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THE DETECTION OF POTENTIAL GENETIC HAZARDS IN COMPLEX ENVIRONMENTAL MIXTURES USING PLANT CYTOGENETICS AND MICROBIAL MUTAGENESIS ASSAYS

Milton J. Constantin and Karen Lowe, The University of Tennessee/Comparative Animal Research Laboratory, Oak Ridge, Tennessee, and T. K. Rao, Frank W. Larimer and James L. Epler, Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee

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DISCLAIMER

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INTRODUCTION

The overall evaluation of the health and environmental effects of modern technology has been expanded over recent years to include consideration of the long-term consequences of exposure to potential mutagens and carcinogens. The realization that the effluents and wastes from industry could pose health hazards for future generations has led to considerable research and the potential for regulation of these releases. The Research Conservation and Recovery Act (RCRA) specifically addresses the potential health and environmental hazards of solid wastes. The approach that we have taken with an array of complex mixtures from the technological world involves a coupling of chemical characterization and preparation with short-term bioassays.

The number of specific compounds and complex effluents and streams in need of biotesting and characterization in any technology is overwhelming and would tax the scientific community's whole-animal testing capabilities; therefore many "screening" (short-term) assays for toxicity, mutagenicity, teratogenicity, and carcinogenicity have been developed. The working hypothesis has been that the rapid tests can serve as predictors of biohazards early in the development of the technology. Thus, process changes might be initiated and/or definitive

whole-animal testing considered. Conceivably, savings in both time and money on the technology and the biological testing would be gained.

The possibility of using short-term biological tests to isolate and identify the potential hazard of complex materials has been investigated with various coupled chemical and biological approaches. The work has usually involved chemical analyses and preparation for the biological testing (e.g., cytotoxicity, mutagenicity, and carcinogenicity assays). As a prescreen for potential mutagenesis and/or carcinogenesis, the genetic test for Salmonella histidine reversion [Ames test; see review (1)] has been widely used. Subsequent fractionation procedures to aid in isolating and identifying the mutagens in the material are carried out, with the bioassay being used as a tool to trace the biological activity and guide the separations. The biological tests function as (1) predictors of long-range health effects such as mutagenesis, teratogenesis, and/or carcinogenesis, (2) predictors of toxicity for man and his environment, (3) a mechanism to rapidly isolate and identify a hazardous agent in a complex material, and (4) a key to relative biological activity through the correlation of control data with changes in environmental or process conditions.

Plant systems may offer an additional means to evaluate complex mixtures. According to Kihlman (9) root tips are the ideal plant tissue in which to study the effects of chemicals on chromosomes, because (1) they are readily available throughout the year, (2) they are inexpensive,

(3) they are easy to handle, (4) they provide data within a few days, (5) they are directly exposed to the chemical in aqueous solutions of known concentrations, (6) they provide numerous dividing cells for analysis, and (7) they offer the advantages of few, relatively large chromosomes.

Barley, Hordeum vulgare (L) emend Lam., has 2n = 14 chromosomes that range from 6 to 8 μm in length. The barley embryo has from 5 to 7 seminal roots that yield numerous dividing cells for analysis within 24 to 48 hrs after the initiation of germination. Effects of chemical and physical agents can be assessed in terms of cytogenetic and genetic endpoints in the same population of plants following seed treatments. This unique advantage has made barley a useful test organism for studies of mutagenesis.

The most frequently used method for studying the effects of chemicals on the chromosomes of barley root tip cells is the analysis of anaphases for detectable aberrations (16). The assay has been used in studies of chemicals either suspected or known to be mutagens; generally the results have been in agreement with those derived from other test organisms for those compounds tested in common. The assay has not been used widely to test complex mixtures.

In this report, we have carried out a comparative study with microbial mutagenicity assays (Salmonella and yeast) and plant cytogenetic

assays. The materials utilized were aqueous extracts from a fly ash sample and an arsenic-contaminated ground water sample (Provided by the U. S. Environmental Protection Agency). The studies aid in evaluating the usefulness of short-term bioassays with complex environmental mixtures.

