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Ames *Salmonella* Mutagenicity Assays on Water: Dichloromethane Extracts versus Preparation of Growth Media with Test Samples

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A variety of procedures have been described for the preparation of samples in Ames *Salmonella*/microsome mutagenicity assays on water. Many of the techniques are aimed at concentrating mutagens from large volumes of water. Among these are liquid-liquid extraction, resin adsorption, flash evaporation and membrane filtration (DUTKA *et al.* 1981, GRABOW *et al.* 1981). SAXENA & SCHWARTZ (1979) followed a different approach and used a direct test method in which the water under investigation was decontaminated by membrane filtration and then used to prepare the bottom agar layer for plate incorporation assays. Tester strains with or without S9 liver homogenate were incorporated in the top layer. This procedure made it possible to incorporate up to 20 mL of test sample in each plate.

The different techniques for the preparation of test samples all have their own advantages and disadvantages (SAXENA & SCHWARTZ 1979, GRABOW *et al.* 1980, 1981, DUTKA *et al.* 1981). This paper describes a comparison of a direct test procedure resembling that of SAXENA & SCHWARTZ (1979) to testing dichloromethane liquid-liquid extracts. The latter proved to represent one of the most successful concentration techniques (DUTKA *et al.* 1981, GRABOW *et al.* 1981). The results are essential for comparing data reported in different studies, the selection of appropriate techniques for specific purposes, and the development of standard procedures.

MATERIALS AND METHODS

Samples. The laboratory tap water, and settled sewage, activated sludge and biofilter effluents of the Daspoort wastewater purification works in Pretoria have been described (GRABOW *et al.* 1980, 1981). Grab samples were processed within 4 h after collection.

Liquid-liquid extraction. Dichloromethane liquid-liquid extraction at neutral, acidic and basic pH levels was done as described by GRABOW *et al.* (1981). The extracts of 20 L samples of settled sewage and secondary treated wastewater, and 100 L tap water, were all evaporated to dryness and resuspended in 5 mL dimethyl sulfoxide (DMSO) for plate incorporation assays (AMES *et al.* 1975, GRABOW *et al.* 1981). All extracts were tested undiluted and in DMSO dilutions of 8:10, 6:10, 4:10 and 2:10.

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Direct testing of water samples. Test samples were used directly to prepare the glucose and salt solutions required for the bottom agar layer in plate incorporation assays. The two solutions were autoclaved separately (120°C/15 min), mixed, cooled to about 50°C and 20 mL quantities were poured into 90 mm diameter Petri dishes. The top layer containing tester strains TA98 or TA100 in the presence or absence of S9 liver preparation was prepared and added as described by AMES *et al.* (1975) and GRABOW *et al.* (1981). In some experiments the top layer was also prepared by directly using test samples. Some drinking-water samples were concentrated 2.75 times by reverse osmosis using an Ajax unit (Santa Barbara, California) with cellulose acetate spiral wrap modules, prior to the preparation of bottom layers. The concentration of histidine in water samples was evaluated by means of an amino acid analyzer and a growth test. The latter was performed by streaking *Salmonella* tester strains on bottom agar prepared from test samples without the addition of histidine. All tests were done at least in triplicate, and included positive, negative, toxicity and sterility controls (AMES *et al.* 1975, GRABOW *et al.* 1981). Negative controls in the direct test consisted of assays in which the bottom layer was prepared from distilled water instead of test sample.

RESULTS

Results of mutagenicity assays are expressed as the mutagenicity ratio (MR) which is the ratio of revertants on test plates (spontaneous + induced revertants) to those on negative control plates (spontaneous revertants). An MR of 2.0 or more is generally considered a statistically significant indication of mutagenic activity, provided all controls conform to specifications (AMES *et al.* 1975). Tester strain TA98 had 15 to 50 spontaneous revertants per plate and tester strain TA100, 120 to 200.

The sample of settled sewage collected on 1981-06-22 contained 0.52 mg/L histidine, and the samples of activated sludge and humus tank effluents collected on the same day contained 0.04 mg/L histidine. These concentrations are well below 1 mg/L which was found not to affect the spontaneous reversion rate of *Salmonella* tester strains (SAXENA & SCHWARTZ 1979). These data are in agreement with the results of growth tests which were negative for all samples tested.

