Evaluation of Selected Membrane Filtration and Most Probable Number Methods for the Enumeration of Faecal Coliforms, *Escherichia coli* and Enterococci in Environmental Waters

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Abstract. Selected methods recommended in national and international water quality guidelines were compared in tests on environmental waters with different levels of faecal pollution. The following methods yielded no statistically significant differences in counts of faecal coliforms and *Escherichia coli* in raw sewage, semi-treated effluent, polluted urban run-off and stored potable water: Membrane filtration (MF) using MFc Agar or Chromocult Coliform Agar containing X-glucuronide, or a miniaturised microtitre-plate Most Probable Number (MPN) assay using a liquid growth medium containing chromogenic 4-methyl-umbelliferyl-β-D-glucuronide. Significant differences were, however, found between the Chromocult and the other methods for unpolluted river water. Counts of faecal enterococci in raw sewage, semi-treated effluent and polluted urban run-off, obtained by the following methods did not differ significantly: MF using M-Enterococcus Agar, Bile-Esculin Agar or Enterococcus Selective Agar, or a microtitre-plate MPN method with a liquid growth medium containing chromogenic 4-methyl-umbelliferyl-β-D-glucoside. Significant differences were, however, found between the MPN and the other methods for unpolluted river water and stored potable water. MF using Chromocult Coliform Agar had useful benefits for the simultaneous enumeration of coliforms and *E coli*. However, in view of cost and practical considerations, MF using MFc Agar or M-Enterococcus Agar proved the methods of choice for respectively enumerating faecal coliforms and *E coli*, or faecal enterococci, in analyses for general water quality surveillance purposes.

Key words: microbiological analyses methods, membrane filtration, most probable number (MPN), indicator organisms, faecal coliforms, *Escherichia coli*, enterococci, environmental water

1. Introduction

The use of bacterial indicator organisms to assess the microbiological quality of water is well established and has been practised for almost a century (IAWPRC, 1991; Grabow, 1996). The primary objective for using indicator organisms and methods commonly related to their examination is to indicate the degree of water contaminated by faecal wastes (Grabow, 1996; Standard Methods, 1998).

Faecal coliforms are widely used to evaluate the quality of waste water effluents, environmental fresh water sources, sea water at bathing beaches, raw water for drinking water supply and recreational waters (ISO, 1990 and 1995; Standard Methods, 1998). According to the South African Water Quality Guidelines, faecal coliforms indicate the possible presence of pathogens responsible for the transmission of infectious diseases such as gastroenteritis, salmonellosis, dysentery, cholera and typhoid fever (DWAF (Department of Water Affairs and Forestry) 1996). The group consists of members of the genera *Enterobacter*, *Klebsiella*, *Citrobacter*, and *Escherichia* (DWAF, 1996).

For practitioners in the fields of environmental health and engineering operating basic laboratory facilities, the most suitable indicator would be a single organism group requiring a single-step detection method. Such a group would be favoured for the simplicity and reliability (accuracy and selectivity) of the particular detection method. A small laboratory will not have access to the advanced technology and skills required for complex methods of specific indicator detection (DWAF, 1996). Reliable methodologies that can be carried out in a basic facility or are affordable when using analytical contractors are therefore a primary requirement.

Methods generally used for detecting faecal coliforms are not complex and provide relatively quick assessment of faecal water pollution, making it an ideal indicator for use in a basic laboratory. Standard faecal coliform membrane filtration procedures are well developed and are well established and widely used (Standard Methods, 1998). These procedures are relatively simple and can be performed with relative accuracy by a non-expert analyst.

Escherichia coli is a member of the faecal coliform group and is a more specific indicator of faecal pollution than faecal coliforms (South African Bureau of Standards (SABS), 1984; Graboio, 1990; International Organization for Standardization (ISO), 1995). Simultaneous detection of faecal coliforms and *E coli* would, therefore, have practical benefits such as confirming whether faeces from warm-blooded animals had caused pollution of water resources (Grabow, 1996). The presence of *E coli* in water also presents useful indication of the risk of infection to users. However, additional steps are required to detect *E coli* in water samples. Smaller water laboratories in South Africa, while being able to test for faecal coliforms, might not have sufficient facilities for confirming *E coli* (DWAF, 1996). The tendency among non-expert analysts would therefore be to use only faecal coliform detection to assess faecal pollution of surface waters.

