The long-term survival of *Escherichia coli* in river water

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Escherichia coli introduced into autoclaved filtered river water survived for up to 260 d at temperatures from 4° to 25°C with no loss of viability. Survival times were less in water which was only filtered through either a Whatman filter paper or a 0·45 μm Millipore filter or in untreated water, suggesting that competition with the natural microbial flora of the water was the primary factor in the disappearance of the introduced bacteria. Survival was also dependent upon temperature with survival at 4°C > 15°C > 25°C > 37°C for any water sample. Direct counts showed that bacterial cells did not disappear as the viable count decreased. The possession of the antibiotic resistance plasmids, R1*drd*-19 or R144-3, did not enhance survival nor cause a faster rate of decay, indicating that the metabolic burden imposed by a plasmid was not a factor in survival under starvation conditions. There was no evidence of transfer of either plasmid at 15°C or of loss of plasmid function during starvation.

The survival of a micro-organism in an environment in which it is not indigenous depends upon its ability to tolerate an alien set of biological, physical and chemical conditions. For faecal bacteria, this is important from a public health point of view but also, as there is the possibility that genetically-manipulated organisms might be released into the aquatic environment, it is necessary that the factors which influence the long-term survival of non-indigenous micro-organisms are understood.

There have been many studies on the survival of *Escherichia coli* and other indicators of faecal pollution in freshwater, marine and estuarine habitats. In most of the marine studies, *E. coli* disappeared rapidly because of the influence of temperature (Orlab 1956), sedimentation (Rittenberg *et al.* 1958) or the bactericidal effects of u.v. light (Gameson & Saxon 1967; Fujioka & Narikawa 1982), although it is more likely that a combination of these and other factors were responsible (Mitchell 1968; Mitchell & Chamberlain 1975). In estuarine environments, these bacteria survived for longer periods, mainly because of the decreased effect of u.v.

light which is partially adsorbed by the more turbid waters. Predation (McCambridge & McMeekin 1980) or temperature (Faust *et al.* 1975) are also suggested as the major factors in the disappearance of *E. coli*.

In freshwater, it is less clear which are the major factors involved in the disappearance of faecal bacteria. Indeed, *E. coli* has been shown to grow in diffusion chambers suspended in both the aerobic and anaerobic layers of a warm monomitic reservoir receiving thermal effluent from a nuclear power station (Gorden & Fliermans 1978). Survival times for *E. coli* of 1–5 d were noted in well water (McFeters *et al.* 1974) and up to 6 weeks in a laboratory stream (Cherry & Guthrie 1975). Mancini (1978) has suggested that temperature is the major factor in the disappearance of faecal bacteria in freshwater. Other studies have revealed that *E. coli* introduced into aquatic environments suffered from sub-lethal stress within 1 week (McFeters *et al.* 1982). This made the enumeration of *E. coli* on selective media difficult and subject to error (Bissonnette *et al.* 1975, 1977; Zaske *et al.* 1980) suggesting that the reported

disappearance of E. coli was because they became non-culturable rather than dying.

We report here on the long-term survival of E. coli in river water samples and on some of the factors, e.g. temperature, competition for nutrients and possession of plasmids which might affect the survival of these bacteria in laboratory microcosms.

Materials and Methods

STRAINS

Three strains were used in this study: E. coli nal-R (a nalidixic acid-resistant mutant of E. coli K12), E. coli R1drd-19 (E. coli K12 with the transfer derepressed plasmid R1drd-19 of the F incompatibility group) and E. coli R144-3 (E. coli K12 with the I incompatibility plasmid R144-3). All the strains were originally obtained from Dr E. Meynell (University of Kent) and have been described in more detail elsewhere (Harden & Meynell 1973).

The organisms were routinely maintained on Nutrient agar (Oxoid) slopes at 4°C. The antibiotic resistance of the strains carrying plasmids was checked before use. Escherichia coli nal-R was routinely used as the recipient in the plasmid transfer experiments.

MEDIA

Nutrient broth (Oxoid) and Nutrient agar (Oxoid) were made according to the manufacturers' instructions. Antibiotics (all obtained from Sigma) were added at final concentrations of 10 mg/l except for nalidixic acid which was added at 50 mg/l. All agar plates were dried and stored at 4°C until used.