The preparation of top layer from test samples did not have a significant effect on MR values, regardless of whether or not the bottom layer was also prepared from test samples. This is probably because the top layer has a volume of only 2 mL .

The results in Table 1 show that direct tests yielded positive results (MR of 2.0 or more) for all three settled sewage samples, one of three humus tank effluent samples, two of three activated sludge effluent samples, and two of five tap water samples, while reverse osmosis concentrates yielded significantly higher values than unconcentrated samples. There was no consistent pattern of

TABLE 1. Mutagenicity Ratios for a Variety of Waters Tested by the Direct Test Procedure.

Sample	Date	Mutagenicity Ratio (MR)			
		TA98		TA100	
		-S9	+S9	-S9	+S9
Settled sewage	81-06-15	3.1	1.9	2.3	2.4
	81-06-22	2.1	1.7	2.5	2.8
	81-06-28	1.9	1.8	2.0	1.8
Humus tank effluent	81-06-15	2.2	1.6	1.3	1.0
	81-06-22	1.6	1.1	1.8	1.5
	81-06-28	1.5	1.6	1.3	1.3
Activated sludge effluent	81-06-15	1.9	1.4	2.1	1.7
	81-06-22	1.4	1.2	1.7	1.6
	81-06-28	1.5	2.0	1.2	1.1
Tap water	81-06-15	1.6	1.9	1.9	1.7
	81-06-22	1.3	1.3	1.5	1.7
	81-06-28	1.2	2.1	1.6	1.7
	81-08-24	1.0	1.0	1.6	1.3
	81-09-07	1.7	1.5	2.0	2.9
Tap water x 2.75 (reverse osmosis)	81-08-24	1.0	1.0	2.0	1.9
	81-09-07	2.4	1.5	3.5	3.1

MR values for TA98 or TA100 in the presence or absence of S9 liver preparation for any of the waters.

The direct test method generally yielded higher MR values for settled sewage, and humus tank and activated sludge effluents than tests on dichloromethane extracts of homologous samples (Table 2). In the case of tap water, extracts yielded higher MR values but direct testing also yielded positive results for both samples. Extracts occasionally yielded no result because of toxic effects while such interference was never detected in direct tests. Dilutions of extracts never had toxic effects, but they never yielded higher MR values than undiluted extracts, or positive results for undiluted samples which were toxic. The results display no correlation between MR values of direct tests and tests on extracts for any of the tester strains in the presence or absence of liver preparations. However, an outstanding feature of the results is that TA100 generally yielded higher MR values for direct tests while TA98 generally had higher MR values for tests on extracts.

DISCUSSION

Compared to liquid-liquid extraction, the direct test method has various useful practical advantages. Among these are that much

TABLE 2. Mutagenicity Ratios for a Variety of Waters Tested by the Direct Test Procedure and Dichloromethane Extraction.

Sample and date		Mutagenicity Ratio (MR)			
		TA98		TA100	
		-S9	+S9	-S9	+S9
Settled sewage					
1981-11-16	Neutral extract	T	T	T	T
	Acidic extract	1.2	1.6	0.8	0.7
	Basic extract	1.0	1.5	-	-
	Direct test	3.3	3.2	3.5	2.6
1982-01-25	Neutral extract	1.5	1.2	0.9	1.0
	Acidic extract	1.7	1.1	1.1	0.7
	Basic extract	0.9	1.2	1.2	1.1
	Direct test	2.0	0.9	2.3	1.6
Humus tank effluent					
1981-11-16	Neutral extract	1.5	1.1	1.8	1.4
	Acidic extract	1.4	1.1	1.0	0.7
	Basic extract	0.8	1.3	-	-
	Direct test	2.3	2.1	2.4	1.3
1982-01-25	Neutral extract	1.3	1.0	0.9	0.9
	Acidic extract	1.3	0.7	0.9	0.8
	Basic extract	1.2	1.2	1.5	1.5
	Direct test	2.1	1.6	1.6	1.4
Activated sludge effluent					
1981-11-16	Neutral extract	1.0	1.7	T	2.3
	Acidic extract	1.3	1.4	1.0	0.7
	Basic extract	0.8	1.3	-	-
	Direct test	2.1	2.9	2.8	1.5
1982-01-25	Neutral extract	1.4	1.6	1.2	1.1
	Acidic extract	2.8	1.1	1.1	0.9
	Basic extract	1.1	1.1	1.1	1.2
	Direct test	2.3	1.6	2.1	1.6
Tap water					
1981-11-02	Neutral extract	2.2	1.1	-	1.1
	Acidic extract	4.7	2.4	1.7	1.3
	Basic extract	1.0	1.1	0.9	0.9
	Direct test	2.0	1.2	2.8	1.7
1981-11-09	Neutral extract	2.8	2.1	1.4	1.4
	Acidic extract	7.9	3.2	1.7	1.1
	Basic extract	1.3	1.2	1.1	0.9
	Direct test	2.5	1.6	3.4	2.7

