National Toxicology Program
Toxicity Report Series
Number 35

NTP Technical Report
on Toxicity Studies of

A Chemical Mixture of 25 Groundwater Contaminants

Administered in Drinking Water
to F344/N Rats and B6C3F$_1$ Mice

National Toxicology Program
Post Office Box 12233
Research Triangle Park, NC 27709

NIH Publication 93-3384
August 1993

These studies were supported in part by funds from the Comprehensive Environmental Response, Compensation, and Liability Act trust fund (Superfund) by an interagency agreement with the Agency for Toxic Substances and Disease Registry, U.S. Public Health Service.

United States Department of Health and Human Services
Public Health Service
National Institutes of Health

Foreword

The National Toxicology Program (NTP) is made up of four charter agencies of the United States Department of Health and Human Services (DHHS):

- the National Cancer Institute (NCI) of the National Institutes of Health;
- the National Institute of Environmental Health Sciences (NIEHS) of the National Institutes of Health;
- the National Center for Toxicological Research (NCTR) of the Food and Drug Administration; and
- the National Institute for Occupational Safety and Health (NIOSH) of the Centers for Disease Control.

In July 1981, the Carcinogenesis Bioassay Testing Program was transferred from NCI to NIEHS. NTP coordinates the relevant Public Health Service programs, staff, and resources that are concerned with basic and applied research and with biological assay development and validation.

NTP develops, evaluates, and disseminates scientific information about potentially toxic and hazardous chemicals. This knowledge is used for protecting the health of the American people and for the primary prevention of disease.

NTP designs and conducts studies to characterize and evaluate the toxicologic potential of selected chemicals in laboratory animals (usually two species, rats and mice). Chemicals selected for NTP toxicology studies are chosen primarily on the bases of human exposure, level of production, and chemical structure. Selection *per se* is not an indicator of a chemical's toxic potential.

The studies described in this toxicity study report were performed under the direction of NIEHS and were conducted in compliance with NTP laboratory health and safety requirements. These studies met or exceeded all applicable federal, state, and local health and safety regulations. Animal care and use were in accord and compliance with the Public Health Service Policy on Humane Care and Use of Animals.

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United States Department of Health and Human Services
Public Health Service
National Institutes of Health

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This NTP report on the toxicity studies of a chemical mixture of 25 groundwater contaminants is based primarily on 26-week studies that took place from September 1988 through April 1989.

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ABSTRACT

A Chemical Mixture of 25 Groundwater Contaminants

Toxicity studies were performed with a chemically defined mixture of 25 groundwater contaminants, using dose levels considered to have environmental relevance. The mixture contained 19 organic compounds and six metals (shown on page 8); the selection of these compounds was based primarily on the frequency of their occurrence in United States Environmental Protection Agency surveys of groundwater contamination in the vicinity of hazardous waste disposal sites. This report focuses primarily on 26-week drinking water toxicity studies with male and female F344/N rats and $B6C3F_1$ mice. The endpoints evaluated included histopathology, clinical pathology, neurobehavior, and reproductive toxicity. Additional studies using this same chemical mixture are briefly reviewed in this report and include an evaluation of spermatogenesis in $B6C3F_1$ mice exposed to the chemical mixture for 13 weeks, a continuous breeding study with Sprague-Dawley rats and CD-1® Swiss mice, studies of myelotoxicity in $B6C3F_1$ mice exposed to the chemical mixture for up to 31.5 weeks, studies of immunosuppression in $B6C3F_1$ mice exposed for up to 13 weeks, *in vitro* mutagenicity assays in *Salmonella typhimurium* and *Escherichia coli*, and measures of genetic damage in bone marrow and peripheral blood of F344/N rats and $B6C3F_1$ mice in 2-week drinking water studies.

In a 26-week drinking water study in which rats were administered the chemical mixture at composite contaminant concentrations of 0, 11, 38, 113, and 378 ppm, no deaths occurred, but the body weight gain of high-dose males was slightly less than that of the controls. Water consumption decreased with dose and was 24% to 28% less than that by the controls at the highest concentration. Changes in organ weights occurred primarily in high-dose rats and included increased absolute and relative liver and kidney weights in females, increased relative kidney weight in males, and decreased absolute and relative thymus weights in males and females. Hematologic assessments indicated that rats receiving 378 ppm developed a microcytic anemia consistent with that accompanying iron depletion. Multiple foci of inflammation occurred in the liver of exposed rats. In high-dose females, these liver lesions were especially prominent and included bile duct and oval cell hyperplasia. Inflammation also occurred in the mesenteric lymph nodes, the adrenal

gland, and the spleen. The amount of hemosiderin in the spleens of rats receiving the higher concentrations of the chemical mixture was less than normal.

Components of a Chemical Mixture of 25 Groundwater Contaminants

Component	EPA Survey Average[1] (ppm)	Target Concentration (ppm)			
Acetone	6.9	1.59	5.30	15.9	53.0
Aroclor 1260	0.21	0.0003	0.001	0.003	0.01
Arsenic	30.6	0.27	0.9	2.7	9
Benzene	5.0	0.375	1.25	3.75	12.5
Cadmium	0.85	1.53	5.1	15.3	51
Carbon tetrachloride	0.54	0.012	0.04	0.12	0.4
Chlorobenzene	0.1	0.003	0.01	0.03	0.1
Chloroform	1.46	0.21	0.7	2.1	7
Chromium	0.69	1.08	3.6	10.8	36
1,1-Dichloroethane	0.31	0.042	0.14	0.42	1.4
1,2-Dichloroethane	6.33	1.2	4	12	40
1,1-Dichloroethylene	0.24	0.015	0.05	0.15	0.5
1,2-*trans*-Dichloroethylene	0.73	0.075	0.25	0.75	2.5
Di(2-ethylhexyl) phthalate	0.13	0.0005	0.0015	0.0045	0.015
Ethylbenzene	0.65	0.009	0.03	0.09	0.3
Lead	37.0	2.1	7	21	70
Mercury	0.34	0.017	0.05	0.17	0.5
Methylene chloride	11.2	1.125	3.75	11.25	37.5
Nickel	0.5	0.204	0.68	2.04	6.8
Phenol	3.27	0.87	2.9	8.7	29
Tetrachloroethylene	9.68	0.102	0.34	1.02	3.4
Toluene	5.18	0.21	0.7	2.1	7
1,1,1-Trichloroethane	1.25	0.06	0.2	0.6	2
Trichloroethylene	3.82	0.195	0.65	1.95	6.5
Xylenes	4.07	0.048	0.16	0.48	1.6
Total concentration (ppm)	131.05	11.3428	37.8025	113.128	378.025

[1] Average concentration of 14 to 3011 analyses for each compound; based on a July 1985 survey conducted for the United States Environmental Protection Agency by Lockheed Engineering and Management Services Company, Inc. Adapted from Yang and Rauckman, 1987.

In a 26-week study in which mice were exposed to the chemical mixture at concentrations of 0, 11, 38, 113, and 378 ppm in drinking water, there were no clear adverse effects noted in survival, weight gain, clinical pathology parameters, or histopathologic evaluations. Water consumption decreased with increasing dose, and water consumption by high-dose mice was approximately 40% less than that by the controls.

In neurobehavioral assessments, no clear treatment-related effects were observed in measures of forelimb and hindlimb grip strength, hindlimb footsplay, motor activity, response to a thermal stimulus, or startle response in rats or mice evaluated at 6-week intervals throughout the 26-week drinking water studies.

There were no effects on sperm morphology or motility or on estrous cycle length in rats or mice receiving the chemical mixture during the 26-week studies. Sperm concentration was decreased in F_1 CD-1® Swiss mice during continuous breeding studies, although there were no clear adverse effects on the fertility of Sprague-Dawley rats or CD-1® Swiss mice in these studies. Pup weight, the number of live males, and the number of male pups per litter were slightly decreased in dosed rats in the continuous breeding study; the number of live female mouse pups in litters born of the F_0 and F_1 generations was decreased in the 378 ppm group. The significance of these observations, if any, is not known. F_1 mice receiving 378 ppm had increased incidences of hepatic inflammation compared to the controls.

In female B6C3F_1 mice that received the chemical mixture in drinking water at concentrations as high as 756 ppm for 2 weeks or 378 ppm for 13 weeks, assessments of immune function showed suppression of hematopoietic stem cells and antigen-induced antibody-forming cells. This was manifested by impaired resistance to challenge with a nonlethal strain of mouse malaria, *Plasmodium yoelii*. Additional evidence of an adverse effect on hematopoietic stem cells was demonstrated by decreases in the *in vitro* colony-forming ability of granulocyte-macrophage progenitor cells and erythroid precursor cells isolated from female mice that had received the chemical mixture at a concentration of 378 or 756 ppm in 31.5-week studies. Potential genotoxic effects of the chemical mixture to the bone marrow of F344/N rats and B6C3F_1 mice were assessed in 2-week drinking water studies with concentrations as high as 756 ppm. Small increases in sister chromatid exchanges and micronucleated polychromatic erythrocytes occurred in the bone marrow of dosed male mice, and micronucleated polychromatic erythrocytes were also increased in dosed female mice.

The chemical mixture did not induce mutations in *Salmonella typhimurium* strain TA98 or TA100 and did not induce DNA damage in *Escherichia coli* with or without metabolic activation.

In summary, rats receiving drinking water containing a mixture of 25 common groundwater contaminants at levels of potential environmental relevance developed inflammatory lesions in the liver, spleen, lymph nodes, and adrenal gland, as well as evidence of an iron deficiency anemia. The inflammatory lesions could not be predicted based on the known toxic effects of the individual components of the chemical mixture.

Mice exposed to similar concentrations of the chemical mixture did not show adverse effects in a standard toxicity study but developed deficits in bone marrow function, evidence of genetic damage, hepatic inflammation, and immunosuppression in other studies that generally included exposures to higher concentrations or exposures of longer duration. The no-observed-adverse-effect level for histologic injury (granulomatous inflammation of the liver) was 11 ppm in rats; however, no clear evidence of histologic injury was seen in mice exposed to concentrations of the chemical mixture as high as 378 ppm in a standard 26-week study.

PEER REVIEW PANEL

The members of the Peer Review Panel who evaluated the draft report on the toxicity studies of a chemical mixture of 25 groundwater contaminants on December 2, 1992, are listed below. Panel members serve as independent scientists, not as representatives of any institution, company, or governmental agency. In this capacity, panel members act to determine if the design and conditions of the NTP studies are appropriate and to ensure that the toxicity study report presents the experimental results and conclusions fully and clearly.

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SUMMARY OF PEER REVIEW COMMENTS

On December 2, 1992, the Technical Reports Review Subcommittee of the Board of Scientific Counselors for the National Toxicology Program met in Research Triangle Park, North Carolina, to review the draft technical report on toxicity studies of a chemical mixture of 25 groundwater contaminants.

Dr. Raymond Yang, Colorado State University, and Dr. John Bucher, NIEHS, introduced the short-term toxicity studies of the chemical mixture by reviewing the rationale for the study, the experimental design, and the results.

Dr. Carlson, a principal reviewer, said that in several places the document was not sufficiently clear in pointing out that the levels and the nature of the chemical contaminants studied would not be encountered by the public through consumption of finished drinking water. He felt that the expression "environmentally realistic doses" was misleading. Dr. Carlson also questioned the stated NOAEL of 11 ppm based on the severity of granulomatous inflammation in female rats.

Dr. Davis, a second principal reviewer, thought that the approach of the NTP in the study of chemical mixtures could be more clearly spelled out in the introduction, and a clearer statement of how the doses of the individual chemicals compared to the actual groundwater survey results could be made. He disagreed with the speculation that increased erythrocyte counts in rats, in conjunction with decreases in most other parameters, could be related to dehydration. He asked that the term "granulomatous inflammation" be introduced earlier in the report in description of the liver lesions.

Dr. Ward, a panel member, asked how many of the chemicals were mutagenic (five, as answered by Dr. Errol Zeiger, NIEHS) and if the NTP was contemplating performing 2-year studies on this mixture. Dr. van Zwieten, another panel member, asked that more complete information on the toxicity and carcinogenicity of the various components of the mixtures be included.

Dr. Bucher responded by agreeing to Dr. Carlson's suggestion to clarify the fact that finished drinking water would not be expected to contain contaminants at the levels evaluated in these studies. Concerning the NOAEL, Dr. Bucher and Dr. Michael Elwell, NIEHS, discussed the difference between the inflammatory liver lesions found in the

control and low-dose rats and the lesions in rats receiving doses of 38 ppm or greater. Dr. Bucher agreed to attempt to clarify several of the sections of the report according to Dr. Davis's suggestions and agreed to reexamine the speculations concerning the cause of the polycythemia. He responded to Dr. Ward by indicating that the NTP had no plans to perform 2-year studies on the chemical mixture. Dr. Bernard Schwetz, NIEHS, responded to Dr. van Zwieten's suggestion by pointing out the magnitude of the toxicology information currently published on the 25 chemicals that composed the mixture and stating that many of the toxic effects differed with dose and route, making the task exceptionally complicated.

After a short discussion of errors and editorial corrections to the report, Dr. Klaassen accepted the report with the suggested changes on behalf of the peer review panel.

INTRODUCTION

Human exposure to chemicals, whether environmental or occupational, is rarely limited to a single chemical. People are exposed daily to a variety of potentially toxic synthetic and natural chemicals in food, drink, air, and personal hygiene products as well as to chemicals in the workplace. In recent years, various environmental problems have led to increased concern about potential toxicity from exposure to multiple chemicals, including those associated with hazardous waste disposal sites (Yang and Rauckman, 1987; Yang *et al.*, 1989a,b).

Although people are typically exposed to mixtures of chemicals, toxicological testing is most often conducted using single chemicals because of the expense and complexity of studies designed to evaluate the toxicity of chemical mixtures. Extensive characterization of some mixtures, such as diesel exhaust, cigarette smoke, and some petroleum products, has been undertaken (Schwetz and Yang, 1990). Studies have also been performed on soil and water containing multiple contaminants (Silkworth *et al.*, 1984).

Because of the immense number of potential mixtures of natural and synthetic chemicals in the environment and the complexity of interactions of components in a given mixture, testing even a small sampling of all potential mixtures is, of course, unfeasible. One assumption frequently made to estimate the potential toxicity of a chemical mixture is that the effects of the components are additive. However, because various types of potential interactions, including potentiation, synergism, and antagonism, may also occur, predicting the toxicity of a mixture based on the toxicity of the individual components may not be appropriate (Schwetz and Yang, 1990). A committee assembled by the National Research Council/National Academy of Sciences (NRC/NAS) at the request of the National Institute of Environmental Health Sciences (NIEHS) reviewed toxicological and epidemiological data from studies of well-characterized chemical mixtures and concluded that a new approach for characterizing the effects of mixtures was necessary (NAS, 1988; Schwetz and Yang, 1990). In response, the NIEHS and NTP have selected two approaches to determine the potential magnitude of the toxicological problems presented by chemical mixtures. The first approach is to characterize the toxicity of well-defined complex chemical mixtures. The second approach is to evaluate mechanisms responsible for effects that deviate from additivity. This second approach combines the testing of specific

mixtures with a conceptual evaluation that allows information obtained from the testing of individual chemicals and combinations of chemicals to be applied to other mixtures (Schwetz and Yang, 1990). The current study of 25 groundwater contaminants is a part of the first approach, *i.e.*, to explore the potential for toxic interactions among chemicals in complex mixtures.

Occurrence and Exposure

One of the most critical environmental concerns today is groundwater contamination. Approximately 50% of the population of the United States and 75% of American cities rely on groundwater as a partial or primary source of drinking water; about 95% of the rural U.S. population depends on groundwater for all of their water needs (Yang and Rauckman, 1987; Yang *et al.*, 1989c; NTP, 1990). Groundwater contamination may occur in a number of ways, with hazardous waste disposal practices being a major contributor.

To prepare a representative mixture of groundwater contaminants, the results of surveys of groundwater near hazardous waste disposal sites conducted by Lockheed Engineering and Management Services Company, Incorporated, and the Mitre Corporation for the U.S. Environmental Protection Agency (USEPA) were used (Yang and Rauckman, 1987). A mixture of 25 components, including 19 organic compounds and six metals, was selected for the groundwater contaminants studies, based on the frequency of occurrence in groundwater sources, known toxic potential, large commercial production volume, potential environmental impact, or representation of certain chemical classes. Although cyanide, 2,4-dichlorophenoxyacetic acid, and vinyl chloride are frequently found in groundwater, these chemicals were excluded from the chemical mixture because of the formation of toxic vapor, interactions with other chemicals, or volatility (Yang and Rauckman, 1987).

The highest concentrations of many of the groundwater contaminants added to the drinking water used in the current studies were near the average concentrations found in groundwater near hazardous waste disposal sites in the USEPA surveys (Table 1; Yang *et al.*, 1989a,b; Schwetz and Yang, 1990). With the exception of three metals (cadmium, chromium, and nickel) for which the maximum concentrations used in these studies were more than 10 times higher than the average concentrations in the USEPA surveys, the concentration of each individual component in the chemical mixture was within a factor of 10 of the average concentration found in the surveys; the concentrations of eight

chemicals were below the averages in the surveys (Yang *et al.*, 1989a). It should be noted that these contaminant levels represent worst-case situations, and most are several orders of magnitude higher than those detected in unfinished drinking water sources throughout the U.S. Finished drinking water typically contains much lower levels of contaminants.

TABLE 1 Components of a Chemical Mixture of 25 Groundwater Contaminants

Component	CAS Number	EPA Survey Average[1] (ppm)	Target Concentration (ppm)			
Acetone	67-64-1	6.9	1.59	5.30	15.9	53.0
Aroclor 1260		0.21	0.0003	0.001	0.003	0.01
Arsenic trioxide[2]		30.6	0.27	0.9	2.7	9
Benzene	71-43-2	5.0	0.375	1.25	3.75	12.5
Cadmium acetate hydrate[2]		0.85	1.53	5.1	15.3	51
Carbon tetrachloride	56-23-5	0.54	0.012	0.04	0.12	0.4
Chlorobenzene	108-90-7	0.1	0.003	0.01	0.03	0.1
Chloroform	67-66-3	1.46	0.21	0.7	2.1	7
Chromium chloride hexahydrate[2]		0.69	1.08	3.6	10.8	36
1,1-Dichloroethane	75-34-3	0.31	0.042	0.14	0.42	1.4
1,2-Dichloroethane	107-06-2	6.33	1.2	4	12	40
1,1-Dichloroethylene	75-35-4	0.24	0.015	0.05	0.15	0.5
1,2-*trans*-Dichloroethylene	156-60-5	0.73	0.075	0.25	0.75	2.5
Di(2-ethylhexyl) phthalate	117-81-7	0.13	0.0005	0.0015	0.0045	0.015
Ethylbenzene	100-41-4	0.65	0.009	0.03	0.09	0.3
Lead acetate trihydrate[2]		37.0	2.1	7	21	70
Mercuric chloride[2]		0.34	0.017	0.05	0.17	0.5
Methylene chloride	75-09-2	11.2	1.125	3.75	11.25	37.5
Nickel acetate tetrahydrate[2]		0.5	0.204	0.68	2.04	6.8
Phenol	108-95-2	3.27	0.87	2.9	8.7	29
Tetrachloroethylene	127-18-4	9.68	0.102	0.34	1.02	3.4
Toluene	108-88-3	5.18	0.21	0.7	2.1	7
1,1,1-Trichloroethane	71-55-6	1.25	0.06	0.2	0.6	2
Trichloroethylene	79-01-6	3.82	0.195	0.65	1.95	6.5
Xylenes	1330-20-7	4.07	0.048	0.16	0.48	1.6
Total concentration (ppm)		131.05	11.3428	37.8025	113.128	378.025

[1] Average concentration of 14 to 3011 analyses for each compound; based on a July 1985 survey conducted for the United States Environmental Protection Agency by Lockheed Engineering and Management Services Company, Inc. Adapted from Yang and Rauckman, 1987.

[2] The concentrations shown are for the metal ion, not the salt.

Absorption, Disposition, Metabolism, and Excretion

Little is known about the pharmacokinetics and metabolism of chemical mixtures. Although it is unlikely that any of the 25 groundwater contaminants in the chemical mixture in the current studies would approach the saturation kinetic level if taken singly at the average concentration found in the groundwater surveys, acute exposure at a very high concentration or repeated or chronic exposure at a lower concentration might have different results (NRC, 1989). Also, the accumulation of metals or persistent organics in the body over time could pose a health threat. Development of a pharmacokinetic model may require an uncertainty factor to anticipate synergism or antagonism. Induction or inhibition of mixed-function oxidases or conjugating enzymes may occur and may be highly dose dependent, thus affecting the metabolism of other components of the mixture. For humans exposed to contaminated groundwater, pharmacokinetic models may also have to take into consideration alternate routes of absorption; for example, dermal exposure would result from bathing, and inhalation of compounds that volatilize could also occur (NRC, 1989).

Study Rationale and Design

The design for the studies summarized in this report evolved from lengthy debates about the best approach for predicting the potential hazards associated with exposures to mixtures of environmental contaminants. Existing studies of chemical mixtures were largely focused on investigations of toxicologic interactions of high-dose binary mixtures, primarily dealing with acute toxicity, or on extremely complex mixtures such as diesel exhaust, tobacco smoke, synfuel fractions, or soil samples from Love Canal, where the actual identity of the chemical components was only partially known. There was a lack of toxicity studies of defined, yet relatively complex, mixtures using environmentally relevant doses that were applicable to more than one or a few exposure areas or target populations. Therefore, the decision was reached to study a chemically defined mixture given for relatively long exposure periods at environmentally realistic doses. It was decided to investigate possible adverse effects of a known chemical mixture of groundwater contaminants. This decision was based on knowledge of contaminants found in samples collected in groundwater near numerous hazardous waste disposal sites and on the potential for consumption of contaminated water supplies by the public.

Detailed information concerning the guidelines for the selection of chemicals for the mixture and the dose levels chosen have been published (Yang and Rauckman, 1987; Yang *et al.*, 1989a). A mixture of 25 components, including 19 organic components and six metals, was selected for toxicologic characterization. Concentrations of individual components were based on concentrations found in groundwater in a USEPA survey and on acute toxicity data obtained from the Registry of Toxic Effects of Chemical Substances, 1980; drinking water was chosen as the route of administration because this is the primary route of potential human exposure. F344/N rats and B6C3F$_1$ mice were used in the 26-week studies. The studies performed included reproductive system, clinical pathology, neurobehavioral, and histopathologic evaluations. Hematopoietic and immune system toxicity and genetic toxicity studies have been reported (Table 2).

TABLE 2 Summary of Toxicity Studies of a Chemical Mixture of 25 Groundwater Contaminants

Study	Sex and Species	Reference
Toxicity	Male and female rats and mice	This report
Sperm morphology and vaginal cytology	Male and female rats and mice	Appendix C
Spermatogenesis	Male mice	Appendix C; Chapin *et al.*, 1989
Reproductive assessment by continuous breeding	Male and female rats and mice	Appendix D; Heindel *et al.*, 1993
Genetic toxicity	Male and female rats and mice	Appendix E; Shelby *et al.*, 1990
Neurobehavioral evaluations	Male and female rats and mice	Appendix G
Hematopoietic system toxicity	Female mice	Appendix H; Hong *et al.*, 1991, 1992
Immunotoxicity	Female mice	Appendix I; Germolec *et al.*, 1989
Interaction with carbon tetrachloride	Male rats	Simmons *et al.*, 1989

MATERIALS AND METHODS

Characterization of a Chemical Mixture of 25 Groundwater Contaminants

Stock solutions of groundwater contaminants were prepared by Midwest Research Institute (MRI; Kansas City, MO). Stock A consisted of 18 organic components, excluding phenol; Stock B was an aqueous solution of six metal salts and phenol. To prepare Stock A, a toluene-based stock was prepared by mixing di(2-ethylhexyl) phthalate (DEHP), Aroclor 1260, and toluene with carbon tetrachloride, chlorobenzene, 1,1-dichloroethylene, and ethylbenzene. The remaining 11 organic components were then mixed with the toluene-based stock. To prepare Stock B, arsenic trioxide was added to reagent-grade water. The solution was boiled until the arsenic trioxide dissolved, then cooled, and the remaining five metal salts and phenol were added. The organic and metal components and the target concentration of each component in Stocks A and B are listed in Table 3. These concentrations were based on the results of a USEPA survey of contaminants in groundwater around hazardous waste disposal sites, toxicity data of individual components, and solubility of the individual components in the matrix of the aqueous solution of the 25 chemicals (Appendix F; Yang *et al.*, 1989c).

The organic and inorganic compounds used in the chemical mixture were of the highest purity available commercially; reagent-grade water was used in the drinking water solutions. The identity and purity of each compound was analyzed prior to preparation of the drinking water solutions by elemental analysis, infrared and nuclear magnetic resonance spectroscopy, gas chromatography, and spark source mass spectroscopy (Appendix F).

The stability of the drinking water solutions of the 25 components, stored under animal room conditions for up to 96 hours or sealed in amber glass bottles in the dark at 5° C or room temperature for up to 21 days, was tested by MRI. To prepare the drinking water solutions for the stability studies, Stock B was diluted with water, magnetically stirred, and sonicated. The solution was then further diluted with water to obtain the desired concentration, Stock A was added, and the solution was mixed. Fifteen organic components [all of the organic components except acetone, Aroclor 1260, di(2-ethylhexyl) phthalate, and phenol] were analyzed by gas chromatography/mass spectroscopy. The

concentrations of Aroclor 1260 and acetone were determined using two different gas chromatographic systems; the concentrations of phenol and di(2-ethylhexyl) phthalate were determined using two different high-performance liquid chromatographic systems. The drinking water solutions were analyzed for the six metal components by inductively coupled plasma emission spectroscopy.

TABLE 3 Components in the Stock Solutions of a Chemical Mixture of 25 Groundwater Contaminants

Component	Lot Number	Target Concentration[1] (ppm)
STOCK A		
Acetone	AN228	530
Aroclor 1260	13-23D	0.1
Benzene	862604	125
Carbon tetrachloride	01615EM	4
Chlorobenzene	258806 386	1
Chloroform	03225DP	70
1,1-Dichloroethane	10129X	14
1,2-Dichloroethane	B101984	400
1,1-Dichloroethylene	02720LM, 01322KP	5
1,2-*trans*-Dichloroethylene	C011885	25
Di(2-ethylhexyl) phthalate	PM401	0.15
Ethylbenzene	U050286	3
Methylene chloride	01720EP	375
Tetrachloroethylene	2628LL	34
Toluene	63318PM, 9817LL	70
1,1,1-Trichloroethane	TA821004-1	20
Trichloroethylene	TB08039AA	65
Xylenes	A102186	16
STOCK B		
Arsenic trioxide	F102186	238
Cadmium acetate hydrate	1313CM	1209
Chromium chloride hexahydrate	A010888, 00905 EP	2467
Lead acetate trihydrate	855991	1281
Mercuric chloride	256923 586	6.76
Nickel acetate tetrahydrate	01627HM	229
Phenol	264049 886	290

[1] Concentration of component in undiluted stock solution (1.7 mL of the organic substock; 1 L of the metal salt and phenol substock).

Results of these studies showed that a drinking water solution containing 25 groundwater contaminant components can be prepared and the components of the solution remain visibly dissolved; however, target concentrations for some of the components could not be achieved for multiliter quantities due to volatility and affinity of some of the compounds

for glassware. Additionally, stability studies of dose formulations stored for 96 hours or 21 days showed losses of organic components, particularly Aroclor 1260 and di(2-ethylhexyl) phthalate, for which recovery was extremely low at zero time; most losses occurred within the first 24 hours. Most of the organic components were stable for 7 days when stored in the dark at 5° C; however, tetrachloroethylene, trichloroethylene, 1,2-*trans*-dichloroethylene, and 1,2-dichloroethane showed losses at higher concentrations (100X), and tetrachloroethylene and trichloroethylene also showed significant losses at lower concentrations (10X). The phenol and metal components were stable under all conditions tested, and there was no evidence of chemical interactions in the stock solutions. During the studies, the stock solutions were stored in glass containers, refrigerated, in the dark.

Dose Formulations

Periodically, the study laboratory received shipments of Stock A (organic components) and Stock B (metal salts and phenol) that were prepared by MRI. High-dose solutions (378 ppm total contaminants) were prepared by mixing 1.1 L Stock B with 9.9 L deionized water and adding 1.87 mL Stock A. Lower concentrations (11, 38, and 113 ppm total contaminants) were prepared by diluting the 378 ppm solutions with deionized water. Dose formulations were prepared three times per week. Initially, the drinking water solutions were stored in amber glass bottles at 5° C; later in the studies, the solutions were stored at room temperature to maintain solubility of the organic components.

The study laboratory monitored the drinking water formulations for lead acetate (all exposure concentrations) with atomic absorption spectroscopy and for the organic markers methylene chloride, acetone, 1,2-dichloroethane, benzene, and toluene (38, 113, and 378 ppm concentrations) with gas chromatography. The concentrations of lead acetate and acetone in the 38, 113, and 378 ppm formulations were within 10% of the target concentrations. All animal room samples of the 113 and 378 ppm formulations and 8 of 12 animal room samples of the 38 ppm formulations were also within 10% of the target concentration of lead acetate; all animal room samples of the 378 ppm concentration and 11 of 12 animal room samples of the 113 ppm concentration were within 10% of the target concentration for acetone. There was a high degree of variation in the concentrations of other organic markers in the dose formulations prior to and after dosing. This variability was attributed to high volatility or to the low solubility or miscibility of the organic markers in water as well as to the complexity of the analysis methods employed.

For the 11 ppm formulations, three of seven dose formulations and 4 of 12 animal room samples contained lead acetate concentrations within 10% of the target concentration. Variability in the results of the lead acetate analyses may have been due to the formation of a carbonate derivative or to the dose formulations being at the limit of detection for the spectrometer.

Toxicity Study Designs

26-WEEK BASE STUDIES

Male and female F344/N rats used in these studies were obtained from Simonsen Laboratories (Gilroy, CA); male and female B6C3F$_1$ mice were obtained from Taconic Farms (Germantown, NY). Rats were 31 days old and mice were 32 days old at receipt; rats and mice were quarantined 11 days and were approximately 6 weeks old when the studies began. Blood samples were collected from five rats and five mice per sex at the beginning and end of the studies. The sera were analyzed for antibody titers to rodent viruses (Boorman *et al.*, 1986; Rao *et al.*, 1989a,b); all results were negative. Additional details concerning the study design are provided in Table 4.

The exposure levels selected for the 26-week studies were based on potential human exposure through consumption of unfinished drinking water (based on USEPA survey results for groundwater contamination), on solubility studies, and on acute toxicity data for each of the 25 components in the mixture used in these studies. Additionally, palatability and mortality were evaluated in 2-week studies conducted at Battelle Columbus Laboratories (Columbus, OH). In these studies, administration of the chemical mixture to rats and mice at concentrations of 0, 38, 374, and 756 ppm daily for 15 days did not affect survival. However, water consumption decreased with increasing exposure concentration, and the decrease was accompanied by decreased body weight gains in high-dose rats and in mice in the two highest dose groups. Based on these results, concentrations of 0, 11, 38, 113, and 378 ppm were selected for rats and mice in the 26-week studies. Groups of 20 rats and 20 mice per sex per exposure level were administered drinking water containing a mixture of 25 groundwater contaminants daily. During Week 14, 10 animals per group were evaluated; because these animals exhibited no clinical signs of toxicity, the remaining animals continued to receive dosed drinking water for another 12 weeks.

Rats were housed five per cage by sex and mice were housed individually. NIH-07 Open Formula Diet (Zeigler Brothers, Inc., Gardners, PA) in pellet form was available *ad libitum*. Animal rooms were maintained at 21° to 24° C and 35% to 65% relative humidity, with 12 hours of fluorescent light per day and at least 10 room air changes per hour.

Complete necropsies were performed on all base-study animals. The brain, heart, right kidney, liver, lungs, right testis, and thymus from animals surviving to Week 26 were weighed. Organs and tissues were examined for gross lesions and fixed in 10% neutral buffered formalin. Tissues to be examined microscopically were trimmed, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Complete histopathologic examinations were performed on all animals in the 0 and 378 ppm groups. Gross lesions in rats and mice and the adrenal glands, liver, lungs, mesenteric lymph nodes, and spleen of rats in the lower exposure groups were examined. Tissues examined microscopically are listed in Table 4. Perl's iron, PAS with and without diastase, Masson's trichrome, acid-fast, and Giemsa stains were applied to additional sections of liver, spleen, and mesenteric lymph node. Samples of liver from formalin-fixed wet tissues were post-fixed in Fowler's fixative and processed for transmission electron microscopy examination.

Upon completion of the laboratory pathologist's histologic evaluation, the slides, paraffin blocks, and residual wet tissues were sent to the NTP Archives for inventory, slide/block match, and wet tissue audit. The slides, individual animal data records, and pathology tables were sent to an independent pathology laboratory where quality assessment was performed. Results were reviewed and evaluated by the NTP Pathology Working Group (PWG); the final diagnoses represent a consensus of contractor pathologists and the PWG. Details of these review procedures have been described by Maronpot and Boorman (1982) and Boorman *et al.* (1985).

SUPPLEMENTAL EVALUATIONS

Summaries of the continuous breeding, genetic toxicology, hematopoietic system toxicity, and immunotoxicity studies are given in Appendices D, E, H, and I, respectively.

Clinical Pathology

Clinical pathology studies were performed on base-study mice and on rats designated for clinical pathology testing. Ten animals per sex and exposure level were evaluated. For

rats, blood for hematology evaluations was collected on Days 95 (females), 99 (males), and at the end of the study (Day 183). Blood for clinical chemistry evaluations was collected on Days 7, 19, 49, 95 (females), 99 (males), and 183. Urine samples were collected on Days 5, 17, 47, 93 (females), 95 (males), and 181. For mice, blood for hematology and clinical chemistry evaluations was collected on Days 95 and 183. For the hematology and clinical chemistry evaluations, animals were anesthetized with a CO_2:O_2 gas mixture, and blood was drawn from the retroorbital sinus. Samples for hematology analysis were placed in serum separator tubes (Microtainer®, Beckton-Dickinson and Co., Rutherford, NJ) containing sodium EDTA; samples for clinical chemistry evaluations were placed in similar tubes devoid of anticoagulant. The latter samples were allowed to clot at room temperature; the samples were then centrifuged and serum was removed. All hematologic and biochemical analyses were performed on the day of sample collection.

Hematologic determinations were made using an Ortho ELT-8 hematology analyzer (Ortho Instruments, Westwood, MA). The parameters that were evaluated are listed in Table 4. Differential leukocyte counts were taken and morphologic evaluations of blood cells were made from blood smears stained with Wright-Giemsa. Smears made from blood samples stained with new methylene blue were examined microscopically with a Miller disc for the quantitative determination of reticulocytes.

Clinical chemistry variables were measured with a Hitachi 704® chemistry analyzer (Boehringer Mannheim, Indianapolis, IN). The parameters that were evaluated are listed in Table 4. Reagents for assays of sorbitol dehydrogenase were obtained from Sigma Chemical Company (St. Louis, MO); other reagents were obtained from the equipment manufacturer.

Urine samples were collected overnight from fasted rats individually housed in metabolism cages (Lab Products, Inc., Maywood, NJ). After volume was measured, the following urinalysis variables were measured with a Hitachi 704® chemistry analyzer: urine glucose, total protein, alkaline phosphatase, and aspartate aminotransferase. Specific gravity was determined with an American Optical Refractometer/Total Solids Meter (American Optical, Buffalo, NY).

Sperm Morphology and Vaginal Cytology in Rats and Mice

Vaginal cytology and sperm morphology evaluations were performed on base-study rats and mice (10 animals per sex) from the 0, 38, 113, and 378 ppm exposure groups at the 14-week interim evaluations and at the end of the 26-week studies. The parameters that were evaluated are listed in Table 4. Methods were those described by Morrissey *et al.* (1988). Briefly, for the 12 days prior to interim or terminal sacrifice, the vaginal vaults of 10 females of each species per dose group were lavaged and the aspirated lavage fluid and cells were stained with toluidine blue. Relative numbers of leukocytes, nucleated epithelial cells, and large squamous epithelial cells were determined and used to ascertain estrous cycle stage (*i.e.*, diestrus, proestrus, estrus, and metestrus).

Sperm motility was evaluated at necropsy in the following manner. The left epididymis was isolated and weighed. The tail of the epididymis (cauda epididymis) was then removed from the epididymal body (corpus epididymis) and weighed. Test yolk (rats) or Tyrode's buffer (mice) was applied to slides and a small incision was made at the distal border of the cauda epididymis. The sperm effluxing from the incision were dispersed in the buffer on the slides, and the numbers of motile and nonmotile spermatozoa were counted for five fields per slide.

Following completion of sperm motility estimates, each left cauda epididymis was placed in buffered saline solution (0.9%). Cauda epididymides were gently minced and the tissue was incubated in the saline solution and then heat fixed at 65° C. Sperm density was then determined microscopically with the aid of a hemacytometer. To quantify spermatogenesis, testicular spermatid head count was determined by removing the tunica albuginea and homogenizing the left testis in phosphate-buffered saline containing 10% dimethyl sulfoxide. Homogenization-resistant spermatid nuclei were counted with a hemacytometer.

Neurobehavioral Studies

Neurobehavioral evaluations were performed on rats and mice designated for this purpose. Ten animals per sex received 0, 11, 38, 113, or 378 ppm for 26 weeks. Tests were performed on animals 1 to 3 days before the first day of dosing and during Weeks 6, 12, 18, and 24. The tests that were performed are listed in Table 4.

Motor activity (horizontal and rearing movement) was measured with a Figure 8 Photobeam Activity System (San Diego Instruments, San Diego, CA) over five continuous 3-minute intervals. Startle responsiveness was measured using an SR-LAB Startle Response System (San Diego Instruments) with four chambers equipped with a sound generation system and tactile (15 to 20 psi air-puff) stimuli relay. Eighty trials were conducted, with 8-second intervals between trials. Twenty startle trials with the tactile stimulus (20 msec per trial) were followed by 40 trials in which a pre-pulse of 80 to 90 db(A) white noise preceded the tactile stimulus by 100 msec; the final 20 trials were tactile stimulus trials. Startle response for each trial, including data for initial reactivity, habituation, and pre-pulse inhibition of the startle response, was recorded after each tactile stimulus was turned off.

Forelimb and hindlimb grip strength were measured using a method similar to that described by Meyer *et al.* (1979). Each animal was allowed to grip a triangular ring with its forepaws and was gently pulled back along a platform until its grip was broken. While the backward motion continued, the animal was allowed to grasp a T-shaped bar with its hindpaws, then forced to release the bar by continued pulling. The maximum strain required to break the forelimb and hindlimb grip was recorded with Chatillon push-pull strain gauges (Kew Gardens, NY). Five trials were administered, with less than 1 minute between trials, so that the degree of habituation or fatigue could be observed.

Hindlimb footsplay was tested with a modification of the method described by Edwards and Parker (1977). Animals with inked hind feet were held horizontally at a height of 32 cm (rats) or 22 cm (mice) and released; the distance between the outer digits of the two hind feet was measured for each of three trials.

Thermal sensitivity was measured by determining the time for animals to respond to a 55° C heat stimulus on a Model 550 Analgesia Meter (Omnitech Electronics, Inc., Columbus, OH). The response was characteristically a vigorous licking of the hindpaws; animals failing to respond within 30 seconds were removed immediately and assigned a score of 30 seconds.

TABLE 4 Experimental Design and Materials and Methods in the 26-Week Drinking Water Studies of a Chemical Mixture of 25 Groundwater Contaminants

EXPERIMENTAL DESIGN	
Study Laboratory	Battelle Columbus Laboratories (Columbus, OH)
Size of Study Groups	Base Studies: 20 males and 20 females per species per exposure group Clinical Pathology Study: 10 male and 10 female rats per exposure group Neurobehavioral Studies: 10 males and 10 females per species per exposure group
Route of Administration	Drinking water
Doses/Duration of Dosing	Rats and mice: 0, 11, 38, 113, or 378 ppm daily for 14 or 26 weeks
Date of First Dose	Rats: 3 October 1988 (males), 4 October 1988 (females) Mice: 26 September 1988 (males), 27 September 1988 (females)
Date of Last Dose	Base Studies: 14-Week interim: Rats: 5 January 1989 (males), 6 January 1989 (females) Mice: 29 December 1988 (males), 30 December 1988 (females) Study termination: Rats: 3 April 1989 (males), 4 April 1989 (females) Mice: 27 March 1989 (males), 28 March 1989 (females) Neurobehavioral Studies: Rats: 30 March 1989 Mice: 10 March 1989
Necropsy Dates	14-Week interim: Rats: 5 January 1989 (males), 6 January 1989 (females) Mice: 29 December 1988 (males), 30 December 1988 (females) Study termination: Rats: 3 April 1989 (males), 4 April 1989 (females) Mice: 27 March 1989 (males), 28 March 1989 (females)
Type and Frequency of Observation	Animals were observed twice daily and were weighed at the beginning of the studies, weekly thereafter, and at necropsy. Clinical observations were recorded weekly. Water consumption by cage was measured weekly.
Necropsy and Histologic Examinations	Complete necropsies were performed on all animals in the base studies. Histopathologic evaluations were performed on all animals in the control and 378 ppm groups and on all animals that died early. The following tissues were examined: adrenal glands, brain (three sections), clitoral glands, esophagus, eyes (if grossly abnormal), femur and marrow, gallbladder (mice only), gross lesions and tissue masses, heart, kidneys, large intestine (cecum, colon, rectum), liver, lungs, lymph nodes (mandibular and mesenteric), mammary gland, nasal cavity and turbinates (three sections), ovaries, pancreas, parathyroid glands, pharynx (if grossly abnormal), pituitary gland, preputial glands, prostate gland, salivary gland, seminal vesicle, small intestine (duodenum, jejunum, ileum), spinal cord/sciatic nerve (if neurological signs were present), spleen, stomach (forestomach and glandular stomach), testes (with epididymis), thigh muscle, thymus, thyroid gland, trachea, urinary bladder, uterus, and vagina (females in vaginal cytology studies only). The adrenal glands, liver, lungs, mesenteric lymph nodes, and spleen of rats and gross lesions in rats and mice in the lower dose groups were examined.

TABLE 4 Experimental Design and Materials and Methods in the 26-Week Drinking Water Studies of a Chemical Mixture of 25 Groundwater Contaminants (continued)

Supplemental Evaluations	Clinical Pathology Studies: Blood for hematology and clinical chemistry evaluations was collected from the retroorbital sinus of rats designated for the clinical pathology study and mice designated for the base study. For rats, blood for hematology evaluations was collected at the end of Weeks 14 and 26; blood for clinical chemistry evaluations was collected during Weeks 1, 3, 7, 14, and 26. Urine samples were collected from rats overnight during Weeks 1, 3, 7, 14, and 26. For mice, hematology and clinical chemistry samples were collected from base-study animals evaluated at 14 weeks or 26 weeks. Hematology parameters included hematocrit (Hct), hemoglobin (Hgb) concentration, erythrocyte (RBC) count, reticulocyte count, nucleated erythrocyte count, mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), platelet count, and leukocyte (WBC) count and differentials. Clinical chemistry parameters included creatinine, albumin, alanine aminotransferase (ALT), creatine kinase (CK), and sorbitol dehydrogenase (SDH). Urinalysis parameters included glucose, protein, alkaline phosphatase, aspartate aminotransferase (AST), volume, and specific gravity. Sperm Morphology and Vaginal Cytology Evaluations: Sperm morphology and vaginal cytology evaluations were performed on base-study animals at the 14-week interim evaluations and at the end of the 26-week studies. Animals in the 0, 38, 113, and 378 ppm groups were evaluated. Male rats and mice were evaluated for necropsy body and reproductive tissue weights, spermatozoal data, and spermatogenesis. Females were evaluated for necropsy body weight, estrous cycle length, and the percent of cycle spent in the various stages. Neurobehavioral Studies: Rats and mice (10 per sex per group) designated for the neurobehavioral studies were subjected to a battery of neurobehavioral tests prior to the beginning of the studies and during Weeks 6, 12, 18, and 24. The tests included motor activity, startle responsiveness, forelimb and hindlimb grip strength, hindlimb footsplay, and thermal sensitivity. Rats and mice received 0, 11, 38, 113, or 378 ppm.
ANIMALS AND ANIMAL MAINTENANCE	
Strain and Species	F344/N rats B6C3F$_1$ mice
Animal Source	Rats: Simonsen Laboratories (Gilroy, CA) Mice: Taconic Farms (Germantown, NY)
Time Held Before Study	11 days
Age When Study Began	6 weeks
Age When Killed	19 weeks (14-week interim); 32 weeks (26-week termination)
Method of Animal Distribution	Animals were weighed and were randomized using a computer program (Xybion®).
Diet	NIH-07 Open Formula Diet (Zeigler Brothers, Inc., Gardners, PA) in pellet form and deionized, filtered water (City of Columbus) were available *ad libitum*.
Animal Room Environment	Rats were housed five animals per cage and mice were housed individually. The temperature was maintained at 21° to 24° C and relative humidity at 35% to 65%, with at least 10 air changes per hour. Fluorescent light was provided for 12 hours per day.

Statistical Methods

ANALYSIS OF CONTINUOUS VARIABLES

Two approaches were employed to assess the significance of pairwise comparisons between dosed and control groups in the analysis of continuous variables. Organ and body weight data, which are approximately normally distributed, were analyzed with the parametric multiple comparisons procedures of Williams (1971, 1972) or Dunnett (1955). Clinical chemistry, hematology, and sperm morphology data, which typically have skewed distributions, were analyzed with the nonparametric multiple comparisons methods of Shirley (1977) or Dunn (1964). Jonckheere's test (Jonckheere, 1954) was used to assess the significance of dose-response trends and to determine whether a trend-sensitive test (Williams, Shirley) was more appropriate for pairwise comparisons than a test capable of detecting departures from monotonic dose response (Dunnett, Dunn). If the P-value from Jonckheere's test was greater than or equal to 0.10, Dunn's or Dunnett's test was used rather than Shirley's or Williams' test.

The outlier test of Dixon and Massey (1951) was employed to detect extreme values. No value selected by the outlier test was eliminated unless it was at least twice the next largest value or at most half of the next smallest value. The extreme values chosen by the statistical test were subject to approval by NTP personnel. In addition, values indicated by the laboratory report as being inadequate due to technical problems were eliminated from the analysis.

ANALYSIS OF VAGINAL CYTOLOGY DATA

Because the data are proportions (the proportion of the observation period that an animal was in a given estrous state), an arcsine transformation was used to bring the data into closer conformance with normality assumptions. Treatment effects were investigated by applying a multivariate analysis of variance (Morrison, 1976) to the transformed data to test for simultaneous equality of measurements across dose levels.

ANALYSIS OF NEUROBEHAVIORAL DATA

Neurobehavioral data were analyzed by applying an analysis of variance followed by the least significant difference test.

Quality Assurance

The animal studies of the chemical mixture of 25 groundwater contaminants were performed in compliance with U.S. Food and Drug Administration Good Laboratory Practices regulations (21 CFR 58). The Quality Assurance Unit of Battelle Columbus Laboratories performed audits and inspections of protocols, procedures, data, and reports throughout the course of the studies.

RESULTS

26-Week Drinking Water Study in F344/N Rats

No exposure-related deaths occurred in rats during the 26-week study; however, one control female died during Week 8 (Tables 5 and 6). The final mean body weight and mean body weight gain of male rats in the 378 ppm (high-dose) group were notably lower than those of the controls by Week 14 and remained so throughout the study (Figure 1). Water consumption by males and females decreased with increasing dose (Tables 5 and 6). No clinical signs of toxicity in male or female rats were noted.

TABLE 5 Survival, Weight Gain, Water Consumption, and Compound Consumption Data for F344/N Rats at the 14-Week Interim Evaluation in the 26-Week Drinking Water Study of a Chemical Mixture of 25 Groundwater Contaminants

Dose (ppm)	Survival[1]	Mean Body Weight (grams)			Final Weight Relative to Controls[3] (%)	Average Water Consumption[4] (g/day)	Average Dose[4] (mg/kg/day)
		Initial	Final	Change[2]			
MALE							
0	10/10	109	361	252		19.4	
11	10/10	103	344	241	95	18.5	0.93
38	10/10	117	352	236	98	17.5	3.00
113	10/10	104	345	241	96	16.4	8.49
378	10/10	103	322	220	89	14.9	26.59
FEMALE							
0	9/10[5]	93	188	94		15.1	
11	10/10	93	191	98	102	14.6	1.11
38	10/10	93	192	99	102	13.4	3.55
113	10/10	99	194	95	103	12.0	9.49
378	10/10	98	194	96	104	10.8	28.69

[1] Number surviving at 14 weeks/number of animals per dose group.
[2] Mean weight change.
[3] (Dose group mean/control group mean) × 100.
[4] Nominal composite dose of 25 groundwater contaminants. Average of individual consumption values for Weeks 1-14 for all animals in the base, neurobehavioral, and clinical pathology studies; average dose based on Week 13 (male) or Week 14 (female) body weights.
[5] Week of death: 8.

