Comparison of Colilert-18 with miniaturised most probable number method for monitoring of *Escherichia coli* in bathing water

Ananda Tiwari, Seppo I. Niemelä, Asko Vepsäläinen, Jarkko Rapala, Seija Kalso and Tarja Pitkänen

ABSTRACT

The purpose of this equivalence study was to compare an alternative method, Colilert-18 Quanti-Tray (ISO 9308-2) with the European bathing water directive (2006/7/EC) reference method, the miniaturised most probable number (MMPN) method (ISO 9308-3), for the analysis of *Escherichia coli*. Six laboratories analysed a total of 263 bathing water samples in Finland. The comparison was carried out according to ISO 17994:2004. The recovery of *E. coli* using the Colilert-18 method was 7.0% and 8.6% lower than that of the MMPN method after 48 hours and 72 hours of incubation, respectively. The confirmation rate of presumptive *E. coli*-positive wells in the Colilert-18 and MMPN methods was high (97.8% and 98.0%, respectively). However, the testing of presumptive *E. coli*-negative but coliform bacteria-positive (yellow but not fluorescent) Colilert-18 wells revealed 7.3% false negative results. There were more false negatives in the naturally contaminated waters than in the samples spiked with waste water. The difference between the recovery of Colilert-18 and the MMPN method was considered not significant, and subsequently the methods are considered as equivalent for bathing water quality monitoring in Finland. Future bathing water method equivalence verification studies may use the data reported herein. The laboratories should make sure that any wells showing even minor fluorescence will be determined as positive for *E. coli*.

Key words | alternative method, bathing water, Colilert-18, comparison, *E. coli*, miniaturised most probable number method

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ABBREVIATIONS

ANOVA	One-way analysis of variance
D	Relative difference from zero
E. coli	*Escherichia coli*
Fluor44	β-glucuronidase test
Gas44	Gas production from lactose test
Ind44	Indole test
ISO	International Organization for Standardization
MMPN	Miniaturised most probable number
MMPN-48	MMPN method after 48 hours of incubation
MMPN-72	MMPN method after 72 hours of incubation
Ox	Oxidase test
U	Expanded uncertainty of the measurement

INTRODUCTION

Regular monitoring of bathing water is needed for protecting the health of bathers and ensuring safe water quality (WHO 2006). European bathing water directive (2006/7/EC) is a major legally-binding and guiding standard for monitoring of bathing water quality in the European Union member

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states (European Union 2006). This directive sets criteria for classifying bathing water as excellent, good, sufficient and poor on the basis of microbial quality (European Union 2006). It specifies the acceptable counts of *Escherichia coli* (*E. coli*) and intestinal enterococci (as counts/100 mL), that will be monitored as microbiological parameters. The microbial threshold values of bathing water are different for inland and coastal waters (European Union 2006). As an EU member country, Finland has implemented the European bathing water directive since the beginning of 2006 and monitors its bathing sites as guided by the directive. According to the European Environment Agency (EEA 2012), there are 320 bathing sites in Finland, of which 74% are inland bathing sites.

There is a significant epidemiological linkage between poor bathing water quality and gastrointestinal illness among bathers (WHO 2006; Mansilha *et al.* 2009). Bathing water may contain different faecal pathogenic bacteria, viruses and protozoa (WHO 2006; Hokajärvi *et al.* 2013). The use of indicators of faecal contamination simplifies the water quality monitoring at bathing sites, as it is not practical or even possible to detect all pathogens in daily monitoring activities (WHO 2006; Valente *et al.* 2010). *E. coli* is the best-known indicator of pathogenic microbes with faecal origin in water (WHO 2001). *E. coli* has exclusively faecal origin, and its presence in water confirms the faecal contamination (WHO 2006).

The standard methods for monitoring of *E. coli* as specified in European Bathing Water Directive (2006/7/EC) are the membrane filtration method (ISO 9308-1 2000) and miniaturised most probable number (MMPN) method (ISO 9308-3 1998). Various studies have reported that the membrane filtration method (ISO 9308-1 2000) is a suitable technique only for the monitoring of high quality drinking water or disinfected waters, because the highly sensitive culture medium fails to suppress the growth of background flora (Rompre *et al.* 2002; Bonadonna *et al.* 2007; Pitkänen *et al.* 2007; Fricker *et al.* 2008). The MMPN method (ISO 9308-3 1998) is recommended for monitoring surface water and waste water, and has been used in studies of activated sludge at wastewater treatment plants (Orruno *et al.* 2014). There are only a few studies that report the use of the MMPN method for monitoring of surface water. Lebaron *et al.* (2005) used this method as a reference method for testing of a fluorometer method for real-time operational monitoring of *E. coli* from seawater. Mansilha *et al.* (2009) reported the results of an equivalence study conducted in Portugal between the multiple-tube fermentation method and the MMPN method for enumerating faecal coliform bacteria and *E. coli*.

