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RESUSCITATION AND QUANTIFICATION OF STRESSED *ESCHERICHIA COLI* K12 NCTC8797 IN WATER SAMPLES

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SUMMARY

The aim of this study were to investigate the impact on numbers of using different media for the enumeration of *Escherichia coli* subjected to stress, and to evaluate the use of different resuscitation methods on bacterial numbers. *E. coli* was subjected to heat stress by exposure to 55^{o}C for 1 h or to light-induced oxidative stress by exposure to artificial light for up to 8 h in the presence of methylene blue. In both cases, the bacterial counts on selective media became below the limits of detection whereas on non-selective media colonies were still produced. After resuscitation in non-selective media, using a multi-well MPN resuscitation method or resuscitation on membrane filters, the bacterial counts on selective media matched those on non-selective media. Heat and light stress can affect the ability of *E. coli* to grow on selective media essential for the enumeration as indicator bacteria. A resuscitation method is essential for the recovery of these stressed bacteria in order to avoid underestimation of indicator bacteria numbers in water. There was no difference in resuscitation efficiency using the membrane filter and multi-well MPN methods. This study emphasises the need to use a resuscitation method if the numbers of indicator bacteria in water samples is not to be under-estimated. False negative results in the analysis of drinking water or natural bathing waters could have profound health effects.

Key Words: Resuscitation, *Escherichia coli*, Water samples

INTRODUCTION

Bacteria belonging to the coliform group have been used for many years in order to assess the hygienic quality of water. The isolation of *Escherichia coli* or other coliform organisms from a water sample is sufficient to class it as unsatisfactory. This will initiate a program of resampling and checks on treatment processes in the case of drinking water. The detection of coliform organisms and/or *E. coli* is taken as an indication of the potential presence of faecally-derived bacterial pathogens. It is of the utmost importance, therefore, to detect the maximum numbers of indicator species, including those individuals (probably the majority of the population) that have been sub-lethally damaged (White *et al*. 1984).

Indicator bacteria, including coliforms, may become stressed or injured in waters, seawater and waste waters (Bjergbaek and Roslev 2005). These injured bacteria can be incapable of growth and colony formation because of structural or metabolic damage (Ozkanca *et al*. 2002). They also become injured and nonculturable through oxidation and thermal stress (McCleery and Rowe 1995; Dukan *et al*. 1997). More than 90% of the indicator bacteria in a sample may not be detected because of stress (McFeters *et al.* 1986; Dukan *et al*. 1997). These false negative bacteriological findings could result in an inaccurate definition of water quality, or even worse, lead to the erroneous acceptance of a potentially hazardous condition resulting from the presence of pathogens (LeChevallier and McFeters 1985; Reisssbrodt *et al*. 2002).

Stressed organisms are present under ordinary circumstances in chlorinated drinking water, polluted natural waters, and relatively clean surface waters. High numbers indicator bacteria may be injured when confronted with partial or inadequate disinfection and the presence

of metal ions or other toxic substances. These, and other factors, including extremes of temperature and pH and solar radiation, may lead collectively to significant underestimation of the number of viable indicator bacteria (American Public Health Association 1992; Ozkanca *et al*. 2002). Enteropathogenic bacteria are less susceptible than coliforms to injury under conditions similar to those in drinking water and these injured pathogens retain the potential for virulence (LeChevallier and McFeters 1985; McFeters *et al*. 1986; Dukan *et al*. 1997).

'Viability' is often defined as the capacity to form colonies on an agar medium suitable for the growth of the organism, and 'non-viable' when this ability to form colonies is lost. That such a definition is unsatisfactory has been demonstrated by showing that although there may be large numbers of bacteria actively respiring, the great majority of these failed to form colonies on nutrient agar (Ozkanca and Flint 1997; Ozkanca *et al*. 2002; Bjergbaek and Roslev 2005). This under-estimation of the true population of viable cells is made worse when using selective media, traditionally employed in standard culture procedures, because the inhibitory substances included in the formulation are optimised for the growth of populations from samples rich in nutrients and can be highly damaging for organisms adapted to low nutrient conditions (Ray 1979). The common observation in low-nutrient habitats of finding large bacterial numbers by direct microscopy but corresponding low numbers if measured by culture methods suggests that large portions of microbial populations are indeed dormant or undergoing a shift in metabolism in response to changing environmental conditions (Jannasch and Jones 1959; Bjergbaek and Roslev, 2005). Taking all the evidence into account, it has been concluded that living, metabolically active, bacterial populations exist that do not form colonies on agar plates in the laboratory and, therefore, exhibit no 'viable count'. However, this may be a seasonal phenomenon for many bacteria, which may exist in a non-culturable form at certain times of the year but remain viable

and, when appropriate conditions occur, the bacteria return to a culturable state (Colwell *et al*. 1985; Bjergbaek and Roslev, 2005).

