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Application of a real-time reverse transcription polymerase chain reaction for rapid detection of *Escherichia coli* in drinking water: an EU representative study

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ABSTRACT

The microbiological quality of water intended for human consumption in the EU is regulated by the recast Drinking Water Directive 2020/2184 (DWD), which sets strict parametric values for intestinal enterococci and *Escherichia coli* (*E. coli*), allowing no more than zero colony-forming units per 100 mL. Detection and enumeration of *E. coli* typically rely on culture-based reference methods or the most probable number approach, which require 1–2 days to produce results—potentially delaying timely action during contamination events. In contrast, molecular techniques can deliver results within hours. The DWD permits the use of alternative methods if they are as reliable as the reference method and developing and validating such methods requires multiple laboratory trials in line with ISO standard 16140-2. Following this, we conducted a representative EU study to validate a molecular method based on real-time reverse transcription polymerase chain reaction for rapid *E. coli* detection in drinking water. In a concerted action, the first of its kind for drinking water, nineteen laboratories across ten Member States participated. To ensure consistency, drinking water was artificially contaminated with *E. coli*. The alternative method showed slightly lower sensitivity than the reference method (91.1 % vs. 97.2 %) but delivered much faster results, making it a valuable screening tool. It can support rapid decision-making during contamination events, reducing the risk of waterborne outbreaks and protecting public health. For reliable routine performance, appropriate training in this alternative method is strongly recommended.

1. Introduction

In the European Union, the microbiological quality of water intended for human consumption is tightly controlled by the recast Drinking Water Directive (DWD) 2020/2184 (EC, 2020) and its transposition into national regulations by Member States (MS). It sets strict limits on the presence of intestinal enterococci, *Escherichia coli (E. coli)* and coliform bacteria allowing no more than zero colony-forming units per 100 mL. Developing rapid microbial water testing for *E. coli* detection in drinking water is crucial for public health, as it enables timely identification of faecal contamination, helping to prevent potential outbreaks of waterborne diseases and ensuring the safety of drinking water supplies.

E. coli is a bacterial species belonging to the coliform group that inhabits the gut of warm-blooded animals and humans. With some exceptions, *E. coli* generally does not survive well outside of the intestinal tract, making it an accurate faecal indicator organism for water quality evaluation (Odonkor and Ampofo, 2013; Nowicki et al., 2021), along with intestinal enterococci (WHO, 2022) and coliform bacteria (Odonkor and Ampofo, 2013). Therefore, its presence in environmental samples, food or water usually indicates recent faecal contamination or poor sanitation practices in food-processing facilities which is associated with the potential existence of a broad range of pathogens.

The reference method for *E. coli* detection under the DWD is described in ISO 9308-1 (ISO-Standard, 2014) or ISO 9308-2 (ISO-Standard, 2012). The first specifies a method involving membrane filtration followed by enumeration on agar plates suitable for waters with low bacterial background flora, while the latter specifies a semi-quantitative method based on growth of target organism in a liquid medium, with the calculation of the most probable number (MPN). However, both methods require 21–24 h to yield results, which can significantly delay public authorities' response to potential drinking water contamination issues.

To address these limitations, the DWD allows the use of alternative methods for microbiological water quality assessment provided they demonstrate results at least as reliable as those produced by the methods specified in the DWD. Alternative methods, including molecular techniques, can substantially reduce analysis time to just a few hours. Real-time polymerase chain reaction (PCR) is particularly advantageous for rapid and specific detection of bacteria. However, for regulatory acceptance, these methods must undergo rigorous validation studies in multiple laboratories, according to standardised protocols like ISO 16140-2 (ISO-Standard, 2016). In alignment with this requirement, the Joint Research Centre (JRC) launched a call for expressions of interest to participate in an EU trial study aimed at developing and validating a molecular approach for rapid *E. coli* detection in drinking water distribution systems. The advantage lies in a quicker detection of a contamination event that would decrease the exposure to a potentially hazardous situation for consumers. To meet DWD requirements, the method has to match the reference method in sensitivity. The sensitivity is referred to the ability of the method to detect *E. coli*, as defined by ISO 16140. The approach followed by the JRC is based on a real-time reverse transcription (RT) PCR method targeting the *E. coli* 16S rRNA gene, which has already been developed and validated by Dutch and Flemish drinking water laboratories (Heijnen et al., 2017, 2024), according to ISO 16140-2 (ISO-Standard, 2016). This method allows the detection of *E. coli* in drinking water within 3 h instead of 21–24 h at a sensitivity comparable to the reference culture method. As indicated by Abberton et al. (2016), the detection of 16S rRNA transcripts is a stronger indication of viable *E. coli* than DNA alone, as the presence of *E. coli* 16S rRNA transcripts in drinking water indicates the potential for protein synthesis within these cells. Moreover, 16S rRNA is highly abundant in viable cells, enabling robust detection of *E. coli* at the required sensitivity (1 CFU/100 mL).

In this study, the method of the Dutch and Flemish drinking water laboratories was slightly modified by the JRC to make it less restrictive in terms of the RNA extraction process. This adjustment allowed greater flexibility and made the method more accessible to all participating laboratories, ensuring broader applicability and ease of use. The method can be conducted within a single day, demonstrating the potential of rapid and accurate *E. coli* detection using molecular methods.

Then, the JRC conducted two EU-wide interlaboratory studies, involving 18 and 19 participating laboratories, respectively, with the aim of validating this modified molecular approach on an EU-representative scale and evaluating its suitability for the rapid *E. coli* detection in drinking water. The results were compared with those of the reference method (enumeration on chromogenic coliform agar (CCA) plates). The first interlaboratory study was a preparatory step, while the second one incorporated protocol improvements based on the outcome of the first interlaboratory study. This article describes and discusses mainly the results of the second interlaboratory study.

The objectives of these studies were: i) to involve a geographically broad and representative participation of laboratories across EU Member States (MS); ii) to test the modified molecular based method as an alternative detection method for routine *E. coli* detection on a representative EU scale. To our knowledge, this is the first EU-wide validation of a molecular method for *E. coli* detection in drinking water; iii) to identify key components of the alternative method to meet the equivalency criteria with the standard method, according to ISO 16140-2 (ISO-Standard, 2016); and iv) to propose further refinements to overcome the identified weaknesses of the alternative method.

