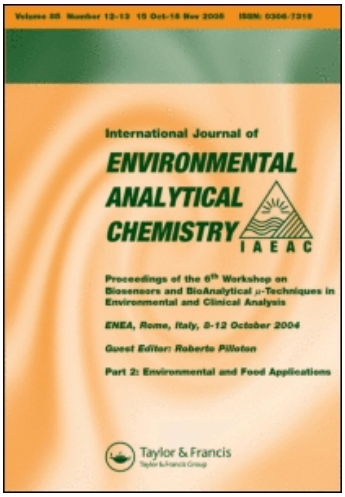

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Analytical Isolation, Separation and Identification of Mutagens from Nonvolatile Organics of Drinking Water

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A general procedure has been developed for the concentration/fractionation of mutagenic residue organics from small, <50 L, and large, to 1200 L, volumes of drinking water obtained from a variety of sources. This procedure features concentration of the residue organics chromatographically by passage of the water through XAD-2 and XAD-7 resins in specially designed columns, details of which are given. The residue organics are eluted from the resins via organic solvents, followed by solvent removal and subsequent bioassay for mutagenicity. Then the residue organics are fractionated via a coupled bioassay/analytical fractionation method which progressively focuses to the bioactive constituents of the complex mixture of residue organics. In this report, results for the optimal operation and validation of the concentration system are given, using drinking water derived from an industrially polluted river system, a wilderness river system and a major aquifer system. The predominant type of mutagenesis observed for the residue organics isolated from these samples was direct-acting to the *Salmonella* tester strain, TA98, which was decreased by the addition of the metabolic activation system from the livers of rats previously

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treated with Arochlor 1254. Some TA100 direct-acting mutagenesis was observed for all samples. Fractionation of the residue organics indicated the mutagens to be nonpolar. Samples of residue organics collected over a period of a year from each type of drinking water showed no discernable pattern of mutagenesis versus season. The methodologies described in this paper provide a comprehensive approach for the concentration/isolation of residue organics from drinking water for studies to identify biohazardous compounds and to characterize these compounds biologically.

KEY WORDS: Drinking water, mutagenicity, environmental mixtures, groundwater, HPLC, XAD resins.

INTRODUCTION

Detection of chemical carcinogens in drinking water,[1] plus epidemeologic evidence suggestive of increased cancer due to consumption of certain water supplies[2,3,4] has stimulated world wide research for the toxicological assessment of drinking water organics. Most of these latter studies have applied short term mutagenic/carcinogenic bioassays to the water, either directly, or more generally to complex organic mixtures extracted from the samples.[5,6] However it has become increasingly clear that any definitive assessment of risks due to water borne organics must be predicated upon the ability to detect, isolate and identify such compounds.[7] In this paper we report our analytical approach toward achieving this goal.

This task is complicated by the fact that the thousands of organics present typically occur only in the part per million or lower range. Operationally, the great diversity of naturally occurring and synthetic chemicals which find their way into water utilized for drinking purposes divides into two categories:[8,9] (a) compounds of low solubility and with a volatility sufficient for rapid separation and identification; (b) relatively soluble compounds with low volatility which are not readily separated but are collected by extraction or concentration from water as complex mixtures of residue organics. The volatile organics comprise only about 10% of the total organic material by weight in drinking water,[9] and the vast majority of these (>90%) have been identified and can be quantitated. The Health Effects Research Laboratory of the U.S. Environmental Protection Agency recently evaluated methods for the sampling and analysis of volatile organics in drinking water, and recommended the Grobe closed-loop-stripping techniques as the method of choice.[10,11] Our

efforts have been directed to the far more prevalent, nonvolatile constituents.

Isolation, identification and risk assessment of these residue organics is a far more difficult task. Any sampling method for obtaining these organics from aqueous solutions should be reasonably quantitative and free of artifacts of isolation, thus yielding concentrated mixtures representative of the original dilute solution. Since the vast majority of these compounds are unknown, it is impossible to test the absolute efficiency of any one procedure, so a combination of complementary, broad range methods is preferable. Kopfler,[12] Jolley[13] and the National Academy of Science[14] have reviewed the strategies and methods employed in the sampling of this category of organics. No clear choice of methodology has emerged although many laboratories are utilizing some form of resin based chromatography.[5,6,15-23]

Our implementation of an XAD resin procedure was influenced by our experience in the separation for identification of mutagens from pre-prepared organic residues of drinking water. Those studies used residues of repeat drinking water samples prepared by reverse osmosis (RO)[24] as provided by the USEPA,[25] and a mixture of activated carbon–chloroform extracted organics from drinking water (CCEO) processed years earlier and provided by F. M. Middleton.[26,27] It was demonstrated that such residue mixtures are too complex for conventional chemical analysis,[28] and indeed required extensive manipulation by capillary GC/MS/DS to reveal the identity even a small fraction of the total constituents.[29] On the other hand for mutagenic analysis such mixtures often required at least partial fractionation in order to reveal mutagenic capacities masked by toxic effects in the parent mixtures.[24]

Based upon our work with both the RO and CCEO residues we proposed a general procedure for the analysis of such complex mixtures.[30] That method combines the *Salmonella* mutagenesis assay with reverse phase HPLC separation of mutagenic subfractions, leading to the chemical separation and identification of bioactive constituents. In considering the collection of residues from new samples of drinking water, our results with the RO residues had documented the value of access to repeat samples on a short term basis; success with the CCEO residue was facilitated by its unique magnitude, about 125 g obtained by the processing of 50,000 gal of

drinking water. This report describes our development and application of an XAD-2/XAD-7 resin procedure which combines simplicity of processing with broad, relatively representative recovery of nonvolatile organics. Although on a much more modest scale than would accommodate 50,000 gallon samples, it is nevertheless designed to yield quantities of residues sufficient for extensive bioassay and mutagen separation. We describe here our process with this approach toward our overall goal in these studies—an understanding of mutagens and carcinogens among nonvolatile organics of drinking water; their prevalence, concentration, chemical structure, origin, and mechanism of biological effects.

EXPERIMENTAL

Concentration apparatus

The general design of the apparatus for the large scale concentration of organic residues from drinking water is shown in schematic form in Figure 1, and details of the fabrication of this assembly are shown in Figure 2. The pressure regulating gauge was obtained from Veriflo (Richmond, CF); the Whitey flow regulating valve, the 0.25, 0.5 and 1.0 inch tubing of 316 stainless steel, SWAGELOK® and other stainless steel fittings, and the 40 micron sintered stainless steel frits were obtained from Cincinnati Valve and Fitting (Cincinnati, OH); AP40 fiberglass, Durapore 0.45 micron filters, and the 47 mm, and 142 mm filter assemblies were purchased from the Millipore Corporation (Bedford, MA). Machining of the column frits and fittings was by Tri-State Controls, Inc. (Cincinnati, OH). Two versions of the apparatus were constructed, one for small volume (<50 L) samples and one for large volume (>50 L) samples. The large volume columns of 200 cc bed volumes were constructed as shown in Figure 2. Small volume columns, of 25 cc bed volumes, were constructed similarly from 0.5 by 13 inch 316 stainless steel tubing fitted with 0.5 inch Swagelok® fittings. Silanized glass wool was obtained from Supelco (Bellefonte, PA). The XAD-2, -4, -7 and -8 resins, obtained from the Applied Science Division of the Milton Roy Co. (State College, PA) via The Munhall Co. (Worthington, OH), were prepared and evaluated according to USEPA criteria.[31] Gas chromatographic analyses of each lot of resin were run according to these

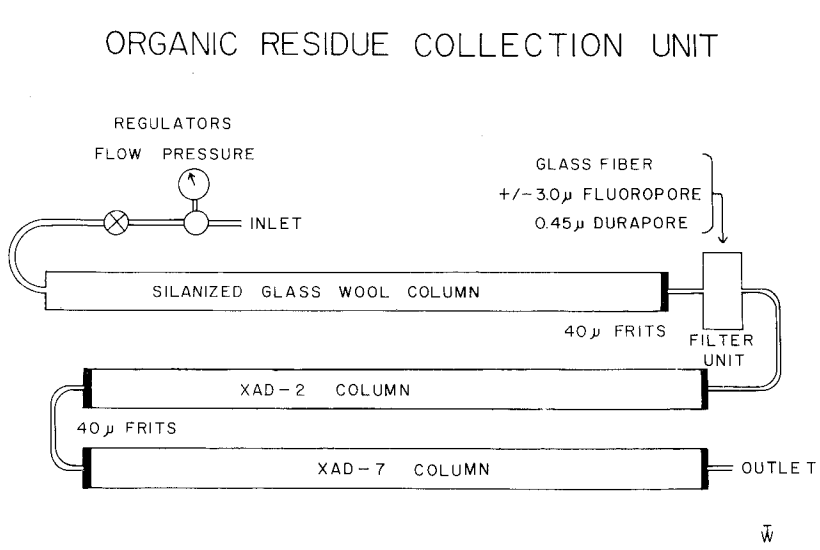

FIGURE 1 Schematic of the apparatus for large scale concentration of residue organics.

