AN INVESTIGATION OF THE TEMPORAL VARIATION OF MUTAGENIC ACTIVITY IN DRINKING WATER

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November 1987

RESTRICTION:

PERMISSION: This work was funded by the Department of the Environment whose permission to publish has been obtained

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Drinking water samples were collected each month for a period of one year from one lowland river, water treatment works. Each sample was concentrated by freeze-drying and extracts were tested for mutagenicity in the bacterial fluctuation assay. The study revealed significant variations in the mutagenic potency of samples but there was no obvious seasonal pattern.

Several other parameters, including chlorine dose, were also measured but there were no correlations with variation in mutagenicity.
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1. INTRODUCTION

Concentrated extracts of drinking waters derived from surface sources have been consistently shown to be mutagenic when tested in bacterial assays (Nestmann et al 1979, Loper 1980, Kool et al 1981, Fawell et al 1986). The major proportion of mutagenic activity in drinking water samples is produced when the water is chlorinated for disinfection purposes.

It has been noted that if concentrated extracts are prepared from a particular sampling site on different occasions, some variation in the mutagenic potency of the extracts is observed. Grimm-Kibalo et al (1981) investigated seasonal variation in mutagenic activity in samples of raw and finished water concentrated by XAD adsorption. Mutagenic activity was detected more often and at higher levels in finished water than in raw water. In general, mutagenic activity was greatest during spring, summer and early autumn and decreased during the winter. A similar seasonal pattern of mutagenic activity was observed by Slooff and van Kreijl (1981) with concentrated extracts prepared from raw water samples taken from the River Rhine. These authors noted that mutagenic activity appeared to be influenced by the flow rate of the river, with highest activity occurring during periods of low river flow.

Another possible explanation for the increase in mutagenic activity in water samples during summer months is that the formation of mutagens during chlorination may be increased when the water temperature is higher. In support of this hypothesis, Rook (1974) reported that formation of trihalomethanes (THMs) during chlorination was temperature dependent and hence may vary with the season of the year. Change of temperature is also
an important factor in the development of algal blooms. Algal products may represent a source of precusor material for THMs and also possibly the chlorine-derived mutagens detected in drinking water extracts.

In order to investigate temporal variation of mutagenic activity, drinking water samples were collected each month for a period of one year from one lowland river, water treatment works. Each sample was concentrated by freeze-drying and extracts were tested for mutagenicity in the bacterial fluctuation assay.

2. MATERIALS AND METHODS

2.1 Sample collection

Final water samples were collected from a conventional, slow-sand filtration, treatment works. This works was selected because previous studies had shown that the treated water consistently gave a mutagenic response in the fluctuation assay. At this works, water is abstracted from a lowland river into a settling reservoir, where it is stored for about one day. Between May and October, the intake water may be prechlorinated with about 2.5 mg/l chlorine to reduce algal growth in the reservoir and to prevent mussel growth in the pipework from the reservoir to the rapid gravity filters. The water is then filtered through coarse rapid gravity sand filters and dechlorinated with sulphur dioxide before passage through slow sand filter beds. The filtered water is then chlorinated to give a total residual of about 3 mg/l. Finally, the water is dechlorinated with sulphur dioxide to leave a total residual of about 0.4 mg/l prior to distribution.

In the present study, 20 litre samples of final water were collected in stainless steel vessels from a tap in the chlorination house. One litre
samples were also collected into chlorine "demand-free" bottles for accurate chlorine determinations. Immediately on returning to the laboratory the pH of the water sample was recorded and chlorine residuals determined using the DPD titrimetric method. Samples were taken each month over a one-year period.

2.2 Preparation of extracts

Each sample (16 litres) was concentrated by freeze-drying in an Edwards Minifast freeze-drier. This machine processed 8 litres of water per cycle (about 26 hours). Consequently, two runs of the freeze-drier were required for each sample, the water awaiting processing being stored in the dark at 4 °C until the first run was completed. The freeze-dried solids obtained from the two runs were combined and extracted three times with HPLC grade methanol. The volume of methanol extract was then reduced by rotary evaporation at 30 °C and finally under a stream of nitrogen to give an overall concentration factor of $10^4$ (100 μl of extract was equivalent to 1 litre of original water). Full details of sample preparation are described in Appendix A.

