THE EFFECT OF STORAGE ON THE COLIFORM AND *BACTERIUM COLI* COUNTS OF WATER SAMPLES

OVERNIGHT STORAGE AT ROOM AND REFRIGERATOR TEMPERATURE

By the Public Health Laboratory Service Water Sub-committee*

Frequent and regular bacteriological examination of water supplies at all stages from source to consumer is recognized as essential, and standard methods have been elaborated for the collection and examination of such samples. There is, nevertheless, considerable disagreement both on the maximum permissible time interval between sampling and bacteriological examination and on the method of storing the sample during this period.

If the sample cannot be examined immediately after collection, it should be maintained as nearly as possible in its original state until the examination can be made. This is possible only when the storage period is short, for as soon as the sample is collected the biological equilibrium is upset and changes in the bacterial flora begin. At temperatures over 10° C. the indigenous water bacteria may multiply and outgrow other more hygienically important forms, or those bacterial species for which the environment is unsuitable may die out.

The Franklands (1894) record the instance of a deep-well water in which the bacteria increased from seven per ml. to almost half a million per ml. after storage for 3 days at 20° C. Whipple (1901) studied the multiplication of bacteria in waters, and showed that a slight reduction in the number present, lasting perhaps for 4–6 hr., preceded the great increase noted by earlier observers. He also demonstrated that multiplication was greater during storage at high temperatures. It is probable that there is a constant increase of typical water bacteria which is not at first apparent because of a reduction in other forms for which the environment is unsuitable. ZoBell (1943) considers that this multiplication is due to the adsorption and concentration of nutrient substances upon the surface of the sampling bottle, so that the food becomes more readily available than when dispersed in low concentration throughout the water. He was unable to demonstrate this effect when the water contained an amount of food material excessive in relation to the bacteria present. The work of Taylor & Collins (1949) adds weight to this view.

It is now generally recognized that, to minimize bacterial multiplication, not only must samples be examined shortly after collection, but they must also be kept cool during the interval between collection and testing. Caldwell & Parr

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(1933) found that coliform organisms died off more or less rapidly on storage regardless of the temperature. Storage on ice generally reduced the death-rate of coliform organisms and of course prevented rapid multiplication of bacteria growing at 22 and 37° C. Webster & Raghavachari (1935) compared the coliform content of a variety of Madras waters which had been in transit, iced and un-iced, for 20–48 hr. before examination. They found that, whereas nineteen iced samples yielded typical faecal *coli*, the corresponding un-iced portions did not: the reverse occurred in only one sample. Prescott, Winslow & McCrady (1946) state that, if a fairly pure water is cooled with ice and kept at a temperature between 6 and 10° C., there will usually be little material increase in the bacterial count in 12 hr.

Water samples reach atmospheric temperature fairly rapidly unless adequate precautions have been taken. Even cold weather cannot always be relied on to keep samples at a low temperature: a period of a few hours in a warm train or motor-car may effect a radical change in the temperature of the sample. Current practice advocated by various authorities on this subject is as follows:

The Bacteriological Examination of Water Supplies (Ministry of Health, 1939, Report no. 71) recommends that samples should be despatched to the laboratory by the quickest route immediately after collection, the time occupied in transit being preferably less than 6 hr. If a longer period is likely to elapse, the sample should always be packed in ice. Thresh, Beale and Suckling (Taylor, 1949) recommend that the sample be kept cool with ice or other refrigerant if more than 2 hr. is likely to elapse between collection and examination. *Standard Methods for the Examination of Water and Sewage* (American Public Health Association and American Waterworks Association, 1946) recommends that the period between sampling and examination should not exceed 6 hr. for impure waters and 12 hr. for relatively pure waters; during transit the temperature should be kept between 6 and 10° C. The P.H.L.S. Water Application Form states that samples should reach the laboratory within 6 hr. of collection.

These opinions show a wide variation in the permissible time limits for storage before testing and an even wider variation in the conditions of storage during transit. Little more than preliminary studies on the influence of time and storage temperature on the coliform content of water samples have been recorded in the literature on the subject. The observations of Platt (1935), besides indicating 'the complexity of the factors determining the death of bacteria under natural conditions', demonstrated the rapid death-rate of coliform bacteria in samples kept at room temperature, and emphasized the importance of the ice-chest temperature for favouring the survival of faecal *coli*. Cox & Clayborne (1949) carried out a series of comparative tests on waters from various sources, including rivers and a swimming-pool, stored at refrigerator and at room temperature (about 26° C.). Refrigerated samples showed little change in the coliform counts after storage for up to 48 and sometimes 96 hr. or more. Samples stored at room temperature showed erratic and unpredictable fluctuations in the coliform count, but generally the counts fell sharply, reaching a level of about a quarter to a half of the original population within 24 hr. The swimming-pool samples (the pool was filled with chlorinated mains water which was not further chlorinated during use)

showed an increase in the coliform population at room temperature, which was even more rapid when samples were stored at 35° C. It should be remarked that the absolute number of coliform organisms in these samples was very high, the lowest count recorded being 390 per 100 ml.

