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TOXICITY OF LEACHATES

INTERIM PROGRESS REPORT

APRIL 1, 1978 TO JANUARY 1, 1979

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D. A. Brown, Environmental Sciences
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Oak Ridge National Laboratory
Oak Ridge, Tennessee 37830

Operated by
Union Carbide Corporation
for the
U.S. Department of Energy

Prepared for the
Office of Solid Waste
U.S. Environmental Protection Agency

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CONTENTS

ABBREVIATIONS

AAS	atomic absorption spectrometry
ANPR	Advance Notice of Proposed Rulemaking
Ar	Aroclor
As	arsenic
ASTM	American Society for Testing Materials
BAP	benzo(a)pyrene
DMSO	dimethylsulfoxide
EP	Extraction Procedure
EPA	Environmental Protection Agency
GC	gas chromatography
ORNL	Oak Ridge National Laboratory
PAH	polyaromatic hydrocarbon
PCB	polychlorinated biphenyl
∅B	phenobarbital
RCRA	Resource Conservation and Recovery Act
SLT	Standard Leaching Test
VOA	Volatile Organics Analysis

1. EXECUTIVE SUMMARY

Under Subtitle C of the Resource Conservation and Recovery Act (RCRA) of 1976 (PL 94-580), the Environmental Protection Agency (EPA) is required to promulgate regulations for the management of hazardous waste. To assist the EPA in developing characteristics for identifying wastes which, due to their toxic nature, pose a potential hazard to human health and the environment, the Oak Ridge National Laboratory (ORNL) has conducted studies on (1) leaching of toxicants from waste materials, (2) analytical procedures for characterizing extracts of wastes, and (3) screening bioassays for evaluating the toxicity of extracts. This report summarizes work during the period April 1, 1978 through January 1, 1979.

Experimental work during this reporting period has concentrated on:

(1) extracting four wastes (fly ash, scrubber sludge, soybean process cake,* and bottom ash) by use of the procedure proposed in the Federal Register on December 18, 1978 (43 FR 58956) in order to uncover potential problem areas;

(2) evaluating various analytical methodologies selected during ORNL's earlier work to determine their suitability for analyzing wastes and waste extracts;

(3) evaluating methodologies for preparing concentrates of the organic materials present in the extracts suitable for mutagenicity testing;

(4) evaluating short-term in vitro mutagenicity bioassays;

(5) evaluating screening assays for toxicity to aquatic organisms and terrestrial plants;

(6) determining the utility of these procedures by use of the extracts prepared from the four wastes and from a sample of groundwater contaminated with arsenic.

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*The soybean process cake was evaluated because earlier work indicated that waste from edible oil processing may be mutagenic.

Extraction Procedure (EP). Extractors of the type described in Figure 1 of 43 FR 58961 were used to perform the extractions on the wastes. They were constructed of polymethylmethacrylate resin and fabricated by the Environmental Engineering Department of the University of Tennessee. However, while these extractors satisfactorily agitated the wastes, there was occasional jamming during extraction of bottom ash. The extractor was redesigned to improve the alignment of the rotor and container so as to minimize jamming, lessen the possibility of abrasive damage to the unit during extraction of hard materials, and allow the use of organic solvent containing waste materials. This new extractor was similar to the previous unit. By changing the materials of construction to Type 316 stainless steel, improving the bearing design, lessening manufacturing tolerances, and redesigning the container and motor mount, we were able to increase both the versatility of the extractor and the operator productivity.

A study was also conducted to compare atomic absorption spectrometry (AAS) with other analytical methods for the measurement of metals in the EP extracts. (Wherever the "EP extract" is specified, this is actually a combination of an acidic aqueous extract of the solid portion of the waste and the original liquid present in the sample.) The AAS methods used were those in the then current EPA Standard Methods for Water and Waste (EPA-62516-74-003), except that to increase sensitivity a graphite furnace was used in place of the conventional flame. Analytical techniques included spark source mass spectrometry, inductively coupled plasma emission spectrometry, optical emission spectroscopy, and neutron activation analysis. The results indicate that AAS compares well with other methods for metal analysis. The uncertainty of the analytical procedures for the heavy metals of interest ranged from 1 to 10 percent in the concentration range of concern (0.1 to 10 mg/liter).

When these wastes were evaluated, the groundwater contaminated with arsenic (As) was found to be the only one which exceeded the threshold for a toxic waste as defined in 43 FR 58956 because it contained both 412 mg/liter of arsenic and 0.49 mg/liter of cadmium. The only other waste which might be labeled toxic by these criteria would be the fly ash by virtue of its borderline extract cadmium

level (0.1 mg/liter). However, taking into account the blank (0.01 mg/liter) would drop the level to marginally below the threshold.

Aquatic Toxicity. The objective of the aquatic screening tests was to determine the toxicity and potential hazard of waste extracts to aquatic biota. The tests used were acute (short-term lethality) tests and chronic (long-term) tests designed to measure sublethal effects on reproduction. The test organism was the cladoceran Daphnia magna, an aquatic organism sensitive to most classes of toxic chemicals.

The As-contaminated groundwater exhibited toxic effects in both acute and chronic aquatic bioassays; however, based on the threshold for a toxic waste described in the Advance Notice of Proposed Rulemaking (ANPR) 43 FR 59022 it would not be ranked as a toxic waste. On the other hand, the soybean process waste exhibited neither acute nor chronic toxic effects in the test employed. The three power plant wastes (fly ash, flue gas desulfurization sludge and bottom ash) showed only marginal toxicity, well below the aforementioned threshold levels.

The 28-day chronic toxicity test appears to show the presence of toxic materials in the As-contaminated groundwater. Daphnia reproduction, however, is greatly influenced by the food supply, and the extracts often contain high concentrations of acetate. The acetate may be a substrate for the bacteria, which are, in turn, fed on by the daphnids, and which in turn would result in an increase in number of young produced. For the controls in which acetic acid was used, it was found that the production of young was in fact significantly higher than when dilution water only was used.

Acute toxicity tests (48-hr, LC_{50}) were conducted on extracts before and after storage at 4 C for 28 days. Because of the low toxicities of the extracts, reliable 90 percent fiducial limits could not be obtained. However, the data indicated no statistically significant change in toxicity.

Mutagenicity. The mutagenicity protocol is intended to serve as an indicator of the chronic hazards of mutagenicity and carcinogenicity. Because of systemic differences in reactivity to mutagenic substances, a battery of assays has

been employed by ORNL with eukaryotic and prokaryotic organisms to detect both point mutation specifically and DNA damage generally. The vast majority of known chemical mutagens are organic in nature. Because the concept of threshold is ill-defined for genetic activity, and because the EP extract of solids was anticipated to be low in organic character, it was deemed advisable to examine a concentrated extract of the organic constituents of the EP extract in addition to the extract itself.

Mutagenicity as defined in the proposed regulations 43 FR 58965 (for delisting) and the ANPR 43 FR 59023 requires the application of three assays for determination of whether or not a waste is a hazardous waste: (1) gene (point) mutations in bacteria; (2) gene mutations in eukaryotes, either in mammalian somatic cells in culture or in fungal microorganisms; (3) recombinogenic or repair-related phenomena. The tests used in the ORNL program for each category were: (1) the Salmonella/microsome assay, (2) the Saccharomyces $\underline{can^r/his^+}$ dual assay, and (3) the Salmonella $\underline{uvrB}$ repair assay.

Arsenic-contaminated groundwater, four EP extracts, and XAD-2 concentrates of all five samples were tested in each assay both with and without metabolic activation. Both Aroclor- (Ar) and phenobarbital- (ϕB) induced rat liver S-9 mix were used in the studies with metabolic activation. The XAD-2 concentrates of the EP extracts achieved a 250-fold (v/v) concentration of the organic material, while, because of sample limitations, only a 12.5-fold concentration could be obtained on the contaminated groundwater.

None of the tested samples displayed toxicity in the Salmonella/microsome assay in the dose ranges tested, and only in the case of the As-contaminated groundwater was mutagenic activity found. It was slightly mutagenic with the frameshift strain TA98, but only upon mutagenic activation with Ar-induced S-9. The XAD-2 concentrate, however, was mutagenic with the frameshift strains TA1537, TA98, and TA100. It did not require metabolic activation and, in fact, the addition of the S-9 mix (either Ar- or ϕB-induced) reduced the mutagenic activity.

In the Saccharomyces mutation assay the As-contaminated groundwater sample was not mutagenic. However, the XAD-2 concentrate was mutagenic without metabolic activation after a 24-hr exposure. Metabolic activation appeared to reduce the mutagenic potential of the concentrate. None of the power plant or soybean process wastes or concentrates exhibited mutagenic activity either with or without activation. In addition, none of the test materials appeared to be toxic to the test organism.

The As-contaminated groundwater exhibited moderate toxicity to the organisms in the DNA repair assay. However, none of the test samples displayed mutagenic activity either with or without S-9 activation.

Of the five wastes discussed in this report, only the As-contaminated groundwater possessed detectable mutagenic activity. For purposes of bioassay, the mutagenic principal in the undiluted waste water is at the limit of resolution, the XAD-2 concentrate being necessary to conclusive demonstration of mutagenic activity. Work is in progress to determine if the o-nitroaniline known to be in the waste accounts for the mutagenic properties.

The response of the Salmonella/microsome assay to the As waste implies a frameshift mutation mechanism which requires the addition or deletion of DNA base-pairs. This is supported by the yeast results with this waste, which show a moderate preponderance of induced forward mutation to can^r, relative to induced reversion of the his^+ base-pair substitution. This is a typical response (by this system) to a frameshifting agent.

The As-contaminated groundwater and its XAD-2 concentrate failed to elicit a response from the bacterial DNA repair assay. There are two key considerations: (1) the overall mutagenic potency of the waste is moderate, (2) validation studies have shown that the repair assay is particularly insensitive (although not unreactive) to frameshifting agents. Hence, it may not be significant to obtain a negative result in this context.

With regard to the negative results obtained with the power plant wastes and the soybean process cake, the aqueous extracts and XAD-2 concentrates of these

materials are extremely deficient in organic character. The majority of organic mutagens are not detectable at the parts-per-billion level. Furthermore, inorganic mutagens (e.g., metals and metal complexes) which might be present are commonly not detectable by the bioassays in question.

Phytotoxicity. One objective of this project was to develop a short-term assay for screening wastes for phytotoxicity. Two tests were studied: a 48- and 72-hr root (radicle) elongation test for radish and sorghum, respectively, and a 2-week seedling growth study for wheat and soybean. Test seeds or plants were exposed to various concentrations of the EP extracts or As-contaminated groundwater to determine their toxicity, the ultimate objective being to see if a 10 percent concentration of the extract is significantly toxic to the plants. This concentration is that which the EPA scenario indicates could be used for irrigation. The As-contaminated groundwater was found to be highly phytotoxic, causing a 33 percent growth reduction in the radish roots even at a 2 percent concentration; however, at a 0.1 percent concentration there was a slight stimulation of root growth. None of the waste extracts showed toxic effects in all tests. The scrubber sludge was toxic to radish seeds in the root elongation test even at a 10 percent concentration, but the same concentration was not toxic to sorghum. Fly ash, soybean process cake, and bottom ash were only slightly toxic to radish and sorghum at concentrations exceeding 10 percent. In the seedling growth studies, the fly ash and soybean wastes caused a slight reduction of root weight but not shoot weight.

In both the short- and long-term tests with the soybean process cake, the dicotyledons (radish and soybeans) were not affected, whereas the roots of the monocotyledons (wheat and sorghum) were reduced significantly.

One significant potential problem relates to the presence of high concentrations of acetate in extracts of highly basic wastes. Early results confirm literature reports that acetate is phytotoxic. While this did not cause difficulties in the samples evaluated during this period, work is under way to further define the magnitude of the potential problem.

2. INTRODUCTION

2.1. Background

Under the terms of the RCRA of 1976 (PL 94-580), EPA is required to promulgate regulations for the management of hazardous waste. Section 3001 of Subtitle C of RCRA further requires EPA to develop and promulgate criteria for identifying the characteristics of hazardous waste and for listing particular wastes which are subject to the regulations. To assist the EPA in developing characteristics for identifying wastes which, due to their toxic nature, pose a potential hazard to human health and the environment, ORNL entered into an interagency agreement with EPA in 1977. Under the terms of this agreement ORNL would conduct studies to evaluate leaching and bioassay tests, recommend toxicity test protocols, and perform analytical, biological, and environmental sciences testing to establish a preliminary toxicity data base on leachates.

The work is a cooperative effort between ORNL's Biology, Environmental Sciences, and Analytical Chemistry Divisions.

2.2. Scope of Work

Samples of various wastes have been provided to ORNL by the Hazardous Waste Management Division, Office of Solid Waste, EPA; the American Society for Testing Materials (ASTM); and other groups.

Based on the needs of EPA's Office of Solid Waste, emphasis during this work period has been on:

(1) evaluating the EP proposed in the Federal Register on December 18, 1978 (43 FR 58956) to measure the tendency of toxicants to migrate from a waste under "improper" disposal conditions,

(2) evaluating the following short-term, inexpensive screening tests to determine whether the introduction of such extracts to the environment might pose a substantial present or potential hazard to human health or the environment: (1) 48-hr *D. magna* LC_{50}; (2) 28-day *D. magna* reproduction; (3) Salmonella/microsome bacterial reversion; (4) *Saccharomyces* mutation; (5) Salmonella-uvrB repair; (6) Radish, wheat, and sorghum radicle length; (7) Wheat and soybean seedling growth.

Experimental work during this reporting period has concentrated on:

(1) testing a series of wastes using the EP;

(2) evaluating various analytical methodologies selected during ORNL's earlier work to determine their suitability for analyzing waste extracts (with emphasis on identifying and determining the concentration in the extracts of those species on the National Interim Primary Drinking Water Standards list and the Priority Pollutants list);

(3) evaluating methodologies for preparing concentrates of the organic materials present in the extracts suitable for mutagenicity testing;

(4) developing and evaluating short-term in vitro mutagenicity bioassays;

(5) developing and evaluating screening assays for toxicity to aquatic organisms and terrestrial plants;

(6) determining the utility of these procedures by use of extracts prepared from various wastes.

Earlier work under this contract leading up to the current EP will be published as part of the final project report scheduled for early fall of 1979.

3. SAMPLING

3.1. Sample Handling

Figure 3-1 shows the stepwise disposition of a given waste as it proceeds through analysis at ORNL. The Environmental Sciences Division (ESD) executes the EP on the waste and distributes the resulting EP extract to the Environmental Sciences Division for phytotoxicity and aquatic screening assays and to the Analytical Chemistry Division (ACD) for further processing. The Analytical Chemistry Division performs inorganic and volatile organic analyses on the EP extract; prepares XAD-2 resin concentrates of the EP extract suitable for polynuclear aromatic hydrocarbons, polychlorinated biphenyls, and pesticide analyses following chemical fractionation; and provides aliquots of the EP extract and its XAD-2 resin concentrate to the Biology Division for mutagenicity bioassay.

3.2. Sample Description

The following materials, covering wastes expected to be both hazardous and nonhazardous, were supplied by EPA or ASTM for use in this project: (1) As-contaminated groundwater, (2) organoarsenical waste, (3) metal cleaning waste, (4) dyestuff manufacturing sludge, (5) latex carpetbacking sludge, (6) plating sludge, (7) soybean process charcoal filtration cake; (8) raw shale, (9) shale retort waste, (10) fludized bed combustion waste, (11) municipal sewage sludge, (12) coal-fired power plant fly ash, (13) coal-fired power plant bottom ash, (14) flue gas desulfurization sludge, (15) API separator sludge.

This report describes the work conducted on the extraction and testing of the following materials: (1) As-contaminated groundwater, (2) fly ash, (3) scrubber sludge, (4) bottom ash, (5) soybean process charcoal filtration cake.

Four of the wastes were extracted by use of the EP described in the proposed regulations (Appendix I). A previous publication[1] described the philosophical basis of the EP. Three of the wastes represent different solid coal combustion residues.

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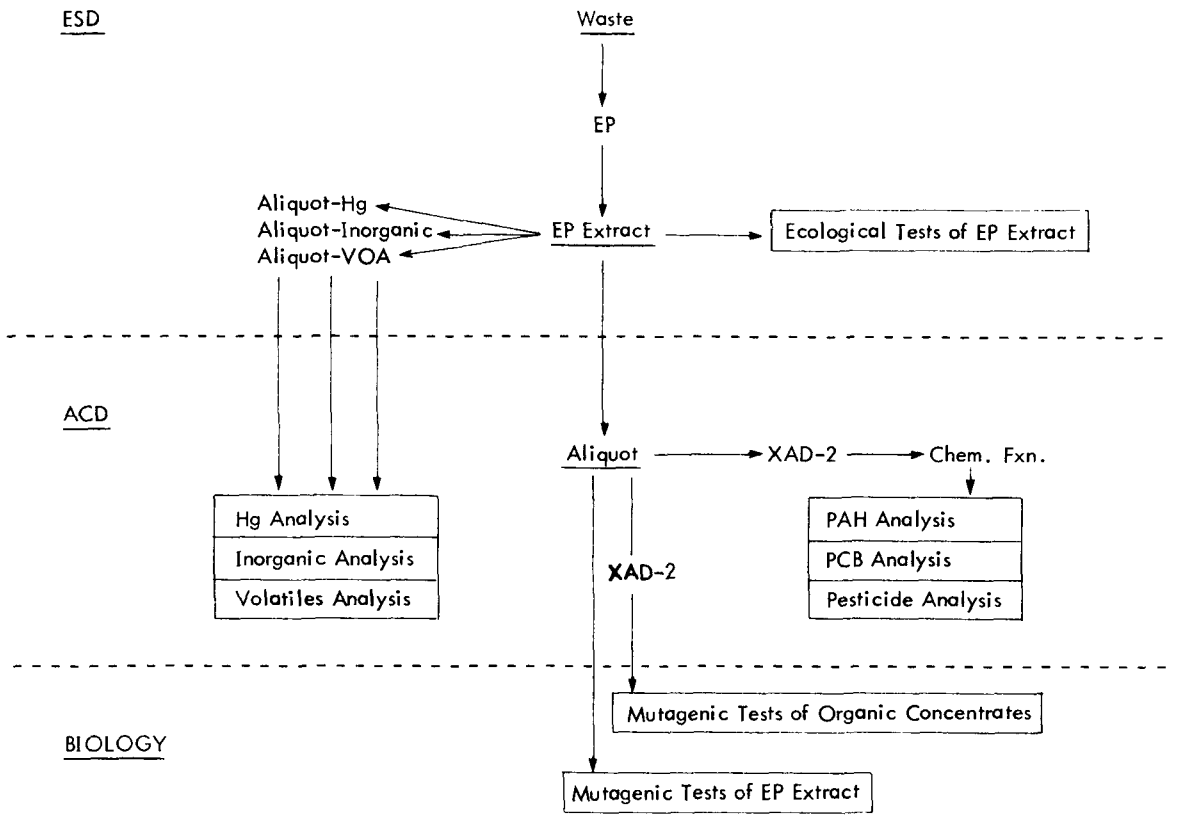

Figure 3-1. Sample handling procedures within ORNL.

These wastes were fly ash, scrubber sludge, and bottom ash. The fourth waste, soybean process cake, is a solid disposed of after the bleaching of corn syrup; it was selected for use in this program as a result of the observation during an earlier study[2] that an edible oil processing waste showed mutagenic activity. The As-contaminated groundwater sample was distributed for testing as received, since it was a liquid and did not have to be extracted by the EP. Detailed information regarding the chemical and physical characteristics of the wastes was not provided with the samples. The results from studies conducted on the remaining samples will be reported in the final project report.

All waste samples received for testing were placed outdoors in an open shed. This was a safety precaution deemed necessary due to the lack of information concerning the nature of the samples. The representativeness of the waste sample is not assumed, since the waste was stored for an extended period of time and other collection and storage factors were unknown, such as the procedures used to collect the samples (i.e., ORNL did not know whether the sampling protocols cited in the proposed hazardous waste regulations were used), temperature changes prior to arrival at ORNL, and possible sample-container interactions. It is unreasonable to assume or predict changes (biological, chemical, or physical) that may occur in a sample over time. These topics, though, are a necessary consideration in the final version of the hazardous waste characterization procedure. Therefore, it should be noted that the waste testing being conducted was to assess the adequacy of the testing protocols cited in the hazardous waste regulations, rather than to determine whether the wastes were hazardous.

Additionally, a standard procedure was not applicable in obtaining a representative subsample of the wastes for extraction. The types of wastes to be tested ranged from a dry powder to sludge and slurry. Therefore, upon examination of each waste, a judgment was made as to how an aliquot of the sample would be taken. In most cases thorough mixing or quartering was utilized.

It is our recommendation that more specific sampling guidelines be incorporated into the EP itself, rather than into the Appendix of the regulations. The

draft report, Handbook for Sampling Hazardous Waste (Research Grant R-804692010), seems to be an adequate reference source for this information.

3.3. Preliminary Sample Data

Preliminary laboratory data collected by ORNL on the waste samples included:

(1) Solids content, calculated as the weight in grams of the sample after drying divided by the original weight in grams of the sample.

(2) General physical description including color, odor, and consistency.

(3) Preliminary titration of a 5.0-g sample with 0.5 N acetic acid.

A 1:16 solid:solution ratio was used to give an indication of the amount of acid required for pH adjustment during the sample extraction. Table 3-1 summarizes these data for the four waste samples. While these wastes were of varying moisture content, ranging from a low of 1 percent moisture to a high of 59 percent, they were all what would normally be considered to be solids. One area worthy of comment concerns the need for care to be taken during all phases of the sampling and testing phases to prevent loss of sample moisture. This is especially important for high moisture content wastes. A change in moisture content due to evaporation of water from the sample from 90 percent to 80 percent would change the contribution of the original liquid phase to the final EP extract from 31 percent to only 20 percent. Thus it is very important to ensure that the sample tested is truly representative of the waste sampled.

TABLE 3-1

SAMPLE CHARACTERISTICS AND TREATMENT

Waste	Solids content*	Preliminary titration of sample (mEq 0.5 N acetic acid per g sample)†	Physical state	Sample treatment before extraction
Fly ash	0.9923	0.00	dark grey, powder-like, dead insects and dried plant materials mixed with waste; odorless	none
Scrubber sludge	0.6844	0.43	dense, light grey sludge topped with small amount of standing liquid; cement-like odor	supernatant removed; solid:liquid ratio 6.5:1 (by wt)
Bottom ash	0.7442	0.03	dark grey, heterogeneous mixture of large and small particles; odorless	sized to pass 3/8 in. seive
Soybean process cake	0.4090	0.07	dark grey, moist, soil-like consistency and odor	none

*Weight (g) of sample after drying per original weight (g) of sample.

†Milliequivalents of acetic acid required to initially adjust the solution to pH 5.

4. EXTRACTION PROCEDURE

4.1. Problem Definition

Prior to initiation of this study, EPA had commissioned a study by Dr. R. Ham and his co-workers at the University of Wisconsin to develop a Standard Leaching Test (SLT). The purpose of this test was to devise a procedure which would model waste disposal in a landfill in admixture with a decomposable organic waste such as refuse.

Specifically EPA was interested in determining what components of the waste would be likely to migrate out of the waste under such a disposal environment. It was the intent of ORNL and EPA that the ORNL work would be geared toward developing methodology to measure whether the leachate posed a potential hazard to either man or the environment.

However, soon after initiation of the work at ORNL, it was learned that while the SLT did a good job of modeling landfill conditions, the inherent toxicity of the SLT extraction fluid was such as to preclude determination of the additional toxicity caused by material migrating from the waste and thus make SLT unsuitable for the planned use of bioassay procedures at some later date. Thus the ORNL work program was modified by EPA to include testing of a second-generation EPA leaching method and to determine if it was compatible with bioassay procedures for determining the toxicity of the waste. It should be noted that EPA's intent in developing the EP was (1) to "model" improper management based on wastes creating a problem through migration of chemicals out of a disposal site, and (2) to use this with the resultant analysis as a screen to separate out those wastes which required special handling. The contamination scenario has been developed for definition purposes only; it is not meant to address actual site specific disposal methods which might be used for a particular waste.