2. Materials and methods

2.1. Participating laboratories and study design

The JRC, as the organising laboratory, developed a procedure for the validation of the alternative method and performed the preliminary tests in-house in a dedicated study described in the Supplementary Information (intralaboratory study).

To meet the requirements of ISO 16140-2 (ISO-Standard, 2016), a list of participating collaborators and laboratories was compiled for the interlaboratory studies (Table 1). The alternative method for drinking water was tested by a total of 18 organisations involving 20 laboratories from 11 MS and the JRC. The first interlaboratory study was launched in October 2021, followed by a second one, conducted in March 2023. Eight of the participating laboratories listed in Table 1 routinely use the alternative method for *E. coli* detection in drinking water.

2.1.1. First interlaboratory study

The first interlaboratory study was coordinated by the organising laboratory (JRC), which did not participate in the testing itself. During the first interlaboratory study, both the reference and alternative methods for drinking water were tested by 16 organisations involving 18 laboratories from 11 MS. The comparison focused on determining the sensitivity-limit of *E. coli* detection and reproducibility for the reference and alternative methods. For this purpose, each participating laboratory analysed 100 mL of drinking water in triplicate using the respective

Table 1
Laboratories participating in the interlaboratory studies. Member States (MS), organisation, laboratory and methods used for RNA extraction and real-time reverse transcription PCR (RT-PCR) are included. N/A, not applicable.

Member State	Organisation	Laboratory	Extraction method	Real-time RT-PCR
Austria (AT)[a]	AGES Austrian Agency for Health and Food Safety	Reference Laboratory for *E. coli* including VTEC	ZymoBIOMICS DNA/RNA Mini kit	SensiMIX II Probe kit Roche, LightCycler 480
Belgium (BE)	Pidpa	N/A	NucliSens extraction Biomerieux (no bead beating)	One-step mix - Evoscript Roche Biorad CFX96
	De Watergroep, Heverlee[2]	N/A	Qiagen RNeasy Powerwater (50) Lot 172037190	BioRad CFX96 Deep Well Real-Time System + C1000 Touch Thermal Cycler
Croatia (HR)	IPH Institute for Public Health in the Primorje-Gorski Kotar County	N/A	ZymoBIOMICS DNA/RNA Mini kit	Meridian bioscience Appliedbiosystems Quantstudio 5
	IOF Institute of Oceanography and Fisheries	Laboratory of Microbiology	ZymoBIOMICS DNA/RNA Miniprep Kit	SensiMIX II Probe kit Roche, LightCycler 480
Germany(DE)	NLGA Public Health agency of Lower Saxony	N/A	ZymoBIOMICS DNA/RNA Miniprep Kit	SensiMIX II Probe kit Bio-RAD CFX96
Finland (FI)	Finnish Institute for Health and Welfare	Laboratory of Water Microbiology, Microbiology Unit	ZymoBIOMICS DNA/RNA Miniprep Kit Modifications to the RNA extraction protocol. Total nucleic acid extraction, followed by TURBO DNA-free™ Kit to remove DNA to obtain pure RNA.	SensiMIX II Probe kit QuantStudio™ 6 Flex Real-Time PCR System, 96-well (Applied Biosystems)
Italy (IT)	Italian National Institute of Health	National Center For Water Safety	ZymoBIOMICS DNA/RNA Miniprep Kit	SensiMIX II Probe kit Bio-RAD CFX96 C1000 Touch
The Netherlands (NL)	WLN	N/A	ZymoBIOMICS DNA/RNA Miniprep Kit	HawkZ05 Fast One-step RT-PCR Kit Quantstudio 5 Thermo Fisher Scientific
	Vitens N.V. Water Expertise Centre	N/A	NucliSens extraction Biomerieux	SensiMIX II Probe kit CFX96 Touch Real-Time PCR
	AQZ	N/A	NucliSens extraction Biomerieux	SensiMix II Probe kit QuantStudio™
	Het Waterlaboratorium	N/A	NucliSens extraction Biomerieux Filters and RNA shield fluid were transferred into Lysisbuffer after which our own protocol was followed.	Roche EvoScript RNA Probes Master Bio-Rad CFX96 C1000 Touch
	KWR Water Research Institute	N/A	NucliSens extraction Biomerieux	Roche EvoScript RNA Probes Master Bio-Rad CFX Opus 96 Real-Time PCR System
Poland (PL)	Voivodship Sanitary and Epidemiological Station, Unit of the State Sanitary Inspection	Interdisciplinary Laboratory of Molecular Diagnostics in Katowice	ZymoBIOMICS DNA/RNA Miniprep Kit	SensiMIX II Probe kit LightCycler 480 II
		Laboratory of Microbiological Analysis in Bydgoszcz	ZymoBIOMICS DNA/RNA Miniprep Kit	SensiMIX II Probe kit Rotor-Gene® Q MDx CE
		Laboratory of Environmental and Food Analysis in Olsztyn	ZymoBIOMICS DNA/RNA Miniprep Kit	SensiMIX II Probe kit Roche LightCycler96
Portugal (PT)	National Institute of Health Doutor Ricardo Jorge	Department of Environmental Health	ZymoBIOMICS DNA/RNA Miniprep Kit	SensiMIX II Probe kit Bio-Rad CFX96
Slovakia (SK)	WRI Water Research Institute	National Water Reference Laboratory in Slovakia	ZymoBIOMICS DNA/RNA Miniprep Kit	SensiMIX II Probe kit Brilliant III Ultra-Fast QPCR Master Mix AriaMX, Agilent, real-time PCR
Slovenia (SI)	NLZOH National Laboratory of Health, Environment and Food	N/A	ZymoBIOMICS DNA/RNA Miniprep Kit	SensiMIX II Probe kit Bio-Rad CFX96
European Commission (EC)[b]	Joint Research Centre (JRC)	Laboratory of Molecular Ecology; Water and Marine Resources	ZymoBIOMICS DNA/RNA Miniprep Kit	SensiMIX II Probe kit Applied Biosystems 7900 HT

[a] Participated only in the first interlaboratory study.
[b] Participated only in the second interlaboratory study.

reference and alternative methods. As the two methods do not share the same initial step, different test portions prepared from the same batch solution were used for the two methods (unpaired study). Sample analysis was carried out according to the standard operating procedures (SOPs) previously distributed to all participants by the JRC (see Supplementary Information for details).

