DANISH MINISTRY
OF THE ENVIRONMENT
Environmental
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Coliform bacteria and *E. coli* in drinking water. Comparison of EU reference method with alternative methods

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Content

Annex A – Summary of Danish Colilert P/A Study

Background

Coliform bacteria and ***E. coli*** are two important parameters for control of the quality of drinking water. The Council Directive on the Quality of Water intended for Human consumption (98/83/EF) specifies in Annex III, part 1 the method for analysis of these parameters: EN ISO 9308-1:2000. This method is based upon the use of Lactose TTC agar with sodium heptadecylsulphate (Tergitol 7), also called the TTC-Tergitol method.

The Directive states that Member States may use alternative methods, providing the provisions of Article 7, part 5 are met. The Article states that:

5a) Member States shall comply with the specifications for the analyses of parameters set out in Annex III of the Directive.
5b) Methods other than those specified in Annex III, Part 1, may be used providing it can be demonstrated that the results obtained are at least as reliable as those produced by the methods specified. Member States which have recourse to alternative methods shall provide the Commission with all relevant information concerning such methods and their equivalence.

The large European study with the Council Directive reference method for coliform bacteria and ***E. coli*** in drinking water has shown that the EU reference method fails to detect a significant proportion of coliforms and ***E. coli*** in drinking water (Niemela, Lee & Fricker, 2003). Especially water with high heterotrophic counts may cause problems with competing flora in drinking water. These types of water, e.g. water from wells or contaminated mainwater, are precisely the types of water in which coliform bacteria and ***E. coli*** are looked for and often should be found. It should be noted that in the EU study ***E. coli*** were analysed by direct incubation at 44 °C which is not in compliance with the EN ISO standard. Nevertheless this does not change the conclusions regarding coliform bacteria at 37 °C. It might affect the results for ***E. coli*** due to on one hand lower findings of competing flora leading to higher counts of ***E. coli*** but on the other hand stressed ***E. coli*** due to the combination of maximum temperature at the same time as growing on a selective agar ("hurdle-effect").

EN ISO 9308-1:2000 states specifically in the scope that: "The Standard Test has a low selectivity, allowing the detection of injured bacteria. Due to the low selectivity, background growth can interfere with the reliable enumeration of coliform bacteria and ***E. coli***, for example in some drinking waters, like shallow well waters, that have not been disinfected and yield a high background growth. This part of EN ISO 9308 is therefore especially suitable for disinfected water and other drinking waters of low bacterial numbers". Later in the scope it is concluded that the method is "… applicable to other kinds of water provided that suspended matter or background flora does not interfere with filtration, culture and counting".

Based on this information stated in the Standard and on the studies done on comparison of Colilert™ with the EU reference method for the analysis of drinking water for coliform bacteria including ***E. coli*** (Niemela, Lee & Fricker, 2003) Denmark decided not to conduct a fully equivalency study but instead verify the existing knowledge by testing Danish drinking water of different microbiological quality in a limited equivalency study. It was also decided that it should be aimed to validate and approve a method that could be used for water with as well as

without background flora. Most of the Danish drinking water from public water supplies has a very good microbiological quality with a low content of heterotrophic microorganisms. In Denmark however there are many small private water supplies e.g wells with higher levels of background flora than normally found in the public water supplies and this background flora may affect the detection of coliform bacteria. Furthermore will the good quality water normally contain very high levels of background flora when it is contaminated with coliform bacteria including ***E. coli*** and the method used shall therefore be valid for this purpose as well.

In the end of 2003 an equivalency study was conducted in one laboratory with the specific purpose to:

- document that the already known problems with using EN ISO 9308-1:2000 for drinking water with high heterotrophic counts also were valid for Danish drinking water
- compare other well-known methods to EN ISO 9308-1:2000 (without deviations from the Standard)
- support a Danish equivalency study from 2000/2001 (chapter 2)
- demonstrate the effect of Colilert as a quantitative method as Colilert is approved for qualitative testing (P/A) of Danish drinking water in case of testing contaminated water to find the source of contamination (annex A).

Colilert is already used as a supplementary test in several microbiological laboratories in Denmark for quantitative testing and seems to be a robust and well-known method that needs validation.

It was decided to use only one microbiological testlaboratory (Danish Environmental Protection Agency's (EPA) Reference Laboratory) for this equivalency study as it is a requirement for other laboratories to demonstrate verification of the method before using it routinely. This meant that there was no need to use more laboratories to verify potential problems with the methods as problems found by the reference laboratory when analysing Danish drinking water will be enough to discriminate the method.

The equivalency studies were performed on the EN ISO 9308-1:2000 (designated "EU reference method" in this report) against five other internationally recognized methods: Lauryl Sulphate Agar (LSA), Membrane Lactose Glucuronide Agar (MLGA), Chromogenic agar, Chromocult and Colilert. Results from the equivalency studies are given in chapter 3 and 4.

Sammenfatning og konklusioner

Coliforme bakterier og ***E. coli*** er to vigtige parametre i forbindelse med kontrollen af dansk drikkevand. Rådets direktiv om kvaliteten af drikkevand (98/83/EF) specificerer i Bilag III, del 1 referencemetoden til disse to parametre: EN ISO 9308-1:2000. Metoden er baseret på brugen af Laktose TTC agar med natrium heptadecylsulfat (Tergitol 7), også kaldet TTC-Tergitol metoden.

Direktivet angiver, at medlemsstaterne kan bruge alternative metoder, forudsat at kravene i artikel 7, stk. 5 overholdes. Artiklen angiver, at:

5a) Medlemsstater skal overholde specifikationerne for analyse af parametrene der er anført i Direktivets Bilag III.
5b) Der kan anvendes andre metoder end de i Bilag III, del 1, anførte, såfremt det kan påvises, at de resultater, der opnås herved, er mindst lige så pålidelige som dem, der kan opnås ved de angivne metoder. Medlemsstater, der anvender alternative metoder, meddeler Kommissionen alle relevant oplysninger vedrørende disse metoder og deres ækvivalens.

På baggrund af oplysningerne i Standarden (EN ISO 9308-1:2000) om interferens fra følgeflora samt studier, der har sammenlignet Colilert™ med EU referencemetoden for analyse af drikkevand for coliforme bakterier, herunder ***E. coli*** (Niemela, Lee & Fricker, 2003), besluttede Danmark at undlade at foretage et fuldt ækvivalensstudium og i stedet verificere den eksisterende viden ved at teste dansk drikkevand af varierende mikrobiologisk kvalitet i et begrænset ækvivalens-studium. Det blev også besluttet at sigte mod at validere og godkende en metode, som kunne anvendes til vand såvel med som uden kraftig følgeflora.

