ELSEVIER

International Journal of Food Microbiology 40 (1998) 57–64

International Journal of Food Microbiology

An interlaboratory study to find an alternative to the MPN technique for enumerating *Escherichia coli* in shellfish

I.D. Ogden[a,*], G.C. Brown[b], Susan Gallacher[c], P.H. Garthwaite[b], M. Gennari[d], M. Pilar Gonzalez[e], L.B. Jørgensen[f], B.T. Lunestad[g], Marion MacRae[a], M. Celeste Nunes[h], A.C. Petersen[i], J.T. Rosnes[j], J. Vliegenthart[k]

[a]*Department of Medical Microbiology, University of Aberdeen, Foresterhill, Aberdeen, AB25 2ZD, UK*
[b]*Department of Mathematical Sciences, University of Aberdeen, Aberdeen, AB24 3QY, UK*
[c]*Marine Laboratory, SOAEFD, Victoria Road, Aberdeen, AB11 9DB, UK*
[d]*Instituto di Ispezione delgi Alimenti di Origine Animale, Sez. 1, Via Celoria 10, 20133 Milan, Italy*
[e]*Instituto de Investigacions Marinas, r/ Eduardo Cabello, 6. Vigo-36208, Spain*
[f]*MAF, Danish Institute of Fisheries Research, DK-2800 Lyngby, Denmark*
[g]*Central Laboratory, Directorate of Fisheries, Strandgaten 229, 5002 Bergen, Norway*
[h]*Instituto Português de Investigação Maritima, Avenue de Brasilia, 1400 Lisboa, Portugal*
[i]*Cork Regional Technical College, Rossa Avenue, Bishopstown, Cork, Ireland*
[j]*Norconserv, Institute of Fish Processing and Preservation Technology, PO Box 327, N-4001, Stavanger, Norway*
[k]*Inspectie Gezondheidsbescherming, Evertsenstraat 17, 4461 XN Goes, Netherlands*

Received 9 June 1997; received in revised form 8 December 1997; accepted 28 December 1997

Abstract

Nine laboratories in eight countries tested 16 batches of common mussels (*Mytilus edulis*) over a 32 week period in order to find an alternative to the Most Probable Number (MPN) technique to enumerate *E. coli*. The alternatives investigated included the 3M Petrifilm system, the Merck Chromocult agar method and a Malthus conductance technique. The Petrifilm was found to be unsuitable and was subsequently dropped from the trial. After 669 analyses, a correlation of 0.83 was observed for log *E. coli* counts between the MPN and Chromocult methods and there was no significant evidence that either method tended to give higher readings than the other. The MPN was slightly better than the Chromocult method for repeatability but the Chromocult was slightly better for reproducibility. However, the observed differences are probably too small to be of practical importance. On the basis of these data therefore, the two methods appear equally suitable for *E. coli* enumeration in shellfish. There were poor correlations between these methods and the Malthus technique. A small but significant number of samples tested positive on the Malthus instrument but were recorded negative on the MPN and Chromocult tests. Subsequent analysis positively identified *E. coli* from these Malthus assays. After statistical analysis, errors were noted in both the MPN and Chromocult methods but it was found that there would be no statistical differences if the Chromocult agar were used as an alternative to the MPN technique. Published by Elsevier Science B.V.

Keywords: Interlaboratory study; *Escherichia coli*; MPN; Chromogenic media; Conductance; Shellfish

*Corresponding author.

PII S0168-1605(98)00016-6

1. Introduction

This work presents the results of an European Union funded study entitled 'Study to be carried out on the quality of water for the production of bivalve molluscs' (94/C 179/09/ XIV/D/1/QUALEAUX).