In an aquatic environment, *E. coli* cells may remain viable but lose the ability to grow on selective media. These cells may still be culturable on non-selective nutrient medium (Bissonnette *et al*. 1975). Microcosm studies with river, estuarine and sea water (Xu *et al*. 1982; Flint 1987; Bjergbaek and Roslev, 2005) have demonstrated that some samples will contain viable but non-culturable cells which will not grow even on non-selective media. Direct culture is routinely used to detect coliform bacteria which act as indicators of faecal contamination. The occurrence of stressed *E. coli* suggests that results from direct selective culture of water samples must be interpreted with caution. Sublethal damage and toxic inhibition raise two important questions. The first is whether the methods that are used routinely are sensitive enough to detect all viable bacteria, or whether merely a small proportion is detected. The second question is whether sublethally damaged cells are important, both because they can be reactivated and multiply in food which has become contaminated, or because they are able to cross the acid pH barrier of the stomach and reactivate and multiply in the gastro-intestinal tract.

There are some major problems posed by the reactivation of viable bacteria. The media recommended in various official standard methods for examination of water vary widely in their efficiencies of recovery. In such cases, it is disturbing that the effective level of compliance of water samples with standards can be altered, merely by changing the medium and recovery technique. The changes in the viable count with time in studies on bacterial survival can be affected by the procedure for dilution and enumeration, since the effect of the primary stress will be increased by an unsuitable enumeration method.

A major part of research has been on resuscitation using agar or broth culture techniques. *E. coli* cells which were non-culturable on agar medium containing selective agents recovered culturability after incubation on non-selective media (Bissonnette *et al*. 1975). Membrane filtration is widely used to concentrate micro-organisms from water samples and membrane filters can be conveniently moved from resuscitation on agar medium to a selective agar medium for colony growth and counting (Bissonnette *et al*. 1977; Reissbrodt *et al*. 2002). In broth culture, resuscitation is best attempted under conditions in which culturable cells, which are present in the sample, do not grow. Effective resuscitation may require specific conditions suitable for the particular micro-organism and the cause of non-culturability.

In this study, a micro-MPN method was evaluated as a means of resuscitation of sub-lethally stressed bacteria, produced by illumination under defined, reproducible conditions. A period in non-selective broth was used to allow recovery of damaged bacteria, prior to the addition of selective agents (e.g. lauryl sulphate). These experiments could have considerable significance in terms of the validity of conventional agar based selective methods for enumeration of indicator bacteria.

MATERIAL AND METHODS:

ORGANISM

Escherichia coli K-12 NCTC 8797 was obtained from the University of Northumbria stock collection.

MEDIA

Nutrient broth (NB), Nutrient agar NA), Membrane Lauryl Sulphate Broth (MLSB) and Membrane Lauryl Sulphate Agar (MLSA) were obtained from Merck and made following the manufacturer's instructions. Eosin Methylene Blue agar (EMBA) and MacConkey agar (MCA) were obtained from Oxoid and made following the manufacturer's instructions.

Preparation of stressed cells.

Filtered-autoclaved lake water (FALW) samples were prepared by collecting lake water, passing it through a 0.22 μm Millipore filter and autoclaving the filtrate at 121^{o}C for 15 minutes. The bacterial cultures were grown in nutrient broth (100 ml) overnight at 37 ^{o}C. Cells were harvested by centrifugation at 10,000 x g for 15 mins. The pellet was washed with FALW (100 ml) and centrifuged before being resuspended in FALW to give 10^6 cfu ml^{-1}. These resuspended cells (1 ml) were exposed to light (100 watt m^{-2}) for 3 h in the presence of 0.2 ml methylene blue (4 mg ml^{-1}) or heated to 55^{o}C for 1 h in a stationary water bath to give light- and heat-stressed cells respectively.