COUNTING

Routine viable counts were determined using the surface spread plate technique on nutrient agar. Serial dilutions were prepared in quarter-strength Ringers solution (Cruickshank et al. 1975) and 0·1 ml samples were plated in quadruplicate. The plates were incubated at 37°C overnight and counted manually.

Direct total counts were made using a light microscope and a Neubauer counting chamber.

At least 100 squares were examined on three separate slides to obtain the count.

Viable and direct counts are expressed as mean ± 95% confidence limits of the mean.

WATER SAMPLES

One litre samples of river water were collected in sterile bottles from two sites on the River Sowe near the Finham sewage works outfall, Coventry (Ordnance Survey ref. SP 339753 and SP 332734 for the samples collected above and below the sewage works, respectively).

Samples were collected from above and below the sewage works outfall to provide representative samples from a typical 'clean' West Midlands river and one polluted with organic effluent. The average BODs of the samples were 1 and 6 mg/l, respectively (data obtained from the Severn Trent Water Authority).

SURVIVAL EXPERIMENTS

The river water samples were treated to produce four different samples as shown in Fig. 1. River water (100 ml) was added to a 250 ml sterile flask wrapped in aluminium foil. Each flask was inoculated with 0·1 ml of an overnight culture of E. coli in nutrient broth to give an initial viable count of approximately 10^6 cells/ml. The flasks were incubated in the dark without shaking at 4°, 15°, 25° and 37°C. Samples (0·5 ml) were removed at each sample time and serially diluted for the viable count determination. The sample times varied from daily to every 5 d depending upon the incubation temperature and the water treatment.

THE EFFECT OF INITIAL VIABLE COUNT ON SURVIVAL

To study the effects of the initial viable count on survival, a slightly different inoculation procedure was used to prevent the addition of excessive amounts of nutrient broth to the river water flasks. Escherichia coli was grown overnight in nutrient broth and harvested by centrifugation as aseptically as possible. The cell pellet was washed with sterile river water and resuspended to the appropriate cell concentration such that 0·1 ml of the final sample added

Sample	Comment
Untreated river water	Contains protozoa, suspended material, bacteria, phage

Whatman No. 1 filter

↓

| Whatman-filtered water | Contains phage, bacteria |

0·45 μm Millipore filter

↓

| Millipore-filtered water | Contains phage, ultramicrobacteria |

Autoclave 121°C/15 min

↓

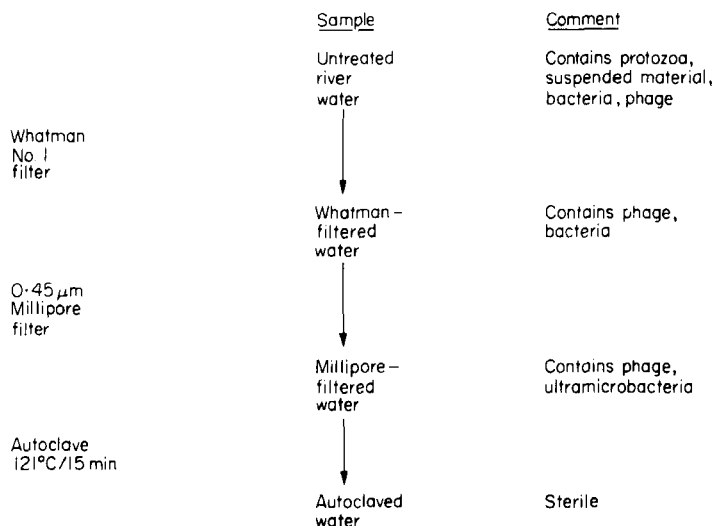| Autoclaved water | Sterile |

Fig. 1. Scheme for the preparation of the various water samples used in the survival experiments.

to 100 ml river water gave an initial viable count of approximately 10^6, 10^7 or 10^8 cells/ml of river water.