Whilst this paper was being prepared for publication, a preliminary summary of the effect of overnight refrigeration of samples on the bacteriological examination of farm water supplies was recorded by Jones, Franklin & Thomas (1950). About 20 % of water samples examined 20 hr. after sampling, following 18 hr. storage at 3–5° C., had a significantly lower coliform content than when examined 2 hr. after collection. Samples with more than fifty coliform organisms per 100 ml. at the initial testing seldom showed an increased content after refrigeration. Colony counts at 22 and 37° C. did not reflect the reduction in numbers experienced in the coliform test.

The coliform test is now universally recognized as the most delicate and most dependable test for determining the safety of drinking water, and the formulation of a practicable standardized procedure for transportation and storage of samples between collection and examination should render the test even more valuable. It was with this end in view that the following work was undertaken.

METHODS

The standard method for determining the number of coliform organisms in a sample of water described in the Ministry of Health's Report (1939) is subject to a large error, both because of the small number of tubes examined at each dilution, and because of the large dilution factor. Halvorson & Ziegler (1933) state that when five tubes are used for each of three tenfold diminishing volumes of water the most probable number of coliform organisms given by probability tables may be too high by 260 % of the real value or too low by 70 %. It was, therefore, realized that some more accurate method would be required before any alteration in the number of coliform organisms detected after storage could be regarded as a real change and not merely an expression of the scatter of the standard test. Accordingly, a method recommended by Fisher & Yates (1943) and using seventy tubes was adopted. Ten tubes were inoculated with each of seven twofold diminishing volumes of the water sample, and the total number of tubes was noted in which acid and gas were produced after 48 hr. incubation at 37° C. For the interpretation of results, a table (see Appendix I) was prepared from which, given the largest volume of water inoculated and the number of tubes fermented, the most probable number (M.P.N.) of coliform bacteria in 100 ml. of the sample could be derived. The method was considered to have the greatest reliability when the number of positive (fermented) tubes roughly equalled the number of negative tubes.

In replicate tests of the same sample a difference of nine tubes or more in the number fermented would be expected to occur by chance only once in twenty times (5 %), increases or decreases occurring equally frequently, i.e. each once in forty times (2·5 %). As a working criterion a difference of nine tubes or more between the sample tested as soon after collection as possible, and the same

sample tested after storage for 20–24 hr.—either at room temperature or in the refrigerator—was taken to indicate a statistically significant change in the number of coliform organisms present. An increase of nine positive tubes corresponds approximately to a doubling of the number of organisms present per 100 ml. and an equal decrease to a halving of the number.

Choice of samples for examination

Samples were collected from rivers, reservoirs, lakes, springs and wells, both shallow and deep. Wells were classified as deep only when the water-bearing area lay beneath an impervious stratum.

A total of 151 samples were collected, from eighty sampling points scattered over the following counties of England and Wales: Bedfordshire, Berkshire, Caernarvonshire, Cambridgeshire, Cheshire, Denbighshire, Essex, Hampshire, Hertfordshire, Kent, Lancashire, Monmouthshire, Northamptonshire, Oxfordshire, Surrey and West Suffolk. The samples were examined at seven laboratories—Birkenhead, Cambridge, Conway, London, Manchester, Newport (Mon.) and Oxford. To allow for a possible seasonal variation, the samples were collected over a complete year from February 1950 to January 1951.

When possible, waters were selected whose regular coliform content was known, from repeated routine testing, to lie between ten and ninety per 100 ml. In many instances, preliminary examination by the standard method (Report no. 71) helped in determining the suitability of a water. When routine sampling showed an unpredictable variation in the coliform content of a water, the number of volumes used in the test proper was increased from seven to nine or ten, and the series of seven twofold dilutions which most nearly conformed to the standard required was chosen retrospectively.

Sampling

To avoid chance differences in coliform content which might have occurred in collecting three successive portions, samples from sources considered suitable were collected in sterile Winchester bottles filled to the brim in accordance with the technique for collection of samples laid down in Report no. 71, and transported to the laboratory without delay.

Storage

On arrival at the laboratory, the Winchester was inverted several times and a portion of the sample, approximately one-tenth, discarded. From the Winchester, after re-stoppering and vigorous shaking, three similar sterile bottles each holding at least 20 oz., labelled *A*, *B* and *C*, were filled completely. Bottle *A* was available for immediate examination by the seventy-tube method. Bottle *B* was stored in the dark at room temperature, bottle *C* was stored in the refrigerator. From the remainder of the sample in the Winchester, the pH and the oxygen absorbed from permanganate were determined; a standard (Report no. 71) coliform examination was also set up, and duplicate poured plates of 1 ml. of water were prepared to ascertain the bacterial plate counts in nutrient agar at 22 and 37° C.

Temperatures

Temperatures were observed: (1) of the sample at the time of collection; (2) of the room temperature where sample *B* was stored; (3) of the refrigerator temperature where sample *C* was stored.