Størstedelen af Danmarks drikkevand fra almene vandforsyninger er af god mikrobiologisk kvalitet med lavt indhold af heterotrofe mikroorganismer. I Danmark er der imidlertid en hel del lokale vandforsyninger, som har højere indhold af følgeflora, som kan påvirke påvisningen af coliforme bakterier. Dertil kommer, at også drikkevand, der normalt er af god mikrobiologisk kvalitet, typisk vil indeholde meget høje niveauer af følgeflora i de tilfælde, hvor det forurenes med coliforme bakterier, herunder ***E. coli***, og den anvendte metode skal også kunne anvendes til disse situationer.

Det danske studium blev baseret på Niemela, Lee & Frickers artikel fra 2003 og på et tidligere dansk studium fra 2000/2001. Der blev udført to begrænsede ækvivalensstudier i praksis, heraf ét med podede vandprøver og ét med naturlige vandprøver. Formålet med det danske studium var at:

- dokumentere, at de allerede kendte problemer med anvendelsen af EN ISO 9308-1:2000 på drikkevand med højt indhold af heterotrofe mikroorganismer også gjaldt for dansk drikkevand
- sammenligne andre velkendte metoder med EN ISO 9308-1:2000
- undersøge effekten af Colilert som en kvantitativ metode, da denne metode er godkendt til kvalitativ prøvning (P/A) af dansk drikkevand (se bilag A).

Det danske studium blev udført på EN ISO 9308-1:2000 (betegnet "EU reference metode" i denne rapport) og inkluderede i alt fem internationalt anerkendte metoder: Lauryl Sulfat Agar (LSA), Membran Laktose Glucuronid Agar (MLGA), Chromogen agar, Chromocult, Colilert samt den danske referencemetode indtil nu: DS 2255:2001 med MPN i MacConkey-bouillon.

Den samlede konklusion på resultaterne fra det danske studium med både podede og naturlige prøver af drikkevand er som følger:

Colilert viste sig at være ækvivalent til EU's referencemetode til påvisning af coliforme bakterier og ***E. coli*** i podede prøver med lav baggrundsflora. Det samme studie bekræftede, at EU's referencemetode gav problemer i form af overvoksede membranfiltre ved analyse af vand med højt indhold af heterotrofe mikroorganismer. Colilert viste god genfinding af coliforme bakterier og ***E. coli*** i prøver med såvel lavt som højt indhold af heterotrofe mikroorganismer (baggrundsflora).

Studiet viste, at det samme problem med overvoksede filtre forekom tilsvarende for tre af de andre membranfiltreringsmetoder: LSA37, Chromogen agar og Chromocult.

MLGA viste forholdsvis god genfinding af både coliforme bakterier og ***E. coli*** i podede prøver, men det blev valgt at udelukke metoden fra det videre arbejde på grund af nogle tekniske faktorer med inkubationstider, der blev vurderet at være uhensigtsmæssige for de fleste danske laboratorier.

Den hidtidige danske referencemetode (DS 2255:2001) indgik i den del af studierne, der omfattede naturlige prøver. Det blev fundet, at metoden gav dårligere resultater end de øvrige metoder, idet der ikke blev påvist coliforme bakterier med DS 2255 i én af de 15 prøver, der blev fundet positiv med én eller flere af de andre metoder (EU referencemetoden, Chromogen agar og Colilert).

Miljøstyrelsen har på denne baggrund besluttet at implementere EU referencemetoden EN ISO 9308-1:2000 med Colilert som en alternativ metode til undersøgelse af dansk drikkevand for coliforme bakterier og ***E. coli***.

Summary and conclusions

Coliform bacteria and ***E. coli*** are two important parameters for control of the quality of drinking water. The Council Directive on the Quality of Water intended for Human consumption (98/83/EF) specifies in Annex III, part 1 the method of analysis of these parameters: EN ISO 9308-1:2000. This method is based upon the use of Lactose TTC agar with sodium heptadecylsulphate (Tergitol 7), also called the TTC-Tergitol method.

The Directive states that Member States may use alternative methods, providing the provisions of Article 7, part 5 are met. The Article states that:

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Based on information stated in the Standard (EN ISO 9308-1:2000) about interference of background growth and on studies done on comparison of Colilert™ with the EU reference method for the analysis of drinking water for coliform bacteria including ***E. coli*** (Niemela, Lee & Fricker, 2003) Denmark decided not to conduct a fully equivalency study but instead verify the existing knowledge by testing Danish drinking water of different microbiological quality in a limited equivalency study. It was also decided that it should be aimed to validate and approve a method that could be used for water with as well as without background flora.

Most of the Danish drinking water from public water supplies is of very good microbiological quality with low heterotrophic counts. In Denmark however there are quite many private water supplies, e.g. wells with higher levels of background flora than normally found in public water supplies and this background flora may affect the detection of coliform bacteria. Furthermore will the good quality water normally contain very high levels of background flora when it is contaminated with coliform bacteria including ***E. coli*** and the method used shall therefore be valid for this purpose too.

The Danish equivalency study was based on Niemela, Lee & Frickers publication (2003) and one earlier Danish study from 2000/2001. Two limited practical equivalency studies were undertaken on spiked respectively natural water samples with the specific purpose to:

- document that the already known problems with using EN ISO 9308-1:2000 for drinking water with high heterotrophic counts also were valid for Danish drinking water
- compare other well-known methods to EN ISO 9308-1:2000
- demonstrate the effect of Colilert as a quantitative method as this method is approved for qualitative testing (P/A) of Danish drinking water (annex A).

The equivalency studies were performed on the EN ISO 9308-1:2000 (designated “EU reference method” in this report) and included in total five other internationally recognized methods: Lauryl Sulphate Agar (LSA), Membrane

Lactose Glucuronide Agar (MLGA), Chromogenic agar, Chromocult, Colilert and the Danish national reference method until now: MPN in MacConkey broth (DS 2255:2001).

The final conclusion on the data from the Danish equivalency studies with spiked as well as natural drinking water samples are summarized as follows:

Colilert was shown to be equivalent to the EU reference method for the detection of coliform bacteria and ***E. coli*** in spiked samples with a low background flora.

Furthermore the same study with spiked samples confirmed well-known problems by using the reference method for detection of coliform bacteria and ***E. coli*** in waters with high heterotrophic counts due to overgrowth of the membrane filters. Colilert showed good recoveries in the same samples.

The study demonstrated that the problem with overgrown filters was also seen for three other membrane filtration methods: LSA37, Chromogenic agar and Chromocult.

MLGA showed relatively good recoveries in spiked samples, but the method was excluded from the further studies due to technical reasons.

The Danish reference method until now (DS 2255:2001) was included in testing of natural drinking water samples where it was found not to be able to detect coliform bacteria in any of 15 samples where one or more of three other methods (EU Reference method, Colilert and Chromogenic agar) detected coliform bacteria.

The Danish EPA has therefore decided to implement the EU Reference method EN ISO 9308-1:2000 with Colilert as an alternative quantitative method for the examination of coliform bacteria and ***E. coli*** in Danish drinking water.

