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Validation and comparison of methods for enumeration of faecal coliforms and *Escherichia coli* in bivalve molluscs

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Abstract

Validation and comparison of methods for enumeration of faecal coliforms and *Escherichia coli* in bivalve molluscs

The main result of the validation study to show the equivalence of two methods for the enumeration of faecal coliforms in bivalve molluscs is that the plate count method on Mac Conkey agar was indeed found to be equivalent to the MPN method. Meaning that the Netherlands fulfilled the demands as stated in Council Directive 91/492/EC.

The quality requirements of bivalve molluscs, intended for human consumption, are laid down in European legislation. One of the requirements of Council Directive 91/492/EC is that bivalve molluscs must contain less than 300 faecal coliforms or less than 230 *Escherichia coli* per 100 g mollusc flesh and intravalvular liquid. According to this Directive, the microbiological analyses should be performed with a Most Probable number (MPN) method, 'or any other bacteriological procedure shown to be of equivalent accuracy'. Up to 2005, the Netherlands preferred a plate count method on Mac Conkey agar. To meet the requirements it was necessary to perform a national validation study. Additional to this validation study, four procedures for the enumeration of *Escherichia coli* were compared. This comparison was performed as in the new European Regulation 854/2004 the quality of bivalve molluscs is only based on the number of *Escherichia coli* and not longer on faecal coliforms. The conclusion from this comparison study is that a plate count method can be considered as a possible alternative method for the newly prescribed MPN method for the enumeration of *Escherichia coli* in bivalve molluscs.

Key words: Bivalve molluscs, faecal coliforms, *Escherichia coli*, validation study, comparison of methods.

Rapport in het kort

Validatie en vergelijking van methoden voor de telling van fecale bacteriën van de coligroep en *Escherichia coli* in tweekleppige weekdieren

Het belangrijkste resultaat van de validatiestudie waarin gelijkwaardigheid van twee methoden voor de telling van fecale bacteriën van de coligroep in tweekleppige weekdieren werd aangetoond, was dat de telplaatmethode op Mac Conkey agar inderdaad gelijkwaardig werd bevonden aan de MPN-methode. Dit betekent dat Nederland voldeed aan de eisen van Richtlijn 91/492/EC.

De kwaliteitseisen van tweekleppige weekdieren, bedoeld voor menselijke consumptie, zijn vastgelegd in Europese wetgeving. Eén van de vereisten van Richtlijn 91/492/EC is dat tweekleppige weekdieren minder dan 300 fecale bacteriën van de coligroep of minder dan 230 *Escherichia coli* per 100 g weekdiervlees en vocht mogen bevatten. Volgens deze Richtlijn moet de microbiologische analyse uitgevoerd worden met een Meest Waarschijnlijke Aantal (MPN) methode, ‘of een andere bacteriologische procedure van welke gelijke nauwkeurigheid is aangetoond’. Tot 2005 prefereerde Nederland het gebruik van een telplaatmethode op Mac Conkey agar. Om aan de eisen te voldoen was het nodig om een nationale validatiestudie uit te voeren. Naast deze studie werden ook vier procedures voor de telling van *Escherichia coli* vergeleken. Deze vergelijking werd gedaan omdat in de nieuwe Europese Verordening 854/2004 de kwaliteit van tweekleppige weekdieren alleen gebaseerd is op het aantal *Escherichia coli* en niet langer op fecale bacteriën van de coligroep. De conclusie van deze vergelijkingsstudie is dat een telplaatmethode een mogelijke alternatieve methode kan zijn voor de nieuw voorgeschreven MPN-methode voor de telling van *Escherichia coli* in tweekleppige weekdieren.

Trefwoorden: Tweekleppige weekdieren, fecale bacteriën van de coligroep, *Escherichia coli*, validatiestudie, vergelijking van methoden

Contents

Summary

The quality requirements of bivalve molluscs, intended for human consumption, are laid down in European legislation. One of the requirements of Council Directive 91/492/EC is that bivalve molluscs must contain less than 300 faecal coliforms or less than 230 *Escherichia coli* per 100 g mollusc flesh and intravalvular liquid. According to this Directive, the microbiological analyses should be performed with a (five-tube, three dilutions) Most Probable number (MPN) method, 'or any other bacteriological procedure shown to be of equivalent accuracy'. Up to 2005, the Netherlands preferred a plate count method on Mac Conkey agar. To meet the requirements it was necessary to perform a national validation study, in which the plate count method on Mac Conkey agar was compared to an MPN method which was valid by that time ('MPN-classical'). Additional to this validation, four procedures for the enumeration of *Escherichia coli* were compared: MPN-classical (ISO 7251), MPN-Donovan (ISO 16649-3), Mac Conkey plate count (Annex 1) and TBX-plate count (ISO 16649-2). Fifty shellfish samples with different contamination levels were prepared by contaminating live mussels with different amounts of sewage sludge. Before analyses, the samples were homogenised, after which the homogenate was analysed with the four different methods. The results were statistically analysed according to the procedure as described in ISO 16140 and by using Bland-Altman analysis.

The out come of the analyses is summarised below.
Faecal coliforms:

- Mac Conkey (Plate count) vs. ISO 7251 (MPN-classical): not significantly different.

Escherichia coli

- Mac Conkey (Plate count) vs. ISO 7251 (MPN-classical): differences on the boundary of significance;
- Mac Conkey (Plate count) vs. ISO 16649-3 (MPN-Donovan): significantly different;
- Mac Conkey (Plate count) vs. ISO 16649-2 (Plate count-TBX): differences on the boundary of significance;
- ISO 7251 (MPN-classical) vs. ISO 16649-3 (MPN-Donovan): not significantly different;
- ISO 7251 (MPN-classical) vs. ISO 16649-2 (Plate count-TBX): not significantly different;
- ISO 16649-2 (Plate count-TBX) vs. ISO 16649-3 (MPN-Donovan): not significantly different.

The results of the validation study show that the method used in the Netherlands (Mac Conkey) is equivalent to the classical MPN method (ISO 7251) for the enumeration of faecal coliforms in bivalve molluscs. This means that the Netherlands fulfilled the demands as stated in Directive 91/492/EC.

In the new legislation (Commission Regulation (EC) 854/2004) the quality of bivalve molluscs is no longer based on the number of faecal coliforms, but on the number of *Escherichia coli*. For this, the Mac Conkey method can not be used as it gives significantly lower results than the prescribed MPN method (ISO 16649-3). Both MPN methods (ISO 7251 and 16649-3) and the plate count method on TBX (ISO 16649-2) gave equivalent results for the enumeration of *Escherichia coli* in mussels. Therefore, this study has also shown that the plate count method on TBX (ISO 16649-2) can be considered as a possible alternative method for the prescribed Donovan MPN method (ISO 16649-3) for enumeration of *Escherichia coli* in bivalve molluscs. For acceptance of alternative methods at international level, a full validation according to the procedure as described in EN/ISO 16140 is needed.