PLASMID TRANSFER EXPERIMENTS

Both plasmid R1*drd*-19 and plasmid R144-3 transfer at a high frequency in liquid mating experiments. Test-tubes containing 1 ml of nutrient broth were inoculated with the donor colonies and incubated at 37°C for 6 h without shaking. One ml of an exponential phase culture of the recipient strain, *E. coli* nal-R, was added and the test-tube incubated for a further 20 h without shaking at 37°C. Samples were streaked onto nutrient agar plates containing both kanamycin and nalidixic acid. Plasmid transfer was detected simply by the observation of growth on the dual antibiotic plates.

Results

THE EFFECTS OF TEMPERATURE AND FILTRATION ON THE SURVIVAL OF *E. coli* nal-R

The disappearance of *E. coli* in these experiments was expressed in two ways: firstly as the value, *k*, which is the decay constant (the gradient of the linear portion of the plot of log viable count against time) and secondly as the t_{99} value (the time taken for a two log drop in

viable count from the original inoculum size). This second value allowed the inclusion of the lag, the time before there was any drop in bacterial numbers. The term disappearance is used as there is no evidence in any survival experiments that *E. coli* actually died.

Escherichia coli nal-R grown in nutrient broth was added to the river water samples and the flasks were incubated at 4°, 15°, 25° and 37°C for up to 260 d. Figure 2 shows the survival curves for *E. coli* in the water samples collected from above the sewage works outlet and Fig. 3, those in the water samples collected from below the sewage works. The survival parameters for the two water samples, the four treatments and the four incubation temperatures are given in Table 1 and, for comparison, the value of *k* calculated using the equation given in Mancini (1978) is also listed.

