	NR	NR	NR	NR	NR
Molybdenum	~2500	67	51	23	12	110	33
Neodymium	9.8	1.1		45	49	47	55

[a]Reported as parts per million by weight, except as noted

*IS: Internal Standard

**Not Reported

***Mercury Observed

†( ) Reported by independent laboratory (EMSL/RTP)

TABLE 3
ELEMENTAL ANALYSIS OF PARTICULATE SAMPLES AS DETERMINED BY SPARK SOURCE MASS SPECTROMETRY [a]
(CONCLUDED)

Element	Copper Smelting 3-10 Micron	Aluminum Smelter 1-3 Micron	Aluminum Smelter 3-10 Micron	Ceramics Plant 1-3 Micron	Ceramics Plant 3-10 Micron	Sludge Incinerator, 1-3 Micron	Sludge Incinerator, 3-10 Micron
Nickel	760	≈2800	≈3800	88	36	≈4500	≈3000
Niobium	6.7	1.8	0.48	29	32	30	31
**Nitrogen	NR**	NR**	NR**	NR**	NR**	NR**	NR**
Osmium							
**Oxygen	NR**	NR**	NR**	NR**	NR**	NR**	NR**
Palladium							16
Phosphorus	≈1300	0.76%	≈2000	0.61%	0.67%	10.0%	10.0%
Platinum							
Potassium	1.0%	≈4100	840	4.0%	4.0%	1.0%	1.0%
Praseodymium	2.0	0.08	0.03	13	9.9	9.4	11
*Rhenium	IS*	IS*	IS*	IS*	IS*	IS*	IS*
Rhodium							
Rubidium	43	98	6.1	460	370	22	24
Ruthenium							
Samarium	3.6			2.5	3.6	1.1	2.6
Scandium	1.7	<0.17	<0.08	15	40	3.8	4.4
Selenium	≈3400	22	14	42	20	73	52
Silicon	3.0%	740	230	40.0%	30.0%	10.0%	8.0%
Silver	≈2200	2.0	1.1	0.57	0.64	600	500
Sodium	7.0%	8.0%	27	20.0%	15.0%	10.0%	8.0%
Strontium	82	9.4	50	≈1200	≈1400	≈1300	≈1500
Sulphur	2.0%	3.0%	0.6%	≈3800	≈4700	0.8%	0.57%
Tantalum	1.3	1.5	0.91	0.54	0.60	1.4	3.4
Tellurium	≈1300	26	4.3	2.7	1.3	0.29	0.34
Terbium				0.36	0.53	0.21	0.30
Thallium	1100	3.0	0.19	10	5.3	2.5	5.3
Thorium	1.1	<0.24	<0.12	10	11	2.3	3.1
Thullium				0.12	0.14	0.10	0.08
Tin	≈4600	100	33	36	9.2	1.0%	0.5%
Titanium	≈1100	130	140	0.51%	0.67%	0.53%	≈3800
Tungsten	79	21	20	17	3.7	35	44
Uranium	2.4	1.3	0.40	10	5.6	11	31
Vanadium	98	0.51%	≈1500	540	210	94	55
Ytterbium				1.1	1.8	0.29	0.37
Yttrium	8.1	2.2	1.4	61	38	18	21
Zinc	0.7%	160	56	360	220	0.56%	0.56%
Zirconium	11	5.3	1.5	35	38	210	120

[a] Reported as parts per million by weight, except as noted

*IS: Internal Standard

**Not Reported

†( ) Reported by independent laboratory (EMSL/RTP)

TABLE 4
CARCINOGENIC AND STRUCTURALLY SIMILAR POLYCYCLIC ORGANIC CONSTITUENTS IN PARTICULATE SAMPLES AS DETERMINED BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY [a]

SOURCE / PARTICLE SIZE	OPEN HEARTH FURNACE			COKE OVEN HEATER			BASIC OXYGEN FURNACE			IRON SINTERING			OIL FIRED POWER PLANT		
	1-3μ	3-10μ	FILTER[c]	1-3μ	3-10μ	FILTER[c]	1-3μ	3-10μ	FILTER[c]	1-3μ	3-10μ	FILTER[c]	1-3μ	3-10μ	FILTER[c]
IDENTIFIED COMPONENT															
ANTHRACENE/PHENANTHRENE	2.7	0.4	6786 ng	3.9	(B)	830 ng	24.8	6.9	163 ng	289.5		5929 ng	(B)	(B)	
METHYL ANTHRACENES										27.4					
FLUORANTHENE	1.3	0.2	2455			132	7.4	1.4		31.2		1982			
PYRENE	0.4		222			190	2.9	1		16.1		1542			
METHYL PYRENE/FLUORANTHENE										18.0					
CHRYSENE/BENZ(A)ANTHRACENE	0.4					200			131	1.9		127			
METHYL CHRYSENES															
BENZO FLUORANTHENES	1.6	0.5	284			140	1.6		444	1.8		633			
BENZO(A)PYRENE	0.9	0.3	29			73				4.3		191			
BENZO(E)PYRENE	3	2	98			500	1.8		685	9.0		2030			
3-METHYLCHOLANTHRENE															
INDENO(1,2,3,-CD)PYRENE	1.1								448	100		620			
BENZO(GHI)PERYLENE	0.4								321	0.5		343			
DIBENZ(A,H)ANTHRACENE	4.3											493			
DIBENZO(C,G)CARBAZOLE															
DIBENZO(A,I AND A,H)PYRENES	1.5											538			
CORONENE															

SOURCE / PARTICLE SIZE	CERAMICS PLANT			COPPER SMELTER			ALUMINUM SMELTER			SLUDGE INCINERATOR		
	1-3μ	3-10μ	FILTER[c]	1-3μ	3-10μ	FILTER[c]	1-3μ	3-10μ	FILTER[c]	1-3μ	3-10μ	FILTER[c]
IDENTIFIED COMPONENT												
ANTHRACENE/PHENANTHRENE	22		426 ng	0.5			24.7	172.6	14216 ng			
METHYL ANTHRACENES							9	51.3	4706			
FLUORANTHENE	6.2						34.1	137.2	20948			
PYRENE	4.1		36				42.9	155.8	24738			
METHYL PYRENE/FLUORANTHENE	3.9		82				32.5		27222			
CHRYSENE/BENZ(A)ANTHRACENE			23				93.6	287.3	26198			
METHYL CHRYSENES			57				25	79.7	7266			
BENZO FLUORANTHENES	5.8		19				248	786.3	59016			
BENZO(A)PYRENE	5.8		29				46.5	405.6	124238			
BENZO(E)PYRENE	13.8						807.2	1014	250692			
3-METHYLCHOLANTHRENE							59.5	23.5	27459			
INDENO(1,2,3,-CD)PYRENE	3.6						341.4	249.2	39959			
BENZO(GHI)PERYLENE	0.8						426	786.3	49180			
DIBENZ(A,H)ANTHRACENE	4.5						1387	1213.3	151225			
DIBENZO(C,G)CARBAZOLE							84.7	77.6	6988			
DIBENZO(A,I AND A,H)PYRENES							352	216.1	1172			
CORONENE							87.3	50.2	6824			

[A] REPORTED AS PARTS PER MILLION BY WEIGHT

[B] NOT ANALYZED

[C] ONE QUARTER OF FILTER MATERIAL ANALYZED
(NOTE: FILTER ANALYSES REPORTED AS NANOGRAMS PRESENT)

results, one should note that if collection cyclones were operating at temperatures above 350 F, much of the organic fraction would have been lost as vapor (see Table 1 for sampling logistics).

Table 5 indicates results from the high resolution mass spectra of the vaporized constituents of the particulate samples, and the hydrocarbon structures identified from those spectra. Since studies of pure 4-, 5-, and 6-ring aromatic hydrocarbons have shown that their rate of vaporization varies with both the number of aromatic rings and the type of condensation (peri or cata), the aluminum smelter samples were subjected to additional mass spectral scans made over the period of time during which the samples continued to yield vaporization products. The semi-quantitative results, presented in Table 6, are more representative of the hydrocarbon constituents of those samples than obtainable by the routine analytical method. Eighty-seven percent of the aromatic hydrocarbon content of these two samples was concentrated in 4- to 6-ring aromatic systems.

Mass spectra from all samples analyzed by HRMS were screened for nine precise masses indicating the possible presence of carcinogenic polycyclic organic hydrocarbons (see Table 7). Since HRMS alone cannot determine the isomeric form of a compound with a given mass number, the presence of a compound with the precise mass corresponding to a known carcinogen is not conclusive evidence of the carcinogen's presence. The mass spectra of both aluminum smelter samples (1-3μ and 3-10 μ) indicated the possible presence of all carcinogens listed in Table 7. The precise masses indicating possible carcinogens were not detected in any of the other samples analyzed.

TABLE 5
CONSTITUENTS OF PARTICULATE SAMPLES AS DETERMINED BY HIGH RESOLUTION MASS SPECTROMETRY[a]

SAMPLE ORIGIN	PARTICLE SIZE (MICRONS)	PERCENT OF SAMPLE VAPORIZED	GASES EVOLVED (@3000°C, 10^{-6} torr)	HYDROCARBON STRUCTURES IDENTIFIED[b]
Open Hearth Furnace	3-10	2.1	HCN, CH_3CN, HCl, CO_2, NO_2, EtOH, CH_3COOH, SO_2, COS, CO	Pyridine, C_5-C_7, aliphatic radicals, trace oxygenates
Open Hearth Furnace	1-3	0.12	HCN, CH_3CN, HCl, NO, NO_2, H_2S, CO_2, EtOH, CO, COS, SO_2, CS_2	Pyridine, Me-pyridine; aliphatic hydrocarbon radicals through C_7 Six unidentified mass peaks (< mass 102; trace oxygenates
Coke Oven Heater	1-3	5.3	HCN, CH_3CN, CO, NO, H_2S, CO_2, NO_2, SO_2, COS, C_6H_6, CS_2	Pyridine, Me-pyridine, C_7-C_8 naphthenes, aliphatic radicals through C_6: unidentified mass peaks (< mass 109)
Basic Oxygen Furnace	1-3	3.2	HCN, CH_3CN, CO, NO, HCl, CO_2, NO_2, EtOH, SO_2, COS	Pyrrole possible, trace oxygenates, trace hydrocarbons through C_9
Iron Sintering Plant	3-10	7.3	HCN, CH_3CN, CO, NO, H_2S, HCl, CO_2, NO_2, EtOH, SO_2, COS	C_6H_6, $C_{10}H_8$, $C_{11}C_{10}$, $C_{14}H_{10}$
Iron Sintering Plant	1-3	37.8[c]	HCN, CH_3CN, CO, NO, HCl, CO_2, NO_2, EtOH, SO_2, COS, H_2S	C_6H_6, $C_{10}H_8$, $C_{11}H_{10}$, $C_{14}H_{10}$, C_6H_6O
Copper Smelter	1-3	n.a.[d]	SO_2, CS_2, CO, CO_2	As_4O_6
Copper Smelter	3-10	n.a.[d]	HCN, CO, HCl, NO_2, SO_2, CS_2, COS	As_4O_6
Aluminum Smelter	1-3	1.3	CHN, CO, NO, HCl, CO_2, NO_2, SO_2, COS, H_2S	Aromatic hydrocarbons; nitro- and sulfur heterocyclics
Aluminum Smelter	3-10	5.2	CHN, CO, NO, HCl, CO_2, NO_2, SO_2, COS	Similar to 16-3 in composition; slightly lower carbon number distribution for all classes of compounds
Ceramics Plant	1-3	2.4	HCN, CO, NO. MeOH, HCl, SO_2, NO_2, H_2S, COS	Trace organics through C_{10}
Ceramics plant	3-10	2.1	HCN, CO, NO, HCl, CH_3CN, CO_2, EtOH, NO_2, SO_2, COS, H_2S	Pyridine, trace organics through C_{10}
Municipal Incinerator	1-3	4.0	HCN, CO, NO, HCl, CH_3CN, CO_2, NO_2, SO_2, CS_2	Pyrrole, phenol, aromatics through C_{10} aliphatic radicals through C_8
Municipal Incinerator	3-10	0.5	HCN, CO, NO, H_2S, HCl, CH_3CN, CO_2, NO_2, COS, SO_2, CS_2	Trace hydrocarbon