PURPOSE

The research was designed to assess the mutagenic potential of complex environmental mixtures (extracts of solid wastes) in terms of cytogenetic effects in a higher plant and of gene mutations in microbial species. A complex environmental mixture that is capable of inducing both endpoints warrants a close scrutiny as a potential health hazard to humans.

MATERIALS AND METHODS

Microbial Mutagenicity Assays

Preparation of liver-homogenate (S-9). The microsomal preparation was made according to Ames et al. [1]. The liver of Sprague-Dawley rats was washed in an equal volume of 0.15M NaCl, minced with sterile scissors, and homogenized in 3 vols. of 0.15M KCl with a Potter-Elvehjem apparatus. The homogenate was centrifuged at 4°C for 10 min at 9000 x g. The supernatant was collected and stored at -80°C. The activation system (S-9) mix contained per ml: 0.3 ml of liver homogenate,

8 μmol $MgCl_2$, 33 μmol KCl, 5 μmol glucose 6-phosphate, and 4 μmol nicotinamide adenine dinucleotide phosphate in 100 μmol of sodium phosphate buffer (pH 7.4).

Bacterial mutagenicity assay. Among various bacterial mutagenicity test systems, the assay developed by Ames utilizing the histidine auxotrophic strains of Salmonella typhimurium is very widely used as a prescreen in the detection of potential genetic and carcinogenic hazards. The standard set of four tester strains are generally applied in the assays. The missense strains TA-100 and TA-1535 are designed to detect base pair substitutions while strains TA-1537 and TA-98 are used to detect frameshift mutations. The general procedure was described by Ames et al. [1]. A bacterial suspension (2×10^8/ml) was added in 2 ml of molten top agar (45°C) containing test substance. S-9 mix (0.5 ml/plate) was added when required to incorporate metabolic activation. The top agar was overlayed on Vogel- onner [19] minimal media and revertant colonies were counted after 2 days of incubation at 37°C. Known chemical mutagens as positive controls and solvent controls were routinely run.

Yeast assay. The Saccharomyces assay utilizes both a forward and a reverse mutation scheme. Forward mutation is detected by the inactivation of the arginine permease gene (CAN1), leading to resistance (can^r) to the toxic antimetabolite canavanine. Reverse mutation is monitored by use of a histidine auxotroph (his 1-7) which reverts by base-pair substitution.

The test strain used in these experiments was constructed from stocks of Saccharomyces cerevisiae maintained at ORNL and from stocks obtained from Berkeley collection. Strain XL7-10B has the genotype α p^+ CAN1 his 1-7 lys 1-1 ura 1.

Details of the preparation of media, rat liver homogenates, and the mutagenicity assay have been described [12,13]. The test material was mixed with yeast cells in buffer or rat liver homogenate (S-9) in buffer and incubated for 3 or 24 h. at 30°C with shaking before it was plated on selective media. Mutant clones were scored on selective plates after 5 days incubation at 30°C. For determination of survival, dilutions were plated on yeast extract-peptone-dextrose plates and scored after 2 days at 30°C.

Plant cytogenetic assay. Seeds of Himalaya barley from R. A. Nilan, Washington State University, Pullman, stored under refrigeration, were hand-picked for quality just prior to each experiment. Approximately 25 seeds were sown embryo-side-up on Whatman #1 filter paper in glass petri plates. The filter paper was thoroughly saturated by adding 7.5 ml of either double-distilled water or a test solution at pH 7.0. Each treatment was done in triplicate; plates were placed in sealed polyethylene bags, and cultured for 42-46 h at 25°C under 10-15 μ EMS fluorescent light.

Germinating seeds were killed and fixed in acetic ethanol (3:1 ethanol glacial acetic acid) and stored in the refrigerator until the roots were processed. Excised roots were hydrolyzed for 9 min in 1 N HCl at 60°C,

reacted with Schiff's Reagent for at least 15 min, treated with pectinase (0.4% in water, pH 4.0) for at least 30 min, squashed in aceto-carmine, and Deckglaskitt was used to seal around the coverslip. Cells were observed at 1000X magnification for the presence of bridges and fragments at anaphase.

