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Evaluation of Four Concentration/Extraction Procedures on Waters and Effluents Collected for Use with the *Salmonella typhimurium* Screening Procedure for Mutagens

B. J. Dutka, A. Jova, and J. Brechin

Microbiology Laboratories Section, Analytical Methods Division, National Water Research Institute, Canada Centre for Inland Waters, Department of Environment, P.O. Box 5050, Burlington, Ontario, Canada, L7R 4A6

With the great influx of laboratories (over 1000) into mutagen screening activities (MAUGH 1978) and the great variety of extraction/concentration procedures available, there now exists the problems of standardization of methods and reproducibility of results. Several workers, TAHAGI et al. (1977) and NAGAO et al. (1977) have found that some mutagens such as dimethylnitrosamine and the pyrrolizidine alkaloids can not be properly detected by the standard Salmonella typhimurium mammalian microsome plate incorporation assay. They have found that by incubating the test chemical with the S-9 mix (microsomes) and bacterial culture for varying periods before adding the soft agar overlay they could enhance the sensitivity of the Salmonella typhimurium mutagen screening test.

In this study, four concentration/extraction procedures; solvent extraction, adsorption onto XAD resins, flash evaporation and membrane filtration, were compared for their ability to concentrate and extract potential mutagens from river, lake and effluent samples. In conjunction with these concentration procedures, three variations of the Salmonella typhimurium test were also assessed. Details of those studies are presented below.

MATERIALS AND METHODS

A total of 33 water samples were collected for mutagen content assessment. The sample collection sites are shown in Figure 1. The U.S. EPA procedure for the solvent extraction of organic compounds from effluent samples was used with the extractions being performed at both pH 2 and 11. Ten litres of sample were extracted to produce a base/neutral fraction and an acid (phenol) fraction, each in 25 mL of DMSO.

Flash evaporation by means of a rotary evaporator with a 45°C water bath was used to concentrate 600 mL of water or effluent to 60 mL for 10X concentration testing.

A fifty mL aliquot of sample was passed through a 0.20 micron Nalgene filter unit. The sterile filtrate was the 1X sample. The membrane filter was removed from the Nalgene holder, and dissolved in 5 mL of DMSO by just bringing to a boil three times in a microwave oven. The resultant suspension was tested for sterility

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before being tested for mutagenic activity. Sixty mL of 10X flash evaporated concentrate was passed through a 0.20 micron Nalgene filter unit. The sterile filtrate was the 10X concentrate for mutagen testing. The membrane filter was removed from the Nalgene holder and treated as above. Negative controls for the membrane filter studies were 0.2 μ Nalgene membrane filter units through which had been passed 50 mL of distilled water and the filter treated as above.

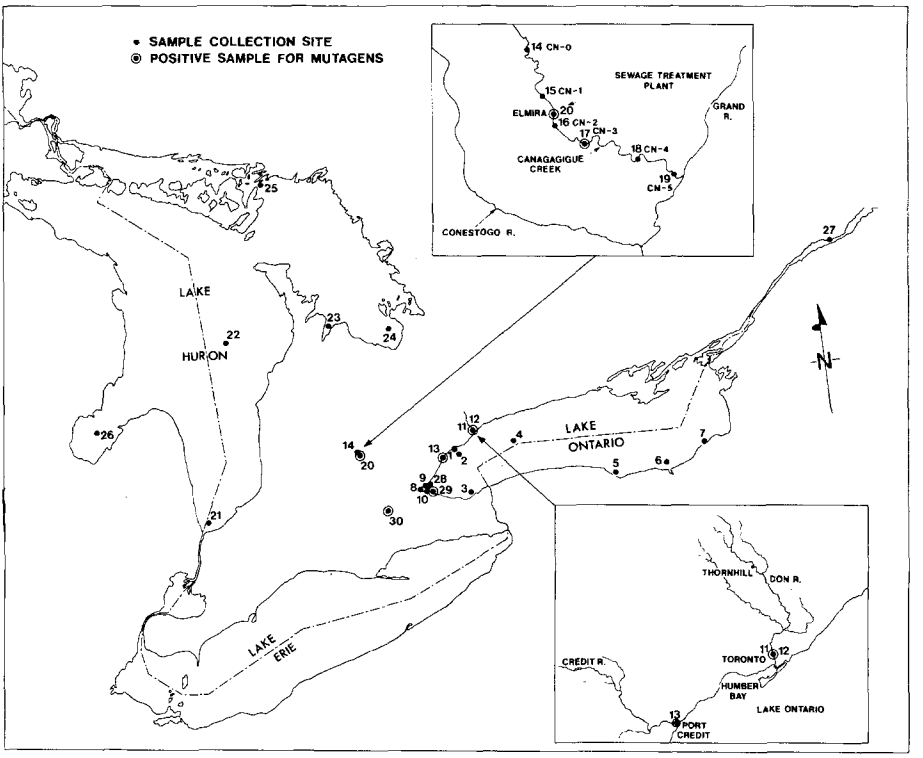

Figure 1. Site of Sample Collection for Mutagen Tests

Procedures were the same for XAD-2 and XAD-7 resins except where noted. The resins were purified by Soxhlet extraction and stored under methanol until required. Five L water samples were passed through the resin columns at a flow rate of 40 to 50 mL/min. Samples were adjusted to pH 1 to 2 with H_2SO_4 before passage through XAD-7.

