Preliminary Investigations into Analysis of Stressed Bacteria

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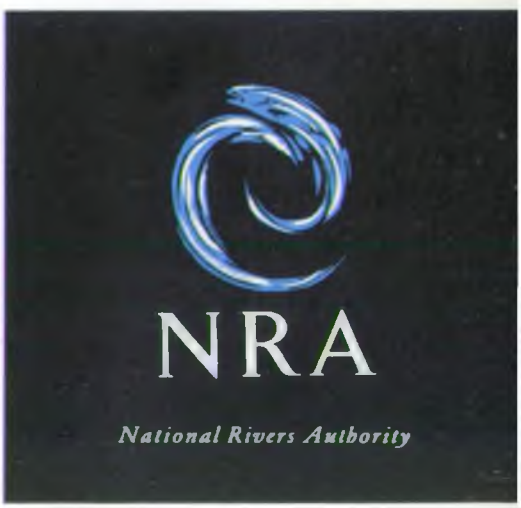

PRELIMINARY INVESTIGATIONS INTO ANALYSIS OF STRESSED BACTERIA

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Author: T E Irving

Contract Manager: G Stanfield

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WRc Medmenham, Henley Road, Medmenham, PO Box 16, Marlow, Buckinghamshire SL7 2HD. Telephone: Henley (0491) 571531

SUMMARY

I OBJECTIVES

To assess by review and experimentation the viable but non-culturable state of bacteria, and methods for enumeration of stressed organisms.

II REASONS

There is concern that culture-based methods do not detect the presence in water of bacteria which, though not culturable, may nevertheless still be intact and viable, and potentially able to cause disease.

III CONCLUSIONS

Literature claiming to demonstrate a stable state in which bacteria are viable but non-culturable, is not completely convincing, and the public health significance of such a state has yet to be determined. Further research is needed.

IV RECOMMENDATIONS

Further work is needed on methods for assessing bacterial viability and activity, which do not involve culturing. Alternatively, it may be useful to investigate improvements in culturing methods. Work is needed to determine whether viable but non-culturable bacteria pose a public health threat.

V RESUME OF CONTENTS

Literature on the viable but non-culturable state is reviewed, and mention is made of other factors affecting survival of bacteria in the environment.

Results are reported of tests to show the efficiency of some currently used culturing methods for recovery of stressed bacteria. Preliminary results are given of work on comparison of culture-based and microscopic methods for bacterial enumeration, on assessment of viability by microscopic observation, and on restoration of colony-forming ability of stressed cells.

Recommendations for further research are made, and a bibliography of 46 references is included.

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CONTENTS Continued

SECTION 1 - REVIEW OF PUBLISHED WORK ON VIABLE, NON-CULTURABLE BACTERIA

1.1 INTRODUCTION

The phenomenon of bacterial mortality has occupied the minds of researchers for many years, both from a theoretical and practical point of view. While life and death are comparatively easy to define for higher organisms, the same is not true of the microbes.

For practical purposes, viability of bacteria has mostly been defined in terms of colony-forming ability, or the ability to cause visible growth in liquid culture media. However, much comparative work has shown that the choice of culture medium can profoundly affect the apparent "number" of viable cells in a water sample, and so the simple description of a bacterial cell as "alive" or "dead" cannot be made. Rather we should say that the organisms concerned either can or cannot by cultured under the conditions of the particular test used.

That bacteria can be recovered on one growth medium, but not another, means that the results of a single test cannot preclude the undetected presence of potentially viable organisms. By using the most efficient recovery procedures available, and by applying consistent conditions of analysis to all samples, one may obtain useful comparative data. This allows the relative densities of various types of organisms to be inferred within and between samples, but does not give information on absolute numbers. This may be acceptable in some situations. The incidence of pollution indicator organisms in various waters, for example, may provide useful information. Densities of these organisms in different samples can be compared, and relative levels of pollution can be inferred. Also, acceptable indicator densities, and numerical standards and guidelines have all been set on the basis of the results of culture-based methods of enumeration. However, in tests for the presence of pathogenic organisms, one may wish to say with more certainty whether or not a particular type of microbe is present, and here the culture-based methods may be theoretically less satisfactory.

A negative result in such a test can never prove that the organism being sought is definitely not present. Also information on the minimum numbers of the pathogens required to cause infection, and the necessary conditions required, must be obtained for a judgement to be made on the safety of the water for any particular purpose.

1.2 THE VIABLE, NON-CULTURABLE STATE

It was in 1987 that major interest in the subject was expressed in the UK. This followed a paper given in March of that year at the International Conference on Environmental Protection of the North Sea, by Professor R R Colwell, of the University of Maryland, USA[1].

This was in fact a review paper, and most of its contents would have been familiar to those who had been following publications on the subject over the previous 5 years. However, extensive media coverage, both directly of the conference, and indirectly of comments on its contents by environmental pressure groups, led to wide public interest in the subject. The contention was that pathogenic organisms were present in coastal waters, and while conventional microbiological techniques would fail to demonstrate their presence, they could nevertheless be shown to be viable, and potentially able to cause infection.

Professor Colwell said that these findings had implications for the future of sewage disposal to the sea, and for the problems of pollution, and that a re-evaluation of current practices was needed. Improvement of waste treatment and management, especially aimed towards removal of pathogenic organisms, was said to be a highly recommended path of action. This was reported in rather more sensational terms by the popular media.

Publications on this subject from Professor Colwell's group, and from her collaboration with others, started in 1982 with a report by Xu and others[2] on survival and viability of non-culturable Escherichia coli and Vibrio cholerae in the estuarine and marine environment. This

stated that plating methods for indicator organisms and pathogens had severe limitations for estimation of viable populations of these organisms.

The work involved the use of microcosms to study the decline in bacterial numbers under various conditions. The "microcosms" were conical flasks containing filter-sterilised water of known salinity, which was inoculated with a suspension of a pure culture of the bacteria being studied, then incubated and sampled periodically. Enumeration techniques used to study the survival included viable counts (plating on selective and non-selective agars, and most-probable number counts using fluid culture media), and direct microscopic counts (acridine orange direct count, direct viable count and fluorescent antibody direct count).

Acridine orange direct counts (AODC) followed the method of Hobbie and others[3], and used track-etched polycarbonate membrane filters to concentrate the cells. Direct viable counts (DVC) were made using the methods of Kogure and others[4,5]. This involved incubating the sample with a nutrient source (yeast extract) and the antibiotic nalidixic acid, a compound which inhibits DNA synthesis and prevents division of Gram-negative bacteria. The result is that viable, metabolising bacteria are seen microscopically as unnaturally elongated cells, owing to their growth but inability to divide. Substrate-responsive populations can therefore be estimated directly.

The fluorescent antibody technique was said to give a measure of the outer membrane integrity of the cells. The antibodies used were directed towards the lipopolysaccharide components of the cell envelope, and this was claimed to be proof that cells binding the fluorescent antibody remained intact, and were therefore viable.

Results of microcosm experiments using E. coli, waters of 5 and 25 g/kg salinity, and storage temperatures of 10 and 25 °C, showed rather inconsistent results. Descriptions in the text of results shown in tables indicate that there may have been some misprints. For example,

at one point where differences between total, active and culturable populations are stated to be greater after extended incubation, the opposite appears true from the tabulated results.

It is notable that in several places, especially with the higher salinity value, viable counts by the plating method are higher than those estimated by the direct viable count. This is not commented upon. Nevertheless, the acridine orange total counts are always shown to be greater than the viable counts by culturing methods, and culturability appears to be reduced more at higher temperatures and salinities.

Similar experiments using Vibrio cholerae showed increases in counts by all methods with storage, except in the case of 25 g/kg salinity at 25 °C. This was thought to be due to carry-over of nutrients in the bacterial inoculum.

A further series of experiments is reported, these being carried on for rather longer than the 96 h of the first set. Here culturable numbers of E. coli are shown to decline, while the total count by acridine orange and fluorescent antibody methods remains roughly constant. The direct viable count is said in the text to show an initial increase, but remain stable thereafter. However, in the accompanying figure, the direct viable count is shown to lie between the total and viable counts, and to decline to roughly the same degree as the viable counts, that is by a factor of about 10 during storage at 4-6 °C for 13 days.

Far more dramatic results were seen with suspensions of Vibrio cholerae in estuarine water (salinity 11 g/kg). Here viable counts by four methods were reduced by over 5 orders of magnitude to undetectable levels in less than 10 days, while direct counts by acridine orange and fluorescent antibody techniques remained roughly constant. The apparent die-off was fastest with the most selective culturing method.

Overall this paper did not give an unequivocal view of viability and culturability, and some of the conclusions drawn are hard to accept. This is especially true of the claim that total, active and culturable

cells of V. cholerae have greater persistence in the estuarine environment than cells of E. coli. This is based on values which, it is implied in the text, are an artefact of the experimental technique. Indeed, exactly the opposite appears to be so in the second, more credible, set of experiments described. Also there is little to substantiate the claim that the fluorescent antibody direct count detects only viable cells. Nevertheless, the persistence of intact cells in saline and estuarine water, when the viable counts are seen to be much reduced, was clearly shown in the case of V. cholerae.

A later paper by Roszak and others[6], using an environmental strain of Salmonella enteritidis from the Potomac River, showed that cells in river water microcosms could not be recovered by culturing methods after 3 days. The methods used were plate counts on veal infusion (non-selective), and xyline-lactose-deoxycholate (selective) agars, and a most probable number (MPN) test using dulcitol broth for primary enrichment, followed by subculture to selenite cystine broth and XLD agar. As in the previous work, direct counts were made using acridine orange or fluorescent antibody treated preparations, and the direct viable count using acridine orange staining after incubation with yeast extract and nalidixic acid.

At the same time as the viable counts dropped to below the level of detection, direct counts remained roughly constant. The fluorescent antibody count was 20% lower than the acridine orange direct counts, and the direct viable count was some 40% lower.

Four days after culturability ceased, it was found possible to resuscitate the cells by addition of nutrient (identity and concentration not stated). Small atypical colonies could be grown on plating media 25 hours after this addition, and normal colony-forming ability was regained after 52 hours. However, 21 days after culturability ceased, it was not possible to achieve resuscitation by this method, although the direct viable count remained unchanged.

