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Investigations into the use of the bacterial species *Bacillus stearothermophilus* and *Escherichia coli* (H_2S positive) as tracers of groundwater movement

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INVESTIGATIONS INTO THE USE OF THE BACTERIAL SPECIES *Bacillus stearothermophilus* AND *Escherichia coli* (H_2S POSITIVE) AS TRACERS OF GROUNDWATER MOVEMENT

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Ministry of Works and Development
Christchurch

WELLINGTON 1980

Investigations into the use of the bacterial species *Bacillus stearothermophilus* and *Escherichia coli* (H_2S positive) as tracers of groundwater movement

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Studies on the use of two bacterial species *Bacillus stearothermophilus* and *Escherichia coli* (H_2S^+) as tracers of groundwater movement were conducted at the Water and Soil Division groundwater study area at Burnham. Both species were used to trace groundwater movement over a total distance of 920 m. Both were recovered from groundwater samples by membrane filtration methods, permitting a lower detection threshold of one bacterial cell/100 ml of groundwater.

B. stearothermophilus was found to occur naturally in the Burnham groundwater system and concentrations increased following rainfall. This limited the use of the species as a tracer to periods of dry weather. The species was also detected in sewage effluent. *E. coli* (H_2S^+) was not detected in uncontaminated soils or groundwater systems in the Lincoln-Burnham area but was found to occur intermittently in sewage effluent. Neither *B. stearothermophilus* nor *E. coli* (H_2S^+) were considered to be suitable as tracers in sewage-polluted groundwater.

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1 INTRODUCTION

1.1 General

The Canterbury Plains groundwater system constitutes an extensive and important resource from which large quantities of water are extracted for irrigation, stock watering, domestic consumption, and industrial processes. Recently the chemical and microbial quality of this groundwater has become a source of concern to local authorities. For example, since 1963, an average increase in nitrate nitrogen levels from 2.9 to 9.5 g/m^3 has been recorded in some wells in the Christchurch area (Minnery, pers. comm.). Micro-organisms indicative of faecal contamination have also been recorded in wells at Burnham and Templeton downstream of effluent irrigation schemes (Martin and Noonan 1977).

Water contaminated with faecal material is regarded as a health hazard because of the possible presence of pathogenic micro-organisms, including members of the *Salmonella* and *Shigella* genera, *Vibrio comma, Entamoeba hystolytica,* and enteric viruses (Gates 1967). It is possible to detect these pathogens in water but the laborious techniques and expensive equipment involved in their isolation and identification generally make routine sampling for these organisms impracticable. However, pathogenic organisms in faeces or sewage are almost always outnumbered by other species that are easier to detect and may be used as indicators of faecal pollution. The micro-organisms commonly chosen for this purpose are the coliform group of bacteria. Other pollution indicators include faecal streptococci and *Clostridium perfringens.*

The coliform group of bacteria contains a number of species that may have their origins in other than faecal material. However, one species, *Escherichia coli,* is of undoubted faecal origin, being restricted in its natural habitat to the intestinal tract of warm-blooded animals, including man. Enumeration of "faecal coliform" bacteria, although less exacting than enumeration of *E. coli*, is often undertaken because of the relative simplicity and rapidity of the procedures involved.

A number of organisations, including the Water and Soil Division of Ministry of Works and Development, Department of Scientific and Industrial Research, Paparua County Council, North Canterbury Catchment Board, and Lincoln College are now involved in the identification of areas in and around Christchurch in which coliform and faecal coliform bacteria are known to be present in the groundwater. The possible sources of this type of contamination are of particular concern to these organisations. In an area such as the Canterbury Plains these include sewage-polluted river recharge zones, border-dyked effluent disposal areas, unsealed or poorly sealed oxidation ponds, septic tank soak pits, offal pits, refuse pits, and leaking sewer mains. Although enumeration of coliform and faecal coliform bacteria, faecal streptococci, and *C. perfringens* may provide clues to the proximity and size of the contamination source, it does not normally allow the positive identification of a particular source of faecal material. Techniques are needed to permit the "labelling" of suspected sources of faecal contamination with highly distinctive "tracer" organisms which can be identified and counted if they enter the groundwater system.

1.2 Groundwater tracers

Both living and non-living tracers are used to determine the direction of movement of groundwater, its dilution and residence time in an aquifer, and its spatial dispersion. Alternatively, the characteristics and behaviour of the tracer itself may be of importance. In particular, the rate of movement and dispersion of bacterial tracers may be used to predict the probable behaviour of pathogenic species of bacteria in groundwater systems.

There are three main types of non-living water tracer:

1. Radioactive isotopes
These include tritiated water and isotopes of the halide ions. In the case of the latter group, the selection of the ion to be used normally depends on availability, price and half-life. Most commonly chosen are I-125, I-131, I-132, Br-82, and Cl-36 (White 1976).

2. Chemicals
Since mineral lattices are negatively charged, anions such as fluoride, chloride, and bromide are favoured as chemical tracers. The use of cations is normally limited to lithium which is not readily adsorbed (White 1976). Petrol was used successfully in vertical tracer studies at Heretaunga by Thorpe (1977).

3. Fluorescent dyes
The most commonly used fluorescent dye is fluorescein (or uranine). This dye is very suitable for direct observation in clear water because its fluorescence spectrum peaks at green wavelengths where the colour response of the human eye is most sensitive. Smart and Laidlaw (1977) compared eight fluorescent dyes (amino G acid, photine CU, fluorescein, lissamine FF, pyranine, rhodamine B, rhodamine WT, and sulpho rhodamine B) in terms of sensitivity, minimum detectability, photochemical and biological decay rates, adsorption losses, and other characteristics. They recommended rhodamine WT, lissamine FF, and amino G acid as the most suitable for quantitative tracing studies.

The most commonly used living tracers are bacteria. Their use is attractive because the ability to isolate a single cell from a large volume of samples yields a sensitivity of detection which may exceed that possible with radio-isotopes (White 1976). Coliform bacteria are themselves

remarkably sensitive indicators of faecal pollution and may be detected a considerable distance from the source of contamination. However, in cases where a particular source of pollution is to be identified, a tracer organism not normally found in sewage, such as *Serratia marcescens,* may be used.

Serratia marcescens, which produces distinctive red pigmented colonies, was used to study sewage dispersion around a marine outfall by Putman *et al.* (1956) and in Oslofjord by Omerod (1964). The identification of *S. marcescens* colonies tends to be hampered by the growth of other aerobic species, particularly in sewage-polluted samples. This problem was partially solved when Rippon (1963) used an antibiotic resistant mutant to trace river water movements. The strain used possessed resistance to certain antibiotics which were applied to reduce the growth of other species during incubation. The performance of antibiotic resistant *S. marcescens* as a tracer of sewage dispersal was compared to that of *Bacillus niger* by Pike *et al.* (1969) who conducted experiments at two coastal submarine outfall sites. *B. niger* is a spore-forming bacterium which produces a black pigment on media containing tyrosine. Both species were found to provide information on longshore and offshore dispersion.

Other bacterial species with potential as tracer organisms include urease positive and hydrogen sulphide positive (H_2S^+) strains of *E. coli* and the thermophile *Bacillus stearothermophilus*. *E. coli* (H_2S^+) was used to monitor vertical and horizontal groundwater movement in conjunction with rhodamine WT dye at Heretaunga by Thorpe (1977). *B. stearothermophilus* is considered to have potential as a tracer because of its ability to grow at 71 °C, a temperature at which growth of all other species is prevented. However, attempts by Noonan (1976) at using this species to check the seal provided by concrete pads around investigation wells at Burnham were unsuccessful.

Because the detailed geological structure of a groundwater study area will normally be unknown, it is important that the tracer organism selected accurately represents the behaviour of the water or substances in solution or suspension. The success of a species used as a sewage tracer in soil and groundwater studies will depend, not only on the interaction between the species and the sewage medium, but also on the same range of environmental factors which affect the normal sewage biota. The number of wastewater micro-organisms in groundwater downstream of a source of contamination will depend on:

a. The type of sewage disposal structure and application method (e.g., surface applied oxidation pond effluent, subsurface applied septic tank effluent).

b. The quantity of effluent, rate of application, and the number of micro-organisms in the effluent.

c. The characteristics of the non-microbial material in the effluent.

d. The penetration and survival of micro-organisms in the strata above the groundwater.

e. The distance to the groundwater.

f. The rate of movement of the groundwater and the the amount of dilution by uncontaminated groundwater.

g. The survival of micro-organisms in groundwater.

h. The filtering action of strata containing groundwater.

Present knowledge on the behaviour of wastewater micro-organisms in soil and groundwater has been reviewed by Gerba *et al.* (1975), Noonan (1976) and others. However, little information is available on the penetration and survival of specific tracer organisms in soil and groundwater systems.

Although the health risks inherent in the use of bacterial species as tracers are considered to be low (White 1976), these risks should not be ignored. *S. marcescens* has been implicated in cases of septicaemia by Dobson (1968). *B. stearothermophilus,* although not considered pathogenic, can proliferate in foodstuffs when stored at high temperatures and may contribute to food spoilage (Sumner, pers. comm.). The problem of transferable antibiotic resistance between strains of *E. coli* (some of which are pathogenic) means that use of this organism as a tracer should also be approached with caution. Some H_2S strains of *E. coli* have been found to exhibit resistance to certain antibiotics, principally tetracycline, and have the ability to transmit both the H_2S production and tetracycline resistance characters (Orskov and Orskov 1973).

Micro-organisms other than bacteria have been used successfully as water tracers. Wimpenny *et al.* (1972) noted that the specificity of a bacteriophage for its host is such that different phages can be used simultaneously to identify different pollution sources as the organism is completely inert in the absence of its hosts. They compared *S. marcescens* with a lambda-like phage of *E. coli* and two yeasts as tracers in river water and found the phage to be the most suitable. Lycopodium spores (25 nm diameter) may be coloured by dyeing and have been used by Smith and Atkinson (1974) to investigate underground flow rates in cavernous and fissured limestones.