The long analysing time (48–72 hours) of ISO 9308-3 and the unsuitability of ISO 9308-1 for surface water testing are the major drawbacks of the reference methods specified in the European bathing water directive. Alternative methods are needed since fast, sensitive, simple and quantitative monitoring techniques are considered ideal for quality monitoring of bathing water (Lebaron *et al.* 2005; Bonadonna *et al.* 2007). The Colilert-18 (ISO 9308-2 2012) method gives a result within 18 hours. It is an emerging technique for the monitoring of water (Niemelä *et al.* 2003; Buckalew *et al.* 2006). Various studies in the past, such as Fricker *et al.* (1997), Eckner (1998), Niemelä *et al.* (2003), Buckalew *et al.* (2006), Pitkänen *et al.* (2007) and Kämpfer *et al.* (2011) also showed that Colilert-18 gives at least as, or even more, reliable results for monitoring drinking water quality than the membrane filtration method (ISO 9308-1).

In both the Colilert-18 and MMPN methods, the detection of *E. coli* is based on the fluorogenic reaction (positive for β-glucuronidase) (Lebaron *et al.* 2005; Valente *et al.* 2010). The Colilert-18 method detects coliform bacteria on the basis of a chromogenic reaction (positive for β-galactosidase) and the *E. coli* result is based on the chromogenic and a fluorogenic reaction in the same well (Niemelä *et al.* 2003; Lebaron *et al.* 2005; Valente *et al.* 2010). The MMPN method only monitors *E. coli*, not other species belonging to the coliform group of bacteria (Lebaron *et al.* 2005; Valente *et al.* 2010).

The European member states may use an alternative method for the monitoring of bathing water but they have to prove it as reliable as the reference method (European Union 2006). It means the recovery of target organisms from the alternative method should not significantly differ from the result obtained with the reference method. The comparison of quantitative microbial procedures is specified in ISO 17994, which has recently been revised (ISO 17994 2014).

The analytical methods for testing *E. coli* from bathing waters in Finland as specified in the Bathing Water

Directive (2006/7/EC) were not considered optimal choices. There was very limited experience of the MMPN method in Finland, and the high limit of detection gave rise to objections to its use for Finnish clean bathing waters. The unsuitability of the membrane filtration ISO 9308-1 method for bathing water monitoring furthermore encouraged the organisation of an alternative method comparison study. The purpose of this study was to compare the alternative Colilert-18 method against the MMPN method specified as the reference method in the bathing water directive (2006/7/EC) and to evaluate the suitability of these methods for the determination of *E. coli* in Finnish bathing waters. To our knowledge, a comparison trial between Colilert-18 and MMPN methods for use in bathing water quality monitoring has not been done before.

MATERIALS AND METHODS

Organisation of the collaborative trial

A total of six laboratories located in the cities of Kuopio, Helsinki, Seinäjoki, Mikkeli, Jyväskylä and Lappeenranta participated in this study. This ensured diverse geographical variation and selection of bathing water sample types relevant in Finland. The comparison covered samples from both inland and coastal bathing water locations. The planning, implementation and reporting of this study was carried out by an expert panel with a statistician and representatives of the participating laboratories. The written protocol of the study was agreed in detail and tested on a training day among all the participating laboratories. All the laboratories who participated in this study had a recognised quality assurance system in place.

Samples

A total of 267 bathing water samples were analysed in this study. Among the samples studied, 225 (84%) were collected from inland fresh water bathing sites and 42 (16%) were from coastal brackish water bathing sites. The sample material included 111 undiluted water samples (42%); 36 samples were diluted prior to testing (13%), and 120 samples were spiked with sewage prior to testing (45%). Each laboratory aimed to test at least 40 water samples during a bathing season. The participating laboratories were responsible for collecting the water samples needed for their study. Separate sample bottles obtained at the same bathing site were considered as separate samples for the purposes of this study. As soon as the samples were received in the laboratory, a preliminary test was conducted using the Colilert-18 method to find the *E. coli* count in the sample. Only the water samples with *E. coli* counts of 5–20 MPN/10 mL were used in the real comparison test, otherwise the samples were spiked or diluted to fulfil the requirements.

For the dilution, the preferred water was bathing water from a bathing area exhibiting a low count of *E. coli* (less than 5 MPN/10 mL) and stored up to 3 days at $5 \pm 3\,^{\circ}C$. In the case of spiking, the samples were spiked with sewage samples collected from local sewage treatment plants. The *E. coli* count in the sewage was determined on the arrival day of the samples using spread-plating on the Chromocult Coliform medium (Merck, Darmstadt, Germany) at $36 \pm 2\,^{\circ}C$ for 18–21 hours. The spiking volume was determined with a target of 5–20 MPN/10 mL based on the prior *E. coli* count information. When necessary, intermediate dilutions of the sewage into fresh bathing water or sterile water were made.

