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ABSTRACT

Non-volatile mutagens resulting from the chlorination of freshwater were detected using the Ames/Salmonella/microsome assay with strain TA 100 in a non-liver activated system. Results indicated that dissolved organic material <2000 MW was primarily responsible for non-volatile mutagen formation. The level of detectable mutagenic activity (following chlorination)decayed rapidly with a half-life of 1-5 days.

INTRODUCTION

One of the various environmental factors which may cause genetic changes in humans is contamination of drinking water sources (1-5). Surveys by the U. S. Environmental Protection Agency have revealed various trace organohalide contaminants in domestic water supplies (6,7). Although in some cases such compounds may result from industrial pollution, it is now recognized that the water treatment process, primarily chlorination, results in the formation of a number of these compounds (8,9). Many of these products remain to be identified (8), but of those which have, many are considered to be mutagenic and/or carcinogenic (12,13). Haloform compounds have probably been studied the most (8), but the formation of numerous non-volatile organohalides resulting from water chlorination is becoming recognized as a widespread phenomenon (3, 8,10-12,14-16).

At the Savannah River Plant, the possible buildup of non-volatile mutagens in a closed-loop cooling reservoir, Par Pond, prompted our studies. Cooling water from a production nuclear reactor is continuously cycled through the pond. In an attempt to keep down scum formation on the heat exchangers, water which passes through the heat exchangers is chlorinated daily to about 4 ppm (1.5 ppm total residual) for a one hour period. The purpose of this study was to determine the influence of the molecular weight of organic material in water on non-volatile mutagen formation during chlorination. The persistence of non-volatile mutagenic activity caused by water chlorination and the possible accumulation of mutagenic activity in Par Pond were also examined. The Salmonella microsome assay as described by Ames et al. (17) was used to detect mutagenic activity. Changes in the back mutation frequencies in the histidine operon of a specially modified bacterial strain are used to detect mutagenic compounds. Strain TA-100 in a non-liver activated system was used in this study. Earlier work (11,12,14,16) has shown that this strain is sensitive to non-volatile mutagens.

METHODS AND MATERIALS

Molecular Weight Fractionation

Twenty-five liters of raw, unfiltered water from Par Pond was centrifuged at 15,000 rpm (27750 xG) in a Sorvall® (trademark of E. I. du Pont de Nemours & Co., USA) continuous centrifuge at a flow rate of 100 mL/min. Microscopic counts by epifluorescence miscroscopy (18) of the supernatant showed a 70 to 90% reduction in bacteria (>0.2μm^3 in volume), which corresponded to a decrease in dry weight of about 75% in particulates which were $\geq$0.45 µm in diameter. The water was fractionated further by Amicon hollow fiber ultrafiltration membranes (Amicon Corp., Lexington, Mass., USA) with nominal molecular weight cutoffs of 100,000 MW (H1P 100) and 2000 MW (H1P 2). Twenty-five liters of supernatant were passed first through the H1P 100 and then through the H1P 2. The scheme outlined resulted in four fractions: (1) particulate, (2) $\geq$ 100,000 MW, (3) $\leq$ 100,000 MW and > 2000 MW, (4) $\leq$ 2000 MW. All fractions were stored at 4°C until chlorination.

Chlorination, Adsorption, and Concentration

Fractions 1, 2, and 3 were diluted to one liter in chlorine-demand-free water (CDFW). Fraction 4 remained in the original water volume (25 L). Each of the fractions were chlorinated to 4 ppm by adding 4.5% sodium hypochlorite solution. The fractions were chlorinated at room temperature at a pH of 7-8 for about 2 hours. To stop the reaction, a 1% solution of ferrous sulfate (freshly prepared) was added. The pH was reduced to less than 3 to prevent ferric precipitates. Reduced pH also resulted in better recovery of non-volatile mutagenic activity in the extracts. Following chlorination, the samples were run by gravity flow, first through a cotton wool prefilter and then through a 50 cc column of XAD-2®, 20 to 50 mesh resin (Rohm & Haas, Phila., PA, USA) at 40 to 50 mL/min. The resins were previously cleaned by methods of Junk et al. (20). Their methods were modified by substituting acetone for acetonitrile.

The XAD-2 columns were then dewatered using a stream of nitrogen gas and the adsorbed material was eluted from the columns with 20 mL of acetone followed by 100 mL of diethyl ether. This resulted in a two phase extract. The column was re-extracted with an additional 50 ml of ether, and the two ether extracts (the ether phase from the first extract plus the second ether extract) were combined and brought to dryness under N_2 at reduced temperature. The resulting material was then redissolved in 1 mL of dimethylsulfoxide (DMSO) for use in the Ames assay (17). Containers and prefilters were also extracted with acetone and ether to ensure that activity was not being lost by adsorption.