1.3 The Burnham experimental site — existing knowledge

The Water and Soil Division Burnham experimental site is adjacent to an area used for the disposal of oxidation pond effluent by border dyke irrigation. The effects of the disposal area on microbial quality of the groundwater at Burnham were investigated by Noonan (1976) using an array of investigation wells (Fig. 1). He reported that, following effluent application to two border dyke

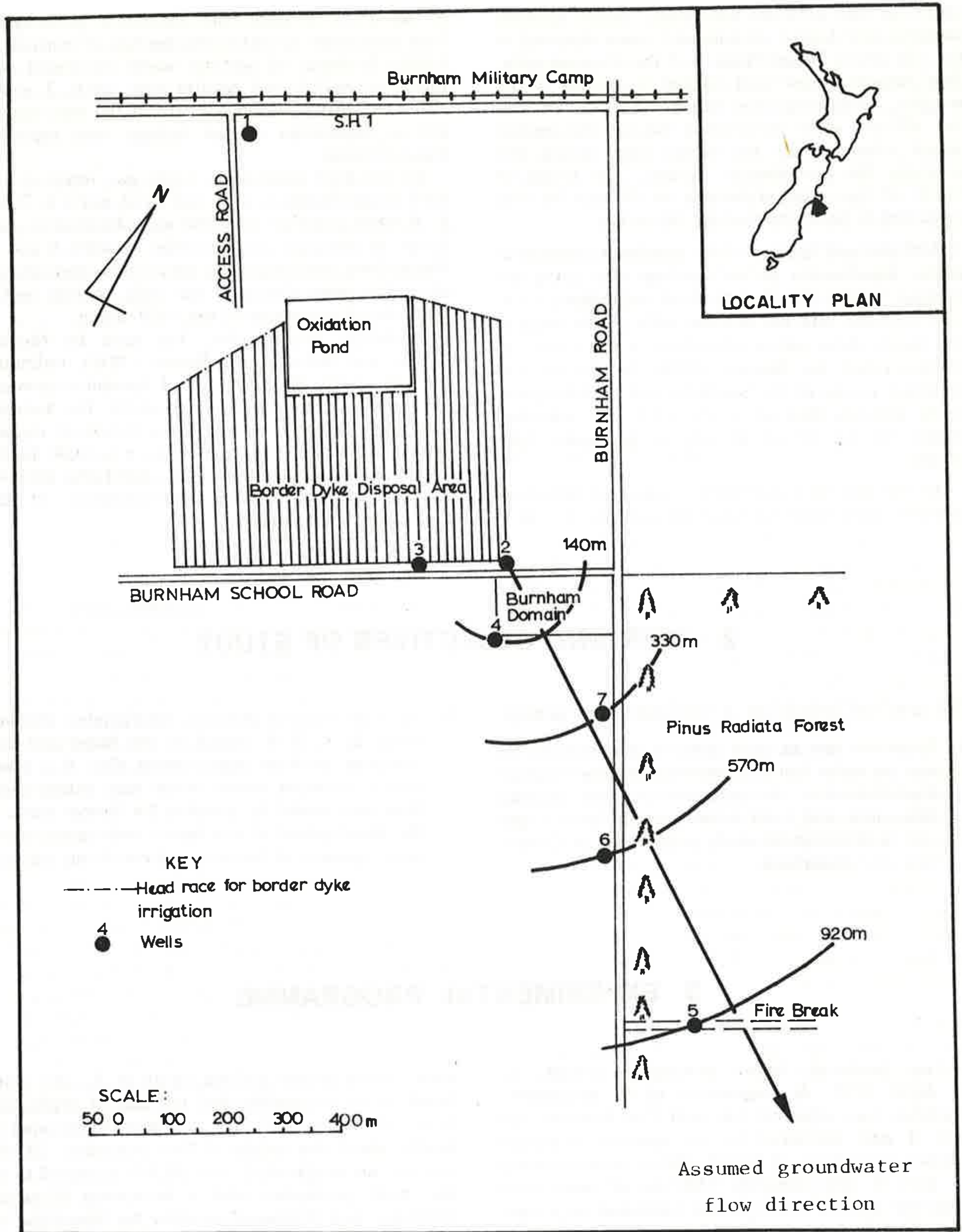


Figure 1 Burnham wastewater disposal area, associated investigation wells, and radial distance of wells 4, 7, 6, and 5 from well 2

strips in line with the well array, faecal coliform bacteria and faecal streptococci were detected in the six wells "downstream" of the disposal area. This response was most marked in periods of dry weather. It was concluded that micro-organisms from the effluent were percolating through the porous gravel strata under the border dyke strips and entering the groundwater system. The speed of travel of the micro-organisms in the aquifer was estimated to be approximately 160 m/day.

McCabe and Rowse (1976) conducted a series of tracer experiments at the Burnham site using the isotope Iodine-131. In the first experiment I-131 was injected into the effluent race at the head of the border dyke strips identified as the source of contamination by Noonan (1976). No tracer was recorded in any of the monitored wells. Subsequent tests indicate that all of the I-131 was adsorbed within the top 50 cm of soil on the border dyke strips.

In the second experiment, single-well filtration velocity tests were conduced on wells 5, 2, and 3 in an effort to determine horizontal groundwater flow velocities by observing the rate of removal of I-131. Evidence of vertical water movement was found in well 5 and results from wells 2 and 3 indicated that low permeability at the well casing and/or adsorption of the isotope was impeding tracer removal.

In the third experiment, I-131 was injected into well 2 and detectors were set up in wells 4, 7 and 6. A small positive response was observed in well 4 but no response was recorded in wells 6 and 7. The authors concluded that results obtained indicated a low permeability of the well casings and a high rate of adsorption of the I-131 tracer.

Although inconclusive, the work by Noonan (1976) and McCabe and Rowse (1976) indicated the possibility of some form of hydraulic connection between wells 2, 4, 7, 6, and 5. The Burnham site was therefore chosen for a series of experiments using bacterial tracers as a prelude to developing techniques useful in identifying specific sources of groundwater contamination in the Canterbury Plains region.

2 AIMS AND OBJECTIVES OF STUDY

The aims and objectives of this study were twofold:

a. To select one or more species of bacterium for use as water tracers, investigate their survival characteristics in groundwater, and develop laboratory and field techniques for their future use in groundwater study programmes in Canterbury and elsewhere.

b. To investigate a possible relationship between wells 2, 4, 7, 6, and 5 at the Water and Soil Division Burnham experimental site. If a connection between these wells was established, this site would be suitable for further work in the development of new tracer techniques, using other species of bacteria and non-living tracers.

3 EXPERIMENTAL PROGRAMME

The bacterial tracer programme commenced in April 1977. A suspension of *B. stearothermophilus* was injected into well 2 at Burnham and well 4 was monitored for the species. Although numbers in excess of 10 000/100 ml were detected in well 4, approximately 150/100 ml were also detected in well 1 which was monitored as a control. This high background level of *B. stearothermophilus* meant that the species was initially considered to be unsuitable as a tracer. However, a twice monthly sampling programme was initiated to establish "background noise" levels of *B. stearothermophilus* at Burnham and the sampling continued for a year. By February 1978, numbers had fallen to below 5/100 ml and the species was accordingly reconsidered for use as a tracer.

From May to September 1977, various other species were investigated as potential tracers. For technical reasons, *B. niger, S. marcescens*, and a urease postive strain of *E. coli* were found to be unsuitable and the use of antibiotic-resistant strains of *E. coli* was rejected because of doubts about the safety of this procedure. Of the species investigated, *E. coli* (H_2S^+) appeared to be the most promising and a membrane filtration technique was developed to allow the identification of this strain in water samples and to distinguish it from non-H_2S producing *E. coli* strains (see Section 4.1.2.3.).

In November 1977, tests were conducted on a Water and Soil Division vacuum chamber water sampler (Martin 1976) to determine its suitability for use in bacterial tracer studies. It was found that large numbers of both *B. stearothermophilus* and *E. coli* cells could adsorb to the inside walls of the sampler chamber and/or the "blow-out" hose (unpublished data, Water and Soil Division, Christchurch). These organisms, even after repeated

flushing with sterile water, continued to appear in subsequent samples. An additional problem was the possibility of growth of certain bacterial species on the equipment when left suspended in the well water for long periods (Burman and Colbourne 1976). The vacuum chamber sampler was therefore considered unsuitable for use in bacterial tracer work.

An alternative system of well water sampling was devised using weighted glass bottles (Fig. 2). This system allowed the use of a new sterile bottle for the collection of each sample, reducing the possibility of cross-contamination between samples.

In a preliminary experiment in November 1977, approximately 2.1 x 10^{10} *E. coli* (H_2S^+) cells were injected into well 2 at Burnham and were later detected in wells 4 and 6. This was followed in February 1978 by a more detailed investigation using both *E. coli* (H_2S^+) and *B. stearothermophilus*. Details of this last experiment are presented in Sections 4 and 5.

In May 1978, experiments were set up to investigate the survival or "die-off" characteristics of *E. coli* (H_2S^+) and *B. stearothermophilus*.

To determine whether or not *E. coli* (H_2S^+) would be suitable as a tracer species in sewage polluted waters, sewage effluents from Lincoln and Burnham were sampled and analysed for the presence of H_2S^+ faecal coliform bacteria and *E. coli* (H_2S^+).

4 METHODS AND MATERIALS

4.1 Laboratory techniques

4.1.1. *Bacillus stearothermophilus*

4.1.1.1. **Characteristics.** *Bacillus stearothermophilus* Donk, 1920 is a rod shaped spore forming bacterium. The spores are strongly heat resistant and the vegetative cells of the selected strain have the ability to grow at 70–71 °C on tryptone glucose extract agar.

4.1.1.2 **Preparation of culture.** *B. stearothermophilus* cells were grown by means of a "batch culture" technique in a Virtis "Moduculture" 5 litre fermenter. Two 5 l batches were grown for injection into the Burnham aquifer. The composition of the nutrient medium used is given in Table 1.

Tryptone, sodium chloride, and yeast extract were added to water in the fermenter. The solution was mixed, sterilised at 121°C for 15 minutes and allowed to cool. The glucose, calcium chloride, thiamine, and methionine were mixed, filtered to sterility through a Gelman GA–8 0.2nm 22mm membrane filter and added to the solution in the fermenter under sterile conditions.