TABLE 6 Survival, Weight Gain, Water Consumption, and Compound Consumption Data for F344/N Rats in the 26-Week Drinking Water Study of a Chemical Mixture of 25 Groundwater Contaminants

Dose (ppm)	Survival[1]	Mean Body Weight (grams) Initial	Final	Change[2]	Final Weight Relative to Controls[3] (%)	Average Water Consumption[4] (g/day)	Average Dose[4] (mg/kg/day)
MALE							
0	10/10	102	421	320		18.7	
11	10/10	100	419	319	99	18.0	0.78
38	10/10	104	408	304	97	17.2	2.54
113	10/10	98	402	304	95	16.1	7.28
378	10/10	103	393	290	93	14.3	22.31
FEMALE							
0	10/10	94	220	126		14.4	
11	10/10	96	219	122	99	13.9	0.97
38	10/10	96	215	119	98	12.9	3.17
113	10/10	95	216	121	98	11.7	8.48
378	10/10	96	214	119	97	10.3	25.80

[1] Number surviving at 26 weeks/number of animals per dose group.
[2] Mean weight change.
[3] (Dose group mean/control group mean) × 100.
[4] Nominal composite dose of 25 groundwater contaminants. Average of individual consumption values for Weeks 1-26 for all animals in the base, neurobehavioral, and clinical pathology studies.

There were a number of changes in absolute organ weights and in organ weights relative to body weight in rats (Table A1). The absolute kidney weight of high-dose females was increased compared to that of the controls, and relative kidney weights were increased in high-dose males and in females in the 113 and 378 ppm groups (Table 7). Absolute and relative liver weights of high-dose females were greater than those of the controls, and relative thymus weights were decreased in high-dose females and in males in the 113 and 378 ppm groups. There were no histopathologic findings in the kidney or thymus that would account for the weight changes in these organs; however, physiologic changes associated with decreases in water consumption by dosed animals may have affected kidney weights.

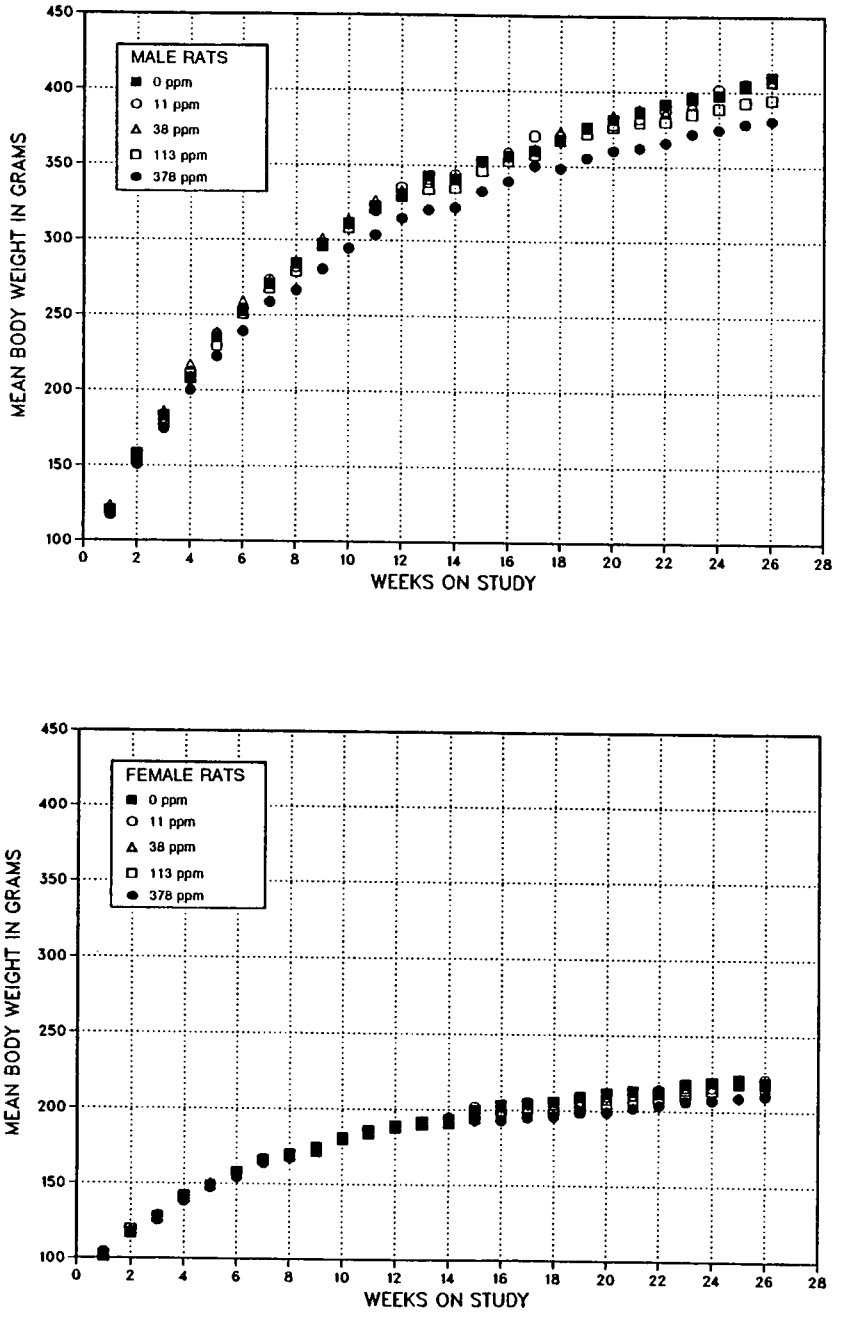

FIGURE 1 Body Weights of F344/N Rats Administered a Chemical Mixture of 25 Groundwater Contaminants in Drinking Water for 26 Weeks

TABLE 7 Selected Organ Weights and Organ-Weight-to-Body-Weight Ratios for F344/N Rats in the 26-Week Drinking Water Study of a Chemical Mixture of 25 Groundwater Contaminants[1]

	Concentration (ppm)				
	0	11	38	113	378
MALE					
n	10	10	10	10	10
Necropsy body wt	428 ± 5	426 ± 5	415 ± 9	406 ± 7*	399 ± 7**
Right kidney					
Absolute	1.416 ± 0.034	1.485 ± 0.033	1.412 ± 0.044	1.411 ± 0.045	1.462 ± 0.029
Relative	3.31 ± 0.08	3.49 ± 0.07	3.40 ± 0.04	3.47 ± 0.07	3.67 ± 0.05**
Liver					
Absolute	16.495 ± 0.386	18.279 ± 0.571	17.054 ± 0.559	16.279 ± 0.568	15.656 ± 0.374
Relative	38.55 ± 0.68	42.93 ± 1.15**	41.05 ± 0.82	40.05 ± 0.96	39.27 ± 0.75
Thymus					
Absolute	0.340 ± 0.017	0.326 ± 0.015	0.307 ± 0.017	0.261 ± 0.016**	0.263 ± 0.011**
Relative	0.79 ± 0.03	0.77 ± 0.04	0.74 ± 0.03	0.65 ± 0.04*	0.66 ± 0.03*
FEMALE					
n	10	10	10	10	10
Necropsy body wt	221 ± 3	220 ± 3	215 ± 3	218 ± 4	211 ± 3*
Right kidney					
Absolute	0.793 ± 0.013	0.809 ± 0.016[2]	0.808 ± 0.016[2]	0.839 ± 0.020	0.938 ± 0.022**
Relative	3.60 ± 0.08	3.69 ± 0.03[2]	3.77 ± 0.06[2]	3.84 ± 0.06*	4.45 ± 0.09**
Liver					
Absolute	7.714 ± 0.144	8.146 ± 0.183	7.924 ± 0.132	7.966 ± 0.215	8.940 ± 0.427**
Relative	34.94 ± 0.52	37.09 ± 0.66	36.87 ± 0.56	36.54 ± 0.93	42.43 ± 1.92**
Thymus					
Absolute	0.236 ± 0.013	0.224 ± 0.013	0.195 ± 0.009	0.217 ± 0.015	0.171 ± 0.007**
Relative	1.07 ± 0.05	1.02 ± 0.05	0.91 ± 0.04	0.99 ± 0.06	0.81 ± 0.03**

[1] Organ weights and body weights are given in grams; relative organ weights (organ-weight-to-body-weight ratios) are given as mg organ weight/g body weight (mean ± standard error).
[2] n=9.
* Significantly different (P≤0.05) from the control group by Williams' test.
** Significantly different (P≤0.01) from the control group by Williams' or Dunnett's test.

Changes in hematology and clinical chemistry parameters occurred at several exposure levels and time points (Appendix B). In general, the data that follow were interpreted as consistent with a treatment-related microcytic anemia and paradoxical polycythemia in male and female rats in the 378 ppm groups at 14 and 26 weeks. At Week 26, there was a hematopoietic response to the anemia.

In male and female rats, changes in hematology parameters at 14 weeks (Day 99 for males and Day 95 for females) and at the end of the study were generally limited to the 113 and 378 ppm groups (Table B1). At Week 14, hematocrit (Hct), hemoglobin (Hgb) concentration, mean cell hemoglobin (MCH), and mean cell volume (MCV) were significantly decreased in high-dose male rats; mean cell hemoglobin concentration (MCHC) and the nucleated erythrocyte (RBC) count were significantly increased. Hct and Hgb concentration were also significantly decreased in males exposed to 113 ppm. By Week 26, Hgb concentration was decreased in all dosed groups of male rats; MCV, MCH, and MCHC were significantly decreased in the high-dose group, and reticulocyte counts were increased and Hct remained decreased in the 113 and 378 ppm groups. In high-dose female rats, decreases in Hct, Hgb concentration, MCH, and MCV were accompanied by increased RBC counts at 14 weeks and at 26 weeks. Additionally, Hgb concentration, MCH, and MCHC were significantly decreased in females in the 113 ppm group at Week 14, and MCH was also decreased in this group at Week 26. At the end of the study, platelet counts were significantly decreased in all dosed groups of female rats. In high-dose females, MCHC was significantly decreased and the reticulocyte count was significantly increased at Week 26.

A review of the blood smears for RBC morphology indicated the presence of schizocytosis (red cell fragments) and keratocytosis (red cells with hornlike projections) in high-dose animals at 14 weeks and at 26 weeks. The occurrence of these RBC abnormalities would be consistent with a process leading to traumatic RBC injury resulting in fragmentation. RBC fragments may have contributed to the increased RBC counts and decreased MCH and MCV.

At Week 14, there were increases in the leukocyte (WBC) and lymphocyte counts of female rats in the 113 and 378 ppm groups. Increases in WBC and lymphocyte counts were also observed in high-dose females at the end of the study. The increases in WBC and lymphocyte counts are consistent with a chronic inflammatory process.

In general, alanine aminotransferase (ALT) activities were significantly increased in male and female rats at the highest exposure levels throughout the study (Table B2). However, only the increase in the high-dose group was statistically significant at the end of the study. Additionally, sorbitol dehydrogenase (SDH) activities were significantly increased in females receiving 113 or 378 ppm, beginning at Week 7. Other changes in female rats

included significant decreases in albumin concentration in the 113 and 378 ppm groups. In high-dose male rats, albumin concentration was decreased at Weeks 7, 14, and 26; SDH activity was significantly increased at Week 26. These results are consistent with hepatocellular injury and decreased hepatic function.

The only significant changes in urinalysis parameters were sporadic increases in urine specific gravity and decreases in urine volume in male and female rats in the 113 and 378 ppm groups at various time points (data not shown). These findings were attributed to poor palatability and decreased water intake. There were no treatment-related increases in the measured urine metabolite concentrations or enzyme activities when the data were normalized for the 16-hour urinary output of these constituents.

No gross lesions that were observed after 14 weeks were attributed to exposure to the chemical mixture, but at the end of the 26-week study, 2 of 10 high-dose females had enlarged pancreatic/mesenteric lymph nodes. Treatment-related microscopic lesions were present in the liver, spleen, and mesenteric lymph nodes of rats examined at 14 weeks or 26 weeks; treatment-related lesions were also present in the adrenal gland of male and female rats at 26 weeks (Table 8).

Histopathologic changes in the liver consisted of multiple foci of inflammation of minimal to marked severity. The severity increased with concentration and with length of exposure. In general, the severity was slightly greater in females. Minimal inflammation occurred in a few control rats and in rats in the lower dose groups. This consisted of a minimal periportal accumulation of lymphocytes; a few macrophages were sometimes present. While the morphology of this minimal hepatic inflammation was similar in the 0, 11, and 38 ppm groups, the incidence was slightly greater in dosed females than in the controls. This minimal inflammation was considered to be within normal limits for the inflammatory cell infiltrates typically seen in the liver of control rats. However, in a single female rat in the 38 ppm group, there was mild inflammation with a morphology that was clearly different from that of the inflammatory lesions in the controls, and this enhanced granulomatous inflammation also occurred with dose-related increases in severity in males and females receiving 113 or 378 ppm. At a mild or higher severity grade, the hepatic inflammation was characterized by the presence of multiple foci of irregularly rounded aggregates of histiocytes (macrophages) with abundant pale eosinophilic cytoplasm. These foci were often surrounded by a prominent infiltrate of mature lymphocytes (Plate 1).

These inflammatory foci were most often in the periportal portion of the hepatic lobules, adjacent to bile ducts (Plate 2), or in the connective tissue stroma around the larger hepatic veins. In rats with markedly severe inflammation, there was often degeneration or necrosis of individual hepatocytes. In addition, the number of mitotic figures in hepatocytes was increased, and mild bile duct or oval cell hyperplasia was also present. This marked severity of inflammation in the liver was seen in 5 of the 10 female rats exposed to 378 ppm for 26 weeks. Electron microscopic examination of the inflammatory foci in the liver demonstrated macrophages with numerous phagolysosomes in the cytoplasm, which contained round, electron-dense droplets. Nonstaining crystalline structures were also present in the cytoplasm and within phagolysosomes of the macrophages (Plate 3).

The incidence of inflammation in the mesenteric lymph nodes was also increased in males and females in the 113 and 378 ppm groups evaluated at 14 or 26 weeks. Inflammation consisted of multiple foci of histiocyte (macrophage) aggregates in the parafollicular portion of the cortex and in the paratrabecular and medullary sinuses. These foci of macrophages were similar to those which often occur in the lymph nodes of control rats but were increased in incidence and severity in dosed rats. Because of the abundant number of mature lymphocytes normally present in the lymph nodes, infiltration of lymphocytes around the foci of histiocytes was not apparent.

The incidence of inflammation in the spleen was slightly increased in dosed rats, particularly females, after 26 weeks. This inflammation consisted of scattered foci of histiocyte (macrophage) aggregates in the red pulp and marginal zone along the periarteriole lymphoid sheaths (Plate 4). In addition, the amount of golden brown hemosiderin pigment present in the fixed histiocytes or macrophages within the red pulp of the spleen was decreased (Table 8). This finding was best demonstrated by the Perl's stain for iron, which showed a marked reduction for iron staining in the splenic red pulp of rats in the 378 ppm groups at 14 weeks and at 26 weeks (Plates 5 and 6).

TABLE 8 Incidence and Severity of Selected Lesions in F344/N Rats in the 26-Week Drinking Water Study of a Chemical Mixture of 25 Groundwater Contaminants[1]

	Concentration (ppm)				
	0	11	38	113	378
WEEK 14					
Male					
Liver					
Inflammation	6/10 (1.0)	0/10	3/10 (1.0)	6/10 (1.3)	10/10 (1.6)
Lymph node (mesenteric)					
Inflammation	1/10 (1.0)	0/10	1/10 (1.0)	5/9 (1.4)	9/10 (1.9)
Spleen					
Inflammation	0/10	0/10	0/10	2/10 (1.0)	1/10 (1.0)
Hemosiderin depletion	0/10	0/10	0/10	6/10 (1.0)	10/10 (1.0)
Female					
Liver					
Inflammation	3/10 (1.0)	4/10 (1.0)	8/10 (1.0)	9/10 (1.7)	10/10 (2.4)
Lymph node (mesenteric)					
Inflammation	0/8	2/10 (1.0)	2/9 (1.5)	2/8 (2.0)	7/10 (1.4)
Spleen					
Inflammation	0/9	0/10	0/10	1/10 (1.0)	2/10 (1.0)
Hemosiderin depletion	0/9	0/10	1/10 (1.0)	6/10 (1.2)	10/10 (2.0)
WEEK 26					
Male					
Liver					
Inflammation	5/10 (1.0)	7/10 (1.0)	10/10 (1.0)	10/10 (1.2)	10/10 (2.3)
Lymph node (mesenteric)					
Inflammation	1/10 (1.0)	2/9 (1.0)	2/9 (1.0)	7/10 (2.0)	8/10 (2.4)
Spleen					
Inflammation	0/10	0/10	0/10	0/10	2/10 (1.5)
Hemosiderin depletion	0/10	0/10	1/10 (1.0)	2/10 (1.0)	10/10 (1.0)
Adrenal cortex					
Inflammation	0/10	0/10	0/10	3/10 (1.3)	5/10 (1.0)
Female					
Liver					
Inflammation	4/10 (1.0)	8/10 (1.0)	9/10 (1.1)	10/10 (1.9)	10/10 (3.5)
Lymph node (mesenteric)					
Inflammation	3/10 (1.7)	1/8 (1.0)	0/9	7/9 (1.1)	8/10 (1.8)
Spleen					
Inflammation	1/10 (1.0)	0/10	0/10	2/9 (1.0)	8/10 (1.4)
Hemosiderin depletion	0/10	0/10	1/10 (1.0)	5/9 (1.0)	10/10 (1.5)
Adrenal cortex					
Inflammation	0/10	1/10 (1.0)	1/10 (1.0)	7/10 (1.3)	4/10 (1.0)

[1] Incidences are given as number of animals with lesions/number of animals examined microscopically. Average severity (in parentheses) is based on the number of animals with lesions: 1=minimal, 2=mild, 3=moderate, and 4=marked.

A number of special histologic stains and electron microscopy were used to better characterize the inflammatory lesions in the liver, spleen, and lymph nodes. Giemsa, periodic acid-Schiff (PAS) stains with and without diastase pretreatment, and acid-fast stains did not indicate the presence of fungal or bacterial organisms within the histiocytic foci. There was minimal but variable PAS-positive staining granules in histiocytic foci, which is consistent for lipofuscin. Perl's stain for iron demonstrated an occasional iron-positive granule in the cytoplasm of macrophages. Masson's trichrome stain demonstrated a minimal amount of collagen (fibrosis) in the inflammatory foci in the liver.

In male and female rats evaluated at 26 weeks, small foci or multifocal accumulations of histiocytes or histiocytes and lymphocytes were observed in the adrenal cortex, primarily in the zona reticularis but also in the zona fasciculata; these foci of inflammation were similar to but much smaller and less numerous than those seen in the liver (Table 8).

Sperm morphology and vaginal cytology evaluations were performed on base-study rats from the 0, 38, 113, and 378 ppm groups at 14 weeks and at 26 weeks (Appendix C). At the 14-week evaluation, the mean left testis weight of high-dose males was significantly less than that of control males, but the change was not significant when the left testis weight was expressed relative to body weight. There were no other changes in males or females at 14 weeks or 26 weeks.

Continuous Breeding Study in Sprague-Dawley Rats

A continuous breeding study was conducted in Sprague-Dawley rats administered the chemical mixture at concentrations of 0, 38, 189, and 378 ppm in drinking water for 7 days prior to breeding and for 112 days while housed in breeding pairs (Appendix D). Little evidence of significant adverse effects on reproductive competence was observed. The number of live male pups, the number of males per litter, and live pup weights were slightly decreased for breeding pairs in the high-dose group. For the final F_1 litters, the body weights of exposed pups were decreased. The body weights of F_1 rats exposed to 189 or 378 ppm remained lower than those of the controls throughout the study. The mean F_2 pup weight in the high-dose group was 12% lower than that of the controls. Sperm morphology and vaginal cytology parameters were not affected by exposure to the chemical mixture.

26-Week Drinking Water Study in B6C3F$_1$ Mice

All mice survived to the end of the study. The final mean body weights and mean body weight gains of dosed and control groups were similar at 14 weeks and at the end of the study (Tables 9 and 10 and Figure 2). Water consumption by dosed males and females was decreased, particularly for animals in the 378 ppm (high-dose) groups.

There were no significant changes in absolute or relative organ weights of males or females (Table A2). No clinical findings or gross lesions were considered related to consumption of the chemical mixture. No treatment-related microscopic lesions occurred in male or female mice.

TABLE 9 Survival, Weight Gain, Water Consumption, and Compound Consumption Data for B6C3F$_1$ Mice at the 14-Week Interim Evaluation in the 26-Week Drinking Water Study of a Chemical Mixture of 25 Groundwater Contaminants

Dose (ppm)	Survival[1]	Mean Body Weight (grams) Initial	Final	Change[2]	Final Weight Relative to Controls[3] (%)	Average Water Consumption[4] (g/day)	Average Dose[4] (mg/kg/day)
MALE							
0	10/10	24.5	39.5	15.0		5.7	
11	10/10	24.4	39.6	15.2	100	5.2	1.77
38	10/10	24.2	39.7	15.5	101	5.3	6.35
113	10/10	24.7	40.0	15.3	101	5.1	18.21
378	10/10	24.2	39.0	14.8	99	3.5	42.20
FEMALE							
0	10/10	20.1	33.9	13.8		7.0	
11	10/10	20.2	34.6	14.4	102	6.9	2.66
38	10/10	20.2	35.7	15.6	106	6.4	8.56
113	10/10	20.6	35.3	14.7	104	5.4	21.49
378	10/10	20.3	34.7	14.4	103	4.1	56.05

[1] Number surviving at 14 weeks/number of animals per dose group.
[2] Mean weight change.
[3] (Dose group mean/control group mean) × 100.
[4] Nominal composite dose of 25 groundwater contaminants. Average of individual consumption values for Weeks 1-14 for all animals in the base and neurobehavioral studies; average dose based on Week 13 (male) or Week 14 (female) body weights.

TABLE 10 Survival, Weight Gain, Water Consumption, and Compound Consumption Data for B6C3F$_1$ Mice in the 26-Week Drinking Water Study of a Chemical Mixture of 25 Groundwater Contaminants

Dose (ppm)	Survival[1]	Mean Body Weight (grams)			Final Weight Relative to Controls[3] (%)	Average Water Consumption[4] (g/day)	Average Dose[4] (mg/kg/day)
		Initial	Final	Change[2]			
MALE							
0	10/10	24.3	48.9	24.6		5.0	
11	10/10	24.8	49.4	24.6	101	4.8	1.46
38	10/10	24.0	48.3	24.4	99	4.9	5.24
113	10/10	24.5	46.5	22.0	95	4.7	15.26
378	10/10	24.4	46.9	22.5	96	3.4	36.98
FEMALE							
0	10/10	20.1	45.4	25.3		6.3	
11	10/10	20.0	45.7	25.7	101	6.2	2.10
38	10/10	20.6	45.3	24.7	100	5.6	6.68
113	10/10	20.5	47.0	26.5	104	5.0	17.44
378	10/10	20.1	44.7	24.6	99	4.0	49.09

[1] Number surviving at 26 weeks/number of animals per dose group.
[2] Mean weight change.
[3] (Dose group mean/control group mean) × 100.
[4] Nominal composite dose of 25 groundwater contaminants. Average of individual consumption values for Weeks 1-26 for all animals in the base and neurobehavioral studies.

Statistically significant changes in a few hematology parameters occurred sporadically in high-dose male and female mice at 14 weeks and 26 weeks (Table B3). These changes were minor and were not considered to be treatment related. No significant changes occurred in clinical chemistry parameters at 14 weeks or 26 weeks (Table B4).

Sperm morphology and vaginal cytology evaluations were performed on base-study mice from the 0, 38, 113, and 378 ppm groups at 14 weeks and 26 weeks (Appendix C). At the 14-week evaluation, the left epididymal weights of males in the 38 and 378 ppm groups were significantly less than those of control males; however, the decreases were not dose related. There were no other significant changes in males or females at 14 weeks or 26 weeks. More extensive characterizations of the effect of consumption of the chemical mixture on spermatogenesis are presented in Appendix C, page C-7. The results of these studies were largely negative.

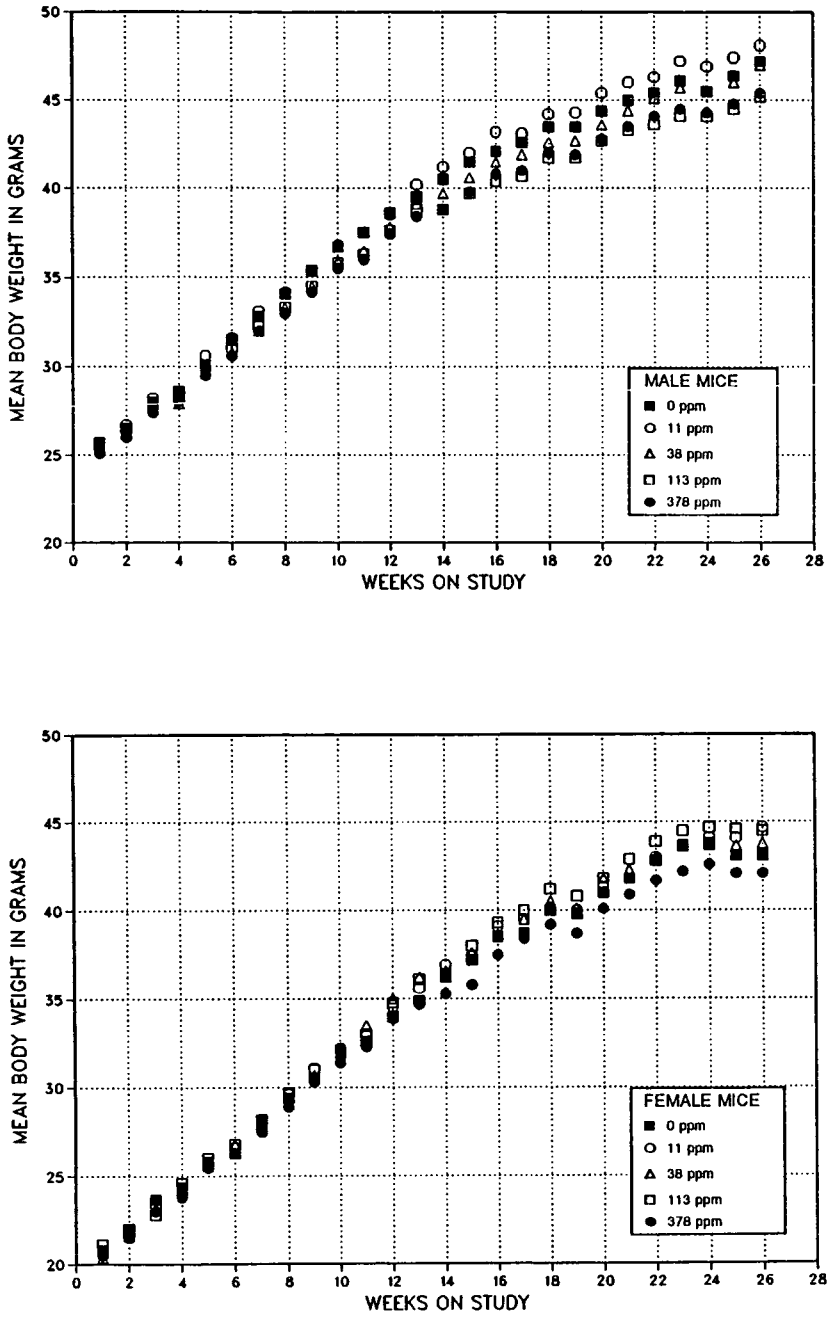

FIGURE 2 Body Weights of B6C3F$_1$ Mice Administered a Chemical Mixture of 25 Groundwater Contaminants in Drinking Water for 26 Weeks

Continuous Breeding Study in CD-1® Swiss Mice

A continuous breeding study was conducted in CD-1® Swiss mice administered the chemical mixture at concentrations of 0, 38, 189, and 378 ppm in drinking water for 7 days prior to breeding and for 98 days while housed in breeding pairs (Appendix D). Water consumption by mice exposed to 189 or 378 ppm was decreased. Overall, few adverse effects were seen in these studies; however, the number of female pups per litter was decreased in the 189 and 378 ppm groups. Body weights, spermatid head counts, and estrous cycle lengths of exposed and control F_0 mice were similar.

For F_2 pups, the pup weight in the high-dose group was 9% lower than that of the control group when pup weights were adjusted for litter size. The number of live F_2 female pups per litter was significantly decreased in the high-dose group. Sperm concentration was decreased in high-dose male mice. Water consumption by exposed mice was decreased. Exposed F_1 females had a greater incidence and severity of liver inflammation (control, 3/29; 378 ppm, 19/19), and the incidence, but not the severity, of nephropathy was increased in exposed F_1 mice (males: control, 4/20, 378 ppm, 9/20; females: control, 5/20, 378 ppm, 17/20).

Genetic Toxicity Studies

The chemical mixture of 25 groundwater contaminants was tested for mutagenicity and prophage induction in bacteria and for cytogenetic effects in bone marrow cells of rats and mice (Appendix E; Shelby *et al.*, 1990). The mixture was negative for induction of mutations in *Salmonella typhimurium* strains TA98 and TA100 with and without S9; no DNA damage, as indicated by the induction of prophage lambda, was observed in *Escherichia coli* with or without Aroclor 1254-induced rat liver S9. In 2-week studies with concentrations of up to 20% (756 ppm) of the stock solution, small but significant increases in sister chromatid exchanges and micronucleated polychromatic erythrocytes (PCEs) occurred in the bone marrow of male mice; micronucleated PCEs were also slightly increased in female mice. Additionally, the mitotic index was increased and average cell generation time was decreased in male and female mice. The percentage of PCEs in bone marrow was decreased in female mice, and the percentage of PCEs in peripheral blood was increased in males and females. The only effects observed in rats were increased PCE frequencies in the peripheral blood of males and in the bone marrow of males and females. The genetic toxicity studies are summarized in Appendix E.

Neurobehavioral Studies

Neurobehavioral effects of the chemical mixture were evaluated by testing the forelimb and hindlimb grip strength, hindlimb footsplay, total horizontal activity, pawlick latency, and startle response in rats and mice and total vertical activity in rats (Appendix G). Very few changes in these parameters were observed. Pawlick latency was significantly lengthened for high-dose female mice at 24 weeks. During Week 6, the startle response profiles for female mice were significantly decreased in the 113 and 378 ppm groups; these parameters had returned to normal values by Week 12. Other statistically significant changes were random or were not dose related.

Hematopoietic and Immune System Toxicity Studies

Residual damage to the hematopoietic system and alterations in hematopoietic responses in female B6C3F$_1$ mice exposed to the chemical mixture of 25 groundwater contaminants in drinking water were evaluated by Hong *et al.* (1991, 1992); these studies are summarized in Appendix H. Mice receiving 5% or 10% (189 or 378 ppm) of the chemical mixture stock in drinking water for 108 days showed suppressed marrow granulocyte-macrophage progenitors (CFU-GMs). The suppression disappeared during a 10-week recovery period. Mice exposed to 200 rads whole-body irradiation during the second and ninth weeks of the recovery period had significantly slowed recoveries of bone marrow progenitors compared to a control group receiving radiation only, with no prior chemical treatment (Hong *et al.*, 1991).

In female B6C3F$_1$ mice exposed to 0%, 1%, 5%, or 10% (0, 11, 189, or 378 ppm) solutions of the chemical mixture for 17 days to 31.5 weeks, there were no significant effects on bone marrow cellularity. However, CFU-GMs and erythroid precursors were significantly suppressed in females exposed to 5% or 10% solutions for 15.5 or 31.5 weeks. Erythrocyte mean cell volume was decreased in mice exposed to a 10% solution for 15.5 weeks or to a 5% or 10% solution for 31.5 weeks.

Two-week and 13-week studies of the immunotoxic effects of the chemical mixture in female B6C3F$_1$ mice were conducted by Germolec *et al.* (1989) and are summarized in Appendix I. No mice developed overt signs of toxicity; however, females in the high-dose group (20% or 756 ppm in the 2-week study and 10% or 378 ppm in the 13-week study) exhibited effects on rapidly proliferating immune function cells, including suppression of

granulocyte-macrophage progenitor cells and antigen-induced antibody-forming cells. No effects on T cell function or T and B cell numbers were observed in any exposed groups. Resistance to challenge with the infectious agent *Plasmodium yoelii* was decreased in high-dose females; this finding correlated with the immune function changes.

PLATE 1

Liver from a female rat administered 378 ppm chemical mixture in drinking water for 13 weeks contains multiple subcapsular foci of macrophages (arrows) surrounded by a dense infiltrate of lymphocytes. H&E, 160×.

PLATE 2

Higher magnification of liver from a female rat administered 378 ppm chemical mixture in drinking water for 13 weeks shows a single focus of macrophages (arrow) with pale-staining cytoplasm adjacent to a tangential section of bile duct (D). Note the prominent lymphocytic infiltrate. H&E, 400×.

PLATE 3

Electron micrograph of macrophage from a focus of inflammation within the liver from a female rat administered 378 ppm chemical mixture in drinking water for 26 weeks. Cytoplasm of macrophage contains multiple, round, electron-dense granules within larger, paler-staining phagolysosomes. Note the presence of nonstained, needle- or rod-shaped crystalline material within the cytoplasm of macrophage as well as phagolysosomes (arrows). TEM, 6000×.

PLATE 4

Spleen from a female rat administered 378 ppm chemical mixture in drinking water for 26 weeks. Note the pale-staining foci of macrophages (arrows) in the marginal zone and red pulp of the spleen. H&E, 160×.

PLATE 5

Spleen from a control female rat demonstrates the typically abundant black-staining, iron-positive aggregates of hemosiderin pigment granules within macrophages of the red pulp and marginal zones of the spleen. Splenic capsule (C) is at top of photomicrograph and a periarteriole lymphoid sheath (S) is at lower right. Compare with spleen from treated rat in Plate 6. Perl's iron, 160×.

PLATE 6

Spleen from a female rat administered 378 ppm chemical mixture in drinking water for 26 weeks. Note the marked reduction in the amount of hemosiderin pigment granules compared to that seen in the control in Plate 5. Capsule (C) is at top of photomicrograph and a periarteriole lymphoid sheath is at the lower right. Perl's iron, 160×.

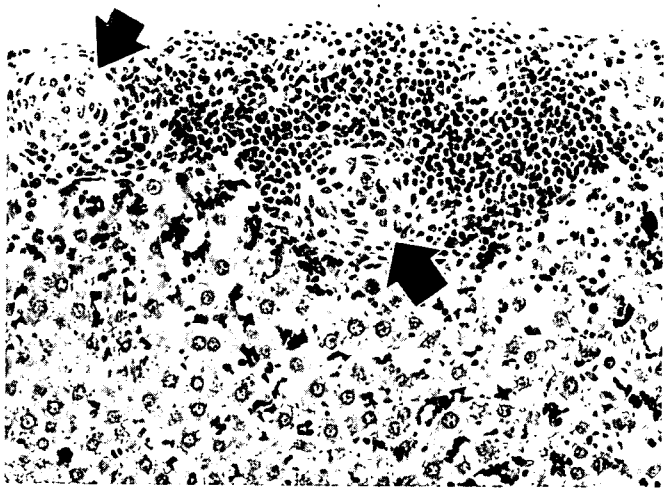

PLATE 1

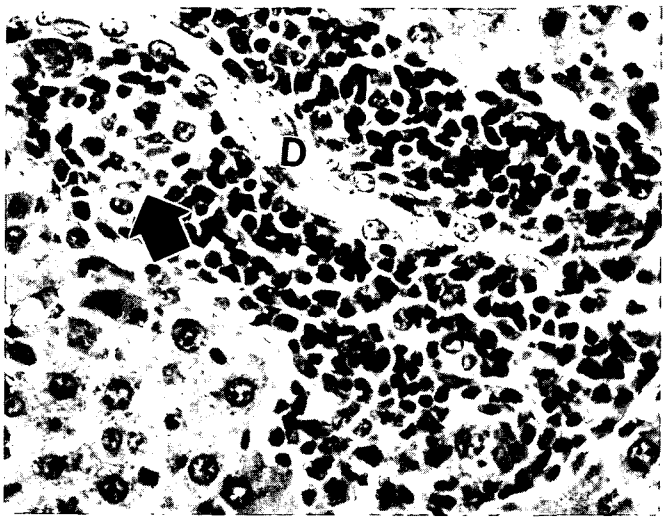

PLATE 2

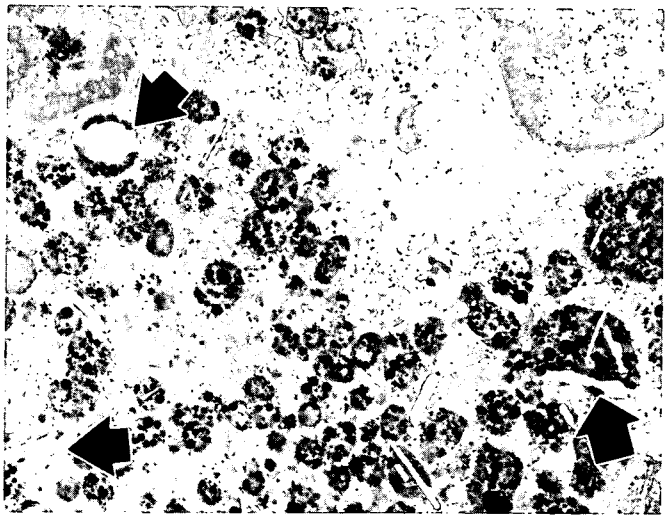

PLATE 3

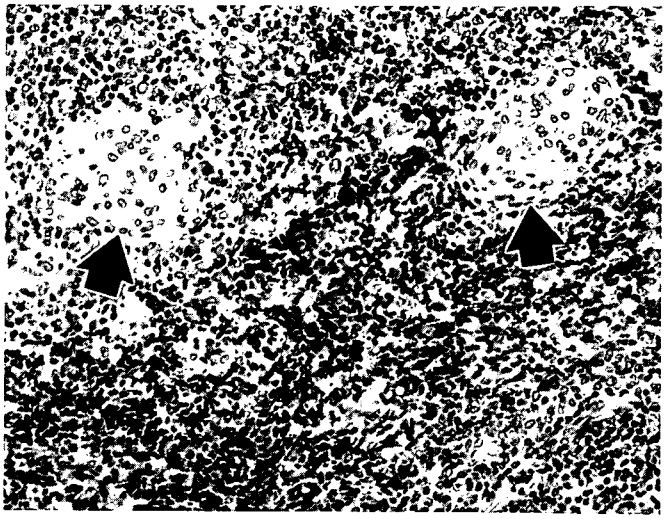

PLATE 4

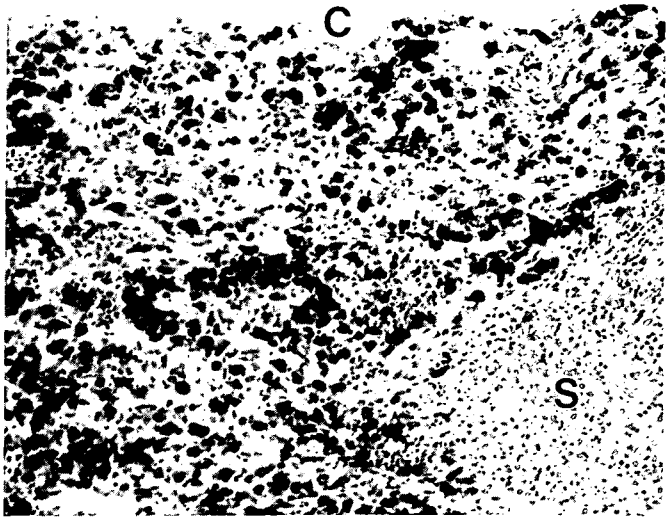

PLATE 5

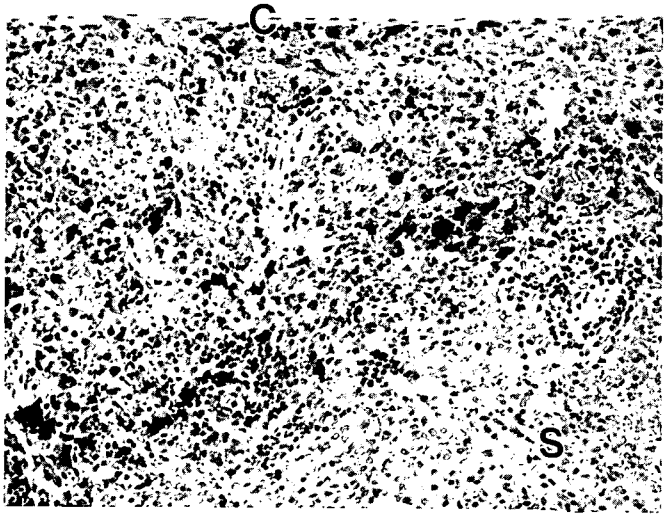

PLATE 6

DISCUSSION

In preliminary animal studies, the highest concentration of the chemical mixture of 25 groundwater contaminants used was 5- or 10-fold more concentrated than the most concentrated mixture ultimately used in the studies in this report. This mixture, with a high concentration of about 3780 ppm, was unpalatable. The concentrations of most of the components of this concentrated mixture were at approximately 90% of their saturation limits in this particular matrix. The concentrations of the 25 chemical components in the dilutions used in these studies (0.3% or 11 ppm, 1% or 38 ppm, 3% or 113 ppm, and 10% or 378 ppm) are compared with the average United States Environmental Protection Agency survey levels in Table 1. With the exception of three metals which are present at more than 10-fold the respective survey levels (arsenic, cadmium, and nickel), the components are quite close to or somewhat below the levels measured in the environment and thus represent a reasonable simulation of a "worst-case" encounter with polluted groundwater. For comparison purposes, examples of polluted groundwater include reports of trichloroethylene at a concentration of 2.8 ppm in potable water in the Silicon Valley area of California (Ames *et al.*, 1987) and carbon tetrachloride at concentrations of 0.061 to 18.7 ppm in wells in Hardeman County, Tennessee (Meyer, 1983). The highest concentrations of these compounds in the chemical mixture used in these studies were 6.5 ppm for trichloroethylene and 0.4 ppm for carbon tetrachloride.

In the 26-week studies that are the primary focus of this report, no mortality due to administration of the chemical mixture occurred, and there were only minor decreases in body weight gain. Although several of the metal components of the chemical mixture are recognized neurotoxins, there was no evidence of the development of neurologic or neurobehavioral effects. Many of the solvents, including phenol, chloroform, and carbon tetrachloride, are known to be central nervous system depressants at exposure levels higher than those used in these studies.

In rats, consumption of the chemical mixture resulted in a dose-related occurrence of multiple foci of inflammation consisting of aggregates of macrophages within the liver, lymph nodes, spleen, and adrenal glands. The lesions were generally more severe in females and had the morphologic features of granulomatous inflammation. A similar

lesion was observed in the liver of female CD-1® Swiss mice in the 378 ppm group in the F_1 generation in the continuous breeding studies.

While there is considerable variation in the morphologic appearance and causes of granulomatous inflammation, it is generally a cellular infiltrate consisting predominantly of macrophages with lymphocytes and/or plasma cells. Multinucleated (foreign body or Langhans' type) giant cells, neutrophils, and sometimes eosinophils and fibrosis are variable components of granulomatous inflammation which were not present in the foci of inflammation seen in these studies. In addition to numerous infectious agents (*Mycobacterium* sp., fungal organisms, etc.), inert particles, lipids, and complex polysaccharides may cause a granulomatous inflammatory response (Adams, 1976; Williams and Jones Williams, 1983). A number of xenobiotics have been reported to produce hepatic granulomas in humans (McMaster and Hennigar, 1981) and laboratory animals (Cabral and Galendo, 1990; Mesfin *et al.*, 1992). Granulomatous inflammation has also been produced in the liver of rats as the result of hepatocellular necrosis following ligation of a hepatic vein (Weinbren *et al.*, 1981). An animal model for chronic granulomatous hepatitis has been developed for rats by giving a single intravenous injection of porcine intestinal alkaline phosphatase (Harms *et al.*, 1992). In this procedure, acute inflammation and necrosis are followed in 2 weeks by persistent granulomatous foci that are morphologically identical to those induced with the chemical mixture, as described in this report.

In the present study, the granulomatous inflammation seen in rats in the 113 and 378 ppm groups was clearly related to administration of the chemical mixture; this lesion was also present in one female rat in the 38 ppm group. The elevated leukocyte and lymphocyte counts were consistent with the inflammation seen histologically. The tissue sites where the inflammation occurred in this study were the same as those reported for rats administered the insecticide fenvalerate (Okuno *et al.*, 1986a). However, there was a prominent lymphoid component in almost all of the inflammatory foci in the liver of rats administered the chemical mixture. This is in contrast to the granulomas seen with fenvalerate in rats and mice (Parker *et al.*, 1983; Okuno *et al.*, 1986a,b) or with drug-induced hepatic granulomas in monkeys (Mesfin *et al.*, 1992), where lymphocytes were generally not a component of the inflammatory lesion. A lymphocytic and/or plasmacytic component has been reported as a part of drug-induced granulomas in humans (McMaster and Hennigar, 1981). In male and female rats, administration of a

chlorinated paraffin mixture by gavage resulted in similar granulomatous lesions in the liver and the mesenteric and pancreatic lymph nodes after 2 years. These inflammatory lesions were present in the liver of female rats by 13 weeks; by 6 months, the lesions were present in males, but were less severe than the lesions in females (NTP, 1986; Bucher *et al.*, 1987). Thus, the results of the chlorinated paraffin mixture study are similar to those of the chemical mixture study in that the lesions in rats were slightly more prominent in females.

There were no consistent morphologic features of the macrophages, based upon results of a number of special histologic stains. Ultrastructural features included the presence of needle- or rod-shaped crystalline material in the cytoplasm and phagolysosomes identical to those seen in the hepatic granulomas induced in mice with fenvalerate (Okuno *et al.*, 1986a,b). The cause of these granulomas in mice was demonstrated to be a cholesterol ester derived from an isomer of fenvalerate (Kaneko *et al.*, 1986; Okuno *et al.*, 1986a). Twelve months after treatment was stopped, there was partial resolution of fenvalerate-induced granulomas in mice (Okuno *et al.*, 1986b). The authors speculated that the granulomatous response more closely resembled a foreign-body reaction than a response to an immunologic stimulus.

Certain components in the chemical mixture, such as cadmium (Faeder *et al.*, 1977) and carbon tetrachloride (Popp and Cattley, 1991), are hepatotoxins when administered individually at higher concentrations than were used in the mixture, and preexposure of rats to the chemical mixture resulted in an enhancement of the hepatotoxicity of carbon tetrachloride (Simmons *et al.*, 1989; Yang *et al.*, 1989a). However, there have been no reports of granulomatous lesions resulting from administration of these chemicals to rats or mice. Thus, the granulomatous lesions appear to be a unique response to the mixture *per se*.

The other histologic finding of interest in rats was an apparent iron (hemosiderin) depletion in the spleen. This finding, coupled with evidence of a microcytic, or iron-deficiency, anemia, suggests development of a chronic iron-deficiency state.

The reason for the apparent iron deficiency in rats is not known, but several of the component chemicals of the mixture interfere with iron absorption from the gastrointestinal tract. In particular, cadmium has been shown to inhibit the mucosal

uptake of duodenally infused ^{59}Fe in mice when administered at concentrations as low as 0.2 to 0.8 mmol (22.5 to 100 ppm) in the drinking water (Hamilton and Valberg, 1974). Lead has also been shown to interact with iron absorption (Flanagan *et al.*, 1979). As indicated, a microcytic anemia was observed in male and female rats in the 378 ppm groups at 14 and 26 weeks. There was a hematopoietic response at Week 26, but the anemia was nonresponsive at Week 14. An unusual finding was the elevated erythrocyte (RBC) counts in these affected animals. Usually, microcytic anemias are associated with an ineffective erythropoiesis, and decreases in RBC counts are expected. The presence of schizocytes and keratocytes indicates a process causing red cell fragmentation (Jain, 1986). Schizocytes are irregular red cell fragments resulting from traumatic red cell injury in the circulation. Keratocytes are irregular red cells with one or more hornlike projections. They are believed to result from rupture of cellular vacuoles and are often seen in conditions associated with schizocyte formation. Schizocytes occur in disseminated intravascular coagulation, microangiopathic hemolytic anemia, glomerulonephritis, burns, congestive heart failure, myelofibrosis, and neoplasia (Jain, 1986). It is quite likely that red cell fragmentation contributed to the elevated RBC counts and decreased mean cell volume. However, there was no evidence indicating the cause of red cell trauma. Additionally, there was histologic evidence, discussed above, indicating iron depletion in the tissues. Iron deficiency can result in a microcytic anemia, but iron deficiency anemia would not account for the increased RBC count. There was evidence (increased urine specific gravity and decreased urine volume) that the high-dose animals found the dosed water poorly palatable and had a decreased water intake. If these animals became dehydrated, the decrease in plasma volume could have contributed to the polycythemia and masked an actual decrease in red cell numbers.