Storage temperatures

Room temperature. This varied according to the time of year, but usually lay between 16 and 23° C.

Refrigerator temperature. This was consistent throughout the year between the limits of 2 and 5° C.

Examination

Time

Bottle *A* was examined immediately after filling, bottles *B* and *C* after 20–24 hr. storage.

Technique

In accordance with the technical procedure described in Report no. 71, volumes of 32, 16, 8 and 4 ml. of the sample were pipetted into tubes containing the same volumes of double-strength MacConkey broth; volumes of 2, 1, 0·5 and 0·25 ml. into tubes containing 5 ml. single-strength MacConkey broth. Sample volumes of less than 1 ml. were obtained by dilution of the water sample with sterile quarter-strength Ringer solution.

Inoculated tubes were incubated at 37° C. and were examined after 24 hr. Those showing acid and gas production were subcultured and incubated at 44° C. to test for the presence of faecal *coli*. Tubes not producing acid and gas at 24 hr. were incubated for a further period of 24 hr. and if at the end of that period acid and gas were produced these tubes were then subcultured and incubated at 44° C.

In this way it proved possible to compare the content of coliform organisms and faecal *coli* in a water tested within 2 hr. of collection and after 20–24 hr. storage both at room temperature in the dark and at refrigerator temperature.

Method of analysis

The principal technique used for the statistical appraisal of the results was the analysis of variance, which enables the average response of waters to storage to be assessed. In this and the analyses of the variability in response (see Appendix II) the actual magnitude of the changes in the number of positive tubes was used, as it is a more sensitive indicator than the 'all or nothing' working criterion, i.e. a difference of nine positive tubes or more between the sample tested as soon after collection as possible, and the same sample tested after storage for 20–24 hr. either at room temperature or in the refrigerator. For simplicity in Tables 1–3 and 5–8 the findings have been presented in terms of the working criterion (a difference of at least nine in the total number of positive tubes), but the tests of significance upon which the text is based were made from the full analysis.

EXPERIMENTAL

Technique controls

To determine how far the expectations based on the seventy-tube method described are fulfilled in practice, an experiment was carried out in four laboratories to assess whether the chance variability in the total number of positive tubes accorded with that predicted.

Twenty-two samples of water were tested in triplicate for presumptive *coli*, as soon after collection as possible, and of these fourteen were further examined for faecal *coli* (Table 1).

Table 1. *Comparison of triplicate tests on samples of a water*
(Changes of nine positive tubes or more.)

		Between first and second test Number showing			Between first and third test Number showing		
Type of test	No. of samples	Increase	No change	Decrease	Increase	No change	Decrease
Presumptive	22	0	22	0	0	21	1
Faecal	14	1	13	0	0	14	0

One of the forty-four comparisons for presumptive coliform organisms exceeded the working criterion of nine tubes difference; so did one of the twenty-eight comparisons for faecal *coli*. The chance variability was thus rather less than that expected of one in twenty. In other words, the theoretical foundation upon which later conclusions are based is a sound one.

Table 2. *Effect of overnight storage upon the presumptive coliform content of water samples*
(Changes of nine positive tubes or more.)

		Number showing			Percentage showing		
Storage	No. of samples	Increase	No change	Decrease	Increase	No change	Decrease
Room temperature	151	23	99	29	15·2	65·6	19·2
Refrigerator (2–5° C.)	151	10	115	26	6·6	76·2	17·2
		Theoretical percentage			2·5	95·0	2·5

Presumptive coliform count

The number of samples tested for presumptive coliform organisms was 151. The figures in Table 2 present the findings in terms of a difference of nine positive tubes or more between the sample examined as soon after collection as possible and after 20–24 hr. storage at room temperature and in the refrigerator.

Storage at room temperature

On storage there was a tendency for the number of organisms in the water to change. 15·2 % or approximately one sample in seven showed a significant increase, and 19·2 % or approximately one in five a significant decrease. This

increase or decrease means at least a doubling or a halving of the most probable number of coliform organisms originally present in the sample.

Storage at refrigerator temperature

6·6 % or approximately one sample in fifteen showed a significant increase and 17·2 % or approximately one in six a significant decrease (see Table 2). These results show less variation than those at room temperature, but it is evident that real changes in the presumptive count can take place even in the refrigerator. At both room and refrigerator temperatures decreases occurred more frequently than increases.

Faecal coli *count*

The number of samples tested was 111. Table 3 shows the effect of overnight storage at room temperature and in the refrigerator on the faecal *coli* content of water samples.

Table 3. *Effect of overnight storage upon the faecal* coli *content of water samples*

(Changes of nine positive tubes or more.)

		Number showing			Percentage showing		
Storage	No. of samples	Increase	No change	Decrease	Increase	No change	Decrease
Room temperature	111	8	74	29	7·2	66·7	26·1
Refrigerator (2–5° C.)	111	7	89	15	6·3	80·2	13·5
		Theoretical percentage			2·5	95·0	2·5

Storage at room temperature

7·2 % of samples or approximately one in fourteen showed a significant increase, and 26·1 % or approximately one in four a significant decrease in the faecal *coli* content, after overnight storage.