The conclusion from these results was that there existed "a linear sequence of responses by bacterial cells to conditions of nutrient depletion, the end result of which yields an inactive (dormant-like), but viable, cell"[6]. The first stage of this process was considered reversible, as shown by the recovery of cells on addition of nutrient, but "protracted nutrient limitation requires more than simple addition of nutrient, since the DVC (direct viable count) was stable up to 60 days".

This last statement is fascinating, for the direct viable count method, which supposedly indicates that cells are viable, is itself merely a simple addition of nutrient (yeast extract), followed by incubation for 6 hours. Nalidixic acid has to be used to prevent the cells from dividing, and Kogure[5] states that, even in the presence of the antibiotic, cells will begin to divide if the incubation is prolonged.

It would be interesting to know if any tests had been done with the direct counting method, but omitting the nalidixic acid. Would the elongating cells have divided, and if so, would they have been able to form colonies if conditions were favourable?

Work published by Colwell and others in 1985[7] used pure cultures of E. coli, Shigella sonnei, and a toxin-producing strain of Vibrio cholerae. Microcosm experiments showed that V. cholerae became non-culturable after 1 day, while total counts by the acridine orange method remained constant. Fluorescent antibody direct counts were initially lower than the total counts, but rose slightly to the same level, while the direct viable counts were some 2 orders of magnitude lower than the total counts, and remained constant.

Acridine orange direct counts of Sh. sonnei also remained constant, while viable counts on a selective medium (MacConkey agar) were reduced to undetectable levels in 13 days. At this time, though, 100 viable cells per ml were seen in plating tests using a non-selective medium (tryptone soya agar), and culturable cells were still present at the end of 3 weeks incubation. Fluorescent antibody counts were similar to the

acridine orange counts and the direct viable count declined by over an order of magnitude during the experiment.

Interestingly, the experiments using E. coli showed that it survived rather better. Viable counts were reduced by 3 orders of magnitude in 19 days, and appeared to be levelling off. The acridine orange direct counts were stable, and the direct viable count declined by an order of magnitude during the course of the incubation.

Experiments were also done in membrane chambers suspended in semitropical sea water (salinity 38 g/Kg, temperature 25 °C). These chambers were enclosed vessels, fitted with polycarbonate membrane filters of pore size 0.4 μm, to allow diffusion of substances in and out. This was to provide natural marine conditions, while also containing the bacteria under test. Although not discussed extensively in the text, the results of these experiments were rather different from those of the laboratory microcosms. All fluorescent antibody, direct viable, and plate counts of V. cholerae dropped to zero (time period not specified). Culturable E. coli could not be detected after 13 hours, but fluorescent antibody and direct viable counts of this organism stayed constant at between 10^5 and 10^6 per ml. The direct viable count in this experiment appears to have been carried out with fluorescent antibody staining, instead of acridine orange, to make it species-specific. This is not discussed in detail, and it seems that the standard acridine orange method was used in the microcosm experiments.

Acridine orange direct counts were 2 orders of magnitude higher than the direct viable counts, but dropped suddenly between 2 and 4 days. This was the same strain of E. coli which showed extended culturability in the laboratory microcosms, but those had been at rather lower salinity (15 g/kg).

No discussion is given of the disappearance of V. cholerae, but it is possible that the cells may have been able to pass through the pores of the membrane. It is mentioned that marine bacteria were found to be able to enter the membrane chamber.

Returning to the microcosms, additional test were done to show that the non-culturable V. cholerae cells were still able to cause infection, by injection of the test water into tied-off loops of rabbit ileum. Distension with fluid, and haemorrhaging were seen, even though the microcosms contained no viable cells. However, it should be noted that the microcosm contents were concentrated by centrifugation for the rabbit tests, so that the material inoculated into the ileal loops contained 10^8 total cells per ml. It is not stated if the concentrates were checked for culturable cells.

The non-culturable E. coli from the membrane chambers was also able to cause fluid accumulation. Fluid aspirated from the positive ileal loops was found to contain viable, culturable bacteria, biochemically indistinguishable from the initial inocula.

In discussion, the authors stated that pathogens remain viable in the environment for longer periods than had previously been thought, and persist in a dormant state until suitable conditions for growth return. This was said to have implications for public health, and for the deliberate release of genetically-engineered microbes into the environment. In the latter case, plating methods would be inadequate for monitoring these organisms.

A later paper by Grimes and Colwell[8] concerns survival of E. coli in membrane chambers in semi-tropical sea water. This is an extended examination of the results given in the last-mentioned paper[7], but with no mention of the disappearing V. cholerae cells, other than that one membrane chamber was inoculated with this organism.

At roughly the same time of publication, another paper examined survival of a pure culture of Campylobacter jejuni biotype 2, and hypothesised that these organisms could survive for extended periods in natural aquifers, after deposition by animal hosts[9].

Much of the paper is concerned with the growth kinetics and survival of culturable campylobacters, which are fastidious in their requirements

for nutrients, and especially for oxygenation. Microaerophilic conditions are required for growth, and therefore the organisms do not survive well in fast-moving aerated water. However, for stationary stream water microcosms, a comparison is shown of enumeration by the acridine orange and direct viable counting methods, with viable counts on 5% sheep blood agar. Acridine orange direct counts and direct viable count results show a slow decline over the course of measurement (about an order of magnitude in 12 days), while in 10 days the culturable cells were reduced from 10^9 per ml to none detectable in 1 ml. It was stated that preliminary results with animal passage showed that these non-culturable cells retained viability, showing that non-culturability on agar could not be equated with non-viability. The authors state that "extrapolating these findings to the natural environment, it is concluded that the present methods used to detect campylobacters do not provide adequate quantification. This hypothesis is corroborated in point source outbreaks of campylobacteriosis in which no organism can be isolated from the suspected transmission vehicles"[9].

Later, microcosm experiments were also used to assess viability and recovery by culture of Legionella pneumophila, the causal agent of the pneumonia known as Legionnaire's disease[10]. Here again, a viable but non-culturable state was hypothesised, although direct viable counts by the nalidixic acid method could not be made, as the bacteria did not survive the conditions of this test. However, a test for lethality to chick embryos was used, and microcosm contents containing less than 1 culturable cell proved positive by this method. Yolk sac tissue from infected embryos was also shown to contain large numbers of legionellae, both by cultural methods and by direct microscopy.

Microcosm experiments again showed a decline in cells recoverable by culturing methods, while direct counts by the acridine orange and fluorescent antibody methods remained constant, or declined to a lesser extent. Again the viable but non-culturable state was said to account for the inability in some cases to recover legionellae from environmental samples during outbreaks of Legionnaire's disease.

A paper published in 1987 by Brayton and others[11] again looked at Vibrio cholerae, this time in natural waters in Bangladesh. Here a monoclonal antibody for the V. cholerae 01 antigen was used, which proved highly specific, with no cross-reactivity towards any other bacteria tested. This was used for fluorescent antibody total counts and for fluorescent antibody direct viable counts, and shoved viable V. cholerae in all 13 river and pond water samples tested, at between 10^2 and 10^4 cells per ml. Culturing tests using an MPN method were negative (ie <0.3 organisms per 100 ml) except for one, which yielded 2 organisms per 100 ml. This was again said to demonstrate the viable but non-culturable state of V. cholera and the fluorescent antibody direct viable count method was recommended for screening village water sources, rather than culture-based methods.

Other papers published around this time were more concerned with the direct viable count method than with the viable but non-culturable state. Roszak and Colwell[12] used the direct viable count method modified with radiolabelled substrates and microautoradiographic analysis, to assess viability of E. coli and Salm. enteritidis in microcosm experiments. This work shoved that substrate responsiveness by elongation of cells in the direct viable count test is not the only proof of viability, for the autoradiographic results shoved cells which were metabolically active, but which were not elongated. It was suggested that these cells were incorporating substrate for maintenance functions, but not for growth. This was seen as a further progression in the steps towards a "somnicell", or dormant cell, produced in a strategy for starvation survival.

Kogure and others[13] were concerned with use of the direct viable count method to indicate heterotrophic activity of marine bacteria, and this work differed from earlier studies in using natural populations, and a wide variety of substrate preparations rather than just the non-defined general nutrient mixture provided by yeast extract. Of the nutrient mixtures used, a combination of 6 carbohydrates was less effective than yeast extract or a combination of 10 amino acids. Yeast extract was concluded to be the best substrate, but curiously was said to be equal

to the amino acid mixture, since no significant difference between the two could be found.

1.3 DISCUSSION AND COMMENT

1.3.1 Survival of bacteria under unfavourable conditions

This review has been deliberately limited to discussion of the viable but non-culturable state, and has consequently not covered a vast amount of literature on survival of bacteria under adverse conditions.

Roszak and Colwell[14] have extensively reviewed work on "survival strategies" of bacteria in the natural environment, and this paper is a valuable source of additional references, especially on published techniques for distinguishing viable from non-viable bacteria.

While much of the work on this subject has been concerned with enteric bacteria (because of their status as pollution indicators), and pathogens (because of their public health significance), mention should be made of studies on natural aquatic bacteria, and especially of the work of Morita on "starvation-survival" of heterotrophic bacteria[15]. The physiological and biochemical changes seen in marine bacteria under starvation conditions may well have great relevance to survival of non-marine species when exposed to unfamiliar environments. Examples are reduction of metabolic processes to a dormant state, production of very small cells, and reductive cell division on starvation[16-19].

Closely controlled laboratory conditions have also been used to test the survival of starved cultures, and for this subject the work of Postgate should be investigated[20-23]. Other workers have also investigated problems of viable counting methods for damaged or starved bacteria[24,25], and mechanisms of inactivation and survival[26,27].

An important factor in bacterial mortality (or non-culturability) in marine water is solar radiation, a subject which does not seem to have been considered in published work on the viable but non-culturable

state. Indeed, inactivation due to sunlight was largely discounted until the experiments of Gameson and Saxon[28] showed convincingly the destructive effects of solar radiation. Later Gameson and Gould[29] reported the results of extensive experiments, both small-scale shore-based tests (in what would later have been called microcosms), and large-scale in situ tests carried out in the sea with labelled discharges of sewage.

Gameson and Gould[29] found no simple relationship between rates of apparent mortality and solar radiation recorded, but it was clearly shown that reduction in viable counts was much greater during the day than at night. Chamberlin and Mitchell[30] however, used their results and others to develop a mathematical model of light-dependent coliform decay. With hindsight, and considering the highly complex factors involved in loss of culturability of bacteria, this approach may seem misguided, and the authors themselves comment that "not all species are equally sensitive to light, nor is the sensitivity of a given clone necessarily the same from one experiment to another"[30].