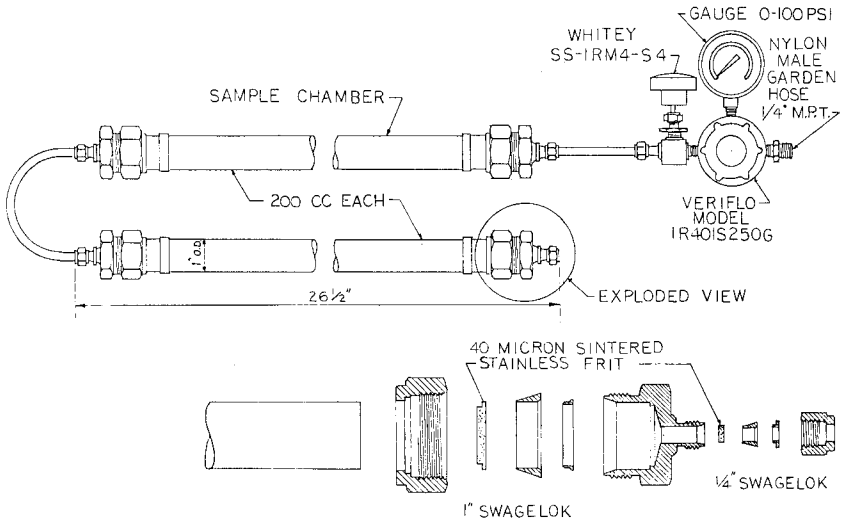

FIGURE 2 Details of fabrication for the large scale concentrator.

criteria, and the results and certifications of analyses were provided by the supplier with each order. Resins were stored until use in amber bottles under HPLC grade methanol, and columns were formed by gravity from slurries in the same solvent.

Instrumentation

High performance liquid chromatography (HPLC) separations were performed on a Waters Associates (Milford, MA) Model ALC/GPC 204 fitted with components as previously described.[26] For preparative scale (mg level) separations, a Waters radial compression model (RCM) column unit was utilized. The RCM was fitted with an 8 mm by 10 cm column packed with 10 μm silica particles bonded with octadecylsilane for reverse phase separations; normal phase separations utilized RCM columns containing 10 μm silica (PORASIL®).

Gas Chromatography (GC) analyses were performed on a Hewlett-Packard (Palo Alto, CA) Model 403 flame ionization unit fitted with a glass 6 ft by 0.25 in i.d. column containing 5% OV17 on 100/120 mesh Gas Chrom Q (Applied Science). Data were continuously collected and analyzed using a Spectra Physics Autolab 1 Computing Integrator (Santa Clara, CA), and chromatograms were displayed on a 10 mV recorder.

Mass spectrometry (MS) analyses were performed on a Kratos MS80 (Manchester, U.K.) high performance mass spectrometer as previously described.[32] These data were continuously collected during MS runs and processed on a Data General (Westboro, MA) Nova/4C DS-55 data system. Computer interaction, data display and output were via Hewlett-Packard Models 2649C graphics terminal and 9876A printer systems. Samples introduced via GC utilized a Carlo Erba Series 4160 GC (Milan, Italy) fitted with a SE54 30 m by 1 mm fused silica, film thickness of 0.25 μM, capillary column.

Drinking water samples and other chemicals

Finished drinking water was obtained from two U.S. cities which draw upon environmentally distinct surface waters. For one city, Source I, raw water from a river impacted by chemicals from numerous industrial, municipal and agricultural sources is treated by a series of settling, coagulation and floculation steps, with the final

product being chlorinated to a residual level of 1 to 2 mg/L. The second city, Source II, draws raw water from a network of streams and rivers that principally drain wilderness regions. The water is settled in a series of reservoirs, then chlorinated to a residual level of 2 to 4 mg/L prior to distribution. Additionally, finished drinking water from a major U.S. aquifer system, Source III, was concentrated. Water recharge of the aquifer in this area is primarily by stream bank infiltration from a river. The river is subject to multiple points of contamination, including industrial, municipal and agricultural. Preliminary experiments to evaluate the concentration apparatus utilized drinking water previously shown (Loper and Tabor, upublished) to contain mutagenic nonvolatile organics.

American Society for Testing Materials (ASTM) Type I water[33] for HPLC and for the preparation of other aqueous solutions was purified using a Continental Millipore Water Conditioning System (El Paso, TX) as previously described.[26] The HPLC solvents methanol, methylene chloride and acetonitrile were obtained from Fisher Scientific (Cincinnati, OH). All HPLC solvents were degassed immediately prior to use by 15 min of sonication while under reduced pressure. Hexane, diethyl ether, methylene chloride and acetone for elution of the XAD columns were of pesticide grade obtained from Matheson Coleman and Bell (Cincinnati, OH). Diethyl ether was extracted with acidic aqueous 5% ferrous sulfate immediately prior to use to eliminate peroxides. The base-neutral nonvolatile pollutant standards (USEPA Method 625) were obtained from Supelco.

4-Nitrothiophenol from Aldrich Chemicals (Milwaukee, WI), was recrystallized according to the procedure of Barnett and Jencks,[34] and stock solutions were maintained as recommended by Cheh and Carlson.[35] Sodium sulfate (Reagent Grade, Fisher Scientific) was muffled at 500°C $\geqq$ 4 hrs priors to use. All other chemicals were of reagent grade and were used without further purification.

General operation of concentration apparatus

The apparatus was assembled as shown in Figure 1. A 142 mm bacterial filter unit was used for high total organic carbon ($>$20 ppm) or particulate laden water, or in situations where $>$200 L of water were to be passed through the system; otherwise a 47 mm bacterial filter unit was applied. All collections employed a 200 cc

prefilter column, packed with silanized glass wool. When the line pressure of the drinking water source was <40 psi, one of two alternative modes of operation was utilized. The first alternative used a controlled volume TFE diaphragm pump (model MR-117S, Milton Roy Co.), which was connected between the water source and the concentration apparatus. This mode of operation was generally used for sample volumes larger than 60 L. The second alternative was to collect the drinking water samples in 20 L stainless steel reservoirs (model RS20, Amicon Corporation, Lexington, MA). The collection apparatus was connected to the outlet of the reservoir, and a cylinder of water-pumped purified nitrogen was connected to the inlet of the reservoir. Then the sample was forced under 30 psi pressure through the concentration apparatus. Routinely, the volume of water passed through any of these systems was measured by collection of the final effluent in calibrated containers, usually 55 gal drums.

Following concentration of residue organics, each component, i.e. columns and filters, was extracted with a hexane:acetone solvent system, 85:15 by volume, according to the methods of LeBel *et al.*[20] Eluates were dried over sodium sulfate. Preliminary experiments were conducted to evaluate other solvent extraction methods[36,37] to assess mutagen recoveries.

Each system component extract was reduced in volume by rotovaporation under reduced pressure while warming the flask at 40°C. Further reduction in volume was accomplished by evaporation under a stream of purified nitrogen while warming the container at 40°C. As solutions were concentrated, some constituents occasionally came out of solution. In these cases a small volume of acetone was added to keep components in solution until sufficient evaporation of the solvent had accomplished the removal of the remaining hexane from the sample via an azetrope. Usually three to four additions of acetone were required. Final volumes of the acetone concentrates were recorded and the samples were stored in teflon capped amber vials at 4°C until bioassay. At the time of mutagenicity testing, aliquots of the solution were removed, added to an appropriate volume of dimethyl sulfoxide (DMSO) and the acetone was removed via gentle evaporation under a stream of purified nitrogen while warming the container at 40°C.

Preliminary residue organic concentration experiments

Preliminary evaluation of the XAD resins was performed using a 1.0 cm by 25 cm glass column fitted with Teflon® fittings (Glenco, Houston, TX). These small scale columns were packed by gravity using methanol slurries of a particular XAD resin. Each column was flushed with two volumes of Type I water to displace the methanol prior to each pilot experiment. A finished drinking water sample known to contain *Salmonella* mutagens (Loper and Tabor, unpublished), contained in a 20 L stainless steel reservoir, was forced over the column using pressure from a cylinder of purified nitrogen. The gas pressure was adjusted to maintain a flow of 120 ml/min. Following sample concentration, the column was eluted with 100 ml of hexane:acetone (85:15 by volume). The elute was concentrated and further processed for bioassay as described in the previous section. This procedure was conducted for all four XAD resins evaluated.