Commercial shellfish production in the EU has great economic benefits with output exceeding one million tonnes annually. Approximately one half of this is common mussel (*Mytilus edulis*) production. The Council Directive 91/492/EEC, 1991 outlines the health rules governing the production and marketing of live bivalve molluscs. The microbiological status of the molluscs determines whether the food may be sold for direct human consumption. If bacterial (indicator organisms) numbers exceed a specific value, the shellfish must be discarded or resettled in clean water to reduce these numbers. The microbiological quality of production areas is assessed by estimating the number of faecal coliforms or *Escherichia coli* per 100 g of shellfish tissue. The recommended method is the five tube Most Probable Number (MPN) technique which is time consuming, labour intensive and recognised to be limited in its accuracy. Many member countries are developing improved methods but there have been few inter-country validation studies. The objective of this project was to test alternatives to the MPN technique to enumerate *E. coli*.

Nine laboratories in eight different countries compared the MPN method with the 3M Petrifilm technique. A preliminary round of sampling identified deficiencies in the Petrifilm technique, the main one being an apparent inhibition of the blue colouration (this being the colour of developing *E. coli* colonies) by the undiluted mussel homogenate. A complete investigation of this problem was outside the scope of this project and therefore the Petrifilm was replaced by Merck Chromocult agar which had shown initial promise during in-house trials in the CSL laboratory. This medium contains two separate chromogens; colourless compounds which give either blue coloured colonies if metabolised by bacteria possessing the enzyme β-glucuronidase or red coloured colonies by those with β-galactosidase. *E. coli* possesses both enzymes and produces dark blue colonies on this medium (Kilian and Bülow, 1976; Manafi and Kneifel, 1989). Few micro-organisms other than *E. coli* exhibit β-glucuronidase activity (Hofstra and Huis in't Veld, 1988).

In addition, a third method based on a Malthus conductance technique to specifically detect *E. coli* in foods was tested by three of the eight participating laboratories having the appropriate equipment.

2. Materials and methods

2.1. Collection, distribution and sampling

A total of 32 mussel samples were sent by each laboratory to all partners (four times throughout an eight month sampling period). Collections of mussels were made on Mondays where possible. Approximately fifty large mussels were placed in plastic bags and stored in cool boxes prior to dispatch to each laboratory. Sufficient freezer gel packs were added to maintain a temperature of approximately 4°C during transit. Care was taken not to place the packs directly against the samples to avoid freezing. A 25 ml plastic bottle containing water/antifreeze or water/ethanol was included near the mussels, and its temperature was recorded on arrival in the receiving laboratory. Results of analyses of mussel samples that were >8°C on arrival were flagged for later scrutiny and any >15°C were rejected. Cool boxes were sent by 24 h courier for delivery on Tuesday and the shellfish stored at chill temperature prior to testing on Wednesdays.

In all laboratories, each batch of mussels was split into three 100 g samples (meat + liquor) for analysis in triplicate. Using protective gloves, 15–30 mussels were selected for testing depending on size. Those gaping or showing obvious signs of damage were discarded. The selected shellfish were scrubbed clean under running tap water of potable standard prior to opening using a sterile shucking knife. The liquor and meat were collected in a weighed sterile bag or beaker, transferred to a Colworth stomacher and homogenised for 2–3 min in a filter bag. Decimal dilutions were prepared in sterile 0.1% peptone water (Oxoid CM9).

2.2. Methods of E. coli enumeration

2.2.1. MPN method

The MPN method under test was that used by the UK Ministry of Agriculture, Fisheries and Food, Department of Health and Public Health Laboratory Service Working Group for the examination of

shellfish for *E. coli* (PHLS, 1992) . First stages were performed in minerals modified glutamate medium (MMG, Oxoid CM607 + sodium glutamate (Oxoid L124) and ammonium chloride). This medium is prepared as double and single strength in bottles containing inverted Durham tubes to detect gas production. Ten millilitre volumes of the 1:10 homogenate were inoculated in each of five tubes of double strength MMG which is equivalent to 1 g tissue/tube. One millilitre of the same dilution was introduced to five tubes of 9 ml single strength MMG volumes, equivalent to 0.1 g tissue/tube. One millilitre of a 1:100 dilution was inoculated into five tubes of 9 ml single strength MMG equivalent to 0.01 g tissue/tube. All tubes were incubated at 37±1°C for 18–24 h in a waterbath or incubator after which the production of gas was recorded. Bottles showing no reaction were re-incubated for a further 24 h at 37°C and then re-examined. Absence of gas constituted a negative result for *E. coli*. Tubes showing gas production were examined further by sub-culturing into (a) 5 ml volumes of brilliant green bile broth (BGBB) and (b) 5 ml volumes 1% tryptone water (TPW), both incubated at 44±0.5°C for 24 h. The following control cultures were used:

NCTC 10418 *E. coli*	gas	+ ve	indole positive
NCTC 9528 *Klebsiella aerogenes*	gas	− ve	indole negative

BGBB tubes were examined for gas production and TPW for indole production using Kovac's reagent. Positive reactions in BBGB and TPW indicated the presence of *E. coli* and the numbers were calculated from appropriate MPN tables. Results were expressed as the number of *E. coli*/100 g of shellfish.

2.2.2. Chromogenic method

Chromocult agar was prepared according to the manufacturers instructions taking care not to overheat when dissolving. The plates were dried fully before use. Aliquots (0.5 ml, this being the maximum volume that was easily dried on a 10 cm plate) of the undiluted homogenate were inoculated on to six separate Chromocult plates and spread with a sterile implement. If the shellfish under test had little or no associated liquor, 100 ml chilled sterile dilution fluid (0.1% peptone) was added prior to the stomacher operation. Note of this 1:1 dilution was taken into account when assessing the final count and when making the 1:10 dilution. Volumes (0.5 ml) of the 1:10 homogenate were spread on six further Chromocult plates. The plates were incubated at 37±0.5°C. Coliform colonies appear red and *E. coli* colonies blue after 24 h incubation. The numbers of *E. coli* were recorded and the plates re-incubated for a further 24 h when the numbers of blue colonies were recounted. The number of *E. coli* per 100 g shellfish was calculated from this number. Blue colonies were subcultured, purified and tested in tryptone water and MacConkey broth at 44°C for the presence of indole and acid/gas production from lactose respectively. These tests confirmed the correct recognition of *E. coli*. This confirmation was dropped when operators became familiar with the technique.

2.2.3. Conductance method

Conductance measurements were made on Malthus 2000 instruments (IDG Group, Bury, UK) capable of holding 10 ml electrodes in an incubator controlling at 37±0.05°C. Laboratories in Denmark, Ireland and the UK participated in this exercise. The molluscs were collected, opened and homogenised as described above and triplicate 5 ml volumes of the undiluted homogenate were aseptically inoculated into 5 ml double strength glucuronide/trimethylamine-oxide (GT) broth (Ogden, 1993). Conductance changes of >400 μS are produced by *E. coli* reducing trimethylamine oxide to trimethylamine. This reaction occurs only in the presence of a fermentable carbohydrate, this being D-glucuronic acid in GT broth. Trimethylamine is highly charged resulting in an easily observed large conductance change. The conductance tubes were scanned every 6 min and data recorded on a personal computer. Results were available numerically and graphically. The start of growth on the conductance graph (ΔG versus time) is referred to as the detection time (DT), and was measured as the time (h) when three consecutive 1 microsiemen (μS) changes occurred.

2.3. Shellfish categorisation

Shellfish are categorised (Council Directive 91/492/EEC, 1991) by the number of *E. coli*/100 g tissue as shown in Table 1. Category A can be sold for direct human consumption while those from category B must be relaid to reduce the bacterial load before harvesting. Category C shellfish cannot be

Table 1
Definition of shellfish category areas by MPN and Chromocult methods

Category	*E. coli*/100 g	MPN/100 g	Chromocult/100 g
		from MPN tables	from Chromocult plates
A	<230	4 2 0 = 200	6 colonies = 200
B	230–4600	4 2 1 – 5 5 1 = 250–3500	7–138 colonies = 233–4600
C	>4600	5 5 2 = 5400	139 colonies = 4633

used for direct consumption. Calculations from MPN tables can be used directly for categorisation.