4.2. Extracting Apparatus

Extractions were performed with extractors of the type described in 43 FR 58961 at the direction of the Project Officer. To save time, prototype extractors

constructed of Plexiglas were borrowed from the Environmental Engineering Department of the University of Tennessee. However, due to the possible problems of abrasive damage during extraction of hard materials, the solubility of the Plexiglas in organic media present in some wastes, and the possibility of jamming (due to misalignment), an improved version of the extraction equipment was designed and fabricated at ORNL from Type 316 stainless steel. Details of materials and design can be found in Appendix II (Figures II-1 through II-11).

As shown in Appendix II, the most important improvements of the new design are the use of Type 316 stainless steel in all parts which are exposed to the waste or extract, as requested by EPA, and the unitizing of the vessel, supporting frame, and stirring motor. Type 316 stainless steel, a high quality steel, was considered an improvement because of its inherent strength and resistance to abrasion, a factor which is especially important when dealing with hard granular wastes such as bottom ash. The integration of the vessel, support stand, stirring rod, and stirring motor is seen to be the best way of assuring positive alignment of the agitator blade and vessel bottom. This feature minimizes the probability of waste particles binding and scraping between them and assures a smooth mixing action. A high-torque, low-rpm stirring motor and its accompanying solid-state controller have proven more than adequate for the job and provide positive, variable-speed propulsion of the stirring rod. The conical bearing surface for the stirring rod on the bottom of the vessel is an important improvement in that it assures positive centering of the stirring rod in the vessel and minimizes grinding action and clogging between the stirring rod end and the vessel bottom. This unitized system allows close tolerances to be maintained between the edges of the stirring blade and the bottom and sides of the extraction vessel, thereby assuring thorough mixing of the solid and liquid phases during the EP.

One potential problem remains to be resolved regarding the possible leaching of metals from the walls of the vessel and stirring rod. The extent of this problem is being verified at this time. A possible alternative is to have the vessel and stirrer fabricated out of Tefzel (a DuPont registered trademark).

Due to the lack of additional specification in the EP, the laboratory practices and utilization of reagents cited in Appendix II was deemed more than adequate. We recommend the specification of water and chemical grades as an addition to the EP.

4.3. Results and Discussion

Data recorded during the extraction of the four wastes are represented in Table 4-1. The following parameters were recorded for each individual extraction: initial pH, final pH, amount of 0.5 N acetic acid initially added to adjust the solution to pH 5, total 0.5 N acetic acid added during the 24-hr extraction period, and electrical conductivity of the final extract. The solution pH was automatically adjusted during all extractions.

Following extraction, the solid and liquid phases were again separated by use of the Millipore filtration system. A 0.45-μm pore size filter was utilized for final filtration. In all cases filtration was accomplished by means of a vacuum filter which proved to be satisfactory for all wastes extracted to date. It should be noted that the EP extract is a combination of the original liquid from the waste sample and the liquid resulting from the extraction.

Data recorded upon completion of the extractions are given in Table 4-2. The following parameters were recorded for the extract in its final form: pH, electrical conductivity, total 0.5 N acetic acid added, color, and remarks on unusual appearance or occurrence.

Upon completion of the EP, aliquots of the extract were delivered to the appropriate groups at ORNL for analysis. When delivery could not be accomplished immediately after completion of the procedure, all extract samples were refrigerated at 4 C.

We recommend that standardized procedures for storing the EP extract prior to analysis be incorporated into the final protocol.

TABLE 4-1

EXTRACTION DATA

Waste	Extraction	Initial pH*	Final pH*	Acetic acid initially added (mEq/g sample extracted)	Total acetic acid added (mEq/g sample extracted)	Electrical conductivity (μmho/cm)
Fly ash	1	4.5	5.0	0.00	0.156	2000
	2	5.5	4.9	0.05	0.207	1980
	3	5.0	4.9	0.00	0.254	1960
	4	5.0	5.0	0.00	0.146	2000
	mean	5.0	4.9	0.013	0.191	1985
Scrubber sludge	1	9.8	5.1	0.415	0.490	3380†
	2	9.8	5.1	0.413	0.478	3070†
	3	10.1	5.1	0.420	0.516	3270†
	mean	9.9	5.1	0.416	0.495	3240
Bottom ash	1	9.6	5.1	0.011	0.026	141
	2	9.4	5.2	0.010	0.027	151
	3	9.2	5.2	0.010	0.023	150
	4	9.2	5.1	0.011	0.026	162
	mean	9.3	5.1	0.011	0.026	151
Soybean process cake	1	8.2	5.0	0.063	0.077	284
	2	8.6	5.1	0.071	0.082	265
	3	8.6	5.1	0.063	0.077	235
	4	8.6	5.2	0.062	0.073	255
	mean	8.5	5.1	0.065	0.077	260

*1:16 solid:solution ratio.

†Supernatant not added.

TABLE 4-2

EXTRACT DATA

Extract	pH	Electrical conductivity (μmho/cm)	Total acetic acid added (mEq/g sample extracted)	Color
Fly ash	4.8	1910	0.191	transparent, colorless
Scrubber sludge	4.9	2340	0.429	transparent, colorless (white precipitate formed after addition of supernatant to leachate the precipitate was filtered out, and both were sent separately to Analytical Chemistry Division)
Bottom ash	5.0	154	0.026	transparent, colorless
Soybean process cake	5.2	240	0.077	transparent, yellow

5. CHEMISTRY

5.1. Problem Definition

Due to the need of the Office of Solid Waste for rapid, reliable analytical methods for the analysis of the 14 substances (i.e., eight heavy metals and six pesticides) for which threshold levels have been proposed (Table 5-1), the Analytical Chemistry Division of ORNL has been concerned with the validation of analytical techniques and quantitative analysis of the extracts obtained from the EP. In addition, ORNL has also been concerned with the validation of analytical techniques for the concentration extraction, and quantitative analysis of the Priority Pollutants (Table 5-2) in extracts obtained from the EP. The Priority Pollutants fall into several distinct chemical classes: polyaromatic hydrocarbons (PAH's), phenols, pesticides and polychlorinated biphenals (PCB's), volatile halo-organics, phthalates, various nitro and halo-substituted benzenes and toluenes. The objective of this work has been to identify and measure in a cost-effective manner as many of these compounds as possible at one-tenth the level indicated in Table 5-1 or 1 mg/liter for those species listed in Table 5-2.

In addition to characterization and measurement, an additional aspect has been to develop methodology for concentrating the organic constituents of the extract in a manner suitable for testing in the short-term bioassays described in the section on mutagenicity testing.

5.2. Scope of Work

Many instrumental techniques are available for the measurement of metals in a liquid waste or EP extract. A study was conducted to compare the commonly used AAS with other techniques. The AAS methods used were those specified in the current EPA Standard Methods for Water and Wastes (as specified in the proposed regulations) except that a graphite furnace was employed instead of the conventional flame. This change has since been incorporated in the Standard Methods. Analytical techniques examined for analyzing extracts include spark source mass

spectrometry (including isotope dilution where applicable), inductively coupled plasma emission spectrometry, optical emission spectroscopy, and neutron activation analysis. A collaborative AAS analysis of an EP extract also was carried out with the Illinois State EPA, and two EPA Laboratory Performance Evaluation Standards supplied by the EPA Environmental Monitoring and Support Laboratory (Cincinnati) were used to test the accuracy of our AAS methodology.

Preparation of Organic Concentrates. Our initial attempts to assay the aqueous EP extracts directly were unsuccessful from the standpoints of both organic analytical characterization and mutagenicity bioassay because of the low concentrations of the extracted constituents. Thus, we conducted an investigation of available methods for concentrating the organics present in the extracts. Methods for separating the extracts into chemically simpler fractions and for analyzing volatile organic compounds and metals also were examined.

TABLE 5-1

PROPOSED EP EXTRACT THRESHOLDS FOR DESIGNATING A WASTE AS A TOXIC WASTE

Substance	Threshold level (mg/liter)
Ag	0.5
As	0.5
Ba	10.0
Cd	0.1
Cr	0.5
Hg	0.02
Pb	0.5
Se	0.1
2,4-D	1.0
Lindane	0.04
Methoxychlor	1.0
Toxaphene	0.05
2,4,5-TP Silvex	0.1
Endrin	0.002

TABLE 5-2

EPA PRIORITY POLLUTANT LIST

Acenaphthene
Acrolein
Acrylonitrile
Aldrin/Dieldrin
Antimony and compounds
Arsenic and compounds
Asbestos
Benzene
Benzidine
Beryllium and compounds
Cadmium and compounds
Carbon tetrachloride
Chlordane (technical mixture and metabolites)
Chlorinated benzenes (other than dichlorobenzenes)
Chlorinated ethanes (including 1,2-dichloroethane, 1,1,1-trichloroethane, and hexachloroethane)
Chloroalkyl ethers (chloromethyl, chloroethyl, and mixed ethers)
Chlorinated naphthalene
Chlorinated phenols (other than those listed elsewhere; includes trichlorophenols and chlorinated cresols)
Chloroform
2-Chlorophenol
Chromium and compounds
Copper and compounds
Cyanides
DDT and metabolites
Dichlorobenzenes (1,2-, 1,3-, and 1,4-dichlorobenzene)
Dichlorobenzidine
Dichloroethylenes (1,1- and 1,2-dichloroethylene)
2,4-Dimethylphenol
Dinitrotoluene
Diphenylhydrazine
Endosulfan and metabolites
Endrin and metabolites
Ethylbenzene
Fluoranthene
Haloethers (other than those listed elsewhere; includes chlorophenylphenyl ethers, bromophenylphenyl ether, bis(dischloroisopropyl) ether, bis(chloroethoxy) methane, and polychlorinated diphenyl ethers)
Halomethanes (other than those listed elsewhere; includes methylene chloride, methylchloride, methylbromide, bromoform, dichlorobromomethane, trichlorofluoromethane, trichlorodifluoromethane)
Heptachlor and metabolites
Hexachlorobutadiene
Hexachlorocyclohexane (all isomers)
Hexachlorocyclopentadiene
Isophorone
Lead and compounds
Mercury and compounds
Naphthalene
Nickel and compounds
Nitrobenzene
Nitrophenols (including 2,4-dinitrophenol, dinitrocresol)
Nitrosamines
Pentachlorophenol
Phenol
Phthalate esters
Polychlorinated biphenyls (PCB's)
Polynuclear aromatic hydrocarbons (including benzanthracenes, benzopyrenes, benzofluoranthene, chrysenes, dibenzanthracenes, and indenopyrenes)
Selenium and compounds
Silver and compounds
2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD)
Tetrachloroethylene
Thallium and compounds
Toluene
Toxaphene
Trichloroethylene
Vinyl chloride
Zinc and compounds

Solvent partitioning by use of cyclohexane and dichloromethane, and macroreticular resin extraction with XAD-2 (refs. 3,4) were investigated as methods for preparing organic concentrates. While determining that the resin method offered the most promise as a cost-effective method, we realized that the pH and ionic strength of the EP extract had to be controlled if reproducible concentrates were to be obtained.

Fractionation and Analysis of Organic Concentrates. Because the expected complex nature of the organic concentrates would seriously limit our ability to directly identify and measure the various classes of toxicants, we required a means of separating the organic concentrate into simpler, more easily analyzed fractions. A modified version of a fractionation scheme[5] developed and validated in this laboratory for isolation of polyaromatic hydrocarbons from environmental materials was the most cost-effective approach, and we examined it for its ability to separate the various classes of toxicants prior to their analysis by gas-liquid chromatography.

Analysis of Volatile Organic Compounds. Because volatile organic compounds are not readily determined in the organic concentration/fractionation method, they must be determined by a separate method. The advantages of the purge and trap method over solvent extraction and other methods for the determination of volatile organic compounds in water have been well established.[6] We evaluated a manual version of this method[7] for its applicability to EP extract analysis.

5.3. Results

Table 5-3 is a complete listing of all the EPA Priority Pollutant and Interim Drinking Water Standard constituents which are specifically determined by this chemical protocol. The application of this protocol to the EP extracts and groundwater sample is discussed below.

TABLE 5-3

SPECIFIC COMPOUNDS SELECTED FOR MEASUREMENT IN EP EXTRACTS AND As-CONTAMINATED GROUNDWATER SAMPLE

PAH*	Volatile	Element	PCB/pesticide
Acenapthene	1,1-Dichloroethylene	Ag	PCB (Ar 1242)
Fluoranthene	Methylene chloride	As	Lindane γ-isomer
Naphthalene	trans-1,2-Dichloroethylene	Ba	Methoxychlor
1,2-Benz(a)anthracene	Acrolein	Be	Endrin
Benzo(a)pyrene (BAP)	Dichlorobromomethane	Cd	Toxaphene
Chrysene	Tetrachloroethylene	Cr	
Anthracene	Bromoform	Cu	
1,12-Benzoperylene	Bis-[2-chloroethyl] ether	Hg	
Fluorene	Acrylonitrile	Ni	
Phenanthrene	1,1-Dichloroethane	Pb	
Dibenz(a,c and a,f)anthrenes	Chloroform	Sb	
Pyrene	1,2-Dichloroethane	Se	
	Trichloroethylene	Tl	
	2-Chloroethyl vinyl ether	Zn	
	1,1,2-Trichloroethane	F	
	Chlorodibromomethane		
	S-Tetrachloroethane		

*Plus 31 other PAH's not included on the Priority Pollutant List.

Analysis for Metals. Because of the availability and widespread use of AAS, it was compared with five other instrumental methods of metals analysis by assaying an EP extract of sewage sludge. Analyses were conducted in quadruplicate. The results shown in Table 5-4 indicate that AAS compares well with other methods of metals analysis. Further evidence for the accuracy of our AAS method was obtained through a cooperative study arranged with Mr. Frank Schmidt of the Illinois State EPA. The results of quadruplicate analyses of a sewage sludge EP extract are

TABLE 5-4

COMPARISON OF METALS ANALYSES OF SEWAGE SLUDGE EP EXTRACT

Metal	Average concentration ± S.D. (mg/liter) for method*					
	AAS	ID-SSMS	SSMS	ICPS	OES	NAA
Ag	0.0002 ± 0.00001	—	< 0.02	<0.02	—	<0.01
As	0.03 ± 0	—	< 0.02	—	—	0.08 ± 0.003
Be	0.0004 ± 0.00002	—	—	—	< 0.1	
Cd	1.2 ± 0	1.1 ± 0	—	0.8 ± 0.08	—	1.03 ± 0.03
Cr	0.03 ± 0.0008	<0.2	—	<0.5	—	0.06 ± 0.006
Cu	0.7 ± 0.01	0.75 ± 0.07	—	0.7 ± 0.07	—	—
Hg	0.00003 ± 0.000001	—	—	—	—	—
Ni	3.4 ± 0.2	4.1 ± 0.27	—	3.0 ± 0.3	—	—
Pb	0.03 ± 0.003	<0.05	—	<0.1	—	—
Sb	0.10 ± 0.002	—	~0.01	—	—	0.041 ± 0.005
Se	<0.002	—	≤ 0.01	—	—	<0.02
Tl	0.01 ± 0.001	—	≤ 0.01	—	—	—
Zn	36.7 ± 0.68	39.0 ± 2.2	—	45.0 ± 4.0		55 ± 1.5

*AAS, atomic absorption spectrophotometry; ID-SSMS, isotope dilution-spark source mass spectroscopy; ICPS, inductively coupled plasma emission spectrometry; OES, optical emission spectrometry; NAA, neutron activation analysis.

shown in Table 5-5. Four metals are footnoted as being determined in a second study; except for these four, the results of the two laboratories in the first study were in good agreement. The data reported for the second study also agree well, which implies that a contamination problem (probably incurred during packaging the EP extract for shipment) caused the disparity of results for these four metals in the first study.

Finally, two EPA Laboratory Performance Evaluation Standards were analyzed for their content of As, Be, Cd, Cr, Cu, Hg, Ni, Pb, and Se. According to Dr. D. E. Sanning, the EPA Project Officer, the Be results by AAS were biased slightly

TABLE 5-5

COMPARISON OF ORNL AND ILLINOIS STATE EPA ANALYSES OF SEWAGE SLUDGE EP EXTRACT

Metal	Average concentration ± S.D. (mg/liter) in study by	
	ORNL	Illinois State EPA
Ag*	0.0003	<0.005
As*	0.007 ± 0.0002	0.007
Ba*	<0.5	0.4
Be	0.0004 ± 0.00002	<0.005
Cd	1.2 ± 0	1.1 ± 0
Cr	0.03 ± 0.0008	0.04 ± 0.005
Cu	0.7 ± 0.01	0.8 ± 0.05
Hg*,†	0.027	<0.03
Ni	3.4 ± 0.2	4.0 ± 0
Pb	0.03 ± 0.003	0.04 ± 0.001
Zn	36.7 ± 0.7	48.5 ± 1.7

*Result of second study.

†Single measurement of Hg.

low, but were sufficiently accurate and precise for this study. Thus, we felt that AAS was validated as an accurate and precise method of metals analysis, and we adopted it for our standard method.

Preparation of Organic Concentrates. Experiments were conducted to compare the efficiency of XAD-2 resin with that of solvent extraction (with both cyclohexane and methylene chloride) in their ability to extract and concentrate organic contaminants present in an EP extract. For the spiking experiments, a synthetic EP extract was prepared from 3.4 ml of glacial acetic acid/liter of triple-distilled water. This "extract" was then spiked with 6×10^5 to 12×10^5 decompositions/min/liter of the ^{14}C-labeled organic compounds listed in Table 5-6, the pH was adjusted to 6.8 with Na_3PO_4, and the conductance was adjusted to 20 mmho/cm with NaCl. These samples were then extracted with five 100 ml portions of organic solvent or passed through 4 g of XAD-2 by the method [after ref. 3] described in Appendix III. Our results are shown in Table 5-6. Methylene chloride was the most efficient solvent for recovering the organics. However, its use in mutagenic bioassay is questionable. It should also be noted that while the solvent extractions provide reliable extraction for most of the organics, the XAD-2 also provides a direct 100-fold concentration. Furthermore, as many as 15 EP extracts may be extracted simultaneously by XAD-2 if a simple peristaltic pump is used, eliminating considerable technician time. XAD-2 has also been employed successfully elsewhere[4] for isolating mutagens for short-term bioassay. Thus the XAD-2 method appeared to be the most cost-effective approach to the preparation of organic concentrates for analysis or mutagenic bioassay and was adopted as our routine concentration method.

Fractionation and Analysis of Organic Concentrates. The most cost-effective means of fractionating the organic concentrates into simpler, more readily analyzed fractions was felt to be a modification of our existing method[5] for isolation of PAH's from environmental materials. The modified fractionation scheme is shown in Figure 5-1, along with the actual fractionation recoveries achieved with the cyclohexane extracts from the organic concentration studies. Specific details of the

TABLE 5-6

EFFICIENCIES OF SOLVENT EXTRACTION AND XAD-2 RESIN FOR CONCENTRATION OF ORGANICS FROM A MOCK EP EXTRACT

Compound	Cyclohexane* (%)	Methylene chloride* (%)	XAD-2† (%)
PCB (Ar 1254)		~100	95
BAP	~100	~100	82
Naphthalene	~100	~100	~100
Hexadecane	~100	~100	93
Indole	41	95	84
Phenol	3	99	70
Stearic acid	~100	99	91
Stearyl alcohol	96	95	90
Succinic acid	0	0	0
Sitosterol	~100	~100	91

*Extraction efficiency only.

†Actual recovery after adsorption/desorption and concentration to 5.0 ml.

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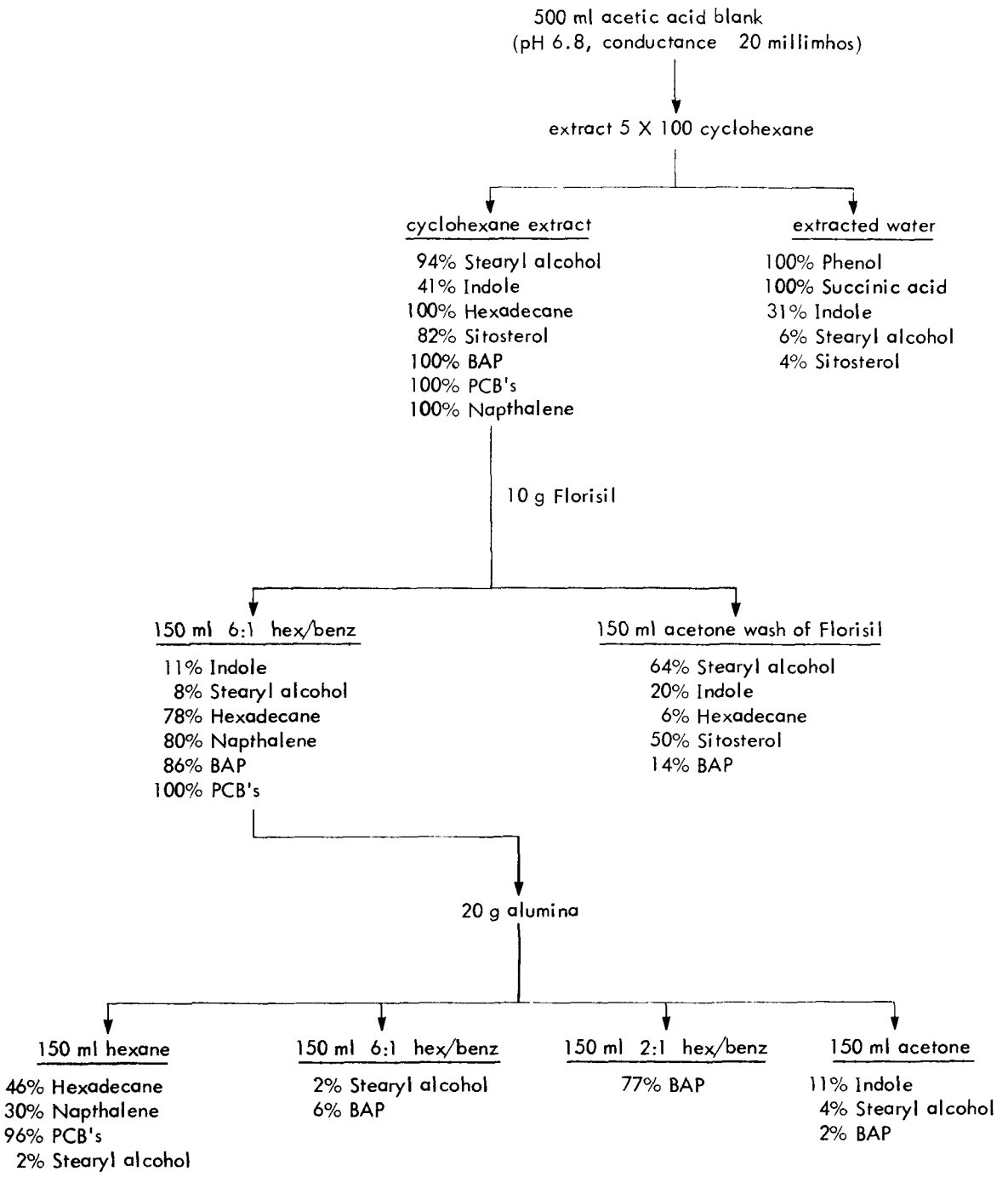

Figure 5-1. Recoveries of tracers in fractionation procedure.

fractionation procedure are included in Appendix III. Our results with tracer compounds indicate that nonpolar materials from the EP extracts are separated from polar materials by adsorption column chromatography on Florisil. The nonpolar organics then are separated on an alumina column onto a PCB pesticide mono- and diaromatic fraction, a polyaromatic fraction, and a heteroaromatic fraction. Each fraction is analyzed by gas chromatography (GC) at the conditions noted in Appendix III. Recoveries of PAH's from environmental materials are virtually quantitative.[5] PCB recoveries are being evaluated with Standard Solutions supplied by the EPA (Cincinnati).

The groundwater sample was known to be contaminated with o-nitroaniline. Because the XAD-2 procedure is not particularly efficient for concentration of ionized organics, a separate solvent extraction procedure was employed (Appendix III) to extract and concentrate the o-nitroaniline for GC analysis. Four methylene chloride extractions were sufficient to remove all solvent-extractable color from the groundwater sample.