List of abbreviations

BGLB	Brilliant Green Lactose Broth
CEN	European Committee for Standardization
cfu	colony forming units
CRL	Community Reference Laboratory
EC broth	*Escherichia coli* broth
ISO	International Organization for Standardization
LTB	Lauryl Sulphate Tryptose broth
MMGB	Mineral Modified Glutamate Broth
MPN	Most Probable Number
NRL	National Reference Laboratory
PCR	Polymerase Chain Reaction
PS	Peptone saline solution
RIKILT	Institute of Food Safety
RIVM	National Institute for Public Health and the Environment
RIVO	Netherlands Institute for Fisheries Research
RSV	RIKILT Standaard Voorschrift (RIKILT Standard Operation Procedure)
TBX	Tryptone Bile Glucuronic agar

1. Introduction

Council Directive 91/492/EC (1991) prescribes an MPN-method for the enumeration of faecal coliforms or *Escherichia coli* for the classification of bivalve molluscs production areas. This MPN method is described in general terms. No reference is made to a standard method, like described by ISO or CEN. The Directive approves the usage of alternative methods when 'shown to be of equivalent accuracy'. Many alternative methods (e.g. plate count methods, impedance methods) are available for the analysis of the indicator bacteria faecal coliforms and *Escherichia coli*. Since speed and reduction of labour are important issues for financial and legislative reasons, several EU Member States prefer other methods than the MPN method. However, not all methods are validated for bivalve molluscs.

In the Netherlands, a plate count method on Mac Conkey agar for the enumeration of faecal coliforms has been used for the classification of shellfish production area's since the eighties. The method was validated at that time. However, the results were not published and at present not retrievable. Therefore a proper validation of the Mac Conkey plate count method against the (by that time) prescribed MPN method (MPN-classical) was required to verify the legitimacy of the method used at national level.

Since 2004 new legislations for, amongst others, bivalve molluscs have been published. These legislations (Commission Regulations (EC) No 853/2004, 854/2004 and 2073/2005) prescribe the enumeration of *Escherichia coli* by using an MPN method as reference method. The detection of faecal coliforms as prescribed by Directive 91/492/EC has been abandoned. In Commission Regulation (EC) No 2073/2005, a specification of this MPN-method is given, being the MPN method described by Donovan *et al*. (1998), which is currently described in ISO/TS 16649-3 (Anonymous, 2005). However, it is allowed to use alternative methods, when they are validated against the prescribed MPN method. According to the same Regulation, the validation studies should follow the procedure as described in EN/ISO 16140, 'or other internationally accepted similar protocols'.

Since the Netherlands have a shellfish monitoring system, which is based on rapid decisions on opening, closing and declassification of production areas, there is a need for fast and reliable methods. In order to select a proper method, which is fully accepted, different methods were compared to the prescribed methods (MPN-classical and MPN-Donovan).

The main objective of the studies described in this report was the national validation of the Mac Conkey plate count method against the prescribed classical MPN method (ISO 7251) for the enumeration of faecal coliforms in bivalve molluscs, to show the legitimacy of the use of the Mac Conkey plate count method in the Netherlands.

An additional objective was the comparison of methods for the enumeration of *Escherichia coli* in bivalve molluscs: the Mac Conkey plate count method, the TBX plate count method (ISO 16649-2), the classical MPN method (ISO 7251) and the Donovan MPN method (ISO 16649-3).

A summary of the studies as well as the main conclusions were reported to the Dutch Food and Consumer Product Safety Authority in 2005. The finishing of the full report was delayed due to the fact that four staff members involved in the studies at RIKILT and at the RIVM changed jobs or retired since early 2005.

2. Materials and Methods

2.1 Sample preparation

Mussels (*Mytilus edulis*) were collected from the estuary the Oosterschelde, the Netherlands from May to October 2004. The mussels were not tested for contamination prior to the experiment. However, the area in which they were collected was classified as Category A according to Council Directive 91/492/EC. Therefore the assumption could be made that the shellfish did not contain faecal coliforms above 300 cfu/ 100 g shellfish meat and moist. After collection, the mussels were stored in a basin with flow-through clean seawater. Since the experiment was performed in a half-year period, mussels with a different lipid composition could be analysed.

The mussels were artificially contaminated using active sludge derived from the Water Treatment Plant in Waarde (Zeeland, the Netherlands). The active sludge was collected within 12 h prior to contamination of the mussels and stored in the refrigerator (2 °C - 8 °C) until use. The active sludge was estimated to contain 10^9-10^{10} *Escherichia coli* per ml.

Five different rounds of contaminations were performed. For each contamination level, 1 kg of mussels was placed into a polyethylene basket. The basket was placed in a 5 litre bucket containing 2 litre seawater, contaminated with 1 ml to 1 litre of active sludge to achieve different levels of contamination. The accumulation of microorganisms was performed over 6-16 h. In order to keep the mussels as healthy and active as possible, the bucket was aerated (see Picture 1).

Picture 1 Mussel contamination experiment (faecal coliforms and Escherichia coli)

2.2 Microbiological methods

2.2.1 Preparation of primary homogenate

Artificially contaminated mussel were transported to the laboratory on melting ice and examined within 12 h.
Mussels were cleaned under running water and aseptically opened to collect internal meat and moist. Hundred gram of the meat and moist was homogenised in 200 ml peptone saline solution (PS: peptone 1 g/L and NaCl 8.5 g/L) by using a Stomacher homogeniser (1 min). This primary homogenate was further diluted in PS depending on the requirements of the method (see below).

2.2.2 Classical MPN (ISO 7251)

To distinguish the two MPN procedures in this report, they are denoted as classical MPN or MPN-classical (ISO 7251; Anonymous, 1993) and as Donovan MPN or MPN-Donovan (ISO 16649-3; Anonymous, 2005).

The classical MPN method was performed according to ISO 7251 (Anonymous, 1993). The procedure is summarised below. The media used are described in ISO 7251.

Sixty ml of the primary homogenate was diluted in 140 ml PS to obtain a 10^{-1} dilution of the sample material in PS. This 10^{-1} dilution was used to prepare 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} dilutions.

Dilutions were inoculated in 5-fold as follows:
0: 10 ml 10^{-1} dilution in 10 ml double-strength enrichment medium Lauryl Sulphate Tryptose Broth (LTB);
10^{-1}: 1 ml 10^{-1} dilution in 10 ml single-strength enrichment medium LTB;
10^{-2}: 1 ml 10^{-2} dilution in 10 ml single-strength enrichment medium LTB;
10^{-3}: 1 ml 10^{-3} dilution in 10 ml single-strength enrichment medium LTB;
10^{-4}: 1 ml 10^{-4} dilution in 10 ml single-strength enrichment medium LTB;
10^{-5}: 1 ml 10^{-5} dilution in 10 ml single-strength enrichment medium LTB.

The tubes were incubated at (37 ± 1) °C and examined for gas production after (24 ± 2) h and after (48 ± 4) h. Tubes in which gas was produced were recorded as positive for the presence of faecal coliforms.
For confirmation of the presence of *Escherichia coli*, one loopful of broth material from each tube with gas formation was inoculated into a tube containing 10 ml of *Escherichia coli* (EC) broth. EC broth was incubated at (44 ± 0.5) ^{o}C for (48 ± 2) h and checked for the presence of gas production. One loopful of broth material from each positive EC tube was inoculated into a tube containing 5 ml of Tryptone water and incubated at (44 ± 0.5) ^{o}C for (24 ± 2) h. After incubation, 1 ml of Kovacs reagent was added to the tube. The appearance of a red circle within 1 minute after addition of the Kovacs reagent indicated a positive indole reaction. Samples showing gas formation in both LTB broth and EC broth and showing a positive indole reaction, were recorded as positive for the presence of *Escherichia coli*.

The number of positive tubes in each dilution resulted in an MPN code. From this MPN code the Most Probable Number of *Escherichia coli* and/or faecal coliforms was derived using the 5-fold MPN-tables (De Man, 1983).

