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A membrane filtration method for the enumeration of *Escherichia coli* in bathing water and other waters with high levels of background bacteria

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ABSTRACT

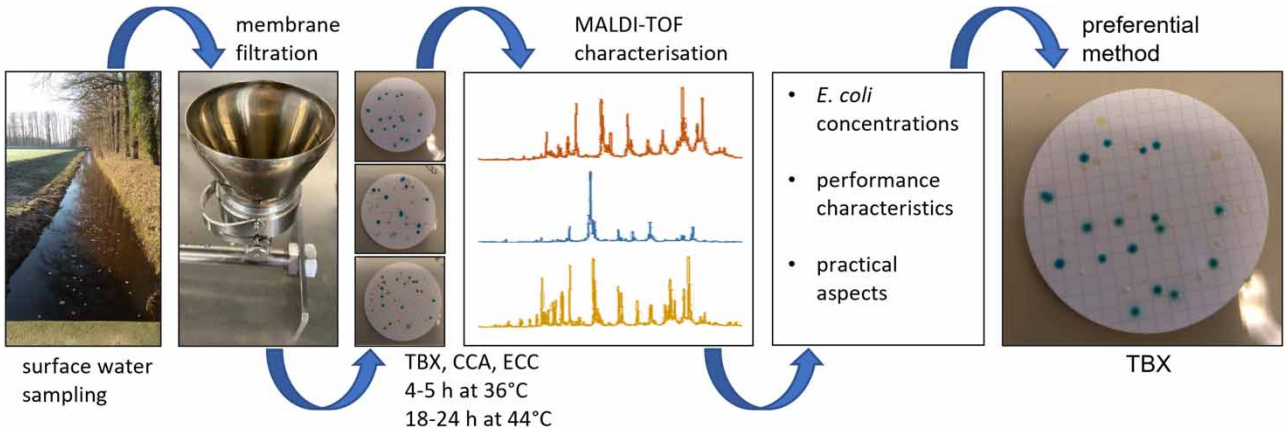
The presence and level of faecal indicator bacteria are important factors in estimating the microbiological quality of surface water and the risk of human infection upon exposure to this water. Until 2014, ISO 9308-1:2000 was available and used to enumerate faecal indicator *Escherichia coli* in bathing water. In 2014, this ISO was technically revised and replaced by ISO 9308-1:2014. This ISO introduced a less selective method for enumeration of *E. coli* that allows non-specific growth from waters containing high levels of bacteria, such as surface waters. This implies that currently there is no suitable reference membrane filtration method for the compliance monitoring of official bathing sites for *E. coli* according to the European Bathing Water Directive. Here, the performance characteristics of three chromogenic culture media, namely Tryptone Bile X-glucuronide (TBX) agar, Chromogenic Coliform Agar (CCA), and CHROMagar *E. coli/Coliform* (ECC) were investigated at 44 °C for water with varying levels of bacteria according to ISO 13843:2017. Based on performance characteristics, colony counts, and practical usage, TBX appeared the most suitable culture medium for the enumeration of *E. coli* in bathing water and other waters with high levels of background bacteria, such as surface water in agricultural areas and wastewater discharge points.

Key words: bathing water, comparative study, *Escherichia coli*, performance characteristics, surface water

HIGHLIGHTS

- Performance characteristics at 44 °C, after resuscitation at 36 °C, were comparable for the tested chromogenic culture media TBX, CCA, and ECC.
- Based on performance characteristics, colony counts, and practical usage, TBX was selected as the preferential culture medium.

