WATER RESEARCH

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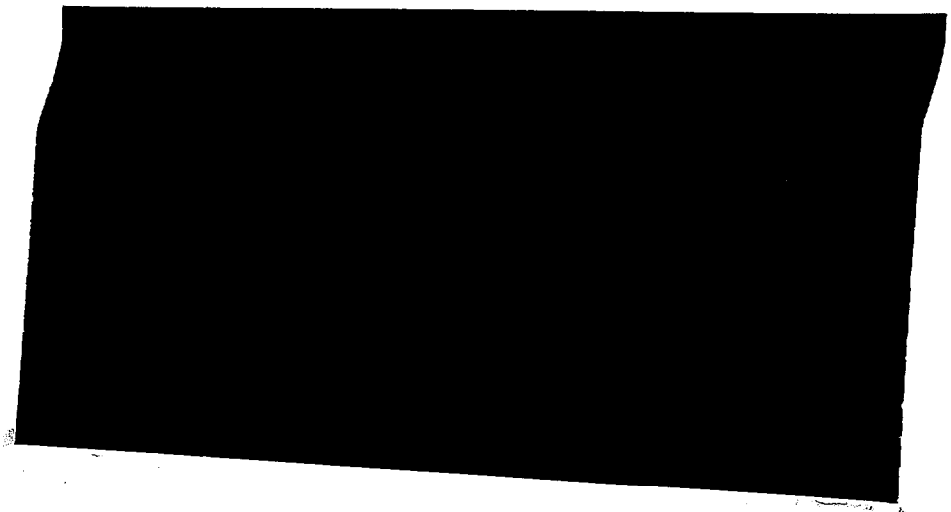

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IMPROVED METHODS OF BACTERIOLOGICAL SAMPLING AND ANALYSIS (ME 9116 SLD)

Final report to Department of the Environment
April 1985 - May 1986

DoE Contract Reference: PECD 7/7/160
Contract Duration: April 1985-May 1987

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PREFACE

On 1 April 1985, the Department of the Environment placed a contract (Reference No PECD 7/7/160) with the Water Research Centre to investigate improved methods of bacteriological sampling and analysis. This contract, which was originally planned to end on 31 March 1987, was extended by two months and finally ended on 31 May 1987.

This contract report, which covers the whole period from April 1985 to May 1987, completes the work done under this contract.

SUMMARY

This report describes work in the period April 1985 to May 1987 on the Department of the Environment's research contract Improved Methods of Bacteriological Sampling and Analysis (Reference PECD 7/7/160).

Methods for rapidly detecting and enumerating bacteria are well established in the food and pharmaceutical industries and have many clinical applications. Although these methods could offer manpower, time and cost savings in the analysis of water their adoption into routine use has been hindered by lack of evaluation and their incompatibility with existing procedures. This report evaluates the application of several rapid bacteriological techniques to water quality monitoring and assesses the needs for sample storage and preservation procedures.

Two commercially available instruments (the Bactometer and the Malthus Growth Analyser) which detect bacteria by measuring changes in the electrical properties of culture media have been evaluated. The performance of test procedures developed by the instrument manufacturers and WRc for detecting coliform bacteria in recreational and drinking waters was assessed. The advantages and disadvantages of the techniques are discussed as is their potential role in routine water quality monitoring.

A rapid means of confirming the presence of E. coli by detecting the presence of the enzyme, Beta-glucuronidase, has been evaluated. A method in which this test is combined with the traditional membrane filtration procedure to give confirmed E. coli counts in 18 h is described.

The results of the studies with the Beta-glucuronidase test and electrical techniques are being used to prepare detailed test procedures. These will be submitted for approval as "tentative procedures" for incorporation in the next edition of Report 71.

Determination of the adenosine tri-phosphate (ATP) content of water samples and direct counting of bacteria by epifluorescence microscopy have been investigated as replacements for the standard plate count technique. Using suitable concentration and resuscitation procedures concentrations as low as four organisms per ml could be detected by ATP measurement. Although a working, direct-count procedure was developed automated counting of the bacteria by image analysis proved infeasible.

Study of the effect of storage on the bacterial content of water samples was hindered by many problems which could not be overcome during the period of the contract. The problems encountered, results obtained and a recommendation for future approaches to this problem are discussed.

Overall conclusions based on the studies are given as are recommendations for further research.

CONTENTS

1. INTRODUCTION

Techniques for the bacteriological examination of water, apart from changes such as the introduction of membrane filters, have remained essentially unchanged for more than 50 years. In the examination of foods, pharmaceuticals and clinical specimens, the position is quite different. Here several innovative methods have been developed and adopted into routine practice where beneficial.

Many of these new procedures have potential benefits for the water industry in terms of reduced cost and manpower, as well as assisting in the provision of water of improved and consistent quality.

This report covers work carried out between 1 April 1985 and 31 May 1987 under the Department of the Environment's research contract 'Improved Methods of Bacteriological Sampling and Analysis' (Reference PECD 7/7/160). The initial objectives of this study were:

1. to evaluate, in the context of water analysis, recently developed rapid automated bacteriological techniques. To develop these techniques further in terms of ease of use, sensitivity and specificity and to assess their value in routine water analysis;

2. to develop a simple means of preserving the bacterial content of water samples for a period of not less than 24h with storage at 4 °C and 20 °C.

During the period of the contract a need was identified for additional topics to be studied in connection with Objective 1. It was agreed that this should be done by diverting research effort

from Objective 2 in which unforeseen difficulties were hindering progress. Thus, although the objectives divided the programme into two separate topics, improved methods of analysis, and preservation of samples, the former was the subject of most study.

1.1 Improved methods of analysis

In water quality monitoring two microbiological parameters are most often used. These are detection of faecal indicator bacteria, coliform organisms and Escherichia coli, and determination of total bacterial content using the standard plate count technique. Of these, analysis for coliform organisms and E. coli is the most important with in excess of 1.5 million determinations being carried out by UK water undertakers each year.

This frequent use of coliform organisms and E. coli in the water and also the food and pharmaceutical industries has stimulated the development of several novel methods of analysis. However, at the start of the contract only one of these, electrometry, seemed to have potential applications in water quality monitoring. Two instruments are available commercially for detecting bacteria by electrometry, the Bactometer manufactured by Bactomatic Inc, Princeton, New Jersey and the Malthus Growth Analyser, Malthus Instruments, Stoke-on-Trent.

Previous work by WRc had shown that, of these, the Bactometer had many advantages for research applications. Accordingly, initial research effort was directed at evaluating and developing further methods for detecting coliform organisms and E. coli in water using this instrument. Later, as envisaged, similar studies were carried out using the Malthus Growth Analyser. Studies with both instruments seemed desirable if harmonisation and

standardisation of future water quality monitoring was to be achieved. The work carried out on the evaluation and development of electrometric techniques is described fully in Section 4.

Like the Bactometer and Malthus Growth Analyser, most 'new' or automated microbiological methods are based on the use of computer controlled instruments and have a large sample handling capacity. These instruments are expensive in comparison with the equipment used in traditional bacteriological techniques and some water undertakers will be unable to justify this expenditure without centralisation of analytical services. There is, therefore, a demand for rapid bacteriological techniques which do not involve the use of expensive or large capacity instruments. One way of satisfying this demand is to modify traditional procedures so that they require less manpower and produce results more quickly. An example of an existing achievement of this type is the inclusion in Report 71 (DoE et al 1983) of the single tube confirmation test for E. coli as a valid alternative to the traditional two tube procedure.

During the first months of the contract, discussions with water authorities revealed that the 24 h delay between obtaining a presumptive detection of a coliform organism or E. coli and its subsequent confirmation could be a problem by delaying decisions on remedial action. If action is taken on a presumptive detection which does not subsequently confirm, manpower and resources will not have been used cost-effectively. Continued false alarms of this kind can also lead to diminished respect for the results of monitoring amongst those required to carry out remedial action. On the other hand, if action is postponed until the presumptive detection is confirmed, the consumer may have been at risk because of this

delay. Any modification to existing techniques which would confirm the presence of E. coli within at most a few hours of the presumptive detection would be extremely useful.

Beta-glucuronidase is an enzyme thought to be exclusive to E. coli (94% of strains) and some shigellas and salmonellas. This enzyme can be detected in broth cultures or colonies of the organism suspected of being E. coli in about 4 h. Reagents for carrying out this test are commercially available and some workers, especially in the USA, have advocated adoption of the technique for rapid confirmation of E. coli. However, the test has not been thoroughly evaluated in the UK and, as a consequence, there has been little discussion about its use as a replacement for the single or two-tube confirmation procedure recommended in Report 71. Since the adoption of this technique would be of operational and cost benefit to the water industry an evaluation of the technique was included in these studies. Evaluation of the Beta-glucuronidase test as a rapid confirmation procedure for E. coli and possible extension of its use to incorporation in a selective isolation medium is reported in Section 5.

Although of little sanitary significance, measurement of the total bacterial content of water indicates the efficacy of water treatment processes and the integrity of distribution systems. The traditional analytical procedure, the standard plate count, is time consuming and takes 72 h before a result is known. Furthermore, it is inefficient in that it detects only about 10% or less of the viable bacteria present.

The inherent inefficiency of the plate count technique and absence of a viable alternative has

undoubtedly engendered a lack of appreciation and understanding of the value of determining the total bacterial loading of drinking water. Armed with an efficient, more rapid procedure, the water microbiologist would be able to observe deterioration in quality earlier and possibly gain a better understanding of the seasonal occurrences of organisms of nuisance rather than public health significance.

As part of the contract, two novel alternatives to the standard plate count were investigated; determination of the adenosine tri-phosphate (ATP) content of water and direct bacterial counting procedures using epifluorescence microscopy. Semi-automated instruments were used in both these procedures.

The purpose of the studies was to seek a correlation between these methods and the standard plate count technique and develop them further for routine use. Studies with epifluorescence microscopy are reported in Section 2 and those with ATP in Section 3.

1.2 Preservation of samples

Although saving manpower, most of the recently developed bacteriological analysis techniques involve relatively high capital expenditure. A means of off-setting this cost is to rationalise all analytical facilities in a central laboratory, to which all routine samples are conveyed. However, where transit distances are great, it would be difficult to ensure that samples are analysed within the recommended 6-h period (DoE et al 1983) from collection. The ability to preserve or 'fix' bacteriological samples for even up to 24 h would allow the cost benefits of centralisation to be realised without prejudicing the quality of the data obtained.

The purpose of the contract studies was therefore to investigate possible means of 'fixing' or maintaining the bacterial content of water samples so that, if necessary, they could be analysed after a maximum of 24 h storage. The approach taken and problems encountered in these studies are described in Section 6.

2. DIRECT COUNTING USING EPIFLUORESCENT MISCROSCOPY

2.1 Introduction

Direct counting of bacteria using light-microscopy has always been regarded as one of the most valid methods of determining the total bacterial content or biomass of materials. However, traditional methods are laborious and of limited sensitivity, since only small (0.01 ml) sample volumes can be used.

In recent years, several workers have combined the established skills of microscopy with the more modern technologies of membrane filtration and image analysis to develop direct-counting techniques, which are more sensitive and less laborious. Of these a procedure developed by Pettipher (1983) called the Direct Epifluorescent Filter Technique (DEFT) has attracted most interest. Although originally developed for applications in the dairy industry, extension of its use to water analysis has been suggested.

DEFT is not the only epifluorescence technique available, since epifluorescence microscopy has been used by marine and fresh water microbiologists for a number of decades. However, a review of the relevant literature (see Appendix A) showed that there was a myriad of recommendations on all stages of the procedure and equipment that should be used. In the context of the present contract, an evaluation of these was both infeasible and

inappropriate and it was decided that the DEFT technique should form the basis of the studies. Furthermore, the reagents and equipment needed for the DEFT technique were already available commercially and it was considered that this made it attractive for routine applications in the water industry.

2.2 The direct epifluorescent filter technique (DEFT)

2.2.1 General description

In this technique the bacteria in a water sample are concentrated on a black polycarbonate membrane filter. The surface of the filter is then treated with a solution of acridine orange (AO), a fluorochrome which stains the nucleic acid of the bacterial cells. Subsequently, the surface of the filter is observed microscopically using incident illumination at a wavelength, which excites the dye, causing it to fluoresce with a green or orange colour. The metabolic activity of the cells governs, to a degree, the intensity and colour of the fluorescence. The fluorescing cells in a statistically significant number of fields of view are counted and, from this, the concentration of bacteria per unit volume of sample can be calculated. By use of an image analyser, the counting of the bacteria can be automated, the output from the image analyser being passed to a micro-computer for calculation of the concentration of bacteria in the sample.

2.2.2 Equipment used at WRc

Throughout the studies a Nikon Labophot microscope was used, linked to an AMS 40-10 television image analyser. Although less sophisticated (and also less expensive) than the Optomax System III image

analyser used by Pettipher (1983), the manufacturers (Analytical Measuring Systems, Saffron Walden) claimed that it was suitable for the DEFT technique and they had developed software specifically for this purpose. The AMS system, complete with epifluorescence microscope cost in the region of £11 000 (1985 prices).

In later stages of the study some minor modifications were made to the system. The first of these was the use of a Vickers 100x/NA 1.3 oil immersion microscope objective, which had a theoretical light transmission twice that of the original objective. This allowed brighter fluorescent images to be seen both visually and by the image analyser. For studies with fluorochromes other than acridine orange, a different filter block, excitation filter and absorption filter had to be used. The characteristics of the filters used are shown in Table 2.1.

Table 2.1. Characteristics of filters used with the Nikon microscope

Block	Main wavelength	Excitation filter	Dichroic mirror	Absorption filter
UV	365 nm	UV 330-380	DM 400	420K
Blue	495 nm	IF 420-485	DM 510	520-560
Green	546 nm	IF 535-550	DM 580	580

2.2.3

Initial studies with DEFT

Initial trials with the DEFT technique proved to be extremely disappointing. Almost all the bacteria treated with acridine orange fluoresced green with the fluorescence fading rapidly. This rapid fading combined with poor contrast with the background made automatic counting unreliable.

These results disagreed with those of Pettipher (1983), who reported that, after staining with

acridine orange, bacteria in milk which were actively metabolising fluoresced orange, with dead or moribund cells fluorescing green. Presumably because of the very low concentration of utilisable carbon in water, bacteria are metabolising at such a low rate that in the context of milk they would be considered moribund.

Although this is a logical explanation for the discrepancy in colour of fluorescence, it did suggest that the DEFT technique, as reported by Pettipher (1983), was unsuitable for the analysis of water. As a consequence two different lines of study were initiated. The first was to study all aspects of the DEFT staining technique to see if brighter fluorescent images could be produced. The second was to survey rapidly other fluorochromes to see if any gave a better response than acridine orange.

2.3 Investigation of DEFT staining procedures

These studies took the form of purely qualitative observation with the aim of attaining the best contrast between bacteria and the background, the least fading of fluorescence and minimum debris fluorescence. The results therefore show a degree of subjectivity, which must be considered in their interpretation.

2.3.1 Procedures studied

The various stages of three staining procedures using acridine orange were investigated with special reference to the effect of different concentrations of the fluorochrome and rinsing procedures.

The first procedure was that reported by Fry and Davies (1985), in which the stain is added to 10 ml of the sample (or dilution of it) to give a final concentration of 5 mg/l. After 3 minutes the

sample is filtered and the membrane rinsed with filter-sterilised, distilled water. After drying in air the filter is mounted in sterile paraffin oil before viewing.

The second method was the DEFT in which, after filtration of the sample, the membrane is overlayed with 2.5 ml of acridine orange (2.5 mg/l in pH 6.6 citrate buffer). After 2 minutes contact time the stain is removed by vacuum filtration. The filter is then rinsed twice, once with 2.5 ml of buffer at pH 3 and once with 2.5 ml of iso-propanol.

The third procedure was that of Zimmerman and Meyer-Reil (1974), which is similar to the DEFT in that staining is carried out on the filter. However, a lower concentration of acridine orange is used and after 3 minutes contact the filter is rinsed with xylene, then iso-propanol and mounted in cinnamaldehyde and eugenol, which are reported to quench fluorescence of debris, but not of bacteria.

In addition to comparing the relative performance of each of these procedures, certain stages were interchanged to see if optimum staining could be achieved.

2.3.2
Results

Neither the technique of Fry and Davies nor that of Zimmerman and Meyer-Reil produced images that were clearer than those obtained with the DEFT. The method of Fry and Davies gave brighter images when acridine orange concentrations greater than 5 mg/l were used and with these the period between illumination and fading to obscurity was much longer. However, background fluorescence increased with higher concentrations of the fluorochrome, although this problem could be overcome by use of 'on the membrane' staining as in the DEFT.

Increased concentrations of acridine orange had little effect on images produced by the DEFT and Zimmerman and Meyer-Reil procedures.

If rinsing was omitted a bright green background fluorescence was produced, which faded quite rapidly to reveal relatively dull green fluorescent bacteria and debris. This background could be reduced with the use of pH 3 citrate buffer but returned with prolonged illumination. If pH 6 citrate buffer was used, background fluorescence was obtained and debris became more prominent. Rinsing with filter-sterilised distilled water had little effect, results being similar to those obtained without rinsing.

If only the iso-propanol rinse was used with the DEFT procedure, a good contrast with the background fluorescence was produced, but both red and green particles were difficult to identify as bacteria. The rate of filtration was markedly reduced with the Zimmerman and Meyer-Reil technique causing the xylene rinse to have a contact time greater than their recommendation of 30 seconds. With this technique, background fluorescence was bright yellow/green and very stable. The cinnamaldehyde and eugenol mountant caused the filter to become transparent which it is thought aggravated the effects of background fluorescence. Similar effects were observed when the mountant was used with the DEFT.

Since the DEFT and Zimmerman and Meyer-Reil techniques had slight similarities, such as 'on filter staining' and use of buffer solutions and iso-propanol, certain aspects of these procedures were combined and studied in a series of further experiments.

The DEFT acridine orange solution was used with the Zimmerman and Meyer-Reil rinses and the filter mounted in liquid paraffin. This gave a decrease in visible debris with some relatively stable bright red/orange fluorescent bacteria. Green fluorescent bacteria were rather dull and indistinct. In another trial the DEFT was used complete, with an added 1 ml xylene rinse. Debris were bright orange/red on a blotchy background. Many red bacteria-sized particles and a few green fluorescent bacteria were present, although in general the fields of view were uncountable. Finally the DEFT rinses were changed to a 2 minute contact time followed by 1 ml of xylene applied for 30 seconds. This induced a slight increase in background fluorescence with few green and some red bacteria visible, which appeared particularly bright. Generally it was considered that too much destaining had occurred and this illustrates just how critical rinses are in the staining procedure.

2.3.3
General conclusions

It is difficult to draw conclusions from these types of experiments because of their subjective nature but the following points can be made. Excessive contact with pH 3 citrate buffer results in bacterial destaining and also in increased background fluorescence. Destaining is therefore possibly a result of pH rather than washing. The use of phosphate buffers with AO produced generally less satisfactory results than with citrate buffer. The improvement in numbers of green bacteria with lower concentrations of AO in phosphate buffer was generally counteracted by poorer contrast and increased fading rate.

Fixing of the stain with iso-propanol aids in reducing fading. Xylene was generally found to be ineffective and seems to reduce the number of green bacterial particles present in a sample. Mounting

with cinnamaldehyde and eugenol was unsuccessful due to clearing of the polycarbonate filter. It is probable that Zimmerman and Meyer-Reil did not use this type of filter, but their publication does not make this point clear.

2.4 Survey of other fluorochromes

A small survey of other fluorochromes was carried out to see if they produced better distinction between bacteria and debris, and images which were more suited to automatic counting. Of those tested three were selected for further study; these were 4'6-diamidino-2-phenylindole (DAPI), ethidium bromide and propidium iodide. The studies with these fluorochromes are reported in Sections 2.6 and 2.9.

2.5 Image analysis with acridine orange stained bacteria

Regardless of the staining procedure used the intensity of fluorescence of bacteria from water was found to be too low and of too short a duration to allow accurate counting by image analysis.

Although this failure was due mainly to the poor response of water bacteria to the staining procedure, limitations of the image analyser were contributing factors. For instance, the monochrome television cameras used in image analysers are more sensitive to red than they are to green light and so red images need not be of such a high intensity. Because of their greater metabolic activity, bacteria in milk give a red/orange fluorescence when stained with acridine orange and are generally larger than bacteria in water, which give green fluorescent images. It is not surprising, therefore, that Pettipher (1983) was able to use image analysis to automate the counting of bacteria in milk.

The non-specific binding of acridine orange to debris also hindered automated counting since the image analyser was unable to discriminate between fluorescent particles of similar sizes but differing types (bacteria or debris).

2.6 Studies with the fluorochrome DAPI

DAPI has not been used extensively in the UK but has found favour with limnologists in Germany and South Africa. The procedure for its use is similar to the DEFT although a different excitation wavelength has to be used. With this fluorochrome bacteria fluoresce bright blue against the dark background of the filter.

Compared with acridine orange, DAPI gave improved stability and intensity of fluorescence. Although a satisfactory direct count could be obtained if the bacteria in each microscope field were counted by direct observation, automated counting by image analysis was still not possible. It was realised, however, that automatic counting was essential if the technique was to be capable of carrying out the large number of determinations required in future studies. Similarly, the technique would be of limited value to a water undertaker, if visual counting had to be employed.

With acridine orange the main factor preventing automatic counting was low intensity and rapid fading of fluorescence. This was not a problem with DAPI, since bright, relatively stable images were produced. However, the problems encountered with particles of debris noted in the work with acridine orange became of more importance when DAPI was used.

It is inevitable that when a water sample is filtered to remove bacteria, other particles will also be retained on the surface of the filter. If

the filter is then treated with a fluorochrome and viewed with epifluorescent illumination, this debris can often fluoresce naturally, or as a consequence of the staining procedure. This effect has been observed consistently in the present studies and has hampered automatic counting.

Using the DAPI staining procedure bacteria give a bright blue, fluorescent image, whereas debris normally appears yellow. With visual counting the yellow particles can be ignored and do not affect the results obtained. However, the image analyser is unable to discriminate between differently coloured particles and consequently results are influenced by the size and quantity of debris present.

To try to minimise the effect of these non-bacterial particles, two ways of increasing the intensity of the blue fluorescence given by the bacteria were studied. The first was to increase the concentration of DAPI in the staining solution, and the second was the use of surface-active agents to aid penetration of the dye into the cell. Differential filtration was also studied as a means of removing the non-bacterial particles, but it was found that unacceptable losses of bacteria occurred with filters that removed the desired quantity of debris.

2.6.1 Effect of DAPI concentration

For visual counting, little enhancement in terms of intensity of fluorescence or cell count was achieved with concentrations of DAPI in excess of 0.1 mg/l (Figure 2.1). At concentrations of 5 mg/l or above the background gave a brown/yellow fluorescence which reduced the contrast with the fluorescent cells, and made counting more difficult. However, it was found that if the stain was applied at a different point in the procedure,

DAPI concentrations in excess of 15 mg/l could be used without producing this background fluorescence.

Figure 2.2 shows the effect, using the revised staining procedure, of different concentrations of DAPI on the visual and automatic counts. Again, increased concentrations of the fluorochrome had little effect on the counts obtained visually. In contrast, those obtained by the image analyser increased with increasing concentrations of DAPI. Above 20 mg/l the counts obtained using the image analyser exceeded those obtained manually. These results suggest that the fluorescence of both the bacteria and the non-bacterial debris was enhanced by the higher concentrations of DAPI.

2.6.2 Use of surface-active agents

It has been reported that use of surface-active compounds improves DAPI staining by making the cell envelope more permeable to the fluorochrome, more of which can then react with the nucleic acid within the cell. Triton X-100, used by others, was found to remove the black dye from the membrane filters, giving unacceptably high blue background fluorescence, thus reducing overall contrast.

Sodium dodecyl sulphate (SDS) did not show this effect, but appeared to react with DAPI in solution to give a flocculent precipitate. However, this could be avoided by using the revised staining procedure in which the filtration and staining steps are separated. Treatment of the sample with 10 mg/l of SDS before filtration produced enhanced bacterial fluorescence and noticeably better overall contrast. However, this enhancement was not great enough to enable the image analyser to discriminate between the two types of fluorescent particle present.

2.6.3

Discussion

The studies reported above led to an improved manual counting procedure. This procedure, using a higher concentration of DAPI and treatment with SDS, gave clearer, stable fluorescent images, which are necessary for obtaining accurate counts.

Attempts to automate the counting procedure continued to be unsuccessful due to the fluorescent debris encountered in most samples. This is likely to be a problem with most automated counting techniques for water bacteria, since the instruments used are unable to judge as well as the human eye whether a particle is a bacterium or not.

The revised and recommended counting procedure is described fully in Section 2.7

2.7

Procedure for the direct counting of bacteria using DAPI

The technique given in this section has been found to be a satisfactory means of obtaining a direct count of the bacterial content of a water sample.

2.7.1

Fluorochrome

DAPI-(Sigma Chemical Co Ltd) is supplied desiccated in 10 mg vials which must be stored below 0 °C in the dark. A stock solution of 1 g/l is prepared by dissolving 10 mg of DAPI in 10 ml of filter-sterilised distilled water (fdw). The prepared solution is stored in a darkened vessel at 4 °C. All glassware to be used in the preparation of the fluorochrome solution must be rinsed with fdw and then sterilised.

2.7.2

Membrane filters

Nuclepore, polycarbonate, 25 mm diameter membrane filters with a pore size of 0.2 µm are used. To attain the dark background essential for fluorescence microscopy the filters are dyed black before use. The dye used is Irgalan Black (Acid

Black No 107, Ciba Geigy Corp). This is used at a concentration of 2 g per litre of 2% v/v glacial acetic acid (BDH) in fdw. Before use, filters are immersed in this solution for 10 minutes prior to rinsing in fdw.

Note: Although black polycarbonate filters are commercially available some have been found to have large hydrophobic areas and are therefore not suitable for use in this technique.

2.7.3 Sample filtration and staining

The volume of sample filtered depends on the number of bacteria thought to be present in water. This can be predicted from a graph showing the bacteria per ml against the volume of sample to be filtered to obtain the chosen number of organisms in each field of view (unit area of filter). This is discussed more fully in Section 2.8.

Figure 2.3 illustrates the volume of water to be filtered to count an average of 4-5 bacteria per field. However, since the volume that can be filtered also depends on the amount of non-bacterial matter present in the sample this type of graph can only acts as a rough guide. This aspect of the technique is discussed further in Section 2.8.

Samples are usually taken using sterile, plastic, screw-top containers (Sterilin) of 30, 150 or 250 ml capacity. Ideally, samples should be analysed immediately after collection (within 1-2 hrs). If this cannot be done the samples should be 'fixed' by the addition of formaldehyde (1% v/v) or refrigerated.

Millipore filtration apparatus (25 mm diameter, 30 ml volume) with glass frit filtration bases are

used. A stainless steel manifold enables the vacuum to be applied to individual filter towers and allows several samples to be filtered at the same time. All glassware is rinsed with fdw and sterilised prior to use.

The DAPI stock solution is diluted to a concentration of 1 mg/l prior to each experiment. The sample is filtered through the pre-dyed membrane filter with a vacuum of approximately 20 mm Hg. The vacuum is released as soon as possible to prevent excess drying and stressing of the membrane. Approximately 2 ml of DAPI solution is then applied to the surface of the membrane and left for a contact time of 10 minutes before re-applying the vacuum.

As soon as the dye solution has passed through, the membrane is placed on a thin film of filter sterilised liquid paraffin on a clean, glass slide. A Pasteur pipette assists the formation of the film of liquid paraffin. Glass slides should be cleaned with methylated spirits before use. A drop of liquid paraffin is placed on the surface of the filter and a coverslip (previously cleaned with methylated spirits) added, care being taken to exclude air bubbles.

2.7.4
Microscopy

The microscope system used must be equipped for epifluorescence. With DAPI, an IF 330-380 excitation filter and a DM 400 dichroic mirror are required. In the studies at WRc the objective lens used was a Vickers 100x with a numerical aperture of 1.30; this gave a final magnification of x1250 with the microscope system used. A non-fluorescent immersion oil was used (eg Cargille type VDF).

2.7.5
Counting

Using ultraviolet excitation, DAPI bound to bacterial DNA fluoresces blue/white. DAPI not bound to DNA may emit weak, yellow fluorescence when bound to debris. Only the blue/white bacteria-shaped particles are counted.

The field of view is divided up by a gridded eyepiece graticule. Usually, four squares within this grid are chosen as the area for counting (242 μm^2) and each area should contain between three and 15 bacteria.

If there are less than this, a larger counting area may be used (usually 25 squares) or a larger volume of sample filtered (Section 2.8 'Limits Of Sensitivity'). The field of view is moved randomly over the surface of the filter, a separate count being taken at each new position. Between 20 and 40 separate fields should be counted per slide. However, the number of fields used depends both on the total number of cells counted and the accuracy required. For example if a total of 400 cells is counted 95% confidence limits of $\pm$10% of the mean will be obtained. If there are less than 3-15 bacteria per field of view a lower degree of accuracy would be obtained unless more than 40 fields were counted.

2.7.6
Calculation of the number of cells per unit volume

The area of the eyepiece graticule must be calibrated to represent a known area of the filter. This is achieved using a stage graticule with known divisions so that a calibration factor can be calculated. Once this is done the number of cells per ml can be calculated using the following formula:

$$\frac{\text{(Mean count per field) x (Filtration area)}}{\text{(Area of counting field) x (Volume of sample in ml)}}$$

2.8 Limits of sensitivity of DAPI technique

In determining the limit of sensitivity of the direct counting technique there are several parameters which must be considered. The parameters used in calculating the number of bacteria per ml of sample, as shown in the formula in Section 2.7.5, are of major importance.

With a fixed 'mean count per field', 'filtration area' and 'area of counting field' the number of cells per ml of sample is proportional to the volume of sample filtered. A log graph of sample volume against cells per ml produces a straight line relationship from which the maximum limit of sensitivity can be deduced.

Figure 2.3 illustrates this relationship using values calculated using the formula. This graph can be used to estimate the volume of sample to be filtered if an approximate number of cells per ml is known. It can, however, only be used if the area of the counting field is to be 4 squares on the eyepiece graticule (2.42×10^{-4} mm^2 of filter) and with a mean of 4.5 cells per field. Figure 2.4 shows a similar graph produced from experimental data when there were between four and five cells per field. This agreement between a theoretical and an observed relationship is surprising since in the latter variations due to experimental factors, such as the presence of debris, are to be expected.

To increase the limit of sensitivity a larger counting area may be selected. In practice, this has been an area of 25 squares on the eyepiece graticule (1.51×10^{-3} mm^2 of filter). This is illustrated in Figure 2.5.