Storage at refrigerator temperature

6·3 % of samples or approximately one in sixteen showed a significant increase and 13·5 % or approximately one in seven a significant decrease in the faecal *coli* content (see Table 3).

At both room and refrigerator temperatures, decreases in the coliform and faecal *coli* content of water samples, as compared with the coliform and faecal *coli* content of the unstored samples, occurred more frequently than increases. Storage in the refrigerator caused less variation than storage at room temperature, but even on storage in the refrigerator, the tendency of the coliform and faecal *coli* content of water samples to vary is still substantial.

Extent of changes in coliform content of water samples

Tables 1–3 show that real changes take place in the presumptive coliform and the faecal *coli* content of water samples after storage. It is of considerable practical interest and importance to know the probable extent of these changes, and Table 4 presents this information. There were seven instances (with six separate

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samples of water) of at least a sevenfold increase in the coliform or faecal *coli* content of the water; there were twenty-one instances (with eighteen separate samples of water) of a decrease in the most probable number to one-seventh of its original value, or less. To illustrate the changes which may occur after overnight storage, some specific examples have been selected.

Presumptive coli *test*

Oxford no. 6. A decrease from seventy-nine per 100 ml. to six at room temperature.

Newport no. 4. A decrease from fifty per 100 ml. to three at refrigerator temperature.

Faecal coli *test*

Manchester no. 9. A decrease from twenty-six per 100 ml. to two at room temperature.

Cambridge no. 3. An increase from ten per 100 ml. to seventy-nine at refrigerator temperature.

But perhaps the most remarkable result was for Newport no. 7. This sample had an initial count of 100 presumptive coliform organisms per 100 ml. The effect of storage at room temperature was to increase the most probable number to over 500, whereas on storage in the refrigerator the value fell to 11. This particular water contained virtually no faecal *coli*.

Initial coliform content of water samples

At one stage of the investigation it had seemed that the response to storage might depend upon the initial density of the organisms present. This possibility was investigated and the results are shown in Table 5.

The percentage of waters which showed no change after overnight storage appeared to be independent of the original coliform or faecal *coli* content. It will be seen that, in the waters which had less than fifty presumptive *coli* or twenty faecal *coli* per 100 ml. originally, the increases and decreases occurred with approximately equal frequency. The more heavily polluted waters, however, showed less tendency to increase, and more tendency to decrease their content of organisms. Approximately one in four of the heavily polluted waters showed a significant decrease of organisms after storage, whether at room temperature or in the refrigerator.

Source of water

Waters were classified into three broad types: surface, including river, reservoir and lake water; underground shallow, combining shallow wells and springs; and underground deep, comprising deep well waters. The figures in Table 6 show that a high percentage of samples from surface and underground shallow sources varied on storage, but that the response of underground deep waters differed little from that to be expected by chance. This suggestion that waters from underground deep sources do not vary on storage would require to be confirmed by the examination of a very much larger number of samples.

Table 4. *The extent of variations in the most probable number of coliform or faecal* coli *organisms after storage for* 20–24 *hr.*

		Increases							Decreases					
Type of test	Temperature	7-fold or more	6- to 7-fold	5- to 6-fold	4- to 5-fold	3- to 4-fold	2- to 3-fold	No significant change	2- to 3-fold	3- to 4-fold	4- to 5-fold	5- to 6-fold	6- to 7-fold	7-fold or more
Presumptive *coli*	Room temperature	1	0	4	1	7	10	99	8	5	2	3	3	8
	Refrigerator	1	0	1	0	2	6	115	14	4	2	1	2	3
Faecal *coli*	Room temperature	3	0	0	0	1	4	74	12	4	1	2	1	9
	Refrigerator	2	0	0	0	2	3	89	10	3	1	0	0	1

Table 5. *Effect of storage upon the number of coliform organisms and faecal* coli *in water samples classified according to the mean density of organisms before storage*

(Changes of nine positive tubes or more.)

				No. showing			Percentage showing		
Examination	Temperature of storage	Mean no. of organisms per 100 ml. before storage	No. of samples	Increase	No Change	Decrease	Increase	No Change	Decrease
Presumptive *coli* count	Room temperature	Under 50*	80	13	54	13	16·2	67·5	16·2
		50 and over*	71	10	45	16	14·1	63·4	22·5
	Refrigerator	Under 50	80	6	64	10	7·5	80·0	12·5
		50 and over	71	4	51	16	5·6	71·8	22·5
Faecal *coli* count	Room temperature	Under 20*	59	5	45	9	8·5	76·3	15·3
		20 and over*	52	3	29	20	5·8	55·8	38·5
	Refrigerator	Under 20	59	5	52	2	8·5	88·1	3·4
		20 and over	52	2	37	13	3·8	71·2	25·0

* These counts were arbitrarily chosen because they yield contrasting groups of approximately equal size.