Experiments to evaluate artifact leaching from the XAD resins were conducted similarly, using both small scale and large scale columns. Type I water, 20 L, was passed through each resin, the columns eluted and the hexane:acetone eluate concentrated. These extracts were examined via capillary GC/MS and packed column GC using flame ionization detection, and concentrates of the eluates were examined for mutagenic artifacts by bioassay.

Chlorinated artifact formation

The effect of chlorine on artifact leaching from the XAD resins was assessed as follows. Type I water was spiked with a chlorine standard (2200 mg free chlorine per liter, obtained from USEPA-HERL, Cincinnati, OH) to a final concentration of 1.1 mg total residual chlorine per L. The actual residual chlorine concentration in each sample was verified by USEPA Method 330.2.[38] A 40 L sample of the chlorinated Type I water was passed through the large scale concentration apparatus, Figure 1, composed of tandem columns of XAD-2 resin followed by XAD-7 resin. In a parallel control experiment, a 40 L sample of unchlorinated water was similarly passed through the XAD columns. The columns were individually eluted with 1250 ml of the hexane/acetone solvent (85:15 vol/vol). The

eluates were dried over sodium sulfate, concentrated to 1.2 ml by rotovaporation under reduced pressure at 35°C, and stored at 4°C in teflon-lined, crimp-capped, amber vials that had been purged with nitrogen prior to sealing. The residue organics then were examined for mutagenesis to the tester strains TA98 and TA100 in the absence and presence of microsomal activation as described below. In a repeat experiment, 80 L samples of nonchlorinated and chlorinated Type I water were examined in a similar manner.

Organics recovery from XAD-2

The system was evaluated for extraction efficiency by passing Type I water, spiked at 75 μg/L with a standard mixture of base neutrals, across the pilot scale column packed with XAD-2 resin. The column was eluted with five volumes of hexane:acetone (85:15) solvent. The eluate was concentrated by rotovaporation as described before, then transferred to a vial. Following the addition of 100 μl of methanol, the sample was further concentrated to 50 μl using a gentle stream of dry nitrogen and warming the vial at 30°C. Individual constituents were examined via GC by injection of 4 μl of the concentrate. Quantitation was by computed peak areas and comparison to those obtained similarly on chromatography of the standard mixture of base neutrals. For verification of peak identities, 10 μl of the methanol solution of the column eluate was diluted with 90 μl of water, then extracted with 10 μl of methylene chloride. One microliter of this extract was injected into the capillary GC and peaks were identified by MS.

Column operation parameters

Both the small volume, 25 cc, and large volume, 200 cc, stainless steel columns were evaluated via a series of experiments to determine operation parameters and system capacity. A finished drinking water, known to contain mutagenic residue organics, was used as a sample source. In these experiments, flow rates and sample volumes were varied using columns connected in different configurations, e.g. two columns in parallel, or two or more columns in series, and packed with either XAD-2, XAD-7 or both resins. Following extraction of the residue organics onto the resins from the drinking water, the

columns were eluted with eight column volumes of hexane:acetone (85:15 by vol). The eluates were processed as described previously for bioassay.

Biological analysis

Tester strains TA98 and TA100 of the *Salmonella* microsome mutagenicity assay system were provided by Ames. Characteristic properties of the bacteria were verified for each fresh stock and their mutagenic properties were again verified using positive and negative controls as part of each experiment.[39,40] The nitroreductase deficient strains, TA98 NR and TA100 NR, were obtained from H. Rosenkranz,[41] and utilized in the *Salmonella* microsome mutagenicity assay as described by Cheh *et al.*[35] Microsomal activation requiring mutagenesis tests utilized polychlorinated biphenyl mixture Aroclor 1254 induced rat liver microsomes, $9000 \times g$ supernatant fraction (S9), from Litton Bionetics (Kensington, MD). Mutagenesis assays, without (−S9) and with (+S9) microsomal activation, were conducted as previously described.[24] The detection of mutagenesis in experimental samples was based upon a dose dependent response exceeding the zero dose (spontaneous control value) by at least two fold, i.e. the ratio of total revertant colonies per plate to spontaneous colonies per plate was $\geqq 2$. In some situations involving HPLC subfractions where the amount of sample was limiting, semi-quantitative determinations of mutagenesis were made as previously described.[15] All recoveries of bioactivity from concentrated or fractionated residue organic samples were based upon an expression of mutagenesis per liter equivalent, representative of the original water sample. The mean revertant colony counts, ±standard error, obtained from each group of spontaneous plates and positive control plates were as follows (number of plates appears in parenthesis): TA98 spontaneous, −S9, 15±3 (45); TA98 spontaneous, +S9, 28±6 (31); TA98 with 2 μg 1-amino-2-carboxy-4-nitroanthraquinone (ACNA), −S9, 1686±295 (30); TA98 with 2 μg 2-aminoanthracene (2-AA) and +S9, 1575±327 (16); TA100 spontaneous, −S9, 122±10 (48); TA100 spontaneous, +S9, 126±14 (32); TA100 with 1 μl methylmethanesulfonate (MMS), −S9, 1282±163 (32); TA100 with 2 μg 2-AA, +S9, 1460±340 (16); TA98 NR spontaneous, −S9, 15±4 (27); TA98 NR with 2 μg ACNA, 1785±436; TA100 NR spon-

taneous, −S9, 106±18 (36); TA100 NR with 2 μg ACNA, 1374± 114 (24).

4-Nitrothiophenol experiments

Experiments to inactivate direct-acting mutagens via reaction with the nucleophile, 4-nitrotheophenol (NTP), were conducted according to the recommendations of Cheh and Carlson.[35] Stock solutions of NTP, 10 to 20 mg/ml, were maintained at −20°C as 50:50 aqueous ethanol solutions acidified to pH 4.5. Concentrations were determined by dilution of the stock solution 1:1000 into 0.1 M sodium phosphate buffer, pH 7.4, measurement of the absorbance of this solution at 410 nm, and calculation using the reported[35] molar absortivity of $13{,}700\,M^{-1}\,cm^{-1}$. Working solutions of NTP were prepared by diluting the stock solution into 0.05 M sodium phosphate buffer, pH 7.4, to a concentration range of 6.5×10^{-4} M to 2.6×10^{-2} M.

Reaction of NTP with samples containing direct-acting TA98 and TA100 mutagens was accomplished by incubating the sample with NTP at room temperature for 30 minutes. Such mixtures were bioassayed for mutagenesis with strains TA98 NR and TA100 NR, using ethanol solutions of 2-chloromethyl naphthylene and 1,3-dichloroacetone respectively as positive mutagen controls for the NTP reaction, as described by Cheh and Carlson.[35]

Gas chromatography and mass spectroscopy

Gas chromatographic analyses of HPLC subfractions of SEP-PAK® concentrates were performed by the slow injection of 2 to 7.5 μl of the appropriate hexane/acetone, acetonitrile/water or methylene chloride solution. The nitrogen carrier gas was flowing at 70 ml/min, and the temperatures of the injector and detector were 220°C and 320°C respectively. A linear temperature program from 80°C to 270°C at 7.5°C/min was initiated at the time of injection. Weight values for sample constituents were calculated based on peak areas compared to those obtained from chromatography of repeated injections of 2.0 μl of a 2.6 mg/ml methanol solution of standard base neutral mixture.

High resolution mass spectrometry analyses were performed in the presence of the internal mass standard perfluorokerosenes (PFK). The conditions for EI spectra were as follows: ionizing current, 1×10^{-4} A; ionizing energy, 40 eV; accelerating voltage, 4 kV; scan range, 20–600 m/e; scan speed of 5.5 s for 3000 resolution and 13 s for 7500 resolution; scan internal, 1 s. Samples were introduced via direct insertion probe or capillary GC. For the former, 5 to 50 μl of a methylene chloride solution of sample was evaporated on a capillary sample holder which was placed in a shaft tip of the direct insertion probe. The probe was air cooled to 20°C, the air shut off, and then the temperature of the probe was increased at 10°C/min while continuously monitoring the total ion current and collecting spectral data. Capillary introduction of samples was under the following GC conditions: injection temperature, 50°C; oven temperature at 50°C for 75 S after injection then programmed at 250°C at 10°C/min; separator temperature, 250°C; helium carrier gas at 20 ml/s with a 20 ml/min flow make-up to the separator. Five hundred nanoliters of methylene chloride or hexane/acetone solutions of sample were introduced by the cold on-column splitless injection technique of Grobe and Neukom.[42] After a 70 S solvent divert, the valve to the MS was opened and data were collected. Isobutane chemical ionization (CI) spectra at 1000 resolution were obtained on samples introduced by capillary GC at an ionization current of 1.5×10^{-3} A. Other GC/MS conditions were as described above.

RESULTS AND DISCUSSION

Two types of technical problems related to the apparatus and the concentration procedure were resolved during the design and preliminary experimental stages. The first entailed design considerations to reflect the diversity of physical properties of the various types of water from which organics were to be concentrated. The second type was more experimental, and dealt with chemical and biological questions associated with the concentration of organics from dilute aqueous solution.