2.3 Bacterial mutagenicity assay

The concentrated extracts were tested for mutagenic activity in the bacterial fluctuation assay (Green et al 1976, 1977) using the Salmonella tester strains developed by Ames. Extracts were tested at dose levels of 5, 10 and 20 μl/ml of test medium (equivalent to 50, 100 and 200 ml of original water/ml) in Salmonella typhimurium TA98 and TA100. Each assay was carried out both in the presence and absence of a microsomal fraction (S9) derived from Aroclor-induced rat liver. Positive and negative controls were included in each assay and all tests were repeated at least once on a different day. The results of the mutagenicity assays were
analysed using the GLIM statistical package. Further details of the fluctuation assay and the method of statistical analysis are given in Appendix B.

2.4 Analytical determinations

Each sample was submitted for the following chemical determinations:

1. Trihalomethanes (THM μg/l)
2. Total organic carbon (TOC mg/l)
3. Adsorbable organic halogen (AOX μg/l)
4. Ammonia (NH₃ mg/l)
5. Nitrate (NO₃ mg/l)

Staff at the treatment works supplied data on the water temperature on the day of sampling, the amount of chlorine added to the water and the chlorophyll-a content of the water leaving the holding reservoir. This latter parameter gives some indication of the amount of algal growth in the holding reservoir. However, the chlorophyll determinations were not carried out every day and the values given were those closest to the day of sampling.

3. RESULTS

3.1 Mutagenicity data

The results obtained with the various samples when tested in the absence of S9 are summarised in Table 1 and are shown graphically in Figures 1 and 2. The data are plotted as the estimated number of revertants per well (which can be calculated from the number of positive wells) against the dose of extract tested. The statistical analysis indicates the position of the best-fitted straight line through these points. The slope value of this line can be used as an estimate of mutagenic potency. The results obtained with the positive control
mutagens are not given but on all occasions gave the expected results indicating that the assay was functioning satisfactorily.

In strain TA100 in the absence of S9, all of the samples showed mutagenic activity (all significant at 0.1% level). The slope values of the lines ranged from 3.95 to 15.32 with highest activity in February and lowest activity in January. There was no obvious seasonal pattern for mutagenicity in this strain.

In strain TA98 in the absence of S9, 8 of the 12 samples were mutagenic (that is significant at 0.1% level), the March sample was only weakly mutagenic while three samples (the samples taken in July, August and October) were negative. The slope values of the positive samples ranged from 3.38 in June to 14.56 in July. There was again no obvious seasonal pattern for mutagenicity, though with the exception of the September sample, there was some tendency for the summer and autumn samples to show lower levels of mutagenic activity than the winter and spring samples. Generally, the slope values obtained with TA98 were lower than those seen with TA100. This is in accord with previous experience of drinking water samples (Fawell et al 1986).

In the presence of S9, mutagenic activity was invariably weaker and many of the samples were toxic to the bacterial strain. We have since shown that S9 enzymes can convert methanol (the solvent used for the extracts) to formaldehyde. When the level of formaldehyde detected in the methanol extracts/S9 mix was added directly to a bacterial culture, toxicity was observed. It would appear that the toxicity observed with the water samples in the presence of S9 may have been due, at least in part, to the solvent used rather than particular organic compounds in the sample.
3.2
Analytical data

Histograms of the various analytical determinands and other parameters are shown in Figures 3-9. The pH of the water samples (Figure 3) was fairly constant throughout the year at about 7.6. The water temperature rose from 4 °C in February to 18 °C in August. The TOC values (Figure 4) varied around 4 mg/l with no obvious seasonal pattern. AOX (Figure 4) appeared to peak in May and June but the reason for this early summer peak is unknown. Total THM levels (Figure 5) were highest in August which may reflect increased reaction rates at higher water temperatures. Analysis of individual THMs indicated possible differences in seasonal variation with individual compounds (Figure 6). The level of chloroform peaked in early summer (April, May and June) and then decreased, whereas the levels of brominated THMs appeared to peak in late summer. This may reflect a seasonal change in bromide concentration in the raw water.