The dilution series for the methods being tested were prepared directly before use. The tests to compare the methods were always made from the same sample bottle. An equal sample volume of 10 mL, which is the maximum sample volume of the reference method using one dilution step, was used for analysis of all samples using both methods. Attention was given to work order. For every other sample, the inoculation of the MMPN plate was carried out first, and for the other samples, the Colilert-18 tray was filled in first. Incubation was carried out simultaneously, immediately following the sealing of the plate in the MMPN method and the tray in the Colilert-18 method.

Methods compared

Alternative method: Colilert-18

The Colilert-18 test (ISO 9308-2 2012) was carried out by mixing a 10 mL sample (from the same bottle as used for the reference method; undiluted/diluted/spiked) with

90 mL of sterile deionised water. Following this, the Colilert-18 reagent (IDEXX Laboratories, Inc., Westbrook, ME, USA) and 1–2 drops of antifoam solution (IDEXX) were added and the solution was mixed again. The filled and sealed 51-well Quanti-tray (IDEXX) was incubated at 36 ± 2 °C for 18–21 hours before counting the yellow and fluorescent wells exhibiting colour equal to or greater than the Quanti-tray comparator (IDEXX). The *E. coli* MPN count was the MPN value corresponding to the number of yellow wells that also exhibited fluorescence under 365 nm UV-light.

Reference method: miniaturised MPN method

In the MMPN method (ISO 9308-3 1998), half dilution of the sample was prepared by mixing a volume (such as 25 mL) of the sample to be studied (undiluted/diluted/spiked fresh or brackish water) with the same volume (25 mL) of special dilution water (synthetic sea salt, Bio-Rad Laboratories, France). With a single dilution, the pipetting of an entire microtiter plate (Bio-Rad Laboratories Inc., France) required $96 \times 200\ \mu l = 19.2$ mL of liquid, half of which, 9.6 mL, was from the sample. This sample volume equates to the sample volume of 10 mL used in the Colilert-18 method. The microtiter plates were sealed using a plastic film and incubated at 44 ± 0.5 °C, first for 45–51 hours and then the incubation was continued to 69–72 hours. The incubation was conducted for two time intervals to test the note in ISO 9308-3 stating that reading may be carried out any time after 36 hours, as the fluorescence does not alter with time. The time intervals were selected based on practical reasons. The *E. coli* MPN counts were the MPN values corresponding to the numbers of fluorescent wells recorded after 45–51 hours (MMPN-48) and 69–72 hours of incubation (MMPN-72).

Confirmation tests

Confirmation tests were conducted to investigate the false positive rate. All *E. coli*-positive (fluorescent) wells of both methods (Colilert-18 and MMPN-72) were confirmed. In addition, the false negative rate of the Colilert-18 method was investigated by selecting five yellow wells without fluorescence per tray randomly for confirmation tests. The MMPN method does not include coliform bacteria analysis corresponding to the yellow wells without fluorescence in Colilert-18 and therefore the false negative rate of the MMPN method was not investigated.

The sub-cultures from the wells were incubated overnight on Chromocult Coliform Agar medium (Merck, Germany) at 36 ± 2 °C. The purity of the isolates was ensured by second sub-culturing overnight on tryptone soya agar (TSA, Oxoid Ltd, UK) at 36 ± 2 °C. The oxidase test was conducted on all the colonies. All the oxidase-negative colonies were tested in Fluorocult Lauryl Sulfate Broth (Merck, Germany) tubes containing a Durham tube for gas collection at 44 ± 0.5 °C for 21 ± 3 hours. The fluorescence of the Fluorocult tubes was recorded under UV light, gas formation was studied and the indole test was carried out using Kovac's reagent. The oxidase-negative strains that were positive for indole and fluorescence tests in the Fluorocult tubes were considered as confirmed *E. coli* bacteria. Formation of gas at 44 °C and purple colour on the Chromocult Coliform medium also supported this interpretation, but were not required to determine the presence of *E. coli*.

The confirmed *E. coli* count in Colilert-18 and MMPN-72 methods was calculated by subtracting the false positive results from the original counts. The corrected *E. coli* count of the Colilert-18 method was obtained by adding the estimated false negative results to the confirmed *E. coli* result. In the MMPN-72 method, the confirmed and corrected *E. coli* count was the same.