GRAPHICAL ABSTRACT



INTRODUCTION

The presence and the level of faecal contamination are important factors in estimating the microbiological quality of surface water and the risk of human infection upon exposure to this water. *Escherichia coli* (*E. coli*) is the most frequently used indicator for faecal pollution in water, such as bathing water. Methods for the enumeration of *E. coli* in water are described in the ISO 9308 series, which currently consists of three parts: ISO 9308-1:2014 (Anonymous 2014), ISO 9308-2:2012 (Anonymous 2012), and ISO 9308-3:1998 (Anonymous 1998). ISO 9308-1:2014 describes a membrane filtration and colony count method, using the coliform and *E. coli*-specific Chromogenic Coliform Agar (CCA) (Lange *et al.* 2013). ISO 9308-2:2012 and ISO 9308-3:1998 are both most probable number (MPN) methods, in which the concentration of the target organisms is derived from the number of tubes showing growth in a liquid medium (Tiwari *et al.* 2016). In all three methods, the identification of *E. coli* is based on the selective ability of *E. coli* to use β-glucuronide as a substrate (Kilian & Bulow 1976). The media used in ISO 9308-1 and 9308-2 are additionally suitable for the enumeration of total coliforms, while ISO 9308-3 is a method for the enumeration of *E. coli* only.

The membrane filtration method described in ISO-9308-1:2014 is a technical revision of, and therefore replaces, ISO 9308-1:2000 (Anonymous 2000), in which two membrane filtration methods were described: (1) a Standard Test, using Lactose TTC Tergitol (LTTC) agar, which was applicable for monitoring of very clean and/or disinfected water for both coliforms and *E. coli*, and (2) a Rapid Test for the enumeration of *E. coli*, using Tryptone Soy Agar (TSA) and Tryptone Bile Agar (TBA). The Rapid Test was additionally suitable for water with high levels of (background) bacteria because it was more selective due to the addition of bile salts and an incubation temperature of 44 °C. Some European Union (EU) Member States have been using the Rapid Test for routine monitoring of *E. coli* in bathing water according to the European Bathing Water Directive 2006/7/EC (Anonymous 2006). Like the old method using LTTC agar, the new membrane filtration method using CCA is incubated at 36 °C, thus optimising the growth of coliform species, but also resulting in relatively low selectivity and non-specific growth of bacteria from waters containing high levels of other bacteria, such as surface water, including bathing water and discharged wastewater (Jozić *et al.* 2018). The growth of bacteria other than coliforms and *E. coli* may interfere with the reliable enumeration of the target bacteria, but also, the growth of coliforms may interfere with the reliable enumeration of *E. coli*. ISO 9308-1:2014 is, therefore, only meant for the enumeration of coliforms and *E. coli* in waters with low bacterial numbers.

The updated reference to ISO standards in the Bathing Water Directive requires the use of the latest version of these standards, which in this case implies that currently there is no suitable official membrane filtration method for compliance monitoring of official bathing sites for *E. coli*. Moreover, if member states would want to use an alternative membrane filtration method for the enumeration of *E. coli* in bathing water, a reference method is no longer available for comparison in a validation study. Previously, Jozić *et al.* (2018) performed a study in which they modified the method described in ISO 9308-1:2014 by using an elevated incubation temperature of 44 °C (and resuscitation at 36 °C). The method was assessed as sufficiently sensitive, specific, and efficient. The elevated incubation temperature suppressed the growth of non-specific thermo-intolerant (coliform) bacteria and improved the selectivity of the method. Despite this improvement, the enumeration of *E. coli* might still be further enhanced by using an *E. coli*-specific chromogenic culture medium, such as Tryptone Bile X-Glucuronide agar (TBX), rather than coliform-specific agar CCA. TBX is already being used for the assessment of food safety according to official guidelines for quantification of *E. coli* in products intended for human consumption or animal feed, applying ISO 16649-1:2018 or ISO 16649-2:2001 (Anonymous 2001; Anonymous 2018b), and its use for the enumeration of *E. coli* in water has been investigated before (Vergine *et al.* 2017; Jozić *et al.* 2019). In these previous studies, however, results were variable (Jozić) or 37 °C was used (Vergine). The aim of this study was to investigate the relative performance of TBX for the enumeration of *E. coli* in natural waters with a high level of background bacteria at a selective incubation temperature of 44 °C, preceded by a resuscitation step at 36 °C. The value of a resuscitation step to increase the recovery of environmentally stressed or weakened *E. coli* when using selective membrane filtration methods has been shown before (Dufour *et al.* 1981; Havelaar & During 1988; Özkanca *et al.* 2009; Jozić *et al.* 2019). Performance characteristics of TBX were compared with those of CCA and CHROMagar *E. coli*/Coliform (ECC).