[a] From low ionizing voltage mass spectra data, as reported by ERDA/PERC

[b] All isomeric structures are possible

[c] Measurement doubtful

[d] n.a. - not available

TABLE 6

SEMI-QUANTITATIVE MASS SPECTRAL ANALYSIS OF PARTICULATE MATTER COLLECTED AT THE ALUMINUM SMELTER[a]

Particle Size	1-3μ	3-10μ
Quantity Analyzed	100.9 mg	100.0 mg
Percent Vaporized	4.5	3.7
Structural Types	**Percent of Total Ionization**	
3-ring aromatics	2.3	0.8
Phenylnaphthalenes	2.3	5.4
4-ring, peri-condensed	6.5	7.5
4-ring, cata-condensed	7.0	7.0
5-ring, peri-condensed	20.4	12.4
Phenylanthracenes	3.5	12.0
5-ring, cata-condensed	12.6	13.3
6-ring, peri-condensed (mass 276)	17.1	3.7
6-ring, peri-condensed (mass 302)	3.5	2.5
7-ring, peri-condensed (Coronene)	1.1	0.9
Dinaphthothiophene	1.5	1.6
Azapyrene + Benzocarbazole	12.2	12.5
Benzacridine	1.3	5.7
Carbazole	1.0	1.9
Acridine	1.2	3.2
Dibenzocarbazole	1.4	0.4
Dibenzacridine	0.8	3.1
Azabenzo(ghi)perylene	0.03	2.1
Azaperylene	4.3	4.0

[a]High resolution mass spectrometry; quantitation by integrated peak height versus time

TABLE 7

POSSIBLE DETECTION OF CARCINOGENIC POLYCYCLIC ORGANIC MATERIAL FROM HIGH RESOLUTION MASS SPECTROMETRIC ANALYSIS

MASS	FORMULA	POSSIBLE COMPOUNDS	CARCINOGENIC POTENTIAL*
228	$C_{18}H_{12}$	Benzo(c)phenanthrene	+++
252	$C_{20}H_{12}$	Benzo(b)fluoranthene	++
		Benzo(j)fluoranthene	++
		Benzo(a)pyrene	+++
254	$C_{20}H_{14}$	Benz(j)aceanthrylene (cholanthrene)	++
256	$C_{20}H_{16}$	7,12-Dimethylbenz(a)anthracene	++++
267	$C_{20}H_{13}N$	Dibenzo(c,g)carbazole	+++
268	$C_{21}H_{16}$	3-Methylcholanthrene	++++
278	$C_{22}H_{14}$	Dibenz(a,h)anthracene	+++
279	$C_{21}H_{13}N$	Dibenz(a,j)acridine	++
		Dibenz(a,h)acridine	++
302	$C_{24}H_{14}$	Dibenzo(a,h)pyrene (Dibenzo(b,def)chrysene)	+++
		Dibenzo(a,i)pyrene	++

*+ = carcinogenic; ++, +++, ++++ = strongly carcinogenic (Reference 11)

5.2 BIOASSAYS

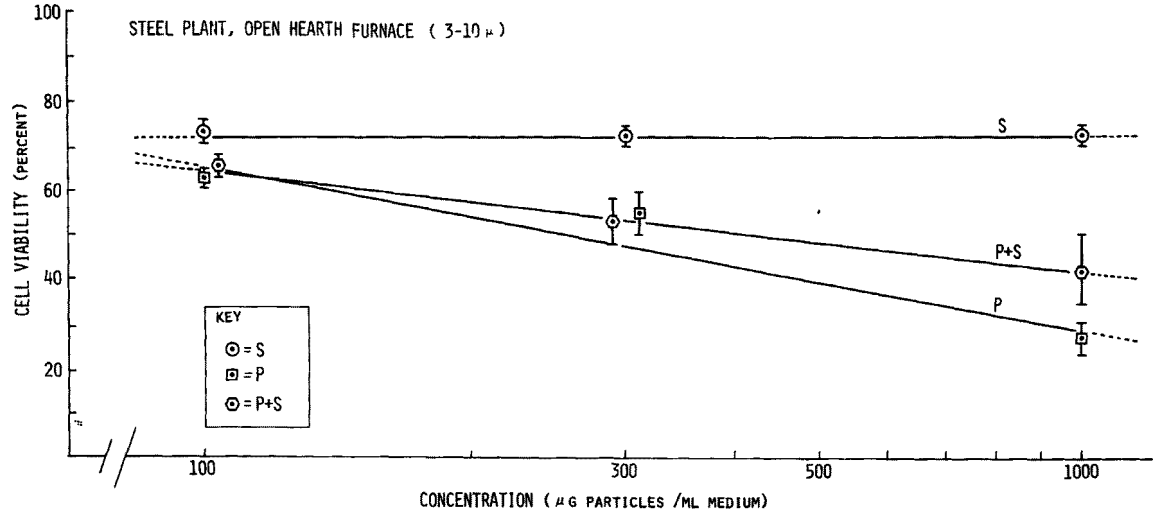
Preliminary concentration-response data from the RAM cytotoxicity bioassay are presented in Figures 4-11. In order to linearize the concentration-response data, cell response, expressed as viable cells as a percent of control and reported as mean values ± 1 standard deviation, is plotted against the common logarithm (base 10) of the particle concentration in the culture medium. The cell responses to the three types of particle exposure are provided (see Section 3.2.1): particles plus supernatant fraction (P+S), particle fraction (P), and supernatant fraction (S). Linear dose-response regression lines were fitted to the data, using a least squares solution.[12] Particulate samples collected at the basic oxygen furnace were found to be nontoxic at the highest concentration (i.e., 1000µg particles/ml medium), so no further runs were performed. Due to sample mass limitations, the particle plus supernatant (P+S) fraction of the coke oven heater 3-10µ sample was tested only at the 1000 µg/ml concentration.

Additional concentration-response tests were conducted on selected fractions of two particulate samples, to determine the cellular response over a greater particulate concentration range than previously tested. In Figure 12, the RAM viability is noted after exposure to the supernatant fraction (S) of the 3-10µ copper smelter sample at concentrations ranging from 1 µg/ml to 40 µg/ml; the preliminary concentration-response data (Figure 8) are also provided for comparison. In Figure 13, the RAM viability is noted after exposure to the particles plus supernatant fraction (P+S) of the 1-3µ sludge incinerator sample at concentrations ranging from 1 µg/ml to 200 µg/ml; preliminary concentration-response data from Figure 10 are included for comparison.

Results of the cytotoxicity bioassay on the filter material used to collect the sub-micron particles at the industrial facilities are provided in Table 8. The mean value of two observations is presented.

Results of the mutagenic bioassay, as conducted by Litton-Bionetics, Inc., are presented in Table 9. The aluminum smelter 1-3μ particles indicated mutagenic activity on two of the three bacterial strains tested. The copper smelter 1-3μ particles indicated possible mutagenic activity on one of the three bacterial strains. The remaining particulate samples indicated no mutagenic activity on the bacterial strains, under the test conditions. Table 10 provides dose-related response data for the two samples possessing mutagenic activity.

Research Triangle Institute (RTI) conducted limited additional mutagenic screening tests on aliquots of three of the eleven particulate samples tested by Litton-Bionetics, Inc. (i.e., copper smelter 3-10μ sample, ceramics plant 3-10μ sample, and sludge incinerator 3-10μ sample). Similar laboratory procedures were used, except that RTI employed freshly prepared mouse liver microsomal fractions for metabolic activation studies (rather than frozen preparations), and conducted all screening tests (e.g., toxicity determinations, positive and sterility controls, and the mutagenic screening test) for a given sample on the same day, rather than on separate days. Results of the RTI mutagenic screening tests are presented in Table 11; the average of two observations per treatment level is presented.



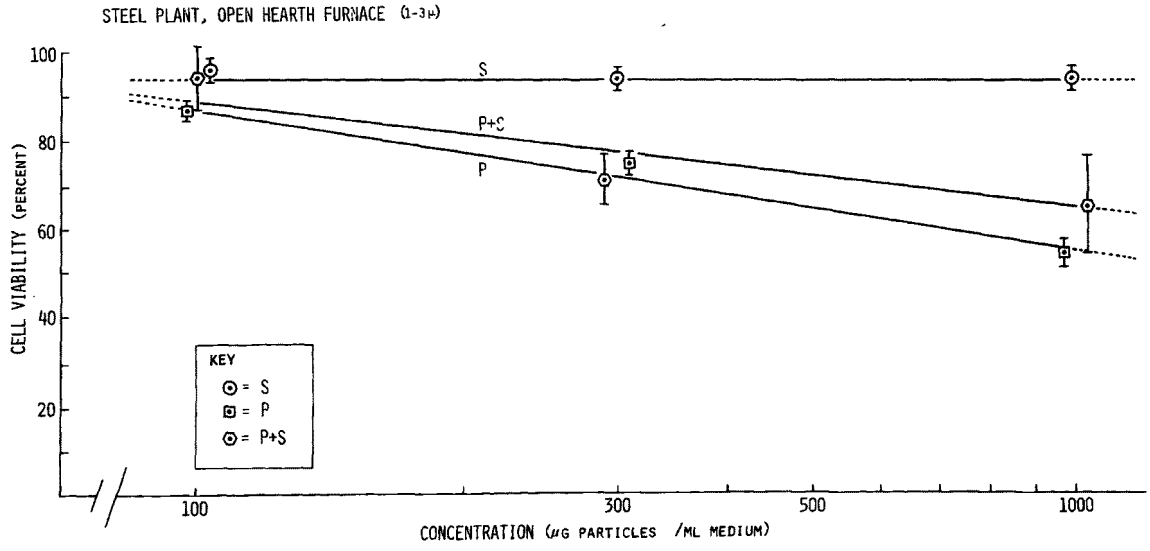
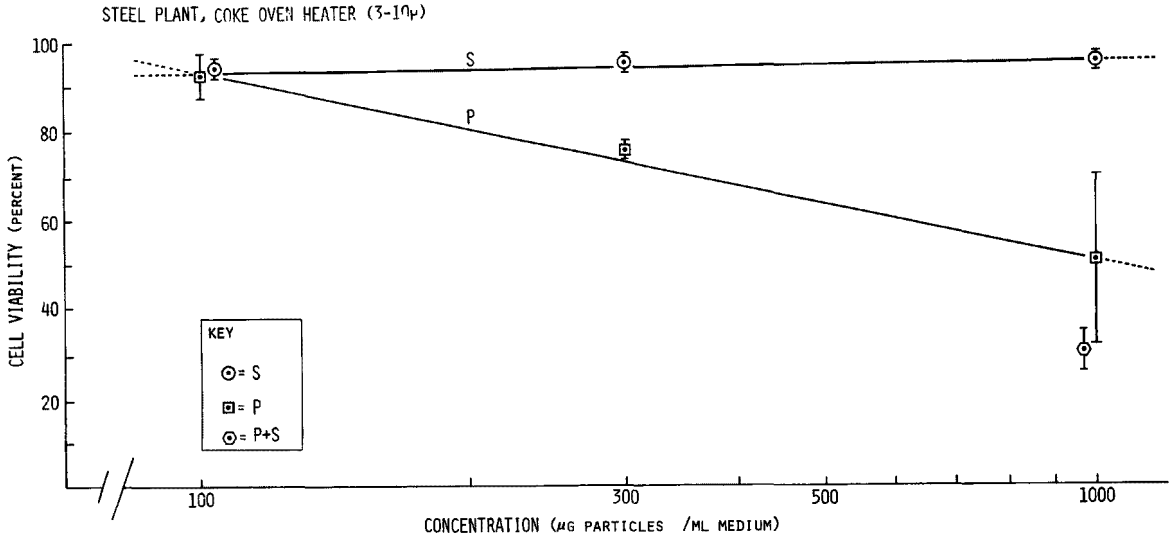


