Arch. Environ. Contam. Toxicol. 16, 531–537 (1987)

Archives of Environmental Contamination and Toxicology

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Mutagenic Activity of Surface Waters Adjacent to a Nuclear Fuel Processing Facility

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Abstract. Surface waters adjacent to a nuclear fuel processing facility were extracted, using XAD-resin adsorption followed by solvent elution, and the extracts were assayed for mutagenic potential by the Ames *Salmonella*-mammalian microsome test. Dose-related mutagenic responses with TA102 (+S9) were produced with the extracts of water samples obtained from a creek receiving wastewater from the processing facility (specific mutagenic activities of 7,250 to 8,250 net revertants per L equivalent of water). The creek water extracts were not mutagenic with TA102 in the absence of S9, or with any other tester strain (i.e., TA97, TA98, TA100, and TA1535) in the presence or absence of S9. Surface water samples downstream and upstream of this creek were not mutagenic; apparently indicating the lack of persistence of the observed mutagenicity. The major constituent in the mutagenic creek water extracts was identified as tributylphosphate (TBP) by gas chromatography-mass spectrometry. However, TBP was not mutagenic with TA102 (+S9) at doses ranging from 196 μg/plate to 9.8 ng/plate. Because tester strain TA102 detects oxidative mutagenesis due to x-rays and ultraviolet radiation, it is possible that the observed mutagenicity of creek water extracts was due to radionuclides complexed to TBP.

Significant mutagenic activity has been detected in a variety of industrial wastewater effluents and sludges. Among mutagenic industrial discharges are petroleum refinery effluents (Metcalfe *et al.* 1985), petroleum refinery sludges (Donnelly *et al.* 1985), coke-oven effluents (Van Hoof and Verheyden 1981; Van Hoof and Manteleers 1983), chlorinated pulp mill effluents (Rannug 1980), textile manufacturing effluents (Möller *et al.* 1984), foundry effluents (Somani *et al.* 1980), and wastewaters from the production of nitrobenzoic acids (Sundvall *et al.* 1984), nitrotoluenes (Spanggord *et al.* 1982; Sundvall *et al.* 1984), acetonitrile (Brown and Donnelly 1984) and vinyl chloride (Rannug and Ramel 1977). Mutagenic industrial wastewaters discharged to municipal sewer systems are major contributors to the observed mutagenicity of municipal wastewaters (Ellis *et al.* 1982; Meier and Bishop 1985; Rappaport *et al.* 1979; Reinhard *et al.* 1982). Moreover, the release of mutagenic industrial and/or municipal wastewater effluents into surface waters has produced measurable mutagenic activity in these systems (Maruoka and Yamanaka 1982, 1983; Moore *et al.* 1980; Van Hoof and Verheyden 1981).

There is an urgent need to characterize the mutagenic potential of many, yet untested, industrial wastewater effluents, and of surface waters into which they are discharged. The present study focuses on the mutagenic activity, as determined by the Ames *Salmonella*-mammalian microsome test, of surface waters (*i.e.*, Nolichucky River Basin, upper-east Tennessee) receiving wastewater from a nuclear fuel processing facility (*i.e.*, Nuclear Fuel Services, Inc, Erwin, Tennessee—NFS). At this plant, highly enriched uranium-235 hexafluoride gas is used to manufacture the fuel employed in the reactors of U.S. Navy nuclear submarines. An extensive literature search failed to provide any other

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published research on the mutagenic activity of process wastewaters from the nuclear industry. Fallon and Fliermans (1980) did, however, report significant mutagenic activity of chlorinated cooling water from a production nuclear reactor.

Materials and Methods

Water Sampling Sites and Protocol

Eight sampling sites were established on the Nolichucky River Basin in the vicinity of the NFS plant (Erwin, TN) as depicted in Figure 1. Water samples were collected from selected sites at three different times (Series A, B, and C). Water grab samples were collected from approximately midstream and mid-depth in amber-glass bottles. All glassware was washed with Alconox detergent (Baker Chem.) and rinsed with tapewater, distilled-deionized water, and methanol. Unless otherwise specified, the organic solvents were purchased from Fisher Scientific, and were either of pesticide grade or were glass distilled in our laboratory. All water samples were stored at 4°C for no more than 48 hr before they were extracted.