Using a sterile syringe, a suspension of *B. stearothermophilus* (containing approximately 5x10^6 cells) was injected into the nutrient medium through a self-sealing septum.

The inoculated solution was mixed continuously by means of an electric stirrer and sterile air was bubbled through the medium. The fermenter was incubated at 55 °C for 24–36 hours.

Table 1 Composition of nutrient medium for the batch culture of *B. stearothermophilus*. (From Reanney, pers. comm.)

	Quantity per litre of tap water
Tryptone	10 g
Sodium Chloride	8 g
Yeast extract	10 ml
Calcium chloride 1 M	1 ml
Thiamine	0.01 g
Methionine	0.01 g

To avoid the problem of introducing bacterial growth media into the injection well, the solution was then centrifuged in a Sorvall RC2–B centrifuge at 4000 rpm (2600 gs) to precipitate the *B. stearothermophilus* cells. The culture medium was decanted and the cells were re-suspended in a 0.85% solution of NaCl. No attempt was made to induce sporulation of the *B. stearothermophilus* cells before injection.

4.1.1.3 **Detection in groundwater samples.** *B. stearothermophilus* cells in groundwater samples were detected by membrane filtration using a Gelman magnetic filter funnel apparatus and Gelman GN–6 GRID 0.45 nm membrane filters. Dilution series were prepared when required.

After filtration, the membrane was placed, grid uppermost, onto a poured plate of tryptone glucose extract agar (TGEA). The plate was then inverted and a sterile Gelman 47 mm absorbent pad was placed on the inside of the lid to absorb excess moisture.

The plates were incubated at 70°C ± 0.1°C for 14–16 hours. All light brown colonies were then counted as *B. stearothermophilus* and results were expressed per 100 ml of sample.

4.1.2 *Escherichia coli* (H_2S^+)

4.1.2.1 **Characteristics.** The *E. coli* (H_2S^+) strain used in the Burnham experiment has the ability to produce hydrogen sulphide from thiosulphate and tetrathionate in contrast to typical *E. coli*. Aside from this ability, the strain exhibited the biochemical properties characteristics of typical *E. coli*. The characteristics and significance of *E. coli* (H_2S^+) have been described by Orskov and Orskov (1973) and Layne *et al.* (1971).

4.1.2.2 **Preparation of culture.** *E. coli* (H_2S^+) was batch cultured in a Virtis "Moduculture" fermenter, as described for *B. stearothermophilus*, for injection into the Burnham aquifer. Although nutrient

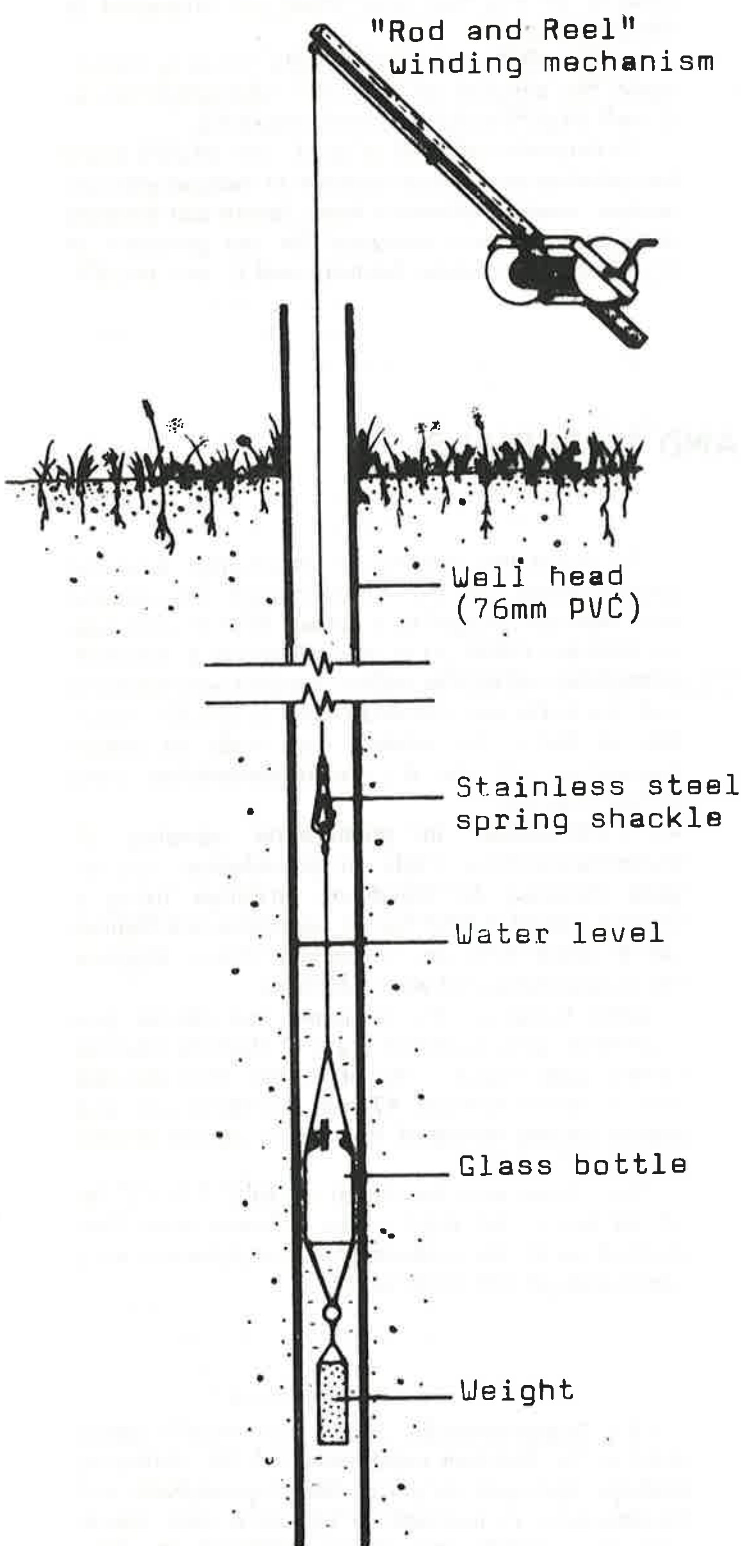


Figure 2 Schematic diagram of well water sampling apparatus (not to scale)

broth is suitable for *E. coli* (H_2S^+) growth, the more selective minerals modified glutamate medium (MMG) was used to minimise the possibility of contamination.

A 5 l batch of the medium was prepared, added to the fermenter, sterilised at 121°C for 15 minutes and allowed to cool. As described for *B. stearothermophilus*, the MMG was inoculated with approximately 5×10^6 actively growing *E. coli* (H_2S^+) cells, and the medium was aerated and stirred. The fermenter was incubated at 37°C for 36 hours.

The solution was centrifuged and the precipitated cells were resuspended in 0.85% NaCl solution and stored at 5–6°C until required for injection.

4.1.2.3 **Detection in groundwater samples.** *E. coli* (H_2S^+) cells in groundwater samples were detected by membrane filtration using a Gelman magnetic filter funnel apparatus and Gelman GN–6 0.45 nm membrane filters. Dilution series were prepared when required.

After filtration, the membrane was placed on a Gelman GN–6 sterile absorbent pad soaked with membrane enriched teepol broth (Oxoid) as described in Great Britain Department of Health and Social Security, Report No. 71 (1969). The filters and pads were then incubated at 30°C ± 0.5°C for 4 hours and for a further 14 hours at 44.5°C ± 0.5°C. All yellow colonies were then counted as faecal coliform bacteria.

To distinguish H_2S^+ colonies from the others, the filters were then placed on poured plates of triple sugar iron agar (Oxoid) and incubated for a further 4–5 hours at 44.5°C ± 0.5°C. The H_2S^+ strain produced a black ring around the perimeter of the colony. The non-H_2S^+ strains remained yellow. All black-ringed colonies were counted and results were expressed per 100 ml of sample.

The described technique identified H_2S positive "faecal coliform" bacteria and not necessarily *E. coli* (H_2S^+). Some strains of other species of coliform bacteria have the ability to produce H_2S from thiosulphate. However, in samples taken in the three weeks prior to the February 1978 experiment no H_2S^+ faecal coliform bacteria were identified in the groundwater at Burnham although they were subsequently isolated from the oxidation pond effluent (Section 4.6). For the purposes of the main tracer experiment, all H_2S^+ faecal coliform were counted as *E. coli* (H_2S^+).

4.2 Field sampling techniques

Well water samples were collected by means of weighted glass bottles. Thirty bottles were arranged in two rectangular tins (15 in each) and were sterilised by autoclaving. During intensive sampling, one tin was being sterilised in the laboratory while the other was in use in the field.

Using a "rod and reel" system (Fig. 2) each sterile bottle was manoeuvred into the open well head without being handled. The bottle was then lowered until the weight struck the water surface. The line was then lowered a further 1 m to place the open neck of the bottle approximately 0.5 m below

the water level. As each bottle was attached to its own 1 m length of cord, the main cord remained uncontaminated. This procedure was followed at each well to ensure that the samples were always taken from the same depth.

After about 30 seconds, audible bubbling in the well ceased. The bottle was then raised and the sample was poured into a sterile collection bottle and stored in ice for transport to the laboratory.

To avoid cross-contamination between wells, the operator's hands were carefully washed in absolute alcohol before and after handling each full bottle. The system proved effective and reliable throughout the sampling programme.

4.3 Twelve month sampling programme – *Bacillus stearothermophilus*

As noted in Section 3, a sampling programme designed to establish the natural "background" levels of *B. stearothermophilus* in Burnham groundwater commenced in April 1977 and continued until March 1978. The sampling programme was largely confined to wells 1,2,3, and 4 but was expanded to include wells 5, 6, and 7 in January 1978. Sampling dates and wells sampled are listed in Table 2.

4.4 Experimental design – Burnham tracer study

A 20 l "mix" containing approximately 2.58 x 10^9 *B. stearothermophilus* cells and 2.36 x 10^{11} *E. coli* (H_2S^+) cells was injected into well 2 at the Burnham site. A length of sterile rubber hose was lowered approximately 3 m below the static well water level, a sterile funnel inserted into the top end of the hose and the mix poured in.