Surface Spread Method

Suspensions of *E. coli* were prepared by serial decimal dilution and 100 μl spread on agar plates using a surface spread plate technique. Plates were incubated at 37^{o}C or 44^{o}C as appropriate.

Multi-well Plate (Micro-MPN) Method

Serial decimal dilutions of the sample were made using a multi-pipettor. Each well of the multi-well plate contained 150 µl of growth medium (1.6 x normal strength NB) and water sample (100 µl) was added to each well. The multi-well plates were incubated at 37°C for 24 h. The number of positive results per dilution was counted. The MPN was calculated from the probability table of Tillett (1987).

Resuscitation of *Escherichia coli* by membrane filter technique:

Water samples (10 ml) containing stressed *E. coli* were filtered through sterile membrane filters (Gelman GN-6, 0.45 µm pore size). The membrane filter was placed on the surface of an agar plate to allow resuscitation to take place. Two different media were used for this experiment, NA as a non-selective medium and MLSA as a selective medium. The membrane filter was applied to a NA plate for up to 8 hours at 37°C to resuscitate injured *E. coli*. After this pre-incubation, the membrane was transferred to MLSA and incubated at 44°C. The number of *E. coli* cells were able to produce colonies were counted. Controls consisted of membrane filters incubated on NA plates only.

Resuscitation of *Escherichia coli* by Multi-well plates

Resuscitation by a non-selective medium (NB) was carried out at 37°C for up to 8 hours. Samples were analysed every hour during this time period. The decimal dilutions were prepared using a multi-pipettor on multi-well plates and 50 µl NB added to each well. In the test cases, 100 µl MLSB was added to each well after the appropriate incubation period and the multi-well plate transferred to 44°C for a further 24 h. The 96-well MPN value was compared with the MPN

count obtained from samples where NB (100 µl) was added instead of the selective medium, followed by incubation at 44°C.

To minimize experimental error, all experiments were replicated twice. Figures 1 to 4 show the mean of the data from each experiment with the error bar indicating the range of values obtained.

RESULTS

In experiments to determine which media may be the best for the recovery of stressed indicator bacteria, *E. coli* was subjected to stress through the application of heat and light. It is reported here the inability of bacteria to grow on selective media when exposed to two separate stresses, heat and light-induced oxidiative stress, was examined alongside the use of two methods, a multi-well MPN assay and a membrane filter assay to allow resuscitation of bacteria. The results are shown in table 1. At present experiment, the incubation time was chosen because this gave a decrease in viable count from approximately 10^6 cfu ml^{-1} to around 50 cfu ml^{-1} (a suitable number to determine on membrane filters). After this exposure time, there were no colonies present on any of the selective agar plates, although colonies could still be detected on the non-selective NA plates. Samples of water were filtered through the membrane filters and these were incubated on a NA plate for differing periods of time before being transferred to one of the three selective agars MCA, EMBA and MLSA which are commonly used as selective media. As Table 1 shows MLSA was the only medium to show recovery of the viable count of *E. coli* after resuscitation for 6 to 8 h. Resuscitation using this method could not be carried out for longer than 16 h because colonies became visible on the filter on the NA plate after this time period. The final viable count was not significantly different from that obtained on NA alone rather than on the NA/MLSA combination.

In subsequent experiments a different stress, visible light, was used to see if MLSA also worked as a recovery medium with this stress. The water samples containing *E. coli* were exposed to 100 watt m^{-2} light in the presence of methylene blue and the changes in the viable count measured over a period of 5 h. The viable count in the samples was determined using the surface spread plate method on the selective medium, MLSA, and on the non-selective medium,

NA. The plates were incubated at two temperatures, 37°C routinely used for the analysis of coliform organisms and 44°C which is more selective for thermotolerant coliforms, such as *E. coli*. The results are shown in Figure 1 for incubation at 37°C and in Figure 2 for incubation at 44°C on the two media. The figures represent results from a typical experiment.

