WATER RESEARCH

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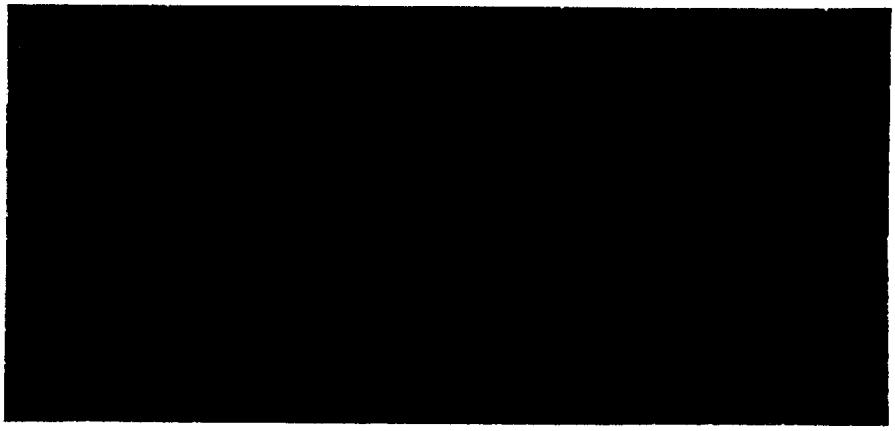

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EFFECTS OF STORAGE ON MUTAGENIC ACTIVITY OF WATER SAMPLES

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UNRESTRICTED

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SUMMARY

A limited study was conducted to investigate whether bankside storage of water prior to treatment had any beneficial effects with respect to the mutagenic activity of water since it is possible that the concentration of precursor compounds in the raw water may decrease on storage, thus reducing the potential of the water to form mutagens on chlorination.

After short-term storage (7 days) there was no apparent change in the mutagenic activity of the water either before or after chlorination. There was some evidence that long-term storage (60 days) may reduce the potential of the water to form mutagens on chlorination, although further studies would be needed to substantiate this observation. The construction of large, raw-water reservoirs, however, would not be a feasible treatment option at the majority of water works.

CONTENTS

		Page
SUMMARY		
1.	INTRODUCTION	1
2.	MATERIALS AND METHODS	1
2.1	Preparation of concentrated extracts	1
2.2	Mutagenicity assays	3
2.3	Chemical analysis	3
2.4	Experimental	4
2.4.1	Reservoir storage for seven days	4
2.4.2	Reservoir storage for 60 days	4
3.	RESULTS	4
3.1	Reservoir storage for seven days	5
3.2	Reservoir storage for 60 days	5
3.3	Analytical data	6
4.	DISCUSSION	6
REFERENCES		7
TABLES		
FIGURES		
APPENDIX A -	STUDIES ON LABORATORY STORAGE OF WATER SAMPLES	
APPENDIX B -	PREPARATION OF CONCENTRATED EXTRACTS	
APPENDIX C -	BACTERIAL FLUCTUATION ASSAY USING SALMONELLA TYPHIMURIUM TESTER STRAINS	

1. INTRODUCTION

It has been shown that the major proportion of the mutagenic activity of drinking water is produced when the water is chlorinated for disinfection purposes (Nestmann et al 1979, Maruoka and Yamanaka 1980, 1983, Kool et al 1981, Fawell et al 1986). The precursor compounds which are transformed to mutagens by chlorination are probably naturally-occurring, organic compounds, such as humic acids (Meier et al 1983, Kringstad et al 1983, Horth et al 1987).

At certain works, water is stored in reservoirs prior to treatment for periods ranging from a few days to several months depending on the size of the reservoir and the demand for water. Retention of water in a storage reservoir may affect the level of these precursors and thus alter the potential of the water to show mutagenic activity after chlorination.

A limited suvey was therefore conducted to investigate whether reservoir storage of raw water had any effect on mutagenic activity. Experiments are described in which water samples were tested before and after storage in reservoirs both with and without laboratory chlorination.

2. MATERIALS AND METHODS

2.1 Preparation of concentrated extracts

Freeze-drying was chosen as the method of concentrating water samples prior to mutagenicity testing. Freeze-drying is a relatively non-selective technique which is therefore particularly appropriate when dealing with samples of unknown and variable composition.

Samples were collected in clean, stainless-steel containers and transported to the laboratory. On arrival, samples were filtered through Whatman GF/C grade filters. Those samples which were not being laboratory chlorinated were sterilised by filtration through Oxoid, 0.45 µm, cellulose acetate membranes.

Prior to laboratory chlorination, samples were adjusted to pH 7.0 using either Analar hydrochloric acid or sodium hydroxide. The water was then chlorinated using a single, fixed dose of sodium hypochlorite (5 mg/l) and mixed with a magnetic stirrer for a contact time of one hour. The samples were then dechlorinated by addition of sodium sulphite solution to leave a free chlorine residual of 0.5 mg/l. Previous work at WRc has shown that partial dechlorination to this level does not substantially affect the mutagenic activity of the sample (Wilcox and Denny 1985).

Samples were concentrated by freeze-drying in an Edwards Minifast freeze-drier. However, this machine processed only 8 litres of water per cycle (about 26 hours) and hence it was necessary to store portions of samples for short periods in the laboratory prior to concentration. A series of experiments were therefore performed in order to confirm that short-term laboratory storage of samples did not significantly affect their mutagenic activity or mutagen-forming potential on chlorination. A description of these studies can be found in Appendix A.

Following freeze-drying, the residual solids were extracted three times with methanol and the solvent extracts concentrated by rotary evaporation at 30 °C and then blown down to dryness under a stream of nitrogen. The residual solids were redissolved in dimethyl sulphoxide (DMSO) prior to testing. Full details of the concentration procedure are given in Appendix B.

2.2 Mutagenicity assays

Extracts were tested for mutagenic activity in two-step, bacterial, fluctuation assays using Salmonella typhimurium TA98 and TA100 both in the presence and absence of rat liver S9 fraction. All assays were repeated at least once on a different day.

Extracts were tested at doses equivalent to 0.02, 0.05 and 0.1 litres of original sample/ml of test medium. Appropriate negative and positive controls were included in each assay.

The results of the fluctuation assays were analysed using the Generalised Linear Interactive Model (GLIM) statistical package, which tests whether the variability exhibited by the observed number of revertant-bearing wells is significantly greater than would be expected on the assumption of binomial variation. Full details of the mutagenicity assay and statistical analysis are given in Appendix C.

2.3 Chemical analysis

Before freeze-drying, aliquots from the various water samples were removed and submitted for chemical analysis for the following determinands:

pH
Total organic carbon (TOC, mg/l)
Adsorbable Organic Halogen (AOX, µg/l)
Total Trihalomethane (TTHM, µg/l)

2.4 Experimental

2.4.1 Reservoir storage for seven days

Samples were collected from a treatment works which abstracts water from a lowland river into a bankside reservoir with a nominal retention time of seven days. In an attempt to sample comparable water, two samples were taken, the first at the raw water inlet to the reservoir and the second (one week later) at the reservoir outlet as the water passed into the treatment works.

2.4.2 Reservoir storage for 60 days

To investigate the effects of long-term storage on the mutagenic activity of water, samples were taken from a large, storage reservoir where water was held for approximately 60 days before treatment. Samples were collected on the same day at the point of abstraction on the river and also from the reservoir outlet as the water passed into the treatment works.

3. RESULTS

The results of the mutagenicity assays are shown graphically in Figures 1 to 8 and are summarised in Tables 1 and 2. In the graphs, 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 a crude estimate of mutagenic potency. The results obtained with the positive control mutagens in each assay are not given, but on all occasions these gave the expected results indicating that the mutagenicity assay was functioning satisfactorily. Full procedural blanks and negative controls were also performed and found to be satisfactory.

3.1 Reservoir storage for seven days

Raw water extracts prepared before and after reservoir storage for seven days were non-mutagenic in TA100 and showed very weak activity in strain TA98 in the absence of S9 (Figures 1 and 2). In the presence of S9, there was evidence of weak activity in raw water with both strains. There was no obvious effect of storage on the activity observed in the raw water.

The samples prepared after laboratory chlorination showed much higher levels of mutagenic activity in both TA100 and TA98 in the absence of S9 (Figures 3 and 4). Storage in the reservoir for seven days appeared to have no effect on the level of mutagenic activity observed after chlorination.

3.2 Reservoir storage for 60 days

The extracts prepared from raw water collected at the intake and outlet of the reservoir were not mutagenic in either TA100 or TA98 in the absence of S9 (Figures 5 and 6). In the presence of S9, however, there was evidence of activity in both strains. In strain TA100, the slope values of lines before and after storage were not significantly different, whereas with TA98 the slope value of the stored water was significantly lower than that of the intake water ($p < 0.01$).

The laboratory chlorinated water samples both showed clear activity in TA100 in the absence of S9 (Figure 7). However, activity was significantly lower in the stored sample; the slope values of the intake and outlet water being 19.87 and 8.41, respectively. This may indicate that long-term reservoir storage of raw water may reduce the potential of the water to form mutagens on chlorination. In the presence of S9, activity of both samples was reduced.

The laboratory chlorinated water samples were also mutagenic in strain TA98 in the absence of S9 (Figure 8) and again activity appeared to be significantly reduced after storage ($p < 0.05$). The slope values for the intake water and the stored water were 5.34 and 2.46, respectively. In strain TA98 in the presence of S9 both samples showed comparable levels of activity.

3.3 Analytical data

The analytical data collected as part of the storage experiments are given in Table 3.

Where samples were analysed before and after chlorination, the expected increase in TTHMs and AOX on chlorination was observed.

4. DISCUSSION

In this limited study, storage of raw water in a bankside reservoir for seven days did not appear to alter the potential of the water to form mutagenic compounds on chlorination. The TOC value showed little change as did the TTHM level after chlorination. This probably indicates that there was no significant decrease in the level of precursor compounds during the period of storage.

After 60 days storage in a reservoir there did appear to be a reduction in the mutagenic activity of the extracts prepared from the water after it had been chlorinated. However, it must be borne in mind that these were "spot samples" and before any firm conclusions can be reached about the effect of long-term storage on the mutagenicity of water samples, a series of samples would need to be taken on a regular basis throughout the storage period. However, the value of such a study may be dubious since, even if long-term reservoir storage proved to have beneficial effects in reducing the mutagenic activity of final water, it could not be regarded as a practical treatment alternative for the majority of water works.