To obtain maximum sensitivity, the whole field of view can be used as the counting area. Figure 2.6 represents the theoretical data obtained counting

an average of one cell per field and using the whole area of view as the counting area (0.0108 mm^2 of filter). A volume of 350 ml of mains tap water can easily be filtered in half an hour through 25 mm diameter filter with a pore size of 0.2 µm. From the regression equation of the line in Figure 2.6 the number of bacteria per ml can be calculated for 350 ml of sample. This is calculated to be 47 bacteria per ml of sample. However, to obtain 90% confidence intervals of ±10% of the mean then approximately 260 fields (or 260 cells) must be counted (Cassell 1965).

2.9 Studies with other fluorochromes

Propidium iodide and ethidium bromide were studied to ascertain if an improved distinction between bacteria and debris could be achieved using image analysis. With both fluorochromes the bacteria fluoresced more brightly than with DAPI or acridine orange. Fluorescence faded very slowly, possibly due to the longer excitation wavelength. The rate of fading of ethidium bromide was greatest when staining the sample in solution prior to filtration. With staining 'on the membrane' the rate of fading increased if sodium dodecyl sulphate or Triton X-100 pretreatments were used.

Both bacteria and debris fluoresced red and although brightness and contrast with the background were superior to other fluorochromes, this factor made counting by image analysis infeasible and visual counting difficult and subjective.

2.10 Discussion and conclusions

A direct, bacterial-counting technique has been developed which uses the fluorochrome DAPI. For the analysis of water samples this technique is considered to give clearer, more stable fluorescent images than procedures using acridine orange.

Attempts to automate the counting procedure have been unsuccessful due to the fluorescent debris encountered in most samples. This is likely to be a problem with most automated counting techniques for water bacteria since the instruments used are unable to judge, as well as the human eye, whether a particle is a bacterium or not.

In the automated direct-count technique reported by Pettipher (1983) for applications in the dairy industry, the fluorochrome acridine orange is used. This fluorochrome gives bright orange fluorescence with bacteria which are actively metabolising and green fluorescence with quiescent or moribund cells. The type of monochrome television camera used in the AMS 40-10 system is most sensitive to red/orange light. As a consequence, when acridine orange is used the image analyser is able to discriminate bacteria which are metabolising (orange), from debris or dead cells (green). However, when this technique is used with water samples the majority of the bacteria present appear green since they are not metabolising as actively as those found in milk. In earlier studies at WRc, acridine orange was found to give images which faded too quickly for reliable counts to be obtained by manual or automatic means. For visual direct counting of bacteria from water samples the DAPI technique reported above is to be preferred to one using acridine orange.

It is likely that more sophisticated image analysers would be capable of discriminating between bacteria and debris in a DAPI preparation. However, since the cost of such systems would be difficult to justify in terms of operational benefits to a water undertaker this topic was not pursued further.

The limit of sensitivity of the DAPI technique is influenced by many factors. Of major importance is the amount of non-bacterial debris present in the water to be analysed. If this is large, filtration of sufficient water to give a countable number of bacteria in each field of view becomes difficult. Similarly, this debris can mask the presence of bacteria making counts inaccurate. If the water contains little debris the limits of sensitivity can be extended to about 50 bacteria per ml assuming that 300-400 ml of water can be filtered. Although possible, filtration of larger volumes (up to 1 litre) is consider impractical due to the time required (about 1 h).

A limit of sensitivity of about 50 bacteria/ml makes the direct counting procedure less sensitive than the traditional 22 °C standard plate count (1 colony forming unit/ml). However, the ratio of direct-counts and colony counts, obtained using the standard plate count technique, is unlikely to be 1:1 and is probably in the range of 10-100:1. As a consequence, a direct count of between 50-100 bacteria/ml may be representative of a 22 °C plate count less than 10 colony forming units/ml. Since most distribution waters have plate counts in excess of this, the limit of sensitivity of the direct counting procedure may be adequate for routine monitoring of water quality.

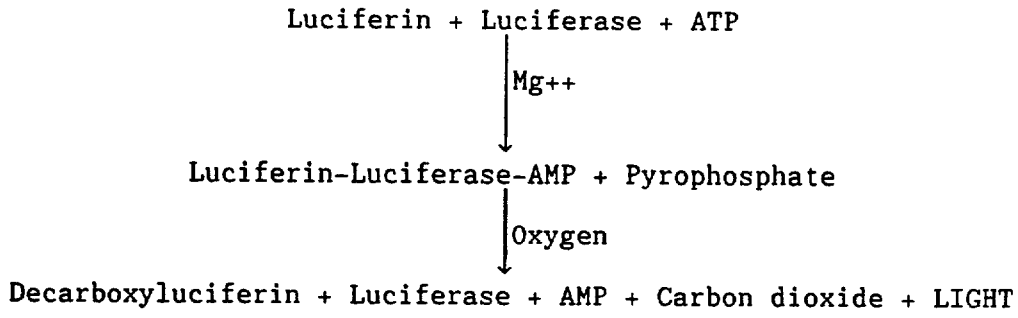
3. ADENOSINE TRIPHOSPHATE

3.1 Introduction

Adenosine triphosphate (ATP) is the labile, high-energy store of all living organisms. ATP technology has been reviewed by Karl (1980). In this review he shows that ATP concentration can be considered a good measure of the biomass or viability of a biological system. Accordingly, ATP measurement should be capable of estimating the

total viable bacterial content with the added advantage of measuring all the active bacteria present. This is in contrast to viable bacteria counting techniques, such as the standard plate count, which only detect that portion of the bacterial population that is able to grow under the the chosen cultural conditions, normally only 10% or less. The speed of ATP determination is an additional benefit, since it takes only minutes per sample compared with the days required for incubation of plate counts.

ATP is analysed using the Firefly light-emitting reaction, where the oxidation of luciferin in the presence of luciferase and ATP results in the generation of light. ATP acts as the energy source for this reaction, and approximately one photon of light is generated per molecule of ATP utilised. The reaction can be much simplified as follows:



In recent years high sensitivity, microprocessor controlled luminometers have become commercially available. This coupled with the development of much purer ATP monitoring reagents has made the determination of the ATP content of a sample into a relatively simple assay.

A good correlation between ATP content and viable bacterial count has been reported (Karl 1980), and the assay has been successfully applied by workers

in food, brewing, limnology, water and clinical fields. However, direct measurement of the ATP content of a water sample to give an estimate of its microbial content can be limited by the sensitivity of ATP determination. Current ATP measurement systems are only capable of measuring down to the equivalent of 100-1000 bacteria per ml.

Since potable water normally contains less than this number of bacteria, a method to concentrate the bacteria in a water sample would be required before ATP determination could be carried out. Membrane filtration is a normal procedure for concentrating bacteria, but it can stress the cells and thus result in an underestimate of the ATP content.

In the studies reported here the relationship between direct ATP analyses and viable plate counts for both mixed cultures of naturally-occurring bacteria and pure cultures of organisms isolated from natural waters has been investigated. Also reported is a method of sample concentration involving filtration followed by resuscitation, to alleviate any stress to the bacteria. The techniques and results of these experiments are described below.

3.2 Methods

All ATP analyses were carried out using Lumac ATP monitoring reagents and an LKB 1251 Luminometer.

3.2.1 Direct ATP analysis

Analyses were carried out on samples of sterilised water which had been inoculated with a seed of bacteria. Samples were taken over a period of days to cover the growth response of the inoculated bacteria to the nutrients naturally occurring in the water. This source of samples was chosen so that different concentrations of the same culture

of bacteria could be studied without the need for either dilution or concentration before ATP analysis. This was necessary because the ATP content of a bacterium is known to be affected by changes in the environment in which it is suspended. The experiments were carried out using mixed and pure cultures of bacteria as an inoculum. The mixed inoculum was a sample of River Thames water, and the pure cultures were isolates obtained from a river water.

ATP analyses were carried out by direct extraction of the ATP with Lumac NRB (Bacterial ATP release agent) and measurement of the released ATP by Lumac Lumit-PM (Luciferase-luciferin reagent). Both these reagents are directly injected into the sample within the luminometer. Counts of viable bacteria were carried out by the pour plate technique using yeast extract agar. Samples were serially diluted in 0.1% Peptone broth. Plates were incubated at 20 °C, and colonies counted after 3, 7 and 14 days incubation.

3.2.2 Results and discussion

The results of the colony counts after 14 days incubation have been used, because it was found that the standard three day count often gave a serious underestimate of the potential colony forming units. The degree of underestimation was associated with both the concentration of bacteria present and the source of the sample. With high counts the error was not serious. However, with lower concentrations the 3-day count was often around 50% of that achieved by Day 14. Counts carried out on chlorinated distribution samples often had no colonies visible after three days incubation, but did achieve a count after 14 days.

The results of the ATP content and 14-day plate counts of the samples are plotted against each

other in Figures 3.1 to 3.4. The results of the mixed culture experiment are shown in Figure 3.1. The other three figures represent three different pure cultures. A better relationship between ATP content and cell numbers is demonstrated for the pure cultures compared with the mixed populations. There is also an obvious difference in the apparent ATP/cell value between the mixed and pure cultures. The pure cultures have values around the 1 fg/cell level, this being the commonly accepted average value for ATP/bacterial cell. Variations around this value are normally accounted for by differences in cell size. However the value for the mixed culture plot is nearer 10 fg/cell. This apparently high value is probably caused by an under estimate of the viable count which normally occurs in plate counts on mixed cultures. A value of 10% is often quoted as the expected recovery, and this would account well for the apparent high reading.

3.2.3 Concentration technique

A known volume of a water sample was filtered through a pasteurised, 50 mm diameter, 0.2 µm Nuclepore polycarbonate membrane filter, care being taken to ensure the membrane did not dry out at the end of filtration. The membrane was then transferred to a sterile 60 mm plastic petri dish containing 0.5 ml of 0.1% peptone broth. The peptone broth acted as a resuscitant to the organisms which had been stressed during filtration. The membrane was left in contact with the peptone broth for approximately 30 minutes at room temperature, after which time 0.5 ml of ATP extractant NRB (Lumac) was added to release ATP from the bacterial cells. The ATP content of the extract was then determined.

Experiments have been carried out mainly using tap water, but in some the effluent from a slow sand

filter was used, since a higher bacterial population would be expected in this type of sample. In all experiments a range of volumes of sample were filtered. Measurements of the viable count, and the ATP content of the neat, unfiltered sample were carried out as described in Section 3.2.1 except that in these experiments it was found necessary to extend the incubation period of the plate count procedure from 3 to 14 days. The reason for this was that with some samples no colonies were visible after three days incubation even though the ATP concentration suggested that viable bacteria were present. After extended incubation colonies did develop on these plates. Subsequently, all plates were incubated for 14 days with colony counts also being made after three days. Direct counts of the neat, unfiltered samples were also determined using the procedure described in Section 2.7.

3.2.4

Results and discussion

The results obtained are presented in Table 3.1 and graphically in Figure 3.5. By extrapolation of the line of best fit through the results in Figure 3.3 an ATP content per unit volume of original sample can be obtained. These values and the correlation coefficient of the line of best fit, plus the results of analyses carried out on the unextracted samples are presented in Table 3.2.

These results (Table 3.2 and Figure 3.5) demonstrate that there is a good correlation and proportionality between the volume filtered and the amount of ATP extracted from the resuscitated micro-organisms. The results indicate that a reasonable limit of detection using this technique is about 10 ng/l.

For unconcentrated tap water samples the viable and direct counts and ATP measurements were below the

Table 3.1. Extracted ATP content from a range of sample volumes

Sample volume filtered (ml)	Sand-filtered water	ATP content (ng/l) Tap-waters 1	2	3	4	5	6
0	1	0	2	0	2	5	1
10	128	12	8	ND	ND	ND	ND
25	236	4	11	ND	ND	ND	ND
50	535	8	20	4	10	10	ND
75	638	ND	ND	ND	ND	ND	ND
100	1048	11	33	5	14	3	11
200	ND	ND	ND	8	ND	ND	ND
250	2077	31	70	ND	33	12	16
300	ND	ND	ND	15	ND	ND	ND
500	4179	68	173	21	59	11	34
1000	7274	126	242	44	135	31	67
2000	ND	173	ND	ND	217	44	143
Viable count (cfu/ml)	100*	<1*	1*	6	28	4	28

* 3 d plate count (others 14 d)
ND Not determined

Table 3.2. Results of total and viable counts, and ATP analyses on neat and concentrated samples

	Sand-filtered water	Tap-waters 1	2	3	4	5	6
Correlation coefficient	0.997	0.999	0.995	0.994	0.998	0.990	0.999
ATP equiv (ng/l)	14.65	0.083	0.504	0.057	0.190	0.040	0.050
ATP Neat (ng/l)	13.00	<0.1	<1	<0.1	0.22	<0.1	<0.5
Plate count (cfu/ml)	100*	<1*	1*	6	28	4	28
Direct count (/ml)	200 000	<10 000	<10 000	ND	ND	ND	ND

* 3 d plate count (others 14 d)
ND Not determined

limits of detection. The slow sand-filtered water sample had an ATP content approximately two orders of magnitude greater than that of the tap water.

This corresponds well with the viable count of 100 cfu/ml obtained from this sample. ATP values obtained for both neat and concentrated samples of the slow sand filtered water were also in close agreement. This indicated that there were no significant losses due to the concentration procedure used.

With most of the samples of tap water no colonies had developed after the recommended incubation period of three days, although using the resuscitation procedure, the ATP values indicated that low concentrations of bacteria were present. Extending the incubation period to 14 days confirmed that this was so and implies that the ATP technique can be more sensitive than the standard 22 °C plate count.

3.3 Conclusions

The results obtained from direct analyses of ATP from neat samples of water gave a reasonable correlation between ATP content and viable plate count for samples derived from pure cultures, with a poorer correlation for the mixed culture samples. The theoretical sensitivity of ATP analyses of around 1000 bacteria/ml is confirmed by these results, almost all counts below 1000 cfu/ml having an ATP value below detection level.

These experiments suggest that ATP measurements carried out on samples of concentrated, resuscitated water can give a rapid estimation of bacterial concentrations as low as 4 cfu/ml. This procedure is very unselective, and micro-organisms other than bacteria will be included in the estimate, so that care will be required in the

expression of results obtained. However, an increase in the ATP concentration of a sample of water taken in distribution would indicate an increase in biomass in that water resulting from a lowering of quality. The procedure could therefore be used in a rapid, early warning system.

Despite this reservation, the increased sensitivity indicated by these studies suggests that ATP measurement may have other applications in water quality monitoring. One of these is its possible use as a presence or absence test for selected groups of organisms.

The technique would involve concentration of the bacteria in the sample by membrane filtration followed by incubation, for 6 h, on a selective medium which contains no ATP. Any increase in ATP after the incubation period would be attributable to growth of the target organism. However, the test would rely on the growth medium being totally selective for the target organism. In the past, methods like this have been suggested by the manufacturers of ATP reagents, but have been dismissed because of doubtful sensitivity. Further work is needed to evaluate this type of technique since it could provide a rapid and operationally valuable presence or absence test for E. coli.

4. ELECTROMETRIC METHODS

4.1 Introduction

It was discovered at the end of the last century that the growth of bacteria could produce changes in the electrical conductivity of culture fluids, and from that time papers appeared sporadically in the literature on related topics. These are summarised by Cady (1978) and Firstenberg-Eden and Eden (1984).

In the mid-1970s, Ur and Brown (1975) and Cady (1975), working independently, first described the use of continuous impedance monitoring as a technique of wide potential use in clinical microbiology. Later Richards et al (1978) developed similar instrumentation with different operating parameters. The technology was subsequently taken up by commercial manufacturers: Ur and Brown's instrument became the "Bactobridge" (no longer made), Cady's was developed into the "Bactometer", and that of Richards et al formed the basis of the Malthus instrument.

Electrometric instruments have found wide acceptance, mainly in the food, dairy and brewing industries, where they are used for quality control of raw materials and products, sterility testing, checking viability of starter cultures, hygiene control, and prediction of shelf life. They are also used by some producers of toiletries and cosmetics for quality control and sterility testing. Despite interest from the water industry, the technique has not yet been adopted for routine testing of water, owing to a lack of information on its suitability for this task.

4.1.1 Choice of electrometric instrument

When this study started, only two manufacturers were offering suitable electrometric machines; Malthus Instruments of Burslem, Stoke-on-Trent, now owned by Radiometer, and Bactomatic Ltd of Henley-on-Thames, a subsidiary of an American parent company, which has itself become part of the McDonnell-Douglas Corporation.

WRc had previously tested an 8-sample Malthus instrument and a 32-sample Bactometer machine but these were considered inadequate for this study. The instrument finally chosen was the Bactometer M123 Microbial Monitoring System, a

computer-controlled analyser capable of handling 128 samples simultaneously, and with the potential to be expanded to take up to 512 samples. This machine was selected because it appeared to be more versatile than the equivalent Malthus instrument, but this does not imply a recommendation, and subsequently a Malthus instrument was also obtained for comparison. Advantages of the Bactometer M123 were that it could measure three different electrical parameters in the test vessel, and that samples could be analysed in disposable plastic culture modules (sample size 1-2 ml), or a variety of glass bottles (up to 250-ml capacity) which are incubated remotely from the growth monitor.

4.1.2
Description of the Bactometer M123 system

The system consists of a "Bactometer Processing Unit" (BPU), a computer and peripherals. The BPU has two forced-air convection incubators (temperature range 10 °C below ambient to over 50 °C), each of which is independently controlled, and accepts four disposable culture modules. The module is a polystyrene moulding with 16 wells, each of which has two small stainless steel electrodes in the base. The rear tongue of the module forms an edge connector which plugs into one of the eight positions in the BPU. One BPU thus takes up to 128 samples, and the computer system used can control up to four units, giving a maximum capacity of 512 samples.

When the BPU is running, each of the wells is switched into the measuring circuit in turn, an alternating current is applied across the electrodes, and the measurements are passed to the controlling computer. After each complete scan of the 128 wells, which takes six minutes, the data are stored on disk. Thus ten data points per hour are collected for each test well.

A simple menu program allows the operator to select the appropriate detection algorithm and the electrical parameter to be measured. The computer program detects the point on the curve where an accelerating change in the electrical parameter occurs. This is called the "detection point" and the period from inoculation to this point is termed the "detection time". This detection time reflects the activity of the culture and the size of the initial inoculum.

Results can be displayed as detection times on the instrument's colour monitor or as hardcopy from a graphics printer. It is also possible to store all collected data on floppy disk for future reference and the complete impedance curves can be displayed on the monitor, or obtained as hardcopy from the printer or digital plotter.

The instrument has been operated throughout the study using the manufacturer's standard software.

4.2 Initial studies using pure cultures

A collection of pure cultures was used to give an initial assessment of the performance of the instrument, the suitability of currently-available growth media and the applicability of the various signals that the machine could measure. Twenty strains (shown in Table 4.1) were used in conjunction with four growth media specifically developed for use with electrometric growth analysers, one standard medium normally used in the membrane filter technique and one modification of this. Although of different composition (Appendix B) these all comprised a nutrient base with bile salts or synthetic surface-active compounds as selective agents. The media are listed in Table 4.2. DSLS medium is modified from 0.1% SLS medium such that the level of selective agent is unchanged, but all nutrients are present at

half-strength. This medium was used in order to check whether the high levels of nutrients present in 0.1% SLS medium were necessary for satisfactory results.

Table 4.1. Cultures used in media trials

Species	Identification
Aeromonas hydrophila	NCIB 9240
Citrobacter freundii	NCIB 3735 NCIB 11490
Citrobacter intermedius	NCIB 4143 NCIB 6071 NCIB 7271
Enterobacter aerogenes	NCIB 10102
Enterobacter cloacae	NCIB 5920
Escherichia coli	NCIB 86 NCIB 8269 NCIB 8879 NCIB 9481 NCIB 9485 NCIB 10243 NCIB 10544 ATCC 13706
Klebsiella pneumoniae	NCIB 418 NCIB 8258 NCIB 8267
Serratia marcescens	NCIB 10644

Table 4.2. Growth media used in electrometry trials

Medium	Reference
0.1% sodium lauryl sulphate (SLS)	DoE et al (1983)
Diluted SLS (DSLS)	
CM	Firstenberg-Eden and Klein (1983)
M20	Hepburn (1984)
Modified M20	Hepburn (1985)
Malthus coliform medium	Malthus Instruments (undated)
Anderson medium	Anderson (1984)

On each occasion that a particular combination of organism and medium was used, the test was carried out three times, a different electrical signal being monitored in each case. Users of electrometric growth analysers have found that advantages may be gained by measuring a particular signal in certain circumstances. The choice of signal is therefore an important parameter to be considered in developing electrometric methodology.

In addition all tests were duplicated and run at two different incubation temperatures, this being possible as the Bactometer instrument has two independent incubators. While it has been suggested that tests can be improved by manipulation of incubation temperatures (Firstenberg-Eden and Eden 1984), it was decided to start, at least, by using the temperatures normally employed in the UK for standard methods, namely 37 and 44 °C.

Cultures of the test organisms were grown overnight in nutrient broth (Oxoid) at 37 °C. They were then diluted 1000-fold, and 10-µl portions were added to 0.5 ml of growth medium in disposable Bactometer culture modules. To avoid alteration of the medium formulations, all dilutions were made using the particular medium under test as the diluent. Because of the high dilutions employed, however, it was not considered necessary to wash and resuspend the cultures, as the carry-over of nutrient broth from the overnight culture was negligible (less than 0.002% of the final test mixture by volume).

4.2.1
Selection of culture medium

The three media which gave the best overall results, in terms of short detection times and good curve quality, were the CM medium developed by Bactomatic Ltd (Firstenberg-Eden and Klein 1983), Sodium lauryl sulphate (SLS) medium (0.1%) (DoE

et al 1983), as used in the membrane filter test for coliform organisms in the UK, and DSLS, a more dilute version of 0.1% SLS. These all gave clear, well-formed curves, with sharp acceleration points which were detected reliably by the instrument's software. Detection times were slightly, but consistently, shorter with 0.1% SLS medium. DSLS medium was roughly comparable with CM.

The medium developed by Malthus Instruments (undated) gave generally satisfactory results, though with longer detection times than any of the three media already mentioned. The Malthus medium also did not contain a pH indicator, so it was impossible to check visually that fermentation of lactose had occurred in any particular culture well. Also reasonably effective was the medium of Anderson (1984), but occasional failures to detect coliform growth were encountered with it, this probably being due to weak overall signal changes.

The least satisfactory medium tested was M20 (Hepburn 1984). This had been developed for use with the Malthus Microbial Growth Analyser and proved incompatible with the Bactometer instrument. M20 medium was later modified by Hepburn (Appendix B), lactose and bromocresol purple being omitted, and 0.4% w/v sodium lauryl sulphate being substituted for Bile Salts No 3. This still gave unreliable results in the Bactometer. Sometimes there was no adverse effect, but on other occasions dramatic and unpredictable signal changes were seen. Further investigation of this showed that the bile salts were responsible for the poor performance of the original formulation of M20 and that the new formulation would work reasonably satisfactorily in the Bactometer instrument if the lactose content was restored. This seems to stabilise the system, without apparently contributing to the electrical signal being measured.

Thus full-strength SLS medium was found to be the most satisfactory overall and other comparative tests later in the study confirmed this. This indicated that for natural samples, the test should be carried out by adding the sample to an equal volume of double-strength SLS medium, so that the final incubation mixture contained the nutrients and selective agents at normal concentration.

4.2.2
Choice of signal to be measured

Reasonable detection times were usually obtained with all three signals (conductance, capacitance and impedance), but conductance curves usually showed the smallest overall signal changes during bacterial growth and also longer detection times than with either of the other signals.

Capacitance curves consistently gave the greatest overall signal change with the growth media used, but were generally "noisier" and less stable. This normally gave no trouble, but occasionally disruption of the baseline before the detection point could give a false detection. This did not occur with any of the media selected as being most promising, so would probably not be a problem in practice.

Impedance curves were generally clear and stable, with detection times roughly similar to the equivalent capacitance curves. For reliable performance, then, the impedance signal is probably the best to measure, though with a suitable choice of growth medium, there is no reason why the capacitance signal should not be used if desired. With the operating parameters of the Bactometer M123, the conductance signal is the least useful for detecting growth of coliform organisms.

4.2.3
Investigation of growth medium components

As noted earlier, one of the changes made to M20 medium by Hepburn (1985), was the removal of lactose from the formulation, as this was considered unnecessary for detection of coliform organisms by electrical methods. It was therefore decided to investigate the most successful medium in the present study, 0.1% SLS, to see if all the components were necessary for its satisfactory use. Media were therefore prepared, all containing sodium lauryl sulphate and phenol red indicator, but with all the other components present singly or in all possible combinations, as follows:

1. Peptone
2. Lactose
3. Yeast extract
4. Peptone and lactose
5. Peptone and yeast extract
6. Lactose and yeast extract
7. Peptone, lactose and yeast extract (complete medium)

When all media were tested with identical samples, only Medium 2 (lactose alone) failed to give any signal change in the Bactometer instrument. Peptone alone gave a generally satisfactory impedance curve, while yeast extract alone gave a biphasic curve with an unstable baseline. The combination of peptone and yeast extract gave a stronger signal change than peptone alone, but with the combinations of peptone and lactose, and yeast extract and lactose, the overall signal changes were similar to when lactose was not present. The curves were smoother and more stable, however. The complete medium gave the shortest detection times, with the most stable and least disrupted impedance curves.

Thus lactose is not essential for the detection of coliform organisms by this technique, but its inclusion is beneficial in stabilising the instrument's response. Also, if lactose is present along with a pH indicator, a simple visual check can be made after incubation that fermenting organisms are present in the culture.

4.3 Tests on environmental samples

Tests using the Bactometer instrument lend themselves particularly well to monitoring of environmental samples, where the levels of pollution measured are higher than in drinking water. For example, the total coliform standard for bathing waters as laid down in the appropriate EC Directive (European Communities 1976) is 10 000 per 100 ml or 100 per ml. Using 1-ml sample volumes, this level of pollution would fall comfortably within the range of sensitivity of the instrument, and tests could be quickly and easily set up using disposable culture modules. No dilution of the sample would be necessary as long as it contained less than about one million organisms per ml.

Samples of river water were tested using three growth media, 0.1% SLS, CM and diluted 0.1% SLS (DSLS). Samples were tested at 37 and 44 °C, and both impedance and capacitance signals were monitored.

The results obtained were in good agreement with previous ones obtained with the Bactometer B32 instrument. One hundred coliform organisms per ml (at 37 °C incubation) were detected in about 7 hours and the calibration curve eventually produced for 0.1% SLS medium (see Section 4.4) indicated that a single organism in a 1-ml sample would be detected in about 10 hours. Detection times were shorter, for the same concentration of organisms,

in tests carried out at 44 °C, presumably because the generation times for the bacteria which could grow at this temperature were proportionately shorter.

A limited number of tests were carried out using marine water. The presence of higher salt concentrations in the marine samples does not seem to affect the operation of the Bactometer instrument, and detection times obtained appear to fit the same calibration curve.

4.3.1
Samples at the limit of detection

As mentioned above, if one coliform organism is present in a mixture being tested by electrometry, a detection would be expected in about 10 hours at 37 °C. However, the situation sometimes arises when a detection time longer than this is seen. This is normally due to slow-growing non-coliform organisms and would therefore be regarded as a negative result. During the development stage of this method, it was considered important to check the contents of the culture wells when the test was completed, to identify the organism or organisms which caused the detection. This was done with wells giving detection times indicative of less than about five coliforms per ml, as even when coliforms are present at such levels, sampling errors may give spurious results. In this way it was hoped to gain information on the specificity of the culture media being used, especially as the usual visual assessment of factors such as colony morphology could not be made using electrometric methods.

4.3.2
Identification of bacteria from electrometric tests

After the electrometric test was over, the contents of appropriate wells were subcultured to a solid medium and incubated overnight. Sometimes it was then necessary to repeat this process to separate

the components of mixed cultures, but in many cases it appeared that one strain predominated, and eliminated others present by competitive selective pressures. Purified cultures were then tested using the API 20E identification system, which provides a battery of 20 separate biochemical tests, and a computerised "profile index" for identification.

A number of cultures were also tested using the "Coli-Fast" test kit (Cherwell Laboratories, Bicester, Oxon). This is a fluorescent assay for the presence of the enzyme Beta-glucuronidase (see Section 5). In the tests carried out using both identification systems, excellent agreement was seen, with cultures showing a positive fluorescent reaction in the "Coli-Fast" test confirming as E. coli by the API 20E tests. Only one discrepancy was seen, with a culture which gave an ambiguous profile in the API test, and could not be identified.

It must be said, however, that the API 20E and "Coli-Fast" tests do not identify bacteria on the same basis as the established water analysis tests, described in Report 71. These require that a culture must ferment lactose with gas production in 24 hours at 44 °C and produce indole from tryptophan in 24 hours, also at 44 °C, to be classified as E. coli. With the phenotypic variation commonly seen within a single species of bacteria, it is inevitable that some strains of E. coli will not display both of these characteristics and will thus fail to confirm by the Report 71 methods. Nevertheless, they are likely to confirm by the API or "Coli-Fast" tests and their sanitary significance is not necessarily reduced by their inability to perform certain biochemical reactions at elevated incubation temperatures.

While no clear picture emerged as to which types of organisms normally gave late detections, the following observations on the available data can be made. If a detection time is longer than 10 hours at 37 °C incubation temperature, then E. coli is unlikely to be present in the sample. On only two occasions was E. coli identified with longer detections (11.5 and 13.5 hours). These isolations were from saline water samples and additional stressing factors may have been present. However, a number of strains of E. coli were isolated from tests incubated at 44 °C with detection times longer than 10 hours, the longest being 16.5 hours. It would appear that the additional stress of incubation at elevated temperatures can adversely affect electrometric coliform tests and it may be that the media used are too selective under these conditions.

At an incubation temperature of 37 °C only five isolations of organisms other than E. coli were made from tests with detection times shorter than 10 hours, and in all these cases the detection times were between 9.3 and 9.9 hours. The indication, then, is that if a detection is made before 10 hours at 37 °C, E. coli is likely to be present in the culture. If the detection occurs after 10 hours, E. coli is unlikely to be present, and between 9 and 10 hours there may be some interference from other organisms.

These results hold only for 1-ml samples incubated in disposable culture modules in 0.1% SLS medium. Different combinations of media and inoculum size would give a slightly different time scale (see Section 4.8). However, the most important observation to be made from these tests is that there is probably little value in carrying out an elevated temperature test (ie at 44 °C) to give an indication of the presence of E. coli. The results

indicate that the presence of E. coli may be inferred from the detection time seen at 37 °C. However, extensive tests with a wide variety of environmental samples would be needed to validate this observation.