Recent developments in chromogenic substrate testing for coliforms have facilitated testing for *E coli* in one simple step by means of a highly selective agar medium (Merck, 1996), to be used with membrane filtration, as well as a miniaturised microtitre plate most probable number (MPN) method for detection of *E coli* (ISO, 1994a; Sanofi[R], 1995).

Although faecal coliforms are generally used as principal indicators of faecal pollution in waters of the target catchment, enterococci were also included in this study. These organisms are excreted in faeces by man and other animals and are useful indicators to complement assessment of faecal pollution of water. Enterococci generally have a longer life span than faecal coliforms in the aquatic environment and can therefore be used to detect faecal pollution for longer periods after pollution incidents (Guillemin *et al.*, 1991). Since the international tendency for assessing the health-related microbiological quality of recreational water is towards the use of enterococci, the South African Water Quality Guidelines (DWAF, 1996) have included enterococci in target water quality guidelines for recreational waters. These organisms also provide useful information regarding the risk of infection posed to recreational users of water.

Various methods based on selective media and membrane filtration can be used to detect faecal enterococci in water. The selectivity of the media used with the various methods appears to be less than ideal (Dionisio and Borrego, 1995). Additional confirmation-methods prescribed by Standard Methods (1998) and ISO (1996) are therefore required. Figueras *et al.* (1996) proposed a fast method to confirm faecal enterococci after initial enumeration with the conventional methods. Such additional steps discourage the use of faecal enterococci in laboratories with limited testing facilities. Reliable, one-step, or abbreviated second-step, direct-testing methods for faecal enterococci would therefore greatly improve the ability of field operators to assess faecal pollution in water resources.

Recent developments such as the one-step Enterococcus Selective Agar[®] (Merck, 1997) method as well as a miniaturised liquid most probable number (MPN) (ISO, 1994b) method are now available commercially (Sanofi[®], 1995). These new methods were included in this study in addition to established enterococci detection methods.

This study compared the reliability and efficiency of the new methods for *E coli* detection with conventional faecal coliform detection methodology as well as those for faecal enterococci.

Materials and Methods

Water types

Samples from different water sources were examined as the reliability of the various detection methods was expected to vary for different levels and types of faecal pollution. Five water types were selected from the Modder River catchment in the Free State Province, South Africa to represent varying levels of faecal pollution: (1) raw wastewater; (2) semi-treated wastewater effluent; (3) polluted urban run-off, (4) river water not subjected to pollution from urban discharges and (5) domestic water supplies stored in household containers.

Colony verification and false positives

Between 12 % and 40 % of all the target colonies cultured on the various media were randomly selected for colony confirmation. Verification was done with multi-test identification system galleries (API® by bioMérieux®). Any colouration caused by the selectivity of the growth medium was removed from the selected colonies to eliminate all possible interference with the functions of the API test strips. This was done by streaking out and growing single colonies from the selective media on plate count agar (Standard Methods, 1998). Colonies were picked up with sterile swabs to exclude possible interference from metal inoculum needles with some of the tests used in the strips. The results obtained from sample analyses of the various waters were adapted to reflect only the true positive numbers detected by the methods.

Positive wells from the MPN method did not require confirmation, as the enzymes in the selective media used in the wells are considered highly selective (Pourcher *et al.*, 1991).

Statistical analyses

Sample size: For this study, 15 samples for each indicator group per water type were used as a crude initial sample size estimate (Standard Methods, 1998). After assessing the first 15 samples, the mean differences of each data set were used to estimate the final minimum sample size (Helsel and Hirsch, 1995). This step was necessary to confirm that sample sizes of 15 per water type were sufficient for statistical analyses in this study.

ANOVA: The Kruskal-Wallis test (Helsel and Hirsch, 1995) was used to compare the data of the various groups. The Tukey multiple-comparison test was used to identify the method that differed significantly from the others (Helsel and Hirsch, 1995; SigmaStat®, 1997).