The ammonia (Figure 7) and nitrate levels (Figure 8) in the final water were low throughout the year. However, nitrate levels tended to be higher in winter, reaching 14.9 mg/l in November, and dropping to 4.61 mg/l in June. This seasonal pattern in river nitrate levels has been attributed to agricultural run-off and high flows during winter months (Hill 1984).

The chlorophyll-a content of the reservoir water (Figure 9) tended to increase in the summer, presumably due to increased algal growth. However, there was no correlation with THMs or mutagenicity.

The amounts of chlorine added to the water on the day the samples were collected and the corresponding residual chlorine levels are shown in Table 2. The total chlorine residual of the final water ranged from 0.26 mg/l in December to
0.71 mg/l in July. The amount of chlorine applied ranged from 2.77 mg/l in February to 7.11 mg/l in September.

4. DISCUSSION

In this study there were significant differences in the level of mutagenic activity in final drinking water samples taken from the same treatment works over a period of one year but there was no obvious seasonal pattern. Variation in mutagenic activity was not associated with the extent of chlorination used during water treatment or with any of the water quality parameters which were measured.

However, it is conceivable that there are many factors which affect mutagenic activity and hence only one sample per month may be insufficient to detect trends. Day to day changes in the operation of the treatment works may also have affected results. A study of raw water samples which are chlorinated to a standard procedure in the laboratory may give a more accurate picture of temporal variation of mutagenic activity of drinking water.
REFERENCES


Table 1. Mutagenic activity of freeze-dried extracts prepared from drinking water samples taken each month from the same treatment works, in Salmonella typhimurium TA100 and TA98 in the absence of S9

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<tr>
<th>Sampling date</th>
<th>TA100</th>
<th>TA98</th>
</tr>
</thead>
<tbody>
<tr>
<td>January</td>
<td>3.95 ++</td>
<td>7.41 ++</td>
</tr>
<tr>
<td>February</td>
<td>15.32 ++</td>
<td>6.52 ++</td>
</tr>
<tr>
<td>March</td>
<td>6.83 ++</td>
<td>2.44 +</td>
</tr>
<tr>
<td>April</td>
<td>12.35 ++</td>
<td>7.05 ++</td>
</tr>
<tr>
<td>May</td>
<td>6.46 ++</td>
<td>6.98 ++</td>
</tr>
<tr>
<td>June</td>
<td>5.25 ++</td>
<td>3.38 ++</td>
</tr>
<tr>
<td>July</td>
<td>4.97 ++</td>
<td>2.03 ns</td>
</tr>
<tr>
<td>August</td>
<td>8.36 ++</td>
<td>2.31 ns</td>
</tr>
<tr>
<td>September</td>
<td>8.72 ++</td>
<td>14.56 ++</td>
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<tr>
<td>October</td>
<td>5.71 ++</td>
<td>0.31 ns</td>
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<tr>
<td>November</td>
<td>9.01 ++</td>
<td>5.07 ++</td>
</tr>
<tr>
<td>December</td>
<td>9.60 ++</td>
<td>4.49 ++</td>
</tr>
</tbody>
</table>

Slope values were derived from dose response plots with each sample in the fluctuation assay

ns not significant
+ significant at 5% level
++ significant at 1% level
+++ significant at 0.1% level

Table 2. Chlorination doses used during water treatment on day of sampling (mg/l)