Several of the chemicals in the mixture, most notably lead, are known to affect hematopoiesis. The anemia of chronic lead poisoning is usually mild to moderate and results from impaired heme production and shortened RBC survival. The anemia is usually normocytic-normochromic, but with heme production impaired, erythropoiesis is ineffective, so there is a tendency for the anemia to be slightly microcytic-hypochromic. A mild reticulocyte response may also occur. In other species, red cells with basophilic stippling are found and are used as a marker of lead poisoning. No basophilic stippling was noted in a review of blood smears from rats in the current study. The lead in the chemical mixture could have been associated with the microcytic anemia noted but would not have been expected to produce the red cell fragmentation.

No adverse effects of the chemical mixture were observed in the screening assays for reproductive system parameters in the 26-week studies in rats or mice, although there was evidence of reduced sperm concentration in CD-1® Swiss mice of the F_1 generation in the continuous breeding studies. In separate studies, Chapin *et al.* (1989) observed no effects of the chemical mixture on spermatogenesis, and overall, it appeared that consumption of the chemical mixture did not adversely affect fertility in the continuous breeding studies, even in the presence of maternal toxicity. There were, however, some unusual findings in the continuous breeding studies relating to reduced numbers of male rats and female mice born in particular generations, but the significance of these findings, if any, is not known. Although many of the components of the chemical mixture are known reproductive toxicants at higher doses than those used in these studies, there was little evidence of consistent adverse effects on reproduction, and there was no evidence of a significant synergistic effect of the various chemicals.

Although there was no clear evidence of toxicity in mice in the 26-week study, specific studies of immunotoxicity (Appendix I; Germolec *et al.*, 1989), myelotoxicity (Appendix H; Hong *et al.*, 1991, 1992), and genetic damage (Appendix E; Shelby *et al.*, 1990) showed adverse effects in $B6C3F_1$ mice exposed for 2 weeks to 31.5 weeks to concentrations similar to those used in the 26-week studies. Proliferating cells in the bone marrow and thymus appeared to be targets of the chemical mixture. As was the case with the liver in rats, a number of the components of the mixture (Aroclor 1260, benzene, phenol, toluene, xylene, and the metal components) are known to affect these organs at doses higher than those used in these studies (Germolec *et al.*, 1989; Hong *et al.*, 1991). The fact that studies with the typical toxicologic endpoints revealed no significant evidence of toxicity in mice suggests that conventional approaches are inadequate to fully assess more subtle toxicities resulting from long-term, low-level exposures to environmental contaminants (Phillips and Silbergeld, 1985; Yang *et al.*, 1989a,b; Yang, 1992). Treated animals may appear clinically normal, but a subclinical state providing evidence of enhancement or potentiation of toxicity resulting from exposure to novel chemical, physical, or biological agents may exist. Although studies of these specific and more sensitive endpoints were not performed with rats, a reduction in thymus weights was noted in exposed animals in the 26-week studies. This response was stronger in rats than in mice, suggesting possible deficits in immunocompetence.

In summary, rats receiving drinking water containing a mixture of 25 common groundwater contaminants at levels of potential environmental relevance developed inflammatory lesions in the liver, spleen, lymph nodes, and adrenal gland, as well as evidence of an iron-deficiency anemia. The inflammatory lesions could not be predicted based on known toxic effects of the individual components of the chemical mixture. Mice exposed to similar concentrations of the chemical mixture did not show adverse effects in a standard toxicity study but developed deficits in bone marrow function, evidence of genetic damage, hepatic inflammation, and immunosuppression in other studies that generally included exposures to higher concentrations or exposures of longer duration. The no-observed-adverse-effect level for histologic injury (granulomatous inflammation of the liver) was 11 ppm in rats; however, no clear evidence of histologic injury was seen in mice exposed to concentrations of the chemical mixture as high as 378 ppm in a standard 26-week study.

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APPENDIX A

Organ Weights and Organ-Weight-to-Body-Weight Ratios

TABLE A1 Organ Weights and Organ-Weight-to-Body-Weight Ratios for F344/N Rats in the 26-Week Drinking Water Study of a Chemical Mixture of 25 Groundwater Contaminants[1]

	0 ppm	11 ppm	38 ppm	113 ppm	378 ppm
MALE					
n	10	10	10	10	10
Necropsy body wt	428 ± 5	426 ± 5	415 ± 9	406 ± 7*	399 ± 7**
Brain					
Absolute	1.991 ± 0.018	2.009 ± 0.020	1.972 ± 0.016	1.969 ± 0.022	1.988 ± 0.016
Relative	4.66 ± 0.06	4.73 ± 0.07	4.77 ± 0.10	4.86 ± 0.07	4.99 ± 0.06**
Heart					
Absolute	1.153 ± 0.019	1.134 ± 0.024	1.110 ± 0.030	1.100 ± 0.024	1.093 ± 0.024
Relative	2.70 ± 0.04	2.66 ± 0.05	2.67 ± 0.04	2.71 ± 0.04	2.74 ± 0.04
Right kidney					
Absolute	1.416 ± 0.034	1.485 ± 0.033	1.412 ± 0.044	1.411 ± 0.045	1.462 ± 0.029
Relative	3.31 ± 0.08	3.49 ± 0.07	3.40 ± 0.04	3.47 ± 0.07	3.67 ± 0.05**
Liver					
Absolute	16.495 ± 0.386	18.279 ± 0.571	17.054 ± 0.559	16.279 ± 0.568	15.656 ± 0.374
Relative	38.55 ± 0.68	42.93 ± 1.15**	41.05 ± 0.82	40.05 ± 0.96	39.27 ± 0.75
Lungs					
Absolute	2.192 ± 0.044	2.063 ± 0.054	2.062 ± 0.074	1.914 ± 0.103*	2.035 ± 0.058*
Relative	5.13 ± 0.11	4.85 ± 0.13	4.97 ± 0.15	4.72 ± 0.24	5.10 ± 0.13
Right testis					
Absolute	1.468 ± 0.039	1.553 ± 0.020	1.464 ± 0.028	1.401 ± 0.035	1.467 ± 0.029[2]
Relative	3.44 ± 0.10	3.65 ± 0.04	3.53 ± 0.04	3.45 ± 0.05	3.65 ± 0.07[2]
Thymus					
Absolute	0.340 ± 0.017	0.326 ± 0.015	0.307 ± 0.017	0.261 ± 0.016**	0.263 ± 0.011**
Relative	0.79 ± 0.03	0.77 ± 0.04	0.74 ± 0.03	0.65 ± 0.04*	0.66 ± 0.03*
FEMALE					
n	10	10	10	10	10
Necropsy body wt	221 ± 3	220 ± 3	215 ± 3	218 ± 4	211 ± 3*
Brain					
Absolute	1.787 ± 0.021	1.770 ± 0.011[2]	1.803 ± 0.019[2]	1.749 ± 0.017	1.771 ± 0.013
Relative	8.10 ± 0.10	8.09 ± 0.12[2]	8.44 ± 0.18[2]	8.03 ± 0.12	8.42 ± 0.13
Heart					
Absolute	0.715 ± 0.020	0.686 ± 0.019	0.691 ± 0.011	0.658 ± 0.015	0.671 ± 0.018
Relative	3.24 ± 0.07	3.12 ± 0.07	3.22 ± 0.05	3.02 ± 0.05	3.19 ± 0.09
Right kidney					
Absolute	0.793 ± 0.013	0.809 ± 0.016[2]	0.808 ± 0.016[2]	0.839 ± 0.020	0.938 ± 0.022**
Relative	3.60 ± 0.08	3.69 ± 0.03[2]	3.77 ± 0.06[2]	3.84 ± 0.06*	4.45 ± 0.09**
Liver					
Absolute	7.714 ± 0.144	8.146 ± 0.183	7.924 ± 0.132	7.966 ± 0.215	8.940 ± 0.427**
Relative	34.94 ± 0.52	37.09 ± 0.66	36.87 ± 0.56	36.54 ± 0.93	42.43 ± 1.92**
Lungs					
Absolute	1.343 ± 0.066	1.397 ± 0.030	1.311 ± 0.045	1.247 ± 0.042	1.353 ± 0.046
Relative	6.07 ± 0.26	6.37 ± 0.17	6.10 ± 0.21	5.73 ± 0.21	6.43 ± 0.22
Thymus					
Absolute	0.236 ± 0.013	0.224 ± 0.013	0.195 ± 0.009	0.217 ± 0.015	0.171 ± 0.007**
Relative	1.07 ± 0.05	1.02 ± 0.05	0.91 ± 0.04	0.99 ± 0.06	0.81 ± 0.03**

[1] Organ weights and body weights are given in grams; relative organ weights (organ-weight-to-body-weight ratios) are given as mg organ weight/g body weight (mean ± standard error).

[2] n=9.

* Significantly different (P≤0.05) from the control group by Williams' test.

** Significantly different (P≤0.01) from the control group by Williams' or Dunnett's test.

TABLE A2 Organ Weights and Organ-Weight-to-Body-Weight Ratios for B6C3F$_1$ Mice in the 26-Week Drinking Water Study of a Chemical Mixture of 25 Groundwater Contaminants[1]

	0 ppm	11 ppm	38 ppm	113 ppm	378 ppm
MALE					
n	10	10	10	10	10
Necropsy body wt	49.2 ± 1.0	49.9 ± 1.1	48.7 ± 0.7	47.3 ± 1.5	47.3 ± 1.3
Brain					
Absolute	0.440 ± 0.005	0.447 ± 0.006	0.433 ± 0.005	0.440 ± 0.004	0.440 ± 0.003
Relative	8.97 ± 0.18	8.97 ± 0.17	8.91 ± 0.18	9.40 ± 0.34	9.36 ± 0.27
Heart					
Absolute	0.213 ± 0.011	0.213 ± 0.010	0.192 ± 0.005	0.203 ± 0.007	0.210 ± 0.007
Relative	4.33 ± 0.23	4.28 ± 0.20	3.95 ± 0.13	4.31 ± 0.14	4.43 ± 0.11
Right kidney					
Absolute	0.374 ± 0.021	0.369 ± 0.010	0.380 ± 0.015	0.385 ± 0.021	0.393 ± 0.016
Relative	7.56 ± 0.29	7.40 ± 0.14	7.81 ± 0.32	8.11 ± 0.27	8.28 ± 0.19
Liver					
Absolute	2.486 ± 0.167	2.591 ± 0.114	2.365 ± 0.054	2.407 ± 0.156	2.292 ± 0.131
Relative	50.18 ± 2.40	51.75 ± 1.49	48.56 ± 0.69	50.52 ± 1.77	48.11 ± 1.64
Lungs					
Absolute	0.323 ± 0.018	0.315 ± 0.016	0.307 ± 0.013	0.308 ± 0.011	0.332 ± 0.025
Relative	6.62 ± 0.44	6.34 ± 0.32	6.32 ± 0.30	6.53 ± 0.23	7.06 ± 0.53
Right testis					
Absolute	0.117 ± 0.002	0.119 ± 0.002[2]	0.116 ± 0.002	0.119 ± 0.003	0.120 ± 0.003
Relative	2.38 ± 0.03	2.40 ± 0.06[2]	2.38 ± 0.04	2.54 ± 0.08	2.54 ± 0.07
Thymus					
Absolute	0.079 ± 0.007	0.072 ± 0.008	0.078 ± 0.006	0.073 ± 0.007	0.072 ± 0.006
Relative	1.62 ± 0.15	1.44 ± 0.16	1.60 ± 0.11	1.51 ± 0.13	1.51 ± 0.11
FEMALE					
n	10	10	10	10	10
Necropsy body wt	45.8 ± 1.4	46.4 ± 1.1	45.7 ± 2.1	47.6 ± 1.7	44.7 ± 1.0
Brain					
Absolute	0.456 ± 0.005	0.451 ± 0.003	0.451 ± 0.004	0.457 ± 0.006	0.451 ± 0.006
Relative	10.05 ± 0.33	9.76 ± 0.22	10.06 ± 0.42	9.72 ± 0.41	10.13 ± 0.28
Heart					
Absolute	0.169 ± 0.005	0.164 ± 0.004	0.161 ± 0.006	0.175 ± 0.007	0.175 ± 0.008
Relative	3.72 ± 0.13	3.56 ± 0.14	3.54 ± 0.08	3.71 ± 0.18	3.90 ± 0.16
Right kidney					
Absolute	0.244 ± 0.006	0.245 ± 0.007	0.239 ± 0.007	0.250 ± 0.008	0.247 ± 0.006
Relative	5.36 ± 0.13	5.30 ± 0.18	5.28 ± 0.15	5.25 ± 0.06	5.54 ± 0.16
Liver					
Absolute	1.859 ± 0.060	1.910 ± 0.035	1.869 ± 0.074	1.928 ± 0.070	1.812 ± 0.048
Relative	40.64 ± 0.79	41.22 ± 0.49	41.15 ± 1.01	40.54 ± 0.64	40.51 ± 0.56
Lungs					
Absolute	0.264 ± 0.012	0.286 ± 0.017	0.273 ± 0.019	0.275 ± 0.016	0.291 ± 0.014
Relative	5.80 ± 0.28	6.12 ± 0.26	5.99 ± 0.31	5.79 ± 0.30	6.52 ± 0.34
Thymus					
Absolute	0.069 ± 0.008	0.065 ± 0.008	0.059 ± 0.005	0.071 ± 0.004	0.054 ± 0.005
Relative	1.51 ± 0.17	1.42 ± 0.18	1.30 ± 0.11	1.51 ± 0.11	1.21 ± 0.13

[1] Organ weights and body weights are given in grams; relative organ weights (organ-weight-to-body-weight ratios) are given as mg organ weight/g body weight (mean ± standard error). Differences from the control group for body weights, organ weights, and relative organ weights are not significant by Williams' or Dunnett's test.

[2] n=9.

APPENDIX B

Hematology and Clinical Chemistry Results

TABLE B1 Hematology Data for F344/N Rats in the 26-Week Drinking Water Study of a Chemical Mixture of 25 Groundwater Contaminants[1]

	0 ppm	11 ppm	38 ppm	113 ppm	378 ppm
MALE					
n					
Week 14	8	10	9	9	8
Week 26	9	10	9	10	10
Hematocrit (%)					
Week 14	47.7 ± 0.3	48.0 ± 0.3	47.1 ± 0.3	46.5 ± 0.3*	40.9 ± 0.6**
Week 26	47.5 ± 0.2	46.5 ± 0.4	47.1 ± 0.6	46.5 ± 0.6*	42.7 ± 1.3**
Hemoglobin (g/dL)					
Week 14	15.4 ± 0.1	15.6 ± 0.1	15.2 ± 0.1	15.0 ± 0.1*	13.7 ± 0.2**
Week 26	15.1 ± 0.1	14.7 ± 0.1**	15.0 ± 0.1*	14.7 ± 0.2**	13.3 ± 0.4**
Erythrocytes (10^6/μL)					
Week 14	8.99 ± 0.07	8.99 ± 0.06	8.81 ± 0.04	8.79 ± 0.09	10.11 ± 0.19
Week 26	9.15 ± 0.04	8.82 ± 0.07*	8.99 ± 0.11	8.90 ± 0.12	9.48 ± 0.15
Reticulocytes (10^6/μL)					
Week 14	0.14 ± 0.01	0.17 ± 0.01	0.16 ± 0.01	0.17 ± 0.02	0.16 ± 0.01
Week 26	0.18 ± 0.01	0.18 ± 0.01	0.18 ± 0.01	0.21 ± 0.01*	0.29 ± 0.03**
Nucleated erythrocytes (10^3/μL)					
Week 14	0.02 ± 0.02	0.02 ± 0.01	0.03 ± 0.01	0.05 ± 0.03	0.12 ± 0.04*
Week 26	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.03 ± 0.03
Mean cell volume (fL)					
Week 14	53.0 ± 0.2	53.4 ± 0.2	53.4 ± 0.2	52.9 ± 0.3	40.5 ± 0.9**
Week 26	51.9 ± 0.3	52.7 ± 0.3	52.4 ± 0.2	52.3 ± 0.3	45.3 ± 1.8**
Mean cell hemoglobin (pg)					
Week 14	17.1 ± 0.1	17.3 ± 0.1	17.2 ± 0.1	17.1 ± 0.1	13.6 ± 0.3**
Week 26	16.5 ± 0.0	16.6 ± 0.1	16.6 ± 0.1	16.5 ± 0.1	14.1 ± 0.6**
Mean cell hemoglobin concentration (g/dL)					
Week 14	32.3 ± 0.1	32.4 ± 0.1	32.2 ± 0.2	32.3 ± 0.1	33.4 ± 0.2**
Week 26	31.8 ± 0.2	31.6 ± 0.2	31.8 ± 0.2	31.5 ± 0.2	31.1 ± 0.2*
Platelets (10^3/μL)					
Week 14	564.0 ± 7.0	575.1 ± 14.8	523.1 ± 14.5	537.4 ± 23.9	624.4 ± 16.9
Week 26	616.3 ± 16.3	618.2 ± 9.3	591.8 ± 20.5	588.7 ± 14.8	668.0 ± 36.3
Leukocytes (10^3/μL)					
Week 14	9.80 ± 0.32	9.63 ± 0.29	9.36 ± 0.52	11.06 ± 0.34*	10.96 ± 0.59
Week 26	8.10 ± 0.46	7.26 ± 0.27	7.33 ± 0.55	7.74 ± 0.60	13.74 ± 1.99
Segmented neutrophils (10^3/μL)					
Week 14	1.60 ± 0.17	1.35 ± 0.12	1.45 ± 0.16	1.94 ± 0.30	1.76 ± 0.17
Week 26	1.61 ± 0.21	1.58 ± 0.12	1.40 ± 0.18	1.53 ± 0.24	1.62 ± 0.12
Lymphocytes (10^3/μL)					
Week 14	8.11 ± 0.26	8.12 ± 0.26	7.73 ± 0.42	8.98 ± 0.22*	9.10 ± 0.58
Week 26	6.35 ± 0.39	5.57 ± 0.26	5.81 ± 0.41	6.06 ± 0.57	11.94 ± 2.01
Monocytes (10^3/μL)					
Week 14	0.04 ± 0.02	0.02 ± 0.01	0.06 ± 0.03	0.03 ± 0.02	0.06 ± 0.02
Week 26	0.06 ± 0.02	0.08 ± 0.03	0.06 ± 0.02	0.09 ± 0.02	0.11 ± 0.03
Eosinophils (10^3/μL)					
Week 14	0.05 ± 0.02	0.13 ± 0.03	0.12 ± 0.03	0.11 ± 0.04	0.05 ± 0.03
Week 26	0.08 ± 0.03	0.03 ± 0.01	0.06 ± 0.02	0.07 ± 0.03	0.07 ± 0.03

TABLE B1 Hematology Data for F344/N Rats in the 26-Week Drinking Water Study of a Chemical Mixture of 25 Groundwater Contaminants (continued)

	0 ppm	11 ppm	38 ppm	113 ppm	378 ppm
FEMALE					
n					
Week 14	10	9	10	10	10
Week 26	10	10	10	10	10
Hematocrit (%)					
Week 14	47.9 ± 0.5	47.4 ± 0.4	47.4 ± 0.8	46.1 ± 0.7	44.4 ± 0.7**
Week 26	47.3 ± 0.5	47.2 ± 0.4	47.1 ± 0.5	46.1 ± 0.7	41.7 ± 0.5**
Hemoglobin (g/dL)					
Week 14	15.5 ± 0.1	15.4 ± 0.1	15.4 ± 0.3	14.7 ± 0.2*	14.3 ± 0.2**
Week 26	15.4 ± 0.2	15.5 ± 0.1	15.3 ± 0.2	15.0 ± 0.2	13.0 ± 0.2**
Erythrocytes (10^6/μL)					
Week 14	8.45 ± 0.09	8.35 ± 0.07	8.40 ± 0.14	8.16 ± 0.12	9.03 ± 0.16*
Week 26	8.55 ± 0.09	8.51 ± 0.07	8.50 ± 0.06	8.50 ± 0.11	9.43 ± 0.18**
Reticulocytes (10^6/μL)					
Week 14	0.14 ± 0.01	0.13 ± 0.01	0.14 ± 0.01	0.16 ± 0.01	0.15 ± 0.01
Week 26	0.14 ± 0.01	0.15 ± 0.01	0.13 ± 0.01	0.14 ± 0.01	0.20 ± 0.02**
Nucleated erythrocytes (10^3/μL)					
Week 14	0.03 ± 0.01	0.02 ± 0.01	0.04 ± 0.02	0.00 ± 0.00	0.03 ± 0.02
Week 26	0.00 ± 0.00	0.02 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01
Mean cell volume (fL)					
Week 14	56.7 ± 0.2	56.8 ± 0.2	56.4 ± 0.2	56.7 ± 0.3	49.3 ± 0.6**
Week 26	55.6 ± 0.2	55.4 ± 0.2	55.4 ± 0.3	54.3 ± 0.7	44.3 ± 0.8**
Mean cell hemoglobin (pg)					
Week 14	18.4 ± 0.1	18.4 ± 0.1	18.3 ± 0.1	18.1 ± 0.0**	15.8 ± 0.1**
Week 26	18.1 ± 0.1	18.2 ± 0.1	18.0 ± 0.1	17.6 ± 0.2*	13.8 ± 0.3**
Mean cell hemoglobin concentration (g/dL)					
Week 14	32.4 ± 0.1	32.5 ± 0.2	32.4 ± 0.1	32.0 ± 0.1*	32.1 ± 0.2
Week 26	32.6 ± 0.2	32.8 ± 0.1	32.5 ± 0.2	32.5 ± 0.1	31.2 ± 0.3**
Platelets (10^3/μL)					
Week 14	570.2 ± 26.2	577.7 ± 11.7	631.1 ± 32.4	616.9 ± 54.4	504.3 ± 32.3
Week 26	684.2 ± 42.5	587.9 ± 15.6*	615.6 ± 14.5*	575.7 ± 9.8**	591.5 ± 14.6**
Leukocytes (10^3/μL)					
Week 14	6.50 ± 0.48	6.42 ± 0.29	8.97 ± 1.53	8.73 ± 0.61**	8.66 ± 0.60*
Week 26	6.81 ± 0.58	6.20 ± 0.26	6.79 ± 0.75	8.24 ± 0.80	15.87 ± 1.92**
Segmented neutrophils (10^3/μL)					
Week 14	1.56 ± 0.20	1.30 ± 0.15	2.49 ± 0.81	1.38 ± 0.23	1.87 ± 0.21
Week 26	1.62 ± 0.30	1.02 ± 0.12	1.77 ± 0.34	1.50 ± 0.35	1.79 ± 0.28
Lymphocytes (10^3/μL)					
Week 14	4.85 ± 0.34	5.00 ± 0.25	6.31 ± 0.70	7.25 ± 0.40**	6.70 ± 0.44**
Week 26	5.06 ± 0.46	5.02 ± 0.27	4.93 ± 0.46	6.55 ± 0.57	13.96 ± 1.74**
Monocytes (10^3/μL)					
Week 14	0.02 ± 0.01	0.02 ± 0.02	0.09 ± 0.05	0.01 ± 0.01	0.05 ± 0.02
Week 26	0.07 ± 0.02	0.06 ± 0.03	0.04 ± 0.01	0.09 ± 0.03	0.09 ± 0.03
Eosinophils (10^3/μL)					
Week 14	0.08 ± 0.02	0.11 ± 0.02	0.09 ± 0.03	0.08 ± 0.02	0.05 ± 0.02
Week 26	0.06 ± 0.02	0.10 ± 0.02	0.05 ± 0.01	0.11 ± 0.05	0.03 ± 0.02

[1] Data are given as mean ± standard error.
* Significantly different (P≤0.05) from the control group by Dunn's or Shirley's test.
** Significantly different (P≤0.01) from the control group by Shirley's test.

TABLE B2 Clinical Chemistry Data for F344/N Rats in the 26-Week Drinking Water Study of a Chemical Mixture of 25 Groundwater Contaminants[1]

	0 ppm	11 ppm	38 ppm	113 ppm	378 ppm
MALE					
n	10	10	10	10	10
Creatinine (mg/dL)					
Week 1	0.66 ± 0.02	0.63 ± 0.02	0.67 ± 0.02	0.67 ± 0.02	0.61 ± 0.02
Week 3	0.56 ± 0.02	0.57 ± 0.02	0.59 ± 0.01	0.56 ± 0.02	0.57 ± 0.02
Week 7	0.61 ± 0.01	0.60 ± 0.02	0.60 ± 0.00	0.60 ± 0.00	0.57 ± 0.02*
Week 14	0.65 ± 0.02	0.66 ± 0.02	0.64 ± 0.02	0.68 ± 0.02	0.65 ± 0.02
Week 26	0.70 ± 0.00	0.67 ± 0.02	0.72 ± 0.02[2]	0.69 ± 0.01	0.67 ± 0.02
Albumin (g/dL)					
Week 1	4.1 ± 0.0	4.0 ± 0.0	4.1 ± 0.1	4.1 ± 0.1	4.1 ± 0.0
Week 3	4.2 ± 0.1	4.2 ± 0.0	4.3 ± 0.0	4.1 ± 0.1	4.1 ± 0.0
Week 7	4.7 ± 0.1	4.7 ± 0.1	4.7 ± 0.1	4.7 ± 0.1	4.5 ± 0.1*
Week 14	4.8 ± 0.1	4.8 ± 0.0	4.8 ± 0.0	4.7 ± 0.1	4.6 ± 0.1*
Week 26	4.9 ± 0.1	4.6 ± 0.1*	4.8 ± 0.1[2]	4.7 ± 0.1	4.5 ± 0.1**
Alanine aminotransferase (IU/L)					
Week 1	43 ± 2	42 ± 1	48 ± 1*	45 ± 1	55 ± 1**
Week 3	41 ± 1	38 ± 1	42 ± 1	47 ± 2*	51 ± 1**
Week 7	50 ± 3	48 ± 1	70 ± 9	53 ± 2	63 ± 2**
Week 14	48 ± 2	53 ± 2*	59 ± 3**	57 ± 1**	76 ± 5**
Week 26	70 ± 3	59 ± 4	76 ± 4[2]	81 ± 7	232 ± 52**
Creatine kinase (IU/L)					
Week 1	468 ± 92	549 ± 86	558 ± 76	451 ± 31	476 ± 58
Week 3	1145 ± 360	638 ± 123	872 ± 146	755 ± 110	971 ± 148
Week 7	512 ± 40	401 ± 64	528 ± 73	427 ± 50	560 ± 83
Week 14	389 ± 56	322 ± 35	517 ± 178	458 ± 89	658 ± 165
Week 26	192 ± 51	186 ± 32	218 ± 34[2]	219 ± 31	140 ± 16
Sorbitol dehydrogenase (IU/L)					
Week 1	24 ± 1	24 ± 1	26 ± 1	23 ± 1	24 ± 1
Week 3	21 ± 1	21 ± 1	21 ± 1	19 ± 1	17 ± 1**
Week 7	23 ± 1	20 ± 1	31 ± 3	20 ± 1	23 ± 1
Week 14	20 ± 1	20 ± 1	23 ± 1	23 ± 1	27 ± 4
Week 26	22 ± 2	23 ± 2	24 ± 1[2]	25 ± 2	110 ± 31**

TABLE B2 Clinical Chemistry Data for F344/N Rats in the 26-Week Drinking Water Study of a Chemical Mixture of 25 Groundwater Contaminants (continued)

	0 ppm	11 ppm	38 ppm	113 ppm	378 ppm
FEMALE					
n	10	10	10	10	10
Creatinine (mg/dL)					
Week 1	0.69 ± 0.02	0.69 ± 0.01	0.69 ± 0.01	0.68 ± 0.01	0.66 ± 0.02
Week 3	0.59 ± 0.01	0.57 ± 0.02	0.56 ± 0.02	0.57 ± 0.02	0.58 ± 0.01
Week 7	0.61 ± 0.01	0.61 ± 0.02	0.60 ± 0.02	0.59 ± 0.01	0.59 ± 0.01
Week 14	0.61 ± 0.01	0.61 ± 0.01	0.59 ± 0.02[2]	0.60 ± 0.02	0.59 ± 0.01
Week 26	0.72 ± 0.01	0.71 ± 0.01	0.72 ± 0.01	0.71 ± 0.02	0.66 ± 0.02*
Albumin (g/dL)					
Week 1	4.4 ± 0.1	4.3 ± 0.1	4.4 ± 0.1	4.4 ± 0.0	4.3 ± 0.0
Week 3	4.6 ± 0.0	4.6 ± 0.1	4.6 ± 0.0	4.5 ± 0.1	4.4 ± 0.1*
Week 7	4.6 ± 0.1	4.5 ± 0.1	4.5 ± 0.1	4.5 ± 0.1	4.4 ± 0.1
Week 14	4.9 ± 0.1	4.9 ± 0.1	4.6 ± 0.2	4.7 ± 0.1	4.4 ± 0.1**
Week 26	5.3 ± 0.1	5.2 ± 0.1	5.0 ± 0.1	4.6 ± 0.2**	4.3 ± 0.1**
Alanine aminotransferase (IU/L)					
Week 1	37 ± 1	41 ± 1*	39 ± 1	43 ± 2**	48 ± 2**
Week 3	36 ± 1	37 ± 1	37 ± 1	36 ± 1	44 ± 1**
Week 7	37 ± 1	34 ± 1	35 ± 1	41 ± 2	48 ± 1**
Week 14	41 ± 2	45 ± 2	46 ± 3	49 ± 2*	57 ± 3**[2]
Week 26	45 ± 2	49 ± 3	45 ± 1	66 ± 11*	233 ± 16**
Creatine kinase (IU/L)					
Week 1	511 ± 54	478 ± 70	429 ± 21	451 ± 43	570 ± 99
Week 3	401 ± 27	471 ± 90	536 ± 97	377 ± 32	374 ± 41
Week 7	333 ± 39	361 ± 26	427 ± 74	431 ± 87	484 ± 86
Week 14	251 ± 29	197 ± 29	225 ± 29[2]	234 ± 29	245 ± 32
Week 26	142 ± 27	172 ± 48	136 ± 12	178 ± 20	143 ± 19
Sorbitol dehydrogenase (IU/L)					
Week 1	32 ± 1	30 ± 1	30 ± 1	30 ± 1	29 ± 1
Week 3	21 ± 0	22 ± 0	21 ± 1	21 ± 0	18 ± 1**
Week 7	22 ± 1	22 ± 1	23 ± 1	26 ± 1*	27 ± 1**
Week 14	18 ± 1	19 ± 1	21 ± 1[2]	22 ± 2	34 ± 4**[2]
Week 26	16 ± 1	19 ± 1	17 ± 1	43 ± 14**	255 ± 18**

[1] Data are given as mean ± standard error.
[2] n=9.
* Significantly different (P≤0.05) from the control group by Shirley's test.
** Significantly different (P≤0.01) from the control group by Shirley's test.

TABLE B3 Hematology Data for B6C3F$_1$ Mice in the 26-Week Drinking Water Study of a Chemical Mixture of 25 Groundwater Contaminants[1]

	0 ppm	11 ppm	38 ppm	113 ppm	378 ppm
MALE					
n					
Week 14	10	8	10	10	10
Week 26	10	10	10	10	10
Hematocrit (%)					
Week 14	48.6 ± 0.4	49.3 ± 0.4	48.4 ± 0.5	48.6 ± 0.7	48.9 ± 0.4
Week 26	49.4 ± 0.4	48.9 ± 0.3	49.7 ± 0.6	48.8 ± 0.4	49.6 ± 0.5
Hemoglobin (g/dL)					
Week 14	16.1 ± 0.1	16.3 ± 0.0	15.9 ± 0.2	16.1 ± 0.2	16.2 ± 0.1
Week 26	15.8 ± 0.1	15.8 ± 0.1	15.8 ± 0.1	15.7 ± 0.1	15.9 ± 0.1
Erythrocytes (10^6/μL)					
Week 14	10.02 ± 0.10	10.11 ± 0.07	10.00 ± 0.10	10.04 ± 0.16	10.22 ± 0.09
Week 26	9.92 ± 0.06	9.78 ± 0.10	9.80 ± 0.13	9.61 ± 0.20	10.08 ± 0.12
Reticulocytes (10^6/μL)					
Week 14	0.20 ± 0.01	0.18 ± 0.01	0.19 ± 0.01	0.18 ± 0.01	0.20 ± 0.01
Week 26	0.16 ± 0.01	0.14 ± 0.01	0.15 ± 0.01	0.15 ± 0.01	0.13 ± 0.01
Mean cell volume (fL)					
Week 14	48.6 ± 0.2	48.8 ± 0.3	48.4 ± 0.3	48.5 ± 0.2	47.9 ± 0.2*
Week 26	49.9 ± 0.3	50.1 ± 0.4	50.6 ± 1.1	50.9 ± 1.2	49.0 ± 0.5
Mean cell hemoglobin (pg)					
Week 14	16.1 ± 0.1	16.2 ± 0.1	15.9 ± 0.1	16.1 ± 0.1	15.9 ± 0.1
Week 26	16.0 ± 0.1	16.1 ± 0.1	16.2 ± 0.3	16.4 ± 0.4	15.8 ± 0.1
Mean cell hemoglobin concentration (g/dL)					
Week 14	33.1 ± 0.2	33.1 ± 0.2	32.9 ± 0.1	33.2 ± 0.2	33.2 ± 0.2
Week 26	32.0 ± 0.3	32.3 ± 0.2	31.9 ± 0.2	32.2 ± 0.2	32.1 ± 0.2
Platelets (10^3/μL)					
Week 14	1000.8 ± 39.4	970.8 ± 38.0	1064.5 ± 23.6	1009.0 ± 38.3	1049.2 ± 42.5
Week 26	1069 ± 27	1035 ± 28	1025 ± 33	1042 ± 40	1050 ± 27
Leukocytes (10^3/μL)					
Week 14	6.68 ± 0.38	6.15 ± 0.30	6.08 ± 0.22	6.09 ± 0.50	5.55 ± 0.30*
Week 26	7.14 ± 0.51	7.18 ± 0.26	7.10 ± 0.32	6.28 ± 0.43	6.80 ± 0.36
Segmented neutrophils (10^3/μL)					
Week 14	1.07 ± 0.12	1.04 ± 0.14	0.91 ± 0.07	0.91 ± 0.11	0.79 ± 0.05
Week 26	1.01 ± 0.14	1.34 ± 0.12	1.11 ± 0.11	1.21 ± 0.12	1.25 ± 0.10
Lymphocytes (10^3/μL)					
Week 14	5.45 ± 0.38	5.04 ± 0.25	5.05 ± 0.22	5.05 ± 0.43	4.57 ± 0.31
Week 26	5.96 ± 0.45	5.65 ± 0.20	5.81 ± 0.22	4.92 ± 0.42	5.41 ± 0.34
Eosinophils (10^3/μL)					
Week 14	0.17 ± 0.05	0.07 ± 0.02	0.12 ± 0.03	0.12 ± 0.03	0.17 ± 0.04
Week 26	0.17 ± 0.06	0.19 ± 0.02	0.19 ± 0.04	0.15 ± 0.04	0.14 ± 0.03

TABLE B3 Hematology Data for B6C3F$_1$ Mice in the 26-Week Drinking Water Study of a Chemical Mixture of 25 Groundwater Contaminants (continued)

	0 ppm	11 ppm	38 ppm	113 ppm	378 ppm
FEMALE					
n	10	10	10	10	10
Hematocrit (%)					
Week 14	48.8 ± 0.4	49.2 ± 0.4	48.9 ± 0.3	48.8 ± 0.3	49.4 ± 0.7
Week 26	49.3 ± 0.4	49.1 ± 0.5	49.1 ± 0.3	49.0 ± 0.4	49.9 ± 0.7
Hemoglobin (g/dL)					
Week 14	15.8 ± 0.1	15.9 ± 0.1	15.9 ± 0.1	15.9 ± 0.1	16.1 ± 0.2
Week 26	15.9 ± 0.1	15.9 ± 0.1	15.9 ± 0.1	16.0 ± 0.1	16.0 ± 0.2
Erythrocytes (10^6/μL)					
Week 14	9.94 ± 0.06	9.96 ± 0.09	9.87 ± 0.07	9.90 ± 0.09	10.19 ± 0.13
Week 26	9.84 ± 0.06	9.83 ± 0.08	9.91 ± 0.08	9.91 ± 0.08	10.14 ± 0.14
Reticulocytes (10^6/μL)					
Week 14	0.181 ± 0.009	0.176 ± 0.010	0.153 ± 0.010	0.157 ± 0.010	0.146 ± 0.012*
Week 26	0.15 ± 0.01	0.15 ± 0.01	0.16 ± 0.01	0.15 ± 0.01	0.13 ± 0.01
Mean cell volume (fL)					
Week 14	49.1 ± 0.2	49.5 ± 0.2	49.5 ± 0.2	49.3 ± 0.3	48.5 ± 0.2
Week 26	50.2 ± 0.3	50.1 ± 0.3	49.7 ± 0.3	49.4 ± 0.3	49.2 ± 0.2*
Mean cell hemoglobin (pg)					
Week 14	15.9 ± 0.1	16.0 ± 0.1	16.1 ± 0.1	16.1 ± 0.1	15.8 ± 0.1
Week 26	16.2 ± 0.1	16.1 ± 0.1	16.1 ± 0.1	16.1 ± 0.1	15.8 ± 0.1*
Mean cell hemoglobin concentration (g/dL)					
Week 14	32.4 ± 0.1	32.3 ± 0.2	32.4 ± 0.2	32.7 ± 0.2	32.6 ± 0.2
Week 26	32.3 ± 0.2	32.3 ± 0.2	32.4 ± 0.2	32.6 ± 0.3	32.2 ± 0.2
Platelets (10^3/μL)					
Week 14	937.1 ± 29.2	931.6 ± 25.8	913.6 ± 26.7	886.4 ± 40.9	917.9 ± 36.3
Week 26	874.7 ± 16.0	931.5 ± 70.1	918.3 ± 32.7	870.8 ± 38.2	903.8 ± 45.2
Leukocytes (10^3/μL)					
Week 14	5.54 ± 0.37	5.26 ± 0.40	5.21 ± 0.32	5.51 ± 0.29	4.72 ± 0.29
Week 26	4.94 ± 0.20	5.28 ± 0.55	5.14 ± 0.33	4.59 ± 0.29	5.16 ± 0.42
Segmented neutrophils (10^3/μL)					
Week 14	0.89 ± 0.11	0.68 ± 0.07	0.78 ± 0.08	0.79 ± 0.11	0.62 ± 0.04
Week 26	0.85 ± 0.06	0.93 ± 0.11	0.92 ± 0.14	0.81 ± 0.11	1.04 ± 0.14
Lymphocytes (10^3/μL)					
Week 14	4.59 ± 0.28	4.48 ± 0.35	4.33 ± 0.29	4.59 ± 0.24	4.00 ± 0.28
Week 26	4.00 ± 0.19	4.25 ± 0.48	4.15 ± 0.31	3.67 ± 0.27	3.98 ± 0.33
Eosinophils (10^3/μL)					
Week 14	0.04 ± 0.01	0.11 ± 0.03	0.09 ± 0.02	0.12 ± 0.03	0.09 ± 0.03
Week 26	0.09 ± 0.03	0.10 ± 0.03	0.07 ± 0.02	0.12 ± 0.03	0.14 ± 0.04

[1] Data are given as mean ± standard error.

* Significantly different (P≤0.05) from the control group by Shirley's test.

TABLE B4 Clinical Chemistry Data for B6C3F$_1$ Mice in the 26-Week Drinking Water Study of a Chemical Mixture of 25 Groundwater Contaminants[1]

	0 ppm	11 ppm	38 ppm	113 ppm	378 ppm
MALE					
n					
Week 14	8	8	10	10	9
Week 26	10	10	9	10	9
Creatinine (mg/dL)					
Week 14	0.39 ± 0.01	0.38 ± 0.02	0.37 ± 0.02[2]	0.34 ± 0.02	0.37 ± 0.02
Week 26	0.35 ± 0.02	0.38 ± 0.02	0.33 ± 0.02	0.35 ± 0.02	0.32 ± 0.02
Albumin (g/dL)					
Week 14	3.5 ± 0.0	3.7 ± 0.1	3.5 ± 0.1	3.6 ± 0.1	3.5 ± 0.1
Week 26	3.9 ± 0.1	3.9 ± 0.1	3.8 ± 0.1	3.8 ± 0.1	3.7 ± 0.1
Alanine aminotransferase (IU/L)					
Week 14	35 ± 4	67 ± 18	31 ± 2	37 ± 5	33 ± 2
Week 26	55 ± 10	85 ± 22	76 ± 14[3]	103 ± 27	84 ± 31[3]
Creatine kinase (IU/L)					
Week 14	339 ± 87	675 ± 120	264 ± 48[2]	664 ± 202	443 ± 167
Week 26	531 ± 162	1369 ± 433	859 ± 283	1390 ± 554	516 ± 201
Sorbitol dehydrogenase (IU/L)					
Week 14	57 ± 2	69 ± 3	60 ± 3	60 ± 3	58 ± 2
Week 26	70 ± 7	65 ± 2	59 ± 2[3]	63 ± 5	61 ± 2[3]
FEMALE					
n	10	10	10	10	10
Creatinine (mg/dL)					
Week 14	0.39 ± 0.02	0.38 ± 0.01	0.37 ± 0.02	0.39 ± 0.01	0.37 ± 0.02
Week 26	0.37 ± 0.02	0.37 ± 0.02	0.38 ± 0.01	0.39 ± 0.01	0.37 ± 0.02
Albumin (g/dL)					
Week 14	4.1 ± 0.1	4.0 ± 0.1	4.0 ± 0.0	4.0 ± 0.0	4.0 ± 0.1
Week 26	3.9 ± 0.1	3.9 ± 0.1	3.9 ± 0.1	4.0 ± 0.0	3.9 ± 0.1
Alanine aminotransferase (IU/L)					
Week 14	39 ± 6	27 ± 2	35 ± 5	36 ± 5	42 ± 8
Week 26	31 ± 3	32 ± 5	38 ± 7	36 ± 5	30 ± 4
Creatine kinase (IU/L)					
Week 14	718 ± 245	433 ± 118	406 ± 99	511 ± 133	469 ± 126
Week 26	386 ± 99	297 ± 67	501 ± 119	660 ± 196	398 ± 93
Sorbitol dehydrogenase (IU/L)					
Week 14	53 ± 2	50 ± 2	50 ± 2	53 ± 2	53 ± 2
Week 26	42 ± 1	40 ± 1	42 ± 1	42 ± 2	42 ± 2

[1] Data are given as mean ± standard error. Differences from the control group were not significant by Dunn's or Shirley's test.
[2] n=9.
[3] n=10.

APPENDIX C

Reproductive Tissue Evaluations and Estrous Cycle Characterization

TABLE C1 Summary of Reproductive Tissue Evaluations in Male F344/N Rats at the 14-Week Interim Evaluation in the 26-Week Drinking Water Study of a Chemical Mixture of 25 Groundwater Contaminants[1]

Study Parameters	0 ppm	38 ppm	113 ppm	378 ppm
n	10	10	10	10
Weights (g)				
Necropsy body weight	370 ± 6	363 ± 4	355 ± 4*	331 ± 6**
Left epididymis	0.457 ± 0.009[2]	0.454 ± 0.007	0.436 ± 0.006	0.447 ± 0.008
Left cauda epididymis	0.137 ± 0.003	0.148 ± 0.006	0.136 ± 0.004	0.143 ± 0.003
Left testis	1.55 ± 0.03	1.56 ± 0.02	1.50 ± 0.02	1.44 ± 0.04*
Spermatid measurements				
Spermatid heads (10^7/g testis)	11.25 ± 0.33	10.45 ± 0.53	10.60 ± 0.39	11.28 ± 0.52
Spermatid heads (10^7/testis)	17.41 ± 0.48	16.29 ± 0.73	15.88 ± 0.55	16.21 ± 0.76
Spermatid count (mean/10^{-4} mL suspension)	87.05 ± 2.42	90.25 ± 6.81	79.40 ± 2.77	81.05 ± 3.79
Epididymal spermatozoal measurements				
Motility (%)	81.97 ± 1.78	83.56 ± 1.87	80.60 ± 1.47	82.13 ± 1.89
Concentration (10^6/g cauda epididymal tissue)	553 ± 30	594 ± 40	645 ± 42	611 ± 46

[1] Data are presented as mean ± standard error. Differences from the control group for epididymal and cauda epididymal weights and spermatid and spermatozoal measurements are not significant by Dunn's or Shirley's test.
[2] n=9.
* Significantly different (P≤0.05) from the control group by Shirley's test.
** Significantly different (P≤0.01) from the control group by Shirley's test.

TABLE C2 Summary of Reproductive Tissue Evaluations in Male F344/N Rats in the 26-Week Drinking Water Study of a Chemical Mixture of 25 Groundwater Contaminants[1]

Study Parameters	0 ppm	38 ppm	113 ppm	378 ppm
n	10	10	10	10
Weights (g)				
Necropsy body weight	428 ± 5	415 ± 9	406 ± 7*	399 ± 7**
Left epididymis	0.459 ± 0.007[2]	0.459 ± 0.008	0.457 ± 0.010	0.452 ± 0.005
Left cauda epididymis	0.172 ± 0.004	0.170 ± 0.004	0.169 ± 0.008	0.167 ± 0.006
Left testis	1.56 ± 0.02	1.55 ± 0.03	1.53 ± 0.04	1.53 ± 0.02
Spermatid measurements				
Spermatid heads (10^7/g testis)	8.19 ± 0.49	7.26 ± 0.33	7.31 ± 0.75	7.80 ± 0.30
Spermatid heads (10^7/testis)	12.70 ± 0.66	11.22 ± 0.51	11.19 ± 1.16	11.93 ± 0.52
Spermatid count (mean/10^{-4} mL suspension)	63.50 ± 3.28	56.10 ± 2.53	55.93 ± 5.82	59.63 ± 2.61
Epididymal spermatozoal measurements				
Motility (%)	71.70 ± 1.32	70.53 ± 1.94	71.21 ± 2.79	70.91 ± 2.61
Concentration (10^6/g cauda epididymal tissue)	655 ± 29	703 ± 27	724 ± 51	605 ± 70

[1] Data are presented as mean ± standard error. Differences from the control group for epididymal, cauda epididymal, and testis weights and spermatid and spermatozoal measurements are not significant by Dunn's test.
[2] n=9.
* Significantly different (P≤0.05) from the control group by Williams' test.
** Significantly different (P≤0.01) from the control group by Williams' test.

TABLE C3 Summary of Estrous Cycle Characterization in Female F344/N Rats at the 14-Week Interim Evaluation in the 26-Week Drinking Water Study of a Chemical Mixture of 25 Groundwater Contaminants[1]

Study Parameters	0 ppm	38 ppm	113 ppm	378 ppm
n	9	10	10	10
Necropsy body weight	192 ± 4	196 ± 6	197 ± 1	197 ± 2
Estrous cycle length (days)	5.00 ± 0.08	4.95 ± 0.16	4.85 ± 0.11	4.95 ± 0.28
Estrous stages (% of cycle)				
Diestrous	35.2	40.8	40.0	42.5
Proestrus	18.5	13.3	15.0	13.3
Estrus	32.4	27.5	27.5	28.3
Metestrus	11.1	16.7	16.7	14.2
Uncertain diagnoses (%)	2.8	1.7	0.8	1.7

[1] Necropsy body weight and estrous cycle length data are presented as mean ± standard error. Differences from the control group for necropsy body weight and estrous cycle length are not significant by Dunn's test. By multivariate analysis of variance, exposed groups do not differ significantly from the control group in the relative length of time spent in the estrous stages.

TABLE C4 Summary of Estrous Cycle Characterization in Female F344/N Rats in the 26-Week Drinking Water Study of a Chemical Mixture of 25 Groundwater Contaminants[1]

Study Parameters	0 ppm	38 ppm	113 ppm	378 ppm
n	10	10	10	10
Necropsy body weight	221 ± 3	215 ± 3	218 ± 4	211 ± 3*
Estrous cycle length (days)	5.00 ± 0.07	5.05 ± 0.24	5.05 ± 0.05	5.15 ± 0.15
Estrous stages (% of cycle)				
Diestrous	40.0	37.5	38.3	45.8
Proestrus	14.2	11.7	10.8	15.8
Estrus	25.8	31.7	33.3	21.7
Metestrus	20.0	17.5	16.7	16.7
Uncertain diagnoses (%)	0.0	1.7	0.8	0.0

[1] Necropsy body weight and estrous cycle length data are presented as mean ± standard error. Differences from the control group for estrous cycle length are not significant by Dunn's test. By multivariate analysis of variance, exposed groups do not differ significantly from the control group in the relative length of time spent in the estrous stages.

* Significantly different ($P \leq 0.05$) from the control group by Williams' test.