Analysis of Volatile Organics. The apparatus we employed in the purge and trap collection of volatile organics is shown in Figure 5-2. The entire collection and analysis procedure was evaluated with a standard made up in ethylene glycol with the following toxicants at the 1 mg/ml level: 1,1-dichloroethane; 1,2-dichloroethane; hexachloroethane; trichloroethane; tetrachloroethane; 2-chloroethyl vinyl ether; chloroform; 1,1-dichloroethylene; 1,2-trans-dichloroethylene; 1,2-dichloropropane; 1,3-dichloropropylene; (bis)-2-chloroisopropyl ether; bromoform; dichlorobromoethane; trichloroethylene; and dichloromethane. Because of the high volatilities of these toxicants, aliquots of the standard were added to a mock EP extract only immediately prior to purging. Thermal desorption and GC analysis of the trapped standards indicated an average precision of ± 20 percent. While the absolute recovery of each toxicant is unknown, recoveries should be highly reproducible for standards and samples treated in the same manner. Sensitivity is approximately 0.1 mg/liter when a 1.0-ml sample is analyzed. Greater sensitivity could

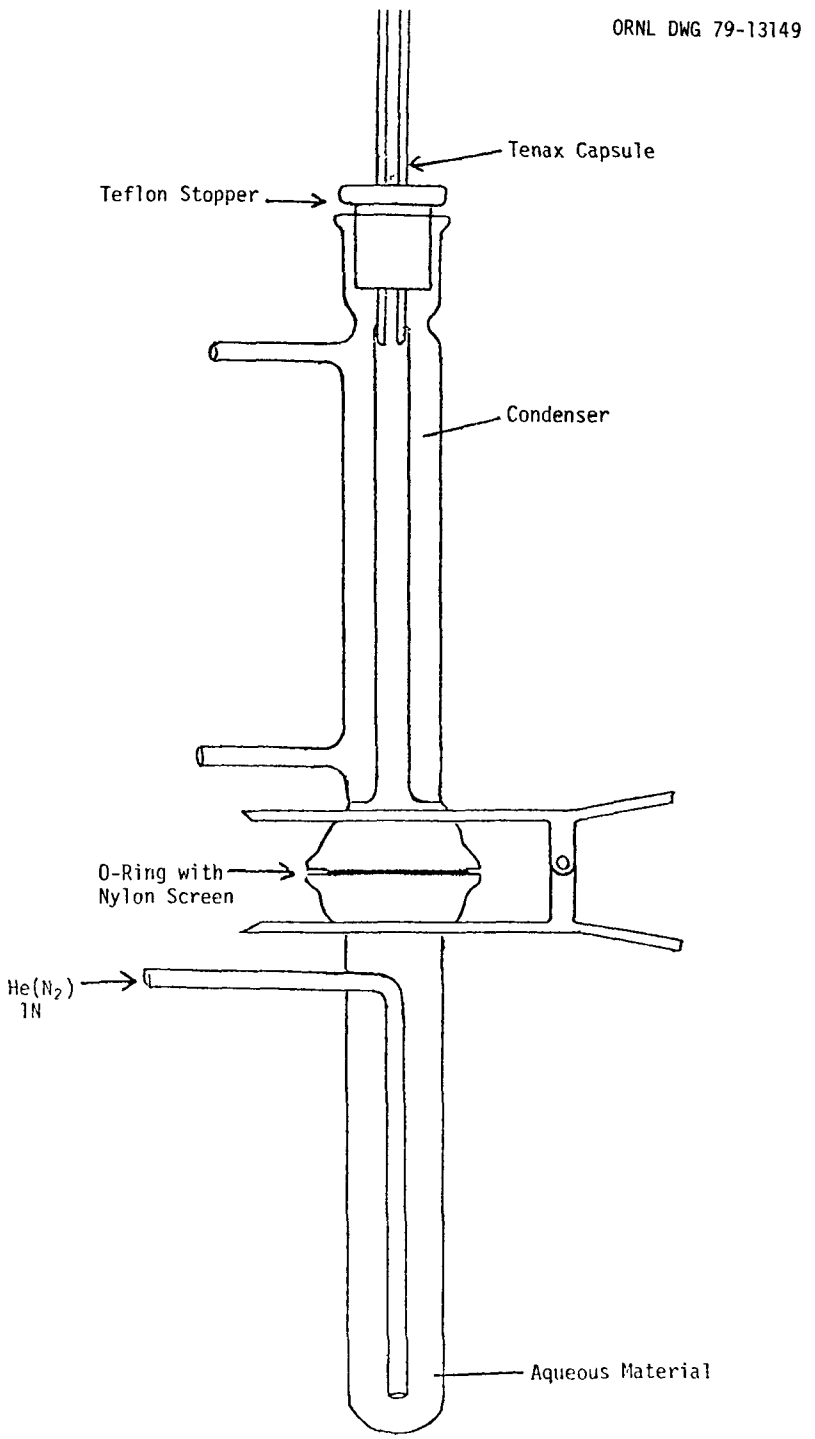

Figure 5-2. Purge and trap apparatus.

be obtained by use of a larger sample. Further evaluation of this method with a EPA Evaluation Standard is in progress.

5.4. Discussion

Inorganic Analyses. The results of the inorganic analyses are presented in Table 5-7. The uncertainty of the analyses at this concentration level typically ranges from ±1 to ±10 percent. In combination with the organic analyses, the results seem to indicate that the main constituents of the EP extracts are ionic in character, as would be expected from the use of a dilute acidic aqueous extractant fluid. On the basis of 10 times the EPA Primary Drinking Water Standard as the maximum permissible concentrations (Table 5-1) of dissolved metals in the EP extracts, the As-contaminated groundwater sample would be labeled toxic from its content of As and Cd. Considering the unknown history of this sample, our result for As of 412 mg/liter compares well with the value (480 mg/liter) labeled on the original sample container, apparently from an analysis conducted elsewhere in June, 1978. Handling and storage history prior to our receipt of the sample are unknown, and may contribute to this difference.

Of the remaining four EP extracts, the only one which may be labeled toxic by these criteria is fly ash, by virtue of its borderline Cd level. The proposed definition of a hazardous waste does not include a threshold for fluorine. However, fluoride in the fly ash EP extract was five times the Primary Drinking Water Standard, or one-half a "10 times standard" level.

It is interesting to note the increasing concentration trend in the EP extracts of bottom ash < scrubber sludge < fly ash for several of the elements. This observation is consistent with the preferential accumulation[8] of many of these elements in fly ash vs. bottom ash.

Metals analyses also were performed on the EP extract blanks, as shown in Table 5-7. All of the metals in the blanks were far below their maximum permissible concentrations, except for Ba and Zn, which were significant in comparison with the levels in some of the EP extracts. Thus although concentrations in these blanks were

TABLE 5-7

TRACE ELEMENTAL ANALYSES OF As-CONTAMINATED GROUNDWATER SAMPLE, EP EXTRACTS, AND BLANK

Element	Concentration (mg/liter) in:					
	As-contaminated groundwater	EP extracts of:				Blank
		Fly ash	Scrubber sludge	Bottom ash	Soybean process cake	
Ag	<0.01	<0.01	<0.01	<0.01	<0.01	<0.001
As	412	<0.01	0.05	<0.01	<0.01	<0.001
Ba		<0.50	<0.50	<0.50	<0.50	<0.500
Be	<0.01	0.01	<0.01	<0.01	<0.01	<0.001
Cd	0.49	0.10	0.01	<0.01	<0.01	0.001
Cr	<0.01	<0.01	0.01	<0.01	<0.01	0.001
Cu	0.01	0.05	0.02	0.01	0.07	0.004
Hg	<0.01	<0.01	<0.01	<0.01	<0.01	<0.001
Ni	0.94	0.66	0.14	0.02	0.02	0.013
Pb	0.12	<0.01	<0.01	<0.01	<0.01	<0.001
Sb	0.30	0.04	0.03	<0.01	<0.01	<0.002
Se	0.01	<0.01	0.03*	<0.01*	<0.01*	<0.001
Tl	7.72	0.02	0.01	<0.01	<0.01	<0.001
Zn	0.25	1.55	0.24*	0.03*	0.11*	0.283
F		8.00	3.0	<0.10	<0.10	<0.100

*Single determination.

low relative to RCRA thresholds, they still could influence a borderline case, as for example, the fly ash EP extract.

An optical emission spectroscopic survey analysis was also performed on a precipitate which formed in the EP extract of scrubber sludge shortly after generation. As might be expected from the limestone employed in the scrubber, the major element in the precipitate was Ca. Approximately 0.1 mg/g of Mg and B also were detected.

Organic Analyses. The first five samples, consisting of one arsenic-contaminated groundwater sample and extracts from four wastes, were subjected to the analytical protocols detailed in Appendix III and discussed above. In addition to these wastes, four EP extract blanks were taken through the same analytical scheme to evaluate the contribution of the blank to the composition of the EP extracts. Detection limits for a 500-ml sample were: for PAH's, 2 μg/liter; for volatiles, 0.01 ng/liter; for PCB's, 0.1 ng/liter; for pesticides, 0.1–1 ng/liter. No PAH's, volatiles, or pesticides were detected in groundwater or any of the extracts or blanks. PCB's were detected at a concentration of ~0.2 ng/liter for the As-contaminated groundwater and all four extracts; they were detected at a concentration of ~1 ng/liter for all four blanks. The identification of PCB's in EP extracts is tentative.

For the analysis of purgeable volatiles, a few milliliters of EP extract were taken immediately after generation, bottled, and refrigerated in order to preserve any volatiles present in the fresh extract. No volatiles could be detected in these samples above the 0.1 ng/liter level. Even when the extracts were frozen immediately after generation, no volatiles were present. This result is not suprising in view of the fact that the wastes were stirred vigorously in open containers for 24 hr, so any volatiles present probably were removed to the atmosphere during the EP. To obtain meaningful data on the content of the volatile organic species in wastes it will be necessary to analyze the wastes directly. An alternative would be to modify the EP apparatus so as to continuously purge the system through a Tenax adsorbent

trap during the waste extraction. In any case, the presence of high levels of volatile Priority Pollutants in wastes conceivably could present a significant health/environmental hazard which would not be detected by the EP protocol.

Interestingly, the As-contaminated groundwater sample did not have a detectable level of volatile organics either. This result may have been influenced by the handling of the sample prior to our receipt. The groundwater sample was received in a partially filled plastic jug 3 months after collection. The sample collection, storage, and shipping conditions prior to receipt are unknown.

The analyses conducted in search of PAH's and other organics revealed that the EP extracted little or no organic material in the classes analyzed, within the defined detection limits (1 mg/liter). In scans for PCB's, pesticides, and other halogenated compounds there was evidence of electron-capturing material at a concentration of approximately 0.2 ng/liter in the EP extract. The similarity in the profiles of each corresponding sample fraction suggests background material and not the compounds characteristic of the waste or groundwater. A further examination of the PCB fraction of the As-contaminated groundwater by GC–mass spectroscopy revealed that any material present was below the detection limits. The material indicated by the electron-capture detector, although unidentified at present, is nonetheless from 100 to 1000 times lower than the maximum allowable concentrations suggested for pesticides.

Electron-capture detector GC profiles (Figure 5-3) of the PCB/pesticide fractions of three samples and a PCB standard equivalent to a concentration of 0.2 ng of PCB per liter of EP extract serve to illustrate the low concentrations of organics present in the EP extracts. Very little material is visible in these profiles, except for two prominent peaks in the fractions of the blank and soybean process cake. These constituents were, however, too low in concentration for mass spectrometric identification.

The o-nitroaniline in the As-contaminated groundwater sample was the only organic constituent quantified from these samples. A specific measurement indicated

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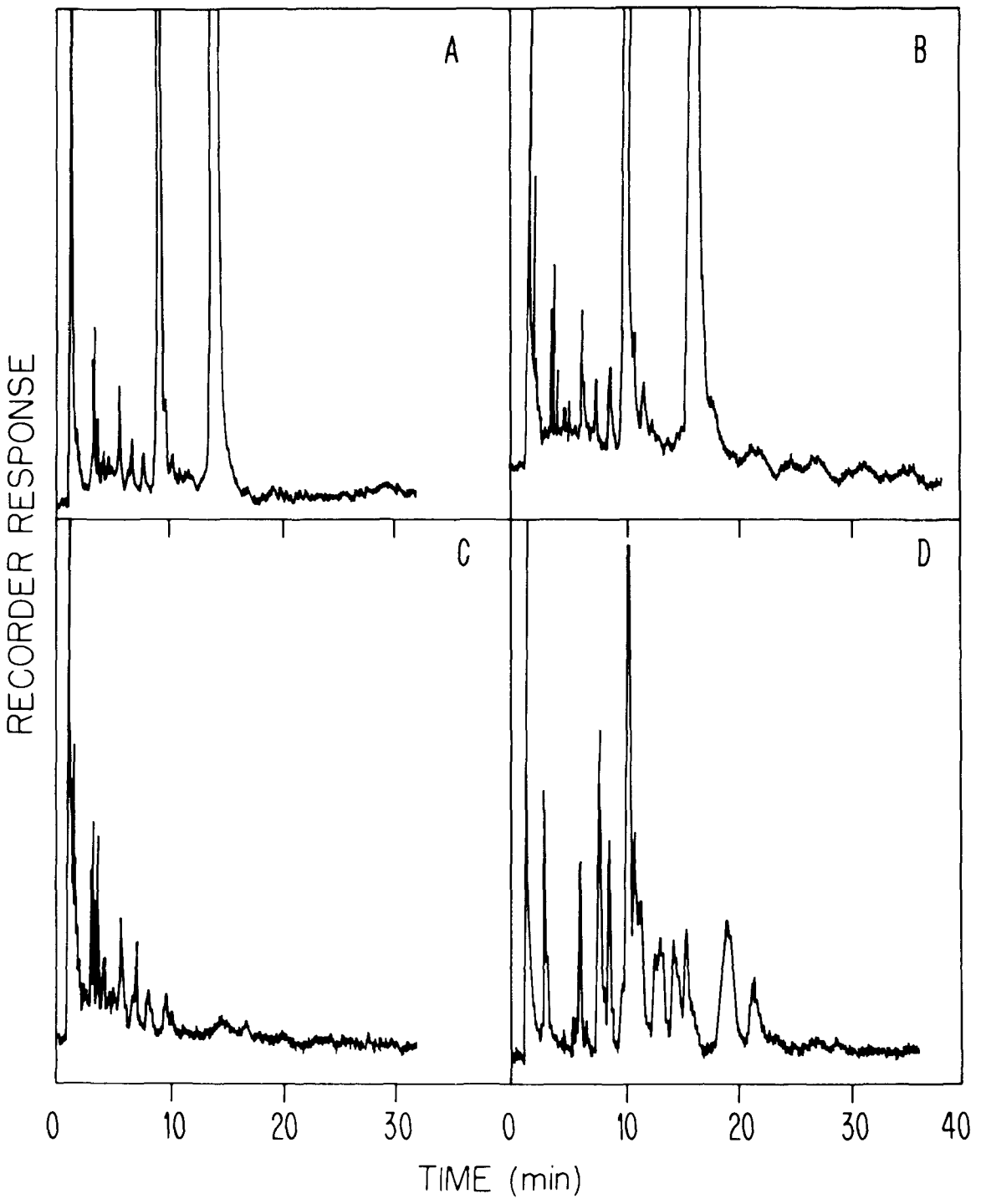

Figure 5-3. Electron-capture GC analysis of PCB/pesticide fraction of EP Extracts of (A) Blank 3, (B) soybean process cake, (C) scrubber sludge, and (D) a PCB 1242 standard equivalent to 0.2 ng/liter of PCB in an EP Extract.

a concentration of 320 mg/liter. No other compounds were even detected in the measurement of o-nitroaniline, supporting the results of the analysis of the XAD-2 organic concentrates of the EP extracts.

5.5 Conclusions

Because the EP is basically an aqueous extraction of the waste, it would not be expected to be an aggressive extractor of nonpolar organic species present in the waste. Furthermore, since the extraction is carried out in an open system, volatile organic toxicants (e.g., chloroform, trichloroethylene) would be expected to vaporize from the system.

Many organic wastes are actually liquids; therefore there may not be a need for a more aggressive extractant, since for a fluid waste the extract is, in fact, the waste itself. In the case of a multiphase waste, the final EP extract contains both the original liquid portion of the waste and the extract from the solid portion. Thus this liquid may function as a cosolvent and greatly increase the apparent aggressiveness of the extraction process.

If further work shows this to be a real problem, alternative equipment and extractants might be used for the assessment of organic contaminants which may be released in the landfill disposal of wastes. One attractive alternative may be steam distillation.

For inorganic species we feel that the present acidic extraction is adequate. The addition of extract blanks to the protocol is advisable to allow corrections for background contamination due to either equipment or reagents used in the various procedures. In some instances not running a blank may result in a false classification of the waste as hazardous.

A comparison of the EP extracts with actual leachate samples taken from landfills containing mixed wastes would also be desirable as a further means of evaluation of the EP.

6. AQUATIC TOXICITY

6.1. Problem Definition

The objective of the aquatic screening tests was to determine the toxicity and potential hazard of extracts from solid wastes to aquatic biota. The tests used were acute (short-term lethality) tests and chronic (long-term) tests designed to measure sublethal effects on reproduction. The test organism was the cladoceran, D. magna, an aquatic organism sensitive to most classes of toxic aqueous chemicals.

6.2. Scope of Work

The two tests had different purposes. The acute test estimated the concentration of the extract that was lethal to D. magna during a short exposure period of 48 hr, whereas the chronic test determined whether continuous exposure to extract dilutions of 1:100 and 1:1000, impaired reproduction of D. magna. The chronic test did not, therefore, establish a dose-response relationship for the extract, such as an LC_{50}, but only determined whether or not the extract was toxic at the concentrations tested.

The procedures used are described in Appendix IV.

The parameters measured to assess the effects of chronic exposure of D. magna to the extracts were length of life, day of first brood release, number of broods of young produced, number of young per brood, and total number of young produced per daphnid. Based on the results of the chronic exposure tests, one or more of these parameters were selected as criteria for identifying those extracts potentially hazardous to aquatic biota. The sensitivities of the parameters to chronic exposure to the extracts were compared by analysis of variance combined with Duncan's Multiple Range Test to identify significant differences between individual treatment means. A type I error level (α) of 0.05 was set to identify significant differences between treatment means.

6.3. Results

The As-contaminated groundwater exhibited both acute and chronic toxicity. In the two tests, the 48-hr LC_{50} occurred at dilutions of 2.2 and 1.7 percent (Table 6-1). The results of the chronic toxicity tests are shown in Table 6-2. No significant effects on reproduction or survival occurred at the 1:1000 dilution, but at the 1:100 dilution, significant effects were found for three of the five parameters measured. The mean number of young produced per adult and the mean number of young per brood were both reduced by 88 percent compared with the dilution water controls. Also, the mean day (age) of first brood release was increased from 11.3 for the controls to 13.7 for the 1:100 dilution.

In tests of the three wastes from a power plant, all of the extracts from the wastes had indicated low acute toxicities (Table 6-1). The 48-hr LC_{50}'s ranged from 60 to 94 percent. Chronic toxicity effects on daphnid survival and reproduction were also small (Table 6-2).

The fly ash extract produced no significant adverse effects on any of the five test parameters at either test dilution.

The scrubber sludge extract produced some significant inhibition in the number of broods and number of young per brood when compared with the acetic acid control; but, those effects were not significant when compared with the dilution water controls. The inhibition in the number of young produced (compared with the acetic acid controls) was 27 percent at the 1:1000 dilution and 18 percent at the 1:100 dilution, while the inhibition in number of young per brood was 32 percent and 16 percent, respectively.

The bottom ash extract was the only one of the five extracts tested to have an apparent effect on survival of the animals in the chronic tests. At the 1:100 dilution, survival (measured as an average length of life) was 14 percent less than for the acetic acid controls. These effects were not seen at the 1:1000 dilution. The number of broods of young, the number of young, and the number of young per brood were also reduced in both test dilutions, but these effects were not statistically significant.

TABLE 6-1

ACUTE TOXICITIES OF As-CONTAMINATED GROUNDWATER SAMPLE AND EP EXTRACTS TO D. magna

Extract	48-hr LC_{50}* (%) (95% fiducial limits)	
	First test	Second test
As-contaminated groundwater	2.2†	1.7†
Fly ash	90 (71 to >100)	69†
Scrubber sludge	85 (51 to >100)	60†
Bottom ash	94†	60†
Soybean process cake	‡	§

*Concentration of the extract necessary to kill or immobilize 50% of the test organisms in 48 hr.

†Approximate value; 95% fiducial limits could not be calculated.

‡Undiluted extract had no effect on organisms.

§Less than 50% of the test organisms were killed or immobilized in 100% of the extract.

TABLE 6-2

CHRONIC TOXICITY OF As-CONTAMINATED GROUNDWATER AND EP EXTRACTS TO *D. magna*[*]

Extract and concentration	Life length during study (days)	Day of first brood release	No. of broods of young/adult	No. of young produced/adult	No. of young/brood
As-contaminated groundwater					
Control	$27.2^{a} \pm 1.8$	$11.3^{a} \pm 1.0$	$5.2^{a} \pm 0.8$	$90.3^{a} \pm 15.4$	$17.8^{a} \pm 4.1$
1:1000	$26.1^{a} \pm 4.2$	$12.5^{a} \pm 1.6$	$4.8^{a} \pm 1.1$	$79.5^{a} \pm 24.3$	$16.5^{a} \pm 3.4$
1:100	$28.0^{a} \pm 0.0$	$13.7^{b} \pm 1.0$	$5.0^{a} \pm 0.8$	$11.2^{b} \pm 10.8$	$2.1^{b} \pm 1.7$
Fly ash					
Control	$25.8^{c} \pm 4.7$	$10.8^{c} \pm 0.6$	$5.5^{c} \pm 1.5$	$106.1^{c} \pm 36.2$	$20.0^{c} \pm 0.9$
Acetic acid control[†]	$25.2^{c} \pm 5.9$	$11.0^{c} \pm 0.0$	$5.5^{c} \pm 2.0$	$116.0^{c} \pm 46.3$	$20.5^{c} \pm 3.4$
1:1000	$27.0^{c} \pm 3.2$	$11.0^{c} \pm 0.0$	$5.7^{c} \pm 1.1$	$134.3^{c} \pm 25.3$	$23.6^{c} \pm 2.8$
1:100	$27.5^{c} \pm 1.6$	$11.0^{c} \pm 0.0$	$5.7^{c} \pm 1.3$	$133.0^{c} \pm 20.2$	$23.8^{c} \pm 3.2$
Scrubber sludge					
Control	$28.0^{d} \pm 0.0$	$9.0^{d} \pm 0.0$	$6.8^{d} \pm 0.4$	$139.8^{e} \pm 6.2$	$20.7^{e} \pm 2.0$
Acetic acid control[†]	$28.0^{d} \pm 0.0$	$10.7^{d} \pm 2.3$	$6.8^{d} \pm 1.0$	$186.0^{d} \pm 39.2$	$27.0^{d} \pm 3.1$
1:1000	$27.8^{d} \pm 0.4$	$9.0^{d} \pm 1.1$	$7.2^{d} \pm 0.4$	$134.8^{e} \pm 25.0$	$18.8^{e} \pm 3.6$
1:100	$28.0^{d} \pm 0.0$	$9.0^{d} \pm 0.0$	$6.6^{d} \pm 0.9$	$151.6^{e} \pm 12.6$	$23.4^{e} \pm 4.0$
Bottom ash					
Control	$23.2^{f} \pm 8.2$	$8.6^{g} \pm 4.7$	$5.0^{f} \pm 2.8$	$84.2^{f} \pm 53.0$	$13.5^{f} \pm 8.5$
Acetic acid control[†]	$24.9^{f} \pm 6.9$	$12.0^{f} \pm 4.9$	$4.6^{f} \pm 2.3$	$72.0^{f} \pm 40.9$	$13.5^{f} \pm 7.5$
1:1000	$24.5^{f} \pm 4.3$	$12.8^{f} \pm 0.6$	$4.4^{f} \pm 1.5$	$62.1^{f} \pm 33.2$	$13.6^{f} \pm 5.1$
1:100	$21.4^{f} \pm 6.8$	$12.1^{f} \pm 2.1$	$3.7^{f} \pm 2.3$	$45.4^{f} \pm 42.3$	$10.2^{f} \pm 4.6$
Soybean process cake					
Control	$27.4^{h} \pm 1.3$	$11.3^{h} \pm 1.0$	$6.6^{h} \pm 1.0$	$99.2^{i} \pm 16.3$	$15.1^{i} \pm 1.9$
Acetic acid control[†]	$27.5^{h} \pm 1.6$	$11.0^{h} \pm 0.0$	$6.5^{h} \pm 0.7$	$118.5^{h,i} \pm 23.2$	$18.2^{h,i} \pm 2.0$
1:1000	$27.7^{h} \pm 1.0$	$11.0^{h} \pm 0.0$	$6.8^{h} \pm 1.0$	$111.8^{h,i} \pm 31.4$	$16.7^{h,i} \pm 4.9$
1:100	$27.5^{h} \pm 1.6$	$11.0^{h} \pm 0.0$	$6.5^{h} \pm 1.2$	$131.7^{h} \pm 23.7$	$20.5^{h} \pm 3.5$

[*]Values given are mean ± S.E. Means with the same letter are not significantly different ($\alpha = 0.05$).