Water Extraction Procedures

Water samples were extracted by XAD-resin adsorption followed by solvent elution. A mixture of Amberlite® synthetic ion exchange resins XAD-2 and XAD-7 (Rohm and Haas) was employed as described by Rappaport *et al.* (1979). The XAD-2 and XAD-7 resins were prepared as described by Honer *et al.* (1980) and Dressler (1979), respectively. Both resins were stored under methanol at 4°C prior to use. Glass chromatography columns (19-mm ID × 300-mm length) were loaded with 33 cm^3 of prepared XAD-2 resin followed by 33 cm^3 of prepared XAD-7 resin; small plugs of glass wool were placed above and below the resin bed. Each fresh resin bed was eluted with 2 bed volumes (140 mL) of acetone and 4 bed volumes of methylene chloride. The combined eluate (negative control) was processed and assayed for mutagenic activity as described below for water sample extracts. This control ensured that no solvent-elutable mutagenic activity was present in the fresh resin bed.

Prior to loading a water sample, each resin bed was washed with 1 L of distilled-deionized water to remove solvent residues. The pH of each water sample was adjusted (pH 2.0, 7.0, or 11.0) prior to extraction with concentrated hydrochloric acid or concentrated ammonium hydroxide. Series A samples were extracted at pH 2.0, 7.0 and 11.0, whereas Series B and C samples were extracted at pH 7.0 only. Water samples (4.0 to 20 L) were allowed to pass through the columns by gravity flow, at a flow rate of approximately 30 mL/min. After residual sample water had drained from the columns, the resin beds were loaded with acetone and allowed to equilibrate for 10 min. Subsequently, the columns were eluted with 2 bed volumes of acetone followed by 4 bed volumes of methylene chloride. The combined organic eluate from each column was concentrated to approximately 10 mL, using a Kuderna-Danish evaporative concentrator (Lab Glass Co.). A 2.5-mL aliquot of each 10-mL concentrated eluate was stored at −20°C for possible chemical analysis later (see below), and the remaining 7.5 mL were transferred to a ceramic evaporating dish and evaporated to dryness in a 45°C water bath.

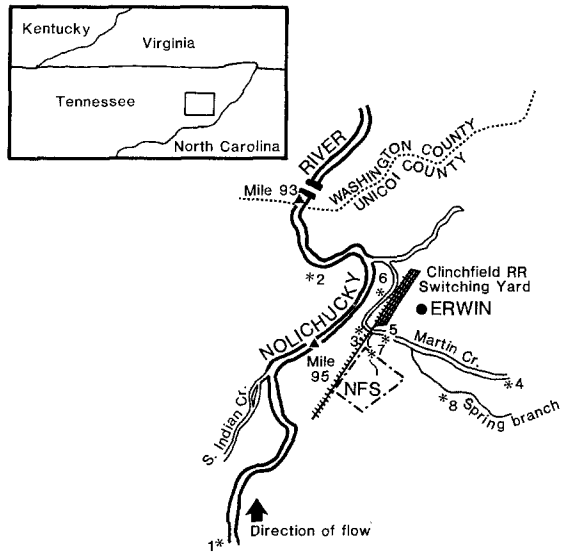

Fig. 1. Location of eight sampling sites within the Nolichucky River Basin. The sampling sites are depicted with asterisks (see Table 1 for site descriptions) and the location of the Nuclear Fuel Services (NFS) plant is shown

The resulting residues were weighed, resuspended in 4 mL of dimethyl sulfoxide (Me_2SO; spectrophotometric grade, Aldrich Chemical Co.), and stored at −20° C until assayed for mutagenic activity.

Mutagenicity Assay

The Ames *Salmonella*-mammalian microsome assay was used to determine the mutagenicity of the samples described above, and of tributylphosphate (TBP; 100% purity, Ticar Chemical Co.). The protocol followed was that of Maron and Ames (1983), with the modifications recommended by Batzinger *et al.* (1978). These modifications consisted of adding biotin and trace amounts of histidine to the bottom agar, rather than top agar, and replacing the NaCl in the top agar with Vogel-Bonner minimal E medium. These modifications were intended to maximize the sensitivity of the assay, and are also reported to render the test less sensitive to exogenous histidine.