TABLE C5 Summary of Reproductive Tissue Evaluations in Male B6C3F$_1$ Mice at the 14-Week Interim Evaluation in the 26-Week Drinking Water Study of a Chemical Mixture of 25 Groundwater Contaminants[1]

Study Parameters	0 ppm	38 ppm	113 ppm	378 ppm
n	9	10	10	10
Weights (g)				
Necropsy body weight	40.4 ± 0.8	40.7 ± 0.6	40.8 ± 0.7	40.6 ± 0.9
Left epididymis	0.050 ± 0.001	0.046 ± 0.001*	0.047 ± 0.002	0.045 ± 0.002*
Left cauda epididymis	0.016 ± 0.001	0.016 ± 0.001	0.015 ± 0.001	0.015 ± 0.001
Left testis	0.119 ± 0.002	0.116 ± 0.003	0.121 ± 0.002	0.117 ± 0.002
Spermatid measurements				
Spermatid heads (10^7/g testis)	20.26 ± 0.49	20.42 ± 0.58	20.51 ± 0.70	20.99 ± 0.42
Spermatid heads (10^7/testis)	2.40 ± 0.05	2.37 ± 0.07	2.47 ± 0.07	2.45 ± 0.06
Spermatid count (mean/10^{-4} mL suspension)	75.06 ± 1.68	74.08 ± 2.13	77.13 ± 2.33	76.53 ± 1.91
Epididymal spermatozoal measurements				
Motility (%)	78.51 ± 1.40	78.45 ± 3.02	77.18 ± 2.75	81.80 ± 1.64
Concentration (10^6/g cauda epididymal tissue)	1459 ± 140	1196 ± 157	1268 ± 200	1294 ± 196

[1] Data are presented as mean ± standard error. Differences from the control group for necropsy body weight, cauda epididymal and testis weights, and spermatid and spermatozoal measurements are not significant by Dunn's test.

* Significantly different (P≤0.05) from the control group by Shirley's test.

TABLE C6 Summary of Reproductive Tissue Evaluations in Male B6C3F$_1$ Mice in the 26-Week Drinking Water Study of a Chemical Mixture of 25 Groundwater Contaminants[1]

Study Parameters	0 ppm	38 ppm	113 ppm	378 ppm
n	10	10	10	10
Weights (g)				
Necropsy body weight	49.2 ± 1.0	48.7 ± 0.7	47.3 ± 1.5	47.3 ± 1.3
Left epididymis	0.050 ± 0.002	0.049 ± 0.001[2]	0.050 ± 0.001	0.052 ± 0.001
Left cauda epididymis	0.015 ± 0.000	0.014 ± 0.001	0.015 ± 0.001	0.016 ± 0.000
Left testis	0.115 ± 0.002	0.113 ± 0.002[2]	0.118 ± 0.002	0.117 ± 0.003
Spermatid measurements				
Spermatid heads (10^7/g testis)	12.95 ± 0.74	12.10 ± 0.86[2]	12.82 ± 0.93	12.09 ± 1.05
Spermatid heads (10^7/testis)	1.49 ± 0.08	1.39 ± 0.07	1.52 ± 0.12	1.41 ± 0.11
Spermatid count (mean/10^{-4} mL suspension)	46.63 ± 2.60	43.30 ± 2.32	47.43 ± 3.65	44.08 ± 3.57
Epididymal spermatozoal measurements				
Motility (%)	68.13 ± 4.21	73.36 ± 2.92	73.35 ± 2.59	74.68 ± 2.68
Concentration (10^6/cauda epididymal tissue)	1152 ± 108	1251 ± 139	1277 ± 108	1324 ± 102

[1] Data are presented as mean ± standard error. Differences from the control group for necropsy body weights are not significant by Dunnett's test; differences from the control group for epididymal, cauda epididymal, and testis weights and spermatid and spermatozoal measurements are not significant by Dunn's test.

[2] n=9.

TABLE C7 Summary of Estrous Cycle Characterization in Female B6C3F$_1$ Mice at the 14-Week Interim Evaluation in the 26-Week Drinking Water Study of a Chemical Mixture of 25 Groundwater Contaminants[1]

Study Parameters	0 ppm	38 ppm	113 ppm	378 ppm
n	10	10	10	10
Necropsy body weight	35.1 ± 1.3	36.2 ± 1.3	35.3 ± 1.2	35.2 ± 1.1
Estrous cycle length (days)	4.25 ± 0.13	4.10 ± 0.10	4.35 ± 0.13	4.30 ± 0.15
Estrous stages (% of cycle)				
Diestrous	30.0	29.2	25.0	29.2
Proestrus	12.5	21.7	20.8	16.7
Estrus	36.7	26.7	32.5	35.8
Metestrus	20.8	22.5	21.7	18.3

[1] Necropsy body weight and estrous cycle length data are presented as mean ± standard error. Differences from the control group for necropsy body weight and estrous cycle length are not significant by Dunn's test. By multivariate analysis of variance, exposed groups do not differ significantly from the control group in the relative length of time spent in the estrous stages.

TABLE C8 Summary of Estrous Cycle Characterization in Female B6C3F$_1$ Mice in the 26-Week Drinking Water Study of a Chemical Mixture of 25 Groundwater Contaminants[1]

Study Parameters	0 ppm	38 ppm	113 ppm	378 ppm
n	10	10	10	9
Necropsy body weight	45.8 ± 1.4	45.7 ± 2.1	47.6 ± 1.7	44.7 ± 1.0[2]
Estrous cycle length (days)	4.95 ± 0.05	4.85 ± 0.20	4.85 ± 0.11	4.83 ± 0.17[3]
Estrous stages (% of cycle)				
Diestrous	25.8	28.3	27.5	40.0
Proestrus	17.5	10.8	15.8	16.7
Estrus	40.8	40.8	40.0	28.3
Metestrus	15.8	20.0	16.7	15.0

[1] Necropsy body weight and estrous cycle length data are presented as mean ± standard error. Differences from the control group for necropsy body weight are not significant by Dunnett's test. Differences from the control group for estrous cycle length are not significant by Dunn's test. By multivariate analysis of variance, exposed groups do not differ significantly from the control group in the relative length of time spent in the estrous stages.

[2] n=10.

[3] Estrous cycle longer than 12 days or unclear in 1 of 10 animals.

Toxicology Studies of a Chemical Mixture of 25 Groundwater Contaminants

III. Male Reproduction Study in B6C3F$_1$ Mice

Robert E. Chapin, Jerry L. Phelps, Bernard A. Schwetz, and Raymond S. H. Yang

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Toxicology Studies of a Chemical Mixture of 25 Groundwater Contaminants

III. Male Reproduction Study in B6C3F$_1$ Mice

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Toxicology Studies of a Chemical Mixture of 25 Groundwater Contaminants. III. Male Reproduction Study in B6C3F$_1$ Mice. CHAPIN, R. E., PHELPS, J. L., SCHWETZ, B. A., AND YANG, R. S. H. (1989). *Fundam. Appl. Toxicol.* **13**, 388–398. A mixture of chemicals has been developed that models contaminated groundwater around hazardous waste sites. We investigated the effects of this mixture on spermatogenesis in B6C3F$_1$ mice. The animals consumed three different concentrations of this mixture for 90 days, after which time they were euthanatized. Although there was a concentration-related decrease in the amount of fluid consumed at the higher two concentrations, there were no differences in body weight among the groups. Similarly, there was no effect of mixture consumption upon the histology of liver, kidney, testis, epididymis, or seminal vesicles or upon the absolute organ weights of these organs. Kidney weight relative to body weight was increased in the high dose group. Epididymal sperm number and testicular spermatid count were not affected by treatment. These studies show that, at exposure levels that decrease fluid intake and increase adjusted kidney weight, there were no effects of this mixture on gametogenesis in male mice. © 1989 Society of Toxicology.

The previous decade has seen a large increase in our knowledge of the cellular and intercellular events involved in reproduction (Knobil and Neill, 1988, for review). The knowledge base for reproductive toxicants has also expanded (e.g., Waller *et al.*, 1985) and has included some insightful mechanistic studies. Virtually all of these studies have focused on the toxic effects of individual chemicals and ways to ameliorate or exacerbate those effects. While this approach permits a clear definition of the effects of one toxicant, most human situations involve exposure to multiple chemicals simultaneously or sequentially. Groundwater contamination, especially from toxic waste dump sites, is one example of exposure to multiple compounds. Because more than half of the United States population consumes groundwater, the long-term low dose intake of chemical mixtures is an urgent problem which has only just recently been addressed. The National Toxicology Program has developed a model mixture of the 25 contaminants (19 organics and 6 inorganic salts) most frequently found in groundwater leachate from toxic dumps, and is conducting toxicology studies with this model mixture. The guidelines used in selecting the chemicals and the theoretical exercises leading to the setting of target concentrations have been published (Yang and Rauckman, 1987). More concrete information on the final composition, formulation, and analysis of the mixture is reported in a study by Yang *et al.* (1989).

Many of the chemicals in this mixture are known to adversely affect reproduction when

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TABLE 1

Chemical	Refs.
Cadmium	Aoki and Hoffer (1978) Kotsonis and Klaassen (1978) Danielsson *et al.* (1984) Laskey *et al.* (1984) Der *et al.* (1976)
Carbon tetrachloride	Kalla and Banansol, 1975
Chloroform	Land *et al.* (1981)
DEHP	Gray and Gangolli (1986)
1,2-Dichloroethane	Igwe *et al.* (1986)
Lead	Sokol *et al.* (1985) Wiebe *et al.* (1983) Der *et al.* (1976)
Mercury	Lee and Dixon (1975)
Nickel	Mathur *et al.* (1977a,b)
Trichloroethylene	Land *et al.* (1981) Zenick *et al.* (1984) Nelson and Zenick (1986)
o-Xylene	Washington *et al.* (1983)

administered to laboratory animals (Table 1). These studies generally are designed to demonstrate the potential toxicity of a chemical, and this toxicity may be limited to a specific route (e.g., Cd) or large doses (DEHP). The studies cited in Table 1 are not low dose, long-term designs, but often use a maximum chemical challenge and expose the animal to a single compound. Relatively little is known, then, of the possible synergistic or antagonistic effects of administering several of these compounds simultaneously. One example of a synergistic effect was reported by Der *et al.* (1976) who demonstrated that coadministration of lead and cadmium (25 μg/day ip × 70 days) produced greater testicular damage than seen after greater doses of either compound given alone. These data and the lack of studies evaluating multiple compound exposure at environmentally realistic low doses for subchronic exposure periods prompted us to assess the effects of this mixture on gametogenesis in male mice.

METHODS

Animals. Male B6C3F$_1$ mice were received from Charles River Breeding Laboratories at 6 weeks of age and were acclimated to the NIEHS facility for 2 weeks. They were housed one per cage, with hardwood chip bedding at 70 ± 2°F, 50 ± 10% relative humidity. Feed was NIH-31 chow *ad lib;* water during acclimation and for controls during the 90-day study was deionized water, provided *ad lib.* At the end of the acclimation period, the animals were randomized by body weight into four groups of eight animals each. During the 90-day study, they were housed individually in ventilated cage racks (Lab Products).

Chemical mixture. The specifics of the formulation, the analytical challenges, and the stability and chemical speciation under normal laboratory conditions are in an accompanying article (Yang *et al.*, 1989). For ease of handling, stocks were prepared of both the organics and the inorganic metal salts. The final mixture was then formulated by adding the organic stock to the inorganics. The flask, with minimal headspace, was capped and mixed by gentle stirring in the dark for 2 hr or until the organic phase had become incorporated. Dilutions were made using repeating pipetors, avoiding bubbling, into deionized water. These methods had been shown to produce solutions of consistent chemical composition (Yang *et al.*, 1989). A preliminary pilot study was performed to determine the palatability of the mixture. At 20% and above, fluid consumption decreased and body weights declined more than 10%. Based on these data, concentrations for the 90-day study were set at 0, 100-, 20-, and 10-fold dilutions of the technically achievable stock of the mixture. These are referred to below as 0, 1%, 5%, and 10%. The concentrations of the chemicals for the highest dose group can be found in Table 2.

The mixture was pipetted into 60-ml amber glass bottles, leaving a minimum head space, topped with a Teflon-lined septum, and a stainless-steel sipper tube. These bottles were emptied, refilled, and weighed on Monday, Wednesday, and Friday. Full bottle weights were recorded and were weighed again 24 hr later (Tuesday and Thursday). The mixture was available over weekends, but consumption was not recorded. Body weights were recorded weekly. Although some loss of volatile organics was inevitable, quantitative analyses of the dosing solutions (Yang *et al.*, 1989) indicated that the chemical mixture was sufficiently stable under the experimental conditions to provide adequate estimation of the exposure concentrations (Table 2).

Necropsy and tissue handling. At the end of the 90-day treatment period, mice were killed by asphyxiation with CO_2, weighed, and the following organs were dissected and weighed: left kidney, left and right testis, right epididymis, right cauda epididymis, and seminal vesicles. The right cauda epididymis was placed in a plastic petri dish containing 4.5 ml phosphate-buffered saline (PBS), minced gently with razor blades, and swirled to dissociate clumps of sperm and epididymal tissue. After 5 min, the suspension was fixed with 0.5 ml of 50% glutaraldehyde and stored at room temperature until counted. The right testis was weighed and frozen in a labeled jar on dry ice

and was used later for spermatid head count. Whole seminal vesicles, bisected left kidney, and a section of liver were fixed in 4% neutral-buffered formalin (NBF). Left testis and epididymis were also fixed in NBF after nicking the capsule of the testis. All tissues except testis/epididymis were fixed for 24 hr and then moved to 70% ethanol prior to embedding in paraffin, and sections were stained with hematoxylin and eosin. Testis/epididymis was rinsed in 0.1 M phosphate-buffered saline for 24 hr, then an equatorial section of testis and a longitudinal section of epididymis were embedded in glycol methacrylate (JB4 kit from PolySciences, Warrington, PA). Sections (2 μm) were cut and stained with periodic acid–Schiff's and hematoxylin. Tissue sections were evaluated on a Nikon Optiphot microscope.

Cauda epididymal sperm were counted on a calibrated Electrozone/Celloscope cell counter (Particle Data Corp., Elmhurst, IL). Only counts in the 30 to 43 μm^3 size range were used as sperm (based on previous in-house studies). Separated heads and tails were not included in the final counts. Results are expressed as 10^6 cells/mg cauda epididymis.

Testicular spermatid head counts were made by detunicating the frozen testis, reweighing, and homogenizing in 10 ml PBS. This was diluted 1:6 with 0.1% trypan blue in PBS, and the nuclei in eight secondary corner squares on the hemocytometer were counted. Results are expressed both as total nuclei per testis and as the number of nuclei per gram of testis.

Statistics. Equality of variances was measured by Bartlett's test. The means for each endpoint was compared across treatments by ANOVA; repeated pairwise comparisons were made by Student's t test to determine differences between treated and control groups. The relationship of kidney weight to fluid consumption was tested by analysis of covariance. Group means were considered significantly different from controls at $p < 0.05$.

RESULTS

Chemical mixture. Table 2 is a summary of the average EPA survey concentrations for the 25 component chemicals in this mixture; it also shows the target concentrations for the individual components at the highest dose level, and the results of actual analyses of all 25 chemicals from the freshly prepared dosing solution (high dose), as well as a sample of that same solution after being on the cages for 48 hr. All volatile organics were analyzed by the purge and trap method using gas chromatography–mass spectrometry. Because this is a multistep procedure for the analysis of 15 chemicals at one time, we expected larger than usual variations in the results. The occasionally higher recoveries in the 48-hr samples (Table 2), particularly those with >100% recovery, are probably due to this limitation. In earlier studies, the recovery of Arochlor 1260 and DEHP was poor, possibly due to their binding onto the glass water bottles; they were not measured for this study. Similarly, lead was found in previous determinations to be consistently present at 98–102% of its nominal values (see, for example, Yang *et al.*, 1989) and was not measured in these analyses.

Body weight and water consumption. Total body weight was not significantly reduced by consumption of this mixture; data from representative weeks are shown in Fig. 1A. Water consumption was decreased in the upper two dose groups at all time points; data from representative weeks are shown in Fig. 1B. The treated and control animals did not exhibit any adverse clinical signs.

Organ weights, sperm, and spermatid data. Mean unadjusted organ weights are shown in Table 3. Absolute kidney weights show an increase that is not statistically significant. When corrected for body weight, the kidney weight changes in the middle and high dose are significant. Analysis of covariance showed that kidney weights (absolute or adjusted) were not affected by the volume of fluid consumed ($p = 0.16$). No other organ weights, adjusted or unadjusted, were different from controls.

Cauda epididymal sperm density was increased in the lower dose group relative to controls (Fig. 2). Sperm density was not decreased in any dose group. Similarly, the testicular spermatid head count, expressed either as total number per testis or as number per gram of testis, was not affected by treatment (Fig. 3).

Histopathology. There was considerable glycogen extraction from all livers during fixation and embedding. Nonetheless, liver sections of control and treated animals were indistinguishable; nuclear size, number of cells with large nuclei, bile duct, and vasculature appearances were similar in all groups.

TABLE 2

COMPARISON OF CONCENTRATIONS OF THE COMPONENTS OF THE 25 CHEMICAL MIXTURE WITH CALCULATED CONSUMPTION AND EPA SURVEY RESULTS

	Average EPA survey (ppm)	High dose (10%) concentrations[a] (ppm)			Calc. consump. high dose[b] (μg/mouse/day)
		Target	Found (% target)		
			Fresh prep	48 hr	
Acetone	6.9	53.0	79	82	131
Arochlor 1260	0.21	0.01	ND	ND	—
Arsenic	30.6	9.0	108	107	28.1
Benzene	5.0	12.5	106	102	37.6
Cadmium	0.85	51	98	98	152.0
Carbon tetrachloride	0.54	0.4	93	88	1.02
Chlorobenzene	0.10	0.1	108	99	0.32
Chloroform	1.46	7	105	98	19.3
Chromium	0.69	36	102	101	107
DEHP	0.13	0.015	ND	ND	—
1,1-Dichloroethane	0.31	1.4	109	107	4.29
1,2-Dichloroethane	6.33	40	103	106	104
1,1-Dichloroethylene	0.24	0.5	83	92	1.35
1,2-trans-Dichloroethylene	0.73	2.5	105	110	7.73
Ethylbenzene	0.65	0.3	106	113	1.06
Lead	37.0	70	—	—	—[c]
Mercury	0.34	0.5	91	91	1.35
Methylene chloride	11.2	37.5	96	91	102
Nickel	0.5	6.8	102	98	18.6
Phenol	34.0	29	95	98	86.4
Tetrachloroethylene	9.68	3.4	96	89	9.04
Toluene	5.18	7	103	102	21.6
1,1,1-Trichloroethane	1.25	2	110	107	6.37
Trichloroethylene	3.82	6.5	111	112	21.8
Xylenes	4.07	1.6	101	100	4.35
Total concentration of all chemicals	131.05	375.025			

Note. DEHP, Di(2-ethylhexyl) phthalate; ND, not detectable; —, not analyzed.

[a] The technically achievable stock formulation of the 25 chemical drinking water mixture, based on EPA survey results, toxicity, and solubility of the component chemicals, was not palatable for animals. Therefore, the highest experimental dose is the 10-fold dilution of the stock. The "fresh prep" column refers to the freshly prepared dosing solution, sampled immediately, shipped via express mail, and analyzed approximately 24 hr later; the "48 hr" column refers to the drinking water samples that remained in the bottles after a 48-hr exposure period and were then sampled and analyzed similarly. See Yang and Rauckman (1987) and Yang *et al.* (1989) for details.

[b] Consumption data are means of four 24-hr periods for each mouse in the last 2 weeks of the study. The values are calculated based on the weight change of the water bottle and the concenration at 48 hr for each compound.

[c] Although the lead content was not determined because of the previous quantitative recovery, assuming its presence at 100% target concentration yields an estimated average intake of 208 μg/mouse/day.

Kidney appearance was similarly not different between groups; there was no increase in cell death or vascularity. Cortical structure and glomeruli were equivalent in treated and control kidney sections. Sections of testis showed normal tubular structure; all stages of spermatogenesis were present, with no apparent alterations in cell associations or spermia-

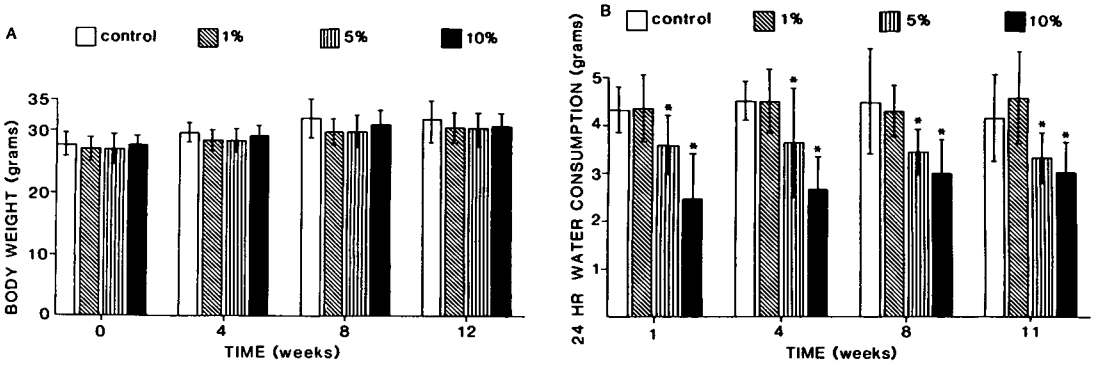

FIG. 1. (A) Mean (±SD) body weights of mice consuming filtered tap water or different concentrations of complex mixture; Week 0 is taken prior to exposure; Week 12 is taken at death. The mean weights of the treated groups are not different from controls (n = 8 all groups). (B) Mean (±SD) water consumption over 24 hr, measured twice weekly during the weeks indicated, for control and mixture-exposed mice (n = 8, all groups). *Different from control group, $p < 0.05$.

tion. There was no evidence of Sertoli cell vacuolation or Leydig cell hyperplasia (Fig. 4). Epididymides from control animals contained one to four immature germ cells per longitudinal tissue section (30–40 tubular cross-sections); this was not increased in treated animals nor was epididymal tubular epithelium visibly affected at the light microscopic level (Fig. 5). There was a small apparent increase in PAS-positive material in epididymal lumena in caput epididymis in four of eight animals (Fig. 5); the significance of this is not clear, but would be consistent with a functional change in the secretory activity

TABLE 3

BODY AND ORGAN WEIGHTS TAKEN AT DEATH FROM CONTROL AND MIXTURE-EXPOSED MICE

	Mixture concentration			
	Control	1%	5%	10%
Body weight (g)	31.80 ± 3.26 (7)	30.66 ± 2.56 (8)	30.42 ± 2.53 (8)	30.81 ± 2.18 (8)
Right testis weight (mg)	121 ± 12 (8)	118 ± 11 (8)	111 ± 12 (8)	112 ± 6 (8)
Right epididymal weight (mg)	44.8 ± 4.4 (7)	42.5 ± 3.1 (7)	41.6 ± 5.0 (6)	42.2 ± 3.2 (8)
Right cauda epididymal weight (mg)	16.8 ± 2.5 (7)	15.9 ± 1.9 (6)	16.1 ± 1.4 (5)	15.5 ± 2.5 (7)
Seminal vesicle weight (mg)	397 ± 41 (8)	366 ± 43 (8)	361 ± 51 (8)	356 ± 44 (8)
Kidney weight (mg)	289 ± 25 (8)	294 ± 34 (8)	300 ± 39 (8)	304 ± 24 (8)
Kidney weight as % of body weight	0.902 ± 0.041 (7)	0.956 ± 0.067 (8)	0.985 ± 0.061* (8)	0.985 ± 0.058* (8)

Note. Cauda epididymides were improperly dissected in seven mice, and body weight was improperly recorded for one animal.

* Different from control at $p < 0.05$.

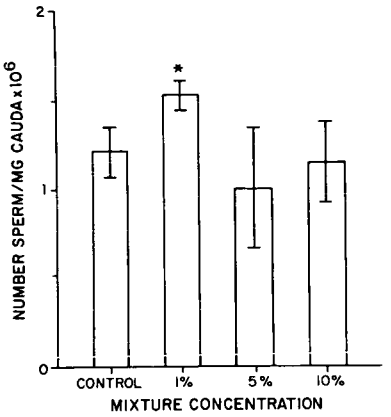

FIG. 2. Mean (±SD) number of sperm per mg cauda epididymis from control and mixture-exposed mice. *Different from control group at $p < 0.05$ (n = 7, 6, 5, 7 for control, 1%, 5%, and 10% groups, respectively).

of the epididymal secretory cells. Seminal vesicles from all groups were characterized by a cuboidal or columnar epithelium overlayed on a linear or deeply invaginated basal lamina. There was no observable cell death or karyorrhexis. All seminal vesicles were filled with a PAS-positive cell-free matrix, the appearance of which was indistinguishable between groups.

DISCUSSION

The exposure of mice to this mixture of 25 chemicals in the drinking water did not alter the microscopic structure of the liver, kidney, testis, epididymis, prostate, or seminal vesicles. Similarly, spermatid production by the testis was unaltered, providing quantitative support for the qualitatively negative histopathology findings. These endpoints were normal despite a concentration-dependent decrease in the amount of fluid consumed, which reached significance at the middle and highest concentrations. Despite this, body weights in the treated groups did not differ from controls at any time point measured. Previous reports of the effects of water restriction on teratology (Schwetz *et al.*, 1977) and male reproductive hormone levels (Armario *et al.*, 1987) concluded that the adverse effects of decreased water availability were probably linked to decreased food consumption. We cannot address this possible confounder because we did not measure food consumption.

Absolute kidney weights appeared to be increased, though not significantly, in the two highest dose groups. However, when expressed per body weight, these increases became significant. This suggests an enlargement of the kidneys in these treated animals, although microscopy was unable to define any differences between the groups. The lack of significant association between fluid volume and kidney weight suggests that these weight changes were a result of exposure to the mixture and not to dehydration.

It must be noted that while the analytical results of the dosing solution samples yielded very good recoveries for most of the chemicals in this mixture, not all of the recoveries were as good, particularly for the volatile organics. In general, phenol and all six inorganics were consistently recovered quantitatively. Recoveries of Arochlor 1260 and DEHP were consistently poor. Volatile organics varied in recovery; factors causing

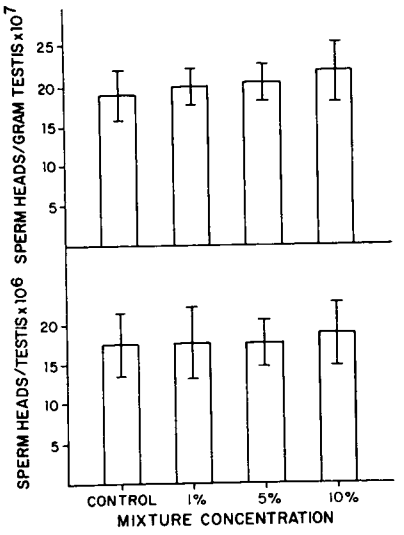

FIG. 3. Mean (±SD) number homogenization-resistent testicular spermatid nuclei (heads), expressed as per g testis, or total number per testis (n = 8, all groups).

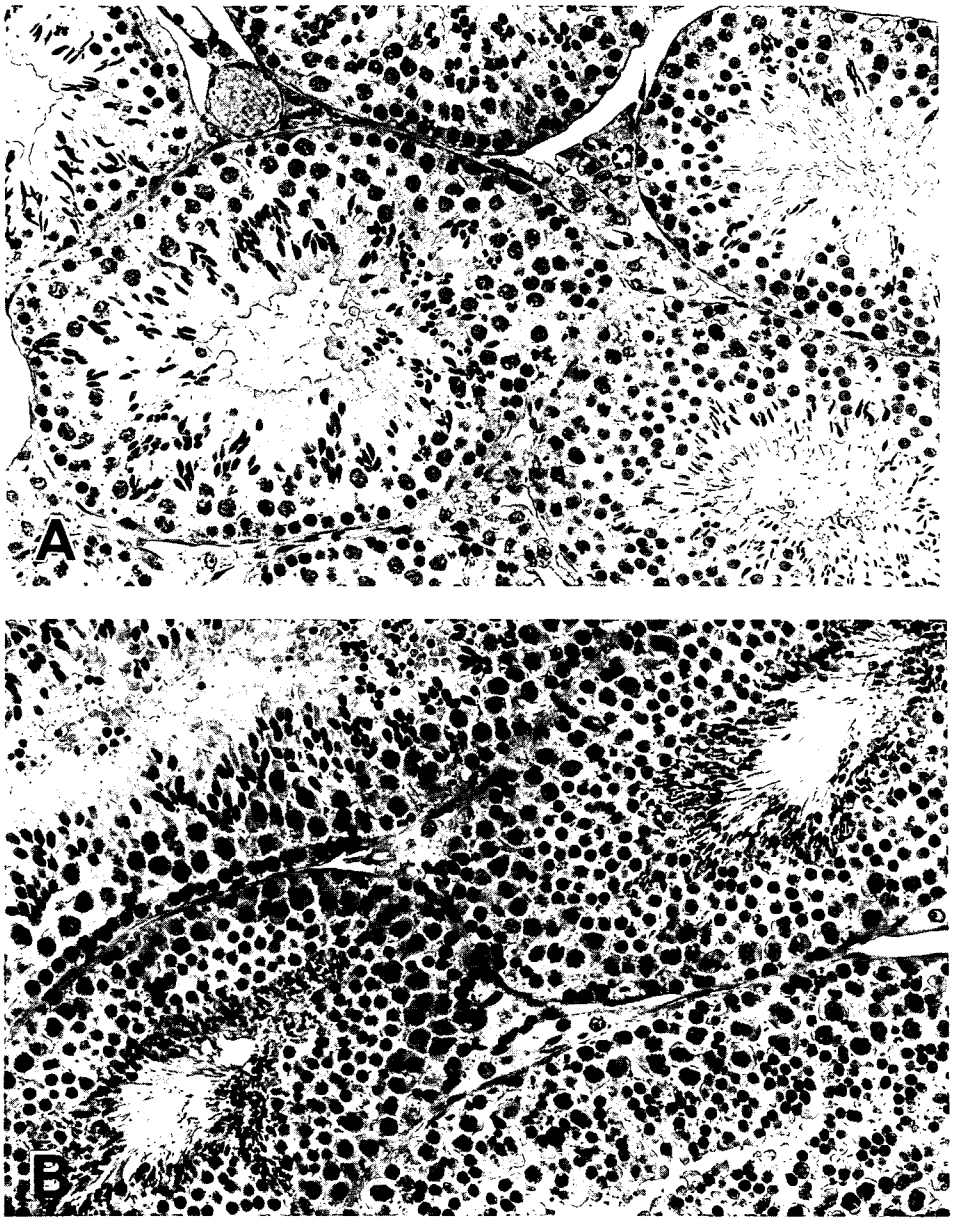

FIG. 4. Sections of testis from control (A) and 10%-treated (B) mice. There is no increase in germ cell death, epithelial vacuolation, or change in the germ cell associations (which would indicate altered kinetics of maturation). ×500.

such variations might include interpersonnel variations in preparing the dosing solutions, sampling techniques (degree of aeration during aliquoting), and analytical differences.

Several of the chemicals in this mixture have been reported to have toxic effects on the male reproductive system of rats or mice when administered alone at sufficient concentrations. Some representative reports are listed in Table 1. For the majority of these compounds, the relevant site(s) of action or mechanism(s) is unknown. Cadmium appears to affect pri-

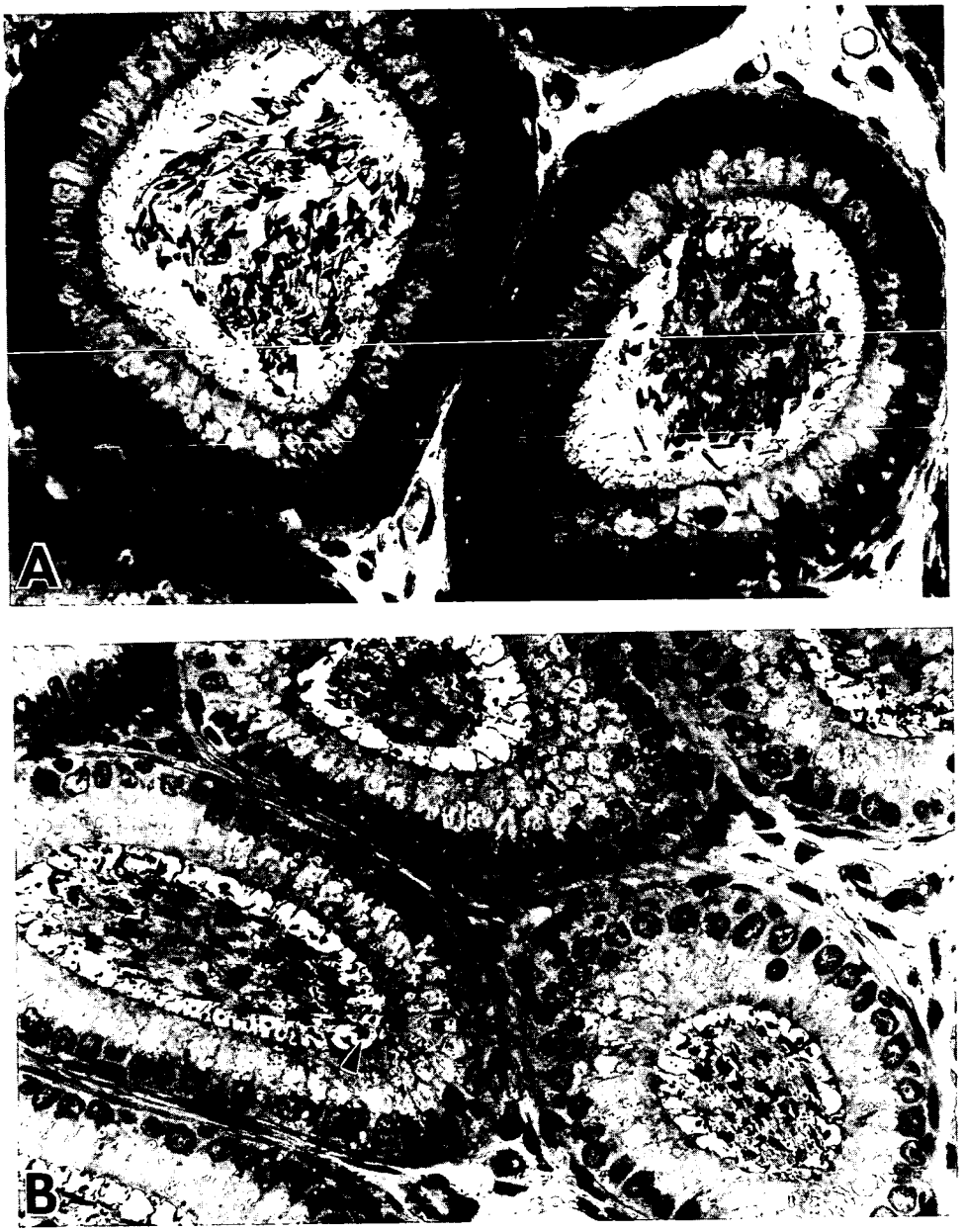

FIG. 5. Sections of caput epididymis from control (A) and 10%-treated (B) mice. Note the slight increase in intraluminal PAS-positive material (arrowhead). ×500.

marily the testicular vasculature (Aoki and Hoffer, 1978), while lead apparently has multiple sites of action in the male reproductive system, both central and peripheral (Sokol *et al.*, 1985; Wiebe *et al.*, 1983, respectively). Trichloroethylene may act at endogenous opioid receptor sites, thus primarily affecting sexual functioning and behavior rather than spermatogenesis per se (Nelson and Zenick, 1986; Zenick *et al.*, 1984).

That this mixture containing known reproductive toxicants apparently did not affect

spermatogenesis prompts consideration of several factors. The treatment period was sufficiently long to cover the entire cycle of spermatogenesis and epididymal sperm maturation in the mouse (reviewed by Clermont, 1972) so the nonexposure of a vulnerable germ cell age cannot explain this lack of effect.

Other considerations would be those of dose, route, or species. It is possible that the top dose used in our studies was insufficient to produce reproductive toxicity. However, the pilot study showed that at concentrations greater than 10%, water consumption decreased sharply and the animals became moribund from dehydration. The top dose used was the maximum that could be employed and still maintain animal viability. Testing at higher concentrations would have required a different route of administration, which would have subverted the intent of the study.

In most of the studies listed in Table 1, the animals were exposed by intraperitoneal injection or peroral intubation. An administered bolus dose produces different peak blood levels than does a similar dose consumed in the drinking water over 24 hr. While the *in vivo* studies cited for cadmium, lead, mercury, and trichloroethylene used daily doses that were similar on a milligrams per killigram basis (within a factor of 2) to those reported herein, all used bolus injections or intubations into rats. Additionally, Boekelheide and Eveleth (1988) have shown that dose *rate* is important for at least one testicular toxicant (2,5-hexanedione): the same total dose administered over short or long periods of time produced more or less testicular damage, respectively. This may also relate, in the present study, to the differences in total dose given, e.g., (1) DEHP requires 1–2 g/kg/day for effects, while our mice received virtually none; (2) 1,2-dichloroethane was toxic at 150 mg/kg/day (Igwe *et al.*, 1986), while our mice consumed, at most, 4.08 mg/kg/day; and (3) mercury produced reproductive damage at 1 mg/kg/day ip (Lee and Dixon, 1975), while the mice in our study received 0.064 mg/kg/day. Thus, there are considerable differences in administered dose. When combined with different species responses and presumed differences in kinetics *in vivo*, the disparate results may not be surprising.

Another consideration is the time required to reach a steady state body burden of a chemical, for the greatest exposure of critical target cells occurs when steady state is achieved. This is a balance of absorption and elimination; for the various chemicals in this mixture, the times to reach steady state vary from minutes to years. For example, peak blood levels of chloroform after one oral dose occur within 1 hr in several species (Brown *et al.*, 1974; Taylor *et al.*, 1974; Fry *et al.*, 1972). Benzene is rapidly and extensively absorbed (Sabourin *et al.*, 1986) and rapidly eliminated (Rickert *et al.*, 1979) with an elimination half-life of less than 2 hr.

In contrast, it is unlikely that an *in vivo* steady state was achieved during this 90-day dosing period for cadmium. This metal is very poorly absorbed from the gut (Foulkes, 1984) and excretion is slow, with a half-life of 17–38 years in humans. Chronic exposure is not required for testicular damage, though, if the dose is sufficient, a single or several administrations of ca. 100 mg $CdCl_2$/kg will damage the rodent testis (Kotsonis and Klaassen, 1978). In contrast, Zenick *et al.* (1982) supplied male rats with water containing up to 68.8 mg cadmium/liter for 70 days, a concentration which would have produced a daily dose of ca. 7 mg/kg/day. They saw no effect on sperm count, morphology, or reproductive success. Additionally, any Arochlor 1260 consumed by the mice in this study would not be near steady state. The half-life of this mixture of polychlorinated biphenyls is ca. 460 day (Wyss *et al.*, 1986). Thus, it is clear that a few of the components of this mixture could not have reached biological equilibrium. A small possibility exists that continued dosing with this mixture would result in observable reproductive tract damage. Particularly for the chemicals having long biological half-lives, the likelihood of causing an adverse effect is a function of dose level *and* duration of exposure.

In other studies, this mixture has been shown to be immunosuppressive at concentrations similar to those used in the present study (Germolec *et al.*, 1989), and the repeated dosing of male rats with this mixture has been found to inhibit state-3 hepatic mitochondrial respiration (Kermani *et al.*, in preparation). For the present report, we did not assess sperm function. However, in the continuous breeding study using the same levels as those reported above, the fertility of mice was unchanged in either the first or second generation (J. Heindel *et al.*, unpublished) supporting the lack of reproductive toxicity of this mixture in this species. Additional continuous breeding studies are ongoing with rats.

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APPENDIX D

Continuous Breeding Studies

CONTINUOUS BREEDING STUDIES

Materials and Methods

CONTINUOUS BREEDING STUDIES

To determine the effects of a chemical mixture of 25 groundwater contaminants on reproduction, continuous breeding studies were performed in Sprague-Dawley rats and CD-1® Swiss mice (Heindel *et al.*, 1993). Male and female VAF Crl:CD BR outbred Sprague-Dawley rats and COBS Crl:CD-1 (ICR) BR VAF/Plus™ outbred albino mice used in the continuous breeding studies were obtained from Charles River Breeding Laboratories (Portage, MI for rats and Raleigh, NC for mice). Rats were 8 weeks old and mice were 9 weeks old at receipt. All rats and mice were quarantined for 2 weeks before the studies began. Blood samples were collected from two rats and two mice of each sex and were analyzed for antibody titers to rodent viruses; all results were negative.

For the continuous breeding studies, groups of 20 breeding pairs received the chemical mixture at a concentration of 38, 189, or 378 ppm in drinking water. For each study, 40 untreated breeding pairs were maintained as controls. The animals were housed separately for 7 days, then housed in breeding pairs for 112 days (rats) or 98 days (mice), while receiving dosed drinking water. NIH-07 Open Formula Diet (Zeigler Brothers, Inc., Gardners, PA) in pellet form was available *ad libitum*. Clinical findings, body weights, fertility, number of litters per pair, number of live pups per litter, proportion of pups born alive, sex of live pups, pup body weights within 24 hours of birth, and feed and water consumption were recorded. Vaginal cytology evaluations of control and high-dose female mice were performed; body and testis weights of control and high-dose male rats and mice were recorded, and a testis was frozen for spermatid head count.

The final litter of pups born to each breeding pair of mice and each control and high-dose breeding pair of rats after the continuous breeding period was reared. Siblings were housed two (rats) or three (mice) per cage by sex, and received the same doses as the F_0 animals. After weaning, 20 nonsibling F_1 rats or mice of each sex were housed in breeding pairs for up to 7 days and then housed singly through delivery of pups. Body weights, fertility, number of litters per pair, number of live pups per litter, proportion of pups born alive, sex of live pups, pup body weights within 24 hours of birth, and feed and water consumption were recorded. At the end of the study, F_1 mice were necropsied. Selected organs were fixed in 10% neutral buffered formalin (ovaries were fixed in Bouin's fixative) and embedded in paraffin, and sections were stained with hematoxylin and eosin. Male reproductive tissues were embedded in glycol

methacrylate treated with periodic acid-Schiff's reagent and counterstained with hematoxylin. Sperm motility was assessed for rats and mice.

STATISTICAL METHODS

For fertility data, which are expressed as proportions, the Cochran-Armitage test (Armitage, 1971) was used to test for dose-related trends. Each dose group was compared to the control group with Fisher's exact test. The number of litters and the number of live pups per litter were determined per fertile pair and then treatment group means were determined. The proportion of live pups was defined as the number of pups born alive divided by the total number of pups produced by each pair. The sex ratio was expressed as the number of male pups born alive divided by the total number of live pups born to each fertile pair. Dose group means were analyzed for overall differences by the Kruskal-Wallis test (Kruskal and Wallis, 1952) and for ordered differences by Jonckheere's test (Jonckheere, 1954). Pairwise comparisons were made with the Wilcoxon-Mann-Whitney U test (Mann and Whitney, 1947).

An analysis of covariance (Neter and Wasserman, 1974) was performed to correct for the potential effect of number of live and dead pups per litter on average pup weight. Least-squares were tested for overall equality with an F test and for pairwise equality with a *t*-test. Possible sex differences were controlled by analyzing data for each sex and then for males and females, combined. For organ weights, analysis of covariance was also used to adjust for body weight; unadjusted weights were analyzed with the Kruskal-Wallis and Wilcoxon-Mann-Whitney U tests. Possible dose-related trends were tested by Jonckheere's test.

Results

RATS

No clinical signs of toxicity were noted in exposed F_0 rats. Mean body weights of rats exposed to 378 ppm were slightly less than those of the controls but were within 10% of the control weights throughout the 18-week period of study. Water consumption by males and females decreased with increasing dose. Fertility, number of litters per pair, live pups per litter, proportion of pups born alive, number of female pups per litter, and cumulative days to litter were not affected by administration of the chemical mixture. The number of male pups, male pups per litter, and live pup weights were slightly decreased in the high-dose group (Table D1).

For the F_1 generation, initial body weights of exposed pups were decreased, and the body weights of F_1 rats in the 189 and 378 ppm groups remained lower than those of the controls throughout weaning

(Table D2); mean body weights of rats exposed to 378 ppm remained lower than those of the controls throughout adulthood. For F_1 rats, fertility, mating, and pregnancy indices, number of live F_2 pups per litter, proportion of live pups, and sex of live pups were not affected by exposure. The mean F_2 pup weight in the high-dose group was 12% lower than that of the controls (Table D3). Epididymis and testis weights of males in the 378 ppm group were less than those of the controls but were appropriate for the lower body weights in this group (Table D4). Sperm motility and morphology data and estrous cycles of exposed and control rats were similar.

MICE

There were no clinical signs of toxicity in mice; however, water consumption by mice exposed to 189 or 378 ppm was decreased. There were no significant differences in fertility, number of litters per pair, live male pups per litter, proportion of pups born alive, and pup weight between exposed and control groups (Table D5). The number of female pups per litter was decreased in the 189 and 378 ppm groups (Table D6). The cumulative days to litter and the parental body weights, testis weight, spermatid head count, and estrous cycle length of exposed and control mice were similar.

For the F_1 pups, postnatal survival, organ and body weights, feed consumption, mating and fertility indices, number of live F_2 pups per litter, and sex ratio were not affected by administration of the chemical mixture (Tables D7 and D8). Water consumption by exposed mice was decreased at all dose levels. The pup weight of the high-dose group, when adjusted for litter size, was 9% lower than that of the control group. The number of live F_2 female pups per litter was significantly decreased in the high-dose group. Sperm concentration was decreased in high-dose males (Table D8). Exposed F_1 females had a greater incidence and severity of liver inflammation (control, 3/29; 378 ppm, 19/19), and the incidence, but not the severity, of nephropathy was increased in exposed mice (males: control, 4/20, 378 ppm, 9/20; females: control, 5/20, 378 ppm, 17/20). The liver lesions consisted of foci of inflammatory cells (sometimes associated with necrotic or degenerative hepatocytes), which often had a central aggregate of macrophages forming a typical microgranuloma.

Discussion

The results indicate that exposure of CD-1 Swiss mice or Sprague-Dawley rats to the chemical mixture had only slight effects on reproductive competence in animals that also had evidence of maternal toxicity, including increased incidences of hepatic inflammation and nephropathy in mice in the F_1 generation and poor growth of F_1 rat pups during the preweaning period.

Some specific effects deserve comment. The number of F_1 male rat pups in the highest dose group was statistically lower than that in the controls, and pup weights were reduced. However, the total number of pups per litter was not different than that in the controls; this effect on the number of male pups was not seen in the offspring of the F_1 generation and thus may not be biologically significant. In the mouse study, there were decreases in the numbers of female pups in both the F_0 and F_1 generation litters. As in the rat study, these decreases did not result in overall decreases in the total number of pups per litter and did not increase in magnitude in the F_1 generation litters, which suggests that the effect may not be biologically important.

Although many of the chemicals in the mixture are known to adversely affect reproduction when administered in sufficient amounts, there is little evidence for consistent adverse reproductive effects under the conditions of these studies. These findings extend and are in general agreement with those of Chapin *et al.* (1989), who found few or no effects of consumption of the chemical mixture on spermatogenesis in B6C3F_1 mice.

TABLE D1 Fertility and Reproductive Performance Data for F_0 Sprague-Dawley Rats in the Continuous Breeding Study of a Chemical Mixture of 25 Groundwater Contaminants[1]

Study Parameters	0 ppm	38 ppm	189 ppm	378 ppm
Fertile pairs/cohabiting pairs	40/40	20/20	20/20	19/20
Litters/pair	4.3 ± 0.2	4.3 ± 0.2	4.2 ± 0.3	4.6 ± 0.2
Live pups/litter	13.7 ± 0.3	12.5 ± 0.4*	13.2 ± 0.4	12.8 ± 0.6
Males/total pups	0.50 ± 0.01	0.50 ± 0.02	0.50 ± 0.02	0.45 ± 0.02*
Proportion of pups born alive	0.96 ± 0.01	0.94 ± 0.02	0.96 ± 0.01	0.96 ± 0.02
Live pup weight (g)	6.42 ± 0.06	6.47 ± 0.07	6.18 ± 0.13	6.07 ± 0.09*

[1] Except for number of fertile pairs/cohabiting pairs, data are presented as mean ± standard error. Pairs were considered fertile if they produced one or more litters.
* Significantly different (P<0.05) from the control group.