Design of the concentration apparatus

The system is constructed of readily available stainless steel components with Swagelok® fittings, see Figure 2. A glass wool pre-

column was found to be useful for concentrating water containing diatomaceous and/or clay particulate matter. In the concentration of finished drinking water from one surface source, this column had to be changed every 50 L due to the high particulate content, while a low particulate ground water source allowed for the collection of more than 1200 L without changing any of the filtration media.

Another filtration feature of the system is the use of a 0.45 micron filter for general disinfection purposes. Two sizes have been utilized: a 142 mm filter assembly for large sample volumes and/or for high total organic carbon (>20 ppm) or particulate laden water; a 47 mm filter assembly for small sample volumes and/or for low total organic or low particulate laden water. Since columns are transported long distances between cities for some collections, this filter was included to minimize microbial metabolism of organics concentrated on the resin columns. An 0.22 micron filter was not considered essential for this purpose and its use would have impeded overall flow rates. Filtration residues from both the glass wool and microbial filter were extracted with the hexane:acetone solvent as described later, and the concentrates were examined for *Salmonella* mutagenesis. No mutagenesis was observed in these extracts obtained from a variety of water sources.

An additional design feature of the system is the choice of resin column physical parameters to obtain efficient collection of a broad range of classes of organic compounds. Studies of XAD column design and chromatographic parameters by Malcolm *et al.*,[43] Thurman *et al.*[44] and Leenheer and Huffman[45] were considered in arriving at columns of 200 cc bed volume with a length to diameter ratio of 30 to 1 for the concentration of up to 1500 L of low (<3 ppm) total organic carbon (TOC) water. Apparent capacity factors, k', reported for XAD-8,[46] were also considered in balancing sample size versus bed volume. The same considerations[43–46] were employed in the design of the 25 cc bed volume columns for use in the concentration of smaller sample volumes, i.e. <50 L.

Preliminary experiments for choice of XAD resins

Pilot concentration experiments were conducted using a finished drinking water known to contain *Salmonella* mutagens (Loper and Tabor, unpublished). The ultimate choice of XAD resins was also

based upon considerations discussed by van Rossum and Webb[37] in their experimental evaluation of these resins. Successive concentration experiments of 20 L of finished drinking water on separate pilot columns (20 cc bed vol.) of XAD-2, -4, -7, and -8 were conducted. The columns were eluted with 5 bed volumes of hexane:acetone solvent (85:15) and each eluate was concentrated for mutagen assays. The maximum recovery of mutagenic activity was found in the drinking water residue organics eluted from drinking water with XAD-2 and with XAD-7. Parallel experiments were conducted with Type I water for an initial assessment of artifacts leaching from each resin type. Columns were eluted with hexane:acetone and the eluates concentrated; concentrates were examined via temperature programmed gas chromatography on a 5% OV17 column. A minimum number of artifacts were obtained with XAD-2 and XAD-7; the maximum amount of artifacts were observed with XAD-4. Subsequent examination of these control water extracts via capillary GC/MS showed them to be principally hydrocarbons (C_8 to C_{24}) with the occurrence of some phthalate aliphatic esters. These results are consistent with the nature of artifacts from eluates of XAD-2 observed by James *et al.*[47] and Care *et al.*[48]

The possible interference of artifacts in the bioassay system was examined also. Cheh *et al.*[49] have reported that the organic mixture eluted from XAD-4 following the passage of distilled water containing free chlorine at 0.7 mg/l was mutagenic to TA100. To examine the possible occurrence of such artifacts in our system, portions of Type I water, with and without chlorination to 1.0 mg/L, were processed separately through each component of the dual XAD-2/XAD-7 column system. Organics were eluted from the columns via hexane:acetone, and the eluates concentrated and tested for mutagenic activity using TA98 and TA100. No mutagenic effects were apparent in these residues from either the control water sample or the chlorinated water samples, Figure 3. In subsequent experiments other such residue organics were separated by an initial reverse phase HPLC (as described below) and the resultant subfractions were bioassayed. The results were again negative, thus eliminating the likelihood that mutagenic artifacts were present but undetected in the parent residue due to the presence of other competing components.

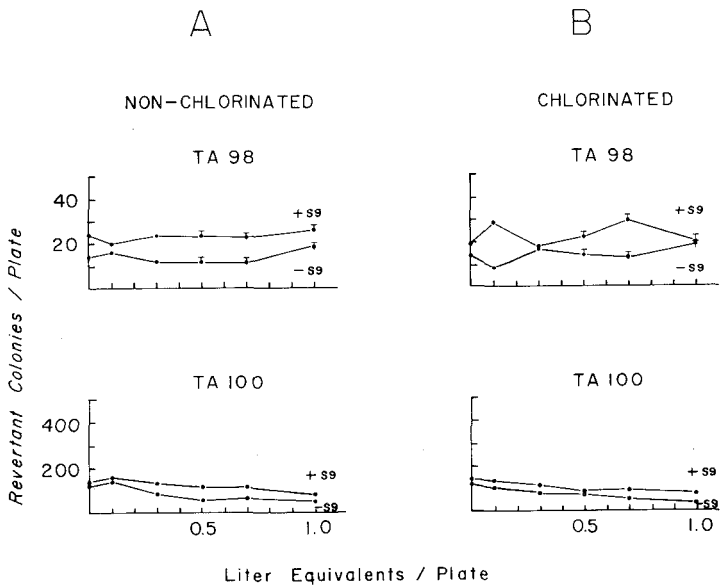

FIGURE 3 Mutagenic activity of residue organics eluted from XAD-2 after processing samples of Type I water and Type I water containing 1 mg/L residual chlorine. Mutagenesis assays were conducted in the absence (−S9) and presence (+S9) of metabolic activation. In this and subsequent figures, liter equivalents per plate values refer to the volume of original water sample proportional to the fractional volume of the residue organics concentrate on the plate.

The next question concerning the experimental aspects of the concentration system was to examine the operational efficiency of the XAD resins for removal of organics from dilute aqueous solution. In previous studies of mutagenic residues from drinking water,[15, 24, 26, 30, 32] we and others (see reviews 5, 6) had shown the bioactive organics to be principally nonpolar organic compounds. This is consistent with the argument of Yamasaki and Ames[50] who stated that organic compounds, which will exert biological effects and thus are capable of passing through biological membranes, are likely to be apolar or lipophilic compounds of low polarity. A broad range of this class of compounds was demonstrated by Chriswell *et al.*[51] to be extracted on XAD-2 from dilute aqueous solutions. In their examination of mixtures of model compounds, only aliphatic

hydrocarbons, some short-chain aliphatic carboxylic acids and some polar compounds such as phenols were not effectively recovered. These results have been confirmed by others[16-19,22,23] in more extensive studies incorporating a combination of XAD resins into the collection system. The use of methyl acrylate ester resins, XAD-7 or -8, in combination with the polystyrene-divinylbenzene resins, XAD-2 or -4, increased the overall efficiency of the system by extracting those classes of compounds poorly recovered in the original system of Chriswell *et al.*[51] XAD-7 and XAD-8 differ only in pore size, 80 Å versus 160 Å, and surface area, 450 m^2/g versus 200 m^2/g; their concentration abilities for types of organics are functionally equivalent (Baird, personal communication, Nov. 1980). Based upon its capacity, XAD-7 was chosen over XAD-8.

Results of our extraction efficiency experiment were in agreement with other reports[37,43-46] in that the average recovery was 70% from XAD-2 for a group of representative compounds. In this experiment, a 2 L sample of Type I water, spiked with 150 μg of a standard mixture of base neutral extractables consisting of the compounds shown in Table I, was passed through the XAD-2

TABLE I

Recovery of base/neutral organics from water with a pilot-scale XAD-2 column system.

Compound	% Recovery[a]
1,3-Dichlorobenzene	25
1,2-Dichlorobenzene	28
Bis-(2-chloroethoxy)-methane	2
Naphthalene	100
Hexachlorobutadience	66
Acenapthene	77
2,4-Dinitrotoluene	109
Fluorene	112
Diethylphthalate	47
Hexachlorobenzene	90
Anthracene	98
Pyrene	103
Chrysene	113
Benzo(a)anthracene + dibenzo(a,h)anthracene	65

[a]Average of duplicate determinations.

column. Following elution with hexane:acetone and subsequent concentration, the residue organics were analyzed and quantitated via gas chromatography. Assignment of peaks was verified by separation on capillary GC and identification of the eluting compounds via MS. Recoveries for most of these compounds were in the 50 to 100% range. Exceptions were the relatively low recoveries of the dichlorobenzenes and of the ether, bis-(2-chloroethoxy)methane; this was probably due to losses occurring during the concentration of the eluate since these compounds are more volatile than the others in the series.

