Environmental Complex Mixture Toxicity Assessment

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Trichloroethylene (TCE) was found as a contaminant in the well supplying water to an aquatic testing laboratory. The groundwater was routinely screened by a commercial laboratory for volatile and semivolatile compounds, metals, herbicides, pesticides, and polychlorinated biphenyls using U.S. Environmental Protection Agency methods. Although TCE was the only reportable peak on the gas chromatograph, with average concentrations of 0.200 mg/l, other small peaks were also present, indicating the possibility that the contamination was not limited to TCE alone. A chronic 6-month carcinogenicity assay was conducted on-site in a biomonitoring trailer, using the Japanese medaka fish (Oryzias latipes) in an initiation-promotion protocol, with diethylnitosamine (DEN) as the initiator and the TCE-contaminated groundwater as a promoter.

Study results indicated no evidence of carcinogenic potential of the groundwater without initiation. There was, however, a tumor-promotional effect of the groundwater after DEN initiation. A follow-up laboratory study was conducted using reagent grade TCE added to carbon-filtered groundwater to simulate TCE concentrations comparable to those found in the contaminated groundwater. Study results indicated no promotional effects of TCE. These studies emphasize the necessity for on-site bioassays to assess potential environmental hazards. In this instance, chemical analysis of the groundwater identified TCE as the only reportable contaminant, but other compounds present below reportable limits were noted and may have had a synergistic effect on tumor promotion observed with the groundwater exposure. Laboratory toxicity testing of single compounds can produce toxicity data specific to that compound for that species but cannot take into account the possible toxic effects of mixtures of compounds. — Environ Health Perspect 106(Suppl 6):1299–1305 (1998). http://ehpnet1.niehs.nih.gov/docs/1998/Suppl-6/1299-1305gardenarbstract.html

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Chemical contamination of the earth’s water supplies continues, often with alarming consequences. To characterize and assess the magnitude and extent of environmental hazards on-site and to subsequently remediate these problems requires an integrated approach utilizing both chemical analyses and biologic testing. Standard bioassay testing of single compounds often yields toxicity data of limited value. In reality, most environmental contaminants exist as a mixture of compounds, not as single entities. Even if elaborate chemical profile analyses are done on samples from contaminated sites to identify the contaminants, the data generated often do not reflect the possible synergistic or antagonistic toxic effects of these chemicals on biologic systems. The cost of testing potential combinations of environmental contaminants is an issue as well. How the nation addresses the real-world public health issues of mixture toxicity, risk assessment, and risk management remains extremely important (1).

The studies presented here demonstrate the value of using an in situ biologic approach for assessing a contaminated groundwater (GW) supply. The accurate assessment of the toxicity of potentially hazardous chemicals on-site is the key to determining the method and extent of the cleanup of that site (2). Historically, single organisms ranging from clams (3) to duckweed plants (4) have been used as biomonitor in streams where effluents from industrial sources or power plants may be threatening the aquatic flora and fauna. In many instances, however, an underground aquifer is the sink of contamination, in which case surface-water sentinel organisms cannot be used on-site for biomonitoring. Our laboratory has specifically designed and used mobile biomonitoring laboratories to conduct toxicity tests on-site (2,5,6) to evaluate the toxic hazard of contaminated GW. Briefly, the biomonitoring laboratory is an 8×24 ft trailer equipped with aquaria and flow-through diluter systems; on-site GW is pumped in for conducting carcinogenicity, ventilatory response, and other short-term aquatic assays using a variety of fish, invertebrates, and frog embryos. Equipment and exposure facilities in the mobile labs are similar to those in the fixed laboratory.

The Japanese medaka fish (Oryzias latipes) is the model used for the carcinogenicity assays both in mobile and fixed laboratories. Although a variety of fish species have been used in cancer research (7–10), the Japanese medaka has been the subject of significant research and manifests many of the characteristics of a good cancer model (11–16). Medaka are easily cultured, have a short time-to-tumor response, are relatively easy to maintain, have low spontaneous tumor formation, and can be easily evaluated histologically because a section of the entire animal can be placed on one slide (17–19). Finally, the small size of the medaka affords the opportunity to conduct bioassays with greater statistical power, and

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Abbreviations used: BDL, below detection limits of the method; DEN, diethylnitosamine; MS-222, ethyl 3-amino-2-nitro-benzoate; methanesulfonic acid salt; GC, gas chromatograph; GW, groundwater; HP, Hewlett-Packard; TCE, trichloroethylene; U.S. ACEHR, U.S. Army Center for Environmental Health Research.
the hardness of the animal allows direct assessment of complex environmental contamination in mobile laboratories (2).