TABLE D2 F_1 Sprague-Dawley Rat Pup Weights in the Continuous Breeding Study of a Chemical Mixture of 25 Groundwater Contaminants[1]

Age (days)	0 ppm	38 ppm	189 ppm	378 ppm
MALE				
0	7.10 ± 0.15	7.01 ± 0.14	6.90 ± 0.17	6.54 ± 0.21*
4	12.65 ± 0.43	12.36 ± 0.47	11.03 ± 0.48*	10.30 ± 0.58*
7	18.94 ± 0.77	18.13 ± 0.67	16.66 ± 0.96	14.39 ± 0.99*
14	36.50 ± 1.44	34.47 ± 1.52	31.52 ± 1.71*	26.78 ± 2.44*
21	59.72 ± 2.42	56.64 ± 2.51	50.18 ± 2.79*	41.57 ± 2.99*
Necropsy[2]	589 ± 16	—	—	497 ± 11*
FEMALE				
0	6.60 ± 0.14	6.57 ± 0.14	6.55 ± 0.17	5.97 ± 0.19*
4	11.62 ± 0.39	11.09 ± 0.34	10.43 ± 0.45	9.77 ± 0.66*
7	17.57 ± 0.70	15.61 ± 0.56	15.67 ± 0.69	13.75 ± 1.08*
14	34.47 ± 1.38	30.40 ± 1.38	30.47 ± 1.33	26.60 ± 2.10*
21	55.55 ± 2.27	50.20 ± 1.95	48.68 ± 2.29*	39.98 ± 2.88*
Necropsy	381 ± 9	—	—	336 ± 7*

[1] Pup weights are given in grams (mean ± standard error). For controls, n=23 to 25; for exposed groups, n=12 to 17.
[2] Necropsy at 90 ± 10 days for control and 378 ppm groups; other dose groups were terminated at Day 21.
* Significantly different (P<0.05) from the control group.

TABLE D3 Fertility and Reproductive Performance Data for F_1 Sprague-Dawley Rats in the Continuous Breeding Study of a Chemical Mixture of 25 Groundwater Contaminants

Study Parameters	0 ppm	378 ppm
Females with copulatory plugs/cohabiting pairs	18/20	14/20
Mating index (%)	90	70
Fertile pairs/females with copulatory plugs	16/18	13/14
Fertility index (%)	88	92
Live pups/litter[1]	16.3 ± 0.4	15.9 ± 0.6
Proportion of pups born alive[1]	0.98 ± 0.01	1.00 ± 0.0*
Males/total pups[1]	0.52 ± 0.03	0.46 ± 0.05
Live pup weight[1] (g)	5.99 ± 0.14	5.28 ± 0.25*
Adjusted live pup weight[1] (g)	6.05 ± 0.06	5.22 ± 0.18*

[1] Data are given as mean ± standard error.
* Significantly different (P<0.05) from the control group.

TABLE D4 Organ Weights and Sperm Parameters for F_1 Sprague-Dawley Rats in the Continuous Breeding Study of a Chemical Mixture of 25 Groundwater Contaminants[1]

Study Parameters	0 ppm	378 ppm
MALE		
Necropsy body wt (g)	589 ± 17	498 ± 12*
Testis wt (mg)	1822 ± 75	1662 ± 43*
Epididymis wt (mg)	583 ± 10	536 ± 13*
Sperm concentration (10^6/g cauda epididymal tissue)	586 ± 15	589 ± 22
Sperm motility (%)	84 ± 2	86 ± 2
Abnormal sperm (%)	0.8 ± 0.1	0.7 ± 0.1
FEMALE		
Necropsy body wt (g)	381 ± 9	336 ± 7*
Ovary wt (mg)	56.6 ± 2.7	54.9 ± 2.7

[1] Data are presented as mean ± standard error. For control and exposed groups, n=20.
* Significantly different (P<0.05) from the control group.

TABLE D5 Fertility and Reproductive Performance Data for F_0 CD-1® Swiss Mice in the Continuous Breeding Study of a Chemical Mixture of 25 Groundwater Contaminants[1]

Study Parameters	0 ppm	38 ppm	189 ppm	378 ppm
Fertile pairs/cohabiting pairs	38/40	19/20	19/20	19/20
Litters/pair	4.8 ± 0.1	4.8 ± 0.1	4.9 ± 0.1	4.9 ± 0.1
Live pups/litter	13.4 ± 0.4	13.6 ± 0.6	13.0 ± 0.5	12.1 ± 0.5*
Males/total pups	0.49 ± 0.01	0.49 ± 0.01	0.52 ± 0.01*	0.53 ± 0.01*
Proportion of pups born alive	0.95 ± 0.01	0.98 ± 0.01	0.98 ± 0.01	0.97 ± 0.02
Live pup weight (g)	1.58 ± 0.01	1.60 ± 0.03	1.60 ± 0.03	1.56 ± 0.03

[1] Except for number of fertile pairs/cohabiting pairs, data are presented as mean ± standard error. Pairs were considered fertile if they produced one or more litters.
* Significantly different ($P<0.05$) from the control group.

TABLE D6 Average Number of Live F_1 Female CD-1® Swiss Mouse Pups per Litter in the Continuous Breeding Study of a Chemical Mixture of 25 Groundwater Contaminants[1]

Litter	0 ppm	38 ppm	189 ppm	378 ppm
1	5.5 ± 0.4	5.8 ± 0.5	5.9 ± 0.4	5.3 ± 0.4
2	6.6 ± 0.4	6.6 ± 0.4	6.0 ± 0.5	5.7 ± 0.4
3	7.3 ± 0.3	7.5 ± 0.6	6.3 ± 0.5	6.0 ± 0.5*
4	7.8 ± 0.4	7.9 ± 0.8	6.6 ± 0.5	5.5 ± 0.4*
5	7.5 ± 0.7	7.4 ± 0.7	6.2 ± 0.7	6.1 ± 0.6
Combined	6.9 ± 0.2	7.0 ± 0.3	6.2 ± 0.3*	5.7 ± 0.3*

[1] Data are presented as mean ± standard error. n=38 for controls; n=19 for exposed groups.
* Significantly different ($P<0.05$) from the control group.

TABLE D7 Fertility and Reproductive Performance Data for F_1 CD-1® Swiss Mice in the Continuous Breeding Study of a Chemical Mixture of 25 Groundwater Contaminants

Study Parameters	0 ppm	378 ppm
Females with copulatory plugs/cohabiting pairs	16/20	14/19
Mating index (%)	85	74
Fertile pairs/females with copulatory plugs	17/16	15/14
Fertility index (%)	106	107
Live pups/litter[1]	12.4 ± 0.9	11.5 ± 0.6
Proportion of pups born alive[1]	1.00 ± 0.0	1.00 ± 0.0
Males/total pups[1]	0.44 ± 0.03	0.50 ± 0.03
Live pup weight[1] (g)	1.58 ± 0.05	1.47 ± 0.03
Adjusted live pup weight[1] (g)	1.58 ± 0.03	1.44 ± 0.03*

[1] Data are given as mean ± standard error.
* Significantly different (P<0.05) from the control group.

TABLE D8 Organ Weights and Sperm Parameters for F_1 CD-1® Swiss Mice in the Continuous Breeding Study of a Chemical Mixture of 25 Groundwater Contaminants

Study Parameters	0 ppm	378 ppm
MALE		
Necropsy body wt (g)	37.8 ± 1.0	36.4 ± 0.9
Seminal vesicle wt (mg)	341 ± 14	374 ± 21
Testis wt (mg)	129 ± 4	136 ± 4
Epididymis wt (mg)	37.9 ± 0.9	36.2 ± 1.7
Sperm concentration (10^6/g cauda epididymal tissue)	895 ± 55	715 ± 52*
Sperm motility (%)	62 ± 6	62 ± 5
Abnormal sperm (%)	7.0 ± 0.8	8.2 ± 0.8
Spermatid head count (10^7/g testis)	10.5 ± 0.5	8.3 ± 0.5*
FEMALE		
Body wt (g)	29.9 ± 0.6	29.4 ± 0.9
Uterus wt (mg)	261.0 ± 19.0	291.0 ± 21.0
Ovary wt[2] (mg)	42.60 ± 2.27	46.48 ± 3.79

[1] Data are presented as mean ± standard error. For control and exposed groups, n=20.
[2] Ovaries were weighed with oviduct attached.
* Significantly different (P<0.05) from the control group.

APPENDIX E

Genetic Toxicology

Toxicity and Mutagenicity of a Mixture of 25 Chemicals Found in Contaminated Groundwater

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Lyon, International Agency for Research on Cancer

Toxicity and mutagenicity of a mixture of 25 chemicals found in contaminated groundwater

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Summary. A defined mixture of 25 chemicals that are often found in contaminated groundwater was prepared as an aqueous solution and studied for mutagenicity in bacteria, for prophage induction in bacteria, for palatability and effect on weight-gain in rats and mice, and for cytogenetic effects in bone marrow cells of rats and mice. The bacterial mutation and prophage induction tests were negative. Exposure to the mixture in drinking water for two weeks resulted in a concentration-related decrease in water consumption in male and female rats and mice. Concentration-related decreases in weight gain were observed in male and female mice; in rats, only the high-dose groups showed decreased weight gains. A small but significant increase in sister chromatid exchanges was seen in male mice and a similar weak effect on micronucleated polychromatic erythrocytes (PCE) in the bone marrow of males and females. Also in bone marrow of male and female mice, an increase in mitotic index and a decrease in average cell generation time was observed. The % PCE in bone marrow was decreased in female mice only, while the % PCE in peripheral blood was increased in both sexes. In rats, the only effects observed in the cytogenetic studies were increased PCE frequencies in the peripheral blood of males and in the bone marrow of males and females. These results indicate that the 25-chemical mixture studied is not genotoxic in bacteria and that a concentration-dependent effect on its palatability to rodents leads to reduced water consumption, food consumption and weight gain. Although the bone marrow effects may be associated with disruptions of normal erythropoiesis that, in turn, alter the cytogenetic end-points reported, elevations of SCE and MN-PCE in mice suggest the 25-chemical mixture, under the conditions of administration, leads to cytogenetic damage in the bone marrow. Such damage may indicate a potential health hazard.

Introduction

One of the most critical environmental issues today is groundwater contamination (NRC/NAS, 1984; OTA, 1984a,b; EPA, 1986; Valiulis, 1986; Urbain, 1987).

Approximately 50% of the population in the United States depends upon groundwater as its primary source of drinking water and domestic water supplies. Despite the importance of groundwater as a vital resource, and the growing awareness of the serious level of groundwater contamination, little or no information is available on the health effects of the contaminants, particularly as a multiple chemical mixture.

Under an interagency agreement with the Agency for Toxic Substances and Disease Registry, the National Toxicology Program (NTP) has been studying chemicals and chemical mixtures that are found at or near hazardous waste disposal sites. Because it is not possible to obtain a representative sample of the more than 25 000 known hazardous waste disposal sites, the NTP is studying the health effects of a chemically defined mixture of groundwater contaminants frequently detected near hazardous waste disposal sites, to complement the efforts by other laboratories that concentrate their effort on binary chemical mixtures or on complex chemical mixtures of uncertain composition.

The origin of a toxicology programme on chemical mixtures simulating groundwater contaminants at the National Institute of Environmental Health Sciences (NIEHS)/NTP, the rationale of investigating a chemically defined mixture containing 25 organic and inorganic compounds, the guidelines used to select the chemicals, the theoretical exercises leading to the setting of target dose levels, and the experimental approach and design have been reported in detail previously (Yang & Rauckman, 1987). Results from initial work on the chemistry and on the suitability of the mixture for toxicological studies, immunotoxicity, male reproductive toxicology and other aspects of health effects have been published elsewhere (Chapin *et al.*, 1989; Germolec *et al.*, 1989; Hong *et al.*, 1989; Simmons *et al.*, 1989; Yang *et al.*, 1989a,b,c,d) and are summarized by Schwetz and Yang in this volume.

We present here the results of tests for mutagenicity and prophage induction in bacterial cells and 14-day studies of water and food consumption, weight gain and cytogenetic effects in bone marrow cells of rats and mice.

The mixture was evaluated for mutagenic activity in the *Salmonella* plate-incorporation assay (Maron & Ames, 1983) using strains TA98 and TA100 in the presence and absence of Aroclor 1254-induced rat liver S9. In addition, the ability of the mixture to cause DNA damage was examined by determining the ability of the mixture to induce prophage lambda in *Escherichia coli* using a micro-suspension assay developed by Rossman *et al.* (1984). Previous studies (Houk & DeMarini, 1987, 1988) have shown that this assay responds to some carcinogenic chlorinated compounds that are not detected in the conventional *Salmonella* assay.

For assessment of possible genotoxic or cytotoxic damage in bone marrow of mice and rats, endpoints evaluated included the frequency of chromosomal aberrations (CA), of sister chromatid exchanges (SCE) and of micronucleated (MN) erythrocytes, as well as the rate of cellular proliferation and of erythropoiesis. This spectrum of endpoints has been used previously by the NTP to characterize the *in vivo* genotoxic/cytotoxic potential of single agents such as methyl isocyanate (Tice *et al.*, 1986) and butadiene (Tice *et al.*, 1987).

Table 1. Comparison of concentrations of the components of the 25 chemical mixture dosing solutions with EPA survey results

	Average EPA Survey (ppm)	Dose levels[b] (ppm)		
		Low dose (1% stock)	Medium dose (10% stock)	High dose (20% stock)
Acetone	6.9	5.3	53	106
Arochlor 1260	0.21	0.001	0.01	0.02
Arsenic	30.6	0.9	9	18
Benzene	5.0	1.25	12.5	25
Cadmium	0.85	5.1	51	102
Carbon tetrachloride	0.54	0.04	0.4	0.8
Chlorobenzene	0.1	0.01	0.1	0.2
Chloroform	1.46	0.7	7	14
Chromium	0.69	3.6	36	72
Diethylhexyl phthalate	0.13	0.0015	0.015	0.03
1,1-Dichloroethane	0.31	0.14	1.4	2.8
1,2-Dichloroethane	6.33	4	40	80
1,1-Dichloroethylene	0.24	0.05	0.5	1
1,2-*trans*-Dichloroethylene	0.73	0.25	2.5	5
Ethylbenzene	0.65	0.03	0.3	0.6
Lead	37.0	7	70	140
Mercury	0.34	0.05	0.5	1
Methylene chloride	11.2	3.75	37.5	75
Nickel	0.5	0.68	6.8	13.6
Phenol	34.0	2.9	29	58
Tetrachloroethylene	9.68	0.34	3.4	6.8
Toluene	5.18	0.7	7	14
1,1,1-Trichloroethane	1.25	0.2	2	4
Trichloroethylene	3.82	0.65	6.5	13
Xylenes	4.07	0.16	1.6	3.2

[b] The concentrations of each of the 25 components in the dosing solutions presented here are nominal values. With the exception of acetone, phenol and all six metals, all other chemicals had less (approximately 40% or less) than quantitative recoveries. Therefore, the actual intake by the animals is less than these theoretical values presented. For details of analytical results and the basis for using this mixture in animal studies, see Yang *et al.* (1989a,d).

[a] Survey conducted for the US EPA by Lockheed Engineering and Management Services Co., Inc., in July 1985. The data-base includes analyses of groundwater samples in the vicinity of 180 hazardous waste disposal sites located throughout the USA.

Materials and methods

Chemical mixture

The formulation, composition and analysis of a stock solution in deionized water of the 25-chemical mixture were given in detail by Yang *et al.* (1989a). The concentrations of individual components in this stock are approximately at their respective 90% saturation levels. This stock solution is too concentrated to be used for animal experimental work because of low palatability (leading to a 90% decrease in water

consumption) and toxicity (Yang *et al.*, 1989d). Therefore, in the present studies, a 20% solution of the stock was the highest dose used; the other doses were 10% and 1% of the stock (Table 1).

For the present studies, two substocks were prepared by Midwest Research Institute (a chemistry support contractor for the NTP), Kansas City, MO, and shipped to Battelle Columbus (the laboratory conducting animal toxicology studies) in multiple sets of containers (all amber glass, Teflon construction). Substock A contained all organic components except phenol and substock B contained the metal salts plus phenol. To obtain the high-dose solution (20%), the contents of a bottle (1 litre) of substock B were poured into a glass container and diluted to 5 litres with deionized water. A glass vial containing the neat organic substock (substock A) was uncapped, its contents were gently added to the 5 litres of diluted substock B solution, and the substock A vial was immersed in the mixture to ensure quantitative transfer of the contents. The container, selected to contain 5 litres to minimize headspace and prevent the loss of volatile organic compounds, was immediately sealed. The mixture was stirred (magnetic stirrer) until all organic components were dissolved (approximately 1 to 2 h). The lower doses (10% and 1%) were prepared by diluting the high dose (20%) solution with deionized water.

Dose analysis

One set of samples which consisted of all three mixture concentrations for both freshly prepared samples and samples kept in animal cages for 48 h were analysed for three marker chemicals, acetone, 1,2-dichloroethane and lead acetate. Earlier analytical studies of solutions under simulated animal cage conditions revealed that some loss of organic volatiles occurred during mixing of the substocks and during the first 24 h after preparation. However, the concentations of acetone, phenol, and all of the metals remained constant during preparation. Solutions held under simulated animal cage conditions for 96 h showed losses of the organic volatiles, the majority of which occurred within the first 24 h (Yang *et al.*, 1989a).

Animals and animal care

Fischer 344 rats and B6C3F$_1$ mice were produced under barrier conditions at Taconic Farms Inc. (Germantown, NY) under NTP contract. They were transferred to Battelle (Columbus Division, Columbus, OH) at approximately four weeks of age. Following quarantine and quality control assessment, the animals were placed on studies at 5 to 6 weeks of age. Each animal was identified with a unique number by toe clipping. A computerized randomization process, based on animal body weights, was employed to distribute animals into treatment groups.

The animals were housed five per group (rats) or individually (mice) in polycarbonate cages with hardwood chips. Feed (Ziegler Bros. NIH-07 Open Formula diet) and water were given *ad libitum*. Animal rooms were maintained at 21–24°C and 35–65% relative humidity. There were a minimum of 10 changes of room air per hour and a fluorescent light cycle of 12 h per day.

Dose levels and study design

Groups of 10 animals of each sex were given drinking water containing 0%, 1%, 10% or 20% of the mixture stock (see Yang *et al.*, 1989a) as shown in Table 1. Drinking water exposure was continuous for two weeks using dark glass bottles fitted with Teflon-lined caps and stainless steel sipper tubes. The head space was kept to a minimum. Body weight of each animal was recorded on days 0, 1, 4, 7 and 14 of the study. Fresh dosing solutions or deionized water (for the control group) were prepared and changed every Monday, Wednesday and Friday. Twice per week, the 24-hour water consumption for each animal was measured. Food consumption of animals was measured weekly. Clinical observations were made twice daily for all animals. Except in the cytogenetic studies, these animals were not necropsied nor did they undergo histopathological examinations.

***Salmonella* assay**

The *Salmonella* plate-incorporation assay was performed as described by Maron and Ames (1983). Bacterial strains were obtained from Dr Bruce N. Ames (Department of Biochemistry, University of California, Berkeley, CA). The 20% stock of the mixture was filter-sterilized (0.45 μm filter) and assayed twice, each in duplicate, in strains TA98 and TA100 in the presence and absence of Aroclor 1254-induced Sprague–Dawley rat liver S9 (1 mg of protein/plate). Positive controls were dissolved in dimethylsulfoxide (DMSO) and consisted of 2-aminoanthracene at 0.5 μg/plate in the presence of S9 for both strains, 2-nitrofluorene at 3 μg/plate for TA98 in the absence of S9, and sodium azide at 3 μg/plate for TA100 in the absence of S9. Solvent controls consisted of DMSO (100 μl/plate) for comparison to the positive controls and H_2O (100 μl/plate) for comparison to the mixture.

Prophage-induction assay

The Microscreen prophage-induction assay developed by Rossman et al. (1984) was performed essentially as described by Houk and DeMarini (1987, 1988). The lambda lysogen WP2$_s$(λ) (*lon*$_1$, *sulA*$_1$, *trpE*$_{65}$, *uvrA*$_{155}$, *lamB*$^+$) was derived from *E. coli* B/r and was obtained from Dr Evelyn M. Witkin via Dr Anne C. Frazer (Department of Microbiology, New York University Medical Center, New York). The indicator strain TH-008 (streptomycin^r) was derived from *E. coli* C and was obtained from Dr Frazer. Single-colony isolates of each strain were grown overnight in nutrient broth (Oxoid No. 2) to stationary phase. For prophage induction, an aliquot of an overnight culture of WP2$_s$(λ) was diluted 1:40 in Vogel–Bonner minimal medium supplemented with 0.2% glucose and 20 μg/ml of tryptophan and grown to mid-log phase (2×10^8 cells/ml). Cells were then centrifuged at 10 000 rpm for 10 min and resuspended in 10 times the original volume of fresh supplemented minimal medium to give a final concentration of $\sim 2 \times 10^7$ cells/ml.

The microsuspension assay was then performed as described by Houk and DeMarini (1988) except that four layers of plastic wrap (Saran Wrap, Dow Chemical Co.) were placed over each microtitre plate before the lid was placed tightly on each

plate. Plates were not placed in plastic bags. The S9 used for the *Salmonella* assay was also used for the prophage-induction assay; however, the S9 mix contained only 2.5% S9.

The microtitre plates were incubated overnight at 37°C, after which the concentration of lambda phage was determined by sampling 50 μl from each well of the microtitre plate and diluting the sample in 5 ml of supplemented minimal medium. After vortexing the tubes, 100 μl from each tube was added to top agar (supplemented with 10 mM $MgSO_4$) along with 200 μl of log-phase indicator cells (TH-008), which had been prepared by diluting an overnight culture of TH-008 1:40 in Oxoid No. 2 and growing the culture until it reached a concentration of $\sim 2 \times 10^8$ cells/ml. The contents of the tubes were vortexed and poured onto bottom agar made of half-strength tryptone medium supplemented with streptomycin (100 μg/ml) to select against the lysogen. Streptomycin was used instead of chloroform, which had been used previously (Houk & DeMarini, 1987, 1988) to kill the lysogen. Plates were incubated overnight at 37°C, and plaques were counted by hand.

Positive controls were dissolved in acetone and consisted of 2-aminoanthracene at 1.25 μg/ml in the presence of S9 and 2-nitrofluorene at 150 μg/ml in the absence of S9. Solvent controls consisted of acetone (5%) for comparison with the positive controls and distilled water for comparison with the mixture. Dilution tubes were sampled in duplicate, and experiments were performed twice. Results for each experiment were expressed as the mean of two plates ± S.E.M.

Cytogenetic studies

The following additional chemicals were used in the cytogenetic studies: bromodeoxyuridine (BrdUrd) tablets (Boehringer Mannheim), colchicine (Eli Lilly), Hoechst 33258 (American Hoechst), Giemsa (Harleco), Metofane® (Pitman-Moore, Inc.), reagent grade corn oil from Sigma, and 7,12-dimethylbenzanthracene (DMBA; CAS No. 56-55-3; Lot No. C9A) from Eastman Kodak.

In mice, a 50 mg BrdUrd tablet coated partially (70%) with paraffin (McFee *et al.*, 1983) was implanted subcutaneously as described by Tice *et al.* (1986, 1987). Two hours before kill time, each animal was injected intraperitoneally with 2 mg/kg colchicine. Mice were killed by cervical dislocation, the rats by CO_2 asphyxiation. Bone marrow for SCE and/or CA evaluation was obtained and processed as described by Tice *et al.* (1986, 1987). Peripheral blood and bone marrow smears for MN and/or polychromatic erythrocyte (PCE) determinations were prepared and fixed as described by Tice *et al.* (1986, 1987). Mouse bone marrow slides for SCE or CA evaluation were differentially stained using a modified (Tice *et al.*, 1978) fluorescence-plus-Giemsa technique (Goto *et al.*, 1978); rat bone marrow slides were stained for 10 minutes with 4% Giemsa. Slides to be evaluated for MN and/or for %PCE were stained with acridine orange as described by Kato (1974). Bone marrow proliferation kinetics (based on 100 randomly selected metaphase cells per mouse; 10 mice per group), SCE frequency (based on 25 metaphase cells per mouse; 8 mice per group), CA frequency (based on 50 metaphase cells per animal; 8 animals per group), the mitotic index (MI; based on 1000 nucleated cells per animal; 10 animals

per group). MN incidence (based on 1000 PCE and/or normochromatic erythrocytes (NCE) per animal; 10 animals per group), and %PCE (based on 1000 peripheral blood erythrocytes or 200 bone marrow erythrocytes per animal; 10 animals per group) were scored as described by Tice *et al.* (1986, 1987). All scoring was conducted without knowledge of the agent or treatment dose. Slides obtained from mice treated with DMBA were included in the study to evaluate scorer reliability.

Statistical analysis

The statistical analyses of the cytogenetic data are described in detail by Tice *et al.* (1986, 1987). Briefly, the level of significance was established at an alpha of 0.05. To determine if data from male and female animals could be pooled, a two-way Brown–Forsythe analysis of variance (ANOVA), which is based on separate group variances, was conducted with dose and sex as factors. If a nonsignificant sex difference was obtained, data were pooled between sexes, otherwise, male and female data were analysed separately. A one-tailed trend test (Margolin *et al.*, 1986), based on individual animal responses, was used to determine if a treatment-related increase occurred for SCE or for the percentage of metaphase cells containing at least 1 chromosomal aberration (excluding gaps). A two-tailed trend test, also using individual animal data, was used to evaluate the effect of treatment on bone marrow cell proliferation (evaluated as the average generation time; AGT), MI or the percentage of PCE in bone marrow or peripheral blood. For MN data, the number of MN-PCE or MN-NCE were summed across animals within each exposure group and analysed by a one-tailed trend test (Margolin & Risko, 1988). If a significant response was obtained, pairwise comparisons between each treatment group and the concurrent control group were conducted to determine the minimal effective dose, using the appropriate one or two-tailed Student's *t*-test (for responses based on individual animal data) or a one-tailed Pearson's chi square test (for MN data). To verify scorer reliability, slides from a study in which mice were treated either once (for SCE and CA) or twice (for MN) with DMBA (100 mg/kg) were randomly included in the appropriate analyses.

Results

Salmonella

Table 2 shows that the mixture was not mutagenic in strains TA98 or TA100 of *Salmonella* in the presence or absence of Aroclor 1254-induced rat-liver S9. Although the mixture did not cause an increase in the number of revertants/plate, the 20% stock was cytotoxic at 1000 μl/plate in the absence of S9, as evidenced by a thinning of the bacterial lawn.

Prophage induction

Table 3 shows that the mixture did not cause DNA damage in *E. coli*, as indicated by its inability to induce prophage lambda in either the presence or absence of Aroclor

Table 2. Mutagenicity of the NTP 25-chemical mixture in *Salmonella*

Dose[a] (μl plate)	Revertants/plate							
	Experiment 1				Experiment 2			
	TA98		TA100		TA98		TA100	
	+S9	−S9	+S9	−S9	−S9	−S9	−S9	−S9
0	33 ± 2.3	19 ± 3.0	131 ± 4.8	120 ± 5.8	42 ± 0.7	24 ± 4.5	134 ± 2.5	125 ± 11.7
5	34 ± 4.0	20 ± 1.0	125 ± 8.0	79 ± 7.5	49 ± 1.0	26 ± 5.5	127 ± 16.5	136 ± 5.0
10	37 ± 0.5	23 ± 4.5	131 ± 6.0	114 ± 12.0	40 ± 1.5	27 ± 5.0	127 ± 15.0	127 ± 17.0
25	35 ± 3.0	24 ± 7.0	129 ± 15.5	111 ± 5.5	42 ± 2.5	20 ± 0.5	146 ± 2.0	121 ± 12.5
50	35 ± 1.5	19 ± 1.5	143 ± 17.0	117 ± 16.5	53 ± 4.5	30 ± 9.0	126 ± 12.5	137 ± 5.5
100	44 ± 4.5	22 ± 5.0	132 ± 7.0	102 ± 1.0	49 ± 8.5	27 ± 6.5	123 ± 11.0	136 ± 21.0
250	35 ± 0.5	31 ± 11.0	109 ± 8.0	128 ± 3.0	60 ± 6.5	29 ± 1.0	143 ± 2.0	129 ± 12.5
500	35 ± 4.5	21 ± 1.5	138 ± 1.5	124 ± 6.5	44 ± 8.0	31 ± 2.5	140 ± 2.0	114 ± 1.5
1000					44 ± 5.5	14 ± 5.0[b]	139 ± 2.0	115 ± 15.0[b]
DMSO (100 μl)	30 ± 2.5	20 ± 2.5	125 ± 2.5	119 ± 2.0	40 ± 2.0	25 ± 3.4	140 ± 3.0	123 ± 3.4
2-AA (0.5 μg)	328 ± 13.3		704 ± 1.5		947 ± 13.4		709 ± 71.1	
2-NF (3μg)		195 ± 4.0				280 ± 5.3		
NaN_3 (3 μg)				1160 ± 15.3				1106 ± 17.6

[a] A 20% stock solution of the mixture was used: results are the average ± S.E.M. of two plates per dose.
[b] Toxic.
2-AA = 2-aminoanthracene; 2-NF = 2-nitrofluorene.

Table 3. Induction of prophage lambda by the NTP 25-chemical mixture

Dose [a] (%)	Plaques/plate			
	Experiment 1		Experiment 2	
	+S9	−S9	+S9	−S9
0	22±2.3	50±6.6	47± 3.0	32± 2.5
0.078	25±4.5	51±6.0	32± 5.0	61± 1.0
0.156	27±1.0	64±6.5	26± 8.0	51±11.5
0.312	13±1.0	38±0.0	27± 2.5	70± 3.5
0.625	12±1.5	57±1.0	18± 2.0	58± 1.5
1.25	15±3.0	Toxic	25± 3.0	91± 9.5
2.5	25±2.5		39± 2.0	Toxic
5.0	31±1.5		49± 3.0	
10.0	35±1.5		56±10.0	
Acetone (5%)	29±4.0	32±0.0	35±7.0	28±2.0
2-AA (1.25 μg/ml)	1188±93.0		1538±26.5	
2-NF (150 μg/ml)		1714±31.5		567±55.5

[a] A 20% stock solution was used; results are the average ± S.E.M. of two plates per dose.
2-AA = 2-aminoanthracene; 2-NF = 2-nitrofluorene.

Table 4. Body weight changes, water and food consumptions of rats and mice exposed to the NTP 25-chemical mixture for 14 days

Parameters	Control	Values as % of control		
		Low dose (1% stock)	Medium dose (10% stock)	High dose (20% stock)
Male rats				
Body weight gain	62.1 g	101.1%	101.6%	83.6%
Water consumption	19.1 g	85.3%	61.3%	48.2%
Food consumption	15.1 g	100%	96%	86.1%
Female rats				
Body weight gain	37.6 g	89.4%	99.2%	80.6%
Water consumption	13.9 g	94.2%	73.4%	55.4%
Food consumption	12.5 g	96.8%	96.8%	87.2%
Male mice				
Body weight gain	2.7 g	96.3%	77.8%	25.9%
Water consumption	4.5 g	84.4%	60.0%	46.7%
Food consumption	4.3 g	100%	95.3%	90.7%
Female mice				
Body weight gain	2.1 g	95.2%	71.4%	4.8%
Water consumption	4.1 g	97.6%	56.1%	39.0%
Food consumption	3.6 g	102.8%	100%	94.4%

1254-induced rat-liver S9. Although the 20% stock mixture did not cause a reproducible, dose-related increase in the number of plaques/plate, it was cytotoxic at 1.25–2.5 μg/ml in the absence of S9, as evidenced by clear, non-turbid wells at these doses in the microtitre plate.

Palatability study

Neither mortality nor clinical signs of toxicity were observed in the animals in these 14-day studies. The data on body weight changes, water and food consumption for rats and mice are summarized in Table 4. Decreases in body weight gain were seen in a dose-dependent manner in mice; marked reduction (74–95%) of body weight gain was seen in the high-dose group. In rats, only the high-dose group had a lower (16–19%) body weight gain. This body weight effect is probably associated with the stress sustained by the rats and mice as a result of a dose-related reduction in the rate of water consumption (Table 4) and somewhat lower rate of food consumption in the high-dose groups. These results indicate that the animals found the chemical mixture solution unpalatable at relatively high levels (i.e., 20% or 10% stock). However, as illustrated in other studies (Germolec *et al.*, 1989), the 10% solution employed as the highest dose level had little effect on body or organ weights in longer-term studies (i.e., 13 weeks).

Chromosomal aberrations

Mouse

Table 5 presents the CA data, providing information on the types of CA observed, the number of CA per cell and the percentage of metaphase cells with at least one aberration. Based on a significant difference in response for CA data between male and female mice ($p = 0.02$), the data were kept separate when evaluated by one-tailed trend test analysis. In both sexes, the chemical mixture did not induce a significant increase in the percentage of damaged cells. The positive control, DMBA at 100 mg/kg, induced a significant increase in aberrations.

Rat

Due to the lack of a significant difference in response for clastogenic damage between male and female rats ($p = 0.64$), the data were pooled and then evaluated by one-tailed trend test analysis (Table 5). When gaps are excluded from the analysis, the increase in CA was non-significant.

Table 5. *In vivo* chromosomal aberration tests in animals exposed to the NTP 25-chemical mixture

Species	Sex	*N*	Dose	Total aberrations G'/G''	B'/B''	RR	CA/cell (−G)	DC (−G)
Mouse	F	8	0	15/0	12/1	2	0.037 ± 0.010	3.75 ± 1.031
		8	1%	32/0	17/0	2	0.047 ± 0.01	3.75 ± 0.881
		8	10%	20/0	8/0	1	0.022 ± 0.010	3.25 ± 0.959
		8	20%	18/0	11/3	1	0.037 ± 0.011	3.00 ± 0.655
Trend *p* value							0.2774	0.1920
Mouse	M	8	0	6/0	5/0	1	0.017 ± 0.010	1.50 ± 0.732
		8	1%	8/0	9/1	0	0.025 ± 0.008	2.50 ± 0.824
		8	10%	15/1	10/2	0	0.030 ± 0.013	2.75 ± 1.065
		8	20%	7/0	1/0	0	0.002 ± 0.002	0.25 ± 0.250
Trend *p* value							0.0873	−0.0740
Rat	F+M	16	0	5/0	9/1	0	0.012 ± 0.004	1.25 ± 0.403
		16	1%	6/0	7/1	1	0.011 ± 0.004	1.12 ± 0.364
		16	10%	11/0	11/3	0	0.017 ± 0.004	1.75 ± 0.403
		16	20%	13/0	11/0	1	0.015 ± 0.006	1.38 ± 0.473
Trend *p* value							0.1366	0.1818
DMBA positive control (mg/kg)								
Mouse	M	8	0	14/0	8/0	0	0.020 ± 0.008	2.25 ± 0.701
		8	100	10/3	43/2	13	1.112 ± 0.163	35.00 ± 2.204
Student *t* test *p* value							0.0001*	0.0001*

G', G'' = chromatid and isochromatid gap, respectively; B', B'' = chromatid and isochromatid break, respectively; RR = rearrangement; CA/cell (−G) = number of aberrations per cell (excluding gaps); %DC (−G) = percentage of cells containing at least one aberration (excluding gaps).
Data are presented as group totals (50 cells were scored per animal) or as group means ± S.E.M.
*p value significant at $\alpha = 0.05$.

Table 6. Micronucleus test results in animals exposed to the NTP 25-chemical mixture

Species	Sex	Dose	MN-PCE/1000 PCE	*N*	Total MN-PCE
Bone marrow					
Mouse	F+M				
MN-PCE		0	1.30 ± 0.231	20	26
		1%	1.70 ± 0.325	20	34
		10%	1.65 ± 0.335	20	33
		20%	2.50 ± 0.394	20	48*
Trend *p* value					0.0079**
Rat	F+M				
MN-PCE		0	1.15 ± 0.300	20	23
		1%	1.45 ± 0.303	20	29
		10%	1.35 ± 0.284	20	27
		20%	1.10 ± 0.261	20	22
Trend *p* value					0.2946
DMBA positive control					
Mouse	M	0	2.40 ± 0.367	5	24
		100 mg/kg	31.40 ± 6.420	5	314
p value					0.0000*
			MN-cell/1000 cells	*N*	Total MN-cells
Blood					
Mouse	F				
MN-PCE		0	0.70 ± 0.153	10	7
		1%	1.50 ± 0.428	10	15
		10%	1.90 ± 0.314	10	19
		20%	1.20 ± 0.359	10	12
Trend *p* value					0.2799
Mouse	M				
MN-PCE		0	2.70 ± 0.517	10	27
		1%	2.40 ± 0.521	10	14
		10%	0.90 ± 0.379	10	9
		20%	1.70 ± 0.300	10	17
Trend *p* value					−0.0218
Mouse	F+M				
MN-NCE		0	1.85 ± 0.284	20	37
		1%	1.85 ± 0.254	20	37
		10%	2.30 ± 0.459	20	46
		20%	2.15 ± 0.342	20	43
Trend *p* value					0.1813

MN-PCE = micronucleated polychromatic erythrocyte; MN-NCE = micronucleated normochromatic erythrocyte. Data are presented as a group means ± S.E.M. among animals or as total micronucleated cells among pooled cells. 1000 cells per animal were scored in each case, except for positive controls (2000/animal).

*One-tailed chi-square test, *p* value significant at $\alpha = 0.05$.

**One-tailed trend test using pooled animal data, *p* value significant at $\alpha = 0.05$.

Bone marrow micronucleated erythrocytes

Mouse
Since there was not a significant difference in response for bone marrow MN-PCE levels between male and female mice ($p = 0.59$), the data were pooled and then evaluated by a one-tailed trend test (Table 6). The frequency of MN-PCE was increased slightly but significantly in these animals, with only the data at the highest dose being elevated significantly over control data. The positive control, DMBA at 100 mg/kg injected twice, induced a significant increase in MN-PCE.

Rat
In rats, bone marrow MN-PCE data (Table 6) for males and females could again be pooled, in the absence of a significant sex difference ($p = 0.36$). Exposure to the chemical mixture in drinking water did not induce a significant increase in MN-PCE.

Peripheral blood micronucleated erythrocytes
MN scoring in peripheral blood was limited to mice (Table 6). On account of a significant interaction between sex and dose for bone marrow MN-PCE data ($p < 0.01$), these data were kept separate when evaluated by a one-tailed trend test analysis. In neither sex did exposure to these chemicals in drinking water result in a significant increase in MN-PCE. Combined peripheral blood MN-NCE data for male and female mice ($p = 0.51$) also did not show a significant increase after exposure.

Bone marrow %PCE

Mouse
On account of a significant interaction between sex and dose for bone marrow %PCE data ($p < 0.01$), the data were kept separate when evaluated by a two-tailed trend test analysis (Table 7). The chemical mixture at all three doses induced a significant decrease in the percentage of PCE in female mice, while having no effect in male mice. The positive control, DMBA at 100 mg/kg injected twice, induced a significant depression in the percentage of bone marrow PCE in mice.

Rat
Similarly, in rats, the data (Table 7) were analysed separately by a two-tailed trend test analysis due to the presence of a significant sex difference in response ($p = 0.01$). Exposure induced a significant increase in bone marrow %PCE in both sexes, with male rats exhibiting a greater response. In a pairwise comparison, only the increase at 20% stock concentration was significantly different from control data.

Peripheral blood %PCE

Mouse
On account of a significant difference in response for the percentage of PCE in peripheral blood between male and female mice ($p < 0.01$), the data were analysed separately by a two-tailed trend test (Table 7). Exposure induced a significant

Table 7. Effect of the NTP 25-chemical mixture on %PCE

Species	Dose	%PCE Female	%PCE Male
Bone marrow			
Mouse	0	68.80 ± 1.995	59.05 ± 3.156
	1%	49.25 ± 2.663*	56.85 ± 2.498
	10%	50.35 ± 3.142*	51.35 ± 3.362
	20%	46.65 ± 4.237*	58.30 ± 2.111
Trend *p* value		0.0048**	0.8058
Rat	0	39.20 ± 1.571	39.60 ± 1.926
	1%	34.95 ± 2.526	40.50 ± 3.433
	10%	41.50 ± 3.189	45.35 ± 2.692
	20%	48.55 ± 1.831*	57.20 ± 0.285*
Trend *p* value		0.0002**	0.0000**
DMBA positive control			
Mouse	0		59.90 ± 4.570
	100 mg/kg		47.30 ± 2.905
p value			0.0242*
Peripheral blood			
Mouse	0	2.22 ± 0.217	2.62 ± 0.209
	1%	2.14 ± 0.156	2.64 ± 0.124
	10%	3.42 ± 0.273*	4.78 ± 0.290*
	20%	3.47 ± 0.324*	5.70 ± 0.275*
Trend *p* value		0.0001**	0.0000**
Rat	0	3.55 ± 0.217	5.11 ± 0.241
	1%	3.25 ± 0.156	5.23 ± 0.162
	10%	3.76 ± 0.273	5.47 ± 0.356
	20%	3.44 ± 0.324	6.09 ± 0.329*
Trend *p* value		0.7403	0.0087**
DMBA positive control			
Mouse	0		59.90 ± 4.570
	100 mg/kg		47.30 ± 2.905
p value			0.0242*

%PCE = percentage of polychromatic erythrocytes, 200 erythrocytes scored per animal.
Data are presented as group means ± S.E.M.
*Two-tailed Student *t* test, *p* value significant at $\alpha = 0.05$.
**Two-tailed trend test, using individual animal data, *p* value significant at $\alpha = 0.05$.
Number of animals was 10 in each case except for positive control experiments, where 5 animals were used.

increase in peripheral blood % PCE in both sexes ($p < 0.01$). Pairwise comparisons indicated a significant difference from control data with both the 10% and 20% stock concentrations.

Rat

Due to a significant difference in response for the percentage of PCE in peripheral blood between male and female rats ($p < 0.01$), the data were analysed separately by a two-tailed trend test analysis. Exposure induced a non-significant increase in peripheral blood % PCE in female rats and a significant increase in male rats, with the minimal effective dose being the 20% stock (Table 7).

SCE and proliferation kinetics

An evaluation of SCE and proliferation kinetics was limited to the bone marrow of mice (Table 8). Due to a significant sex difference in response (SCE, $p < 0.01$; average generation time, $p = 0.04$), the data were evaluated separately by a trend test analysis. Exposure to these chemicals in drinking water induced a slight but statistically significant increase in SCE/cell frequency ($p = 0.02$; significant at alpha = 0.0250) in males, when the highest dose was omitted from the analysis. In female mice, the increase in SCE was non-significant ($p = 0.08$). The positive control, DMBA at 2.5 mg/kg, induced a significant increase in SCE. Mice of both sexes exhibited significant decreases in average generation time with the 10% and 20% stock solutions.

Table 8. Results of SCE and AGT studies of the NTP 25-chemical mixture

			SCE/cell		AGT (h)	
Species	Sex	Dose	Mean ± S.E.M.	*N*	Mean ± S.E.M.	*N*
Mouse	F	0	7.12 ± 0.462	8	14.58 ± 0.347	10
		1%	7.44 ± 0.549	8	14.20 ± 0.448	10
		10%	5.91 ± 0.601	8	12.23 ± 0.264*	10
		20%	9.16 ± 1.282	8	12.69 ± 0.361*	10
Trend *p* value			0.0756		0.0003**	
Mouse	M	0	4.62 ± 0.235	8	15.43 ± 0.268	10
		1%	5.88 ± 0.277*	8	15.10 ± 0.328	10
		10%	5.84 ± 0.319*	8	13.08 ± 0.505*	10
		20%	5.23 ± 0.469	8	12.32 ± 0.466*	10
Trend *p* value			0.2625		0.0001**	
Trend *p* value		(−20%)	0.0195**			

SCE = sister chromatid exchange, 25 cells scored per mouse; AGT = average generation time in hours, 100 metaphase cells scored per mouse.

Data presented as group means ± S.E.M. among *N* animals.

*For SCE data, one-tailed *t*-test; for AGT data, two-tailed *t*-test. *p* value significant at $\alpha = 0.05$

**For SCE data, one-tailed trend test; for AGT data, two-tailed trend test, *p* value significant at $\alpha = 0.05$.

Table 9. Effect of the NTP 25-chemical mixture on mitotic index

Species	Sex	Dose	*N*	Mitotic index (%)
Mouse	F	0	10	2.38 ± 0.232
		1%	10	3.07 ± 0.240
		10%	10	2.84 ± 0.400
		20%	10	3.62 ± 0.221*
Trend *p*-value				0.0176**
Mouse	M	0	10	1.42 ± 0.101
		1%	10	2.07 ± 0.162*
		10%	10	2.69 ± 0.400*
		20%	10	3.38 ± 0.312*
Trend *p*-value				0.0001**
Rat	F+M	0	20	3.20 ± 0.355
		1%	20	3.78 ± 0.481
		10%	20	3.76 ± 0.282
		20%	20	2.34 ± 0.421*
Trend *p*-value				0.0450†

1000 nucleated cells were scored per animal.
Data are presented as group means ± S.E.M. among *N* animals.
*Two-tailed *t* test, *p* value significant at $\alpha = 0.05$.
**Two-tailed trend test, *p* value significant at $\alpha = 0.05$.

Mitotic index

Mouse

Due to a significant difference in response for MI data between male and female mice ($p < 0.01$ as determined by a two-way ANOVA), the data were evaluated separately by a two-tailed trend test analysis (Table 9). Exposure induced in both male and female mice a significant increase in mitotic index ($p < 0.01$ and $= 0.02$, respectively). By pairwise comparison, the lowest effective doses were 20% and 1% for female and male mice, respectively.

Rat

In rats (Table 9), male and female mitotic index data could be pooled due to the lack of a sex difference in response ($p = 0.13$ as determined by a two-way ANOVA). By a two-tailed trend test, drinking contaminated water induced a slight but significant decrease in mitotic index ($p = 0.04$). Only the data at the 20% stock concentration were significantly different from the control group data.

Discussion

The negative results in *Salmonella* (Table 2) were not unexpected because none of the compounds present in the mixture is mutagenic in *Salmonella* when tested alone in the plate-incorporation assay. Similarly, the negative results in the prophage-induction assay in *E. coli* (Table 3) suggest that the chemical mixture is not genotoxic in microbial systems. Although only a few of the individual chemicals present in the

mixture have been tested in the prophage-induction assay, none of those previously tested was positive at the concentrations tested here (Rossman *et al.*, 1984; Houk & DeMarini, 1987; D. M. DeMarini, unpublished results).

Results of the cytogenetic studies are summarized in Table 10. The responses obtained indicate the complexity of *in vivo* systems. Drinking the chemical mixture for 14 days resulted in indications of cytotoxic damage, the nature of which was sex- and species-dependent. In the bone marrow, the average generation time measures the cell cycle duration for the proliferating cell populations, whereas the mitotic index is a measure of the proportion of proliferating cells. The percentage of PCE in bone marrow is a measure of the rate of medullary erythropoiesis, while the percentage of PCE in peripheral blood, at least in the mouse, is a measure of the total rate of erythropoiesis (medullary and extramedullary). In the bone marrow of mice drinking the mixture, the shorter cell cycle duration and increased number of proliferating cells suggest that bone marrow mobilization occurred in response to toxic damage induced early in the treatment period. The % PCE data in peripheral blood and bone marrow of mice are more interesting. While the % PCE data in peripheral blood clearly indicate an increased rate of erythropoiesis, the bone marrow exhibits no change (in male mice) or a depression in erythropoiesis (in female mice). A possible explanation of this apparent discrepancy is the tendency in mice toward extramedullary haematopoiesis under conditions of haematopoietic stress (Boggs *et al.*, 1969; Tice *et al.*, 1987). The extent of this cytotoxic damage is also clearly sex-dependent, with male mice exhibiting a greater response. In rats, the bone marrow exhibited an increased rate of erythropoiesis in both sexes, with the increase in % PCE in peripheral blood being evident in male rats only. Also, in the bone marrow the mitotic index was significantly depressed. A correlation analysis of the percentage of PCE in bone marrow versus peripheral blood revealed the lack of a significant correlation for both male ($r = 0.06$, $p = 0.69$) and female ($r = 0.07$; $p = 0.9$) mice, although in rats there was a significant positive correlation for males ($r = 0.52$, $p < 0.01$) but not for females ($r < 0.01$; $p = 0.97$)

The extent of genotoxic damage was also sex- and species-dependent. There was a slight but significant increase in SCE frequency in male mice and, based upon data pooled between male and female mice, a slight but significant increase in MN-PCE. However, the frequency of chromosomal aberrations was not elevated. MN can arise from both clastogenic and aneuploid-related processes (Heddle *et al.*, 1983), so it is not clear whether in mice the increase in MN in the absence of an increase in CA indicates that the MN arise from aberrant cytokinesis or, considering the magnitude of the MN response, results from differences in the statistical power of the two assays. In rats, the frequency of aberrations, SCE and MN were not significantly increased. However, it should be noted that if gaps were included in the statistical analysis of rat bone marrow clastogenic damage, exposure to these chemicals in drinking water induced a significant increase in the percentage of damaged cells ($p = 0.02$).

In conclusion, the results of this study indicate that the chemical mixture studied was not genotoxic in bacteria. Exposure to dosed drinking water did not produce mortality or clinical signs of toxicity in rats or mice but reductions in weight gain were observed in both species. In cytogenetic studies, manifestations of genotoxic and

Table 10. Summary of cytogenetic test results from mice and rats exposed to a mixture of 25 chemicals in drinking water

Species, sex	BM CA	BM MH-PCE	PB MN-PCE	PB MN-NCE	BM SCE	BM AGT	BM MI	BM %PCE	PB %PCE
Mouse									
F	0		0		0	−	+	−	+++
M	0		0		+[a]	−	+++	0	+++
M+F		++		0					
Rat									
F								+++	0
M								+++	++
M+F	0	0					−		

[a] Highest dose omitted from trend test analysis.