Table 6. *Effect of overnight storage upon the number of coliform organisms in water samples classified according to source*

(Changes of nine positive tubes or more.)

Source	Presumptive *coli*		Faecal *coli*	
	No. of comparisons before and after storage	Percentage showing increase or decrease	No. of comparisons before and after storage	Percentage showing increase or decrease
Surface	98	27·6	80	38·8
Underground shallow	184	32·6	128	21·1
Underground deep	20	5·0	14	7·1
Total	302	29·1	222	26·6

Laboratory

Waters were classified according to the laboratory in which they had been examined (Table 7). The variability of the samples in response to storage was more marked at some laboratories than at others. This variability may well have arisen from the type of water sampled, possibly emphasized by repeated sampling of a particular supply.

Table 7. *Effect of overnight storage upon the number of coliform organisms in water samples according to laboratory*

(Changes of nine positive tubes or more.)

Laboratory	Presumptive *coli*		Faecal *coli*	
	No. of comparisons before and after storage	Percentage showing increase or decrease	No. of comparisons before and after storage	Percentage showing increase or decrease
Birkenhead	28	32·1	6	50·0
Cambridge	38	23·7	38	21·1
Conway	54	24·1	26	19·2
London	44	15·9	44	34·1
Manchester	40	40·0	28	28·6
Newport	38	39·5	36	16·7
Oxford	60	31·7	44	31·8
Total	302	29·1	222	26·6

Season of sampling

Table 8 shows that the waters sampled from January to March varied more on storage than samples taken at other seasons. Detailed analysis by months suggests that this does not arise from a straightforward relation with the time of year.

Table 8. *Effect of overnight storage upon the number of coliform organisms in water samples according to season*

(Changes of nine positive tubes or more.)

	Presumptive *coli*		Faecal *coli*	
Season of sampling	No. of comparisons before and after storage	Percentage showing increase or decrease	No. of comparisons before and after storage	Percentage showing increase or decrease
January to March	38	44·7	24	45·8
April to June	88	29·5	58	29·3
July to September	94	27·7	72	30·6
October to December	82	23·2	68	13·2
Total	302	29·1	222	26·6

DISCUSSION

It was decided at the outset to limit this investigation to drinking water supplies which were naturally contaminated with coliform bacteria. Experiments such as those of Fricker (1944), who artificially contaminated samples of drinking water with coliform cultures, were, it was felt, less likely to reflect what might actually occur in normal practice. The losses he demonstrated might be at least partly due to the use of fresh cultures, whereas the coliform organisms still present at the time of collection in a sample of naturally contaminated water might well represent the hardier survivors of a much larger original population and might, therefore, more easily persist through the projected period of storage.

Furthermore, the seventy-tube method, which is cumbersome and quite unsuitable for routine use, was chosen only after deliberate consideration of the alternatives. The use of a solid medium (Harold, 1936) was considered, but for the purpose of this investigation it was felt essential to use a liquid medium in a technique resembling as closely as possible that in ordinary use. The standard (Report no. 71) method, even if replicated several times, would be statistically less satisfactory than the seventy-tube method. Once the 'technique controls' had substantiated the expected accuracy of the seventy-tube method it became clear that this was the best available method for the purpose, in that, though elaborate, it closely resembled the standard method but offered the necessary accuracy.

The tabulated results show that in a substantial number of waters the presumptive coliform content and the faecal *coli* content are significantly altered by overnight storage either at room temperature or in the refrigerator, decreases being more frequent than increases. There was less variation in the refrigerator than at room temperature (Tables 2 and 3), but even in the refrigerator the changes were substantial.

That the variation could be regarded as real depended upon the accuracy of the method of examination which was used. Had the standard (Report no. 71) method been used, some of the real variations in coliform content that did occur might have been regarded as falling within the normal scatter of the test. It

may well be that some of the conflicting views already expressed on this subject have arisen from the use of statistically less sensitive methods.

So far as the present investigations go, the coliform and faecal *coli* content of waters from all sources seem to change on storage, except perhaps waters obtained from beneath an impervious stratum. Heavily polluted waters showed more tendency for their content of organisms to decrease than to increase. No correlation was apparent between the change in the coliform and faecal *coli* content and the pH, oxygen absorbed, or the plate counts in nutrient agar. This is less surprising when it is considered that successive samples, from the same source and showing no striking differences on collection, frequently behaved quite differently on storage. For example, a land spring on first sampling showed considerable loss of both presumptive and faecal *coli* after storage at room temperature, but no change after storage in the refrigerator. On second sampling there was no change in the presumptive count at either temperature, but a significant loss of faecal *coli* at both temperatures. On sampling for the third time, only a week later, no changes were found in either count at either temperature. Or again, a loss of faecal *coli* with no change (or even a gain) in the presumptive count at either temperature might be found. Altogether, all possible permutations were encountered.

The most extreme change of all, a significant drop to zero on storage (a result previously observed in practice—G. S. Wilson, personal communication), occurred only four times in this series—once with the presumptive count and three times with faecal *coli*—in all cases after storage at room temperature.