4.4 Calibration of the Bactometer system

One use of electrometric instruments is in quantitative estimation of bacterial loading, rather than a simple "presence or absence" test. This requires the system to be "calibrated", by carrying out a number of tests using a conventional microbiological assay as well as the electrometric technique. The following gives examples of such calibration, though as stated later, the scientific basis of such calibrations is questionable.

A large number of suitable results has been obtained, but as a variety of different media and test methods have been used during the study, it has not been possible to compile very large sets of truly comparable data.

Figures 4.1 and 4.2 show two sets, each of 58 samples, using 0.1% SLS medium in the Bactometer M123. All membrane counts were carried out using traditional methodology and 0.1% SLS medium. One set of results is for tests carried out using full strength medium and the other is for diluted medium (ie where the sample is added to its own volume of full strength medium to give a final culture of half strength medium. For the "full strength" test the sample is added to its own volume of double strength medium.) The figures show impedance detection times plotted against counts by membrane filtration, and calculated lines of best fit are drawn through the data points. The correlation coefficients for the two regression analyses are 0.94 and 0.91. These data sets are somewhat unsatisfactory, as nearly all the membrane results

lie between 10 and 1000 organisms per ml. Using such samples alone, a misleading regression line would be obtained, owing to the tendency for the points to tail off at low concentrations (ie detection times are longer than expected). In each case only four points were obtained with very high bacterial levels, and these are particularly important to obtain the correct slope for the regression line (see Firstenberg-Eden and Eden (1984)).

The line equations for the two sets of data were similar, so for interest they were combined to give the plot shown in Figure 4.3. Here the correlation coefficient was 0.92 and the line equation was little altered.

To obtain a more satisfactory distribution of results for the calibration, these data were combined with earlier ones obtained with the same growth medium and experimental conditions, but using a smaller Bactometer instrument. Figure 4.4 shows the plot for all data combined, and the correlation coefficient is now 0.95 for the 258 points. While there is some scatter of points, this plot compares well with calibration curves obtained (and published) for the analysis of foodstuffs - see for example, Fleisher et al (1984), Firstenberg-Eden and Klein (1983), Firstenberg-Eden and Tricarico (1983) and Firstenberg-Eden et al (1984).

The results shown here should be viewed with caution. The plots refer only to 1-ml samples processed in the Bactometer and the membrane results are presumptive counts at an incubation temperature of 37 °C. Different experimental conditions would require different calibrations. Also there is a fundamental problem in deciding what should be correlated with the impedance

detection times. Should it be a presumptive membrane count, or a confirmed count of either coliform organisms or E. coli? In no case is the answer clear, since the two techniques are measuring different things. The membrane count gives an indication of the number of viable bacteria present, while the impedance technique is measuring the overall metabolic activity in the sample, which reflects both the number of organisms present and their individual metabolic rates.

It is frequently possible to correlate parameters which have a systematic variation with concentration, and obtain apparently good results, but in many cases this does not reflect good scientific reasoning.

4.5 Samples with low-level contamination

The electrometric methods described so far have been restricted to sample volumes of 1 ml or less. This is ample for routine monitoring of recreational waters, or other situations where more than one coliform organism per ml of sample is expected, and allows the use of disposable plastic culture modules with the Bactometer M123 instrument. These are convenient and not excessively expensive (25p per sample), but for drinking water or other samples where the presence of low levels of coliform organisms must be measurable, the restricted sample volume is unacceptable.

There are two main ways in which this problem can be approached, the first and more obvious being to incubate larger volumes of water. This can be done with the Bactometer instrument using various sizes and shapes of bottles, each having two stainless steel electrodes passing through the cap or lid. The bottles are placed in a special basket which also contains suitable connectors for the bottle

electrodes. The basket assembly is placed in an accurately controlled air incubator, or submerged in a thermostatted water bath, and a ribbon cable with suitable termination is used to connect the remote basket to the Bactometer processing unit. The largest bottles available hold about 250 ml, allowing a 100-ml sample to be added to 100 ml of double-strength medium, to give the same inoculation mixture as would be used in a disposable module, but of 100 times greater volume. However, in practice it was found that double strength medium offered no advantages using these larger electrodes, so for economy, single strength medium is recommended, but with an added 1 g/l of sodium lauryl sulphate. Addition of 100 ml of sample to an equal volume of this medium thus gives a culture containing SLS at 0.1% w/v, but with nutrients at half-strength compared with normal 0.1% SLS medium.

Parallel tests indicate that detection times are slightly longer (by 0.5 to 1 hour) using bottles than when the same sample is treated similarly in a module. This is not unexpected, as during development of the electrometric technique it was found that detection times were shorter if the electrodes were fixed in the bottom of the test vessel, as with the disposable modules, than if they were suspended from the top of the vessel, as with the bottle electrodes (Firstenberg-Eden and Eden 1984). Also it takes rather longer for the signal to stabilise after inoculation, due to the larger volume which must be brought to a stable incubation temperature. This would only cause problems with very highly contaminated samples, where the detection would fall within the stabilisation period of the first 1 or 2 hours. Incubation of the bottle baskets in water baths rather than air incubators would reduce this stabilisation time, due to the more rapid thermal transfer.

Apart from these observations, signals from bottle electrodes appear to give stable curves and reliable detections. The main disadvantages are the larger volumes of medium needed, the high capital cost of cables, baskets, bottles and electrodes (the electrodes have an expected life of 20-30 uses), and the inconvenience of sterilising, washing and recycling the electrodes themselves.

4.6 Pre-incubation of samples

An alternative approach for samples containing low levels of micro-organisms is to use a pre-incubation stage, followed by transfer of a small subsample of this culture to a disposable module. This has been tried using two non-selective pre-incubation media known to promote recovery and rapid growth of even fastidious organisms (brain-heart infusion and tryptone-soya broth).

A measured volume of water sample is added to its own volume of double-strength medium and incubated at 37 °C. Initially a 3-hour period was used to allow cultures to be inoculated during a good proportion of the working day. Small volumes (1 ml) of these cultures are then added to 1 ml of selective growth medium in a disposable module and placed in the Bactometer instrument as usual.

Ideally the technique must allow the growth of a single coliform organism in a 100-ml sample, to such a density that a 1-ml subsample would contain enough organisms (preferably at least 10) to give a reliable detection in the electrometric instrument. When reference was made to the calibration curves obtained for 0.1% SLS medium, it was realised that a 3-hour incubation period would be inadequate. On theoretical grounds, with a generation time of 25 minutes, it would take 4 hours 10 minutes to ensure adequate growth of a single organism in 100 ml, to

give ten organisms per ml for subculture. In practice, however, one cannot rely on this growth rate and it is likely that a lag phase of non-replication will occur. A 4-hour incubation therefore proved inadequate. Better results were obtained using a 6-hour period, but this reduces the practicality of the method for the average laboratory operating a normal working day.

This aspect of the technique was therefore not investigated in depth. However, tests showed that tryptone soya broth was the more effective enrichment medium, and interesting results were obtained when the pre-incubation mixture was subcultured to two Bactometer wells, one incubated at 37 °C and the other at 44 °C. The detection at 37 °C gives an indication of the loading of coliform organisms or other related types which are bile-tolerant ("enteroforms"), while a detection at 44 °C indicates that thermotolerant types such as E. coli are present. A number of tests emerged with positive detections at 37 °C but none at 44 °C, indicating the presence of coliform organisms (or "enteroforms"), but the absence of E. coli.

4.7 Use of the Malthus Microbial Growth Analyser

As mentioned earlier, growth media developed for the Malthus instrument proved unsatisfactory in the Bactometer. It was thus likely that the methods developed during this study would not suit the Malthus system. As the UK water industry had shown particular interest in the Malthus instrument (because of its ability to handle large sample volumes), and Wessex Water had already obtained a system, it was considered important that the present study should include work on the Malthus machine. After negotiation with Malthus Instruments, an ex-demonstration system was obtained for a six-month hire period.

4.7.1
Description of the Malthus instrument

The Malthus system used during this study had one incubator to take 28 100-ml bottle electrodes and one to take 128 samples in electrode bottles with capacities of 2 or 10 ml. The system could be expanded by adding extra incubators, up to a maximum of 256 samples. The incubators consisted of thermostatic water baths with the necessary wiring and connectors for the sample electrodes. Very fine temperature control was said to be needed for stable signals to be obtained and this was achieved using low-wattage heaters. The electrodes consisted of ceramic fingers with two thin strips of platinum deposited on the surface. This provided adequate electrode surface area using the minimum amount of metal, but meant that the electrodes had to be handled with considerable care to avoid breakages.

Data storage, handling and display were carried out by a desk-top microcomputer with graphics printer, and this communicated with an "Intelligent Control System" (ICS), which was the central part of the system. The ICS was a second processor which constantly collected measurements from the incubators and stored them in its memory. It was interrogated at intervals by the computer, and passed the data to it for permanent storage on floppy disk. The advantage of this system was that the computer could be taken off-line and used for other purposes, such as statistical analysis of data or reviewing earlier results, while data collection was in progress. As long as the computer was placed back on-line before the ICS's memory was full (in practice about 12 hours' data could be held), no information was lost and a continuous stream of readings was obtained. The measuring systems were protected against mains failure by an uninterruptable power supply.

The system used a driving frequency of 10 KHz and conductance was the only parameter which could be measured. Various aspects of the detection routine could be changed by the operator and program software was provided to allow different routines to be applied to previously collected data, to see which best suited the conductance curves obtained. The standard water-testing software allowed two lengths of test to be used, 18 hours duration with each sample measured every 6 minutes, or 36 hours duration with 12-minute test intervals. Other test periods would be possible with alternative program disks from the manufacturer.

4.7.2
Initial trials and comparison with the Bactometer M123

The output from the Malthus instrument was similar to that of the Bactometer, in that "detection times" were displayed for each channel, and the data from a completed test could be stored, and displayed as a curve on the screen or printer. Certain differences existed between the two systems, but essentially the information which could be obtained from both was the same. However, the operating parameters of the two electrical monitoring systems had considerable differences, which meant that methods for one machine could not readily be transferred to the other.

Earlier work (Section 4.2.1) had shown that the M20 medium originally developed by Wessex Water for the Malthus system gave erratic results in the Bactometer instrument, and that a later modification of the medium, though slightly better, was still unreliable. The modified medium was again tried in comparative trials of the two machines and the previous findings were confirmed. However, Malthus Instruments also supply a coliform growth medium for use in their machines, which is basically the same as the modified M20 medium, but contains no yeast extract and has 0.5 g of lactose

added per litre. This gave better results in the Bactometer than did the modified M20 medium, although the curves displayed a sharp downward turn, following the initial accelerating upward change. Although this disruption of the curve is undesirable, it did not appear to interfere with detection and the curves were more stable than those obtained with M20 medium. Detection times were generally similar to those obtained with 0.1% SLS medium, although agreement between replicate tests appeared less consistent.

Both the Malthus coliform medium and modified M20 medium gave similar results when used in the Malthus instrument, but here 0.1% SLS medium gave problems. When conductance curves could be obtained, they were reasonably satisfactory, with clear acceleration points, but frequently the instrument indicated that the test mixture was outside the acceptable conductance range and would not accept it as a valid sample. Investigation showed that 0.1% SLS medium is not sufficiently conductive to fall within the instrument's range with some samples, a problem which did not occur with the Bactometer because of the different operating parameters. Adding a suitable amount of sodium chloride to the medium removed this problem, but the medium is basically unsuited to tests where conductance is measured. Dilute media with comparatively low nutrient content (such as M20) are more effective in such cases.

Thus existing media developed for one machine cannot reliably be used in the other and it is doubtful whether a universal medium could be formulated which would give optimum results on both systems.

4.8 Collaborative trials using samples from Wessex Water

Several comparative trials have been carried out using duplicate samples from Wessex Water. The samples, from various parts of the Wessex region, were collected and transported to WRc Medmenham and to the Wessex Regional Scientific Centre at Saltford near Bristol. At an agreed time they were then tested at both locations by the conventional membrane filter technique, and also using electrometric instruments, the Malthus instrument at Saltford and the Malthus and Bactometer instruments at WRc.

The samples examined were of generally good quality water, ranging from no detectable coliforms to several hundred per 100 ml. Tests with the Malthus instrument used the latest modification of M20 medium (supplied by Wessex Water), with incubation at 37 °C. One hundred-ml samples were added to bottle electrodes containing 10 ml of 10x strength medium. In the Bactometer tests, 100-ml samples were added to 100 ml of SLS medium as described earlier, in 250-ml electrode bottles. These were incubated remotely from the analyser in a forced-air convection incubator. Samples were also analysed using the conventional membrane filter method with 0.1% SLS medium and final incubation at 37 and 44 °C. Confirmation of the identity of colonies was carried out when necessary, tests being the fermentation of lactose at 37 and 44 °C, and the production of indole from tryptophan at 44 °C. Isolates from the electrometric tests were also confirmed by the same scheme and cultures which did not confirm by the conventional tests were identified by the API 20E system.

The results were examined to see if it was feasible to predict the presence of E. coli in a sample from the detection time observed. For the Bactometer, only one sample out of 16 where E. coli proved to

be present had a detection time longer than 12 hours. Conversely, of 17 tests which detected, but where E. coli was absent, only one had a detection time less than 12 hours. For the Malthus machine, three out of 24 E. coli positive tests had detections longer than 12 hours, and four out of 22 where E. coli was absent had detection times of less than 12 hours. Thus for these samples, a detection time of less than 12 hours appears to give a good indication of the presence of E. coli, and a detection time longer than 12 hours suggests its absence.

Using this value, an analysis was made of the respective results of the electrometric tests and the membrane results from the 44 °C incubation tests (ie presumptive and confirmed E. coli tests). The findings are shown in Tables 4.3 and 4.4. Two separate tables are given, as the data are not strictly comparable for the two electrometric instruments. This is because the number of observations is greater for the Malthus machine than for the Bactometer, owing to more replication during two of the trials. Thus there is a total of 48 results for the Bactometer and 64 for the Malthus instrument. A positive result using an electrometric test means a detection time of less than or equal to 12 hours, a negative one meaning longer than 12 hours. A positive membrane test means that the presence of E. coli was confirmed, while a presumptive positive means that at least one yellow colony was seen on the membrane, but did not confirm as E. coli.

Combining Rows 1, 3, 5 and 6 from the tables shows that 47% of the samples tested with the Malthus instrument gave presumptive positive membrane results at the 44 °C incubation temperature and 50% of the Bactometer samples. The Malthus machine itself gave 39% positive results (Rows 1+4+6) and

the Bactometer 50%. There was some disagreement between the two methods. Rows 3+5 give the percentage of the total samples which showed at least a presumptive positive membrane result, but which were missed by the electrometric technique: 16% for the Malthus and 6% for the Bactometer. Conversely Row 4 gives the samples where the electrometric technique gave a positive detection, but nothing was isolated on the membrane: 8% for the Malthus and 6% for the Bactometer samples.

Table 4.3. Analysis of Malthus and membrane results

1.	Confirmed +ve on membrane, electrical test +ve	31%
2.	Negative by both tests	45%
3.	Presumptive +ve on membrane, electrical test -ve	11%
4.	Membrane test -ve, electrical test +ve	8%
5.	Confirmed +ve on membrane, electrical test -ve	5%
6.	Presumptive +ve on membrane, electrical test +ve	0%
		100%

Table 4.4. Analysis of Bactometer and membrane results

1.	Confirmed +ve on membrane, electrical test +ve	40%
2.	Negative by both tests	44%
3.	Presumptive +ve on membrane, electrical test -ve	4%
4.	Membrane test -ve, electrical test +ve	6%
5.	Confirmed +ve on membrane, electrical test -ve	2%
6.	Presumptive +ve on membrane, electrical test +ve	4%
		100%

Such discrepancies are inevitable with samples which are at the limit of detection by both techniques, but overall the Bactometer gave the same number of positives as the membrane method, while the Malthus gave 8% fewer (difference = (3+5)-4). The Malthus results to some extent reflect problems which were seen with some of the electrodes (see next section) and it may be that new ones would have given better results.

Thus it would appear feasible to use electrometric instruments for testing drinking water for faecal indicator organisms, but extensive comparisons with conventional methods under operational conditions would be needed before unequivocal recommendations could be made. A further problem is that bottle electrodes are no longer being supplied by Bactomatic Ltd. While it might be possible to have alternative items made, it means that at the moment only Malthus can supply an "off the shelf" system capable of handling 100-ml sample volumes, while the experience of this study has tended to favour the Bactometer for versatility, ease of use and quality of results.

4.9 Reliability of electrometric instruments

The ability to process large numbers of samples is no advantage if the instrument carrying out the analysis is not reliable. Indeed the potential amount of information which could be lost due to a failure is very large. Peripheral devices such as printers may not be essential to the running of the system, but the analyser itself and the controlling computer must be fully functional.

The Bactometer M123 instrument suffered the failure of both disk drives shortly after delivery. This was possibly due to incorrect wiring during manufacture. The drives were replaced, and the system has run for the balance of the 2-year contract period with only routine servicing maintenance.

The Malthus analyser suffered failure of one disk drive three months after installation. This was replaced, but failed again at the end of the 6-month hire period. In its defence, it must be said that the system was not new, but an ex-demonstration machine.

The original disposable modules provided by Bactomatic Ltd occasionally had slight moulding imperfections which could cause one or two of the 16 wells to make poor contact with the measuring circuit, but this seems to have been cured. The reusable bottle electrodes have given excellent results. With the Malthus instrument some of the bottle electrodes have been unsatisfactory, but again these were not new when delivered. Unstable baselines were sometimes seen, and some electrodes gave increasing linear signal changes which could mask detections. Differences in electrode performance were sometimes seen when tests were duplicated, with drifting baselines and differences in the overall signal changes.

4.10 Conclusions

The impedance method has potential for testing environmental and recreational water samples for coliform organisms, where a 1-ml sample volume is sufficient. Large numbers of tests may be set up quite quickly, although overall test time would still extend beyond a normal working day unless pollution levels in the samples were very high. The theoretical sensitivity of the method is one organism per ml, and this would correspond to a detection time of about 10 hours. Quantitative results can be obtained after suitable calibration, but there will be considerable scatter on the calibration curve at densities below about ten organisms per ml, due to sampling errors and late detections by interfering organisms.

There may also be potential for testing drinking water, but it would be more difficult to obtain quantitative information at this extreme limit of the instruments' sensitivity. There would be little saving in time over conventional methods, but automated data collection and handling would be available and one machine could perform a large number of tests.

The choice between the two available machines will depend largely on the intended use. For small-volume samples up to 1 ml, the Bactometer offers greater ease of use, and the advantage and convenience of disposable culture vessels. For larger volumes of sample up to 100 ml, only Malthus can currently offer a suitable system, although the performance of the Bactometer with such samples, using the bottle electrodes which are no longer available, was equally good, if not slightly better.

Growth media which give the best results with one machine have been found to be incompatible with the other. Thus a single method to cover tests using either instrument cannot be given. With the Bactometer, 0.1% SLS medium has proved to be the most satisfactory. Using disposable modules, 1 ml of sample is added to an equal volume of double-strength medium, while for large volume tests, 100 ml of sample is added to 100 ml of single strength medium containing an additional 1 g/l of selective agent as described earlier. For tests with the Malthus instrument, the coliform medium supplied by Malthus Instruments, or the M20 medium developed by Wessex Water may be used. For drinking water samples, a 100-ml volume of water is added to 10 ml of medium prepared at 10 times normal strength.

5. BETA-GLUCURONIDASE TEST

5.1 Introduction

The tests outlined in Report 71 (DoE et al 1983) for identifying E. coli are the production at 44 °C, of indole from tryptophan and acid and gas from lactose (AGI tests), after a 24 hour incubation. They are both time consuming and laborious and have also been reported to be inadequate, especially with regards to stressed

cells (Evans et al 1981). Other criteria, such as anaerogenic and non-lactose fermenting E. coli strains and lactose fermenting non-coliforms further contribute to the inefficiency of these tests. The use of enzymic tests to identify bacteria is an attractive alternative to existing methods due to their speed, sensitivity and specificity.

The enzyme, Beta-glucuronidase (Beta-D-glucuronide glucuronosohydrolase, EC 3.2.1.31), has been found in various species of bacteria including corynebacteria, streptococci and staphylococci, but among the Vibrionaceae and Enterobacteriaceae its presence is exclusive to E. coli and some Shigella and Salmonella strains. The high frequency of this characteristic among E. coli strains (94%-97%) indicates that the Beta-glucuronidase (BG) test has a valuable potential for simplifying the differentiation of E. coli from other Enterobacteriaceae. The false identification of the occasional Beta-glucuronidase-positive/ lactose-fermenting Salmonella or Shigella strains as E. coli would still be of advantage in water quality monitoring.

This study was undertaken to compare the Beta-glucuronidase test with the conventional methods for confirming the presence of E. coli, to evaluate the use of some of the commercially available Beta-glucuronidase diagnostic kits and to develop, if necessary, new, Beta-glucuronidase assay techniques.

5.2 Studies on commercially available test kits

The enzyme, Beta-D-glucuronidase, hydrolyses conjugated glucuronides by the equation:

Beta-D-glucuronide → Alcohol + D-glucuronic acid

It can be readily identified in bacteria by the use of commercially available chromogenic or fluorogenic synthetic substrates. These substrates are based on natural Beta-D-glucopyranosiduronates and comprise a Beta-D-glucuronide attached to a chromogenic or fluorogenic alcohol. The two main alcohols used for the Beta-glucuronidase test are 4-nitrophenol and 4-methylumbelliferol. When hydrolysed 4-nitrophenyl-Beta-D-glucuronide (NG) releases the yellow pigment 4-nitrophenol whereas 4-methylumbelliferyl-Beta-D-glucuronide (MUG) releases 4-methylumbelliferol which fluoresces under long-wave UV light.

The test kits studied are diagnostic tablets produced by A/S Rosco of Denmark (Lab-M Manchester), Coli-Fast kits manufactured by PBI International in Italy (Cherwell Laboratories, Oxon), and Coli-MUG tubes from Hach, Colorado, USA (Camlab Ltd, Cambridge).

5.2.1
Rosco diagnostic tablets

The Rosco tablets contain the substrate 4-nitrophenyl-Beta-D-glucopyranosiduronic acid and a large amount of a white, inert powder. Beta-glucuronidase hydrolyses this substrate to release the yellow nitrophenol which is easily observed against the white suspension formed by the inert, bulking agent.

The test is performed in a tube by preparing a dense milky suspension (McFarland 5 or 6) in 0.25 ml of saline from a fresh overnight culture of the organism to be tested. One reagent tablet is added and the tube is agitated vigorously. The test culture is then incubated at 35 or 37 °C for 4 h. The development of a yellow colour is indicative of a positive reaction.

5.2.2

Coli-Fast reagent

Coli-Fast is a fluorescent assay which is sold in kits comprising bottles of substrate, buffered medium and intensifier solution. The substrate is probably 4-methylumbelliferyl-Beta-D-glucuronide, as a positive reaction to this test is shown by the presence of fluorescence visible under long-wave (365 nm), UV radiation. The manufacturer's instructions detail two ways in which the test can be performed.

The first uses the cultures produced by the coliform, multiple tube procedure. In this test 0.5 ml of broth culture from positive tubes is transferred to clean glass test tubes. Two drops of the reconstituted substrate and 0.5 ml of buffered medium are added. The contents of the tubes are mixed and incubated for 4-6 h at 37 °C. The tubes are then exposed to long-wave UV radiation. Beta-glucuronidase positive cultures fluoresce bright blue whereas negative tubes show no fluorescence.

In the second procedure the colonies that have developed on membrane filters incubated on media selective for coliform organisms are used. The membrane filter bearing coliform colonies is transferred to an absorbent pad previously soaked with the buffered substrate solution. This is incubated at 37 °C for 4-6 h. The filter is then exposed to UV and the fluorescent colonies counted. If the results are doubtful, or negative, the filter is flooded with 1 ml of intensifier solution. After 10 min the membrane is again exposed to UV radiation and the blue fluorescent colonies counted. The identity of the intensifier solution is unknown.

5.2.3

Hach Coli-MUG tubes

The Hach Coli-MUG method has been developed for the semiquantitative determination of total presumptive coliform organisms and specific presence of E. coli in 10-ml water samples. The method is basically a presence or absence test although if sufficient tubes were inoculated they could be used for a Most Probable Number (MPN) determination. However the cost of such a method would almost certainly prohibit its routine use for analysis of large numbers of samples.

The Hach Coli-MUG tubes are ready-to-use screw-capped fermentation vials containing lauryl tryptose broth and MUG reagent. After addition of the 10-ml water sample to one of these tubes, and a 24 hour incubation at about 35 °C, the presumptive presence of coliform organisms in the sample is shown by the collection of gas in a Durham tube and cloudiness in the broth. The presence of fluorescence in the tube when exposed to UV light, after the same incubation, indicates the presence of E. coli. As with the standard MPN technique a further incubation of up to 24 hours may be necessary in some cases.

5.3

Evaluation of the Beta-glucuronidase test as a confirmation procedure for E. coli

In the following tests, strains of E. coli were isolated from different sources, including rivers, reservoirs and the sea.

5.3.1

Method

The standard membrane filtration procedure for isolating faecal coliform organisms, using 0.1% lauryl sulphate membrane broth (0.1% SLS) was employed for obtaining the strains of E. coli from the water samples (DoE et al 1983).

All colonies picked off the membranes were streaked onto membrane lauryl sulphate agar and incubated at 44 °C for 24 hours, to check purity and provide an inoculum for further tests. These cultures were checked for purity and then coded. If the isolates were not pure, the different colonies present were subcultured and each given a new code number. When purity was assured the cultures were tested by the conventional E. coli confirmation procedures of acid and gas production from lactose and indole production from tryptophan (AGI tests), and also the Rosco and Coli-Fast Beta-glucuronidase tests. Any strains that could be identified as E. coli by either the conventional or Beta-glucuronidase methods, but not both, were identified using API 20E identification kits. Furthermore the purity of these cultures was checked again by subculture from the lactose fermentation test to a plate of SLS agar.

Strains which were identified as E. coli by the API 20E system, were tested again by the AGI and BG techniques. However, since BG tests were reported to work more quickly with freshly grown cultures, these were prepared on SLS agar to provide a suitable inoculum. As an additional precaution all BG tests were incubated for up to 24 hours. Any strains that did not produce detectable BG activity within this time were classed as BG negative.

Using these results a comparison could be made between the different E. coli confirmation procedures and the relative merits of the two types of BG test assessed. The proportion of E. coli that did not conform to all of the identification procedures could also be determined.

5.3.2
Results

In total 558 E. coli cultures have been investigated for their possession of

Beta-glucuronidase activity and their ability to produce indole from tryptophan and acid and gas from lactose. The results obtained are shown in Table 5.1 and summarised in Table 5.2.

It should be stressed at this point that the way this study was carried out precluded false detection of E. coli by the BG test alone, ie the only false positives would have to be BG +ve, acid and gas +ve, and indole +ve. The relevance of this is that if any false positives were unknowingly encountered, it would still not affect a comparison between the two procedures.

From Table 5.2 it can be seen that over 97% of the total strains studied were BG positive after a 24 hour incubation, as compared with 91.2% of the strains that displayed the conventional test properties for E. coli after the same incubation period. In addition even though the 4 hour Beta-glucuronidase test did yield some false negative reactions (2.5%), it still identified a larger percentage (92.1%) of the total E. coli than did the conventional tests after 24 hours.

5.3.3 Discussion and conclusions

It can be concluded from these results that both the negative strains and those strains positive only after 24 hours for Beta-glucuronidase make up a smaller percentage of the total E. coli (7.9%) than do the strains that do not display the conventional test properties (8.7%).

It should also be mentioned that on three occasions, strains of Klebsiella oxytoca were isolated and identified. They produced both acid and gas from lactose and indole from tryptophan. However, they did not produce Beta-glucuronidase activity and in this respect the Beta-glucuronidase test appears to better than conventional methods as

Table 5.1. Results of comparing confirmation methods for *E. coli*

Characteristics of *E. coli*	Numbers obtained	Percentage of total
BG +VE Acid +ve Gas +ve Indole +ve	465	83.3
BG WEAKLY +VE Acid +ve Gas +ve Indole +ve	30	5.4
BG -VE Acid +ve Gas +ve Indole +ve	14	2.5
INDOLE -VE BG +ve Acid +ve Gas +ve	9	1.6
GAS -VE BG +ve Acid +ve Indole +ve	36	6.5
ACID -VE Gas -ve BG +ve Indole +ve	4	0.7

BG = Beta-glucuronidase

Table 5.2. Summary of results of comparing confirmation methods for *E. coli*

Identification procedure	Nos positive (%)* after incubation 4 hr		24 hr	
	Nos	%	Nos	%
Conventional	---	----	509	91.2
Alternative (BG method)	514	92.1	544	97.5

* The percentages shown are those of the total number of isolates identified as *E. coli* using either API 20E, or by both positive Beta-glucuronidase and conventional AGI tests

no false positives, ie strains that were not E. coli but were Beta-glucuronidase positive were obtained.

Despite the fact that reliance on a single characteristic for bacterial identification is usually a hazardous principle, the results have shown that the Beta-glucuronidase test gives fewer misidentifications than the conventional tests for E. coli. Also Beta-glucuronidase tests are less time-consuming, less laborious and cheaper than the conventional tests recommended in Report 71 (DoE et al 1983) for the confirmation of the presence of E. coli. There are considerable savings that could be made, not only in time that would normally be spent preparing and sterilising media, but also in the time spent incubating the tests. Given these facts, the Beta-glucuronidase test does appear to be at least a supplement, and possibly a successor, to the already accepted tests for identifying E. coli. However much more extensive and intensive work needs to be performed first.

5.4 Comparison of Coli-Fast and Rosco tablets

After working with both Coli-Fast and Rosco Beta-glucuronidase tablets, a number of comparisons can be drawn between the two. To be of any use, any Beta-glucuronidase diagnostic test must be rapid, accurate, simple to perform and inexpensive. Both tests appear to detect E. coli with equal rapidity. However, it is easier to determine the result of a test with the Rosco tablets than with the Coli-Fast. This is because it is easier to detect a slight change of colour than to determine a similar change in the intensity of fluorescence of a culture. Also Beta-glucuronidase negative Coli-Fast cultures may fluoresce slightly and glass test tubes show various degrees of natural fluorescence. In contrast, any formation of a yellow colouration with the Rosco tablets is easily

detectable due to the presence of the tablet's inert white bulking agent. The Rosco test is slightly simpler to perform as it does not require the mixing of any solutions, nor does it need a UV lamp.

In terms of cost, the Rosco tablets are more appealing than the Coli-Fast. A bottle of 25 Rosco tablets costs £2.90; one test will, therefore, cost just under 12p. The Coli-Fast reagent kits for 80 fluorogenic assays costs £61.65, making the cost of one assay about 77p (1987 prices).