AGT = average generation time; BM = bone marrow; CA = chromosomal aberrations; MN-NCE = micronucleated normochromatic erythrocytes; MN-PCE = micronucleated polychromatic erythrocytes; PB = peripheral blood; %PCE = percentage of polychromatic erythrocytes; MI = mitotic index; SCE = sister chromatid exchange.

0 = not significant at $\alpha = 0.05$; −,+ = a significant decrease or increase, respectively, $0.05 < \alpha < 0.01$; −−,++ = a significant decrease or increase, respectively, $0.01 < \alpha < 0.001$; −−−,+++ = a significant decrease or increase, respectively, $\alpha < 0.001$.

cytotoxic damage were observed, the type and magnitude of which were species-, sex- and dose-dependent. These findings are consistent with other studies of the 25-chemical mixture that identified the bone marrow as a target tissue for the toxic effects of the mixture (Germolec *et al.*, 1989; Hong *et al.*, 1989). The significant decrease in the AGT, the increased MI and the tissue-dependent increase in the percentage of PCE all suggest a rebound effect from initial toxic damage induced early in the exposure period and that extramedullary haematopoiesis is responsible for the increase in the %PCE in the peripheral blood of mice. Although the chromosomal effects observed might be associated with disruptions of normal erythropoiesis that may, in turn, alter the cytogenetic endpoints reported, slight elevations of SCE and MN PCE in mice suggest that exposure to the 25-chemical mixture leads to cytogenetic damage in the bone marrow. Such effects would not be unexpected with a mixture containing known clastogens such as benzene and phenol. The possibility of detecting the effects of these compounds in the genetic toxicity assays employed could be estimated from the lowest effective doses in the genetic activity profiles presented in this volume by Waters *et al.*

The analysis of multiple cytogenetic endpoints, indicative of both genotoxic and cytotoxic damage, provides a comprehensive assessment of the biological activity of the 25-chemical mixture and adds to the evidence of its haematological effects.

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This manuscript has been reviewed by the Health Effects Research Laboratory, US Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Agency, nor does mention of trade names or commercial products constitute endorsement of recommendation for use.

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APPENDIX F

Chemical Characterization and Dose Formulation Studies

Toxicology Studies of a Chemical Mixture of 25 Groundwater Contaminants

I. Chemistry Development

Raymond S. H. Yang, Thomas J. Goehl, Richard D. Brown, Allan T. Chatham, Dora W. Arneson, Ronald C. Buchanan, and Roger K. Harris

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Toxicology Studies of a Chemical Mixture of 25 Groundwater Contaminants

I. Chemistry Development

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National Toxicology Program, National Institute of Environmental Health Sciences, P.O. Box 12233, Research Triangle Park, North Carolina 27709, and †Midwest Research Institute, 425 Volker Boulevard, Kansas City, Missouri 64110

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Toxicology Studies of a Chemical Mixture of 25 Groundwater Contaminants. I. Chemistry Development. YANG, R. S. H., GOEHL, T. J., BROWN, R. D., CHATHAM, A. T., ARNESON, D. W., BUCHANAN, R. C., AND HARRIS, R. K. (1989). *Fundam. Appl. Toxicol.* **13**, 366–376. As part of an effort to evaluate the toxicology of a chemically defined mixture of 25 frequently detected groundwater contaminants, we report here the formulation and analytical chemistry of this mixture. Many problems were anticipated, including limitation of solubility, chemical interactions, and extreme volatility in the aqueous solution of 25 chemicals. The final technically achievable stock solution was prepared based on EPA survey concentrations of these chemicals in groundwater around hazardous waste disposal sites, their toxicity information, and solubility of the individual compounds in the matrix of the aqueous solution of these 25 chemicals. Because the anticipated animal studies were to be conducted at various laboratories, for ease of handling and maximum stability, the stock solution was stored or shipped as two substock solutions: an organic substock with 18 neat organic chemicals in a glass vial sealed with minimum headspace and an aqueous substock solution with 6 metals of various salt forms and phenol. The concentrations of the solutions were such that direct mixing of the organic and aqueous substocks produced the desired high dose level for the animal experiments. Analyses of all 25 chemicals in the drinking water mixture required six different chromatographic and spectroscopic methods. Some loss of organic volatiles during mixing of the substocks and during the first 24 hr following preparation did occur. However, the concentrations of acetone, phenol, and all the metals remained constant during preparation. Solutions held under simulated animal cage conditions for 96 hr showed losses of the organic volatiles; the majority of which occurred within the first 24 hr. This study shows that it is possible to conduct animal experiments on an aqueous mixture containing 25 groundwater contaminants. Furthermore, a reasonable estimate of intake of individual chemicals can be achieved provided that dosing solutions are prepared fresh at frequent intervals (e.g., 48 to 72 hr) and that comprehensive analyses are carried out.

One of the most critical environmental issues today is ground water contamination (NRC/NAS, 1984; OTA, 1984a,b; JAWWA, 1986; Valiulis, 1986; Urbain, 1987). Approximately 50% of the population in this country depends upon groundwater as its primary source of drinking water and domestic water supplies; about 95% of rural Americans depend on groundwater for all their water needs; nearly 75% of American cities derive their supplies, in total or in part, from groundwater (Pye *et al.*, 1983; NRC/NAS,

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1984; OTA, 1984a; Hanson, 1985; Valiulis, 1986). Despite the importance of groundwater as a vital national resource and the gradual awareness of the seriousness of contamination of groundwater, little or no information is presently available on the health effects of groundwater contaminants, particularly as a multiple chemical mixture.

The effort described herein on the formulation and analysis of a 25-chemical mixture represents the initial phase of a toxicology program on chemical mixtures at the National Toxicology Program (NTP). The background information on the genesis of such a program at the National Institute of Environmental Health Sciences (NIEHS), the rationale of investigating a chemically defined mixture containing 25 organic and inorganic compounds, the guidelines used to select the chemicals, the theoretical exercises leading to the setting of target dose levels, and the experimental approach and design were reported elsewhere (Yang and Rauckman, 1987; Yang *et al.*, 1989b,c). The two companion papers report our findings on the immunotoxicology (Germolec *et al.*, 1989) and male reproductive studies (Chapin *et al.*, 1989) on this chemical mixture. Other information regarding enhancement by this mixture of myelotoxicity from irradiation (Hong *et al.*, 1989; Yang *et al.*, 1989b) and of hepatotoxicity from carbon tetrachloride treatment (Simmons *et al.*, 1989; Yang *et al.*, 1989c) have also been reported.

Prior to conducting toxicity studies on a chemical mixture simulating groundwater contaminants, chemistry developmental work had to be initiated to make a stable and fully characterized test sample relevant for toxicological studies. The scope of this initial phase included the formulation of a chemical mixture containing 25 frequently detected groundwater contaminants, dose analysis of each of the 25 chemicals, and stability and chemical speciation studies of the mixture under normal laboratory conditions. This report, a summary of chemical and analytical experiments, is intended to serve as an information base for other investigators interested in this area.

MATERIALS AND METHODS

Reagents

Chemicals used were purchased from various suppliers and they are at the highest purities commercially available in the quantities required. Prior to use, each chemical was analyzed qualitatively to confirm its identity and purity. Analytical methods employed in these bulk chemical analyses included (1) elemental analysis, (2) Fourier Transform nuclear magnetic resonance spectroscopy (FT-NMR), (3) gas chromatography (GC), (4) infrared spectroscopy, (5) Karl Fischer titration, and (6) spark source mass spectrometry. Reagent grade water (18 megohm-cm) was used throughout the formulation process.

Formulation of the Stock Mixture

The stock mixture was prepared from two substocks. The organic substock mixture contained all organic components except phenol. The aqueous substock contained the metal salts plus phenol.

A. Preparation of organic substock solution. First, a solution of the organic components present at target levels less than 10 ppm (Yang and Rauckman, 1987) in the drinking water solution was prepared in toluene. The ratio of components was adjusted so that the final concentration of each would be correct in the drinking water dosing solution at the time of the animal experiment. The toluene solution was prepared by weighing and transferring 160 mg of di(2-ethylhexyl) phthalate (DEHP) and 110 mg of Arochlor 1260 into a 100-ml septum vial. Toluene (88 ml) was added to the vial before the vial was sealed with a Mininert valve. The following volumes of neat chemicals were than added to the toluene mixture using volumetric pipets and/or gas-tight microliter syringes: 2.74 ml carbon tetrachloride, 0.99 ml chlorobenzene, 4.48 ml 1,1-dichloroethylene, and 3.77 ml ethylbenzene. The vial was sealed by closing the Mininert valve; the contents were then dissolved by shaking the vial vigorously.

Second, a solution of all the organic components (except phenol) was prepared by combining the following components in a 50-ml septum vial and sealing with a Miniert valve: 13.42 ml acetone, 2.85 ml benzene, 0.94 ml chloroform, 0.24 ml 1,1-dichloroethane, 6.48 ml 1,2-dichloroethane, 0.40 ml 1,2-*t*-dichloroethylene, 5.65 ml methylene chloride, 0.42 ml tetrachloroethylene, 1.83 ml toluene solution (prepared above), 0.30 ml 1,1,1-trichloroethane, 0.89 ml trichloroethylene, and 0.37 ml xylenes.

Portions (3.33 ml/each) of the organic substock were transferred to individual 4-ml screw-cap vials. The vials were sealed with Teflon-lined screw caps, then packed for storage.

B. Preparation of aqueous substock solution. Arsenic(III) trioxide (720 mg) was transferred to a 2-liter flask containing 1000 ml of water. The mixture was heated and gently boiled for about 45 min until the arsenic trioxide dissolved; the solution was then allowed to cool to room temperature before being diluted to volume with water. The solution was then quantitatively transferred to a 9-liter glass carboy using 1 liter of water. The following chemicals were weighed and transferred to the carboy: 7.20 g cadmium(II) acetate hydrate, 11.10 g chromium(III) chloride hexahydrate, 7.80 g lead(II) acetate trihydrate, 40.8 mg mercuric(II) chloride, 1.80 g nickel(II) acetate tetrahydrate, and 1.80 g phenol. The volume of mixture was brought up to 6.00 liters by the addition of water. A magnetic stirring bar was added to the mixture, which was then stirred overnight. The resulting solution was transferred to 32-oz amber screw-cap (lined with Teflon) bottles for storage.

C. Preparation of the stock mixture by mixing the substocks. To prepare a 2000-ml portion of the chemical mixture stock at the highest technically achievable concentrations (designated hereafter as stock; all dilutions are expressed as percentage stock), two bottles of the aqueous substock (2 liters) were transferred into a large (3 or 4 liter) Erlenmyer flask containing a magnetic stirring bar. After starting the stirrer, one 4-ml vial of the organic substock (containing 3.33 ml) was then emptied into the aqueous substock, followed by repeated rinsing of the vial with the aqueous substock. The organics, upon entering into the aqueous solution, sank to the bottom as a globule. Whitish or cloudy streaks were seen in the globule. Stirring broke the globule up into many droplets which surfaced and formed "oil slicks." It required about a 2-hour stirring (with stopper or cap on) to achieve complete solution. The pH of this solution is approximately 4.5; the solution has a blue and violet tint. No problems were encountered in diluting the stock to obtain lower dose levels.

Analytical Methods for the Drinking Water Mixtures

Each sample was analyzed in two to six replicates by six analytical systems for the 25 mixture components. The analytical systems described below were used to analyze the following groups of components in the chemical mixture stock 96-hr stability study: volatile organics, metals, Aroclor 1260, acetone, phenol, and DEHP. Portions of control samples (i.e., deionized water), when available, were also analyzed for volatile organics and metals only. For other stability studies (i.e., 21-day stability studies) or lower concentration chemical mixtures, minor adjustments of the methods were necessary. The instrument systems were evaluated for linearity of response on each analysis day with standard solutions containing each component at concentrations ranging from about 40 to 120% of the target concentration for the component. The correlation coefficient calculated for each component always exceeded 0.99. The concentrations of the components (μg/ml) in each sample were determined from the linear regression equations computed for the standards, using the internal standard method of calculation. The equations related the concentrations (μg/ml) of each component to the corresponding instrument response (ppm) for that chemical. The calculations were based on the concentrations of chemicals present in the solutions. The concentrations of the standards were verified with independently prepared matrix standards.

A. Volatile organics. The method used for analyzing the volatile organic components in the samples was a modification of EPA Method 624 (Federal Register, 1984). The method involves a helium purge of the water sample, during which the volatile organics are trapped on an adsorbent column. The organics are then flash-vaporized onto a Carbopak B/1% SP-1000 column for gas chromatographic separation and mass spectrometric detection. Primary ions for each volatile organic are monitored, and extracted ion current profiles (EICPs) are used to quantitate the area of each component. The 15 organic components analyzed by this method and their respective retention time in minutes were: benzene (16.0), carbon tetrachloride (12.7), chlorobenzene (23.4), chloroform (10.4), 1,1-dichloroethane (9.1), 1,2-dichloroethane (11.1), 1,1-dichloroethylene (7.9), 1,2-*t*-dichloroethylene (9.9), ethylbenzene (25.1), methylene chloride (5.1), tetrachloroethylene (21.0), toluene (22.3), 1,1,1-trichloroethane (12.3), trichloroethylene (15.5), and xylenes (28.9).

Each sample (25 ml) was mixed with 10 ml of internal standard (benzene-d_6, 97.72 μg/ml in methanol, retention time = 15.8 min) and they were then analyzed using a gas chromatographic system interfaced to a mass spectrometer, as described below:

Gas chromatography: Perkin-Elmer Sigma-3
Column: 1% SP-1000 on 60/80 mesh Carbopak B; 1.8 m × 2 mm i.d., glass
Oven temperature: 45°C for 3 min, then increased from 45 to 220°C at 8°C/min
Detector: Finnigan MAT OWA mass spectrometer, linked via a single-stage glass jet separator to the gas chromatograph
Electron multiplier voltage: −1400 V
Temperatures
Injector port: 220°C
Separator (helium): 250°C
Ion source: 70°C
Scan range: 35–250 amu
Scan rate: 3 sec/scan
Data system: Incos 2400 Data System
Carrier gas: Helium

Flow rate: 30 ml/min
Purge and trap system: Tekmar LSC-2 liquid sample concentrator
Purge gas: Helium
Purge time: 11 min
Trapping adsorbent: Tenax and silica gel
Flash temperature: 180°C
Injection volume: 20 μl

B. Metals. Drinking water samples were analyzed for the following metals by inductively coupled plasma (ICP) emission spectrophotometry: arsenic, cadmium, chromium, lead, mercury, and nickel. Ultrapure concentrated nitric acid (200 μl) was added to each 20-ml portion to make the solutions approximately 1% acid. The solutions were then analyzed by the ICP system described below:

Instrument: Jarrell-Ash Model 1155A ICP-AES
Forward power: 1.15 kW
Reflected power: less than 5 W, minimized
Observation height: Position of maximum signal/noise ratio for cadmium (manufacturer recommended)
Nebulizer type: Fixed crossflow
Coolant gas flow: 15.1 liter/min
Auxiliary gas flow: 0.1 liter/min
Sample gas flow: 0.6 liter/min
Solution uptake: 1.8 ml/min

C. Aroclor 1260. Aliquots (100 ml) of the samples were pipetted into individual 250-ml separatory funnels. Methylene chloride (6 ml) was added to each funnel, and the mixture was shaken for 2 min. The layers were allowed to separate and the organic layer was collected in a 30-ml vial. The extraction procedure was repeated twice with 6-ml vol of fresh methylene chloride, and the organic layers were combined. Three 100-ml portions of undosed water individually spiked with 6 ml of methylene chloride containing Aroclor 1260 at about 1.7 μg/ml level for recovery determination and a 100-ml vol of undosed water were also extracted with the samples. The combined extracts from each individual sample were evaporated to dryness using heat (Pierce Reacti-Therm and Reacti-Vials) and a gentle stream of nitrogen. After the vials cooled to room temperature, 8 ml of hexane and 2 ml of internal standard solution (pentachlorobenzene, 0.4 μg/ml in hexane) were added to the vials, which were then vortexed for 30 sec. Portions of these final solutions were analyzed using the gas chromatographic system described below:

Instrument: Varian 3700 gas chromatograph with autosampler
Column: 3% SP-2250 on 100/120 Supelcoport; 1.8 m × 2 mm i.d., glass
Detector: Electron capture ^{63}Ni
Attenuation: 64 × 10 frequency units/mV
Data System: Nelson 4400 Data System
Temperatures
Injector: 250°C
Oven: 160°C for 4 min, then 160 to 250°C at 10°C/min, hold at 250°C for 11 min
Detector: 300°C
Carrier gas: Nitrogen
Flow rate: 30 ml/min
Injection volume: 6.2 μl
Retention times
Pentachlorobenzene (Internal Standard): 3 min
Aroclor 1260: Many Aroclor 1260 peaks eluted over the time from 11 to 23 min. A single, well-resolved peak eluting at about 16.6 min was chosen for the calculations.

D. Acetone. Aliquots (25 ml) of the samples were mixed with 5 ml of internal standard solution (methyl ethyl ketone, 0.789 mg/ml in water) and diluted to 50 ml with water. Portions of the final diluted solutions were analyzed using the gas chromatographic system described below:

Instrument: Varian 3700 gas chromatograph with autosampler
Column: Porapak Q, 80/100 mesh; 1.8 m × 2 mm i.d., glass
Detector: Flame ionization
Attenuation: 32×10^{-11} A/mV
Data System: Nelson 4400 Data System
Temperatures
Injector: 200°C
Oven: 155°C for 15 min, then 155 to 200°C at 15°C/min, hold at 200°C for 9 min
Detector: 220°C
Carrier gas: Nitrogen
Flow rate: 30 ml/min
Injection volume: 4.8 μl
Retention times
Acetone: 4.1 min
Methyl ethyl ketone (Internal Standard): 9.5 min

E. Phenol. An aliquot (5 ml) of each sample was pipetted into a vial and sealed with a Teflon-lined septum cap. A 100-μl aliquot of internal standard solution [acetophenone, 0.1% (v/v) in methanol] was injected into the solution. Portions of these solutions were filtered (0.45-μm pore size) and analyzed on the high-performance liquid chromatographic system described below:

Instrument: Varian 5020 HPLC System
Injector: Waters WISP 710B
Guard column: Whatman CO:PELL ODS, 20 mm × 2 mm i.d.
Analytical column: Altex Ultrasphere ODS, 5 μ; 250 mm × 4.6 mm i.d.
Mobile phase: Water:methanol:glacial acetic acid (60:40:1, v/v/v)
Flow rate: 1.0 ml/min
Detector: Waters 440

Detection wavelength: 254 nm
Attenuation: 0.2 AUFS
Data System: Nelson 4400 Data System
Injection volume: 20 μl
Retention times
Phenol: 7.9 min
Acetophenone (Internal Standard): 15.4 min

F. DEHP. Aliquots (100 ml) of the samples, in which the pH of the solution was adjusted to over 11 with 1 N sodium hydroxide, were transferred into individual 250-ml separatory funnels. A 10-ml portion of methylene chloride (previously saturated with water) was added to each funnel, and the mixture was shaken for 1 min. The layers were allowed to separate and the organic layer was collected in a vial. The extraction procedure was repeated twice with 10-ml vol of fresh methylene chloride and the organic layers were combined. Three 100-ml portions of undosed water individually spiked with 1 ml of acetone containing DEHP at the 0.015 mg/ml level, for recovery determination, and a 100-ml volume of undosed water spiked with 1 ml of acetone were also extracted with the samples. The combined extracts from each individual sample were evaporated to dryness using heat (Pierce Reacti-Therm and Reacti-Vials) and a gentle stream of nitrogen. After the vials cooled to room temperature, 0.5 ml of acetonitrile and 0.5 ml of internal standard solution (di-*n*-octyl phthalate, about 0.04 mg/ml in acetonitrile) were added to the vials, which were then vortexed for 30 sec. Portions of these final solutions were analyzed using the high-performance liquid chromatographic system described below:

Instrument: Waters M6000A
Injector: Waters WISP 710B
Guard column: Whatman CO:PELL ODS; 20 mm × 2 mm i.d.
Analytical column: Waters Resolve C-18, 5 μ; 150 mm × 3.9 mm i.d.
Mobile phase: Water:acetonitrile (12:88, v/v)
Flow rate: 1.0 ml/min
Detector: Waters 440 with Waters extended wavelength module
Wavelength: 229 nm
Attenuation: 0.1 AUFS
Data system: Nelson 4400 Data System
Injection volume: 35 μl
Retention times
DEHP: 9.5 min
Di-*n*-octyl phthalate (Internal Standard): 11.7 min

Stability Studies on Organic Substock

To assess the stability of organic components under storage conditions, a neat solution of all organic components (except phenol) was prepared and stored at 5°C in 3.7-ml vials, filled to the top and sealed with Teflon-lined screw caps. These samples were stored for 43 and 64 days, respectively, before analysis. A zero-time stock solution was prepared fresh for each analysis day. On each of these analysis days, aliquots (1 ml) of the zero-time and stored solutions were mixed with internal standard (1,2-dichloropropane, 40 μl per 25 ml *n*-undecane) and diluted to 25 ml with *n*-undecane (99%). A gas chromatographic system as described below was used for analyses.

Instrument: Varian 3700 gas chromatograph
Column: DB-624 Megabore, 30 m × 0.53 mm i.d., fused-silica
Film thickness: 3.0 μm
Injection mode: Flash vaporization
Detector: Flame ionization
Temperatures
Injector: 200°C
Oven program: 15°C for 10 min, then increase to 150°C at 5°C/min
Detector: 250°C
Carrier gas: Nitrogen
Flow rate: 10 ml/min
Injection volume: 1.5 μl

DEHP, Arochlor 1260, chlorobenzene, and carbon tetrachloride were not analyzed. Acetone and 1,1-dichloroethylene coeluted in the chromatographic system; therefore, they were treated as one component for calculation purposes. The same was true for chloroform and 1,1,1-trichloroethane, and benzene and 1,2-dichloroethane.

The metals and phenol were chemically stable in the aqueous substock. No specific stability studies were conducted.

Stability Studies on Drinking Water Mixture: 96-hr Test

To simulate the conditions of laboratory animal studies, two chemical mixture water solutions (mixture stock and 1% stock) were placed in inverted 250-ml screw-cap amber glass drinking water bottles fitted with Teflon-lined rubber septa and stainless-steel sipper tubes at room temperature (20–25°C). There was little or no headspace in these filled bottles. At zero time (immediately upon mixing the solution), 24, 48, and 96 hr, the samples were analyzed for all 25 chemicals. Each of the 24-, 48-, and 96-hr samples were from a pooled solution of three bottles. With the exception of the zero-time samples which were analyzed in replicate of six per each sample, all other analyses were performed in duplicate.

Stability Studies on Drinking Water Mixture: 21-Day Test

To investigate the stability of the chemical mixture under two storage conditions [5°C, sealed, in the dark; and

room temperature (20–25°C), sealed, in the dark], two chemical mixture water solutions (50 and 1% stock), following zero-time sampling, were stored under the above two conditions in 1-liter amber glass screw-cap bottles and analyzed periodically. The bottles were sealed, with minimal headspace, with Teflon-lined caps. On Days 7, 14, and 21, samples (previously unopened bottles) from both room temperature and 5°C storage were analyzed for all 25 chemicals. With the exception of the zero-time samples which were analyzed in replicate of six per each sample, all other analyses were performed in duplicate.

RESULTS AND DISCUSSION

Preliminary Attempts and Problems Encountered

Originally, a mixture of 28 chemicals was selected based on surveys of groundwater contamination in the vicinity of hazardous waste disposal sites for EPA (Yang and Rauckman, 1987). In addition to the 25 chemicals listed in Table 1, cyanide, 2,4-dichlorophenoxyacetic acid (2,4-D), and vinyl chloride were also included. At the beginning of this study, the major thrust of the chemistry developmental work was to assess the feasibility of preparing such a mixture and to determine the maximum solubilities of the 28 compounds to be mixed in water.

After looking at the wide variety of components proposed for the mixture, a decision to group the 28 compounds into different subsolutions based upon their physical and chemical properties was made. These subsolutions would then be combined into a final mixture. Throughout feasibility studies, an effort was made to adhere to the ratios of components originally proposed (Yang and Rauckman, 1987). At this stage, the primary method of monitoring insolubilities or incompatibilities was visual observation.

Problems were encountered during initial attempts to formulate the mixture. These included (A) formation of lead chromate because of chemical interaction between lead and chromium trioxide; (B) incompatibility of cyanide (potassium salt) because of evolution of hydrogen cyanide gas in an acidic medium (i.e., the mixture); adjustment of pH to basic range led to the formation of insoluble metal hydroxides; (C) limitation of solubility of most chemicals, particularly 2,4-D, Arochlor 1260, and DEHP; (D) poor analytical recoveries of Arochlor 1260 and DEHP; and (E) the extreme volatility of vinyl chloride. These problems and limitations eventually led to the deletion of cyanide, 2,4-D, and vinyl chloride from the mixture. Because of their ubiquitous presence at the trace level, Arochlor 1260 and DEHP were retained in the mixture at very low concentrations despite the solubility and recovery problems.

Technically Achievable Mixture Stock

Since it was apparent that the originally proposed high target levels (Yang and Rauckman, 1987) were unattainable due to solubility limitation, a final workable mixture solution containing all 25 chemicals was prepared by setting the concentrations of all chemicals at the 90% level of the highest analytically confirmed values from any of the aqueous mixtures prepared during the initial attempts. In most instances, these levels represented the 90% of the saturation levels for the respective chemicals in this deionized water-based mixture. The list of chemicals of this mixture and the final achievable concentrations for each of the 25 chemicals are shown in Table 1. This formulation is the result of the combined consideration of the EPA survey concentrations of these chemicals in the groundwater around hazardous waste disposal sites, the toxicity of individual components, and the solubility of the individual compounds in this unique matrix. We feel that it represents the technically achievable solution and it may be the worst-case scenario as far as drinking water contamination is concerned.

Stability of Organic Substock Under Storage Conditions

As shown in Table 2, analyses of an organic substock solution stored for 43 and 64 days

TABLE 1

A TECHNICALLY ACHIEVABLE STOCK CHEMICAL MIXTURE IN DEIONIZED WATER AND THE CONCENTRATIONS FOR ITS COMPONENTS

	Technically achievable stock chemical mixture (ppm)
Acetone	530
Arochlor 1260[a]	0.1
Arsenic(III)	90
Benzene	125
Cadmium(II)	510
Carbon tetrachloride	4
Chlorobenzene	1
Chloroform	70
Chromium(III)	360
DEHP[b]	0.15
1,1-Dichloroethane	14
1,2-Dichloroethane	400
1,1-Dichloroethylene	5
1,2-trans-Dichloroethylene	25
Ethylbenzene	3
Lead(II)	700
Mercury(II)	5
Methylene chloride	375
Nickel(II)	68
Phenol	290
Tetrachloroethylene	34
Toluene	70
1,1,1-Trichloroethane	20
Trichloroethylene	65
Xylenes[a]	16

[a] These chemicals are mixtures themselves but in this paper they are considered as individual entities.
[b] Di(2-ethylhexyl)phthalate.

at 5°C in screw-cap vials with no headspace showed little changes in the concentrations of the components, as compared to the zero-time samples. Chromatographic profiles of the aged samples and freshly prepared samples appeared to be identical. The profiles of coeluting peaks did not suggest evidence of losses of either component.

Stability of Drinking Water Mixtures Under Simulated Animal Experimental Conditions: 96-hr Test

Table 3 is a summary of the results from the 96-hr stability test of the mixture stock under the simulated rat cage experimental conditions at room temperature. In reviewing the data in Table 3 (and Table 4), it must be emphasized that the zero-time values were derived from the ratios of the analytical findings over their respective theoretical concentrations, while the values of the other time points were derived from comparing the analytical findings at these time points with their respective average zero-time concentrations. Thus, for instance, the zero-time benzene concentration (Table 3) was 89.3% of the theoretical value of 125 μg/ml and the 24-hr benzene concentration was 76.8% (86 μg/ml) of the zero-time concentration (112 μg/ml). In general, the between sample variability among replicates was very small; in the case of the six replicates of zero-time analysis, the standard deviations (SDs) were no more than 4.7% for any of the 25 chemicals (Table 3). While eight chemicals (acetone, phenol, and all six metals) appeared to be stable in water under the testing conditions for 96 hr, target concentrations cannot be maintained for the others. Volatility is certainly a factor for the loss of most organics. Poor recoveries for Arochlor 1260 and DEHP, even at zero time, may be due to their affinity for glassware as reported by Pope and Byrne (1980). The unusually high zero-time concentrations of 147.2 and 127.9% for mercury and trichloroethylene, respectively, may be a reflection of the difficulties in accurately weighing or measuring a small amount of chemicals during the preparation stage. In considering the overall findings in Table 3, the fact that the lowest recovery at 96 hr was 55.6% (Arochlor 1260) of the zero-time concentration and the fact that all of the chemical concentrations remained rather stable between 24 to 96 hr made us believe that this drinking water mixture solution may be used for animal toxicology studies.

Table 4 is a summary of the 96-hr stability study of a diluted drinking water chemical mixture (1% stock). As might be expected, dilution rendered at least three chemicals (Arochlor 1260, DEHP, and mercury) to be at or

TABLE 2

STABILITY OF ORGANIC SUBSTOCK STORED AT 5°C

	Retention time (min)	Percentage of zero time	
		Day 43	Day 64
Acetone and 1,1-dichloroethylene	3.5	97.0	97.9
Methylene chloride	4.3	96.9	96.6
1,2-trans-Dichloroethylene	4.9	97.8	98.4
1,1-Dichloroethane	6.1	101.5	101.2
Chloroform and 1,1,1-trichloroethane	10.1	94.1	95.6
Benzene and 1,2-dichloroethane	12.1	98.6	97.8
Trichloroethylene	14.4	97.1	102.8
Toluene	18.2	98.0	99.8
Tetrachloroethylene	19.6	97.7	100.3
Ethylbenzene	21.8	89.8	96.4
Xylenes	22.2–23.5	99.6	98.6

below detection limits. With the exception of mercury (below detection limit) and some variability of recovery of acetone, the same chemicals (i.e., the other five metals and phenol) which recovered 96% or more in the stock mixture solution at 96 hr (Table 3) also had quantitative recovery in the diluted mixture solution (1% stock). For most other chemicals, there appeared to be better recovery rates at 96 hr in the more diluted mixture solution; probably because all of these chemicals were well below the saturation levels in water. The between sample variability among replicates was larger than those of the mixture stock above; presumably due to difficulties of analyzing some of the chemicals at very low levels. Even though three replicates of zero-time analysis had a SD as high as 21.1% in one case, nevertheless, 14/23 chemicals analyzed had SDs less than 5% (three to six analyses), with an overall range of SDs between 0.2 and 21.1%.

Storage Stability of Drinking Water Mixtures: 21-Day Test

Twenty-one-day stability studies of a chemical mixture at 50% stock and a diluted drinking water mixture at 1% stock were also conducted. The test solutions were kept in sealed amber glass bottles, in the dark, at room temperature (i.e., 20 to 25°C) and at 5°C. Since we already obtained preliminary information on palatability problems in rats and mice with the chemical mixture stock (Yang *et al.*, 1989a), it was considered unlikely that future stock preparation would be made at the full strength. Therefore, the 21-day storage stability study for higher concentration samples was represented by the results from a sample at 50% stock. Because of the huge amount of information (i.e., analytical results for 25 chemicals at two different temperatures and four time points), to conserve space, no data will be presented here. The salient findings are summarized below.

While there were no apparent differences between room temperature and 5°C for the storage of the 50% stock, at lower concentrations (i.e., 1% stock) of the drinking water mixture, the 5°C storage condition appeared to be better. The stability of various chemicals in the drinking water mixture seemed to be better when the chemical concentrations are low (i.e., 1% stock) because 21 out of 23 chemicals (DEHP and mercury not analyzed because of low concentrations) had over 90% recovery at 5°C for 21-day storage. In con-

TABLE 3

SUMMARY OF THE MIXTURE STOCK 96-hr STABILITY STUDIES[a]

	Found/theoretical or zero time (%)[b]			
	Zero time	24 hr	48 hr	96 hr
Acetone	102.8	99.0	100.2	98.8
Arochlor 1260	70.0	83.0	63.6	55.6
Arsenic	100.9	101.4	99.9	99.0
Benzene	89.3	76.8	85.3	78.2
Cadmium	99.5	99.7	99.2	101.6
Carbon tetrachloride	81.3	55.7	75.4	70.5
Chlorobenzene	101.9	81.0	87.2	86.1
Chloroform	94.3	77.3	86.7	81.0
Chromium	98.3	101.3	100.6	102.7
DEHP[c]	56.5	82.5	79.5	77.7
1,1-Dichloroethane	88.6	74.0	85.8	78.1
1,2-Dichloroethane	92.7	82.1	85.4	79.6
1,1-Dichloroethylene	74.7	59.6	74.2	63.1
1,2-trans-Dichloroethylene	83.1	69.6	82.9	73.7
Ethylbenzene	85.2	74.7	84.5	79.8
Lead	99.7	99.9	99.2	101.1
Mercury	147.2	100.2	104.0	96.3
Methylene chloride	100.6	78.3	91.4	83.2
Nickel	98.0	102.7	101.3	101.8
Phenol	99.9	100.2	99.0	98.4
Tetrachloroethylene	77.8	66.6	81.5	74.4
Toluene	90.7	77.2	86.5	80.6
1,1,1-Trichloroethane	88.8	66.1	78.1	69.4
Trichloroethylene	127.9	75.7	88.9	81.5
Xylenes	74.7	78.6	86.1	79.0

[a] To simulate the conditions of laboratory animal studies, the chemical mixture water solutions were placed in inverted 250-ml screw cap amber glass drinking water bottles fitted with Teflon-lined rubber septa and stainless-steel sipper tubes at room temperature (20–25°C). There was little or no headspace in these filled bottles. At zero time (immediately upon mixing the solution), 24, 48, and 96 hr, the samples were analyzed for all 25 chemicals. Each of the 24-, 48-, and 96-hr samples were from a pooled solution of three bottles. With the exception of the zero-time samples which were analyzed in replicate of six samples, all other analyses were performed in duplicate.

[b] The zero-time samples were compared with the theoretical values to derive the percentage values presented here; all other time points were compared to the zero-time values. With the exception of the zero-time samples which were analyzed in replicate of six samples (standard deviations of six analyses ranged between 0.3 and 4.7%), all other analyses were performed in duplicate. For each chemical, a pooled standard deviation and an average test variability limit (95%) were calculated in the stability study; the range for the former is 0.4–4.0% and that for the latter is 1.0–10.8%.

[c] Di(2-ethylhexyl) phthalate.

trast, only 10 out of 25 chemicals had over 90% recovery with the 50% stock chemical mixture at 5°C for 21-day storage. Once again, the same 8 chemicals (acetone, phenol, and all six metals) which were stable under the simulated animal experimental conditions were stable in water under the testing conditions for 21 storage days. In addition, 1,2-dichloroethane and xylenes were also stable under the 21-day storage conditions for the 50% stock.

Since the 21-day stability studies indicated the loss of certain components (mainly volatile organics) over time, in our subsequent an-

TABLE 4

SUMMARY OF THE 1% STOCK 96-hr STABILITY STUDIES[a]

	Found/theoretical or zero time (%)[b]			
	Zero time	24 hr	48 hr	96 hr
Acetone	100.9	89.4	91.4	120.3
Arochlor 1260	24.2	ND	ND	ND
Arsenic	97.9	106.7	102.2	100.5
Benzene	92.4	66.0	91.0	89.3
Cadmium	101.0	105.4	101.9	100.7
Carbon tetrachloride	80.0	36.7	77.6	91.6
Chlorobenzene	102.4	72.1	85.3	80.9
Chloroform	91.9	71.3	93.4	92.5
Chromium	99.8	104.2	100.2	100.0
DEHP[c]	ND	ND	ND	ND
1,1-Dichloroethane	89.5	68.4	94.8	93.2
1,2-Dichloroethane	101.0	79.6	92.1	94.8
1,1-Dichloroethylene	88.6	42.8	97.4	83.1
1,2-trans-Dichloroethylene	96.3	54.2	105.8	82.8
Ethylbenzene	85.5	62.3	83.6	77.9
Lead	97.9	106.9	100.0	101.7
Mercury	ND	ND	ND	ND
Methylene chloride	93.6	—[d]	102.7	92.6
Nickel	98.8	106.1	101.2	100.9
Phenol	101.6	108.3	106.3	103.4
Tetrachloroethylene	94.2	44.7	78.5	69.2
Toluene	96.1	63.4	86.5	82.6
1,1,1-Trichloroethane	91.8	52.2	87.2	93.7
Trichloroethylene	100.9	54.0	84.6	77.5
Xylenes	80.8	60.9	82.2	77.9

[a] To simulate the conditions of laboratory animal studies, the chemical mixture water solutions were placed in inverted 250-ml screw cap amber glass drinking water bottles fitted with Teflon-lined rubber septa and stainless-steel sipper tubes at room temperature (20–25°C). There was little or no headspace in these filled bottles. At zero time (immediately upon mixing the solution), 24, 48, and 96 hr, the samples were analyzed for all 25 chemicals. Each of the 24, 48, and 96 hr samples were from a pooled solution of three bottles. With the exception of the zero-time samples which were analyzed in replicate of six samples, all other analyses were performed in duplicate.

[b] The zero-time samples were compared with the theoretical values to derive the percentage values presented here; all other time points were compared to the zero-time values. With the exception of the zero-time samples which were analyzed in replicate of three to six samples (standard deviations of three to six analyses ranged between 0.2 and 21.1%), all other analyses were performed in duplicate. For each chemical, a pooled standard deviation and an average test variability limit (95%) were calculated in the stability study; the range for the former is 0.2–17.2% and that for the latter is 0.6–49.9%. ND, not determined or calculated because of low concentrations.

[c] Di(2-ethylhexyl) phthalate.

[d] Samples not quantified because of high background.

imal experiments, we chose to store only the organic and aqueous substocks and to use the freshly prepared mixtures for no more than 72 hr.

Conclusions

An aqueous mixture of 25 groundwater contaminants was formulated based upon EPA survey concentrations of these chemicals in the groundwater around hazardous waste disposal sites, their toxicity information, and their solubility in this unique matrix. Analytical methods have been developed for all 25 chemicals in the drinking water mixture. Although some losses of certain organic chemicals were inevitable, the stabil-

ity of this mixture at different concentrations under simulated animal experimental conditions and under storage was such that it is possible to conduct animal experiments. In addition, we believe that reasonable estimates of the intake of individual chemicals may be achieved provided that fresh dosing solutions are prepared at 48- to 72-hr intervals and that comprehensive analyses are carried out.

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APPENDIX G

Neurobehavioral Studies

TABLE G1 Forelimb and Hindlimb Grip Strength Data for F344/N Rats in the 26-Week Drinking Water Study of a Chemical Mixture of 25 Groundwater Contaminants[1]

	0 ppm	11 ppm	38 ppm	113 ppm	378 ppm
MALE					
n	10	10	9	10	10
Forelimb Grip Strength					
Prestudy	0.36 ± 0.02	0.36 ± 0.02	0.39 ± 0.02[2]	0.40 ± 0.02	0.41 ± 0.01
Week 6	0.89 ± 0.03	0.87 ± 0.04	0.94 ± 0.03	0.90 ± 0.03	0.87 ± 0.02
Week 12	1.22 ± 0.03	1.19 ± 0.02	1.24 ± 0.02	1.23 ± 0.02	1.18 ± 0.04
Week 18	1.41 ± 0.02	1.44 ± 0.02	1.46 ± 0.04	1.44 ± 0.04	1.40 ± 0.04
Week 24	1.09 ± 0.05	1.11 ± 0.05	1.16 ± 0.06	1.12 ± 0.03	1.09 ± 0.04
Hindlimb Grip Strength					
Prestudy	0.16 ± 0.01	0.16 ± 0.01	0.18 ± 0.01[2]	0.19 ± 0.01	0.18 ± 0.01
Week 6	0.39 ± 0.01	0.41 ± 0.02	0.41 ± 0.01	0.40 ± 0.01	0.38 ± 0.01
Week 12	0.72 ± 0.03	0.71 ± 0.02	0.75 ± 0.02	0.76 ± 0.03	0.69 ± 0.02
Week 18	0.85 ± 0.03	0.83 ± 0.02	0.86 ± 0.02	0.85 ± 0.01	0.82 ± 0.02
Week 24	0.72 ± 0.03	0.66 ± 0.04	0.71 ± 0.02	0.68 ± 0.03	0.70 ± 0.03
FEMALE					
n	10	10	10	10	10
Forelimb Grip Strength					
Prestudy	0.39 ± 0.02	0.41 ± 0.01	0.39 ± 0.02	0.41 ± 0.02	0.40 ± 0.03
Week 6	0.79 ± 0.03	0.82 ± 0.02	0.79 ± 0.02	0.83 ± 0.03	0.81 ± 0.02
Week 12	0.87 ± 0.02	0.89 ± 0.03	0.88 ± 0.03	0.90 ± 0.03	0.81 ± 0.03
Week 18	1.26 ± 0.01	1.24 ± 0.03	1.26 ± 0.02	1.26 ± 0.02	1.23 ± 0.03
Week 24	0.97 ± 0.02	0.89 ± 0.05	0.84 ± 0.04	0.91 ± 0.05	0.94 ± 0.04
Hindlimb Grip Strength					
Prestudy	0.16 ± 0.01	0.17 ± 0.01	0.18 ± 0.01	0.16 ± 0.01	0.17 ± 0.01
Week 6	0.30 ± 0.01	0.31 ± 0.01	0.34 ± 0.02	0.31 ± 0.02	0.31 ± 0.01
Week 12	0.61 ± 0.01	0.65 ± 0.02	0.71 ± 0.03*	0.68 ± 0.03*	0.63 ± 0.02
Week 18	0.69 ± 0.02	0.69 ± 0.02	0.70 ± 0.01	0.69 ± 0.01	0.68 ± 0.02
Week 24	0.64 ± 0.01	0.62 ± 0.02	0.69 ± 0.03	0.65 ± 0.03	0.63 ± 0.02

[1] Data are given as kg grip strength (mean ± standard error of five trials).
[2] n=10.
* Significantly different (P≤0.05) from the control group by analysis of variance followed by the least significance difference test.

TABLE G2 Hindlimb Footsplay Data for F344/N Rats in the 26-Week Drinking Water Study of a Chemical Mixture of 25 Groundwater Contaminants[1]

	0 ppm	11 ppm	38 ppm	113 ppm	378 ppm
MALE					
n	10	10	9	10	10
Prestudy	5.1 ± 0.2	5.8 ± 0.3	5.1 ± 0.2[2]	5.2 ± 0.2	4.8 ± 0.2
Week 6	7.2 ± 0.4	7.1 ± 0.4	7.4 ± 0.3	6.9 ± 0.3	6.5 ± 0.3
Week 12	8.7 ± 0.3	8.3 ± 0.3	8.5 ± 0.4	8.1 ± 0.3	7.8 ± 0.3
Week 18	8.1 ± 0.7	8.4 ± 0.4	7.6 ± 0.4	7.2 ± 0.3	6.7 ± 0.4
Week 24	8.3 ± 0.6	8.0 ± 0.4	7.8 ± 0.7	7.6 ± 0.4	7.2 ± 0.4
FEMALE					
n	10	10	10	10	10
Prestudy	4.9 ± 0.2	4.8 ± 0.1	4.7 ± 0.2	5.0 ± 0.2	4.8 ± 0.2
Week 6	4.9 ± 0.1	5.2 ± 0.1	5.2 ± 0.2	5.5 ± 0.3	5.2 ± 0.3
Week 12	6.0 ± 0.2	6.3 ± 0.3	6.5 ± 0.4	6.3 ± 0.3	5.8 ± 0.3
Week 18	5.2 ± 0.2	5.6 ± 0.3	5.8 ± 0.3	5.7 ± 0.4	5.7 ± 0.4
Week 24	5.2 ± 0.3	5.8 ± 0.2	6.5 ± 0.4	6.3 ± 0.3	5.7 ± 0.4

[1] Data are given as cm footsplay (mean ± standard error of three trials). Differences from the control group were not significant by analysis of variance followed by the least significance difference test.

[2] n=10.

TABLE G3 Total Horizontal and Vertical Activity Counts for F344/N Rats in the 26-Week Drinking Water Study of a Chemical Mixture of 25 Groundwater Contaminants[1]

	0 ppm	11 ppm	38 ppm	113 ppm	378 ppm
MALE					
n	10	10	9	10	10
Total Horizontal Activity Count					
Prestudy	129 ± 12	135 ± 12	115 ± 16[2]	128 ± 6	134 ± 14
Week 6	197 ± 11	191 ± 9	194 ± 12[2]	217 ± 8	199 ± 9
Week 12	154 ± 16	164 ± 24	140 ± 20	155 ± 18	147 ± 10
Week 18	129 ± 18	142 ± 23	138 ± 14	112 ± 14	103 ± 16
Week 24	98 ± 15	63 ± 8	92 ± 11	89 ± 10	81 ± 10
Total Vertical Activity Count					
Prestudy	23 ± 5	21 ± 4	17 ± 4[2]	28 ± 5	21 ± 5
Week 6	36 ± 3	44 ± 4	43 ± 4[2]	55 ± 7	39 ± 5
Week 12	27 ± 5	33 ± 9	27 ± 5	24 ± 4	22 ± 4
Week 18	14 ± 2	16 ± 3	16 ± 3	10 ± 3	13 ± 3
Week 24	7 ± 1	8 ± 2	10 ± 2	10 ± 2	10 ± 2
FEMALE					
n	10	10	10	10	10
Total Horizontal Activity Count					
Prestudy	131 ± 10	128 ± 9	123 ± 10	120 ± 11	131 ± 10
Week 6	187 ± 8	183 ± 5	181 ± 12	167 ± 10	159 ± 16
Week 12	168 ± 10	181 ± 11	168 ± 8	175 ± 7	174 ± 14
Week 18	125 ± 13	147 ± 10	141 ± 14	157 ± 11	141 ± 13
Week 24	130 ± 10	131 ± 10	133 ± 12	115 ± 13	91 ± 10
Total Vertical Activity Count					
Prestudy	29 ± 4	28 ± 4	27 ± 4	28 ± 4	25 ± 4
Week 6	56 ± 7	45 ± 3	43 ± 7	46 ± 7	38 ± 5
Week 12	43 ± 5	49 ± 5	47 ± 7	44 ± 7	42 ± 5
Week 18	28 ± 5	31 ± 5	30 ± 6	35 ± 6	25 ± 4
Week 24	25 ± 7	25 ± 4	28 ± 3	19 ± 4	17 ± 5

[1] Data are given as mean ± standard error; total for five intervals. Differences from the control group were not significant by analysis of variance followed by the least significance difference test.

[2] n=10.

TABLE G4 Pawlick Latency Data for F344/N Rats in the 26-Week Drinking Water Study of a Chemical Mixture of 25 Groundwater Contaminants[1]

	0 ppm	11 ppm	38 ppm	113 ppm	378 ppm
MALE					
n	10	10	9	10	10
Prestudy	6.1 ± 0.4	8.3 ± 1.5	7.2 ± 0.7[2]	5.6 ± 0.4	6.7 ± 0.8
Week 6	5.3 ± 0.3	5.5 ± 0.2	5.5 ± 0.3	5.4 ± 0.2	5.2 ± 0.2
Week 12	4.9 ± 0.2	5.7 ± 0.3	5.0 ± 0.3	5.2 ± 0.2	5.4 ± 0.2
Week 18	5.7 ± 0.4	5.7 ± 0.4	5.9 ± 0.3	6.2 ± 0.3	6.5 ± 0.4
Week 24	5.3 ± 0.3	4.9 ± 0.3	4.7 ± 0.2	5.2 ± 0.2	5.6 ± 0.6
FEMALE					
n	10	10	10	10	10
Prestudy	5.5 ± 0.5	5.9 ± 0.3	5.3 ± 0.2	5.7 ± 0.6	5.6 ± 0.4
Week 6	5.9 ± 0.3	6.3 ± 0.3	6.4 ± 0.4	5.9 ± 0.3	6.0 ± 0.3
Week 12	5.5 ± 0.3	6.1 ± 0.6	6.1 ± 0.4	5.5 ± 0.3	5.6 ± 0.4
Week 18	6.5 ± 0.3	6.9 ± 0.4	6.6 ± 0.3	6.4 ± 0.4	6.3 ± 0.4
Week 24	5.2 ± 0.3	5.7 ± 0.4	5.8 ± 0.5	5.3 ± 0.2	5.5 ± 0.4

[1] Data are given in seconds (mean ± standard error). Differences from the control group were not significant by analysis of variance followed by the least significance difference test.

[2] n=10.