- = not done; T = toxic.

smaller volumes of test samples are required, and that time-consuming, expensive and complex extraction procedures are eliminated. In the case of settled sewage and secondary treated wastewater the direct test method proved even more sensitive than tests on extracts because it yielded positive results more often and was not affected by toxic effects (Table 2). This phenomenon is probably due to the disadvantage of extraction procedures of also concentrating interfering toxicants. As can be expected, the sensitivity of the direct test method could not match that of tests on the highly concentrated extracts (concentration factor 2×10^4) of the tap water samples (Table 2). However, it is important that the direct test method yielded positive results for a high proportion of the tap water samples (Tables 1 and 2). No limits have as yet been adopted or proposed for mutagenic activity of drinking-water, and since the tap water concerned is known to be of excellent quality, including mutagenic activity (GRABOW *et al.* 1980, 1981), it may very well turn out that the sensitivity of the direct test method is sufficient to screen drinking-water supplies. Dose-response data can be obtained by appropriate dilution of test samples with distilled water.

The results in Tables 1 and 2 as well as data reported in earlier studies (GRABOW *et al.* 1980, 1981) indicate that dichloromethane extraction recovers frameshift mutagens more efficiently than base-pair substitution mutagens because MR values of extracts were generally higher for TA98 than for TA100. This tendency is not reflected by MR values of direct tests. It is of course known that the recovery efficiency of liquid-liquid extraction for highly polar organic mutagens and inorganic mutagens is low (GRABOW *et al.* 1980, 1981). In extraction procedures where extracts are evaporated to dryness and the dry extract resuspended in DMSO for Ames testing, as was done in this and other studies (GRABOW *et al.* 1980, 1981, DUTKA *et al.* 1981), volatile compounds are also lost. An important source of error in the direct test method is the autoclaving step. The high temperature inevitably results in the loss of volatile compounds and may even affect the chemical structure and activity of non-volatile mutagens. However, the results in Tables 1 and 2 indicate that surprisingly many mutagens withstand autoclaving and that the direct test method actually yields a more representative picture of mutagenic activity in water than tests on dichloromethane extracts. Membrane filtration of water samples for direct testing as was done by SAXENA & SCHWARTZ (1979) would not seem advisable because it probably removes a high proportion of mutagens (GRABOW *et al.* 1980, DUTKA *et al.* 1981). If it should prove necessary to concentrate water for direct testing, it would probably be best to use a non- selective procedure such as reverse osmosis (Table 1) or flash evaporation (DUTKA *et al.* 1981).

The fluctuating MR values and absence of a fixed pattern or correlation of MR values for TA98 and TA100 in the presence or absence of S9 liver preparation in both direct tests and tests on extracts (Tables 1 and 2), confirms earlier observations that the

content of mutagens in water is generally highly complex and fluctuates continually with regard to composition and concentration (GRABOW *et al.* 1980,1981, DUTKA *et al.* 1981). This implies that the routine screening of water for mutagenic activity should be done at high frequency by an assay system which covers a wide spectrum of mutagens. The direct test method would seem to be well suited for this purpose because it is simple, quick, economical, requires small volumes of test water, and limits selective detection of mutagens to a minimum.

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