This paper examines the potential hepatocarcinogenicity of trichloroethylene (TCE) in a GW mixture and alone as a pure compound. A ubiquitous environmental contaminant and rodent carcinogen (20–25), TCE is a member of a family of chemicals classified as volatile organic compounds, which are known to cause cancer in laboratory animals (26–28). TCE is transformed in anaerobic conditions in water to vinyl chloride, a known human carcinogen. From World War II until 1980, 13 billion pounds of TCE was produced in the United States, primarily for use as a degreasing agent in machine shops, including those at Department of Defense installations. During this period TCE disposal was unregulated, resulting in contamination of many GW supplies. It is the chemical contaminant most likely to be found at designated Superfund sites and associated GW plumes. More than 30,000 of these TCE-contaminated sites have been identified. Contaminated water supplies typically contain an average of 0.001 to 0.002 mg/l TCE. During routine chemical screening, TCE was found in the well that supplied water to our aquatic testing laboratory—at concentrations as high as 0.400 mg/l, with an average concentration of 0.200 mg/l. Risk assessment studies have determined a safe level of TCE in the drinking water to be 0.005 mg/l (25,26). Epidemiologic studies of people exposed to high levels of TCE in the drinking water or ambient air of the workplace suggest an increase in cancer incidence (29). Human results are inconclusive, however, because other chemicals also present with the TCE may have contributed to the increased cancer rates.

The two TCE studies presented in this paper were designed to evaluate the potential for TCE to act as either a complete carcinogen or as a tumor promoter in the medaka when administered as a pure compound or as a component of GW containing other low-level contaminants. Diethylnitrosamine (DEN), a known and well-studied hepatic carcinogen in mammals (30–35) and medaka (36–39), was used as the initiating agent in the medaka prior to exposure to the potential promoting agents in GW or TCE.

Materials and Methods

Chemicals

Diethylnitrosamine and TCE were obtained from Sigma Chemical Company (St. Louis, Missouri) and stored at room temperature. A stock solution of 9.4 g/l DEN was prepared in American Society for Testing and Materials Type I water on the day of exposure. TCE stock solutions of 0.50 g/l were prepared biweekly in processed and filtered well water and stirred for 48 hr prior to being dispensed into the diluter test system. Ethyl 3-aminobenzoate, methanesulfonic acid salt (MS-222) was dissolved in laboratory well water at a concentration of 200 mg/l for euthanizing the fish.

Experimental Animals

This study was conducted in compliance with the Guide for the Care and Use of Laboratory Animals (40) and was approved by the U.S. Army Center for Environmental Health Research (U.S. ACEHR) Institutional Animal Care and Use Committee. U.S. ACEHR facilities are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Fourteen-day-old Japanese medaka were used for both studies. These were obtained from the U.S. ACEHR in-house medaka culturing tanks. The culture tanks, as well as the test tanks, were held in a 25° ± 1°C water bath with daily temperature verification. Water for both tests was drawn from a 500-ft well located on-site. Water for the contaminated GW test was pumped directly into the trailer from the well. The water used for the laboratory TCE test and the water used as the diluent for the trailer test was processed through a series of carbon and particle filters, as well as through reverse osmosis, aeration, and ultraviolet sterilization prior to use in the tests. A photoperiod of 16 hr light and 8 hr dark was maintained. Fish were fed flake TetraMin (TetraWerke, Melle, Germany) and live 24-hr-old brine shrimp nauplii. Test fish were housed in 5-gal aquaria in a continuous flow environment with five to six tank turnovers per day for both studies. The only exception was the initial 48-hr DEN exposures, where the fish were held under static conditions in 1.0-liter mesh-bottom glass beakers suspended in borosilicate glass animal jars containing 1.5 liters of the test water.