The recovery efficiency of chloroethers on the XAD collection system was of particular interest since we recently isolated, from an old drinking water residue, a potent promutagen that has been assigned a confident structure as 3-(2-chloroethoxy)-1,2-dichloropropene.[32] Fifty micrograms of this relatively nonvolatile mutagen was added to 2 L of Type I water and concentrated on an XAD-2 column. Following elution and concentration of the eluate, the residue organics were assayed for mutagenesis using TA100. Greater than 95% of the mutagenic activity of this compound was recovered, thereby showing that XAD-2 would effectively extract this compound and that the elution/concentration procedure allowed for its recovery.

Evaluation of solvent systems for eluting residue organics from XAD columns

Several solvent systems have been reported for eluting drinking water residue organics from XAD columns. Junk *et al.*[36] originally reported the use of diethyl ether to elute XAD-2, while van Rossun and Webb[37] reported their evaluation of several solvent systems, including acetone and methylene chloride for the various XAD resins that they evaluated. Additionally, LeBel *et al.*[20] employed a hexane:acetone solvent system for the elution of XAD resins in their studies of drinking water mutagens. Reports of other solvent systems for eluting XAD columns have included those by Baird *et al.*,[16,17] Kool *et al.*,[22,23] and Noodsij *et al.*[18,19]

To evaluate alternative solvents for the elution of residue organics, three 25 cc columns, packed with XAD-2, were connected in parallel to the prefiltration glass wool column and bacterial filter. Fifty liters

of drinking water were passed through each column at a flow rate of 200 ml/min. One XAD-2 column was eluted with 200 ml of hexane:acetone (85:15 by volume),[20] the second with 200 ml of diethyl ether,[36] and the third with 40 ml of acetone followed by 160 ml of methylene chloride.[37] The acetone and methylene chloride eluates from the latter column were combined prior to further processing. The eluates from the three columns were concentrated to 1.0 ml and the concentrates were processed further for bioassay. Control experiments consisted of passing 14 L of Type I water through two different 25 cc XAD-2 columns. One column was eluted with hexane:acetone and the other was eluted with diethylether. The eluates were concentrated and were processed for bioassay.

Residues obtained using Type I water in these procedures were assayed minus S9 while the others were tested both minus and plus S9. Results of mutagenicities in these experiments are summarized in Table II. For either TA98 and TA100, the least mutagenicity was

TABLE II

Mutagenesis of drinking water residue organics eluted by various solvents from small volume (25 cc) XAD-2 columns.

Elution solvent water sample	Net revertants/liter equivalent			
	TA98		TA100	
	−S9	+S9	−S9	+S9
Hexane:acetone[a]				
Type I	—[c]	ND	—[c]	ND
Drinking	33	12[d]	125	86[d]
Diethylether				
Type I	117	ND	38[d]	ND
Drinking	113	35	253	105
Acetone/methylene chloride[b]				
Type I	ND	ND	ND	ND
Drinking	24	7[d]	54[d]	22[d]

[a]A hexane:acetone, 85:15 by volume, solvent mixture.
[b]Acetone followed by methylene chloride.
[c]Same as spontaneous rate.
[d]Not significant. Does not equal nor exceed a doubling above spontaneous reversion rate.

recovered in the residue organics eluted from the XAD-2 column by acetone followed by methylene chloride. Mutagenicity of the drinking water residue organics eluted by diethyl ether was the highest for the three solvent systems examined. However, the experiments using Type I water showed that diethyl ether extracted significant artifactual mutagenicity for both TA98 and TA100, thereby raising doubt as to the reality of the mutagenesis of the drinking water residue organics extracted by this solvent.

The hexane:acetone solvent system showed the best combination of sensitivity and specificity. Using drinking water, this system recovered mutagenicity for both TA98 and TA100 at higher levels than were obtained using acetone followed by methylene chloride. Additionally the residue organics for the Type I water system blanks using this eluting solvent system did not show any mutagenesis. Therefore, the hexane:acetone solvent system was chosen for the elution of drinking water residue organics from XAD resins in all subsequent experiments.

Preliminary experiments to establish operation parameters

To establish the functional comparability of the small, 25 cc XAD-2 columns versus the large 200 cc columns, the parallel recovery of mutagenic residue organics from a drinking water sample was evaluated. The valves, glass wool column and filters were assembled as shown in Figure 1. A tee, fitted on the outlet ends with flow regulating valves, was installed in the system following the filter. A 200 cc column packed with XAD-2 was attached to one valve and a 25 cc column of XAD-2 was attached to the other. To start, water was passed through the parallel columns at the same flow rate, approximately 100 ml/min. After 14.2 L of water had passed through the 25 cc column, that valve was shut off, the column was detached and extracted with hexane:acetone (85:15) for mutagenesis testing of the residue organics. The flow through the larger column was maintained at the same rate until 125 L of water had passed. During passage of the last 25 L, a second 25 cc column was attached to the vacant valve and 15.8 L of water was passed through the column. The 200 cc column and the second 25 cc column were extracted with hexane:acetone (85:15) and the eluates were processed for muta-

genesis testing. Mutagenic activity was recovered from each of the three columns (data not shown), with no discernible difference in the per liter recovery of mutagenesis between the large and small columns.

Effects of water sample flow rate through the collection system on the recovery of mutagenesis was evaluated via the following experiment. Three 25 cc columns containing 12.5 cc of XAD-2 each, and designated columns A through C respectively were connected via flow regulating valves to the same pre-column glass wool and bacterial filters. Drinking water was passed through the system according to the following protocol: column A ran continuously at 50 ml/min; column B ran the first 30 min of each hr at 100 ml/min; and column C ran the first 15 min of each hr at 200 ml/min. This protocol gave flow rates for each of the columns, A–C, of 4 bed vol/min, 8 bed vol/min and 16 bed vol/min respectively. Following the passage of 27 L of drinking water through each column, the columns were eluted with the hexane:acetone solvent and the eluates were processed for bioassay. Results are presented in Figure 4. No appreciable differences in the levels of mutagenic activity to either TA98 or TA100, minus and plus S9, were found. Similar results were obtained in separate experiments when flow rates of 1, 2 and 4 bed vol/min were utilized, data not shown.

The question of extraction capacity for mutagenic residue organics from drinking water was addressed in the next series of experiments. Twenty-five cubic centimeter bed volume columns were connected to the drinking water source through a common pre-filtration glass wool column and bacterial filter. The configuration of columns was as shown in Figure 5. Two 25 cc XAD-2 column sets, 1 and 3, were attached in parallel. An additional column set, 2, was attached in series to column set 1. Column set 1 was not removed during the entire course of the experiment. However, at 30 L intervals, the columns for sets 2 and 3 were replaced with freshly packed 25 cc XAD-2 columns. A total of 210 L of drinking water was passed through column set 1 plus 2, thus set 2 consisted of 7 columns total, i.e. 2A through 2G. Likewise, 210 L of water was passed through the 7 columns of set 3, i.e., 3A through 3G. Drinking water residues recovered from each column were tested for mutagenicity using strains TA98 and TA100 minus S9. Representative data shown in Figure 6(a) are for TA98 with residues from column 1, which

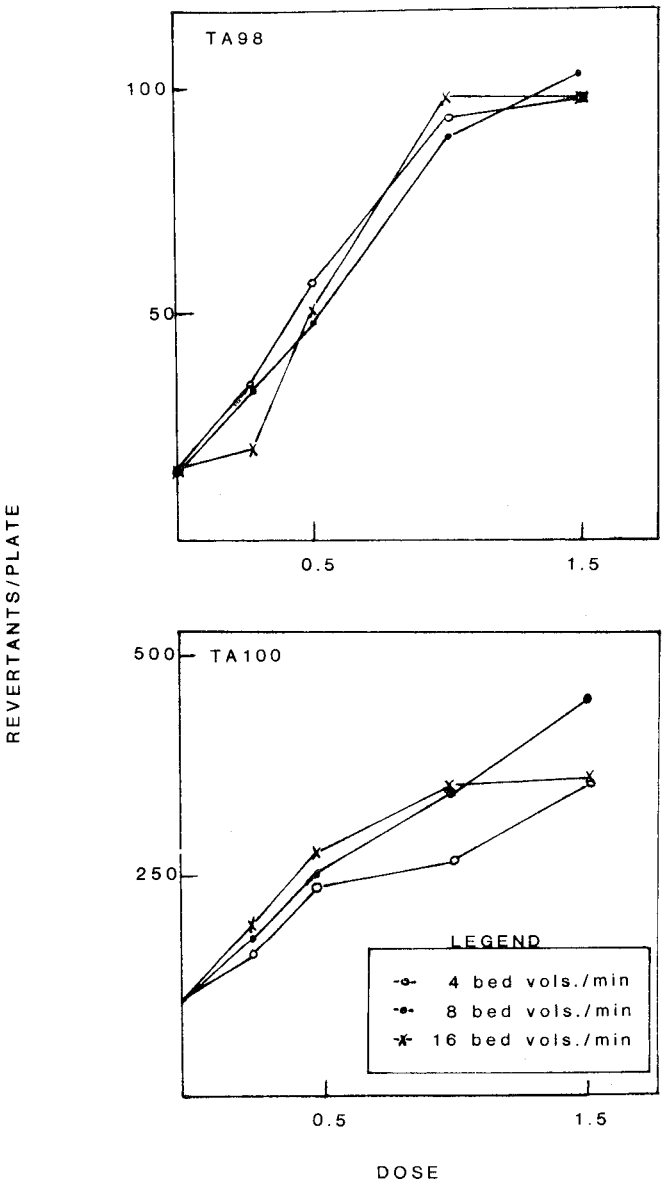

FIGURE 4 Direct-acting (−S9) mutagenic activity or residue organics eluted from XAD-2 after processing samples of drinking water from Source I through the concentration apparatus at the flow rates indicated in the box.