Data processing

The comparison of the relative recovery of the methods was based on the procedure of the ISO 17994 (2004) standard (recently revised as ISO 17994 (2014); our study was already completed before the new version of the standard was available). The relative differences of the non-zero counts between alternative (Colilert-18) and reference (MMPN) methods were calculated after natural logarithm transformation (Niemelä *et al.* 2003; Fricker *et al.* 2008). The number of results with zeros was low, and the sample was discarded if it had a zero count on either one of the methods to avoid mathematical problems. The expert panel of this collaborative study decided to set up the maximum

acceptable deviation of the one-tailed relative difference from zero (D) to be 20% (in accordance with ISO 17994 2014).

Further processing of the relative deviations included three main steps: (1) studying the normality of the data, (2) examining the differences between the laboratories and sample types and (3) determining the relative differences between methods. To determine the normality of relative differences, visual evaluation of frequency distributions was used. The equality of the MMPN results after 45–51 and 69–72 hours of incubation was tested using the Wilcoxon signed rank test. The differences between the original, confirmed and corrected results were tested using the paired-samples t-test and the related-samples Friedman's two-way analysis of variance by ranks. The variation of the relative deviations of laboratories and sample categories were tested with one-way analysis of variance (ANOVA), and evaluation was supported by the non-parametric Kruskal–Wallis test. The statistical software used in the study was IBM SPSS version 22. All statistical test outcomes were regarded as significant at $P < 0.05$.

RESULTS

Descriptive statistics of the study data

A total of 263 out of 267 sample pairs analysed in this study met the criteria for the comparison analysis. The distribution of the relative difference of the natural logarithm of *E. coli* (MPN count) from original, confirmed and corrected results followed normal distribution when visually investigated using a histogram plot (Figure 1).

Most of the samples (238 out of 263; 90.5%) resulted in the same *E. coli* MPN count in the MMPN method after 48 and 72 hours of incubation. However, for 25 samples, the MMPN-72 count was higher than the MMPN-48 count. Original MMPN-48 results did not differ significantly from the confirmed MMPN results ($P = 0.827$) whereas there was a significant difference between the original MMPN-72 results and confirmed MMPN results ($P = 0.021$). As the increase in *E. coli* counts over the incubation time was statistically significant ($P < 0.001$), the method comparison results using both original counts (MMPN-48 and MMPN-72) are presented.

Differences between the laboratories and sample types

The types of samples included natural undiluted and diluted samples and waters spiked with waste water. The sample selection varied between the laboratories. More samples were taken from inland fresh waters than from coastal brackish waters (Table 1(a)). The differences between the mean relative recovery values between Colilert-18 and MMPN when using natural and spiked inland and coastal samples were not statistically significant ($P = 0.487$–0.691; Table 1(a)). The mean relative difference of original and confirmed counts between Colilert-18 and MMPN methods was close to zero when analysing inland samples spiked with waste waters (Table 1(a)). When analysing natural inland and coastal samples, Colilert-18 seemed to result in lower original and confirmed MPN values than the MMPN method.

The laboratory means of the relative difference of the original counts between the methods varied from −24.0% to +12.6% (Table 1(b)). Laboratory 5 seemed to produce higher *E. coli* MPN values when using the Colilert-18 method than the MMPN method. In laboratory 4 the situation was the opposite, with higher MMPN counts compared to Colilert-18 counts. However, the differences between the mean values of the various laboratories were not significant when compared with the internal variation of each laboratory ($P = 0.105$–0.734; Table 1(b)). Owing to the fact that statistically significant differences were not found between sample categories or laboratories ($P > 0.05$), the results of all the sample categories and laboratories were combined for further evaluation.

False positive and false negative rates

The isolation of cultures for confirmation was carried out successfully in all laboratories. The cultures from 99.95% of the observed fluorescent wells of the Colilert-18 method and from 99.61% of fluorescent tubes in the MMPN method were isolated. A total of 2,184 fluorescence-positive wells from Colilert-18 were further cultivated and tested. Of these, 2,130 were confirmed as *E. coli* (biotypes 1 and 2; Table 2(a)). Of the 2,507 fluorescence-positive wells of the MMPN method tested, 2,458 were confirmed as *E. coli* (Table 2(b)). 2.5% and 3.3% of the isolates from Colilert-18 and MMPN methods, respectively, were β-glucuronidase positive but did not produce gas from the lactose (biotype