1 International equivalency studies and approved methods

Many countries have during the latest years made a lot of studies of the equivalency between the EU reference method for drinking water (EN ISO 9308-1:2000) and other internationally recognized methods for coliform bacteria and ***E. coli***. Due to this fact The Danish Environmental Protection Agency (EPA) decided to use these results as basic knowledge for the national Danish studies.

This chapter gives an overview over international equivalency studies and approved methods. This overview does not aim to give detailed information on the studies but to highlight the conclusions used for the Danish work.

1.1 EU equivalency study

Niemela, Lee & Fricker published in 2003 a study which aimed to demonstrate the use of ISO CD 17994 2001, the Equivalency standard stating criteria for comparing two methods. They demonstrated it specifically for the comparison of Colilert and the EU reference method for detection of coliform bacteria and ***E. coli***.

They compared results from 20 laboratories in 13 European countries (including Denmark) and concluded that the Colilert detected significantly more coliform bacteria as well as ***E. coli*** than the EU reference method did. This means that the EU reference method fails to detect a significant proportion of coliform bacteria including ***E. coli***. Furthermore Niemela, Lee & Fricker (2003) concluded that confirmation of positive wells in Colilert was not necessary.

As mentioned in "Background" the EU Study by Niemela, Lee & Fricker (2003) deviated from the EU Standard by incubation of plates for ***E. coli*** directly at 44 °C, which may affect part of the conclusion. The main-problem with growth of background flora will nevertheless be of increasing influence at lower temperature.

As possible explanations for the better recovery of coliform bacteria with Colilert than with the EU reference method Niemela, Lee & Fricker (2003) pointed out that:

- background flora may disturb the reading of the TTC-Tergitol plates and may inhibit the growth of coliform bacteria on the plates leading to false negative findings
- detection of β-D-galactosidase activity in Colilert on primary isolation where the same organisms often do not express their ability to ferment lactose if inhibitory agents are present as in TTC-Tergitol agar
- stressing of the bacteria due to membrane filtration
- more suitable composition of nutrients in Colilert than in TTC-Tergitol agar with excessive content of nutrients.

Finally they concluded that the findings of Colilert as superior to the EU reference method is limited to Colilert as a defined substrate with limited amounts of nutrients and not to all methods using β-D-galactosidase and β-D-glucoronidase as detection principle.

1.2 Equivalency studies and accepted methods in other countries

Based on experience from other European countries the status for methods either accepted or approved as alternative methods to the EU reference method in some other countries is outlined below.

1.2.1 The Netherlands

According to RIVM and KIWA (2001): "Comparison between NEN-EN-ISO 9308-1 and an alternative method for the enumeration of coliform bacteria and ***Escherichia coli"*** it has been demonstrated that LSA performs equally, or sometimes even better (the recovery is the same or higher) than TTC-Tergitol for the enumeration of coliform bacteria and ***E. coli***. The study demonstrated thus that detection and enumeration of coliform bacteria and ***E. coli*** with LSA (and confirmation in accordance with the EU reference method) is at least as reliable as with the EU reference method. The data have been submitted to the Commission.

1.2.2 UK

The UK has compared Membrane Lauryl Sulphate broth (MLSB) to TTC-Tergitol. UK has undertaken sufficient equivalency testing for approving MLSB as an alternative method for coliform bacteria and ***E. coli*** and the data have been submitted to the Commision.

1.2.3 Germany

In Germany the Federal Environmental Agency's drinking water Commission has approved Colilert-18/Quanti-Tray as an alternative method to the EU reference method without having undertaken a specific equivalency study.

1.2.4 Colilert International Approvals

Colilert is approved or accepted in 24 countries for official drinking water analyses (amongst these 24 countries four EU Member States: Germany, Hungary, Czech and Italy and two non-EU-Member States: Iceland and Norway have approved the method according to the new drinking water directive, whilst UK and Ireland have approved the method according to the old directive).

1.3 Methods for the Danish equivalency study

As mentioned above the Danish equivalency study intended to verify results from other European countries when used for Danish drinking water. The methods were chosen on basis of:

- The EU equivalency study with Colilert (preliminary results before publication from Niemela, Lee & Fricker, 2003)

- Presentations of equivalency studies from the Netherlands and UK at the first EMAG meeting, 11 April 2003:

  the Netherland study where TTC-Tergitol was compared with the method with LSA

  the UK study where MLSB and Membrane Lactose Glucunoride Agar (MLGA) were compared to Tergitol-TCC

- The inclusion of two commercially available chromogenic media for coliform bacteria and ***E. coli*** also using membrane filtration as technique:
  - Chromogenic agar (Oxoid)
  - Chromo Cult (Merck).

MLSB was not included in the Danish study. The technique used for MLSB with soaking a pad in broth is well-known in UK laboratories, but not routine in Danish laboratories. The technique is not difficult but deviating from the normal flow in the laboratories. Therefore it was assessed to be time consuming in the laboratories because of changing between different techniques for membrane filtration in the daily work.

2 Danish equivalency study 2000/2001 on MPN (DS 2255) against Colilert

In 2000/2001 a Danish equivalency study was performed between the existing national reference method (DS 2255:1983) and Colilert. Both methods are MPN-methods but with two different detection principles. DS 2255 defines coliform bacteria as bacteria able to ferment lactose to acid and gas at 37 °C in MacConkey broth and ***E. coli*** as coliform bacteria furthermore able to ferment lactose at 44 °C in MacConkey broth and producing indole from tryptophane at 44 °C. In Colilert coliforms are defined by the activity of ß-galactosidase and ***E. coli*** by the activity of ß-glucuronidase.

Fricker, Niemela & Lee (2000) states that it is clear the many coliform bacteria are unable to ferment lactose within 48 hours whereas they will be able to demonstrate the activity of ß-galactosidase. Therefore differences between the two methods may be expected as fully agreeable results may not be achieved by two different detection principles.

The different principles however does not discriminate per se one method for another as there are more different definitions of the group of coliform bacteria and coliform bacteria and ***E. coli*** detected with either of the methods are unwanted in drinking water.

2.1 Materials and methods

A total of 64 samples of drinking water were analysed in two laboratories using both the Danish national MPN method and Colilert for coliform bacteria and ***E. coli***. The results from both methods were then compared.

2.2 Results

Of the 64 samples examined most (51) were negative using both methods. The results of the remaining 13 tests are given in Table 1.

