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# Autoradiographic detection of radioactive bacteria introduced into sea water and sewage

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The agency of water in the dissemination of bacteria and viruses has long been recognized, but though measures have been taken to control this hazard, water still plays a large role in spreading pathogens such as enterovirus and salmonella. A simple and reliable method for studying dissemination of this kind would therefore be most useful.

The presence in water of thermostable coli bacteria is generally considered an index of fæcal contamination. Unfortunately, their detection in a water sample is a laborious procedure which requires growing cultures, often on different media, for confirmation. Furthermore, the significance of one particular source of contamination when several are present cannot be determined with current bacteriological technique. The problem has been previously attacked by using mutants of the contaminating micro-organisms, or indicator organisms such as *Serratia* (Robson, 1956). The tracer bacteria are added to the water at the source of contamination and water samples are taken for bacteriological examination.

Radioactive substances and dyes have also been used to study the spread of contamination (Montens, 1954; Ely, 1957). Because of rapid dilution, high levels of radioactivity are necessary to follow dispersion in large volumes of water. In addition, the action of biological factors on the survival of contaminating bacteria cannot be ascertained with these materials alone.

Bacteria which have been radioactively labelled can be used as tracer organisms. If they can be detected by a method which avoids the necessity of culturing water samples and if the method is sufficiently sensitive, the use of radioactive bacteria offers several advantages. In the present paper such a method is described, for use in studying the spread of bacteria in water. Water samples were filtered through a Millipore filter and an autoradiogram of the filter was prepared in which the bacteria appeared as black spots. The accuracy and sensitivity of the method were estimated and a test made under field conditions.

## METHODS

Strain B *Escherichia coli* were used. The bacteria were grown overnight at 37° C. in agitated 500 ml. flasks on Andersson's synthetic medium.* The medium was modified by replacing $MgSO_4$ with $MgCl_2$ to eliminate carrier sulphur. One micro-

* $MgSO_4$, 0·1 g.; $Na_2HPO_4$, 6 g.; $KH_2PO_4$, 3 g.; NaCl, 5 g.; $NH_4Cl$, 1 g.; glucose, 4 g.; $H_2O$, ad 1000 ml.; pH = 7.

curie $^{35}S$, carrier-free, in 2 ml. isotonic saline solution, was added per 100 ml. medium. This synthetic medium does not provide for optimal growth but the low sulphur content is necessary for the bacterial production of amino acids labelled with radiosulphur. The presence of organic sulphur compounds would prevent the incorporation in the bacteria of radiosulphur in sufficient amounts. A culture containing $3 \times 10^8$–$5 \times 10^9$ bacteria/ml., as determined by direct counting under the microscope, was generally used in the experiments.

The number of bacteria in water samples to which a known number of bacteria had been added was determined both by viable count and by the autoradiographic technique. The water samples were prepared from the bacterial culture by dilution with 0·9 % NaCl to an estimated concentration of 10 (sample B 1) and 100 (sample B 2) bacteria per ml. A control sample (C) was made with sterile radioactive media to the same dilution as B 2.

The viable count was performed by inoculating 1 ml. from each water sample in nutrient agar and incubating at 37° C. for 24 hr.

The autoradiographic determination of bacteria was made by diluting 1 ml. from each water sample in 500 ml. of 0·9 % NaCl and filtering the solution through a Millipore filter (type HA, pore size 0·45 $\mu$). The filters were dried at 50° C. for 20 min. and applied to X-ray film (Gevaert Osray). After 10 days' exposure the films were developed. Black spots (Pl. 1) which appeared similar in structure to autoradiographic images of bacteria obtained by other authors (e.g. Stonier, 1956) were taken to be bacteria. The spots were counted under × 10 magnification, but some films were also counted under higher magnification. The number of spots on unexposed films from the same package as the exposed films was also determined.

Ten determinations were made from all water samples and the autoradiograms from different samples were counted independently of each other so that the technician making the counts was not influenced by previous totals. Stainless steel funnels were used for filtration and were washed twice with 0·9 % NaCl at the end of every filtration to remove any radioactive medium present. Before they were developed, the films were separated from the filters under water to avoid electrostatic phenomena which might cause black spots on the films.

The possibility that a thin layer of sludge on the filter interfered with the number of spots was investigated by filtering water samples obtained by diluting the culture of labelled bacteria with heavily polluted water instead of 0·9 % NaCl.