The final washing of the XAD-2 resin was with benzene and the XAD-7 resin with petroleum ether. The extracts were evaporated to dryness in a rotary evaporator, dissolved in a small volume of DMSO and then made up to 25 mL in DMSO.

The Salmonella typhimurium plate incorporation assay for mutagen screening, outlined in "Methods for Microbiological Analysis of Waters, Wastewaters and Sediments" (1978) was used in this study. S. typhimurium strains TA98, TA100, TA1535, TA1537 and TA1538 were

used with positive and negative controls and (a) with the addition of rat liver microsomes (S-9 mix) (b) without the addition of S-9 mix and (c) with a 20 minute 37°C preincubation of sample, cells and S-9 mix before the addition of the soft top agar. For extracts in DMSO a minimum of three sample volumes, 10, 20 and 200 µL were tested with the 10 and 20 µL volumes being made up to 200 µL with DMSO. For unconcentrated samples, volumes up to 1 mL were used. All tests were done in triplicate and all suspected positives were repeated for confirmation. Positive and negative controls were included in each experiment.

RESULTS AND DISCUSSION

Table 1 is a summary of the samples showing mutagenic activity. This table also indicates the concentration/extraction procedures that produced the highest number of extracts showing mutagenic activity, as well as which strains of S. typhimurium reacted most frequently with the mutagens. In this study a positive test was one in which the number of revertants were at least double the negative and DMSO controls as well as being statistically significant at the 1.0% level. Typical dose response curves obtained from samples displaying mutagenic activity are shown in Figure 2. In no instance did any filtrates, 1X or 10X, display mutagenic activity, however, with the exception of sample 29, all samples in Table 1 showed mutagenic activity with either the 1X or 10X membrane filters dissolved in DMSO. In all instances the control membrane filters dissolved in DMSO produced negative results.

Several of the samples produced a mutagenic effect with the 1X DMSO dissolved membrane filter and were inturn negative with the 10X DMSO dissolved membrane filter solution an indication that the 10X solution may have been toxic to the tester strains. Dose responsive relationships could not be established for the DMSO-dissolved membrane filter solutions because 4.5 mL of the original 5.0 mL of each solution were used in the initial experiment.

Of the nine positive samples, only six were positive by the solvent extraction procedure. All six of these had mutagenically active base/neutral fractions and three also had mutagenically active acid fractions.

The XAD resin extracted samples showed mutagenic activity in only four of the nine positive samples, three XAD-2 extracts and two XAD-7 extracts with sample 20C being positive in both extracts.

Based on the samples tested in this study, the DMSO dissolved membrane filter concentration procedure was the most sensitive concentration procedure for mutagenic activity on two counts. One being that this procedure produced the highest number of positive results (Table 1) and the second being that the positive reactions were obtained from smaller volumes (0.9 mL and 9.0 mL) of original sample. In contrast, doses of solvent extracts equivalent to 60 to 80 mL of original sample and doses of XAD extracts equivalent to 40 mL of original sample were required, to produce a mutagenic

Table 1. Summary of Samples Showing Mutagenic Activity by One or More Concentration/Extraction Procedures

	Concentration Procedures					Salmonella strains and Microsome Treatment											
					Membrane												
Sample	Solvent		XAD Resin		Filter	TA98			TA1538			TA1537			TA1535		
No.	Acid	Base	2	7	1X 10X	-S9	+S9	INC*	-S9	+S9	INC	-S9	+S9	INC	-S9	+S9	INC
11		+					+		+	+			+				
11					+											+	
11					+												
12		+					+			+			+				
12					+					+							
13					+											+	
13					+					+							
17b				+		+			+								
17b					+		+										
20a	+						+			+							
20a		+					+			+							
20a					+					+							
20b	+									+							
20b		+					+			+							
20b			+				+			+	+						
20b					+	+	+			+							
20c	+									+							
20c		+					+			+							
20c			+				+			+	+						
20c				+		+	+			+							
20c					+				+	+							
29		+					+			+							
29			+								+						
30					+					+							
Total	3	6	3	2	6 4	3	12	0	3	19	3	0	2	0	1	1	0

* Salmonella cells + sample + S-9 mix incubated 20 min. at 37°C prior to addition of top agar.