FIGURE 4
VIABILITY OF RABBIT ALVEOLAR MACROPHAGES EXPOSED TO VARIOUS FRACTIONS OF PARTICULATE SAMPLES COLLECTED AT THE OPEN HEARTH FURNACE



***sample mass limitations precluded complete assay of the P+S fraction**

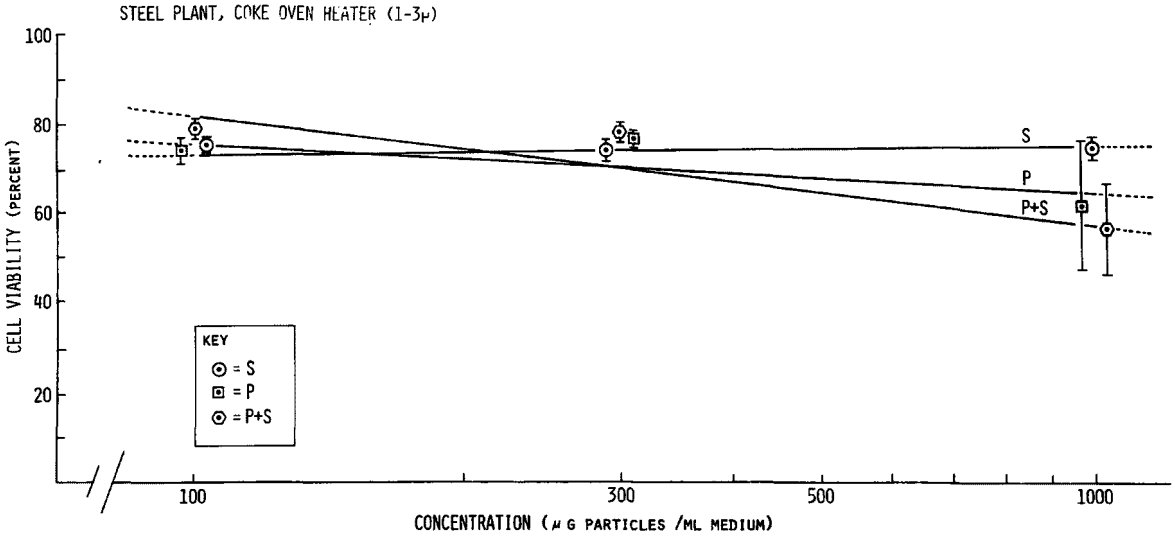
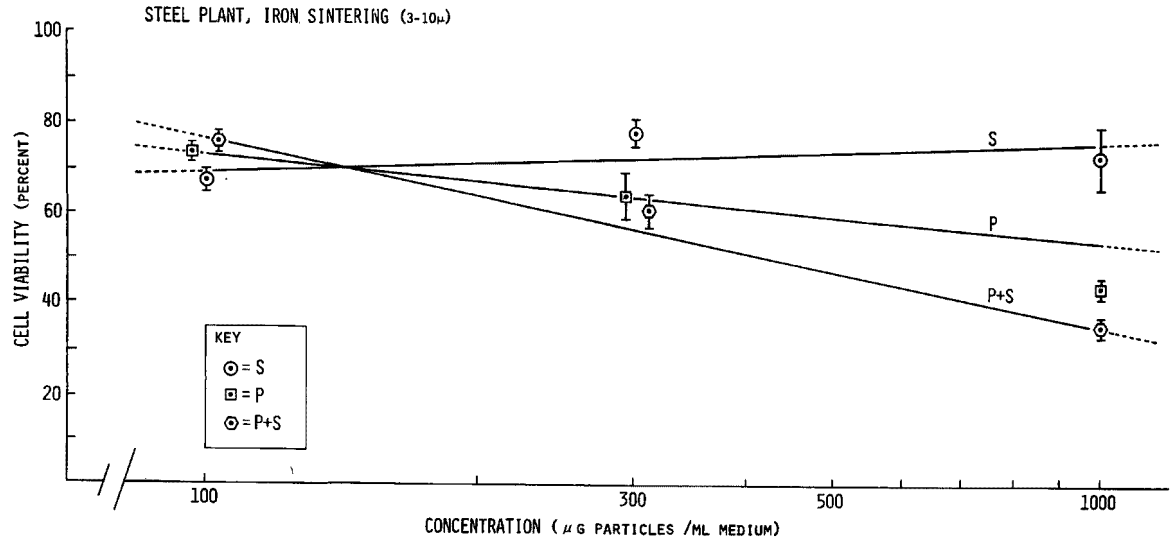


FIGURE 5
VIABILITY OF RABBIT ALVEOLAR MACROPHAGES EXPOSED TO VARIOUS FRACTIONS OF PARTICULATE SAMPLES COLLECTED AT THE COKE OVEN HEATER



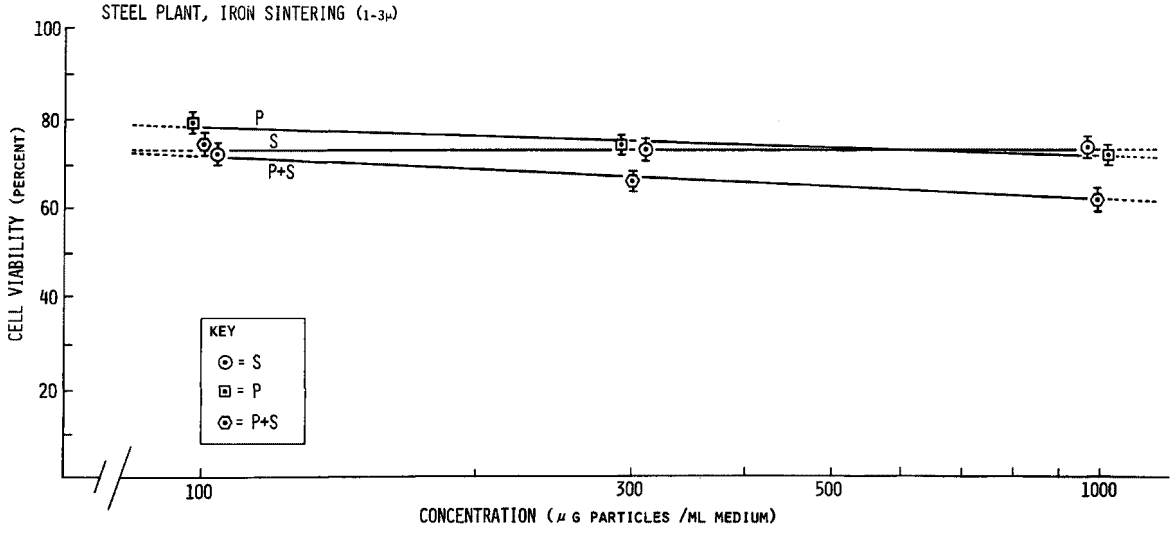


FIGURE 6
VIABILITY OF RABBIT ALVEOLAR MACROPHAGES EXPOSED TO VARIOUS FRACTIONS OF PARTICULATE SAMPLES COLLECTED AT THE IRON SINTERING PLANT

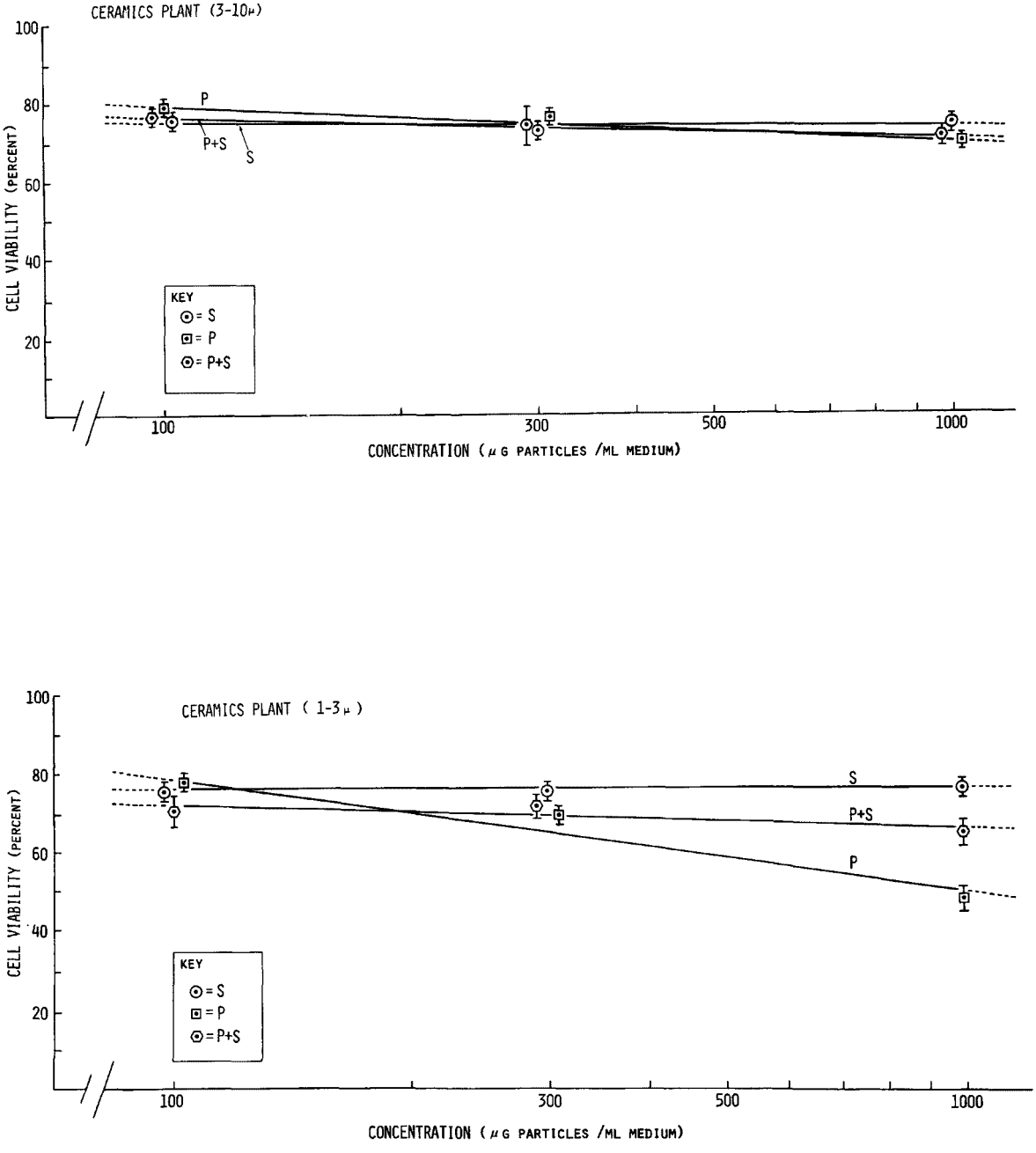
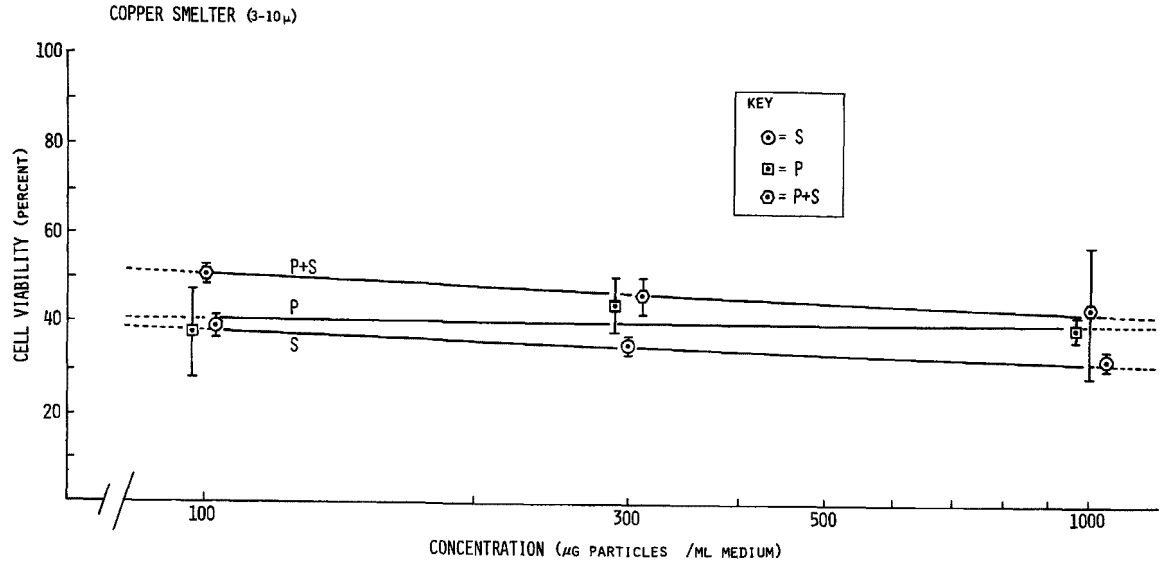