Categorisation from Chromocult plates was made by adding together the number of blue colonies (*E. coli*) observed on all six plates of the same dilution. Category A shellfish contain less than seven *E. coli* from the six plates of the zero dilution (equivalent to $<230/100$ g as shown in Table 1). If the total number of *E coli* from the same six plates was between 7–138, this is equivalent to a B classification. When the total number of *E. coli* from the six plates from the 1:10 homogenate was between 14–139, this is equivalent to category C.

To determine bacterial numbers from conductance data, a calibration curve is constructed plotting log numbers versus DT. Calibration curves of DT's versus MPN and Chromocult were constructed. From these curves the detection time limits of areas A, B and C could be determined.

2.4. Statistical methods

With both MPN and Chromocult data being skewed, natural logarithms were taken of both to correct to approximate normality. Correlations between counting methods, the strength of their relationship, were first calculated. Regression analyses were then performed to adjudge the accuracy of prediction of one from the other.

Calculations of inter-laboratory repeatability and intra-laboratory reproducibility required analysis of variance of the data, (the variance ratios being compared to the *F* distribution to test their significance). These measures gave indications of how well each method could be repeated on the same data within one laboratory, and how close results were between two laboratories analysing the same data. Cochran's test for extreme standard deviations and Dixon's test for outlying means were then used to check the data.

Repeatability, reproducibility, Cochran's and Dixon's test are all defined (ISO, 1981). All computational analysis was carried out using Genstat 5, Release 3.1.

3. Results

Each laboratory received two batches for analysis every two weeks throughout the 32 week testing period. With all laboratories performing the MPN and Chromocult techniques, there was a maximum of 768 possible data sets. With three laboratories having conductance instruments, there could be a maximum of 288 associated DT's. There were 93 missing values for the MPN, 99 for the Chromocult and 103 for the Malthus data. Explanations for missing values included temperature too high or low (frozen) on arrival, frozen mussel beds, experimental error and intermittent instrument error. When comparing bacterial counting methods it is important that a wide numerical range is obtained, this being representative of all categories for bivalve mollusc definition. Observed counts ranged between zero and $10^4/100$ g in the MPN technique and zero and $10^5/100$ g in the Chromocult method.

The relationship between the log MPN and log Chromocult data is presented in Fig. 1. The correlation coefficient was 0.83. Fig. 2 and Fig. 3 show the scatter plots of the log MPN versus Malthus DT's and log Chromocult versus Malthus DT's respectively. Their correlations are -0.093 and -0.071 respectively. Values of zero were changed to 1 and values $>18\,000$ were altered to 18 000 in the MPN and Chromocult data so they could be transformed to the log scale.

It is of interest to note how well the MPN readings could be modelled by the new Chromocult data. The squared correlation has a value of 0.78 and date of collection, collecting laboratory and testing labora-

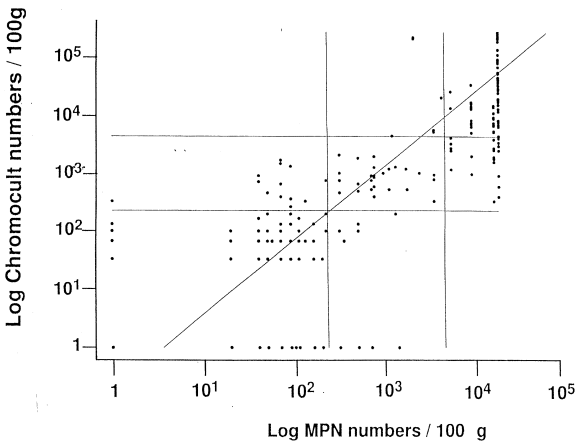

Fig. 1. Relationship between log MPN and log Chromocult numbers.