Sample Collection and Treatments

Twenty-five liters of water were sampled biweekly at three locations along the temperature gradient in Par Pond. Extraction and concentration procedures were carried out as described above. Water used for the mutagen persistence experiments was taken from Upper Three Runs Creek, a thermally unaltered, black-water stream located on the SRP site, or from the ambient temperature end of Par Pond. For the mutagen persistence experiments, water was held in 50-liter polyethylene carboys. Hypochlorite was added to produce a chlorine concentration of 4 ppm. Water (with temperature and pH conditions as described earlier) was then allowed to stand in the carboys exposed to the atmosphere. Residual chlorine was undetectable within 48 hours in all cases. Water was sampled periodically for about 2 weeks, and extraction and concentration of non-volatile mutagens were done using XAD-2 as described above.

Mutagenic Activity

The mutagens were tested following the procedures of Ames et al. (17). Strain TA-100 was grown overnight at 37°C in nutrient broth (Oxoid, Basingstoke, England). All testing was done on plastic petri dishes containing 25 mL of glucose-mineral salts medium (7.0 g K_2HPO_4, 3.0 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.5 g $Na_2C_6H_5O_7$, 1.0 g $(NH_4)_2SO_4$, 15 g agar, and 20 g glucose in 1 liter of solution). The mineral salts agar was overlaid with 2 mL of top agar (5 g NaCl and 6 g agar in 1 liter of solution). Extracts dissolved in DMSO were added to the molten top agar in volumes ranging from 5 to 200 µL. All tests were done in duplicate. Sodium azide (2 µg/plate) and methyl methane sulfonate (0.7 µg and 1.4 µg/plate) were used as positive controls for strain TA-100 (17,19), and strain markers were routinely checked. Non-chlorinated water samples and distilled water controls were checked periodically to ensure that the reagents and procedures used did not induce mutagenic activity in the extracts. A response of twice the background reversion rate or greater was considered to be significant (17).

RESULTS

Molecular Weight vs. Mutagen Formation

Four series of experiments were conducted to investigate which molecular weight fractions when chlorinated were important in non-volatile mutagen formation (Figure 1). The low molecular weight fraction (<2000 MW) showed the highest specific mutagenic activity (reversion rate/volume equivalents added). The higher molecular weight fractions showed some activity, but only at much higher volumes (Table 1). The results consistently showed that chlorination of the <2000 MW fraction resulted in the highest specific mutagenic activity. Results from Experiments 1 and 4 (Figure 1 and Table 1) also showed that the majority of the mutagenic activity which resulted from chlorination of the unfractionated, raw water could be accounted

TABLE 1

Results of Mutagenicity Tests Using TA-100 in a Non-Liver Activated System with Concentrates from Different Molecular Weight Fractions

Exp. No.	FRACTION	2 Particulates	1 >100,000	5 <100,000 >2,000	4 <2,000	Whole Water	SR	Azide 10 µg	MMS 0.1 µg
1	XBKG / Vol	5.5 / 2.6	1.6 / 2.2	2.2 / 2.3	6.4 / 0.625	9.8 / 0.625	88	634	740
	mg C/L	1.2	0.8	0.3	15.3	17.2			
2	XBKG / Vol	5.8 / 5.0	2.8 / 5.0	3.0 / 2.5	3.4 / 0.34	ND	80	426	712
	mg C/L	0.6	0.7	0.8	7.2	9.2			
3	XBKG / Vol	2.1 / 5.0	2.4 / 5.0	1.6 / 5.0	4.3 / 0.8	ND	96	395	738
	mg C/L	0.7	0.8	0.5	4.1	5.8			
4	XBKG / Vol	1.3 / 5.0	1.5 / 5.0	4.7 / 5.0	9.1 / 0.95	8.8 / 1.25	97	555	ND
	mg C/L	0.9	0.9	0.6	5.1	7.5			

XBKG = level above background = $\frac{\text{colony number on experimental plate at peak of response curve}}{\text{colony number on background plate}}$

Vol. = volume (L) of water to which the extract added was theoretically equivalent.

mg C/L = total organic carbon concentration (normalized to original volume in each fraction).

SR = standard revertants = colony number on background plate.

Azide = X BKG on azide control plate.

MMS = X BKG on methyl methane sulfonate plate.