Table 1 Results of coliform and *E. coli* analyses from 13 samples of water using the Danish national method (DS 2255:1983) and Colilert

DS 2255:1983		Colilert	
Coliform bacteria	*E. coli*	Coliform bacteria	*E. coli*
<1	<1	2	<1
1	1	10	<1
<1	<1	16	<1
>161	<1	>201	<1
<1	<1	1	<1
<1	<1	4	<1
1	<1	<1	<1
<1	<1	1	<1
1	<1	1	<1
1	<1	1	<1
49	49	53	14
70	46	47	8
23	23	64	13

2.3 Conclusions

There were no significant differences between the number of coliform bacteria found using the two different methods when comparing (Students t-test) paired results for coliform bacteria when detected by both methods. However Colilert detected coliform bacteria in 12 samples and the Danish reference method in only eight. This indicates that the use of Colilert will increase the number of samples found to contain coliform bacteria compared to the use of the Danish MPN-method. It is likely, based on data generated during the European study that also the actual number of coliform bacteria detected with Colilert will be higher in some samples than that detected by traditional methods.

For ***E. coli*** there was complete agreement between the two methods regarding which samples were positive (excluding one sample with the finding of 1 ***E. coli***/100 ml with the Danish reference method and <1 /100 ml with Colilert, which is found to be within the statistical uncertainty). However, the total numbers of ***E. coli*** were higher using the Danish reference method (three samples; n = 13) although non-significant (Students t-test). The most likely explanation for the higher counts is the possible misidentification of ***E. coli*** using traditional detection principles. Traditional methodologies identify ***E. coli*** based on its ability to grow at 44 °C, ferment lactose and produce indole from tryptophan. Some other coliform species (notably strains of ***Klebsiella oxytoca***) also possess these characteristics and may lead to false positive results for ***E. coli***.

In conclusion, the use of Colilert is likely to give rise to an increase in the number of samples found to contain coliform bacteria. Furthermore Colilert may lead to an increase in the actual number of coliform bacteria detected in a given sample over that detected using the Danish standard method. For ***E. coli***, the number of samples found to be positive by Colilert is likely to be similar to the findings with DS 2255:1983, but the actual number of ***E. coli*** in the samples may be slightly lower using Colilert.

3 Danish equivalency study on TTC-Tergitol (EN ISO 9308-1:2000) compared to five other methods – spiked samples

This equivalency study included five different methods (four membrane filtrations and one MPN) to be compared with TTC-Tergitol when analysing samples of drinking water spiked with the coliform bacteria ***Enterobacter aerogenes*** and ***E. coli***.

Besides the comparison of methods, two different brands of membrane filters were tested as it has been shown (Ossmer, Schmidt & Mende, 1999) that different types of filters may affect the results significantly. Furthermore the samples were analyzed right after spiking and again after 24 hours at 0 – 5 °C which should show the possible effect of refrigerated storage as it might be used in normal sampling procedures.

The choice of methods is described in details in "Background" and in part 1.3.

This study aimed to verify for Danish drinking water the problems seen with the EU Reference Method when background flora interferes as well as the suitability of Colilert as demonstrated in the EU trial published by Niemela, Lee & Fricker (2003). At the same time the Danish study included other internationally tested methods to examine their performance for Danish drinking water.

3.1 Materials and Methods

3.1.1 Water samples

Two different levels of microbiological quality of drinking water were used:
a) drinking water from a public water supply ("Public")
b) drinking water from a private water supply (well) with high heterotrophic counts at 15.000 cfu/ml ("Well").

Both types of water were inoculated with ***Enterobacter aerogenes*** as well as ***E. coli***. The cultures were grown overnight in Brain Heart Infusion broth (BHI; Merck 1.10493) at 37 ºC respectively 44 ºC after which they were stored in a water/ice-bath. Immediately after placing in water/ice the concentrations of the cultures were determined for each of the cultures by pour plating in Yeast Extract Glucose agar as double spreadings (YEA; EN/DS 6222:2002) incubated at 37 ºC overnight. The cultures were kept on ice (0 ºC) overnight and used for dilution and inoculation of water samples. Dilutions used for inoculation were calculated from the YEA counts.

Four litres of each type of water were inoculated with 15 ml diluted mixed culture to give a final concentration on 10 – 25 cfu/100 ml.

Exact concentrations of the used inoculums were determined as described above for the BHI-tubes, immediately after spiking the water samples.

Inoculation was done while the water was homogenized on a magnetic stirrer. After inoculation the water was left on the stirrer for 30 minutes to ensure a homogenous sample. Hereafter the water samples were distributed as follows:

i) 1300 ml were analyzed immediately in portions of 100 ml (six membrane filtrations on two different filters and one MPN technique)
ii) 1300 ml were stored at 0 – 5 ºC for 24 hours before analysing in portions of 100 ml

1400 ml were diluted with 1400 ml non-inoculated water of the same type (public or well). From these 2800 ml:
iii) 1300 ml were analyzed immediately in portions of 100 ml
iv) 1300 ml were stored at 0 – 5 ºC for 24 hours before analysing in portions of 100 ml

Table 2: Designation of the different samples

	Public water supply	Private water supply
Analyzed immediately	Public 0	Well 0
Analyzed after refrigeration for 24 hours	Public 24	Well 24
Diluted and analyzed immediately	Public(1:1) 0	Well(1:1) 0
Diluted and analyzed after refrigeration for 24 hours	Public(1:1) 24	Well(1:1) 24

3.1.2 Membranfilters

Two different brands of membrane filters (0,45 µm cellulose-mixed-esters) were tested:
MF1 = Gelman 66278 – GN 6. White filter.
MF2 = Millipore HAWG04700. Black filter.

3.1.3 Methods

The tested methods are listed in table 3.

Table 3: Methods tested in the equivalency study.

Designation	Technique	Substrate type	Substrate Brand
M1	Membrane filtration. EN ISO 9308-1:2000	TCC-Tergitol	Oxoid CM793 + SR 148a
M2 – 37	Membrane filtration	LSA	Scharlau 01-524
M2 – 44	Membrane filtration	LSA	Scharlau 01-524
M3	Membrane filtration	MLGA	Oxoid CM 1031
M4	Membrane filtration	Chromogenic medium	Oxoid CM 1046B
M5	Membrane filtration	ChromoCult	Merck 1.10426.0500
M6	Most Probable Number (MPN)	Colilert (Quanti Tray)	Idexx

All combinations of type of water, dilution, type of filter and methods were tested immediately as well as after 24 hours.

For every combination 100 ml of sample was analyzed.

3.2 Results and discussion

In table 4 is shown the results of the determination of concentrations of the inoculums used for spiking the water samples.

Table 4: Colony counts of inoculum used for spiking determined in YEA 37 °C. Counts from both spreadings are given.