5.5 The integration of a Beta-glucuronidase test and a standard membrane filtration coliform count

In many of the old techniques and media developed recently for identifying E. coli rapidly, the Beta-glucuronidase test has been used as a secondary identification or confirmation procedure. Very little interest seems to have been shown in using this test as a diagnostic feature in isolation media in the UK. The possibility of integrating a Beta-glucuronidase test with the standard coliform medium was therefore investigated.

In the context of water quality monitoring this is extremely attractive, since it would allow a confirmed E. coli count to be obtained at the same time as the presumptive total or thermotolerant coliforms counts, after only an 18-hour incubation period. The lengthy secondary confirmation procedures for E. coli could be avoided and allow remedial action to be taken with greater conviction.

The possibility of incorporating either MUG or NG into the recommended membrane filtration coliform medium has therefore been studied. The medium used throughout the study was 0.1% lauryl sulphate membrane broth (Oxoid).

5.5.1
Tests using 4-methylumbelliferyl-Beta-D-glucuronide

MUG was incorporated into the SLS broth at a concentration of 100 mg/l before the medium was dispensed onto sterile incubating pads. Following filtration, the membranes were incubated on the pads for 4 hours at 30 °C followed by 14 hours at 44 °C. After incubation the presumptive thermotolerant coliform organisms were identified by the usual colony characteristics based on acid production from lactose (ie by yellow colouration), and the presence of E. coli confirmed by exposing the membrane to long wavelength UV. E. coli colonies could be readily distinguished by the appearance of fluorescence or fluorescent haloes around the colonies.

With this method, the second incubation of 4 hours at 37 °C associated with the Coli-Fast fluorescent membrane filter test and observed colony mixing and spreading which may result from this could be omitted, the mixing of colonies causing difficulty in relating Beta-glucuronidase activity to individual colonies.

Thus by this MUG membrane filter Beta-glucuronidase test, a thermotolerant coliform count (based on acid production from lactose at 44 °C) and an E. coli count (based on fluorescence) could be made on the same membrane, but more importantly at the same time.

5.5.2
Tests using 4-nitrophenyl-Beta-D-glucuronide

Preliminary studies showed that the incorporation of nitrophenyl glucuronide into existing coliform media would not be as straightforward as it had been with 4-methylumbelliferyl-Beta-D-glucuronide. Difficulties arose because acid is produced during growth of coliform organisms on media containing lactose, such as 0.1% SLS. This turns the phenol red in the medium from red to yellow and gives

coliform colonies their diagnostic colouration. Although 4-nitrophenol is yellow above pH 7.0, at acid pH it is colourless. As a consequence even in the absence of any colour masking by the phenol red indicator, the NG would not be detectable in the acid conditions prevailing at the end of a coliform test.

Since the purpose of the study was merely to augment the recommended coliform procedure without radically changing it, omission of lactose and phenol red was rejected as a possible solution. Instead a means of increasing the pH of the medium after development and counting of the yellow coliform colonies was considered. If this could be achieved, the appearance of colonies would be influenced at alkaline pH by a combination of both the yellow 4-nitrophenol and the deep red phenol red. Hopefully this would give distinguishable brown colonies.

Experimentally, this method has proved very successful. In the procedure NG is added to the SLS medium prior to its sterilisation. The studies have shown that the optimum concentration of NG in SLS is between 0.4 and 0.6 g/l. At concentrations below this colour difference between BG positive and negative colonies is poor and at higher concentrations a haze of the substrate product around each positive colony causes difficulty in relating Beta-glucuronidase activity to individual colonies and colony growth seems inhibited.

After incubation of the membranes, a standard coliform count is made based on the production of acid from lactose, before the development of the Beta-glucuronidase test. In order to examine the existence of any free 4-nitrophenol that may exist in the colonies, due to the enzymatic hydrolysis of the NG by Beta-glucuronidase, the pH of the

colonies has to be raised above 8.4. To do this the membrane filter and incubating pad together are placed aseptically in a 90 mm petri dish, into which are placed a few drops of ammonia solution (sg 0.88). The membrane is kept with its incubating pad to prevent the former drying out. The ammonia should be put at the edge of the dish so that only the vapour comes into contact with the colonies on the membrane. The petri dish lid must be replaced immediately after its addition.

The membrane is ready to examine as soon as the pH shift has caused the necessary colour changes in the colonies. In practice this takes about 30 seconds. The membrane turns red as do the BG -ve colonies and the presumptive E. coli (ie Beta-glucuronidase +ve colonies) turn a distinctive golden brown colour.

5.5.3
Comparison of MUG and NG "combined coliform count and Beta-glucuronidase test"

It is apparent from these studies that both these substrates can be incorporated into membrane lauryl sulphate broth (SLS) to provide a rapid, single step procedure for confirming the presence of E. coli. Presumptive coliform organisms can be identified by the usual colony characteristics and the presence of E. coli confirmed by exposing the membrane to either UV when using MUG or to ammonia vapour when using NG. By both methods, Beta-glucuronidase positive and negative colonies can be clearly distinguished from each other.

In any Beta-glucuronidase test, difficulties may arise in distinguishing weakly positive from negative strains. However the numbers of false negative E. coli encountered by the Beta-glucuronidase method does not appear to detract from the value of this test when compared with the numbers of false identifications encountered with the conventional methods for

identifying E. coli. Any colonies that are not clearly positive or negative should be subcultured and examined further.

Despite the problems associated with changing the pH of the colonies on a membrane filter, it has become apparent that NG is a better and more practical substrate than MUG for use in such a Beta-glucuronidase test. This substrate gives a more defined and easily recognisable colour difference between Beta-glucuronidase positives and negatives, which is particularly useful when trying to identify weakly positive colonies. NG is better than MUG in this respect as it is easier to determine a colour change than it is to determine a similar change in the intensity of fluorescence of a colony. The complications of working with or from a membrane which has to be viewed under an UV lamp must also be considered when using MUG. However MUG is more versatile than NG for use in different media as it does not interfere with any indicators that may be present.

Another consideration is the comparison in cost between the two substrates. Prices for NG vary between £38.30 per gram (BDH) and £73.90 (Sigma), whereas MUG costs £142.80 per gram (Sigma). At these prices, sufficient NG to make one litre of medium would cost between £19.15 and £36.95. As MUG is used at a lower concentration, its cost for one litre of medium is £14.28. A litre of medium is enough to incubate between 400 and 500 membranes.

Neither substrate poses any problems as regards its storage requirements. Both must be kept at below 4 °C and NG must also be kept in the dark.

5.6 Conclusions

It has been shown in this study that despite relying on a single characteristic, the Beta-glucuronidase test is a worthy alternative to the conventional methods (AGI tests) for identifying E. coli. Over 97% of the strains studied were BG positive as compared with the 91% that demonstrated the conventional test results after the same incubation time. More than 92% of the strains demonstrated BG activity after only 4 hours.

Beta-glucuronidase assays have also been proven to be applicable to routine coliform analyses, in particular the coliform count by membrane filtration. By incorporating MUG or NG into SLS medium, the ability to detect E. coli is improved with considerable savings in time, media and labour. Although NG appears to be the better substrate for use with SLS in membrane filtration methods, MUG is more versatile and can be easily used in many types of media, ie MPN determinations and presence/absence tests.

It had been proposed to study the incorporation of NG in other coliform media and also the use of a third substrate phenolphthalein glucuronide. However only preliminary trials could be performed before the contract period expired.

6. SAMPLE STORAGE AND PRESERVATION

The storage of samples before analysis has been a subject of concern for many years, and one which has not been satisfactorily resolved. Various effects of time and temperature of storage on the concentration of faecal indicators and total bacterial content of samples have been reported. The magnitude of such effects was dependent not only on the bacteria studied, but also on the type

of water sampled (river, potable, marine). A bibliography of relevant reports is given in Appendix C.

The study of this subject under the terms of the contract was delayed to allow suitable development of the direct-count and ATP determination techniques. When these were available, it was decided to start work on the effects of storage using water known to contain coliform bacteria and have relatively high 22 °C standard plate counts. River water was used as it would possess these characteristics and contain enough organic material to support the growth of plate count organisms. This would allow the growth potential of waters in storage to be assessed and any possible antagonistic effects of high plate count populations on recovery of coliform bacteria to be observed.

6.1 Method

Two 5-litre samples of River Thames water were collected in sterile glass bottles. The samples were returned to the laboratory immediately and analysed for the following: total and thermotolerant coliform organisms, 22 °C plate count, ATP content and direct count by epifluorescence microscopy, using the fluorochrome DAPI (see Section 2).

Subsequently, one of the samples was stored at 4 °C and the other at 20 °C, both in the dark. These mixtures were subsampled six times during the subsequent 52-hour storage period. All determinations were carried out in triplicate.

6.2 Results

The results obtained from two storage experiments of this type are shown in Tables 6.1 and 6.2.

Table 6.1 Results from first river water storage equipment

Storage period	Bound ATP ng/l		Free ATP ng/l		Plate count 10^4/ml		Direct microscopic count 10^6/ml		Coliform organisms /100 ml		Thermotolerant coliform organisms /100 ml	
(hours)	4 °C	20 °C	4 °C	20 °C	4 °C	20 °C	4 °C	20 °C	4 °C	20 °C	4 °C	20 °C
0	182	165	7	12	1.26	1.65	1.05	1.47	23 000	22 900	1 800	2 000
4	196	211	21	17	0.80	0.46	1.29	1.88	13 000	17 700	1 700	1 800
23	197	195	12	13	2.73	5.66	1.45	1.86	20 300	22 500	1 300	1 200
28	137	153	18	62	2.96	9.56	1.45	2.19	11 600	17 000	1 400	1 200
47	192	149	10	72	9.50	2.09	1.25	1.94	19 300	11 100	1 300	400
52	123	147	21	76	2.12	2.12	1.05	1.54	14 300	7 100	1 100	200

Table 6.2 Results from second river water storage equipment

Storage period	Bound ATP ng/l		Free ATP ng/l		Plate count 10^4/ml		Direct microscopic count 10^6/ml		Coliform organisms /100 ml		Thermotolerant coliform organisms /100 ml	
(hours)	4 °C	20 °C	4 °C	20 °C	4 °C	20 °C	4 °C	20 °C	4 °C	20 °C	4 °C	20 °C
0	1 066	1 247	5	15	0.95	0.94	3.71	3.96	11 400	13 100	1 150	1 240
4	1 063	908	38	38	0.41	0.86	4.48	4.75	11 500	10 600	1 150	1 170
24	1 538	1 350	12	25	1.12	0.83	4.23	4.06	12 500	7 500	970	610
29	741	1 415	42	147	1.31	0.82	4.58	3.55	8 500	6 400	880	430
48	1 512	846	20	76	0.94	0.60	4.55	3.55	9 600	2 200	830	100
53	1 385	605	51	245	1.35	1.00	4.51	3.03	9 300	1 500	720	50

6.2.1

ATP

There was a difference in the overall level of ATP measured between the two experiments. In the second, the concentration was considerably higher, and this was reflected in the higher total bacterial count by microscopy. However, viable counts were lower in the second experiment, possibly indicating the presence of large numbers of inactive or moribund cells which were nevertheless structurally intact.

In both experiments there appears to be an initial slight increase in the level of bound ATP at both storage temperatures, suggesting some growth of the bacteria present, but over the full storage period there was a net decrease in bound ATP levels, except in the sample stored at 4 °C in the second experiment. Free ATP levels increased overall, though to a variable extent. This release of ATP into the water would suggest some bacterial die-off and structural breakdown.

The results shown are averages of replicate determinations, but there was a large amount of variability among individual replicate analyses - much greater than that obtained from ATP standard solutions. Unlike standard solutions of ATP, these contained settleable solids. As only 0.1 ml of sample was used in each ATP determination sampling error would have been inevitable. The ATP analysis technique used is not selective, and organisms other than bacteria (algae, diatoms and protozoa) would have been included in the results. The presence or absence of a single clump of algal cells would have had a significant effect on the ATP result obtained. As a consequence, the changes in bound ATP levels could simply be a result of sampling error or inclusion of organisms other than bacteria.

6.2.2
Standard plate counts

The plate counts results show a net overall increase in each case, but this is of doubtful significance. In the first experiment the counts rose to a peak then declined during the storage period, while in the second experiment this effect was not observed. There were unexpectedly large changes in numbers between morning and afternoon samples, which suggests that uniform mixing of the sample was not obtained, presumably because of the amount of particulate material in the sample.

6.2.3
Direct microscopic counts

The direct counts gave more consistent results, possibly because larger volumes of sample were used in this analysis, removing the error associated with low volume sampling. The results indicate little overall change in numbers at both storage temperatures. However, as mentioned above, this count does not discriminate between viable and inactive organisms, and in the second experiment in particular the results are thought to reflect a large number of moribund cells.

6.2.4
Coliforms and thermotolerant coliforms

The results for coliform organisms and thermotolerant coliform organisms are plotted in Figures 6.1 and 6.2. The regression lines indicate that there was little mortality at the 4 °C storage temperature, but that it was more marked at 20 °C.

6.3
Discussion

In these experiments variations between consecutive samples hindered interpretation of the results obtained. It is thought that the presence of particulate material prevented a homogeneous sample from being produced and that, as a consequence, sampling errors occurred.

Results indicate that whereas at the higher temperature the population of indicator organisms decreases, at 4 °C, there is little change in the bacterial population of the samples, suggesting that storage for up to 24 h at this temperature may be possible.

6.4
Tests on water of low nutrient content

A similar series of tests was carried out using the same method as described above, but with river water diluted 10-fold in dechlorinated tap water. It was hoped that this would be representative of a supply derived from a poorly-treated surface water

6.5
Results

The full results are shown in Table 6.3 and the mortality curves for indicator bacteria are plotted in Figure 6.3. The 22 °C plate counts decreased by about an order of magnitude during storage at 4 °C, but at 20 °C, they increased by nearly two orders. Levels of indicator bacteria declined in a similar manner to the previous experiments, and the direct microscopic counts increased slightly at both temperatures. The ATP results may be misleading, as the concentration of viable bacteria was below the limit of detection of the ATP assay. The figures in the Table are probably indicative of the presence of non-bacterial ATP, and no reliance should be placed on any apparent trends.

6.6
Discussion

The changes in bacterial populations seen here are similar to those observed by other workers. At 20 °C, but not at 4 °C the plate count organisms are able to utilise the assimilable organic carbon in the water for growth. Although coliform organisms may also be able to do this at 20 °C faecal organisms cannot compete with the indigenous water bacteria for the limited quantity of nutrients present. As a consequence they decrease in number.

Table 6.3 Results of storage experiment using low nutrient-content water

Storage period	Bound ATP ng/l		Free ATP ng/l		Plate count 10^4/ml		Direct microscopic count 10^6/ml		Coliform organisms /100 ml		Thermotolerant coliform organisms /100 ml	
(hours)	4 °C	20 °C	4 °C	20 °C	4 °C	20 °C	4 °C	20 °C	4 °C	20 °C	4 °C	20 °C
0	164	76	69	95	2.43	1.8	0.65	0.71	150	100	15	18
4	179	147	41	48	0.77	0.73	0.82	0.85	25	30	11	10
24	78	67	33	1	0.37	4.63	1.01	1.14	35	80	10	4
28	96	79	31	2	0.40	4.80	1.11	1.13	26	6	8	4
48	92	88	14	<1	0.37	67.7	0.94	1.15	19	6	3	2
54	68	76	8	1	0.27	159.0	0.99	1.62	19	8	3	1

Previous workers have suggested that the increased number of plate count organisms which occur in stored samples can inhibit the analysis for coliform bacteria. There was little, if any, evidence of this happening in the experiments reported here, except that the apparent decline of coliform organisms was slightly faster in the sample stored at 20 °C in which the plate count organisms grew.

Since these experiments were carried out, the method of creating samples has been questioned. The dilution of river water with tap water is equivalent to inoculating a weak nutrient solution (tap water) with a new spectrum of bacteria which may be capable of growth in this environment. However, in samples of water from a distribution system the growth of the bacteria present will have been restricted by exhaustion of the nutrients in the water which that particular population requires. As a consequence little or no further growth would be expected when samples of this water were stored. Such criticism is justified, and indicates the difficulty of creating a representative model to study microbial ecosystems.

6.7 Simple overnight storage experiments

After the difficulties encountered in the experiments described above, rather more simple tests were carried out to see whether storage of water samples for 24 hours at refrigerator temperature would adversely affect the results of bacteriological assays. Table 6.4 shows results for four samples taken from different stages of treatment of a surface-abstracted water. Two of the samples were of effluent from a upflow rapid sand filter, used as preliminary treatment of the raw water, and the others were of effluent from slow sand filters, representative of the finished water before disinfection.

Table 6.4 Results of overnight storage experiments

		Sample A		Sample B		Sample C		Sample D	
		Start	24 h	Start	24 h	Start	24 h	Start	24 h
Coliform organisms	cfu/100 ml	1650	4700	2700	3300	3	1	2	1
Thermotolerant coliform organisms	cfu/100 ml	310	350	620	250	<1	<1	<1	<1
37 °C plate count	cfu/ml	420	640	340	390	10	10	11	10
22 °C plate count	cfu/ml	3000	2500	2160	2750	24	21	340	660

Samples were taken in sterile glass bottles, and returned to the laboratory immediately. They were then tested for total and thermotolerant coliform organisms and standard plate counts incubated for 48 hours at 22 °C and 24 hours at 37 °C. The bottles were then stored at 4 °C overnight and all tests were repeated.

The table shows that some of the measured parameters showed an increase on storage, some a decrease and some were unaltered. However, the changes were fairly small in all cases, indicating that a 24-hour delay in analysing samples might be acceptable. An increase in bacterial count over this period might produce false positive results, but on theoretical grounds would be more acceptable than a decrease, which might lead samples to be classified as acceptable, when they would have been unacceptable if analysed without delay.

Opinion is divided on the acceptability of sample storage. For example Standridge and Lesar (1977) considered that samples of non-potable water could be stored at 4 °C for 24 hours without adversely affecting thermotolerant coliform counts. Standridge and Delfino (1983) reported that storage of potable water for 48 hours at room temperature had little effect on the public health significance of most samples containing more than two coliform organisms per 100 ml. On the other hand, McDaniels and Bordner (1983) concluded that, because of coliform losses during storage, samples with counts which were initially unacceptable, would have been reported as acceptable if held for 24 hours at refrigerator temperature. They recommended that samples should be stored on ice and analysed within 6 hours.

Assessment of storage procedures would require the testing of large numbers of representative samples,

and this has not been possible within the period of this contract. The inherent variability of bacterial counts means that clear-cut answers are unlikely to be obtained, and the continuing disagreement between various authorities on the effect of delayed analysis, is ample demonstration of the problems involved in investigation of this subject.

7. WORK ELSEWHERE

During the period of the contract staff have endeavoured to make contact with other workers involved in similar studies. This has been done mainly through the Society of Applied Bacteriology Rapid Methods Group and the reformed DoE/PHLS Standing Committee of Analysts.

Visits were made to workers at the University of Reading who developed the Direct Epifluorescent Filter Technique. Similarly, at the Institute of Marine and Environmental Research (IMER), a sophisticated IBAS image analyser is being used to count the number and mass of bacteria in marine waters. Discussion with both these groups of workers confirmed the view that more expensive and sophisticated systems were required if the automated direct-counting procedure was to be of value in water quality monitoring.

Good relationships were forged with Wessex Water, who are using a Malthus Instrument, and Thames Water who have a Bactometer M123 and the Vitek system for automating bacterial identification. Some collaborative work has been carried out.

During the contract period the Department of Trade and Industry (DTI) brought together a team of experts to formulate a development programme for ATP technology. The programme is based on the

setting up of four consortia to deal with the following work areas; reagents, clinical applications, food and beverages and industrial applications. WRc is a member of the industrial applications consortium and with other members has devised a programme of work which is to be part-funded by the DTI. The programme aims to assess existing methods and, where necessary, to develop novel methods for extracting ATP from bacterial cells and to increase the sensitivity of ATP analysis.

The Project Leader visited the United States over the period 16-26 November 1986, funded by WRc. He attended the Water Quality Technology Conference of the American Water Works Association (AWWA) (Portland, Oregon 16-20 November) and presented a paper 'Rapid and semi-automated microbiological analyses for water quality' mainly covering research covered by this contract (Pike et al 1987). He also had discussions with staff of the Seattle Water Department, and with microbiologists and epidemiologists of the Water Engineering Research Laboratory and Health Effects Research Laboratory of the US Environmental Protection Agency at Cincinatti. The general impression was that work under the present contract was not being duplicated in the USA, rather that developments there were complementary.

8. GENERAL DISCUSSION

During the past five years, many rapid bacteriological techniques have been developed for specific applications and subsequently marketed as being suitable for a wider range of uses. The purpose of the present studies was to evaluate some of these methods and assess their suitability for application to the routine bacteriological examination of water. From the results and

discussions given in previous sections of this report it is apparent that, without modification and further development, few of the techniques chosen for study were suitable for this purpose.

The results obtained with the Direct Epifluorescent Filter Technique (DEFT) were particularly disappointing. The observed performance of the technique for the analysis of water was very much inferior to that reported for the detection of bacteria in milk, for which the method was originally developed. Despite much modification and use of different fluorochromes, repeatable, unequivocal results could not be obtained. Undoubtedly some of the observed shortcomings of the automated technique could be attributed to deficiencies of the image analyser used, even though this system is marketed as being suitable for the DEFT. Additionally, however, the inherent limits of sensitivity of the method and inconsistent response of the bacteria in drinking water to staining with the fluorochromes, suggest that the technique is unlikely ever to be of value in routine water quality monitoring.

The limit of sensitivity of ATP measurement was always likely to restrict the use of this technique to the analysis of waters containing in excess of 1000 organisms per ml. With suitable concentration and resuscitation procedures a limit of sensitivity of around four organisms per ml has now been achieved. The procedure, however, is very unselective and micro-organisms other than bacteria will be included in the analysis, and a good correlation with standard bacterial plate counts cannot be relied upon. An increase in the ATP concentration of a distribution water would, of course, indicate an increase in the biomass in that water resulting from a general lowering of quality. ATP measurement could, therefore, have operational

benefits in providing a more rapid warning of deterioration in quality than existing standard plate count techniques. Despite its limitations, ATP measurement is proving of value in other areas of microbiology and the Department of Trade and Industry is part funding the development of more sensitive reagents and instruments in the hopes of establishing a bioluminescence industry in the UK.

After evaluation of existing, and development of new coliform test procedures for use with the Bactometer M123, it was concluded that this technique did offer time and manpower savings for the testing of waters where a 1-ml sample was sufficient. At the theoretical limit of sensitivity of the method (one coliform per ml) a positive result could be recorded within 10 h and with suitable calibration quantitative results could be obtained.

Both the Malthus Instrument and the Bactometer M123 may also have potential for the testing of drinking water. However, at a limit of sensitivity of one coliform per 100 ml it would be more difficult to obtain reliable quantitative information. As a consequence, it is suggested that for drinking water analysis these instruments are used as a means of performing a "rapid" presence or absence test, with subsequent confirmation by traditional methods. This would allow any operational benefits of the new technique, such as manpower and cost savings, to be realised whilst satisfying the traditionally orientated legislative requirements of the UK and EC drinking water standards.

Detailed procedures for using the Malthus and Bactometer instruments in presence or absence tests are now being written. These will be submitted to the joint DoE/PHLS Standing Committee of Analysts Working Group 2 for inclusion as "tentative procedures" in the next edition of Report 71.

Studies with the Beta-glucuronidase test indicate that it is a suitable replacement for the traditional single and two tube E. coli confirmation procedures. The test requires only 4-6 h to complete and its use would allow any remedial action to be taken with more conviction. The possibility of including this test with the electrometric presence or absence procedure is a further attraction, as is its incorporation into the standard membrane filtration method for E. coli. This latter application would permit a confirmed E. coli count to be obtained within 18 h of analysis compared with the 42 h required for traditional methods. Again the results of these studies will be submitted to the joint DoE/PHLS Standing Committee of Analysts Working Group 2 for consideration as "tentative procedures" in the next edition of Report 71.

Although it is accepted that new methods must be rigorously evaluated before they can be accepted as standard or recommended procedures, there appear to be no official guidelines on the amount of comparative data required. Furthermore, existing definitions of coliform organisms and E. coli are based on a restricted number of biochemical characteristics (such as acid and gas from lactose) which few, if any, of the new methods use to demonstrate the presence of these organisms. Unless more flexibility is allowed in the definition of the coliform and E. coli a new method not reliant on the detection of the established characteristics can never be shown to be equivalent to the traditional procedures. Faced with this dilemma bacteriologists are being forced to seek artificial correlations between the old and new techniques. Electrometric techniques are an example. Here the detection times obtained for samples are correlated with the number of colony-forming units obtained from analysis of the

same samples by traditional membrane filtration techniques. Although this approach can indicate a degree of equivalence between methods it cannot demonstrate the superiority of one method over another. Similarly, in the studies reported here, the Beta-glucuronidase test has been shown to be a more efficient procedure (compared with traditional methods) for confirming the presence of E. coli. However, since E. coli is defined in terms of the acid and gas from lactose and indole reactions theoretically the Beta-glucuronidase test cannot be used. It would appear, therefore, that if the benefits of new techniques are to be obtained a radical appraisal of the coliform and E. coli definitions will be required. Similarly, new units for expressing the bacteriological quality of water will have to recognised since the results of ATP analysis and electrometric methods should not be expressed in terms of colony-forming units.

Work on the preservation of samples was hindered by many problems which could not be overcome during the period of the contract. Provision of a supply of samples known to contain low numbers of coliform bacteria was particularly important. Much time was spent in trying to identify a suitable source or in devising a means of creating samples by dilution of more polluted waters. This latter approach, although providing samples with a known coliform content, caused several additional complications. The presence of debris and particulates prevented the production of a homogeneous sample and, as a consequence, large sampling errors occurred. The presence in these samples of relatively high levels of assimilable organic carbon led to the growth of non-coliform organisms at storage temperatures above 4 °C. Since this would not necessarily occur in samples of drinking water this approach was considered unrealistic.

It is concluded, therefore, that assessment of storage effects requires the testing of large numbers of real samples. This was not possible in the present study. However, the limited data obtained suggests that a 24 h delay for samples kept at 4 °C could be acceptable. Certainly this should be investigated more thoroughly before the costly and lengthy procedure of developing a suitable preservative is undertaken.

9. OVERALL CONCLUSIONS

The following conclusions can be made on the basis of the studies reported in previous sections.

1. Few of the techniques chosen for study were suitable, without modification or development, for use in routine water quality monitoring.

2. For the analysis of relatively polluted waters, (more than 100 coliform organisms/100 ml) electrical methods offer time and manpower savings compared to traditional bacteriological techniques, and quantitative results can be obtained after calibration of the instrument with a traditional procedure.

3. At lower concentrations of coliform organisms, such as might be present in drinking water, electrical methods give unreliable quantitative results and they should be used only for presence or absence testing. However, even when used in this way, electrical methods give little or no time advantage over conventional procedures, since it will be at least 12 h before the absence of coliform bacteria can be proven.

4. The Beta-glucuronidase test seems to be a suitable, rapid alternative to the traditional

procedures for confirming the presence of E. coli. Usually, this test requires only 4 h to complete (compared to the 24 h required for traditional procedures) and it identifies, correctly, a higher proportion of E. coli than the recommended diagnostic criteria of acid and gas from lactose and indole production.

5. The Beta-glucuronidase test can also be incorporated into the membrane filtration technique for isolating total coliform organisms, allowing the presence of E. coli to be detected and confirmed within 18 h of the sample being analysed. This is a significant advance on the 48 h required for the currently recommended procedures.

6. Direct-counting of bacteria by epifluorescence microscopy is unlikely to be of value in drinking water quality monitoring because of the inherent limits of sensitivity of the technique, and inconsistencies in the response of bacteria, in water, to staining.

7. The need for special storage conditions or preservation procedures for bacteriological samples can only be assessed thoroughly by testing large numbers of 'real' samples that contain low numbers of coliform organisms. Atypical results will be obtained if samples are created by diluting samples of relatively polluted water.

10. RECOMMENDATIONS FOR FUTURE RESEARCH

Since the beginning of the contract, water industry interest in rapid bacteriological techniques has increased significantly and many water undertakers have expressed views about the relevance of current developments to current and future operational

practices. These views have been taken into consideration in formulating the following recommendations for future research.

10.1 Drinking water monitoring

Despite much discussion and criticism total coliform organisms and E. coli (thermotolerant coliform organisms) are likely to remain the primary indicators of water quality for the foreseeable future. As a consequence, research should concentrate on these parameters with the aim of making monitoring more operationally useful. In this context, reducing the time taken to obtain a confirmed detection of the presence of coliform organisms is the main aim with results obtained in the same 'working day' as the target for increased protection of public health and reduction of operational costs. However, to achieve this, technical advancement is required in both the time taken to obtain a presumptive detection of coliform organisms and their subsequent detection.

It had been hoped that electrical methods would provide the means of improving significantly on the time taken to obtain a presumptive result. However, the studies carried out by WRc strongly suggest that this is not possible, and that at best, these techniques offer only a 'rapid', automated presence or absence test for coliform organisms. It should also be mentioned that electrical techniques were only of practical interest to the larger water undertakers who could offset the high capital cost of the necessary instrumentation by centralisation of their analytical services.

Of course, presence or absence testing may be all that is required for the vast majority of drinking water samples analysed each year in the UK for coliform organisms. If this were accepted, the

possibility of obtaining presumptive detections of coliform organisms within the 'working day' would be more feasible since, with conventional techniques, it is the requirement of obtaining quantitative results which causes delay. With presence or absence testing a larger volume of the water sample, or a concentrate, could be added to a selective growth medium and incubated in the usual way. This would be monitored, either manually or automatically, at regular intervals until a diagnostic change was observed. The more contaminated samples would produce this change most rapidly ensuring that remedial action could be taken, more quickly, where it was urgently required.

Although not a new idea, presence or absence testing is worthy of reconsideration and further development to increase the rapidity with which results can be achieved.

For confirming the presence of total coliform organisms and E. coli the situation, with regard to rapidity, seems more promising. Here detection of the enzyme Beta-galactosidase is being advocated as a rapid means of confirming coliform organisms and detection of Beta-glucuronidase for identifying strains of E. coli. Both these tests have been studied by a number of workers and some commercial test kits (of non-UK origin) are available. However, evaluation of the potential of these tests for use in water quality monitoring has been fragmented and is largely unpublished. Additionally, although a recent survey carried out, by one of the authors of this report, on behalf of the Joint PHLS/DoE Standing Committee of Analysts Working Group 2 has revealed that some water microbiologists are already using these tests, there would appear to be no easy way of collating all the information which they have obtained.