There was little difference between the survival of *E. coli* in the untreated and Whatman-filtered water from above or below the sewage outfall, indicating that protozoa and attachment to particles appear to have little influence on the disappearance of *E. coli* in these river water samples. Survival was greatly enhanced, however, at temperatures of 25°C and below in the samples which had been Millipore-filtered to remove most of the bacteria which would otherwise be competing for nutrients with the *E. coli*. The eventual decline in bacterial numbers after 8 d in the sample from below the sewage outfall
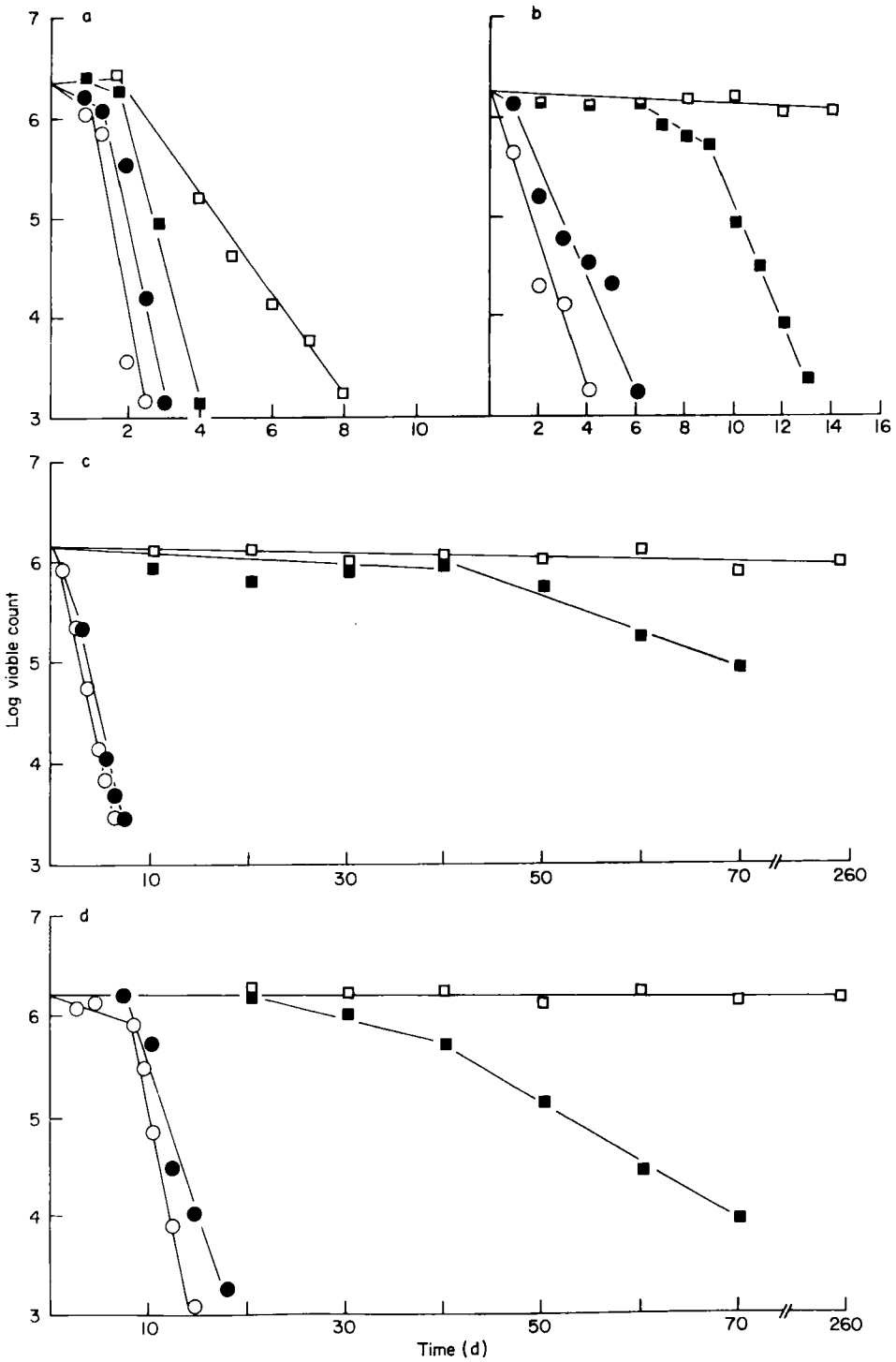