In both cases, there was gradual decline in the viable count because of the stress damage caused by the combined effect of light and methylene blue. At the end of the five-hour incubation period, the viable count had declined from a starting value of 1.6×10^6 cfu ml^{-1} to undetectable on the selective agar plates MLSA and to a value of 63 cfu ml^{-1} on the non-selective agar plates NA. In repeat experiments the same pattern was followed with *E. coli* becoming undetectable on the selective agar at 37°C within 5 h of exposure to the stress. A very similar pattern emerged at 44°C with the viable count becoming undetectable after 5 h of exposure on MLSA plates but not on NA plates. There was no significant difference between the time for 99% or 99.9% loss in viable count when comparing the same media at the different temperatures.

These experiments showed that sub-lethally stressed or damaged bacteria could not grow on selective media although viable cells were still present in the culture when detected on non-selective agar plates. Therefore, in order to try to resuscitate the bacteria so that they would respond to the selective medium, the bacteria were cultured on a membrane filter on an agar plate in the membrane resuscitation method as used above or in a non-selective liquid medium in the multi-well method.

In the multi-well most probable number method, MLSB selective medium was added to the wells at time intervals. Analysis of the data enable the number of 'viable' bacteria in the sample to be determined as the recovery process takes place. Figure 3 shows the relevant results. Viable cells able to grow in MLSB at 44°C were found in the culture after 3 h of pre-incubation

in NB followed by the addition of MLSB. By 8 h of incubation in NB followed by MLSB, the viable count was the same as that seen when only NB was used for the resuscitation. The final MPN value in these samples was not significantly different from that at the end of the incubation of the samples analysed on NB rather than on MLSB.

The results obtained using the membrane filter method for resuscitation is shown in Figure 4. In this experiment water samples were filtered through membrane filters and placed on NA plates to allow resuscitation and subsequent growth on MLSA or NA was recorded. There was no resuscitation detected until after incubation on NA plates for 3 h. After this time, there was an exponential increase in numbers to the same level seen in colonies enumerated simply on NA. Again, the final viable count was not significantly different from that obtained on NA alone rather than on the NA/MLSA combination.

DISCUSSION

Several important factors including the availability of nutrients, presence of toxins, and the physical stresses imposed by sunlight and oxidative stress have an influence on the survival of bacteria in aquatic environments. Many stresses lead to the formation of a dormant or viable but non-culturable phase in the bacterial cells. This state leads to the apparent loss of viability through the loss of the ability of the bacteria to produce visible colonies when grown on selective and non-selective agars. However, these cells retain the ability to carry out respiration as observed through the use of fluorescence microscopy of stained samples (Özkanca *et al*. 2002). These bacteria often retain the ability to cause disease (LeChevallier and McFeters 1985; McFeters *et al*. 1986; Reissbrodt *et al*. 2002).

At present study reported here the inability of bacteria to grow on selective media when exposed to two heat and light-induced oxidiative stress, was examined alongside the use of two methods, a multi-well MPN assay and a membrane filter assay to allow resuscitation of bacteria. Pagel *et al*. (1982) have previously shown higher recoveries of stressed bacteria are possible on membrane filters incubated on agar medium rather than on liquid broth media. It is assumed that there is a diffusion limitation of nutrients from the solid medium which prevents stressed organisms dying through exposure to high concentrations of nutrients too quickly. Lim and Flint (1996) have previously shown that *E. coli* can recover from the effects of heat treatment in a starvation medium. In their experiments, they heated *E. coli* to temperatures of up to 65^{o}C for up to 6 h and then allowed the bacteria to recover in the dark at 15^{o}C. The original viable count on nutrient agar was recovered in a period of up to 6 days. They showed that mild heat treatment did not kill *E. coli* but rather it stressed *E. coli* so that they were unable to grow even on non-

selective NA agar plates. The exposure of bacteria to heat stress induces the heat shock response which enables cells to protect themselves from destruction by heat and aids in the recover process once the stress has been removed (Reissbrodt *et al*. 2002). The addition of extra nutrients did not increase the rate at which bacteria recovered from the heat stress suggesting that it is not a process that is dependent upon the presence of nutrients but rather one that requires time and a suitable environment. In the experiments reported here, *E. coli* similarly was inactivated by heat at 55^{o}C so that the cells failed to grow on selective media and the viable count was reduced on the non-selective medium. The bacteria were able to recover viability when exposed first to NA on a membrane filter and then to the selective medium MLSA, but not on the other selective media, EMBA and MCA. The bacteria recovered to the same count on NA within 12 h (Table 1) and went on to recover to the original inoculum size on NA and on MLSA in 5 d (data not shown) as in the experiments reported by Lim and Flint (1996). The reasons why recovery is possible on some selective media but not others are still open to question, but must be related to the selective agents used in the different media. If the stressed cells remain more permeable to some agents than others, then this will influence their recovery.