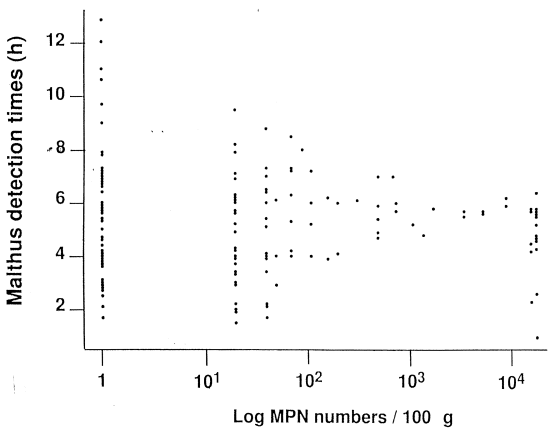

Fig. 2. Relationship between log MPN numbers and Malthus detection times.

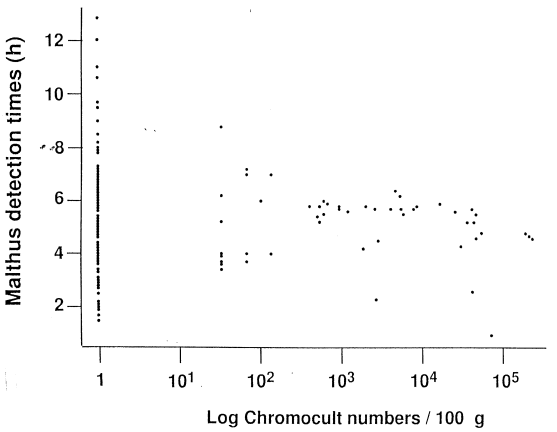

Fig. 3. Relationship between log Chromocult numbers and Malthus detection times.

Table 2
Repeatability and reproducibility by method and sampling round

Sampling round	log MPN		log Chromocult		Malthus DT	
	r	*R*	*r*	*R*	*r*	*R*
1	2.76	4.17	0.70	6.70	1.75	5.25
2	3.08	5.10	2.03	7.40	1.32	5.83
3	3.83	6.67	1.72	2.43	1.72	2.60
4	4.14	7.85	4.56	9.61	1.43	4.27
5	2.50	15.98	1.03	15.40	1.60	–
6	3.14	5.87	3.77	10.00	3.84	8.08
7	3.04	4.57	3.07	6.72	0.77	4.42
8	3.36	4.97	1.16	4.20	2.99	6.15
9	3.47	5.67	3.09	8.91	0.81	1.68
10	3.20	6.16	3.82	9.93	1.16	1.48
11	1.81	4.61	1.48	2.08	2.75	6.40
12	2.49	5.20	2.33	7.22	3.41	5.41
13	2.95	7.32	1.59	6.52	1.00	2.15
14	3.74	7.44	2.64	4.13	1.35	19.33
15	3.32	6.55	2.31	12.63	0.84	6.38
16	2.59	5.27	2.54	10.54	1.82	3.31
All	3.16	6.65	2.65	8.16	2.09	5.50

R = intra-laboratory reproducibility.
r = inter-laboratory repeatability.

tory are all significant ($P < 0.001$ for each factor). Ignoring other factors, the squared correlation between the logarithms of MPN and Chromocult is 0.72.

The inter-laboratory repeatability (r) and intra-laboratory reproducibility (R) were calculated at each time and over all times and the values are shown in Table 2. The repeatability provides a measure of variation within each laboratory (between the three replicates in a laboratory) and the reproducibility shows the variation between laboratories for each batch of mussels. Repeatability will always be at least as big as reproducibility as r is part of the formula for R (ISO, 1981). From these results it seems that MPN is more reproducible between laboratories but less repeatable within laboratories than the Chromocult method. However, although the difference between the overall values of r for MPN and Chromocult (3.16 and 2.65 respectively) is statistically significant ($F_{450,446} = 1.42$, $P < 0.001$), the difference is probably not large enough to be of practical importance. Similarly, the difference be-