2.1.2. Second interlaboratory study

The second interlaboratory study was conducted with the aim of standardising the activities of each laboratory as much as possible in respect to the first interlaboratory study. Therefore, several modifications were made to the SOP aiming to improve the sensitivity of the alternative method based on the outcomes of the first interlaboratory study (see Supplementary Information for details, Table S26). Briefly, the organising laboratory (JRC) carried out all the filtrations, mixed cellulose filter were used for the reference method while polycarbonate filter membranes were used for the alternative method. Samples were distributed to the participating laboratories in polycarbonate filter membranes covered with an RNA preservative solution to be analysed by the alternative method. A vortex was recommended for the cell lysis step, and a real-time PCR master mix (including ROX concentration adequate for each PCR instrument) was distributed to the participating laboratories. The participating laboratories analysed the filters only by the alternative method.

The organising laboratory (JRC) also participated in the second interlaboratory study. During this study, the alternative method was tested by 17 organisations, involving 19 laboratories from 10 MS and the JRC, while the reference method was only tested by the JRC.

This paper focuses primarily on the methodology and the results from the second interlaboratory study.

2.2. Sample preparation and shipment

2.2.1. First interlaboratory study

Experimentally contaminated water samples were prepared by the organising laboratory (JRC) using validated preparation protocols to ensure homogeneity among samples. Although preferred, the use of drinking water samples naturally contaminated with *E. coli* was not feasible for this study, as their rarity poses significant sampling difficulties.

Artificial drinking water samples were prepared by spiking 250 mL of tap water (drinking water) with *E. coli* obtained from Vitroids™ (WDCM00090, Sigma-Aldrich) at five contamination levels (Table 2). Prior to spiking, water was treated with sodium thiosulfate ($Na_2S_2O_3$, 0.1 %) to remove or neutralise residual chlorine. Vitroids containing approximately 50 CFU/disc were first diluted in phosphate-buffered saline (PBS) (1 Vitroid/mL) to prepare a stock solution used to spike the dechlorinated tap water.

Each method (reference and alternative) required two volumes of 100 mL filtrations of the water sample for an analysis that was performed in triplicate. Accordingly, six filtrations were performed per laboratory at each contamination level. Considering the number of participating laboratories, a batch of 300 samples was prepared in 250 mL screw-top sterile coliform water sample bottles (Thermo Fisher Scientific) comprising 60 samples per contamination level. Additionally, 30 blank samples (one for each laboratory) containing only DNA/RNA/DNase/RNase-free water were included as well as three positive controls (control A, B and C). The positive controls A, B and C served as quality check for those steps that are unique for the alternative method, i.e. RNA extraction (or isolation), cDNA synthesis and real-time PCR, respectively (Table 2).

The positive controls were prepared as follows.

- control A, a 1 mL E. *coli* liquid culture containing 104 cells/mL (prepared from Vitroids™ WDCM00090, Sigma-Aldrich) was centrifuged at 8000 rpm for 2 min and resuspended with 750 μL DNA/RNA Shield™ (Zymo Research);
- control B, the RNA was extracted from the resuspended pellet (control A) using ZymoBIOMICS DNA/RNA Miniprep Kit (Zymo Research) following the manufacturer's instructions;
- control C, the extracted RNA (control B) was retrotranscribed into cDNA using the SensiFAST cDNA Synthesis kit (Bioline) as indicated in section 2.4.3 (control C).

Each participating laboratory received 15 samples, 1 blank control and the positive controls (A, B, and C). The samples and control A were shipped in isothermal boxes at 4 °C within 24–48 h (there was an unexpected delay in the shipment of some samples). Positive controls B and C were shipped in dry ice.

Upon arrival, samples were filtered and both methods were preferably performed on the same day. When this was not possible, samples were analysed by the reference method, and filters were stored to be analysed subsequently by the alternative method.

The JRC provided the materials and reagents to the laboratories that did not routinely use the alternative method. The master mix used for the real-time PCR was shipped at high ROX concentration in the first interlaboratory study (Table S26), which proved to be unsuitable for some real-time PCR instruments. Using higher ROX concentrations than recommended can, in fact, reduce performance and lead to inaccurate results.

2.2.2. Second interlaboratory study

In the second interlaboratory study, the JRC prepared the samples as described in section 2.2.1. In this case, the JRC filtered all samples and the filters were distributed to the participants preserved in ZR BashingBead™ Lysis Tubes (Zymo Research) and covered with 750 μL DNA/RNA Shield™ (Zymo Research) at 4 °C (Fig. S1).

From each 250 mL sample, 100 mL were filtered using mixed cellulose filter membranes (MF-Millipore™) and analysed by the reference method (CCA plates, see 2.3). The remaining 100 mL were filtered by polycarbonate filter membranes (Track-Etch Membrane, Nucleopore™, Whatman™, Cytiva) to be analysed by the alternative method (real-time RT-PCR, see 2.4). The JRC carried out all filtrations and analysis by the reference method, while the participating laboratories analysed the samples only by the alternative method starting from polycarbonate filter membranes. This approach was adopted to preserve sample stability during the shipment and the homogeneity of the study, as suggested by the comparison study on recreational waters by Aw et al. (2019). This method minimises or prevents loss of rRNA (Natarajan et al., 2021; Trivedi et al., 2022).

Each laboratory received 18 polycarbonate membrane filter samples, including the blank control, as well as positive controls A, B, C (Table 2).

The polycarbonate filter samples, together with positive control A, were shipped in isothermal boxes containing cooling blocks and delivered by express-courier within 24–48 h. A temperature of ≤8 °C was

Table 2
Samples distributed to each participating laboratory: blank control, three positive controls and drinking water samples per triplicate at five different levels of contamination.

Sample	Description
Blank control	DNA/RNA/DNase/RNase-free water
Level 0 (L_0)	Drinking water 0 CFU/100 mL
Level 1 (L_1)	Drinking water 1 CFU/100 mL
Level 2 (L_2)	Drinking water 3 CFU/100 mL
Level 3 (L_3)	Drinking water 5 CFU/100 mL
Level 4 (L_4)	Drinking water 10 CFU/100 mL
Positive control A	*E. coli* pellet (from a liquid culture of approximately 10^4 cells/mL)
Positive control B	RNA isolated from *E. coli* pellet
Positive control C	cDNA reverse transcribed from the RNA isolated from the *E. coli* pellet

maintained. Positive controls B and C were shipped in dry ice.