2.2.3 Donovan MPN (ISO 16649-3)

The Donovan-MPN method was performed according to ISO 16649-3 (Anonymous, 2005). The procedure is summarised below. The media used are described in ISO 16649-3.
Sixty ml of the primary homogenate was diluted in 140 ml PS to obtain a 10^{-1} dilution of the sample material in PS. This 10^{-1} dilution was used to prepare 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} dilutions.

Dilutions were inoculated in 5-fold as follows:
0: 10 ml 10^{-1} dilution in 10 ml double-strength enrichment medium Mineral Modified Glutamate Broth (MMGB);

10^{-1}: 1 ml 10^{-1} dilution in 10 ml single-strength enrichment medium MMGB;
10^{-2}: 1 ml 10^{-2} dilution in 10 ml single-strength enrichment medium MMGB;
10^{-3}: 1 ml 10^{-3} dilution in 10 ml single-strength enrichment medium MMGB;
10^{-4}: 1 ml 10^{-4} dilution in 10 ml single-strength enrichment medium MMGB;
10^{-5}: 1 ml 10^{-5} dilution in 10 ml single-strength enrichment medium MMGB.

After incubation at (37 ± 1) °C for (24 ± 2) h, the MMGB tubes were examined for acid production (yellow coloration)[1] and for lactose fermentation (gas production). From each tube showing acid production[1], a loopful of material was streaked on a plate containing Tryptone Bile Glucoronic agar (TBX). TBX plates were incubated at (44 ± 1) °C for (21 ± 3) h. The presence of characteristic blue colonies on TBX indicated the presence of *Escherichia coli* in the original MMGB tube.

The number of positive tubes in each dilution resulted in an MPN code. From this MPN code the Most Probable Number of *Escherichia coli* was derived using the 5-fold MPN-tables (De Man, 1983).

2.2.4 Plating method on TBX (ISO 16649-2)

The plating method on Tryptone Bile Glucoronic agar (TBX) was performed according to ISO 16649-2 (Anonymous, 2001). The procedure is summarised below. The media used are described in ISO 16649-2.
Sixty ml of the primary homogenate was diluted in 140 ml PS to obtain a 10^{-1} dilution of the sample material in PS. This 10^{-1} dilution was used to prepare 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} dilutions.
Fifteen ml of the primary homogenate was distributed over 8 Petri dishes (each with a diameter of 9 cm). Subsequently, 15 ml of freshly prepared and molten TBX agar was added to each dish. Furthermore, duplicates of 1 ml of the 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} dilutions were inoculated into Petri dishes and mixed with molten TBX agar.
After solidification, TBX plates were resuscitated at (37 ± 1) °C for (4 ± 0.5) h, followed by incubation at (44 ± 1) °C for (18 ± 2) h. Typical blue (ß-glucuronidase-positive) colonies were counted and the number of *E. coli* in the original sample was calculated.

2.2.5 Plating method on Mac Conkey

The plating method on Mac Conkey agar was performed according to an in-house procedure of RIKILT (RSV A0741). The procedure is summarised below.

[1] Text amended (in red) on 09-07-2007

Sixty ml of the primary homogenate was diluted in 140 ml PS to obtain a 10^{-1} dilution of the sample material in PS. This 10^{-1} dilution was used to prepare 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} dilutions.
Fifteen ml of the primary homogenate was distributed over 8 Petri dishes (each with a diameter of 9 cm). Subsequently, 15 ml of freshly prepared and molten Mac Conkey agar (Annex 1) was added to each dish. Furthermore, duplicates of 1 ml of the 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} dilutions were inoculated into Petri dishes and mixed with molten Mac Conkey agar. After solidification, Mac Conkey agar plates were resuscitated at (37 ± 1) °C for (2 ± 0.5) h, followed by incubation at (44 ± 1) °C for (24 ± 2) h. Typical red colonies were counted and the square root of the total number of typical colonies ($\sqrt{n}$) was confirmed by inoculation in Brilliant Green Lactose Broth (BGLB; Annex 1). Tubes were incubated at (44 ± 1) °C for (24 ± 2) h. Gas production indicated the presence of faecal coliforms. When tubes were also positive for the indole reaction in Tryptone water (see 2.2.2), the presence of *Escherichia coli* was assumed.
Taking into account the confirmation results, the numbers of faecal coliforms and *Escherichia coli* in the original samples were calculated.

2.3 Statistical analyses

The results were analysed following the procedure as described in ISO 16140 (Anonymous, 2003). Additional to the orthogonal regression analysis on the estimated most probable numbers according to ISO 16140, an alternative analysis was performed. Assuming Poisson distributed numbers of bacteria in the suspended sample, both the plate counts and presence-absence data can be used to estimate most probable numbers and corresponding (95 %) confidence intervals for the numbers of colony forming units (cfu) in all samples. For direct plate counts (ISO 16649-2) and presence absence in serial dilutions (ISO 16649-3) this is straightforward. In case of additional confirmation (Mac Conkey or ISO 7251) an additional binomial probability (for the fraction *Escherichia coli*) was also estimated. This provides a cfu estimate for each sample, and corresponding confidence intervals, allowing comparison of the error ranges. For equivalence testing of the applied methods, a Bland-Altman analysis (Bland and Altman, 1986) was used for pairwise comparing cfu estimates from all four methods. This involves regression of the difference between two measurements of a sample (as an estimate of their variance) against the sum of the two measurements (as an estimate of its 'true' value). Zero offset then indicates equivalence ('a slope of 1'), whereas a zero slope of the Bland-Altman graph indicates that the variance of the difference between the two methods is independent of their mean (i.e. the estimated 'true' number). This is a very simple procedure, yet provides appropriate information, whereas orthogonal regression analysis (as required by ISO 16140) is more complicated and may lead to biased slope estimates (Dissanaike and Wang, 2003).

3. Results

3.1 General

A total of 70 samples of each 100 g of mussel meat and moist, were analysed according to ISO 16649-2 (Plate count-TBX; Anonymous, 2001) and ISO 16649-3 (MPN-Donovan; Anonymous, 2005). Fifty of the samples were also analysed according to ISO 7251 (MPN-classical; Anonymous, 1993) and the plate count method on Mac Conkey.
The relevant raw data for all methods are given in Annex 2.

In the next subchapters the results of the comparisons between the different methods are presented. For each comparison, the results of the orthogonal regression analysis (ISO 16140) and the Bland-Altman analysis are summarised. For each comparison a figure is presented in which the Log_{10} values found with one method are plotted against the Log_{10} values found with another method. In these figures the 95 % confidence intervals per result and per method are indicated with small lines (based on the assumption that the results are Poisson distributed). For each comparison the results of the Bland-Altman analysis are also summarised in a figure.

3.2 Faecal coliforms (Mac Conkey vs. ISO 7251)

For the faecal coliforms a comparison of the results found with plating method Mac Conkey and with the classical MPN method ISO 7251 was made. Results are summarised in Figures 1 and 2. Figure 1 shows (from the 95 % confidence intervals) that in general the variation within results is higher for the MPN method (ISO 7251). However, for the lower numbers the variation within results becomes comparable for both methods. Furthermore, this figure shows that with ISO 7251 somewhat higher numbers were found compared to the Mac Conkey counts. The figure derived from the Bland-Altman analysis (Figure 2) shows the same. However, both the orthogonal regression analysis and the Bland-Altman analysis showed that the differences between the two methods were not significant (see below).