Four to five doses of each water extract and fifteen doses of TBP, prepared by serial dilutions in Me_2SO, were plated in triplicate at a constant volume of 100 μL. Series A water extracts were tested with *Salmonella* strains TA100 and TA1535, whereas Series B and C water extracts were tested with the strains recommended by Maron and Ames (1983) as the standard tester set (TA97, TA98, TA100, and TA102) and with TA1535. Tributylphosphate was tested with only TA102. All water extracts were tested in the absence (−S9) and presence (+S9) of microsomal activation mix containing the Aroclor® 1254-induced rat liver homogenate fraction S9 at 50 μL S9/plate (prepared in KC1, Litton Bionetics); TBP was assayed only in the presence of microsomal activation (+S9). The genotypes of the individual tester strains were confirmed prior to their use in mutagenicity testing according to established procedure (Maron and Ames 1983). The spontaneous reversion rate, both in the presence and absence of S9 activation, was determined for each tester strain in triplicate,

using Me_2SO as the solvent control. With each assay performed, the tester strains were checked for appropriate responses to known mutagens in Me_2SO (2-aminoanthracene for all strains with S9; without S9, 2-aminoacridine for TA97, 2-nitrofluorene for TA98, sodium azide for TA100 and TA1535, and methyl methanesulfonate for TA102; all from Sigma Chemical Co.). In addition, residues of the solvents used in the extraction of water samples were tested for mutagenic activity (negative controls). All water extracts, known mutagen solutions, solvent controls, and S9 preparations were monitored for sterility.

A positive mutagenic response was defined as a dose-related response with one or more doses producing at least a 2-fold increase in revertant colonies per plate as compared to the concurrent spontaneous count per plate (Maron and Ames 1983). The specific mutagenic activities of the water extracts were each expressed as net revertants per mg of extract residue (± standard deviation), and were based on the slope (± standard deviation of the slope) of the linear portion of the dose-response curve (mean revertants/plate as a function of extract μg assayed/plate) as determined by least-squares regression analysis (Maron and Ames 1983). The statistical significance (probabilities) of the resulting slope and correlation coefficient (r) for the dose-response curve of each extract was determined. The specific mutagenic activities of the water extracts were also each converted to units of net revertants per liter equivalent of original water based on the measured concentration of extractable organics in the water.

Gas Chromatography (GC) and GC-Mass Spectrometry Analyses

Mutagenic water extracts were analyzed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) at the Analytical Chemistry Division, Oak Ridge National Laboratory, Oak Ridge, TN. Extracts (2.5-mL aliquots—see above) were equilibrated with anhydrous magnesium sulfate, to remove all traces of water prior to analysis. Mass spectra were obtained with a Hewlett-Packard 5985A GC-MS, using a 25-m long by 0.24-mm I.D. fused silica capillary column with a bonded methyl (5% phenyl) silicone stationary phase (Quadrex Inc.). Helium was used as the carrier gas at 1.5 kg/cm² head pressure. The splitless injector and GC-MS transfer lines were maintained at 300°C. The oven temperature was programmed to hold at 100°C for 5 min and then ramped at 3°C/min to 300°C. Electron impact spectra were obtained at 70 eV at an ion source temperature of 200°C. Similar column and operating conditions were used to obtain chromatograms with a Perkin-Elmer Sigma I GC equipped with a flame ionization detector operated with hydrogen and air pressures of 1.5 and 2.1 kg/cm², respectively.

Results and Discussion

Mutagenicity results for organic extracts of water samples from the eight sampling sites are summarized in Table 1. The first set of water samples (series A) were all taken on the same day at sites 1 and 2 on the Nolichucky River, and site 3 on Martin Creek. These samples were extracted (4.0-L volumes) at pHs of 2, 7 and 11, and the resulting extracts displayed no detectable mutagenic activity with TA100 and TA1535, with or without metabolic activation.