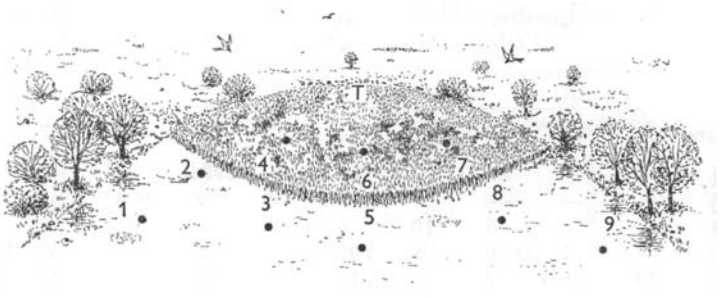
The survival of radioactive bacteria was studied in two water samples. One sample was kept at room temperature and the other at 4° C. Determinations of the number of bacteria were made at regular intervals by using the autoradiographic technique and viable count.

To obtain an idea of the practicability of the autoradiographic method, a study was made of the spread of bacteria from the effluent of a household septic tank which discharged via a sewer into a small inlet of the Baltic Sea near Stockholm. A dense stand of reeds extended for 30–40 m. on both sides of the sewer outlet and in front, for some 20 m. out into water 2 m. deep (Text-fig. 1).

To investigate the presence of substances which might stimulate bacteria and to investigate how this 'background' varied, autoradiograms were prepared from water samples obtained before radioactive bacteria were added to the sewer.

With the aid of a dye a preliminary description of the spread of sewage was obtained, and a number of sites for sampling was selected.

A 200 ml. portion of medium containing radioactive bacteria was then added to the contents of the septic tank by pouring the bacteria into a water closet. For 24 hr. afterwards water samples were taken at varying intervals at the different sampling sites. Two filters were prepared from every sample and the bacteria




Site

| | 1 | | 2 | | 3 | | 4 | | 5 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Film | Film | Film | Film | Film | Film | Film | Film | Film | Film |
| Hours | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 |
| $\frac{1}{3}$ | — | — | — | — | — | — | 123 | 148 | — | — |
| $1\frac{1}{2}$ | — | — | — | — | 616 | 835 | ∞ | ∞ | 46 | 14 |
| 4 | 14 | 6 | 1378 | 1545 | ∞ | ∞ | ∞ | ∞ | 5 | 5 |
| 8 | 55 | 80 | 151 | 160 | 2344 | 2459 | ∞ | ∞ | 45 | 38 |
| 18 | 3 | 1 | 3 | 6 | 93 | 112 | ∞ | ∞ | 38 | 44 |
| 24 | 3 | 6 | 4 | 13 | 287 | 277 | 1029 | 1097 | 73 | 92 |

Site

| | 6 | | T | | 7 | | 8 | | 9 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Film | Film | Film | Film | Film | Film | Film | Film | Film | Film |
| Hours | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 |
| $\frac{1}{3}$ | 54 | 47 | ∞ | ∞ | 30 | 26 | — | — | — | — |
| $1\frac{1}{2}$ | 482 | 493 | ∞ | ∞ | 22 | 27 | 44 | 51 | — | — |
| 4 | ∞ | ∞ | ∞ | ∞ | 3 | 0 | 14 | 15 | 6 | 11 |
| 8 | 20 | 24 | ∞ | ∞ | 7 | 2 | 12 | 19 | 7 | 3 |
| 18 | 59 | 58 | ∞ | ∞ | 6 | 13 | 2 | 0 | 6 | 1 |
| 24 | 33 | 216 | ∞ | ∞ | 9 | 11 | 25 | 44 | 4 | 9 |

Text-fig. 1. Field site, showing sea-shore and stand of reeds (not to scale). The table gives the number of bacteria in autoradiograms from each of ten samples collected at sites indicated, at $\frac{1}{3}$, $1\frac{1}{2}$, 4, 8, 18 and 24 hr. after release of radioactive bacteria into septic tank discharging at T. Symbol ∞ indicates bacteria too numerous to count.

appearing in the autoradiograms were counted. Because of windy weather quite a lot of suspended matter was present in the water and only 50 ml. could be used in each filtration.

In an experiment performed some weeks later, 200 ml. of culture containing radioactive bacteria were released beyond the growth of reeds, on the sea bottom. Samples were taken from a depth of $\frac{1}{2}$ m., at varying distances from the outlet (Text-fig. 2). 200 ml. of each water sample were filtered in this experiment.