These tests remain, therefore, unrecognised officially and this is restricting further use.

Both the Beta-galactosidase and the Beta-glucuronidase tests are extremely rapid, taking between 1 and 4 h to complete, and are of obvious operational benefit. Additionally, incorporation of the Beta-glucuronidase test into the presumptive-total coliform, membrane filtration procedure seems feasible (see Section 5.5) and this may also be possible for the Beta-galactosidase test. Further development work on this aspect would, therefore, seem justified since, if successful, counts of confirmed total coliform organisms and E. coli could be obtained in the same time now required for presumptive isolation. Since these tests would merely be modifications of existing procedures, they would be of immediate benefit requiring little additional expenditure. Similarly, if presence or absence testing were approved, the inclusion of either of these enzyme tests would bring additional benefits.

Developments such as those discussed above may, of course, be of only short-term benefit since recent developments in the field of monoclonal antibodies and gene probes suggest that these may be the methods of the future. Both these techniques are likely to remove or reduce the need for conventional culturing techniques and because of their specificity obviate requirements for confirmation or identification. Since the need for culturing the 'target' organism will be reduced, these techniques will be rapid, providing results on the same day as analysis. Work in both monoclonal antibodies and gene probes needs, therefore, to be encouraged. However, it may be some years before such techniques are developed to an extent which allows their routine use on grounds of reliability and cost.

Although of little sanitary significance determination of the total microbial load of waters in distribution is of operational value. This determinand has, however, received little attention due to shortcomings in the technique used for its measurement; the 22 °C standard plate count. Studies under the terms of the contract indicated that direct-counting techniques were unlikely to be of value in this context, but that ATP measurement had potential, subject to improvement in the sensitivity of the measurement technique. Further research is, therefore, desirable.

10.2 Recreational waters and shellfish

Although this subject may be outside the terms of the present contract, it is worth considering briefly the research needs in these areas. The needs for virological research will not be discussed, even though there is an unquestionable demand for work in this area.

Studies under this contract have shown that electrical techniques offer time and manpower savings in monitoring for compliance with the EC mandatory total coliform and faecal coliform bathing water standards. In the future, however, it seems likely that standards for bathing waters will be based on risk assessment and that the relevant standards will be expressed in terms of faecal streptococci (enterococci). Subject to some clarification of the definition of these indicators, electrical methods are likely to offer considerable time savings in the analysis for this group of organisms. Previous studies at the WRc suggest that using the Bactometer analysis for faecal streptococci requires only 18-24 h compared to the 36-38 h required for membrane filtration techniques. Undoubtedly, a thorough reappraisal of analysis procedures for faecal streptococci is required and this should include electrical techniques.

Bacteriological examination of shellfish tissue has always been hindered by its fibrous consistency and particulate content. Unlike traditional bacteriological techniques, electrical methods are able to detect clumps of bacteria or bacteria bound to particulates. As a consequence, they could provide, for the first time, a method of analysis which is as equally applicable to shellfish tissue as it is to the waters in which shellfish are grown and any adjacent polluting discharges. The value of electrical methods for monitoring the sanitary quality of shellfish needs to be assessed.

10.3 Summary

The research needs identified and discussed in Sections 10.1 and 10.2 can be summarised as follows.

1. Presence or absence testing for coliform organisms and E. coli in drinking water.

2. Use of enzyme tests for confirmation of the presence of coliform organisms and E. coli.

3. Incorporation of specific enzyme detection tests into traditional coliform analysis procedures.

4. The development of monoclonal antibody and gene probe techniques for total coliform organisms and E. coli.

5. Development of ATP measurement as a replacement of the 22 °C standard plate count.

6. Rapid detection of faecal streptococci in recreational waters.

7. Use of electrical methods for analysis of shellfish and shellfish waters.

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FIG 2.1 Effect of DAPI concentration on directly-observed (manual) count.

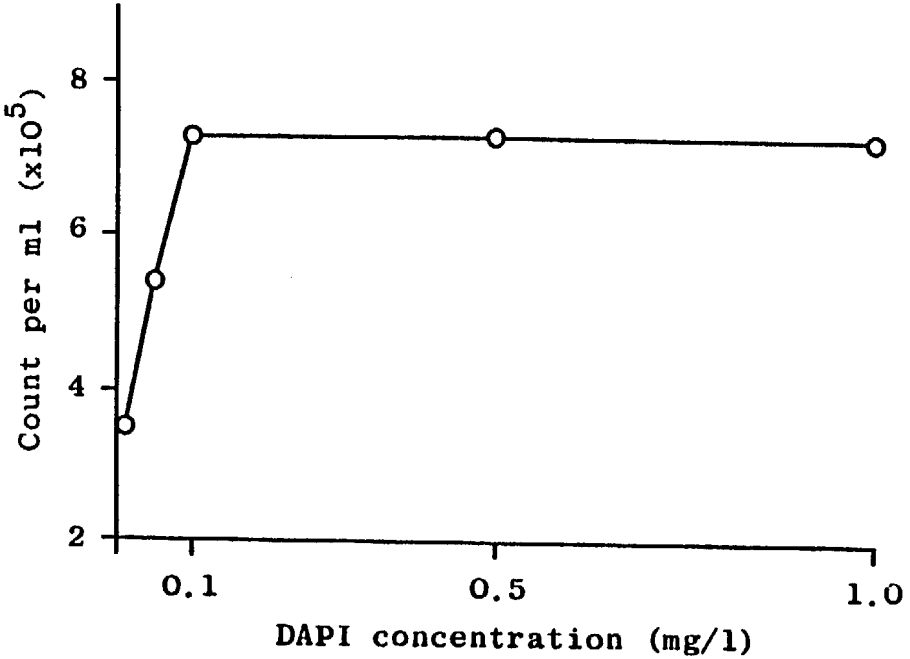


FIG 2.2 Effect of DAPI concentration on manual and automatic counts made on the same sample.

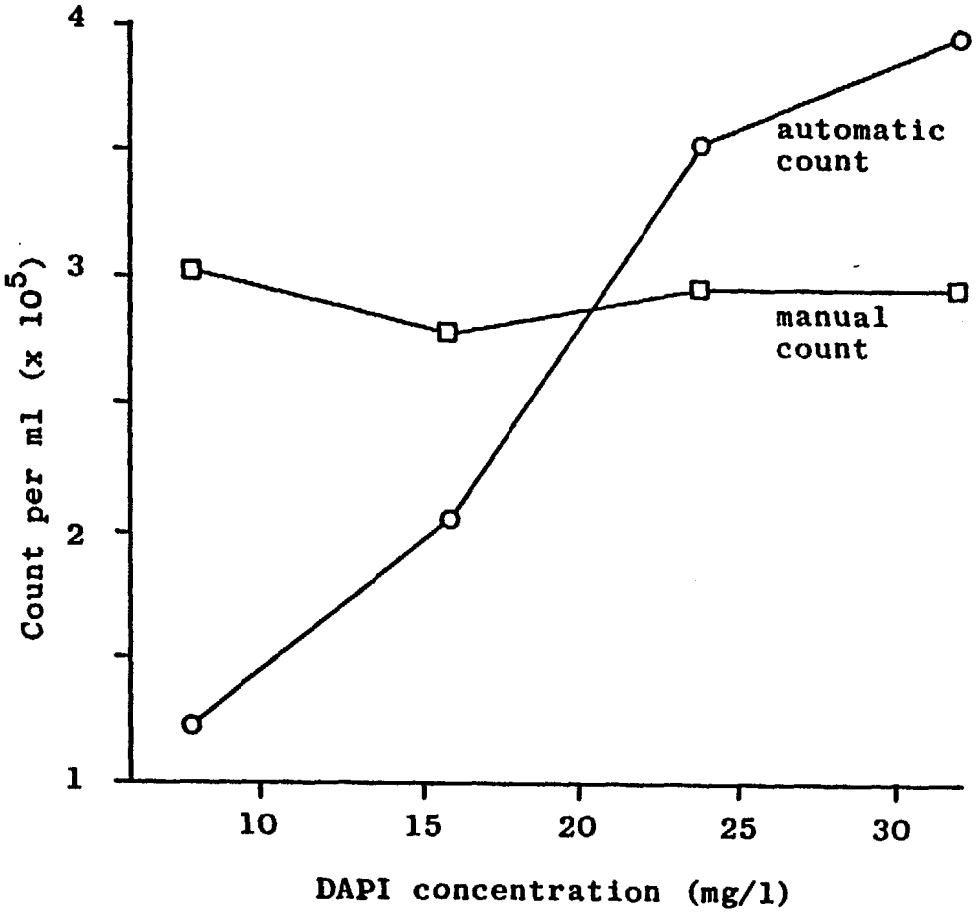


FIGURE 2.3 Theoretical relationship between concentration of bacteria and volume of sample filtered to obtain 4 bacteria in a counting field of 4 squares

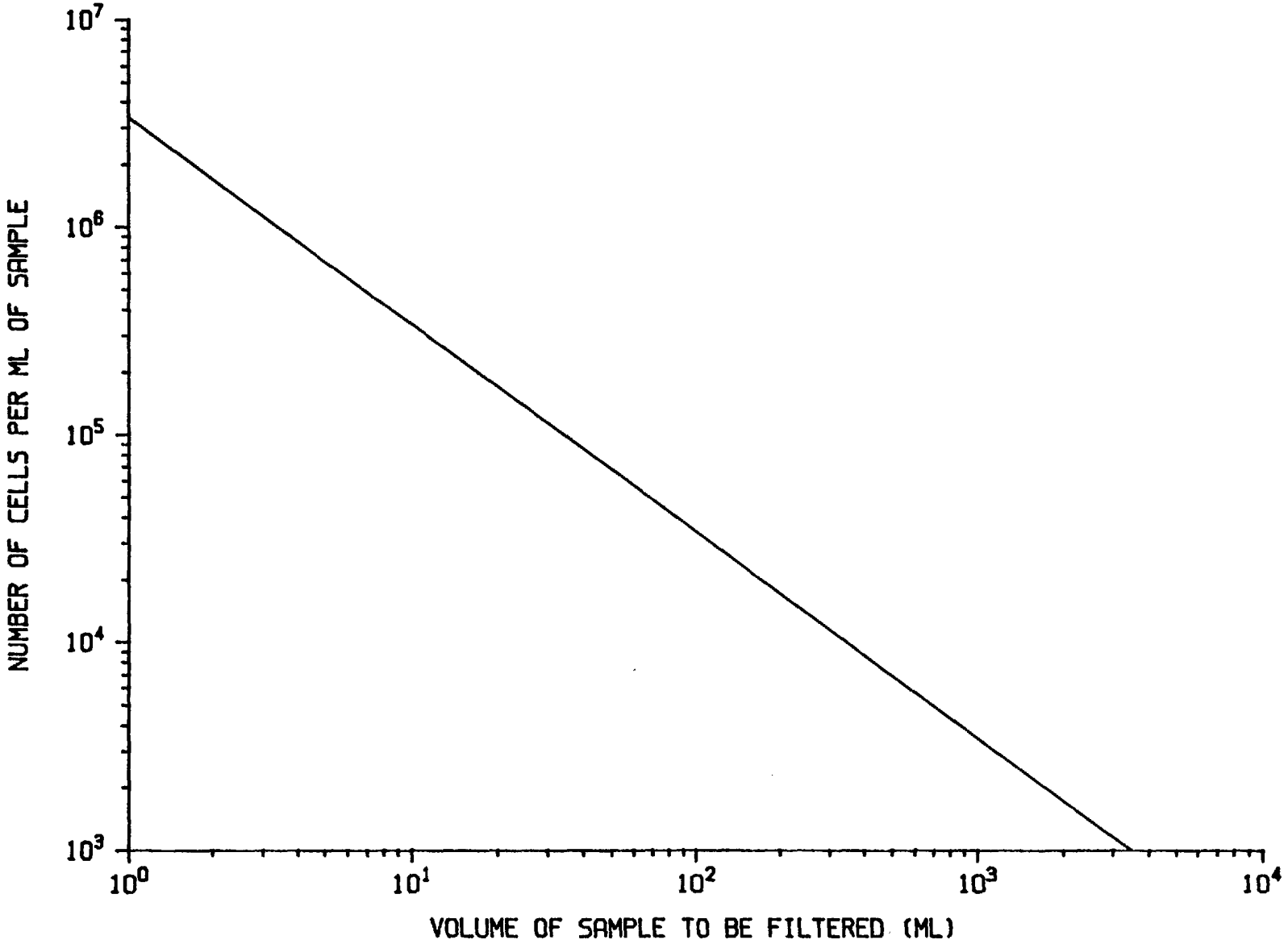


FIGURE 2.4 Observed relationship between concentration of bacteria and volume of sample filtered with 4-5 bacteria countable in a field of 4 squares

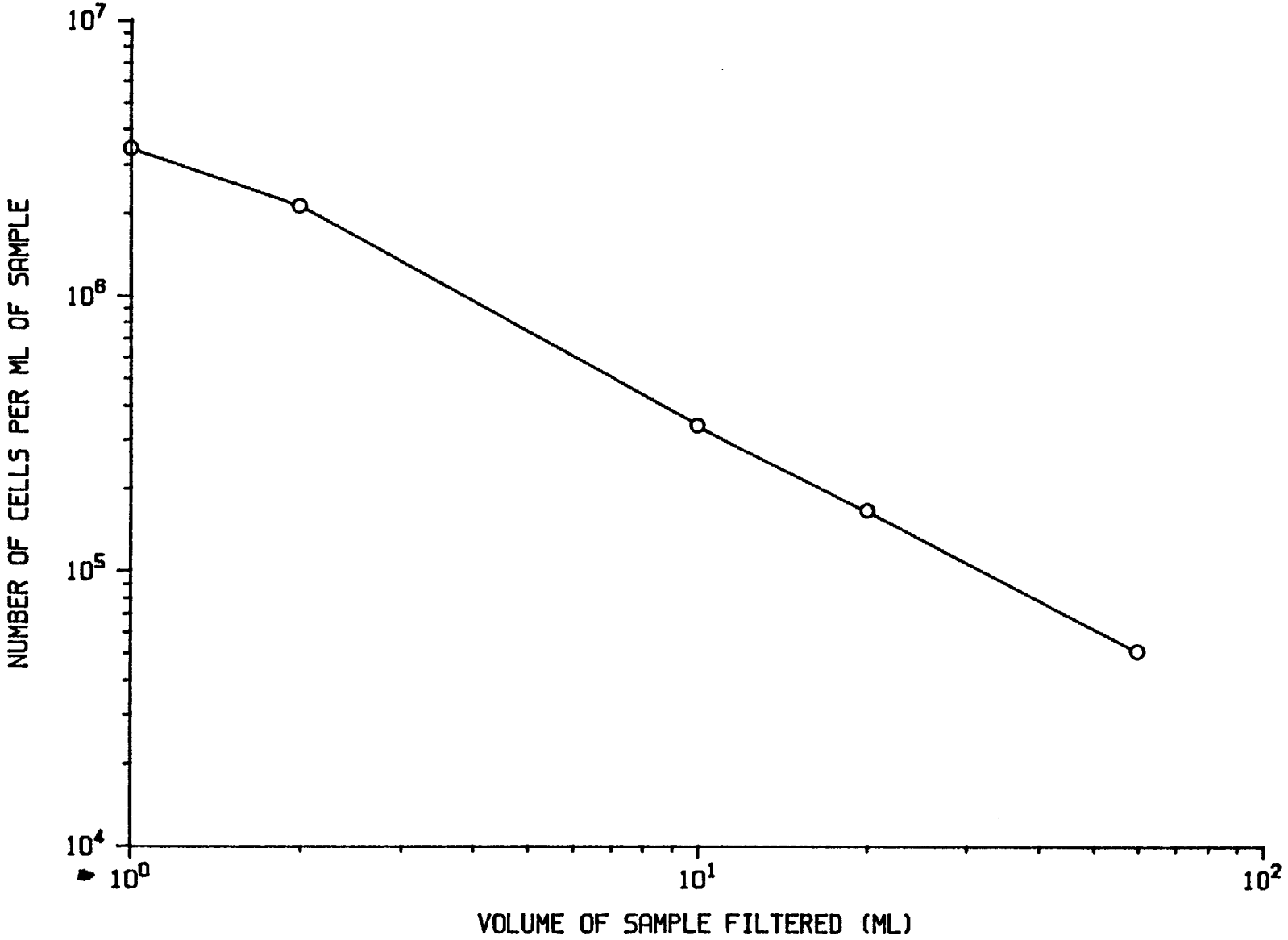


FIGURE 2.5 Theoretical relationship between concentration of bacteria and volume of sample filtered to obtain 4 bacteria in a counting field of 25 squares

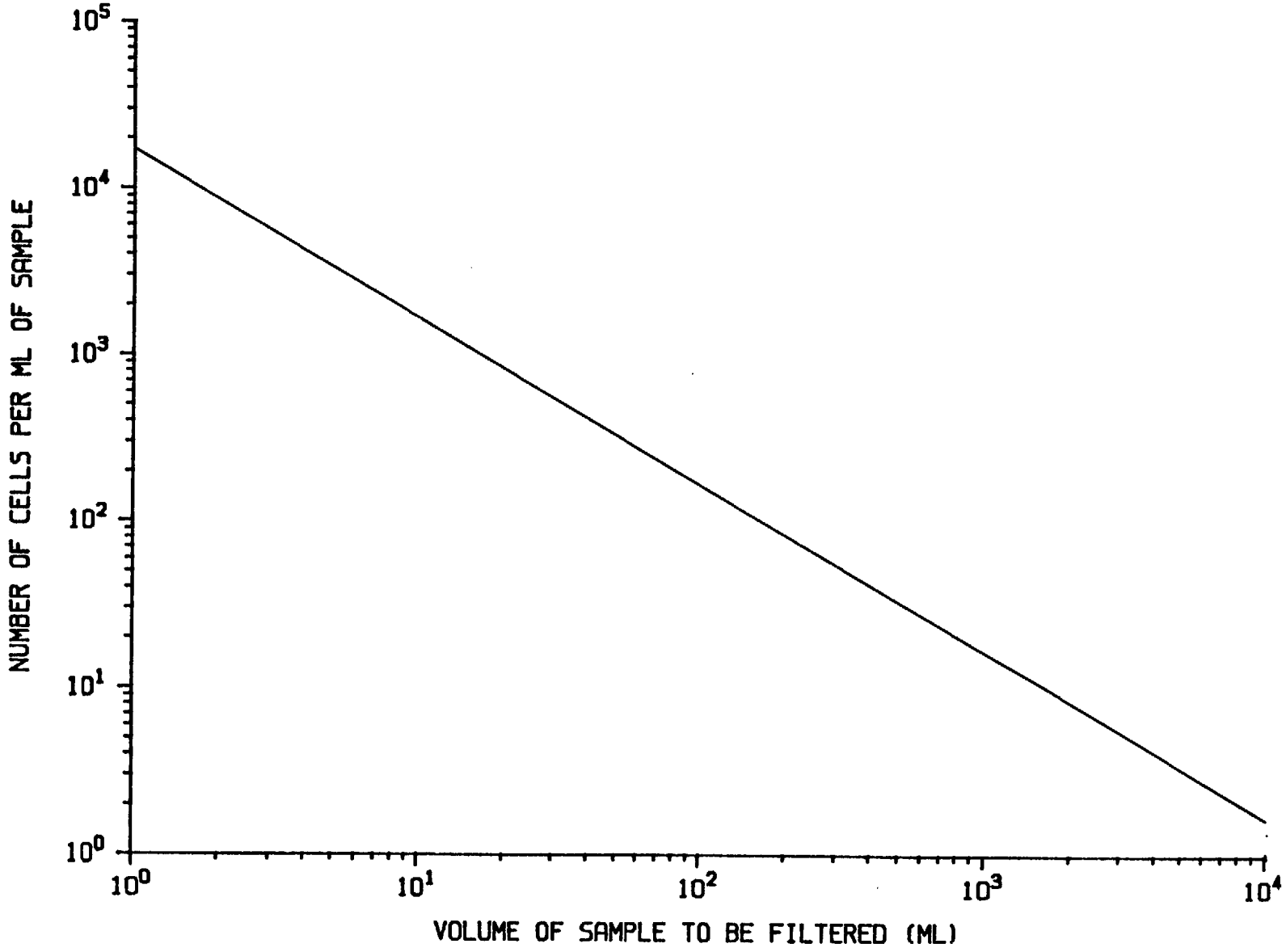


FIGURE 2.6 Theoretical relationship between concentration of bacteria and volume of sample filtered to obtain one bacterium in whole field