Results statistical analyses
Orthogonal regression analysis (ISO 16140):

- Slope = 1.010 (not significantly different from 1)
- Intercept = 0.350 (not significantly different from 0)

Bland-Altman analysis:

- Slope = -0.005, p = 0.84 (not significant)
- Offset = -0.347, p = 0.09 (not significant)

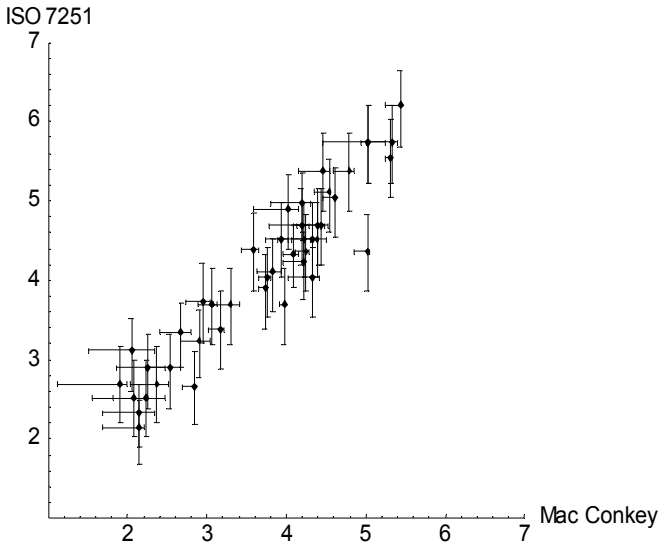

Figure 1 *Faecal coliforms: Log_{10} cfu found on Mac Conkey agar plotted against Log_{10} cfu found with MPN method ISO 7251. Lines indicate the 95 % confidence intervals within a result found with a method.*

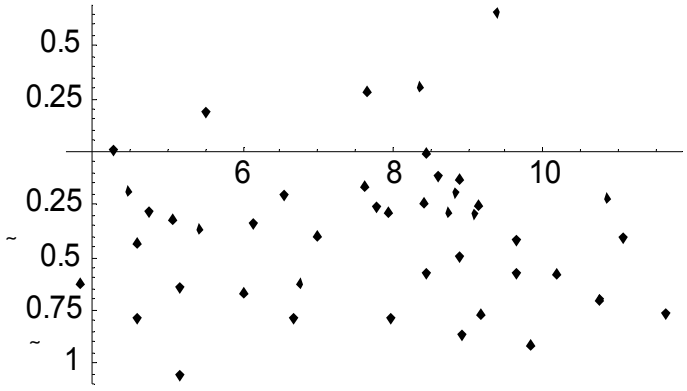

Figure 2 *Faecal coliforms: Bland-Altman analysis of results found with Mac Conkey agar and found with MPN method ISO 7251. On the X-axis the sum of the results found with both methods are indicated, on the Y-axis the differences between the results found with both methods are indicated.*

3.3 *Escherichia coli*

3.3.1 Mac Conkey vs. ISO 7251 (Classical MPN)

The results found with Mac Conkey agar plates and with the MPN method ISO 7251 (classical MPN) for enumeration of *Escherichia coli* are summarised in Figures 3 and 4. The variation within results is comparable for both methods (Figure 3). Similar to the results of the faecal coliforms, the numbers of *Escherichia coli* found with ISO 7251 were higher than the counts found with Mac Conkey agar. This is also reflected in the results of the Bland-Altman test (Figure 4). The differences were on the boundary of significance (see below).

Results statistical analyses
Orthogonal regression analysis (ISO 16140):

- Slope = 0.957 (not significantly different from 1)
- Intercept = 0.401 (just significantly different from 0)

Bland-Altman analysis:

- Slope = 0.025, p = 0.43 (not significant)
- Offset = -0.42, p = 0.06 (just significant)

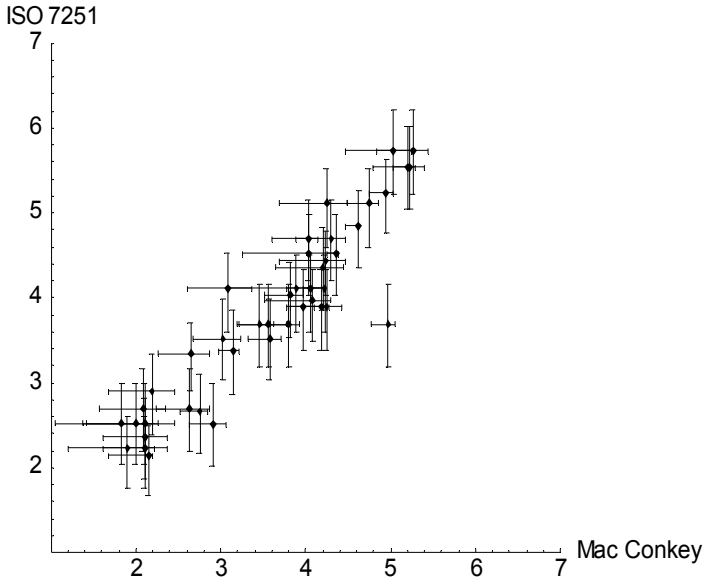

Figure 3 *Escherichia coli: Log_{10} cfu found on Mac Conkey agar plotted against Log_{10} cfu found with MPN method ISO 7251. Lines indicate the 95 % confidence intervals within a result found with a method.*

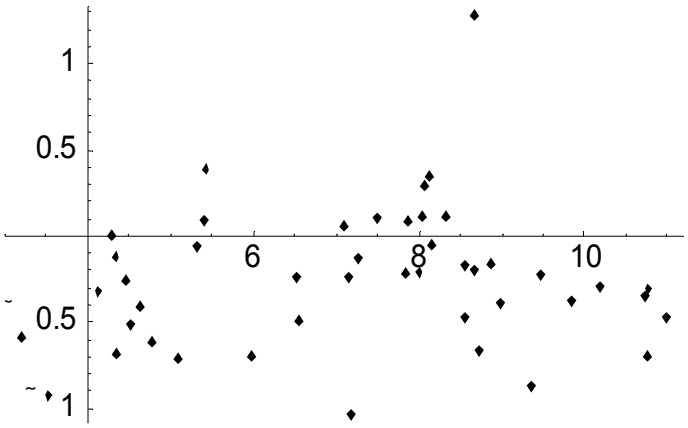

Figure 4 *Escherichia coli: Bland-Altman analysis of results found with Mac Conkey agar and found with MPN method ISO 7251. On the X-axis the sum of the results found with both methods are indicated, on the Y-axis the differences between the results found with both methods are indicated.*

3.3.2 Mac Conkey vs. ISO 16649-3 (Donovan MPN)

The results found with Mac Conkey agar plates and with the MPN method ISO 16649-3 (Donovan MPN) for *Escherichia coli* are summarised in Figures 5 and 6. In general the variation within results is higher for the Donovan MPN method (ISO 16649-3), except for the lower counts where the variation within results of both methods becomes comparable (Figure 5).
Significantly higher results were found with the Donovan MPN method when compared to the results found on Mac Conkey agar.