FIGURE 7
VIABILITY OF RABBIT ALVEOLAR MACROPHAGES EXPOSED TO VARIOUS FRACTIONS OF PARTICULATE SAMPLES COLLECTED AT THE CERAMICS PLANT



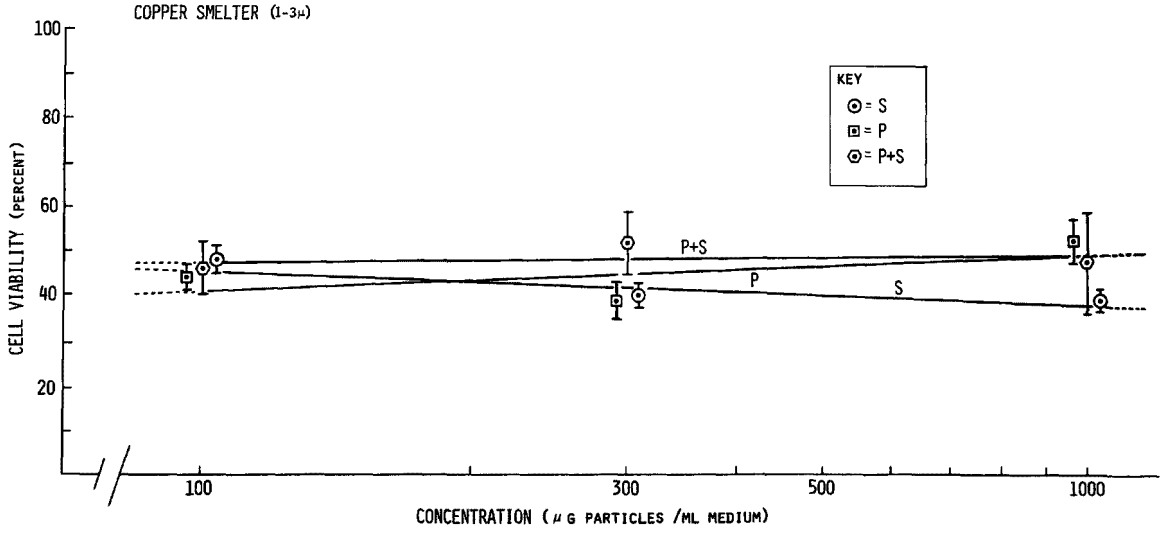


FIGURE 8
VIABILITY OF RABBIT ALVEOLAR MACROPHAGES EXPOSED TO VARIOUS FRACTIONS OF PARTICULATE SAMPLES COLLECTED AT THE COPPER SMELTER

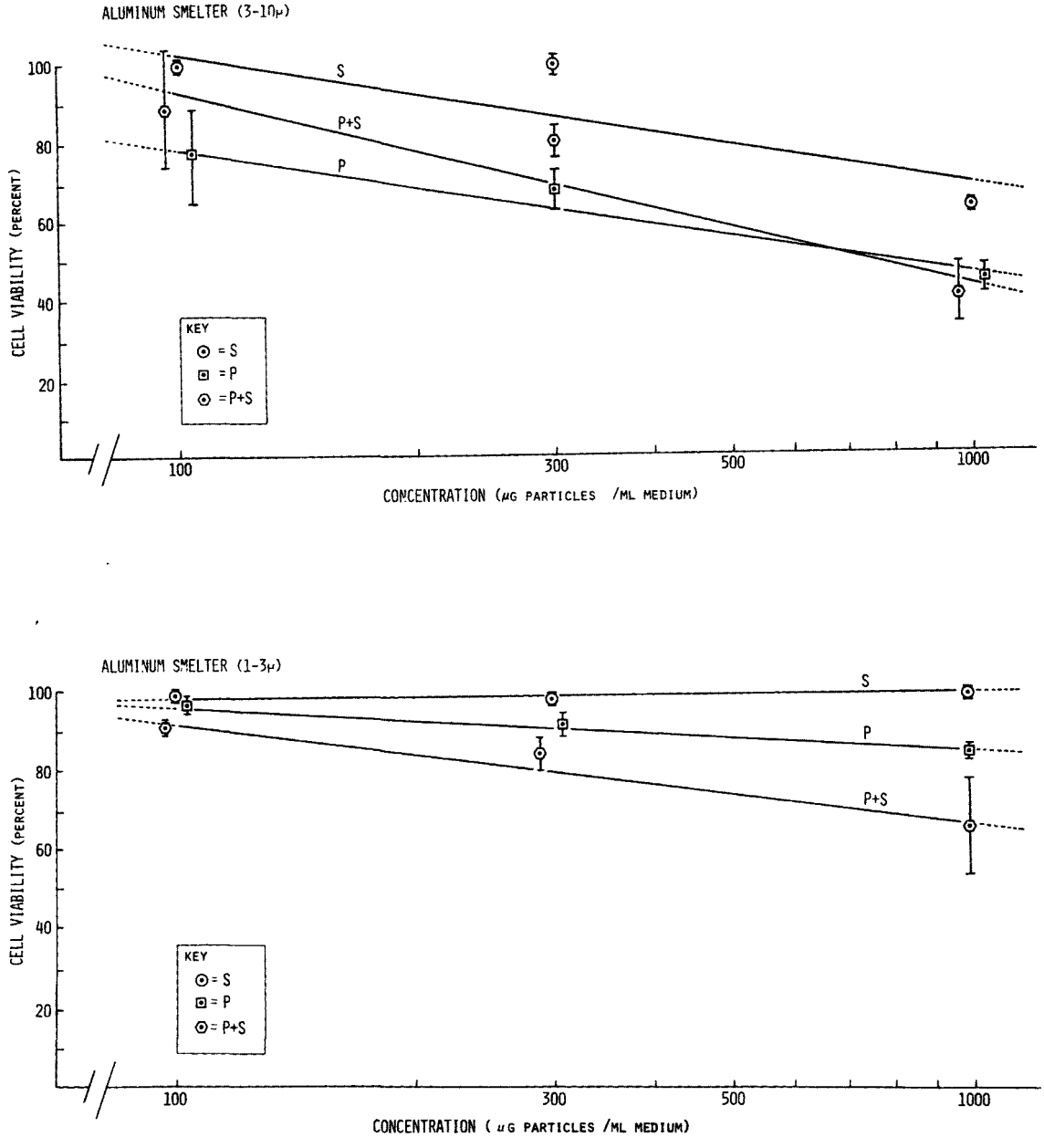


FIGURE 9
VIABILITY OF RABBIT ALVEOLAR MACROPHAGES EXPOSED TO VARIOUS FRACTIONS OF PARTICULATE SAMPLES COLLECTED AT THE ALUMINUM SMELTER