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Table 1. Effect of reservoir storage on the mutagenic activity of water samples in strain TA100 before and after chlorination

Storage conditions	Sample	Slope values[a]			
		Raw water		After chlorination	
		-S9	+S9	-S9	+S9
7-day bankside reservoir	Reservoir inlet	0.04^{NS}	2.95+++	11.75+++	3.49+++
	Reservoir outlet[b]	$<0^{NS}$	3.66+++	14.60+++	6.18+++
60-day storage reservoir	Reservoir inlet[c]	1.40^{NS}	5.23+++	19.87+++	9.21+++
	Reservoir outlet[c]	0.87^{NS}	8.57+++	8.41+++	3.07+++

NS Not significant
++ Significant at 1% level
+++ Significant at 0.1% level
a slope values were determined from the dose response plots in bacterial fluctuation assays
b sampled 7 days after inlet sample
c both samples collected on the same day

Table 2. Effect of reservoir storage on the mutagenic activity of water samples in strain TA98 before and after chlorination

Storage conditions	Sample	Slope values[a]			
		Raw water		After chlorination	
		-S9	+S9	-S9	+S9
7-day bankside reservoir	Reservoir inlet	1.31+	2.16+++	7.54+++	7.92+++
	Reservoir outlet[b]	2.06++	4.23+++	7.45+++	4.94+++
60-day storage	Reservoir inlet[c]	0.85^{NS}	6.09+++	5.34+++	5.57+++
	Reservoir outlet[c]	$<0^{NS}$	2.50+	2.46+	4.54+++

NS Not significant
\+ Significant at 5% level
++ Significant at 1% level
+++ Significant at 0.1% level
a slope values were determined from the dose response plots in bacterial fluctuation assays
b sampled 7 days after inlet sample
c both samples collected on the same day

Table 3. Analytical data

Storage conditions	Day	pH	TOC mg/l	AOX µg/l	TTHMs µg/l
Reservoir storage 7 days					
Raw	0	7.40	4.7	<10	0.5
	7	7.40	4.5	17.5	0.9
Chlorinated	0			77.3	27.8
	7			120	26.6
Reservoir storage 60 days					
Raw	0	7.30	2.2	18.9	1.0
	60	7.30	2.7	17.9	0.8
Chlorinated	0			80.5	17.6
	60			74.0	16.3

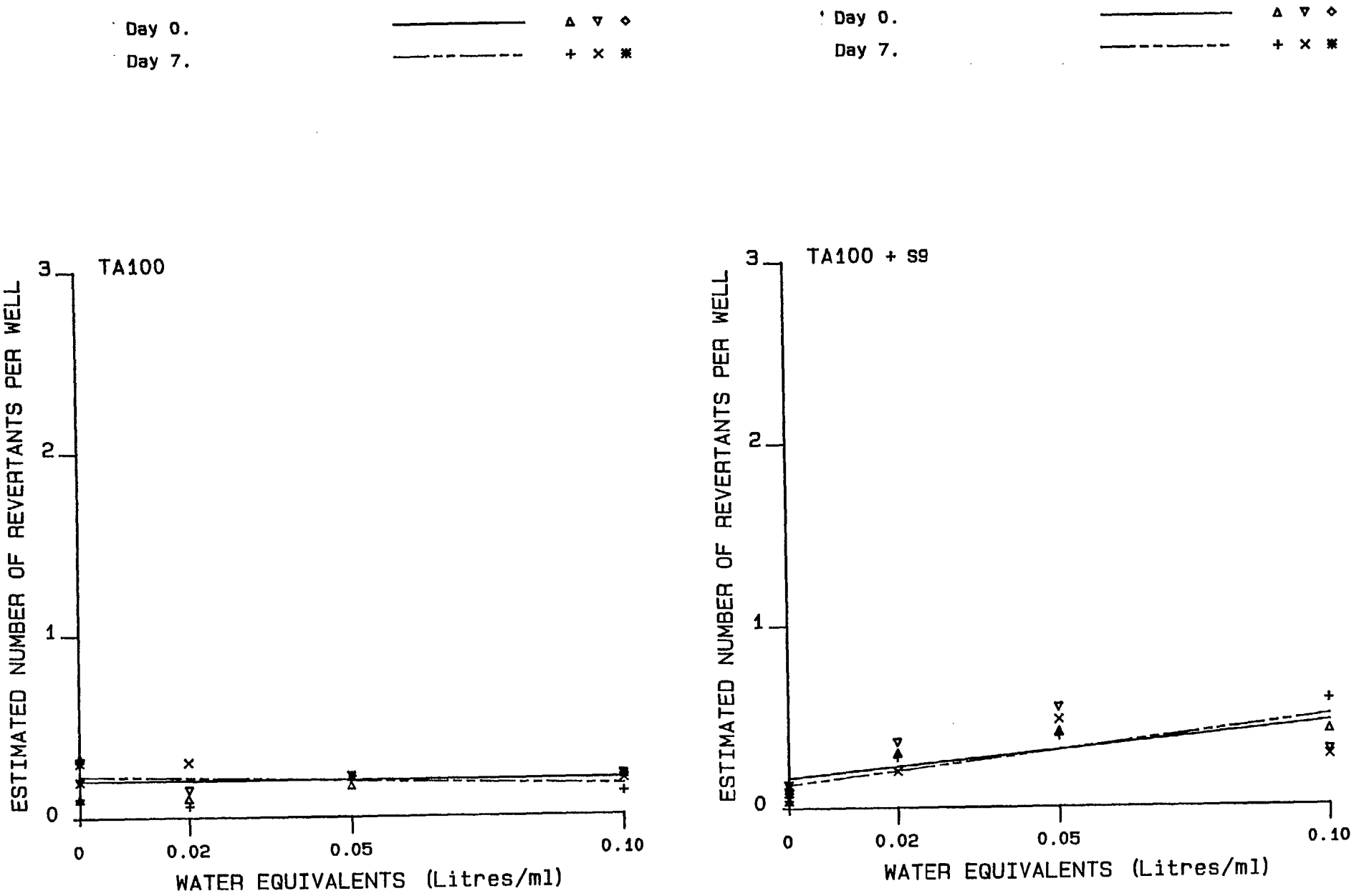

Figure 1. Reservoir storage for 7 days. Raw water.

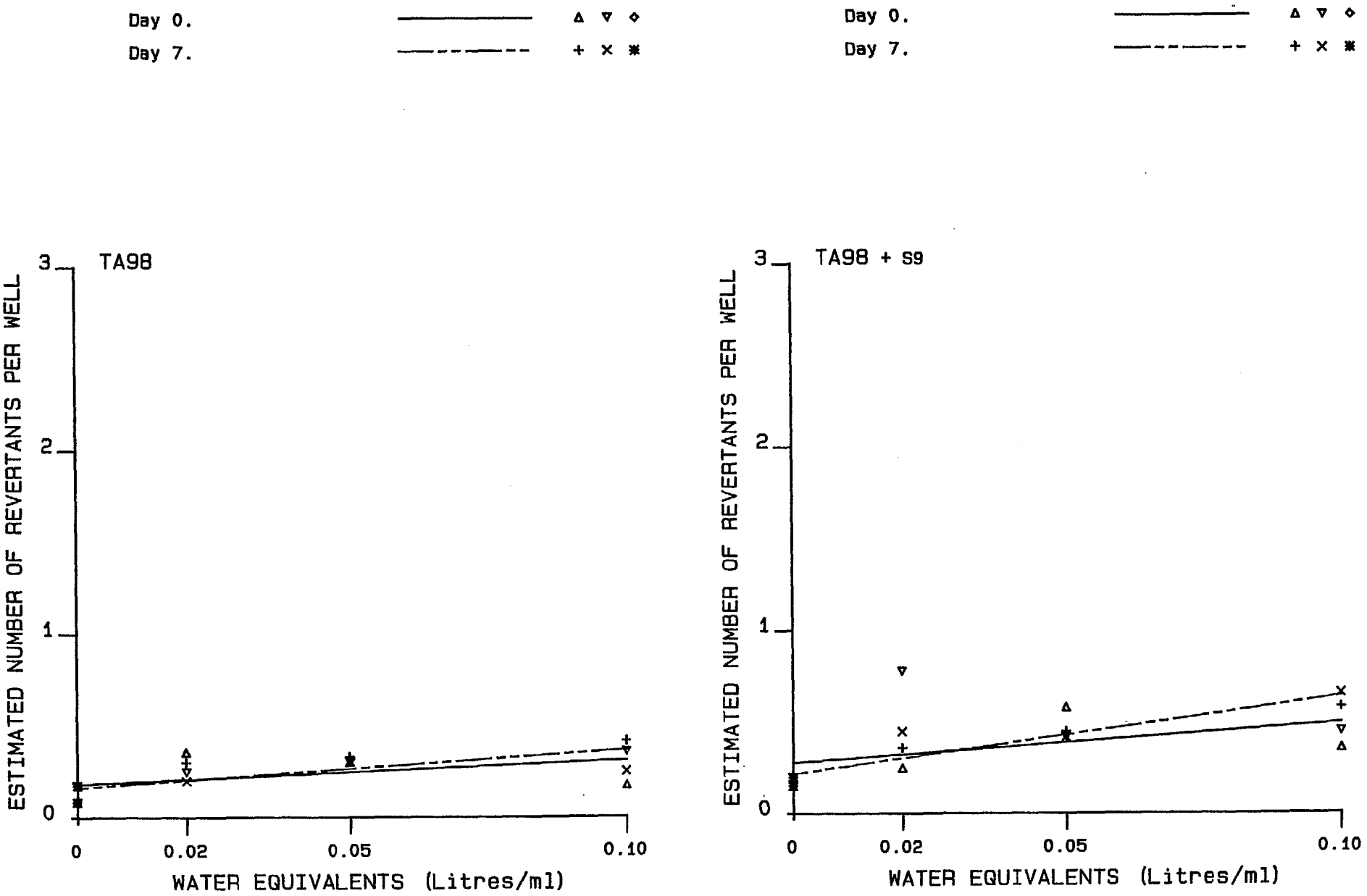

Figure 2. Reservoir storage for 7 days. Raw water.