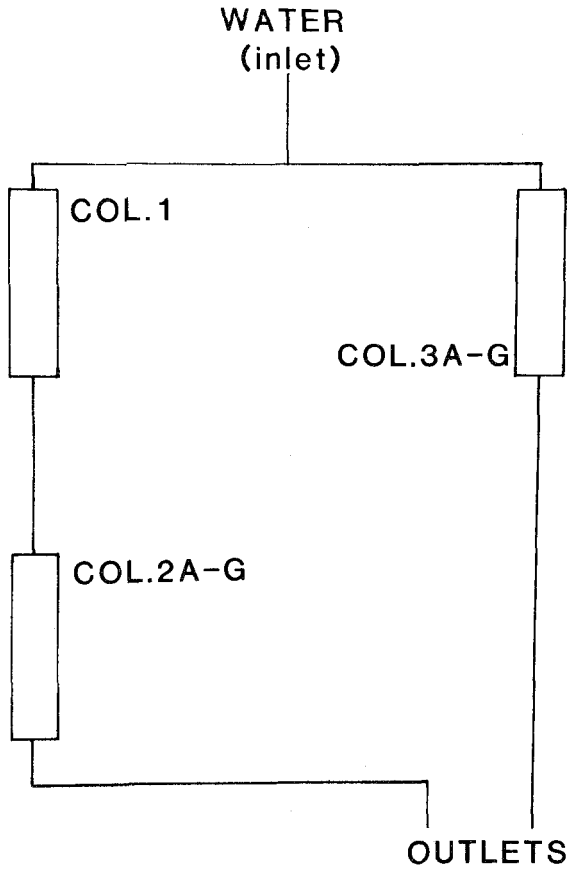

FIGURE 5 Configuration of XAD columns for experiments to determine the adsorption capacity of the resin for mutagenic residue organics from drinking water.

received a total of 210 L, columns 2A, 3A which received 30 L each at the start of the absorption experiment, and columns 2G, 3G, which received 30 L each at the end of the experiment (Figure 6(b). The mutagenic activity on a per liter basis was the highest for residue organics isolated by the columns of set 3, with little difference among them, compare results of 3A and 3G. The activity per liter isolated by column 1 from the entire 210 L was approximately 65% of that for the set 3 series. Little or no mutagenesis was

observed for the residue organics isolated from any one of the columns in set 2, although the rising dose responses obtained were indicative of some activity, see Figure 6. Therefore, a 25 cc XAD-2 column did diminish somewhat the retention of mutagenic residue organics from low (<3 ppm) total organic containing drinking water for a 210 L sample. These results suggest the large column (200 cc) system would be a practical method for recovering mutagens from

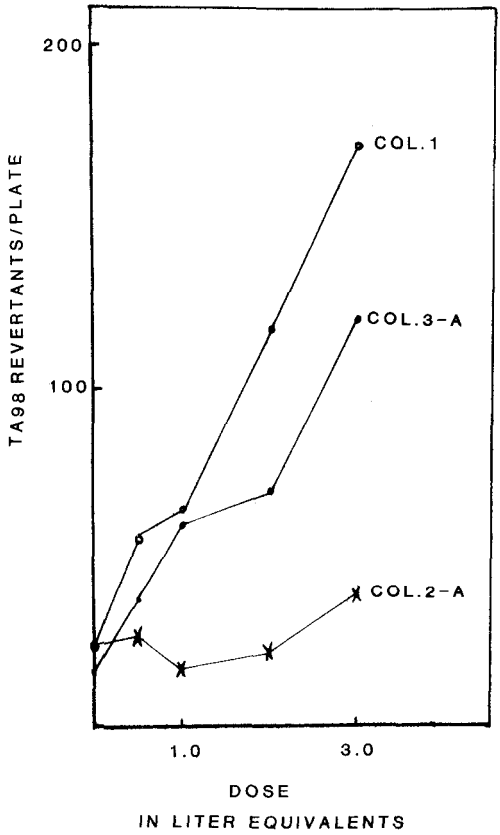

FIGURE 6(a) Direct-acting TA98 mutagenic activity of residue organics eluted from XAD columns 1, 2A and 3A, configured as shown in Figure 5, after the processing of drinking water. Column 1 received a total of 210 L, column 2A received the first 30 L previously passed through column 1, and column 3A received 30 L.

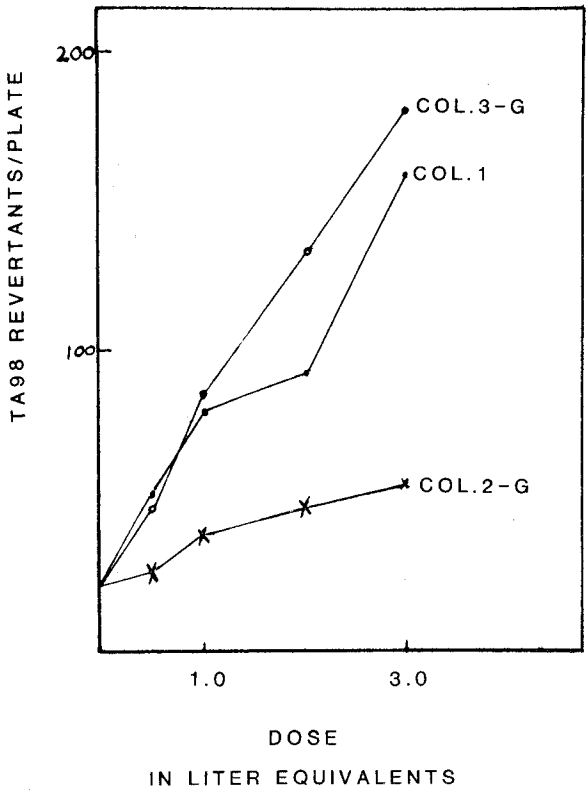

Figure 6(b) Direct-acting TA98 mutagenic activity of residue organics eluted from XAD columns 1, 2G and 3G, configures as shown in Figure 5, after the processing of drinking water. Column 1 received a total of 210 L, column 2G received the final 30 L previously passed through Column 1, and Column 3G received 30 L.

volumes of up to 1680 L of similar drinking water. The original design of the system was based upon a similar capacity limit of 1500 L of drinking water. The 25 cc columns have been used to extract a series of 27 L samples of <3 ppm water.[52]

Experiments to evaluate the exhaustion of the resins in these concentration columns for waters high in total organic carbon, e.g. wastewaters, have not been conducted. However, the 200 cc system has been used in the recovery of mutagenic residue organics from

20 L to 40 of wastewaters containing a total organic carbon of >20 ppm.[53]

With the information from these preliminary experiments, the next step was to apply the collection/concentration system to preparative scale isolation of residue organics samples from current drinking water.

Use of the residue collection system for current drinking waters

The combined XAD-2/XAD-7 residue collection system for large sample volumes was applied to the concentration of nonvolatile organics from the drinking water of three U.S. supplies: two with characteristically different surface water sources and one ground water source. Samples of 70 to 1250 L were concentrated in these studies and the isolated residue organics were tested for mutagenic activity using both TA98 and TA100 tester strains minus and plus S9.

The mutagenesis results for drinking water residue organics from a series of grab samples taken from Source I, that uses an industrial river as a water source, are summarized in Table III. All samples contained direct acting mutagens for both TA98 and TA100; muta-

TABLE III

Mutagenesis of drinking water residue organics isolated from Source I using large volume (200 cc) system.