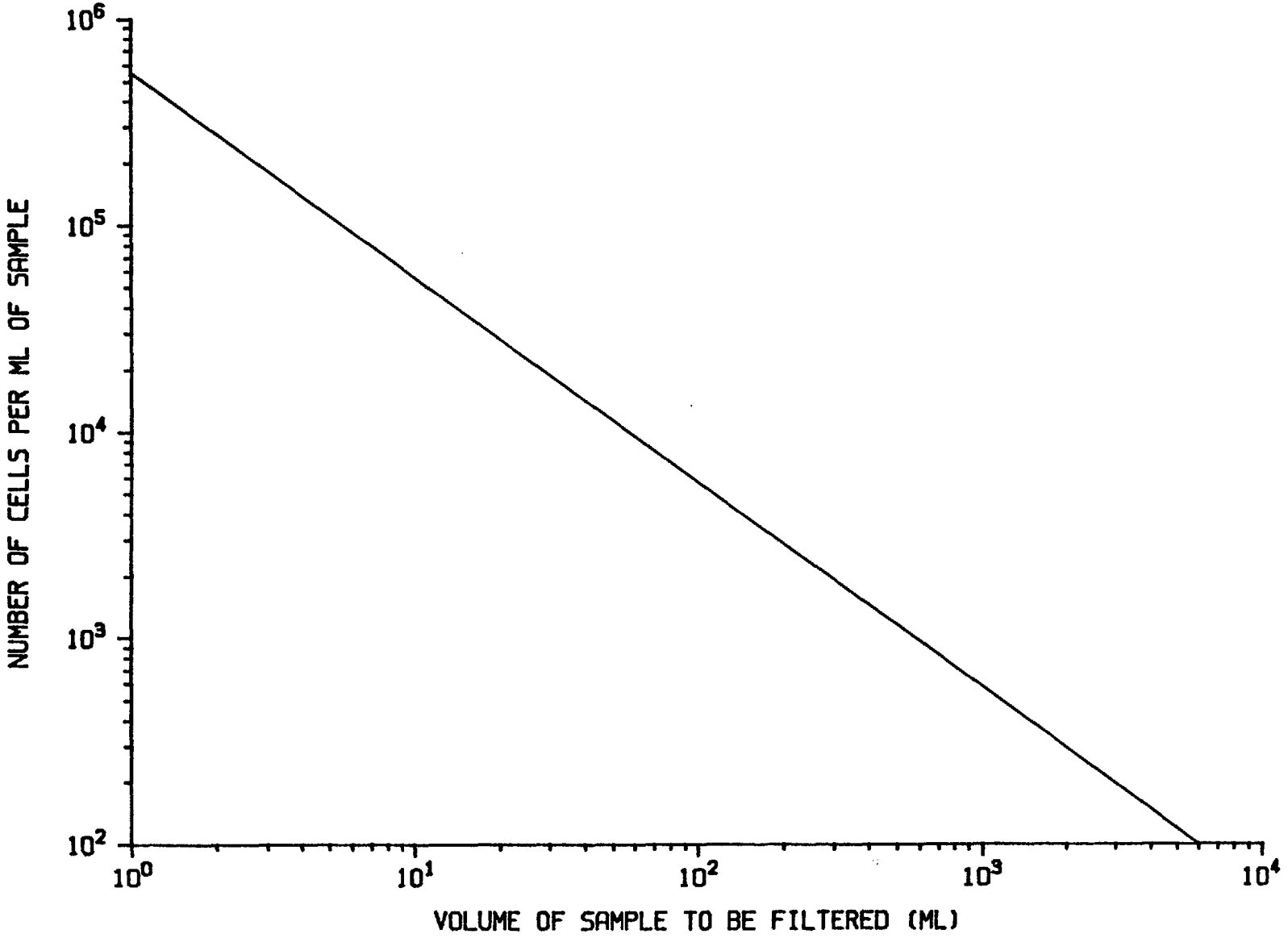


FIG 3.1 ATP CONCENTRATION VS VIABLE COUNT FOR MIXED NATURAL POPULATION OF BACTERIA

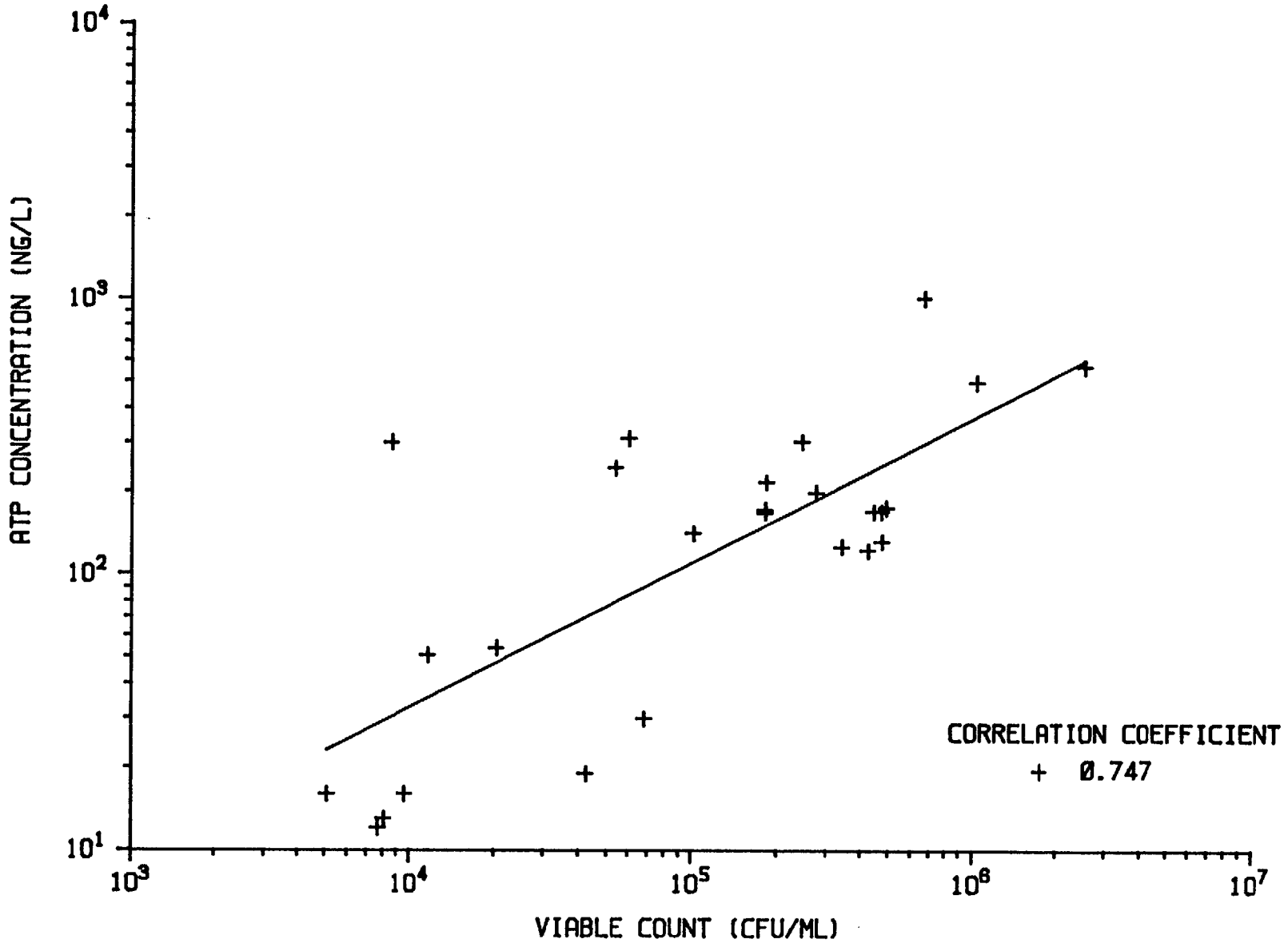


FIG 3.2 ATP CONCENTRATION VS VIABLE COUNT FOR PURE CULTURE W1-4

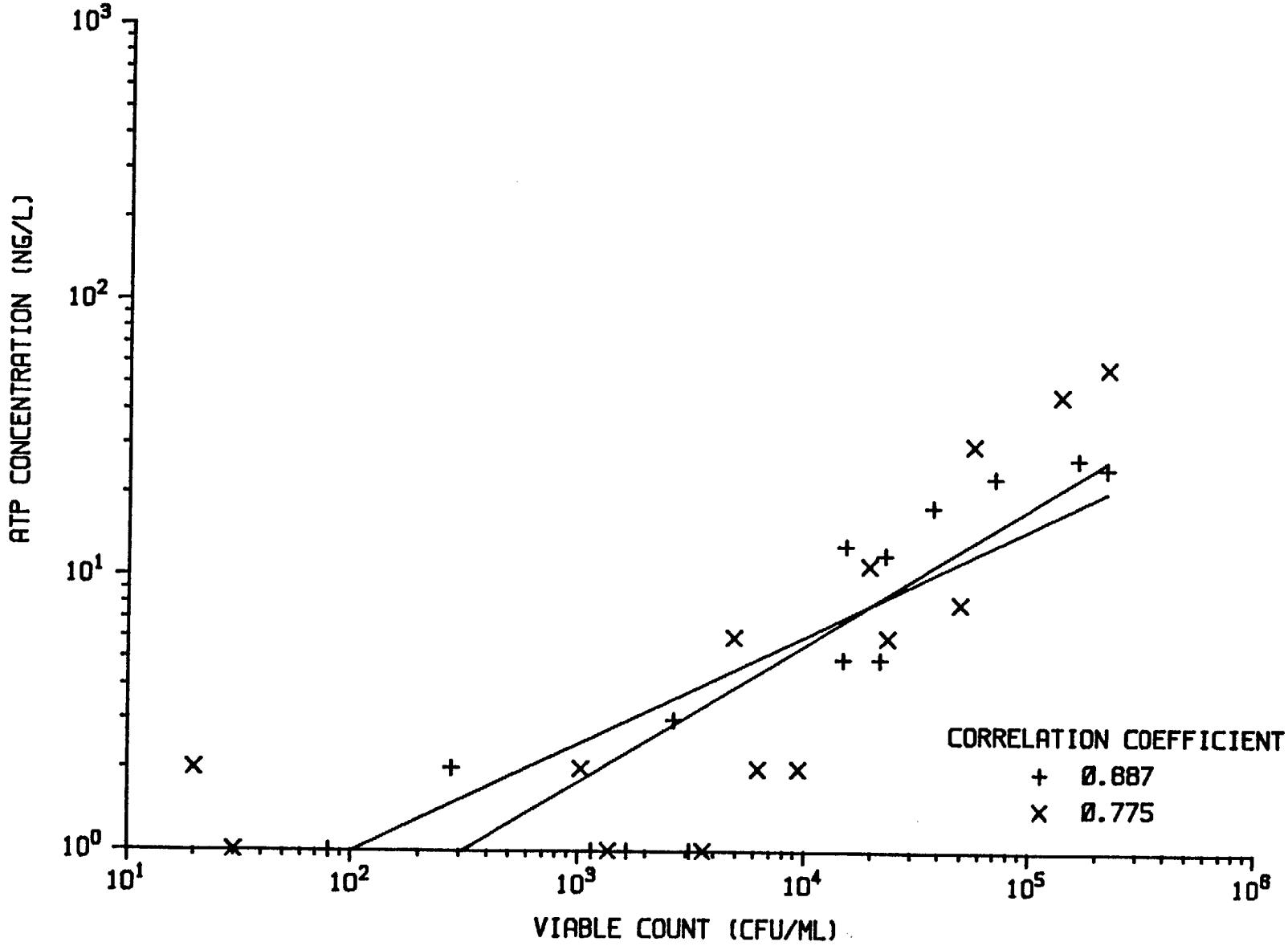


FIG 3.3 ATP CONCENTRATION VS VIABLE COUNT FOR PURE CULTURE W2-2

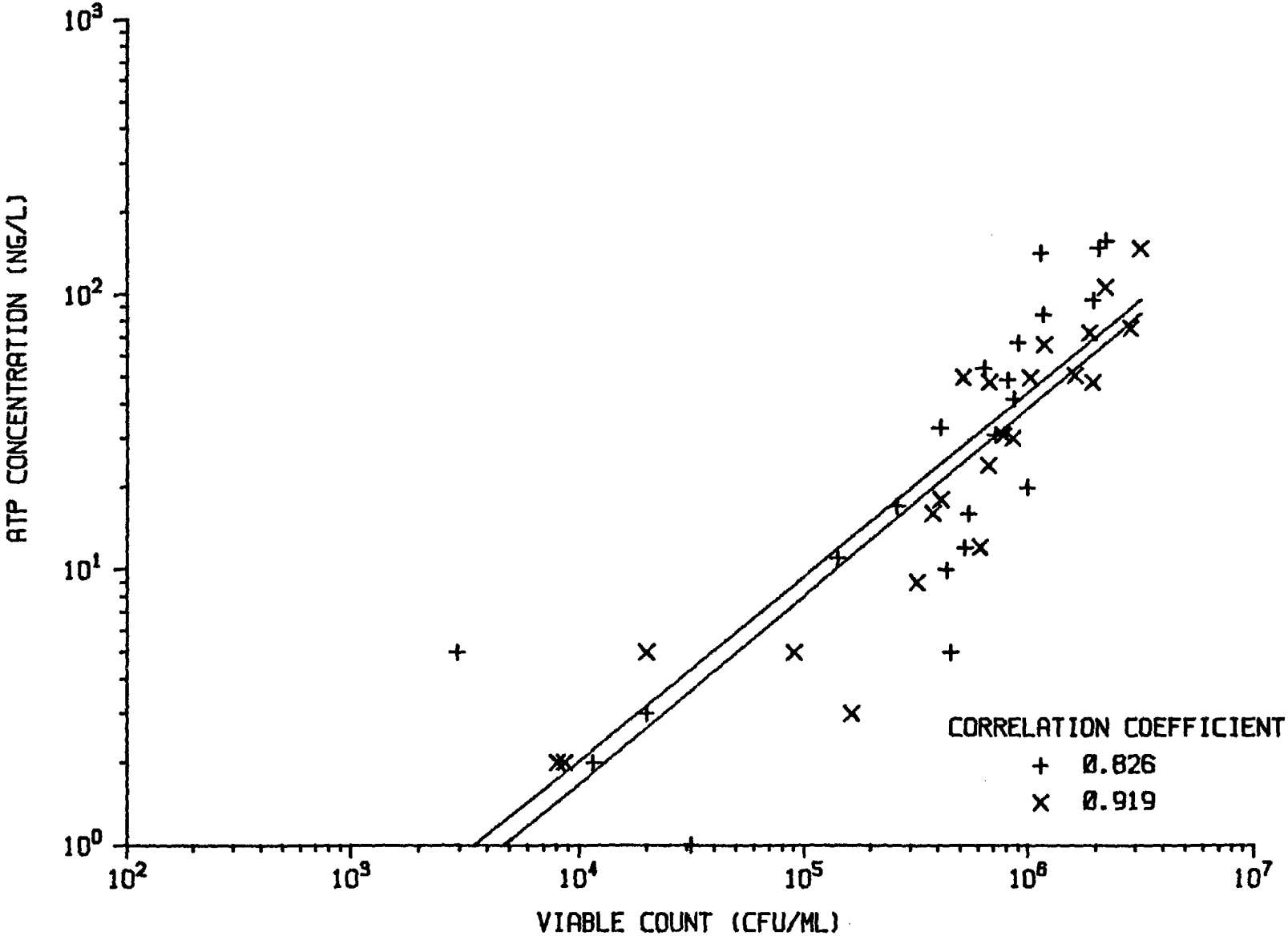


FIG 3.4 ATP CONCENTRATION VS VIABLE COUNT FOR PURE CULTURE W1-10

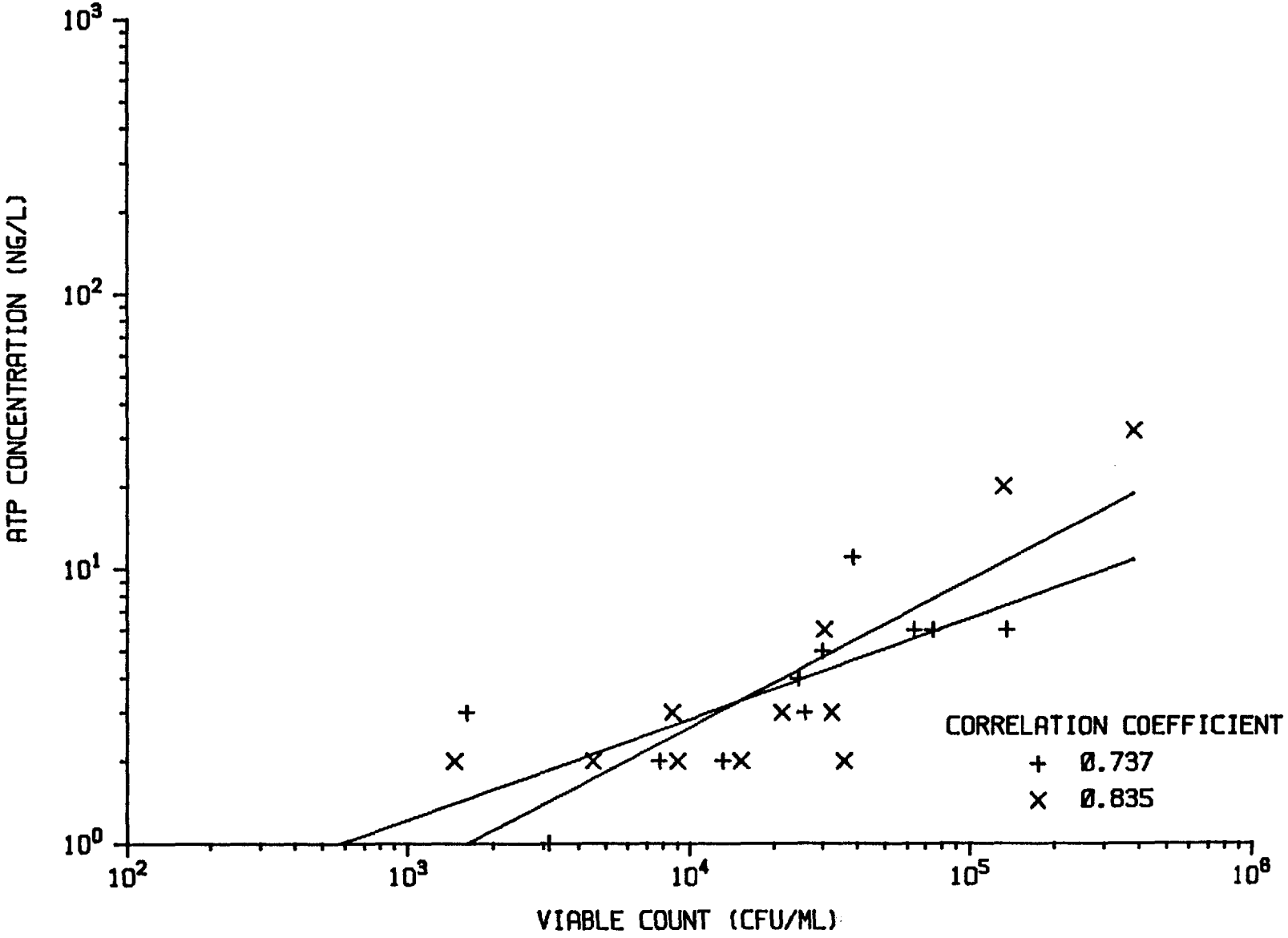
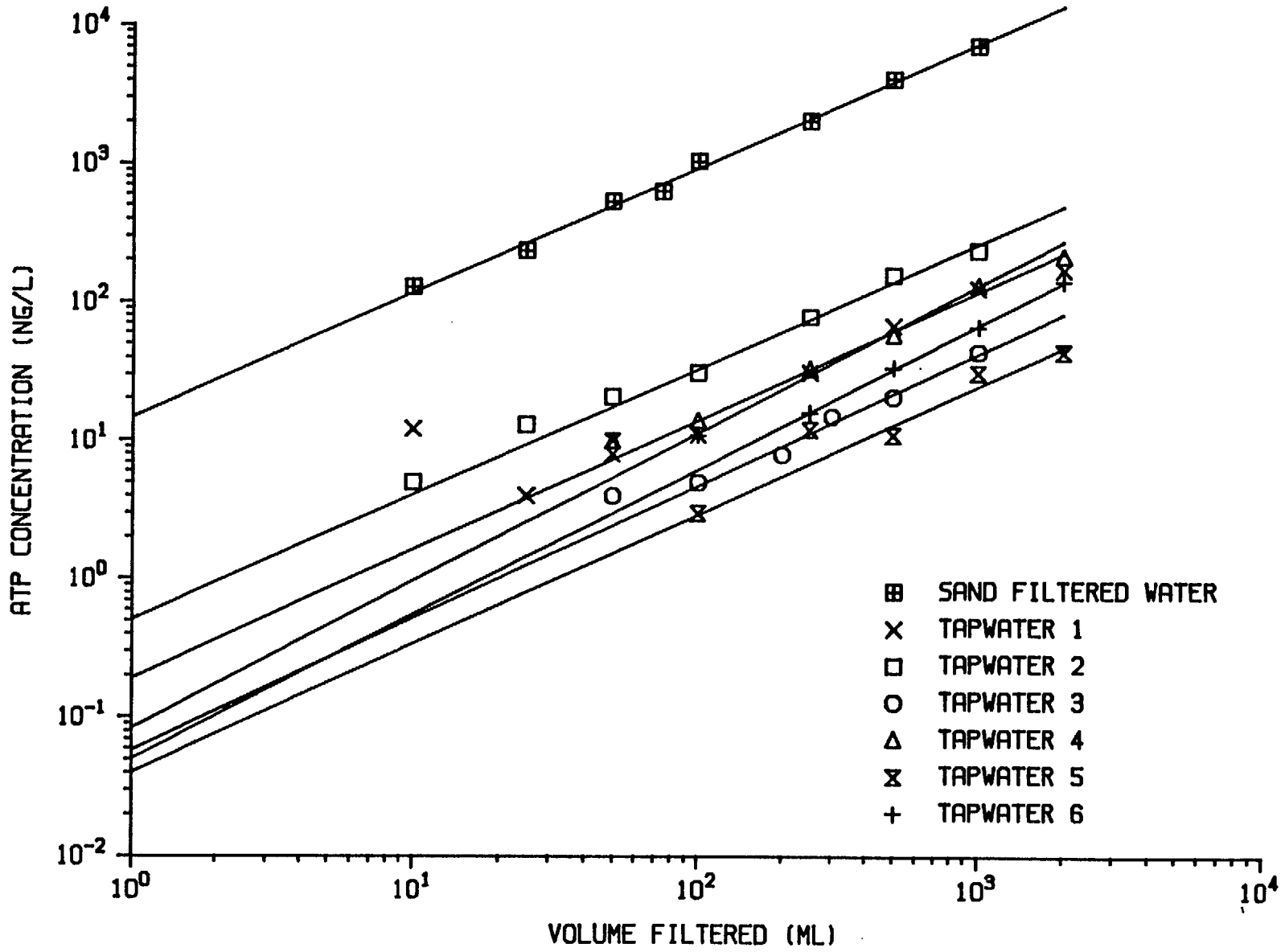


FIG 3.5 RESUSCITATED ATP CONCENTRATION PLOTTED AGAINST VOLUME FILTERED



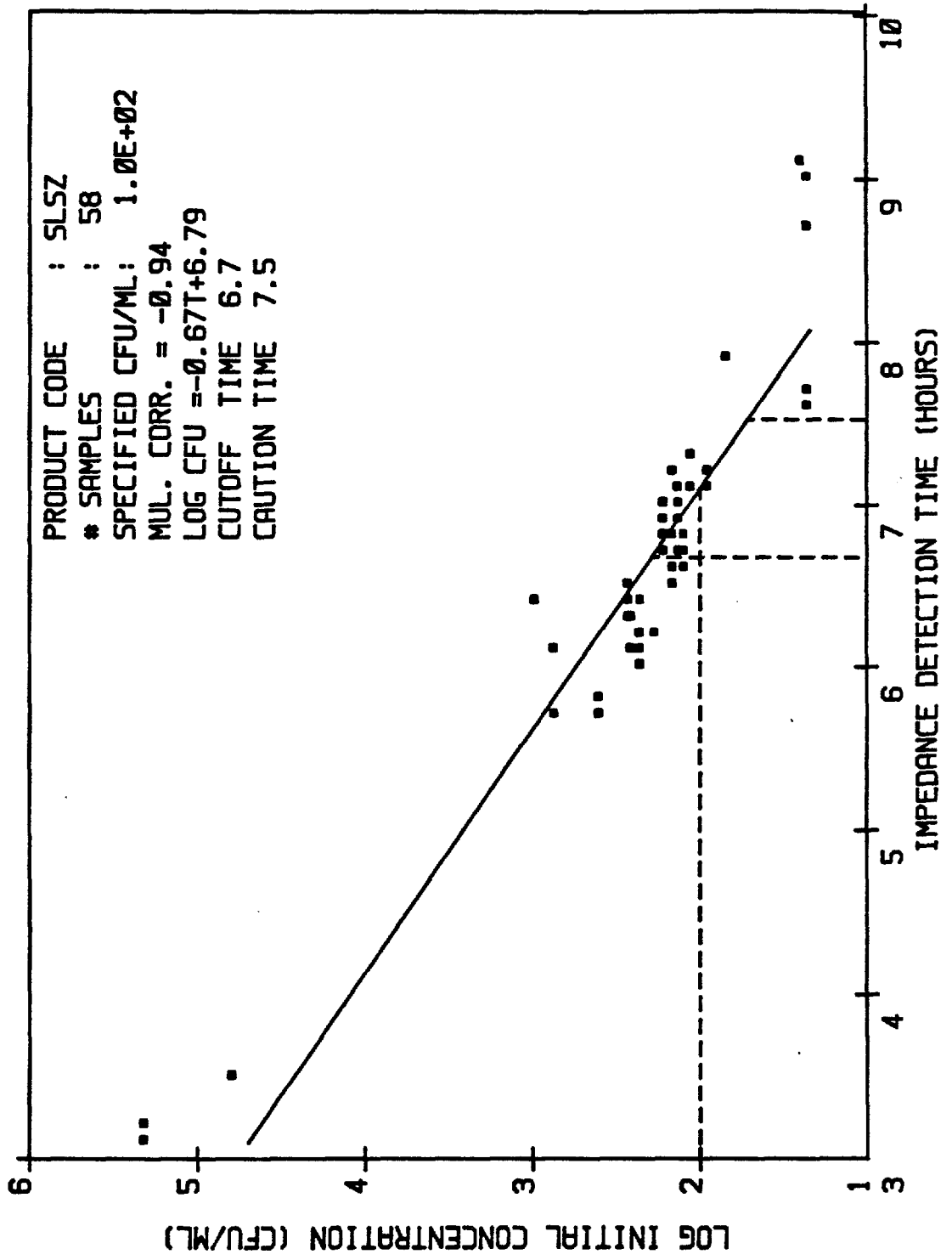


Figure 4.1 Calibration of Bactometer M123 - Diluted SLS medium

Figure 4.2 Calibration of Bactometer M123 – full strength SLS medium

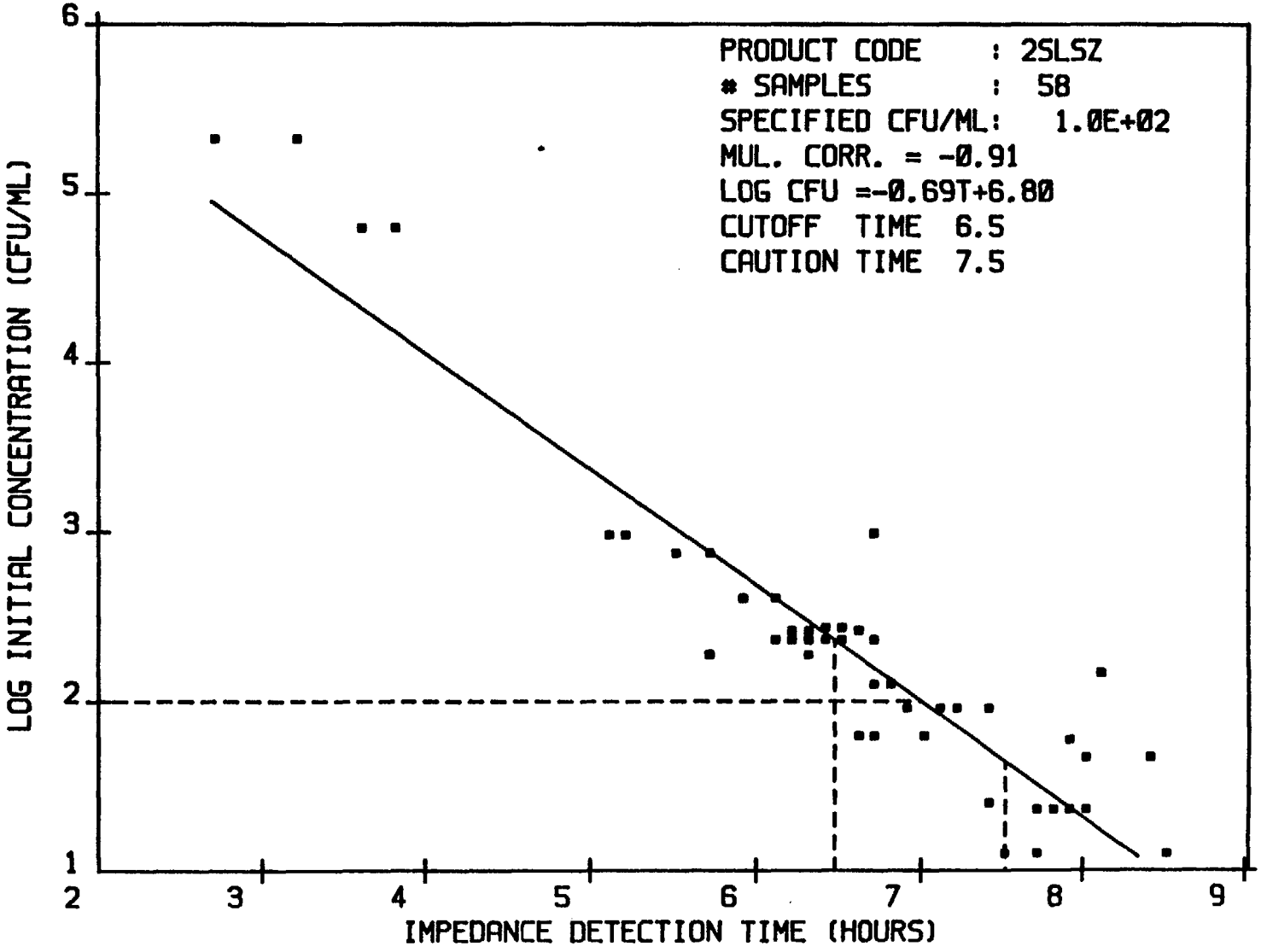


Figure 4.3 Calibration of Bactometer M123 - Results for full-strength and diluted media combined