Faecal coliforms and E coli *enumeration*

Faecal coliform enumeration was carried out by membrane filtration (MF) using MFc Agar (Difco®) according to Standard Methods (1998). After incubation at $44.5\,^{\circ}C \pm 0.2\,^{\circ}C$ for 24 h, colonies exhibiting any shades of blue were counted as faecal coliforms.

Numbers of *E coli* were determined by MF using Chromocult® Coliform Agar containing the substrate X-glucuronide (Merck, 1996). *E coli* possesses the enzyme β-D-glucuronidase, which cleaves the substrate X-glucuronide, causing positive colonies to produce a dark-blue to violet colour (Standard Methods, 1998; Merck, 1996). This one-step identification method for detecting *E coli* by means of a chromogenic response requires little confirmation (Merck, 1996; Lifshitz and Joshi, 1998). After incubation at $37\,^{\circ}C \pm 0.5\,^{\circ}C$ for 24 h, all shades of deep blue-to-violet colonies were counted as *E coli*.

A commercially available (Sanofi®) microtitre-plate most probable number (MPN) method for enumerating *E coli* (ISO, 1994a) was used. The 96-well micro titre plates

containing dehydrated culture medium and chromogenic enzyme substrate 4-methyl-umbelliferyl-β-D-glucuronide (MUG) (Sanofi[®], 1995) specific for *E coli* (ISO, 1994a) were inoculated with appropriate dilutions per sample (ISO, 1994a). The plates were incubated at 44 °C $\pm$ 0.5 °C for a minimum of 36 h and a maximum of 72 h after covering. The numbers of turbid, pale blue fluorescent (positive) wells (detected under a 366 nm UV light) were recorded. A characteristic number of positive dilutions were selected from the recorded readings of the micro titre plate according to ISO (1994a) and the rest discarded. The most probable number of *E coli* present in each sample was then calculated according to Thomas's formula (Standard Methods, 1998) and expressed as organisms per 100 mL. No confirmations were done.

Colonies of faecal coliforms on MFc agar and *E coli* on Chromocult[®] Coliform Agar were selected for confirmation. API[®] 20E test strips were inoculated, incubated and analysed according to the prescriptions contained in the manual provided with the commercial identification kit (bioMérieux[®], 1998). In addition, individual dark blue to violet coloured colonies on the Chromocult Coliform[®] medium were coated with KOVACS' indole reagent as directed in the user manual (Merck, 1996) to confirm *E coli* detection. Cherry-red colouring after some seconds confirmed a positive indole formation and consequently the presence of *E coli*. Quality control tests were done for the various steps as well as on the various batches of test strips using *Klebsiella pneumoniae pneumoniae*, *Proteus vulgaris* and *Pseudomonas aeruginosa* as reference organisms.

Faecal enterococci enumeration

Faecal enterococci were enumerated on M-Enterococcus agar (Difco[®]) according to the Standard Methods (1998) using the MF technique. After incubation at 37 °C $\pm$ 0.5 °C for 44 h, all red, maroon or pink colonies were counted as typical faecal enterococci. The membranes with colonies were then transferred to plates with Bile-Esculin agar (Merck[®]) and incubated for 4 h at 35 °C (Figueras *et al*., 1996). Catalase negative colonies that turned dark brown to black with a typical dark halo (also with colour diffusion into the surrounding media on the membrane) were considered to be faecal enterococci (Standard Methods, 1998; ISO, 1996).

Enterococcus Selective Agar (Merck[®]), a selective medium for faecal enterococci containing bile and esculin, was used with the MF technique as a one-step identification of faecal enterococci. After incubation at 37 °C $\pm$ 0.5 °C for 44 h, all brown to black colonies with a typical dark halo were counted as faecal enterococci (Merck, 1997).

The micro titre plate MPN technique (Sanofi[®]) (ISO, 1994b) method for enterococci, similar to that used as directed for *E coli*, was also used. The micro titre wells contained dehydrated culture medium and the hydrolysable chromogenic enzyme substrate 4-methyl-umbelliferyl-β-D-glucoside (MUD) (ISO, 1994b). After covering, the micro titre plates were incubated at 44 °C $\pm$ 0.5 °C for a minimum of 36 h and a maximum of 48 h. The plates were placed into an UV observation chamber with a wavelength of 366 nm. All wells showing turbidity and blue fluorescence were considered positive and recorded. The most probable number was then calculated (Standard Methods, 1998; ISO, 1994b) and expressed as the number of organisms per 100 mL. No confirmations were done.