[†]Acetic acid, neutralized to pH 7.0 with NaOH, added at same concentration used in the 1:100 dilution of the extract.

The soybean waste had the lowest acute toxicity of the five wastes tested. During the first acute test, no animals were killed or immobilized after an exposure to 100 percent of the extract for 48 hr, and in the second test less than 50 percent were adversely affected. The low toxicity of the extract was also observed in the chronic toxicity tests. No significant reductions in any of the five parameters measured were observed in either of the two test dilutions.

6.4. Discussion

In the chronic tests, the effects of the waste extracts were measured on five daphnid population parameters (Table 6-2). Of these, the most consistently sensitive indicators of chronic toxicity were the number of young per brood and the total number of young produced; length of life, day of first brood release, and number of broods produced were little affected by even the most toxic sample, the As-contaminated groundwater. Since the total number of young produced over a given period is a function of the number of young per brood and the number of broods produced per female, both parameters would be expected to have similar sensitivity to these extracts.

Additional studies, still in progress, on the reproducibility of the 28-day D. magna renewal chronic toxicity test used in this project have demonstrated the following: (1) The mean number of young produced per brood and the mean total number of young produced per daphnid during the 28-day test were again the parameters which were consistently the most sensitive to chemical toxicity. (2) When these parameters were used as criteria for determining chronic toxicity, the 28-day test was highly reliable and consistent in determining (a) the maximum concentration of toxic chemical which produced no significant observed toxic effects and (b) the minimum concentration of the toxic chemical which would produce significant observed toxic effects. (3) When the 28-day test was repeated at intervals of 3 months for a total of three tests, the mean values obtained on each of five daphnid population parameters, measured at five different exposure concentrations of the

toxic chemical plus controls, frequently varied significantly ($\alpha = 0.05$) among the three tests.

Therefore, the criterion selected for identification of extracts potentially hazardous to aquatic biota was a reduction ($\alpha = 0.05$) in the mean number of young produced per daphnid during the 28-day exposure to the extracts when compared with the dilution water control daphnids. Based on this criterion, only one of the five wastes tested, the As-contaminated groundwater, would be classified as potentially hazardous (Table 6-3). Of the remaining four wastes only one, the scrubber sludge, showed any significant chronic toxicity; however, significant effects were only observed when the two test dilutions were compared with the concentrated acetic acid control. It should be pointed out that the toxicity is probably the result of the acetate in the extraction liquids. If the toxicity is compared with the toxicity of dilution water containing the same amount of acetate as is present in the sample, then none of the materials tested would receive a rating of hazardous.

From these results it is not possible to predict whether or not these wastes would be hazardous to natural populations or communities of aquatic organisms. There are severe limitations in the extrapolation of laboratory results based on single-species tests for predicting effects on natural ecosystems. For example, the test systems used in single-species tests are very simple compared with the complexity of natural ecosystems. Thus, a significant toxic effect can be demonstrated in the laboratory, but under natural conditions it may be mitigated, modified or even magnified by the chemical, physical, and biological interactions of the ecosystem. D. magna, however, is sensitive to most classes of toxic chemicals, and results of toxicity tests with it will generally be conservative predictors of potential hazard to other aquatic species.

On the other hand, there are limitations in the EP and in the D. magna chronic toxicity test system which may result in certain types of toxic wastes not being identified as being potentially hazardous in the protocols. All volatile chemicals, for example, are probably lost during the EP. In addition, the

TABLE 6-3

SUMMARY OF THE *D. magna* ACUTE AND CHRONIC TOXICITY TEST RESULTS

Extract	48-hr LC_{50} (%)	Significance of chronic toxicity test results*	
		1:1000 dilution	1:100 dilution
As-contaminated groundwater	2.2	ns	s
Fly ash	90	ns	ns
Scrubber sludge	85	ns†	ns†
Bottom ash	94	ns	ns
Soybean process cake	No significant acute toxicity	ns	ns

*The criterion used to judge significant toxicity was a significant reduction ($\alpha = 0.05$) in the mean number of young produced at either dilution compared with the dilution water controls. ns, no significant toxicity; s, significant toxicity.

†Significant toxicity was not observed when the treatment means were compared with the dilution water control mean; however, significant toxicity was observed when the treatment means were compared with the acetic acid control mean.

renewal-type test procedure used in the chronic toxicity test would be relatively inefficient, compared with a continuous flow-through test system, for detecting nonpersistent chemicals.

In summary, this test should only be used as a screening mechanism to identify hazardous wastes containing toxic chemicals that can be extracted under a specific set of conditions and that are chronically toxic to a sensitive aquatic species under a specific set of test conditions. The test only identifies those wastes that are potentially hazardous.

Acute toxicity tests before and after the 28-day chronic tests were done to determine if the toxicity of the extracts had changed during the chronic tests. Because of the low toxicities of most of the extracts, however, reliable estimates of 48-hr LC_{50}'s and their 95 percent fiducial limits could not be obtained. The results (Table 6-2) suggest that all five wastes tended to increase slightly in toxicity during the 28-day period, but the differences are probably not statistically significant.

6.5. Conclusions

The 28-day chronic toxicity test with D. magna can be used to show the presence of toxic materials in extracts, as shown by the results with the As-contaminated groundwater. Daphnid reproduction, however, is greatly influenced by food supply. The extracts often contain high concentrations of acetic acid, which is neutralized to pH 7.0 in the tests. The acetate may be a substrate for bacteria, which are in turn fed on by the daphnids, resulting in an increase in the number of young produced. In three of the four extracts in which acetic acid was used, the production of young in the acetic acid controls was substantially higher than in the dilution water controls (without acetic acid). In addition, research indicates that the effect of acetate on D. magna varies with concentration. Although at low concentrations acetate tends to increase the reproduction rate, at concentrations equal to or greater than 0.1 percent it becomes acutely toxic. Acetic acid is even more toxic than acetate, with an estimated 48-hr LC_{50} of about 100 mg/liter. The

toxicity of acetic acid, however, is probably more a function of pH than acetate toxicity. What is not known are the effects or interactions that acetic acid (or acetate) may have on other toxic chemicals in the waste materials being extracted. With respect to toxicities to aquatic biota, these effects could possibly by synergistic or antagonistic, and in either case would tend to further obfuscate the interpretation of toxicity test results. We, therefore, recommend that use of acetic acid in the EP for aquatic toxicity be reevaluated as a screening protocol.

7. MUTAGENICITY

7.1. Problem Definition

Because the concept of toxicity includes unnatural genetic activity (including oncogenic, mutagenic, and teratogenic activity), the Office of Solid Waste recognizes the need for rapid and effective methods for the detection of such activity in complex mixtures from the EP.

The bioassay protocol in this project is intended to serve as an indicator of the chronic hazards of mutagenicity and carcinogenicity. Because of systemic differences in the reaction to mutagenic substances, a battery of assays has been employed by the Biology Division. The approach taken involves microbial assays with both eukaryotic and prokaryotic organisms which detect point mutation specifically and DNA damage generally.

The vast majority of known chemical mutagens are organic in nature. Thus, because the concept of threshold is ill-defined for mutagens and carcinogens, and because the EP extract was anticipated to be low in organic character, it was deemed advisable to examine a concentrated extract of the organic constituents of the EP extract as well as the extract itself.

According to the definition of a waste as a hazard (in 43 FR 58961) the application of three assays for genetic activity would be required to delist a waste that is listed as hazardous because of mutagenic activities. The three tests specified are: (1) gene (point) mutations in bacteria, (2) gene mutations in eukaryotes, either in mammalian somatic cells in culture or in fungal microorganisms, (3) recombinogenic or repair-related phenomena. The tests selected in these categories are (1) the Salmonella/microsome assay, (2) the Saccharomyces $\underline{can}^r/\underline{his}^+$ dual assay, and (3) the Salmonella $\underline{uvrB}$ repair assay. The Biology Division of ORNL has had considerable experience applying the Salmonella and Saccharomyces mutation assays to the analysis of complex mixtures, hence their inclusion. Additionally, because of the involvement of the $\underline{uvrB}$ mutation in the design of the Salmonella assay, the Salmonella repair assay was selected for group three.

The Salmonella/microsome assay utilizes a series of histidine-requiring mutants that revert after treatment with mutagens to the wild-type state (histidine-independent). Generalized testing is accomplished by use of two strains, TA1537 and TA98, that detect frameshift mutagens, and two strains, TA1535 and TA100, that detect base-pair substitution mutagens.

The Saccharomyces assay utilizes both a forward and a reverse mutation scheme. Forward mutation is detected by the inactivation of the arginine permease gene, leading to resistance to the toxic antimetabolite canavanine. Reverse mutation is monitored with a histidine auxotroph which reverts by base-pair substitution.

The Salmonella uvrB repair assay does not measure mutation per se, but DNA damage induced by chemical treatment. The test system employs paired, identical strains except that one (TA1978) has normal DNA repair capabilities (uvrB$^+$) and one (TA1538) lacks a specific step (uvrB$^-$) in the enzyme pathways responsible for DNA repair. Preferential killing of the repair-deficient strain by the test substance implies that the material exerts its killing effect by reacting with the cells' DNA, and therefore may be mutagenic.

Full details of the procedures for these assays are given in Appendices V, VI, and VII.

7.2. Results

Four EP extracts, the As-contaminated groundwater sample, and their XAD-2 concentrates have been tested in the Salmonella/microsome assay, the Saccharomyces forward and reverse mutation assay, and the Salmonella repair assay. The assays were applied both with and without metabolic activation (both Ar- and ϕB-induced rat liver S-9 mix were used). The aqueous extracts were tested as received; the XAD-2 concentrates were taken up in 2 ml of dimethylsulfoxide (DMSO), producing a 250-fold (v/v) concentration of the organic material in the aqueous extracts, with the exception of the As-contaminated groundwater, whose concentration factor was 12.5-fold.

Salmonella/Microsome Assay. The As-contaminated groundwater was slightly mutagenic with the frameshift strain TA98, but only upon metabolic activation with Ar-induced S-9 mix (Table 7-1). The sample was not toxic at the concentrations tested. Additionally, the XAD-2 concentrate of As waste was not mutagenic with the missense strain TA1535 (Table 7-2). However, it was mutagenic with the frameshift strains, TA1537 and TA98, and the highly sensitive TA100 strain. It did not require metabolic activation, and the addition of S-9 mix (Ar- or ϕB-induced) reduced the mutagenic activity. It was not toxic at the dose range tested. A dose-dependent response was elicited.

Neither the aqueous extracts nor the XAD-2 concentrates from the power plant wastes or the soybean process cake were mutagenic with or without metabolic activation (Tables 7-3 to 7-6). None of the materials displayed toxicity in the dose range tested.[7]

Saccharomyces Mutation Assay. The As-contaminated groundwater sample was not mutagenic (Table 7-7). However, its XAD-2 concentrate was mutagenic without metabolic activation, given a 24-hr 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.

The extracts and concentrates of the power plant samples and the soybean process cake were not mutagenic with or without metabolic activation (Tables 7-8 to 7-11). None of the material was toxic.

Salmonella DNA Repair Assay. None of the materials displayed activity in this assay, either with or without metabolic activation (Table 7-12). The As-contaminated groundwater was moderately toxic to both test strains (Table 7-12).

7.3. Discussion

Of the five wastes discussed in this report, only the As-contaminated groundwater possessed detectable mutagenic activity. For the purposes of bioassay, the mutagenic principal in the undiluted waste water is at the limit of resolution;

TABLE 7-1

SALMONELLA MUTATION: As-CONTAMINATED GROUNDWATER

Volume (μl)	Revertants/plate	
	TA98	TA100
No activation		
Control	59	152
0.025	NT*	NT
0.050	NT	NT
0.500	55	107
5.000	45	145
φB activation		
Control	67	142
0.025	60	150
0.050	53	105
0.500	67	117
5.000	52	137
Ar activation		
Control	67	142
0.025	87	193
0.050	87	151
0.500	70	190
5.000	96	141

*NT, not tested.

TABLE 7-2

SALMONELLA MUTATION: As-CONTAMINATED GROUNDWATER XAD-2 CONCENTRATE

Volume (μl)	Revertants/plate			
	TA1535	TA1537	TA98*	TA100*
No activation				
Control	12	10	59	152
2.5	NT†	NT	59	151
5.0	27	16	153	215
10	18	41	268	414
25	32	70	500	669
50	29	94	690	963
75	T‡	115	563	828
φB activation				
Control			67	142
2.5			59	120
5.0			96	150
10	NT	NT	NT	226
25			79	288
50			206	414
75			NT	590
Ar activation				
Control	6	7	67	142
2.5	NT	NT	82	155
5.0	11	15	97	162
10	15	27	NT	212
25	22	44	157	243
50	13	52	292	295
75	16	69	NT	508

*Average from two or three independent experiments.

†NT, not tested.

‡T, toxic.

TABLE 7-3

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

Concentration (μl/plate)	Revertants/plate							
	TA1535		TA1537		TA98		TA100	
	EP	XAD-2	EP	XAD-2	EP	XAD-2	EP	XAD-2
No activation								
Control	21	NT*	14	NT	32	57	136	231
50	18	NT	15	NT	27	30	141	180
φB activation								
Control	19	NT	8	NT	28	22	148	227
10	37	NT	17	NT	30	15	154	216
25	28	NT	16	NT	34	40	106	220
50	21	NT	18	NT	19	30	122	232
75	14	NT	16	NT	33	30	177	180
Ar activation								
Control	15	NT	16	NT	46	33	192	209
10	15	NT	14	NT	35	42	108	213
25	8	NT	15	NT	34	28	141	206
50	16	NT	15	NT	53	26	123	206
75	16	NT	18	NT	42	38	172	208

*NT, not tested.

TABLE 7-4

SALMONELLA MUTATION: SCRUBBER SLUDGE EP EXTRACT AND ITS XAD-2 CONCENTRATE

Concentration (µl/plate)	Revertants/plate							
	TA1535		TA1537		TA98		TA100	
	EP	XAD-2	EP	XAD-2	EP	XAD-2	EP	XAD-2
No activation								
Control	20	28	14	19	29	29	140	155
50	21	18	14	18	32	32	136	170
φB activation								
Control	19	15	8	19	28	27	148	192
10	13	19	7	19	19	34	140	182
25	20	16	8	kill	18	28	110	165
50	7	22	9	kill	22	22	120	148
75	15	18	6	kill	14	18	140	137
Ar activation								
Control	15	18	16	28	46	34	192	140
10	19	5	19	14	56	34	137	148
25	26	9	20	19	32	40	146	138
50	17	8	14	9	40	30	125	119
75	17	8	15	14	34	43	156	kill

TABLE 7-5

SALMONELLA MUTATION: BOTTOM ASH EP EXTRACT AND ITS XAD-2 CONCENTRATE

Concentration (µl/plate)	Revertants/plate							
	TA1535		TA1537		TA98		TA100	
	EP	XAD-2	EP	XAD-2	EP	XAD-2	EP	XAD-2
No activation								
Control	16	30	6	15	26	42	77	142
50	8	21	9	16	41	33	106	126
φB activation								
Control	11	26	9	14	19	27	91	112
10	12	22	5	19	31	13	100	125
25	9	17	4	16	18	21	100	119
50	8	20	13	7	24	19	87	117
75	13	19	6	17	30	27	79	146
Ar activation								
Control	12	22	15	18	31	34	99	124
10	7	17	7	13	35	37	82	146
25	10	15	9	17	36	45	76	164
50	13	33	11	16	36	34	75	136
75	9	16	10	14	37	46	78	152

TABLE 7-6

SALMONELLA MUTATION: SOYBEAN PROCESS CAKE EP EXTRACT*

Concentration (μl/plate)	Revertants/plate			
	TA1535	TA1537	TA98	TA100
No activation				
Control	15	13	24	109
50	13	13	24	87
φB activation				
Control	6	4	27	106
10	15	16	37	85
25	13	15	28	87
50	15	15	27	91
75	18	15	26	95
Ar activation				
Control	5	17	30	93
10	15	15	32	82
25	16	15	30	97
50	14	15	29	79
75	19	16	19	83

*Soybean process cake XAD-2 concentrate was highly contaminated. Filter sterilization was not possible.

TABLE 7-7

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

TABLE 7-8

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		16		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

TABLE 7-9

YEAST MUTATION: SCRUBBER SLUDGE 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	22	19	26	13	8	14	14
10		102		96		19		23		9		12
20		97		102		19		21		10		11
50		98		103		18		23		7		8
100	95	101	90	93	15	20	18	18	12	10	11	10
φB activation												
Control	100	100	100	100	14	23	20	21	13	12	11	9
10		104		102		18		19		18		12
20		103		117		21		18		10		14
50		91		96		21		24		16		17
100	110	86	104	89	17	23	12	26	13	12	8	12
Ar activation												
Control	100	100	100	100	21	19	15	27	14	11	10	11
10		102		105		22		27		14		10
20		97		106		20		21		12		11
50		87		97		26		19		13		9
100	130	71	85	83	11	23	16	17	10	14	9	8

TABLE 7-10

YEAST MUTATION: BOTTOM 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	18	19	18	13	9	14	11
10		102		97		19		21		11		9
20		101		91		14		20		8		11
50		99		88		16		16		10		11
100	85	59	107	77	11	28	5	20	5	13	8	8
φB activation												
Control	100	100	100	100	14	17	20	16	13	11	11	8
10		97		98		13		15		9		3
20		91		101		16		15		10		5
50		61		87		15		13		8		5
100	95	45	103	69	18	16	21	22	15	11	14	8
Ar activation												
Control	100	100	100	100	21	13	15	19	14	6	10	10
10		105		97		14		17		8		10
20		72		92		18		21		8		9
50		96		95		9		21		4		7
100	116	55	101	80	9	16	10	16	6	8	13	8

TABLE 7-11

YEAST MUTATION: SOYBEAN PROCESS CAKE 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	18	19	14	13	14	14	9
10		94		89		16		18		11		10
20		103		80		18		20		10		12
50		86		90		15		17		13		8
100	89	105	111	76		14	14	17	7	12	11	15
φB activation												
Control	100	100	100	100	14	19	20	15	13	10	11	11
10		96		102		15		21		13		13
20		109		97		17		15		10		14
50		93		84		19		19		16		11
100	109	87	113	79	17	26	19	17	9	13	17	17
Ar activation												
Control	100	100	100	100	21	15	15	17	14	11	10	12
10		106		102		21		12		13		16
20		109		100		20		17		7		15
50		98		95		28		15		19		12
100	115	91	96	83	14	15	16	13	13	14	7	12

TABLE 7-12

DNA REPAIR IN SALMONELLA*

Sample and activation	% Survival			
	1978 uvrB+		1538 uvrB-	
	EP extract	XAD-2 concentrate	EP extract	XAD-2 concentrate
As-contaminated groundwater				
None	67	97	42	82
φB	70	91	61	78
Ar	40	70	78	92
Fly ash				
None	120	114	109	85
φB	106	101	93	116
Ar	88	90	100	113
Scrubber sludge				
None	117	72	68	46
φB	104	65	85	80
Ar	120	58	92	82
Bottom ash				
None	51	102	95	110
φB	97	74	81	79
Ar	108	80	83	88
Soybean process cake				
None	84	78	126	82
φB	109	75	96	91
Ar	129	81	110	96

*Maximum dose was 100 µl for all samples except fly ash XAD-2, for which it was 50 µl.

the XAD-2 concentration, however, was necessary to conclusively demonstrate mutagenic activity. Work is currently in progress to determine if the o-nitroaniline content of this waste accounts for its mutagenic properties.

The response of the Salmonella/microsome assay to As-contaminated groundwater implies a frameshift mutation mechanism, which requires the addition or deletion of DNA base-pairs. This is supported by the yeast results with this waste, showing a moderate preponderance of induced forward mutation to can^r, relative to induced reversion of the his base-pair substitution. This is typical for a response (by this system) to a frameshifting agent.

The As-contaminated groundwater and its XAD-2 concentrate failed to elicit a response from the bacterial DNA repair assay. There are two key considerations: (1) the overall mutagenic potency of the waste is moderate, (2) validation studies have shown that the repair assay is particularly insensitive (although not unreactive) to frameshifting agents. Hence, it may not be significant to obtain a negative result in this context.

With regard to the negative results obtained with the power plant wastes and the soybean process cake, the aqueous extracts and XAD-2 concentrates of these materials are extremely deficient in organic character. The majority of organic mutagens are not detectable at the parts-per-billion level. Furthermore, inorganic mutagens (e.g., metals and metal complexes) which might be present are commonly not detectable by the bioassays in question.

7.4. Conclusions

Our experience has shown that biological testing — within the limits of the specific system used — can be carried out with complex organic materials but perhaps only when coupled with the appropriate analytical separation schemes. An extrapolation to relative biohazard at this point would be, at least, premature. A number of precautions are given below.

As noted, aqueous materials will generally contain only low amounts of dissolved organics which may be biologically active. If the intent is to determine

whether mutagenic components are present in a given mixture, clearly, a concentration/fractionation scheme must be applied. However, the detection or perhaps the generation of mutagenic activity may well be a function of the chemical fractionation scheme utilized. The inability to recover specific chemical classes or the formation of artifacts by the treatment could well corrupt the results obtained, in addition to the possibility of an inability to detect the specific biological end point chosen. Along with the obvious bias that could accompany the choice of samples and their solubility or the time and method of storage, a number of biological discrepancies can also enter into the determinations. For example, concomitant bacterial toxicity can nullify any genetic damage assay that might be carried out. The dose-response relationship may not be linear, and some other method for a quantitative comparison may be mandatory. The choice of inducer for the liver enzymes involved can be wrong for selected compounds or mixtures. Furthermore, induction of metabolic enzymes of rat liver includes both activating and deactivating enzymes for potential mutagens. Results with mixtures requiring activation can be complex and different from those with pure organic compounds. Mutagenicity studies should include not only proper metabolic activation systems but also appropriate quantitation of the metabolic enzymes (determined by titration studies) in the assays. Mutagenic analyses of complex mixtures of organic constituents activated with crude and complex enzyme homogenates require careful examination and cautious interpretation. The choice of strain in a reversion assay could be inappropriate for selected active components of a mixture; therefore, a battery of tests should be considered, including an assay for forward mutation.

Additionally, the applicability of the generally used Salmonella test to other genetic end points and the validation of the apparent correlation between mutagenicity and carcinogenicity still remains a point to be validated through significant fundamental research. The question of a correlation between mutagenic potency in the Salmonella assay and carcinogenic potency should be treated with caution. Again, the short-term assays chronically show negative results for certain classes of organics. Similarly, compounds involved in or requiring cocarcinogenic

phenomena would presumably go undetected. Recent studies point to synergistic effects between compounds that may further complicate quantitative interpretation of results with complex mixtures.

As a prescreen to aid the investigators in ordering their priorities, short-term testing appears to be a valid approach for complex mixtures. Over-interpretation at this stage of research, especially with respect to relative hazard or negative results, should be avoided.