Data were expressed as aberrations per 100 anaphases and as percent of aberrant anaphases. Statistical inferences were drawn on the bases of analysis of variance, and also 2x2 contingency chi-square tables for aberrant versus normal anaphases in control versus chemical treatments.

Preparation of samples. Aqueous extracts of the fly ash samples were prepared as given in the federal register Vol. 43, No. 243, page 58946 (1978). The As-contaminated sample was used as provided by the Environmental Protection Agency. Resin concentration (XAD-2) procedures were previously described [4]. A 500-ml. aqueous extract is passed through 4 g XAD-2 (Isolab, Inc.) at a flow rate of 1.2 ml/min. The column was rinsed with deionized water, and then eluted with acetone. The acetone eluate was evaporated to dryness and the sample was dissolved in 2 ml. dimethylsulfoxide.

RESULTS AND DISCUSSION

Salmonella assay

Aqueous samples from the As-contaminated ground water and its XAD-2 concentrate (12-5-fold, v/v) were tested for mutagenicity at nontoxic dose range. The results obtained are given in igure 1. The ground

water sample was not mutagenic (Fig. 1 A) even with metabolic activation for strains TA-98 and TA-100. However, the XAD-2 concentrate of ground water (Fig. 1 A) even with metabolic activation for strains TA-98 and TA-100. However, the XAD-2 concentrate of ground water (Fig. 1 B) exhibited a clear dose response with the frameshift strains TA-1537 and TA-98, and the highly sensitive TA-100 strain. The missense strain TA-1535 was not reverted. These results suggest the presence of mutagenic constituent(s) in the aqueous sample whose activity is evident only when a proper concentration method was coupled to the bioassay.

The EP extract and its XAD-2 concentrate from fly ash sample were not mutagenic when tested with the basic set of tester strains and the results are given in Table 1. Addition of metabolic activation system did not influence the result. Lack of mutagenic activity of fly ash from power plant is in conflict with earlier reports of Chrisp, Fisher and Lammert [3] and Kubitschek and Venta [10] that used serum extraction, and Hobbs et al. [8] that used dimethylsulfoxide for extraction. The EP extraction procedure is probably not adequate to extract mutagenic agents from fly ash. When benzene extraction was utilized with a similar fly ash sample obtained from a different power plant, a dose dependent increase in the induction of histidine revertants was observed (unpublished observation). Results obtained with known chemical mutagens (positive controls) are given in Table 1B. The alkylating agents EMS and MMS are

TABLE 1

SALMONELLA MUTATION ASSAY: FLYASH - EP AND ITS CONCENTRATE (XAD-2)

	his$^+$ revertants/plate							
	TA-1535		TA-1537		TA-98		TA-100	
	EP	XAD-2	EP	XAD-2	EP	XAD-2	EP	XAD-2
A. Concentration µl/Plate								
No activation								
Control[a]	21	NT	14	NT	32	57	136	231
50	18		15		27	30	141	180
Ar S-9 activation								
Control[b]	15	NT	16	NT	46	33	192	209
10	15		14		35	42	108	203
25	8		15		34	28	141	206
50	16		15		53	26	123	206
75	16		18		42	38	172	208
B. Positive Controls[c]								
50 µl (5%) EMS	<u>704</u>		11		48		386	
50 µl (5%) EMS	42		13		48		<u>1495</u>	
20 µg 8-AmQ	6		<u>102</u>		56		270	
20 µg B(a)P + S-9	7		<u>122</u>		<u>625</u>		<u>1700</u>	

[a] Solvent Control

[b] Solvent +S-9 control

[c] Numbers underscored represent positive response to mutagens

very specific in reverting TA-1535 and TA-100 respectively while the frameshift strains are reverted by 8-AmQ and B(a)P. 8 AmQ exhibits specificity to the strain TA-1537 and B(a)P is mutagenic only when activated with Ar-S-9 mix.