CONCLUSIONS

After 20–24 hr. storage in a filled sampling bottle either at room temperature or in the refrigerator, approximately one in five samples of water will at least halve its content of faecal *coli* and one in fifteen will at least double it. A rather higher proportion of waters will show corresponding variations in the presumptive coliform count.

The faecal *coli* content is less likely to be altered by overnight storage of the sample in the refrigerator than at room temperature, but even in the refrigerator the tendency to vary is considerable.

This investigation has shown that overnight storage of a water sample, even in the refrigerator, is liable to cause a real change in its coliform and faecal *coli* content.

SUMMARY

This investigation has been concerned with the changes that occur in the coliform and faecal *coli* content of water samples on storage at room and refrigerator temperature for 20–24 hr.

On examination by a seventy-tube method, using twofold diminishing volumes, twenty-three out of 151 samples of water stored overnight at room temperature showed a significant increase in the presumptive number of coliform organisms and twenty-nine a significant decrease. Of the same number stored in the refrigerator, ten showed a significant increase and twenty-six a significant decrease.

Of 111 samples examined for faecal *coli*, eight showed a significant increase on storage at room temperature and twenty-nine a significant decrease. Of the same number stored in the refrigerator, seven showed a significant increase and fifteen a significant decrease. The effects of the source of the water, the time of year and the original number of coliform organisms in the sample were examined.

The present investigation has shown that in a considerable proportion of samples a significant change in the coliform and faecal *coli* content does occur on overnight storage at room or refrigerator temperature. It may, perhaps, be safe to store samples under some conditions for shorter periods, but this is a matter for future investigation.

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APPENDIX I

Expected numbers of organisms per 100 *ml. of water in a test with ten tubes inoculated with each of seven volumes, diminishing twofold, the largest volume of water being respectively,* 32, 16 *or* 8 *ml.*

No. of tubes with positive reaction	Most probable number of organisms per 100 ml. Largest of seven volumes		
	32 ml.	16 ml.	8 ml.
5	1	2	3
6	1	2	4
7	1	3	5
8	1	3	6
9	2	3	7
10	2	4	8
11	2	4	9
12	2	5	10
13	3	5	11
14	3	6	12
15	3	6	13
16	4	7	14
17	4	8	15
18	4	8	17
19	5	9	18
20	5	10	20
21	5	11	21
22	6	11	23
23	6	12	25
24	7	13	27
25	7	14	29
26	8	15	31
27	8	17	33
28	9	18	36
29	10	19	38
30	10	21	41
31	11	22	44
32	12	24	48
33	13	26	51
34	14	28	55

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Appendix I (*cont.*)

No. of tubes with positive reaction	Most probable number of organisms per 100 ml. Largest of seven volumes		
	32 ml.	16 ml.	8 ml.
35	15	30	59
36	16	32	64
37	17	34	69
38	18	37	74
39	20	40	79
40	21	43	85
41	23	46	92
42	25	50	99
43	27	53	107
44	29	58	115
45	31	62	125
46	34	67	135
47	36	73	146
48	39	79	158
49	43	86	171
50	46	93	185
51	50	100	201
52	55	109	218
53	59	119	238
54	65	129	259
55	71	142	283
56	77	155	310
57	85	170	340
58	93	187	373
59	103	206	411
60	114	228	455
61	126	252	505
62	141	282	564
63	158	317	634
64	179	359	718

APPENDIX II

Notes on the statistical analyses

The details of general formulae applicable to this type of dilution test are given by Fisher & Yates (1943). With a dilution factor of two, the average value of the variance of the total number of positive tubes equals the number of tubes at each dilution level—here ten. Thus the standard error of the difference between two totals of positive tubes equals $\sqrt{(10+10)} = 4{\cdot}5$. Twice this standard error represents a difference of nine in the totals of positive tubes, and a difference of this size or more was adopted as a working criterion of a real change in the coliform content of a water.

The sampling variance of the total number of positive tubes is sufficiently stable to be used for calculating fiducial limits and so it is justifiable to use the total of positive tubes without transformation as a variable in an analysis of variance. It may be noted that the sampling variance does not depend upon the volume of water used for the least dilute of the seven levels.

In order to check whether the practical procedure gave results according with the theory, a number of waters were tested in triplicate as soon after collection as possible—twenty-two for presumptive *coli* and fourteen for faecal *coli* (see

Table 9. *Analyses of variance for the technique controls*

	Presumptive *coli*			Faecal *coli*		
Component	Degrees of freedom	Sum of squares	Mean square	Degrees of freedom	Sum of squares	Mean square
Between waters	21	19,665·53	936·45	13	13,093·07	1007·16
Within waters	44	303·33	6·89	28	245·33	8·76
Total	65	19,968·86	—	41	13,338·40	—
Theoretical variance within waters	—	—	10·00	—	—	10·00

Table 1). The resulting analyses of variance are shown in Table 9. The variation between waters, as was expected, considerably exceeds the variation between replicate tests on the same water. That is, the waters differed in their content of coliform organisms. Tests of the mean square 'within waters' against the theoretical sampling variance give the following results:

Test	Value of χ^2	Degrees of freedom	Chance probability
Presumptive *coli*	30·33	44	$P = 0{\cdot}94$
Faecal *coli*	24·53	28	$0{\cdot}7 > P > 0{\cdot}5$

Both mean squares 'within waters' are rather less than the theoretical value, but not to a greater extent than would be expected by chance. There is thus no suggestion that the sampling variance in practice differs from that predicted by theory.