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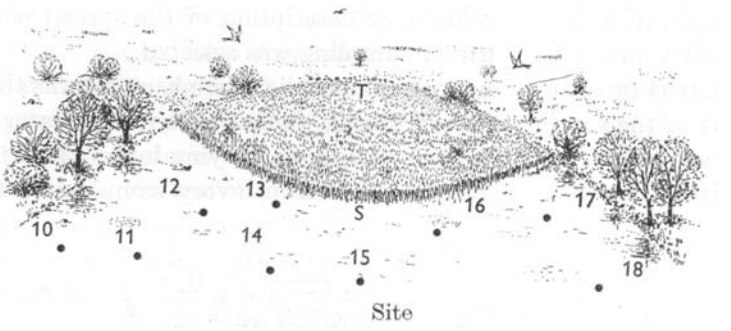


| Hours | 10 | 11 | 12 | 13 | 14 | 15 | S | 16 | 17 | 18 |
|---|---|---|---|---|---|---|---|---|---|---|
| ¼ | — | — | — | — | — | 10 | 2924 | — | — | — |
| ½ | — | — | — | — | 12 | 158 | ∞ | 624 | — | — |
| 1 | — | — | 5 | 15 | 5 | 100 | ∞ | ∞ | ∞ | — |
| 2 | — | 7 | 1869 | 8 | 590 | 2000 | ∞ | ∞ | ∞ | 1176 |
| 8 | 74 | 68 | 7 | — | 17 | 10 | 538 | 7 | 24 | 315 |
| 23 | 9 | 13 | 2 | 19 | 46 | 10 | 11 | 2 | 7 | 4 |

Text-fig. 2. Number of bacteria in autoradiograms ¼, ½, 1, 2, 8 and 23 hr. after release of radioactive bacteria on sea bottom at site S.

## RESULTS

### *Comparison between viable count and autoradiographic determination*

An average of 83 spots (S.D. = 12) and 83 (11) were found in two counts of the autoradiograms prepared from 1 ml. of sample B 1. The number of colonies on the nutrient agar plates from the same sample was 71 (23). Bacterial dilution B 2 gave 612 (44) and 609 (46) spots in two counts of the autoradiograms and 503 (69) colonies on nutrient agar. In the control experiments ten unexposed films showed 1·8 (1·6) black spots. The autoradiograms prepared from 1 ml. of sample C

Table 1

| | Bacteria dilution B 1 | | | Bacteria dilution B 2 | | |
|---|---|---|---|---|---|---|
| | | Autoradiography, 1 ml. diluted in 500 ml. NaCl | | | Autoradiography, 1 ml. diluted in 500 ml., NaCl | |
| Filtration | Viable count, 1 ml. | Count 1 | Count 2 | Viable count, 1 ml. | Count 1 | Count 2 |
| 1 | 72 | 102 | 93 | 420 | 644 | 632 |
| 2 | 87 | 79 | 79 | 428 | 603 | 606 |
| 3 | 50 | 95 | 95 | 519 | 639 | 655 |
| 4 | 59 | 80 | 79 | 512 | 604 | 590 |
| 5 | 71 | 68 | 68 | 563 | 618 | 619 |
| 6 | 62 | 96 | 100 | 469 | 623 | 614 |
| 7 | 130 | 81 | 83 | 590 | 671 | 663 |
| 8 | 70 | 64 | 66 | 403 | 630 | 629 |
| 9 | 53 | 84 | 83 | 573 | 509 | 497 |
| 10 | 57 | 79 | 85 | 556 | 582 | 589 |
| Mean | 71·1 | 82·8 | 83·1 | 503·3 | 612·3 | 609·4 |
| S.D. | 23·4 | 12·0 | 11·0 | 69·0 | 43·9 | 46·4 |

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showed 2·3 (1·4) spots. There was no significant difference between the number of spots on unexposed films and films from sample C. Ten nutrient agar plates from sample C contained a total of two colonies. All data are given in Tables 1 and 2.