While this is not the primary subject of the present report, it is notable that work has continued on attempting to elucidate the effects and mechanisms of bacterial inactivation by visible light[31-33]. It has also been shown that such effects can be sublethal, and analytical results can be influenced by the culturing methods used to enumerate bacteria[34].

1.3.2 Comment

The viable but non-culturable state obviously has serious implications for the standard methods for enumerating bacteria in natural waters. It has clearly been shown that situations can arise where large numbers of intact cells are present in samples where no bacteria can be demonstrated by culturing methods. However, these results have mostly been obtained from laboratory microcosm studies, and the relevance of this to the real environmental situation has not been satisfactorily elucidated.

Whether or not the "viable but non-culturable" state can be formally defined is not clear, and neither is its sanitary significance. The term was first coined as a result of examination of some apparently rather unconvincing results, which shoved that intact cells were present in samples where culturable ones were not, but gave little evidence about viability. Indeed, in that work direct viable counts were often seen to be lower than culture-based counts, and the assessment of viability was based, dubiously, on the ability of cells to be stained by the fluorescent antibody method[2]. Later, Colwell commented that "viability cannot be demonstrated by either the acridine orange direct count or the fluorescent antibody count"[1], a statement which contradicts the earlier contention, but which is considerably more credible.

The situation is clearly different for different types of bacteria, and is likely to depend greatly on the metabolic state of the organisms before exposure to the hostile or nutrient-depleted environment. Vibrio cholerae, for example, appears to lose its culturability particularly easily, but E. coli does not, being apparently more able to adapt to adverse conditions. Also, one cannot say that an organism is non-culturable, without qualification. It may be non-culturable under the conditions of the particular experiment, but that is not to say that a more efficient culturing method might not give a greater degree of apparent viability. This aspect does not seem to have been considered to any great extent in much of the work reviewed on the viable but non-culturable state. Perfectly respectable culturing methods have been used in the reports, but the attempt has always been to demonstrate viability by other means, rather than investigating or trying to correct the inadequacies of the culturing methods.

Great faith appears to have been placed in the direct viable count method of Kogure[4,5], and this test poses an interesting problem. Bacteria which have undergone starvation or other inactivation, and which cannot be recovered by culturing methods, nevertheless appear to respond to a simple addition of yeast extract, and in 6 to 8 hours are actively synthesising cell material, and so that this can be

demonstrated, have to be restrained from dividing by antibiotics. It seems strange that cells which are as active as this in such a short time cannot be cultured given suitable conditions. It needs investigating whether the nalidixic acid-yeast extract treatment can restore culturability, as well as identifying cells as viable by microscopic means. Another aspect of the direct viable count is interpretation of the microscopic images, and it is not clear exactly how this was carried out in the published reports. Recently a report has appeared which proposes an alternative method of interpreting direct viable count tests, which seems to give rather different results to what is termed the "conventional" approach[35]. Also it is notable that some of the work described above showed that some active cells do not respond in the direct viable count test[12].

Another aspect which requires study is the public health significance of viable but non-culturable pathogens in recreational waters. Non-culturable vibrios were shown to be infective if large numbers were injected into ligated loops of rabbit ileum, and non-culturable legionellae killed chick embryos, but this does not indicate whether the vibrios would have been able to cause disease if ingested by mouth, or whether the legionellae would have been able to survive aerosolisation, and infect humans by inhalation. More microbiological and epidemiological evidence is needed.

The work described in this brief review , though not unequivocal, cannot be ignored. More research is clearly needed to substantiate the existence of the viable but non-culturable state, to assess its universality or otherwise, and to discover whether improvements in culturing methods can alter the picture significantly.

SECTION 2. CULTURE MEDIA FOR THE RECOVERY OF STRESSED COLIFORM ORGANISMS

2.1 INTRODUCTION

The culture media used in the UK for the standard tests for coliform organisms in water have been extensively tested using different types of sample material[36]. Earlier work at WRc had shown that the equivalent methods used in the USA were less efficient for growing bacteria which had been stressed by high salinity or sunlight[37]. Subsequently, new American methods were developed to avoid these shortcomings. In particular, the m-TEC method for E. coli[38] and the m-T7 method for total coliforms[39] sought to achieve maximum recovery of cells from different water types. These methods were therefore compared with the standard UK membrane filter technique using "membrane lauryl sulphate medium" (MLS) as described in Report 71[40].

2.2 METHODS

Enumeration of coliform organisms was carried out by the membrane filtration technique[40] using Gelman GN-6 membrane filters (diameter 47 mm, nominal pore size 0.45 μm). Membrane lauryl sulphate medium contained, per litre: peptone, 40 g; lactose, 30 g; yeast extract powder, 6 g; phenol red, 0.2 g; and sodium dodecyl (lauryl) sulphate, 1 g. A dehydrated commercial preparation of this medium was normally used (Oxoid Ltd). The medium was sterilised by autoclaving at 121 °C for 15 min, after which the pH was approximately 7.4. The medium was dispensed on to sterile absorbent pads (Whatman No 17) in 60-mm petri dishes. Lactose-fermenting bacteria which can grow on this medium form yellow colonies. For total coliforms, plates were incubated for 4 h at 30 °C followed by 14 h at 37 °C, while for thermotolerant coliform organisms, the conditions were 4 h at 30 °C followed by 14 h at 44 °C. In subsequent Sections, these methods are referred to as MLS37 and MLS44.

m-T7, for enumeration of total coliform organisms contained, per litre: proteose peptone No 3 (Difco), 5 g; lactose, 2 g; Tergitol 7 (25% active

ingredient), 0.4 ml; polyoxyethylene ether W-1 (Sigma), 5 g; bromothymol blue, 0.1 g; bromocresol purple, 0.1 g; and agar, 15 g. The medium was adjusted to pH 7.4, sterilised for 15 min at 121 °C, cooled to 50 °C and poured into 60-mm petri dishes. After filtration of the samples, plates were incubated for 24 h at 35 °C. Lactose-fermenting organisms form yellow colonies. The medium can be made more selective against Gram-positive bacteria, if necessary, by adding penicillin G to a final concentration of 0.1 mg/l, but this was not done in the tests described below.

m-TEC medium, for enumeration of thermotolerant coliform organisms and E. coli contained, per litre: proteose peptone No 3 (Difco), 5 g; yeast extract, 3 g; lactose, 10 g; sodium chloride, 7.5 g; dipotassium hydrogen phosphate, 3.3 g; potassium dihydrogen phosphate, 1 g; sodium dodecyl sulphate, 0.2 g; sodium deoxycholate, 0.1 g; bromocresol purple, 80 mg; bromophenol red, 80 mg; and agar, 15 g. The medium was sterilised at 121 °C for 15 min, and poured into 60-mm petri dishes. The pH after sterilisation was 7.3. Plates were incubated for 2 h at 35 °C followed by 20 h at 44.5 °C, after which lactose-fermenting organisms appeared as yellow colonies. These may be recorded as presumptive thermotolerant coliforms, but if a presumptive E. coli count is required, an in situ urease tests can be carried out to eliminate urease-positive klebsiellae. For this the membrane is transferred to an absorbent pad soaked with urea substrate. This consists of urea, 2 g, and phenol red, 10 mg, in 100 ml of distilled water. The substrate mixture is adjusted to pH 5.0, when it is a pale straw-yellow colour. After 15 min contact with the substrate, colonies possessing urease activity become a red-purple colour.

2.3 RECOVERY OF TOTAL AND THERMOTOLERANT COLIFORM ORGANISMS FROM DIFFERENT TYPES OF WATER

Over a period of several months, various samples of river water, sea water and sewage were analysed by all four methods simultaneously. Results for the UK method for total coliforms (MLS37) were compared with those for m-T7 medium, and the UK thermotolerant coliform method (MLS44) was compared with the m-TEC procedure.

Table 1 shows mean ratios of counts obtained with the different media from sewage, river water and sea water. The MLS44 method gave higher counts of thermotolerant coliform organisms than m-TEC from all samples, with no difference seen between the results for river and sea water, but with the results for sewage samples showing greater discrepancy between the methods. For total coliforms, m-T7 gave higher counts than MLS37 with fresh-water samples, but with saline samples the situation was reversed. The reason for this is unknown. When first describing m-T7 medium, LeChevallier and others[39] reported that it was superior to their reference method (m-Endo broth) for recovering coliform organisms from drinking water and surface waters, but saline samples do not seem to have been considered.

Table 1. Comparison of media for recovery of total and thermotolerant coliform organisms from different types of water

Sample type	Total coliforms m-T7 : MLS37	Thermotolerant coliforms m-TEC : MLS44
Sewage	1.35	0.14
River water	1.18	0.51
Sea water	0.31	0.52

2.3.1 Short-term river water storage experiments

Some samples of river water were stored overnight and examined again the next day, to see if slight additional stressing of the organisms would change the pattern of results seen. The m-TEC : MLS44 ratio was almost exactly the same at 0.54, but the m-T7 : MLS37 ratio dropped slightly to 1.06. However, in the latter case, the difference in mean counts before and after storage was not statistically significant (Student t test, $p > 0.1$).

2.3.2 Sea water storage experiments

To obtain a greater stressing effect, such as would be experienced by sewage bacteria discharged to the sea, storage experiments were carried out using sea water inoculated with small volumes of sewage. The mixtures, of salinity 35 g/kg, were kept in the dark at room temperature (18-20 °C) and tested periodically by the four methods described above. Numbers of viable bacteria declined with time in all the tests carried out. Experiments lasted for a maximum of 2 weeks, by which time total and thermotolerant coliform levels had dropped by 2-3 orders of magnitude. However, the ratios of counts obtained by the total and thermotolerant coliform methods were very variable. Figures 1 and 2 show scatter plots of the ratios obtained, plotted against the time of storage in sea water. In both cases there does not appear to be any discernible trend in the distribution of points except that, as most points lie below the line of equality (1.0 on the y-axis), the UK methods seem generally more efficient for recovery of coliform organisms from sea water under the conditions of these tests.

2.4 LIGHT MORTALITY EXPERIMENTS WITH SEA WATER

As mentioned in Section 1.3.1, solar radiation appears to be an important factor in enteric bacteria becoming non-culturable in saline water. Therefore if a culturing method is to be used for sea-water samples, especially where the faecal indicator bacteria may have been exposed to the saline environment for some time, it is important that it is as efficient as possible for recovering light-damaged organisms.