	Net revertants/liter equivalent			
	TA98		TA100	
Date	−S9	+S9	−S9	+S9
Aug. '81	100	32[a]	260	250
Sept. '81	80	36[a]	380	130[a]
Nov. '81	140	42	450	140[a]
Feb. '82	380	300	500	200
Apr. '82	52	35[a]	220	95

[a]Not significant. Does not equal nor exceed a doubling above spontaneous reversion rate.

genic activities were decreased by microsomal metabolism. This pattern is typical of many other drinking water samples.[5] The range of specific mutagenic activity, net revertant colonies per liter equivalent dose, was from 4 to 31 times the spontaneous background for TA98, and from 2 to 5 times the spontaneous background for TA100. This would indicate that the use of a grab sample approach over a period will give an indication of trends of general contamination by mutagens in any one water source, but the use of only one or two samples to make specific statements as to the quality of a given water source could be misleading.

Results of bioassays for the residue organics from a series of drinking water samples from Source II, that uses a combined pristine river/stream source, are summarized in Table IV. The finished drinking water from this source, like the one described above, has been chlorinated to 2 to 4 mg/L but the total organic carbon usually is about $\frac{1}{2}$(1 to 2 ppm) that of the first source. The mutagenesis in the seasonal grab samples from the second source generally was lower for TA100 than for the first source.

The results for a series of drinking water samples from Source III, ground water, are summarized in Table V. In general, the level of mutagenesis for the residue organics was lower than that found for either Source I or Source II. Since the aquifier from which the

TABLE IV

Mutagenesis of drinking water residue organics isolated from Source II using large volume (200 cc) system.

	Net revertants/liter equivalent			
	TA98		TA100	
Date	−S9	+S9	−S9	+S9
Fall '80	90	40	300	90[a]
Winter '81	100	—[b]	250	—
Spring '81	70	15	140[a]	—
Summer '81	235	90	150[a]	80[a]

[a]Not significant. Does not equal nor exceed a doubling above spontaneous reversion rate.

[b]Same as spontaneous rate.

TABLE V

Mutagenesis of drinking water residue organics isolated from Source III using large volume (200 cc) system.

	Net revertants/liter equivalent			
	TA98		TA100	
Date	−S9	+S9	−S9	+S9
Winter '82	99	45	341	61[a]
Spring '82	52	33	147[a]	27[a]
Summer '82	17[a]	11[a]	63[a]	17[a]

[a]Not significant. Does not equal nor exceed a doubling above spontaneous reversion rate.

ground water is drawn is recharged principally from a river impacted by industrial discharges, residue organics for mutagenesis assays also were isolated from a 60 L sample of river water. The assay results showed a level of TA98 mutagenesis for the residue organics from the river water, Figure 7(a), which was lower than for the residue organics from the finished drinking water, Figure 7(b). No TA100 mutagenesis was observed for the residue organics from the river water, Figure 7(a), but direct-acting TA100 mutagenesis was measured for the drinking water residue organics, Figure 7(b). Appearance of TA98 mutagenesis from river water prior to chlorination has been observed for other industrially impacted rivers.[22,23] Moreover, the mutagenesis pattern for the finished drinking water, Figure 7(b), is consistent with that seen for other chlorinated drinking water samples.[5,6]

Inactivation of direct-acting TA100 mutagens by NTP

Cheh and Carlson[35] reported studies on the use of 4-nitrothiophenal (NTP) as a trapping reagent for the inactivation of direct-acting mutagens. These studies were based on the hypothesis that most direct-acting mutagens are strong electrophiles and exhibit their mutagenic properties by reacting with nucleophilic sites of the cellular DNA. Therefore, the reaction of a strong nucleophile with

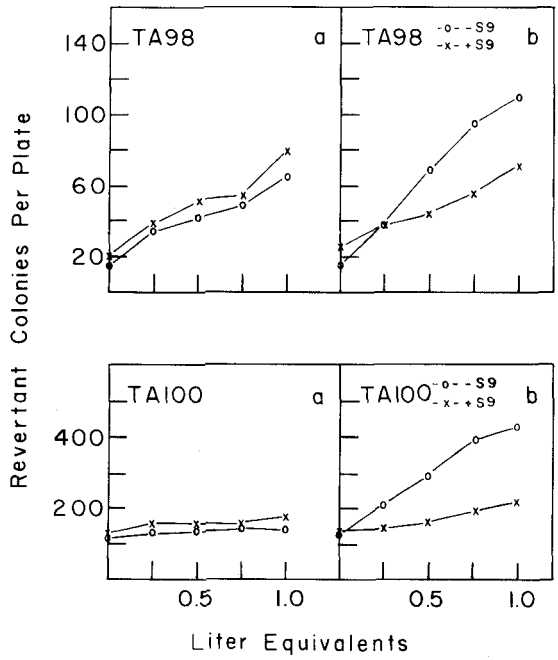

FIGURE 7 Mutagenic activity of residue organics eluted from XAD resins after processing samples of a river-recharge aquifer system: (a) river water; (b) finished drinking water from ground water (Source III). Mutagenic assays were conducted in the absence (−S9) and presence (+S9) of metabolic activation.

mutagenic components in complex mixtures of residue organics would allow for the quantitation of the relative potency of the direct-acting electrophilic mutagens in such mixtures. Also the nucleophile would serve as a trapping agent to stabilize these mutagens for further isolation/identification studies. In order to assess the relative potency of the direct-acting mutagens in our samples of drinking water residue organics, reactions of these samples with NTP were conducted. The results of these experiments for comparable amounts of sample from the three sources are shown in Figure 8. Bioassays were conducted with TA100 NR, a tester strain similar to TA100 but genetically deficient in nitroreductase activity. If present, this enzyme would reduce the nitro group on the NTP to reductive intermediates, e.g. a nitroso compound, thereby producing possible

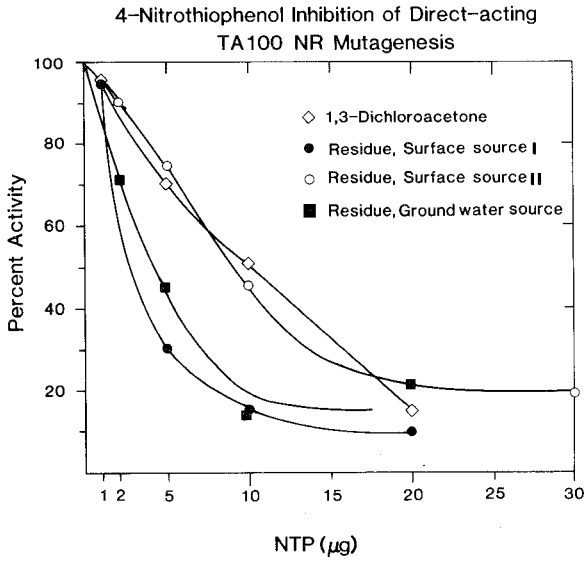

FIGURE 8 Sensitivity of mutagens in residue from different water samples to inactivation by 4-nitrothrophenol (NTP).

mutagenic by-products from the NTP. 1,3-Dichloroacetone, a direct-acting TA100 mutagen, was used as a control. As can be seen, all three sources of residue organics were inactivated by >80% by NTP. However, the mutagenicity of the residue organics from the ground water source (Source III) and from Source I, Figure 8, was trapped as NTP-mutagen adducts at lower levels of the reagent NTP than was required to inactivate the TA100 mutagen in the sample from Source II. These results indicate that the TA100 mutagens in these samples from the ground water source and Source I more readily react with NTP, and suggest that they may be more potent mutagens than those in Source II.

HPLC separation of residue organics from current drinking water

Having isolated mutagenic residue organics from three diverse types of drinking water, a series of experiments were conducted to further

characterize these mixtures as to their complexity and the chemical nature of the mutagens. The mixtures of residue organics were separated via preparative scale reverse phase HPLC using sequential isocratic and gradient step solvent elutions.[26] Eluates were collected in fractions which were bioassayed for both TA98 and TA100 mutagenesis.

Representative chromatograms for the HPLC separation of residue organics from Source I and Source III are shown in Figures 9 and 10, respectively. In general, the distribution of 254 nm absorbing components indicates the mixtures of residue organics from the industrially impacted drinking water, Source I, and the ground water source of drinking water, Source III, contain a variety of organics that range from polar to nonpolar. However, the distribution of direct-acting mutagens, Figures 11 and 12, appears predominately in the nonpolar fractions which elute at a solvent mixture of 40% water:60% acetonitrile, fractions 3 and C in Figures 9 and 10 respectively.