Upon receipt and confirmation of the integrity of the isothermal boxes, membrane filter samples and positive control A were stored at 4 °C at each participant laboratory until RNA extraction was performed, which was carried out within a week. Positive controls B and C were stored at −80 °C. The JRC supplied the necessary materials, kits and reagents to the laboratories that did not routinely use the alternative method. The master mix used for the real-time PCR was shipped at high, low or no ROX concentration depending on the requirements of each laboratory's real-time PCR instrument.

2.3. Reference method

The reference method for detection and enumeration of *E. coli* under the DWD is described in ISO 9308-1, using membrane filtration followed by colony enumeration on agar plates. Briefly, 100 mL of sample volume was filtered using a mixed cellulose membrane filter (MF-Millipore™) of 47 mm diameter, with filtration characteristics equivalent to a rated nominal pore diameter of 0.45 μm and grid lines. After filtration, the membrane filters were placed on CCA ISO Formulation plates (Biolife), ensuring that no air was trapped underneath. The Petri dishes were inverted and incubated at 36 ± 2 °C for 21–24 h, after which the membrane filters were examined and colonies were counted. All dark-blue to violet colonies (positive for β-D-galactosidase and β-D-glucuronidase reactions) were identified as *E. coli*. The reference method results are expressed as CFU/100 mL.

2.4. Alternative method

The detection of *E. coli* in drinking water by real-time RT-PCR is a molecular-based method that most likely detects viable cells, as 16S rRNA has a short half-life compared to DNA (Li et al., 2017). The SOP for the alternative method was set up based on the previous study performed by Dutch and Flemish laboratories (Heijnen et al., 2017, 2024), with some modifications (i.e., column-based RNA extraction method instead of magnetic beads). The JRC tested the method in-house and adapted it to be applied at EU level (Supplementary Information, Part 3, Intralaboratory study).

The duration of the method is 4–6 h and involves four key steps: filtration, RNA extraction, real-time RT-PCR and data interpretation. Table 3 summarises the steps required for the sample analysis using the alternative method.

2.4.1. Filtration

A 0.45 μm pore with 47 mm diameter polycarbonate filter membrane (Track-Etch Membrane, Nucleopore™, Whatman™, Cytiva) was placed in the filtration station using sterile tweezers. During the first inter-laboratory study, mixed cellulose filter membranes (MF-Millipore™) were recommended and used by 12 out of 18 laboratories, while 6 laboratories used polycarbonate filter membranes. Subsequently, 100 mL of water sample were filtered, and the filter-membrane was picked up at opposite edges, using two sets of sterile forceps. The filter was then rolled into a 2 mL cylinder (ZR BashingBead™ Lysis Tube, Zymo Research) tube, with the top side facing inward covered with 750 μL DNA/RNA Shield™ (Zymo Research). To avoid contamination, filtration steps were carried out in sterile conditions, under the biosafety cabinet. This step takes approximatively 0.5 h for eighteen samples.

2.4.2. RNA extraction (or isolation)

RNA was extracted using the ZymoBIOMICS DNA/RNA Miniprep Kit (Zymo Research). The procedure was carried out according to the manufacturer's protocol with some modifications. Briefly, the mechanical homogenisation was carried out using a vortex at maximum speed for at least 20 min and the elution volume was 50 μL. Other kits routinely used by some of the participant laboratories were also accepted (e.g. Biomerieux Nuclisens kit and Qiagen RNeasy Powerwater). The extraction kit/method used by each participating laboratories is described in Table 1. The duration of the RNA extraction procedure was approximately 2.5 h for eighteen samples plus the positive control.

Table 3
Overview of the alternative method steps including a short description and duration calculated for 18 samples and controls.

Step name	Description	Duration (18 samples)
Filtration and filter preservation	Filter 100 mL of each drinking water sample using a polycarbonate filter membrane (0.45 μm pore with 47 mm diameter (Track-Etch Membrane, Nucleopore™, Whatman™, Cytiva). Fold or roll the filter using sterile tweezers and place it into a 2 mL cylinder (ZR BashingBead™ Lysis Tube, Zymo Research)	0.5 h
RNA extraction	Cell lysis and RNA extraction using a commercial extraction kit. ZymoBIOMICS DNA/RNA Miniprep Kit was recommended but NucliSens extraction (Biomerieux) and Qiagen RNeasy Powerwater kits were also accepted	2.5 h
cDNA synthesis	cDNA synthesis from total RNA, with random primers, using the SensiFAST cDNA Synthesis kit (Bioline) (this step was not required in the cases one-step RT-PCR was used, see Table 1)	0.5 h
Real-time PCR	Detection of 16S rRNA gene (*E. coli*), using the primers and probe listed in Table 4. SensiMIX II Probe kit (Bioline), containing dNTPs and all the components for real-time PCR, was used with optional ROX reference dye (no ROX, low ROX or high ROX) depending on the real-time PCR instrument (except in the cases one-step RT-PCR was used, see Table 1)	2–2.5 h

2.4.3. Real time RT-PCR method

The cDNA synthesis was performed from total RNA using random primers, followed by amplification of a specific target gene using the SensiFAST cDNA Synthesis kit (Bioline). The master mix was prepared considering the total number of samples (1 μL of reverse transcriptase, 4 μL of 5x TransAmp Buffer and 10 μL DNAse/RNAse-free water). Then, 15 μL of the master mix were transferred to autoclaved PCR tubes together with 5–10 μL of the corresponding RNA template, in a total volume of 20–25 μL. A RT control containing all components, except the enzyme reverse transcriptase, and a non-template RT blank control containing all components except the RNA template (10 μL of DNase/RNase-free water), were included in each assay run. The reaction was run in a thermocycler (primer annealing for 10 min at 25 °C, reverse transcription for 15 min at 42 °C, inactivation for 5 min at 25 °C). The RT-PCR kit/method and further details on the instruments used by each laboratory are listed in Table 1.

Detection of *E. coli* was carried out by targeting the 16S rRNA gene, using the primers (Thermo Fisher Scientific) and probe (Taqman QSY probe, ThermoFisher Scientific) described by Huijsdens et al. (2002) and listed in Table 4. Some participating laboratories used the same primers and probe from a different supplier.

To this end, the SensiMIX II Probe kit (Bioline), containing dNTPs and all the components for real-time PCR, was used with optional ROX reference dye (no ROX, low ROX or high ROX) depending on the real-time PCR instrument. The real-time PCR mix (for one reaction: 25 μL 2xSensiMIX II Probe kit, 2 μL of 10 μM forward and reverse primers, 0.5 μL of 10 μM probe, and 16.5 μL DNAse/RNase-free water) was prepared for the total number of reactions and replicates.