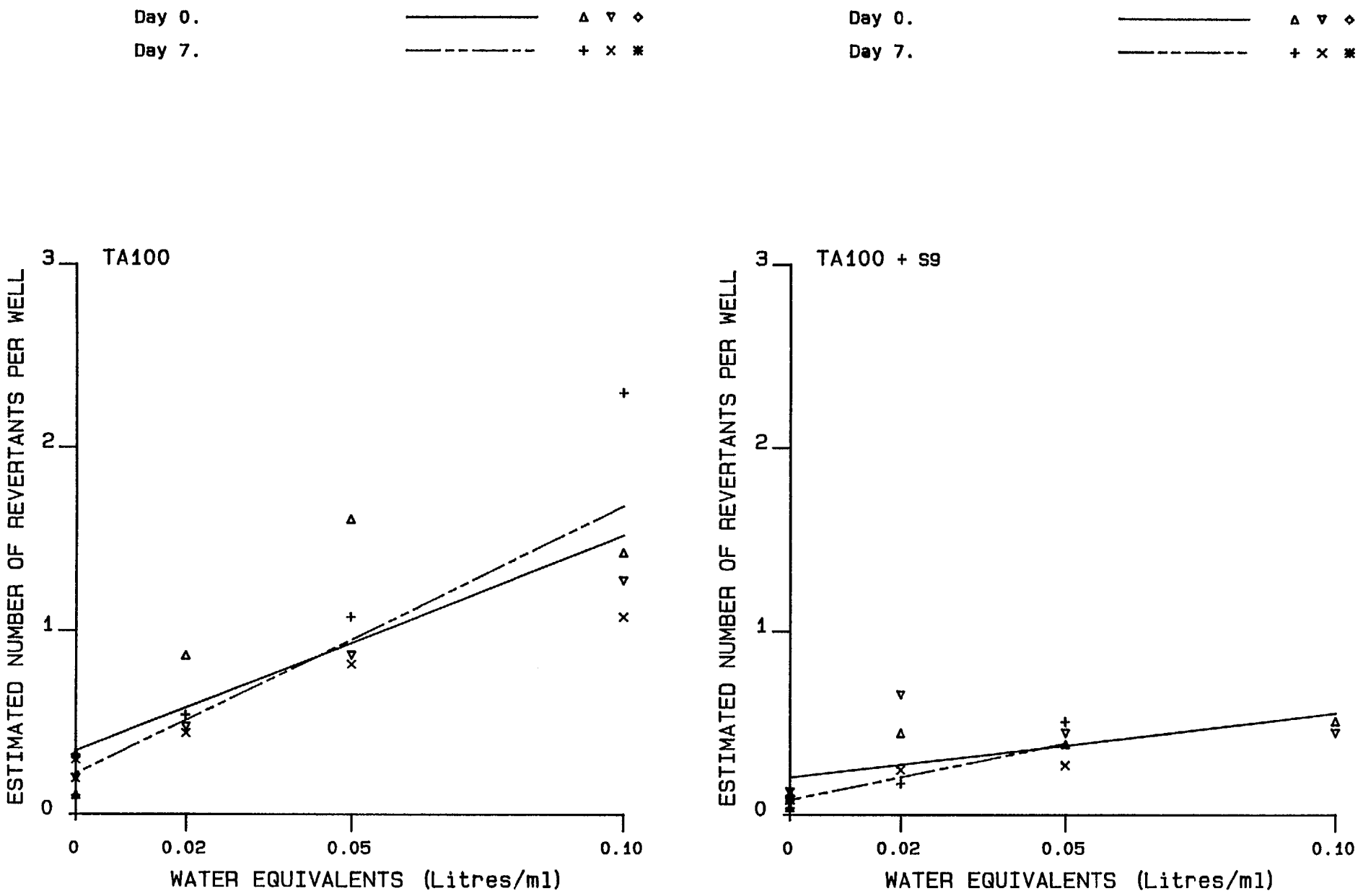

Figure 3. Reservoir storage for 7 days. Laboratory chlorinated water.

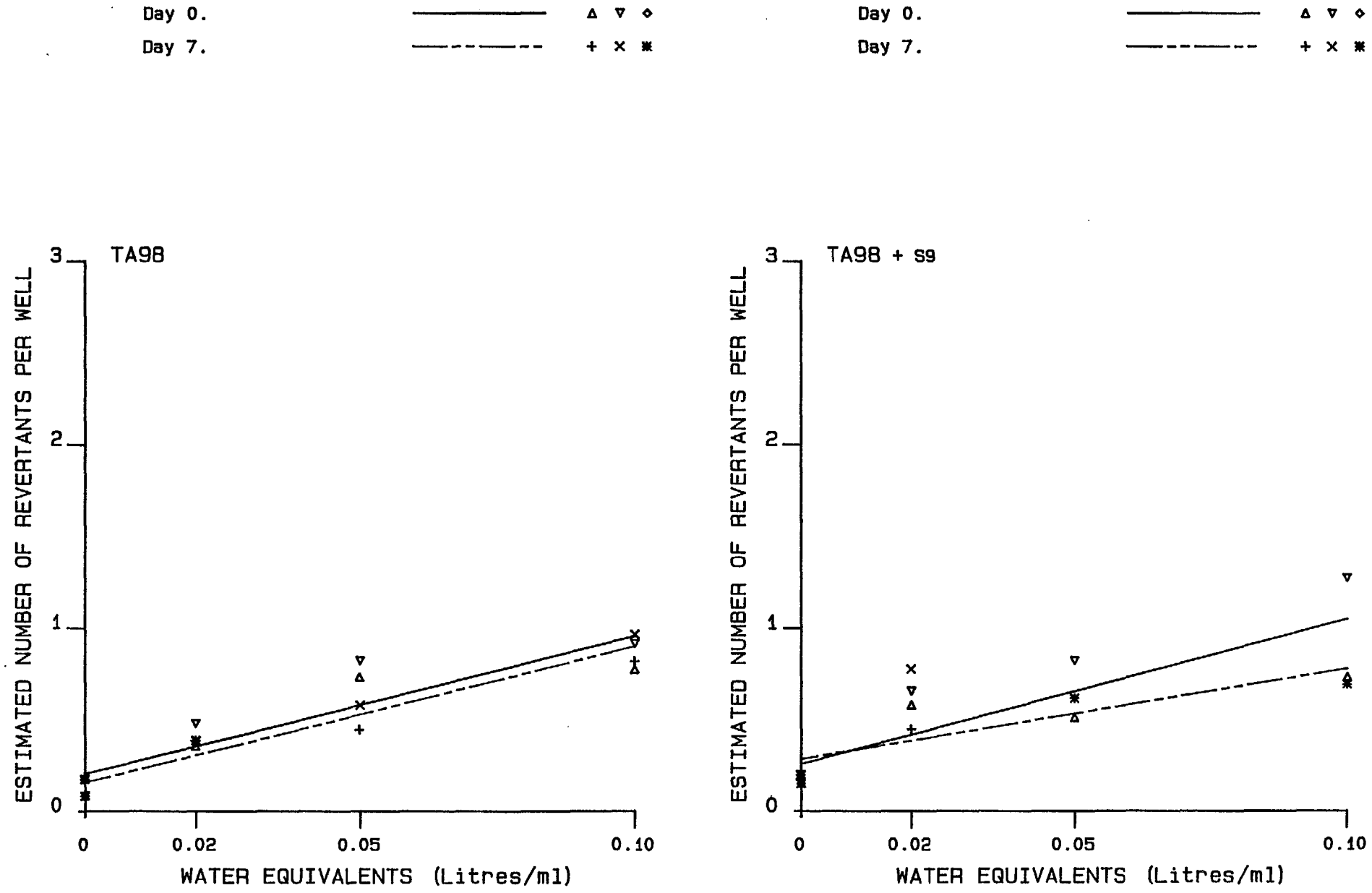

Figure 4. Reservoir storage for 7 days. Laboratory chlorinated water.

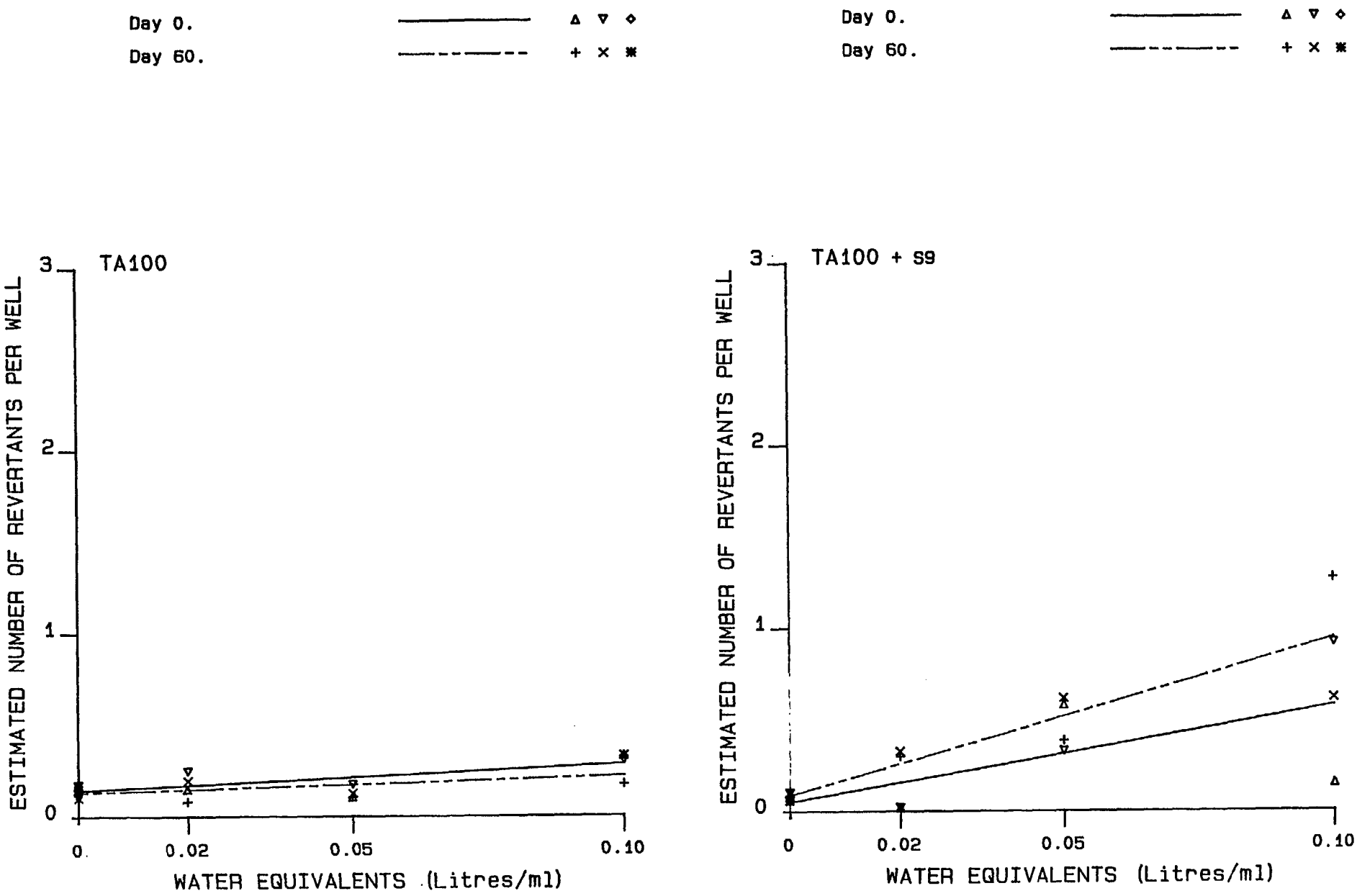

Figure 5. Reservoir storage for 60 days. Raw water.