Dilution	Day –1 (to decide which dilution to use)		Day 0 (counts from inoculum used)	
	Enterobacter	*E. coli*	*Enterobacter*	*E. coli*
10^{-5}	> 300 / > 300	> 300 / > 300	> 300 / > 300	> 300 / > 300
10^{-6}	105 / 117	> 300 / > 300	101 / 103	> 300 / > 300
10^{-7}	13 / 10	31 / 39	9 / 11	51 / 58
10^{-8}	0 / 1	4 / 4	1 / 0	2 / 7
10^{-9}	0 / 0	0 / 0	0 / 0	0 / 0

Based on the results from day -1 it was decided to spike the samples with 15 ml of 10^{-7} of each culture. This resulted in (calculated from the Day 0 results):
[(51 + 58)/2] x 15 ml : 4000 ml x 100 ml = approx. 20 ***E. coli*** pr. 100 ml
[(9 + 11)/2] x 15 ml : 4000 ml x 100 ml = approx. 4 ***E. aerogenes*** pr. 100 ml, i.e. 20 ***E. coli*** + 4 ***E. aerogenes*** = 24 coliform bacteria pr. 100 ml.

These calculated concentrations of coliform bacteria and ***E. coli*** are given as "Expected counts" in table 5.

The results of the comparison of the seven methods are shown in tables 5 and 6. In table 5 results are given as the colony counts or as the most probable number and in table 6 the same results are shown as percentage of the expected counts.

As described in 3.1.2 two filter types were compared. The readings of the plates showed that the black filters (MF2) did not support the differentiation of the different colored colonies as well as the white filters. Furthermore the black filters did not allow a good assessment of the color of the agar underneath the filter. It was therefore decided to use only the counts on MF1 as the counts on MF2 should be treated with some caution. The data from MF2 are not shown in this report.

It should although be noted that the MF2 filters were overgrown in the same samples as MF1.

Table 5: Colony counts resp. most probable numbers from the equivalency study with spiked samples. "C" = coliform bacteria; "EC" = *E. coli*; "OG" = overgrown; "-" = not tested.

	Public 0				Public 24				Well 0				Well 24			
	Undil.		1:1		Undil.		1:1		Undil.		1:1		Undil.		1:1	
	C	EC	C	EC	C	EC	C	EC	C	EC	C	EC	C	EC	C	EC
TTC-Tergitol MF1	5	4	8	8	12	12	6	6	OG	OG	OG	OG	OG	OG	OG	OG
LSA37 MF1	0	0	0	0	5	5	4	4	OG	OG	OG	OG	OG	OG	OG	OG
LSA44 MF1	-	0	-	0	-	0	-	0	-	0	-	0	-	1		5
MLGA MF1	13	13	7	7	9	9	2	2	13	13	9	9	22	19	17	12
Chromogenic MF1	27	12	10	6	12	6	13	8	OG	OG	OG	OG	OG	OG	OG	OG
Chromocult MF1[a)]	23	0	8	0	10	0	8	0	OG	OG	OG	OG	OG	OG	OG	OG
Colilert	12	2	5	1	15	10	10	2	200	19	200	9	200	18	200	11
Exp. Count	24	20	12	10	24	20	12	10	24	20	12	10	24	20	12	10

a) Due to a mistake presumptive *E. coli* were not verified by oxidase test immediately after counting, but after prolonged incubation where the presumptive colonies were found to be oxidase positive, i.e. non-coliforms.

Table 6: Percentage (%) of colony counts resp. most probable numbers compared to the expected counts calculated from the concentration of inoculum. This table converts the results in table 5 to percentage.
"C" = coliform bacteria; "EC" = *E. coli*; "-" = not tested or cannot be calculated due to overgrowth of the plates.

	Public 0				Public 24				Well 0				Well 24			
	Undil.		1:1		Undil.		1:1		Undil.		1:1		Undil.		1:1	
	C	EC	C	EC	C	EC	C	EC	C	EC	C	EC	C	EC	C	EC
TTC-Tergitol MF1	21	20	67	80	50	60	50	60	-	-	-	-	-	-	-	-
LSA37 MF1	0	0	0	0	21	25	33	40	-	-	-	-	-	-	-	-
LSA44 MF1	-	0	-	0	-	0	-	0	-	0	-	0	-	5	-	50
MLGA MF1	54	65	58	70	38	45	17	20	54	65	75	90	92	95	142	120
Chromogenic MF1	113	60	83	60	50	30	108	80	-	-	-	-	-	-	-	-
Chromocult MF1	96	0	67	0	42	0	67	0	-	-	-	-	-	-	-	-
Colilert	50	10	42	10	63	50	83	20	833	95	1667	90	833	90	1667	110

The results in table 5 showed that none of the media TTC-Tergitol, LSA 37, Chromogenic or Chromocult were suitable for water with high heterotrophic counts as the plates were overgrown so reading of typical colonies was not possible.

In the Dutch study (RIVM & KIWA , 2001) it was estimated that much more of the membrane filter was covered with background flora on TTC-Tergitol compared to LSA, which underlines the problem with using TTC-Tergitol for water with high counts of heterotrophic count. In the present Danish study LSA37 was also overgrown when analysing water with high heterotrophic count.

As both LSA37 and LSA44 shall be conducted on each water sample if coliform bacteria as well as ***E. coli*** are to be determined, the performance of LSA44 is of minor interest when LSA37 is found not to be suitable because of overgrowth. Furthermore the results from LSA44 itself were not very convincing as ***E. coli*** spiked in the water samples were not recovered in most of the samples.

MLGA showed reasonable results with a good coherence between undiluted samples and 1:1-diluted samples. The recoveries were from approximately 20 – 70% in spiked public water and 50 – 140% in spiked private water. The higher recoveries in the spiked private water compared to the spiked public water are expected to be caused partly by a natural content of coliform bacteria in the private water and therefore detection of these coliform bacteria as well.

The method using MLGA includes a technical problem as it is incubated at 30 °C for 4 hours prior to 37 °C for 14 hours. This means that samples analysed in the morning have to be read in the night. This implies that the samples have to be moved to 37 °C late in the afternoon/early evening so that reading can be made the next morning without deviations. Contrary the risk is that the reading will not be done at the prescribed time but as the first thing in the morning even if this is after more than 14 hours at 37 °C. Both situations may imply logistic problems causing more expensive analyses due to less flexibility in the planning of sampling as well as in the work in the laboratories. Alternatively it might result in unwanted modifications of the method.

Colilert was the other method able to detect coliform bacteria and ***E. coli*** in the spiked water without being overgrown. It is remarkable that the Colilert detects very high numbers of coliform bacteria in the spiked water from private wells (>> 100% recoveries). This may be due to:

- A high content of coliform bacteria in the well water before spiking. This might also explain the overgrowth of the other media but would not change the conclusion that the overgrown media are not suitable for counting coliform bacteria and ***E. coli*** in this water. Nevertheless the used well water is known to normally having a high heterotrophic count but no coliform bacteria as determined by the Danish national reference method (DS 2255) which is a MPN technique in MacConkey broth. It is possible that the normal findings are false-negative compared to Colilert as the methods uses two different detection principles that may allow detection with Colilert and -D-galactosidase activity, but not with lactose fermentation as principle.
- A false positive reaction in Colilert. This cannot be finally concluded and documented as the growth in the positive wells in Colilert was not identified. Nevertheless literature has shown Colilert to be rather specific for coliform bacteria (Niemela, Lee & Fricker, 2003).