<table>
<thead>
<tr>
<th>Date</th>
<th>Primary</th>
<th>Superchlorinated</th>
<th>Total</th>
<th>Free chlorine</th>
<th>Monochloramine</th>
<th>Residual Total Chlorine</th>
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<td>17.3.83</td>
<td>-</td>
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<td>3.82</td>
<td>0.096</td>
<td>0.108</td>
<td>0.492</td>
</tr>
<tr>
<td>11.4.83</td>
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<td>6.19</td>
<td>0.085</td>
<td>0.125</td>
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<td>3.81</td>
<td>5.93</td>
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<td>0.336</td>
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<td>0.37</td>
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<td>17.11.83</td>
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<td>0.14</td>
<td>0.4</td>
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<td>5.45</td>
<td>0.05</td>
<td>0.16</td>
<td>0.26</td>
</tr>
</tbody>
</table>
Fig. 1. Mutagenic activity of monthly samples in *Salmonella typhimurium* TA100

TA100 – S9

**JAN**

**FEB**

**MAR**

**APR**

**MAY**

**JUNE**

**JULY**

**AUG**

**SEP**

**OCT**

**NOV**

**DEC**

**ESTIMATED NUMBER OF REVERTANTS PER WELL**

**WATER EQUIVALENT (litres/ml)**
Fig. 2. Mutagenic activity of monthly samples in *Salmonella typhimurium* TA98

**TA98 - S9**

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<td>0.2</td>
<td>0.2</td>
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<tr>
<td></td>
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**ESTIMATED NUMBER OF REVERTANTS PER WELL**

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<thead>
<tr>
<th>Month</th>
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<th>SEP</th>
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</table>

**WATER EQUIVALENT (litres/ml)**

**Legend**: ▲, △, □
Fig. 6.
Fig. 9. Chlorophyll - a
APPENDIX A

PREPARATION OF CONCENTRATED EXTRACTS

Eight litres of the water sample were placed in the trays of an Edwards Minifast 3400 freeze-drier and frozen for 3 hours at -40 °C followed by sublimation under vacuum at a shelf temperature of +40 °C. The drying cycle was complete in approximately 24 hours. The residual freeze-dried solids were bulked together and extracted three times with three separate 20 ml aliquots of HPLC grade methanol (Rathburn). The residue and solvent were sonicated and held in ice for 10 minutes and then clarified by centrifugation. The three solvent extracts were combined and the volume reduced by rotary evaporation under a negative pressure (30 mm Hg) at a temperature of 30 °C. Samples were then concentrated further by evaporation at 30 °C under a stream of nitrogen.
APPENDIX B

BACTERIAL FLUCTUATION ASSAY USING SALMONELLA
TYPHIMURIUM TESTER STRAINS

1. STORAGE AND GROWTH OF TESTER STRAINS

The Salmonella typhimurium tester strains were stored as frozen cultures over liquid nitrogen with 10% dimethyl sulfoxide included in the medium to act as a cryoprotective agent. The strains were routinely checked for appropriate genetic markers as described by Ames et al (1975). Broth cultures of the required strain were set up by inoculating 10 ml aliquots of Nutrient Broth No 2 with a scraping from a frozen stock culture; the frozen cultures were not allowed to thaw out during this procedure. The broth cultures were placed in an orbital incubator which was controlled by a timeswitch. At the appropriate time the machine switched itself on and incubated the cultures at 37 °C for 12 h with a shaking speed of 120 revolutions per minute. The 12 h broth cultures were either used immediately or stored in a refrigerator if required later in the day. Cultures of TA100 were diluted 1 to 10 with 1/4-strength Ringers Solution immediately before use; cultures of the other strains were used directly in the mutagenicity assay.

2. MACROSCALE FLUCTUATION ASSAY

The macroscale fluctuation assay devised by Green et al (1976, 1977) was carried out according to the following protocol.
2.1
Tests in the absence of S9

1. To double distilled water was added:

20 µl/ml 20% glucose solution
20 µl/ml Vogel Bonner salts concentrate
10 µl/ml 0.1% Biotin solution.