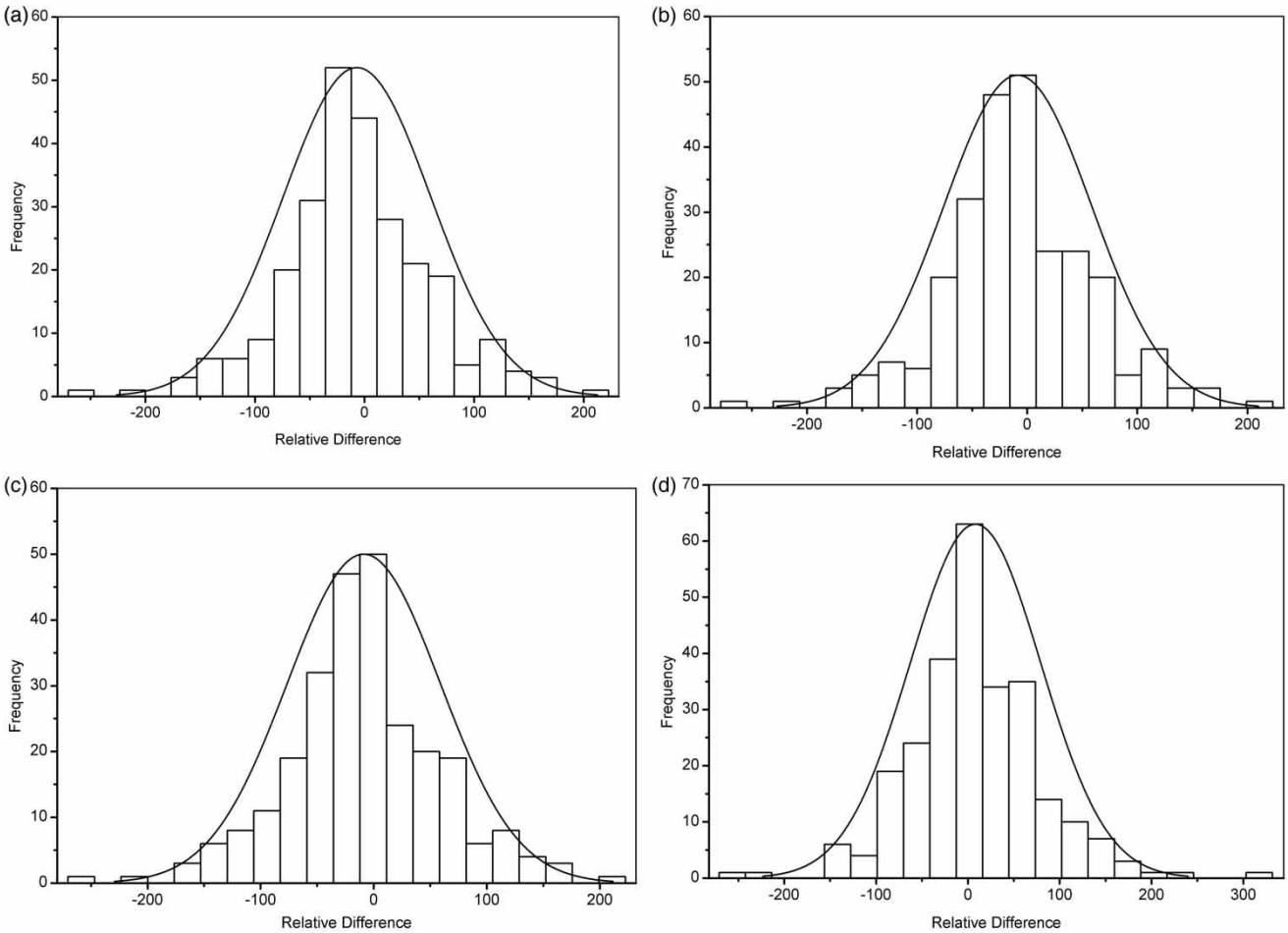

Figure 1 | The distribution of relative difference compared with the normal distribution (curve) for: (a) original Colilert-18 vs. MMPN-48 values; (b) original Colilert-18 vs. MMPN-72 values; (c) confirmed Colilert-18 vs. MMPN-72 values; and (d) corrected Colilert-18 vs. MMPN-72 values.

2; Table 2). The false positive rate of both methods was low, being 2.2% and 2.0% for the Colilert-18 and MMPN methods, respectively. Most of the false positive cultures (28 out of 50 in Colilert-18 and 24 out of 49 in MMPN) were found to be indole negative but fluorescent positive (biotypes 5 and 6; Table 2).

A random selection of the presumptive *E. coli*-negative but coliform bacteria-positive wells in the Colilert-18 trays was isolated for confirmation. In total, 1,244 of the 5,706 yellow but not fluorescent wells were tested (22%). Of these, most cultures were indole and fluorescent negative upon confirmation, but 91 cultures (7.3%) were classified into biotypes 1 and 2 and regarded as positive for *E. coli* (Table 3). The false negative rate was higher than 10% in laboratories 1 and 4, which analysed only natural samples (Table 3). In other laboratories, which also analysed samples spiked with waste water, the false negative rate remained below 10%.