METHODS

Study design

Performance characteristics such as sensitivity, specificity, and selectivity were determined for TBX (BioRad, Hercules, CA, United States of America, 356-4035), CCA (Oxoid, Hampshire, United Kingdom, CM1205B), and ECC (Biotrading,

Mijdrecht, the Netherlands, EF322) according to ISO 13843:2017 (Anonymous 2017). Three different types of surface water were analysed: surface water at discharge points of wastewater treatment plants, narrow waters in agricultural areas (e.g., small rivers and ditches), and water at official bathing sites. These three types of surface water, further referred to as discharged wastewater, agricultural water, and bathing water, represent water with different levels of *E. coli* relative to background bacteria, with expected high, intermediate, and low concentrations, respectively.

Samples

A total of 20 surface water samples were taken in February and March 2018. They included 12 discharged wastewater samples (WW1–WW12), 4 agricultural waters (AW1–AW4), and 4 bathing waters (BW1–BW4). Sample volumes of 1 L were collected in sterile bottles and transported to the laboratory in a cooling box with ice packs (5 ± 3 °C) according to ISO 19458-2007 (Anonymous 2007). Each sample was analysed within 24 h after its collection. One bathing water sample analysis failed, thus leaving 19 samples for the determination of performance characteristics.

Enumeration of *E. coli*

Water samples were filtered through 0.45-μm pore size cellulose ester membrane filters (Millipore, Burlington, Massachusetts, United States of America, no. HAWG047S6). Filtered volumes were 0.01, 0.03, 0.1, and 0.3 ml for discharged wastewater, and 1, 3, 10, and 30 ml for agricultural and bathing water. For volumes less than 10 ml, ≥10 ml of diluent (peptone saline) was added to the filtration funnel before adding the sample, and the contents of the funnel were mixed before filtration (in line with ISO 8199:2018; Anonymous 2018a). All volumes were filtered in triplicate: one filter was placed on CCA, one on ECC, and one on TBX. All culture media were prepared according to the manufacturers' instructions. All plates were incubated at 36 ± 2 °C for 4–5 h followed by incubation at 44 ± 0.5 °C for 21 ± 3 h. After incubation, all characteristic and non-characteristic colonies were counted and categorised based on their colony colour (Table 1). Plates with more than 150 colonies were considered uncountable and omitted from analyses. The *E. coli* concentrations (CFU/ml) were calculated using the formula in ISO 8199-2018 (Anonymous 2018a), from the unconfirmed colony counts, which is according to standard procedures for these methods which do not include a confirmation step, and filtration volumes.

Species identification

For each sample and culture medium combination, one plate with 20–80 colonies was selected for colony confirmation, if available. If only plates with less than 20 colonies were available, the plate with the highest number of colonies was selected. Pictures of the selected plates were taken for future reference. All colonies from the selected plates were subcultured on TSA (Oxoid, Hampshire, United Kingdom, no. PO5012A) and incubated overnight at 36 ± 2 °C.

Colonies were typed by using Matrix-Assisted Laser-Desorption/Ionisation Time-Of-Flight Massa Spectrometry (MALDI-TOF) (Bruker Daltonics GmbH, Bremen, Germany) by using the direct colony method. Applied Main Spectral Profiles (MSP) Libraries were BDAL 5.0.0.0. and SR 1.0.0.0 (Bruker, Daltonic, Germany). Identification score criteria used were those recommended by the manufacturer: a score of ≥2.000 indicated species-level identification, a score of 1.700–1.999 indicated identification to the genus level, and a score of <1.700 was interpreted as unidentifiable based on the MSP libraries used (Alatoom *et al.* 2011). Colonies that failed to produce a score of ≥1.700 were retested. Colonies that did not produce a ≥1.700 score after retesting remained unidentified and were included in data analysis as a non-*E. coli* non-coliform species indicated as 'unidentifiable'.