Fig. 2. The survival of *Escherichia coli* K12 nal-R in River Sowe water collected from above the Finham sewage effluent outfall. All the flasks were incubated in the dark at a, 37°C; b, 25°C; c, 15°C; and d, 4°C. ○, Untreated water; ●, Whatman No. 1-filtered water; ■, 0·45 μm Millipore-filtered water; and □, autoclaved filtered water. In (b) the viable count in the autoclaved water sample remained constant for 260 d.
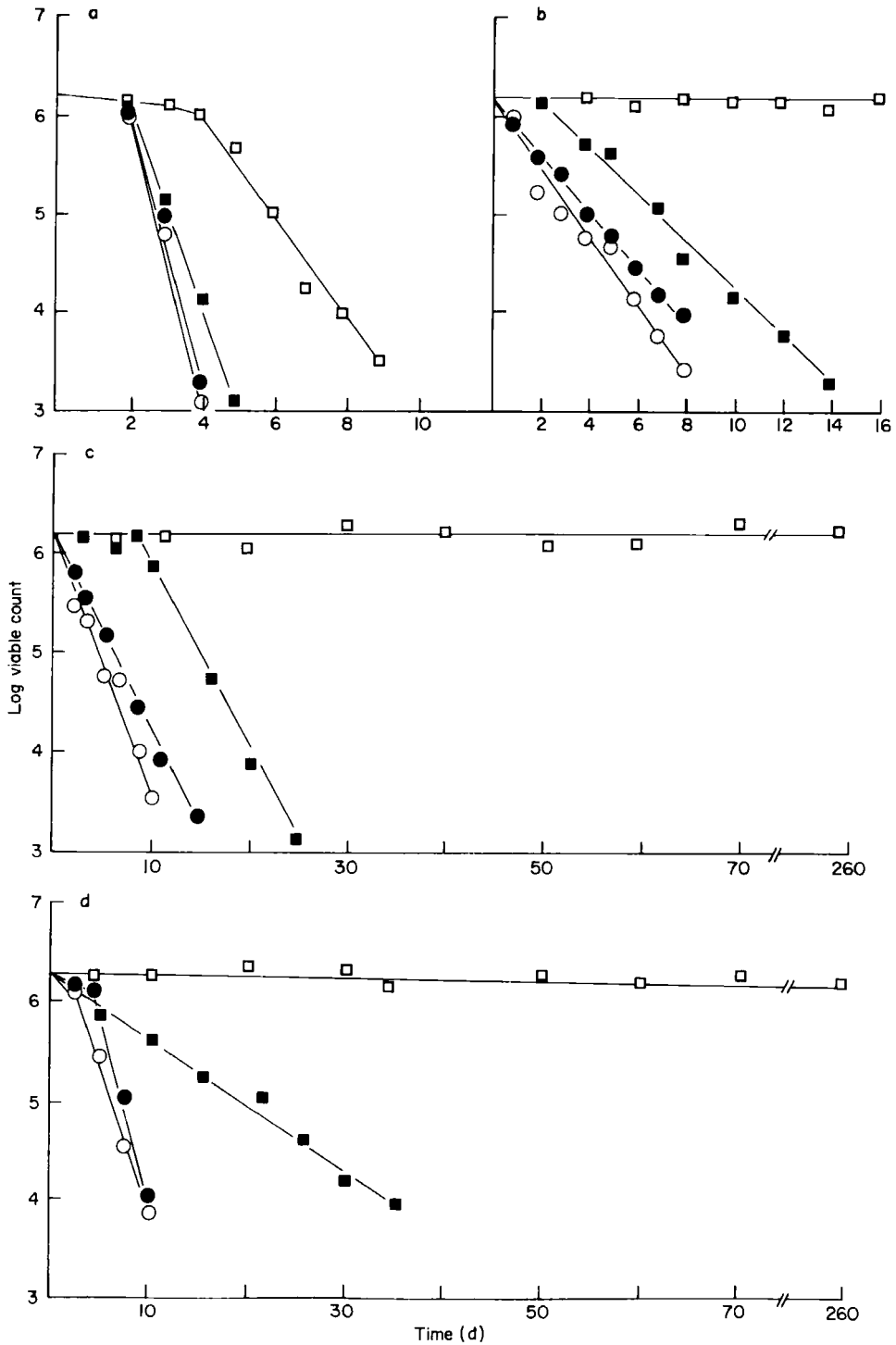
Fig. 3. The survival of *Escherichia coli* K12 nal-R in River Sowe water collected from below the Finham sewage effluent outfall. All the flasks were incubated in the dark at a, 37°C; b, 25°C; c, 15°C; and d, 4°C. ○, Untreated water; ●, Whatman No. 1-filtered water; ■, 0·45 μm Millipore-filtered water; and □, autoclaved filtered water. In (b) the viable count in the autoclaved water sample remained constant for 260 d.