In each assay run, non-template PCR controls were included by adding 46 μL of the reaction mix and 4 μL of DNase/RNase-free water to

Table 4
Primers and probe used for the detection of *E. coli* in drinking water (Huijsdens et al., 2002).

Primers/Probe	Sequence	Start	Stop	Length primer or probe (bp)	Amplicon length (bp)
16S Forward	CATGCCGCGTGTATGAAGAA	393	415	20	
16S Reverse	CGGGTAACGTCAATGAGCAAA	491	471	21	98
Probe[a]	TATTAACTTTACTCCCTTCCTCCCCGCTGAA	468	438	31	

[a] The probe has the fluorophore FAM at 5′ and the quencher QSY at 3′.

dedicated wells. This step was carried out in a different room not contaminated with RNA or DNA, or at least under a different biosafety cabinet. If this was not possible, all surfaces and pipettes were decontaminated using RNase AWAY™ (Thermo Fisher Scientific) to remove all DNA and RNA.

Four μL of the cDNA template were added to the corresponding wells in a DNA-free room and/or a different biosafety cabinet. After covering and sealing the plate, it was mixed by vortexing for 10 s and spun down in microfuge. The samples were analysed in triplicate using the following program: 10 min at 95 °C (polymerase activation), followed by 40 cycles of 10 s at 95 °C (denaturation) and 60 s at 60 °C (annealing/extension). Alternatively, the participants could use other kits or volumes. All information regarding reagents and PCR instruments used by the different laboratories is included in Table 1.

The results of the real-time PCR are expressed as Ct (cycle threshold) or Cq (quantification cycle), which is defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e. exceeds background level).

The approximate duration of cDNA synthesis and real-time PCR was around 2.5–3 h for eighteen samples plus the positive controls.

2.4.4. Data interpretation

The results obtained with the alternative method (Ct or Cq value) were compared with those obtained by applying the reference method (CFU/100 mL) for each sample.

The Ct or Cq value (depending on the real-time PCR cycler used) refers to the number of PCR cycles required to generate a detectable signal. This value is used to determine the presence or absence of *E. coli* in the water samples via the alternative method.

Due to the presence of trace amounts of *E. coli* nucleic acids in the real-time RT-PCR reagents leading to product formation, it is possible that all samples produce a Ct value. For this reason, a specific approach was used to interpret the results.

Indeed, regarding the results of the alternative method, the difference between the Ct value of the blank sample and the Ct value of the drinking water sample is decisive (ΔCt = Ct blank – Ct sample). For suitable reagents, the results in Ct value obtained for a blank sample and a sample containing 1 CFU/100 mL should be 2 cycles apart. A sample is considered positive (*E. coli* present) when: ΔCt >2 with a maximum Ct of approximately 36 (cut-off), while it is considered negative (*E. coli* absent) when: ΔCt ≤2 or Ct value > 36.

The equipment and reagents used by different laboratories may influence the cut-off value used to determine whether *E. coli* is present or absent in the sample. However, a common threshold of 36 (Ct) was recommended, although some laboratories with extensive experience validated their own cut-off value. In these specific cases a threshold of 35 (Ct) was accepted.Some laboratories used a cut-off Ct value of 35 instead of 36, while for one participating laboratory a ΔCt >2 was enough to identify a positive sample (Supplementary Information, Part 2, Table S19).

The validity and quality criteria for determining the acceptability of results were thus:

Ct values of blank samples >35 (to include the laboratories that use 35 as cut-off Ct value).

Ct value of positive controls A, B, C < 30 (determined by the JRC).

2.5. Interlaboratory study

The aim of the interlaboratory study was to compare the reference and the alternative methods when used by different laboratories and determine the applicability of the alternative method (real-time RT-PCR) for rapid *E. coli* detection in drinking water in an EU-representative scale. To achieve this, the performance characteristics (specificity and sensitivity) of the reference and the alternative methods were tested by different collaborators using identical samples (reproducibility conditions). The goal of this was twofold: firstly, to identify the method's critical steps (and those that are more flexible), and secondly, to investigate its performance when applied by different laboratories across EU.

Specificity and sensitivity studies were carried out according to ISO 16140-2 as briefly described in the following sections. Table 5 lists the terminology and definitions used in the formulas described in sections 2.6, 2.7, and 2.8.

2.6. Specificity study

The percentage specificity of the reference and the alternative method was based on the results of contamination level 0 (L_0) as follows:

$$\text{Specificity for the reference method} : SP_{ref=}\left[1-\left(\frac{P_0}{N_-}\right)\right]\times 100\%$$

$$\text{Specificity for the alternative method} : SP_{alt=}\left[1-\left(\frac{CP_0}{N_-}\right)\right]P_0$$

Where N_- is the number of all L_0 tests;

P_0 is the total number of false-positive results obtained with blank samples.

CP_0 is the total number of confirmed false-positive results obtained with blank samples.

Table 5
Terminology used in ISO 12140-2. L_0, contamination level 0.

Abbreviation	Description
AL	Acceptability limit
AM	Alternative method
CP_0	Total number of confirmed false-positive results obtained with blank samples
RM	Reference method
N_-	Number of all L_0 tests
NA	Negative arrangement (RM-/AM-)
ND	Negative deviation (RM+/AM-)
*N*x	Number of samples tested at level x
PA	Positive arrangement (RM+/AM+)
PD	Positive deviation (RM-/AM+)
P_0	Total number of false positive results obtained with blank samples
$(p+)alt$	Number of positive results by the alternative method
$(p+)ref$	Number of positive results by the reference method
RLOD	Relative level of detection
RT	Relative trueness
SEalt	Sensitivity of the alternative method
SEref	Sensitivity of the reference method
SPalt	Specificity of the alternative method
SPref	Specificity of the reference method

2.7. Sensitivity study

The sensitivity study is a comparative study of the results of the reference method (RM) to the results of the alternative method (AM) in artificially contaminated samples. For each of the contamination levels L_1, L_2, L_3 and L_4, the results of all participating laboratories are combined.

$$\text{Sensitivity for the reference method : SEref} = \frac{(PA + ND)}{(PA + ND + PD)} \times 100\%$$

$$\text{Sensitivity for the alternative method : SEalt} = \frac{(PA + PD)}{(PA + ND + PD)} \times 100\%$$

$$\text{Relative trueness : RT} = \frac{(PA + NA)}{N} \times 100\%$$

where PA: positive agreement (AM+/RM+); PD: positive deviation (AM+/RM-); NA: negative agreement (AM-/RM-); ND: negative deviation (AM-/RM+); and N: total number of samples N = (NA + PA + ND + PD).