Results statistical analyses
Orthogonal regression analysis (ISO 16140):

- Slope = 0.938 (not significantly different from 1)
- Intercept = 0.651 (significantly different from 0)

Bland-Altman analysis:

- Slope = -0.03, p = 0.23 (not significant)
- Offset = -0.68, p = 0.002 (significant)

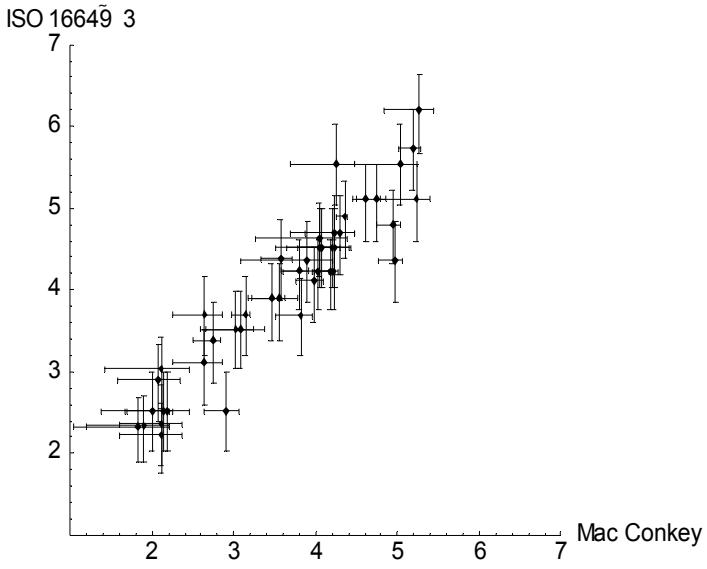

Figure 5 *Escherichia coli: Log_{10} cfu found on Mac Conkey agar plotted against Log_{10} cfu found with MPN method ISO 16649-3 (Donovan). Lines indicate the 95 % confidence intervals within a result found with a method.*

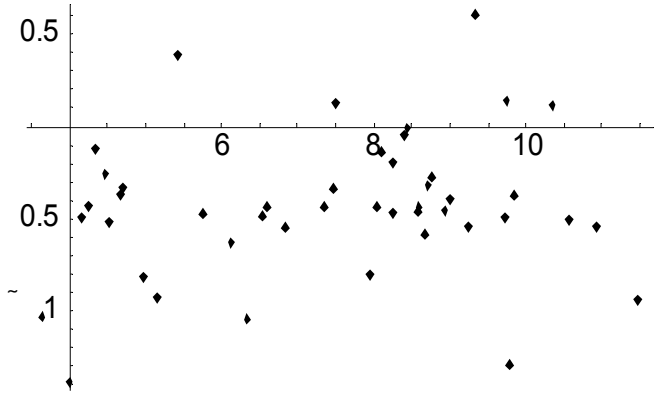

Figure 6 *Escherichia coli: Bland-Altman analysis of results found with Mac Conkey agar and found with MPN method ISO 16649-3 (Donovan). On the X-axis the sum of the results found with both methods are indicated, on the Y-axis the differences between the results found with both methods are indicated.*

3.3.3 Mac Conkey vs. ISO 16649-2 (TBX)

The results found with Mac Conkey agar plates and with the plating method on TBX (ISO 16649-2) for *Escherichia coli* are summarised in Figures 7 and 8.
The variation within results is higher for the results found with Mac Conkey than with TBX (Figure 7).
The results found with TBX are somewhat higher than the results found with Mac Conkey agar, but just on the boundary of significance.

Results statistical analyses
Orthogonal regression analysis (ISO 16140):

- Slope = 1.021 (not significantly different from 1)
- Intercept = -0.305 (just significantly different from 0)

Bland-Altman analysis:

- Slope = -0.01, p = 0.58 (not significant)
- Offset = 0.303, p = 0.037 (significant)

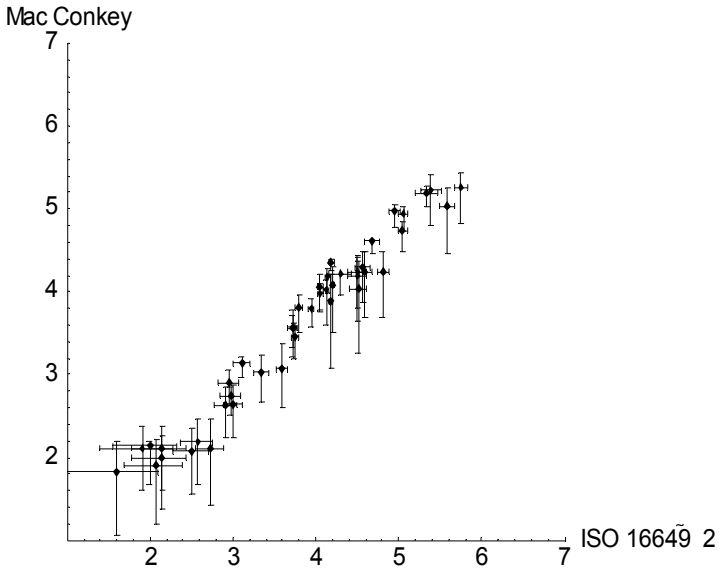

Figure 7 *Escherichia coli: Log_{10} cfu found on Mac Conkey agar plotted against Log_{10} cfu found with plating method ISO 16649-2 (TBX). Lines indicate the 95 % confidence intervals within a result found with a method.*

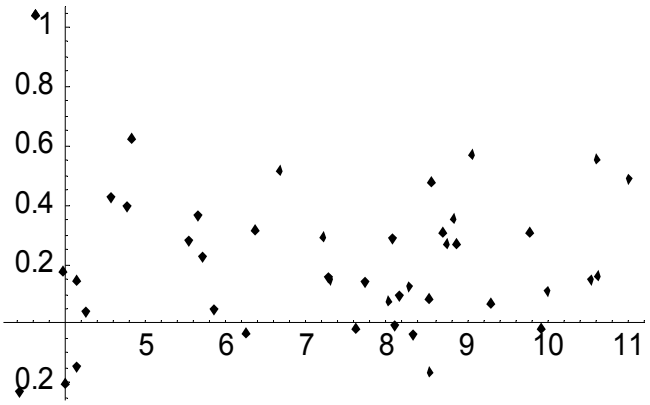

Figure 8 *Escherichia coli: Bland-Altman analysis of results found with Mac Conkey agar and found with plating method ISO 16649-2 (TBX). On the X-axis the sum of the results found with both methods are indicated, on the Y-axis the differences between the results found with both methods are indicated.*

3.3.4 ISO 7251 (Classical MPN) vs. ISO 16649-3 (Donovan MPN)

The results found with the two MPN techniques (ISO 7251 and ISO 16649-3) for *Escherichia coli* are summarised in Figures 9 and 10. With both MPN methods comparable results were obtained. The variation within results was also comparable.

Results statistical analyses
Orthogonal regression analysis (ISO 16140):

- Slope = 1.021 (not significantly different from 1)
- Intercept = -0.264 (not significantly different from 0)

Bland-Altman analysis:

- Slope = -0.01, p = 0.67 (not significant)
- Offset = 0.263, p = 0.182 (not significant)

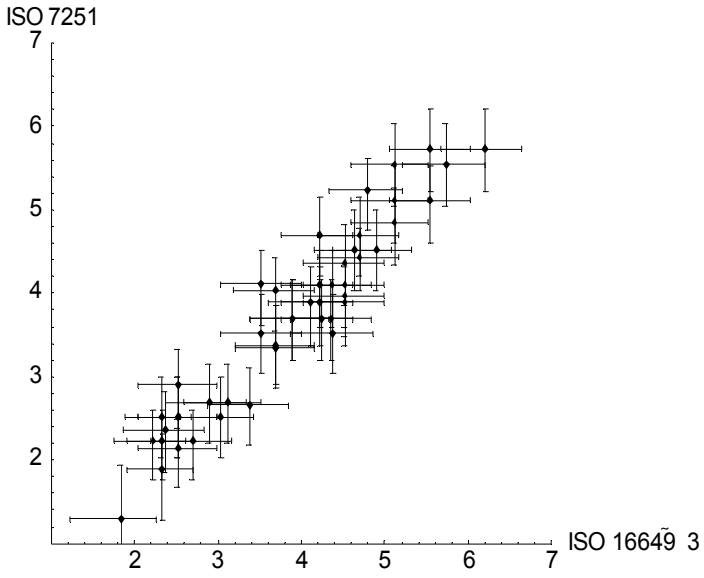

Figure 9 *Escherichia coli: Log_{10} cfu found with MPN method ISO 16649-3 (Donovan) plotted against Log_{10} cfu found with MPN method ISO 7251 (classical). Lines indicate the 95 % confidence intervals within a result found with a method.*