Table 2. *Number of black spots on unexposed films and on autoradiograms from sterile radioactive medium*

| Sample | Unexposed film. Counted spots | Sterile radioactive media C | |
|---|---|---|---|
| | | Viable count | Auto-radiography |
| 1 | 1 | 0 | 1 |
| 2 | 1 | 0 | 3 |
| 3 | 1 | 0 | 2 |
| 4 | 6 | 0 | 4 |
| 5 | 1 | 0 | 5 |
| 6 | 2 | 0 | 1 |
| 7 | 3 | 0 | 3 |
| 8 | 1 | 1 | 1 |
| 9 | 1 | 0 | 2 |
| 10 | 1 | 1 | 1 |
| Mean | 1·8 | — | 2·3 |
| S.D. | 1·6 | — | 1·4 |

Analysis of variance performed on the number of autoradiographic spots showed that with 95 % confidence the population mean lies within ± 26 spots around the sample mean for sample B1 if only one autoradiogram was counted once and within ± 8 spots if ten films were counted. Recounting did not increase the sensitivity. This was also true of sample B2 for which 102 spots was the 95 % confidence limit if one film was counted and 32 if ten films were counted.

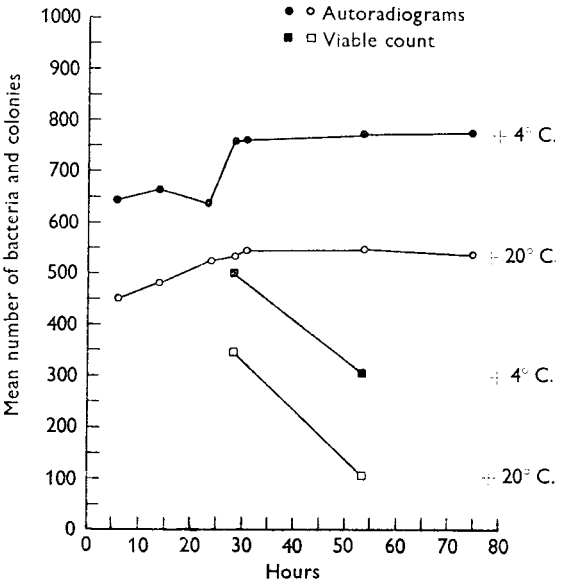
No significant difference in the number of bacteria in the autoradiograms was revealed by comparing the experiments in which 0·9 % NaCl and heavily polluted water were used as diluents.

The results of the survival test show that even after 75 hours storage, the number of bacteria demonstrated in autoradiograms had not decreased but the number of colonies on viable count fell rather rapidly (Text-fig. 3).

### *Field trials*

The number of spots counted on autoradiograms from two natural sea-water samples are given in Table 3. Variance analyses show that the probability of the background exceeding the limit of 12 spots is only 0·1 %. There is a significant difference between the number of spots on autoradiograms from sea water and the number of spots on unexposed films (Tables 2 and 3). This indicates the presence in natural sea water from this source of agents which can produce black spots on the film. Whether these images are the result of radioactivity or of some chemical reaction with the film has not been determined in this experiment.

The data obtained in the field tests are found in Text-figs. 1 and 2. The distribution of bacteria in space and time are consistent with the locations of the sampling sites and the sampling times and also agree well with the fluctuations observed in the dispersion of the dye that was discharged as a control together with the bacteria.




Text-fig. 3. Mean number of bacteria on autoradiograms and colonies on nutrient agar from water samples stored at different temperatures. Each symbol represents the mean of five samples.

Table 3. *Number of spots on autoradiograms from filtrations of natural sea water*

| | Sample A | | Sample B | |
|---|---|---|---|---|
| Filtration | Count 1 | Count 2 | Count 1 | Count 2 |
| 1 | 3 | 3 | 5 | 6 |
| 2 | 2 | 2 | 6 | 6 |
| 3 | 3 | 3 | 4 | 6 |
| 4 | 8 | 8 | 1 | 3 |
| 5 | 5 | 6 | 10 | 7 |
| 6 | 6 | 5 | 2 | 2 |
| 7 | 5 | 6 | 3 | 3 |
| 8 | 8 | 9 | 3 | 2 |
| 9 | 3 | 3 | 1 | 1 |
| 10 | 9 | 7 | 6 | 6 |
| Mean | 5·2 | 5·2 | 4·1 | 4·2 |
| S.D. | 2·5 | 2·4 | 2·8 | 2·2 |

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## DISCUSSION

The presence in autoradiograms of images which resemble but are not caused by radioactive bacteria is one factor which limits the sensitivity of the method described here. These images are presumably found in equal numbers in autoradiograms of filters through which water without radioactive bacteria has been filtered. From Table 3 it is evident that the number of 'simulated bacteria' is fairly constant in this experiment. A statistical analysis, performed on a given number of controls, will give probability limits for the number of bacteria that can be detected with the background present. The background value must be determined in every experiment before the radioactive bacteria are added to the water.