Mixtures of sewage and sea water (salinity 35 g/kg) were exposed to sunlight in pyrex beakers. A Kipp & Zonen solarimeter, with a digital volt-time integrator, was used to measure the solar radiation received, and the mixtures were sampled regularly and analysed by the four methods already described.

Sunlight caused rapid inactivation of the bacteria, and Figure 3 shows a typical plot of the logarithms of colony-forming units per ml against

time of exposure to sunlight. Within the errors likely in the enumeration methods, there is a roughly linear decrease when plotted on these scales, and the slopes of the lines are not equal. Figure 4 shows results of the same experiment, but plotted against the solar radiation received. The two plots are very similar, as the intensity of sunlight was roughly constant throughout the experiment. The reciprocals of the gradients of the lines from the time plot give values for T_{90}, that is the time taken for 90% reduction in colony-forming units. The approximate values for this experiment were: MLS37, 27 min; m-T7, 52 min; MLS44, 42 min; and m-TEC, 29 min. The same calculation from the sunlight plot gives values for S_{90}, the amount of solar radiation needed for 90% inactivation, and the corresponding values for this experiment were: MLS37, 1.1; m-T7, 2.2; MLS44, 1.6; and m-TEC, 1.2, all values in MJ/sq m.

Thus m-TEC is less efficient than MLS44 at the start of the experiment, and becomes less so as the test proceeds. Interestingly, the lines for MLS37 and m-T7 cross during the experiment, so while MLS37 is the more efficient method at the start of the test, the position is reversed as the stressing effect of sunlight increases. This unexpected effect is shown more clearly in Figure 5, where the logarithms of the ratios of m-T7 : MLS37 and m-TEC : MLS44 are plotted against time. The m-T7 : MLS37 ratio rises steadily throughout the experiment, while the m-TEC : MLS44 ratio falls. In the latter case there does not seem to be a simple relationship between recovery ratio and time, but after 60 min exposure the numbers of thermotolerant coliform organisms recovered were so small that the precision of the ratio values is poor.

Figure 6 show a scatter plot of log m-T7 : MLS37 ratios plotted against solar radiation for a combination of several light mortality experiments, and there is a reasonably good linear relationship between the two parameters, with a correlation coefficient of 0.94. The regression line is shown for the relationship:

$$\text{Log (m-T7 : MLS37 ratio)} = (0.41 \times \text{Solar radiation}) - 0.79$$

The explanation for this interesting effect is not clear, but probably results from the total coliform population of the samples being a heterogeneous group of organisms with different survival patterns in sea water exposed to sunlight. In the early stages of exposure, the MLS37 method gives better recovery of bacteria than m-T7. Why this should be so, when the reverse is true in fresh water, is unknown, but the very high nutrient content of the MLS medium may make conditions more acceptable for cells which have been osmotically shocked by suspension in sea water, and have then had the sea water abruptly removed during the membrane filtration procedure. Later in the experiment, when the proportion of different organisms in the surviving population will have changed, it may be that the bacteria present find MLS too selective, and consequently higher recovery is seen on m-T7 medium. Whatever the cause, these results demonstrate the difficulty in trying to formulate a universal culture medium, which is equally effective under all conditions.

SECTION 3 - COMPARISON OF CULTURE-BASED AND DIRECT COUNTING METHODS

3.1 INTRODUCTION

Published work on the viable but non-culturable state revieved earlier, has relied on the simultaneous analysis of samples by culturing methods, and by direct microscopic techniques. It has long been recognised that plating techniques do not recover all the bacteria which can be demonstrated by direct microscopic observation, but because a cell appears intact does not mean that it is necessarily metabolically active, or viable in any definable way. Some method of assessing viability by direct observation is obviously necessary. Observation of microcolony formation is one approach[41,42], but the techniques are difficult and extremely tedious, and cells which are metabolically active, but do not or cannot divide under the conditions of the test, will be recorded as non-viable. The direct viable count method of Kogure[4,5] detects substrate-responsive cells, and this, in one form or another, has been the key method used in demonstrating the viable but

non-culturable state in various types of bacteria. Some assessment of the effectiveness of this technique was obviously needed.

Initial experiments have used a pure culture of *E. coli* type C (ATCC 13706). The culture was grown overnight at 37 °C, harvested by centrifugation, then washed twice with membrane-filtered (0.22 µm) sea water of salinity 35 g/kg. The cells were suspended in filtered sea water to a density of $1\text{-}5 \times 10^6$ per ml, and sampled periodically during storage in the dark at room temperature (18-20 °C).

3.2 METHODS

3.2.1 Culturing methods

Culturable populations were measured by a selective and a non-selective test. The selective test used the MLS37 membrane filter technique described in Section 2.2, while the other used spread-plating on a non-selective lactose agar which contained, per litre: peptone, 10 g; lactose, 10 g; sodium chloride, 5 g; phenol red, 0.025 g; and agar, 12 g.

3.2.2 Direct counting method

Direct counts were made by concentrating the cells from a known volume of water on to the surface of a 25-mm diameter, 0.2 µm pore size, track-etched polycarbonate membrane filter (Nuclepore), which had previously been dyed with Irgalan black. The method used was a modification of those described by Hobbie and others[3] and Pettipher[43], and had been used during previous work at WRc[44].

After filtration of the sample, the vacuum was released, and 1 ml of a 50-mg/l solution of acridine orange in citrate-NaOH buffer at pH 6.6, was pipetted on to the membrane. After 5 minutes contact, the stain was removed by applying vacuum, and the filter was rinsed with three 1-ml volumes of filter-sterilised distilled water. A small drop of filtered liquid paraffin was placed on a clean microscope slide, and spread out

to a thin smear using a second slide. The stained membrane filter was then carefully rolled on to the prepared slide, and another drop of liquid paraffin placed on top. A glass coverslip was lowered slowly on to the membrane, taking care to avoid air bubbles. The slide was viewed under oil immersion at x1250 magnification using a Nikon epifluorescence microscope. The light source was a 100-watt high-pressure mercury arc-lamp, and blue light excitation (420-485 nm) was used, with a DM510 dichroic mirror and 520 nm absorption filter. Depending on conditions, acridine orange-stained cells fluoresced green or orange. Cells were counted with the aid of an eyepiece graticle. To obtain cell densities with 95% confidence limits of ± 10% of the mean count, enough random fields were viewed to ensure that at least 400 cells were counted. Precision was estimated using the graphical method of Cassell[45].

3.2.3 Direct viable count

This followed the revised method of Kogure[5]. A portion of the test mixture was placed in a 30-ml universal container, and nutrient and antibiotics were added to give the following final concentrations: yeast extract, 250 mg/l; nalidixic acid, 20 mg/l; piromidic acid, 10 mg/l; and pipemidic acid, 10 mg/l. This mixture was incubated for 8 h, fixed by addition of formaldehyde (final concentration 2% v/v) and examined by the direct count method described above

3.2.4 Measurement of cell size

Measurement of cells on acridine orange-stained slides was carried out using a Seescan Solitaire-plus image analysis system operating at a resolution of 512 x 512 pixcels. An image of a field of cells was captured into the instrument's frame store using an integrating CCD (charge-coupled device) television camera, operated at an integration rate appropriate to the brightness of the fluorescent image. The digitised image was processed using a linear filter to enhance the contrast, then by a thresholding routine to distinguish the bacteria from the background. Automated routines were used to count the thresholded cells, and to make individual measurements of the longest

feret diameter (the length), shortest feret diameter (width), area, perimeter and aspect ratio of each cell.

3.3 RESULTS OF PRELIMINARY TESTS

A sample taken at the beginning of the first of the survival experiments gave similar results from all the tests, indicating that virtually all the cells present were viable and culturable. The direct viable count showed greatly elongated cells. Figure 7a shows the distribution of cell lengths for the direct count, and Figure 7b the equivalent results for the direct viable count, normalised to the same scale. As the cells on the direct viable count slide were curved or undulating in shape, a simple measurement of the longest feret diameter would have given erroneously low results. Consequently the values used for length in Figure 7b are half the perimeter of each cell. This will be a slight overestimate of the length, owing to the finite thickness of the cells, but will nevertheless be more accurate than a simple length measurement. The measurement was also made by "skeletonising" the cells in the digitised image to a single row of pixcels. Half the perimeter gives an accurate measure of length in this case, but unfortunately the skeletonising process erodes pixcels from the ends of the cells as well as the sides. Length would therefore be underestimated, and as the skeletonising routine was rather slow in operation, its use was abandoned.

Figure 7 indicates the kind of increase in cell size which was seen in the first direct viable count test, but 24 h later, while the total count was virtually unchanged, very few elongated cells were seen on the direct viable count slides. The viable count estimated by this method was about 3 orders of magnitude lower than the total count. This was roughly equal to the culturable count by the selective test, but the non-selective plating method gave counts 100 times greater than this.

Five days later, the total count was stable at 5.7×10^6 cells per ml, the direct viable count showed 6.5×10^3 viable cells per ml, the selective culturing method showed only 1 cfu per ml, and the non-selective method showed 1.3×10^3 cfu per ml.

3.4 FURTHER EXPERIMENTATION

Subsequently a similar but larger-scale experiment was undertaken, and this time rather different results were obtained. Although the same cultures, materials and conditions were used, the strain of E. coli showed greater survival of the culturable population.

Table 2 gives the results of these tests. The total count was seen to rise slightly during the experiment, and larger numbers of very small slender cells were seen as the time of storage increased. While it has yet to be proved, some reductive cell division, as is seen with some native aquatic bacteria[17], cannot be ruled out. For the first 3 samples there was little change in the direct viable count and culturable count results, and the two plating methods did not show the large differences which were previously seen. In the 4th sample the culturable counts had dropped by about an order of magnitude, and a similar decrease was seen in the direct viable count. The direct count did not drop, but about half the cells had become shorter, very slender green-fluorescing rods, about 1.5 x 0.2 μm as against normal cells of 2-3 x 0.8-1.2 μm. Figure 8 shows the difference in cell size distributions from sample 1 and sample 4 direct counts. To indicate the general reduction of size of some of the cells, the cell area has been measured for this Figure.

Table 2. Results of second survival experiment

Sample	Time of storage (h)	Total count	Direct viable count	Culturable count Selective	Culturable count Non-selective
1	0	4.0×10^6	4.3×10^6	2.3×10^6	4.5×10^6
2	4	4.4×10^6	3.7×10^6	2.9×10^6	3.0×10^6
3	24	5.0×10^6	3.6×10^6	1.3×10^6	3.1×10^6
4	48	5.8×10^6	1.9×10^5	1.9×10^5	2.9×10^5

All counts expressed per ml.