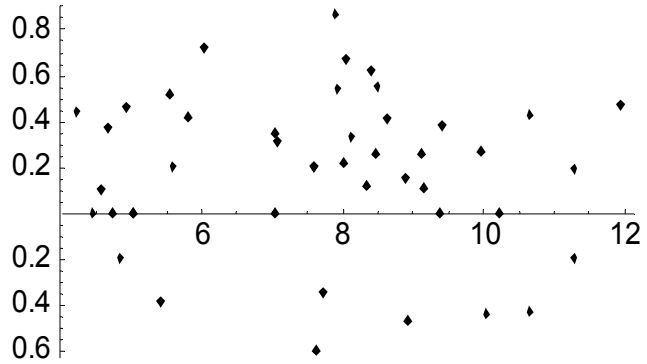

Figure 10 *Escherichia coli: Bland-Altman analysis of results found with MPN method ISO 16649-3 (Donovan) and found with MPN method ISO 7251 (classical). On the X-axis the sum of the results found with both methods are indicated, on the Y-axis the differences between the results found with both methods are indicated.*

3.3.5 ISO 7251 (Classical MPN) vs. ISO 16649-2 (TBX)

The results found with the classical MPN method (ISO 7251) and with the plating method on TBX (ISO 16649-2) for *Escherichia coli* are summarised in Figures 11 and 12. With both methods comparable results were obtained. In general the variation within results is higher for the MPN method (ISO 7251), except for the lower counts where the variation within results of both methods becomes comparable (Figure 11).

Results statistical analyses
Orthogonal regression analysis (ISO 16140):

- Slope = 0.977 (not significantly different from 1)
- Intercept = 0.109 (not significantly different from 0)

Bland-Altman analysis:

- Slope = 0.01, p = 0.63 (not significant)
- Offset = -0.11, p = 0.563 (not significant)

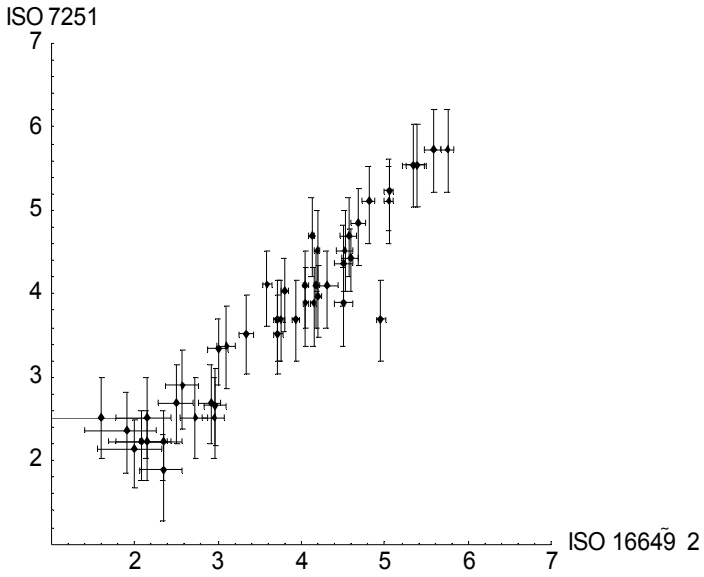

Figure 11 Escherichia coli: Log_{10} cfu found with plating method ISO 16649-2 (TBX) plotted against Log_{10} cfu found with MPN method ISO 7251. Lines indicate the 95 % confidence intervals within a result found with a method.

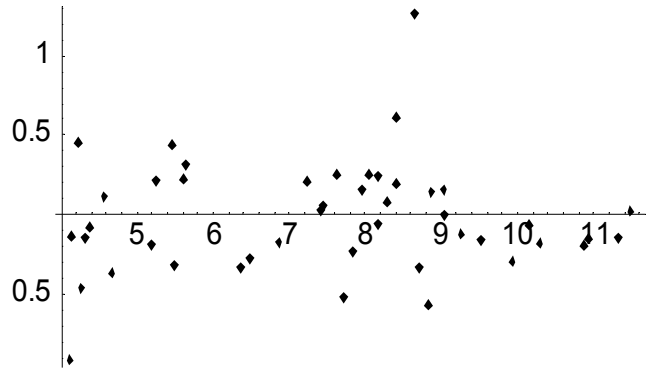

Figure 12 *Escherichia coli: Bland-Altman analysis of results found with plating method ISO 16649-2 (TBX) and found with MPN method ISO 7251. On the X-axis the sum of the results found with both methods are indicated, on the Y-axis the differences between the results found with both methods are indicated.*

3.3.6 ISO 16649-2 (TBX) vs. ISO 16649-3 (Donovan MPN)

The results found with the plating method on TBX (ISO 16649-2) and the Donovan MPN method (ISO 16649-3) for *Escherichia coli* are summarised in Figures 13 and 14. With both methods comparable results were obtained. In general the variation within results is higher for the MPN method (ISO 16649-3), except for the lower counts where the variation within results of both methods becomes comparable (Figure 13).

Results statistical analyses
Orthogonal regression analysis (ISO 16140):

- Slope = 0.979 (not significantly different from 1)
- Intercept = 0.244 (not significantly different from 0)

Bland-Altman analysis:

- Slope = 0.01, p = 0.57 (not significant)
- Offset = -0.24, p = 0.08 (not significant)

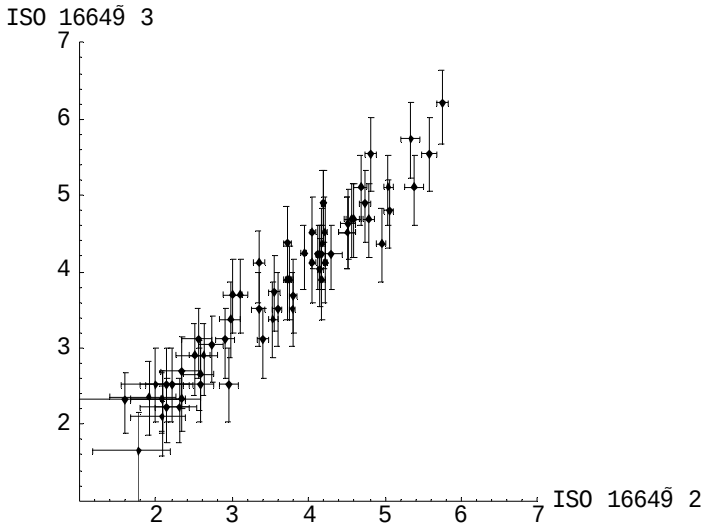

Figure 13 *Escherichia coli: Log_{10} cfu found with plating method ISO 16649-2 (TBX) plotted against Log_{10} cfu found with MPN method ISO 16649-3 (Donovan). Lines indicate the 95 % confidence intervals within a result found with a method.*