Ozkanca *et al.* (2002) also studied the effects of light-induced oxidative stress on bacteria and showed that the presence of photosensitisers, such as toluidine blue, increased the harmful effects of artificial illumination. The type of illumination used in those experiments, as well as in the ones reported here, does not contain the harmful ultra-violet wavelengths that have been shown to be the effective harmful component of sunlight (Gameson and Gould 1975). These artificial sources emit light almost entirely of wavelengths greater than 370 nm, whereas it is the wavelengths of less than 370 nm present in sunlight that have the main harmful effects on bacteria (Davies and Evison 1991). The addition of methylene blue in these experiments was to

act as a photosensitzer through its ability to produce harmful free radicals in the presence of an artificial light source.

In the experiments reported here, *E. coli* were affected by exposure to artificial light in the presence of methylene blue as a photosensitiser. The viable count on the single selective medium tested (MLSA) became undetectable whereas there were still viable cells in the samples when tested on the non-selective NA plates. In experiments shown in Figure 1 and 2, the decline in viable count was not exponential which suggests that the level of stress on the organisms is increasing with increasing exposure. Previous experiments with heat-induced stress showed an exponential decline in viable count (Lim and Flint 1996).

Again, the cells recovered in both the membrane filter method and the multi-well MPN method when NA was used as the resuscitation medium before exposure to the selective agents in the MLSA plates. Data from Multi-well MPN assay suggest that it takes at least 3 hours at 37^{o}C to repair the damage or stress caused by methylene blue/light in order for the cells to become once again resistant to selective agents. Lim and Flint (1996) showed that recovery after exposure to heat took considerably longer under starvation conditions and here recovery from stress caused by heat took longer than recovery from stress caused by light, as viable cells were not detected on the selective media until after 7 h of resuscitation. McCleery and Rowe (1995) showed that recovery of thermal-stressed *E. coli* was increased if cells were treated with catalase and then grown on non-selective Trypticase Soy Agar rather than selective Sorbitol MacConkey Agar supplemented with 4-methylumbelliferyl beta-D-glucuronide.

In these experiments, both sets of results showed that there was no major difference between these two methods for the resuscitation of *E. coli*. Although the multi-well MPN method has certain advantages, such as reducing the time to set up the experiments, reducing the

experimental cost, and is easy to apply, there was no difference between the multi-well MPN method and the plate method in terms of the experimental results. It has been suggested in the past that higher counts of stressed bacteria are obtained using MPN methods because bacteria are able to recover better from stress in a liquid medium rather than on a membrane surface. That was not seen in the experiments reported here. It is possible that the MPN method is better at recovering bacteria which have been exposed to chlorine stress than the membrane filter method but that when heat or light stress is involved there is little if any difference between the efficiency of these two methods.

As the viable count on the selective medium does not exceed that on the NA alone, this suggests that there is a sub-population which is less damaged than the majority of the bacterial cells in the sample. It takes up to 24 h to enable the recovery of a larger proportion of the population. Lim and Flint (1996) showed that recovery could in fact take as long as 7 d. The increase in the viable count is not due to growth from a few unstressed cells as recovery is faster than growth rate under these conditions. There was also no increase in the viable count seen on NA alone, whereas, if this was recovery from a few unstressed cells, one would expect to see an increase in the viable count on NA over the period of resuscitation. The recovery was the same after incubation at 37^{o}C as at 44^{o}C, therefore, it is unlikely that temperature is playing a role in increasing the stress on the organisms. Bjergbaek and Roslev (2005) demonstrated that after exposure to oxidation stress for less than 1% of cells could be detected on agar plates, but after resuscitation more than 20% could be resuscitated to a culturable state.