Colilert was assessed to be the most easy and robust technique to handle including all aspects from preparing to reading and without any verification needed.

3.3 Conclusion on spiked samples

The equivalency study with spiked samples confirmed the problems with using TTC-Tergitol for detection of coliform bacteria and ***E. coli*** in waters with high heterotrophic counts as also stated in the scope of EN ISO 9308-1:2000 (“Due to the low selectivity, background growth can interfere with the reliable enumeration of coliform bacteria and ***E. coli***, for example in some drinking waters, like shallow well waters, that have not been disinfected and yield a high background growth. This part of ISO 9308 is therefore especially suitable for disinfected water and other drinking waters of low bacterial numbers”).

The study demonstrated that the same problem was seen for three other membrane filtration methods: LSA37, Chromogenic agar and Chromocult which were all overgrown as well.

The only membrane filtration method not overgrown was MLGA which showed relatively good recoveries. The negative experience with this method was due to technical facts with incubation periods which means that the use of the method requires quite good logistics if the analyses should not be handled during the night as discussed above. Therefore this method was excluded from the further studies.

Colilert showed varying results for spiked public water compared to TTC-Tergitol with some results higher and some lower than TTC-Tergitol. A Student's t-Test on log 10 converted results for the eight paired set of data for TTC-Tergitol/Colilert in spiked public water showed a non-significant difference (31% probability) which means that the two methods can be considered equivalent. The differences were also found to be non-significant if Students t-test was made on the four paired set of data for coliform bacteria and for ***E. coli*** separately.

It was decided to make a further equivalency study with TTC-Tergitol and Colilert with natural non-spiked water samples of varying microbiological quality. Two othmer methods were included as explained below. The four methods for the equivalency study with natural drinking water were:

- TTC-Tergitol as EU-reference method (EN ISO 9308-1:2000)
- Colilert as the most easy and robust technique to handle, apparently not affected by high heterotrophic counts (at least not for detection of ***E. coli***) and with promising results from extensive international trials.
- Chromogenic agar as the chromogene agar giving the highest recoveries and not requiring verification.
- The Danish reference method DS 2255 (MPN technique using MacConkey broth) to compare the results with the method used today as national method.

4 Danish equivalency study on TTC-Tergitol (EN ISO 9308-1:2000) compared to three other methods – natural samples

As concluded in chapter 3 this study on natural samples was based on the results from the spiked samples and included besides TTC-Tergitol two of the five tested alternative methods , supplemented with the Danish reference method until now (MPN, DS 2255). The natural samples in this part of the equivalency study were drinking water samples from public as well as private supplies.

4.1 Materials and Methods

4.1.1 Water samples

The water samples for this part of the study were the routine samples of drinking water sampled by the EPA reference laboratory from public as well as private water supplies for analysis.

The samples were also tested for aerobic colony counts at 22 °C and for some samples at 37 °C as well according to Danish legislation (DS/EN 6222).

In total 38 samples were analysed.

4.1.2 Methods

The methods chosen for comparison in this part were:

- M1: TCC-Tergitol (EN ISO 9308-1:2000)
- M4: Chromogenic medium (Oxoid CM 1046B)
- M6: Colilert
- M7: MPN with MacConkey broth (DS 2255, 2. ed., 2001).

In this part of the equivalency study verification of presumptive findings with all four methods were verified as described in EN ISO 9308-1:2000 regardless the method used. This should enable a direct comparison of verified findings.

4.2 Results and discussion

Results are shown in table 7 with place of sampling, aerobic colony counts and the results from the comparison.

E. coli was not detected in any of the samples and therefore these results are let out in table 7.

Table 7: Results of analyses of 38 natural samples from public and private supplies for - coliform bacteria (37 °C). In none of the samples *E. coli* were detected.

Sampling site	Colony count pr. ml		Coliform bacteria (37 °C) per 100 ml			
	22 °C	37 °C	DS 2255	TTC-Tergitol	Chromogenic	Colilert
Private supply	18	-	<1	<1	1	1
Private supply	47	-	<1	<1	<1	<1
Private supply	11	-	<1	5	6	4
Public distribution system	3	-	<1	<1	<1	<1
Public distribution system	7	-	<1	<1	<1	<1
Boring	4	<1	<1	<1	<1	<1
Public distribution system	1	-	<1	<1	<1	<1
Water plant supply	<1	<1	<1	<1	<1	<1
Public distribution system	13	-	<1	<1	<1	<1
Water plant supply	4	<1	<1	<1	1	2
Public distribution system	<1	<1	<1	<1	<1	<1
Public distribution system	240	-	<1	39	Overgrown	62
Public distribution system	8	-	<1	<1	16	25
Public distribution system	1	-	<1	1	2	<1
Well for flow measuring	1	-	<1	<1	<1	<1
Public distribution system	<1	-	<1	<1	<1	<1
Well for flow measuring	4	-	<1	<1	<1	<1
Water plant supply	16	1	<1	11	17	25
Water plant supply	32	3	<1	<1	3	3
Public distribution system	13	-	<1	<1	1	<1
Public distribution system	7	-	<1	<1	<1	<1
Public distribution system	10	-	<1	<1	<1	<1
Public distribution system	10	-	<1	<1	1	<1
Public distribution system	6	-	<1	<1	<1	1
Industry	53	-	<1	<1	<1	<1
Private supply	>3000	-	<1	<1	15	<1
Water plant supply	54	3	<1	<1	Uncountable due to iron	<1
Private supply	41	-	<1	<1	<1	<1
Water plant supply	4	<1	<1	<1	<1	<1
Water plant supply	260	35	<1	<1	Uncountable due to iron	4
Private supply	56	-	<1	<1	<1	<1
Private supply	<1	-	<1	<1	<1	<1
Public distribution system	16	-	<1	<1	<1	<1
Public distribution system	43	-	<1	1	<1	<1
Public distribution system	9	-	<1	<1	<1	<1
Public distribution system	6	-	<1	<1	<1	<1
Private supply	8	-	<1	<1	<1	<1
Public distribution system	7	-	<1	<1	1	<1

Table 7 shows that the Danish reference method DS 2255 was not able to detect coliform bacteria in any of the samples. There was not found any presumptive positive findings of coliform bacteria (any colour changes at all) and therefore no ***E. coli***. This means that it is the method it self that is too insensitive, it is not due to any verification steps.

With the other three methods coliform bacteria were detected and verified in five samples with EN ISO 9308-1:2000, in 11 samples with Chromogenic agar and in 9 samples with Colilert.

In total 15 out of the 38 samples were found positive for coliforms although in eight of the 15 samples the counts were only 1 – 2 cfu pr. 100 ml meaning that there is a large uncertainty on these results and also on other lower count.