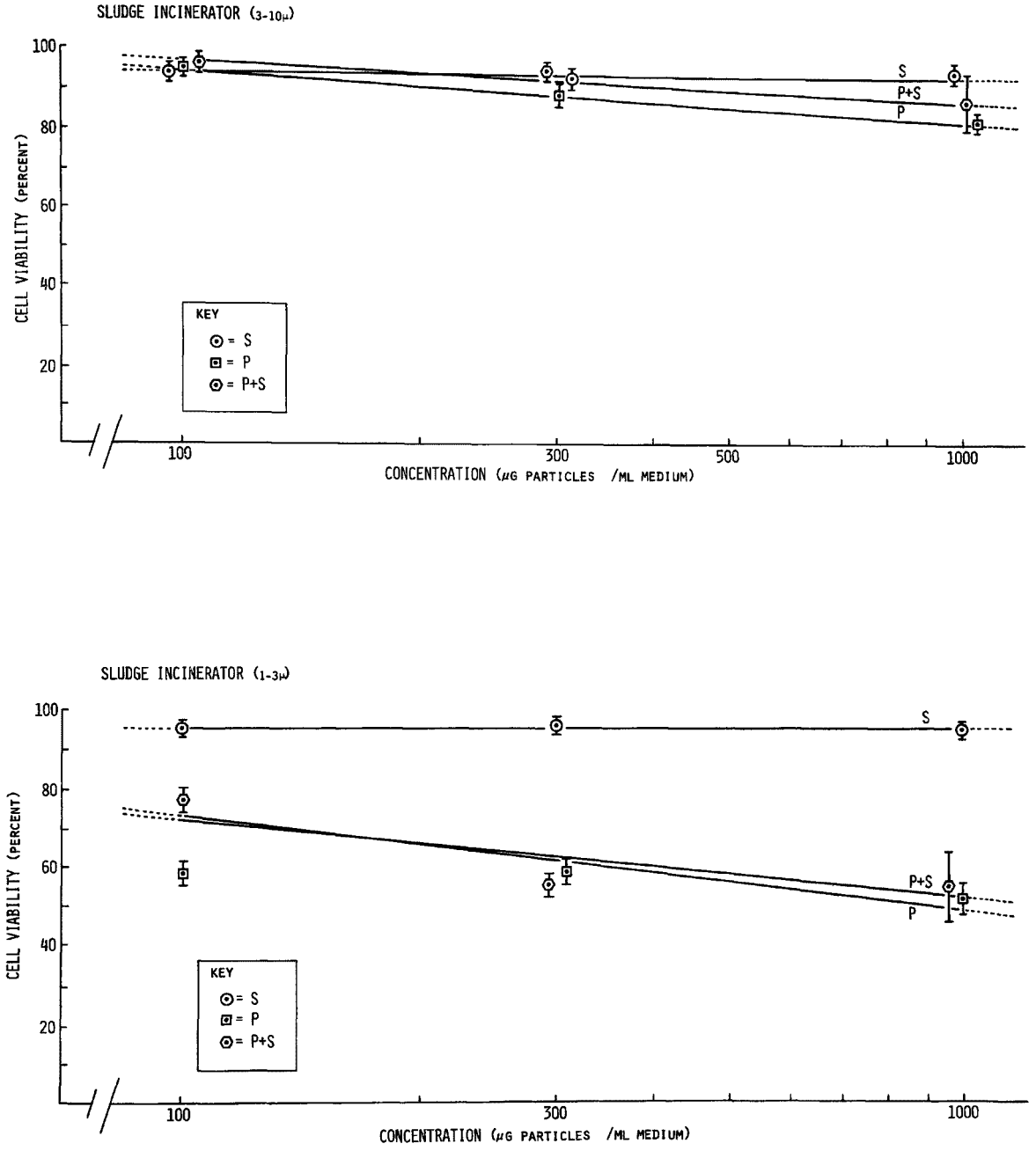


FIGURE 10
VIABILITY OF RABBIT ALVEOLAR MACROPHAGES EXPOSED TO VARIOUS FRACTIONS OF PARTICULATE SAMPLES COLLECTED AT THE SLUDGE INCINERATOR

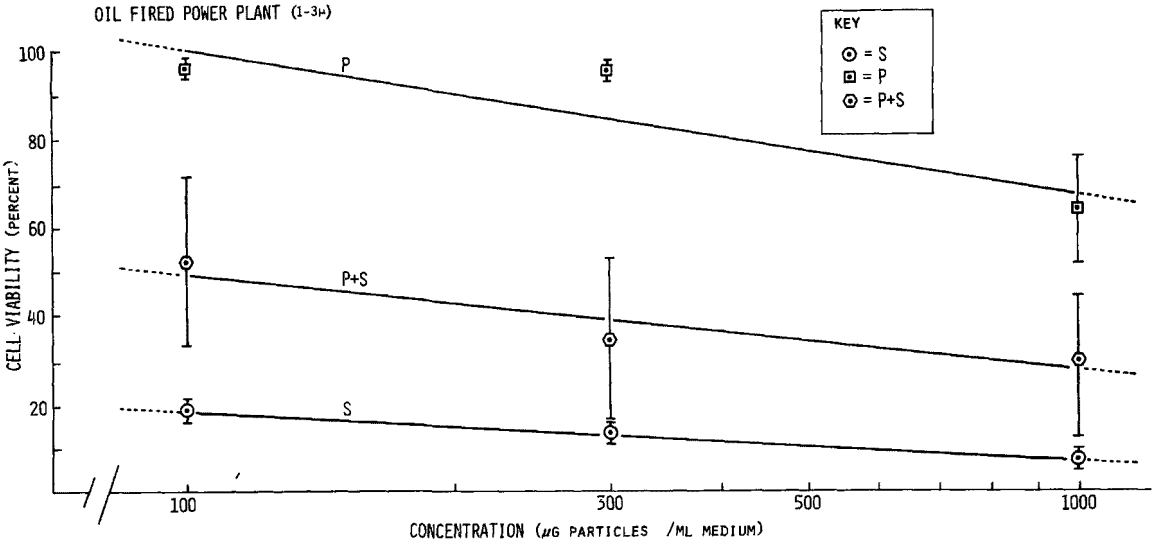


FIGURE 11
VIABILITY OF RABBIT ALVEOLAR MACROPHAGES EXPOSED TO VARIOUS FRACTIONS OF PARTICULATE SAMPLES COLLECTED AT AN OIL FIRED POWER PLANT

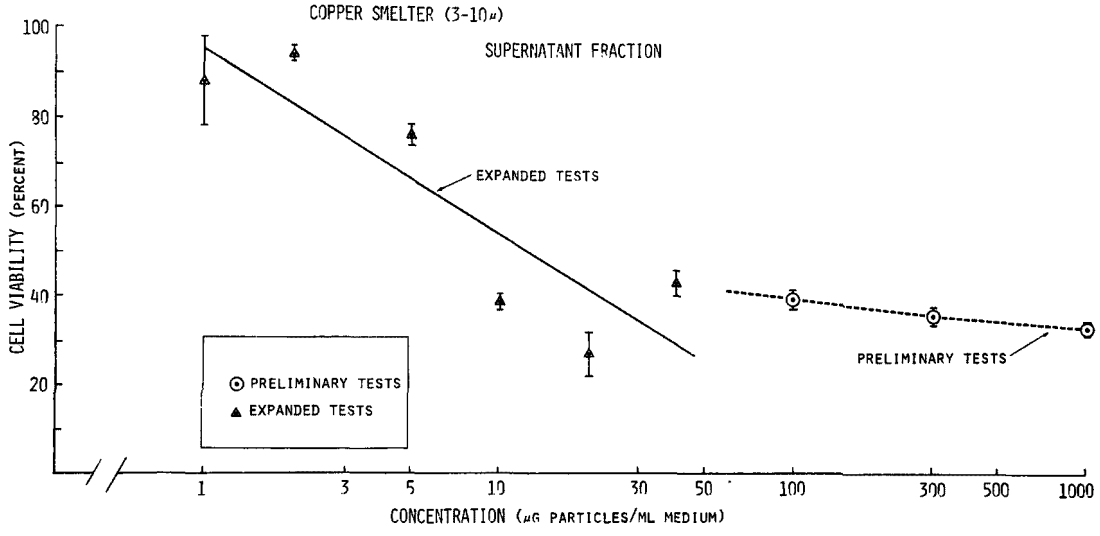


FIGURE 12
VIABILITY OF RABBIT ALVEOLAR MACROPHAGES EXPOSED TO SUPERNATANT FRACTION COLLECTED FROM THE COPPER SMELTER (3-10μ SAMPLE)

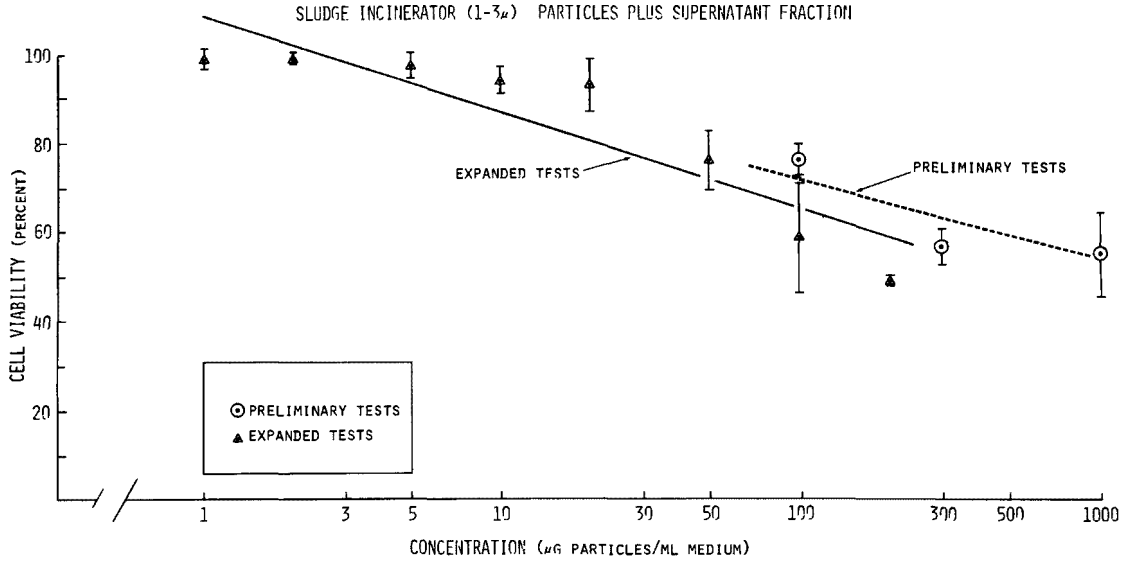


FIGURE 13
VIABILITY OF RABBIT ALVEOLAR MACROPHAGES EXPOSED TO PARTICLES PLUS SUPERNATANT FRACTION COLLECTED FROM THE SLUDGE INCINERATOR (1-3μ SAMPLE)

TABLE 8

VIABILITY OF RABBIT ALVEOLAR MACROPHAGES EXPOSED TO SUB-MICRON PARTICLE FILTERS

FILTER SAMPLE	FILTER PLUS SUPERNATANT	SUPERNATANT	DRIED FILTER
Oil Fired Power Plant	20.4[a]	10.5[a]	30.1[a]
Copper Smelter[b]	26.3	29.5	41.0
Aluminum Smelter[b]	28.2	24.9	86.3
Aluminum Smelter[b]	31.5	32.1	60.8
Iron Sintering[b]	33.0	55.2	95.7
Copper Smelter[b]	37.2	30.6	35.6
Iron Sintering[b]	56.1	58.8	99.5
Paper Mill	56.1	76.7	97.9
Municipal Incinerator	92.1	71.8	93.8
Ceramics Plant	93.0	53.6	98.4
Open Hearth Furnace	95.9	98.8	97.4
Coke Oven	97.6	98.8	99.6
Basic Oxygen Furnace	98.3	98.4	99.6
Teflon Control Filter	NON-TOXIC	NON-TOXIC	NON-TOXIC

[a]Percent viable cells (mean of two observations)

[b]Original filters replaced because of heavy grain loading.