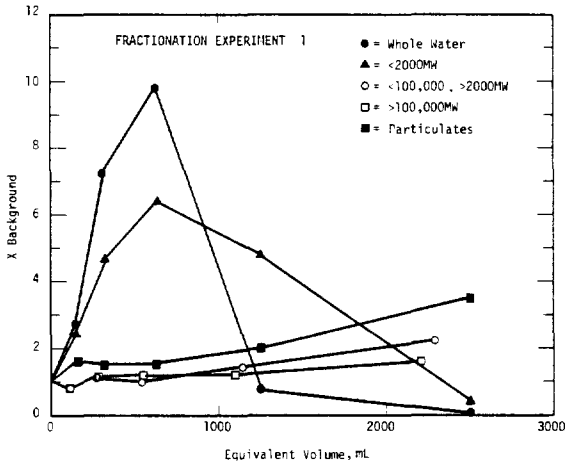

FIG. 1 Mutagenic activity in concentrates from raw water and different molecular weight fractions following chlorination.

X Background = $\frac{\text{colonies on test plate}}{\text{colonies on control plate}}$

Equivalent volume = volume of water to which the concentrate was theoretically equivalent.

for in the activity resulting from chlorination of the <2000 MW fraction. The graphs of colony number versus equivalent volume show that both the <2000 MW fraction and the raw water reached a maximum at intermediate equivalent volumes (Figure 1). The decrease in colony numbers and in the background lawn at equivalent volumes greater than about 1 liter is indicative of toxicity in both raw water and the <2000 MW fraction (17). Curves from the other fractions did not show such toxicity.

Although the higher molecular weight fractions occasionally did show significant increases in reversion rates, this response was noted only at the higher volumes (Figure 1, Table 1). The activity may have been due to retention of some <2000 MW material in the higher molecular weight fractions. The activity may, however, indicate non-volatile mutagen formation by the high molecular weight material. However, the toxicity of the chlorinated <2000 MW fraction would generally mask this contribution to the mutagen load in the whole water sample (Figure 1).

Organic carbon measurements (Table 1) indicated that the <2000MW fraction contained more than half of the carbon in all experiments, ranging from 68 to 89 % of the total organic carbon in the raw water. Organic carbon in any of the other three fractions was less than one-fifth of that in the <2000MW fraction. This observation may partially explain why the low molecular weight fraction was so active in non-volatile mutagen formation.

Par Pond Sampling and Mutagen Persistence

Par Pond was sampled for 5 months, including 3 months when the reactor was not operating. During that 3 months, water was cycled and chlorinated, but it was not heated. Reactor operation was normal for the remaining 2 months. Although mutagenic activity could be detected in the heat exchanger effluent during the daily chlorination period, no significant mutagenic activity was ever detected in Par Pond waters.

The mutagenic activity resulting from chlorination was found to be moderately persistent under the conditions used. Three experiments were done. In two experiments, water from the ambient temperature end of Par Pond was used, while in the third experiment, we used water from Upper Three Runs Creek, a small black-water stream. Figures 2 and 3 show that the slope (reversion rate versus volume) of the straight line portion of the response curve continually decreased with time. Thus, the amount of detectable non-volatile mutagenic activity per volume of water was decreasing. The toxic effects of the extracts also appeared to decrease with time. With increasing time, the colony numbers at the higher volumes increased to 1-2 times background, indicating better survival on the plates. In all cases, the slopes were approaching zero after two weeks (Figure 2), indicating complete loss of detectable mutagenic activity.

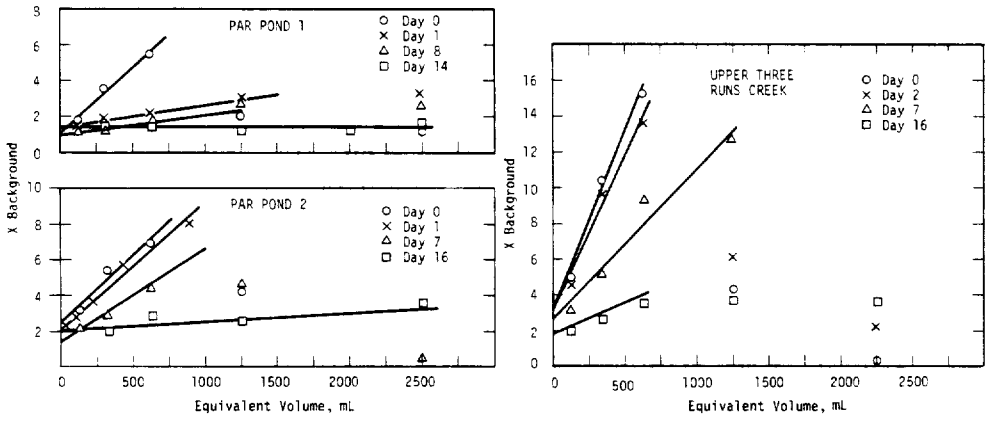

FIG. 2 Changes in mutagenic activity over time in concentrates from raw water which had received a single chlorine dose on day 0.