Mutagenesis for the four fractions isolated from the residue organics of each source was examined more closely. For Source I, the sum of the mutagenesis found in each fraction was considerably greater than the mutagenesis of that found for the mixture of residue organics added to the HPLC for separation, Figure 11. However, for Source III, the sum of the mutagenesis for individual fractions

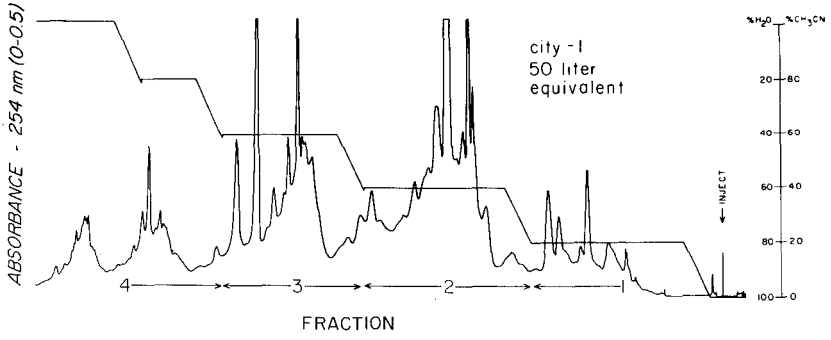

FIGURE 9 Reverse phase HPLC chromatogram of residue organics isolated from drinking water from Source I. Fractions were collected as indicated for mutagenesis bioassays.

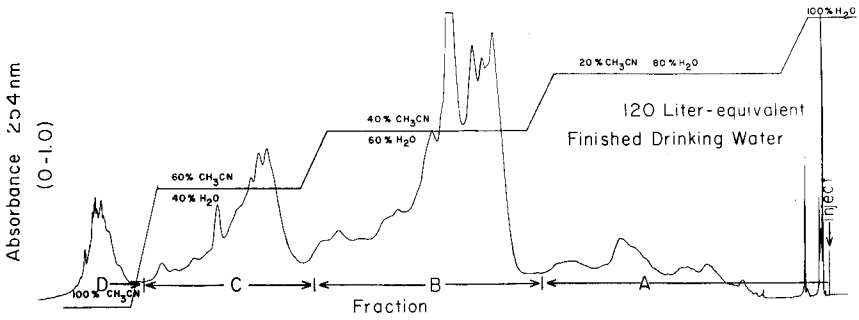

FIGURE 10 Reverse phase HPLC chromatogram of residue organics isolated from drinking water from Source III. Fractions were collected as indicated for mutagenesis bioassays.

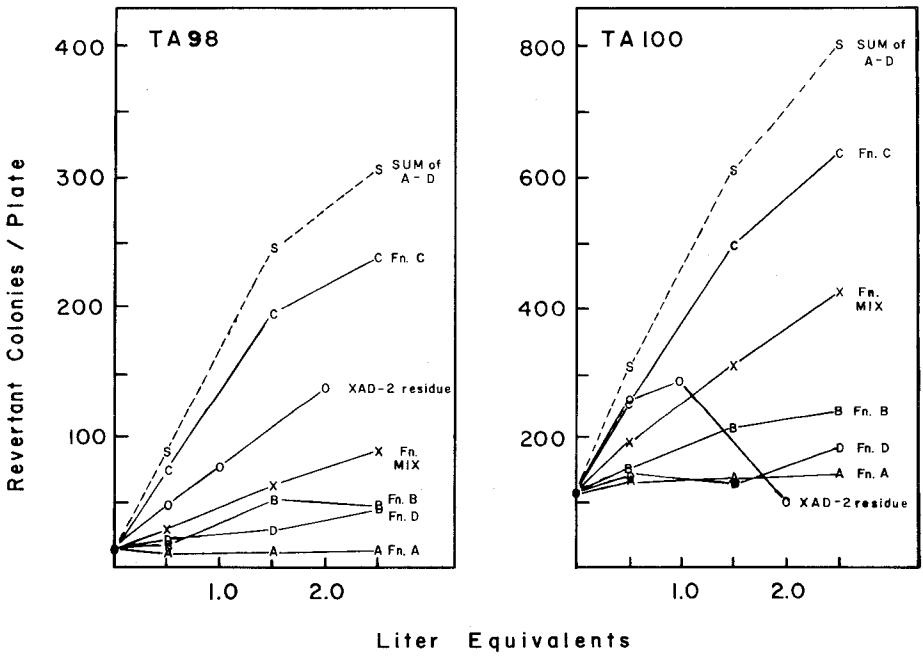

FIGURE 11 Direct-acting mutagenic activity of HPLC fractions (1–4, Figure 9 and shown as Fn. A–D herein) obtain by sequential step elution of drinking water residue organics isolated from Source I. "Sum" is the arithmetic sum of the net revertant colonies for each dose for the four fractions; "Fn. MIX" indicates the mutagenic activity measured for an aliquot of residue organics proportionately reconstituted from the separate fractions; "XAD-2 residue" indicate the mutagenic activity measured on an aliquot of the residue organics injected into the HPLC unit for separation.

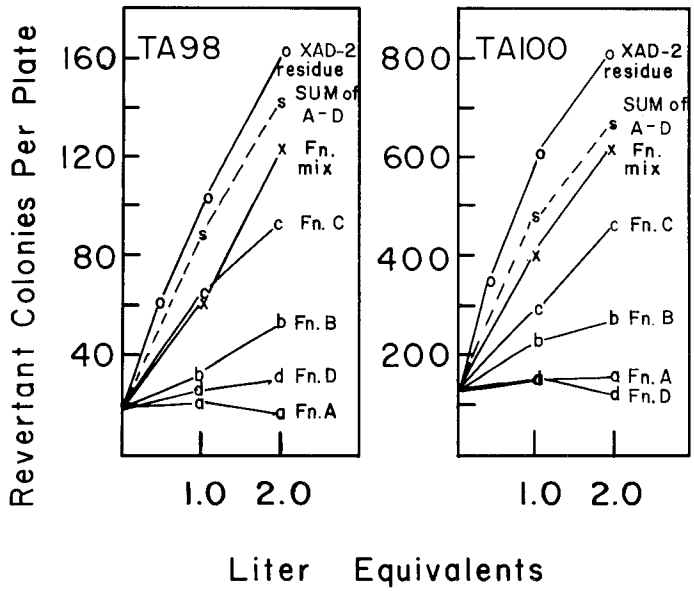

FIGURE 12 Direct-acting mutagenic activity of HPLC fractions (A–D, Figure 10) obtained by sequential step elution of drinking water residue organics isolated from Source III. "XAD-2 residue" indicates the mutagenic activity measured on an aliquot of the residue organics injected into the HPLC unit for separation. "Fn. Mix" indicates the mutagenic activity measured for an aliquot of residue organics proportionately reconstituted from the separate fractions; "Sum" is the arithmetic sum of the net revertant colonies for each dose for the four fractions.

closely approximated that found for the mixture of residue organics added to the HPLC for separation, Figure 12. These results indicate the possibility of toxic compounds or antagonists to mutagenicity in the residue organics from Source I drinking water that are separated from the mutagens via HPLC. This was not the case for the residue organics from Source III. Such results illustrate the importance of the separation of mutagenic mixtures of residue organics in order to better assess the mutagenic potency of such mixtures.

Similar studies of the residue organics from Source II revealed the presence of a potent TA98 direct-acting mutagen in the nonpolar region of components separated via reverse phase HPLC. Application of the coupled bioassay/analytical fractionation procedure[26] to this fraction resulted in the isolation of a component that has

been examined by high-resolution mass spectrometry.[15] The indication was that this mutagenic compound is a previously unidentified substance.

SUMMARY AND CONCLUSIONS

Studies have been conducted to develop a comprehensive approach for the isolation of residue organics from drinking water and the separation of mutagens from these mixtures for compound identification. A methodology for coupled analytical fractionation/bioassay of such mixtures has been reported[26,27] and validated[15,32] in that previously unidentified mutagens have been isolated and characterized.

In the current phase of these studies, methodology for the isolation of residue organics from drinking waters purified from a variety of sources has been detailed. The system developed for this isolation is portable, convenient to use and is readily constructed from widely available materials. The chromatographic resins employed to isolate the residue organics have been shown to concentrate a variety of mutagens from different types of drinking water.

Upon reverse phase HPLC, these mutagens are recovered primarily among the mid to nonpolar fractions. Such compounds may include significant mutagens in that Yamasaki and Ames[50] noted that organic compounds, expected to exert biological effects and capable of passing through biological membranes, are most probably lipophilic compounds of low polarity. Recently Neal presented[54] the same argument, adding that the toxic components were likely to be <500 molecular weight. He has proposed the first priority of compounds for isolation should be lipid soluble in this molecular weight range which are present at concentrations $<1\ \mu g/L$.

The methodology described in this paper addresses such lipid soluble compounds as mutagens. Our studies have provided an economical method which isolates mixtures of residue organics. The combination of Salmonella mutagenesis assays and HPLC chemical fractionation offers a feasible first approach to the isolation of mutagens and potential carcinogens from these mixtures. Such isolated compounds could then be subjected to toxicological evaluation for determination as to their potential adverse health effects.

Where warranted, the complex mixtures and their HPLC subractions also can be examined using other tests of *in vitro* and whole animal toxicity.

Acknowledgements

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