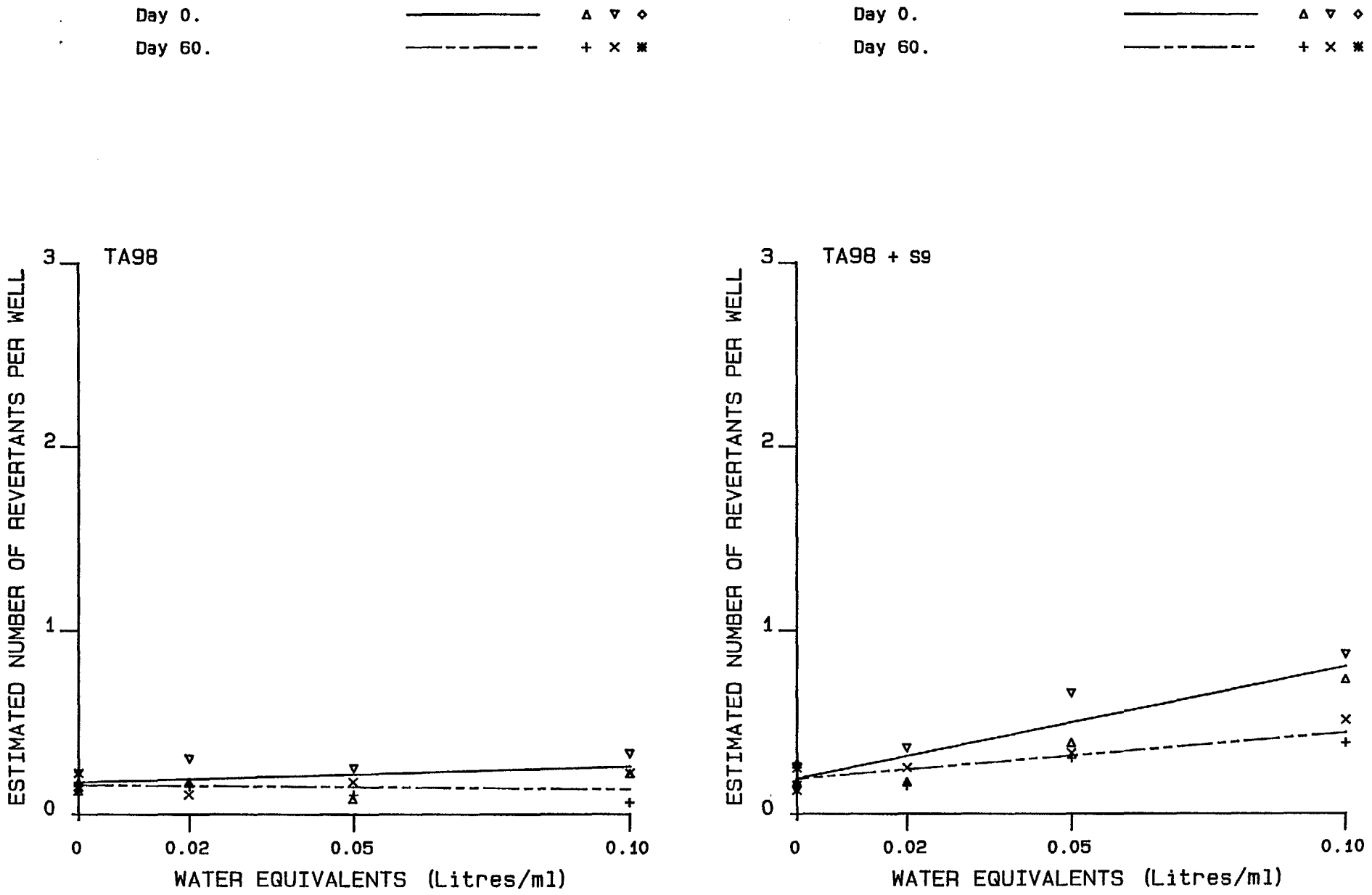

Figure 6. Reservoir storage for 60 days. Raw water.

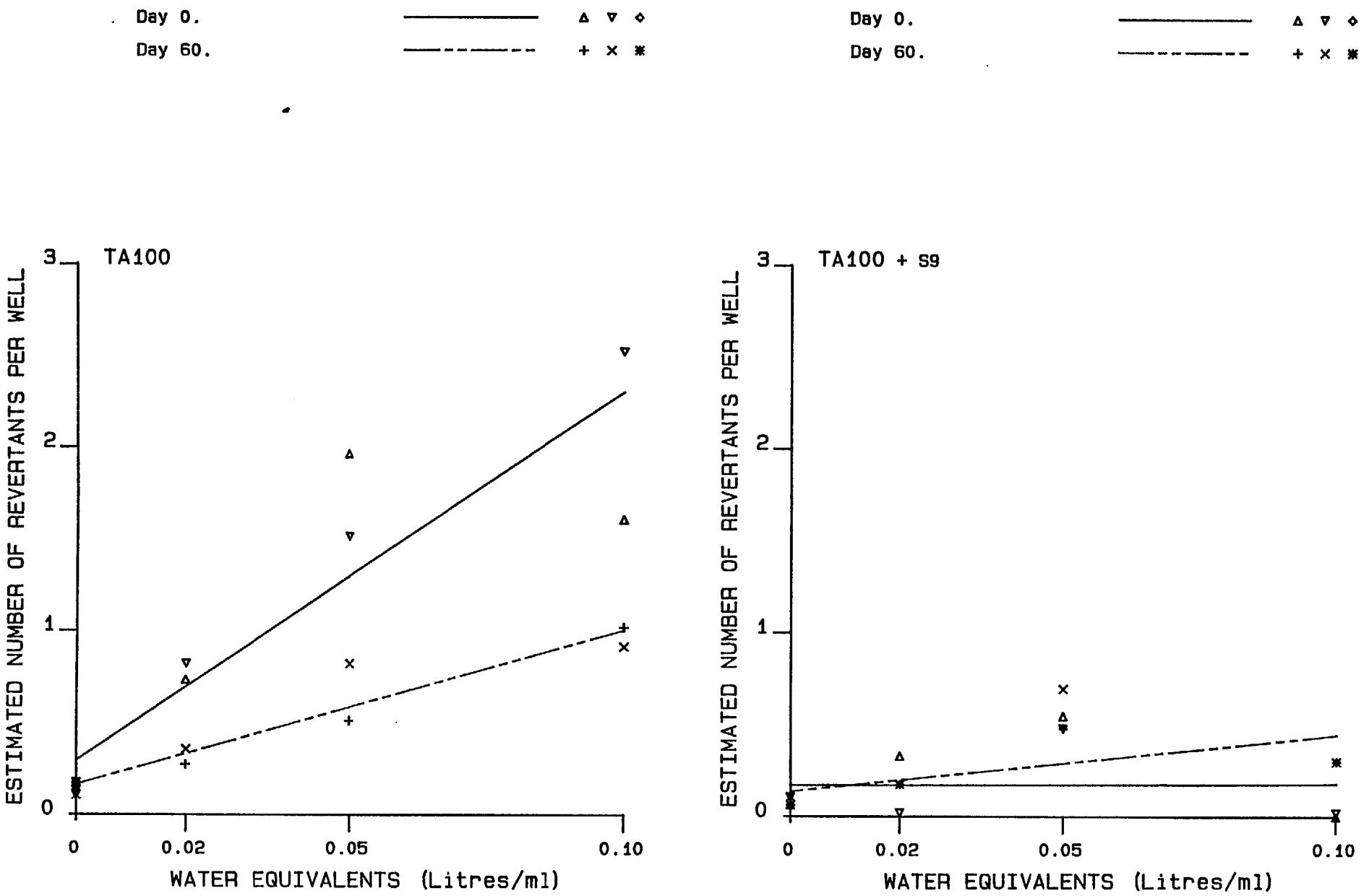

Figure 7. Reservoir storage for 60 days. Laboratory chlorinated water.

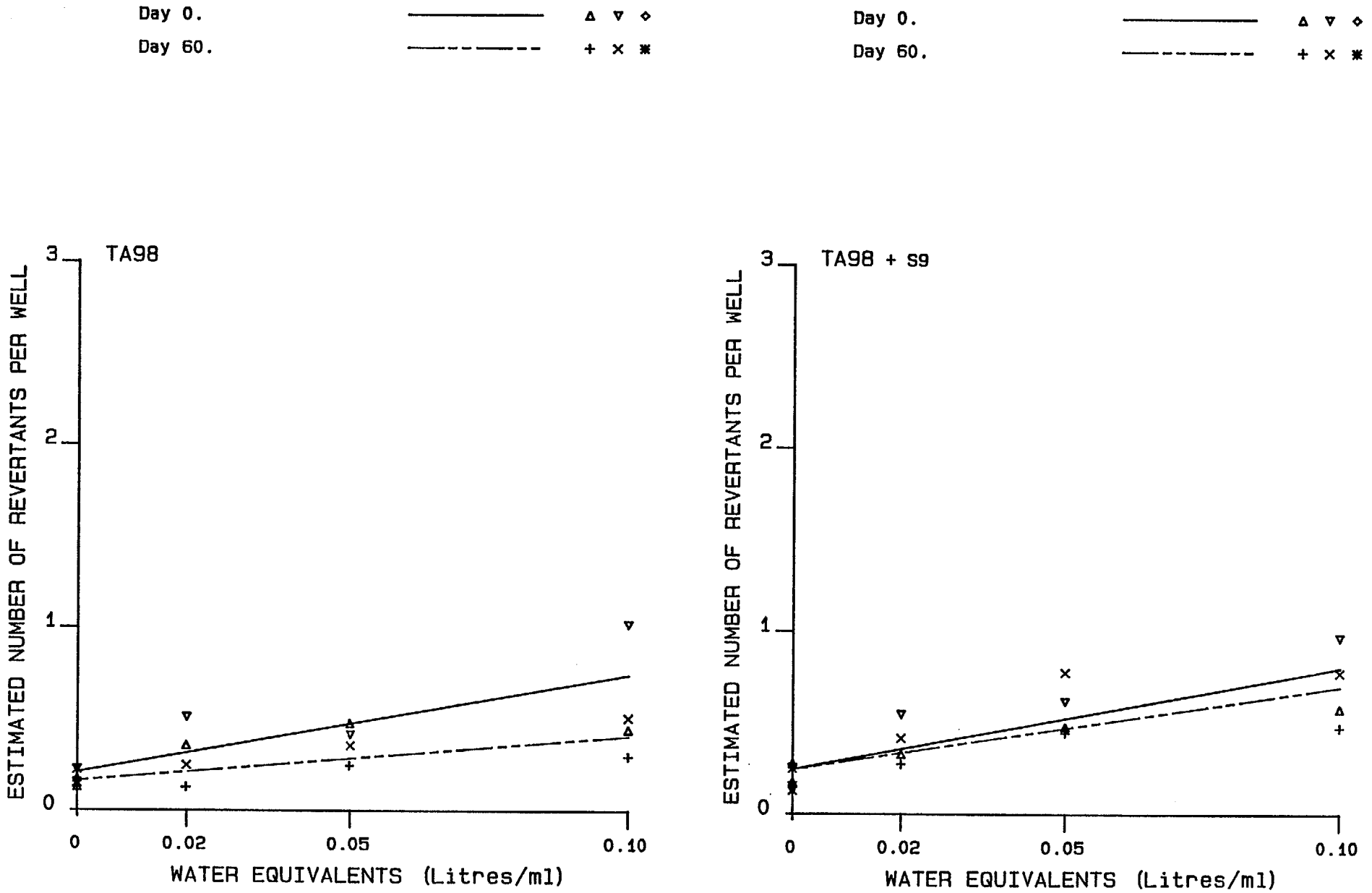

Figure 8. Reservoir storage for 60 days. Laboratory chlorinated water.

APPENDIX A

STUDIES ON LABORATORY STORAGE OF WATER SAMPLES

SUMMARY

A series of experiments was carried out to investigate whether storing water samples in the laboratory prior to concentration affected the mutagenic potential of that water. Short-term storage of raw water at 4 °C did not appear to affect the activity of the sample or its potential to form mutagens on chlorination. Storage of raw water at 20 °C, however, is not recommended.