2. To this mixture stock histidine solution (1 mg/ml) was added to give a final concentration of 0.75 µg/ml for TA100 or 1.5 µg/ml for the other Salmonella strains.

3. This solution was dispensed in 5 ml aliquots into sterile 30 ml Universal bottles.

4. To each 5 ml aliquot 30 µl of a 12 h bacterial culture (diluted 1 to 10 for TA100) was added and the desired amount of concentrated test sample or control substance.

5. Each test included at least three dose levels of the test sample, two untreated controls, a solvent control (the final solvent used for the concentrated extract) and an appropriate positive control. The positive controls used routinely were:

   Sodium azide    (0.05 µg/ml)   TA100 -S9
   2-Aminoanthracene (2.5 µg/ml)  TA100 +S9
   Picrolonic acid  (0.5 µg/ml)   TA98 -S9
   Ethidium bromide (0.05 µg/ml)  TA98 +S9.

6. The mixture was dispensed into a rack of 50 sterile glass test tubes (100 µl/tube) and incubated overnight at 37 °C.
7. On the following day the selection medium was prepared as follows. To double distilled water was added:

- 40 μl/ml 20% glucose solution
- 20 μl/ml Vogel Bonner salts concentrate
- 0.5 μl/ml 1% Bromocresol purple solution.

8. Two millilitres of selection medium was added to each tube in a rack using a Compu-pet automatic dispenser.

9. The tubes were incubated for a further three days at 37 °C and then the number of positive wells was scored.

2.2 Tests in the presence of S9

Aroclor-induced rat liver S9 fraction was obtained from a commercial supplier and stored over liquid nitrogen. S9-mix was prepared immediately before required and kept on ice at all times. The S9-mix comprised:

- 5 ml 0.2 M phosphate buffer pH 7.4
- 1 ml 300 mM KCl solution
- 1 ml 40 mM NADP solution
- 1 ml 50 mM Glucose-6-phosphate solution
- 1 ml 80 mM MgCl₂ solution
- 1 ml S9 fraction.

This mixture was filter-sterilised through a 0.45 μm membrane filter.

The test procedure was similar to that used in the absence of S9, except that the Vogel Bonner medium containing histidine and biotin was dispensed into Universal bottles in 4 ml amounts. The test material and the bacteria were then added followed by 1 ml of S9 mix. This mixture was dispensed into 50 tubes (100 μl/tube) and the rack of tubes placed
immediately into a 37 °C incubator. With some batches of S9 it was necessary to adjust the level of exogenous histidine in order to obtain suitable control values.

3. STATISTICAL ANALYSIS

The results of the fluctuation assays were analysed using the GLIM (Generalised Linear Interactive Modelling) statistical package (Baker and Nelder 1978). Using all the results generated with a specified sample in a particular type of assay (eg tests using strain TA100 in the absence of S9), the statistical program tested whether the variability exhibited by the observed number of positive wells (ie wells containing revertants) was significantly greater than would be expected on the basis of binomial variation. A probability value was calculated that the observed results could have arisen by change in the absence of a genuine dose effect; p-values at or below 0.05 (after adjustment for multiple comparison) were taken to indicate significant mutagenicity.

An extension to the GLIM-based analysis is available that tests for linearity between "dose" and "estimated number of revertants per well" (this latter term can be calculated from the number of positive wells per rack/tray (Venitt 1982). This checks whether each set of data is both significantly and adequately represented by a linear-dose response. The program also indicates the position of the best-fitted straight line through the data and the slope value of this line with 95% confidence limits. Where it is desirable to compare the mutagenic activity of different samples (eg before and after various treatments), a generalised t-test can be applied to these slope values; p-values of 0.01 or below are taken to indicate a significant difference in mutagenic response.
The GLIM-based analysis has been built into a suite of programs, known as 'Sometime', which enables the user to input data from fluctuation assays and the program then analyses and tabulates the results, plots a graph of estimated number of revertants per well against dose and stores the data in a central archive.

REFERENCES


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