Table 3
Table of means

Date	log MPN	Log Chromocult	Difference
5 Feb	3.50	3.69	− 0.08
19 Feb	6.36	5.77	0.59
4 Mar	0.95	0.09	0.86
18 Mar	3.16	2.22	0.94
1 Apr	2.14	1.84	0.30
15 Apr	3.36	1.84	1.52
29 Apr	0.44	0.62	− 0.18
13 May	5.87	4.50	1.37
27 May	2.59	1.40	1.19
10 Jun	6.19	6.10	0.09
24 Jun	0.35	0.08	0.27
8 Jul	6.91	7.02	− 0.12
22 Jul	1.03	0.43	0.76
5 Aug	1.28	0.33	0.94
19 Aug	3.86	2.45	1.53
2 Sep	6.88	6.46	0.55

tween the overall values of R (6.65 and 8.16) is statistically significant ($F_{191,193} = 1.51$, $P < 0.003$) but not large. There are no particular trends over time. The conductance results in Table 2 are not comparable to those for MPN and Chromocult as the units of measurement are not the same. A table of means per date period for log MPN and log Chromocult is presented in Table 3.

Taking the difference between the log MPN and log Chromocult values as a new variate, fitting a regression model with variable dates, collecting laboratory and testing laboratory (all of which are significant), gives a (correlation)^2 value of 0.15. There was more variability in large counts than small counts. The constant term had a t-value of 0.11 ($P > 0.2$), giving no evidence of a bias between the methods i.e. there was no evidence that MPN tended to give either higher or lower values than Chromocult.

4. Discussion

There was broad agreement between the MPN and Chromocult methods in their estimate of *E. coli* numbers and no apparent tendency for one method to give higher estimates than the other. Differences between the methods in their inter-laboratory repeatability and intra-laboratory reproducibility were statistically significant but the size of the differences are quite small (statistical significance arises from the large sample sizes) and one method was better for repeatability while the other was better for reproducibility. Hence, from a statistical viewpoint there is little to choose between the methods and the MPN technique might reasonably be replaced by the Chromocult method. This would inevitably lead to a re-categorisation of some samples. Fifty six samples have a discrepancy in classification in this study and a detailed summary of these is shown in Table 4. Fifty six represents 8.4% of samples tested and is probably not significant when taking into account that some samples were on the border-line between categories. It was significant that no samples resulting in an A categorisation by one method were tested C by the alternative.

Although there is little to choose between the methods on statistical grounds, the authors feel there are several advantages to be gained from using the Chromocult method. The time taken to test mussels by the Chromocult method is considerably quicker and less labour intensive than the MPN technique. An estimate by the UK laboratories suggest that two samples could be analysed by the Chromocult method in the time taken to perform one MPN test. In addition, the results by the Chromocult method are available within 48 h (in practice this is usually 24 h) and little confirmation is necessary when the operator has gained experience in correctly identifying *E. coli* colonies. By comparison the MPN method gives results in 3–4 days. The cost of the Chromocult medium as used in this project was approximately one quarter of the cost of the MPN materials, but an exact price will vary depending on how many MPN tubes are recorded positive and require further analysis. It is possibly less time consuming to produce Chromocult plates than a series of MMG,

Table 4
Number of samples that would be re-categorised if the MPN were replaced by the Chromocult method

Re-categorisation	Number of samples
A → B	14
B → A	10
B → C	6
C → B	22
A → C	0
C → A	0

BGBB and tryptone water tubes for the MPN analysis. Finally, the Chromocult method requires one 37°C incubator whereas the MPN method requires an extra 44°C waterbath for complete *E. coli* confirmation.