Enterococci colonies on M-Enterococcus agar, Bile-Esculin agar and Enterococcus Selective agar were selected for confirmation. The micro-tubes on the prepared API®20 Strep strips (for enterococci testing) were inoculated, incubated and analysed according to the prescriptions contained in the manual provided (bioMérieux, 1997). Quality control tests were carried out using *Streptococcus mutans* and *Enterococcus faecium* as reference organisms.

Results and Discussion

Method selectivity

Table 1 summarises the results from the series of confirmation tests. The various methods yielded considerable percentages of false positives for each organism group. The percentages also varied with the types of water tested. Except for the performance of the enterococci methods in raw sewage and for some enterococci methods in semi-treated effluent, none of the membrane filtration methods produced less than 10 % false positives proposed as the benchmark for indicator method selectivity by Dionisio and Borrego (1995).

Table 1 presents the percentages of true positives for each method and water type. This was done in order to present the percentage true *E coli* tested within the faecal coliform groups. Since the MFc method was not designed to detect only *E coli*, the comparable percentages of true positive *E coli* confirmed within each set of true faecal coliforms are indicated in brackets. The percentages true positive *E coli* confirmed amongst the true positive faecal coliforms indicated that the Chromocult Coliform agar method generally yielded higher numbers of true *E coli* than the MFc method yielded true faecal coliforms or even true *E coli* within the particular faecal coliform group.

The Bile-Esculin confirmation step marginally improved the reliability of the M-Enterococcus result except for poorly treated effluent. The reason for this is unclear. In heavily polluted water, such as raw sewage and poorly treated effluent, the Enterococcus

Table 1. Percentage of true positive indicator organisms detected with various selective methods.

| Indicator groups	Raw sewage	Semi-treated effluent	Polluted urban runoff	Unpolluted river water	Stored potable water
Faecal coliforms on MFc agar	81 % (97)	71 % (79)	88 % (78)	83 % (96)	81 % (10)
E coli on Chromocult Coliform agar	87 %	77 %	78 %	74 %	35 %
E coli with micro titre plate MPN	Confirmation not required				
Enterococci on M-Enterococcus agar	91 %	92 %	82 %	84 %	85 %
Enterococci confirmed on Bile-Esculin Agar	97 %	81 %	85 %	87 %	97 %
Enterococci on Enterococcus Selective Agar	90 %	100 %	77 %	75 %	75 %
Enterococci with micro titre plate MPN	Confirmation not required				
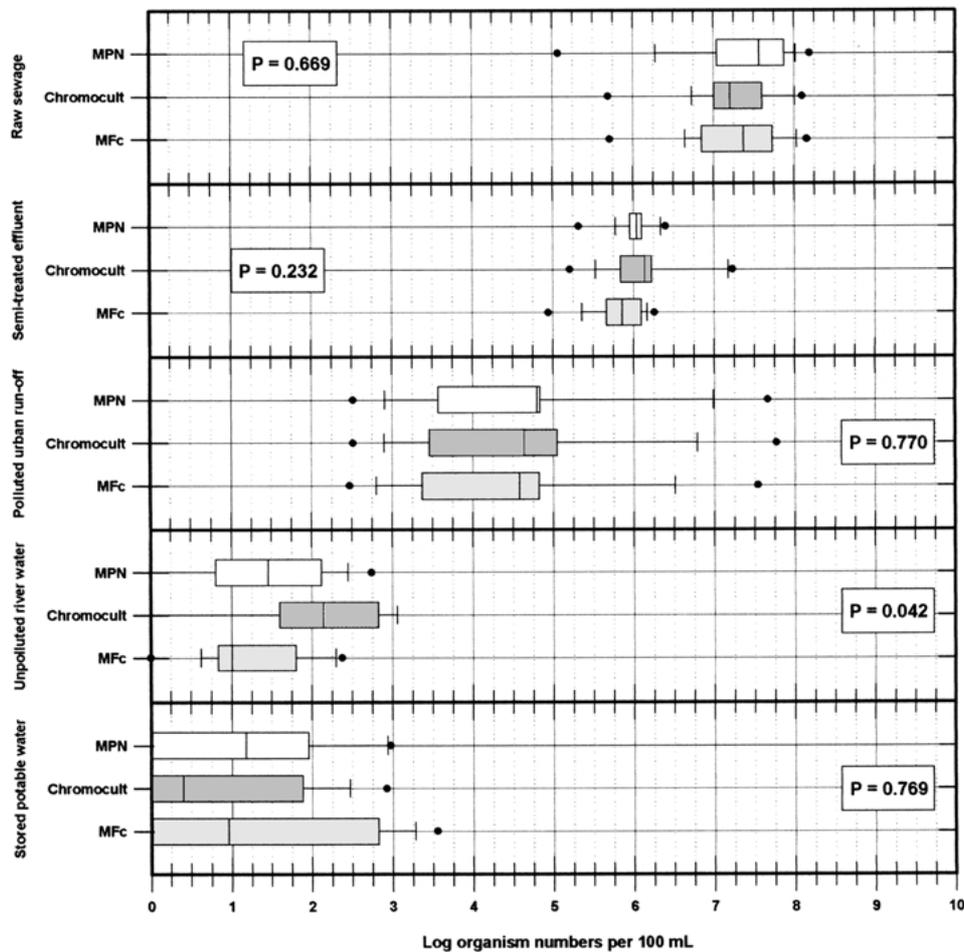
Figure 1. Faecal coliform and *E coli* detection method performance in various water types.