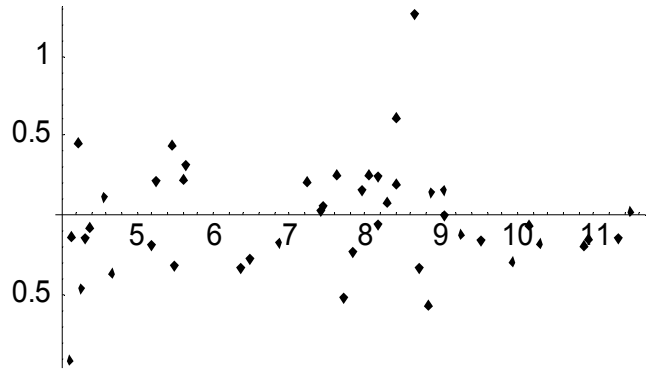

Figure 14 *Escherichia coli: Bland-Altman analysis of results found with plating method ISO 16649-2 (TBX) and found with MPN method ISO 16649-3 (Donovan). On the X-axis the sum of the results found with both methods are indicated, on the Y-axis the differences between the results found with both methods are indicated.*

4. Discussion

The quality of the bivalve molluscs production areas has traditionally been based on the number of faecal coliforms. Although Directive 91/492/EC prescribed the use of an MPN method it also allowed other methods if properly validated. Up to 2005 a plating method on Mac Conkey agar was used for the enumeration of faecal coliforms in bivalve molluscs in the Netherlands. The national validation study has shown that the plate count method on Mac Conkey agar is not significantly different from the (classical) MPN method which is described in ISO 7251 and suggested in Directive 91/492/EC.

In the new legislations (Commission Regulations (EC) No. 853/2004, 854/2004 and 2073/2005) the quality of bivalve molluscs is not longer based on the number of faecal coliforms, but on the number of *Escherichia coli*. The new Regulations describe again an MPN method for the enumeration of *Escherichia coli*.
The comparison studies of the four methods for enumeration of *Escherichia coli* showed in general high variation within results for the MPN methods (to be seen in larger 95 % confidence intervals). This is not an unexpected result as an MPN result is not based on a direct count but on a combination of positive results found with dilutions of the initial sample. At lower counts the variation in results between the MPN methods and the plate count methods became comparable. This is caused by a higher variation in results of plate count methods at lower numbers.
The results of the comparison studies showed that the Mac Conkey agar method is not an acceptable method for the enumeration of *Escherichia coli* in mussels as it gives significantly lower results than the presently prescribed MPN method (ISO 16649-3). Both MPN methods (ISO 7251 and 16649-3) and the plate count method on TBX (ISO 16649-2) gave equivalent results for the enumeration of *Escherichia coli* in mussels. Therefore, this study has also shown that the plate count method on TBX (ISO 16649-2) can be considered as a possible alternative method for the prescribed Donovan MPN method (ISO 16649-3) for enumeration of *Escherichia coli* in bivalve molluscs. For acceptance of alternative methods at international level, a full validation according to the procedure as described in EN/ISO 16140 is needed.

5. Conclusions

The national validation study has shown that the plate count method on Mac Conkey agar for the enumeration of faecal coliforms in bivalve molluscs was not significantly different from the classical MPN method (ISO 7251). Therefore, it can be concluded that the Netherlands have checked the quality of their shellfish production areas in compliance with Directive 91/492/EC.

From the comparisons of the four methods for enumeration of *Escherichia coli* in mussels the following conclusions can be drawn:

- Mac Conkey (Plate count) vs. ISO 7251 (MPN-classical): differences on the boundary of significance;
- Mac Conkey (Plate count) vs. ISO 16649-3 (MPN-Donovan): significantly different;
- Mac Conkey (Plate count) vs. ISO 16649-2 (Plate count-TBX): differences on the boundary of significance;
- ISO 7251 (MPN-classical) vs. ISO 16649-3 (MPN-Donovan): not significantly different;
- ISO 7251 (MPN-classical) vs. ISO 16649-2 (Plate count-TBX): not significantly different;
- ISO 16649-2 (Plate count-TBX) vs. ISO 16649-3 (MPN-Donovan): not significantly different.

Acknowledgements

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Annex 1. Media

Mac Conkey agar No. 3 (Oxoid CM 115).

Composition

Peptone	20	g
Lactose	10	g
Bile salts No. 3	1.5	g
Sodium Chloride	5	g
Neutral red	0.03	g
Crystal violet	0.001	g
Agar	15	g
Water	1000	ml

Preparation
Suspend 51.5 gram in 1 litre of distilled water. Bring to the boil to dissolve completely and sterilise at 121 °C for 15 minutes. Cool down to 47 °C before use. The pH should be 7.1 ± 0.2 (at room temperature).

Brilliant Green Lactose Broth (BGLB)

Composition

Peptone	10	g
Lactose	10	g
Ox Bile (dehydrated)	20	g
Brilliant-green (0.1 % by mass aqueous solution)	13	ml
Water	to 1000	ml

Preparation
Dissolve the peptone in 500 ml of distilled water. Add the 20 g of dehydrated ox bile dissolved in 200 ml distilled water. This solution should have a pH between 7.0 and 7.5. Make up to approximately 975 ml with distilled water. Add the lactose and adjust the pH to 7.4. Add the Brilliant-green solution and make up to 1000 ml with distilled water. Distribute 5 ml volumes in test tubes containing inverted inner fermentation (Durham) tubes and autoclave at 115 °C for 10 min.

Annex 2. Raw data

Table A.2.1 Results faecal coliforms and E.coli found with plating method MacConkey

Sample code	No. of typical colonies[1]	Dilution factor	cfu / 100g	No. of tubes pos. for faecal coliforms	No. of tubes pos. for *E. coli*	Confirmed Faecal coliforms cfu / 100 g	Confirmed *E.coli* cfu / 100 g
M1	426	20	8520	4/15	0/15	2272	<20
M2	173	20	3460	4/15	0/15	923	<20
M3	50	20	1000	0/15	0/15	<20	<20
M4	45	20	900	0/15	0/15	<20	<20
M5	2	20	40	1/2	1/2	20	20
M6	8	20	160	7/8	7/8	140	140
M7	35	20	700	15/15	12/15	700	560
M8	1207	20	24140	15/15	14/15	24140	22531
M9	80	20	1600	14/15	13/15	1493	1387
M10	16	20	320	11/15	6/15	235	128
M11	9	20	180	6/9	5/9	120	100
M12	55	20	1100	12/15	6/15	880	440
M13	15	20	300	9/15	6/15	180	120
M14	11	20	220	7/11	4/11	140	80
M15	17	20	340	5/15	3/15	113	68
M16	16	20	320	8/15	6/15	171	128
M17	23	20	460	11/15	5/15	337	153
M18	3	20	60	2/3	1/3	40	20
M19	5	20	100	4/5	1/5	80	20
M20	32	20	640	11/15	3/15	469	128
M21	54	20	1080	11/15	6/15	792	432
M22	269	20	5380	15/15	8/15	5380	2869
M23	224	20	4480	13/15	4/15	3883	1195
M24	472	20	9440	15/15	10/15	9440	6293
M25	709	20	14180	13/15	10/15	12289	9453
M26	312	20	6240	14/15	9/15	5824	3744
M27	67	20	1340	13/15	9/15	1161	804
M28	457	20	9140	11/15	6/15	6703	3656
M29	133	20	2660	11/15	6/15	1951	1064
M30	714	20	14280	3/4	3/4	10710	10710
M31	20-13	1000	16500	5/5	5/5	16500	16500
M32	39-44	1000	41500	7/7	7/7	41500	41500
M33	93-121	1000	10700	11/11	9/11	107000	87545
M34	61-80	1000	70500	8/9	7/9	62667	54833
M35	36-34	1000	35000	5/6	3/6	29167	17500
M36	32-19	10000	255000	5/6	4/6	212500	170000
M37	20-16	10000	180000	3/5	3/5	108000	108000
M38	19-36	10000	27500	6/6	4/6	275000	183333
M39	184-232	1000	208000	16/16	12/16	208000	156000
M40	22-17	1000	19500	4/5	2/5	15600	7800