There were problems in interpreting the direct viable count slides in this experiment. Using the experimental conditions of Kogure[5], no grossly elongated cells were seen, but rather some were slightly larger than in the total count slides, and the cells fluoresced orange rather than green. The direct viable counts in Table 2 are therefore recorded on the basis of being larger than cells observed in the corresponding direct count slides, but this makes the interpretation of the test very subjective. Table 3 shows numbers of "large" and "small" cells counted in these samples. Again it would appear that there may have been some cell division, either before or during the incubation with yeast extract and antibiotics.

Table 3. Large and small cells observed in direct viable counts

Sample	Large cells	Small cells	Total
1	4.3×10^6	2.3×10^6	6.6×10^6
2	3.7×10^6	2.6×10^6	6.3×10^6
3	3.6×10^6	2.7×10^6	6.3×10^6

All counts expressed per ml.

In an attempt to investigate the lack of elongation of cells, an additional direct viable count test was incubated along with the 4th sample. In this the nutrient content was increased 10 times, to 2.5 g/l, and the tests were incubated for 8 and 24 h. In the test incubated for 8 h the cells had not elongated significantly, but fluoresced orange, mottled with green. After 24 h however, large cells had elongated to lengths between 15 and 50 μm, and interestingly, even some of the very small cells had elongated to form twisted helical structures, indicating that they too were substrate responsive. There did not seem to be evidence of cells having divided in this particular test, although Kogure[5] states that this is a possibility if incubation is extended beyond 8 h.

Clearly this technique is not as straightforward as had been implied, and more evaluation is needed. None of the Colwell reports give any guidance on interpretation of direct viable count slides, other than that elongated cells are counted. This is open to much subjective judgement, especially if mixed populations are being examined. A recent report by Al-Hadithi and Goulder[35] has proposed a different approach to examining such slides, and gives a more systematic, though much more time-consuming way of interpreting the test. They observed natural aquatic bacteria, and obtained elongation of cells to over 20 µm. Interestingly they incubated the tests overnight, which has been found necessary in the present work to achieve significant elongation of cells.

SECTION 4 - PRELIMINARY TRIALS ON RESTORATION OF COLONY-FORMING ABILITY IN OSMOTICALLY-STRESSED BACTERIA

A recent report by Roth and others[25] has proposed the use of betaine (carboxymethyl trimethylammonium hydroxide), an allegedly non-metabolised substance, to restore the colony-forming ability of cells rendered non-culturable by the osmotic stress of exposure to sea water. The following method was therefore tried.

Before analysis of sea water by conventional plating methods, the samples were treated by adding a solution of betaine to a final concentration of 2 mM. As recommended in the paper, small amounts of glucose and ammonium chloride were also added (both 2 mM), and the mixtures were incubated at 25 °C for 2 hours before analysis. Duplicate tests were done, with one of each pair also containing 25 mg/l of chloramphenicol to prevent any possible cell division.

Table 4 lists the results, and it can be seen that the betaine treatment did not significantly increase recovery. Indeed the opposite seemed to be the case for sample 3. For sample 4, the amount of nutrient in the incubation mixture was increased by adding 250 mg/l of yeast extract. Here the viable counts were increased by a factor of about 4. This

indicates that the amount of nutrient in the previous samples had been insufficient to promote recovery of the E. coli strain used, but it is not clear whether the increase in count seen for sample 4 was assisted by the betaine, or whether the yeast extract alone would have had the same effect. Chloramphenicol was not used for this sample, so further tests are also needed to exclude the possibility of cell division during the incubation. Assessment of this technique is continuing.

Table 4. Effect of betaine treatment on culturable E coli.

Sample	Time of storage (h)	Viable count Selective	Viable count Non-selective	Betaine treated count Selective	Betaine treated count Non-selective
1	0	2.3×10^6	4.5×10^6	a) 2.9×10^6 b) 2.1×10^6	4.2×10^6 3.9×10^6
2	4	2.9×10^6	3.0×10^6	a) 2.4×10^6 b) 2.2×10^6	3.9×10^6 3.9×10^6
3	24	1.3×10^6	3.1×10^6	a) 9.3×10^5 b) 6.8×10^5	1.9×10^5 9.8×10^5
4	48	1.9×10^5	2.9×10^5	* 8.6×10^5	1.4×10^6

All counts expressed as cfu per ml.
a) Not treated with chloramphenicol
b) Treated with chloramphenicol
* Nutrient level increased (see text)

SECTION 5 - DISCUSSION AND RECOMMENDATIONS FOR FURTHER WORK

In Section 2, media were compared for their ability to recover coliform bacteria from various types of water. The results obtained for comparison of the m-TEC and MLS procedures for thermotolerant coliform organisms were in accordance with earlier findings at WRc[36]. A similar pattern was seen, with the MLS44 method giving higher counts than m-TEC for all samples, and the discrepancy between the two becoming even greater when the bacteria were stressed by exposure to solar radiation.

m-T7 was not used in earlier WRc studies on methods for enumerating total coliforms[37], as this work was carried out before the medium was first described by LeChevallier and others[39]. The position here is rather confusing, as m-T7 gives better recovery than MLS37 for fresh-water samples, but with saline water the opposite is seen. The matter is further complicated by the results of the light mortality experiments using saline water, where m-T7 is initially less effective than MLS37, but the position is reversed as the stress of exposure to sunlight increases. This is an unusual finding, and indicates that for total coliforms at least, different methods will give better results depending on the degree of stress. However, m-T7 appears superior for fresh-water samples, but confirmation tests to assess its specificity are needed, especially to ensure that it has sufficient selectivity for use with natural samples where numbers of non-coliform organisms may be high.

Experiments involving parallel analyses by culture-based and direct microscopic methods have demonstrated, as expected, that more bacteria can be visualised by total counts than can be grown by culturing methods. However, it has not yet been possible to demonstrate convincingly that a stable population of viable but non-culturable cells has been produced. This is mainly due to unsatisfactory performance of the direct viable counting method, and work is continuing to research this method more fully. This is particularly important since this test has been the key technique used to prove the existence of the viable but non-culturable state. Nutrient concentration and incubation conditions need study, and it is particularly important to develop an objective and systematic way of interpreting the slides. Interestingly, others have come to the same conclusion, and one alternative, though not very convenient, approach to the problem has already appeared in the literature[35]. Simply to count elongated cells is inadequate, as a good deal of subjective judgement is involved, and the danger is that the operator will, intentionally or otherwise, obtain the result which he or she wishes to obtain.

Alternative methods for assessing viability from microscopic observations are clearly needed, and work is currently in progress on the use of INT (2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride) for this purpose. This substance is colourless in solution, but is enzymatically converted by actively metabolising cells to an insoluble red formazan[46]. After suitable incubation with the compound, active bacteria are seen to contain dense red granules. This technique is also being combined with the nalidixic acid-yeast extract direct viable counting method, to see if it aids in interpretation of the test. It is also intended to examine immunofluorescent staining of cells so that selective direct counts can be carried out on mixed populations, and fluorescent antibody direct viable counts will be investigated.

Initial trials with betaine treatment of osmotically-shocked cells have not given convincing results. Nevertheless, this technique deserves further investigation, as do others which approach the problem of demonstrating viability by attempting to improve the culturability of the cells. Enrichment techniques may provide an answer, but the difficulty will be resuscitating the cells without allowing cell division, so that quantitative information is not lost.

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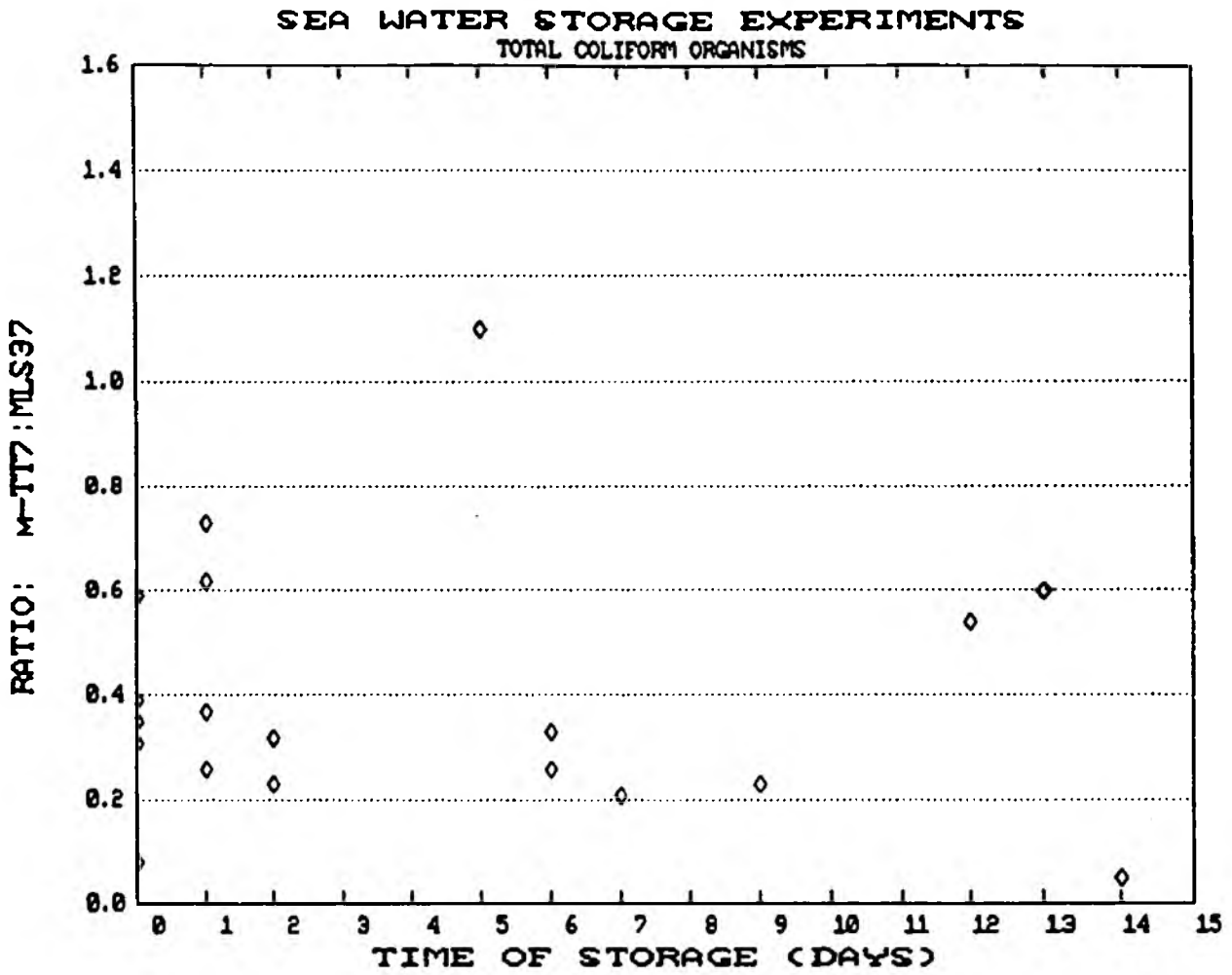


Figure 1. Variation in recovery ratio of two total coliform analysis methods with time of storage of sea water samples.