Saccharomyces mutation assay. The arsenic-contaminated ground water sample was not mutagenic (Table 2). The XAD-2 concentrate of this sample was mutagenic without metabolic activation for a 24 h. exposure. A dose-dependent response was observed. Metabolic activation appeared to reduce the mutagenic potential of the XAD-2 concentrate. Neither of these test materials was toxic. Yeast results show a moderate preponderance of induced forward mutation to can^r relative to induced reversion of the his 1-7 base-pair substitution. This is typical for a response (by this system) to a frameshifting agent.

The fly ash EP extract and its XAD-2 concentrate were nontoxic and nonmutagenic to yeast (Table 3).

Plant cytogenetic assay. Two methods of presentation have been adopted for the cytogenetic effects observed in barley root tip cells; viz., percentage of aberrant anaphases and aberrations per 100 anaphases. Table 4 shows the distribution of anaphases into normal and aberrant classes for negative, positive, and solvent controls versus complex environmental mixtures. The probability values shown in the far right-hand column were calculated by means of a 2 x 2 contingency chi-square table designed to test for interaction.

TABLE 2

YEAST MUTATION: As-CONTAMINATED GROUNDWATER AND ITS XAD-2 CONCENTRATE

Concentration (μl)	% Survival				$can^r/10^7$ survivors				$HIS^+/10^7$ survivors			
	3 hr		24 hr		3 hr		24 hr		3 hr		24 hr	
	Sample	XAD-2	Sample	XAD-2	Sample	XAD-2	Sample	XAD-2	Sample	XAD-2	Sample	XAD-2
No activation												
Control	100	100	100	100	27	24	19	15	6	13	11	6
0.1	71		94		23		16		16		8	
1.0	84		81		20		19		8		8	
10	75	116	85	95	13	19	19	17	13	8	9	9
20		127		98		15		34		8		24
50		138		93		17		69		6		37
100	89	156	99	86	16	17	13	176	9	9	11	55
φB activation												
Control	100	100	100	100	14	17	17	15	12	10	12	8
0.1	107		103		21		14		10		9	
1.0	59		87		27		21		10		10	
10	82	105	103	94	21	18	14	19	14	11	9	12
20		98		86		20		31		13		6
50		103		79		16		89		8		29
100	91	112	119	71	18	21	18	107	12	7	7	24
Ar activation												
Control	100	100	100	100	17	15	21	14	9	9	11	6
0.1	97		94		18		20		11		9	
1.0	92		96		13		27		8		7	
10	87	107	91	108	21	12	22	13	12	10	10	8
20		111		122		25		41		11		9
50		122		91		13		74		5		17
100	88	90	103	82	24	13	22	118	11	8	13	38
EMS, 1% v/v	22				448				1270			

TABLE 3

YEAST MUTATION: FLY ASH EP EXTRACT AND ITS XAD-2 CONCENTRATE

Concentration (µl)	% Survival				$can^r/10^7$ survivors				$HIS^+/10^7$ survivors			
	3 hr		24 hr		3 hr		24 hr		3 hr		24 hr	
	EP	XAD-2	EP	XAD-2	EP	XAD-2	EP	XAD-2	EP	XAD-2	EP	XAD-2
No activation												
Control	100	100	100	100	12	26	19	24	13	10	13	6
0.1	104		101		18		18		5		10	
1.0	109		96		14		13		6		15	
10	94	103	97	96	14	25	12	27	9	6	16	8
20		105		98		27		25		6		7
50		102		86		23		22		8		8
100	97	96	99	92	11	23	16	23	9	9	10	10
φB activation												
Control	100		100	100	17	24	16	23	11	9	9	8
0.1	106		107		20		11		9		8	
1.0	100		102		12		13		3		14	
10	101		98	102	17	25	17	19	9	6	6	9
20				107		23		19		8		11
50				96		18		22		11		7
100	94		109	89	10	29	13	27	6	9	8	11
Ar activation												
Control	100	100	100	100	21	22	14	25	13	6	10	11
0.1	95		93		17		11		6		8	
1.0	93		103		13		10		8		14	
10	103	90	92	102	13	20	19	19	12	9	5	9
20		91		94		21		20		8		12
50		86		92		28		25		8		7
100	109	80	97	84	14	23	13	18	11	6	7	10
B(a)P, 100 µg Ar-Activation			89				181				52	