Table 1. The values of k and t_{99} for the disappearance of *Escherichia coli* nal-R in River Sowe water

Sample	Temp. (°C)	Unfiltered		Whatman filtered		Millipore filtered		Autoclaved		Mancini*
		k	t_{99}	k	t_{99}	k	t_{99}	k	t_{99}	k
Above	4	−0·95	11·5	−0·63	12·2	−0·09	120	0	>260	0·27
sewage	15	−1·15	4·2	−0·96	4·5	−0·08	70	0	>260	0·57
outfall	25	−1·61	2·5	−1·29	3·5	−0·88	11·5	0	>260	1·12
	37	−3·33	1·9	−3·33	2·5	−3·33	3·1	−1·07	60	2·53
Below	4	−0·63	9·0	−0·58	10	−0·14	33	0	>260	0·27
sewage	15	−0·64	8·0	−0·46	10	−0·41	19	0	>260	0·57
outfall	25	−0·86	6·0	−0·66	6·6	−0·52	10·2	0	>260	1·12
	37	−3·40	3·2	−3·17	3·4	−2·23	4·1	−1·15	8	2·53

* Data calculated using the equation $k = 0.8 \times 1.07^{(t-20)}$ where t is temperature in °C (Mancini 1978).

All survival experiments were conducted in the dark in stationary flasks. Viable counts were determined on nutrient agar plates using a surface spread plate technique with incubation at 37°C for 24 h. Values of k and t_{99} were expressed in units of reciprocal days and days, respectively.

and after 40 d in the sample from above the sewage outfall was probably due to the presence of ultramicrobacteria able to pass through the 0·45 μm filter or due to the presence of bacteriophage. Indeed, once decay in numbers had started microscopic examination of the water samples revealed the presence of very small and very motile bacteria which could not be cultivated on nutrient agar plates incubated at 10°, 15°, 25° or 30°C. Bacteriophage were also shown to be present by plaque assays (results not shown) but at very low titres.

Autoclaving the water samples to remove viral predators (bacteriophage) as well as the bacterial competitors gave the longest survival times at all the temperatures for both water samples. No decline in numbers was noted at temperatures of 25°C and below for either water sample over a 260 d experimental period.

The results presented here are from a representative experiment. In repeat experiments, the absolute values of k and t_{99} differed but their order of magnitude and the general pattern of the results remained unchanged. In all experiments conducted to date, survival in autoclaved filtered water samples has been in excess of 260 d (the longest experimental period).

THE EFFECT OF INITIAL VIABLE COUNT
ON SURVIVAL

The previous experiment showed that in autoclaved water at 15°C, *E. coli* survived for a considerable period of time with an initial viable count of 10^6 cells/ml. Figure 4 shows the effect of different initial viable counts on the survival of *E. coli*. Disappearance of *E. coli* was followed by determination of viable counts on nutrient agar and direct counts by light microscopy. In all cases at initial viable counts of 10^6, 10^7 or 10^8 cells/ml the direct count was always higher than the count estimated by viable count. There was no decrease in the viable count or direct count from the initial viable counts of 10^6 or 10^7 cells/ml during the time course of the experiment but at an initial viable count of 10^8 cells/ml there was a decline in the viable count ($k = -0.037$ reciprocal d; $t_{99} = 135$ d), but not in the direct counts.

THE EFFECT OF PLASMIDS ON SURVIVAL

As plasmids have been shown to have an effect on the growth rate of bacteria, *E. coli* carrying plasmid R1*drd*-19 or plasmid R144-3 together with *E. coli* nal-R were incubated at 25° or 15°C, in autoclaved filtered river water samples collected from upstream of the sewage outfall. There was no loss of either the plasmid-carrying or the plasmid-less bacteria as determined by viable counts on nutrient agar with the addition of kanamycin or nalidixic acid, respectively over a 2 month experimental period (Fig. 5). Selection for plasmid transfer on nutrient agar plus both nalidixic acid and kanamycin revealed no evidence for plasmid transfer under these conditions. Colonies from the nutrient agar plus kanamycin plates were examined at the end of
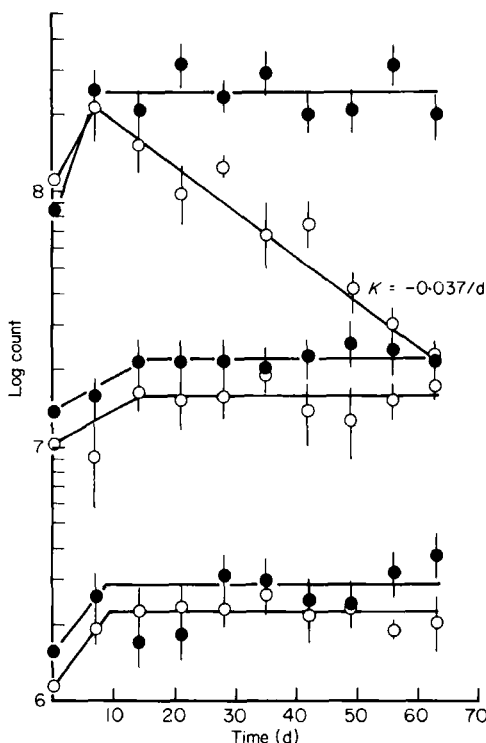
Fig. 4. The survival of different initial viable counts of *Escherichia coli* K12 nal-R in autoclaved filtered River Sowe water collected from above the Finham sewage effluent outfall. Flasks were incubated in the dark at 15°C. O, Viable count on nutrient agar; ●, direct microscope count. The error lines represent the standard error of the mean. *k* is the specific death rate.