Selective method yielded the highest percentage of true positives but as a single-step method, it did not appear to be as reliable as the two-step M-Enterococcus/Bile-Esculin method.

It is not certain to what percentage of true positive results, or conversely, to what extent the MPN methods may have yielded false positive results, as no confirmation tests were required. The performance of these methods could therefore only be measured against the overall performances of the membrane filtration methods in the following figures. The recovery of faecal coliforms and *E coli* using these methods are compared in Figure 1.

The median values, interquartile ranges as well as the outliers beyond the 10th and 90th percentiles of each box plot show the different magnitudes of faecal coliform and *E coli* concentrations found amongst the water types. There were no statistically significant differences between the three groups except for unpolluted river water, where the

Chromocult Coliform agar method yielded significantly higher results than the other two methods. For effluent, a higher median concentration was found using the Chromocult Coliform agar method, although the difference was not statistically significant.

The MFc medium is not designed to detect only *E coli*, therefore one would expect that the wider spectrum of faecal coliforms in a polluted water sample might produce higher means when used for the same water samples as the two *E coli* methods. However, the Chromocult method still produced higher numbers with less variance, indicating its capability to detect higher levels of *E coli* than the MFc method could detect faecal coliforms. This method is thus more useful for specifically determining *E coli* counts. Based on the levels of false positives for both faecal coliforms and the Chromocult method, the faecal coliform method proved to be a more sensitive method for testing presumptive faecal pollution in water stored at home for domestic use, providing a safety margin when evaluating water resources for untreated potable use.

Figure 2 compares the recoveries of the three methods when the data of the various water types were grouped in polluted (raw wastewater, treated effluent, polluted urban runoff) and unpolluted (unpolluted river water, stored water) water categories.

There were no statistically significant differences between results produced by the three methods. The median values, interquartile ranges as well as the outliers beyond the 10th and 90th percentiles of each box plot indicate considerable fluctuation in the data of the polluted water category. This was due to the variation in quality of the three water types in this category.