Table 1 | Morphology of (non-)characteristic colonies

		Non-characteristic colonies	
Culture medium	**Characteristic colonies *E. coli***	**Coliforms**	**Non-coliforms**
TBX	Blue/green	White	White
CCA	Dark blue to violet	Pink/red	Peach/white
ECC	Blue	Pink	White

Determination of performance characteristics

Based on colony colour (i.e., primary confirmatory test) and MALDI-TOF identification (i.e., secondary confirmatory test), data were divided into four categories, according to ISO 13843:2017:

(a) number of characteristic *E. coli* colonies in the primary confirmatory test confirmed as being *E. coli* in the secondary identification test (true-positive rates);
(b) number of non-characteristic colonies in the primary confirmatory test identified as being *E. coli* in the secondary identification test (false-negative rates);
(c) number of characteristic *E. coli* colonies in the primary confirmatory test subsequently shown to not be *E. coli* in the secondary identification test (false-positive rates);
(d) number of non-characteristic colonies in the primary confirmation test confirmed as non-*E. coli* in the secondary identification test (true-negative rates).

Categorical performance characteristics were calculated as follows:

$$\text{Sensitivity} = a/(a + b)$$
$$\text{Specificity} = d/(c + d)$$
$$\text{False-positive rate} = c/(a + c)$$
$$\text{False-negative rate} = b/(b + d)$$
$$\text{Selectivity} = a/n$$
$$\text{Efficiency} = (a + d)/n$$

Statistical analysis

To quantitatively compare the results of the three culture media, a multivariate linear regression analysis was conducted of the relation between the logarithmically transformed concentrations (base 10), the detection method and the type of water by using R (version 3.5.2 (2018-12-20) – 'Eggshell Igloo') and lm (Wilkinson & Rogers 1973; Chambers & Hastie 1992). The model with the lowest Akaike Information criterion (AIC) was selected using the step function (parameter $k = 3.84$). For the graphical presentation of the data package, ggplot2 was used (Wickham 2016).

RESULTS

Morphology

Characteristic colonies were easy to distinguish on each of the culture media (Figure 1). Characteristic colonies were somewhat smaller on ECC compared to the other two culture media. On TBX, the contrast between characteristic (green) and non-characteristic colonies (white) was clearest, because only characteristic colonies displayed a chromogenic reaction. On CCA, the blue *E. coli* colonies displayed a halo around the colony, which complicated colony counting at higher colony numbers.

Concentrations

As expected, average *E. coli* concentrations were higher in discharged wastewater than in agricultural water, which in their turn were higher than average concentrations in bathing water. The model used to compare *E. coli* concentrations obtained with the three different culture media (Supplementary Material, Appendix A, Table S1) showed that there was no statistically significant association between concentrations and culture media, however, there was a statistically significant association between concentrations and water types (Figure 2, Supplementary Material, Appendix B).

Performance characteristics

In total, 899 characteristic colonies and 996 non-characteristic colonies were isolated (Table 2). These 1,895 colonies were further identified. MALDI-TOF identification results for the individual colonies are available in Supplementary Material, Appendix A, Tables S2–S5. In summary, the percentage of colonies that was misidentified based on colony morphology was low and comparable for the three-culture media. All false-positive colonies appeared to be *Citrobacter* species, which indeed can occasionally be β-glucuronidase-positive (Tryland & Fiksdal 1998). This was the case for 4 of 899 characteristic colonies (0.4%), with an almost equal percentage false-positivity for all three-culture media (0.3–0.7%).