The solvent controls (phosphate buffer and acetic acid extraction solution) yielded aberrant anaphases at a frequency that did not differ from that of the negative control, distilled water. In contrast, the frequency of aberrant anaphases was increased in the population of seeds treated with ethyl methanesulfonate, a positive control, and in those treated with the fly ash extract and the arsenic-contaminated ground water. The fly ash extract was used as we received it except for the adjustment to pH 7.0 whereas the arsenic-contaminated ground water was diluted 1:8 and 1:16 with phosphate buffer. The more concentrated solutions were toxic to the germinating seed, i.e., germination was delayed and root growth was inhibited.

Table 5 shows the same experimental data as shown in Table 4 but expressed as aberrations per 100 cells (mean plus or minus one standard deviation for each treatment population). An arc sin percent transformation of the data was done prior to doing the analysis of variance to reduce the nonhomogeneity of the treatment variances. Results of a Student-Neuman-Keul comparison of treatment means [18] showed no difference between the negative control (distilled water) and either of the solvent controls (phosphate buffer and acetic acid extraction solution) whereas the positive control (ethyl methanesulfonate) and the two complex environmental mixtures (fly ash extract and the arsenic-contaminated ground water) induced a significantly increased number of aberrations per 100 anaphases.

Although the two methods of data presentation address different aspects of the cell population's response to seed treatment, the conclusion is the same. That is, that the barley root tip system responded to unknown mutagenic substance(s) in the two complex environmental mixtures in a manner similar to its response to ethylmethanesulfonate, a known mutagen. This response was observed as an increase in the percent of aberrant anaphases and in the number of aberrations per 100 anaphases.

Our results with the barley root tip cytogenetic aberration assay agree with expectations. According to Brewen and Preston (2) structural changes in chromosomes constitute a significant proportion of mutagenic events. Chemicals that induce mutations in eukaryotes invariably also induce chromosomal structural changes (5,11). Fly ash from coal combustion has been reported to be mutagenic in bacteria and known mutagens have been isolated and identified. Arsenic and heavy metals are known to induce both mutations and cytogenetic effects.

Chrisp, Fisher and Lammert (3) reported that horse serum, phosphate-buffered saline, and cyclohexane filtrates of fly ash of a mass median diameter of 2.2 μm, the finest particle size fraction tested, induced an increased number of histidine revertants in Salmonella typhimurium strains TA-98 and TA-1538. The rank order of activity was horse serum >>cyclohexane > saline. In a more recent report, Fisher, Chrisp and Raabe (6)

Table 4 List of seed treatments, the number of normal and aberrant anaphases observed, and the probability for chi-square values for the comparisons of treatment frequencies.

Treatment	Anaphases Observed			P [1]
	Normal	Aberrant		
	N	N	%	
Distilled Water	2182	48	2.15	
Phosphate Buffer [2]	2197	75	3.30	0.05 - 0.10
EMS [3]	2241	184	7.59	< 0.001
Acetic Acid Extraction Solution [4]	3784	88	2.27	0.30 - 0.50
Fly Ash Extract [5]	2874	437	13.20	< 0.001
As-Contaminated Ground Water [6]	1221	190	13.47	< 0.001

[1] 2x2 Contingency tables were used to compute chi-square for interaction; P = probability of difference being due to chance.

[2] Monobasic and dibasic phosphate buffer at pH 7.0.

[3] Ethyl methanesulfonate at 0.025 M in phosphate buffer at pH 7.0; seeds soaked aerobically in water at ∿ 1°C for 16 hrs, 2 hrs in EMS at ∿ 1°C plus 6 hrs at 24°C, rinsed and cultured on a water-saturated Whatman #1 filter.

[4] Weak acetic acid extraction solution as published in 43FR58956

[5] Extract was used as received, i.e., not diluted.