8. PHYTOTOXICITY

8.1. Problem Definition

As part of our work, ORNL has developed and evaluated short-term phytotoxicity tests for use in screening potentially hazardous wastes. These tests were to be performed on a number of wastes extracted by the EP described in Section 3. Thus, we were in a position to identify any problems with the EP which might be reflected in the phytotoxicity tests. Difficulties with phytotoxic effects of acetic acid used in the EP were anticipated because of reports in the literature that acetic acid, even at relatively low levels, can inhibit plant growth.[9]

8.2. Methodology

Short-term (48 and 72 hr) root (radicle) elongation tests were performed with radish and sorghum seeds in a controlled environment. Long-term (2 week) seedling growth studies were carried out in pots under greenhouse conditions with wheat and soybean seeds. Parameters measured were root length in the short-term tests and root-shoot dry weight in the long-term tests. Treated plants were compared with plants grown in distilled water in the case of the short-term tests and a plant nutrient solution in the long-term study. The treated plants received a 10 percent concentration of the EP extract (diluted with nutrient solution) in all the long-term tests to simulate a tenfold dilution that EPA used in its scenario to indicate what could occur as water moves through an underground aquifer. This groundwater could then be used to irrigate crops. In the short-term tests, treated plants received a series of concentrations of the EP extracts. In a few cases concentrations used in these short-term tests did not reach the 100 percent level because of the amount of acetic acid used in the EP. Preliminary results with radish and sorghum treated with different concentrations of acetic acid show that plant growth is inhibited only when the acetic acid concentration exceeds

≃ 5 ml/liter of 0.5 N acetic acid. Further testing is being performed with wheat and soybean.

The plant species selected for these tests are important agricultural species and represent the two major classes (monocotyledons and dicotyledons) of flowering plants. Because of the differences in growth habits of these two classes of plants, they may respond differently to various chemicals in their environment. Of course, this is also true to a lesser extent between individual species of the same class. However, it was not feasible to test large numbers of many different species. Detailed descriptions of the test methods are given in Appendices VIII and IX.

8.3. Results

Arsenic-contaminated groundwater was highly phytotoxic, producing a 33 percent growth reduction of radish roots (radicles) even at a 2 percent concentration. Higher concentrations (10 and 5 percent) reduced growth 70 and 59 percent, respectively. However, at the highest dilution (0.1 percent) there was a slight stimulation of root growth.

Data from other tests are presented in Tables 8-1 and 8-2. Table 8-3 is a summary of the results presented in Tables 8-1 and 8-2.

None of the waste extracts tested showed toxic effects in all the tests. Scrubber sludge was toxic to radish seeds in the root elongation test even at a 10 percent concentration, but the same concentration was not toxic to sorghum. Fly ash, soybean process cake, and bottom ash were only slightly toxic to either radish or sorghum at concentrations exceeding 10 percent. In the seedling growth studies, fly ash and soybean process cake showed only a slight (but significant) reduction of root weight but not shoot weight.

It is interesting to compare results from the short-term and long-term tests with soybean process cake extract. In both tests the dicotyledons (radish and soybean) were not affected. On the other hand, the roots of the monocotyledons (wheat and sorghum) were reduced significantly. Although sorghum showed no

TABLE 8-1

RADISH AND SORGHUM RADICLE LENGTH*

Extract concentration (%)	Radicle length ± S.D. (mm)		% Growth reduction	
	Radish	Sorghum	Radish	Sorghum
As-contaminated groundwater				
Control	27 ± 14			
0.1	31 ± 12		0	
2	18 ± 8		33†	
5	11 ± 7		59†	
10	8 ± 6		70†	
Fly ash				
Control	31 ± 16	17 ± 15	13†	
30	27 ± 15	15 ± 13	12	
Scrubber sludge				
Control	20 ± 15	39 ± 28		
10	16 ± 11	38 ± 30	20†	3
Bottom ash				
Control	22 ± 12	23 ± 13		
100	21 ± 11	20 ± 12	5	13†
Soybean process cake				
Control	22 ± 10	27 ± 12		
50		28 ± 14		0
75	23 ± 10	23 ± 13	0	15†

*Length after 48- and 72-hr growth periods, respectively, at 25 C in the dark. Controls were grown with distilled water and compared with plants treated with the indicated concentration of extract.

†Statistically different from control at a 5% probability level.

TABLE 8-2

WHEAT AND SOYBEAN SHOOT AND ROOT DRY WEIGHT*

Extract concentration (%)	Weight (g)				% Growth reduction			
	Roots		Shoots		Roots		Shoots	
	Wheat	Soybean	Wheat	Soybean	Wheat	Soybean	Wheat	Soybean
Fly ash								
Control	0.33 ± 0.05	0.06 ± 0.03	0.87 ± 0.09	0.43 ± 0.07				
10	0.28 ± 0.04	0.05 ± 0.02	0.83 ± 0.10	0.42 ± 0.02	15†	17	5	2
Scrubber sludge								
Control	0.08 ± 0.01	0.06 ± 0.03	0.13 ± 0.02	0.43 ± 0.07	0			
10	0.08 ± 0.01	0.09 ± 0.03	0.12 ± 0.01	0.41 ± 0.06	0	0	8	5
Bottom ash								
Control	0.09 ± 0.02	0.10 ± 0.02	0.29 ± 0.04	0.17 ± 0.03		10		0
10	0.09 ± 0.01	0.09 ± 0.02	0.31 ± 0.02	0.18 ± 0.04	0	10	0	0
Soybean process cake								
Control	0.08 ± 0.009	0.18 ± 0.05	0.18 ± 0.03	0.29 ± 0.04				
10	0.07 ± 0.009	0.18 ± 0.05	0.21 ± 0.03	0.29 ± 0.04	13†	0	0	0

*Plants grown in sand culture for 2 weeks under greenhouse conditions and watered with a nutrient solution (control) or with the indicated extract diluted with the nutrient solution. Concentration indicates the percent of extract in the solution added to the plants.

†Statistically different from control at a 5% probability level.

TABLE 8-3

SUMMARY RESULTS FROM RADICLE ELONGATION BIOASSAY AND SEEDLING GROWTH STUDIES

Extract and seed type	Concentration (%)	Toxic effects	
		Root	Shoot
Fly ash			
Radish*	30	yes†	—
Radish	10	no	—
Sorghum*	30	no	—
Wheat‡	10	yes†	no
Soybean‡	10	no	no
Scrubber sludge			
Radish	10	yes†	—
Sorghum	10	no	—
Wheat	10	no	no
Soybean	10	no	no
Bottom ash			
Radish	100	no	—
Sorghum	100§	yes†	—
Wheat	10	no	no
Soybean	10	no	no
Soybean process cake			
Radish	75	no	—
Sorghum	75	yes†	—
Sorghum	50	no	—
Wheat	10	yes†	no
Soybean	10	no	no

*Plants used in radicle elongation bioassay.
†5% level of significance, calculated with standard t-test by comparison of results from treated plants with those from controls (Tables 8-1 and 8-2).
‡Plants used in seedling growth studies.
§Significant growth reduction was marginal at the 100% concentration, therefore further tests with more dilute solutions were not performed.

effect at a 50 percent concentration after 72 hr, wheat exposed to extract for a longer period of time was affected even though the solution was more dilute (10 percent concentration).

8.4. Discussion and Conclusions

Because of the refinement of the root (radicle) elongation test, we are quite confident in the test results. Radish and sorghum seeds are best suited for use in the original design of the germination chamber, but the basic chamber can be custom built to accommodate different seed types. We recommend that bioassays using additional plant species be used for a more definitive screening of hazardous materials. The main drawback in the results was caused by toxic interference of acetic acid. Table 8-4 summarizes the effect of acetic acid on radish and sorghum seeds in the radicle elongation bioassay. Fly ash, scrubber sludge, and soybean process cake could not be tested at higher concentrations since extracts had to be diluted to avoid acetic acid effects. As this problem is not inherent to the bioassay procedure, we believe the technique is acceptable for initial screening. However, research on the acetic acid problem is continuing with the long-term seedling growth studies. We anticipate that some solid wastes will require more acetic acid in the EP than will be tolerable in these tests even after a tenfold dilution of the extracts. In such cases it would be necessary to: (1) use more dilute concentrations and consider tests valid only if there is growth inhibition; (2) use only data from other tests for which the acetic acid is not a problem; (3) find suitable test plant species which are less sensitive to acetic acid at concentrations required by the EP; (4) use another EP; or (5) find some method to remove the acetic acid.

As is normally expected with biological systems, variability of the measured parameters was high within and between tests. The "between-test" variation was eliminated by running a control during each test. For radish and sorghum, control means and standard deviations for the five tests reported were 24 ± 5 mm and 27 ± 9 mm, respectively. This high variability could be due to factors other than just biological variability, however. It was not always possible, for example, to

TABLE 8-4

EFFECT OF ACETIC ACID ON RADISH AND SORGHUM SEEDS IN THE RADICLE ELONGATION BIOASSAY*

Volume of acetic acid (ml)	Root length ± S.E. (nm)		% Growth reduction	
	Radish	Sorghum	Radish	Sorghum
Control	40 ± 1.3	47 ± 1.6		
1		48 ± 1.7		0
5	44 ± 1.3	42 ± 1.2	0	11†
10	34 ± 1.1	33 ± 0.8	15†	30†
20	23 ± 0.7	11 ± 0.8	43†	77†

*A 0.5 N acetic acid solution was tested. pH was adjusted to 5.0 for radish only. A second test will be performed with sorghum in which pH will be adjusted.

†Statistically different from control at a 5% probability level.

measure plants at exactly the end of the designated growth period, though this did not vary more than an hour or two. Temperature fluctuation during tests or between tests could have influenced root growth. Tests are presently being performed to quantify temperature effects on root elongation of radish and sorghum. We recommend, however, that controls be run during each test and that care be taken to treat controls and treatment plants exactly the same during each test to avoid experimental errors.

Sand was chosen for the seedling growth study because various types of soil may influence the toxicity of phytotoxic substances depending on the amount of organic matter present.[10] However, the attenuation by soil organic matter of phytotoxic effects of the waste extracts or other potentially toxic substances is an area that needs further research and must be considered in assessing the hazards of toxic substances to the terrestrial environment.

While these data are difficult to interpret in terms of what extracts should be considered hazardous to the environment, they do illustrate the complexity of developing a screening protocol for potentially phytotoxic substances. Because of the variability among plant species in the way they respond to their environment, large numbers of species should be tested. However, even with tests employing a limited number of species and conditions, such as in the test presented here, potentially hazardous chemicals may be identified or flagged for further testing. Since all five materials tested proved to be significantly different from controls in at least one of the tests, they are, in fact, potentially hazardous. However, final determination of whether or not a particular material is hazardous to the environment cannot be decided from phytotoxicity studies alone. For example, damage to plants can arise from mutagenic changes which would not be apparent from the tests used in the phytotoxicity studies.

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APPENDIX I. Extraction Procedure

(A) Equipment

(I) An agitator which, while preventing stratification of sample and extraction fluid, also insures that all sample surfaces are continuously brought into contact with well-mixed extraction fluid.

(II) Equipment suitable for maintaining the pH of the extraction medium at a selected value.

(B) Procedure

(I) Take a representative sample (minimum size 100 g) of the waste to be tested. Separate sample into liquid and solid phases. The solid phase is defined as that fraction which does not pass through a 0.4–0.5 μm filter medium under the influence of either pressure, vacuum, or centrifugal force. Reserve the liquid fraction under refrigeration (1–5 C) for for further use.

(II) The solid portion of the sample, resulting from the separation procedure above or the waste itself (if it is already dry), shall be prepared either by grinding to pass through a 9.5 mm (3/8 inch) standard sieve or by subjecting it to the structural integrity procedure.

(III) Add the solid material from paragraph II to 16 times its weight of deionized water. This water should include any water used during transfer operations. Begin agitation and extract the solid for 24 ± 0.5 hr. Adjust the solution to pH 5 and maintain that pH during the course of the extraction using 0.5 N acetic acid. If more than 4 ml of acid for each g of solid would be required to maintain the pH at 5, then once 4 ml per g of solid has been added, complete the 24-hr extraction without adding any additional acid. Maintain the sample between 20–30 C during extraction.

(IV) At the end of the 24-hr extraction period, separate the sample into solid and liquid phases as in paragraph I. Adjust the liquid phase with deionized water so that its volume is 20 times that occupied by a quantity of water at 4 C equal in weight to the initial sample of solid (e.g., for an initial sample of 1 g, dilute to 20 ml). Combine this liquid with the original liquid phase of the waste. This combined liquid, including precipitate which later forms from it, is the toxicant Extraction Procedure extract.

APPENDIX II. Extractor

Apparatus

Apparatus used in the extraction is shown in Figures II-1 to II-11. Commercial apparatus utilized included: IEC constant-temperature centrifuge; Millipore filtration assembly (cat. no. YY42-142-00, filter cat. no. HAWP-142-50); Chemtrix pH controller (cat. no. 45A); Masterflex tubing pump and pump head (cat. no. 7045-10 and 7013-00); Cole Palmer stirring motor (cat. no. 4558).

Glassware Cleaning and Reagents

Prior to extraction, all glassware and extractors were cleaned with detergent followed by dilute nitric acid, then thoroughly rinsed with autoclaved deionized water from a Millipore Milli-Q water purification system. The deionized water was then autoclaved (15 psig, 127 C, 20 min) before use in cleaning and extracting. In all cases, reagent-grade chemicals were used.

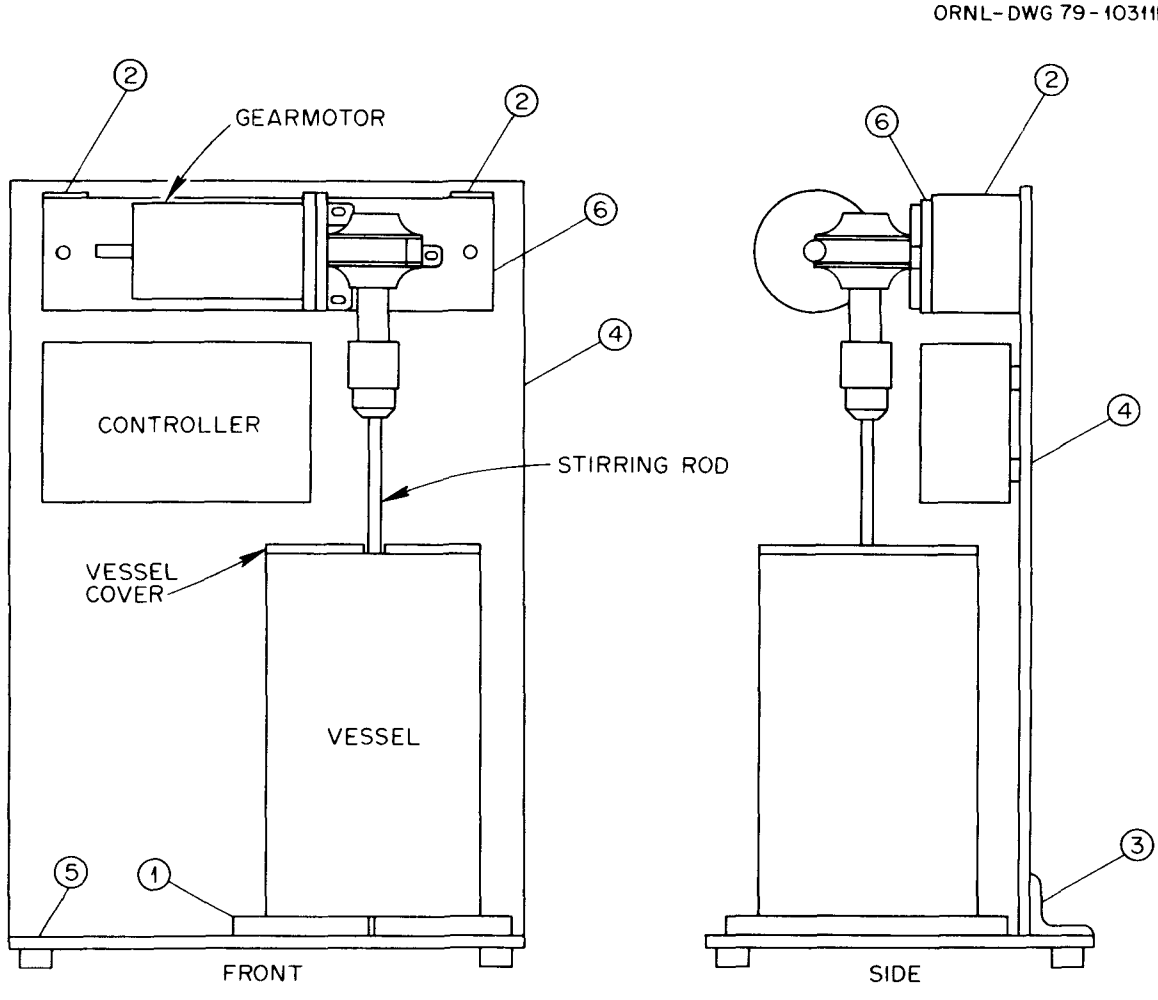


Figure II-1. Extraction apparatus.

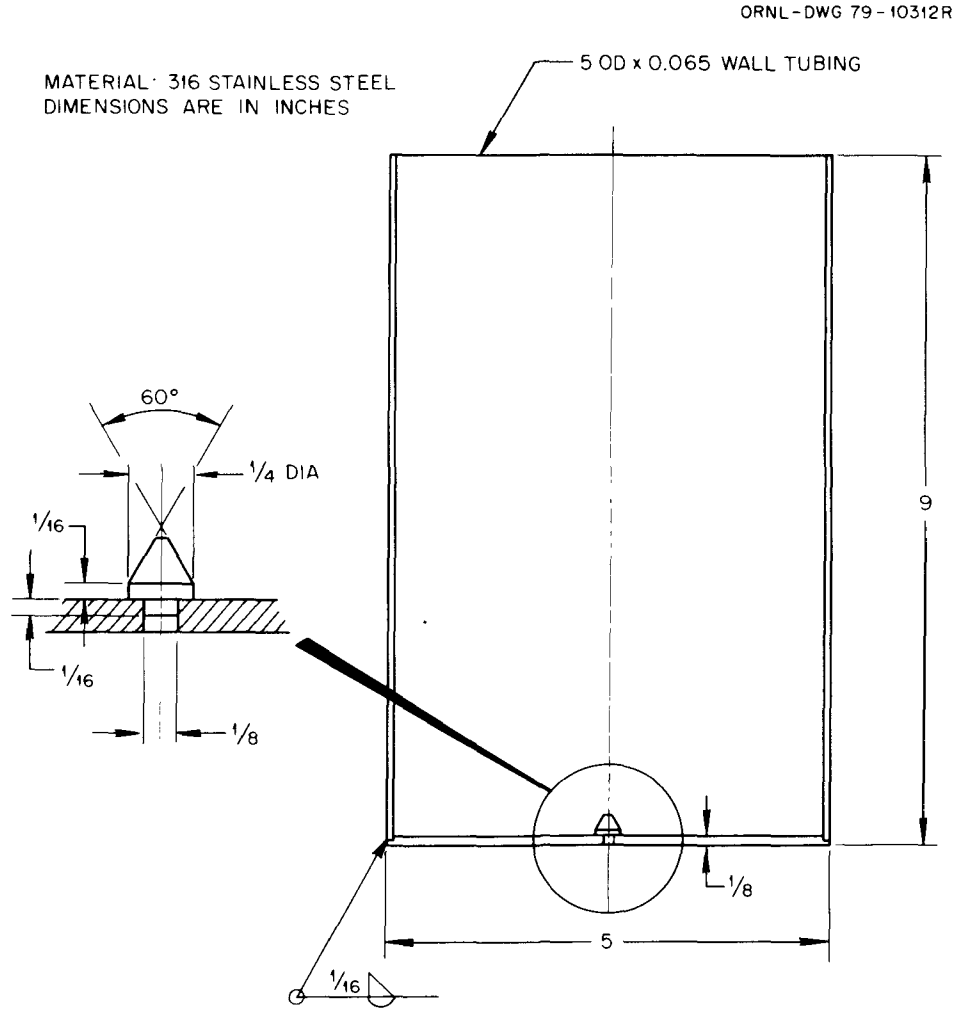


Figure II-2. Extracting vessel.

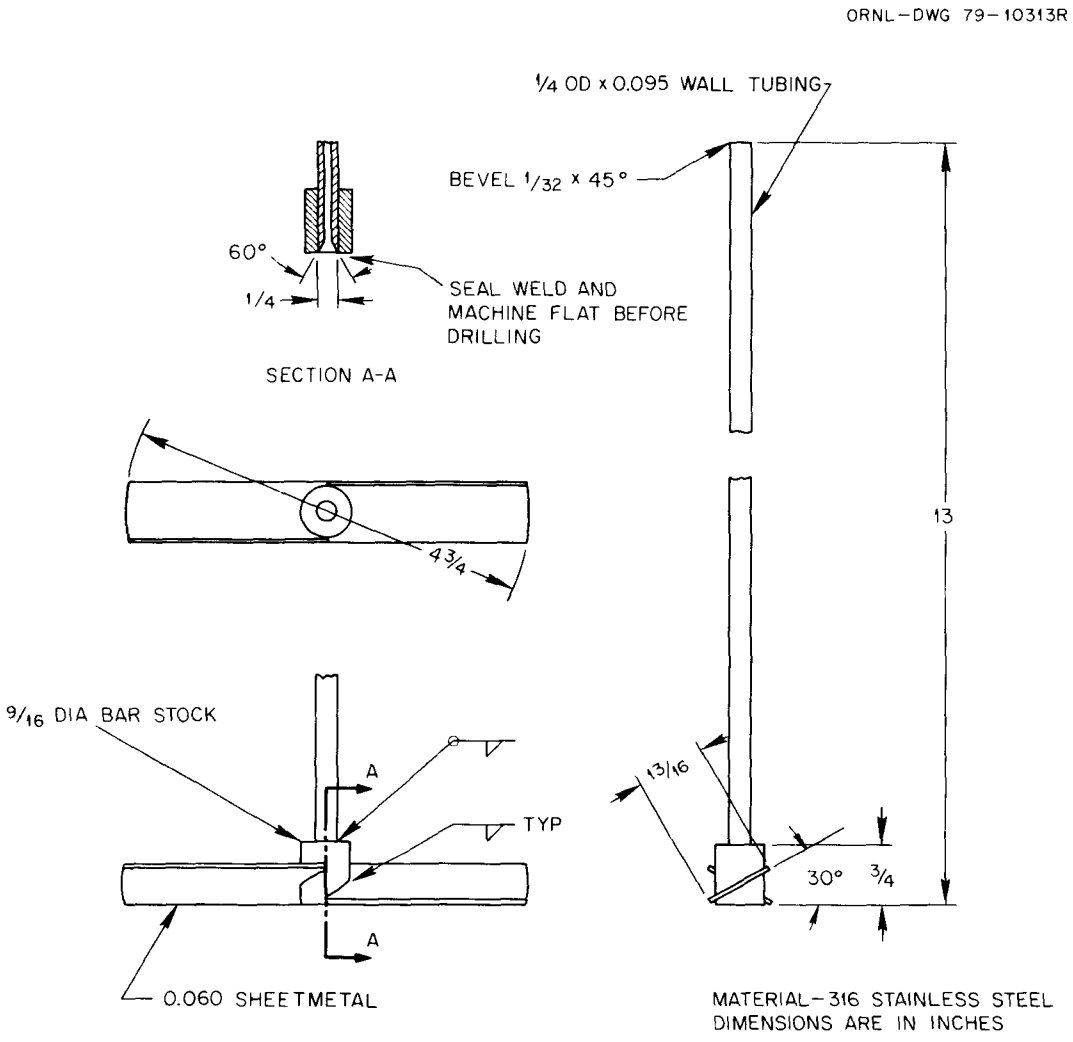


Figure II-3. Stirring rod.