TABLE G5 Startle Response Profiles for F344/N Rats in the 26-Week Drinking Water Study of a Chemical Mixture of 25 Groundwater Contaminants[1]

	0 ppm	11 ppm	38 ppm	113 ppm	378 ppm
MALE					
n	10	10	9	10	10
Prestudy					
Block 1	46 ± 8	57 ± 16	31 ± 4[2]	31 ± 5	42 ± 11
Block 2	35 ± 6	27 ± 4	26 ± 3[2]	23 ± 3	28 ± 4
Block 3	30 ± 5	24 ± 3	21 ± 3[2]	21 ± 3	24 ± 3
Block 4	35 ± 5	26 ± 4	26 ± 3[2]	22 ± 3	25 ± 3
Week 6					
Block 1	33 ± 3	46 ± 7	39 ± 11	34 ± 5	41 ± 7
Block 2	26 ± 3	23 ± 2	27 ± 6	25 ± 3	24 ± 2
Block 3	26 ± 3	23 ± 3	22 ± 3	27 ± 3	26 ± 4
Block 4	27 ± 2	32 ± 7	30 ± 5	33 ± 4	30 ± 3
Week 12					
Block 1	47 ± 11	38 ± 8	29 ± 4	37 ± 6	54 ± 9
Block 2	38 ± 9	30 ± 6	27 ± 5	32 ± 5	33 ± 4
Block 3	35 ± 6	26 ± 4	26 ± 4	27 ± 3	33 ± 3
Block 4	41 ± 7	33 ± 5	36 ± 5	37 ± 5	37 ± 5
Week 18					
Block 1	46 ± 8	32 ± 5	37 ± 5	37 ± 4	33 ± 5
Block 2	35 ± 8	23 ± 3	23 ± 2	25 ± 3	26 ± 2
Block 3	29 ± 6	23 ± 2	21 ± 3	31 ± 8	26 ± 3
Block 4	43 ± 6	35 ± 3	36 ± 4	40 ± 5	42 ± 6
Week 24					
Block 1	29 ± 5	22 ± 4	24 ± 4	21 ± 3	31 ± 5
Block 2	23 ± 4	17 ± 3	18 ± 4	19 ± 3	23 ± 3
Block 3	25 ± 4	16 ± 4	18 ± 4	20 ± 3	23 ± 3
Block 4	32 ± 4	24 ± 3	26 ± 4	25 ± 4	33 ± 5
FEMALE					
n	10	10	10	10	10
Prestudy					
Block 1	34 ± 5	39 ± 4	48 ± 10	42 ± 10	51 ± 9
Block 2	22 ± 4	27 ± 4	31 ± 4	27 ± 5	30 ± 5
Block 3	19 ± 3	20 ± 3	26 ± 5	20 ± 2	25 ± 4
Block 4	21 ± 3	20 ± 2	28 ± 5	24 ± 5	27 ± 5
Week 6					
Block 1	59 ± 8	39 ± 5	46 ± 6	41 ± 8	47 ± 9
Block 2	40 ± 5	30 ± 4	36 ± 3	31 ± 5	41 ± 8
Block 3	29 ± 4	25 ± 2	35 ± 6	30 ± 5	38 ± 10
Block 4	28 ± 3	25 ± 2	28 ± 2	27 ± 3	40 ± 11
Week 12					
Block 1	64 ± 7	40 ± 4*	49 ± 7	39 ± 4*	57 ± 9
Block 2	42 ± 4	31 ± 3	36 ± 5	33 ± 3	43 ± 6
Block 3	38 ± 4	27 ± 3	33 ± 4	33 ± 3	40 ± 5
Block 4	43 ± 4	31 ± 3	37 ± 5	36 ± 3	47 ± 6

TABLE G5 Startle Response Profiles for F344/N Rats in the 26-Week Drinking Water Study of a Chemical Mixture of 25 Groundwater Contaminants (continued)

	0 ppm	11 ppm	38 ppm	113 ppm	378 ppm
FEMALE (continued)					
Week 18					
Block 1	45 ± 5	31 ± 3	37 ± 4	38 ± 5	40 ± 5
Block 2	32 ± 3	27 ± 3	30 ± 3	34 ± 5	33 ± 5
Block 3	31 ± 3	26 ± 3	31 ± 4	32 ± 4	38 ± 7
Block 4	39 ± 4	33 ± 2	32 ± 2	32 ± 3	44 ± 8
Week 24					
Block 1	36 ± 5	28 ± 4	27 ± 5	33 ± 4	36 ± 4
Block 2	30 ± 4	25 ± 4	23 ± 4	26 ± 3	32 ± 5
Block 3	31 ± 4	24 ± 3	25 ± 6	25 ± 3	33 ± 5
Block 4	41 ± 6	29 ± 4	28 ± 6	34 ± 5	40 ± 6

[1] Data are given as mean ± standard error of the average response (amplitude) for each trial. Block 1 = mean of trials 1-20; Block 2 = mean of trials 21-40; Block 3 = mean of trials 41-60; Block 4 = mean of trials 61-80.

[2] n=10.

* Significantly different ($P \leq 0.05$) from the control group by analysis of variance followed by the least significance difference test.

TABLE G6 Forelimb and Hindlimb Grip Strength Data for B6C3F$_1$ Mice in the 26-Week Drinking Water Study of a Chemical Mixture of 25 Groundwater Contaminants[1]

	0 ppm	11 ppm	38 ppm	113 ppm	378 ppm
MALE					
n	10	10	10	10	10
Forelimb Grip Strength					
Prestudy	76 ± 7	64 ± 7	72 ± 6	66 ± 5	70 ± 6
Week 6	133 ± 4	127 ± 4	130 ± 3	128 ± 4	132 ± 3
Week 12	151 ± 4	147 ± 6	147 ± 6	147 ± 4	152 ± 4
Week 18	170 ± 3	168 ± 5	162 ± 6	159 ± 4	172 ± 2
Week 24	152 ± 4	155 ± 3	156 ± 3	157 ± 4	156 ± 3
Hindlimb Grip Strength					
Prestudy	65 ± 2	65 ± 3	69 ± 3	67 ± 3	67 ± 4
Week 6	89 ± 6	88 ± 4	89 ± 4	88 ± 4	88 ± 4
Week 12	114 ± 4	118 ± 5	118 ± 5	109 ± 5	120 ± 5
Week 18	111 ± 2	109 ± 3	107 ± 4	106 ± 3	114 ± 1
Week 24	112 ± 4	115 ± 4	117 ± 5	113 ± 5	117 ± 4
FEMALE					
n	10	10	10	10	10
Forelimb Grip Strength					
Prestudy	73 ± 2	76 ± 2	77 ± 3	73 ± 4	77 ± 2
Week 6	110 ± 7	115 ± 6	106 ± 7	106 ± 7	112 ± 8
Week 12	137 ± 3	143 ± 4	144 ± 3	146 ± 4	140 ± 5
Week 18	141 ± 3	144 ± 4	140 ± 4	141 ± 4	147 ± 4
Week 24	143 ± 3	150 ± 4	147 ± 3	145 ± 3	140 ± 4
Hindlimb Grip Strength					
Prestudy	52 ± 2	56 ± 2	54 ± 2	55 ± 2	55 ± 2
Week 6	79 ± 2	76 ± 2	78 ± 3	80 ± 3	81 ± 3
Week 12	116 ± 4	114 ± 3	119 ± 3	119 ± 4	116 ± 3
Week 18	101 ± 2	105 ± 3	100 ± 4	99 ± 3	99 ± 2
Week 24	116 ± 3	104 ± 3	105 ± 5	109 ± 5	111 ± 4

[1] Data are given as g grip strength (mean ± standard error of five trials). Differences from the control group were not significant by analysis of variance followed by the least significance difference test.

TABLE G7 Hindlimb Footsplay Data for B6C3F$_1$ Mice in the 26-Week Drinking Water Study of a Chemical Mixture of 25 Groundwater Contaminants[1]

	0 ppm	11 ppm	38 ppm	113 ppm	378 ppm
MALE					
n	10	10	10	10	10
Prestudy	4.9 ± 0.1	4.5 ± 0.1	5.0 ± 0.1	4.6 ± 0.1	4.7 ± 0.1
Week 6	4.5 ± 0.1	4.4 ± 0.1	4.6 ± 0.1	4.5 ± 0.1	4.3 ± 0.1
Week 12	5.0 ± 0.1	4.6 ± 0.1*	4.8 ± 0.1	4.5 ± 0.1*	4.8 ± 0.1
Week 18	4.8 ± 0.1	4.6 ± 0.1	4.8 ± 0.1	4.6 ± 0.1	4.7 ± 0.1
Week 24	4.8 ± 0.1	4.8 ± 0.2	4.7 ± 0.1	4.7 ± 0.1	4.8 ± 0.1
FEMALE					
n	10	10	10	10	10
Prestudy	4.6 ± 0.1	4.5 ± 0.1	4.6 ± 0.1	4.6 ± 0.1	4.5 ± 0.2
Week 6	4.3 ± 0.1	4.4 ± 0.1	4.4 ± 0.2	4.4 ± 0.1	4.2 ± 0.2
Week 12	4.7 ± 0.1	4.7 ± 0.1	4.8 ± 0.1	4.6 ± 0.1	4.7 ± 0.1
Week 18	4.5 ± 0.1	4.6 ± 0.1	4.7 ± 0.1	4.6 ± 0.1	4.6 ± 0.0
Week 24	4.6 ± 0.2	4.5 ± 0.1	4.7 ± 0.1	4.7 ± 0.1	4.5 ± 0.1

[1] Data are given as cm footsplay (mean ± standard error of three trials).
* Significantly different ($P \leq 0.05$) from the control group by analysis of variance followed by the least significance difference test.

TABLE G8 Total Horizontal Activity Counts for B6C3F$_1$ Mice in the 26-Week Drinking Water Study of a Chemical Mixture of 25 Groundwater Contaminants[1]

	0 ppm	11 ppm	38 ppm	113 ppm	378 ppm
MALE					
n	10	10	10	10	10
Prestudy	145 ± 10	148 ± 7	162 ± 11	153 ± 7	163 ± 10
Week 6	140 ± 9	143 ± 7	151 ± 8	139 ± 6	142 ± 7
Week 12	122 ± 9	138 ± 9	137 ± 8	140 ± 10	129 ± 7
Week 18	125 ± 9	137 ± 8	134 ± 7	132 ± 5	126 ± 5
Week 24	132 ± 8	128 ± 7	133 ± 6	126 ± 8	130 ± 8
FEMALE					
n	10	10	10	10	10
Prestudy	176 ± 5	171 ± 8	165 ± 7	160 ± 9	172 ± 6
Week 6	163 ± 10	155 ± 8	141 ± 11	145 ± 9	150 ± 7
Week 12	150 ± 9	137 ± 8	130 ± 11	122 ± 16	140 ± 10
Week 18	138 ± 8	139 ± 10	128 ± 6	132 ± 10	139 ± 8
Week 24	126 ± 14	124 ± 7	102 ± 9	107 ± 6	116 ± 11

[1] Data are given as mean ± standard error; total for five intervals. Differences from the control group were not significant by analysis of variance followed by the least significance difference test.

TABLE G9 Pawlick Latency Data for B6C3F$_1$ Mice in the 26-Week Drinking Water Study of a Chemical Mixture of 25 Groundwater Contaminants[1]

	0 ppm	11 ppm	38 ppm	113 ppm	378 ppm
MALE					
n	10	10	10	10	10
Prestudy	11.6 ± 0.9	11.2 ± 1.3	12.6 ± 1.1	9.8 ± 0.9	9.9 ± 0.9
Week 6	8.1 ± 0.4	8.8 ± 0.8	9.4 ± 1.2	9.1 ± 0.7	9.5 ± 1.3
Week 12	10.6 ± 1.2	9.5 ± 1.1	10.6 ± 1.0	10.4 ± 1.2	11.7 ± 1.5
Week 18	11.4 ± 1.1	14.5 ± 2.1	14.0 ± 2.5	11.6 ± 1.4	14.1 ± 2.0
Week 24	9.8 ± 0.6	16.5 ± 2.0*	10.3 ± 0.7	9.4 ± 0.8	12.1 ± 2.1
FEMALE					
n	10	10	10	10	10
Prestudy	10.9 ± 1.1	10.0 ± 0.6	9.0 ± 0.7	9.3 ± 1.1	9.2 ± 0.8
Week 6	8.1 ± 0.8	7.8 ± 0.8	6.5 ± 0.6	7.5 ± 0.6	7.4 ± 0.5
Week 12	10.0 ± 1.1	8.6 ± 0.6	8.4 ± 1.0	9.6 ± 1.1	8.5 ± 1.2
Week 18	11.5 ± 1.3	11.2 ± 1.2	9.4 ± 0.7	9.8 ± 1.0	11.5 ± 1.1
Week 24	8.0 ± 0.7	10.0 ± 1.5	6.8 ± 0.5	8.8 ± 0.9	15.2 ± 2.9*

[1] Data are given in seconds (mean ± standard error).
* Significantly different ($P \leq 0.05$) from the control group by analysis of variance followed by the least significance difference test.

TABLE G10 Startle Response Profiles for B6C3F$_1$ Mice in the 26-Week Drinking Water Study of a Chemical Mixture of 25 Groundwater Contaminants[1]

	0 ppm	11 ppm	38 ppm	113 ppm	378 ppm
MALE					
n	10	10	10	10	10
Prestudy					
Block 1	46 ± 8	69 ± 14	47 ± 6	39 ± 7	51 ± 9
Block 2	31 ± 4	40 ± 7	31 ± 5	21 ± 4	33 ± 6
Block 3	28 ± 4	45 ± 10	34 ± 7	25 ± 5	37 ± 8
Block 4	50 ± 10	56 ± 12	41 ± 8	34 ± 6	42 ± 9
Week 6					
Block 1	54 ± 12	78 ± 16	61 ± 14	45 ± 9	57 ± 10
Block 2	36 ± 10	40 ± 10	51 ± 13	29 ± 7	35 ± 7
Block 3	36 ± 9	41 ± 10	41 ± 10	28 ± 6	35 ± 7
Block 4	46 ± 10	54 ± 12	49 ± 11	35 ± 7	53 ± 10
Week 12					
Block 1	18 ± 2	17 ± 2	17 ± 2	17 ± 2	16 ± 1
Block 2	13 ± 2	12 ± 1	11 ± 1	10 ± 1	10 ± 1
Block 3	12 ± 1	11 ± 1	11 ± 1	10 ± 1	11 ± 1
Block 4	14 ± 1	14 ± 1	14 ± 1	12 ± 1	14 ± 1
Week 18					
Block 1	35 ± 4	39 ± 4	39 ± 4	41 ± 10	32 ± 4
Block 2	23 ± 3	23 ± 2	22 ± 3	27 ± 5	23 ± 3
Block 3	23 ± 2	23 ± 2	23 ± 4	25 ± 3	23 ± 3
Block 4	32 ± 3	30 ± 3	36 ± 5	34 ± 3	32 ± 3
Week 24					
Block 1	36 ± 3	34 ± 4	38 ± 4	38 ± 6	44 ± 4
Block 2	25 ± 2	23 ± 3	23 ± 2	24 ± 4	29 ± 3
Block 3	25 ± 3	28 ± 4	24 ± 2	25 ± 5	32 ± 4
Block 4	36 ± 6	37 ± 5	34 ± 2	32 ± 5	34 ± 4
FEMALE					
n	10	10	10	10	10
Prestudy					
Block 1	59 ± 10	60 ± 8	66 ± 11	51 ± 7	70 ± 12
Block 2	38 ± 4	36 ± 5	51 ± 8	29 ± 3	40 ± 10
Block 3	37 ± 4	39 ± 6	52 ± 8	36 ± 4	39 ± 7
Block 4	49 ± 6	46 ± 8	74 ± 11	51 ± 8	49 ± 9
Week 6					
Block 1	73 ± 8	60 ± 6	61 ± 6	50 ± 4*	50 ± 5*
Block 2	41 ± 4	34 ± 4	47 ± 6	36 ± 5	27 ± 3*
Block 3	47 ± 5	39 ± 4	49 ± 6	38 ± 4	33 ± 4
Block 4	62 ± 7	51 ± 5	59 ± 6	46 ± 6	44 ± 5
Week 12					
Block 1	12 ± 1	13 ± 1	12 ± 0	12 ± 1	14 ± 2
Block 2	9 ± 1	9 ± 1	9 ± 1	8 ± 1	10 ± 1
Block 3	9 ± 1	9 ± 1	9 ± 1	9 ± 1	9 ± 1
Block 4	10 ± 1	11 ± 1	10 ± 1	10 ± 1	11 ± 1

TABLE G10 Startle Response Profiles for B6C3F$_1$ Mice in the 26-Week Drinking Water Study of a Chemical Mixture of 25 Groundwater Contaminants (continued)

	0 ppm	11 ppm	38 ppm	113 ppm	378 ppm
FEMALE (continued)					
Week 18					
Block 1	27 ± 4	30 ± 4	30 ± 6	24 ± 2	32 ± 3
Block 2	18 ± 2	20 ± 3	17 ± 2	17 ± 2	18 ± 2
Block 3	18 ± 1	18 ± 2	16 ± 2	18 ± 2	19 ± 2
Block 4	24 ± 2	25 ± 3	25 ± 3	22 ± 3	25 ± 3
Week 24					
Block 1	35 ± 5	36 ± 5	33 ± 5	31 ± 4	39 ± 5
Block 2	22 ± 3	21 ± 1	22 ± 3	20 ± 2	25 ± 3
Block 3	22 ± 3	22 ± 2	25 ± 3	20 ± 2	25 ± 3
Block 4	28 ± 3	31 ± 4	32 ± 6	24 ± 3	30 ± 4

[1] Data are given as mean ± standard error of the average response (amplitude) for each trial. Block 1 = mean of trials 1-20; Block 2 = mean of trials 21-40; Block 3 = mean of trials 41-60; Block 4 = mean of trials 61-80.

* Significantly different ($P \leq 0.05$) from the control group by analysis of variance followed by the least significance difference test.

APPENDIX H

Hematopoietic Toxicity Studies

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Residual damage to hematopoietic system in mice exposed to a mixture of groundwater contaminants

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SUMMARY

To assess the potential health effects of chemically contaminated groundwater, we initiated a toxicological program on a mixture of 25 frequently-detected groundwater contaminants derived from hazardous waste disposal sites. As part of this program, myelotoxicity studies were conducted. Bone marrow parameters were examined in mice exposed to 0, 1, 5 or 10% of a simulated chemical mixture stock solution of groundwater contaminants in drinking water for 108 days. Mice treated with 5 or 10% of chemical mixture stock solution for 108 days showed suppressed marrow granulocyte macrophage progenitors (CFU-GM); however, this suppression disappeared in 10 weeks following the cessation of treatment. The possible toxicological interaction of groundwater contaminants and radiation on hematopoiesis was investigated by using the number of bone marrow CFU-GM as an index. When mice were exposed to 200 rads whole body irradiation at 2 and 9 weeks during this 10-week recovery period, the combined treatment (i.e., chemical mixture followed by irradiation) group showed a significantly slower recovery of bone marrow progenitors as compared with the control group (i.e., radiation but without prior chemical mixture treatment). This study showed that even 10 weeks after the cessation of chemical mixture treatment when all hematological parameters were normal, a residual effect of the chemical mixture may still be demonstrated as lower progenitor cell numbers following irradiation. Thus, residual damage of hematopoiesis in mice exposed to groundwater contaminants for 108 days renders the mice more sensitive to subsequent irradiation-induced injury.

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INTRODUCTION

Under an interagency agreement with the Agency for Toxic Substances and Disease Registry (ATSDR), the National Toxicology Program (NTP) has been investigating the health effects of a chemically defined mixture of frequently detected groundwater contaminants near hazardous waste disposal sites [1–9]. Bone marrow with rapidly renewing cell populations is one of the most sensitive tissues to cytotoxic agents; studies in laboratory animals have shown that myelotoxicity can be demonstrated at exposure levels that cause no other overt manifestations of toxicity in these animals [10–14]. In humans, bone marrow failure is a significant complication of cancer chemotherapy and has occurred with exposures to drugs and environmental agents [15–20]. Earlier studies at the NTP in mice exposed to a chemically defined mixture of 25 groundwater contaminants have shown the suppression of immune function in 2- and 13-week exposures [4]. Furthermore, a clear dose–response relationship on the depression of marrow granulocyte-macrophage progenitor cells (CFU-GM) and erythroid precursors (CFU-E) was demonstrated in a series of 17-, 108- and 220-day studies on this 25-chemical mixture with little or no change in body weight, bone marrow cellularity, and histopathological and hematological parameters [9]. Other findings suggested an enhancement of carbon tetrachloride hepatotoxicity in male Fischer rats pretreated with a chemical mixture of 25 groundwater contaminants [6,7]. Trainor and Morley [21] reported that a residual marrow damage was detected in female mice as long as 2 months after administration of myelotoxic agents, such as busulfan (BU) and 1,3-bis(chloroethyl)-1-nitrosourea (BCNU). Bone marrow has less reserve in patients with prior exposure to irradiation and it is thus more sensitive to the myelotoxic effects of subsequent treatment of vinblastine [22]. Further, mice exposed to a myelotoxic agent shortly after birth were more sensitive to irradiation 4–5 months later [23]. All the above information suggests that a residual stem cell defect from an earlier treatment of myelotoxic agent may be amplified later by irradiation. Thus, animals exposed to a myelotoxic agent(s) may appear 'normal' clinically or based on conventional toxicological endpoints. However, such a subclinical stem cell defect may provide a basis for enhancement or potentiation of otherwise mild toxic responses from an acute exposure(s) to chemical, physical and/or biological agents [7]. Accordingly, the present study was designed to evaluate potential residual myelotoxic effects of a 25-chemical mixture of groundwater contaminants with subsequent whole body irradiation. Related myelotoxicity studies on the 25-chemical mixture of groundwater contaminants are published elsewhere [9,24].

MATERIALS AND METHODS

Animal treatment

Female B6C3F$_1$ (C57BL/6N × C3H/HeN) mice, 8 weeks of age, were obtained from the National Cancer Institute production contracts (Charles River, Portage,

MI). Animals were housed 10 per cage with NIH-07 rodent diet and water ad libitum for a 2-week acclimation period. Animal room was maintained at 21–22°C and 50–55% humidity. Mice were randomized into experimental groups and given free access to 0, 1, 5, and 10% of the technically achievable chemical mixture stock for 108 days. Water consumption was measured for each cage of 10 mice over a 48-h period at

TABLE I

COMPOSITION AND CONCENTRATION OF COMPONENTS IN THE HIGHEST DOSE LEVEL IN COMPARISON WITH EPA SURVEY VALUES FROM CONTAMINATED GROUNDWATER

	Average EPA survey concentration (ppm)	Maximum EPA survey concentration (ppm)	10% of mixture stock solution (ppm)[a]
Acetone	6.9	250	53
Arochlor 1260	0.21	2.9	0.01
Arsenic	30.6	3680	9
Benzene	5	1200	12.5
Cadmium	0.85	225	51
Carbon tetrachloride	0.54	20	0.4
Chlorobenzene	0.1	13	0.1
Chloroform	1.46	220	7
Chromium	0.69	188	36
Diethylhexyl phthalate	0.13	5.8	0.015
1,1-Dichloroethane	0.31	56.1	1.4
1,2-Dichloroethane	6.33	440	40
1,1-Dichloroethylene	0.24	38	0.5
1,2-*trans*-Dichloroethylene	0.73	75.2	2.5
Ethylbenzene	0.65	25	0.3
Lead	37	31000	70
Mercury	0.34	50	0.5
Methylene chloride	11.2	7800	37.5
Nickel	0.5	95.2	6.8
Phenol	34	7713	29
Tetrachloroethylene	9.68	21570	3.4
Toluene	5.18	110	7
1,1,1-Trichloroethane	1.25	618	2
Trichloroethylene	3.82	790	6.5
Xylenes	4.07	150	1.6
Total concentration of all chemicals (ppm)	161.78		378.025

[a] The highest dose used in the study was prepared as 1 in 10 dilution of the technically achievable stock mixture. For details of EPA surveys and the formulation and dilutions of the chemical mixture stock, see Yang and Rauckman [1]; Yang et al. [2].

2-week intervals during the study. Body weights and clinical observations were recorded once a week throughout the experiment.

Preparation of stock solution

The chemical mixture was formulated with consideration of EPA survey concentrations, and toxicity and solubility of individual component chemicals [1,2]. A final technically achievable stock solution was prepared with 25 common groundwater contaminants found near toxic waste dumps at approximately 90% saturation levels for most chemicals (Table I). In order to minimize possible chemical interactions, the stock solution was stored as 2 substock solutions: an organic substock containing 19 organic chemicals and an aqueous substock containing 6 metals and phenol [2]. Fresh drinking water mixture was prepared from substocks every 48–72 h and analyzed periodically both before and after dosing. Some loss (up to 40%) of organic volatiles during mixing of the substocks and during the first 24 h following preparation did occur; however, the concentrations of acetone, phenol and all metals remained constant in the mixture. Detailed information on the recovery of component chemicals is published elsewhere [2].

Hematology

Mice were anesthetized by CO_2 inhalation and blood samples were obtained from the retro-orbital venous plexus to determine hemoglobin (Hgb), hematocrit (Hct), mean corpuscular volume (MCV), erythrocyte (RBC) and white blood cell (WBC) counts. Measurements were made with a Coulter counter, Model ZB (Coulter Electronics, Inc., Hialeah, FL).

Bone marrow

In an earlier study, we have demonstrated that the chemical mixture of 25 groundwater contaminants affects bone marrow stem cells (CFU-GM and CFU-E) in female mice [9]. In this study, we use colony forming units in granulocyte macrophage progenitors (CFU-GM) as an index for myelotoxicity. CFU-GM from femoral bone marrow cells were assayed using established semisolid culture techniques, a modification of the method by Bradley and Metcalf [25]. Nucleated marrow cells (10^5) were suspended in 1 ml of culture medium containing 20% fetal bovine serum (Hyclone Labs., Ogden, UT), 5% human AB serum (Flow Labs., McLean, VA), 2 mM L-glutamine, 50 μg/ml garamycin, 1.5% methylcellulose, and 10% mouse lung conditioned medium. CFU-GM cultures in quadruplicate were incubated for 7 days at 37°C in 7% CO_2 humidified atmosphere and the plates were stained with methylene blue. The total number of colonies (>40 cells)/plate were counted using a stereomicroscope (Wilk, Heerburg, Switzerland).

Irradiation

To determine if a residual effect of drinking water contaminants on marrow pro-

genitor cells occurred, control and chemical-treated mice received 200 rads of whole-body irradiation (WBI) (utilizing a 137-cesium source) at 2 and again 9 weeks following 108 days of chemical exposure. Hematological and CFU-GM progenitor cells were examined in the non-irradiated and irradiated vehicle control and chemical-treated mice. Although the radiation dose (WBI 200 rads) selected had a profound effect on CFU-GM numbers, it was found to be reversible within 6 weeks (unpublished data).

Statistical analysis

Data were analyzed using the Wilk-Shapiro test for normality, one-way analysis of variance and Dunnett's test for multiple comparison with a control group [26].

RESULTS

Water consumption, body and organ weight changes

A significant decrease in water consumption occurred in mice treated with 5 and 10% stock solution (79 and 72% of the control group respectively) after 1 week, but at the end of treatment the water consumption rates in the treatment groups returned to the control level. Furthermore, previous paired-water studies indicated that myelotoxicity and immune effects were related to 14-day chemical exposure and not to the decreased water intake [4]. As shown in Table II, exposure to the chemical mixture did not affect body weights; there was a slight increase in relative kidney weight and a slight decrease of relative thymus weight at the highest dose level.

Hematology

A statistically significant decrease was seen only in MCV at 10% mixture stock 1 day post-exposure, but not 10 weeks post-exposure (data not shown). The other he-

TABLE II

BODY AND ORGAN WEIGHTS IN FEMALE B6C3F$_1$ MICE FOLLOWING EXPOSURE TO A CHEMICAL MIXTURE OF 25 GROUNDWATER CONTAMINANTS FOR 108 DAYS[a]

Exposure levels % stock mixture	Body wt. (g)	Liver/body wt[b]	Spleen/body wt.	Kidney/body wt.	Thymus/body wt.
0	27.8±0.7	52±3	3.5±0.3	6.7±0.2	1.9±0.1
1	27.8±0.9	51±2	3.5±0.2	7.3±0.2	1.7±0.1
5	27.1±0.6	49±1	3.5±0.1	7.2±0.3	1.9±0.1
10	27.5±0.7	51±1	3.4±0.1	7.7±0.2*	1.5±0.1**

[a] Each value represents the mean ± SEM of 7 mice per group, determined 1 day after the final exposure.

[b] Relative organ weight = organ (mg)/body wt. (g).

*$P<0.05$ versus controls by Dunnett's multiple range test.

**$P<0.01$ versus controls by Dunnett's multiple range test.

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TABLE III

ALTERATIONS OF HEMATOLOGICAL PARAMETERS IN FEMALE B6C3F$_1$ MICE FOLLOWING EXPOSURE TO A CHEMICAL MIXTURE OF 25 GROUNDWATER CONTAMINANTS FOR 108 DAYS[a]

Exposure levels (% stock mixture)	WBC ($\times 10^3/\mu l$)	RBC ($\times 10^6/\mu l$)	HCT (%)	MCV (fl)	Hgb (g/dl)
0	3.6±0.2	8.9±0.1	46.6±0.6	52.4±0.5	14.6±0.2
1	3.0±0.3	8.6±0.2	44.4±1.3	51.6±0.6	14.2±0.2
5	3.5±0.3	8.9±0.1	45.4±0.7	51.0±0.4	14.7±0.2
10	3.2±0.3	8.7±0.1	43.1±0.8	49.5±0.5*	14.7±0.2

[a] Each value represents the mean ± SEM of 10 mice per group, measured 1 day after the final exposure.
*$P<0.01$ versus controls by Dunnett's multiple range test.

matological parameters (i.e. WBC, RBC, Hct, Hgb) remained unaltered when compared to the control group (Table III). Although irradiation has a direct effect on WBC, it caused no dose-related hematological change in animals pretreated with chemical mixture as compared to the irradiated vehicle controls (data not shown).

Bone marrow effects

Functional alterations occurred in the bone marrow, as evidenced by a significant ($P<0.01$) decrease in the number of progenitor cells capable of forming granulocyte-macrophage colonies (CFU-GM) in mice measured 2 d following 108 d of chemical exposure to 5 and 10% of stock mixture. The numbers of CFU-GM returned to normal values 10 weeks after cessation of chemical treatment (Table IV). To study possible residual hematopoietic effects by the chemical mixture during this 10-week recovery period, groups of mice received 2 irradiations at 2 and 9 weeks following the cessation of the 108-d treatment. As shown in Figure 1, mice treated both with chemical mixture (5 and 10% of stock mixture) and irradiation had significantly ($P<0.01$) dose-related suppression of CFU-GM compared to irradiated vehicle controls. Irradiation caused a reduction of marrow progenitor cells in all mice, but the effects were more pronounced in the mice that were treated previously with groundwater contaminants.

DISCUSSION

Bone marrow stem cells are a rapidly proliferating cell population and suppression of hematopoiesis would be predicted in exposure to chemicals that are cytotoxic or chemicals producing DNA-inhibitory effects. Cytotoxic damage to bone marrow cells can be easily related to conditions such as pancytopenia or anemia, and genotoxic damage can be correlated with tumor induction [27–37].

TABLE IV

EVALUATION OF HEMATOIPOESIS IN FEMALE B6C3F$_1$ MICE FOLLOWING EXPOSURE TO A CHEMICAL MIXTURE OF 25 GROUNDWATER CONTAMINANTS FOR 108 DAYS[a]

Parameter	% mixture stock			
	0	1	5	10
2 Days post-exposure				
Cellularity/femur ($\times 10^6$)	21.4±1.0	22.3±0.5	20.3± 1.7	18.2±0.8
CFU-GM/10^5 cells	111.6±1.2	109.1±1.2	89.0± 1.0*	66.1±1.2*
CFU-GM/femur ($\times 10^2$)	238.1±9.2	250.9±4.5	180.0±13.1*	119.9±4.6*
10 Weeks post-exposure				
Cellularity/femur ($\times 10^6$)	20.3±0.7	19.8±0.5	19.9±0.8	19.4±0.4
CFU-GM/10^5 cells	112.6±0.3	112.3±0.2	112.3±0.2	108.3±1.8
CFU-GM/femur ($\times 10^2$)	228.2±8.2	222.1±5.3	223.9±9.2	210.1±1.2

[a] Five mice/group were used to determine colony formation of granulocyte-macrophage progenitor cells (CFU-GM). Results are expressed as mean ± SEM.

*$P<0.01$ versus controls by Dunnett's multiple range test.

In this study, several chemicals that are present in the test chemical mixture of groundwater contaminants are reported to be myelotoxic at relatively high concentrations; these include arochlor 1260, benzene, phenol, toluene, xylene, and heavy metals [29,36–53]. Given the low exposure levels in this study, however, it is not certain that any single chemical by itself can account for the myelotoxicity observed. Within this context, we consider this mixture as a single entity and we explore the health effects of this mixture as a whole without worrying about the contribution from each individual component. Since systematic investigation of all the combinations (33,543,431) of the 25 components of this mixture is impossible, studies on submixtures of those components which are known myelotoxic agents might be fruitful.

Two findings from this study warrant special attention. First, even at the medium dose level (5% chemical mixture stock) a reduction of 25% progenitor cells was observed without any significant change in body and organ weights, bone marrow cellularity, histopathological and hematological parameters after 108 d of treatment. Thus, a reduced functional capacity of the stem cells in the bone marrow to produce progenitors may be one of the more sensitive indicators of low-level, long-term exposure to environmental chemical mixture(s). Second, when mice pretreated with a mixture of chemicals were subjected to subsequent whole body irradiation during the recovery period, there was a significant dose-related CFU-GM difference between the vehicle control and chemical-treated mice.

This suggests a persistent chemical mixture-induced residual damage in marrow stem cells and an increased sensitivity to irradiation stress to the hematopoietic sys-

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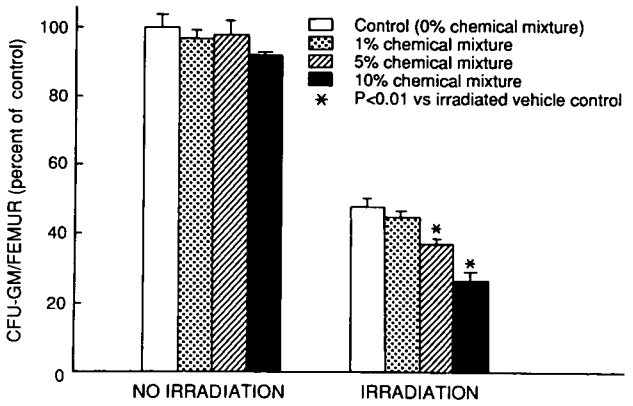

Fig. 1. Residual hematopoietic effects in mice following low-level, long-term exposure to a chemical mixture of 25 groundwater contaminants; whole-body irradiation was necessary to bring forth this subclinical perturbation. Female $B6C3F_1$ mice received 200 rads of whole-body irradiation at 2 and again at 9 weeks following the cessation of 108 d of groundwater contaminants in drinking water at the indicated concentrations. Bone marrow functions were examined in both non-irradiated and irradiated mice (5/group) on week 10 following the cessation of the 108-d treatment. Results are expressed as mean ± SEM percentage of non-irradiated vehicle controls.

tem. Multiple chemical exposures and subsequent irradiation in the present studies suggested that bone marrow damage may be cumulative. Therefore, the CFU-GM assay in combination with irradiation stress may be a useful method for detecting subtle bone marrow injury that may otherwise be missed.

Exposure to chemical mixture at environmental levels will probably not involve acute toxic responses [7]. It is more likely an insidious effect disrupting the homeostasis of the organism. Based on conventional toxicological endpoints, an animal may appear clinically normal; however, final toxicity may be expressed by the enhancement or potentiation of other exposure of chemical, physical, and/or biological agents. This is important to people living in areas where the potential for multiple chemical exposure at higher than normal levels is great. It is not inconceivable that subclinical myelotoxicity due to environmental pollutants in certain sensitive individuals might be exacerbated by subsequent radiological therapy. Similar toxicological interactions might also occur in humans with respect to other chemicals or biological agents when the immunosuppressive effects of this chemical mixture of 25 groundwater contaminants [4] are taken into consideration.

In conclusion, the present studies indicate that female mice treated with a simulated chemical mixture of groundwater contaminants showed significant suppression in granulocyte-macrophage progenitor cells. The numbers of CFU-GM progenitor cells return to normal values within 10 weeks after cessation of chemical mixture treatment. However, irradiation stress in chemical-treated mice demonstrates an interactive, residual effect in the recovery of CFU-GM numbers at a time when marrow

cellularity and peripheral blood counts are not affected by chemical treatment. This suggests that environmental pollutants, acting in concert at a relatively high level, can cause a residual marrow damage in mice with effective compensation to maintain normal circulating leukocyte and erythrocyte levels. Thus, long-term exposure to highly contaminated groundwater represents a potential risk to the hematopoietic system.

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Alterations in Hematopoietic Responses in B6C3F$_1$ Mice Caused by Drinking a Mixture of 25 Groundwater Contaminants

H. L. Hong, R. S. H. Yang, and G. A. Boorman

Myelotoxicity parameters were monitored in female B6C3F$_1$ mice exposed to 0, 1, 5, and 10% of a chemical mixture stock in drinking water for 2.5 to 31.5 weeks. The mixture consisted of 25 groundwater contaminants frequently found near toxic waste dumps, as determined by U.S. Environmental Protection Agency (EPA) surveys. Water consumption, body and organ weights, and hematological and histopathological examinations were conducted. No animals developed overt signs of toxicity after 2.5 weeks of treatment. No significant effect on bone marrow cellularity was observed after 2.5, 15.5, or 31.5 weeks of exposure; however, mice exposed to 5% or higher concentrations of the chemical mixture stock solution for 15.5 weeks showed significant suppression of granulocyte-macrophage progenitor cells (CFU-GM) and erythroid precursors (CFU-E) with no changes in body weight, histopathological or hematological parameters. Decreases occurred in erythrocyte mean corpuscular volume of mice exposed to a 10% solution for 15.5 weeks and to 5 and 10% solutions following 31.5 weeks of treatment. In addition, dose-related decreases were found in body, liver, and thymus weights in the 5 and 10% solutions exposure groups after 31.5 weeks. These results suggest that bone marrow may be a sensitive indicator for long-term, low-level exposure of multiple chemicals in mice. Furthermore, long-term exposure to highly contaminated groundwater may present a subtle risk to the hematopoietic system.

Introduction

The National Toxicology Program (NTP), under the auspices of the Comprehensive Environmental Response, Compensation, and Liability Act (Superfund Act), has been studying chemical mixtures related to hazardous waste disposal sites.[9,19,51,52] Groundwater represents a precious national resource and the contamination of groundwater has become an environmental concern.[10–12,42,46,51] Evidence has accumulated that exposure to certain environmental chemicals, such as halogenated aromatic hydrocarbons, polycyclic aromatics, and heavy metals, can produce myelotoxicity in laboratory animals at dose levels where other manifestations of toxicity are not observed.[2,4,7,13] Bone marrow, with rapidly renewing cell populations, is one of the most sensitive tissues to cytotoxic agents. In humans, bone marrow failure is a significant complication of cancer chemotherapy and has been implicated in exposures to drugs and environmental agents.[14–16,38,40,41,44,48] Examination of colony formation of the hemopoietic cells following exposure to chemicals has proven to be a sensitive indicator of myelotoxicity as well as a means for mechanistic study of the toxicity of various drugs.[23,26,33–37,47] Virtually all of these studies focused on the toxic effects of individual chemicals and the ways to ameliorate or exacerbate those effects. While this approach permits a clear definition of the effects of one toxicant, most human situations involve simultaneous or sequential exposure to multiple chemicals. Groundwater contamination, especially from toxic waste dump sites, is one example of exposure to multiple compounds.

In the NTP chemical mixture toxicology studies, a 25-chemical mixture was prepared based on the EPA survey results of over 1000 known groundwater contaminants near 1061 hazardous waste disposal sites.[51,52] Initial findings on the 25-chemical mixture regarding chemistry development work,[52] immunosuppression in mice,[19] and male reproduction studies in mice[9] have been published. This

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article is a summary of a time-course study using hematopoietic endpoints in B6C3F$_1$ mice to investigate the health effects of exposure to multiple groundwater contaminants. We examined altered patterns of cell proliferation and differentiation induced by a mixture of groundwater contaminants in murine bone marrow hematopoiesis by *in vitro* colony growth assays. Related myelotoxicity studies on this chemical mixture with respect to toxicological interactions with whole body irradiation and its residual damage to the hematopoietic system in mice[53] have been published elsewhere.

Materials and Methods

Animal Treatment

Female B6C3F$_1$ (C57BL/6NxC3H/HeN) mice, 8 weeks of age, were obtained from the National Cancer Institute production contracts (Charles River, Portage, MI). Animals were housed ten each in filter-top polycarbonate cages and were maintained on the NIH-07 diet and water *ad libitum* for a 2-week acclimation period. Room temperature was maintained at 21 to 22°C and 50 to 55% humidity. Mice comprising a weight range of ±1 S.D. from the mean were randomized into experimental groups. Mice were given free access to 0, 1, 5, or 10% of the technically achievable chemical mixture stock for 2.5, 15.5, or 31.5 weeks. Water consumption was measured for each cage of ten mice over a 48-hr period at every 2-week interval during the study. Body weights and clinical observation were recorded once a week on 20 randomly selected mice per group throughout the experiment.

Preparation of Stock Solution

The chemical mixture was formulated with consideration of EPA survey concentrations, and toxicity and solubility of individual component chemicals.[51,52] A final technically achievable stock solution was prepared with 25 common groundwater contaminants found near toxic waste dumps at approximately 90% saturation levels for most chemicals (Table 1). In order to minimize chemical interactions, the stock solution was stored as 2 substock solutions: an organic substock containing 19 organic chemicals and an aqueous substock containing 6 metals and phenol.[52] Fresh drinking water mixture was prepared from substocks every 48 to 72 hr and analyzed periodically both before and after dosing. Some loss of organic volatiles during mixing of the substocks and during the first 24 hr following preparation did occur; however, the concentrations of acetone, phenol, and all the metals remained constant in the mixture. Detailed information on the recovery of component chemicals is given by Yang et al.[52]

TABLE 1. EPA Survey values of frequently detected groundwater contaminants and the NTP chemical mixture.

	Av. EPA Survey Conc (ppm)	Max EPA Survey Conc (ppm)	10% of NTP Mixture Stock Solution (ppm)[a]
Acetone	6.9	250	53
Arochlor 1260	0.21	2.9	0.01
Arsenic	30.6	3,680	9
Benzene	5	1,200	12.5
Cadmium	0.85	225	51
Carbon Tetrachloride	0.54	20	0.4
Chlorobenzene	0.1	13	0.1
Chloroform	1.46	220	7
Chromium	0.69	188	36
Diethylhexylphthalate	0.13	5.8	0.015
1,1-Dichloroethane	0.31	56.1	1.4
1,2-Dichloroethane	6.33	440	40
1,1-Dichloroethylene	0.24	38	0.5
1,2-*trans*-Dichloroethylene	0.73	75.2	2.5
Ethylbenzene	0.65	25	0.3
Lead	37	31,000	70
Mercury	0.34	50	0.5
Methylene chloride	11.2	7,800	37.5
Nickel	0.5	95.2	6.8
Phenol	34	7,713	29
Tetrachloroethylene	9.68	21,570	3.4
Toluene	5.18	110	7
1,1,1-Trichloroethane	1.25	618	2
Trichloroethylene	3.82	790	6.5
Xylenes	4.07	150	1.6
Total Conc of All Chemicals (ppm)	161.78		378.025

[a]The highest dose used in the study was prepared as 1 in 10 dilution of the technically achievable stock mixture. For details of EPA surveys and the formulation and dilutions of the chemical mixture stock, see Yang and Rauckman[51] and Yang et al.[52]

Histopathology

Treated mice were sacrificed following CO_2 anesthesia. Body, liver, spleen, right kidney, and thymus weights were recorded. The tissues were fixed in 10% neutral buffered formalin, processed by standard techniques, and stained with H & E. The lung, heart, liver, kidneys, adrenal glands, spleen, thymus, stomach, uterus, bone marrow (sternum), urinary bladder, and small and large intestines were examined microscopically.

Hematology

Blood samples were obtained prior to sacrifice from the retroorbital venous plexus of the mice. Hematological measurements included hemoglobin (Hgb), hematocrit (Hct), mean corpuscular volume (MCV), red blood cell count (RBC), and white blood cell count (WBC). Cell numbers were determined in a Coulter Counter, Model ZB (Coulter Electronics, Inc., Hialeah, FL).

Bone Marrow Cellularity

Marrow cells were collected from mice by aseptically dissecting both femurs free of attached tissue, cutting the femur at the epiphysis, and flushing the shaft with RPMI-1640 (Gibco, Grand Island, NY). Single-cell suspensions were prepared by successive passage of the cells through 22- and 25-gauge needles. Nucleated cells were enumerated by the Coulter Counter.

Progenitor Cell Formation

Granulocyte-macrophage progenitor cells were assessed for their ability to form colonies (CFU-GM) in semisolid culture techniques, a modification of the method by Bradley and Metcalf.[8] Nucleated femoral marrow cells (10^5) were suspended in 1 mL of RPMI-1640 culture media containing 20% fetal bovine serum (FBS) (Hyclone Labs, Ogden, UT), 5% human AB serum (Flow Labs, McLean, VA), 2 mM L-glutamine, 50 µg/mL garamycin, 1.5% methylcellulose, and 10% mouse lung conditioned medium. Plates in quadruplicate were incubated for 7 d at 37°C in 7% CO_2 humidified chamber. Colonies were stained with methylene blue and counted using a stereomicroscope (Wild, Heerburg, Switzerland).

Erythroid Precursor Formation

Colony-forming units-erythroid (CFU-E) in bone marrow from five mice per group were determined by using a modification of Iscove's method.[28] Nucleated femoral marrow cells (2×10^5) in 1 mL of culture medium, consisting of $1 \times \alpha$ modification of Eagle's medium with Earle's salts (Flow Labs), 30% FBS, 1.5% methylcellulose, 100 µM 2-mercaptoethanol (Sigma Chemical Co., St. Louis), and 0.5 U/mL erythropoietin (Connaught Labs, Swiftwater, PA), were incubated in the CO_2 incubator described above for 6 days. Colonies were stained with a modified benzidine method[18] and counted under the stereomicroscope.

Statistical Analysis

Data were analyzed by RS/1 multicompare procedure, using the Wilk-Shapiro test for normality, one-way analysis of variance, and Dunnett's test for multiple comparison with corresponding controls.[3]

Results

Water Consumption, Body and Organ Weight Changes

A significant decrease in water consumption occurred in mice treated with 5 and 10% solutions (79 and 72% of the control group, respectively) after 1 week, but no significant decrease was seen following 15 and 31 weeks of treatment. Previous paired-water studies indicated that myelotoxicity and immune effects were related to chemical exposure and not to the decreased water intake.[19] Chemical mixture exposure did not alter body and organ weights as compared to controls after 2.5 weeks. However, as the exposure duration lengthened, body and/or organ weights changed. Thus, the thymus weight decreased by about 21% and relative kidney weight (as percentage of body weight) increased by 15% following 15.5 weeks of treatment (Table 2). Furthermore, after 31.5 weeks of exposure, lower body, liver, and thymus weights were seen in the 5 and 10% chemical mixture groups. A significant reduction in relative liver and thymus weights were seen only at the highest dose following 31.5 weeks of treatment. Figure 1 is a plot of the body weight of the highest (10%) and the lowest (1%) dose levels as percentage of respective controls. Interestingly, body weights (as percentage of controls) remain unaffected until after 15 weeks of chemical treatment. While no effect was detected at the low-dose level, the body weights at the high-exposure concentration remained about 12% below those of controls between 15 and 31.5 weeks.

TABLE 2. Effects of a chemical mixture of groundwater contaminants on body and organ weights of female B6C3F$_1$ mice.[a]

Parameter	2.5-Week Exposure				15.5-Week Exposure				31.5-Week Exposure			
	0%[b]	1%	5%	10%	0%	1%	5%	10%	0%	1%	5%	10%
Body Weight (g)	22.2 ± 0.6	21.4 ± 0.6	21.7 ± 0.6	22.3 ± 0.2	27.8 ± 0.7	27.8 ± 0.9	27.1 ± 0.6	27.5 ± 0.7	32.3 ± 1.1	31.2 ± 0.9	28.8 ± 0.8[c]	28.1 ± 0.8[d]
Liver/b.w. (× 10^{-3})	55 ± 1	55 ± 1	55 ± 2	54 ± 1	52 ± 3	51 ± 2	49 ± 1	51 ± 1	48 ± 1	46 ± 1	45 ± 2	42 ± 1[c]
Spleen/b.w. (× 10^{-3})	3.6 ± 0.2	3.5 ± 0.1	3.5 ± 0.1	3.4 ± 0.1	3.5 ± 0.3	3.5 ± 0.2	3.5 ± 0.1	3.4 ± 0.1	3.1 ± 0.2	3.1 ± 0.2	3.2 ± 0.2	3.5 ± 0.1
Kidney/b.w. (× 10^{-3})	7.9 ± 0.2	8.0 ± 0.2	8.1 ± 0.1	8.1 ± 0.2	6.7 ± 0.2	7.3 ± 0.2	7.2 ± 0.3	7.7 ± 0.2[c]	6.4 ± 0.2	6.0 ± 0.3	7.0 ± 0.3	6.7 ± 0.3
Thymus/b.w. (× 10^{-3})	2.9 ± 0.1	2.7 ± 0.1	2.7 ± 0.1	2.6 ± 0.03	1.9 ± 0.1	1.7 ± 0.1	1.9 ± 0.1	1.5 ± 0.03[d]	1.9 ± 0.1	1.8 ± 0.1	1.7 ± 0.1	1.5 ± 0.1[c]

[a]Each value represents the mean ± SEM of seven mice per group.
[b]Percentage of mixture stock solution.
[c]$p < 0.05$ vs. controls by Dunnett's multiple range test.
[d]$p < 0.01$ vs. controls by Dunnett's multiple range test.