Figure 3 compares the four methods used to detect faecal enterococci. For raw sewage, polluted urban run-off and stored potable water, there were no statistically significant differences between the methods at $\alpha = 0.05$. The Enterococcus Selective method had the highest median recovery of enterococci in effluent while for clean river water the MPN method gave significantly different results because of the low numbers recorded.
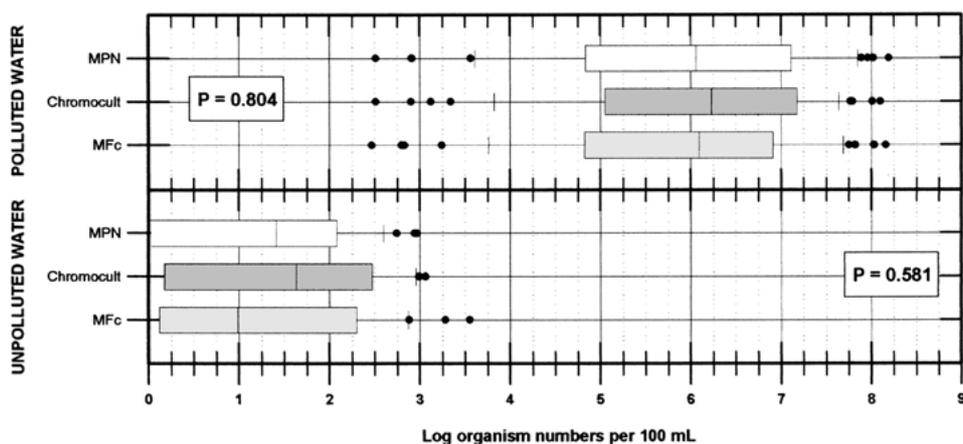

Figure 2.   Faecal coliform and *E coli* detection method performance in polluted and unpolluted water.
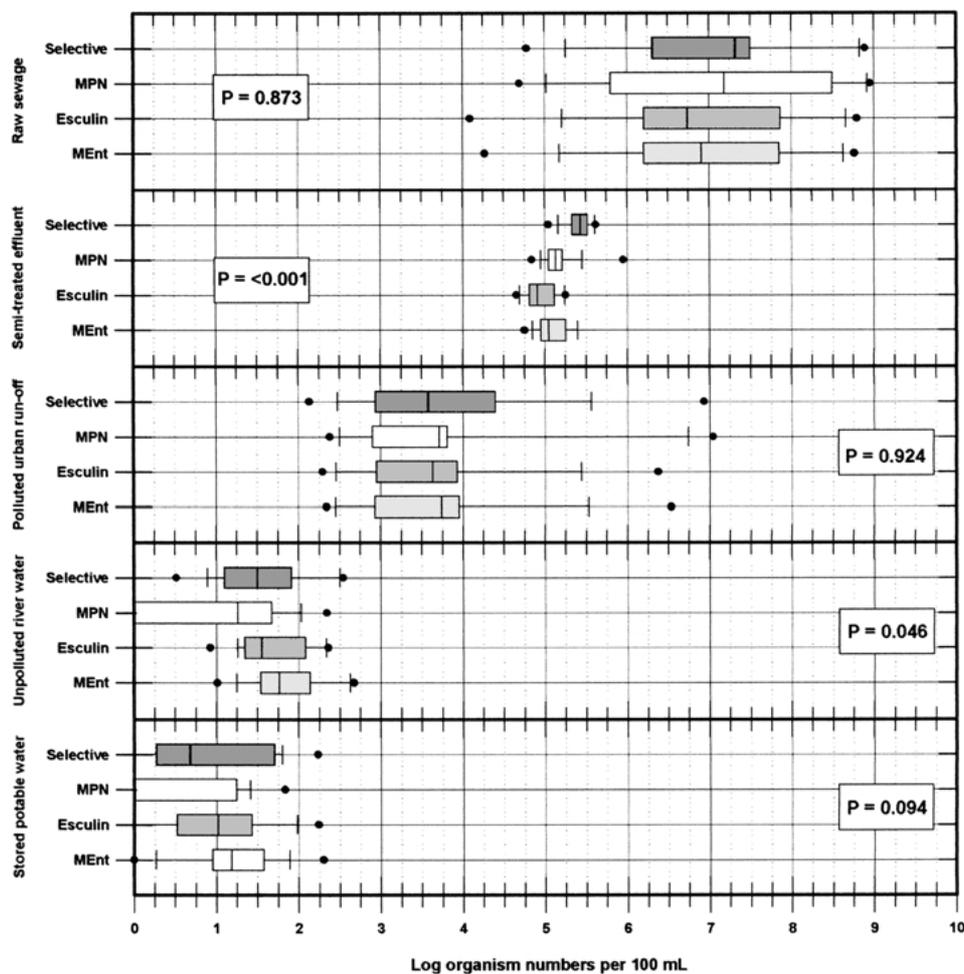
Figure 3. Enterococci detection method performance in various water types.