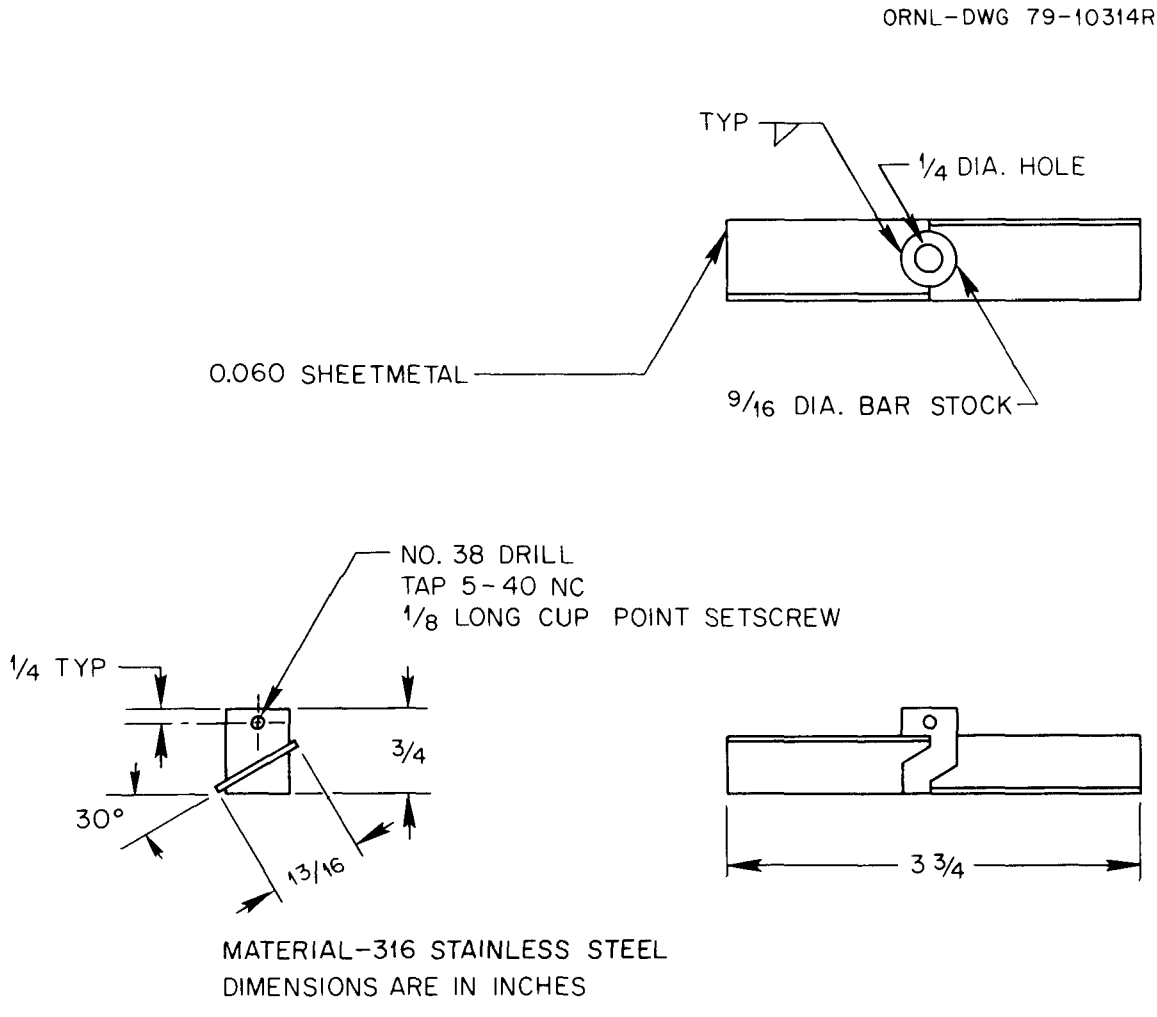


Figure II-4. Upper stirring blade.

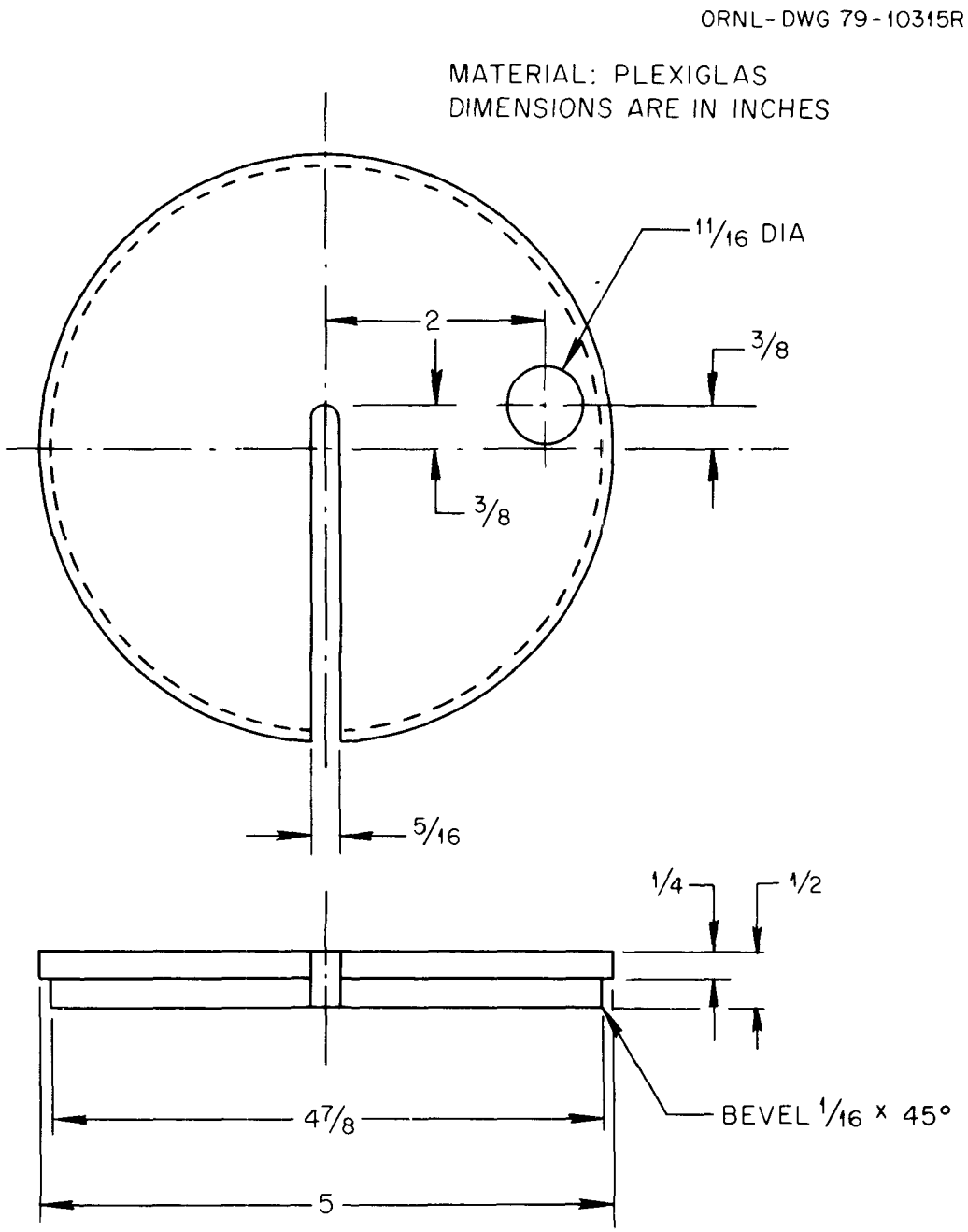


Figure II-5. Vessel cover.

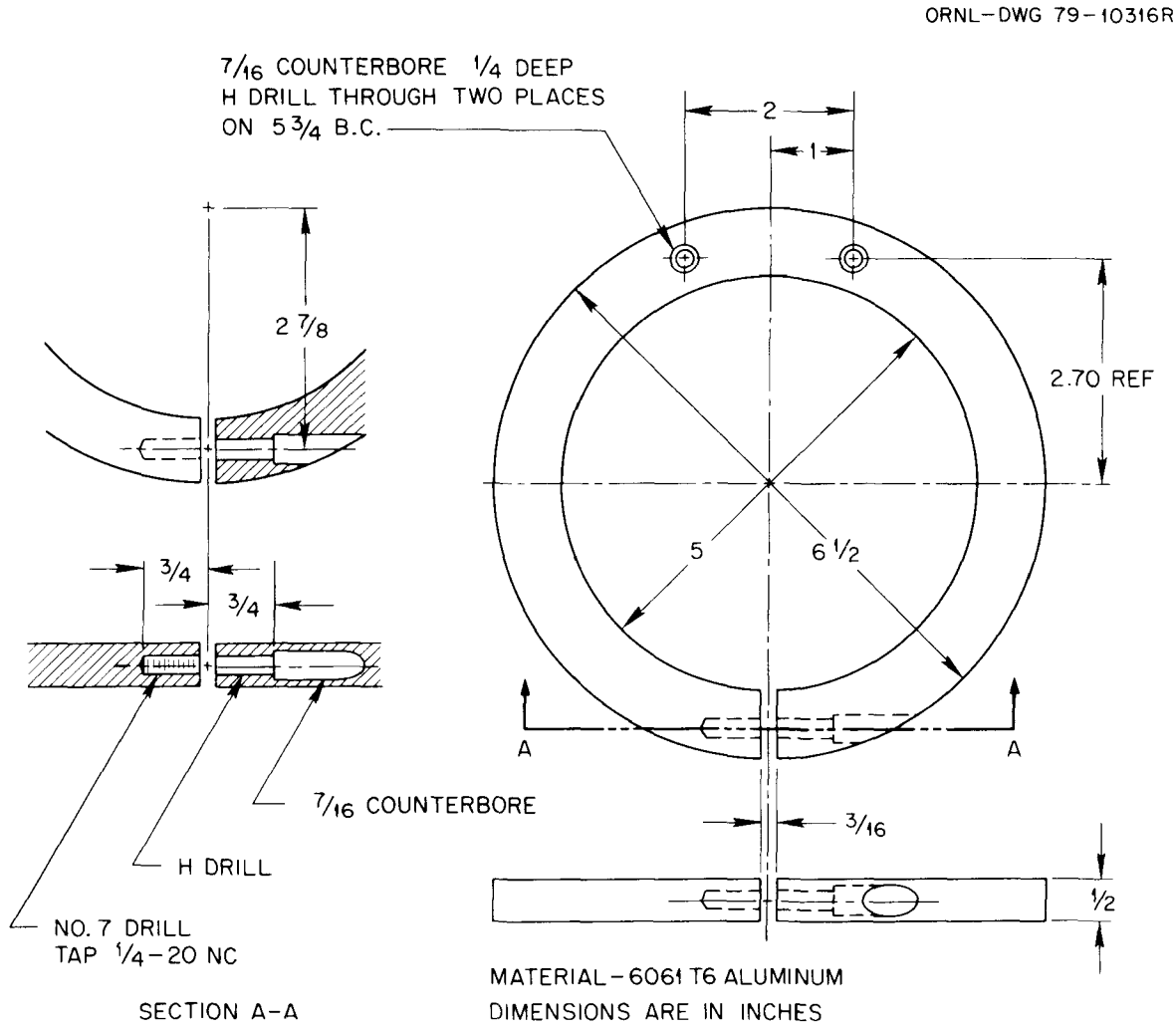
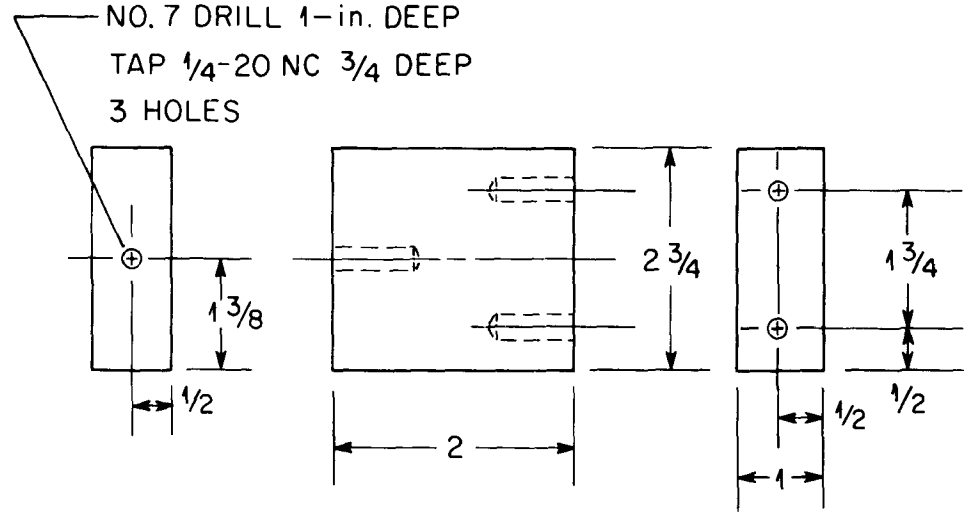


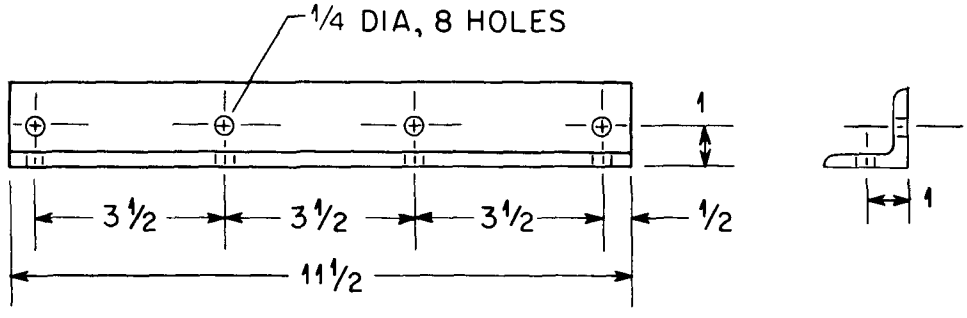
Figure II-6. Part No. 1.

ORNL-DWG 79-10317R



MATERIAL 6061T6 ALUMINUM
MAKE 2

PART NO. 2



MATERIAL - 1 1/2 x 1 1/2 x 1/4
ALUMINUM ANGLE

PART NO. 3

Figure II-7. Parts No. 2 and 3.

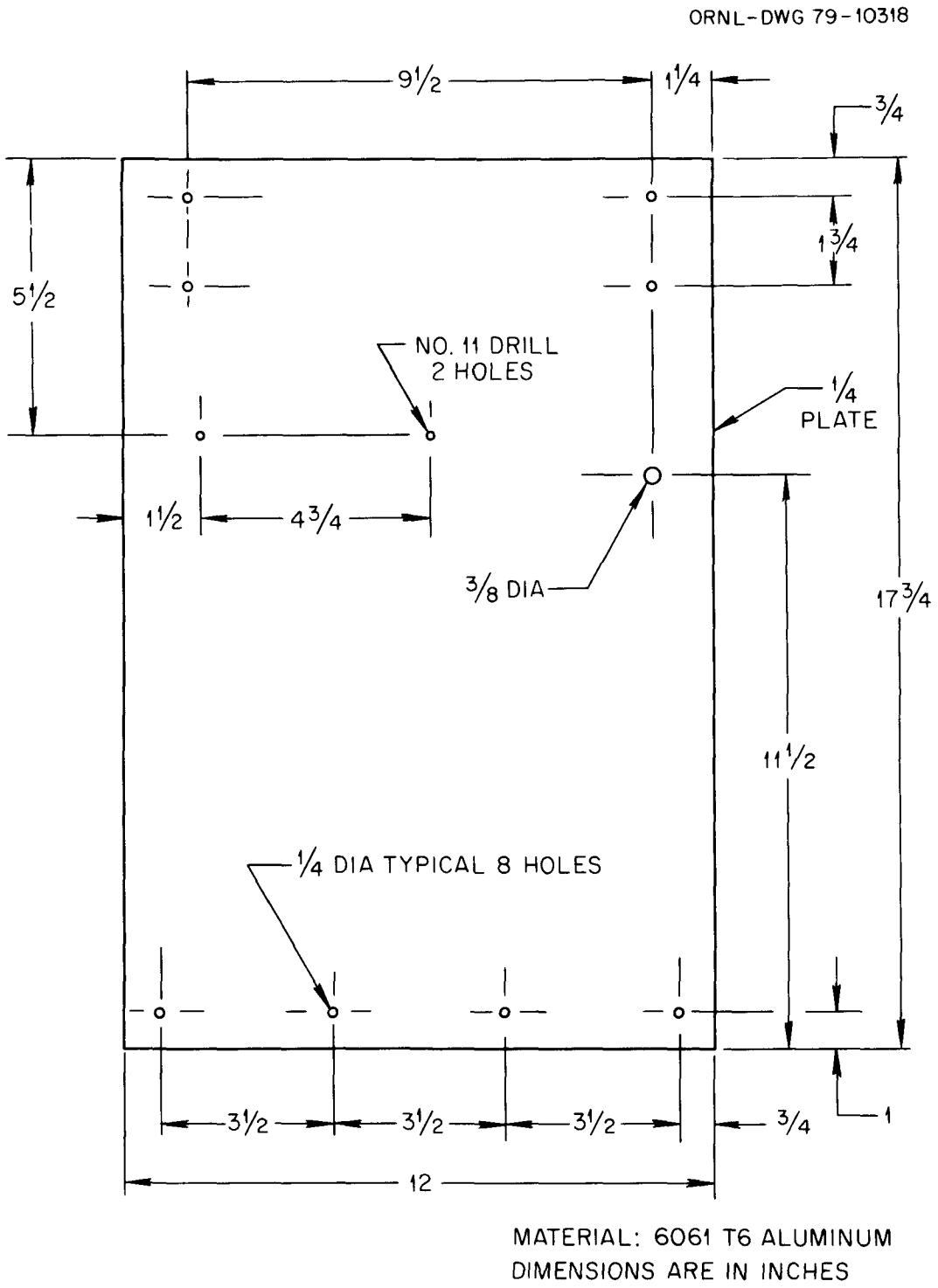


Figure II-8. Part No. 4.

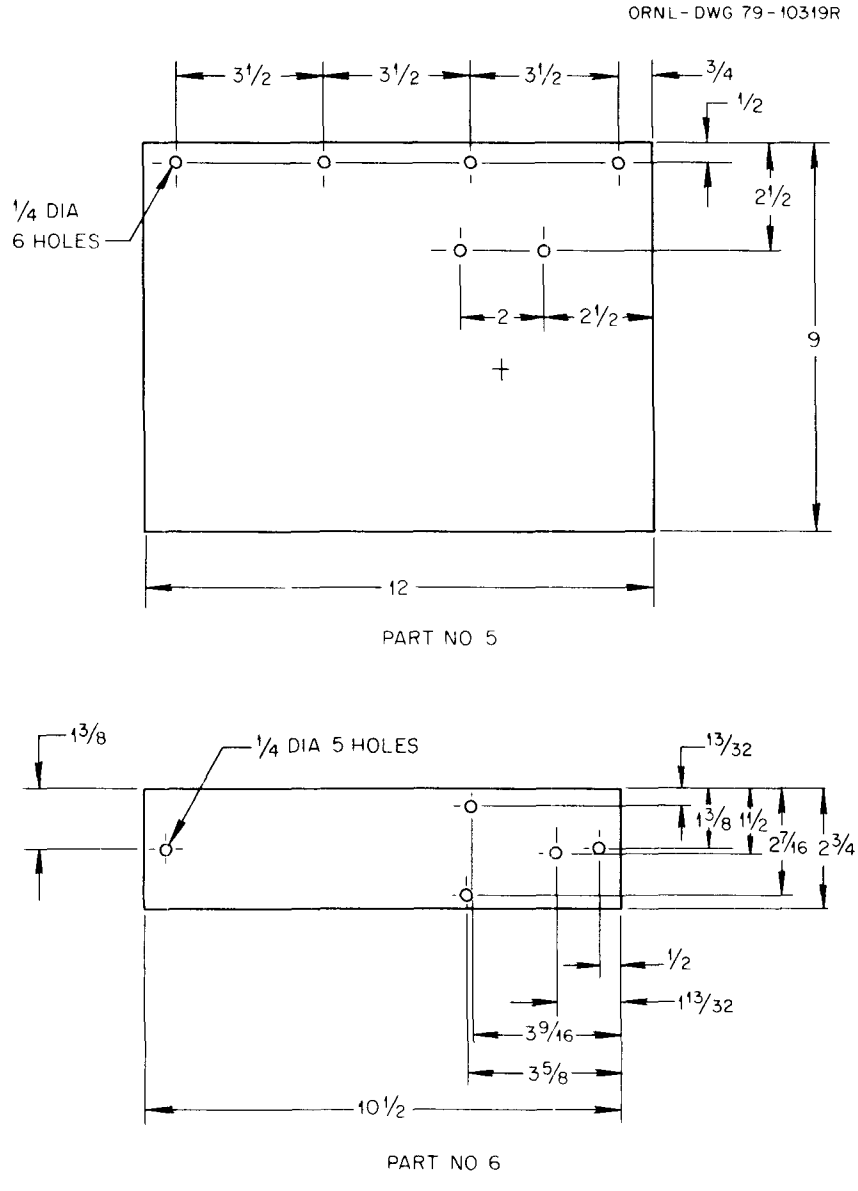


Figure II-9. Parts No. 5 and 6.

0120-79

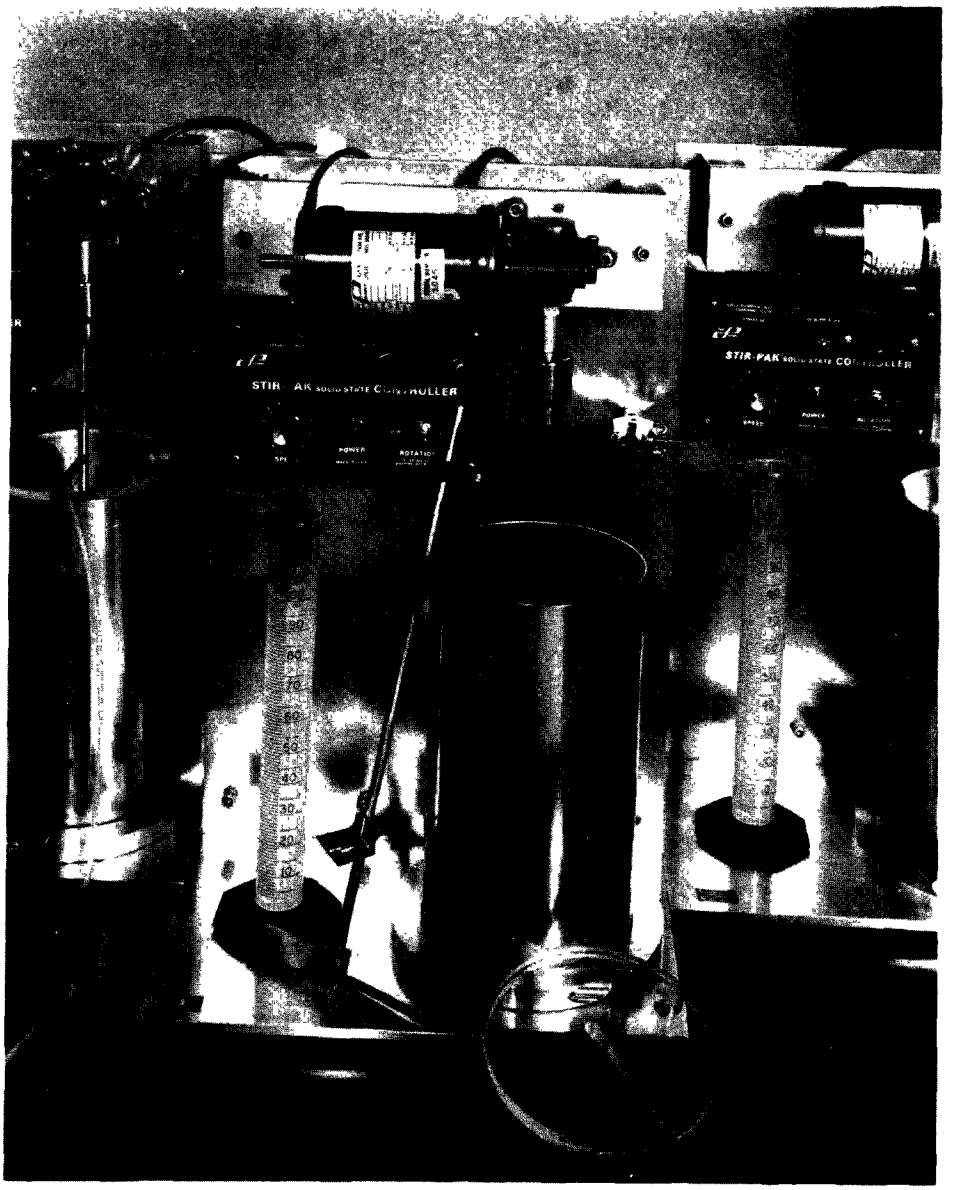


Figure II-10. Extraction apparatus.