Nevertheless there was a natural pattern in the findings with more of the positive samples (eight of 15) positive with two or three methods. For the other seven positive samples cfu was as low as 1 in five of the seven samples. This meant that only two samples were in reality found positive with only one method.

Verification data showed (raw data not shown here) that of 77 presumptive colonies picked from Chromogenic agar only 31 were oxidase negative. This is a verification rate of 40%, where as 100% of the 45 tested positive wells from Colilert were oxidase negative, i.e. verified coliform bacteria.

According to ISO/DIS 17994:2002 the relative differences (RD_i%) were calculated for TTC-Tergitol compared to Chromogenic agar (see table 8) and for TTC-Tergitol compared to Colilert (see table 9). Finally the two alternative methods are compared in table 10.

The results are evaluated as a one-sided evaluation according to ISO/DIS 17994:2002 as it is decided to accept an alternative method whenever its average performance is either quantitatively equivalent (D = 10) or higher than the reference method. In fact the conclusion for the study described in this chapter will be the same regardless of the use of one-sided or two-sided evaluation (details for the two-sided evaluations not shown). According to ISO/DIS 17994:2002 the number of paired data with regular counts – not zero counts – should be higher than produced in this study. As this Danish study however is meant to verify other results it is accepted to use the data from natural contaminated samples and therefore with a higher percentage of very low counts.

Table 8: Results of the comparative study of TTC-Tergitol and Chromogenic agar according to ISO 17994.

TTC-Tergitol per 100 ml	Chromogenic per 100 ml	Relative Difference %	Remarks
<1	1	69,31	b
<1	<1	-	a
5	6	18,23	
<1	<1	-	a
<1	<1	-	a
<1	<1	-	a
<1	<1	-	a
<1	<1	-	a
<1	<1	-	a
<1	1	69,31	b
<1	<1	-	a
39	Overgrown	-	a
<1	16	283,32	b
1	2	69,31	
<1	<1	-	a
<1	<1	-	a
<1	<1	-	a
11	17	43,53	
<1	3	138,63	b
<1	1	69,31	b
<1	<1	-	a
<1	<1	-	a
<1	1	69,31	b
<1	<1	-	a
<1	<1	-	a
<1	15	277,26	b
<1	Uncountable due to iron	-	a
<1	<1	-	a
<1	<1	-	a
<1	Uncountable due to iron	-	a
<1	<1	-	a
<1	<1	-	a
<1	<1	-	a
1	<1	-69,31	b
<1	<1	-	a
<1	<1	-	a
<1	<1	-	a
<1	1	69,31	b

a) Deleted according to ISO 17994, 6.1 as the counts with both methods was zero resulting in <1 cfu per 100 ml or as one or both methods gave results other than a count, e.g. "overgrown".
b) Calculated according to ISO 17994, 6.2.2 as one of the two methods gave count zero. Therefore the constant one (1) is added to these counts before calculation of natural logarithm (ln). Optimal at least 75% of the samples should contain regular count data, which has not been possible here with only 25%.

From the results in table 8 (n = 12) the following values can be calculated:

Mean	92,30	Accord. to ISO 17994, 6.3
Standard deviation	100,00	Accord. to ISO 17994, 6.4
U	57,735	Accord. to ISO 17994, 6.4
LO	34,56	Accord. to ISO 17994, 6.4
HI	150,03	Accord. to ISO 17994, 6.4

From the results in table 8, the calculations above and ISO 17994, 7.3.2 (LO > 0) it is shown that TTC-Tergitol and Chromogenic agar are found to be different in this equivalency study with Chromogenic agar giving the highest counts. From these data Chromogenic agar should then be at least as reliable as TTC-Tergitol. Nevertheless three results for Chromogenic agar were excluded before calculating the equivalency, as colony counts could not be read on this agar due to overgrowth (one sample) and iron residues in the water sample (two samples). Overall it is therefore concluded that Chromogenic agar might give reading problems as also shown with the spiked samples (chapter 3) and Chromogenic agar will therefore not be approved from this Danish study.

Table 9: Results of the comparative study of TTC-Tergitol and Colilert according to ISO 17994.

TTC-Tergitol per 100 ml	Colilert per 100 ml	Relative Difference %	Remarks
<1	1	69,31	b
<1	<1	-	a
5	4	-22,31	
<1	<1	-	a
<1	<1	-	a
<1	<1	-	a
<1	<1	-	a
<1	<1	-	a
<1	<1	-	a
<1	2	109,86	b
<1	<1	-	a
39	62	46,36	
<1	25	325,81	b
1	<1	-69,31	b
<1	<1	-	a
<1	<1	-	a
<1	<1	-	a
11	25	82,10	
<1	3	138,63	b
<1	<1	-	a
<1	<1	-	a
<1	<1	-	a
<1	<1	-	a
<1	1	69,31	b
<1	<1	-	a
<1	<1	-	a
<1	<1	-	a
<1	<1	-	a
<1	<1	-	a
<1	4	160,94	b
<1	<1	-	a
<1	<1	-	a
<1	<1	-	a
1	<1	-69,31	b
<1	<1	-	a
<1	<1	-	a
<1	<1	-	a
<1	<1	-	a

a) Deleted according to ISO 17994, 6.1 as the counts with both methods was zero resulting in <1 cfu per 100 ml or as one or both methods gave results other than a count, e.g. “overgrown”.
b) Calculated according to ISO 17994, 6.2.2 as one of the two methods gave count zero. Therefore the constant one (1) is added to these counts before calculation of natural logarithm (ln). Optimal at least 75% of the samples should contain regular count data, which has not been possible here with only 27%.

From the results in table 9 (n = 11) the following values can be calculated:

Mean	76,49	Accord. to ISO 17994, 6.3
Standard deviation	112,96	Accord. to ISO 17994, 6.4
U	68,117	Accord. to ISO 17994, 6.4
LO	8,37	Accord. to ISO 17994, 6.4
HI	144,61	Accord. to ISO 17994, 6.4

From the results in table 9, the calculations above and ISO 17994, 7.2.2 (LO > 0) it is shown that TTC-Tergitol and Colilert are found to be different in this equivalency study, but with Colilert giving the highest counts. This means that Colilert shows results that are at least as reliable as those found with TTC-Tergitol and therefore Colilert can be regarded equivalent to the reference method.

Table 10: Results of the comparative study of Chromogenic agar and Colilert according to ISO 17994.