Nilsson *et al*. (1991) also showed that *Vibrio vulnificus* could recover viability after exposure to starvation stress when the temperature was shifted from 5^{o}C to 15^{o}C. They showed that the viable count recovered without any increase in total cell count indicating that it is the

recovery of stressed cells that it taking place rather than the growth of a few survivors. Lim and Flint (1996) also showed that there was no increase in total cell count in their heat-treated samples of *E. coli*. Nilsson *et al.* (1991) also showed that recovery required that the cells be able to carry out protein synthesis as cells exposed to chloramphenicol as an inhibitor of protein synthesis did not recover. This may also point to why some selective media allow recovery and not others when cells have to be able to carry out some important metabolic functions to be able to recover from the effects of stress.

Salmonella enteritidis can also recover from a viable but non-culturable state when exposed to high concentrations of nutrients and incubating at sub-optimal temperatures (Roszak *et al*. 1983). Here recovery took place at the normal incubation temperature, 37^{o}C, used for the isolation of coliforms and at 44^{o}C used for the more selective isolation of thermotolerant coliforms such as *E. coli*.

Many methods have been used for the isolation or enumeration of bacteria which are viable but unable to grow on growth media. Radioactive labelling and fluorescent labelling have been widely used (Jones and Rhodes-Roberts 1981; Tabor and Neihof 1982) but these methods cannot be used to differentiate between species and therefore have limited uses in enumerating specific organisms such as the indicator species of coliforms such as *E. coli*. A comparison between the viable count and the count of bacteria capable of respiration activity under starvation conditions gives an indication of how many of the bacterial cells are existing in a dormant state. The counts of a variety of porin-deficient mutants of *E. coli* in filtered-autoclaved seawater showed that, although some were undetectable in water samples by plate counts, they were still capable of respiration (Darcan *et al*. 2003). However, this method is not specific for *E. coli* and cannot,

therefore, be used under circumstances where it is essential to detect *E. coli* to verify the quality of drinking or bathing water.

The selectivity of culture methods is essential for such determinations. Staining methods work well with artificial populations but they are hampered with cells in the natural environment existing in a series of states rather than in a viable or dormant state (Herson and Baker 1982). The factors which determine whether or not *E. coli* survives under starvation conditions in the sea and other aquatic environments are complex. Detection of viable stressed cells on selective media, such as MCA or on EMBA, is usually more difficult and does lead to underestimation because of the known toxic properties of these agars towards stressed cells. The inability to detect enteric bacteria in seawater could be a concern where this organism is used as an indicator of faecal pollution. Any property that affects the survival and, therefore, the ability to detect bacteria by the routinely used plate count methods has to be considered when organisms are being used as an indicator of water quality. We have shown that bacteria survive in different ways depending on water temperature and many other factors (Özkanca and Flint 2002; Özkanca *et al*. 2002). Underestimation of the load of faecal bacteria in a water sample could have potentially hazardous consequences. This would be of even more concern should the same properties be true for bacteria in drinking water supplies or in finished water.

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LEGENDS

Figure 1: Survival of *Escherichia coli* subjected to light-induced oxidative stress [(100 watt m^{-2}) for 3 h in the presence of 0.2 ml methylene blue (4 mg ml^{-1})] and enumerated at 37^{o}C on NA (●) or MLSA (■). Error bars are the range of values from two separate experiments.

Figure 2: Survival of *Escherichia coli* subjected to light-induced oxidative stress [(100 watt m^{-2}) for 3 h in the presence of 0.2 ml methylene blue (4 mg ml^{-1})] and enumerated at 44^{o}C on NA (●) or MLSA (■). Error bars are the range of values from two separate experiments.

Figure 3: Resuscitation of *Escherichia coli* subjected to light-induced oxidative stress [(100 watt m^{-2}) for 3 h in the presence of 0.2 ml methylene blue (4 mg ml^{-1})] using a multi-well Most Probable Number method. Cells were resuscitated by pre-incubation in nutrient broth before enumeration in non-selective nutrient broth (●) or selective Membrane Lauryl Sulphate broth (■). Error bars are the range of values from two separate experiments.

Figure 4: Resuscitation of *Escherichia coli* subjected to light-induced oxidative stress [(100 watt m^{-2}) for 3 h in the presence of 0.2 ml methylene blue (4 mg ml^{-1})] using a membrane filter method. Cells were resuscitated by pre-incubation on nutrient agar before enumeration on non-selective nutrient agar (●) or selective Membrane Lauryl Sulphate agar (■). Error bars are the range of values from two separate experiments.