In the experiments using chlorinated final drinking water, a reduction in mutagenic activity was observed over the eight day storage period. This effect was more apparent at 20 °C than at 4 °C. Storage of final water prior to concentration, therefore, should be avoided whenever possible.

CONTENTS

		Page
SUMMARY		
1.	INTRODUCTION	A3
2.	MATERIALS AND METHODS	A3
2.1	Preparation of concentrated extracts	A3
2.2	Mutagenicity assays	A4
2.3	Chemical analysis	A4
2.4	Experimental	A5
2.4.1	Laboratory storage of raw water at 4 °C	A5
2.4.2	Laboratory storage of raw water at 20 °C	A5
2.4.3	Laboratory storage of chlorinated water at 4 °C	A5
2.4.4	Laboratory storage of chlorinated water at 20 °C	A6
3.	RESULTS	A6
3.1	Laboratory storage of raw water at 4 °C	A6
3.2	Laboratory storage of raw water at 20 °C	A7
3.3	Laboratory storage of final water at 4 °C	A7
3.4	Laboratory storage of final water at 20 °C	A7
3.5	Analytical data	A8
4.	DISCUSSION	A8
TABLES		A10
FIGURES		

1. INTRODUCTION

When concentrating water samples by freeze-drying it is sometimes necessary to store samples in the laboratory for a period since our machine (an Edwards Minifast freeze-drier) can only process 8 litres of water per cycle (about 26 hours). A series of experiments was therefore carried out to investigate whether storage of water samples in the laboratory prior to freeze-drying had any effect on the mutagenic potency of the final, concentrated extract.

2. MATERIALS AND METHODS

2.1 Preparation of concentrated extracts

Samples were collected in clean, stainless-steel containers and transported back to the laboratory as quickly as possible. On arrival at the laboratory, a portion of the sample (either 4 or 8 litres) was removed for immediate concentration (Day 0 of the experiment) and the rest set aside for storage under the appropriate conditions until required.

Before freeze-drying, samples of raw water were filtered through Whatman GF/C grade filters. Those samples which were not being laboratory chlorinated were sterilised by filtration through Oxoid, 0.45 µm, pore diameter cellulose acetate membranes. Samples of final water were not filtered.

Prior to laboratory chlorination, the samples were adjusted to pH 7.0 using Analar hydrochloric acid or sodium hydroxide. The water was then chlorinated using a single, fixed dose of sodium hypochlorite (6.5 mg/l) and mixed with a magnetic stirrer for a contact time of 2 hours.

Samples were concentrated by freeze-drying in an Edwards Minifast freeze-drier. The residual solids were extracted three times with methanol and the solvent extracts concentrated by rotary evaporation at 30 °C and finally under a stream of nitrogen to the required volume. The methanol extracts were tested directly in the mutagenicity assay.

2.2
Mutagenicity assays

Extracts were tested for mutagenic activity in two-step, bacterial, fluctuation assays using Salmonella typhimurium TA98 and TA100. Unless otherwise stated assays were carried out in both the absence and presence of rat liver S9 fraction. All assays were repeated at least once on a different day.

Extracts were tested at doses equivalent to 0.02, 0.05 and 0.1 litres of original sample/ml of test medium. Appropriate negative and positive controls were included in each assay. The results of the fluctuation assays were analysed using the GLIM (General Linear Interactive Model) statistical package.

2.3
Chemical analysis

Before freeze-drying, aliquots from the various samples were removed and submitted for chemical analysis for the following determinands:

Free available chlorine (mg/l)
Total available chlorine (mg/l)
pH
Total organic carbon (TOC, mg/l)
Adsorbable Organic Halogen (AOX, µg/l)
Total Trihalomethane (TTHM, µg/l)

2.4 Experimental

2.4.1 Laboratory storage of raw water at 4 °C

Samples were taken directly from a lowland river at the point of abstraction of a water treatment works. On returning to the laboratory, two aliquots of sample were processed immediately and the remainder was stored at 4 °C in a refrigerator. Further aliquots of the water sample were processed after four and seven days storage at 4 °C. The freeze-dried, methanol extracts were tested in the fluctuation assay against TA98 and TA100. As small amounts of each extract (equivalent to 4.0 litres of original water) were available in this experiment, tests were carried out in the absence of S9 only. Previous work had shown that S9 usually reduces the mutagenicity of water samples, particularly the activity observed after chlorination.

2.4.2 Laboratory storage of raw water at 20 °C

Samples were collected, processed and tested for mutagenicity as described in 2.4.1, but for this experiment the raw water was stored for six days, in the dark, at ambient temperature (ie about 20 °C). Aliquots of raw and laboratory chlorinated water were freeze-dried on the day of sampling and after four and six days storage.

2.4.3 Laboratory storage of chlorinated water at 4 °C

To investigate the effect of storing chlorinated water, a final water sample was collected from the outlet of a water treatment works. The water was stored in a refrigerator in stainless steel containers. Freeze-dried extracts were prepared on Day 0, Day 3 and Day 7 without any further treatment.

2.4.4
Laboratory storage of chlorinated water at 20 °C

This experiment was similar to the previous study except the chlorinated water was stored at ambient temperature in the laboratory. Samples were removed on Day 0, Day 2 and Day 7 and freeze-dried extracts prepared without further treatment.

3. RESULTS

The results of the mutagenicity assays are shown graphically in Figures A1 to A8 and are summarised in Tables A1 and A2.

3.1 Laboratory storage of raw water at 4 °C

Concentrated extracts produced from the raw water did not show significant mutagenic activity in strain TA100 at any of the three storage times (Figure A1). Typically, the extracts produced in the laboratory chlorinated experiments all showed clear mutagenic activity in this strain. Storing the raw water at 4 °C prior to chlorination did not appear to affect the mutagenic activity of the final extract, as all three samples were of similar potency. At the highest dose level tested (0.1 l/ml) the response in the fluctuation assay decreased, indicating that the sample was toxic to the bacteria at this concentration. This dose level, therefore, was not included in estimating the slope values of the dose response plots.

As expected, the raw water extracts showed evidence of weak but statistically significant mutagenic activity for all three samples in strain TA98 (Figure A2). Laboratory chlorination also markedly increased the mutagenic activity of the water in TA98. There was no obvious effect of storage on the activity of the three extracts in this strain.

3.2
Laboratory storage of raw water at 20 °C

In this experiment, extracts of raw water prepared before and after storage for up to six days did not show significant levels of mutagenic activity in either strain TA100 or TA98 in the absence of S9 (Figures A3 and A4). After laboratory chlorination, the samples showed mutagenic activity in both strains, although the level of activity in the chlorinated samples was lower than in the previous study at 4 °C. In the experiments with TA100 the slope value of the dose response plot was higher after four days storage than for the unstored sample or six-day sample. In the experiments with TA98, storage did not appear to affect the mutagenic potential of the water sample.

3.3
Laboratory storage of final water at 4 °C

Mutagenicity was observed with extracts of final drinking water in TA100 without S9 (Figure A5). This activity decreased over the seven day storage period but remained statistically significant throughout. The slope values with the Day 0, Day 3 and Day 7 samples were 5.71, 5.14 and 3.08, respectively. In the presence of S9, the extracts were toxic to TA100 (data are not shown).

The three samples showed little evidence of activity in TA98 in the absence of S9, and were only weakly mutagenic in the presence of S9 (Figure A6).

3.4
Laboratory storage of final water at 20 °C

The extract of final water in this experiment showed significant mutagenic activity in both TA100 and TA98 in the absence of S9 (Figures A7 and A8). Activity in both strains decreased over the seven day storage period. In strain TA100, the slope

values from the dose response plots were 9.34 (Day 0), 4.49 (Day 2) and 1.27 (Day 7). The Day 0 and Day 2 samples were significant at the 0.1% level but the Day 7 sample was non-significant. In strain TA98, the slope values were 6.90 (Day 0), 5.90 (Day 2) and 3.81 (Day 7). All three samples were significant at the 0.1% level.

In the presence of S9, mutagenic activity was reduced in both TA100 and TA98 and there was no clear pattern in the level of activity of the samples with respect to storage.

3.5 Analytical data

The analytical data are given in Table A3. In all cases where samples were analysed before and after chlorination, the expected increase in TTHMs and AOX on chlorination was observed. The other obvious change was a decrease in TTHMs and residual chlorine levels on storing final water at either 4 °C or 20 °C.

4. DISCUSSION

The experiments on water stored in the laboratory prior to concentration by freeze-drying were carried out to investigate whether storage affected the mutagenicity or mutagenic potential of that water. This information was required to assess the effect of storing raw and final water samples before concentration should the necessity arise.

Throughout the studies, extracts produced from raw water stored at 4 °C or 20 °C exhibited no mutagenic activity towards strain TA100 and only weak activity against TA98. When these waters were chlorinated, extracts showed potent mutagenic activity particularly in strain TA100. These

results are in agreement with earlier studies which led to the hypothesis that non-mutagenic precursor compounds in the water are transformed to mutagens on chlorination.

Laboratory storage of raw water at 4 °C did not appear to influence the mutagenic activity of concentrated extracts of the water nor its potential to form mutagenic compounds on chlorination. However, with the storage of raw water at 20 °C the results obtained after laboratory chlorination were ambiguous. The extracts prepared on Day 0 and Day 6 showed similar levels of mutagenic activity, but the extracts prepared on Day 4 exhibited significantly higher activity towards TA100.

Overall, the studies suggested that, if necessary, it is possible to store raw water for up to seven days at 4 °C without significantly affecting its mutagenic activity or mutagenic potential on chlorination. Storage at room temperature would not be advisable due to the possibility that changes may take place in the water at this temperature.

When samples of final drinking water were stored in the laboratory prior to concentration the mutagenic activity of the final extracts towards TA100 in the absence of S9 was reduced, presumably due to breakdown of organic compounds formed during chlorination. There was little change in TOC over the storage period, but TTHM levels were substantially reduced. The total available chlorine also fell from around 0.3-0.4 mg/l to less than 0.02 mg/l over the storage period.

The results of these experiments indicate that final drinking water should not be stored prior to concentration as the mutagenic activity of the sample may decrease on storage.