The acceptability limits (AL) were determined according to ISO 16140-2, section 5.2.4.2. for each contamination level.

The AL is defined as:

$$(ND - PD)max = \sqrt{3Nx \times ((p+)ref + (p+)alt - 2((p+)ref \times (p+)alt))}$$

where $(p+)ref$ is the number of positive samples by the reference method divided by the number of samples tested at level x, Nx; while $(p+)alt$ is the number of positive samples by the reference method divided by Nx.

The difference between PD and ND (PD - ND) was determined for each contamination level. The AL is not met when the observed value for (ND - PD) is higher than the AL.

2.8. Relative level of detection (RLOD)

The relative level of detection (RLOD) was determined using the calculation program version 3 dated 15-08-2015, published on the ISO website (https://standards.iso.org/iso/16140/-2/ed-1/en/), as described by Wilrich and Wilrich (2009, 2015 and 2022) and Mărgăritescu and Wilrich (2019). The calculated RLOD represents the ratio of the level of detection (LOD) of the reference method to the LOD of the alternative method. If this value is less than one, the alternative method is more sensitive than the reference method. The AL for an unpaired study is set at 2.5 meaning that the LOD of the alternative method should not be higher than two times the LOD of the reference method. An LOD value for the alternative method smaller than the LOD value for the reference method is always accepted as this means that the alternative method is likely to detect lower levels of contamination than the reference method.

2.9. Statistical analysis

Descriptive statistical analysis was performed for estimating mean, standard deviation, median and range (minimum and maximum) of continuous variables. The variability of parameters was compared between laboratories at several levels of contamination. Overall, values of $p < 0.05$ were accepted as statistically significant. All statistical analyses were performed using R for Statistical Computing version 4.1.2.

The performance of the participating laboratories were analysed by Z-test. We followed ISO 13528 (ISO-Standard, 2022), first applying Section 7.7 – Consensus value from participant results (arithmetic mean) and then Section 9.4 – Z-scores. This is a standard procedure in proficiency testing which is commonly reported in the literature (Rosario et al., 2008; Ainsbury et al., 2016; Al Bayat et al., 2021; Dhibika et al., 2023).

Whenever possible, the consensus value was determined using participant data by applying Algorithm A and in accordance with ISO 13528 (ISO-Standard, 2022). The Algorithm A provides a robust estimate, namely the robust mean and standard deviation of the data to which it is applied.

The Z-scores were calculated for each level of contamination in accordance with ISO 13528 (ISO-Standard, 2022) by the following formula:

$$Z - score = abs\left(x_{mean\_i-th\ lab} - mean_{all\_samples}\right) \Big/ \sigma_{all\_samples}$$

where *abs*: absolute value; $x_{mean\_i\text{-}th\ lab}$: average of samples of i-th participating laboratory for a given level of contamination; $mean_{all\_samples}$: mean of all samples for a given level of contamination; and $\sigma_{all\_samples}$: standard deviation of all samples for a given level of contamination.

Then, the overall performance of laboratories was concluded by the five levels of average Z-scores described in Table 6 (from excellent to unsatisfactory).

3. Results

For the first interlaboratory study, the JRC prepared a set of artificially contaminated samples (with *E. coli* Vitroids) at 5 contamination levels (0, 1, 3, 5 and 10 CFU/100 mL). Three positive controls (controls A, B and C) and a blank sample were also included in the analysis as indicated in section 2.2.1. These samples were tested by the 18 participating laboratories using both the reference and the alternative methods (Fig. S1). The outcome of the first interlaboratory study is described in detail in the Supplementary Information (Part 1). Eighteen data sets were produced by the participating laboratories but only 14 were considered valid. Four out of 18 laboratories reported problems during the RNA extraction step and were excluded from the analysis. The sensitivity analysis (Table S9) showed a lower sensitivity of the alternative method (65.4 %) compared to the reference method (90.4 %), and the AL were not met (Table S10).

The first interlaboratory study results were also analysed considering only the data set from the participating laboratories that used polycarbonate filter membranes (6 participating laboratories). These participants routinely used the alternative method for *E. coli* detection in their laboratories. It was observed that participating laboratories using polycarbonate instead of mixed cellulose filters reached a higher sensitivity of the alternative method (Table S9) (89.2 %) compared to the reference method (87.7 %). Moreover, in this case, the AL was met for all contamination levels (Table S10). This comparison was not one of the aims of the study but, although other reasons for the differences cannot be ruled out, the results suggest that polycarbonate is preferred. Following the outcome of this study, the SOP was improved and tested in a second interlaboratory study. The main differences between the first and second interlaboratory studies are summarised in the Supplementary Information (Part 4, Table S26). The results of the second interlaboratory study are described in the following sections.

Table 6
Performance of laboratories according to average Z-scores which are calculated considering results from all contamination levels.

Laboratory performance	Average Z-scores
Excellent	0 ≤ Z-score <0.5
Very good	0.5 ≤ Z-score <1
Good	1 ≤ Z-score <2
Satisfactory	2 ≤ Z-score <3
Unsatisfactory	Z-score ≥3

3.1. Variability of the water sample analysis by the alternative method

The variability among the water sample analyses at different contamination levels was estimated for both the alternative and reference methods.

Regarding the alternative method, Fig. 1 summarises the distribution (spread) of Ct values estimated by results of all participating laboratories, for each of the considered five contamination levels. For convenience, the figure is complemented with main results from the descriptive statistics showing in tabular form the Ct-range, median and mean (plus standard deviation) values. Overall, the absence of outliers and a gradually diminishing of median, mean and maximum Ct values (except maximum for the first level of contamination) when contamination levels increased, could be observed.

The average Ct values determined for samples at level of contamination 0 (absence of *E. coli*) were higher than 36, while the Ct values determined for samples at levels 1, 2, 3 and 4 (*E. coli* concentrations 1–10 CFU/100 mL) were lower than 36 as shown in Fig. 1, indicating that the participant laboratories were able to distinguish between negative and positive samples, by using the alternative method. However, two laboratories (4 and 18) showed results below the threshold value of 36 for level 0 and blank samples (Supplementary Information, Tables S13 and S19). Therefore, these laboratories were considered to have generated outliers and for this reason they were excluded from the subsequent specificity and sensitivity studies, in accordance with ISO 1614, part 2 (ISO-Standard, 2016).