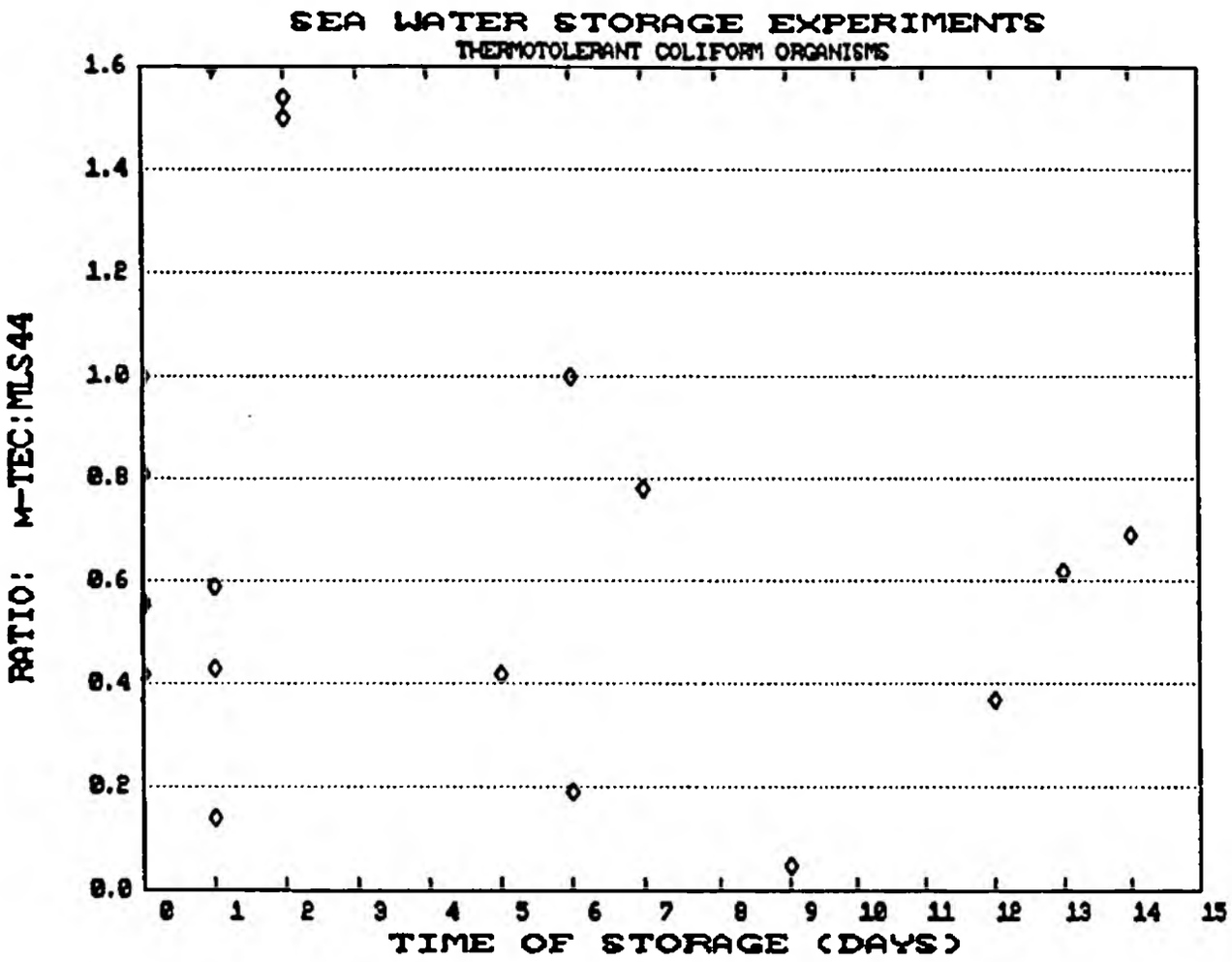


Figure 2. Variation in recovery ratio of two thermotolerant coliform analysis methods with time of storage of sea water samples.

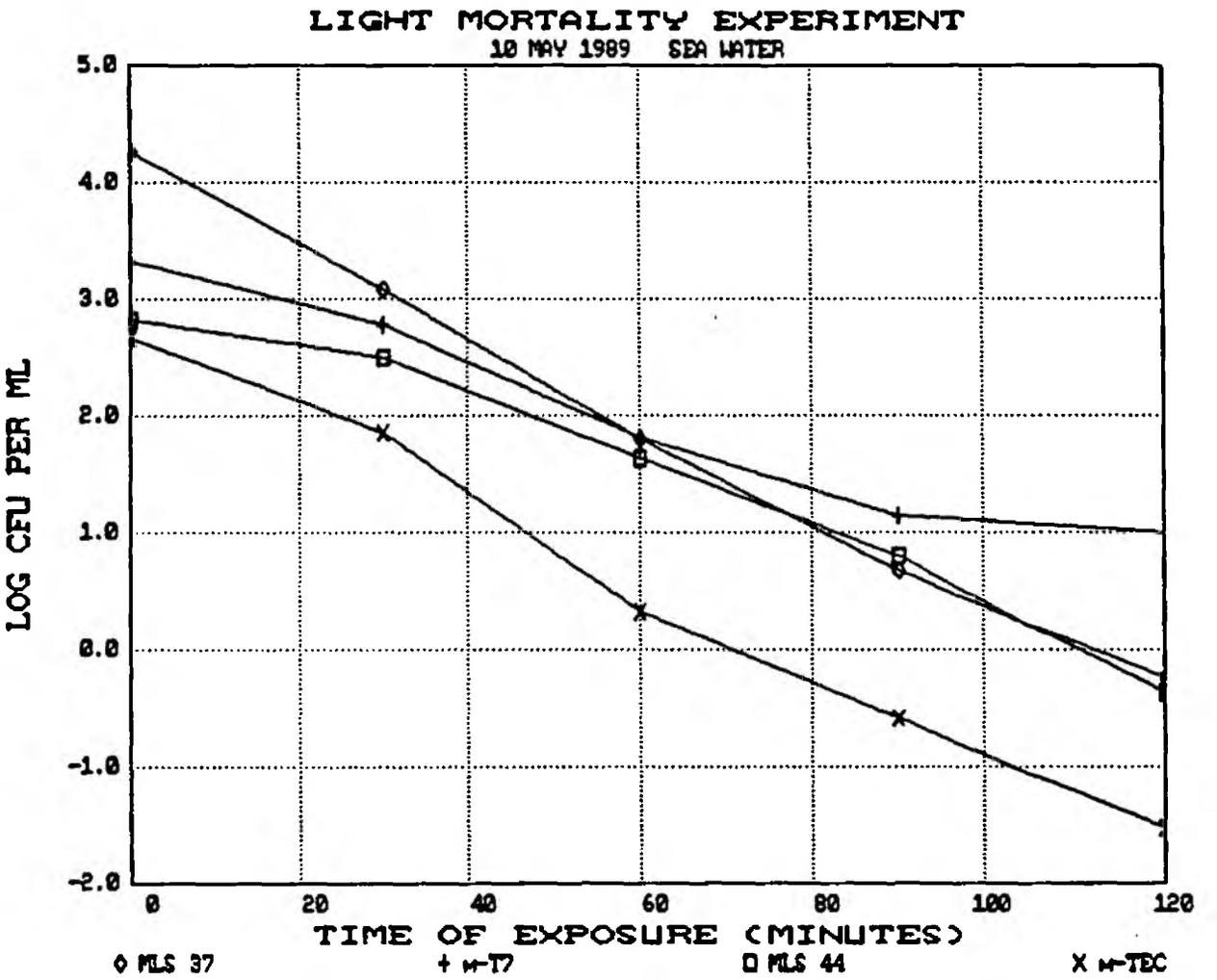


Figure 3. Light-induced inactivation of coliform bacteria measured by four analytical methods. Log cfu per ml plotted against time of exposure to sunlight.