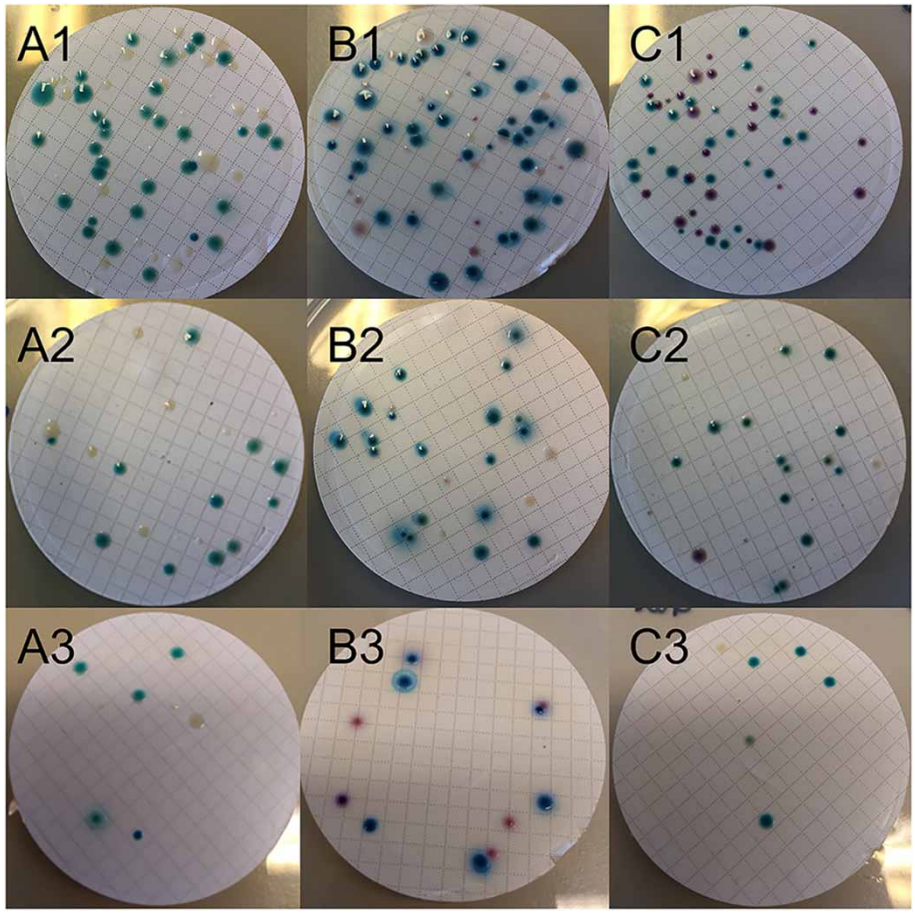


Figure 1 | *E. coli* colonies on TBX (A), CCA (B), and ECC (C), from discharged wastewater (1), agricultural water (2), and bathing water (3) after incubation for 4–5 h at 36 °C followed by 18–24 h at 44 °C. Please refer to the online version of this paper to see this figure in colour: http://dx.doi.org/10.2166/wh.2023.004.

The performance characteristics sensitivity, specificity, false-positive rate, false-negative rate, selectivity, and efficiency were comparable for the three-culture media (Table 3). For all culture media, the sensitivity was higher than 90%, specificity higher than 80%, and selectivity higher than 10%, which is above the threshold values specified in ISO 13843:2017.

DISCUSSION

Due to the replacement of ISO 9308-1:2000 with ISO 9308-1:2014, there currently is no reference membrane filtration method available for the enumeration of *E. coli* in natural waters with a high bacterial background within the legal framework of the EU Bathing Water Directive. Both MPN and membrane filtration methods have long been used for determining water quality, and both procedures have their advantages and disadvantages. The main advantages of membrane filtration are that the method is simple, fast, does not require sophisticated laboratory equipment and allows for the analysis of larger sample volumes than MPN does. Moreover, membrane filtration allows inspection based on colony morphology, gives insight into the level of background bacteria in the water analysed, more easily facilitates species confirmation if this is desired, and offers the possibility to investigate isolate characteristics (e.g., species identification, antibiotic resistance, virulence) for research purposes, as colonies are proportionally representative of the *E. coli* population in the investigated sample. Colony count estimates generally have less variability than estimations based on MPN, where the concentration is derived from a maximum likelihood based on the number of positive tubes (Gronewold & Wolpert 2008). MPN methods, on the other hand, may be more suited for waters with high particle content interfering with direct enumeration and give better recovery from waters