Experimental Design

Test exposures for the medaka were designed as initiation–promotion studies with a 48-hr static acute DEN exposure in the water taking place prior to chronic flow-through exposures to either GW or TCE. DEN exposures were accomplished by filling the glass animal jars with 1.5 liters of laboratory water minus the volume of DEN stock to be added, then adding either 1.6 or 16.0 ml of 9.4 g/l DEN stock solution to the jars to yield test concentrations of 10 and 100 mg/l, respectively. DEN concentrations of 100 mg/l were used only as positive controls for the laboratory study. This concentration of DEN in the water was used in previous studies in our laboratory (38) to produce hepatocellular neoplasms in the medaka. Fourteen-day-old fry were introduced to the test jars by placing the mesh-bottom containers holding the fish into the jars. The jars were then covered with plastic film and placed in opaque plastic containers containing well water, at a height that equaled the height of water in the animal jars, to facilitate heat transfer and temperature equilibration. These containers were then sealed and placed in a 25° ± 1°C water bath. A temperature probe was placed in an extra control container and the remained in the water bath for 48 hr. During the exposure period the containers remained sealed and the fish were not fed. DEN exposure termination occurred by removing the mesh-bottom beaker containing the fish from the containers and transferring the fish to flow-through aquaria for the remainder of the tests.

For the contaminated GW study, 50 fish were used for each treatment tank (Table 1 shows the test design). The medaka fry were exposed to 0 or 10 mg/l DEN for 48 hr, held in clean processed GW under flow-through conditions in the laboratory for 6 days and transferred to a bio-monitoring laboratory where they received dilutions of the raw GW. The raw GW was diluted with processed GW in a flow-through diluter system to obtain concentrations of 10, 50, and 100% contaminated GW by volume, which was then delivered to the test fish. These fish were exposed to contaminated GW for 6 months. Half the fish from each treatment tank were removed for a 3-month interim sacrifice. The remaining 25 fish in each tank remained on study until the 6-month sacrifice point.

For the laboratory exposure, 60 fish per treatment tank were used, with duplicate tanks for each treatment (Table 2 shows the test design). Larger numbers of fish were used in the laboratory exposure to increase the statistical power of the experiment.) The fish were initiated with 0 or 10 mg/l DEN, or treated with 100 mg/l DEN for positive controls, then transferred to test tanks in the laboratory 5 days after the DEN exposure. A stock solution of 0.5 g/l TCE was diluted with processed laboratory well water in a flow-through diluter system.
to achieve test tank concentrations of 0, 0.1, or 1.0 mg/l TCE, which the fish received for 6 months until time of sacrifice. These TCE concentrations were selected to bracket the TCE levels found in the raw GW. There was no 3-month interim sacrifice point in the laboratory study.

**Chemical Analysis**

The concentration of DEN in the exposure jars was determined before and after the fish were exposed. A sample volume of 20 ml was removed from each jar at the start and end of the 48-hr exposure period and analyzed on a Hewlett-Packard (HP) Company (Avondale, Pennsylvania) model 5790A gas chromatograph (GC) equipped with fused silica capillary column (HP-1) 25 m × 0.2 mm id. The capillary column was connected directly to Thermo Electron Corporation (Waltham, Massachusetts) TEA model 543 analyzer, which is a nitrosyl-group-specific detector. Each sample was divided into two 10-ml aliquots, which were then extracted with 2 ml methylene chloride spiked with nitrosomorpholine as an internal standard. The methylene chloride was then placed in an auto sampler vial and injected on the GC using a HP model 7671A autosampler. For TCE analysis, sample volumes of 40 ml were removed biweekly from alternate tanks and analyzed for volatile organic compounds, including TCE, using an HP model 5880A GC equipped with an electron capture detector and a static head space sampler HP model 19395A. The GC was equipped with a capillary column (HP-5) 25 m × 0.2 mm id and model 19392A integrator. A 5-ml sample was placed in a 10-ml headspace vial and equilibrated at least 30 min at 60°C before a 1-ml sample of headspace gas was automatically injected on the GC.