TABLE 9

MUTAGENIC ACTIVITY OF INDUSTRIAL PARTICULATE SAMPLES[a]

Sample	Response With Indicator Strain		
	TA-1535	TA-1537	TA-1538
Open Hearth Furnace, 1-3μ	-	-	-
Basic Oxygen Furnace, 1-3μ	-	-	-
Iron Sintering Plant, 3-10μ	-	-	-
Ceramics Plant, 1-3μ	-	-	-
Ceramics Plant, 3-10μ	-	-	-
Copper Smelter, 1-3μ	-	-	+/-
Copper Smelter, 3-10μ	-	-	-
Aluminum Smelter, 1-3μ	-	+	+/-
Aluminum Smelter, 3-10μ	-	-	-
Sludge Incinerator, 1-3μ	-	-	-
Sludge Incinerator, 3-10μ	-	-	-

a = As determined by Litton-Bionetics, Inc.
- = No response obtained
+ = Positive mutagenic response showing a dose-related increase
+/- = Questionable response or non-dose related positive results

TABLE 10
DOSE RELATED RESPONSE FOR MUTAGENICALLY ACTIVE PARTICULATE SAMPLES[a]

PARTICLE CONCENTRATION (µg/plate)	NUMBER OF REVERTANTS PER PLATE					
	TA-1535		TA-1537		TA-1538	
	NA[b]	A[c]	NA	A	NA	A
Control	14.5 ± 1.3[d]	14.0 ± 0.8	17.0 ± 2.5	19.0 ± 2.5	15.5 ± 2.4	39.3 ± 1.7
Aluminum Smelter (1-3µ)						
0.01	13.0 ± 2.8	11.8 ± 1.3	-	-	16.3 ± 4.2	-
0.1	13.0 ± 2.9	13.3 ± 1.0	14.5 ± 2.7	-	14.0 ± 4.2	40.8 ± 6.1
0.5	14.3 ± 1.7	-	-	-	14.5 ± 5.5	39.8 ± 6.3
1.0	13.8 ± 1.9	14.5 ± 2.4	14.8 ± 3.8	17.5 ± 1.7	14.3 ± 4.9	46.5 ± 7.3
10.0	10.5 ± 2.7	15.3 ± 2.1	15.8 ± 1.9	15.3 ± 1.0	13.5 ± 2.9	48.8 ± 8.2
100.0	-	13.8 ± 3.2	16.5 ± 3.9	21.0 ± 2.2	-	63.8 ± 11.5
500.0	-	-	13.5 ± 3.9	29.3 ± 3.3	-	-
1000.0	-	-	-	38.0 ± 4.7	-	-
Copper Smelter (1-3µ)						
0.01	15.0 ± 2.2	13.0 ± 2.9	14.8 ± 2.1	14.5 ± 1.3	16.5 ± 1.7	39.5 ± 1.7
0.05	14.5 ± 2.9	-	-	-	16.5 ± 2.7	-
0.1	15.0 ± 4.1	16.0 ± 1.2	14.3 ± 2.6	17.8 ± 2.9	14.8 ± 1.9	43.8 ± 6.1
0.5	13.8 ± 2.1	14.0 ± 1.4	17.3 ± 1.1	-	14.3 ± 4.6	44.0 ± 2.6
1.0	13.8 ± 1.0	12.0 ± 1.4	13.8 ± 1.7	14.0 ± 2.2	16.3 ± 3.4	49.5 ± 5.2
10.0	-	15.8 ± 1.7	16.5 ± 1.3	19.8 ± 1.0	-	66.0 ± 5.0
100.0	-	-	-	17.5 ± 2.7	-	-

[a] As reported by Litton-Bionetics, Inc.
[b] Non-activated
[c] Activated
[d] Mean ± 1SD of four observations

TABLE 11
MUTAGENIC ACTIVITY OF THREE SELECTED INDUSTRIAL PARTICULATE SAMPLES[a]

PARTICLE CONCENTRATION (μg/plate)	NUMBER OF REVERTANTS PER PLATE					
	TA-1535		TA-1537		TA-1538	
	NA[b]	A[c]	NA	A	NA	A
Copper Smelter (3-10μ)						
Control	507[d]	174	10	12	31	45
1	385	138	12	22	20	42
10	362	165	13	16	22	45
100	466	120	11	18	16	36
500	259	100	12	8	13	16
1,000	11	59	10	3	0	8
Ceramics Plant (3-10μ)						
Control	322	47	6	8	3	18
1	282	58	8	9	10	16
10	283	66	8	8	11	14
100	202	54	11	12	14	12
500	263	54	10	9	9	14
1,000	233	78	4	6	8	12
Sludge Incinerator (3-10μ)						
Control	490	144	4	10	6	12
1	382	156	8	11	9	16
10	438	132	8	14	8	12
100	452	142	3	12	9	10
500	346	132	11	10	7	14
1,000	305	132	7	8	10	17

[a] As reported by Research Triangle Institute
[b] Non-activated
[c] Activated
[d] Average of two observations

6.0 DISCUSSION

This research effort was undertaken to provide field test validation of selected sampling and testing procedures intended to characterize potential hazards associated with airborne particulate emissions. There are numerous alternate or additional tests that could be used to accomplish this goal; however, only those tests utilized in this research effort are discussed. Any conclusions or recommendations concerning these selected tests are based solely on the test protocol as performed. In some instances, preliminary findings indicated the need to modify the scope of some tests. When time or fiscal constraints permitted, these modifications were undertaken. Otherwise, the test protocols were completed as initially defined and recommendations for additional studies are offered for consideration.

In the subsequent sections, each of the sampling and testing procedures is discussed separately, followed by a section integrating the entire research effort.

6.1 SAMPLE COLLECTION

6.1.1 Sampling Train Performance

The series cyclone sampling train was developed to collect, within one-to-five hours of operation, sufficient quantities of size-classified particulate material from a variety of industrial sources. At the initiation of this project, it was decided that a sample mass equal to 300 mg was sufficient for all subsequent chemical and/or biological testing. In most cases this collection criterion was satisfied, with the exceptions being the open hearth furnace, the basic oxygen furnace, and the coke oven heater (see Table 2). Since

the oil-fired power plant shut down after two hours of sampling train operation, collection of the minimum sample mass was not realized. The stack emissions at the paper mill were substantially reduced on the day of sampling, compared to stack emissions during the site inspection one day earlier, and a negligible amount of particulate material was collected. The cause of this drastic emissions reduction on the day of sampling was undetermined.

The cyclone train also exhibited its versatility by operating successfully under a variety of circumstances. In that instance where cyclone train operation at elevated temperatures was a problem (i.e., sludge incinerator), a water-cooled probe was used. Successful train operation under heavy grain load conditions was also accomplished, although the filters required replacement and the total elapsed sampling time was short. The actual sampling locations at each industrial process provided diverse conditions under which the cyclone train had to operate.

6.1.2 Demonstrated Need for Size-Classified Particles

The need for size-classified particulate material has been established, since the bioassays and chemical analysis have indicated that particulate fractions from the same industrial source do not necessarily possess similar characteristics. The elemental composition (determined by SSMS) of particulate samples collected at the same site often differed (e.g., Na in the aluminum smelter samples, Be and Al in the copper smelter samples, and Ag and U in the basic oxygen furnace samples). The GC-MS analysis of the 1-3μ samples of both the iron sintering plant and the ceramics plant differed substantially from the analyses of the respective 3-10μ samples. The RAM bioassay indicated different particulate toxicities for the aluminum smelter samples and the sludge incinerator samples (i.e., 1-3μ particles

compared to the 3-10μ particles). The mutagenic screening tests conducted by Litton-Bionetics indicated possible mutagenic activity for the aluminum smelter 1-3μ sample and the copper smelter 1-3μ sample, while the respective 3-10μ particles exhibited no mutagenic activity.

6.2 CHEMICAL ANALYSIS

The size-classified particulate material collected at the nine industrial sites was subjected to three types of chemical analysis: SSMS, GC-MS, and HRMS. Aliquots of each particulate sample were analyzed according to the procedures described in Section 4.1. No repetitive determinations were made for any analysis, except for two particulate samples analyzed by SSMS in independent laboratories. The chemical analyses were conducted on intact particulate samples.

6.2.1 Spark Source Mass Spectrometry

Several SSMS scans were conducted on the particulate samples in order to identify trace species as well as major constituents (see Table 3). In addition, two samples (i.e., coke oven heater 1-3μ and copper smelter 1-3μ) were analyzed for major and minor species by an independent laboratory (EMSL/RTP). Since no repetitive analyses were performed by each laboratory, the precision of the data presented in Table 3 has not been determined. The SSMS technique is estimated to be accurate to within 200 to 500 percent.

The SSMS analysis identifies the elemental composition of a given sample, and does not determine the chemical matrix of the sample, nor does it reflect the availability of a constituent to a biological system. It is a useful survey tool, however, in that it can detect the presence of over seventy elements in a particulate sample.

6.2.2 Gas Chromatography-Mass Spectrometry

The GC-MS analysis focused on polycyclic hydrocarbons of known carcinogenic potential or structural analogues. Results were reported as nanograms of a certain species detected in the entire sample, and converted to parts per million by weight (ng species/mg sample). Since an accurate determination of sample mass collected on the filter material could not be made, filter analyses are reported as total nanograms present. All major hydrocarbon peaks were identified in the mass spectra, but there were additional peaks present (but not identified) that did not correspond to known polycyclic hydrocarbon carcinogens. Size-classified particles collected at the same site did not always have comparable analyses (e.g., iron sintering plant, ceramics plant). The aluminum smelter particulate samples contained by far the largest amounts of polycyclic hydrocarbons detected in any of the samples analyzed.

6.2.3 High Resolution Mass Spectrometry

The constituents identified by HRMS in the fourteen samples analyzed are presented in Table 5. Only that portion of the particulate sample that vaporized under the test conditions would be detected in the mass spectra. Interpretation of the mass spectra indicates the mass number of the compounds present but does not specify the isomer. The resulting hydrocarbon masses can then be screened for those precise masses that would indicate the possible presence of known hazardous and/or toxic compounds. This method determines only the presence of a specified mass, and cannot accurately quantify it. The presence of a precise mass indicates only that the suspect structure is possibly present, since the isomeric form having that mass is undetermined.

Analysis of the copper smelter samples indicated an intense spectral peak identified as As_4O_6, the dimer of As_2O_3. The strong As_4O_6 peak prevented further computer analysis of spectra from both samples (1-3μ and 3-10μ). Since studies of pure 4- to 6-ring aromatic hydrocarbon compounds, representative of those detected on the aluminum smelter samples, have shown that their rate of vaporization varies with ring number and type of condensation, the aluminum smelter samples were subjected to an additional semi-quantitative HRMS analysis. Mass spectra from all fourteen particulate samples were screened for those precise masses associated with nine known carcinogenic aromatic hydrocarbons (Table 7). Spectra from the two aluminum smelter samples indicated the presence of all nine precise masses; none of the remaining samples contained any of those nine precise masses.

6.2.4 Comparison of Analytical Results

The elemental composition of two particulate samples (i.e., coke oven heater 1-3μ, copper smelter 1-3μ) analyzed by different laboratories show reasonable agreement, considering the accuracy of the technique. The arsenic concentration in both copper smelter samples is substantially higher than other particulate samples analyzed by SSMS. The intense As_4O_6 spectral peaks obtained by HRMS analysis of the same two samples substantiate the SSMS results. Results from the HRMS of the particulate samples collected at the iron sintering plant appear consistent although the percent of each sample vaporized differed substantially (see Table 5). The GC-MS analysis of the same particulate samples (i.e., iron sintering plant, 1-3 and 3-10μ) detected pocyclyclic organic species in the 1-3μ sample only.

Data presented in Tables 4 and 6 permit comparisons between coronene and dibenzocarbazole concentrations in the 1-3μ and 3-10μ aluminum smelter samples as determined by GC-MS or HRMS analysis. In each

case, the HRMS analysis consistently indicated higher constituent concentrations than determined by GC-MS analysis. With HRMS, coronene was detected at concentrations five times higher and six times higher in the 1-3μ and 3-10μ samples, respectively, than levels determined by GC-MS. Dibenzocarbazole was detected by HRMS at seven and two times the level determined by GC-MS for the 1-3μ or the 3-10μ sample, respectively.

6.3 BIOLOGICAL CHARACTERIZATION

The biological activities of the size-classified particulate samples have been categorized using two in vitro bioassays, one to determine the acute cytotoxic nature of the samples, and the other to determine whether any of the samples are mutagenically active. Both bioassays have indicated their utility in studying the effects of certain pure compounds, but neither has been used extensively to study complex mixtures. The following sections discuss the two bioassays, as performed, and note procedural problems that were encountered during the testing of the complex particulate samples and evaluation of the results. This research study examined the ability of each bioassay to assess specific biological characteristics of complex particulate samples.