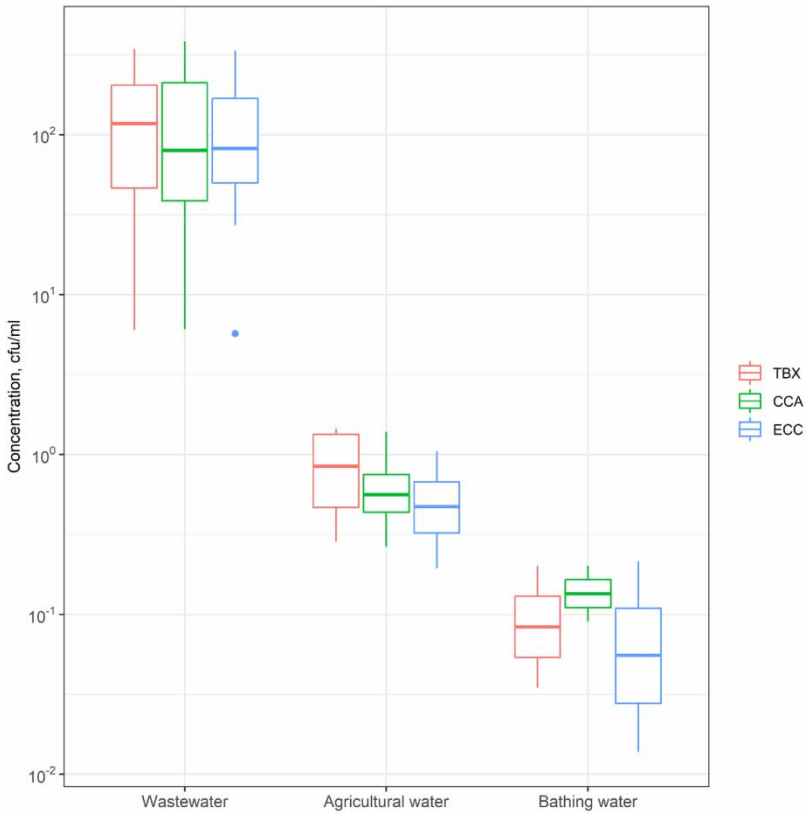


Figure 2 | Median *E. coli* concentrations (CFU/ml) in the examined water types. Boxes indicate median values and 25th and 75th percentile values, whiskers represent minimum and maximum values, and the dot marks represent an outlier.

Table 2 | Total numbers of colonies

	Culture medium							
	TBX		**CCA**			**ECC**		
	E. coli	**Non-coliforms**	***E. coli***	**Coliforms**	**Non-coliforms**	***E. coli***	**Coliforms**	**Non-coliforms**
Colony colour	Green/ blue	White	Dark blue/ violet	Pink/ red	Peach/ white	Blue	Pink	White
Total	304	300	333	308	70	263	286	31
Identified as *E. coli*[a] (percentage)	302 (99)	13 (4.3)	332 (99)	20 (6.5)	3 (4.3)	262 (99)	3 (1.1)	8 (26)

[a]Identified with MALDI-TOF.

with high numbers of injured or stressed cells, such as chlorinated effluents or marine waters (Green *et al.* 1977; Green *et al.* 1980; Rhodes *et al.* 1983).

To select a membrane filtration method for the enumeration of *E. coli* in water with a high level of background bacteria – such as surface water, including bathing water – different aspects of three culture media, i.e., TBX, CCA, and ECC, were considered when incubated at the specific temperature of 44 °C (after resuscitation at 36 °C): performance characteristics, colony counts, and practical usage including colony morphology.