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Figure II-11. Laboratory set-up for extraction procedure.

APPENDIX III. Analytical Methodology

EP Organic Concentrate

This procedure is based on work by Junk et al.[1] and Yamasaki and Ames.[2] A 500-ml aliquot of the EP extract is adjusted to pH 6.8 with Na_3PO_4 and to 20 mmho/cm conductance with NaCl. This preparation is then passed through 4 g of XAD-2 (Isolab, Inc.) at a flow rate of 1.2 ml/min. The column is rinsed with 15–20 ml deionized water, and then adsorbed constituents are eluted with 10 ml redistilled acetone. The acetone eluate is evaporated under N_2 to dryness and brought up in 10 ml cyclohexane for further fractionation. This procedure is carried out in quadruplicate to provide duplicate samples for chemical analysis and mutagenicity testing.

PCB's/Pesticides

The cyclohexane sample resulting from the XAD-2 procedure is fractionated by column chromatography. It is passed through 10 g of Florisil, eluted with 150 ml of 6/1 hexane/benzene, and passed through 20 g of neutral activity III alumina with 150 ml hexane followed by 150 ml of 6/1 hexane/benzene and 160 ml 2/1 hexane/benzene. Each fraction is concentrated to 1 ml with dry, flowing nitrogen under reduced temperature and pressure. PCB/pesticide measurements are made by injecting 5 µl of the hexane concentrate into a gas chromatograph equipped with a 10-ft OV-101 column maintained at 180C [isothermal], and detecting eluting components with an electron capture detector calibrated with external standards.

PAH's

The three fractions obtained from the alumina step in the fractionation scheme contain the diaromatic (hexane and 6/1 hexane/benzene) and polyaromatic (2/1 hexane/benzene) compounds.[3] PAH identifications and measurements are made by injecting 5 µl of each fraction concentrate into a gas chromatograph equipped with a 1/8-inch O.D. x 10-ft 3 percent (w/w) Dexsil 400 on Supelcoport (100/200 mesh)

column and temperature-programming from 100 to 320 C at 2 degrees/min. Flame ionization detection and calibration with external PAH standard are employed.

Other Halogenated or Polar Organics

A scan for the presence of other halogenated compounds is carried out for each of the three fraction concentrates obtained from the alumina step in the fractionation scheme. From each fraction, 5 µl is injected into a gas chromatograph equipped with a 10-ft OV-101 column at 180 C by use of electron-capture detection. Further scans are set up for detection of polar compounds retained by the Florisil and alumina columns during fractionation. These columns are further eluted with 150 ml redistilled acetone, which is then concentrated to 1 ml with dry, flowing nitrogen. This acetone concentrate (5 µl) is injected into a gas chromatograph equipped with a 42-m glass capillary column (0.25 percent Carbowax 20-M) and temperature-programmed from 110 C to 200 C at 2 degrees/min. Flame ionization detection is used.

o-Nitroaniline

A 50-ml aliquot of the arsenic-contaminated groundwater is extracted four times with 20 ml of methylene chloride, and the combined organic layers are concentrated to 1 ml by dry, flowing nitrogen under reduced temperature and pressure. A 0.4-µl sample is analyzed by GC on the Carbowax 20-M capillary column as described above by use of an authentic external standard.

Volatile Organics

The procedure is a modified version of that of Grob.[4] The aqueous sample (5 ml) is purged with N_2 at 100 ml/min into a 1 cm x 1 mm precolumn packed with 2 mg Tenax (60–80 mesh) and 1 mg Florisil, in series. The volatiles are desorbed from the precolumn in the injector (250 C) of a gas chromatograph with the analytical column held at -70 C. The analytical column is a 50-m glass capillary coated with

0.2 percent diethylene glycol succinate. The pollutants are detected by flame ionization during a temperature-programmed run from 0 C to 175 C at 1 degrees/min with a 32-min final hold. Quantification is carried out with external standards treated similarly. Samples are analyzed in duplicate.

Dissolved Metals

Duplicate aliquots of the EP extracts are directly analyzed for metals by flameless graphite furance atomic absorption spectrophotometry. Calibration is conducted with external standards, and one sample aliquot is spiked to check recoveries. Aliquots of EP extracts for Hg determination are preserved by addition to a nitric acid/dichromate solution immediately after generation.

References for Appendix III

1. Junk, G. A., C. D. Chriswell, R. C. Change, L. D. Kissinger, J. J. Richard, J. S. Fritz, and H. H. Svec. Application of resins for extracting organic components from water. Z. Anal. Chem., 282: 331, 1976.
2. Yamasaki, E., and B. N. Ames. Concentration of mutagens from urine by adsorption with the non-polar resin XAD-2. Cigarette smokers have mutagenic urine. Proc. Natl. Acad. Sci. USA, 74: 3555, 1977.
3. Griest, W. H. Multicomponent polycyclic aromatic hydrocarbon analysis of inland water and sediment; Proceedings; International Symposium on Analysis of Hydrocarbons and Halogenated Hydrocarbons in the Aqueous Environment, McMaster University, Hamilton, Ontario, Canada, May 25-27, 1978. In press.
4. Lynch, J. M. Production and phytotoxicity of acetic acid in anaerobic soils containing plant residues. Soil. Biol. Biochem., 10: 131-135, 1978.

APPENDIX IV. Aquatic Toxicity Methodology

The overall protocol consisted of the following: (1) a preliminary 48-hr acute toxicity determination; (2) a definitive 48-hr acute toxicity test; (3) a 28-day life cycle, chronic toxicity test at two dilutions, 1:100 and 1:1000, of the EP extract; (4) a final 48-hr LC_{50} determination (this final determination was used as a check on the EP extract to determine whether its toxicity had changed over the duration of the 28-day life cycle test).

Acute Toxicity Tests

Laboratory cultured first instar Daphnia magna, 12 ± 12 hr old, were utilized for acute toxicity tests. Five D. magna were exposed to 80 ml of extract solution in 100-ml glass beakers covered with watch glasses. Temperature was maintained at 20 ± 0.5 C in an environmental chamber under a 12-hr light/dark regimen. The dilution water used was well water (pH 7.8, alkalinity 119 mg/liter, hardness 150 mg/liter). The EP extracts were neutralized to pH 7.0 with NaOH. The pH of the extract dilutions was measured at the beginning and conclusion of each test.

Serial geometric dilutions with well water were made for each extract, with the concentrations of each extraction solution being 60 percent of each preceding one. All tests were run in triplicate. The range of dilutions was selected to bracket 48-hr LC_{50} values predicted from preliminary toxicity determinations. Additional control beakers were included containing a concentration of neutralized acetic acid equal to the highest concentration used in the acute toxicity tests. Control beakers of dilution water without added extract were also included. Values for 48-hr LC_{50}'s and 95 percent fiducial intervals were obtained by computerized PROBIT analytical procedures.

Chronic Toxicity Tests

Chronic toxicity tests were run at only two dilutions of the extract, 1:100 and 1:1000. First instar D. magna, 12 ± 12 hr old were used. One D. magna was placed in 50 ml of extract solution in a 100-ml beaker covered with a watch glass. Temperature, lighting, and dilution water were the same as for the acute tests. All tests were run with ten replicates, a set of ten controls, and an additional set of controls with neutralized acetic acid added at the same concentration as in the 1:100 dilution. The test organisms were transferred to freshly prepared test solutions and fed 2 mg of prepared food per daphnid every Monday, Wednesday, and Friday. The number of young and the number of broods present in each beaker were then counted. The pH of the extract solutions was measured at the beginning and end of each test. The tests had a duration of 28 days or until all animals had died, whichever came first.

APPENDIX V. Salmonella Mutagenicity Assay

The realization that the list of potential chemical carcinogens is growing faster than our capacity to test the materials and the enormous increase in industrial and technological activities have created an interest in short-term test procedures for the identification of genetic hazards associated with environmental chemical pollutants. Although the health effects of chemicals in the environment are being extensively studied, it is obvious that short-term test procedures are necessary to reduce the study time for evaluating the large number of potentially hazardous substances. To control the problem of environmental carcinogenesis, greater numbers of these compounds are to be screened and assigned priorities for further testing. This appears to be the primary role of the short-term test. Not only should a meaningful short-term test be faster, easier to interpret, more sensitive, and less expensive, but it must also be reliable and relevant to the in vivo assays.

Among the various short-term assays which utilize microbial organisms, the Salmonella test system developed by Ames has been widely used a prescreen for the determination of genetic and potential carcinogenic hazards of complex environmental effluents or products. This test system has been examined more extensively than any other short-term assay for correlating mutagenicity and carcinogenicity.[1] It utilizes a series of histidine-requiring mutants that revert after treatment with mutagens to the wild-type state (histidine independent). Generalized testing of the compound is accomplished by use of the three strains (TA1537, TA1538 and TA98) that detect frameshift mutagens and two strains (TA1535 and TA100) that detect base-pair substitution mutagens. The design of the test is shown in Figure V-1. A recommended protocol outlining the preparation of the components of this test has been published by Ames et al.[2] Some chemicals like dimethylnitrosamine, certain hydrazines, and volatile liquids which are not mutagenic in the standard plate assays are active in the modified procedure, designated the preincubation technique. This modified procedure detects not only these compounds, but also the majority of the compounds that have been shown to be active in the standard plate assay.

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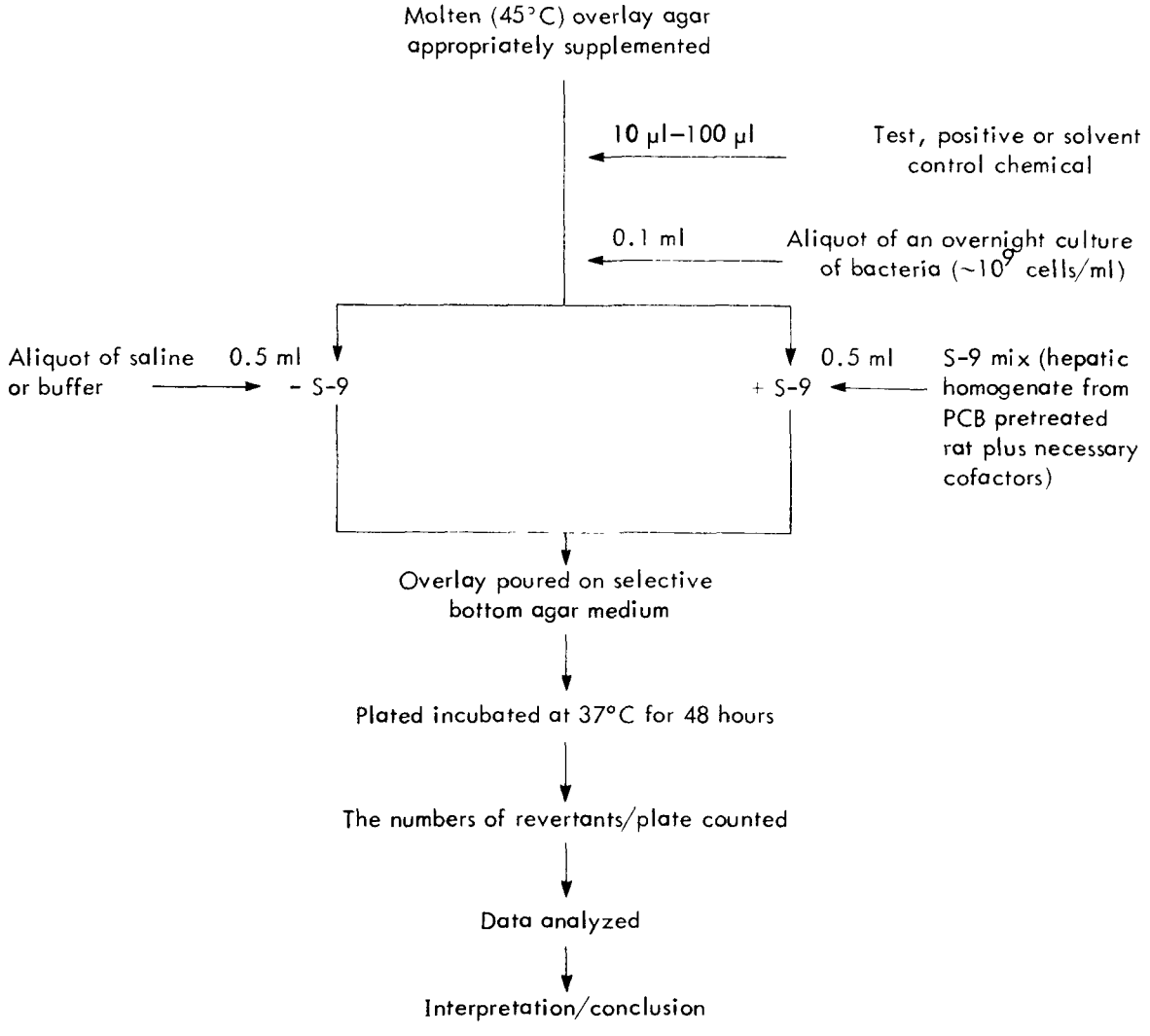

Figure V-1. Reverse mutation assay (agar incorporation method).

Bacterial Strains

Four Salmonella typhimurium indicator strains, TA1535, TA1537, TA98, and TA100, are recommended for screening purposes. TA1535 and TA100 have base-pair substitution mutation in the histidine operon; TA100 also contains an R factor which renders the strain more sensitive to certain mutagens, possibly through error-prone repair. TA1537 and TA98 have frameshift mutation in the histidine operon; TA98 contains an R factor and is more sensitive than TA1538. TA1537 is recommended because of its unique sensitivity to some agents like 9-aminoacridine and certain ICR compounds. The characteristics of these strains are shown in Table V-1.

TABLE V-1

PROPOSED BACTERIA STRAINS*

Strain designation	Gene affected	Additional mutations		
		Repair	LPS	R factor
TA1535	hisG	uvrB	rfa	—
TA1537	hisC	uvrB	rfa	—
TA98	hisD	uvrB	rfa	pKM101
TA100	hisG	uvrB	rfa	pKM101

*See Ames et al.[1] for references.

Storage and Checking of Tester Strains

All strains are initially grown in nutrient broth (8 g Difco-Bacto nutrient broth, 5 g NaCl/liter) at 37 C for 16 hr. The strains are checked for the genetic markers in the following ways:

Histidine Requirement. Streak the cultures on minimal plates both with and without histidine (spread 0.1 ml of sterile 0.1 M L-histidine on the agar surface). Biotin (0.1 ml of 0.5 mM per plate) is also essential for these strains. The strains should grow on plates containing both histidine and biotin.

Deep Rough Character. A sterile filter paper disc containing crystal violet (10 µl of 1 mg/ml) is placed on a nutrient agar petri dish containing 0.1 ml (about 10^8 bacteria) of the nutrient broth culture to be tested in a thin overlay of top agar. After 12 hr incubation at 37 C, a clear zone of inhibition around the disc (about 14- to 18-mm diameter) indicates the presence of rfa mutation.

Presence of Plasmid. The strains with R factor (TA100 and TA98) should be checked routinely for the presence of the ampicillin resistance. Streak a small amount (10 µl of 8 mg/ml in 0.02 N NaOH) of an ampicillin solution across the surface of a nutrient agar plate. After the streak is dry, cultures to be checked are cross-streaked against the ampicillin, and after incubation for 12–24 hr at 37 C, strains which do not contain the R factor will show a zone of growth inhibition around the ampicillin streak, whereas strains containing R factors will not.

Storage. Frozen permanent cultures containing fresh nutrient broth cultures (0.8 ml) with DMSO (0.07 ml) are prepared and maintained in a Revco freezer at -80 C. A working source of these cultures is maintained on masterplates which are prepared as follows:

0.1 ml of sterile 0.1 M L-histidine is spread on the surface of a minimal glucose agar plate. After the histidine solution is absorbed by the agar, 0.1 ml of sterile 0.5 mM biotin is added in the same way. For TA98 and TA100, 0.1 ml of an 8 mg/ml ampicillin solution (in 0.02 N NaOH) is added. By use of a sterile loop, nutrient broth culture of the tester strain is streaked across the agar (for TA98 and TA100, plates with ampicillin are used) and incubated at 37 C for 24 hr. These masterplates with the cultures are stored at 4 C and can be used for several months to grow working cultures.

Preparation of Rat Liver S-9

Male Sprague-Dawley rats (of about 180–200 g weight) are given a single intraperitoneal injection of Aroclor-1254 at a dosage of 500 mg/kg (vehicle, corn oil) 5 days before they are killed. They are fasted 12 hr before they are decapitated and allowed to bleed. The livers are aseptically removed and washed in cold 0.15 M KCl. All steps are performed at 0 to 4 C with cold and sterile solutions and glassware. The livers are minced with sterile scissors in three volumes of 0.15 M KCl (3 ml/g wet liver) and homogenized with a Potter-Elvehjem apparatus with a Teflon pestle. The homogenate is centrifuged for 10 min at 9,000 x g, and the supernatant (S-9) is decanted and stored in convenient aliquots at -80 C. For S-9 from ϕB-induced rat livers, the same procedure as described above is followed except that the rats are given 0.1 percent of sodium phenobarbital in drinking water for 1 week before they are killed.

Media

Top agar (0.6 percent Difco-Bacto agar, 0.5 percent NaCl) is autoclaved and stored in 100-ml bottles at room temperature. Before use, the agar is melted (in an autoclave or in a steam bath), and 10 ml of a sterile solution of 0.5 mM L-histidine-HCl, 0.5 mM biotin is added to the 100 ml of molten agar and mixed thoroughly.

Complete medium (23.5 g BBL standard methods agar in 1 liter of distilled H_2O) is autoclaved and dispensed into 100 x 15 mm plastic petri plates (30 ml/plate).

Vogel-Bonner[3] medium E with 2 percent glucose and 1.5 percent Bacto-Difco agar is used as the minimal medium for mutagenesis assays and is prepared as follows:

Vogel-Bonner Salts (50X)

Warm distilled water	670 ml
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	10 g
Citric acid monohydrate	100 g
Potassium phosphate (K_2HPO_4)	500 g
Sodium ammonium phosphate ($NaHNH_4PO_4 \cdot 4H_2O$)	175 g

The above salts are added to the warm water (45 C) in the specified order. Each salt is dissolved completely before the next is added. When the salts are all dissolved, the solution is cooled to room temperature. About 5 ml of chloroform is added to the solution and stored in a capped bottle at room temperature.

Dissolve 15 g of Difco-Bacto agar in 1 liter of water by autoclaving. Cool to about 60 to 70 C and add 20 ml of 50 x Vogel-Bonner salt solution and 50 ml of sterile 40 percent glucose solution. Mix thoroughly, and dispense into 100 x 15 mm plastic petri plates (30 ml per plate). Other minimal media would presumably also serve the purpose.

Preparation of S-9 Mix (Activation System)

The S-9 mix contains the materials shown in Table V-2.

TABLE V-2

COMPOSITION OF S-9 MIX

	Component	Stock preparation	Volume (µl) of stock added/ ml of final mix	Final concentration of component in mix (µmoles/ml)
1.	NADP	0.1 M	40	4
2.	Glucose-6-phosphate	0.1 M	5	5
3.	Sodium phosphate buffer (pH 7.4)	0.2 M	500	100
4.	$MgCl_2$	0.4 M	20	8
5.	KCl	1.65 M	20	33
6.	Homogenate	standard KCl 9,000 x g supernatant	100	approx. 25 mg of fresh tissue equivalent

*Components 1 and 2 are prepared in sterile distilled water and filter sterilized before using. Components 3–5 are prepared in distilled water, sterilized, and maintained at 4 C. Component 6 is prepared in 0.15 M KCl and stored at -80 C until used.

Positive Control Compounds

Any assay performed should have a control in which the solvent or diluent is employed to see its effect on the rate of spontaneous revertants. In addition to this control, a known direct-acting mutagen and the one that requires metabolic activation should be used to show that the assay system is working.

The positive control compounds shown in Table V-3 could be used in these assays.

TABLE V-3

POSITIVE CONTROL COMPOUNDS

	Concentration (μg/plate)	Activation	Response of strain		
			TA1535, TA100	TA98	TA1537
Sodium azide	2.5	–	+	–*	–*
9-Aminoacridine	10.0	–	–	–	+
2-Anthramine	5.0	+	+	+	+

*Weak responses may be obtained.

Mutagenesis Assay by the Preincubation Method

It may be difficult to detect biological effects with the complex environmental mixtures due to (1) toxicity of the complex mixture or (2) low concentrations of the biologically active components in the complex mixture. The first problem should be dealt with by assaying the complex mixture for general toxicity towards bacterial survival before the mutagenesis assay is performed. The second problem should be dealt with at the level of concentration and fractionation of the complex mixtures. The following protocol is recommended for general toxicity.

Only one strain, TA1537,is used to determine the general toxicity range. Overnight culture in nutrient broth is diluted to obtain about 10^3 cells/ml. To the tubes containing 2 ml standard top agar are added: 0.1 ml of the diluted culture of TA1537, various amounts of the test material (the recommended levels are: 1000, 500, 100, and 10 μl per tube), and 0.5 ml of phosphate buffer, pH 7.4 (for nonactivation) or 0.5 ml of S-9 mix (for activation). The contents are mixed and poured on the surface of a bacterial complete plate. After the agar has hardened, the plates are incubated at 37 C for 48 hr. Survival is compared with a control plate containing solvent but no chemical. Once the toxicity is determined, five dose levels within the 50 percent or greater survival part of the curve are selected for actual mutagenesis assays.

Preincubation Assay

Four tester strains (TA1535, TA1537, TA98, and TA100) described earlier are used in the assay, and each data point is done in duplicate. The assay is conducted as follows:

To the sterile 13 x 100 mm test tubes containing 0.5 ml of the S-9 mix placed in an ice bath, an aliquot of the test compound (or positive control mutagen or solvent or diluent) and 0.1 ml of an overnight bacterial culture are added. S-9 mix should be replaced with 0.067 M phosphate buffer (pH 7.4) in nonactivation tests. The contents are mixed and the tubes are incubated at 37 C in a shaker for 20 min. At the end of the incubation, 2 ml of molten top agar (kept at 45 C) is added per tube and the contents are gently mixed. The contents are then poured onto the surface of a Vogel-Bonner minimal glucose agar plate (appropriately labeled). After the agar has solidified, the plates are incubated at 37 C for 2 days and the his^+ revertants are recorded. Table V-4 shows the results for 2-aminoanthracene, sodium azide, and dimethylnitrosamine tested by the standard plate incorporation method and the preincubation method.

Revertant Confirmation

Randomly selected Salmonella revertants should be picked from plates showing mutagenicity and confirmed for histidine independence by restreaking on minimal plates containing no histidine.

Repeat Tests

The test on each sample should be repeated within 2 weeks following the initial evaluation to confirm the results. The positive results obtained in the initial evaluation with or without PCB-induced rat liver S-9, are to be confirmed in the repeat test. If the results are negative in the initial evaluation in the presence or absence of PCB-induced rat liver S-9, it is suggested that in the repeat tests ϕB-induced rat liver S-9 be included in addition to the PCB-induced rat liver S-9. (It should be noted here that the liver from Ar-induced rats is the most efficient for detecting different classes of carcinogens. The liver from ϕB-induced rats is more efficient for detection of 2-acetylaminofluorene and many other aromatic amines, but it is very inefficient for detection of certain polycyclic hydrocarbons.) If the repeat test results are positive in the presence of ϕB-induced rat

TABLE V-4

MUTAGENICITY OF 2-ANTHRAMINE, SODIUM AZIDE, AND DIMETHYLNITROSAMINE

Concentration of mutagen	Mutagenicity (revertants/plate) in strain					
	TA1535*		TA98		TA100*	
	Plate incorporation	Preincubation	Plate incorporation	Preincubation	Plate incorporation	Preincubation
2-Anthramine (μg)						
0			36	45		
0.01			66	78		
0.1			431	841		
1.0			1994	2104		
10.0			1903	2207		
Sodium azide (μg)						
0	22	15			123	113
1.0	300	274			600	1344
2.5	566	505			865	1902
5.0	867	765			1065	2372
10.0	1643	1100			1275	2746
20.0	2063	1456			1336	3015
50.0	2260	1675			1362	3161
Dimethylnitrosamine (μl)						
0	15	29			113	161
1.0	22	35			176	164
2.5	23	35			182	164
5.0	28	41			176	173
10.0	20	349			178	516
20.0	25	583			209	1090
50.0	2	24			0	122

*Mean of six replicate runs.

liver S-9, they should be reconfirmed by testing the material in the presence of ∅B-induced rat liver S-9 only. If the repeat test results are negative, no further testing is necessary. Figure V-2 gives the general scheme for evaluating the test material in the preincubation assay for four Salmonella tester strains.