[6] Pooled values from treatments using 1:8 and 1:16 dilution (1.25 and 0.625 ml extract plus sufficient water to make 10 ml of solution).

have reported that serum filtrates of the most repirable stack-collected fly ash are mutagenic in Salmonella typhimurium strains TA-98, TA-100, and TA-1538. Serum filtrates of the mutagenic fly ash fraction heated to 350°C were non-mutagenic in the Salmonella assays. They hypothesized that the mutagenic activity of fly ash is associated with organic compounds. Lee, et al. (14) have found dimethylsulfate and its hydrolysis product, monomethylsulfate, at concentrations as high as 830 ppm in fly ash and air-borne particulate matter from coal combustion. These compounds are known mutagens.

In the case of the arsenic-contaminated ground water, numerous metals were present (e.g. Cd-485; Ni-935; Pb-117, Sb-297; Ti-7, 720; and Zn-251 µg/l) (4). Arsenic, especially in the arsenite state, is known to induce mutations and chromosome aberrations (17). Some of the heavy metals are known to be mutagenic and/or carcinogenic (7,15).

CONCLUSIONS

Based on results observed, the following conclusions were reached:

1. The Salmonella and Saccharomyces assays indicated the presence of mutagenic activity in the XAD-2 concentrate of the As-contaminated ground water, but not in the aqueous extract of the fly ash sample.
2. Both assays implicated frameshift mutagenesis as the mechanism involved.
3. The Hordeum root tip assay indicated the presence of mutagenic activity in both complex mixtures tested.

Table 5 List of seed treatments, the number of germinating embryos studied, and mean number of aberrations per 100 cells and their standard deviations. The comparison of treatment means was done using arc sin $\sqrt{\text{percent}}$ transformed data; however, the actual values are shown in this table.*

Treatments	Embryos N	Mean Number of Aberrations /100 cells	Standard Deviation
Distilled Water	60	2.75	3.04
Acetic Acid Extraction Solution	60	2.57	2.85
Fly Ash Extract	60	16.30	9.10
Phosphate Buffer	59	4.37	4.23
EMS (0.025M)	63	11.02	9.73
As-Contaminated Ground Water	63	16.19	9.10

*Footnotes shown in Table 4 regarding treatments apply here as well.

4. Chemical analyses of both complex mixtures show the presence of heavy metals implicating them as the possible cause of chromosomal aberrations.

SUMMARY

Solid wastes have been characterized to determine their potential hazard to humans and the environment. An arsenic-contaminated ground water sample increased the frequency of histidine revertants in Salmonella typhimurium (TA-98) at 0.025 to 5.000 µl per plate with Aroclor-induced S-9 liver microsomes. When 2.5 to 75 µl of the XAD-2 concentrate (12.5-fold, v:v) were used, the mutant frequency was increased in strains TA-98, TA-100, and TA-1537; metabolic activation was not required. Only the XAD-2 concentrate was mutagenic in the Saccharomyces cerevisiae haploid strain XL7-10B; metabolic activation was not required. The mutagenic principal, which is not known, appears to be at the limit of resolution; hence, the XAD-2 concentration is necessary to demonstrate mutagenic activity. The arsenic-contaminated ground water (0.0625 and 0.125 dilutions) and the power plant fly ash extract (undiluted) increased the frequency of bridges and fragments at anaphase in root tip cells of Hordeum. The fly ash sample was negative in the microbial assays. Results emphasize (1) the need for a battery of assays with different organisms and (2) the potential of a simple assay using plant root tip cells to detect mutagenic activity in complex environmental mixtures.

Abbreviations. Ar-S9, Aroclor 1254 (injection) induced rat liver homogenate; B(a)P, Benzo(a)Pyrene; EMS, ethylmethanesulfonate; MMS, emthylmethanesulfonate; 8-AmQ, 8-Aminoquinoline.