The four wells downstream of well 2 (wells 4, 7, 6, and 5) were then monitored for 10 days for the two tracer species. Details of the sampling programme are presented in Appendices 1, 2, 3, 4, and 5.

4.5 Die-off experiments

Four lengths of 28 mm Gallenkamp dialysis tubing were suspended in two specially constructed PVC frames (2 tubes per frame). Each length of tube was 2/3 filled with 200 ml of a mixture of *E. coli* (H_2S^+) and *B. stearothermophilus* suspended in 0.85% NaCl solution. The mixture contained approximately 9.1 x 10^{10} *E. coli* (H_2S^+) cells/100 ml and 7.0 x 10^8 *B. stearothermophilus* cells/100 ml. One frame was suspended in well 1 at Burnham and the second was suspended in well 4. Each frame was suspended approximately 0.5 m below static well water level (Fig. 3).

The dialysis tubes were sampled twice weekly. The frame was pulled up to the well head and was carefully inverted 20 times to re-suspend the bacterial cells which tended to sink to the bottom of the tube. Using the clamped hose outlets shown in Fig. 3, 1 ml samples of the dialysis tube contents were collected in sterile, capped test tubes which were stored on ice for transport to the laboratory. The samples were analysed for *E. coli* (H_2S^+) and *B. stearothermophilus* as outlined in

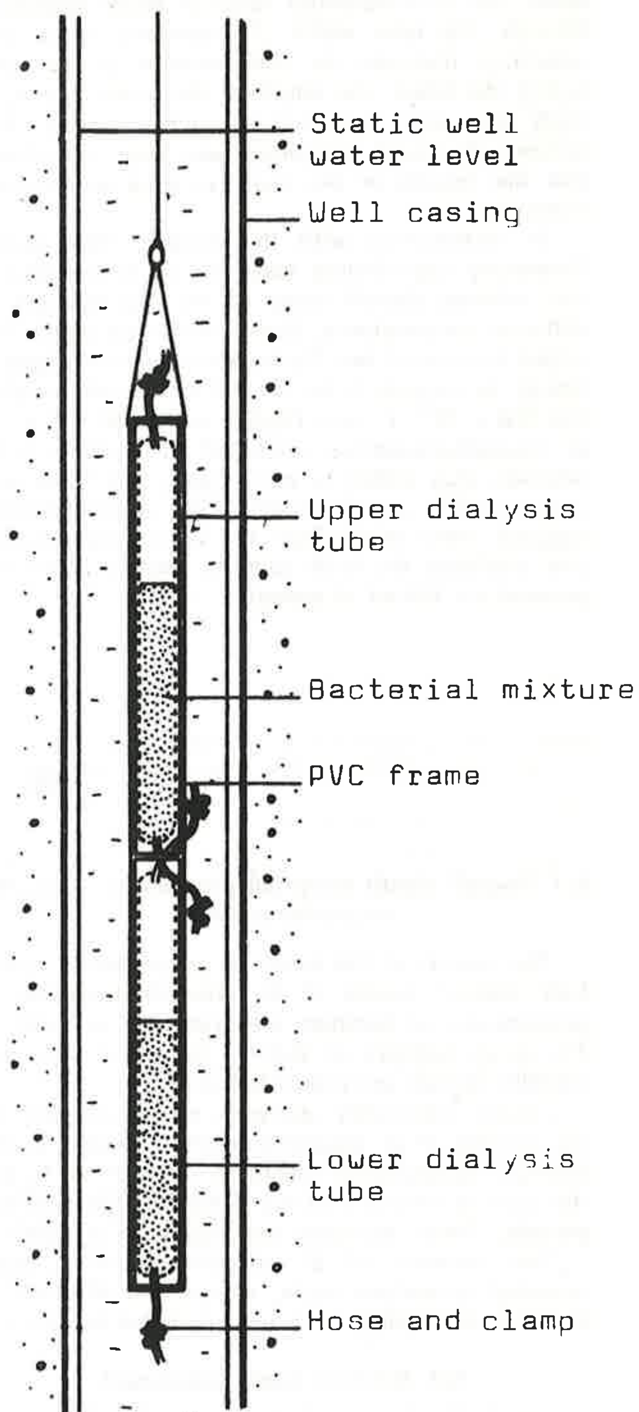


Figure 3 Schematic diagram of dialysis tube apparatus (not to scale)

Sections 4.1.1.3 and 4.1.2.3. The mean bacterial concentration of the four dialysis tube contents was then calculated for both species and the results were expressed per 100 ml of sample.

The amount of liquid in the dialysis tubes tended to decrease during the sampling programme, probably due to differential rates of water movement through the tube walls. To compensate for the resulting increase in concentration of bacteria within the tubes, the length of the water column in each tube was measured at each sampling. The volume of liquid remaining was then calculated and the results of the analysis were accordingly corrected.

In conjunction with the dialysis tube study, laboratory experiments were set up to investigate the relative die-off rates of the two species at different temperatures. One litre of well water was added to each of two flasks which were wrapped in tinfoil to exclude light. A 150 ml mixture containing 3.8×10^{11} *E. coli* (H_2S^+) cells and 4.4×10^9 *B. stearothermophilus* cells/100 ml of 0.85% NaCl solution was added to each flask. One flask was stored at 6°C and the other at 20°C. One-millimetre samples were taken from the flask twice weekly and analysed for both species. Results were expressed per 100 ml of sample.

4.6 Detection of naturally occurring H_2S positive faecal coliform bacteria and *Escherichia Coli* (H_2S^+) in sewage effluents

Samples of treated effluent from the Pasveer ditch treatment plant at Lincoln and the oxidation pond at the Burnham Treatment Plant, together with samples of raw sewage from the Burnham Military Camp were analysed for H_2S producing strains of faecal coliform bacteria as described in Section 4.1.2.3. Colonies that turned black on TSIA were isolated and subcultured into one tube of brilliant green bile broth (to detect lactose fermentation) and one tube of peptone water (for the production of indole) as recommended by the New Zealand Microbiological Society's Committee on Coliform Bacteria (1976). These tubes were incubated at 44.5°C ± 0.2°C for 24 ± 2 hours. To complete the IMVIC series of tests, two tubes of methyl red Voges Proskauer medium (for the detection of *Enterobacter aerogenes*) were inoculated with the H_2S positive faecal coliform isolates and incubated at 30°C for three days as recommended by Great Britain Department of Health and Social Security, Report 71 (1969).

5 RESULTS

5.1 Twelve month sampling programme – *Bacillus stearothermophilus*

The results of the sampling programme to establish natural levels of *B. stearothermophilus* in groundwater at Burnham are presented in Table 2. The mean numbers in wells 1 to 4 and daily and monthly rainfall are presented in Fig. 4.

Linear regression analyses were performed on the number of *B. stearothermophilus* found in the Burnham groundwater (mean of wells 1 to 4) and the rainfall recorded in the preceding 7 and 20-day periods. These analyses are presented in Table 3.

The numbers of *B. stearothermophilus* cells recorded in various soils, waters and effluents in the Lincoln-Burnham area are presented in Table 4.

5.2 Burnham tracer experiment

The raw data from the Burnham tracer experiment (February 1978) are presented in Appendices 1, 2, 3, 4, and 5. These data were graphed and are shown in logarithmic scale in Fig. 5.

The estimated times taken for each species to travel between the injection well and each of the four downstream wells and the calculated velocities for each species based on the radial distances from well 2 to each of the downstream wells are given in Table 5. Arrival time at each well was taken as being half-way between the first time the species was detected and the preceding sampling time.

The relative performance of *B. stearothermophilus* and *E. coli* (H_2S^+) was calculated in terms of the number of cells recovered in proportion to the original number injected (Table 6). The calculation is based on the assumption that the total number of cells of each species to pass through each well in the 10 day sampling programme is approximately represented by the area under the relevant graph (Fig. 5). This area may be expressed in bacteria-time units. Table 6 shows bacteria-time units calculated for each species and each well. The number of bacteria-time units for each species and each well may be expressed as a percentage of the original number of organisms injected into well 2. The relative performance of the two species (i.e., the percentage of *B. stearothermophilus* to pass through each well as opposed to the percentage of *E. coli* (H_2S^+) to pass through that well) may then be estimated and is presented in Table 6 as a "percentage recovery ratio".

5.3 Die-off experiments

The results of the experiments to determine the die-off characteristics of *B. stearothermophilus* and *E. coli* (H_2S^+) are presented on logarithmic scale in Fig. 6. Linear regression analyses performed on the results presented in this figure (well water stored

Table 2 *B. stearothermophilus* levels in groundwater at the Burnham experimental site (cells/100 ml)

Date	Monthly Rainfall (mm)*	Well Number							Mean of wells 1, 2, 3 & 4
		1	2	3	4	5	6	7	
April 11	42.75	34	42	58	22	-	-	-	39
May 10	93.25	52	41	-	36	-	-	-	43
May 26		13	71	55	34	-	-	-	43
June 9	95.00	48	21	101	-	-	-	-	57
June 30		210	108	-	-	-	-	-	159
July 12	205.50	306	207	306	107	-	-	-	245
July 27		270	189	650	134	17	87	30	311
August 4	63.75	120	79	-	75	-	-	-	91
August 23		90	113	-	-	-	-	-	102
September 7	119.25	160	110	200	143	-	-	-	153
September 27		52	206	304	102	-	-	-	166
October 6	30.00	27	-	61	11	-	-	-	33
October 19		-	33	-	21	-	-	-	27
November 3	24.00	29	6	9	3	0	7	2	47
November 24		10	0	-	6	-	-	-	5
December 1	40.50	16	4	31	11	-	-	-	16
December 15		4	0	6	-	-	-	-	3
January 10	36.80	0	12	21	0	0	0	0	8
January 13		1	0	7	0	1	3	0	2
January 18		4	0	17	0	0	0	0	5
January 24		0	0	14	0	0	1	0	4
January 28		2	4	3	0	0	2	0	2
February 1	21.70	2	0	0	-	-	-	-	1
February 2		0	4	10	0	0	1	0	4
February 4		0	3	10	3	0	2	0	4

* Recorded at Templeton Agricultural Research Station

Table 6 Relative performance of *B. stearothermophilus* and *E. coli* (H_2S^+) as groundwater tracers at the Burnham site (expressed as a percentage recovery ratio)