the experiment to determine whether or not any of the plasmid functions had been lost during the survival experiment. Over 200 colonies of *E. coli* R1drd-19 were examined and all were resistant to the same antibiotics (kanamycin, ampicillin, tetracycline and streptomycin/ sulphonamide) as at the start of the experiment.
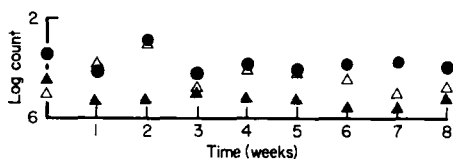

Fig. 5. The effect of plasmids on the survival of *Escherichia coli* in autoclaved filtered River Sowe water collected from above the Finham sewage effluent outfall. Flasks were incubated in the dark at 15°C. ●, R1-19; ▲, R144-3; △, nal-R.

In overnight liquid culture matings in nutrient broth at 37°C, all were able to transfer their plasmid to *E. coli* nal-R although the frequency of transfer was not determined.

Discussion

Escherichia coli has previously been shown to survive for up to 6 weeks in a laboratory stream (Cherry & Guthrie 1975). Temperature and nutrient availability (plus competition for these nutrients) have been suggested as the factors most likely to affect its survival in a laboratory microcosm (Bissonnette *et al.* 1975; Mancini 1978).

In untreated water samples collected from above or below the sewage outfall on the River Sowe, *E. coli* disappeared rapidly with t_{99} values of between 4 and 11 d at temperatures which reflect those which would occur naturally. There was little difference between survival times in the two water samples at 4°C but at 15°, 25° and 37°C survival was for a slightly longer time in the more polluted sample. The survival times are comparable to those from other studies on fresh or estuarine environments (McFeters *et al.* 1974; McCambridge & McMeekin 1981). The rate of decay in the untreated sample depended on temperature, and the slowest decay rates were observed at the lowest temperature, as previously suggested by Faust *et al.* (1975). The values of *k* presented here do not agree closely with those calculated from the data of Mancini (1978) but they fall within the rough area of his values (Table 1). Mancini (1978) comments on the apparent differences in data from experiments conducted using different water samples and this is hardly surprising given the great variation in physical, chemical and biological parameters that there must be between water samples.

In order to study the effects of parts of the biotic component of the water on survival, water samples were filtered in various ways. Filtration through Whatman No. 1 filter paper effectively removed most of the protozoa and the particulate material from the water samples. Both these components have been implicated in the disappearance of *E. coli* from waters, both *in situ* and in the laboratory (Rittenberg *et al.* 1958; Enzinger & Cooper 1976; McCambridge & McMeekin 1980). Here, however, there was

little or no difference in the rate of disappearance of *E. coli* in the Whatman-filtered or untreated samples which would suggest that these factors had little effect on the survival of *E. coli* in these samples, although it must be borne in mind that protozoa could be a major factor in the disappearance of *E. coli* if they were present in large numbers. Again there were only small differences between the survival times in the two water samples, but at 15°, 25° and 37°C survival was for slightly longer in the more polluted sample.

Filtration of the river water through a 0·45 μm Millipore filter did increase the survival times for *E. coli* at all the temperatures tested. Millipore filtration removed most of the bacteria from the water. Once decay in bacterial numbers had started, microscopic examination of the cultures revealed the presence of very small and very motile bacteria which could not be cultured on nutrient agar incubated at temperatures from 10° to 30°C. Consequently, these bacteria could not be identified although visual observation suggested that they could have been a species of *Vibrio*. They must have been able to pass through the 0·45 μm filter, suggesting that they resemble the ultramicrobacteria described in marine environments by Poindexter (1979). Bacteriophage particles were also shown to be present by plaque assays, but at very low titres. The eventual decline in numbers of *E. coli* in the Millipore-filtered samples would probably have been due to the effects of one of these components on *E. coli*.