Although the M-Enterococcus method appeared to yield results quite similar to the Enterococcus Selective method, these results are inflated by false positives (Table 1).

Figure 4 compares counts of faecal enterococci obtained from the grouped polluted water categories by different methods. The results of the Bile-Esculin method have the lower numbers of false positives and should be compared with those of the Enterococcus Selective method instead. These indicate that the Enterococcus Selective method yielded only slightly higher results in polluted water but has the added value of a single step methodology. In polluted water, the MPN method appeared to yield similar results to the membrane filtration methods, but failed to do so in unpolluted water.
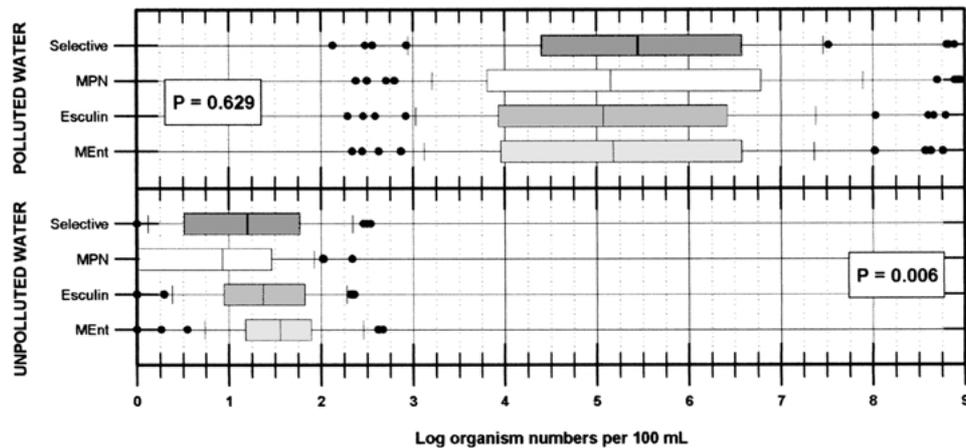
Figure 4.   Enterococci detection method performance in polluted and unpolluted water.

There were no statistically significant differences between the groups in the polluted water category. The Enterococcus Selective method yielded the higher results in a single step. The results yielded by the MPN method for the unpolluted water category were found to be significantly lower than those of the other three methods indicating that this method might not be reliable when monitoring for possible faecal pollution in unpolluted water.

Conclusion

Results obtained in this study indicate that in terms of cost and practical considerations for routine water quality monitoring MF using MFc Agar remains the method of choice for enumerating faecal coliforms in water at a range of levels of faecal pollution. MF using Chromocult Coliform Agar would be the more suitable approach for the specific enumeration of *E coli*.

The latter is more expensive but not excessive when considering that additional *E coli* confirmation steps would add to the cost of the faecal coliform testing method. The Chromocult Coliform method has the added advantage that it can produce total coliform numbers on the same membranes as the *E coli*. This would be a useful tool in instances where monitoring for the larger coliform group may be required.

The MPN method can also be used in the place of the membrane filtration methods. While it appears to be a more expensive method, the cost of membranes, selective media and petri-dishes for the various dilutions as well as the time consumed to do membrane filtration tests, is offset by the neat MPN system which does not require elaborate equipment such as suction pumps and funnels. The enterococci methods could all be used for polluted waters, depending on the purpose of the test. However, for potable water, the traditional M-Enterococcus method with Bile-Esculin confirmation appears to be the more reliable. The MPN should not be used for unpolluted water.

It should be kept in mind that strictly speaking the test methods compared detect different populations of faecal coliforms and faecal enterococci because they are based on the detection of different properties of these organisms. Although this has not been investigated in detail, the results obtained suggest that the populations of organisms detected are closely related and for practical purposes of water quality assessment results obtained by different methods for faecal coliforms and enterococci are comparable.

Acknowledgements

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