Water samples were subsequently taken (Series B) from site 2 on the Nolichucky River, and sites 3 through 7 on Martin Creek (Table 1). For some of these samples, a larger volume (8.0 to 20 L) of water was extracted in order to increase the potential for detecting mutagens. All these samples, however, were extracted only at pH 7 in order to simplify the extraction procedure. This modification was justified based on previous studies that have shown that neutral fractions of drinking waters (Loper 1980) and surface waters (Maruoka and Yamanaka 1982) display the greatest mutagenic activity. Except for the Banner Creek sample (site 7), all water extracts were not mutagenic with any of the tester strains (*i.e.*, TA97, TA98, TA100, TA102, and TA1535), with or without metabolic activation (Table 1). Similarly, the Banner Creek extract was not mutagenic with TA97, TA98, TA100, and TA1535, with or without metabolic activation, and with TA102 in the absence of S9 (Table 1). The Banner Creek extract did, however, produce a dose-related mutagenic response with TA102 in the presence of S9 as shown in Figure 2. The dose-response curve displayed a statistically significant ($p < 0.001$) slope of 2.37 (±0.13 standard deviation of slope) revertants per μg of extract assayed (Figure 2). The two highest doses produced more than a 2-fold increase in revertants over the spontaneous reversion rate; thus, meeting our criteria for positive mutagenesis. The extract of a subsequent water sample from Banner Creek (Series C) was also mutagenic (i.e., dose-related, statistically significant, mutagenic response) with TA102 in the presence of S9 (Table 1 and Figure 2); also note that high doses of this extract proved to be toxic to the tester strains. The specific mutagenic activities of the extracts of these two Banner Creek water samples are shown in Table 2 and are expressed as TA102 (+S9) net revertants per mg of extract residue (1,180 and 2,370) or per liter equivalent of original water (7,250 and 8,250). It should be noted that mutagenicity with TA102 or with any other strain was not detected in Series B and C water samples upstream (*i.e.*, in Martin Creek or Spring Branch) or downstream (i.e., in Martin Creek) of Banner Creek (Table 1).

The spontaneous reversion rate for TA102 (+S9 or −S9) in our laboratory has historically been lower than the rate of 300 ± 60 revertants/plate (−S9) considered typical for the strain (Levin *et al.* 1982). The higher spontaneous reversion rate has been reported for TA102 cultures maintained on master plates containing high levels of tetracycline

Table 1. Mutagenicity of organic extracts of surface water samples

Water sampling site (Tennessee)	Sampling series	Volume of water extracted[a] (L)	Mutagenicity results
1. *Nolichucky River* (upstream of NFS)	A	4.0	ND[b]
2. *Nolichucky River* (downstream of NFS)	A	4.0	ND[b]
	B	20	ND[c]
3. *Martin Creek* (0.1 mile downstream of Banner Creek)	A	4.0	ND[b]
	B	4.0	ND[c]
	C	8.0	ND[c]
4. *Martin Creek* (1 mile upstream of Banner Creek)	B	20	ND[c]
5. *Martin Creek* (immediately upstream of Banner Creek)	B	4.0	ND[c]
6. *Martin Creek* (0.5 mile downstream of site no. 3)	B	8.0	ND[c]
7. *Banner Creek* (immediately outside NFS property line)	B	4.0	ND[d]; mutagenic with TA102 + S9
	C	8.0	ND[d,e]; mutagenic with TA102 + S9
8. *Spring Branch*	C	8.0	ND[c]

[a] Series A samples were extracted at pH 2, 7 and 11; all other samples were extracted only at pH 7
[b] ND = not detectable (*i.e.*, all sample doses produced less than 2-fold increase in revertants as compared to the concurrent spontaneous plates), using TA100 and TA1535, both with and without S9
[c] Not detectable (see b. above) using TA97, TA98, TA100, TA102, and TA1535, both with and without S9
[d] Not detectable (see b. above) using TA97, TA98, TA100, and TA1535, both with and without S9. Also, not detectable with TA102 in the absence of S9
[e] Undiluted extract (1,228 μg residue/plate) was toxic to all five tester strains

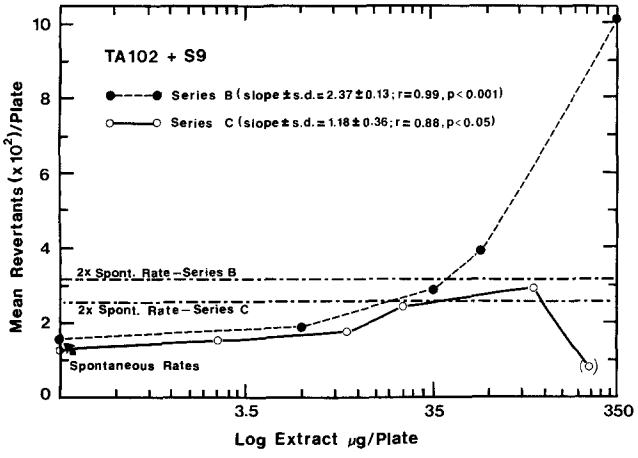

Fig. 2. Dose-related mutagenic responses using TA102 (+S9) of Series B and C water extracts from Banner Creek (site no. 7). Regression analysis data (for mean revertants/plate versus extract μg assayed/plate) are shown which include slope (± standard deviation of the slope), correlation coefficient (r), and probability of significance (p). The point in parentheses was not included in the regression analysis. Results for positive controls as mean revertants (± standard deviation) per quantity of known mutagen assayed: 1203 (±15)/2.0 μg 2-aminoanthracene (TA102 + S9), and 3200 (±346)/1.0 μL methyl methanesulfonate (TA102 − S9)