One clear disadvantage of the Chromocult and Malthus methods when compared to the MPN technique are the small volumes needed for test purposes. The MPN used 55.5 ml volumes compared to 6 ml in the conductance tubes and 3 ml on the Chromocult plates. This would suggest that for samples with low bacterial numbers there would be an improved chance of detection when the MPN was employed. Ways of overcoming this problem would be to use either a greater number than six Chromocult plates or larger plates capable of absorbing greater volumes of sample (e.g. 15 cm which would accommodate slightly more than 1 ml). The inoculation of 50 ml homogenate into 100 ml conductance bottles would improve the Malthus sensitivity but these systems were not available to the authors. All these measures would obviously increase the overall cost of the proposed alternative methods.

It is quite clear that the correlation between conductance detection times and both alternative counting methods is very poor. This is not in accordance with results reported by Ogden (1993) where a correlation of -0.86 was reported from a range of foods tested by the Malthus GT broth and a bromo-chloro-indolyl glucuronide (BCIG) agar (Ogden and Watt, 1991). The poor correlations found here are partly due to the number of occasions that the MPN and Chromocult recorded zero counts when the Malthus method indicated a positive result. Although outside the remit of this project, it was necessary to establish if the mussels tested positive by Malthus contained *E. coli* not recognised by the alternative techniques. Samples of mussels collected during rounds 3 and 8 were therefore tested further. The GT conductance broths (from GT positive, MPN/Chromocult negative samples) were sub-cultured into tryptone waters and incubated at 44°C. Indole was produced in the majority of cases indicating the presence of *E. coli*. After isolation, subsequent tests showed that these *E. coli* were not atypical being lactose positive (and therefore should have been detected by the MPN method) and β-glucuronidase positive, (which should have grown as blue colonies on Chromocult agar). The GT broth contains no inhibitors whereas the other methods contain selective (and therefore possibly inhibitory) agents. The lack of selective compounds may allow a more complete recovery of damaged micro-organisms which could express themselves metabolically as indicated by the positive conductance curves. By comparison, formate is used in the first stage of the MPN method followed by brilliant green and bile salts in subsequent steps. The Chromocult agar incorporates tergitol as the selective agent. There is a possibility that the *E. coli* encountered in the equivocal results are sensitive to these inhibitors. Furthermore, it is well established that damaged organisms prefer aqueous environments (broth cultures) to the drier surfaces of agars (Chromocult test).

5. Conclusions

The statistical analysis of the data suggests that the Chromocult method could replace the MPN technique. We have also shown that the replacement would offer significant savings in both costs and the time taken to produce results. These results show that there is a very poor correlation between the conductance data and the two direct counting methods and further work is required to explain this anomaly.

Acknowledgements

The authors would like to than the European Commission DGXIV (Fisheries) who wholly funded this work, and the numerous laboratory workers who helped with the analyses.

References

Council Directive 91/492/EEC 1991. Laying down the health conditions for the production and the placing on the market of live bivalve molluscs. Official Journal No. L 268, 1.

Hofstra, H., Huis in't Veld, J.H.J., 1988. Methods for the detection and isolation of *Escherichia coli* including pathogenic strains. J. Appl. Bact. Symp. Suppl. 197S–212S.

ISO 1981. International Standard 5725: Precision of test methods

– Determination of repeatability and reproducibility by inter-laboratory tests. 1st edition – 1981-04-01.

Kilian, M., Bülow, P., 1976. Rapid diagnosis of Enterobacteriaceae. 1. Detection of bacterial glycosidases. Acta Pathol. Microbiol. Scand. Sect. B 84, 245–251.

Manafi, M., Kneifel, W., 1989. A combined chromogenic–fluorogenic medium for the simultaneous detection of total coliforms and *E. coli* in water. Zentralbl. Hyg. 189, 225–234.

Ogden, I.D., 1993. A conductance assay for the detection and enumeration of *Escherichia coli*. Food Microbiol. 10, 321–327.

Ogden, I.D., Watt, A., 1991. An evaluation of fluorogenic and chromogenic assays for the direct enumeration of *Escherichia coli*. Appl. Microbiol. 13, 212–215.

PHLS 1992. MAFF, DH and PHLS Working Group: Bacterial examination of shellfish. Public Health Laboratory Service Microbiology Digest 9 (2), 76–82.