Incubation time (h)	NA to NA	NA to MLSA	NA to EMBA	NA to MCA
0	56 (50-62)	0	0	0
1	55 (53-57)	0	0	0
2	50 (45-55)	0	0	1
3	49 (44-54)	0	1 (0-2)	0
5	63 (56-70)	0	0	0
6	65 (58-72)	0	1	0
7	69 (68-71)	19 (15-23)	2 (0-4)	0
8	67 (64-70)	52 (44-60)	0	0
12	56 (51-61)	67 (64-70)	2 (1-3)	5 (0-10)

Table 1. Recovery of heat-stressed *Escherichia coli* on different media using a membrane filter resuscitation and enumeration technique. Values are mean of colony forming units from two separate experiments. Figures in brackets indicate the range in each experiment.

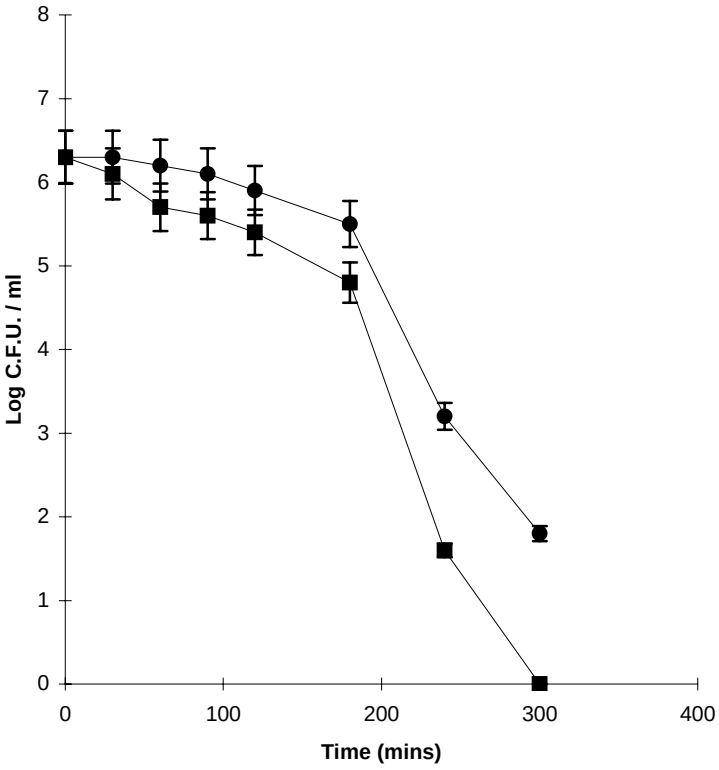

Figure 1

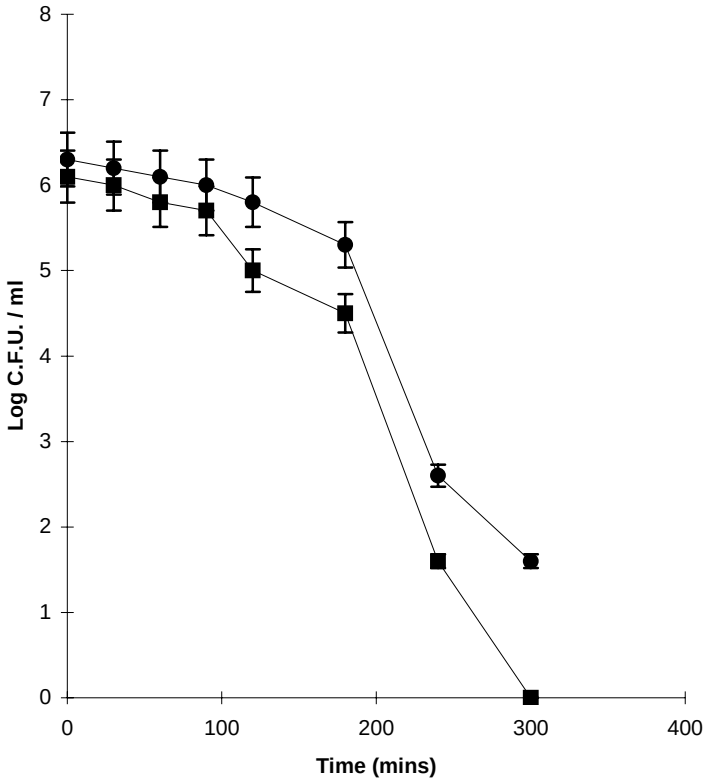

Figure 2

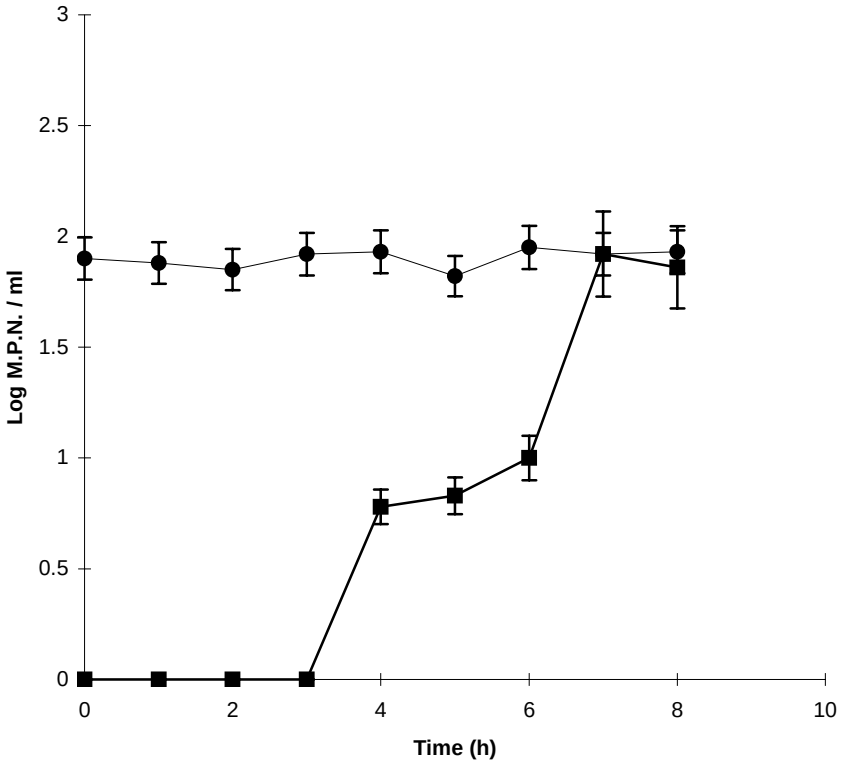

Figure 3

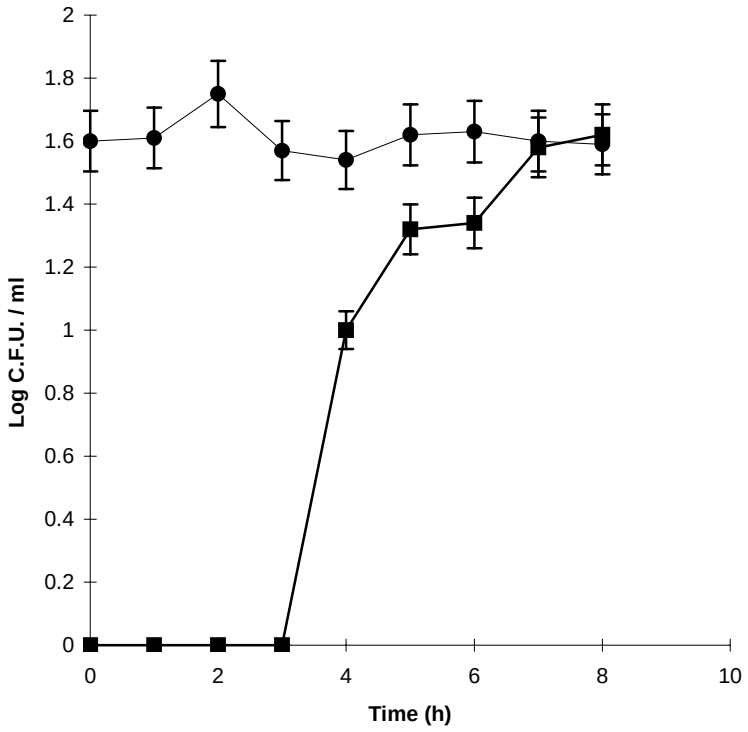

Figure 4