Table 2. Specific mutagenic activities of organic extracts of Banner Creek (Tennessee) water samples

Sample	Extractable organics concentration in the water[a] (mg/L)	TA102(+S9) Net revertants per mg extract residue + s.d.[b]	TA102(+S9) Net revertants per L water equiv. ± s.d.
Series B	3.48	2,370 ± 130	8,250 ± 450
Series C	6.14	1,180 ± 360	7,250 ± 2,210

[a] Determined from the residue weights of the extracts
[b] Represents the slope ± standard deviation of the slope of the initial linear portion of the mutagenic dose-response curve of the extract

(10 μg/mL) (Levin *et al.* 1982). In our study, TA102 test cultures were started directly from frozen permanent stocks containing no tetracycline, as recommended by Levin *et al.* (1982), which may account for the lower spontaneous reversion rate. Levin *et al.* (1982) cautioned, however, that TA102 test cultures with low spontaneous reversion rates may be unacceptable as they may also be insensitive to reversion by mutagens. This, however, was

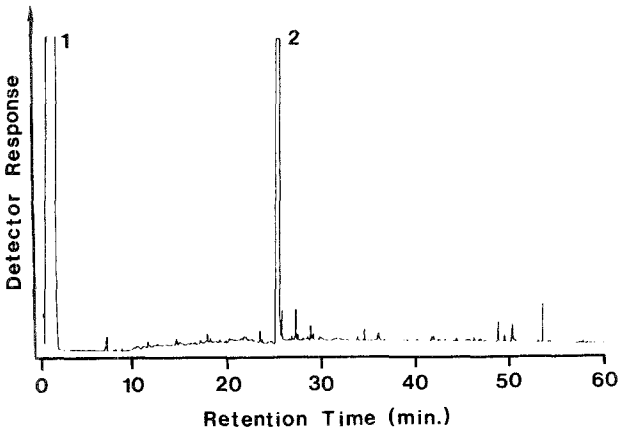

Fig. 3. Gas chromatogram of organic extract of Series C, Banner Creek (site no. 7) water sample. Peak 1 represents the organic solvent, and the mass spectrum of peak 2 is shown in Figure 4

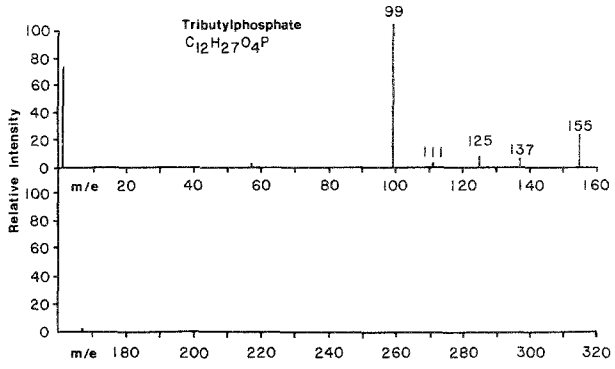

Fig. 4. Mass spectrum of peak 2 in the gas chromatogram shown in Figure 3

not the case for the TA102 cultures used in our study. As shown in the legend to Figure 2, the TA102 test cultures responded with a dramatic increase in revertants in the presence of known mutagens. Moreover, the dose-response curves obtained with water extracts from Banner Creek (Figure 2) are clear evidence of mutagenicity. The mutagenic activity of the Banner Creek water extracts did decline with increasing extract storage time at −20°C. Such a phenomenon has been observed by several investigators working with other water samples (Loper 1980).