Relative difference between alternative and reference method

The comparison between the two methods was made using criteria set by international standard ISO 17994 (2004). The combined original *E. coli* count results from all the laboratories showed that the relative recovery of the Colilert-18 method after 18 hours of incubation was 6.96% and 8.58% lower than that of the MMPN method after 48 hours and 72 hours of incubation, respectively (Table 4). The confirmation changed the relative difference between the methods only slightly, with the average confirmed recovery

Table 1 | A quantitative comparison between the relative differences of the original, confirmed and corrected *E. coli* results obtained using the Colilert-18 method and miniaturised MPN-48 and -72 method (a) when analysing natural and spiked inland and coastal samples and (b) in each laboratory

		Original 18-48		Original 18-72		Confirmed 18-72		Corrected 18-72		
	N	M	SD	M	SD	M	SD	M	SD	Difference
(a) Sample category										
1. Inland – natural	107	−10.42	68.34	−12.10	68.47	−14.15	69.06	6.78	76.42	20.93
2. Inland –spiked	114	−1.17	68.05	−2.99	67.50	−1.44	68.73	13.18	68.83	14.62
3. Coastal –natural	38	−13.22	66.90	−14.31	66.56	−14.34	64.49	1.29	66.93	15.63
4. Coastal – spiked	4	−19.76	13.69	−19.76	13.69	−23.47	6.86	−23.47	6.86	0.00
Total	263	−6.96	67.44	−8.58	67.19	−8.81	67.81	8.30	71.23	17.11
Difference of the mean values, F value		*0.53*		*0.49*		*0.82*		*0.58*		
Probability		*0.663*		*0.691*		*0.487*		*0.628*		
(b) Laboratory										
1	48 *(48,0,0,0)*	−17.30	54.46	−20.41	54.30	−23.67	55.77	5.89	57.11	29.56
2	47 *(7,26,10,4)*	−1.25	65.23	−2.95	65.26	−1.52	65.43	13.92	77.77	15.44
3	48 *(2,46,0,0)*	−7.45	70.38	−11.16	68.50	−11.30	71.62	3.63	79.31	14.93
4	43 *(15,0,28,0)*	−23.56	68.04	−24.04	68.11	−23.48	66.51	−3.94	70.73	19.54
5	36 *(28,8,0,0)*	12.61	80.52	12.61	80.52	12.12	80.27	18.09	86.71	5.97
6	41 *(7,34,0,0)*	−0.58	64.90	−0.58	64.90	0.14	64.69	14.40	53.51	14.26
Total	263 *(107,114,38,4)*	−6.96	67.44	−8.58	67.19	−8.81	67.81	8.30	71.23	17.11
Difference of the mean values, F value		*1.51*		*1.69*		*1.84*		*0.56*		
Probability		*0.188*		*0.138*		*0.105*		*0.734*		

N, number of samples; D, distribution of samples in sample categories (1, 2, 3, 4); M, mean relative difference; SD, standard deviation; Difference, change of the mean value when confirmed results were corrected for the false negatives. Natural samples include both diluted and undiluted samples.

of the Colilert-18 method being 8.81% lower than the MMPN method recovery. After the correction of false negatives, the relative difference between the Colilert-18 and MMPN methods was reversed; the corrected recovery of the Colilert-18 method was 8.30% higher than the recovery of the MMPN method (Table 4).

As the false positive rate was low in both methods, the mean relative difference between the methods was not significantly different when analysing original and confirmed results ($P = 0.151$–0.919) even though the equivalence evaluation according to ISO 19774 principles was different (Table 4). The correction of Colilert-18 false negative results changed the mean relative difference significantly ($P < 0.001$). The correction of the Colilert-18 results with the false negative values resulted in an average 17.1% increase in the mean relative difference compared to the confirmed values (Table 1).

When analysing the results, in light of the expanded uncertainty of the measurement (U), the methods were considered equivalent if zero fell between the lower and higher confidence limit (±20%, ISO 17994 2014). Zero was included in the confidence limit, undoubtedly, when the original Colilert-18 and MMPN-48 results were compared (Table 4). The confidence limits of the original Colilert-18 and MMPN-72 comparison, as well as the confirmed comparison, marginally excluded the zero (Table 4). Therefore, it can be concluded that the detected difference between the recovery of Colilert-18 and the MMPN method is not significant and that these two methods, in practice, can be considered equivalent for bathing water quality monitoring in Finland.