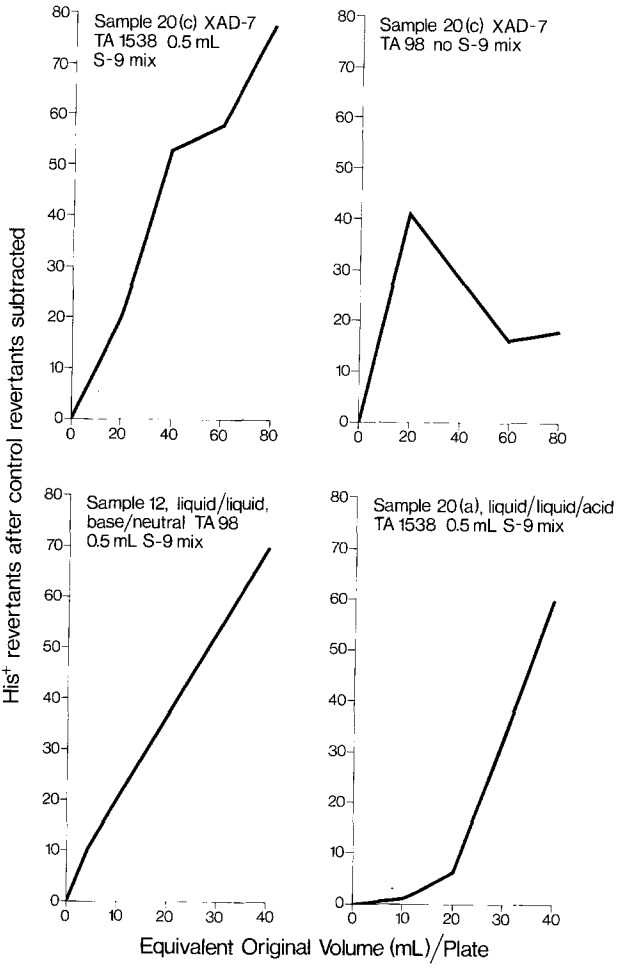

Figure 2. Typical Dose Response Curves

effect. It is probable that if 100 to 500 mL of original sample were membrane filter concentrated (assuming that this concentrate would be non toxic to the tester strains) the number of samples showing positive responses would increase, especially in those samples where slight increases in revertant rates were noted. One observation not detailed in this report was the finding that several of the XAD resin extracts were toxic to the tester strains at a dose equivalent to 40 mL of original sample. The highest was equivalent to 4 mL of original sample. Other, not so obvious toxic effects were observed by microscopic examination of the background bacterial lawn, in several instances, and almost entirely in samples concentrated by XAD resin procedures. For these samples the control revertant rates were usually 20 to 30% higher than the test rates e.g., Sample 24, XAD-7, 41 revertants versus 51 control revertants.

Of the nine samples showing mutagenic activity, seven were positive by strain TA98 and all nine by TA1538. Strain TA98 indicated mutagenic activity in 15 subsamples (Table 1) only three of which were positive without the addition of the S-9 mix. Strain TA1538 indicated mutagenic activity in 25 subsamples of which only 3 samples, were shown to have mutagenic activity when the cells, sample and S-9 mix were preincubated, indicating the presence of substances requiring the additional contact to produce a mutagenic effect. Conversly, it would also appear that in all other mutagenic samples in all strains, the microsomal enzymes were able, during the preincubation step to "detoxify" or inactivate the compound causing the increased rate in revertant colonies in the tester strains. Curiously, all positive results obtained with the preincubation procedure were with XAD-2 extracts, and these were the only positive extracts provided by the XAD-2 procedure. From these data it may be surmised that the XAD-2 resin was able to extract/concentrate some agent which none of the other procedures were able to do.

The data in Table 1 suggests that an investigator studying water and effluent samples from within the Great Lakes Basin, would be able to establish which of those samples are mutagenic in the S. typhimurium test by using strains TA98 nd TA1538 with the routine incorporation of S-9 mix. By using only two strains and routine incorporation of microsomes with triplicate replicates, the work load and expense could be reduced by over 60% per sample.

Table 1 data indicate that there is no single extraction/concentration technique that is uniquely sensitive to all potential mutagenic agents found in the samples tested. If a single concentration procedure had to be chosen, it would have to be the membrane filtration of 1X and 10X flash evaporated samples. The procedure is inexpensive, with $5.00 covering disposables and labour for 1X MF samples and approximately $10.00 for the 10X flash evaporated sample which is then membrane filtered. It is possible to make the membrane filter concentration procedure more effective by filtering a larger volume of sample but toxicity to tester strains may increase.

Based on the data in this study, a quick inexpensive screening procedure for mutagenic activity is available, as part of the battery approach, by using membrane filtered 10X flash evaporated samples and 1X natural samples combined with S. typhimurium tester strains TA98 and Ta1538 with the routine incorporation of S-9 mix. However, it is unrealistic at this stage of experimentation to expect that the above would satisfy all investigators. Therefore, we suggest that the solvent extraction procedure be used as well until sufficient data are collected to confirm the validity of the membrane filter concentration procedure.

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