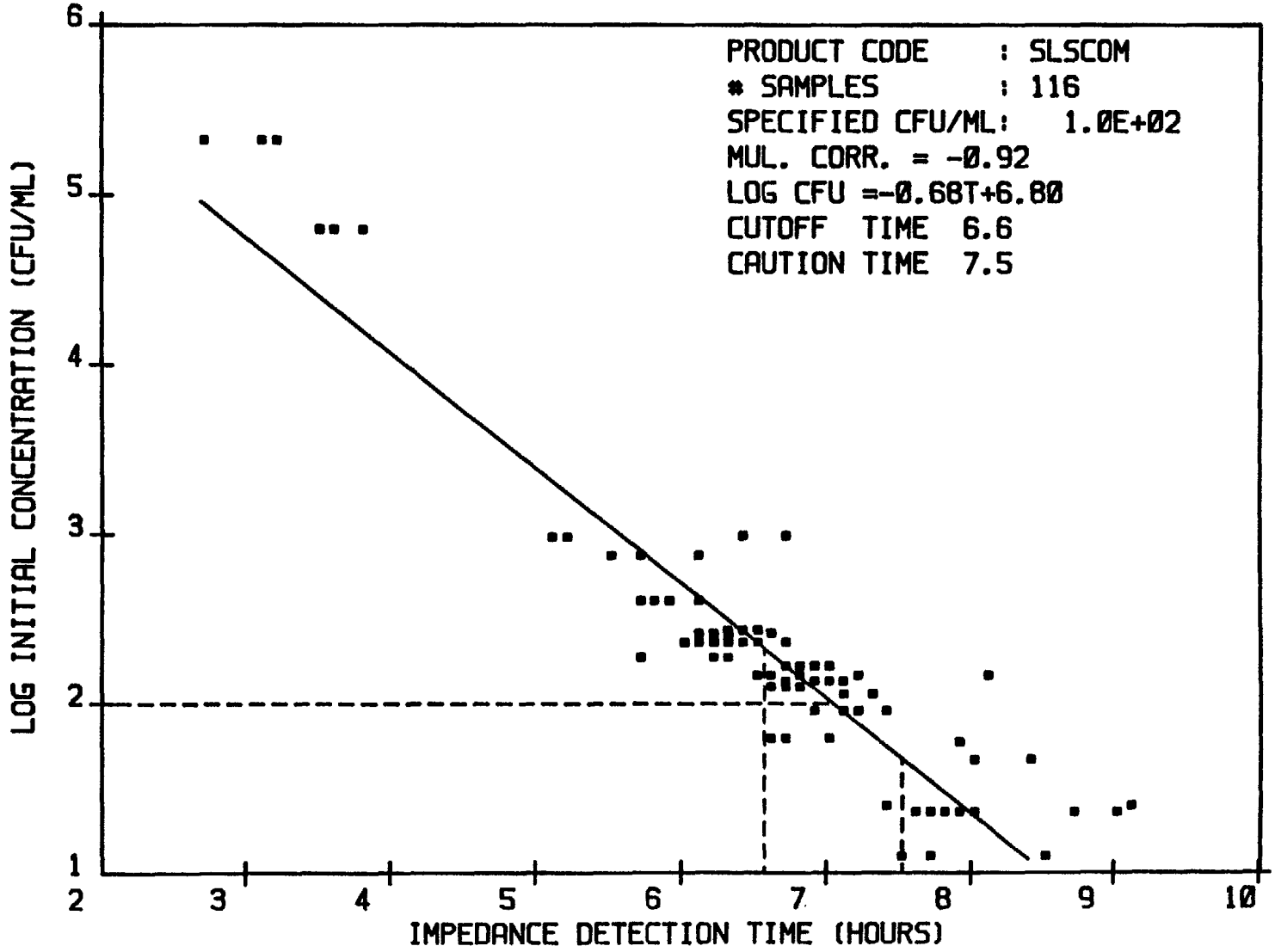


Figure 4.4 Calibration curve for 258 environmental samples

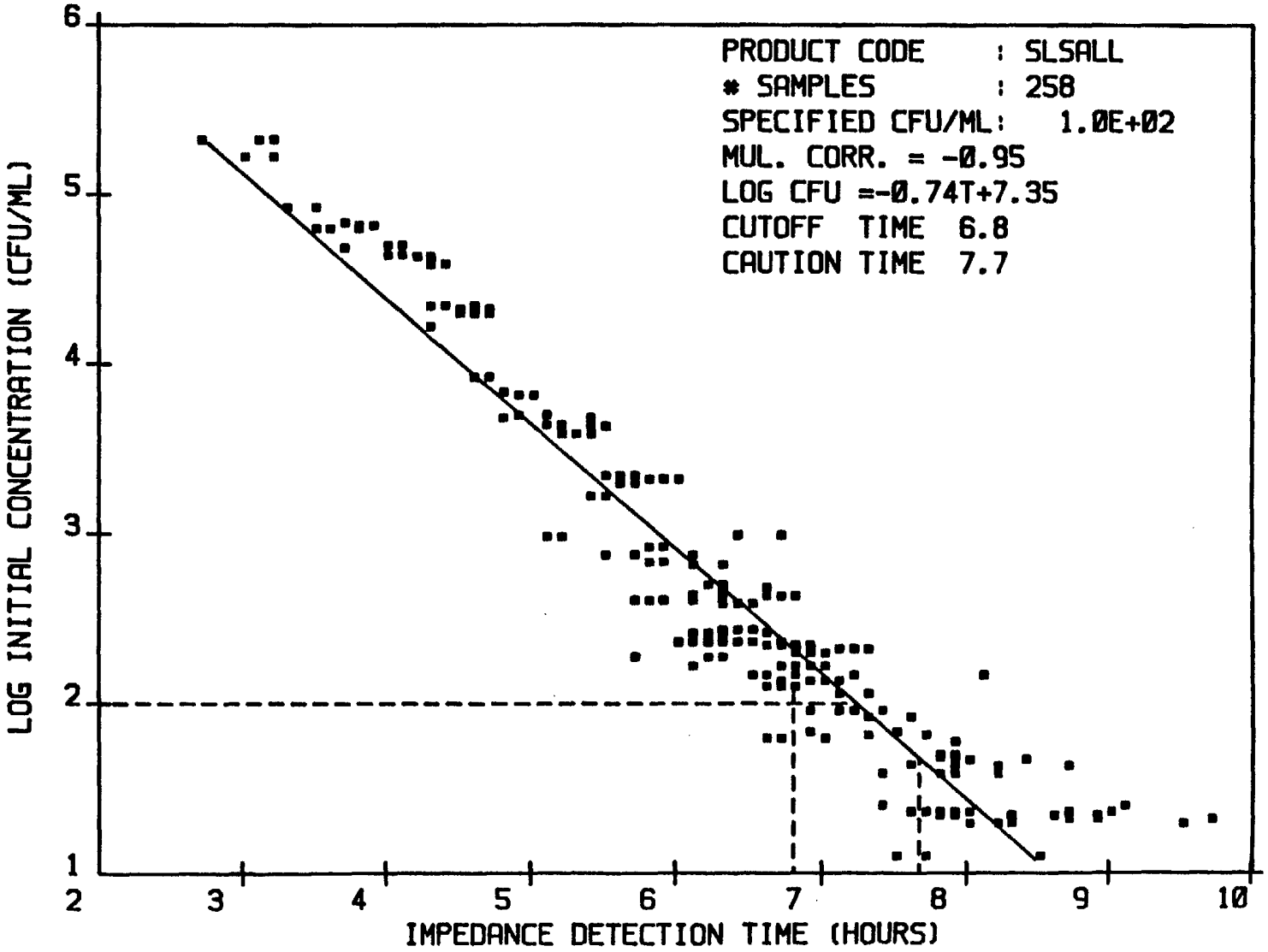


FIG 6.1. FIRST RIVER WATER STORAGE EXPERIMENT

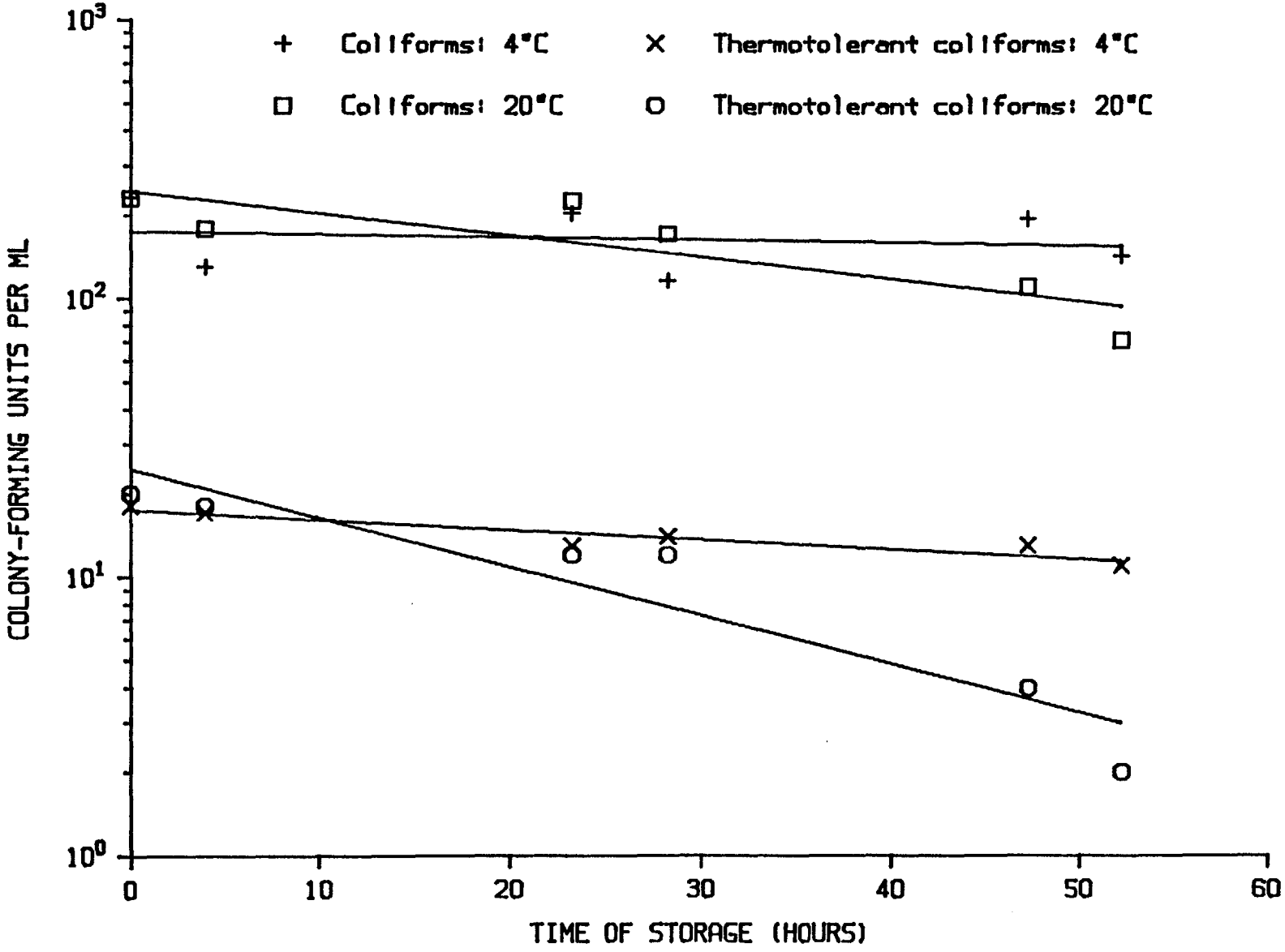


FIG 6.2. SECOND RIVER WATER STORAGE EXPERIMENT

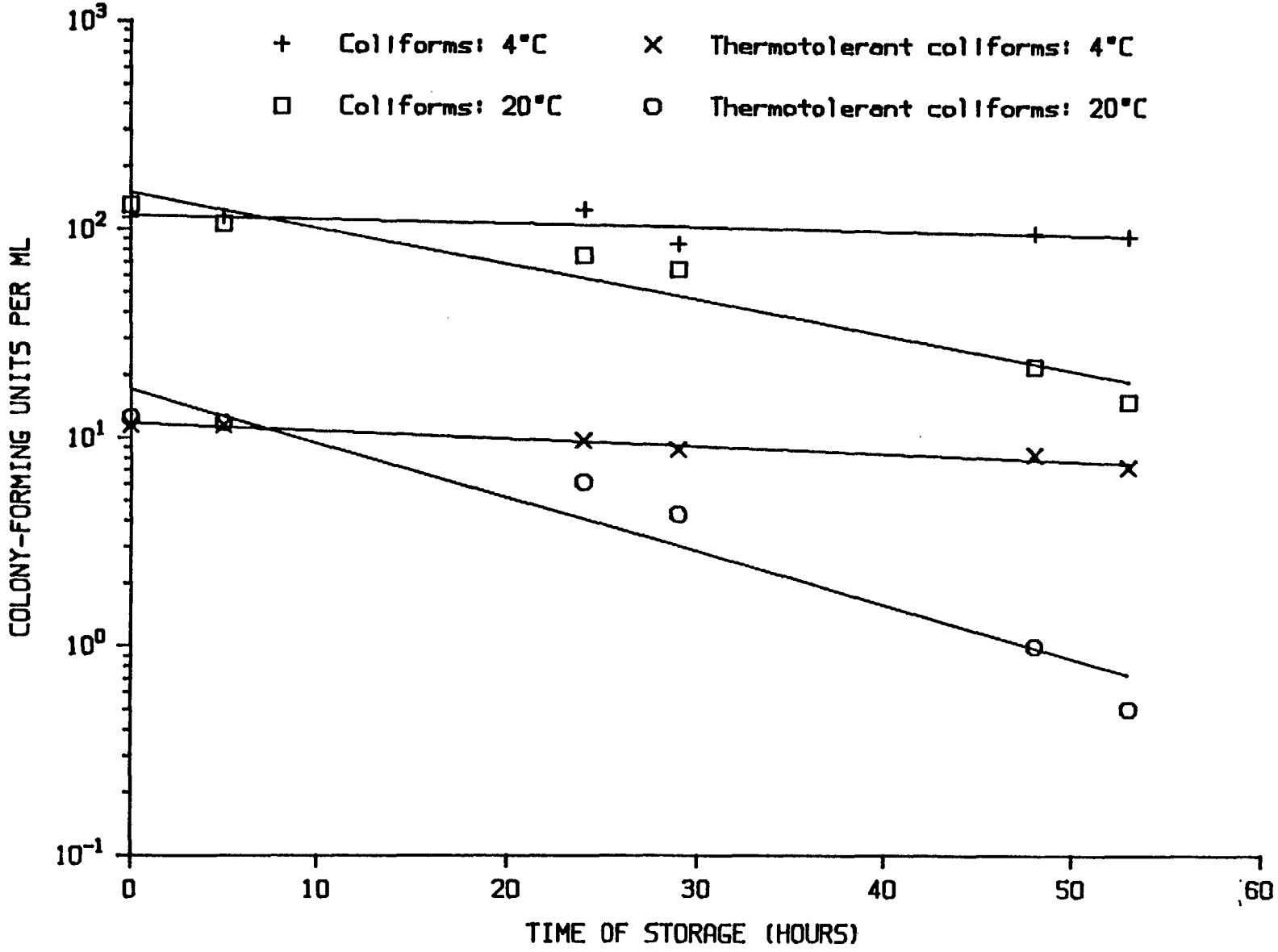
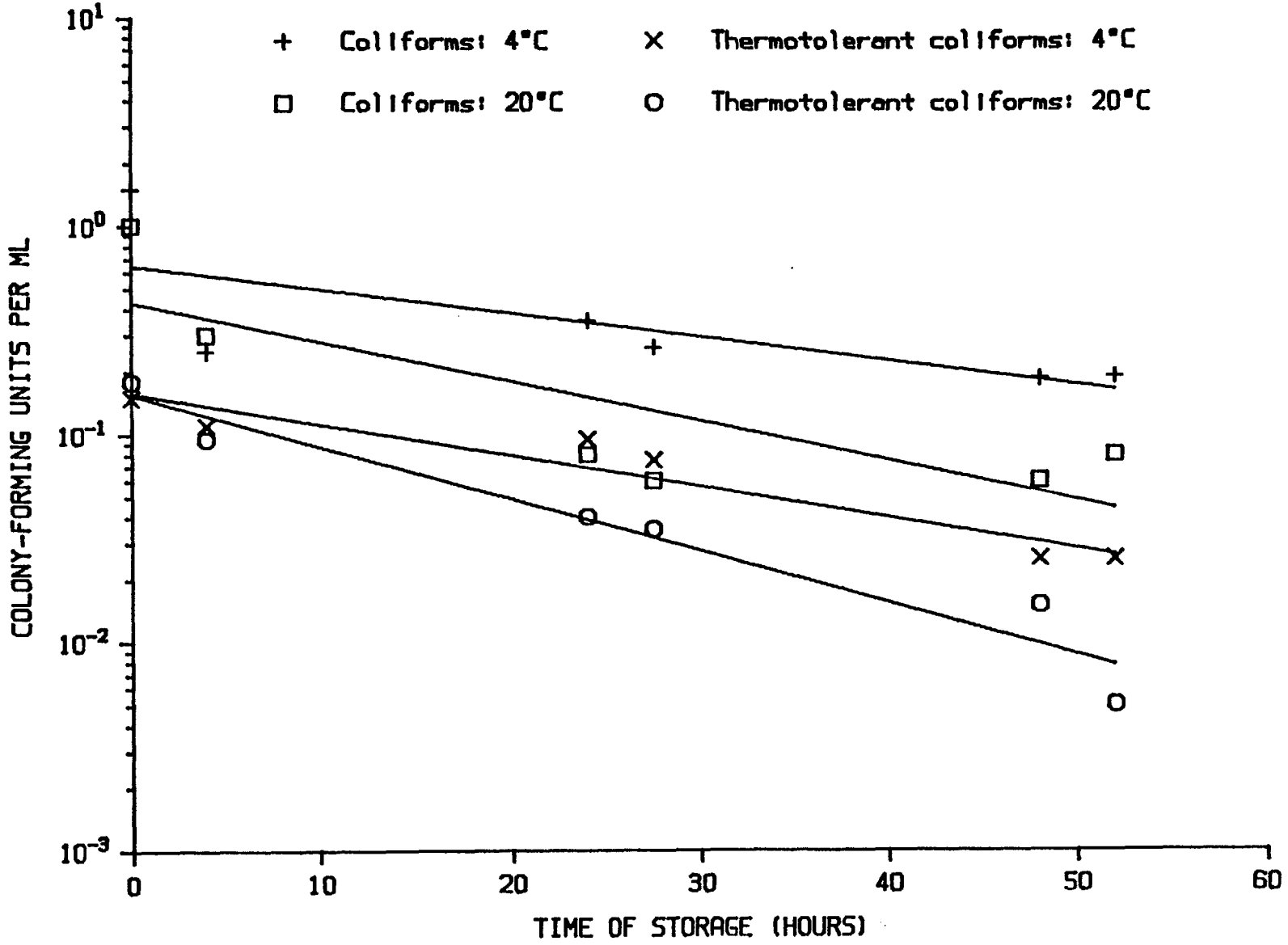


FIG 6.3 STORAGE EXPERIMENT USING LOW-NUTRIENT WATER



APPENDIX A

A REVIEW OF LITERATURE ON DIRECT COUNTING OF BACTERIA BY EPIFLUORESCENCE MICROSCOPY

CONTENTS

1. INTRODUCTION

This review aims to outline developments in the general procedure for the enumeration of aquatic bacteria by epifluorescence microscopy. Variations between workers and their findings will be described within this context.

2. RANGE OF FLUOROCHROMES

The range of fluorochromes available for use in epifluorescence microscopy can be divided up according to their molecular specificity.

2.1 Non-DNA staining fluorochromes include:

Fluorescein diacetate - a fluorescent stain only on hydrolysis to fluorescein by deacetylation with non-specific esterases after transport across the membranes of active cells (Chrzanowski et al 1984, Hobbie et al 1977, Jones and Simon 1975, Paul 1982).

Fluorescein isothiocyanate (FITC) - a protein specific fluorochrome interacting with amino acids (Hobbie et al 1977, Daley 1979, Yentsch et al 1983, Jones 1974, Zimmerman and Meyer-Reil 1974).

Fluorescein mercuric acetate - a nucleoprotein stain (Jones and Simon 1975).

Hoechst 2495 - binds protein (Paul 1982).

o-pthaldialdehyde - binds protein (Yentsch et al 1983).

2.2 DNA specific fluorochromes include:

Acridine Orange (AO) [3,6-bis(dimethylamino) acridine] - a metachromatic fluorochrome binding single and double stranded DNA and RNA (Daley and Hobbie 1975, Hobbie et al 1977, Daley 1979,

Francisco et al 1973, Jones 1974, Taylor and Milthorpe 1980, Pettipher 1983).

Acriflavine - DNA specific fluorochrome used mainly in eukaryotic cells (Bergstrom et al 1986, Paul 1982).

DAPI [4',6-diamidino-2-phenyl indole] - binds A-T (adenosine and tyrosine) rich sequences of the DNA double helix, having no affinity for single DNA or RNA (Allan and Miller 1980, Coleman 1980, Yentsch et al 1983, Porter and Feig 1980, Kapuscinski and Skoczylas 1977, Kapuscinski and Skoczylas 1978, Taylor and Milthorpe 1980)

DCI [4'6-dicarboxyamide-2-phenyl indole] - DAPI analogue, DNA specific (Kapuscinski and Skoczylas 1978).

Ethidium Bromide (EB) [2,7-diamino,10-ethyl, 9-phenyl phenanthridinium bromide] - intercalates between base pairs of the double helix with an affinity for DNA in prokaryotes, in eukaryotic cell nucleii and other cytoplasmic compounds (Roser et al 1984, Kapuscinski and Skoczylas 1978, Taylor and Milthorpe 1980, Paul 1982, Roser 1980).

Ethidium Bromide/Mithramycin (Taylor and Milthorpe 1980).

Euchrysinine-2GNX (E-2GNX) [3,6-diamino-2, 7-dimethyl-9,methyl acridinium chloride] - Acridine Orange derivative (Daley 1979, Jones 1974).

Hoechst 33258 [Bisbenzimide,2'-(4-hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5'-bi-1H- benzimidazole trihydrochloride pentahydrate] - binds A-T rich sequences of DNA, useful in chromosome staining (Bergstrom et al 1986, Sieracki et al 1985, McCoy and Olson 1985, Paul 1982).

Hoechst 33342 [Bisbenzimide,2'-(4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5'-bi-1H-benzimidazole trihydrochloride trihydrate] - binds A-T rich sequences of DNA (Yentsch et al 1983, Taylor and Milthorpe 1980, Paul 1982).

Mithramycin - a fungal antibiotic which requires guanine residues in the DNA (Coleman 1980, Yentsch et al 1983, Taylor and Milthorpe 1980).

Propidium Iodide (PI) [3,8-diamino-5-[3-(diethylmethylammonio)propyl]-6-phenylphenanthridinium diiodide] - intercalates with double stranded DNA and RNA (Porter and Feig 1980, Roser et al 1984, Taylor and Milthorpe 1980, Paul 1982).

Other fluorochromes include auramine (Zimmerman and Meyer-Reil 1974), berberine sulphate (Zimmerman and Meyer-Reil 1974), coriphosphine (Chrzanowski et al 1984), chromomycin (Paul 1982), fluolite XNR (Zimmerman and Meyer-Reil 1974) and 3-o-methyl fluorescein phosphate (Jones and Simon 1975).

Most dyes are relatively large, highly charged and do not readily penetrate viable cells with the exception of Hoechst 33342 and in some cases DAPI. Different staining procedures may facilitate rapid dye penetration. DNA fluorochromes can be ranked by their relative fluorescence: DAPI > EB/Mithramycin > Hoechst 33342 > Mithramycin > EB > AO > PI (Taylor and Milthorpe 1980).

3. EQUIPMENT

3.1 Microscopes

3.1.1 General

The microscope used must be equipped for epifluorescence. It is therefore essential that

the optics of the microscope do not absorb or autofluoresce with the wavelengths of light involved with the fluorochrome in use (Porter and Feig 1980). The type of microscope used is therefore not so critical as the objective lens, excitation and barrier filters, or the light source used. Daley (1979) stated that fading and poor contrast could usually be corrected by minor light filter changes.

Due to the size of aquatic bacteria, the resolution of direct counting is limited by the light microscope (Bowden 1977). Recommended magnification of a water sample ranges between 1000X (Peele and Colwell 1981) and 1562.5X (McCoy and Olson 1985) according to different workers. However the average is around 1250X (Bergstrom et al 1986, Porter and Feig 1980, Ferguson and Rublee 1976, Jones 1974, Paul 1982). The relative size of bacteria in waters and those found in richer nutrient environments, such as wine, can be visualised since the latter require a magnification of only 390X (Cootes and Johnson 1980).

3.1.2 AO studies

The range of microscopes used varies and there have been few comparative studies. Zeiss microscopes have frequently been used with AO (Newell and Christian 1981, Peele and Colwell 1981, Fry and Davies 1985, Porter and Feig 1980, Francisco et al 1973, Zimmerman and Meyer-Reil 1974, Tabor and Neikof 1982, Watson et al 1977, Robarts and Sephton 1981). Nikon Optiphot (Bergstrom et al 1986), Leitz Ortholux (Bowden 1977), Leitz Orthoplan (Daley and Hobbie 1975, Jones 1974, Quinn 1984) and Olympus BHS (Chrzanowski et al 1984, McCoy and Olson 1985) microscopes have also been used with AO and its derivative E-2GNX.

3.1.3

DAPI studies

With DAPI, Zeiss microscopes have again been used successfully (Coleman 1980, Porter and Feig 1980, Tabor and Neikof 1982, Robarts and Sephton 1981). However, the most recent use of DAPI in 1985 compared the Olympus BHT-F, Olympus Vanox, Zeiss Standard 14 and Zeiss Photoinvertoscope IM35 microscopes. The Zeiss IM35 and Olympus BHT-F with their own objectives gave statistically similar counts. However, compared with the Zeiss 14 the Olympus BHT-F gave significantly higher counts using either the Olympus or Zeiss objectives. The aims of this study were directed towards television image analysis and so different objectives, light sources and video camera tubes were also under test. The Olympus BHT-F microscope was finally selected for use. The Olympus Vanox failed to detect bacteria by image analysis, but this may have been due to a less sensitive camera tube in use (Sieracki et al 1985).

3.2 Light sources

3.2.1

AO studies

Jones (1974) suggested that with AO and E-2GNX, results were dependent on the experimental procedure and light source used. Both the intensity and type of illumination appear to affect the fluorescence of a sample. The range of lamp intensities chosen lies between 50 and 200W. The type of lamps used include tungsten, mercury, xenon and quartz iodide. The choice of light source is dependent upon the excitation spectrum of the fluorochrome in use. High pressure mercury vapour lamps have a strong emission at certain wavelengths, eg 336 nm, 405 nm and 435 nm. In contrast xenon lamps give light of a more uniform spectral distribution (Pettipher 1983).

Daley and Hobbie (1975) compared four lamps when using AO; a 100W tungsten halogen lamp, 150W high pressure xenon lamp and two ultra high pressure mercury lamps (50W and 200W). They concluded that the three high intensity lamps gave identical counts with the halogen lamp giving counts lower than controls. Of the three high intensity lamps the 200W mercury lamp was recommended for use with AO. Daley (1979) found AO counts higher than E-2GNX when using a mercury lamp. Jones (1974) found the opposite using a xenon lamp which produced a brighter image than a mercury lamp, although excitation in this case was through two additional heat filters (cutoff at 700 nm). Porter and Feig (1980) found excitation of AO at 490 nm (with a quartz halogen lamp) gave greater fluorochrome stability.

3.2.2
DAPI studies

With DAPI, mercury lamps are generally used since they produce more of the ultraviolet wavelengths necessary for excitation. A 75W xenon and a 100W mercury lamp were used with DAPI by Porter and Feig (1980). Robarts and Sephton (1981) used a 50W mercury lamp and Sieracki et al (1985), comparing microscopes, used three mercury light sources (50W, 100W and 200W). The last of these was selected for the subsequent experiments.

3.2.3
Other fluorochromes

PI and EB experiments have used low intensity (50W) lamps of either the mercury vapour or quartz iodide type (Roser et al 1984, Roser 1980).

3.3
Objective lenses

All the objectives used for the enumeration of bacteria in waters are of 100X magnification, usually oil immersion types and with a numerical aperture of between 1.25 and 1.35.

3.3.1
AO studies

Objectives used with AO include:

100x/NA 1.3 Neofluar oil immersion objective (Peele and Colwell 1980, Francisco et al 1973, Robarts and Sephton 1981).

100x/NA 1.25 Planachromat oil immersion objective (Fry and Davies 1985, Porter and Feig 1980).

100x/NA 1.3 NPL oil immersion objective (Daley and Hobbie 1975).

100x/NA 1.35 Zeiss Planachromat oil immersion objective (Niekof and Tabor 1982).

100x/NA 1.3 glycerol objective (Bergstrom et al 1986).

A water immersion fluorite objective (Jones 1974).

3.3.2
DAPI studies

For DAPI, Porter and Feig (1980) selected the Neofluar 100x/NA 1.3 oil immersion objective without flat field correction, because it possessed less lens coatings, and was therefore optically more transmissive than the Planachromat 100x/NA 1.25 oil immersion objective, which absorbed the excitation range for DAPI. The Neofluar 100x/NA 1.3 oil immersion objective has been used successfully elsewhere with DAPI (Sieracki et al 1985, Tabor and Neikof 1982, Paul 1982). Sieracki et al (1985) recommended the use of a silicone oil immersion objective (SIFL 100/1.25F), with the internal diaphragm open, in preference to the UVFL 100x/NA 1.35 oil objective, as this latter possessed greater field curvature.

3.3.3

Immersion oils

The immersion oil selected for epifluorescence microscopy must be of very low fluorescence. The degree of natural fluorescence induced in the immersion oil will be influenced by the excitation wavelength required for the specific fluorochrome in use, and the intensity and type of light source used. The observations below are therefore general, since specific experimental differences have not been taken into consideration.

Cargille type A immersion oil has been widely chosen because of its optimal contrast (Zimmerman et al 1978, Chrzanowski et al 1984, Hobbie et al 1977, Taylor and Milthorpe 1980, Watson et al 1977). This immersion oil was found to have equivalent performance to Nikon immersion oil, whereas Leitz, Zeiss and Cargille DF and FF oils were found to be less satisfactory (Zimmerman et al 1978). Quinn (1984) recommended the use of Cargille type LF immersion oil, as did Watson et al (1977).

3.4 Membrane filters

3.4.1

General characteristics

To provide satisfactory and reproducible results the membrane filters for total counts of bacteria must comply with several criteria. All bacteria must be trapped on the surface of the filter and lie in one plane, to avoid the need for excessive refocusing. The pores of the filter must be uniformly distributed to ensure there are no regions devoid of cells. To obtain maximum contrast between the filter and the bacteria it may be necessary to reduce the natural fluorescence of the filter by a dying procedure. The observations made here are generally irrespective of fluorochrome used.

3.4.2

Types of filters used

The unique features of polycarbonate filters were first realised in the early 1960s (Fleischer et al 1964). Nuclepore polycarbonate filters have since been widely used in epifluorescence microscopy counting of aquatic bacteria (Zimmerman et al 1978, Bjornsen 1986, Bowden 1977, Newell and Christian 1981, Daley and Hobbie 1975, Hobbie et al 1977, Jones and Simon 1975, Zimmerman and Meyer-Reil 1974, Tabor and Neikof 1982, Watson et al 1977, Quinn 1984, Robarts and Sephton 1981).

Daley and Hobbie (1974) and Ferguson and Rublee (1976) used Sartorius black cellulose membrane filters (0.45 µm pore size) in their experiments. They gave the brightest bacterial fluorescence with darkest background. With white membrane filters bacteria were not seen at all. The Millipore HA black filters (0.45 µm) recommended by Francisco et al (1973) and Jones (1974) were found to have increased background fluorescence following a change in the manufacturing process. Millipore HA black filter surfaces were also more irregular than Sartorius membranes, requiring constant refocusing of the microscope, but were better than the Gelman equivalent. The 0.45 µum pore size was found inadequate to retain bacteria quantitatively on the filter surface. Francisco et al (1973) found 0.2 µm pore size membranes frequently gave higher counts than black 0.45 µm, despite higher background fluorescence. The count on the 0.45 µm membrane averaged about 86% of that using the smaller pore size.

Daley and Hobbie (1974) found that on polycarbonate filters cell distribution and background colour were variable, with large areas devoid of cells. This gave counts up to 60% lower than Sartorius cellulose filters. They noted, however, that recent studies had found 0.2 µm Nuclepore

polycarbonate filters gave counts twice those on 0.45 µm cellulose filters, as no bacteria were trapped within the membrane.

Jones and Simon (1975) obtained highest counts using polycarbonate filters, despite their higher background fluorescence. With cellulose filters they recognised an apparent bacterial/membrane interaction, a decrease in count per unit volume being found with an increase in volume filtered. This was thought to be due to cell penetration into the filter or cell lysis. The effect was not seen when polycarbonate filters were used.

Hobbie et al (1977) found that several drops of a 0.5% surfactant solution above and below the filter decreased hydrophobic areas devoid of cells, although it did increase background fluorescence. Daley (1979) quoted a loss of between 20 and 50% of the bacteria within the thick porous body of a cellulose filter when compared with a polycarbonate membrane of the same pore size. He also reported a change in manufacturing processes so that hydrophobicity of earlier studies was no longer a problem. Consequently the use of surfactants was no longer necessary.

Bowden (1977) found counts on Nuclepore 0.2 µm polycarbonate filters were significantly higher than those on Sartorius 0.2 µm black cellulose filters. AO direct counts on polycarbonate filters were found not to be significantly different to SEM direct counts using polycarbonate filters with numbers of bacteria ranging between 1 and 10 million. However, significant differences were found with the cellulose filters, when counts with SEM and epifluorescence microscopy were compared. These differences were therefore attributed to the filter type.

Zimmerman et al (1978) compared 0.1 µm pore size polycarbonate filters of 5 µm thickness with the more usual 0.2 µm pore size filters in the same experiments. The former were indicated to be better for the retention of very slender rod-shaped bacteria which scanning electron microscopy had shown could pass through 0.2 µm filters. In terms of total biomass, however, this could be ignored unless very oligotrophic water samples were being concentrated. Flow rate through the 0.1 µm filter was effectively reduced by a factor of three compared with the larger pore size.

In experiments to determine numbers of respiring bacteria, Tabor and Neikof (1982) found that Nuclepore polycarbonate filters interfered with the recognition of small INT-formazan deposits. However, Quinn (1984) found that this was related to incubation time. He used 0.2 µm polycarbonate filters stained black with Dylon in INT reduction experiments, and 0.2 µm black nitro-cellulose filters for direct counts.

Zimmerman et al (1978) selected polycarbonate filters for experiments to determine numbers of respiring bacteria. The wetting characteristics of each batch of filters were checked. It was noted in these experiments that adsorption of fluorochromes (in this case AO) to the filter material was a major problem since it decreased contrast. To minimise this effect and enhance contrast two possibilities were considered; the removal of excess fluorochrome with a rinse of iso-propanol (as used by Zimmerman and Meyer-Reil 1974), or prestaining the filters with black dyes (Daley and Hobbie 1977, Jones and Simon 1975). The latter approach was adopted since the former removed the formazan deposits which indicated bacterial activity.

3.4.3
Staining of polycarbonate filters

Zimmerman et al (1978) used filters stained for one day in Sudan black B (1:15,000 w/v) in 50% ethanol, the dye being initially dissolved in 100% ethanol. Filters were subsequently rinsed prior to use. Immersion of the filter in colourless compounds was also reported to saturate active absorption sites of the polycarbonate.

Jones and Simon (1975) recommended the use of Dylon Ebony Black No 8 for polycarbonate filters to reduce their fluorescence. Dye (0.25 g) and 0.25 g NaCl were dissolved in 100 ml of boiling water, the membranes were stained at 60-70 °C for 5 minutes and dried prior to use. Serilene Direct Black CR and Serilene Direct Black BG were also tested. The Dylon staining was reported to give lower control counts, good contrast and lower background fluorescence than other procedures. It was recommended that routine dyeing of membranes should be accompanied by careful examination for even filtration.

Fry and Davies (1985) selected 0.2 µm polycarbonate filters (Unipore) which were prestained in a solution of 6 mg/ml Lanasyn brilliant black A for three minutes.

Irgalan Black (Acid Black No 107 Giegy Corp) staining of Nuclepore polycarbonate filters is the method most widely used to decrease background fluorescence (Bjornsen 1986, Bratbak 1985, Newell and Christian 1981, Chrzanowski et al 1984, Peele and Colwell 1981, Hobbie et al 1977, Daley 1979, Porter and Feig 1980, Sieracki et al 1985, McCoy and Olson 1985, Meyer-Reil 1978, Tabor and Neikof 1982, Watson et al 1977, Robarts and Sephton 1981). The stain is usually prepared at a concentration of 2 g/l in 2% (v/v) acetic acid solution. After staining, filters are rinsed in filter-sterilised

distilled water prior to use. The recommended staining times varied from a minimum of 5 minutes (Newell and Christian 1981). Hobbie et al (1977) recommended staining for 2-24 hours, and Porter and Feig (1980) for 12 hours to decrease natural fluorescence. Bjornsen (1986) used this staining procedure specifically for television image analysis.

3.4.4 Improving bacterial distribution

To aid the random distribution of bacteria over the surface of the filter it has been suggested that the 0.2 µm filter should be overlain on a fine mesh or large pore filter. A 5 µm nylon mesh was suggested by Newell and Christian (1981). Variation in the wetness of a filter can result in a decrease in reproducibility (Daley and Hobbie 1975). To minimise this the use of a 0.8 µm Millipore filter was suggested as a backing pad on the stainless steel filter support. Hobbie et al (1977) suggested a cellulose filter or fine nylon netting. Porter and Feig (1980) used a 0.45 µm Millipore filter on glass filtration bases. Likewise a 0.45 µm Gelman GN6 support filter was chosen by Sieracki et al (1985). Rodrigues and Kroll (1985) replaced stainless steel bases with glass frit bases.

3.5 Mounting of filters

Membrane filter mountants must essentially possess the same fluorescent characteristics as immersion oils. However other factors must be considered as the mountant is in direct contact with the sample and filter. The mountant selected may induce quenching of the bacterial fluorescence, or remove the dye used to stain the membrane filter. Alternatively certain mountants can cause the membrane to become transparent which may in turn produce high background fluorescence obscuring the bacteria.

Mountants may include immersion oils and halogenated compounds, or cinnamic aldehyde to attain a structureless bright-field background. Cargille type A immersion oil (refractive index 1.515) is frequently recommended due to its low fluorescence (Newell and Christian 1981, Peele and Colwell 1981). Mounting of AO-stained samples in Cargille type B immersion oil was suggested by Bjornsen (1986) and also by Porter and Feig (1980) for preparations stained with DAPI.

Breath adhesion of the filter to the slide has sometimes been used (Sieracki et al 1985, Pettipher 1983). Prior to the application of coverslip, however, a mountant is used. Sieracki et al (1985), using breath adhesion, recommended a drop of Cargille type A immersion oil or silicone (refractive index 1.404), depending on the objective used. The coverslip edges were then sealed with paraffin.

Another consideration is whether to mount the filters wet (moist) or dry, although the degree of drying is difficult to quantify. The application of the filtration vacuum until no surface liquid is visible on the filter is probably the most accurate point at which to attain reproducible dryness of the filter (Newell and Christian 1981).

Bergstrom et al (1986) found that when using AO the application of one drop of non-fluorescent immersion oil to the membrane immediately after sample filtration produced stronger fluorescing images than if the filter was allowed to dry. Daley and Hobbie (1974) advocated the use of non-fluorescent immersion oil as a mountant whilst the filter was still damp (see also Jones and Simon 1975, Robarts and Sephton 1981). Fry and Davies (1985) mounted filters in paraffin oil whilst damp. Francisco et al (1973) recommended the draining of

excess liquid prior to mounting in immersion oil by placing the filter on blotting paper, while Daley and Hobbie (1975) found that complete drying of Sartorius 0.45 µm cellulose filters resulted in no bacteria being visible when stained with AO. A decrease in counts were also observed on drying of filters by Jones & Simon 1975. Hobbie et al (1977) recommended moist mounting in immersion oil with Nuclepore polycarbonate membranes. Alternatively Bergstrom et al (1986) found better results with acriflavine if the filters were allowed to dry prior to mounting in immersion oil.