If the background spots are examined under greater magnification, some of them show a structure different from that of the bacterial images. These spots can then be excluded and the sensitivity of the method improved (Table 4). Other background spots which cannot be distinguished from bacteria are probably caused by background radiation. The procedure for examining the spots under 80–150 times magnification is rather tedious but under most circumstances such a high sensitivity will probably not be required.

Table 4. *Images identical with those caused by radioactive bacteria, counted on unexposed films under* 10× *and* ×80 *magnification*

| Film NR | ×10 | ×80 |
|---|---|---|
| 1 | 2 | 1 |
| 2 | 2 | 1 |
| 3 | 1 | 0 |
| 4 | 2 | 1 |
| 5 | 8 | 6 |
| 6 | 3 | 0 |
| 7 | 5 | 3 |
| 8 | 3 | 0 |
| 9 | 4 | 1 |
| 10 | 2 | 1 |
| Mean | 3·2 | 1·4 |

In practice, a count of only a few bacteria on a filter will be of little consequence —only large counts will be important and these are but little affected by the errors discussed above. It is apparent from the statistical analyses that the sensitivity of the method is limited not by the accuracy of counting the spots on the autoradiograms but rather by the uneven dispersion of bacteria in water.

The amount of radioactivity present in the medium is well below the activity required to cause a decrease in outgrowth (Schmidt, 1948; Rubin, 1954). It is reasonable to assume that the radioactive bacteria do not differ from unmarked bacteria in regard to survival in polluted waters.

A larger number of bacteria are found in the autoradiograms compared to the number of colonies found on viable count. This may be due to the presence of aged bacteria which no longer give colonies on nutrient agar but still retain their

structure and thus give autoradiographic images. It is possible that viable counts on a different kind of medium would yield higher counts. It is also possible that two or more bacteria adhere and give rise to only one colony. This could also explain the larger standard variation of colonies in viable count when compared to the number of autoradiographic bacteria (Table 1). As the time between death and lysis of bacteria is rather short (Koch, 1959) it is unlikely that dead bacteria cause a large part of the difference in number between autoradiographically-demonstrated bacteria and counted colonies. The decrease in the number of colonies on viable count after storage of water samples corresponds to the findings of earlier authors (PHLS Water Committee 1953).

Apparently a thin layer of sludge does not absorb enough radiation from the bacteria to interfere with the autoradiographic results. Because the cells are retained *on* rather than *in* the filter absorption of radiation by the filter is negligible.

The present method appears to be suitable for practical use. A small number of bacteria can be detected and the sensitivity of the method increases if there is less sludge in the water. Samples from heavily polluted water may be centrifuged according to the technique described by Rastgeldi (1959) and autoradiograms prepared from the sediment. This modification of the method, however, remains to be developed.

A great advantage of the method described here is that bacteriological handling is eliminated except for the preparation of the test culture. Suction for filtering can be obtained by whatever means are available, e.g. by manual, motor-powered, or Venturi pumps. The filters should be applied to film immediately but they can be sent by mail if their surfaces are protected. Other bacteria such as enterococci can also be marked with radiosulphur and used in tracer experiments.

If large numbers of bacteria are needed, the sulphur content of the medium can be increased by adding carrier sulphur. At concentrations above 0·005 mg. sulphur/ml. medium, however, the uptake of labelled sulphur diminishes (Cowie, Bolton & Sands, 1952) and less favourable conditions for autoradiography occur. A continuous culture apparatus might also be set up near a river or any other location where a great number of bacteria would be needed to perform the test.

## SUMMARY

An autoradiographic method to trace the dissemination in water of radioactive bacteria is reported. It has been tested for accuracy and sensitivity and a field trial has been performed. The method appears suitable for practical use and permits the study of environmental effects on the spreading bacteria.

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## EXPLANATION OF PLATE

### PLATE 1

Detail of an autoradiogram of Millipore filter through which a suspension of radioactive bacteria was passed. Black spots represent bacteria. × 80.



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