Table A1. Effect of storing raw water prior to concentration on the mutagenic activity of the sample before and after chlorination

Storage conditions	Day	Slope values[a]			
		Raw water		After chlorination	
		TA100	TA98	TA100	TA98
4 °C in dark	0	1.55^{NS}	3.77^{+++}	51.71^{+++}	39.36^{+++}
	4	1.43^{NS}	3.93^{+++}	47.45^{+++}	34.36^{+++}
	7	1.05^{NS}	3.01^{+++}	44.54^{+++}	37.51^{+++}
20 °C in dark	0	1.48^{NS}	0.80^{NS}	7.41^{+++}	5.58^{+++}
	4	0.56^{NS}	0.93^{NS}	36.69^{+++}	9.05^{+++}
	6	0.44^{NS}	0.25^{NS}	9.65^{+++}	6.28^{+++}

NS Not significant
+++ Significant at 0.1% level
a slope values were determined from the dose response plots in bacterial fluctuation assays

Table A2. Effect of storing fully treated, chlorinated water, prior to concentration on the mutagenic activity of the sample

Storage conditions	Day	Slope values[a]			
		Without S9		With S9	
		TA100	TA98	TA100	TA98
4 °C in dark	0	5.71+++	0.31^{NS}	TOXIC	3.20++
	3	5.14+++	0.98+	TOXIC	2.49+
	7	3.08+++	<0^{NS}	TOXIC	0.61^{NS}
20 °C in dark	0	9.34+++	6.90+++	1.63++	3.53+++
	2	4.49+++	5.90+++	3.15+++	0.95^{NS}
	7	1.27^{NS}	3.81+++	3.46+++	4.06+++

NS Not significant
\+ Significant at 5% level
++ Significant at 1% level
+++ Significant at 0.1% level
a slope values were determined from the dose response plots in bacterial fluctuation assays

Table A3. Analytical data

Storage conditions	Day	pH	TOC mg/l	AOX µg/l	TTHMs µg/l	Available chlorine (mg/l) Free	Total
Laboratory storage of raw water at 4 °C							
Raw	0	8.10	4.1	19.9	0.9		
	4	8.08	3.9	NT	1.0		
	7	8.07	4.1	NT	1.1		
Chlorinated	0			179	52.1		
	4			218	45.6		
	7			203	70.2		
Laboratory storage of final water at 4 °C							
	0	7.34	3.5	51.5	25.5	0.10	0.37
	3	7.50	3.3	47.8	23.9	<0.02	0.15
	7	7.50	3.0	46.5	12.5	<0.02	0.10
Laboratory storage of final water at 20 °C							
	0	7.20	4.1	111	33.1	0.10	0.42
	2	7.20	3.9	91.1	25.7	<0.02	0.08
	7	7.20	3.9	94.3	18.8	<0.02	<0.02

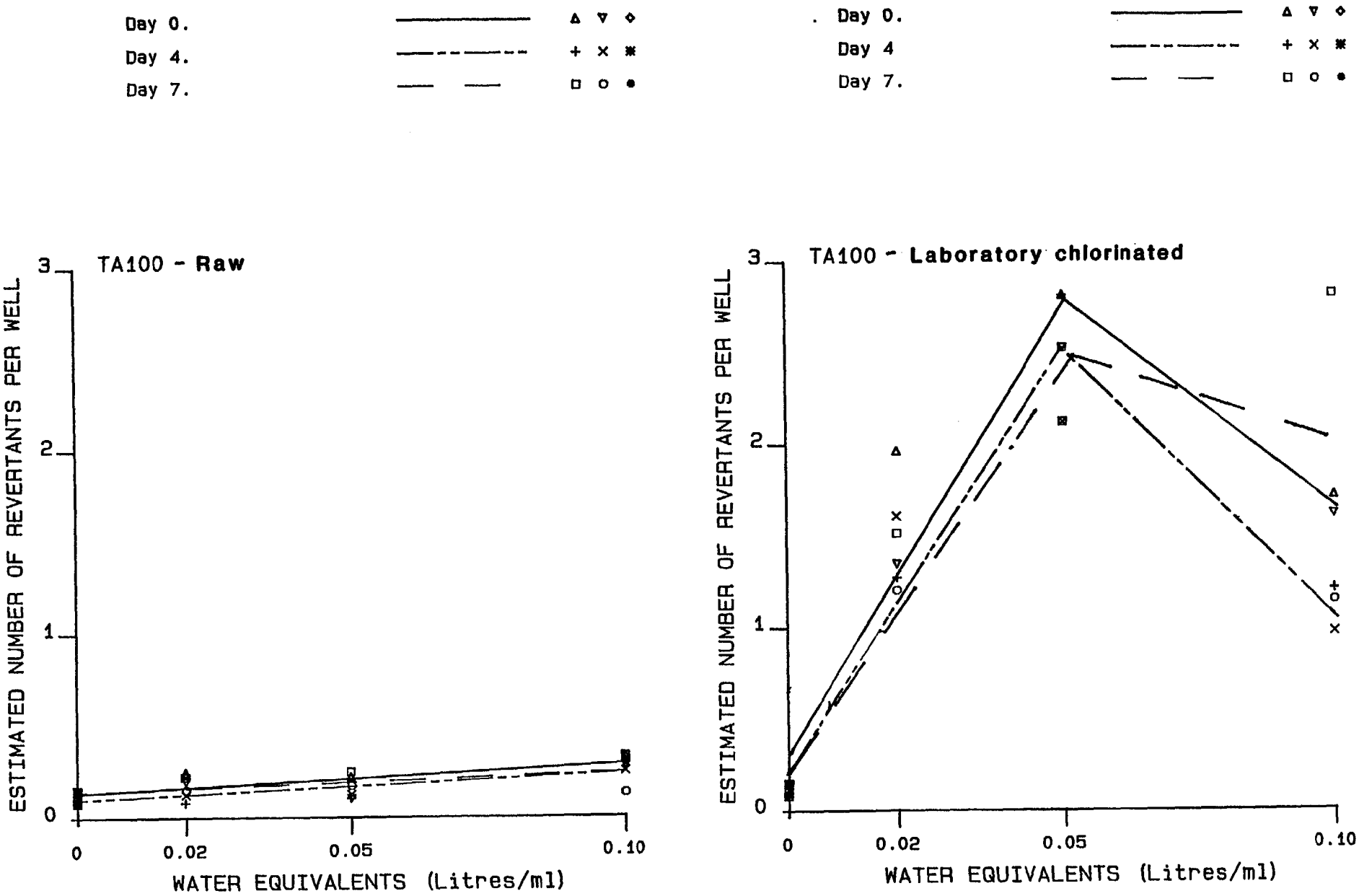

Figure A1. Laboratory storage of raw water at 4°C.

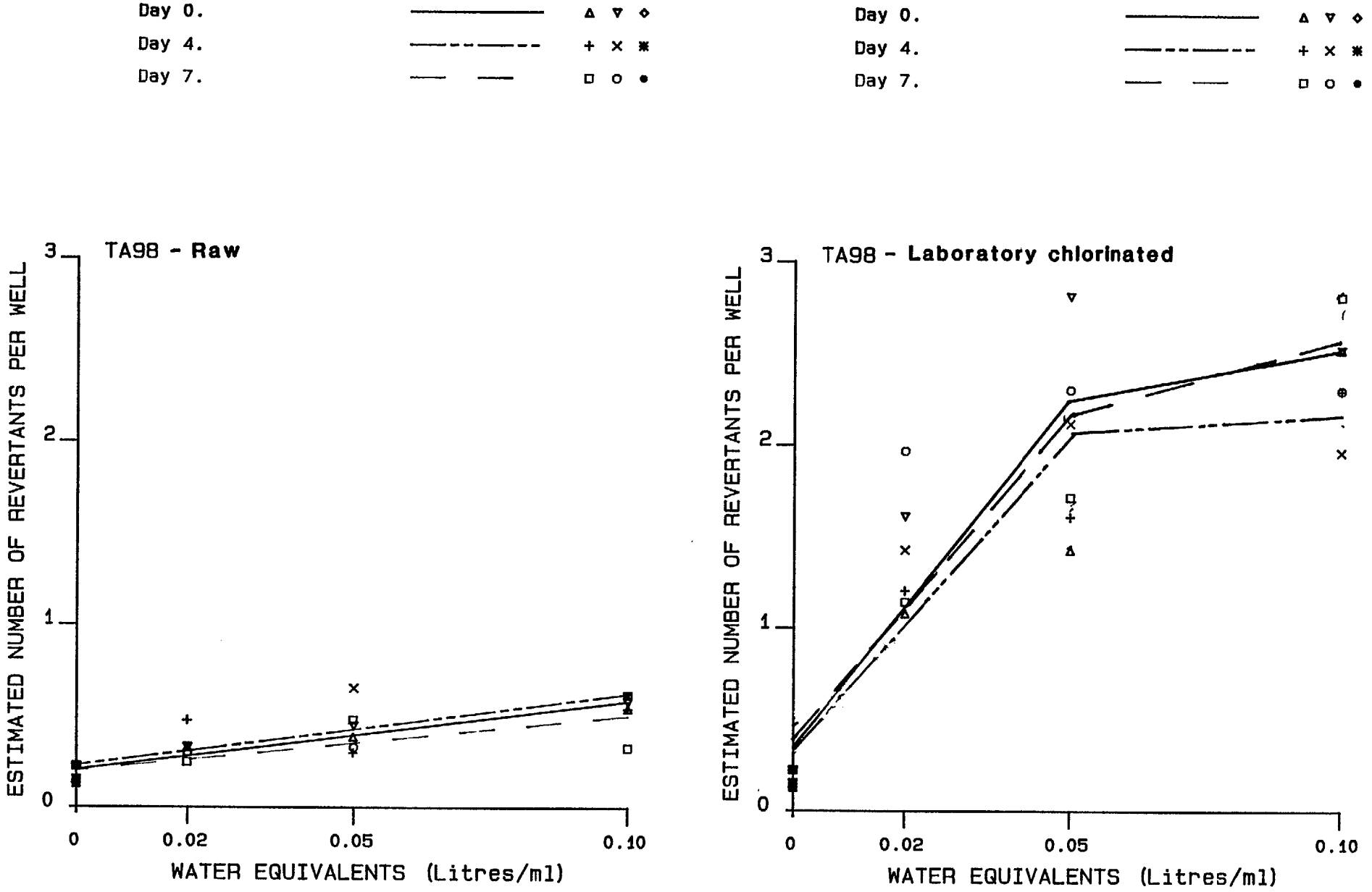

Figure A2. Laboratory storage of raw water at 4°C.