For each culture medium, all performance characteristics determined were above the threshold values specified in ISO 13843:2017 for the approval of alternative culture methods. Importantly, the use of 44 °C as selective incubation temperature

Table 3 | Performance characteristics

	Culture medium		
Performance characteristic (%)	**TBX**	**CCA**	**ECC**
Sensitivity	96	94	96
Specificity	99	100	100
False-positive rate	0.7	0.3	0.4
False-negative rate	4.3	5.9	3.5
Selectivity	50	47	45
Efficiency	99	97	98

increased selectivity relative to that of CCA used according to ISO 9308-1;2014 (Lange *et al.* 2013). For all three media, selectivity was in the same order of magnitude as previously observed by Jozić *et al.* (2018), for inland waters using CCA at 44 °C. When comparing only the results for CCA, the specificity and false-negative rates were slightly higher, and sensitivity and false-positive rates were slightly lower in the current study as compared to Jozić *et al.* (2018). This difference might be related to the difference in the secondary identification tests used: Jozić *et al.* (2018) used Colilert-18 substrate, which is based on the same enzymatic activity as the CCA medium, while MALDI-TOF was used in the current study. Alternatively, differences in these rates might be related to differences in microbial communities in the Dutch and the Croatian samples, as part of the Dutch samples were obtained at wastewater discharge sites, which are likely to have different bacterial communities than bathing water sites.

There were no significant differences in the *E. coli* concentrations obtained on the three-culture media, indicating that TBX, CCA, and ECC can equally be used for the enumeration of *E. coli* in water with high levels of background bacteria when incubated at 44 °C after 4–5 h of resuscitation at 36 °C. These results do not entirely match those reported by Jozić *et al.* (2019), who observed that relative recovery of *E. coli* was superior on temperature-modified CCA as compared to TBX, although the difference they observed between TBX and CCA was small after 4–6 h of resuscitation on a general culture medium (MMGA). However, Jozić *et al.* (2019) analysed spiked samples besides natural samples, which may contribute to this difference. Additionally, performance characteristics may be influenced by geographical and seasonal factors that have an effect on the composition of the background flora (i.e. species composition) and the presence of stressors that may influence the viability of faecal-derived *E. coli* in water, such as nutrient availability, temperature, pH and UV light (Korajkic *et al.* 2019). Jozić *et al.* (2019) took their natural samples during summer in Croatia, whereas in the current study, all samples were taken in the Netherlands and during winter and early spring. Future comparative studies will have to be performed to compare samples from wider geographical areas and different seasons.

Although the performance characteristics and *E. coli* concentrations were similar for all three media, there were differences with respect to the ease of interpretation of the membrane filters. On CCA, *E. coli* colonies had a blue haze, which hampered counting at higher colony numbers. Most importantly, membrane filters on TBX were easier to interpret compared to those on the coliform agars, particularly at higher colony numbers, as only *E. coli* displayed a chromogenic reaction, resulting in a higher contrast between characteristic and non-characteristic colonies. This is particularly relevant in samples with high levels of non-*E. coli* coliforms. As monitoring of bathing water only requires enumeration of *E. coli,* information on coliforms as obtained using CCA or other coliform agars is redundant and may unnecessarily complicate the interpretation of colony counts. For the enumeration of *E. coli*, the use of TBX at a selective temperature of 44 °C and resuscitation at 36 °C therefore offers a good alternative membrane filtration method to ISO 9308-1:2014, to be used in waters with high bacterial background.

CONCLUSION

Based on performance characteristics and colony counts, TBX, CCA, and ECC can be used equally well for the enumeration by membrane filtration of *E. coli* in bathing water and other waters with high levels of background bacteria. However, considering practical aspects as well, TBX may be preferential when the ultimate goal is the enumeration of *E. coli*, and total coliform counts are not desired or required.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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