The Banner Creek water extracts were analyzed by GC and GC-MS in an attempt to identify the mutagenic agent(s) present in these samples. The gas chromatogram of the Series C, Banner Creek water extract exhibited one major peak (peak 2) as shown in Figure 3. By comparing the relative heights of the minor peaks with that of peak 2, it is obvious that the minor constituents represent less than 1% of the total sample. The mass spectrum of peak 2 is shown in Figure 4 and it is identified as that of tributylphosphate (TBP). TBP is a viscous, colorless liquid used extensively in the nuclear industries to extract or purify uranium and plutonium (Schulz and Navratil 1984). As was the case in the present study, TBP has been previously detected, using XAD resins, in potable water supplies (Williams and LeBel 1981) and in municipal wastewater effluents (Ellis *et al.* 1982). In order to determine the mutagenic activity of TBP, fifteen doses (ranging from 98 mg/plate to 9.8 ng/plate) of the same TBP formulation used at NFS were prepared by serial dilutions in Me_2SO and were assayed, using TA102 in the presence of S9. Doses ranging from 98 mg/plate to 0.98 mg/plate were toxic to the TA102 tester strain, whereas doses of 196 μg/plate to 9.8 ng/plate were not mutagenic (*i.e.*, produced less than a 2-fold increase in revertants as compared to the concurrent spontaneous plates) (data not shown).

While TBP itself did not prove to be the mutagenic agent in Banner Creek water extracts, it may well be that the mutagenic response in TA102 (+S9) was induced by radionuclides complexed to TBP. Tester strain TA102 has been demonstrated to detect oxidative mutagens including x-rays and ultraviolet radiation (Levin *et al.* 1982). The primary radionuclides present in NFS wastewater effluents are uranium (234, 235, 236 and 238), thorium (228, 230, 232, and 234), plutonium (238, 239) and tellurium 99 (Blevins *et al.* 1985); thus, the gross radioactivity of NFS effluents is dominated by alpha- rather than beta-emitting radionuclides. Although ionizing alpha and beta radiation are well known to be mutagenic (Unrau 1985), it is not known if TA102 is mutagenically sensitive to these radioactivities. It is also possible that one (or more) of the unknown minor constituents observed in the gas chromatogram of the Series C, Banner Creek water extract (Figure 3) is (are) potently mutagenic. As was the case in the present study, other investigators have also reported the inability of chemical analysis to establish the identity of genotoxic compounds in mutagenic water samples (Donnelly *et al.* 1985; Reinhard *et al.* 1982).

In this study, mutagenic activity was only detected in Banner Creek water samples obtained immediately outside of the NFS property line; apparently indicating the lack of persistence of the observed mutagenicity. Similarly, Fallon and Fliermans (1980) reported the rapid decay of detectable mutagenic activity in the aquatic environment (*i.e.*, in a closed-loop cooling reservoir). Since the only potential pollution source upstream of the Banner Creek sampling site is NFS, it is most

unlikely that the observed mutagenicity in Banner Creek water samples was due to a source other than NFS. Holding ponds on the NFS property containing radioactive wastewater periodically overflow into Banner Creek and may have been the source of the mutagenicity detected in Banner Creek. Moreover, the presence of TBP in the Banner Creek water extract indicates that NFS discharged wastewater into this creek. In Martin Creek below NFS, Blevins *et al.* (1985) detected high levels of gross alpha and beta radioactivities in surface waters, sediments, and aquatic vegetation. Despite concentrating water volumes (4.0 to 20 L) typically used by other investigators to detect mutagenic activity in surface waters via XAD-2 resin extraction (Heartlein *et al.* 1981; Maruoka and Yamanaka 1983), we were unable to measure any significant mutagenicity in the waters of Martin Creek upstream or downstream of Banner Creek. The Banner Creek water samples contained a promutagen which most likely exerted its mutagenic effect through oxidative processes as it was detected with tester strain TA102. Although we were unable to establish the chemical identity of the responsible mutagen(s), the possibility that radionuclides induced the observed mutagenic responses deserves further study.

Acknowledgments. We sincerely thank Dr. Bruce N. Ames, Biochemistry Dept., University of California, Berkeley, for providing us with the *Salmonella* tester strains. We gratefully acknowledge Dr. M. R. Guerin and Michele Buchanan, Oak Ridge National Laboratory, Oak Ridge, TN, for their chemical analyses of mutagenic water extracts. We also acknowledge the technical assistance of Dr. John Babish, Cornell University, and the staff at the Environmental Mutagen Information Center, Oak Ridge National Laboratory.

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Manuscript received December 27, 1986 and in revised form March 21, 1987.