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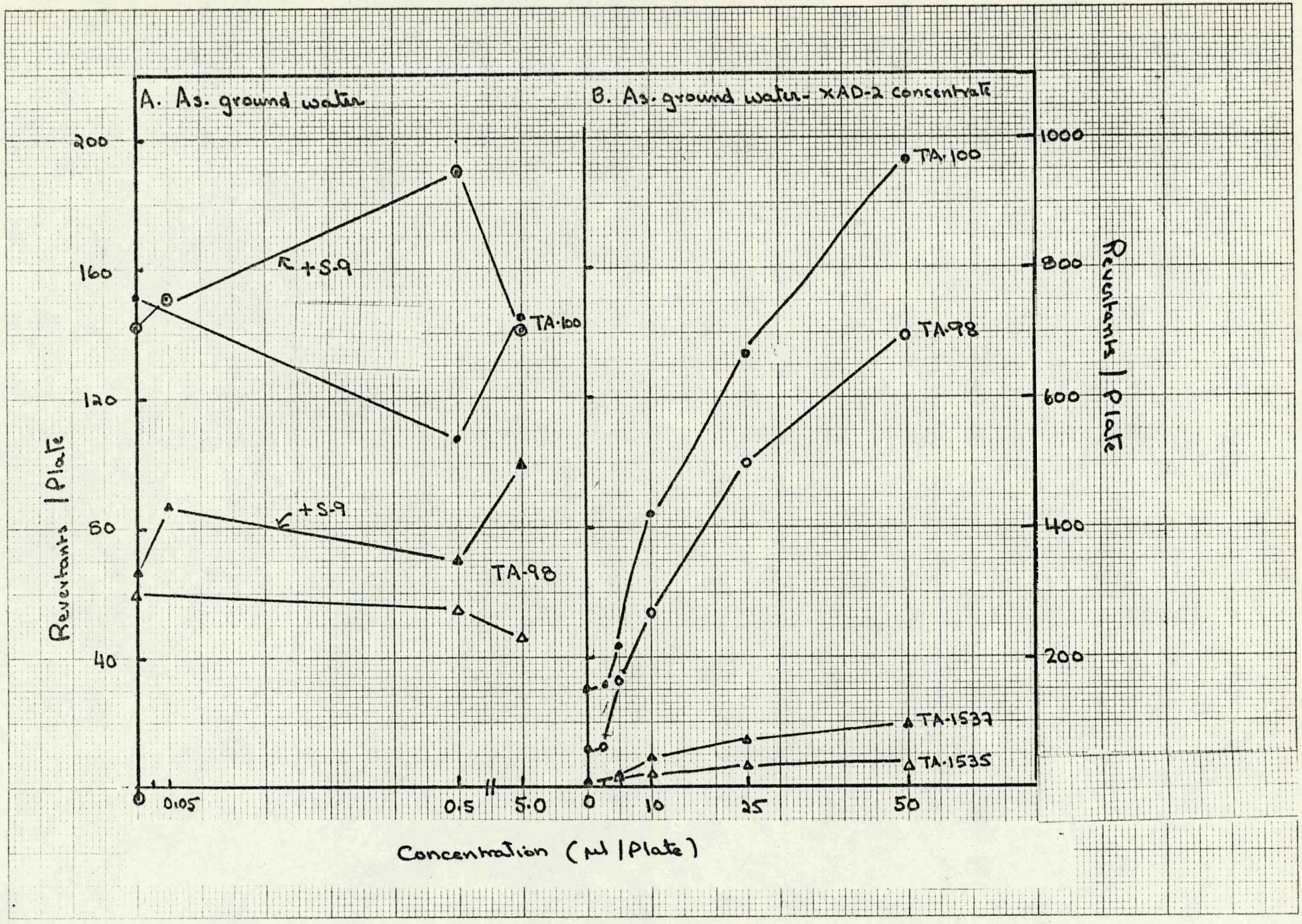
Figure Legend

Mutagenicity of As-contaminated ground water and its XAD-2 concentrate in the Salmonella assay. Each point represents an average of at least three independent experiments.


A. As. ground water
B. As. ground water - XAD-2 concentrate
Revertants / Plate
200
160
120
80
40
+S-9
TA-100
+S-9
TA-98
1000
800
600
400
200
TA-100
TA-98
TA-1537
TA-1535
0.05
0.5
5.0
0
10
25
50
Concentration (μl / Plate)