	Bacillus stearothermophilus	*E. coli* (H_2S^+)
Original Number Injected (well 2)	2 580 000 000	236 000 000 000
Well 4		
Bacteria-time units	271 112	15 668 740
% of number injected	0.010508	0.0066392
% recovery ratio	1.58 : 1	
Well 7		
Bacteria-time units	2 440	4 816
% of number injected	0.0000945	0.0000020
% recovery ratio	46.36 : 1	
Well 6		
Bacteria-time units	1 623	47 855
% of number injected	0.0000629	0.0000202
% recovery ratio	3.10 : 1	
Well 5		
Bacteria-time units	276	2 226
% of number injected	0.0000106	0.0000009
% recovery ratio	11.34 : 1	

Table 4 Number of *B. stearothermophilus* cells recorded in materials in the Lincoln-Burnham area

Material	Locality	Number of *B. stearothermophilus*
A-horizon Wakanui Silt Loam	Lincoln	20 - 1,600/g
A-horizon Lismore Silt Loam	Burnham	14 - 1,300/g
A-horizon Lismore Silt Loam (effluent irrigated)	Burnham	60 - 2,500/g
Race Water (non effluent)	Burnham	10 - 950/100 ml
Pasveer Ditch Effluent	Lincoln	800 - 1,200/100 ml
Oxidation Pond Effluent	Burnham	10 - 1,200/100 ml
Drinking Water	Lincoln College	0 - 40/100 ml

Table 5 Estimated travel times from injection well 2 to each of the downstream wells and calculated velocities

Wells	Radial Distance (m)	*B. stearothermophilus*		*E. coli* (H_2S^+)	
		Estimated Time Taken (h)	Velocity (m/h)	Estimated Time Taken (h)	Velocity (m/h)
2 to 4	140	13.5	10.37	13.5	10.37
2 to 7	330	57	5.79	57	5.79
2 to 6	570	78	7.31	71	8.03
2 to 5	920	117	7.86	95	9.68
		Mean velocity	7.83	Mean velocity	8.47

Table 3 Linear regression analyses for *B. stearothermophilus* numbers (mean of wells 1 to 4) in groundwater at Burnham versus rainfall recorded in preceding 7 and 20-day periods

Rainfall Period	r	r^2	SD	Regression Model
Rainfall in preceding 7 days	0.41*	0.17	20.40	No. B. stearo./100 ml = 27.29 + 2.71 x rainfall in preceding 7 days (mm)
Rainfall in preceding 20 days	0.65**	0.42	44.05	No. B. stearo./100 ml = 5.25 + 1.46 x rainfall in preceding 20 days (mm)

r = correlation coefficient

r^2 = coefficient of determination

SD = standard deviation

* significant at 5% level

** significant at 1% level

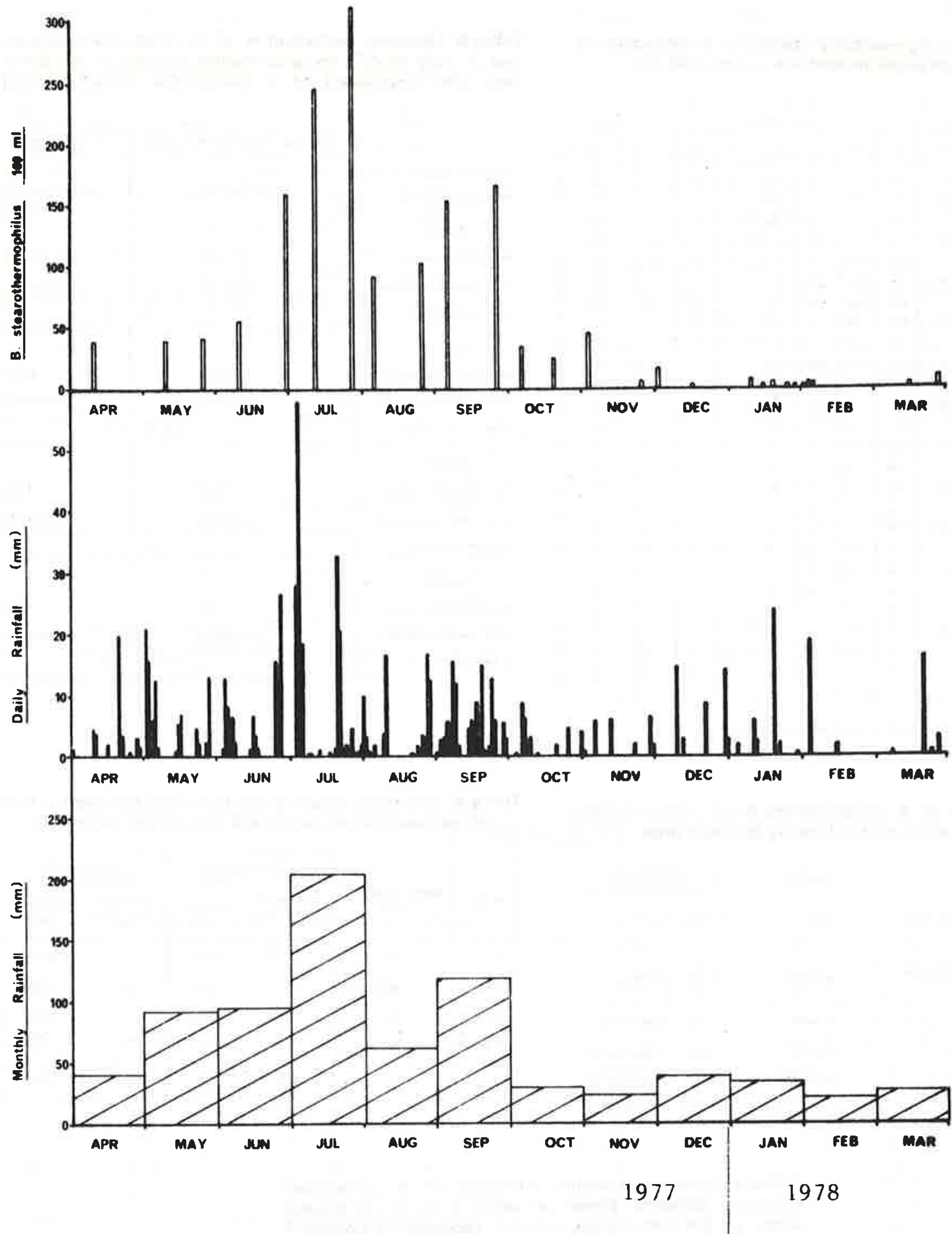
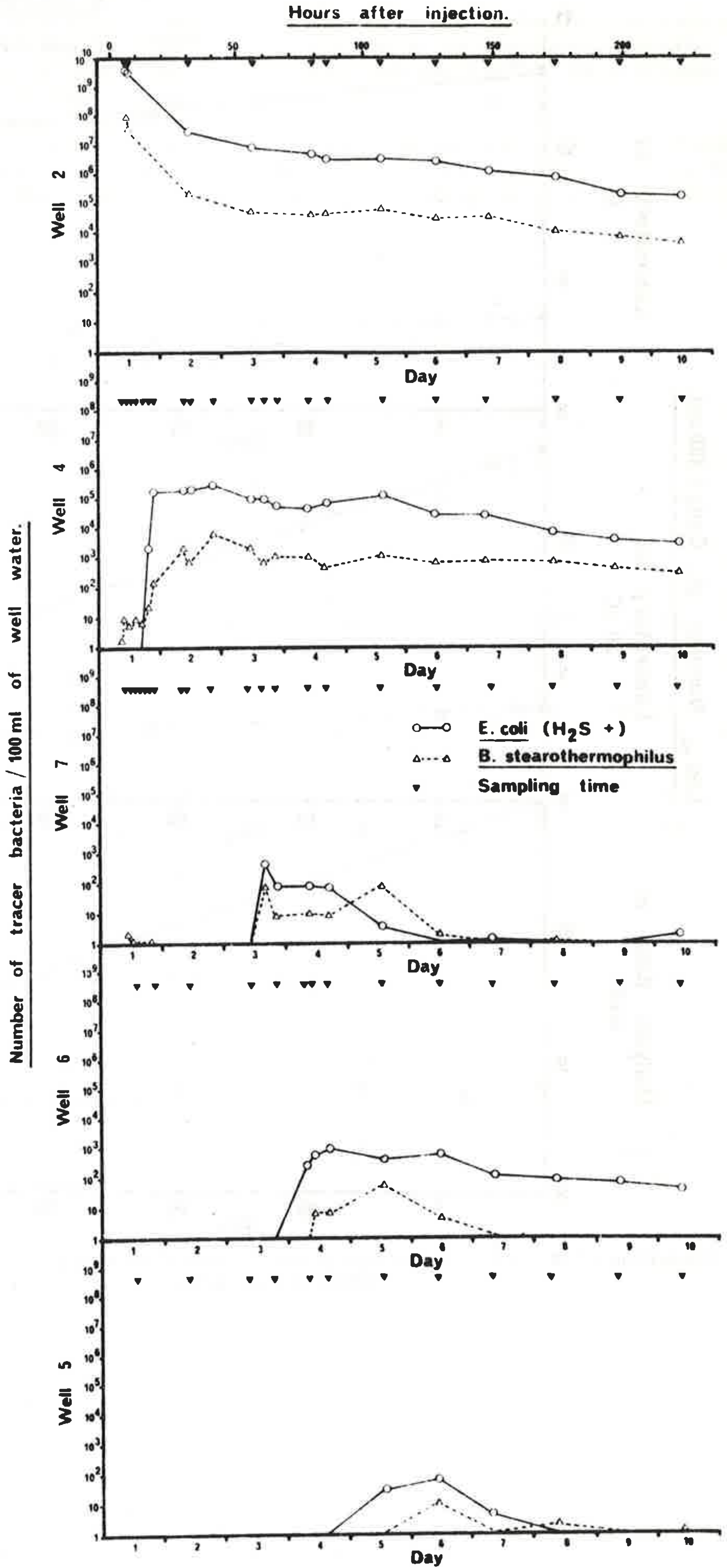