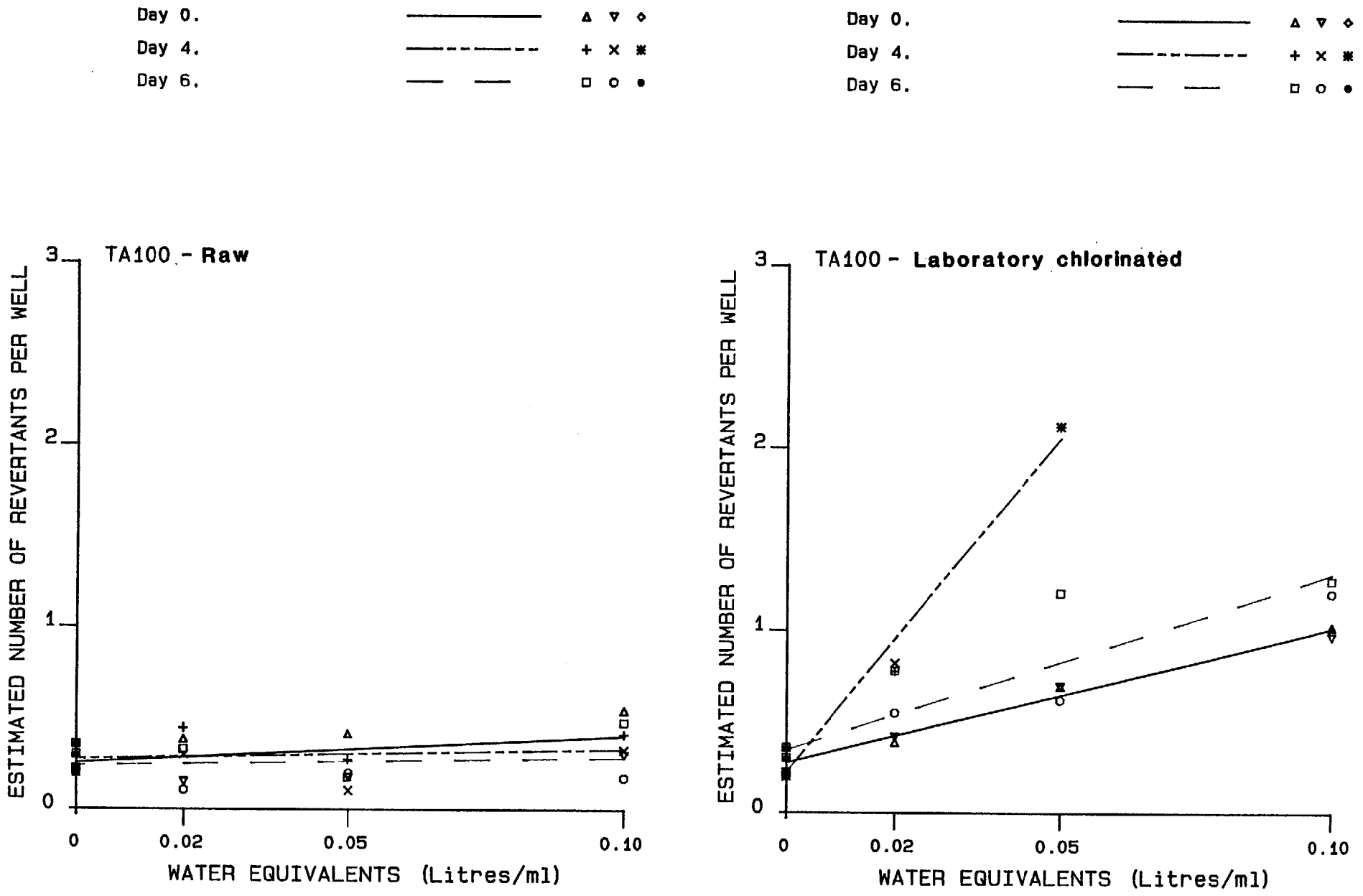

Figure A3. Laboratory storage of raw water at 20°C.

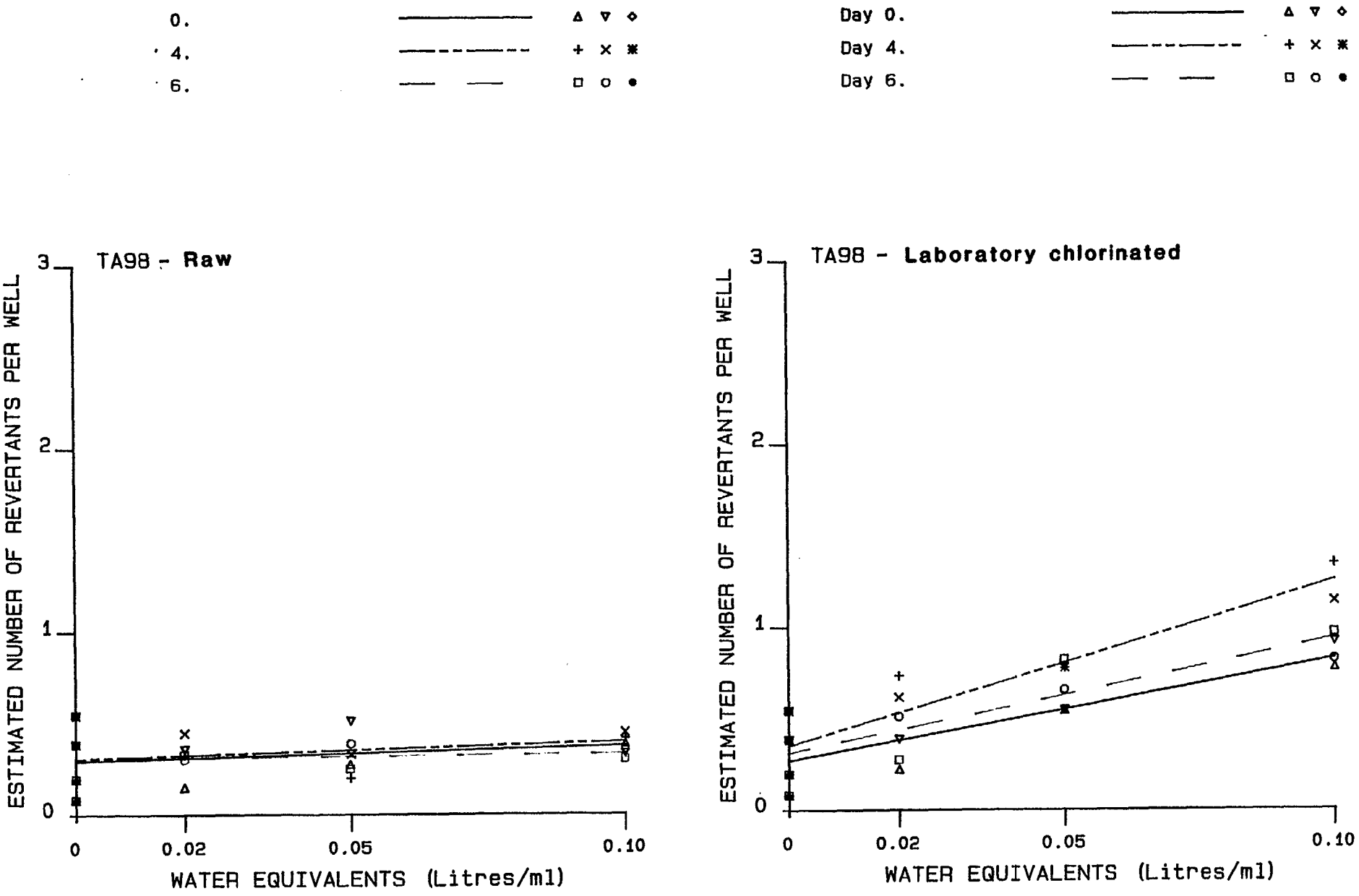

Figure A4. Laboratory storage of raw water at 20°C.

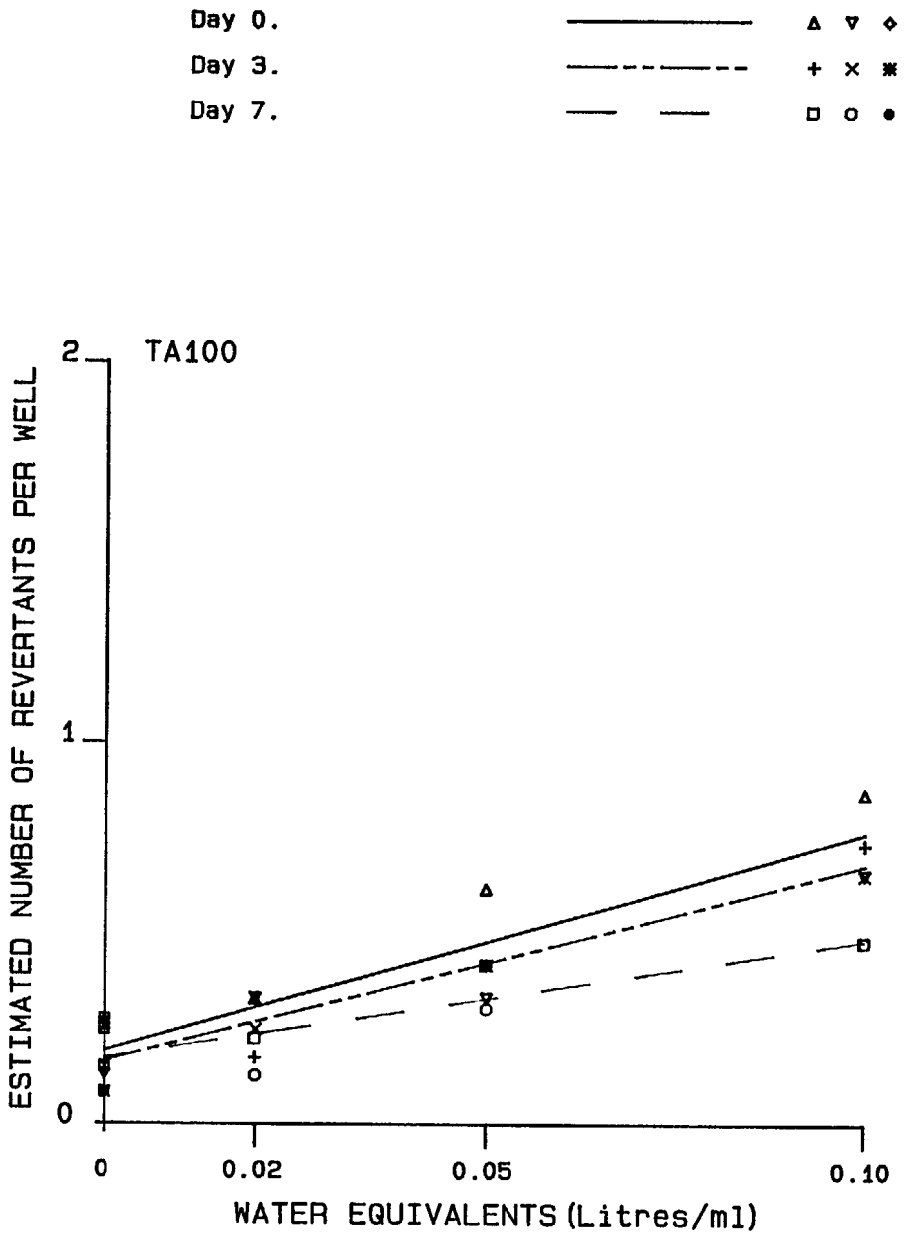

Figure A5. Laboratory storage of final water at 4°C.