**Histopathology**

At the end of both studies, the fish were euthanized with MS-222, weighed, measured, opened along the ventral midline, and placed in Bouin’s fixative. After 24 hr in Bouin’s fixative, the fish were transferred to 70% ethanol for 24 hr, then transferred again to a container of clean 70% ethanol for another 24 hr before being placed finally in 10% neutral buffered formalin. Once in the formalin, the fish were sent to Experimental Pathology Laboratories, Inc. (Herndon, Virginia) to be processed for microtomy, hematoxylin and eosin staining, and histopathologic valuation of preneoplastic and neoplastic lesions. These fish were embedded in Paraplast (Oxford labware, Division of Sherwood Medical, St. Louis, Missouri) with the fish oriented to be sectioned longitudinally from the left side through to the right side. Five section-sheets were cut at a thickness of 4 μm from each fish (two left paramedian, one mid sagittal, two right paramedian). These sections were then mounted on slides, stained with hematoxylin and eosin, and evaluated microscopically for hepatic altered foci formation as well as hepatic tumor development.

**Statistical Analysis**

Chi-square analyses of fish liver foci and neoplasms data were accomplished for each test. Treatment groups within each test were analyzed for differences in total numbers of foci and neoplasms.

**Results**

**Chemistry**

Measured values of DEN before and after the 48-hr exposures varied less than 13% from the nominal values of 10 and 100 mg/l for both studies. For the contaminated GW study, the 6-month average TCE level was 0.125 mg/l for 100% GW. These results are summarized in Table 1. A GC of a sample of contaminated GW with a TCE peak of 0.197 mg/l is shown in Figure 1. Unidentified peaks on the graph indicate the presence of other trace contaminants. For the laboratory study, measured values of TCE for 6 months varied <15% from the nominal values of 0.1 and 1.0 mg/l, with the exception of two of the 1.0-mg/l target tanks, where the TCE values were low (Table 2).

**Figure 1.** Gas chromatograph of contaminated groundwater. Large peak equals 0.197 mg/l TCE.
Histopathology

For the contaminated GW study, there was no hepatocarcinogenic effect of TCE when fish were exposed for 6 months to 0, 25, 50, or 100% contaminated water by volume (Table 3). There was, however, a tumor-promotional effect of the contaminated GW on DEN-initiated fish. Fish receiving 100% contaminated water for 6 months after DEN initiation had significantly more liver neoplasms ($p<0.05$) than fish receiving DEN alone and all other treatments. There was a trend of increasing foci of altered hepatocytes in DEN-treated fish receiving 50 and 100% contaminated water, but these differences were not statistically significant. At the 3-month interim sacrifice point, 2 of 25 fish from the DEN plus 25% GW and 2 of 25 fish from the DEN plus 100% GW had hepatic neoplasia. There were no observed spontaneous deaths from chemical treatments.

In the laboratory study, TCE alone had no apparent effect on hepatic neoplasia or altered foci (Table 4). Fish receiving processed water or exposed to TCE at concentrations of 0.1 and 1.0 mg/l for 6 months showed no significant evidence of preneoplastic focal change or neoplastic lesions as measured by chi-square analysis ($p>0.05$). There was also no evidence of a tumor-promotional effect of TCE in the DEN-initiated fish. There was a very low prevalence of hepatocellular adenomas in all of the 10 mg/l DEN treatment groups, but these were not significantly different from controls. Only the positive control treatment group of 100 mg/l DEN had a significant increase ($p<0.01$) in numbers of foci and tumors over controls and all other treatment groups. The photomicrographs in Figure 2 compare the histopathology of a normal medaka liver to livers exhibiting a treatment-related hepatocellular carcinoma and adenoma.

Discussion

These results demonstrate that although TCE was the major component in a contaminated GW source and therefore appeared to be the lone driving force behind a significant tumor-promotional response in medaka exposed to this source, TCE had no such effect when evaluated in a more controlled laboratory experiment. One possible explanation for this conflicting finding may be the presence of other chemicals in the GW mixture along with TCE. Examination of the chromatograph of GW in Figure 1 reveals at least four smaller peaks that were below reportable limits. Possible synergistic effects of these other contaminants with TCE may explain the results of the GW study. A reexamination of the GW using comprehensive chemical analysis to identify all components, along with another medaka bioassay, may prove helpful in explaining the tumor-promotional response.