References for Appendix V

1. McCann, J., E. Choi, E. Yamasaki, and B. N. Ames. Detection of carcinogens as mutagens in the Salmonella/microsome test: Assay of 300 chemicals. Proc. Natl. Acad. Sci. USA, 72: 5135-5139, 1975.
2. Ames, B. N., J. McCann, and E. Yamasaki. Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. Mutation Res., 31: 347–364, 1975.
3. Vogel, H. J., and D. M. Bonner. Acetylornithinase of Escherichia coli: Partial purification and some properties. J. Biol. Chem., 218: 97-106, 1956.

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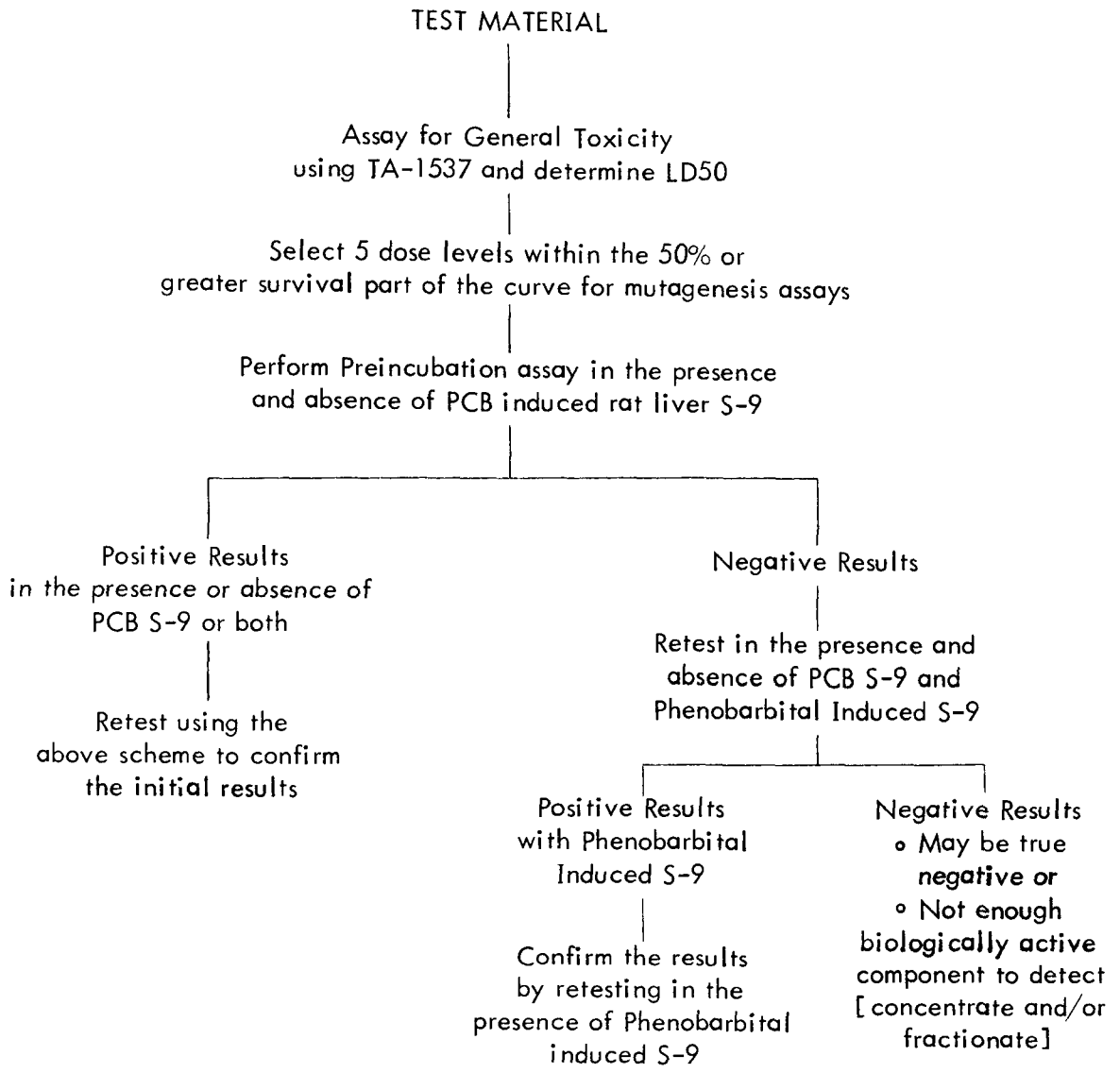

Figure V-2. General scheme.

APPENDIX VI. *Saccharomyces cerevisiae* Gene Mutation Assay

Both forward and reverse mutation can be monitored in the haploid strain XL7-10B.[1] It has the genotype $\alpha\rho^+$ CAN1 his1-7 lys1-1 ura1.

Forward Mutation to Canavanine Resistance (CAN1 → can1)

Canavanine is a toxic arginine analog to which yeast is normally sensitive. Resistance to canavanine has been shown to be almost exclusively due to mutational inactivation of the arginine permease. The permease gene (CAN1) has been estimated to be approximately 7,700 nucleotides long, hence it offers a very large mutational 'target.' CAN1 is mutated by both frameshift and base-pair substitution inducing mutagens; in addition, deletions and chromosomal rearrangements with breakpoints in CAN1 should also be recoverable.

Reversion of his1-7

The his1-7 mutation is a missense mutation resulting from a base-pair substitution in a histidine biosynthesis gene. This mutation confers a requirement for the amino acid histidine. Back mutation by base-pair substitution at the original mutant site removes the histidine requirement. Further, his1-7 reverts by second site mutation — a second base-pair substitution at another site which 'corrects' the original amino acid replacement in the enzyme protein by a second compensatory replacement. Since the reversion event is not limited to a single site, a broader spectrum of base-pair substitutions can be detected. Also, owing to different modes of DNA repair in yeast, his1-7 is reverted by mutagens which have been classified in bacterial systems as acting via a frameshift mechanism.

Both CAN1 and his1-7 mutate readily, and the mutants are subject to a positive selection method. Additionally, this system will tolerate a wide variety of assay conditions (e.g., stationary phase vs. log phase cells or presence or absence of a mammalian microsomal activation system) without requiring modification of the mutant selection procedure or affecting the recovery of mutants.

Supplies and Equipment

YPD, SC-ARG+CAN, and SC-HIS agar plates, prepoured
Steile solution of 0.067 M K_2HPO_4
Sterile solution of 10 percent (w/v) $Na_2S_2O_3$ on ice
Clinical centrifuge and sterile centrifuge tubes
Sterile plastic test tubes with sealing caps (16 x 100 mm if convenient - available from Falcon)
Shaking water bath set at 30 C (rotary preferred)
A supply of sterile 10-, 5-, and 1-ml pipettes and tips for microliter pipettor
Sufficient S-9 mix[2] for activated assays (prepare fresh and hold on ice, maximum 3 hr)
Ice bath for stopping assay
Sterile 0.067 M K_2HPO_4 dilution blanks (in plastic tubes as above)
Glass bacterial spreader and alcohol for flaming
Alcohol or gas burner
Protective gloves for handling test materials
Test material in aqueous or DMSO solution
Hemocytometer and compound microscope

Media

Media have the compositions shown below and are sterilized by autoclaving.

YPD

1% Difco yeast extract	6 g
2% Difco-Bacto-peptone	12 g
2% dextrose	12 g
2% Difco-Bacto-agar	12 g
distilled water	600 ml

For broth leave out agar.

SD

0.67% Difco yeast nitrogen base without amino acid	4 g
2% dextrose	12 g
2% Difco-Bacto-agar	12 g
distilled water	600 ml

A modified synthetic complete is prepared by the following additions to SD (concentrations in mg/liter).

SC

adenine sulfate	20
uracil	20
L-tryptophan	20
L-histidine HCl	20
L-arginine HCl	20
L-methionine	20
L-leucine	30
L-lysine HCl	30

SC-ARG+CAN is prepared by deleting arginine and adding filter-sterilized canavanine sulfate (40 mg/liter) after autoclaving. SC-HIS is prepared by deleting histidine.

Assay Methodology

Suspend a well-formed isolated colony of the appropriate tester strain in 0.067 M K_2HPO_4 and determine the cell concentration using the hemocytometer. Prepare a dilution series and inoculate 25 ml of YPD broth with approximately 200 cells. Grow 2–3 days with vigorous shaking at 30 C until late stationary phase.

Centrifuge the stationary-phase culture and resuspend in buffer. Adjust cell concentration to 2×10^9 cells/ml.

Place sufficient tubes for the assay in the ice bath. To each tube add: up to 0.5 ml of aqueous test material (or up to 100 µl of DMSO solution), 0.4 ml of S-9 mix (for activated assays), and sufficient 0.067 M K_2HPO_4 to bring the volume in each tube to 0.9 ml. Finally, add 0.1 ml of the yeast suspension to each assay tube. Seal the caps.

Without delay, place the assay tubes in the 30 C shaking water bath. At least, a 3-hr and a 20-hr incubation should be performed.

Stop the assay by placing the tubes in the ice bath and adding 1.0 ml of ice-cold 10 percent $Na_2S_2O_3$ to each tube.

Plating

Plate the stopped incubation mixture directly on SC-ARG+CAN and SC-HIS (in duplicate, 0.1 ml/plate.

Dilute the stopped mixture 10^{-5} and plate on YPD to determine survival.

Spread to dryness, flaming the spreader for each plate.

Incubate YPD plates 3 days at 30 C, others 5 days.

Count the plates. Calculate the percent survival and the mutation frequency based on surviving titer and note the mutation yield.

Notes

Activation: ϕB-induced and Ar- (or substitute) induced S-9 are used, as per Ames.[2]

Enzyme titration: after the dose giving 50 percent survival, or the highest dose applicable (if the substance is nontoxic), is determined, the activation system is optimized by titration with varying amounts of S-9.

References for Appendix VI

1. Strain available from F. W. Larimer, Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830.

2. Ames, B. N., J. McCann, and E. Yamasaki. Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. Mutat. Res., 31: 347-364, 1975.

APPENDIX VII. Bacterial DNA Repair Assay

DNA repair tests do not measure mutation per se, but DNA damage induced by chemical treatment of a cell. Microbial test systems measure this damage as cell killing. Test systems employ paired, identical cells, except one has the normal DNA repair capabilities and one lacks a specific step (or steps) in the enzyme pathways responsible for DNA repair. Preferential killing of the repair-deficient strain by the test chemical implies that the chemical exerts its killing effect by reacting with the cells' DNA, and, therefore, may be mutagenic. This implication may not be valid in all cases, since the test cannot separate a purely lethal DNA effect from one that also has a mutagenic component.

The following protocol describes a generalized DNA repair assay which can utilize any of the major bacterial 'repair' strains, i.e., the Bacillus subtilis rec^+-rec^- pair,[1] Escherichia coli $polA^+$-$polA^-$,[2] or Salmonella typhimurium $uvrB^+$-$uvrB^-$.[3] These systems are all based on the hypersensitivity of repair-defective bacteria to the lethal effects of DNA-modifying chemicals.

Strain Maintenance

The source references for the strains chosen give details for the maintenance of master cultures. The repair phenotypes are conveniently verified by checking for UV sensitivity as follows:

The tester strains are parallel-streaked across individual nutrient agar plates and half of each plate is irradiated with a G.E. 15 W germicidal lamp at a distance of 33 cm. The duration of the UV exposure is 6 sec, after which the plates are incubated overnight at 37 C. The repair-deficient strain should show growth only on the unirradiated side of the plate, while the repair-proficient strain should show growth on both sides of the plate.

Supplies and Equipment

Prepoured nutrient agar plates
Sterile solution of 0.067 M K_2HPO_4
Sterile solution of 10 percent (w/v) $Na_2S_2O_3$ on ice
Clinical centrifuge and sterile centrifuge tubes

Sterile plastic test tubes with sealing caps (16 x 100 mm is convenient — available from Falcon)
Shaking water bath set at 37 C (rotary preferred)
A supply of sterile 10-, 5-, and 1-ml pipettes and tips for microliter pipettor
Sufficient S-9 mix[3] for activated assays (prepare fresh and hold on ice, maximum 3 hr)
Ice bath for stopping assay
Sterile 0.067 M K_2HPO_4 dilution blanks (in plastic tubes as above)
Glass bacterial spreader and alcohol for flaming
Alcohol or gas burner
Protective gloves for handling test materials
Test material in aqueous or DMSO solution

Media

Nutrient broth is composed of 8 g Difco-Bacto nutrient broth, 5 g NaCl, and distilled water to 1 liter; sterilization is by autoclaving. Nutrient agar is nutrient broth solidified with 2 percent Difco-Bacto agar.

Repair Assay

Prepare overnight at 37 C nutrient broth cultures of each tester strain; store at 4 C.

0.1 ml of each bacterial culture will be required for each respective assay point. Centrifuge an adequate volume of each culture, discard the broth supernatant, and resuspend the bacteria in a like volume of 0.067 M K_2HPO_4.

Place sufficient tubes for the assay in the ice bath. To each tube add: up to 0.5 ml of aqueous test material (or up to 50 µl of DMSO solution), 0.4 ml of S-9 mix (for activated assays), and sufficient 0.067 M K_2HPO_4 to bring the volume in each tube to 0.9 ml. Finally, add 0.1 ml of the appropriate bacterial suspension to each of the assay tubes. Seal the caps.

Without delay, place the assay tubes in the 37 C shaking water bath. Incubate unactivated assays for 20 min, activated assays for 2 hr.

Stop the assay by placing the tubes in the ice bath and adding 1.0 ml of ice-cold 10 percent $Na_2S_2O_3$ to each tube.

Plating

Prepare the following serial dilutions from each stopped assay tube: 1:100, 1:10, 1:10, 1:10, using the 0.067 M K_2HPO_4 dilution blanks.

For each dilution, pipet 0.1 ml onto duplicate nutrient agar plates. Spread to dryness, flaming the spreader for each plate.

Incubate the plates inverted at 37 C overnight.

Count the plates. Calculate percent survival for each strain at each assay point, relative to untreated controls.

References for Appendix VIII

1. Kada, T., K. Tutikawa, and Y. Sadaie. In vitro and host-mediated "rec-assay" procedures for screening chemical mutagens; and phloxine, a mutagenic red dye detected. Mutat. Res., 16: 165–174, 1972.

2. Slater, E. E., M. D. Anderson, and H. S. Rosenkranz. Rapid detection of mutagens and carcinogens. Cancer Res., 31: 970–973, 1971.

3. Ames, B. N., J. McCann, and E. Yamasaki. Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. Mutat. Res., 31: 347-364, 1975.

APPENDIX VIII. Seed Germination/Radicle Length Assay

Two levels of tests were completed on EP extracts from the following solid wastes: fly ash, scrubber sludge, soybean process cake, and bottom ash. Level one studies consisted of a root elongation bioassay using radish (Raphanus sativus L. 'Early Scarlet Globe') and sorghum (Sorghum vulgare var. saccharatum 'Sugar Drip') seeds. In previous tests seeds were germinated in petri dishes and root (radicle) lengths of treatment and control results were compared. However, the time required to measure root lengths was so great due to their coiled growth pattern that it was not practical to use enough seeds for good statistical comparisons. Therefore, we developed special vertical germination chambers which took advantage of the geotropic growth response of plants resulting in straight hypocotyl growth and a tenfold reduction in measurement time.

One approach to reduce variability was to sieve seeds to separate them into size categories. U.S.A. standard testing sieves numbers 8, 10, and 12 with openings (in millimeters) of 2.36, 2.00, and 1.70, respectively, were used for separation. Within a test only one seed size was used for controls and test dilutions. Although 200 seeds were used for each treatment, only 150 seeds were actually measured. The excess allowed for nongerminating seeds and for radicles which were less than 5 mm long.

The germination chambers were constructed of 3-mm-thick Plexiglas (Fig. VIII-1) with inside dimensions of 10 cm high x 1.5 cm wide x 71 cm long. The size of the chambers was determined by the size of the incubator in which they were to be used. Chambers were mounted on a Plexiglas base support. Two pieces of 3-mm-thick Plexiglas were cut to an appropriate size to fit inside a chamber but extended above the chamber sides about 3 cm for convenience in handling. One hundred depressions (drilled with an electric drill and bit) spaced at 2-cm intervals in staggered rows 2 cm apart across one of the Plexiglas sheets served as seed counters, seed spacers, and to help hold the seeds in place. Seeds were placed on the Plexiglas sheet and brushed into the depressions. A piece of blotter paper was saturated with the solution to be tested and pressed firmly against

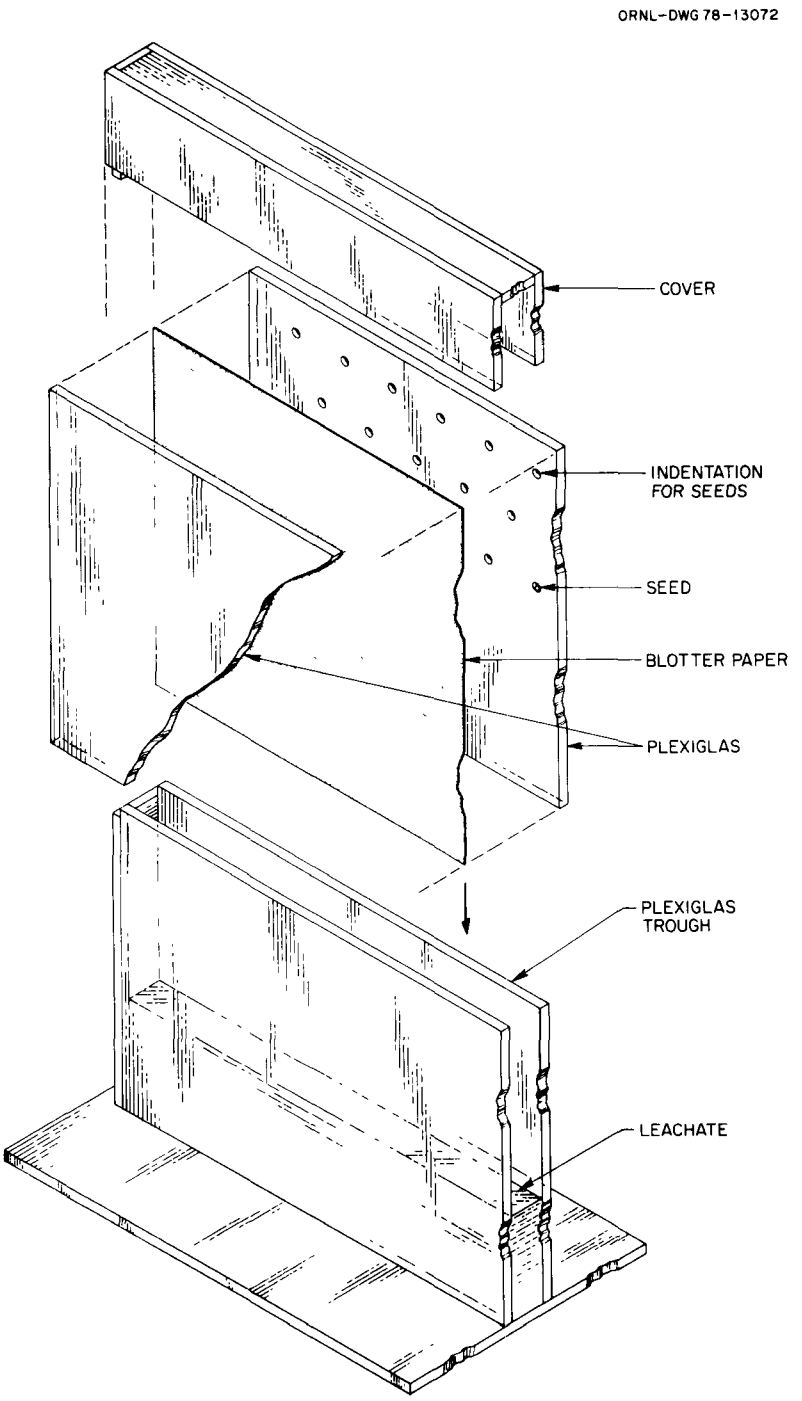

Figure VIII-1. Germination chamber.

the seeds until impressions were seen. Additional test solution, up to a total of 100 ml for 48-hr tests and 125 ml for 72-hr tests, was added to the germination chambers. We recommend that the blotter paper be saturated with the test solution in a flat tray rather than by standing on edge in the chambers, since standing on edge could result in differential movement of chemicals up the paper causing chromatographic separation and variable doses to seeds at different positions. The second Plexiglas sheet was positioned so that the seeds and blotter paper were sandwiched in between the two sheets of Plexiglas, which were then taped securely on the sides and top and placed vertically into the chamber. A Plexiglas lid was placed on top to reduce evaporation. The entire apparatus was then placed in an unlighted incubator set at 25 C. Since the tests reported here were run a small fan has been installed in the growth chamber to exhaust volatiles. The fan affected the chamber temperature, thus thermostat adjustments have been necessary. During this period of adjustment, tests have continued, to avoid delay. Since controls are run with each test, we were not concerned about effects of these temperature differences between tests. However, tests are presently being conducted to quantify temperature effects on root elongation of radish and sorghum. We used two chambers (200 seeds) for each test solution or for each concentration of a particular solution and two chambers containing distilled water as controls. After a predetermined time period (48 hr for radish, 72 hr for sorghum) the chambers were removed from the incubator and the root lengths were measured with calipers.

For cleaning, the chambers were filled with an appropriate cleaning solution (0.1 N HCl) and allowed to stand until their next use, when they were rinsed with distilled water. The rest of the apparatus was washed with two pipette washers, one containing 1 N HCl and one connected to a distilled water supply for rinsing.

Acetic acid was used in the EP to maintain the pH of the extract at approximately pH 5.0. Since acetic acid is toxic to plants, the highest concentration of extract used in the root elongation test was the concentration having less than 5.86 ml/liter of 0.5 N acetic acid. In preliminary tests this and higher volumes of the organic acid were toxic to radish seeds (Table 8-4).

The material referred to as the As-contaminated groundwater sample was not carried through the EP, but was diluted directly from the original solution for the root elongation tests. Since this particular waste was extremely toxic (based on an initial test with radish seeds) and safety problems were not yet resolved, further study was not undertaken.

APPENDIX IX. Seedling Growth Assay

The long-term seedling growth studies were conducted with wheat (Triticum aestiyrum 'Bear') and soybean (Glycine max 'Bedford') by use of the following protocol. Seeds were soaked for approximately 1 hr in deionized water. To 1 liter of sand (white silica sand which passes through a 25 mesh sieve was added 350 ml of a 10 percent concentration of the extract plus one tablespoon Purina Plant Food per gallon of solution; this was to ensure ample exposure of plants to extract. There were 50 wheat seeds in each of 5 containers and 15 soybean seeds in each of 10 containers, giving a total of 250 and 150 seeds, respectively.

Plants were exposed to a 10 percent concentration of extract in droplet form. The dose was sufficient to restore loss by evapotranspiration. The amount of time between each application ranged from every other day to every 3 days. Constant pressure was applied via a compressed air tank to plastic bottles containing the test solution. Solution was forced through Tygon tubing to a polyethylene nozzle (inverted buchner funnel). The volume was regulated with a screw clamp adjusted to a flow rate of 6 ml/sec. Solenoid valves connected to a pushbutton timer delivered the solution for 10 sec when engaged. This design is simple and can be disposed of or acid-washed in order to assure inexpensive, readily available component parts which are easily cleaned between test runs.

Wheat plants were grown for 2 weeks and soybean for 3 weeks. At harvest, sand was washed from the roots, roots and shoots were separated, and dry weights were recorded. To reduce variability between samples 5 soybean plants and 10 wheat plants were consolidated. The N value equaled as many as the number of sample groups available. A standard t-test was used for comparison of treated and control weights.

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