Figure 4 Relationship between *B. stearothermophilus* numbers (mean of wells 1 to 4) in groundwater at Burnham and daily and monthly rainfall

Figure 5 Number of *B. stearothermophilus* and *E. coli* (H_2S^+) cells recorded in 5 investigation wells at Burnham following injection into well 2. (Shown on Log_{10} scale)



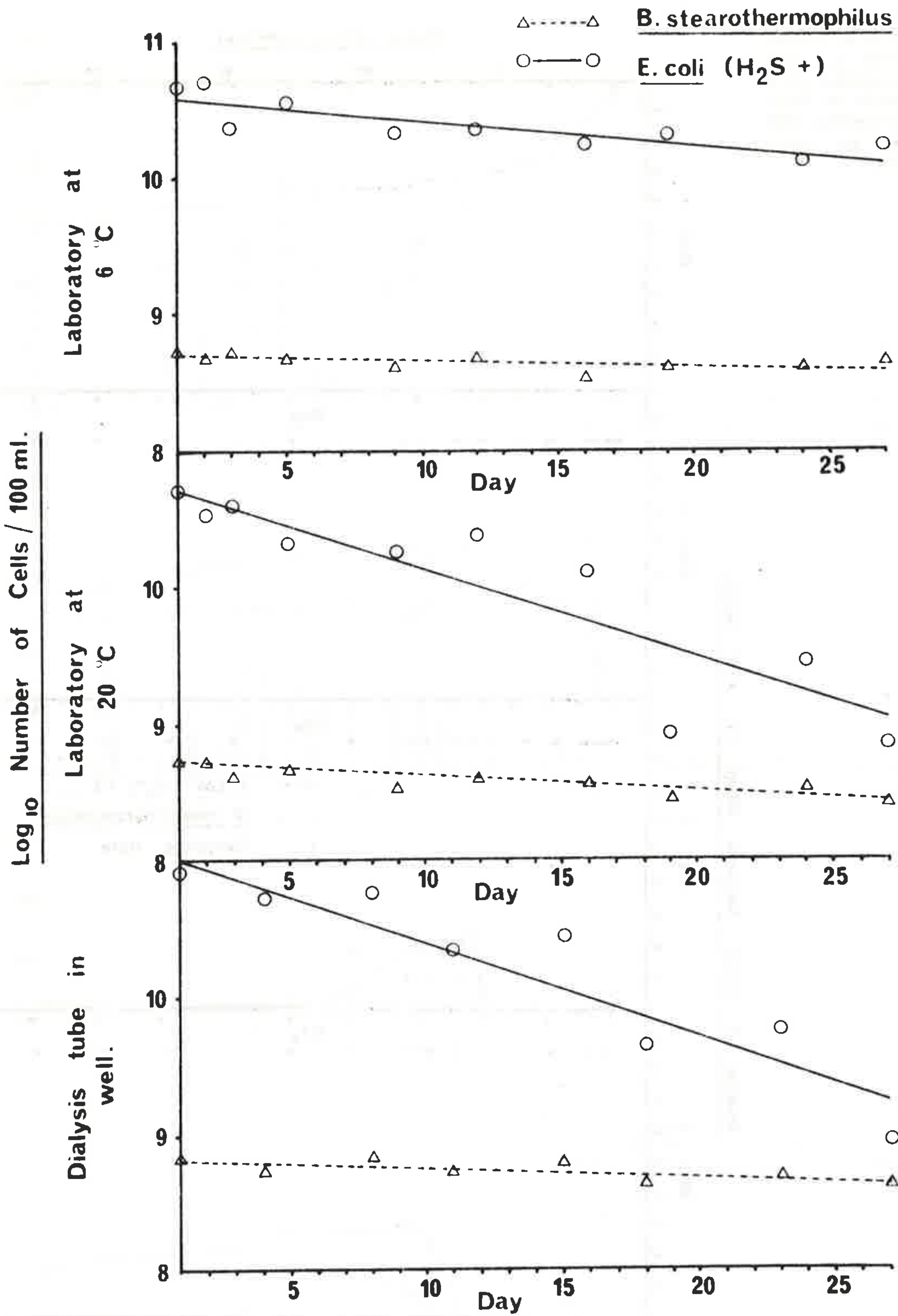


Figure 6 Die-off rates of *B. stearothermophilus* and *E. coli* (H_2S^+) in laboratory and in Burnham wells. (Shown on Log_{10} scale)

in the laboratory at 6°C, well water stored in the laboratory at 20°C, and dialysis tubes in well water at Burnham) are presented in Table 7. Measurements of the well water at Burnham indicated that the groundwater temperature remained at around 11.5°C. Also presented in the table are the estimated T90 times (time taken for 90% of the organisms to die off) for the three experiments.

5.4 Determination of naturally occurring levels of H_2S positive faecal coliform bacteria and *Escherichia coli* (H_2S^+) in sewage effluents

The results of the sampling for H_2S^+ faecal coliform bacteria and the laboratory tests for *E. coli* (H_2S^+) in sewage are presented in Table 8.

Table 7 Linear regression analyses for die-off rates of *B. stearothermophilus* and *E. coli* (H_2S^+).

Experiment	Species	r	r^2	SD	T_{90} Days	Regression Model Log. No. Bacteria/100 ml =
Stored in laboratory at 6°C	B. stearothermophilus	0.73*	0.53	0.65	208	8.71 - 0.005 x time (days)
	E. coli (H_2S^+)	0.85**	0.72	1.26	56	10.60 - 0.018 x time (days)
Stored in laboratory at 20°C	B. stearothermophilus	0.89**	0.79	0.94	100	8.72 - 0.010 x time (days)
	E. coli (H_2S^+)	0.90**	0.81	2.39	15	10.77 - 0.064 x time (days)
In dialysis tubes suspended in well water	B. stearothermophilus	0.79**	0.62	0.75	150	8.83 - 0.007 x time (days)
	E. coli (H_2S^+)	0.94**	0.88	2.44	14	11.17 - 0.072 x time (days)

r = correlation coefficient
r^2 = coefficient of determination
SD = standard deviation
T_{90} = time taken for 90% of the organisms to die-off
* significant at 5% level
** significant at 1% level

Table 8 Numbers of H_2S positive faecal coliform bacteria and *E. coli* (H_2S^+) present in sewage effluents

	Range in concentrations of faecal coliform bacteria/100 ml (yellow colonies on Teepol broth)	Range in concentrations of H_2S producing faecal coliform bacteria 100ml (yellow colonies turning black on TSIA	Percentage of H_2S producing faecal coliform colonies from sewage samples giving positive 44.5°C lactose fermentation and IMVIC results.				
			44.5°C lactose fermentation	Indole	Methyl Red	Vogues Proskauer	Citrate
Raw Sewage - Burnham	8 000 000 - 14 000 000	0 - 65 000	100	100	96	0	0
Oxidation Pond Effluent - Burnham	18 000 - 32 000	0 - 800	100	94	100	0	0
Pasveer Ditch Effluent - Lincoln	71 000 - 220 000	0 - 1 000	98	100	98	0	0
Pure *E. coli* culture (typical response)			100	100	100	0	0

6 DISCUSSION

Several important factors pertaining to the potential use of *Bacillus stearothermophilus* as a tracer organism in groundwater investigations arise from the results of the 12 month sampling programme (Table 2 and Fig. 4). The numbers of *B. stearothermophilus* cells detected in all the wells at the Burnham site increased during the winter months, reaching a peak in July, the wettest month in 1977 (105.5 mm). The lowest concentrations of the species were recorded in February 1978, the driest month (21.7 mm) during the study period. The correlations presented in Table 3 indicate that, during the year of sampling, a positive relationship existed between preceding rainfall and concentrations of *B. stearothermophilus* in the Burnham wells. Rainfall during the previous 20 days was of greater significance than rainfall during the previous 7 days.

The suggested explanation for the observed relationship between *B. stearothermophilus* numbers in groundwater and preceding rainfall may be deduced from Table 4. *B. stearothermophilus* would appear to be an ubiquitous species, cell numbers of up to 1600/g of soil being recorded in the Lincoln area. Similar numbers were found in Burnham soils. Rainfall may tend to wash *B. stearothermophilus* cells down through the soil profile and into underlying groundwater. As demonstrated in the main tracer experiment, the species travels readily in Canterbury groundwater systems, and therefore the cells detected in the Burnham wells following wet weather may have originated from soils a considerable distance upstream. During periods of high rainfall the extensive aquifer systems underlying the Canterbury Plains could be expected to accumulate considerable numbers of *B. stearothermophilus* cells, as indicated by the concentration of 650/100 ml recorded in well 3 in July 1977 (Table 2). A similar relationship between rainfall and concentration in groundwater probably exists for many other species of soil microflora.

The application of oxidation pond effluent containing up to 1200 *B. stearothermophilus* cells/100 ml to border dyke strips containing up to 2500 cells/g of soil, could be expected to cause a greater increase in the concentration of the species in groundwater than the effects of rainfall on non-effluent irrigated soils. However, in samples taken from wells 1, 2, 4, 6, and 7 following effluent application to border dyke strips 31 and 32 (identified by Martin and Noonan (1977) as the source of the microbial contamination in the downstream wells), no increase in the concentrations of *B. stearothermophilus* in the downstream wells was recorded.

The increase in background levels of *B. stearothermophilus* in all wells during the winter months has important implications in terms of the potential of the species as a tracer. The numbers recorded in wells 1, 2, 3, and 4 during the period June to September 1977 (Table 2) are in general far greater than those recorded in wells 4, 5, 6, and 7 during the main tracer experiment (Fig. 5). If the experiment had been conducted during June-September 1977, the arrival of the species in wells 5, 6, and 7 probably could not have been confirmed and the arrival time in well 4 would have been difficult to determine against the high "background noise" level. It appears therefore, that as far as the Canterbury groundwater studies are concerned, the use of *B. stearothermophilus* as a tracer organism would functionally be confined to periods of low rainfall, probably the months November to March. With natural levels in soil of up to 1600 cells/g, the species is also likely to be of limited value as a tracer of vertical water movement through soil profiles.