Table 3. Groundwater study: foci of altered hepatocytes and liver neoplasms.*

| Foci                  | Control | 10 mg/l DEN | 25% GW | 50% GW | 100% GW | 10 mg/l DEN | 25% GW | 50% GW | 100% GW |
|-----------------------|---------|-------------|--------|--------|---------|-------------|--------|--------|---------|
| Basophilic foci       | 0       | 2           | 0      | 1      | 0       | 1           | 3      | 1      |         |
| Eosinophilic foci     | 0       | 1           | 0      | 0      | 0       | 0           | 4      | 4      |         |
| Vacularated foci      | 0       | 2           | 0      | 1      | 0       | 1           | 1      | 3      |         |
| Total number of foci  | 0       | 5           | 0      | 1      | 0       | 2           | 8      | 8      |         |
| Liver neoplasms       |         |             |        |        |         |             |        |        |         |
| Cholangioma           | 0       | 0           | 0      | 0      | 0       | 0           | 0      | 0      |         |
| Cholangiocarcinoma    | 0       | 0           | 0      | 0      | 0       | 0           | 0      | 0      |         |
| Hepatocellular adenoma| 0       | 0           | 0      | 0      | 2       | 0           | 0      | 6      |         |
| Hepatocellular carcinoma| 0     | 1           | 0      | 0      | 1       | 3           | 2      |         |         |
| Total liver neoplasms| 0(26)   | 1(26)       | 0(27)  | 0(25)  | 0(23)   | 3(25)       | 3(22)  | 8* (19)|         |

*Significantly greater number of neoplasms ($p<0.05$) than control or any other treatment groups (chi-square analysis). *Number of liver foci or neoplasms present in 217-day-old medaka sacrificed 6 months after initial 48-hr DEN exposure. **Zero or 10 mg/l DEN administered in the water to 17-day-old medaka for 48 hr, followed by a chronic exposure of 0, 25, 50, or 100% GW by volume contaminated with TCE for 6 months. *Total number of liver foci or neoplasms found in all fish in that treatment group. Number of fish in treatment group in parentheses.

Table 4. Trichloroethylene laboratory study: foci of altered hepatocytes and liver neoplasms.*

| Foci                  | Control | 100 mg/l DEN | 0.1 mg/l TCE | 1.0 mg/l TCE | 10 mg/l TCE | 0.1 mg/l TCE | 1.0 mg/l TCE |
|-----------------------|---------|--------------|--------------|--------------|-------------|--------------|--------------|
| Basophilic foci       | 0       | 1            | 0            | 0            | 0           | 0            | 1            |
| Eosinophilic foci     | 0       | 12           | 59           | 1            | 1           | 12           | 10           |
| Vacularated foci      | 0       | 5            | 76           | 1            | 2           | 13           | 16           |
| Total number of foci  | 0       | 18           | 135*         | 2            | 3           | 25           | 27           |
| Liver neoplasms       |         |              |              |              |             |              |              |
| Cholangioma           | 0       | 0            | 5            | 0            | 0           | 0            | 0            |
| Cholangiocarcinoma    | 0       | 0            | 2            | 0            | 0           | 0            | 0            |
| Hepatocellular adenoma| 0       | 1            | 44           | 0            | 1           | 1            | 3            |
| Hepatocellular carcinoma| 0     | 0            | 22           | 0            | 0           | 0            | 0            |
| Total liver neoplasms| 0(115)  | 1(108)       | 73* (118)    | 0(117)       | 1(111)      | 1(115)       | 3(110)       |

*Significantly greater number of foci and neoplasms ($p<0.01$) than control or any other treatment group (chi-square analysis). *Number of liver foci or neoplasms present in 189-day-old medaka sacrificed 6 months after initial 48-hr DEN exposure. **Zero, 10, or 100 mg/l DEN administered in the water to 14-day-old medaka for 48 hr, followed by a chronic nominal exposure of 0, 0.1, or 1.0 mg/l TCE for 6 months. *Combined total number of liver foci or neoplasms found in all fish in that treatment group. Number of fish in treatment group in parentheses.
seen with the GW. It is also important that any new bioassay done on the contaminated GW should include a cell proliferation assay to help explain any promotional effects of the GW. The use of the thymidine analog 5-bromo-2‘-deoxyuridine to label cells in S-phase of the cell cycle in medaka has been successful in this laboratory (41) and could easily be incorporated into an environmental experiment.