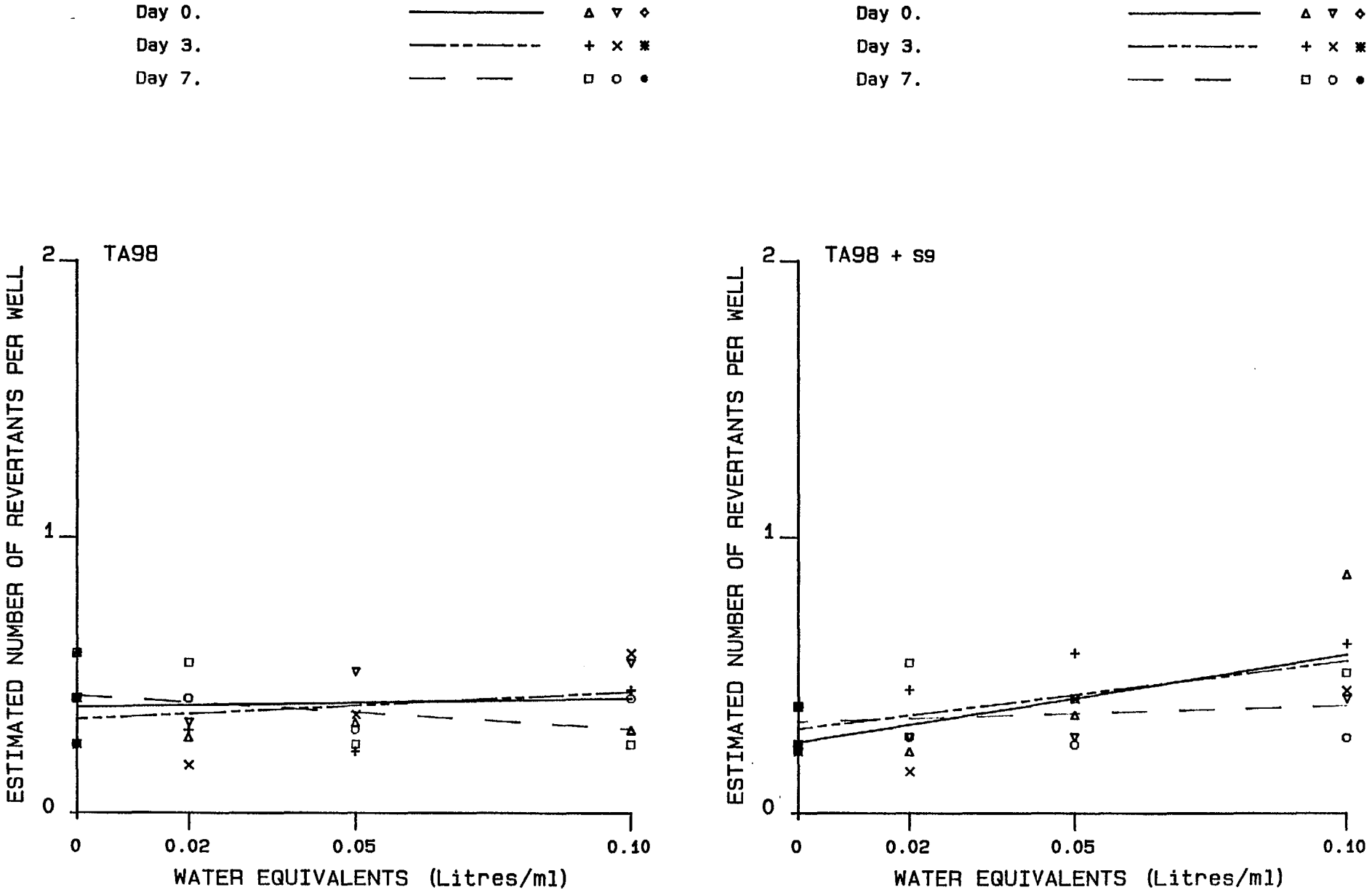

Figure A6. Laboratory storage of final water at 4°C.

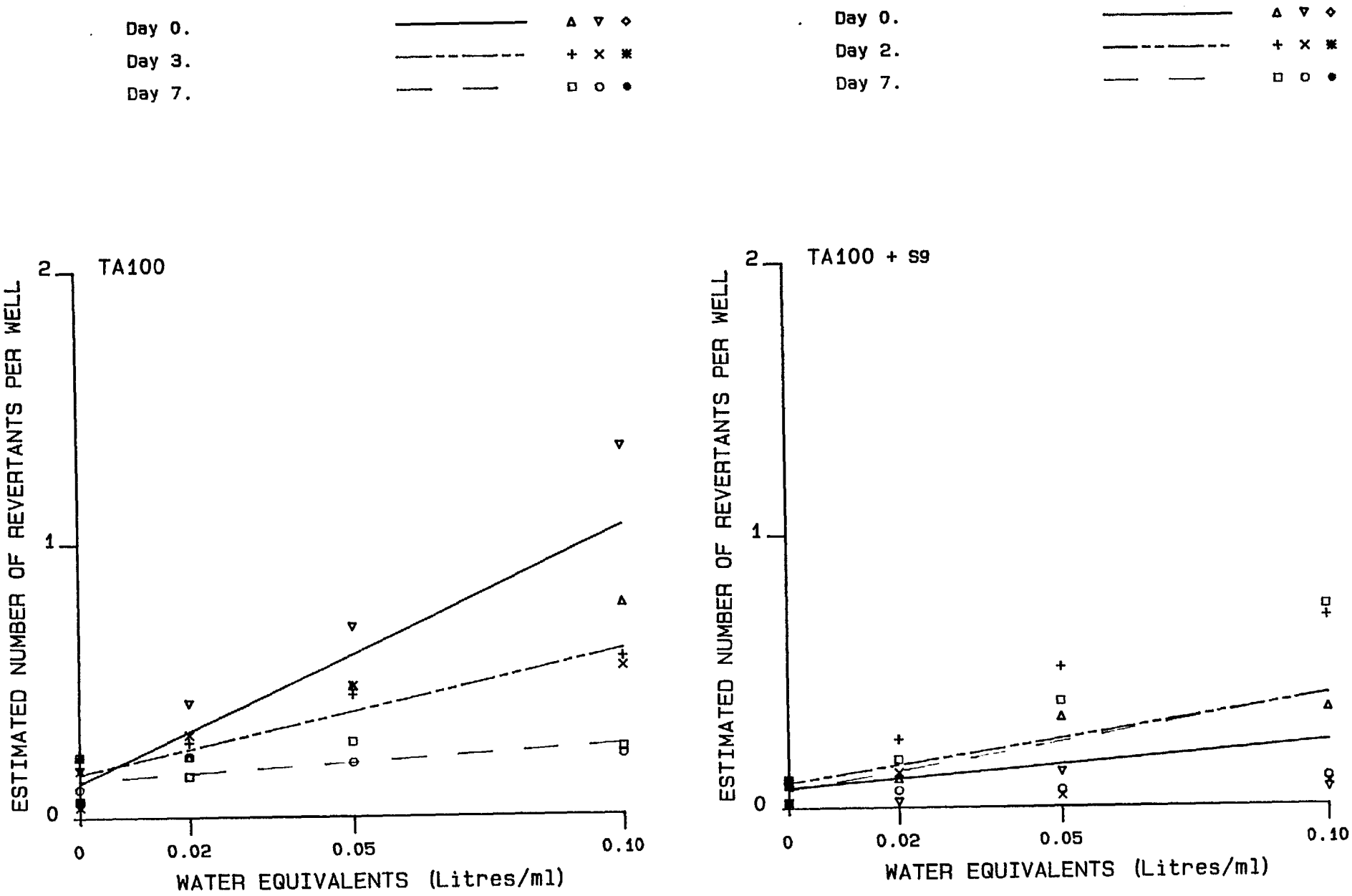

Figure A7. Laboratory storage of final water at 20°C.

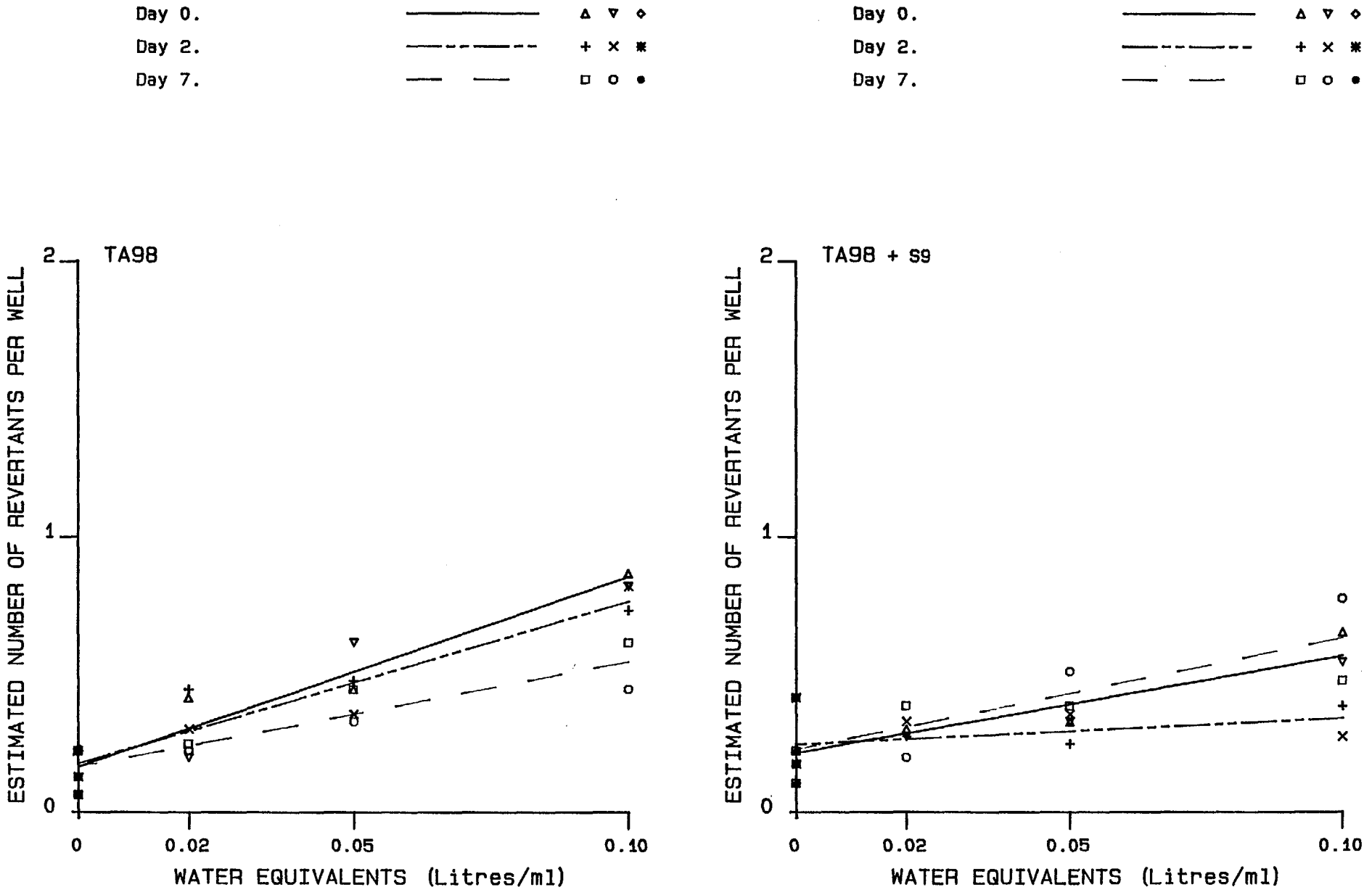

Figure A8. Laboratory storage of final water at 20°C.

APPENDIX B

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 C

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 sulphoxide 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 ¼-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 $MgCl_2$ 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.

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