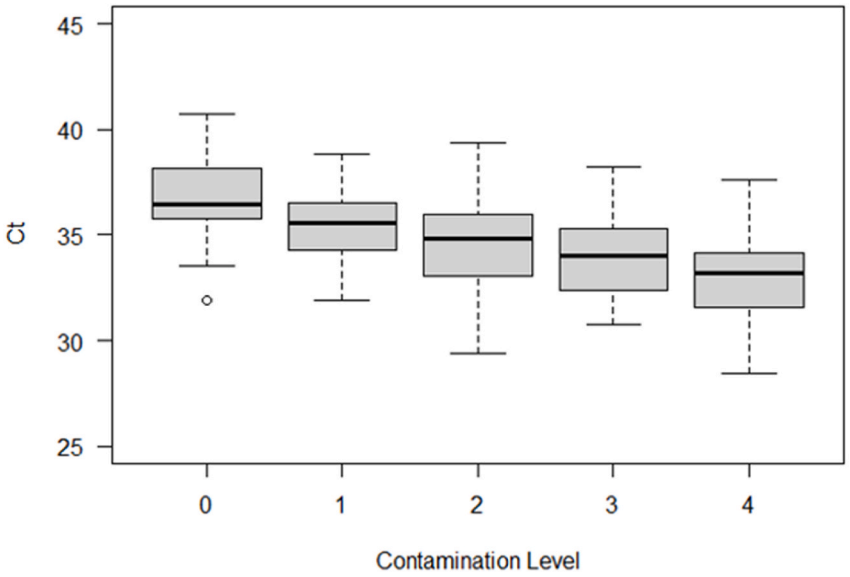
Regarding the reference method, the detailed results of the variability analyses of the sample analysis carried out by the JRC are shown in the Supplementary Information (Table S16, Figs. S13 and S14). Generally, the maximum colony count on the CCA plates for the contamination levels 1–4, varies from 5 to 25 CFU/100 mL.

3.2. Laboratory performance

For the alternative method, the assessment of laboratory performances using a Z-test, based on the derived Ct values, is shown in Fig. 2 for each level of contamination. The performance was evaluated following ISO 13528 (ISO-Standard, 2022), as indicated in section 2.9.

All laboratories showed performance Z-scores for the considered five contamination levels which are below the critical threshold of 2. Only one laboratory reached a maximum Z-score approaching the upper level of a good performance but remaining below the critical threshold of 2. Most participating laboratories (16 out of 19) showed very good or excellent average Z-scores of performance for Ct values (the average Z-scores are calculated considering all contamination levels) which highlights the robustness of the alternative method.

The performance of the drinking water sample analysis by the reference method was evaluated (Supplementary Information section 2.4.3). All samples showed excellent or very good average Z-scores of performance for the considered five contamination levels. About 79 % of



Contamination Level	Min	Mean	SD	Median	Max	% positive samples	% negative samples
0 (0 CFU/100 mL)	31.90	36.69	1.89	36.47	40.76	8.93	91.07
1 (1 CFU/100 mL)	31.90	35.50	1.61	35.58	38.85	63.16	36.84
2 (3 CFU/100 mL)	29.37	34.65	2.32	34.85	39.36	78.95	21.05
3 (5 CFU/100 mL)	30.77	34.00	1.87	34.01	38.20	92.98	7.02
4 (10 CFU/100 mL)	28.46	33.00	1.87	33.24	37.62	94.74	5.26

Fig. 1. Boxplot of cycle threshold (Ct) values estimated by participating laboratories for each of the considered contamination levels. The mean Ct values of all participant laboratories and standard deviation (SD) are included for each contamination level as well as the percentage of samples considered positive and negative. Samples are considered positive when at least two real-time reverse transcription PCR (RT-PCR) replicates show Ct or Cq values < 36 and $\Delta Ct \geq 2$. On the figure, the circles indicate outliers according to the R programming language for Statistical Computing. In the R, the outliers are defined standardly by the interquartile interval (Q3 - Q1) as values outside the range [Q1 - k (Q3 - Q1), Q3 + k (Q3 - Q1)] where k = 1.5, Q1 = 25th percentile and Q3 = 75th percentile of data.

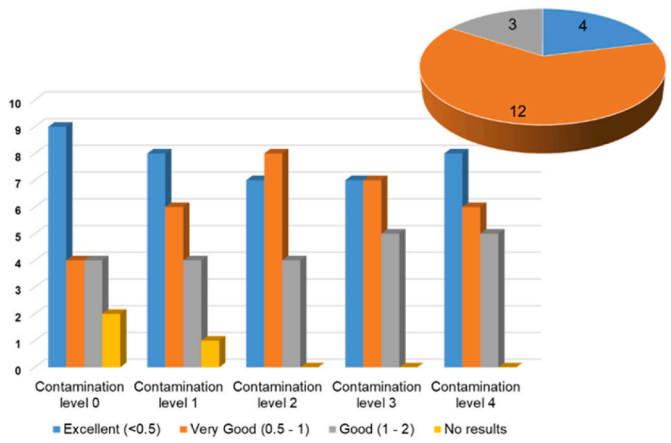


Fig. 2. For alternative method, performance of participating laboratories according to Z-scores for cycle threshold (Ct) estimated under different contamination levels. The pie chart summaries the assessment results for laboratory performances found by the average Z-scores considering all contamination levels. No results = reported non-detected or non-quantified values.

the samples (15 out of 19) showed excellent performance regarding CFU/100 ml estimated for the considered five contamination levels (Supplementary Information Figures S15, S16 and S17). The high Z-score values obtained during the second interlaboratory study for the reference method analysis can be explained because all samples were analysed by the same laboratory (JRC). This approach was taken to minimise the differences observed during the first interlaboratory study due to an unexpected shipment delay. The second interlaboratory study focused on the reproducibility and interoperability of the alternative method among the participating laboratories.

3.3. Specificity and sensitivity studies

The comparison of the alternative and reference method was evaluated through the sensitivity and specificity studies, as described in sections 2.6 and 2.7.