There was considerably longer survival in the Millipore-filtered sample from above the sewage works at 4° and 15°C but not at 25° and 37°C. We have no ready explanation for this difference, bearing in mind that in the Whatman-filtered and untreated water samples survival was longer in the samples from below the sewage works. It is possible that some organic material present in the Millipore filters could have prolonged survival.

In the autoclaved water samples, however, *E. coli* survived in water samples for up to 260 d with no loss of viable numbers, although incubated at temperatures as high as 25°C at which *E. coli* should have been able to grow. Even at 37°C, the optimum growth temperature of this strain, the t_{99} value was up to 60 d in the autoclaved sample compared with 4 d or less in the other samples. These results suggest that it is a

biological component of the water which is primarily responsible for the disappearance of *E. coli* from these water samples and temperature is a secondary, although very important, factor.

Plasmids have been shown to cause a reduction in the growth rate of bacteria which possess them (Zund & Lebek 1980), and to make plasmid-plus strains less competitive in chemostat culture (Godwin & Slater 1979). There was no evidence, however, to suggest that plasmids made the bacteria less competitive under the starvation conditions experienced in these experiments as the plasmid-plus bacteria survived as well as the plasmid-minus bacteria. Although chemostat studies by other workers have suggested that, in the absence of a selective pressure, the plasmid can begin to disintegrate under the effects of nutrient limitation (Godwin & Slater 1979) there was no loss of any of the plasmid functions tested during the starvation period. There was also no evidence for plasmid transfer during the survival experiments in which a plasmid-plus donor and a plasmid-less recipient were incubated together at temperatures of 25°C and below. Although laboratory experiments have shown that plasmid transfer is infrequent below 35°C it has been shown to occur, although infrequently, at 17°C with a derepressed plasmid (Singleton & Anson 1981). These experiments, however, were carried out in a nutrient broth rather than in a starvation medium and the lack of transfer here was most likely due to the absence of a suitable energy source to drive the transfer mechanism.

Morita (1982) suggested that 1% or less of a culture survived for a long period of time without the addition of an exogenous energy yielding substrate. We found, however, that more than 90% of introduced *E. coli* survived in river water for up to 260 d without the addition of any extra carbon source (except that in 0·1 ml of spent nutrient broth in the inoculum) to the water. It is unlikely that these water samples contained sufficient energy supplies to enable the bacteria to meet their maintenance energy requirement for this length of time let alone have sufficient energy to undergo cell division.

The survival of *E. coli* for such a long time without any replication suggests that they may have entered some type of dormant state. Morita (1982) coined the phrase 'starvation-survival' to explain the long-term survival of his marine isolates in the absence of exogenous

nutrient supplies. In their experiments Morita and co-workers (Novitsky & Morita 1976; Morita 1982; Amy & Morita 1983) studied the survival of marine bacteria, particularly marine vibrios, in a mineral salts medium without any added carbon or nitrogen sources. After an initial burst of growth and subsequent decline to a constant viable count, usually less than 0·1% of the initial inoculum, survival times in excess of 8 months were noted under these conditions. Although there was, on occasion, an increase in numbers immediately upon addition of *E. coli* to the river water, the increase was never more than a doubling of the initial inoculum size and certainly not on the scale of the one to two logs increase previously reported (Amy & Morita 1983; Kuruth & Morita 1983). There was no decrease in the size or change in shape of the bacteria noted in our experiments. It is possible that the fragmentation of a culture at the commencement of starvation is a phenomenon associated with marine rather than with freshwater micro-organisms but more non-marine micro-organisms need to be examined before this can be shown categorically to be the case.

Starvation-survival thus becomes an important concept in ecology because it concerns the long-term survival of individuals in unfavourable conditions. Hence the genome will survive enabling the small numbers of bacteria which have survived starvation to recolonize a suitable habitat should favourable conditions return. In the case of these *E. coli*, that would entail re-ingestion into the gut of a suitable animal species. Starvation-survival will also have an important bearing on the dissemination of antibiotic resistance factors as the survival of the plasmid information in an alien environment is as important as the survival of the parental bacterial strains and here we have shown that there was no difference in the survival of plasmid-plus and plasmid-minus strains of *E. coli*.

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