The documentation of a clear discrepancy in bioassay conclusions between a relatively tightly controlled field experimental setting and a laboratory exposure setting potentially have significant implications for carcinogenicity testing and human health risk assessment. The assessment of the low-dose exposures of the human population to single chemicals remains a principal focus of national testing strategies. A true environmental exposure to a human population, however, is more correctly characterized as a mixture scenario.

The use of bioassays to assess mixtures of complex environmental chemical contaminants has involved both in vitro genetic toxicity assays as well as whole animal in vivo assays. Ames mutagenicity assays have been used to evaluate the genotoxicity of metal-contaminated sites in India (42), pulp mill effluent in Canada (43), and, in conjunction with other genotoxicity assays, to assess contaminated environmental sites in the United States (44).

In a previous study, our laboratory used an integrated hazard assessment approach to evaluate the toxic potential of GW contaminated with a mixture of chemicals at a U.S. Army Superfund site (5). A mobile biomonitoring trailer located on-site was used to conduct 10 assays on the GW over a period of 9 months. These assays included acute and chronic toxicity tests utilizing a variety of aquatic organisms (marine bacteria, rotifers, algae, daphnia, fathead minnows, and Japanese medaka), as well as genotoxicity assays (Ames, sister chromatid exchange) and a frog embryo teratogenicity assay-Xenopus. A 9-month initiation-promotion medaka assay similar to the assay described in this paper was also conducted with DEN as the initiator and the GW as the promoter. These assays are described in detail by Twerdok et al. (5) and Burton et al. (6). Comprehensive chemistry profiles of the GW revealed elevated levels of several potentially carcinogenic heavy metals as well as a variety of halogenated solvents. There was not, however, a carcinogenic effect of 1 and 10% GW on the medaka. The negative results of the carcinogenicity assay were in agreement with the negative toxicity of all the other assays conducted. This example of an integrated hazard assessment reinforces the use of integrated assessments performed on-site to provide comprehensive toxicity data for a weight-of-evidence approach to evaluating the media in question.

The data reported in this publication demonstrate that when fish were exposed to an initiating dose of DEN and subsequently exposed to serial dilutions of GW in which only TCE contamination could be demonstrated above U.S. Environmental Protection Agency reportable levels, there was a concentration-dependent trend toward an increase in tumor prevalence. This would seem to argue strongly for TCE acting as a tumor promoter in this study with medaka. However, the subsequent laboratory test did not support such a finding when TCE was evaluated in processed GW, even at TCE concentrations nominally one order of magnitude above contaminated GW levels. The evaluation of the contaminated GW itself is indeed the more significant finding from the perspective of protecting public health, given that this is the material to which people are more likely to be exposed. These and similar findings have led to arguments for increasing the national public health resources being expended on mixture toxicity and risk assessment (45).

The data on TCE in the medaka bioassay, coupled with the evaluation of a TCE-contaminated GW, suggest that TCE alone is apparently not a hepatic carcinogen in this animal model at these environmentally relevant concentrations. Trace contaminants in the GW may have been the driving influence on the overall tumor-promotional effects seen in the TCE GW test, as this effect was not seen in the TCE laboratory test. The use of this model to assist in low-dose extrapolation may in fact provide a real boost to understanding the consequences of low-dose exposure. This research has obvious applications in the assessment of complex aquatic chemical contamination and environmental monitoring. Chemical analyses of a medium can provide information on the identity and levels of environmental contaminants, but these analyses often cannot predict the toxic effects of the mixture. An integrated approach combining chemical analyses and biologic assays conducted on-site may provide a more realistic assessment of the hazards posed by exposure to these environmental mixtures.
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