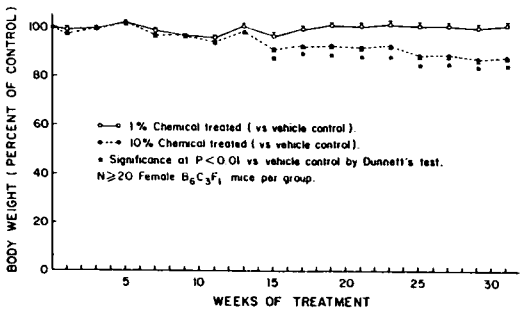

FIGURE 1. Effects of a chemical mixture of groundwater contaminants on body weights. Female B6C3F$_1$ mice were treated with a chemical mixture in drinking water daily for 31.5 weeks. Results are expressed as percentage of vehicle control (means ± SEM of a minimum of 20 mice per group).

Histopathology

The mice contained a variety of mild inflammatory and non-neoplastic lesions that were common for this age and species. These lesions were not noted unless they exceeded the normal spectrum. The forestomach hyperkeratosis appeared to be within normal limits for all exposure groups and was not considered to be treatment related. The inflammation of the liver was characterized by small focal loss of hepatocytes with mild accumulation of mononuclear inflammatory cells. These lesions are very common in mice. This case demonstrated an apparent increase in incidence, but the severity of the lesions was not dose related. Thymic atrophy is considered a treatment effect despite the lack of specific histological lesions because the atrophy is due to a decrease in cortical width and not apparent without morphological techniques unless the lesion is quite severe. Thus, it is possible that the immunological alterations as evidenced by thymic atrophy and the decrease in bone marrow progenitor cells may cause the mice to be more susceptible to the routine inflammatory lesions.

Hematology

Hematological values in mice exposed to the chemical mixture for 2.5 weeks were identical to those in control mice (Table 3). Statistically significant decreases occurred in MCV after 15.5 weeks of exposure to the 10% chemical mixture stock and following 31.5 weeks of treatment with the 5 and 10% chemical mixture stock. The other hematological parameters (i.e., WBC, RBC, Hct, Hgb) remained unaltered compared to controls throughout the study.

Bone Marrow

A significant effect on bone marrow cellularity was not observed; however, functional alterations occurred in the bone marrow as evidenced by a significant dose-related decrease in the numbers of CFU-GM colonies in mice exposed to 5 and 10% chemical mixture stock for 15.5 and 31.5 weeks of treatment (Table 4). In addition, dose-related suppression took place in the colony formation of CFU-E at 5 and 10% chemical mixture stock following either the 15.5- or 31.5-week exposure (Table 5).

Discussion and Conclusions

Hematopoietic cells originate from common stem cells that can give rise to new stem cells as well as generate progenitor cells programmed to differentiate along specific hematopoietic cell lineages. As these cells differentiate into mature cells with specialized functions, a corresponding loss of proliferative capacity occurs. New blood cells belonging to different cell lineages are formed from stem cells during embryogenesis and during the lifetime of the adult. Abnormalities in the normal developmental program for blood cell formation result in various types of hematological diseases. The mechanism of stem cell renewal and commitment is complex. The hematopoietic system is uniquely sensitive to a wide variety of toxic agents and environmental pollutants.[5,6,23,24,35] The injury of stem and progenitor cells can affect the controls that regulate multiplication and differentiation of normal blood cells to different cell lineages.[17,39] Abnormalities of blood cell production may be restricted to a single series, such as granulocyte or erythrocyte precursors, or may involve several hematopoietic lines. Thus, a variety of stages of hematopoietic cell differentiation can be recognized as targets of chemical damage which later results in clinically recognizable disease syndromes. Schurig et al.[43] examined a battery of bone marrow assays in mice using 17 anticancer drugs and demonstrated that colony-forming assays are a very sensitive indicator for altered hematopoiesis, often being suppressed prior to detecting hematological changes. Hematopoiesis represents a well-defined and highly dynamic process that can serve as a sensitive organ system for determining chemically induced cellular and molecular events associated with this injury.[35] The clonal assays provide methods for the simultaneous investigation of myelotoxic effects produced both on cellular proliferation (amplification) and on cel-

TABLE 3. Effects of a chemical mixture of groundwater contaminants on hematological parameter of female B6C3F$_1$ mice.[a]

	2.5-Week Exposure				15.5-Week Exposure				31.5-Week Exposure			
Parameter	0%[b]	1%	5%	10%	0%	1%	5%	10%	0%	1%	5%	10%
WBC (× 10^3/μL)	2.9 ± 0.2	2.9 ± 0.1	3.1 ± 0.2	3.9 ± 0.3	3.6 ± 0.2	3.0 ± 0.3	3.5 ± 0.3	3.2 ± 0.3	3.5 ± 0.2	2.9 ± 0.2	2.8 ± 0.2	4.2 ± 0.2
RBC (× 10^6/μL)	8.2 ± 0.1	8.3 ± 0.1	8.2 ± 0.1	8.1 ± 0.1	8.9 ± 0.1	8.6 ± 0.2	8.9 ± 0.1	8.7 ± 0.1	8.5 ± 0.1	8.5 ± 0.1	8.5 ± 0.1	8.4 ± 0.1
Hct (%)	39.1 ± 0.6	38.8 ± 0.3	38.6 ± 0.7	37.8 ± 0.5	46.6 ± 0.6	44.4 ± 0.6	45.4 ± 0.7	43.1 ± 0.8	43.4 ± 0.9	42.8 ± 0.5	42.2 ± 0.5	40.2 ± 0.8
MCV (fl)	47.7 ± 0.2	46.7 ± 0.2	47.1 ± 0.2	46.7 ± 0.2	52.4 ± 0.5	51.6 ± 0.6	51.6 ± 0.4	49.5 ± 0.5[d]	51.1 ± 0.5	50.3 ± 0.3	49.6 ± 0.4[c]	47.8 ± 0.3[d]
Hgb (g/dL)	14.3 ± 0.2	14.4 ± 0.1	14.4 ± 0.1	14.5 ± 0.2	14.6 ± 0.2	14.2 ± 0.2	14.7 ± 0.2	14.7 ± 0.2	14.3 ± 0.3	14.1 ± 0.1	13.9 ± 0.1	14.1 ± 0.2

[a]Each value represents the mean ± SEM of ten mice per group, measured 1 day postexposure.
[b]Percentage of mixture stock solution.
[c]$p < 0.05$ vs. controls by Dunett's multiple range test.
[d]$p < 0.01$ vs. controls by Dunett's multiple range test.

TABLE 4. Effects of a chemical mixture of groundwater contaminants on granulocyte-macrophage progenitor from bone marrow of female B6C3F$_1$ mice.[a]

	2.5-Week Exposure				15.5-Week Exposure				31.5-Week Exposure			
Parameter	0%[b]	1%	5%	10%	0%	1%	5%	10%	0%	1%	5%	10%
Cellularity/ Femur (× 10^6)	20.0 ± 0.4	18.8 ± 0.7	18.5 ± 0.2	18.6 ± 0.6	21.4 ± 1.0	22.3 ± 0.5	20.3 ± 1.7	18.2 ± 0.8	23.3 ± 0.6	23.0 ± 0.5	22.7 ± 0.5	22.4 ± 0.4
CFU-GM/ 10^5 Cells	112.7 ± 0.3	111.8 ± 0.6	111.8 ± 0.7	111.0 ± 0.3	111.6 ± 1.2	109.1 ± 1.2	89.0 ± 1.0[c]	66.1 ± 1.2[c]	113.3 ± 0.3	111.1 ± 1.0	79.5 ± 0.9[c]	59.4 ± 1.3[c]
CFU-GM/ Femur (×10^2)	225.0 ± 4.6	209.6 ± 7.5	206.7 ± 2.3	206.8 ± 6.7	238.1 ± 9.2	250.9 ± 4.5[c]	180.0 ± 13.1[c]	119.9 ± 4.6[c]	263.1 ± 6.6	255.4 ± 7.5[c]	180.7 ± 4.2[c]	132.8 ± 1.8[c]

[a]Each value represents the mean ± SEM of five mice per group, measured 2 days postexposure.
[b]Percentage of mixture stock solution.
[c]$p < 0.01$ vs. controls by Dunett's multiple range test.

TABLE 5. Effects of a chemical mixture of groundwater contaminants on erythrocyte precursor from bone marrow of female B6C3F$_1$ mice.[a]

	2.5-Week Exposure				15.5-Week Exposure				31.5-Week Exposure			
Parameter	0%[b]	1%	5%	10%	0%	1%	5%	10%	0%	1%	5%	10%
Cellularity/ Femur ($\times 10^6$)	19.2 ± 0.3	19.1 ± 0.5	18.4 ± 1.0	19.4 ± 0.3	21.4 ± 0.9	21.1 ± 0.9	19.3 ± 0.8	19.2 ± 0.7	24.3 ± 0.6	23.6 ± 0.5	24.2 ± 0.5	23.5 ± 0.4
CFU-E/ 2×10^5 Cells	105.5 ± 0.3	104.8 ± 0.4	105.6 ± 1.3	104.2 ± 0.4	105.0 ± 0.8	102.8 ± 0.9	93.6 ± 1.1[c]	65.6 ± 1.1[c]	105.3 ± 1.1	103.1 ± 0.5	84.8 ± 0.8[c]	59.7 ± 0.7[c]
CFU-E/ ($\times 10^2$)	101.4 ± 1.6	100.3 ± 2.1	97.0 ± 4.3	100.8 ± 1.4	112.1 ± 4.0	108.4 ± 3.7	90.1 ± 2.7[c]	62.8 ± 1.1[c]	127.6 ± 2.3	121.8 ± 2.3	102.8 ± 1.4[c]	70.2 ± 0.5[c]

[a]Each value represents the mean ± SEM of five mice per group, measured 3 days postexposure.
[b]Percentage of mixture stock solution.
[c]$p < 0.01$ vs. controls by Dunett's multiple range test.

lular differentiation. In addition, these methods can also be used to evaluate toxic effects on proteins that regulate cell multiplication and differentiation of specific blood cell lineages or populations responsible for their production.

The present studies demonstrated that mice treated with 10% of the chemical mixture (N ≥ 20 mice per group) did not lose body weight until after 15 weeks of treatment, and only a 12% weight reduction was induced compared to the control group at the end of the 31.5-week exposure. The hematological parameters remained unaffected except for a 6% decrease in MCV with the 10% chemical mixture stock after 15.5 and 31.5 weeks of treatment. However, the CFU-GM numbers were reduced to 50% of controls in mice exposed to a 10% chemical mixture for 15.5 and 31.5 weeks. Furthermore, at this concentration (10% chemical mixture), an approximately 45% decrease occurred in the erythroid precursors after 15.5 and 31.5 weeks of treatment. Based on these findings, it is clear that bone marrow parameters are very sensitive toxicological endpoints in mice exposed to a mixture of groundwater contaminants.

Recently, Germolec et al.[19] found that mice exposed to this chemical mixture were immunosuppressed. At a functional level, the hematopoietic effects associated with this treatment were consistent with an antiproliferative mechanism since stem cells and antigen-stimulated B cells are rapidly cycling cell populations and would be expected to represent preferential targets for compounds with antiproliferative effects.[34] In fact, some components of this mixture, including arochlor, benzene, phenol, toluene, and heavy metals, have been shown to cause similar hematopoietic effects in laboratory animals.[1,2,4,7,13,20-22,29,31] Heavy metals such as lead, cadmium, and the polyhydroxy metabolites of benzene are highly reactive and bind to sulfhydryl groups on cell membranes, presumably altering cellular interactions.[32] However, it seems unlikely that the individual contaminants present at such low concentration in this mixture would be solely responsible for the observed effects on hematopoiesis. In several cases, the immunotoxicity of one chemical has been modified by a second chemical that alters xenobiotic metabolism.[45] Metals can either enhance or suppress immune responses.[30] Various environmental chemicals that modify the bone marrow function may also interact with chemical carcinogens, especially since several carcinogens are also myelosuppressive.[49,50] Studies of halogenated aromatic hydrocarbon-induced myelotoxicity indicated that a specific antagonist effectively inhibited receptor binding via competition in bone marrow cells.[26,36] The interactions and mechanisms of action of this chemical mixture are complex and occur at different steps in the overall process. Thus, the effects observed are likely a composite response representing both positive and negative influences caused by the various chemicals and/or their metabolites. The precise mechanism of mixture toxicity is unknown, but the observed hematopoietic effects may be considered an environmental health concern for prolonged exposure to groundwater contaminants.

In conclusion, subchronic or chronic exposure to multiple groundwater contaminants in mice causes suppression of marrow granulocyte-macrophage progenitors and erythroid precursors. The findings imply that bone marrow is a more sensitive endpoint for detecting health effects of groundwater contaminants in mice than histopathological or hematological evaluation. With the potential human exposure to contaminated groundwater, a need exists for greater awareness among clinicians and epidemiologists as to the subtle effects that long-term, low-level exposures of multiple chemicals may bring about. In addition, previous studies from the authors' laboratory have shown that even mild bone marrow injury makes mice more susceptible to a second challenge to the progenitor cells.[25,27] Further study is underway to determine if a mixture of groundwater contaminants might have a long-lasting myelotoxic effect. These results collectively suggest that long-term exposure to highly contaminated groundwater may present a subtle risk to the hematopoietic stem cells.

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APPENDIX I

Immunotoxicity Studies

Toxicology Studies of a Chemical Mixture of 25 Groundwater Contaminants

II. Immunosuppression in B6C3F$_1$ Mice

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FUNDAMENTAL AND APPLIED TOXICOLOGY 13, 377–387 (1989)

Toxicology Studies of a Chemical Mixture of 25 Groundwater Contaminants

II. Immunosuppression in B6C3F$_1$ Mice[1]

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Toxicology Studies of a Chemical Mixture of 25 Groundwater Contaminants. II. Immunosuppression in B6C3F$_1$ mice. GERMOLEC, D. R., YANG, R. S. H., ACKERMANN, M. F., ROSENTHAL, G. J., BOORMAN, G. A., BLAIR, P., AND LUSTER, M. I. (1989). *Fundam. Appl. Toxicol.* **13,** 377–387. Concern over the potential adverse health effects of chemically contaminated groundwater has existed for many years. In general, these studies have focused on retrospective epidemiological studies for cancer risk. In the present studies, immune function was monitored in female B6C3F$_1$ mice exposed to a chemical mixture in drinking water for either 14 or 90 days. The mixture consisted of 25 common groundwater contaminants frequently found near toxic waste dumps, as determined by EPA surveys. None of the animals developed overt signs of toxicity such as body or liver weight changes. Mice exposed to the highest dose of this mixture for 14 or 90 days showed immune function changes which could be related to rapidly proliferating cells, including suppression of hematopoietic stem cells and of antigen-induced antibody-forming cells. Some of these responses, e.g., granulocyte–macrophage colony formation, were also suppressed at lower concentrations of the chemical mixture. There were no effects on T cell function or T and B cell numbers in any of the treatment groups. Altered resistance to challenge with an infectious agent also occurred in mice given the highest concentration, which correlated with the immune function changes. Paired-water studies indicated that the immune effects were related to chemical exposure and not to decreased water intake. These results suggest that long-term exposure to contaminated groundwater may represent a risk to the immune system in humans.

Over the past 15 years evidence has accumulated that exposure to certain chemicals found in the environment (e.g., halogenated aromatic hydrocarbons, polycyclic aromatics, and heavy metals) can produce immune dysfunction in laboratory animals at chemical exposure levels where other manifestations of toxicity are not observed (Koller, 1980; Dean *et al.*, 1986; Luster *et al.*, 1987). These effects are often accompanied by increased susceptibility to challenge with infectious agents or tumor cells. The sensitivity of the immune system to toxic agents probably reflects both the general properties of a xenobiotic (e.g., reactivity with macromolecules) and the complex nature of the immune system. Although limited data are available, there are several examples of humans demonstrating characteristics of immune suppres-

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sion following inadvertent or occupational exposure to xenobiotics, similar to those observed in rodents. These cohorts include, among others: Michigan residents and farmers exposed to polybrominated biphenyls (PBBs) through the consumption of contaminated livestock and dairy products; Chinese and Japanese exposed to polychlorinated biphenyls (PCBs) and dibenzofurans through contaminated rice oil used in cooking; and factory workers with aplastic anemia and leukemia occupationally exposed to benzene (Bekesi *et al.*, 1978; Chang *et al.*, 1981; Snyder, 1984). Despite these selected examples, little evidence is available to suggest that environmental exposure to xenobiotics can influence the immunocompetence of the general population at large. Public concern for possible chemically induced immune disturbances in humans has grown, however, in part from recent studies suggesting T cell abnormalities in women following consumption of aldicarb-contaminated groundwater (Fiore *et al.*, 1986). These concerns have been fueled by suggestions of an immunologic etiology for a recently described disease often referred to as "environmental illness" (Davis, 1985), as well as a rash of court litigations ascribing immunological changes associated with exposure to environmental contaminants (Marshall, 1986; Miller, 1987).

The National Toxicology Program (NTP), under the auspices of the Comprehensive Environmental Response, Compensation and Liability Act (Superfund Act), has initiated a number of toxicology studies on chemicals found in high-priority hazardous waste sites and for which adequate toxicological data are not available (Yang and Rauckman, 1987). As part of this effort, a project on the immunological effects of a chemical mixture of groundwater contaminants was undertaken. Due primarily to potential difficulties in quantitative analysis and data interpretation, immunological studies of complex chemical mixtures have not been reported previously. In the present studies a mixture containing 19 organic and 6 inorganic chemicals was prepared (Table 1). These chemicals represent 25 common contaminants frequently found in groundwater derived from over 1000 known groundwater contaminants identified by the EPA in and around 1061 hazardous waste sites throughout the United States (Yang and Rauckman, 1987; Lockheed Engineering, 1985; Mitre Corp., 1983). Thus, while this specific chemical configuration may not actually occur in the environment, it is representative of a highly contaminated groundwater sample.

METHODS

Preparation of stock solution and experimental design. The chemical mixture was formulated by the Midwest Research Institute (Kansas City, MO) under an NTP contract as described by Yang *et al.* (1989). A final technically achievable stock solution was prepared with consideration of the following three factors: (1) EPA survey concentrations of these chemicals in the groundwater around hazardous waste disposal sites; (2) toxicity of individual components; and (3) solubility of the individual compounds in this unique matrix. This mixture stock, containing 25 chemicals, was prepared by establishing the concentrations of all chemicals at the 90% level of the highest analytically confirmed values from any of the mixtures prepared during the initial attempts. In most instances, these levels represented 90% of the saturation levels for the respective chemicals in a deionized water-based mixture. For ease in handling and to minimize chemical interactions, the stock solution was stored as two substock solutions: an organic substock containing 18 neat organic chemicals and an aqueous substock solution composed of 6 metal salts and phenol. The concentration of the aqueous metal/phenol was such that the direct mixing of the organic and aqueous substocks produced the desired high dose level for animal experiments. The lower dose levels were appropriate dilutions prepared in deionized water. The dose levels for the 14-day study were 0, 0.2, 2, and 20% of the technically achievable stock and for the 90-day study 1, 5, and 10%. The mixture was administered via drinking water to 6- to 8-week-old female C57BL/6 × C3H mice ($B6C3F_1$) obtained through a National Cancer Institute production contract from Charles River (Portage, MI). Control mice received deionized water only. Because of the volatility of some of the organics, fresh drinking water mixtures were prepared from substocks every 48–72 hr. A slight loss of volatile organics occurred; however, quantitative analysis of the dosing solution indicated that the chemical mixture was otherwise stable over this period of time

TABLE 1

EPA SURVEY CONCENTRATIONS OF GROUNDWATER CONTAMINANTS AND COMPOSITION OF A COMPLEX CHEMICAL MIXTURE REPRESENTING A CONTAMINATED GROUNDWATER SAMPLE

	Average EPA survey concentrations (ppm)	Maximum EPA survey concentrations (ppm)	Maximum concentrations used in the study (ppm)[a]
Acetone	6.90	250.0	106.00
Aroclor 1260	0.21	2.9	0.02
Arsenic	30.60	3680.0	18.00
Benzene	5.00	1200.0	25.00
Cadmium	0.85	225.0	102.00
Carbon tetrachloride	0.54	20.0	0.80
Chlorobenzene	0.10	13.0	0.20
Chloroform	1.46	220.0	14.00
Chromium	0.69	188.0	72.00
Diethylhexyl phthalate	0.13	5.8	0.03
1,1-Dichloroethane	0.31	56.1	2.80
1,2-Dichloroethane	6.33	440.0	80.00
1,1-Dichloroethylene	0.24	38.0	1.00
1,2-trans-Dichloroethylene	0.73	75.2	5.00
Ethylbenzene	0.65	25.0	0.60
Lead	37.00	31000.0	140.00
Mercury	0.34	50.0	1.00
Methylene chloride	11.20	7800.0	75.00
Nickel	0.50	95.2	13.60
Phenol	34.00	7713.0	58.00
Tetrachloroethylene	9.68	21570.0	6.80
Toluene	5.18	1100.0	14.00
1,1,1-Trichloroethane	1.25	618.0	4.00
Trichloroethylene	3.82	790.0	13.00
Xylenes	4.07	150.0	3.20
Total concentration of all chemicals (ppm)	131.05		756.05

[a] The highest dose level of the mixture used in the study was a 1:5 dilution (i.e., 20%) of the technically achievable stock mixture which is not shown.

to provide adequate estimation of the exposure conditions. A paired-water study was conducted on a third replicate study using animals exposed for 14 days and provided drinking water equal to that consumed by animals given the high dose (20% technically achievable stock solution).

Pathology and hematology evaluation. Following exposure, mice were killed with CO_2 inhalation, and body, liver, spleen, left kidney, and thymus weights were recorded. The tissues were fixed in 10% neutral-buffered formalin, processed by standard techniques, and stained with hematoxylin and eosin. The lung, heart, liver, kidneys, adrenal glands, spleen, thymus, stomach, uterus, bone marrow (sternum), urinary bladder, and small and large intestines were examined microscopically. Blood samples were obtained prior to death from the retroorbital venous plexus of the mice. Hematological measurements included hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, erythrocyte count (RBC), and total and differential white blood cell count (WBC).

Immune function tests. The antibody response was determined by quantitating the plaque-forming cell (PFC) response to the T-dependent antigen, sheep red blood cells. Mice were immunized by intravenous injection of 2×10^8 sheep red blood cells and IgM PFCs were enumerated in spleen cells 4 days later using a modified Jerne plaque assay in agar (Luster *et al.*, 1988). Lymphocyte proliferation was measured by quantitating [^{3}H]-thymidine ([^{3}H]TdR; spec act = 6.7 Ci/mM, Dupont NEN, Boston, MA) incorporation into splenic lympho-

cytes in response to the mitogens phytohemagglutinin (PHA) and *Escherichia coli* lipopolysaccharide (LPS), in addition to allogeneic leukocytes (mitomycin C-treated lymphocytes from DBA/2N mice) in a mixed leukocyte response (MLR). Conditions for these assays have been previously published (Luster *et al.*, 1988). The natural killer (NK) cell activity of spleen cells was determined using YAC-1 tumor target cells in a ^{51}Cr (spec act = 400 mCi/mM, Dupont NEN) release assay (Reynolds and Herberman, 1981). Cytotoxic T lymphocyte (CTL) activity was measured in spleen cells sensitized *in vitro* with mitomycin C-treated P815 mastocytoma cells (Murray *et al.*, 1985). The anti-tumor activity of peritoneal macrophages was measured against B16F10 tumor cells following *in vitro* activation with lymphokines (supernatants of Con A-stimulated spleen cells) (Varesio *et al.*, 1980).

Lymphocyte enumeration. Splenic lymphocyte subpopulations were quantitated using a Becton Dickinson FACS IV Flow Cytometer (Mountain View, CA). Monoclonal α-Thy 1.2 and α-kappa antibodies were used to enumerate T and B cells, respectively. T cell subsets were measured using α-L3T4 as a T helper cell marker and α-Lyt-2 as a T suppressor/cytotoxic T cell marker as previously described (Rosenthal *et al.*, 1989). Monoclonal antibody reagents were commercially purchased (Becton Dickinson).

Progenitor cell formation. Granulocyte–macrophage progenitor cells were assessed for their ability to form colonies (CFU-GM) in agar as described by Boorman *et al.* (1984). Briefly, bone marrow cells from both femurs were removed, pooled, and passed through a 25-gauge needle to produce single cell suspensions. Aliquots of 1×10^5 cells were added to quadruplicate wells of 6-well tissue culture plates containing 20% FBS (Hyclone Laboratories, Ogden, UT), 5% human AB serum (GIBCO, Grand Island, NY), 2 mM L-glutamine, 0.5 μg/ml gentamycin, and 10% mouse lung-conditioned medium (MLCM). The preparation of MLCM has been previously described (Boorman *et al.*, 1984). Plates were incubated for 7 days at 37°C in a 7.5% CO_2 humidified chamber. Following incubation, colonies containing 40 or more cells were enumerated using an inverted microscope.

Host resistance assays. The resistance to bacterial challenge with *Listeria monocytogenes* (strain L242/73, type 4B) was determined by monitoring mortality following iv injection of 3×10^4 viable bacteria. This concentration approximates an LD_{20} in control mice. A nonlethal strain of malaria, *Plasmodium yoelii* 17XNL, was administered by iv injection of 1×10^6 parasitized syngeneic erythrocytes. The percentage of parasitized erythrocytes, determined by Diff-Quik staining of tail blood, was followed through the course of infection to determine the peak day. The growth of PYB6 tumor cells was monitored for 60 days following sc injection of 5×10^4 viable tumor cells into the right thigh.

Statistical analysis. Statistical significance for immune function tests was determined by the RS/1 Multicompare procedure using the Wilkes–Shapiro test for normality, one-way analysis of variance, and Dunnett's test for multiple comparison with a group control. Data shown are representative of duplicate experiments.

RESULTS

Clinical findings. A decrease in water consumption occurred in mice given the 20% solution (treated mice consumed 52% as much water as control mice) in the 14-day studies as well as in mice given the 5 and 10% solutions in the 90-day studies (73 and 64% of the control group, respectively). Although there were no effects on body weights associated with the decreased water intake, a paired-water group was included to ensure that any clinical or immunological changes observed were not due to the decreased water consumption. This group received an amount of drinking water for 14 days equivalent to that consumed by the group given the 20% concentration, and was based upon daily measurements over the 14-day period. Using these experimental regimens, there were no changes in body, thymus, or liver weights as a result of chemical exposure (data not shown). Furthermore, there were no significant morphological changes in any of the tissues examined between treated and control animals when examined histologically. There were slight increases in kidney weights (15% above controls) in both the 14- and 90-day high dose groups which were not associated with any histogical alterations and may not be related to chemical exposure since a similar effect was noted in the paired-water group. There was a 15% decrease in spleen weight in mice exposed to the high dose for 90 days. Hematological values in mice exposed to the chemical mixture for 14 days were identical to those in control mice (Table 2). In mice exposed for 90 days, however, there were significant dose-related decreases in hematocrit, mean corpuscular volume, hemoglobin, and mean corpuscular hemoglobulin, consistent with a mild microcytic anemia.

TABLE 2

EFFECTS OF A CHEMICAL MIXTURE ON HEMATOLOGICAL PARAMETERS AND QUANTITATIVE CHANGES ON SPLENIC LYMPHOCYTE POPULATIONS

Parameter	Concentration of stock solution used in the 14-day exposure (%)					Concentration of stock solution used in the 90-day exposure (%)			
	0	0.2	2	20	Paired water	0	1	5	10
Hematology									
WBC (1000/μl)	3.2 ± 0.3[a]	3.4 ± 0.4	3.8 ± 0.3	3.2 ± 0.2	2.8 ± 0.3	5.7 ± 0.6	4.8 ± 0.3	4.2 ± 0.3	5.2 ± 0.5
RBC (ml/μl)	9.6 ± 0.1	9.5 ± 0.1	9.7 ± 0.1	9.8 ± 0.1	9.8 ± 0.2	9.6 ± 0.1	9.5 ± 0.1	9.6 ± 0.1	9.6 ± 0.1
Hematocrit (%)	49.3 ± 0.6	49.4 ± 0.3	49.9 ± 0.2	49.7 ± 0.2	50.6 ± 0.9	49.7 ± 0.3	48.6 ± 0.4	48.7 ± 0.5	48.3 ± 0.4[b]
Mean corpuscular volume (fl)	51.7 ± 0.4	51.9 ± 0.3	51.6 ± 0.2	51.1 ± 0.2	51.5 ± 0.2	51.9 ± 0.2	51.3 ± 0.2[b]	50.6 ± 0.2[b]	50.1 ± 0.1[b]
Platelet (1000/μl)	1082 ± 32	1037 ± 14	1040 ± 19	1056 ± 19	1074 ± 24	1079 ± 29	1064 ± 15	1027 ± 20	1016 ± 12
Hemoglobulin (g/dl)	14.8 ± 0.2	14.9 ± 0.1	15.0 ± 0.1	14.9 ± 0.1	15.1 ± 0.2	14.9 ± 0.1	14.6 ± 0.1	14.6 ± 0.1	14.6 ± 0.1
Mean corpuscular hemoglobulin (pg)	15.4 ± 0.1	15.6 ± 0.1	15.5 ± 0.1	15.3 ± 0.1	15.4 ± 0.1	15.5 ± 0.1	15.4 ± 0.1	15.2 ± 0.1[b]	15.2 ± 0.1[b]
Lymphocyte enumeration									
Nucleated cells spleen ($\times 10^6$)	140 ± 10	116 ± 7	134 ± 8.9	152 ± 18	112 ± 9	136 ± 7	127 ± 5	157 ± 8	132 ± 8
% B cells	ND[c]	ND	ND	ND	ND	48 ± 1	50 ± 1	51 ± 1	51 ± 0
% T cells	ND	ND	ND	ND	ND	31 ± 1	30 ± 1	30 ± 1	29 ± 1
% L3T4	ND	ND	ND	ND	ND	25 ± 1	24 ± 1	26 ± 1	24 ± 1
% Lyt 2	ND	ND	ND	ND	ND	15 ± 1	16 ± 1	14 ± 1	14 ± 1

[a] Each value represents the mean ± SEM of a minimum of 10 mice per group.
[b] $p < 0.05$ vs controls by Dunnett's multiple range test.
[c] Not determined.

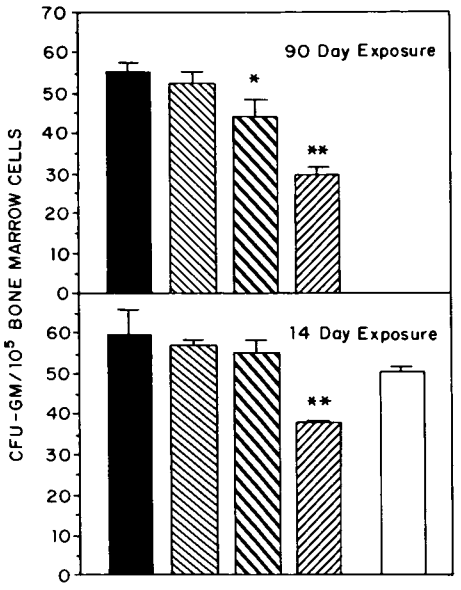

FIG. 1. Myelotoxicity of the chemical mixture as determined by suppression of granulocyte–macrophage progenitor cells (CFU-GM). Bone marrow cells were individually collected from groups of mice (n = 7) following 14 or 90 days of treatment. Dose levels in the 90-day exposure were: ■, vehicle control; ▨, 1%; ▩, 5%; ▨, 10%, and for the 14-day exposure; ■, vehicle control; ▨, 0.2%; ▩, 2%; ▨, 20%, and □, paired water. Bars = SD. As determined using Dunnett's t test, * significant at $p < 0.05$ and ** significant at $p < 0.01$. Both exposure regimens produced a significant dose dependency ($p < 0.05$) for inhibition of colony formation as determined by Jonckheere's test.

Bone marrow and immune parameters. Functional alterations occurred in the bone marrow, as evidenced by a significant decrease in the number of CFU-GM progenitor cells in mice exposed to the 20% concentration for 14 days and in mice exposed to the 5 and 10% concentrations for 90 days (Fig. 1). In mice treated for 90 days, the inhibition of colony formation was accompanied by a decrease in bone marrow cellularity, although hypocellularity was not detected after 14 days of treatment (data not shown). In addition, there was a dose-related suppression in the antibody PFC response to sheep red blood cells at the highest concentration following either the 14- or 90-day exposures (Fig. 2). The suppression of the PFC response was not due to decreased spleen cellularity since the numbers of splenic nucleated cells were similar in all treatment groups (Table 2). It is also unlikely that the mixture produced a reductive effect on any lymphocyte population since there were no significant changes in splenic B or T cell numbers or the number of T cells expressing Lyt-2 or L3T4 surface markers following 14- or 90-day treatments (Table 2).

Since the presumptive major health effect of contaminated groundwater is an increased risk of developing cancer, efforts were focused on examining the effects of the mixture on immunological parameters associated with tumor immunity. The effect of exposure to the mixture on the ability of NK cells, cyto-

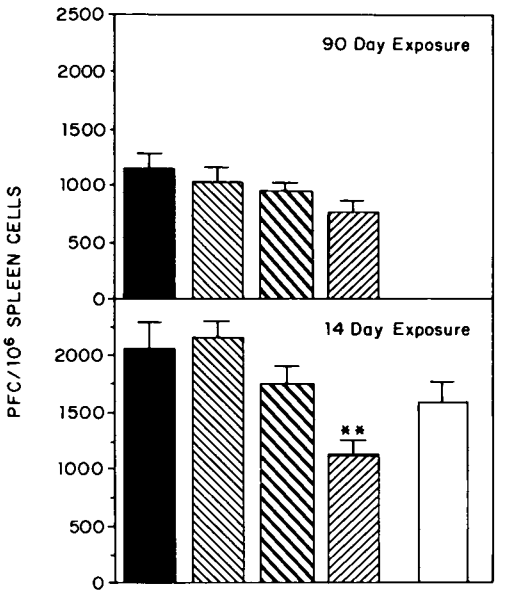

FIG. 2. Suppression of the antibody response to sheep red blood cells in mice exposed to a chemical mixture for 90 or 14 consecutive days. Results represent mean number of plaque-forming cells from individual mice (n = 7 per group). Dose levels in the 90-day exposure were: ■, vehicle control; ▨, 1%; ▩, 5%; ▨, 10%; and for the 14-day exposure: ■, vehicle control; ▨, 0.2%; ▩, 2%; ▨, 20%, and □, paired water. Bars = SD. As determined using Dunnett's t test, ** significant at $p < 0.01$. Both exposure regimens produced a significant dose dependency ($p < 0.05$) for inhibition of plaque forming cells as determined by Jonckheere's test.

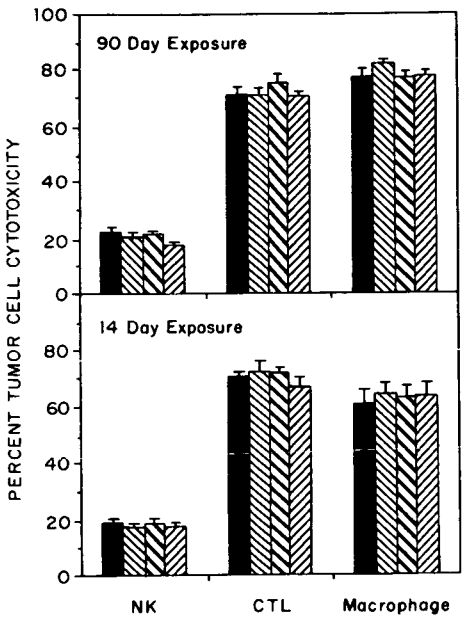

FIG. 3. Effects of 14- or 90-day exposure to a chemical mixture on NK cells, cytotoxic T lymphocyte, and macrophage activity as determined by their ability to lyse appropriate tumor target cells. Each value represents a mean of seven mice per group. Dose levels in the 90-day exposure were; ■, vehicle control; ▨, 1%; ▧, 5%; ▨, 10%; and for the 14-day exposure: ■, vehicle control; ▨, 0.2%; ▧, 2%; and ▨, 20%. Bars = SD. None of these parameters were affected by the chemical mixture.

toxic T lymphocytes (CTL), and lymphokine-activated macrophages to lyse appropriate tumor cell targets is shown in Fig. 3. Immunological alterations associated with T cell, NK cell, or macrophage antitumor activities following exposure to the mixture for 14 or 90 days were not evident.

To determine whether the observed changes were sufficient in magnitude to affect disease resistance following exposure to the chemical mixture, groups of mice were challenged with *L. monocytogenes*, PYB6 syngeneic tumor cells, or *P. yoelii* and the host susceptibility resistance was monitored (Fig. 4). The number of parasitized red blood cells in mice challenged with the malarial parasite *P. yoelii* was increased in mice treated with the highest concentration of the mixture compared to control groups on Days 10, 12, and 14 following infection (only Day 12, which was the day of peak infection, is shown). In contrast, resistance to challenge with *L. monocytogenes* or PYB6 tumor cells was not affected by mixture treatment. Resistance to the latter agents is primarily mediated by macrophages and activated T cells, whereas humoral immunity plays a major role in resistance to malarial parasites (Krier and Green, 1980; Urban *et al.*, 1982; North, 1973).

DISCUSSION

This study demonstrates that exposure to a complex chemical mixture composed of common groundwater contaminants can produce changes in hematopoietic and immune functions. At a functional level, the immunological effects associated with this treatment were consistent with an antiproliferative mechanism since stem cells and antigen-stimulated B cells are rapidly cycling cell populations and would be expected to represent preferential targets for compounds with antiproliferative effects. Similar effects have been associated with a number of cancer chemotherapeutics and toxic environmental contaminants that possess antiproliferative activity, the latter including urethan and chlorinated dibenzo-*p*-dioxins (Luster *et al.*, 1982, 1984; Shand, 1979; Uyeki *et al.*, 1976; Baram *et al.*, 1987). In fact, several of the components of this mixture, including Aroclor, benzene, and heavy metals, have been shown to cause similar immunological and myelotoxic effects in laboratory animals (Koller, 1980; Lutton *et al.*, 1984; Vos and de Roij, 1972; Beran *et al.*, 1983; Bolcsak and Nerland, 1983; Hsieh *et al.*, 1988). However, from general estimates of these earlier studies, it would appear that none of the individual contaminants were present at sufficient concentrations in the chemical mixture to be solely responsible for the observed effects on immune functions. Rather, the effects observed are likely a composite response repre-

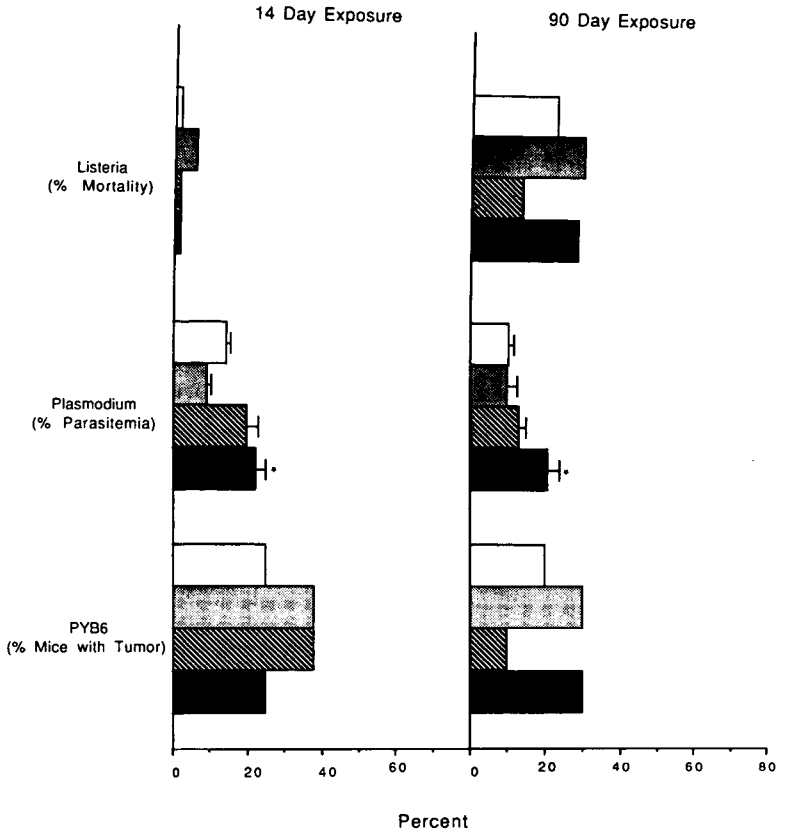

FIG. 4. Changes in host resistance following challenge with PYB6 transplantable tumor cells, *P. yoelii*, or *L. monocytogenes* in mice administered a chemical mixture in drinking water for either 14 or 90 days. Mice were treated with a 0.2 (light shaded bar), 2 (dark shaded bar) or 20% (black bar) dilution of the technically achievable stock for 14 days or a 1 (light shaded bar), 5 (dark shaded bar), or 10% (black bar) dilution for 90 days; control mice received deionized water (open bar). Following infection with *L. monocytogenes* (3×10^4 viable bacteria), mortality was monitored for 14 days ($n = 30$ mice per treatment group). Mortality in a "positive" control group treated with 180 mg/kg cyclophosphamide was 52% (not shown). Infection with *P. yoelii* ($n = 8$ per treatment group) was determined by quantitating the percentage of parasitemia on Days 10, 12, and 14 following injection of 10^6 parasitized erythrocytes. Only the peak day (Day 12) of infection is shown. Note that infection by *P. yoelii* in a group of mice receiving similar amounts of water consumed by the dose group receiving the 20% solution for 14 days was no different than in control animals (not shown). Bars = SD. Tumor growth was monitored for 60 days following subcutaneous injection of 5×10^4 PYB6 tumor cells. * Significantly different from controls at $p < 0.05$ by Dunnett's multiple range test.

senting both positive and negative influences caused by the various chemicals and/or their metabolites.

Although the major effects of the chemical mixture were observed on rapidly proliferating cells, the mechanism(s) responsible for the immunotoxicity and myelotoxicity is only partially understood. Some of the contaminants appear to mediate their effects through very specific events, such as PCBs which act through the Ah receptor (reviewed by Luster *et al.*, 1987), while others may alter lymphocyte function through relatively nonspecific mechanisms. In this respect, heavy metals such as lead and cadmium as well as the polyhydroxy metabolites of benzene are

highly reactive and bind to sulfhydryl groups on cell membranes, presumably altering cellular interactions (reviewed by Luster *et al.*, 1987).

The hematopoietic and immune function changes described in these studies are modest compared to those observed in most primary or acquired immunodeficiency diseases. However, the observed changes appear to be of sufficient magnitude to alter host defense mechanisms when mice were challenged with *P. yoelii*. This is a nonlethal strain of mouse malaria in which humoral immunity (i.e., antibody), in addition to other immune mechanisms, plays a role in resistance (Krier and Green, 1980). Preliminary studies have indicated that immune functions can generally be suppressed by at least 30% prior to observing changes in resistance to infectious agent challenge (Luster *et al.*, 1988). This would be consistent with that observed in the present studies. However, these observations were based on relatively small numbers of animals and it has not been clearly established whether a threshold level actually exists or altered immune function parallels changes in host resistance.

The most common dose-limiting toxicity for many cancer and AIDS therapeutics is myelotoxicity. If severe enough, myelosuppression can result in an increased rate of infectious disease via altered host defense mechanisms. Similar to the antibody response, the reduction in bone marrow colony formation detected in chemically exposed mice was modest, as suggested by normal WBC counts. Although the CFU-GM assay is sensitive, it would appear that the reduction in colony formation was either not of sufficient severity or sufficient duration to produce leukopenia. Schurig *et al.* (1985) have examined a battery of bone marrow assays in mice using 17 anticancer drugs and have demonstrated that colony-forming assays are a very sensitive indicator for altered hematopoiesis, often being suppressed prior to detecting hematological changes.

Concern over the potential adverse health effects of drinking water contaminated with environmental chemicals has existed for many years. In general, however, these studies have focused on retrospective epidemiological surveys for cancer risk and have, for the most part, ignored organ or system toxicities (Shy, 1985; Clark *et al.*, 1986). Although a definitive relationship has, as yet, not been established between the consumption of contaminated groundwater and human health effects, collectively these studies have demonstrated a correlation between increased health risk (e.g., cancer) and exposure. The most common forms of cancer reported from putative exposures include bladder, stomach, colon, and rectum, as well as multiple myelomas (Kuzma *et al.*, 1977; Gottlieb and Carr, 1982). Carcinogenicity can be a relatively insensitive measure of toxicity in which cause–effect relationships are difficult to establish. Thus, it would appear more prudent in future clinical studies to include evaluation of the toxic effects of specific organs/systems, including immune functions in risk assessment. This is supported by recent, but unconfirmed, observations of altered immune function in a small group of individuals in Woburn, Massachusetts exposed to contaminated well water (Ozonoff, personal communication) as well as those living in the Love Canal area near Buffalo, New York (Park, personal communication). While these studies are retrospective, the results suggest the possibility that long-term exposure to contaminated groundwater represents a potential risk to the immune system. Care should be taken, however, when extrapolating meaningful conclusions from experimental data or isolated epidemiological studies for risk assessment of low level human exposure.

The present studies, combined with our current knowledge about the pathogenesis of disease resulting from immunodeficiency and the potential for large scale human exposure to groundwater contaminants, indicate a need for greater awareness among clinicians and epidemiologists as to the subtle effects

that may occur. In addition, careful examination of potentially sensitive targets such as the immune system should be conducted more routinely. The present data should be interpreted with caution since the chemical mixture studied, while approximating concentrations that can be found in environmental samples (see Table 1), was prepared in the laboratory and was designed to mimic a "worst-case scenario." However, given the heterogeneity that would occur when testing the immune system of humans and the limitations in assays that are normally performed in the clinic, it may be difficult to detect subtle immune changes in humans, as occurred here in mice, unless special studies (e.g., stem cell function) are conducted with a large sample population.

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Toxicity Report Number	Chemical	Route of Exposure	Publication Number
1	Hexachloro-1,3-butadiene	Dosed Feed	91-3120
2	*n*-Hexane	Inhalation	91-3121
3	Acetone	Drinking Water	91-3122
4	1,2-Dichloroethane	Drinking Water, Gavage	91-3123
5	Cobalt Sulfate Heptahydrate	Inhalation	91-3124
6	Pentachlorobenzene	Dosed Feed	91-3125
7	1,2,4,5-Tetrachlorobenzene	Dosed Feed	91-3126
8	D & C Yellow No. 11	Dosed Feed	91-3127
9	*o*-Cresol *m*-Cresol *p*-Cresol	Dosed Feed	92-3128
10	Ethylbenzene	Inhalation	92-3129
11	Antimony Potassium Tartrate	Drinking Water, I.P. Inject.	92-3130
12	Castor Oil	Dosed Feed	92-3131
13	Trinitrofluorenone	Dermal, Dosed Feed	92-3132
14	*p*-Chloro-α,α,α-Trifluorotoluene	Gavage (corn oil, a-CD)	92-3133
15	*t*-Butyl Perbenzoate	Gavage	92-3134
16	Glyphosate	Dosed Feed	92-3135
17	Black Newsprint Ink	Dermal	92-3340
18	Methyl Ethyl Ketone Peroxide	Dermal	92-3341
19	Formic Acid	Inhalation	92-3342
20	Diethanolamine	Drinking Water, Dermal	92-3343
21	2-Hydroxy-4-Methoxybenzophenone	Dosed Feed, Drinking Water	92-3344
22	N, N-Dimethylformamide	Inhalation	93-3345
23	*o*-Nitrotoluene *m*-Nitrotoluene *p*-Nitrotoluene	Dosed Feed	92-3346
24	1,6-Hexanediamine	Inhalation	93-3347
25	Glutaraldehyde	Inhalation	93-3348
26	Ethylene Glycol Ethers	Drinking Water	93-3349
28	Tetrachlorophthalic Anhydride	Gavage	93-3351
29	Cupric Sulfate	Drinking Water, Dosed Feed	93-3352
33	2-Chloronitrobenzene 4-Chloronitrobenzene	Inhalation	93-3382
36	Pesticide/Fertilizer Mixtures	Drinking Water	93-3385