General effect of storage on presumptive coli

Details of the analysis of variance are given in Table 10. The highly significant variance ratio 'Between waters' is simply indicative of the differences in initial coliform content of the waters sampled. The component 'Between tests' can be split into two portions, one representing the average effect of overnight storage and the other corresponding to the difference between storage in a refrigerator and storage at room temperature. When these mean squares are compared with their respective interaction terms it is seen that over and above any differences in relative response from water to water there is a significant general effect of storage, which is, however, very small. The average number of positive tubes per test was 37·8 initially and 36·1 after storage. On the other hand, there is no suggestion that the temperature exerts any determining influence upon the response to storage. The mean squares for the interactions 'Waters × storage' and 'Waters × temperature' are each very significantly greater than the theoretical sampling variance of 10.

	Value of χ^2	Degrees of freedom	Chance probability
Waters × storage	702·2	150	$0{\cdot}001 > P$
Waters × temperature	822·3	149	$0{\cdot}001 > P$

That is to say, the response of waters to storage at either temperature is not uniform, but varies substantially from one water to another. This variation is illustrated by the figures in Table 4.

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It is possible that some of this variability in response of waters to storage can be attributed to differences in the average response at different laboratories, or to different average responses of certain classes of water (source), or similarly to the month of sampling, or to the initial content of coliform organisms in the water. The lower part of Table 10 gives certain sums of squares which assist a decision on this point. The 'count' refers to the most probable number of organisms per 100 ml. of water at the initial test, and this was grouped as 0–4, 5–9, 10–19, 20–49,

Table 10. *Analysis of variance—presumptive* coli

Component	Degrees of freedom	Sum of squares	Mean square	Variance ratios
Between waters	150	124,420·63	829·47	16·27*
Between tests	2	372·40	186·20	3·65†
Storage	1	320·66	320·66	6·85†
Temperature	1	51·74	51·74	1:1·07
Waters × tests	299‡	15,244·93	50·99	1·00
Waters × storage	150	7022·17	46·81	1·00
Waters × temperature	149‡	8222·76	55·19	1·00
Labs × storage	6	393·78	65·63	
Sources × storage	2	83·35	41·68	
Months × storage	11	759·48	69·04	
Count × storage	6	773·55	128·92	
Labs × temperature	6	405·10	67·52	
Sources × temperature	2	12·52	6·26	
Months × temperature	11	449·20	40·84	
Count × temperature	6	204·47	34·08	
Total	451‡	140,037·96		
Theoretical sampling variance			10·00	

* Chance probability less than 1 %.
† Chance probability between 5 and 1 %.
‡ One degree of freedom has been subtracted to compensate for one missing reading, which was estimated by the standard procedure.

50–99, 100–249 and 250 and over. Each mean square can be tested separately against the residual of the interaction of which it forms a part, as follows:

	Degrees of freedom	Sum of squares	Mean square	Variance ratio
Storage × count	6	773·55	128·92	2·97*
Storage × other differences	144	6248·62	43·39	1·00
Storage × waters	150	7022·17	—	—

* The variance ratio of 2·97 is significant at the 1 % level.

Thus there are differences in average response according to the degree of initial pollution which predominate over other causes of differences in average response to storage. This is actually the only one of the eight comparisons of this type which attains significance. It must be emphasized that the four factors identified were not built orthogonally into the experiment; thus their effects may be intermingled and cannot be assessed independently. But in view of the non-significance of the other comparisons it seems justifiable to conclude without the labour of a non-orthogonal analysis that the initial content of coliform organisms was the only important correlate of the four with the behaviour of water on storage. When the presumptive coliform count was initially 0–49 per 100 ml. the average decrease

on storage was 0·6 positive tubes. For more heavily polluted waters the average decrease was 3·1 positive tubes. This difference can also be observed from the figures in Table 5.

General effect of storage on faecal coli

Details of the analysis of variance are given in Table 11. The following conclusions may be drawn from it.

There is a slight general tendency for the faecal *coli* content to decrease after storage for 24 hr. The average number of positive tubes per test was 24·5 initially and 21·8 after storage.