DISCUSSION

Several studies in the past have shown the equivalence of the Colilert-18 method (ISO 9308-2) as compared with

Table 2 | Frequencies of the biotypes1–8 based on oxidase test (ox), indole test (ind44), β-glucuronidase test (fluor44) and gas production from lactose test (gas44) in the preliminary positive (fluorescent) wells of (a) the Colilert-18 and (b) MMPN methods

Laboratory	N	1. Ox− ind44+ fluor44+ gas44+	2. Ox− ind44+ fluor44+ gas44−	3. Ox− ind44+ fluor44− gas44+	4. Ox− ind44+ fluor44− gas44−	5. Ox− ind44− fluor44+ gas44+	6. Ox− ind44− fluor44+ gas44−	7. Ox− ind44− fluor44− gas44+	8. Ox− ind44− fluor44− gas44-	False positive rate %
(a) Colilert-18 method										
1	426	411	–	–	1	–	10	–	2	3.1
2	409	376	17	2	1	9	1	–	2	3.7
3	343	312	22	1	1	4	1	–	2	2.6
4	438	424	10	–	–	2	–	–	–	0.5
5	176	162	4	–	9	–	1	–	–	5.7
6	392	391	1	–	–	–	–	–	–	0.0
Total	2,184	2,076	54	3	12	15	13	0	6	
										Average false positive rate of Colilert-18 method: 2.2%
(b) Miniaturised MPN method										
1	490	481	3	5	1	–	–	–	–	1.2
2	465	405	42	2	–	9	4	1	2	3.9
3	408	372	26	1	–	5	3	–	1	2.5
4	563	555	2	–	–	3	–	2	1	1.1
5	176	163	4	8	1	–	–	–	–	5.1
6	405	402	3	–	–	–	–	–	–	0.0
Total	2,507	2,378	80	16	2	17	7	3	4	
										Average false positive rate of miniaturised MPN method: 2.0%

N, number of cultures tested;−, the isolate tested negative; +, the isolate tested positive.
Biotypes 3–8 were considered negative for *E. coli* (false positive).

Table 3 | Frequency of the biotypes 1–8 based on oxidase test (ox), indole test (ind44), β-glucuronidase test (fluor44) and gas production from lactose test (gas44) in the preliminary negative results of the Colilert-18 method

Laboratory	N	1. Ox− ind44+ fluor44+ gas44+	2. Ox− ind44+ fluor44+ gas44−	3. Ox− ind44+ fluor44− gas44+	4. Ox− ind44+ fluor44− gas44−	5. Ox− ind44− fluor44+ gas44+	6. Ox− ind44− fluor44+ gas44−	7. Ox− ind44− fluor44− gas44+	8. Ox− ind44− fluor44− gas44−	Non-coliform (ox+)	False negative rate (%)	Natural samples (%)
1	236	27	1	1	15	–	2	2	115	73	11.9	100
2	223	13	–	2	25	1	1	8	135	38	5.8	37
3	238	7	–	5	28	–	3	10	149	36	2.9	4
4	180	20	3	3	26	–	–	1	90	37	12.8	100
5	176	6	0	6	10	3	3	2	75	71	3.4	78
6	191	14	0	1	6	2	–	1	64	103	7.3	17
Total	1,244	87	4	18	110	6	9	24	628	358	7.3	55

N, number of cultures tested; −, the isolate tested negative; +, the isolate tested positive.
Biotypes 1 and 2 were considered positive for *E. coli* (false negative).

membrane filtration method (ISO 9308-1) for monitoring drinking water quality in many countries, including Finland (Fricker *et al.* 1997; Eckner 1998; Niemelä *et al.* 2003; Pitkänen *et al.* 2007). The present comparison of the relative differences of *E. coli* counts between the alternative Colilert-18 and the reference MMPN (ISO 9308-3) method suggests

Table 4 | A quantitative comparison between the relative differences of the *E. coli* results obtained using the Colilert-18 and MMPN-48 and -72 methods per laboratory according to ISO 17994 (2004). Combined results of six laboratories

Material	N	M	SD	SE	U	LO	HI	Evaluation*
Original 18-48	263	−6.96	67.4	4.16	8.32	−15.28	+1.36	Methods equivalent
Original 18-72	263	−8.58	67.2	4.14	8.29	−16.87	−0.30	Methods different
Confirmed 18-72	263	−8.81	67.8	4.18	8.36	−17.17	−0.45	Methods different
Corrected 18-72	263	+8.30	71.2	4.39	8.78	−0.48	+17.08	Methods equivalent

N, number of samples; M, mean relative difference value (unweighted); SD, standard deviation; SE, standard error of the mean value; U, expanded uncertainty of measurement; LO, lower confidence limit, approximately 95%; HI, higher confidence limit, approximately 95%.

*When evaluating the equivalence of the methods in this comparison, a maximum deviation of D = 20% was used. In a two-sided evaluation, the methods were considered equivalent if neither of the methods gave significantly higher or lower results than the other one.

that the methods can be considered equivalent for monitoring bathing water in Finland (ISO 17994 2014). It is presumable that these results obtained in Finland can also be verified in other countries. For example, Kinzelman *et al.* (2005) and Valente *et al.* (2010) have proposed Colilert-18 as a good technique for monitoring recreational water.