Zimmerman and Meyer-Reil (1974), using AO, promoted drying of the filter by xylene and iso-propanol rinses prior to mounting in Cinnamaldehyde and Eugenol at a 2:1 ratio.

Wynn-Williams (1985) recommended one of three mountants produced by Citifluor Ltd. Citifluor A19 is a glycerol-based photofading retardant for use with AO (Milgram 1986).

4. AO GENERAL PROCEDURAL DIFFERENCES

The procedures for using AO in direct counting are all generally similar with slight modifications. For example these modifications may include AO concentration, contact time, pH or different rinses.

4.1 Chronological developments

Zimmerman and Meyer-Reil (1974) developed an AO staining procedure from methods of earlier workers such as Strugger (1947). It has however been little used. The AO solution was prepared in a phosphate buffer pH 6.7 at a concentration of 1:10000 (w/v) and a contact time of 3 minutes was used. The stain was then drawn through the filter by vacuum, and 3 ml of iso-propanol applied for two

minutes before 1 ml of xylene for 30 seconds. The membrane was then mounted in cinnemaldehyde and eugenol.

Francisco et al (1973) outlined a staining procedure whereby a sample of 1 ml was diluted with 9 ml of filter-sterilised sample water. The pH was adjusted to 8.3 with bicarbonate, and staining was carried out with 1 ml of 0.1% AO solution prepared in bicarbonate buffer. Filtration was immediately followed by a rinse with filter-sterilised sample water. Jones (1974) found the above concentration of AO relatively high with a final concentration of 100 mg/l. He recommended a final concentration of 5 mg/l with staining for 3 minutes, finding no significant increase in count at higher concentrations. Longer contact times resulted in greater background fluorescence. If sample pH was reduced to pH 3.0, counts briefly doubled then decreased, possibly indicating pH sensitivity of some of the organisms. This effect was not reproducible and spectrofluorimetry found no differences in peak fluorescence (450 and 490 nm) at pH 3, 7, 8 and 9. Therefore the pH was thought to affect the binding capacity and intensity of AO. The method of Francisco et al (1973) was also tested and produced significantly lower counts in 50% of trials, with background fluorescence being greater. Jones found that a pH lower than pH 8.3 resulted in unstable fluorescence in bacteria, although in animal cells and trypanosomes maximum colour differentiation was obtained between pH 1.5 and 3.5.

Jones and Simon (1975) recommended dilution of the sample with filter-sterilised sample water if the bacterial content was high. They also found it necessary, on occasions, to rinse the filter with the same diluent. High AO concentrations were found to induce non-specific binding whereas with

low concentrations fewer bacteria were stained. A final concentration of 10 mg/l and 5 minutes staining time was eventually selected for AO. Bacteria may not take up stains easily, and staining in the sample solution may give different results to staining on the membrane after filtration. Chemical pretreatments which possibly increased cell wall/membrane permeability were tested. Toluene, chloroform and lysozyme increased counts by 85, 14 and 82% respectively, although there were variations between filters used. Pretreatments were found to be variable when staining on the membrane and toluene particularly may induce particles to fluoresce.

Daley and Hobbie (1975) selected the technique modified by Jones (1974) using a final concentration of AO of 5 mg/l with a 3-minute staining period, so as to reduce non-specific binding to particles. The staining was followed by a 5-ml rinse with filter-sterilised sample water. This increased contrast, cell brightness, prevented lysis, and increased counts by 65-90%, especially of 0.2-0.5 µm diameter cocci. In marine samples, rinsing with filtered distilled water was recommended as fluorescence was generally duller, because of an increase in adsorption of organics onto the cellulose filter due to the salt content. The method of Zimmerman and Meyer-Reil (1974) was also tested and found to be inferior. Generally contrast was low and the iso-propanol and xylene destaining rendered bacteria dull orange and the background grey.

Differences have been reported between AO brands (Daley and Hobbie 1977, Francisco et al 1973). Fisher AO contained the highest proportion of insoluble particles and together with Sigma was generally lighter in colour. MCB and Baker products occasionally produced high background

fluorescence and predominantly yellow/green cells. All products gave counts not significantly different to those of BDH and Aldrich.

Hobbie et al (1977) and Daley (1979) selected a final AO concentration of 0.01% with 1 to 2 minutes contact time. This AO direct counting procedure (AODC) was subsequently used as a basic method by many workers with slight modifications (Bergstrom et al 1986, Bjornsen 1986, Newell and Christian 1981, Chrzanowski et al 1984, Peele and Colwell 1981, Porter and Feig 1980, Ferguson and Rublee 1976, Maki and Remsen 1981, McCoy and Olson 1985, Paul 1982, Robarts and Sephton 1981).

4.2
Variations in procedures of earlier studies

Porter and Feig (1980) found with the AODC procedure that fading occurred rapidly within a minute. Sample processing was carried out in a dark air hood so as to reduce the fading due to extraneous light and also to reduce contaminants. An AO stock solution was prepared at a concentration of 0.1 mg/l in filtered sterilised distilled water and was stored for several months in the dark at 4 °C. This was diluted to the working concentration prior to use

To reduce the possible effect of contaminants in the AO staining solution Robarts and Sephton (1981) recommended filtering prior to autoclaving and preservation with 2% formaldehyde at 4 °C.

Chrzanowski et al (1984) used AODC in experiments to determine active bacteria using fluorescein diacetate. The only modification was the addition of 5 ml of filtered distilled water after the 2-minute staining prior to filtration.

Bjornsen (1986) increased staining time to 2-5 minutes and followed filtration with five 1-ml

water rinses. He also recommended a sample volume of 1-2 ml.

Bergstrom et al (1986) found high concentrations of humic matter in waters caused precipitation which obscured the bacteria. This was avoided by staining bacteria on the membrane after filtration. The AO staining solution was prepared in citrate/HCl buffer at pH 4.0. A final AO concentration of 0.5 mM gave highest counts. The 0.05 mM concentration produced low counts with weak fluorescence and 5 mM also gave lower counts due to an increase in background fluorescence. Highest counts for humic waters were obtained using 3 minutes contact time, with no significant increase after 10 minutes contact.

McCoy and Olson (1985) used the AODC technique modified in three respects. Firstly, like Bergstrom et al (1986), staining of bacteria was carried out on the membrane filter surface. Secondly, the AO staining solution was made up to 0.01% (w/v) in pH 7.4 sodium phosphate buffer. Finally, after staining for one minute, 1 ml of phosphate buffer was rinsed through the filter.

Other methods for direct counting with AO have also been outlined (Zimmerman et al 1978, Fry and Davies 1985, Kronvall and Myhre 1977, Tabor and Neikof 1982, Watson et al 1977, Quinn 1984).

The method of Zimmerman et al (1978) is analogous to the technique of Zimmerman and Meyer-Reil (1974), in that the AO staining solution is prepared at the same concentration (1:10000 w/v) in a phosphate buffer (pH 6.7; 6.6 mM). Contact time was for 2 minutes on the membrane surface, but with none of the subsequent rinses used by Zimmerman and Meyer-Reil.

Fry and Davies (1985) selected the same AO concentration and staining time as that recommended by Jones (1974) (5 mg/l; 3 minutes). Before staining, the samples were diluted to a volume of 10 ml with filter-sterilised distilled water, and after staining and filtration the same diluent was used to rinse the membranes.

Quinn (1984) chose the staining procedure of Jones (1979). A 20-ml sample was stained with 0.2 ml of AO to give a final concentration of 10 mg/l. This was then shaken for 5 minutes and 6 ml subsampled for filtration.

Kronvall and Myhre (1977) described differential staining of bacteria at low pH with AO in clinical specimens.

Watson et al (1977) used sea water samples for direct counts and biomass estimates. A 0.1% AO (Sigma) stock solution was prepared in 0.02M Tris buffer at pH 7.2. This was added to barium carbonate neutralised samples to give a final AO concentration of 0.01%, staining was carried out for 3 minutes. Other uses of AO with bacteria have been in autoradiography (Meyer-Reil 1978) and flow cytometry (Taylor and Milthorpe 1980). The effect of pH on AO has also been noticed with plant root cortex cells (Roser 1980). Although diverging from bacterial direct counting these other uses of AO may have useful applications.

4.3 Metachromatic staining of AO

4.3.1 Molecular binding

The metachromatic staining of AO is based on its molecular staining characteristics. The excitation wavelength of AO is reported to be 470 nm with a

range of 450-490 nm (Pettipher 1983). Porter and Feig (1980) stated the excitation wavelength of AO to be 436 or 490 nm. Fluorescence of the bound monomer is green at a wavelength of 525 nm with the polymer being red, 650 nm. Electrostatic binding of the basic dye to acidic phosphate occurs with RNA and acidic polysaccharides. Polyanion binding to acidic groups may enable dye/dye interaction forming dimers and polymers accountable for RNA staining. Intercalation into the double helix of DNA between base pairs of the two polynucleotide chains occurs. Negatively charged phosphate groups, electrostatic binding and reactions with bases have been implicated. Generally the DNA molecule is thought to be distorted allowing AO intercalation. This gives rise to orthochromatic green fluorescence. RNA binding with its dye/dye interaction leads to metachromatic red fluorescence.

4.3.2
AO and its link with viability

With mammalian cells there is a general agreement that RNA fluoresces orange/red and DNA green. The orange/red fluorescence is masked by the stronger green DNA fluorescence (Hobbie et al 1977, Pettipher 1983).

In bacterial cells, however, there is no general concensus. Earliest studies stated that the living/dead differentiation was unreliable. Jones (1974) and others found viable bacteria to be green and non-viable orange/red. He found colour to be a reflection of AO concentration and contact time. Pettipher et al (1980) and Hobbie et al (1977) found the opposite to be true.

Pettipher (1983) found that viability was only reflected when the intracellular AO concentration was sufficiently high. Viable cell membranes retard dye penetration whereas non-viable ones

present no barrier. If intracellular AO concentration is high in viable cells then RNA fluorescence is orange/red. If it is low only a small proportion binds to RNA, and then, like DNA, it fluoresces green. Under these conditions viable cells are green. The percentage of green cells increases at low AO concentrations but also at high phosphate buffer concentrations. This may account for results of many workers such as Jones (1974), Quinn (1984) and Daley and Hobbie (1975).

Francisco et al (1973) found green or red fluorescence irrelevant in counting of bacteria and questioned its link with viability. The orange/red shift in highly degraded cells was not definitely linked to viability. Pure cultures of Klebsiella, Escherichia coli and Bacillus subtilis were green and red, while after autoclaving, boiling, formalin or mercury treatment all bacteria were green. During growth experiments the green to orange/red ratio was high in the exponential phase and decreased in the stationary and death phase with E. coli.

Jones (1974) counted green bacteria only and found colour variations to reflect concentration and contact time of AO rather than viability. Daley and Hobbie (1975) also counted green bacteria only. AO of different manufacturers possessed varying colour characteristics. MCB and Baker products produced stains with high background fluorescence and yellow/green cells. They also found that bacteria need not be alive to exhibit green fluorescence but have to be counted in a moist condition.

Hobbie et al (1977) found aquatic samples to contain 95% green bacteria, the rest being red/yellow, reflecting the relative inactivity due to low nutrient concentrations. Green bacteria

appeared easier to count due to their better contrast. Heating of the sample to boiling prior to staining or treatment with acids, drying or organic solvents produced red bacteria. This was due to the breakdown of DNA into the single-stranded form which therefore stained like RNA. Recombination of the DNA was possibly prevented by the aldehyde used in sample fixation. Fixation did not affect the nucleic acids in an untreated sample and therefore viability could be determined after cell death.

4.3.3
AO viability and respiration experiments

Experiments to determine the proportion of respiring bacteria by the reduction of INT to INT-formazan by the electron transport system, found bacterial aggregates well-suited to examining the metachromatic effect of AO. Generally yellow fluorescence of AO prevailed in respiring bacteria containing red formazan deposits. However green AO fluorescence was seen in both formazan positive and negative cells (Zimmerman et al 1978). In similar experiments to determine substrate responsive bacteria in marine samples, the majority of cells were small, coccoid, free-living and green (Peele and Colwell 1981). Quinn (1984), using nalidixic acid to show metabolising bacteria, found 62% of enlarged (nalidixic acid positive) cells after 12 hours incubation to be green. However Kogure et al (1979) reported finding all enlarged cells orange/red.

4.3.4
The Direct Epifluorescence Filter Technique (DEFT)

The DEFT was outlined by Pettipher and co-workers (Rodrigues and Kroll 1985, Pettipher 1983, Pettipher and Rodrigues 1981, 1982a, 1982b). Concentration of AO, contact time and rinses affect the intensity and contrast of the fluorescence.

Maximum AO staining occurs at around neutral pH with maximum visible fluorescence in the acid pH range. Therefore staining at pH 6.6 followed by a rinse at pH 3.0 will attain optimum fluorescence. The buffer selected was 0.1M citric acid/NaOH with the staining buffer containing 0.25 g/l of AO. After the sample has been drawn through the membrane filter, 2.5 ml of staining solution are applied to the surface for 2 minutes before reapplying the vacuum. A rinse of 2.5 ml pH 3.0 buffer is then followed by a 2.5-ml rinse of iso-propanol before mounting the membrane in immersion oil. An ethanol rinse was used initially to speed drying of the membrane, enhance contrast and fluorescence and also decrease the rate of fading. This was later replaced by iso-propanol. The fluorescent brightener Tinopal AN (Uvitex) - now no longer available - was reported to increase contrast. The use of detergents has been investigated with the DEFT. For milk samples Triton X-100 pretreatment with the enzyme trypsin is used. However the use of Tween 80 for pretreatment produces micro-organisms which appear larger than if Triton X-100 is used. In samples containing few or no somatic cells Tween 80 is recommended with the DEFT and would therefore be more appropriate with water samples.

Modifications of the DEFT by Rodrigues and Kroll (1985) include the addition of 10 ml of pH 3.0 citrate buffer to the sample during its filtration and also a 2-ml pH 6.6 citrate buffer rinse. These later experiments were designed to increase the selectivity and sensitivity of the DEFT for milk testing by combining it with the Gram stain.

The DEFT was developed in order to differentiate between viable and non-viable cells. Actively metabolising cells fluoresce orange/red whereas

inactive cells are green in milk. This was found to be independent of Gram type. Generally pasteurisation at 63 °C for 30 minutes or at 72.5 °C for 15 seconds produced orange/red bacteria. This was however dependent on organism type.

4.4
Disadvantages of AO

The major difficulties encountered with AO are the distinction of bacteria from various non-living particles, such as clay and detritus, and colloids which have become stained or are autofluorescent. This problem is greatest in nutrient-rich waters containing large amounts of such matter (Porter and Feig 1980).

Paul (1982) found disadvantages in using AO when enumerating bacteria attached to surfaces which also bind the fluorochrome, such as polystyrene. Other macromolecules were found to bind AO; glucosaminoglycans and galactosaminoglycans, liposomes and negative phospholipids (Peele and Colwell 1981, Daley and Hobbie 1975, Hobbie et al 1977, Daley 1979). AO is cationic, allowing binding to polyanions at low sodium concentrations (eg clays and colloidal flocs). More autofluorescent particles in unstained samples are seen with blue excitation than with ultraviolet, and therefore AO may overestimate direct counts. In low salinity brackish waters autofluorescent particles are approximately 10% of the total count. These particles are possibly small phytoplankton, decaying plant matter or silt or clay particles coated in fluorescent material (Paul 1982).

Bergstrom et al (1986) found AO to precipitate dissolved humic matter producing very bright background fluorescence which masked bacteria. This makes direct counting with AO particularly difficult in humic waters. Four general

disadvantages were outlined for AO. Firstly, AO is not strictly DNA specific and may therefore overestimate the count, and autofluorescent particles may enhance this problem. Secondly AO stained preparations at room temperature were found to fade rapidly and needed immediate examination. Thirdly AO is sensitive to changes in test water and procedure, and finally marked differences have been seen with different brands of AO (Bergstrom et al 1986, Daley and Hobbie 1975, Jones and Simon 1975, Paul 1982).

5. USE OF DAPI

5.1 General staining characteristics

The fluorochrome DAPI is a cytochemical probe for nuclear, mitochondrial and chloroplast DNA and for DNA at previously undetected levels (Porter and Feig 1980). It therefore readily reveals the presence of bacteria and is ideal for the detection of bacterial contaminants in eucaryotic cell cultures (Coleman 1980). DAPI is readily soluble in water and is capable of staining in a range of conditions from pH 4-8 binding almost exclusively to DNA. This specificity is also illustrated by the loss of any staining when slides coated with bacteria were treated with calf thymus DNase before staining.

The fluorescence of bound DNA to DAPI is greatly enhanced over that of the dye alone. The intensity of DAPI-stained cells is sufficient to differentiate small aggregates and can be visualised whilst in solution (Coleman 1980). Porter and Feig (1980) reported an improved count of bacteria less than 1 µm in length. An exposure time of 5-30 minutes to the dye was found to be sufficient to ensure penetration into bacterial cells, either living, or freshly killed with standard fixatives. The fluorochrome cannot be

used to differentiate living from dead bacteria since the DNA retains its staining properties. DAPI does not distinguish Gram positive from negative cells and is not able to penetrate spore walls (Coleman 1980).

The DAPI/DNA complex excitation maximum is at 365 nm although a broad band around this wavelength is usually used. This induces bright blue fluorescence of greater than 390 nm with a maximum at 458 nm (Allan and Miller 1980, Coleman 1980, Porter and Feig 1980, Robarts and Sephton 1981). Unbound and non-DNA bound DAPI may fluoresce a pale yellow which may obscure bacteria. In samples containing large amounts of clays, colloids and detritus, DAPI was advocated for increased accuracy as an alternative to AO due to the relatively weak or non-existent fluorescence of the particles (Porter and Feig 1980, Robarts and Sephton 1981). Sustained excitation of the fluorochrome/DNA complex with UV results in fading to a non-fluorescent form (Allan and Miller 1980). According to Porter and Feig (1980) DAPI-stained preparations are stable for up to 3 minutes, although the degree of fading is largely dependent on the lamp type and intensity of excitation used. For sample preparation Porter and Feig (1980) used a darkened air hood and likewise Robarts and Sephton (1981) recommended the processing of samples in the dark.

5.2 Comparison of DAPI with other fluorochromes

DAPI stains are reported to be more consistent than AO with varying handling time (Porter and Feig 1980). No significant difference was found between DAPI and AO total counts for coccoid, filamentous, attached and unattached bacteria. Robarts and Sephton (1981) reported similar findings. DAPI counts according to Tabor and Neikof (1982) were significantly lower than AO counts using the method

outlined by Hobbie et al (1977). Paul (1982), in a comparison of Hoechst dyes for the enumeration of attached and planktonic bacteria found an overall difference of 6% between AO and Hoechst dyes. However DAPI which possesses similar staining characteristics to Hoechst was found not to illustrate this difference. This discrepancy was accounted for by two possibilities; either AO and DAPI are staining non-microbial particles or Hoechst dyes were underestimating the population.

5.3 Non-specific binding of DAPI

DAPI was found not to be as DNA-specific as the fluorochrome mithramycin since with procaryotes their morphology is definable (Coleman 1980). Non-DNA binding of DAPI is well documented.

DAPI binds mucocyst-trichocyst material in some flagellates and binds very lightly to bacterial slime trails. It also binds to polyphosphate granules, producing in this case brilliant yellow fluorescence (Coleman 1980).

Polyanion polyphosphate is a major constituent of the yeast vacuole, and fluorescent objects complexed with DAPI within the yeast vacuole are thought to be polyphosphate granules. High cation concentrations of S-adenosyl methionine (S-AM) are also present in the yeast vacuole and may influence DAPI fluorescence. The relationship between polyphosphates, S-AM and amino acids with DAPI were investigated by Allan and Miller (1980) using spectrofluorimetry and epifluorescence microscopy.

No fluorescent vacuolar bodies (FVBs) were present when using phosphate buffer mounts. However Tris buffer increased the number of FVBs when compared with water. The DAPI staining solution for microscopy was prepared in 0.025M Tris HCl buffered at pH 7. Nuclei were more prominent in the yeast

cells when pretreated with ethanol, ethanol/acetic acid or NaOH. However these pretreatments, and also formalin and HCl, resulted in no FVBs, whereas glutaraldehyde induced an apparent increase in FVB size.

Cells grown on media containing 6% glucose also possessed more FVBs than from 1% glucose-containing media. Glucose + methionine and glucose + phosphate also resulted in an increase in numbers of FVBs. Other amino acids had little or no effect. A glucose/DAPI relationship was also implicated by Kapuscinski and Skoczylas (1978), producing an increase in fluorescent intensity. DAPI and DCI (a DAPI analogue) showed an increase in fluorescent intensity in sucrose solutions (Kapuscinski and Skoczylas 1977).

Short chain polyphosphates (65 units), when complexed with DAPI, resulted in increased fluorescence and a red shift to peak at 526 nm, and a second scatter peak at 369 nm. S-AM was found not to fluoresce with DAPI. Polyphosphate and DAPI fluorescence was confirmed, although fading was rapid and complete. This fading was decreased by the addition of S-AM, producing an increase in fluorescent intensity at 526 nm. Other polyphosphate chain lengths with DAPI also resulted in an increase in fluorescence on the addition of S-AM.

DAPI interacts with the anionic detergent sodium dodecyl sulphate (SDS) to give an increase in fluorescent intensity (Allan and Miller 1980). The increase in DAPI fluorescence with increasing concentration of SDS was also reported by Kapuscinski and Skoczylas (1978). SDS decomposes DNA complexes of various dyes without changing the DNA structure. The DNA/DAPI complex plus SDS produced an increase in intensity for which the

free dye is responsible. SDS inhibits complex formation and addition of DNA to DAPI/SDS results only in a very slow increase in fluorescent intensity. The effects noted here are even more marked with DCI (Kapuscinski and Skoczylas 1978). Another polyanion dextran sulphate was also reported to produce a broad peak of fluorescence at 456 nm with DAPI (Allan and Miller 1980). In contrast the addition of a cationic detergent such as cetavlon to DAPI was not followed by an increase in fluorescent intensity since they are identically charged (Kapuscinski and Scoczylas 1978).

5.4
DAPI - general procedural differences

DAPI stock and staining concentrations vary between research groups and studies. Generally the fluorochrome is prepared in a concentrated form and diluted to a working concentration on a daily basis or as required. DAPI is usually prepared in a solution of filtered sterilised distilled water. Other diluents used include 0.025M Tris HCl (pH 7) (Allan and Miller 1980). The flow cytometry experiments of Taylor and Milthorpe (1980) used 0.1M Tris HCl/NaOH buffered at pH 7.4 with DAPI at a concentration of 30 mg/l). McIlvaine's buffer (pH 4) was used by Coleman (1980) with DAPI in a range of final concentrations from 0.1 to 0.5 µg/ml.

Porter and Feig (1980) prepared a DAPI (Boehringer) stock solution of 1 mg/ml which could be stored indefinitely at 0 °C. A dilution of this was made to 0.1 mg/l, and this was stable at 4 °C for several weeks. The final concentration used for staining was 0.01 µg/ml, and this was allowed to act for 5 minutes. There was no significant increase in counts using periods longer than 5 minutes, but 2 minutes was insufficient to provide visible fluorescence.

Robarts and Sephton (1981) proposed that a 1 mg/ml stock solution stored in the dark at 0 °C could only be kept for a maximum of two months due to stain deterioration. Staining was performed for 5 minutes in 2 ml samples at a range of concentrations from 0.01 to 0.1 mg/l, with 0.05 mg/l being the adopted final concentration. Using the final concentration selected by Porter and Feig (1980) (0.01 mg/l), Robarts and Sephton found results significantly lower than those obtained by AO.

6. ETHIDIUM BROMIDE AND PROPIDIUM IODIDE

Roser (1980) and Taylor and Milthorpe (1980) selected these fluorochromes for study. The latter workers used eucaryotic cells including leukaemic T cells and blood lymphocytes. PI and EB were prepared at 50 mg/l in a solution of 0.034M trisodium citrate.

Roser (1980) recognised the importance of EB as a cytological stain for cell chromosomes, and found it useful with fungi, and also for detecting killed mammalian cells. Other applications of the fluorochrome had been to measure the DNA of the microbial biomass in soil and dental plaque. EB was very stable when prepared in aqueous solution and could be stored for several months in the dark at 4 °C without any decrease in staining affinity. The stained material fluoresced orange/red when excited by various wavelengths over a wide range. With eucaryotic cells Roser used a range of EB concentrations between 5 and 50 mg/l and staining contact time of between 2 and 10 minutes, followed by a one minute rinse with water. Using procaryotic cells Roser found all except Bacillus sp. endospores stained sufficiently with 50 mg/l for 5 minutes followed by a 1-minute water rinse. Filters were mounted in distilled water or 1% sodium pyrophosphate.

Roser et al (1984) used bacteria with EB and PI to test a method for estimating cell density by measuring the distance of the nth nearest neighbour to the centre of the graticule used. E. coli cells suspended in 25 g/l sterile saline were stained on the filter surface with 100 mg/l of EB for 12 minutes and mounted in pH 9.0 phosphate buffer. PI was used with bacteria dislodged with Triton X-100 from the surface of Hypholoma fasiculare (a basidiocarp fungus). They were then stained with an aqueous solution of PI at 100 mg/l for 10 minutes prior to washing with 'TEN' buffer.

There have been no comparisons between EB or PI and other fluorochromes for direct counting of bacteria in water. However their application to this microbial ecosystem should present no fundamental changes in fluorochrome staining.

7. IMAGE ANALYSIS

Image analysis of microscopic particles was developed in the 1950s and 60s for the detection of coal particles in air and leucocytes in blood. The first application to epifluorescence microscopy was by Pettipher and Rodrigues (1982a, 1982b), using AO for the detection of bacteria and somatic cells in milk. They found standard plate counts, direct counts and automated counts to correlate well, although clumps of cells were counted rather than individual bacteria. An Optomax System III image analyser was used for the work.

Detection and counting of bacteria in water by image analysis has only been developed recently. Sieracki et al (1985) and also Fry and Davies (1985) used image analysis for aquatic bacteria.

Fry and Davies (1985) used two image analysers, the Quantimet 800 and Q10, the latter capable of

digitising the image into binary form, and also enabled editing. Experiments were aimed at determining cell volumes and found no significant difference between the two systems used. However image analysis of photographs was found to be the easiest method and gave cell volumes similar to those obtained by conventional light microscope methods.

Sieracki et al (1985) used an Artek 810 image analyser to decrease the subjectivity of direct counting bacteria in marine samples. The field of view was scanned and then the image was diverted to the camera. The video image was digitised and stored for later editing using a light pen and electronic enhancement. Analysis for total count or cell size and shape could then be carried out. A predetermined number of fields or cells were counted, and a BASIC program was used to display or print the count, number of fields, mean count, standard deviation, coefficient of variation and number of cells per ml of sample. Of the microscopes tested the Olympus BHT-F was selected for use with a chalnicon camera tube and 100W mercury lamp. Calibration of the image analyser for sizing, etc was by means of a stage micrometer of smallest division 10 µm, using transmitted light.

It was found that with the correct microscope and objective, optimum detection, counting and sizing required a higher concentration of DAPI than used for direct counting. Proper focusing of the 100W mercury lamp was found to be essential. The overall time reduction in automated counting over direct counting was estimated at 85%.

Bjornsen (1986) used a more sophisticated image analyser, the IBAS, capable of differentiating particles according to their size, so that large

debris particles could be "sieved" out of the digitised image. Sophisticated enhancement, contrasting and editing was possible, with three video images stored in memory so that alterations could be easily monitored. Calibration and testing for biovolume estimates used fluorescent latex beads of known size and cultured bacteria. The system is capable of measuring 250 cells in 10 minutes for cell number, biovolume, and mean volume with errors at about 5%. Precision was found to be greater than for manual methods.

8. DISCUSSION

Due to the variations in procedure and methods of counting bacteria used by different workers in this field, conclusions are difficult to draw. The aims of any experiment and peculiarities about the sample must be considered when trying to devise an optimum procedure for epifluorescence microscopy.

This review merely outlines the alternatives used within the confines of bacterial counting and epifluorescence microscopy. Findings from other fields have also been considered since effects on staining, for example, may be relevant to this study. General conclusions can be drawn, such as the use of polycarbonate filters in preference to any others due to their generally accepted superiority. Other findings may only be of importance to a specific study, and therefore from the tested procedures and equipment outlined, these can either be simulated or avoided.

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APPENDIX B

FORMULATIONS OF CULTURE MEDIA

0.1% Sodium lauryl sulphate medium

Peptone (Oxoid)	40 g
Lactose	30 g
Yeast extract	6 g
Sodium lauryl sulphate	1 g
Phenol red	0.2 g
Distilled water to	1 litre

pH 7.4 Sterilise at 105 °C for 10 min.

Diluted SLS medium (DSLS)

Peptone (Oxoid)	20 g
Lactose	15 g
Yeast extract	3 g
Sodium lauryl sulphate	1 g
Phenol red	0.2 g
Distilled water to	1 litre

pH 7.4 Sterilise at 105 °C for 10 min.

CM medium

Proteose peptone No 3	10 g
Yeast extract	6 g
Lactose	20 g
Bile salts No 3	1 g
Sodium lauryl sulphate	1 g
Sodium deoxycholate	0.1 g
Bromocresol purple	0.035 g
Distilled water to	1 litre

pH 6.8 Sterilise at 121 °C for 15 min.

Anderson medium

Proteose peptone	20 g
Lactose	5 g
Sodium chloride	5 g
Sodium dihydrogen phosphate	1 g
Bile salts No 3	1 g
Sodium lauryl sulphate	0.3 g
Bromocresol purple	0.02 g
Distilled water to	1 litre

pH 6.8 Sterilise at 115 °C for 10 min.

M20 medium

Lactalbumin hydrolysate	5 g
Yeast extract	2.5 g
Lactose	10 g
Sodium chloride	5 g
Bile salts No 3	1 g
Bromocresol purple	0.02 g
Distilled water to	1 litre

pH 6.8-6.9 Sterilise at 121 °C for 5 min.

Modified M20 medium

Lactalbumin hydrolysate	5 g
Yeast extract	2.5 g
Sodium chloride	5 g
Sodium lauryl sulphate	0.4 g
Distilled water to	1 litre

pH 6.8-6.9 Sterilise at 121 °C for 5 min.

APPENDIX C

A BIBLIOGRAPHY OF REPORTS ON STORAGE AND PRESERVATION OF WATER SAMPLES

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