1: Where two counts are given (xx-yy) duplicate counts were made

Table A.2. continued

Sample code	No. of typical colonies[1]	Dilution factor	cfu / 100g	No. of tubes pos. for faecal coliforms	No. of tubes pos. for *E. coli*	Confirmed Faecal coliforms cfu / 100 g	Confirmed *E.coli* cfu / 100 g
M41	24-16	1000	20000	4/5	3/5	16000	12000
M42	543	20	10860	8/10	6/10	8688	6516
M43	122-107	1000	114500	10/11	9/11	104091	103050
M44	1012	20	20240	10/12	9/12	16867	15180
M45	24-28	1000	26000	5/6	4/6	21667	17334
M46	31-32	1000	31500	4/6	3/6	21000	15750
M47	29-40	1000	34500	6/6	3/6	34500	17250
M48	43-23	1000	22500	5/6	2/6	27500	11000
M49	970	20	19406	11/12	7/12	17789	11320
M50	31-29	1000	30000	5/6	4/6	25000	20000

1: Where two counts are given (xx-yy) duplicate counts were made

Table A.2.2 Results faecal coliforms and E.coli found with MPN method ISO 7251

Sample code	Faecal coliforms MPN-code	*E. coli* MPN-code	Faecal coliforms cfu / 100 g	*E. coli* cfu / 100 g
M1	000	000	<20	<20
M2	000	000	<20	<20
M3	000	000	<20	<20
M4	000	000	<20	<20
M5	100	100	20	20
M6	320	320	140	140
M7	511	511	460	460
M8	55510	55510	33000	33000
M9	550	550	2400	2400
M10	520	410	490	170
M11	510	510	330	330
M12	552	542	5400	2200
M13	530	520	790	490
M14	420	410	220	170
M15	540	510	1300	330
M16	510	500	330	230
M17	530	530	790	790
M18	410	300	170	78
M19	520	410	490	170
M20	542	510	2200	330
M21	541	520	1700	490
M22	5530	5520	7900	4900
M23	5550	5540	24000	13000
M24	5520	5520	4900	4900
M25	55420	55300	22000	7900
M26	55310	55100	11000	3300
M27	5520	5100	4900	330
M28	5540	5520	13000	4900
M29	5520	5510	4900	3300
M30	55530	55520	79000	49000
M31	55410	55400	17000	13000
M32	55531	55521	110000	70000
M33	55552	55541	540000	170000
M34	55550	55540	240000	130000
M35	55550	55540	240000	130000
M36	55552	55551	540000	350000
M37	55552	55552	540000	540000
M38	55554	55552	1600000	540000
M39	55551	55551	350000	350000
M40	55520	55400	49000	13000
M41	55522	55211	94000	9200
M42	55510	55310	33000	11000
M43	55500	55200	24000	4900
M44	55510	55300	33000	7900
M45	55310	55300	11000	7900
M46	55510	55500	33000	24000
M47	55540	55430	130000	27000
M48	55520	55510	49000	33000
M49	55500	55400	24000	13000
M50	55520	55520	49000	49000

Table A.2.3 Results E.coli found with plating method ISO 16649-2 (TBX)

Sample code	No. of typical colonies[1]	No. of typical colonies[1] on next 10 fold dilution	Dilution factor	*E.coli* cfu / 100 g
M1	0		-	<20
M2	0		-	<20
M3	0		-	<20
M4	0		-	<20
M5	0		-	<20
M6	5		20	100
M7	47		20	940
M8	770		20	15400
M9	64		20	1280
M10	7		20	140
M11	7		20	140
M12	51		20	1020
M13	16		20	320
M14	6		20	120
M15	2		20	40
M16	4		20	80
M17	19		20	380
M18	11		20	220
M19	11		20	220
M20	27		20	540
M21	41		20	820
M22	280		20	5600
M23	195		20	3900
M24	437		20	8740
M25	564		20	11280
M26	263		20	5260
M27	45		20	900
M28	263		20	5260
M29	110		20	2200
M30	667		20	13340
M31	20-20		1000	20000
M32	55-42		1000	48500
M33	118-103	14-13	1000	113000
M34	113-112	13-7	1000	111000
M35	62-68		1000	65000
M36	22-27		10000	245000
M37	32-45		10000	385000
M38	45-68		10000	565000
M39	24-20		10000	220000
M40	757		20	15140

1: Where two counts are given (xx-yy) duplicate counts were made

Table A.2.3 continued

Sample code	No. of typical colonies[1]	No. of typical colonies[1] on next 10 fold dilution	Dilution factor	*E.coli* cfu / 100 g
M41	803		20	16000
M42	314		20	6280
M43	70-98	15-15	1000	90000
M44	698		20	13960
M45	32-32		1000	32000
M46	36-28		1000	32000
M47	44-34		1000	39000
M48	36-30		1000	33000
M49	560		20	11200
M50	32-42		1000	37000
M51	6		20	120
M52	172		20	3440
M53	705		20	14100
M54	822		20	16440
M55	63-61		1000	62000
M56	10		20	200
M57	21		20	420
M58	305		20	6100
M59	750		20	15000
M60	50-60		1000	55000
M60A	6		20	120
M61	3		20	60
M62	18		20	360
M64	126		20	2520
M65	178		20	3560
M66	8		20	160
M67	19		20	380
M68	112		20	2240
M69	781		20	15620
M70	754		20	15080

1: Where two counts are given (xx-yy) duplicate counts were made

Table A.2.4 Results E.coli found with MPN method ISO 16649-3 (Donovan)

Sample code	MPN-code	*E. coli* cfu / 100 g
M1	000	< 0.18
M2	000	< 0.18
M3	000	<0.18
M4	000	<0.18
M5	210	68
M6	510	330
M7	550	2400
M8	55530	79000
M9	5520	4900
M10	410	170
M11	510	330
M12	5520	4900
M13	530	790
M14	420	220
M15	411	210
M16	500	230
M17	510	330
M18	420	220
M19	520	490
M20	531	1100
M21	540	1300
M22	5530	7900
M23	5510	3300
M24	5541	17000
M25	5540	13000
M26	5550	24000
M27	510	330
M28	5530	7900
M29	5510	3300
M30	55410	17000
M31	55410	17000
M32	55540	130000
M33	55512	63000
M34	55540	130000
M35	55551	350000
M36	55540	130000
M37	55551	350000
M38	55554	1600000
M39	55552	540000
M40	55500	23000

Table A.2.4 continued

Sample code	MPN-code	*E. coli* cfu / 100 g
M41	55510	33000
M42	55200	4900
M43	555000	23000
M44	55410	17000
M45	55510	33000
M46	55510	33000
M47	55520	49000
M48	55502	43000
M49	55510	33000
M50	55520	49000
M51	400	110
M52	550	2400
M53	5531	11000
M54	5540	13000
M55	55520	49000
M56	410	160
M57	530	750
M58	5510	3300
M59	5530	7900
M60	55530	79000
M60A	4110	210
M61	200	45
M62	540	1300
M64	540	1300
M65	552	5400
M66	510	330
M67	51010	330
M68	5540	13000
M69	55530	79000
M70	55410	17000