The results of the February 1978 tracer experiment at the Burnham site indicate that both *E. coli* (H_2S^+) and *B. stearothermophilus* were able to travel considerable distances in the underlying groundwater system (Fig. 5). Both species were removed from the injection well at the same rate and generally exhibited similar recovery patterns in the downstream wells. In wells 4, 6, and 7 concentrations of *E. coli* (H_2S^+), and to a lesser extent *B. stearothermophilus*, tended to rise relatively rapidly to a peak and to taper off more slowly. This effect was less pronounced in well 5 where relatively low numbers of both species were recorded. The reason for the anomalous second peak of *B. stearothermophilus* cells in well 7 on day 5 is not known. A survey of overseas literature indicates that the distance from well 2 to well 5 of 920 m is the furtherest distance recorded for an injection-recovery operation for bacterial species in groundwater.

The demanding logistics involved in the sampling of a wide range of dilutions from 5 wells for 2 species of bacterium prevented a more accurate estimation of times of arrival for wells 5, 6, and 7. Thus the estimated arrival times, in each case assumed to have occured half-way between the time of first detection and the previous sampling occasion, contain considerable potential error. The mean velocities of 7.66 m/hour or 184 m/day for *B. stearothermophilus* and 8.29 m/hour or 199 m/day for *E. coli* (H_2S^+) (Table 5) are faster than the 160 m/day rate of travel estimated by Noonan (1976). The differences in mean velocities for the two species are not considered to be significant. The lower apparent velocity of *B. stearothermophilus* is probably a function of the lower concentration of the species in the groundwater rather than a result of slower rate of travel, i.e., for each of wells 5, 6, and 7 the numbers of *B. stearothermophilus* passing through the well casing probably took longer to reach the threshold of detection inherent in a 100

ml sampling system than did the higher concentrations of *E. coli* (H_2S^+). For this reason, the data in Table 5 for *E. coli* (H_2S^+) are considered to provide the better estimate of groundwater flow velocity (approximately 200 m/day).

The differences in rates of recovery of the two species, expressed in Table 6 as a "percentage recovery ratio", are of considerable interest. Although relative recovery rates varied markedly from well to well, *B. stearothermophilus* exhibited a higher rate of recovery from groundwater samples than did *E. coli* (H_2S^+). This may have been due to one or more of the following:

a. The membrane filtration technique favoured the recovery of *B. stearothermophilus*.

b. *B. stearothermophilus* has a lower affinity for adsorption in groundwater strata than *E. coli* (H_2S^+).

c. The *B. stearothermophilus* cells sporulated soon after injection and the smaller spores were less affected by the filtering action of groundwater strata than the larger vegetative *E. coli* cells. In the relatively porous Canterbury gravels, this is considered to be unlikely.

d. *B. stearothermophilus* exhibited superior survival characteristics in the Burnham groundwater. This explanation was confirmed by the results of the die-off experiments (Fig. 6, Table 7). In all three experiments *B. stearothermophilus* exhibited a higher T90 than did *E. coli* (H_2S^+). This difference was least marked at 6°C, where *B. stearothermophilus* exhibited a T90 of 208.33 days and *E. coli* (H_2S^+) a T90 of 55.87 days. The 6°C temperature was selected to determine the relative die-off characteristics of the two species under cold storage conditions. The survival rate of both species was possibly enhanced by the presence of residual nutrients, not removed during centrifugation, in the storage flask.

At 20°C, in the storage vessel, the T90 of *E. coli* (H_2S^+) fell to 15.50 days and the T90 of *B. stearothermophilus* to 100.00 days. The die-off rate of *B. stearothermophilus* appeared therefore to be relatively less affected by the higher temperature than *E. coli* (H_2S^+).

Problems such as the differential movement of certain substances, for example proteins, through the dialysis tube walls and bacterial adsorbtion to the inner wall of the tube mean that conditions within the dialysis tube are not entirely representative of natural conditions in the groundwater. However, the dialysis tube experiments clearly indicated the superior survival characteristics of *B. stearothermophilus* (T90 of 150 days compared to 14 days for *E. coli* (H_2S^+)). This was probably due to the spore forming ability of the species.

The results presented in Table 8 indicate that faecal coliform bacteria capable of producing H_2S from thiosulphate occurred naturally in the 3 types of effluent sampled. However, the concentrations of these organisms were extremely variable. H_2S^+ faecal coliform bacteria were detected in the Pasveer ditch and raw sewage effluents on only one of the 7 sampling occasions (samples were collected over a 3 week period). They were detected in the Burnham oxidation pond on 2 of the 8 sampling occasions.

The results of the 44.5°C lactose fermentation and IMVIC tests were also of interest. Although these tests do not categorically delineate *E. coli*, the results presented in Table 8 indicate that most of the H_2S^+ faecal coliform bacteria isolated from the sewage samples were probably strains of *E. coli* able to produce H_2S from thiosulphate.

The significance of the intermittent presence of *E. coli* (H_2S^+) in sewage is not clear. In the case of the oxidation pond, the anaerobic environment in the bottom sediments may enhance the survival rate of H_2S producing micro-organisms, including *E. coli* (H_2S^+). Wind induced turbulence may occasionally carry these strains to the upper layers of the pond. However, the reason for the presence of *E. coli* (H_2S^+) in Pasveer ditch and raw sewage effluents remains unclear.

Because *E. coli* (H_2S^+) appears to occur intermittently in both raw and treated sewage effluents, the species may not be suitable as a tracer in sewage polluted waters. During the main tracer experiment at Burnham, no oxidation pond effluent was applied to the two border dyke strips considered by Martin and Noonan (1977) to be the source of the microbial contamination of wells 2, 4, 7, 6, and 5. Therefore the problem of background levels of *E. coli* (H_2S^+) did not arise in this investigation. However, under prolonged irrigation of the border dyke strips it seems likely that H_2S producing strains of *E. coli* would enter the groundwater system at Burnham.

If *E. coli* (H_2S^+) is unsuitable as a tracer in sewage polluted groundwater, there may be little advantage in using the strain in uncontaminated aquifers. As noted, sewage polluted groundwater may occasionally contain significant numbers of H_2S producing *E. coli*. On the other hand, uncon-contaminated groundwater will contain few, if any, *E. coli* and a non-H_2S producing strain would therefore be suitable. As it would not be necessary to confirm an H_2S^+ character, use of a non-H_2S producing strain would save the 4–5 hours incubation time on TSIA as well as the additional media and equipment required.

The relative rates of recovery of *E. coli* (H_2S^+) and *B. stearothermophilus* have been discussed. The two organisms have other advantages and disadvantages in terms of culture and recovery techniques. The culture medium for *B. stearothermophilus* was the more difficult to prepare and required the addition of traces of thiamine HCl and methionine for optimum growth at the 55°C incubating temperature (Baker *et al.* 1960). At this temperature, oxygen begins to become highly insoluble

in water, and, as the species is strongly aerobic, thorough aeration of the medium was found to be essential for growth to occur. Growth of *B. stearothermophilus* in the fermenter tended to be slower than for *E. coli* (H_2S^+). The former species generally reached less than 25% of the concentration obtainable with *E. coli* (H_2S^+) in double strength MMG for the same 24 hour period.

Following recovery by membrane filtration, colonies of *B. stearothermophilus* could be counted after 12 hours incubation as opposed to up to 24 hours for *E. coli* (H_2S^+) (4 hours at 30°C and 14 hours at 44.5°C on Teepol broth for the initial identification of *E. coli*, plus a further 4 to 6 hours on TSIA for confirmation of the H_2S positive character). However if, as previously noted, a non-H_2S producing strain of *E. coli* is used, incubation time for an *E. coli* tracer could be reduced to around 18 hours.

The *E. coli* (H_2S^+) was supplied by the National Health Institute (1242, *Escherichia coli*, CDC 3441–76, H_2S producing) and was sub-cultured every 30 days. A slight tendency to lose the H_2S^+ character was noted and colonies exhibiting the strongest H_2S character on TSIA were used in renewing the stock culture. The H_2S character proved to be considerably more stable than the urease positive character of a urease positive strain of *E. coli* investigated during 1977.

The principal disadvantage in using bacteria as tracers would appear to be the time required for their identification and enumeration in water samples. With substances such as fluorescent dyes and radioactive isotopes, results can be obtained almost immediately, thereby conferring greater flexibility on a sampling programme. However, the membrane filter technique developed for use in the Burnham experiment provided a very wide range of detectable concentrations of both species, from in excess of 10^{13} cells/100 ml to 1 cell/100 ml. In groundwater samples that are substantially free from suspended solids and extraneous micro-organisms, a lower detection threshold of 1 cell/1000 ml is feasible. This represents a greater level of sensitivity than is possible with either fluorescent dyes or radioactive isotopes. In previous work with microbial tracers, most investigators have relied on plate count methods for recovery of bacteria from water samples, which generally permit a maximum possible sample size of 1 ml. The membrane filtration technique increases lower range sensitivity up to one thousand-fold.

The February 1978 tracer experiment provided useful additional information about the groundwater characteristics of the Water and Soil Division's Burnham experimental site. The suspected connection between wells 2, 4, 5, 6, and 7 was confirmed. As previously noted, the speed of travel was higher than that estimated by Martin and Noonan (1977) but was similar to that estimated by McCabe and Rowse (1976). The shape of the tracer removal curve from injection well 2 (Fig. 5) tends to confirm the suggestion by McCabe and Rowse (1976) that low permeability of the well casing impeded water movement through the well. As the construction of all well casings at the site is identical, this phenomenon may have affected the results recorded in the downstream wells. The high rate of recovery of both species in wells 4 and 6 suggest that the well array 2, 4, and 6 would be valuable for the development of chemical and microbial tracer techniques for groundwater investigations in the Canterbury Plains area.