Table 11. *Analysis of variance—faecal* coli

Component	Degrees of freedom	Sum of squares	Mean square	Variance ratios
Between waters	110	85,993·43	781·76	23·90*
Between tests	2	696·81	348·40	10·65*
Storage	1	545·96	545·96	14·27*
Temperature	1	150·85	150·85	5·57†
Waters × tests	219‡	7,162·52	32·71	1·00
Waters × storage	110	4209·87	38·27	1·00
Waters × temperature	109‡	2952·65	27·09	1·00
Labs × storage	6	539·97	90·00	
Sources × storage	2	38·83	19·42	
Months × storage	10	675·69	67·57	
Count × storage	6	499·10	83·18	
Labs × temperature	6	134·85	22·48	
Sources × temperature	2	3·39	1·70	
Months × temperature	10	136·42	13·64	
Count × temperature	6	268·91	44·82	
Total	331‡	93,852·76		
Theoretical sampling variance			10·00	

* Chance probability less than 1 %.
† Chance probability between 5 and 1 %.
‡ One degree of freedom has been substracted to compensate for one missing reading, which was estimated by the standard procedure.

In addition, there is a slight average benefit of storage in a refrigerator over that at room temperature, which is significant at the 5 % level. The average number of positive tubes after storage in a refrigerator was 22·6, but after storage at room temperature 21·0. No such benefit appeared with presumptive *coli*, but it is clearly of small importance.

Notwithstanding these general tendencies, the response to storage at either temperature varies substantially from one water to another. Each χ^2 test is significant at the 0·1 % level.

For faecal *coli* three of the eight interaction terms are significantly greater than their residuals at the 5 % level, namely those for storage with count, laboratories and months. This illustrates the severe limitations of non-orthogonal data, as a complicated analysis, which was not undertaken, would be needed to disentangle their separate effects. But in view of the findings for presumptive *coli* it is not improbable that for faecal *coli* too the initial content of organisms is the one relevant influence. When the count of faecal *coli* was initially 0–19 per 100 ml.

the average decrease on storage was 0·5 tube. For the more heavily polluted waters the average decrease was 5·1 tubes, which corresponds approximately to a 30% decrease in content of faecal *coli*. Table 5 illustrates this tendency.

The extent of variability in response to storage

The usefulness of the analysis of variance is in assessing the importance of factors which act equally on all waters and so lead to changes in the *average* response of waters to storage. The analyses just given have shown that the average changes which have been detected are of small importance compared with the large fluctuations which may occur in either direction. The *variability* of response thus merits special analysis.

It is important to know if waters show less variability when stored at refrigerator temperature than at room temperature. The difference between the total of positive tubes initially, and after storage in the refrigerator, was taken as a measure of the variation in coliform content occurring at that temperature, and similarly for storage at room temperature. The mean squares of these quantities were:

	Degrees of freedom	Variance		Theoretical sampling variance
		Room temperature	Refrigerator	
Presumptive *coli*	150	117·94	77·32	20·00
Faecal *coli*	110	96·47	45·19	20·00

At both temperatures the variance estimates significantly exceed the sampling value of 20. Since the measures of the effect of storage at the two temperatures are not independent of one another, their variances cannot be compared by the usual variance test. The test derived by Pitman (1939) is the appropriate one, and gives the following results:

	Value of t	Degrees of freedom	Chance probability
Presumptive *coli*	2·90	149	$0{\cdot}01 > P > 0{\cdot}001$
Faecal *coli*	5·44	109	$0{\cdot}001 > P$

Thus the response to storage at refrigerator temperature is significantly less variable than at room temperature, though still substantially greater than would be expected if there were no real changes in coliform content.

It is also important to determine whether there are differences in the variability of response between laboratories or similarly between sources, months or the initial coliform content of the water. As before, these influences are not mutually orthogonal, and so it is not possible to disentangle their several effects. The homogeneity of each set of variances was tested by Hartley's method, using the tables of Thompson & Merrington (1946), and the results are given in Table 12. It is not easy to draw any useful conclusions from this table. There certainly seem to be differences in variability of response between the laboratories; these may have arisen from regional differences, since the nature of the source also appears to affect the extent to which the stored sample may vary. It is evident on examining the variances for the individual months that the differences apparent there do not arise from a straightforward relation with the time of year. There seems to be

Table 12. *Values of M for testing the homogeneity of certain sets of variances*

		Presumptive *coli*		Faecal *coli*	
Set of Variances	Number in set	Room temperature	Refrigerator	Room temperature	Refrigerator
Laboratories	7	22·57*	23·47*	18·96*	14·18*
Sources	3	8·95†	20·89*	11·66*	5·03
Months	11‡	19·28†	19·73†	36·64*	11·70
Count	7	12·78	12·18	12·13	20·54*

* Chance probability less than 1 %.

† Chance probability between 5 and 1 %.

‡ Only one sample was tested in February and so there are no estimates of variance for this month.

little evidence of any important differences between waters with differing degrees of initial pollution.

It is of interest to note that the variances for underground deep waters do not differ significantly from the theoretical value of 20.

	Degrees of freedom	Variance	
		Room temperature	Refrigerator
Presumptive *coli*	9	19·56	7·79
Faecal *coli*	6	20·95	25·62

The numbers of waters tested was small, but this finding suggests that underground deep waters may not show any real changes in content of coliform organisms on storage.

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