"X Background" and "Equivalent Volume" are defined in legend of Fig. 1.

Curves A and B in Figure 3 show a rather constant decrease in the slope value with time after chlorination, while curve C shows a rapid initial drop, followed by a continuing slow decline. Curves B and C are from the ambient temperature end of Par Pond at different times. The experimental temperature was about 10°C higher and the total organic carbon about twice as high in experiment C as compared to experiment B. These differences or other unrecognized variables most likely contributed to the different responses on the two experiments.

DISCUSSION

Our experiments support other investigations which show that water chlorination leads to the formation of non-volatile mutagenic compounds which give a positive Ames test (15,16). Our findings are significant because we found mutagen formation in water with relatively low total organic carbon concentrations (5 to 20 mg C/L). Much of the previous work which identified non-volatile mutagen formation caused by chlorination was done in waters with 5 to 100 times higher organic carbon concentrations (15,16,21).

The abundant, low molecular weight organic compounds are shown to be most important in non-volatile mutagen formation during chlorination. Recently, Schnoor et al. (10) showed that dissolved organic compounds of ≤3000 MW are most important in trihalomethane formation in Iowa River water. Thus, it appears that the low molecular weight organic compounds, which often make up a majority of the organic material in fresh waters (22,23), are the primary precursors to both volatile and non-volatile mutagens caused by chlorination. A great deal of further work will be required to identify which of these, if any, are the compounds responsible for the detected mutagenic activity.

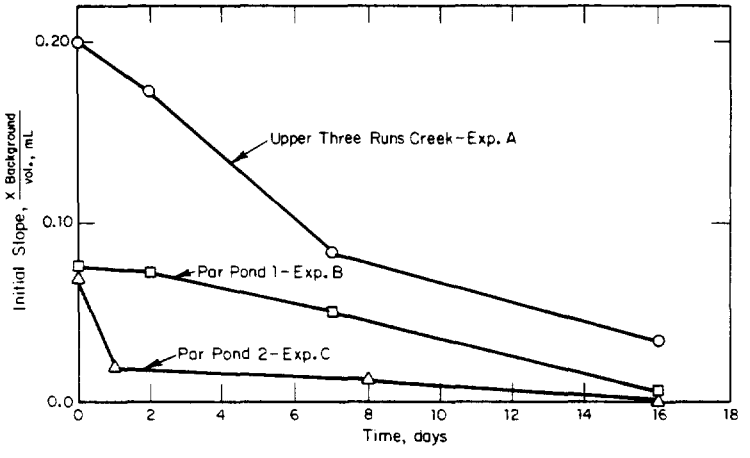

FIG. 3 Changes over time in the initial slope of the response curves shown in Fig. 2. Slope value is based on a least squares best fit of the initial, linear portion of the curves.

Chlorination appeared to increase the toxicity present in concentrated extracts. Although mutagens are generally toxic (24), it is not known whether in these experiments increased toxicity caused by chlorination results solely from mutagenic compounds or whether other toxic products were also created. The toxicity problem will make interpretation of response curves resulting from raw water difficult. Usually, response peaks at intermediate volumes of concentrates. Should the amount of toxicity relative to mutagenicity vary from one sampling to another, the shape of the response curve will change.

The results of the persistence experiments explain the lack of detectable activity in Par Pond. During reactor operation, Par Pond has a turnover time of about 13 days and is chlorinated daily for one hour. Therefore, it would take 312 days for all water to be exposed to full chlorine dosages. However, non-volatile mutagenic activity persisted only about 2 weeks in water from the pond. Thus, activity is diluted below detectability in the pond and does not persist long enough to accumulate and become detectable.

Although no significant accumulation occurred under the conditions described in Par Pond, the mutagenic activity appears to be moderately persistent; therefore, accumulation to significant levels in water supplies may be possible. The persistence of non-volatile mutagenic activity demonstrated here may represent a maximum value since biological activity was probably minimized by the experimental design and, under normal conditions, such activity may contribute to a more rapid rate of decline. However, considering that oxidizing chlorine compounds are the most commonly used water treatment practice for industrial cooling systems in the U.S. (25), areas where water reuse is high may have accumulation of non-volatile mutagenics in drinking water sources.

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