7 CONCLUSIONS

1. The bacterial species *Bacillus stearothermophilus* and *Escherichia coli* (H_2S^+) were both used successfully to trace groundwater movement at Burnham over a total distance of 920 m.
2. Of the two tracer species, *B. stearothermophilus* exhibited the better recovery rate from well water samples. This was probably due to the superior survival characteristics of the species. The species was more difficult to grow in liquid culture than *E. coli* (H_2S^+) but the recovery technique was somewhat simpler and shorter and required less media and equipment.
3. *B. stearothermophilus* was found to be present naturally in Canterbury soils and groundwater systems. Concentrations of the species in groundwater tended to increase following rainfall. This may limit the use of the species as a groundwater tracer to periods of low rainfall, and probably also precludes its use as a tracer of vertical water movement through soil profiles.
4. Neither *B. stearothermophilus* nor *E. coli* (H_2S^+) are considered to be suitable for use as tracers in sewage polluted water as both appear to occur naturally in sewage (although *E. coli* (H_2S^+) is present only intermittently). Accordingly, there may be little advantage in using an H_2S^+ producing strain of *E. coli* as a groundwater tracer. In polluted water, the occasional presence of the strain may preclude its use. In uncontaminated water, a non H_2S producing strain of *E. coli* will suffice.
5. Recovery of *B. stearothermophilus* and *E. coli* (H_2S^+) from groundwater samples by membrane filtration greatly increases the sensitivity of detection over plate count methods.
6. The suspected connection between investigation wells, 2, 4, 5, 6, and 7 at the Water and Soil Division experimental area at Burnham was confirmed. The well array possesses considerable potential as a site for the development of groundwater tracer techniques.

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9 REFERENCES

Baker, H.; Frank, O.; Pasher, I.; Black, B.; Hutner, S.H.; Sobotka, H. 1960: Growth requirements of 94 strains of thermophillic bacilli. *Canadian Journal of Microbiology 6*:557–63.

Burman, N.P.; Colbourne, J.S. 1976: The effect of plumbing materials on water quality. *Journal of the Institution of Plumbing 3*:2.

Dobson, W.H. 1968: *Serratia marcescens* septicemia. *Archives of Internal Medicine 121*:145–50.

Gates, C.D. 1967: The disposal of domestic wastes in rural areas. *In:* "Agriculture and the quality of our environment" (Edited by N.C. Brady). American Association for the Advancement of Science pp. 367–84.

Gerba, C.P.; Wallis, C.; Melnick, J.L. 1975: Fate of wastewater bacteria and viruses in soil. *Journal of the Irrigation and Drainage Division, Proceedings of the American Society of Civil Engineers. 101, No. IR3*:157–74.

Great Britain, Department of Health and Social Security 1969: "The Bacteriological Examination of Water Supplies". Reports on Public Health and Medical Subjects No. 71. Her Majesty's Stationery Office, London. 51 p.

Layne, P.; Hu, A.S.L.; Balows, A.; Davis, B.R. 1971: Extrachromosomal nature of hydrogen sulphide production in *Escherichia coli. Journal of Bacteriology 106(3)*:1029–30.

McCabe, W.J.; Rowse, O.J. 1976: "Underground Water Flow at Burnham." Institute of Nuclear Sciences, DSIR, Bulletin No. INS–R–189.

Martin, G.N. 1976: Water sampling from unpumped wells with static water levels deeper than 10 metres. *Journal of Hydrology (NZ) 15(1)*:41–5.

Martin, G.N.; Noonan, M.J. 1977: "Effects of Domestic Wastewater Disposal by Land Irrigation on Groundwater Quality of the central Canterbury Plains." *Water and Soil Technical Publication* 7. 25 p.

New Zealand Microbiological Society's Committee on Coliform Bacteria 1976: Definition of terms concerning coliform bacteria and recommended methods for their detection. *N.Z. Journal of Science 19*:215–9.

Noonan, M.J. 1976: "Effects of Wastewater Disposal by Land Irrigation on Groundwater Quality in the Rolleston area." Vol. 4, Part A. Unpublished Report, Microbiology Department, Lincoln College.

Omerod, J.G. 1964: *Serratia indica* as a bacterial tracer for water movements. *Journal of Applied Bacteriology 27*:342.

Orskov, I.; Orskov, F. 1973: Plasmid-determined H_2S character in *Escherichia coli* and its relation to plasmid-carried raffinose fermentation and tetracycline resistance characters. *Journal of General Microbiology 77*:487–99.

Pike, E.B.; Bufton, A.W.J.; Gould, D.J. 1969: The use of *Serratia indica* and *Bacillus subtilis* var. *niger* spores for tracing sewage dispersion in the sea. *Journal of Applied Bacteriology 32*:206–16.

Putman, J.L.; Wildblood, A.M.; Robson, J.E. 1956: The use of radioactive and bacterial tracers to follow sewage pollution in the sea. *Water and Sanitary Engineer 6*:99.

Rippon, J.E. 1963: The use of a coloured bacterium as an indicator of local water movements. *Chemistry and Industry (UK) March 1963*:445–6.

Smart, P.L.; Laidlaw, I.M.S. 1977: An evaluation of some fluorescent dyes for water tracing. *Water Resources Research 13(1)*:15–33.

Smith, D.I.; Atkinson, T.C. 1974: Underground flow rates in cavernous and fissured limestone. *In*: "Groundwater Pollution in Europe." Proceedings of a conference, Reading, 1972. The Water Research Centre, Port Washington, 1974 Ed. pp. 409–13.

Thorpe, H.R. 1977: "Movement of Contaminations Into and Through the Heretaunga Plains Aquifer." Unpublished report by MWD for National Water and Soil Conservation Organisation. 148 p.

White, K.E. 1976: Tracer methods for the determination of groundwater residence-time distributions. Preprint of paper presented to the conference on groundwater quality, measurement, prediction and protection, sponsored by Water Research Association, Reading, U.K.

Wimpenny, J.W.T.; Cotton, N.; Stratham, M. 1972: Microbes as tracers of water movement. *Water Resources 6*:731–9.

Appendix 1 Burnham tracer experiment — numbers of tracer bacteria recorded in well 2 (injection well)

Day	Time	Hours after injection	Number/100 ml of well water	
			Bacillus stearothermophilus	E. coli (H_2S^+)
1	10.15 am	6.15	93,000,000	4,030,000,000
	11.15 am	7.15	31,800,000	3,960,000,000
2	11.45 am	31.45	219,000	26,100,000
3	11.40 am	55.40	54,000	9,000,000
4	10.15 am	78.15	39,600	5,100,000
	3.50 pm	83.50	58,000	3,000,000
5	2.10 pm	106.10	63,000	3,200,000
6	12.00 noon	128.00	31,000	2,500,000
7	9.15 am	149.15	32,000	1,350,000
8	10.15 am	174.15	10,000	700,000
9	11.00 am	199.00	7,500	200,000
10	11.15 am	224.15	5,100	163,000

Original "mix" *Bacillus stearothermophilus* 2,580,000,000 cells
E. coli (H_2S^+) 236,000,000,000 cells

Injection time: 4.00 am Day 1.

Appendix 2 Burnham tracer experiment — numbers of tracer bacteria recorded in well 4

Day	Time	Hours after injection	Number/100 ml of well water	
			Bacillus stearothermophilus	*E. coli* (H_2S^+)
1	9.45 am	5.45	2	0
	10.45 am	6.45	10	0
	11.45 am	7.45	6	0
	2.20 pm	10.20	10	0
	4.00 pm	12.00	9	0
	7.40 pm	16.40	25	2,380
	8.40 pm	17.40	210	191,000
2	10.30 am	30.30	2,500	225,000
	11.30 am	31.20	800	237,000
	8.00 pm	40.00	7,500	300,000
3	11.25 am	55.20	2,600	101,000
	3.35 pm	59.35	800	102,000
	8.15 pm	64.15	1,100	60,000
4	9.50 am	76.15	1,180	51,000
	3.45 pm	83.45	510	72,000
5	2.00 pm	106.00	1,400	138,000
6	11.50 am	127.50	760	31,000
7	9.10 am	149.10	810	28,200
8	10.10 am	174.10	880	8,200
9	10.50 am	198.50	510	4,200
10	11.05 am	223.05	340	3,100

Appendix 3 Burnham tracer experiment — numbers of tracer bacteria recorded in well 7

Day	Time	Hours after injection	Number/100 ml of well water	
			Bacillus stearothermophilus	*E. coli* (H_2S^+)
1	11.30 am	7.30	2	0
	12.15 pm	8.51	1	0
	1.55 pm	9.55	0	0
	3.00 pm	11.00	0	0
	4.10 pm	12.10	0	0
	7.30 pm	15.30	1	0
	8.30 pm	16.30	0	0
2	10.20 am	30.20	0	0
	11.20 am	31.20	0	0
	7.50 pm	39.50	0	0
3	11.15 am	55.15	0	0
	3.25 pm	59.25	79	460
	8.05 pm	64.05	8	84
4	9.40 am	77.40	10	87
	3.35 pm	83.35	9	69
5	1.50 pm	105.50	78	3
6	11.45 am	127.45	2	0
7	9.00 am	149.00	0	1
8	10.00 am	174.00	1	0
9	10.40 am	198.40	0	0
10	10.55 am	222.55	0	2

Appendix 4 Burnham tracer experiment — numbers of tracer bacteria recorded in well 6

Day	Time	Hours after injection	Number/100 ml of well water	
			Bacillus stearothermophilus	*E. coli* (H_2S^+)
1	1.45 pm	9.45	0	0
	8.20 pm	16.20	0	0
2	11.10 am	31.10	0	0
3	11.05 am	55.05	0	0
	7.40 pm	63.40	0	0
4	9.30 am	77.30	0	310
	10.55 am	78.55	7	620
	3.20 pm	83.20	7	1000
5	1.40 pm	105.40	61	430
6	11.35 am	127.35	5	630
7	8.50 am	148.50	0	116
8	9.50 am	173.50	0	92
9	10.30 am	198.30	0	73
10	10.45 am	222.45	1	42

Appendix 5 Burnham tracer experiment — numbers of tracer bacteria recorded in well 5

Day	Time	Hours after injection	Number/100 ml of well water	
			Bacillus stearothermophilus	*E. coli* (H_2S^+)
1	1.35 pm	9.35	0	0
2	11.00 am	31.00	0	0
3	10.55 am	54.55	0	0
	7.30 pm	63.30	0	0
4	10.40 am	78.40	0	0
	3.10 pm	83.10	0	0
5	1.30 pm	105.30	0	31
6	11.20 am	127.20	10	64
7	8.40 am	148.40	0	4
8	9.40 am	173.40	2	0
9	10.20 am	198.20	0	0
10	10.30 am	222.30	1	6