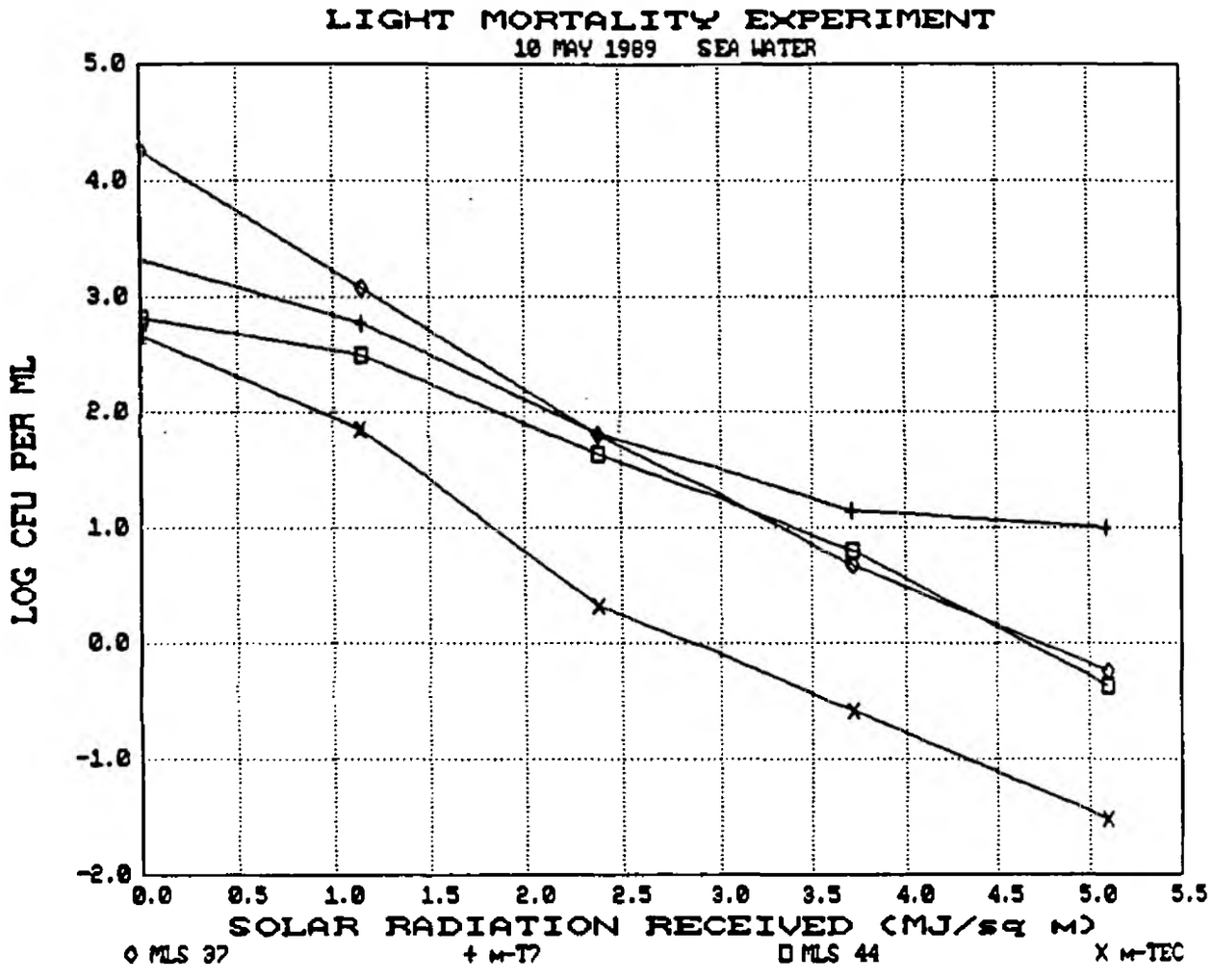


Figure 4. Light-induced inactivation of coliform bacteria measured by four analytical methods. Log cfu per ml plotted against solar radiation received.

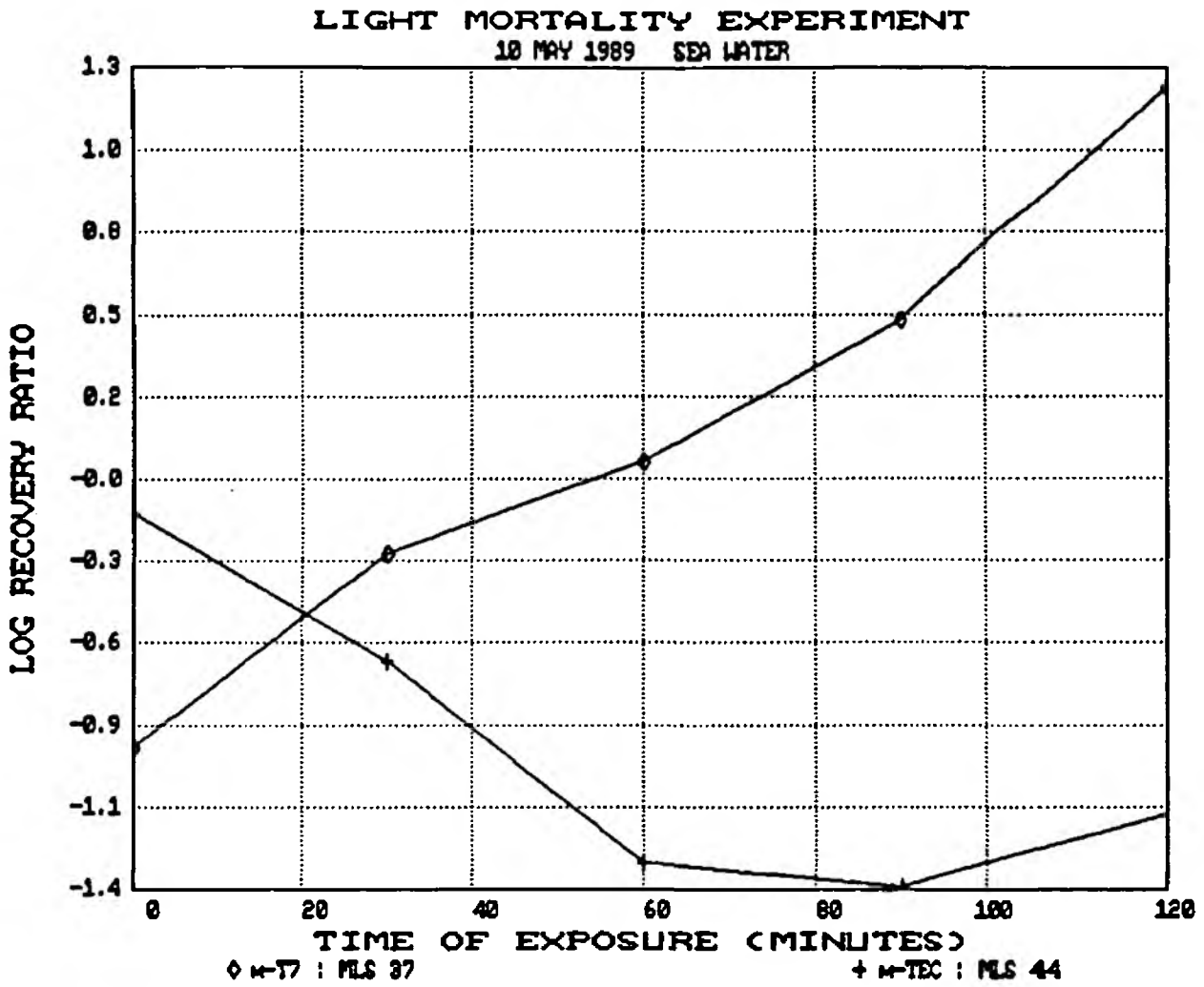


Figure 5. Change in recovery ratios of coliform analysis methods, with exposure to sunlight.

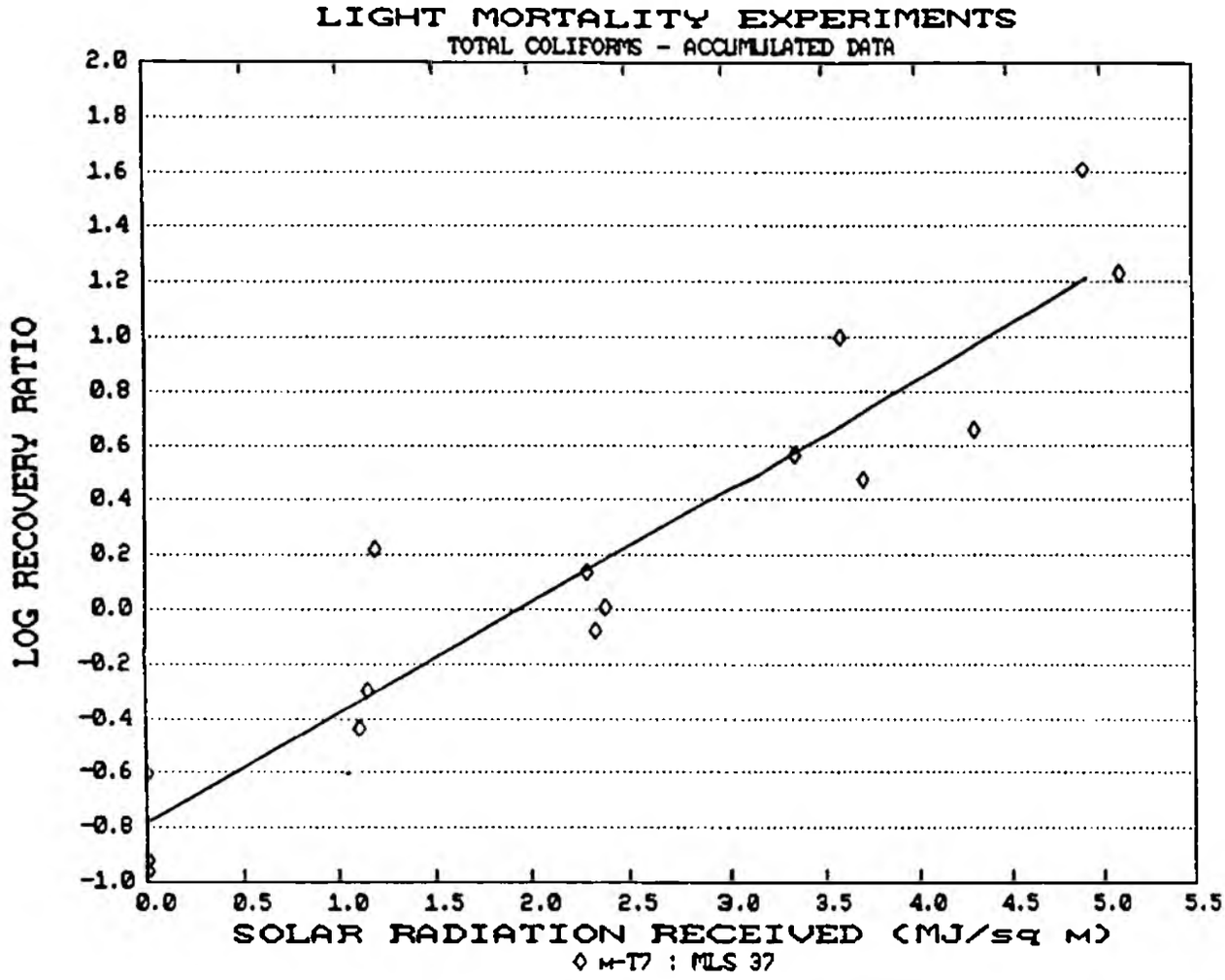
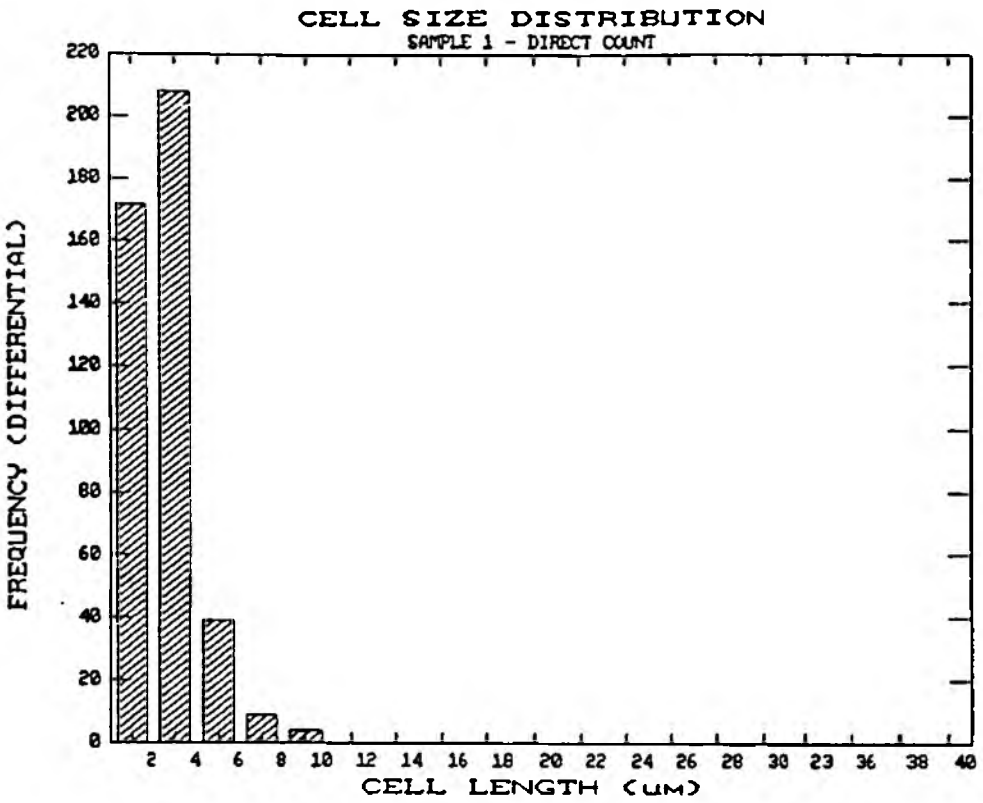