The possibility of false negative results when using the Colilert-18 method has been noticed in past studies. Chao (2006) and Schets *et al.* (2002) reported 11% false negative rate for Colilert-18 values and Warden *et al.* (2011) recorded 7% false negatives. In this study, the false negative rate was 7.3%. When we analysed original and confirmed counts, the relative difference of *E. coli* count between the alternative method and the reference method was negative and changed to positive after correcting the results for false negatives in the Colilert-18 method. False negatives might be due to technical reasons, either differences in work patterns or, more likely, in the interpretation of preliminary positive results. The false negative interpretation of fluorescence in the Colilert-18 method may be caused by weak development of fluorescence during the relatively short 18 hours' incubation time in the Colilert-18 wells, where other coliform bacteria are present in addition to *E. coli*, possibly growing more rapidly (Pitkänen *et al.* 2007).

The prevalence of false negative results was not uniform in the laboratories that participated in this study. The high value of confirmation rate (99.5%) in laboratory 4, for example, would suggest that in this laboratory, only 'certain cases' have been read as presumptive positives. Weak fluorescence has probably been assumed to be negative, leading to a high number of 'hidden' positives or false negatives found during confirmation (rate of false negatives was 12.8%). As a result, the relative difference between these methods became negative. The same explanation is likely to also apply to laboratory 1. In laboratory 5, on the other hand, it seems that even the wells with a weak fluorescence were interpreted as preliminary positive, lowering the confirmation rate to 94% but giving a low percentage of false negatives (3.4%). In laboratories 1 and 4, the correction of false negatives markedly increased the average, whereas in laboratory 5 it was less significant. Based on the results, it can be suspected that the laboratories have interpreted the preliminary positives in different ways. According to the colour comparator provided by the manufacturer for the Colilert-18 method, a certain colour of a very weak fluorescence should be regarded as negative, but our experience from this study suggests that even a minor fluorescence should be interpreted as positive. In addition to the interpretation of the fluorescence results, it is possible that the incubation times of the Colilert trays have varied systematically within the 18 to 21 hours' range in the various laboratories, possibly affecting the results.

It is possible that false negatives would also be present when using the MMPN method. Owing to the high incubation temperature and longer incubation time, however, it is unlikely that there would be as many false negatives as in the Colilert method (Niemi *et al.* 2003). It is therefore quite possible that slightly more *E. coli* grows on the Colilert trays (cf. the corrected results of this study), but because competition and interference by other coliform bacteria might disturb or mask the growth of *E. coli*, it can be supposed that the original Colilert-18 yield remains somewhat lower than in the MMPN method.

Both methods gave the same low range of false positive results in our study, indicating that the β-glucuronidase activity of bacteria other than *E. coli* is not a problem when analysing bathing water in Finland. In other parts of

the world, severe problems have been noted that are related to the false positives occurring when the biochemical reaction of two organisms resulting in yellow colour and fluorescence are overlapped (Pisciotta *et al.* 2002). For example, Chao (2006) reported 36.4% false positive Colilert-18 results in a tropical fresh water study. *Vibrio* species like *Vibrio vulnificus*, *V. cholerae* (Pisciotta *et al.* 2002); *Providencia* spp. (Pisciotta *et al.* 2002); enterobacterial *Shigella* spp. (Caruso *et al.* 2002) and *Aeromonas* spp. (Landre *et al.* 1998) have been reported to cause false positive results in this method.

This comparison was based on the bathing water directive 2006/7/EC, and alternatives were sought for the *E. coli* methods defined in the directive. Of the *E. coli* methods specified in the directive, there had been little experience of using ISO 9308-3 in Finnish laboratories prior to this comparison. In this comparison trial, the alternative methods were not compared against ISO 9308-1, the other reference method stated in the Bathing Water Directive. The comparison caused a considerable workload for the participating laboratories, as all preliminary positive wells were pure cultured in two stages and the strains were subjected to oxidase testing and Fluorocult confirmation. Also a part of the preliminary negative wells of the alternative method were tested. The results of this comparison can be utilised in other countries, who may verify the equivalence between Colilert-18 and MMPN methods in their geographical region to lessen the workload of a method comparison trial.

CONCLUSIONS

The Colilert-18 method yielded results equivalent to those obtained using the reference method of ISO 9308-3 at more than 90% probability. The difference between yields of less than 10% found in this study was not significant. Therefore, the Colilert-18 method can be approved for *E. coli* monitoring in Finnish bathing waters in addition to the methods mentioned in the directive. The speed of the Colilert-18 when compared with the reference MMPN method makes a case for its use when monitoring bathing water quality. It was discovered in this study that approximately 7% of the preliminary negative yellow wells in the Colilert method contained *E. coli*. Certain sample types (natural samples, such as mixtures of surface waters) and possibly also differences in the technical routines between laboratories increased the incidence of false negatives. Based on these results, laboratories using Colilert-18 are guided to interpret even a slight fluorescence in Colilert-18 wells to be *E. coli* positive.

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