6.3.1 RAM Cytotoxicity Bioassay

The effects on rabbit alveolar macrophase (RAM) viability from exposure to particulate material (including soluble and/or insoluble fractions) are provided in Figures 4 through 13. In those figures, RAM cell viability, expressed as percent of controls, is linearly regressed with the common logarithm of the particle concentration in the culture medium. In an attempt to improve the linearity of the experimental data, additional transformations were applied (e.g., probit, logit), but no improvement was evident.

Results from the basic oxygen furnace (1-3μ and 3-10μ) are not reported, since they appeared non-toxic at 1000μg/ml and were not subjected to further testing. The methods used to obtain the particles plus supernatant (P+S) fraction, the particle (P) fraction, and the supernatant (S) fraction are explained in Section 4.2.1. The number of observations per exposure level varied from six observations at 1000 g/ml to three observations at 300μg/ml or 100μg/ml.* In Figures 12 and 13, three observations per exposure level in each expanded concentration-response trial were reported.

The cytotoxic effects of particulate material collected on the filters are provided in Table 8. During the collection of the size-classified particulate material, heavy grain loading conditions existed at three sites and required the replacement of the filters. Original filters and their replacements were subjected to the RAM bioassay procedure. The cytotoxic nature of the sub-micron particles cannot be directly compared to these of the 1-3μ or 3-10μ samples collected at the same industrial site, because the sub-micron particulate concentration to which the RAM cells were exposed could not be quantified (due to thermal degradation of the filter material).

Figures 4-11 indicate that there is a general tendency for the 1-3μ particles to be more toxic than the respective 3-10μ particles collected from the same site (e.g., sludge incinerator, Figure 10).

The toxic nature of the 1-3μ and 3-10μ particulate samples appears to be associated with the particles themselves, since the particles plus

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*In a few instances, only two observations were reported at 100μg/ml.

supernatant (P+S) or the particle (P) fractions are consistently more toxic than the respective supernatant (S) fraction for particles from a specific industrial site (notable exceptions are the copper smelter samples and the oil-fired power plant sample, Figures 8 and 11).

Figures 12 and 13 provide concentration-response data for two particulate samples tested over a greater exposure range than the remainder of the particulate samples. Figure 12 (copper smelter 3-10μ sample, supernatant fraction) indicates that the log concentration-response relationship is not linear over the entire particulate exposure range, while the relationship expressed in Figure 13 (sludge incinerator 1-3μ sample, particles plus supernatant fraction) appears consistent for both preliminary and expanded tests. It would appear that data obtained over a wider than 10-fold exposure range (e.g., 100μg/ml to 1000μg/ml) is required to adequately determine the concentration-response relationships for certain of the particulate samples in the RAM bioassay.

Existing concentration-response data were insufficient to permit a proportional ranking of the particulate samples based on their observed cytotoxic nature. Since the cytotoxic nature of the samples differed widely, any comparison of the particulate samples at a specific level of response (e.g., LD_{50}s*) required extrapolation of the concentration-response lines for some of the samples. Figures 12 and 13 have indicated that extrapolation is ill-advised. If all concentration-response relationships are to be compared, both the slopes and intercepts of the regressions must be considered. Over a limited exposure concentration/response range, the regression

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*that concentration lethal to fifty percent of an exposed population within a specified length of time.

estimates of the slopes and intercepts may not be representative of the relationship over a broader concentration range (see Figure 12). When regression equations describing several particulate samples have unequal slopes (i.e., dose-response lines not parallel), an interpretation of comparative toxicities of the samples depends on the level of response considered.

Particulate samples tested by the RAM bioassay can be ranked, on an ordinal scale, according to observed cytotoxicity, from most to least toxic. Separate rank orders, based on cell viability (mean $\pm$ 1 SD) can be established to include all samples tested at each particular test concentration (i.e., 1000µg/ml, 300µg/ml, 100µg/ml) and for each test fraction (i.e., P+S, P, or S). Non-parametric statistical procedures (Kendall's coefficient of concordance test[13]) indicate that the toxicity rankings established at each test fraction or at each test concentration are not significantly different ($\alpha = 0.01$). Furthermore, using Spearman's rank correlation test or Kendall's tau,[13] all pairs of ranks within test fractions at different particulate concentrations, or between test fractions at the same particle concentration were found to be strongly associated ($\alpha = 0.01$). In effect, these statistical tests indicate that the ordinal ranking of industrial sites based on observed RAM cytotoxicity is independent of test concentration, over the range tested, and independent of testing procedure (i.e., test fraction). All rank orders tended to agree as to which industrial particulate samples were more toxic and which samples were less toxic. In Table 12, the particulate samples have been organized into three toxic categories (i.e., relatively high cytotoxicity, intermediate cytotoxicity, and relatively low cytotoxicity) that reflect the overall performance of each particulate sample in the ordinal rankings for each particulate concentration tested or each test fraction. The order in which each

TABLE 12

RELATIVE CYTOTOXIC NATURE OF THE INDUSTRIAL PARTICULATE SAMPLES[a]

RELATIVELY HIGH CYTOTOXICITY

Oil-Fired Power Plant, 1-3μ

Copper Smelter, 1-3μ

Copper Smelter, 3-10μ

Aluminum Smelter, 1-3μ

Sludge Incinerator, 1-3μ

INTERMEDIATE CYTOTOXICITY

Aluminum Smelter, 3-10μ

Iron Sintering Plant, 1-3μ

Open Hearth Furnace, 1-3μ

Open Hearth Furnace, 3-10μ

Coke Oven Heater, 1-3μ

RELATIVELY LOW CYTOTOXICITY

Iron Sintering Plant, 3-10μ

Ceramics Plant, 1-3μ

Ceramics Plant, 3-10μ

Sludge Incinerator, 3-10μ

Basic Oxygen Furnace, 1-3μ

Basic Oxygen Furnace, 3-10μ

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[a]Based on ordinal ranking of particulate samples at all test concentrations for all test fractions

Order within categories are arbitrary

Coke Oven Heater, 3-10 μ --insufficient testing to permit ranking

Oil-Fired Power Plant, 3-10μ, and paper mill samples--no bioassays conducted

particulate sample appears within each of the three categories is arbitrary and should not be construed to represent differences in relative cytotoxicities.

The RAM bioassay, as performed in this research effort, encountered some procedural problems that required attention. Particles tended to agglomerate when added to the test system. Not only could the effective particle size to which the RAM cells were exposed be changed, but the degree to which various chemical constituents would solubilize in the bioassay medium could be affected. The agglomeration also led to difficulty in counting cells at the termination of a trial (via hemocytometer). The exclusion of trypan blue dye was the criterion by which cell viabilities were estimated. Additional response indicators, including measurements related to membrane integrity and functional impairment, have been developed to add sensitivity to the RAM bioassay and are available for future, more-intensive studies.

6.3.2 Mutagenesis Bioassay

The mutagenic activities of eleven particulate samples on three S. typhimurium bacterial strains under the test conditions specified in Section 4.2.2 are presented in Tables 9 and 10. The screening tests, as conducted by Litton-Bionetics, indicated that one sample, the aluminum smelter 1-3μ material, caused a weak dose-dependent mutagenic response in two of the three bacterial strains (TA-1537, TA-1538), while a second sample, the copper smelter 1-3μ material, indicated possible mutagenic activity in one strain (TA-1538). Each mutagenic response occurred with the mouse liver activation system. All nine remaining particulate samples possessed no detectable mutagenic activity under the test conditions.

The parallel mutagenic screening tests that RTI performed* on three particulate samples (i.e., copper smelter 3-10μ, ceramics plant 3-10μ, and sludge incinerator 3-10μ samples) are presented in Table 11, where mutagenic activity is described in terms of the number of revertants (mutants) per plate. Since only two observations per treatment level were performed, the average number of revertant colonies are reported. No consistent increase in the number of revertants on treated plates relative to controls is evident. Slight increases in the number of revertants per plate resulting from exposure to the ceramics plant 3-10μ sample are inconclusive.

The exposure levels that each laboratory utilized in the mutagenic screening tests were different, often by a factor of 100. Results of those samples tested by both RTI and Litton-Bionetics are comparable. Two samples (i.e., aluminum smelter 1-3μ and copper smelter 1-3μ samples) were identified by Litton-Bionetics as possessing weakly mutagenic activity under the laboratory test conditions.

The mutagenic activity of a compound is usually expressed as the ratio of the number of revertants resulting from treatment relative to the number of spontaneous revertants in suitable controls. Mutagenic activity is indicated as the ratio becomes significantly greater than 1.0. If exposure doses in mutagenic screening tests are sufficient to produce noticeable bacterial toxicity, the number of revertants per plate can be normalized to reflect the anticipated decrease in the number of viable cells exposed to the test compound. If this normalization procedure is attempted, the preliminary toxicity screening test must be sufficient to determine a representative dose-response toxicity relationship.

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*RTI employed a modification of the Litton-Bionetics protocol (see Section 5.2).

The mutagenesis bioassay procedure utilized in this study should be viewed as an initial attempt to screen complex particulate material for mutagenic activity under controlled laboratory conditions. Because of its broad solvent characteristics, DMSO was chosen as the solvent vehicle used to introduce all particulate samples into the test system. The solubility of a particulate sample in DMSO varied depending on the laboratory mixing technique employed (e.g., sonication versus manual). The irregular dose-response relationships between particulate exposure and survival of bacterial populations reflected the difficulties faced when testing a complex sample. The testing protocol employed by Litton-Bionetics and RTI were similar except for those points mentioned in Section 5.2. However, the additional effort expended in following the RTI modifications was substantial. The benefits of each test procedure should be evaluated, and if comparable, the less expensive one (Litton-Bionetics) should be suggested for routine, preliminary screening tests.

6.4 COMPARISON OF CHEMICAL ANALYSIS TO OBSERVED BIOLOGICAL ACTIVITY

An objective of this research was to determine whether the observed biological activity of the particulate samples could be correlated to the identified chemical composition of each sample. Since the chemical analyses are intended for routine screening of large numbers of samples, it is impractical to perform a comprehensive chemical analysis on a given particulate sample, until the need to do so has been identified. The chemical analyses utilized in this research were selected to offer a screening tool with the capacity to provide a substantial amount of chemical information about the intact particulate sample for a relatively small investment. The SSMS technique can rapidly assay for over seventy elements, although the chemical matrix of the sample is not provided. HRMS can suggest the presence of numerous hazardous and/or toxic compounds in the particulate sample, but without additional effort, those compounds cannot be quantified.

The GC-MS analysis has focused on identifying polycyclic hydrocarbons of known carcinogenic potential, and by inference, those with mutagenic potential.

Initial comparisons of the chemical data in terms of observed cytotoxicity involved the determination of the relationship of the individual elemental concentrations in the particulate samples to the observed cytotoxicity of that sample. A best-fit regression line was determined individually for each of the elements by plotting the logarithm of the element's concentration in the total sample versus the observed cytotoxicity (as percent viability) for that sample for each test fraction (i.e., P+S, P, and S) at 1000µg/ml exposure. This statistical approach assumes that no interaction occurs between the various element's effects on observed cytotoxicity. No strong correlation was found between individual elemental concentrations and observed cytotoxicity in a given sample. Since the SSMS data do not provide information as to the biological availability or chemical form of those elements present, strong correlation should not be expected. Two particulate samples could be identical in elemental composition, but due to different chemical compounding, possess vastly different biological activities.