Chromogenic per 100 ml	Colilert per 100 ml	Relative Difference %	Remarks
1	1	0,00	
<1	<1		a
6	4	-40,55	
<1	<1		a
<1	<1		a
<1	<1		a
<1	<1		a
<1	<1		a
<1	<1		a
1	2	69,31	
<1	<1		a
Overgrown	62		a
16	25	44,63	
2	<1	-109,86	b
<1	<1		a
<1	<1		a
<1	<1		a
17	25	38,57	
3	3	0,00	
1	<1	-69,31	b
<1	<1		a
<1	<1		a
1	<1	-69,31	b
<1	1	69,31	b
<1	<1		a
15	<1	-277,26	b
Uncountable due to iron	<1		a
<1	<1		a
<1	<1		a
Uncountable due to iron	4		a
<1	<1		a
<1	<1		a
<1	<1		a
<1	<1		a
<1	<1		a
<1	<1		a
<1	<1		a
1	<1	-69,31	b

a) Deleted according to ISO 17994, 6.1 as the counts with both methods was zero resulting in <1 cfu per 100 ml or as one or both methods gave results other than a count, e.g. "overgrown".
b) Calculated according to ISO 17994, 6.2.2 as one of the two methods gave count zero. Therefore the constant one (1) is added to these counts before calculation of natural logarithm (ln). Optimal at least 75% of the samples should contain regular count data, which has not been possible here with only 50%.

From the results in table 10 (n = 12) the following values can be calculated:

Mean	-34,48	Accord. to ISO 17994, 6.3
Standard deviation	97,15	Accord. to ISO 17994, 6.4
U	56,090	Accord. to ISO 17994, 6.4
LO	-90,57	Accord. to ISO 17994, 6.4
HI	21,61	Accord. to ISO 17994, 6.4

From the results in table 10, the calculations above and ISO 17994, 7.3.4 it is shown that the results from Chromogenic agar compared with Colilert are found to be inconclusive and more samples should be examined before equivalency could be decided. No further studies will though be made as it was not the aim of this study to compare the two alternative methods with each other.

4.3 Conclusion on natural samples

Chromogenic agar will not be approved as an alternative method to the EU Reference method even though the Chromogenic agar generally gave higher counts than the TTC-Tergitol. It was shown that Chromogenic agar gave some of the same problems with reading the plates as was seen with the spiked samples in chapter 3 and this is the reason for not approving the method.

TTC-Tergitol and Colilert was found to be different in this equivalency study with Colilert giving the highest counts for coliform bacteria. This means that Colilert should be considered at least as reliable as TTC-Tergitol and therefore the methods are regarded as equivalent.

Chromogenic agar compared with Colilert was found to be inconclusive for detection of coliform bacteria in this equivalency study and more samples should be examined before equivalency could be decided.

As ***E. coli*** was not detected in any of the samples of natural drinking water conclusion cannot be drawn on this parameter from this part of the practical studies. In the final conclusion results from the spiked samples will therefore be included for this parameter.

5 Final conclusion

In this final conclusion the data from the Danish equivalency studies with spiked as well as natural drinking water samples are summarized.

The overall conclusion is that the Danish EPA approved Colilert as an alternative method to the EU reference method (EN ISO 9308-1:2000) for the examination of drinking water for coliform bacteria and ***E. coli***.

Colilert was shown to be equivalent to the EU reference method for the detection of coliform bacteria and ***E. coli*** in spiked samples with a low background flora. Furthermore the same study with spiked samples confirmed well-known problems by using the reference method for detection of coliform bacteria and ***E. coli*** in waters with high heterotrophic counts due to overgrowth of the membrane filters. Colilert showed good recoveries in the same samples.

The study demonstrated that the problem with overgrown filters was seen also for three other membrane filtration methods: LSA37, Chromogenic agar and Chromocult.

MLGA showed relatively good recoveries in spiked samples, but the method was excluded from the further studies due to technical reasons.

The Danish reference method until now (DS 2255:2001) was included in testing of natural drinking water samples where it was found not to be able to detect coliform bacteria in any of 15 samples where one or more of three other methods (EU Reference method, Colilert and Chromogenic agar) detected coliform bacteria.

6 Literature

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Summary and conclusion from report on P/A methods for detection of coliform bacteria and *E. coli*

Having experienced a series of protracted incidents of polluted potable water the Danish EPA has called for more simple and rapid methods for qualitative screening (P/A) of coliform bacteria, including E. coli in potable water.

The tested methods are not intended to replace the quantitative method(s) approved at all times by the Danish EPA but meant to be used in connection with a case of pollution, with a view to tracking its source/cause.

Commercial rapid methods on the market which have not been approved by the Danish EPA. The intention of the present project was therefore to evaluate the suitability of the methods for Danish potable water and at the same time to evaluate a modification of the method approved by the Danish EPA (DS 2255) in terms of a lowered detection limit.

The testing included 10 samples of Danish potable water of varying quality. All samples were analysed as double determination for coliform bacteria as well as for ***E. coli***. Five of the samples were furthermore analysed for both parameters after inoculation with ***E. coli*** whereas the other five samples were analysed after inoculation with pure cultures of ***Aeromonas hydrophila, Klebsiella pneumoniae*** and ***Citrobacter freundii***.

The tested rapid methods gave positive findings for all the natural water samples (100 ml, 500 ml, 1000 ml) where the reference method detected coliforms and ***E. coli***.

For a few samples the rapid methods gave positive results when the reference method showed negative findings. This means that the rapid methods do not fail to detect polluted water.

The samples inoculated with ***E. coli, A. hydrophila*** (non-coliform) og ***K. pneumoniae*** (coliform) showed full agreement between the tested methods.

For samples inoculated with ***Citrobacter freundii***, however, the results were inconsistent. This may be explained partly by the fact that ***Citrobacter*** is a slow lactose fermenter and partly by the interference between the low concentration of coliforms and the other microorganisms in the water. Atypical reactions are known to appear for ***Citrobacter*** in MacConkey-broth.

On the basis of the results the Danish EPA will permit the use of the tested alternative methods for screening of Danish potable water for coliform bacteria, including ***E. coli***. However, final negative findings with these qualitative methods in cases with polluted drinking water must be verified by quantitative analysis with the method approved at all times by the Danish

EPA. Verification can be done on the same sample within 24 hours from sampling or by testing a new sample from the same source.

The methods which are, thus validated and approved and can be used for qualitative screening, are listed below. The methods cannot be used for the legally required routine public control of potable water, but may form part of tracking the source in case of pollution. Other than the statutory control, operational control of potable water is not under the auspices of the authorities, and the choice of method is free.

The methods are:

Modified DS 2255 with analysis of greater volumes (500 or 1000 ml) by membrane filtration, at which the sensitivity is improved (lowering of the detection limit) and pollutions, if any, may be observed at an earlier stage.

DS 2255 supplemented with direct incubation of MacConkey tubes at 44°C.

The Colilert® systems (18 or 24 hours)

ReadyCULT®.

Reference:
Jeppesen, V.F. & Bagge, L. (2003) Rapid methods for the screening of coliform bacteria and ***E. coli*** in drinking water. Report from the Danish EPA. In Danish.