Figure 6. Relationship between recovery ratio of two coliform analysis methods and solar radiation received.

Log (m-T7: MLS37 ratio) = (0.41 x Solar radiation) - 0.79

Correlation coefficient r = 0.94

a)



b)

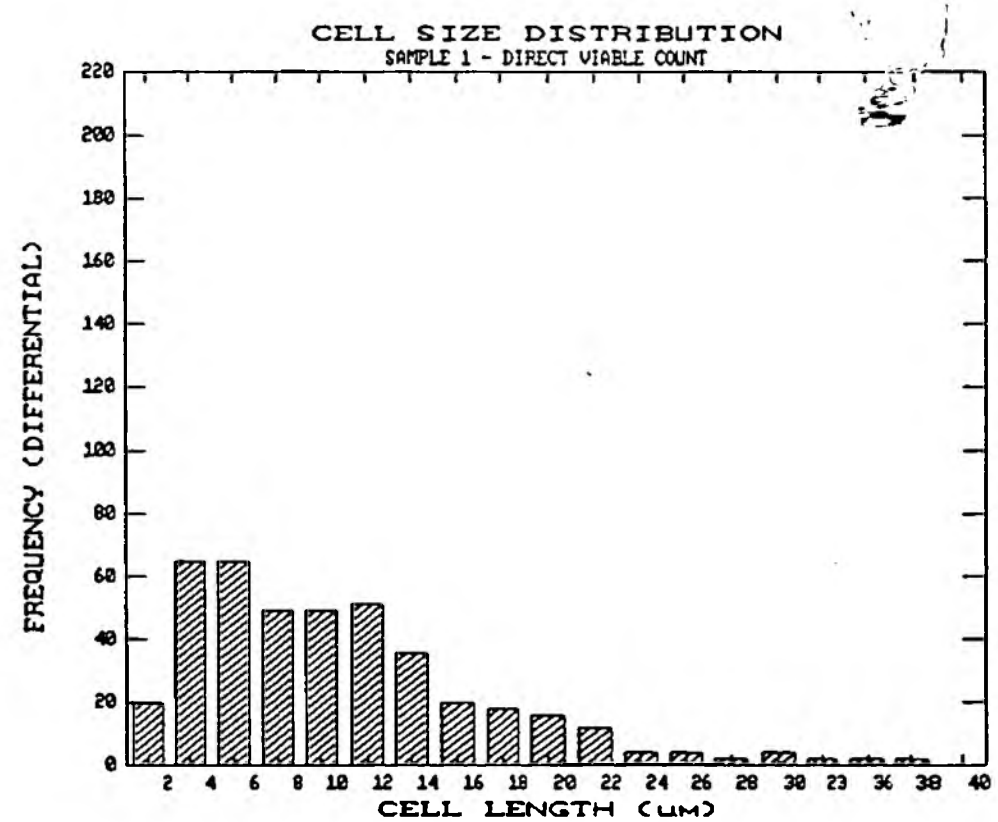
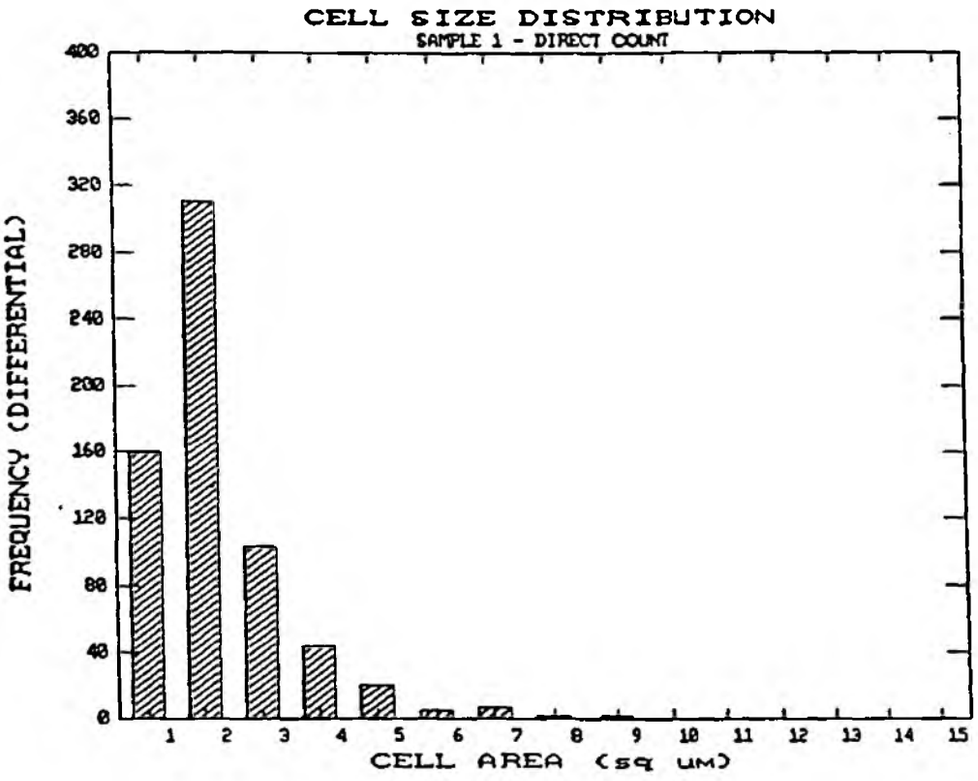


Figure 7. Change in cell size distribution with incubation of the direct viable count method.

a) (Upper plot) before incubation and

b) (Lower plot) after incubation.

a)



b)

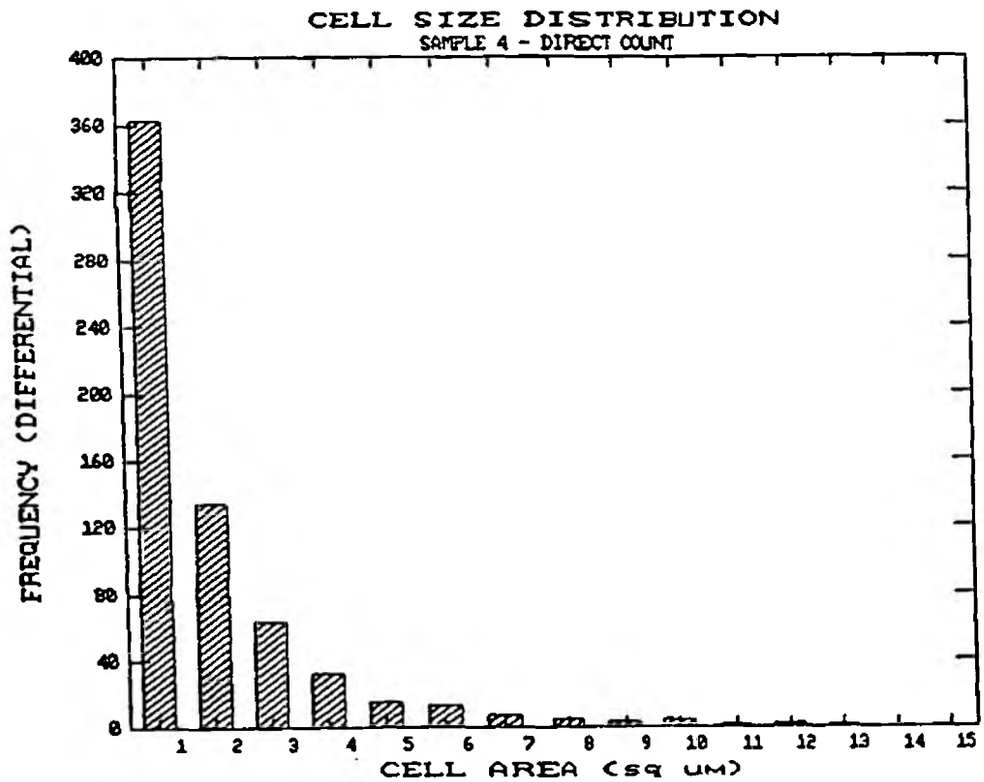


Figure 8. Change in cell size distribution with storage in sea water.

a) (Upper plot) at start of experiment and

b) (Lower plot) after 48 h exposure.