Additional statistical evaluations considered the correlation of observed cytotoxicity with a calculated toxicity index that reflected the relative hazard, on a scale from one to ten, of the chemical constituents (elemental and organic) detected in the particulate samples. The scale presented in Table 13 is based on empirical determination of acute lethality in small laboratory animals.[14] The following equation represents the chemical toxicity index used:

TABLE 13
RELATIVE RANKING OF IDENTIFIED CHEMICAL CONSTITUENTS BASED ON ACUTE TOXICITY

Species	Toxicity Rating[a]	TLV[b]	Species	Toxicity Rating[a]	TLV[b]	Species	Toxicity Rating[a]	TLV[b]
Aluminum	3		Lead*	7	0.15	Thorium	5	
Antimony*	7	0.5	Lithium	5		Thullium	3	
Arsenic*	7	0.5	Lutecium	3		Tin*	4	2.0
Barium	4		Magnesium	3		Titanium*	6	10
Beryllium*	10	0.002	Manganese*	6	5	Tungsten	4	
Bismuth*	6		Molybdenum*	6	5	Uranium	3	
Boron	3		Neodymium	3		Vanadium*	8	0.05
Bromine	3		Nickel*	3	1.0	Ytterbium	3	
Cadmium*	7	0.1	Niobium	2		Yttrium	5	
Calcium	3		Osmium	10		Zinc*	5	5
Cerium	4		Palladium	3		Zirconium	2	
Cesium	1		Phosphorus	7				
Chlorine	6		Platinum	5				
Chromium*	3	0.5	Potassium	3		Anthracene/Phenanthrene	5	
Cobalt*	5	0.1	Praseodymium	3		Methyl Anthracenes	5	
Copper*	3	0.1	Rhodium	3		Fluoranthene	1	
Dysprosium	3		Rubidium	2		Pyrene	1	
Erbium	3		Ruthenium	3		Methyl Pyrene/Fluoranthene	1	
Europium	3		Samarium	3		Chrysene/Benz(a)Anthracene	7	
Fluorine	7		Scandium	4		Methyl Chrysenes	4	
Gadolinium	3		Selenium*	5	0.2	Benzo Fluoranthenes	5	
Gallium	3		Silicon	1		Benzo(a)Pyrene	4	
Germanium	2		Silver*	3	0.01	Benzo(e)Pyrene	2	
Gold	1		Sodium	3		3-Methylcholanthrene	5	
Hafnium	3		Strontium	3		Indeno(1,2,3-cd)Pyrene	5	
Holmium	3		Sulfur	7		Benzo(ghi)Perylene	7	
Iodine	1		Tantalum	1		Dibenz(a,h)Anthracene	3	
Iridium	3		Tellurium*	3	0.1	Dibenzo(c,g)Carbazole	7	
Iron	3		Terbium	3		Dibenzo(a,i and a,h)Pyrenes	7	
Lanthanum	7		Thallium*	8	0.1	Coronene	5	

[a]Toxicity rating based on most probable chemical form, most comparable exposure route, most representative test species; obtained from ref. 14

[b]Threshold limit values (expressed as mg/m^3) from ref. 15

*20 most influential elements

$$TI_{tr} = \sum_{i} TR_i \times [C]_i \qquad \text{eq. (1)}$$

where:

TI_{tr} = Toxicity Index (calculated)

TR_i = Toxicity Rating for the element or organic species

$[C]_i$ = Concentration of individual element or species identified in a particulate sample

Equation (1) represents an extremely simplified approach to a very complex problem. In this equation, no consideration is given to the possible biological availability of the constituents, nor is the solubility of any component factored in. Since specific inorganic species were not identified in the chemical analysis, the toxicity rating required flexibility in ranking the toxic components. In constructing the toxicity rating, lethality data for the most probable form of an element was utilized. The toxicity rating itself is a compromise since it is extremely difficult to compare toxic dosages of a given substance across test species, routes of exposure, or times of exposure. It is acknowledged that a one-to-ten scale does not completely reflect the relative differences in toxicities between chemical compounds.

The common logarithm of the toxicity index (eq. 1) for each particulate sample was plotted versus its observed cytotoxicity, expressed as percent viability, tested at 1000µg/ml. A best-fit regression line was fitted to the data from all particulate samples tested for each bioassay test fraction (i.e., P+S, P, and S). The resulting best-fit regression line possessed a correlation coefficient (r) of -0.09 for the P+S fraction, an r= -0.23 for the P fraction, and an r= -0.23 for the S fraction. Negative correlation coefficients

indicate the expected relationship between macrophage viability and constituent concentration. A correlation is stronger between the variables as the correlation coefficient approaches $\pm$ 1.0.

In examining the data, it was noted that approximately twenty elements in Table 13 consistently provided the largest proportionate impact on the toxicity index (TI) summation for the particulate samples. A second iteration of equation (1) was performed, with the TI values determined solely from those twenty elements. The twenty elements are designated in Table 13. The regression equations possessed the following correlation coefficients: P+S fraction, r= -0.63; P fraction, r= 0.74; and S fraction, r= -0.62.

A second toxicity index was devised to assess the ranking system employed in equation (1), since the toxicity rating of one-to-ten might not be sensitive enough to reflect large differences in toxicity. Using the threshold limit values (TLVs)[15] of the twenty influential elements, the following was performed:

$$TI_{tlv} = \sum_i \frac{[E]_i}{TLV_e} \qquad \text{eq. (2)}$$

where

TI_{tlv} = toxicity index (calculated)

$[E]_i$ = concentration of individual element in a particulate sample

TLV_e = threshold limit value* for the element

The logarithm of the toxicity index (eq. 2) for each particulate sample was plotted versus the observed cytotoxicity (as percent viability) for that sample. The resulting best-fit regression

*TLVs represent the lowest suggested value for the element (or most likely compound (ref. 15)).

line possessed a correlation coefficient (r) of -0.62 for the P+S fraction, an r= -0.62 for the P fraction and an r= -0.65 for the S fraction, comparable to results using equation 1. One should be cognizant of the fact that suggested TLVs present the relative hazard associated with each element; however, the criteria to assess hazards include (among others) inherent toxicity, carcinogenic and mutagenic potential, odor thresholds, and tendency to accumulate in the body.

Several implicit assumptions are made when interpreting the experimental data according to equation (1) or (2). The toxicity index determinations assume a linear dose-response relationship to quantify suggested TR or TLV with observed constituent concentrations. The method assumes strictly additive effects or no interaction between various constituents. In addition, this treatment assumes that all components of toxicological importance have been identified and properly quantified.

Results of SSMS and GC-MS analyses were incorporated in this evaluation. The results from the HRMS analysis could not be directly applied since the presence of known toxic components are only suggested by HRMS.

The improved correlation obtained when considering a toxicity index based on the twenty most influential rather than the total chemical analysis suggest inadequacies in the model. However, this information can be used to provide direction for more intensive studies to determine casual relationships between chemical composition and observed biological activity.

The comparison of mutagenic bioassay results with the chemical analysis of the particulate samples is somewhat limited. Of the

eleven samples tested for mutagenic activity, only two samples (i.e., copper smelter, 1-3μ sample, and aluminum smelter, 1-3μ sample) indicated positive findings. Since the mutagenic screening test performed in this research is relatively insensitive to inorganic mutagens, only the organic analyses were considered. The aluminum smelter samples did contain by far the greatest concentrations of polycyclic hydrocarbons, and both samples possibly contained all nine highly carcinogenic aromatic constituents identified by HRMS (see Table 7). However, both aluminum smelter samples were tested for mutagenic activity, and only the 1-3μ sample tested positive. The copper smelter 1-3μ sample indicated a weak mutagenic response, while tests of the 3-10μ sample were negative. Chemical analysis of the copper smelter samples were again very similar to each other; however, a very high concentration of arsenic was noted in each. Arsenic compounds have caused neoplasms in experimental animals,[14] and some investigators suggest a common mechanism for mutagenisis and carcinogenisis.

6.5 CONSIDERATIONS FOR FUTURE RESEARCH

This research effort should be evaluated in light of its intended goals as well as its programmatic constraints. Several analytical strategies found in PMB's environmental source assessment program were evaluated for their ability to characterize the potential environmental hazards associated with selected industrial particulate emissions. The scope of each analytical tool was designed to be compatible with its companion techniques and the quality of the sample that was assayed. To subject a sample that is not representative of a given source to extensive biological or chemical characterization would be an inefficient use of limited resources. To subject a sample obtained at great expense to a cursory biological testing regime would likewise not be advised. However, suggestions that

reflect the overall compatibility of the sampling and analysis strategy can be made to improve future environmental source assessment programs.

This research effort has recognized the need for a more suitable filter material with which to collect sub-micron particulate material. An ideal filter would be biologically and chemically inert, not subject to thermal degradation, and capable of satisfying the engineering features of the sampling train (e.g., pressure drop). The possible alteration of the particulate samples resulting from collection, handling, and disbursement is being considered in ongoing studies.

Several observations can be made concerning the chemical characterization of the particulate samples. The SSMS technique can be considered a useful survey tool for characterizing the particulate samples, and has the ability to focus interest on those samples with unusually high concentrations of potentially hazardous elements. The GC-MS analysis should not be considered a survey technique, but can quantitatively identify constituents of suspected hazardous samples. The HRMS analysis, although not easily quantifiable, can screen particulate samples for a wide variety of hazardous and/or toxic substances. Inorganic speciation and the partitioning of chemical species within the biological assay media would aid in assessing the potential biological availability of various constituents. Although those determinations were not made in this research effort, they are being considered for appropriate placement in the overall environmental source assessment program.

The two bioassay procedures performed in this research effort should be validated by comparison to currently recognized standard procedures so that routine screening tests can be compared to other studies. If a proportional ranking system is required of the RAM bioassay screening test, the protocol will require expansion to include extended

concentration-response data and additional response indicators (e.g., functional impairment, membrane integrity).

Additional solvent vehicles could be added to the mutagenicity screening test so that chemical constituents with low solubility in DMSO can also be evaluated for mutagenic activity. If normalization of exposed bacterial populations to reflect the inherent toxicity of the sample is envisioned, then consistent concentration-response data must be generated during testing. A comparison of alternate protocols (see Section 6.3.2) can define the better system for a specific testing level (i.e., Levels 1, 2, or 3).

It should be recognized that a complete and comprehensive environmental source assessment program dictates a sampling and analysis strategy whose philosophy and structure permit a maximum use of available resources.

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16. ABSTRACT The report gives results of chemical analyses and cellular biological assays performed on size-classified particulate material collected using a new series cyclone sampling train at nine industrial sites. The exercise was formulated to determine performance of the train and whether the chemical analyses or the bioassays, alone or in combination, were sufficient to characterize the hazards associated with particulate emissions. This program lends support to the view that size-classified particulate matter is needed for the various chemical or biological tests. Elemental analysis and partial organic characterization of the particulate samples have been performed. A cellular bioassay, utilizing rabbit alveolar macrophages, has been used to provide a rank ordering of particulate samples in terms of their observed cytotoxic activity. A bacterial screening technique, utilizing several histidine deficient Salmonella typhimurium strains, has been used to study the mutagenic potential of the particulate samples. Attempts to correlate observed biological activity with chemical analyses are provided.

17. KEY WORDS AND DOCUMENT ANALYSIS

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