The interlaboratory study produced 15 valid data sets contributed by 15 collaborators representing 15 different organisations, in full accordance with ISO 16140-2 (ISO-Standard, 2016). Four sets of results produced by 4 laboratories (4, 14, 15 and 18) from 3 different organisations were excluded from the specificity and sensitivity parameters for the following reasons: laboratories 14 and 15 produced Ct values higher than 36 for all samples, only some replicates at contamination levels 3 and 4 were positive (Supplementary Information, Table S19). Furthermore, positive control B (reverse transcription control) delivered higher Ct values (higher than 30) in respect to controls A and C, indicating a possible problem during the cDNA synthesis step (Supplementary Information, Table S13). Regarding laboratories 4 and 18, the blank samples produced Ct values lower than 35 indicating a possible contamination (Supplementary Information, Table S13).

Excluding the results from the four laboratories as outlined above, the results of the 15 laboratories showed that the specificity of the reference and alternative methods were 100 % and 97.7 %, respectively (Supplementary Information, Table S20). The results of the sensitivity study are shown in Table 7. Overall, the sensitivity of both methods for *E. coli* detection in drinking water was higher than 91 %, with the reference method (97.2 %) being more sensitive than the alternative method (91.1 %). The AL for the sensitivity study were met for contamination levels 0, 1, 3 and 4 as indicated in Table 8. For the aforementioned contamination levels, the difference (ND-PD) was lower than (ND-PD)max. In the case of contamination level 2, the AL is not fulfilled (see section 2.7). For an unpaired study, the AL of RLOD is set at 2.5 according to ISO 16140 part 2 as described in section 2.8 of this

Table 7
Sensitivity of the reference and alternative methods estimated by results of 15 participating laboratories. AM, alternative method; NA, negative arrangement; ND, negative deviation; PA, positive arrangement; PD, positive deviation; RLOD, relative level of detection; RM, reference method.

		Reference method positive (R+)	Reference method negative (R-)
	Alternative method positive (A+)	PA	PD
	Alternative method negative (A-)	ND	NA
Sensitivity	Dataset from 15 laboratories		
	Alternative method positive (A+)	158	5
	Alternative method negative (A-)	16	45
	91.06 % (AM)	97.21 % (RM)	
Relativetrueness	90.63 %		
RLOD	1.787		

Table 8
Acceptability levels (AL) for each contamination level (L_0, L_1, L_2, L_3, L_4). AL are met when ND-PD ≤ AL. ND, negative deviation; PD, positive deviation.

	L_0	L_1	L_2	L_3	L_4
ND-PD	−1	6	5	1	0
AL = (ND-PD)max	2	7	4	2	0

article, meaning that the LOD of the alternative method should not be higher than two times the LOD of the reference. In this study, the RLOD was 1.790, supporting that the reference method is more sensitive than the alternative method, however the alternative method meets the ALfor a method comparison study.

4. Discussion

This study investigated the applicability of an alternative method (real-time RT-PCR) for *E. coli* detection in drinking water at a scale representative of the EU. One major advantage of such a molecular method is that it could reduce the time to yield results from 21-24 h to 4–6 h. Since the early recognition of faecal contamination in distributed drinking water is critical to prevent waterborne outbreaks and protect human health, there is growing interest in the development and application of *E. coli* detection methods for drinking water quality monitoring and management. However, standardisation and validation of those methods is required before implementing them in a legal framework to ensure that they enable *E. coli* detection at the required sensitivity and detection level.

A qualitative method for rapid *E. coli* detection in drinking water based on real-time RT-PCR has been recently validated by Heijnen et al. (2017, 2024), showing that the sensitivity of the method is at least equal to the reference method (RLOD = 0.75). The validation included an interlaboratory study carried out by two Flemish and five Dutch laboratories, showing that the real-time RT-PCR and the culture methods have comparable sensitivities when tested by different laboratories (91.9 % sensitivity of the alternative method). The authors also proved the value of the method as a rapid detection tool to monitor the spread of microbial contamination in two case studies in Belgium and the Netherlands. Yet, these studies were limited to seven laboratories in a confined area of Europe. To provide a wider EU-representative validation of the real-time RT-PCR method, the JRC launched two EU trial interlaboratory studies, aimed at testing the molecular approach for *E. coli* detection in drinking water at EU representative level. Several

large-scale comparison studies applying real-time PCR methods for *E. coli* detection are described in the literature for recreational waters in the United States of America (Aw et al., 2019; Sivaganesan et al., 2019; Haugland et al., 2021).

To our knowledge, the work described in the current article is the only European wide study using the real-time RT-PCR method for *E. coli* detection in drinking water and fulfilling the requirements for validation according to EU regulation (recast Drinking Water Directive, 2020/2184).

The real-time RT-PCR method slightly modified by the JRC (i.e., the recommended extraction method using columns instead of magnetic beads) was first tested in-house, as described in the Supplementary Information (Part 3, Intralaboratory study) showing a higher sensitivity (100 %) than the reference method (83 %) and a RLOD of 0.292 (Supplementary Information Table S25).

Regarding the EU-representative interlaboratory studies described in this article, the participants of the first and second interlaboratory studies were able to follow the SOP and deliver results, even when they were not familiar with the alternative method. The participants that routinely used the alternative method for *E. coli* detection in their laboratories showed better performance and higher sensitivity (89 %) of this method during the first interlaboratory study compared to the other participants that used the alternative method for the first time (48 %). The performance and sensitivity of the method improved during the second interlaboratory study for most of the participating laboratories reaching the 91 % sensitivity, significantly higher than the 64 % sensitivity obtained in the first interlaboratory study and showing that practice is crucial to deliver good quality results. Indeed, this improvement is a result of both protocol refinement and increased operator familiarity with the alternative method. Nevertheless, unexpected sporadic contamination was observed by some experts, the possible sources of which have not been determined yet. The contamination could come from reagents, pipettes or operators. For this reason, to avoid cross-contamination it is important to carry out the different steps of the protocol in different rooms and sterile conditions, if possible, working under safety cabinets. The use of quality controls (positive controls A, B and C) and blank samples was essential to evaluate the validity of the laboratory results and to identify the most critical steps of the alternative method. The first interlaboratory study showed that the best results could be obtained when key operational parameters were optimal (e.g. polycarbonate filter membranes, RNA extraction, real-time RT-PCR reagents, and laboratory experience using the alternative method). Differences in the results among laboratories were seemingly related to the selection of RNA extraction filter membranes, with a better performance when polycarbonate filters were used instead of mixed cellulose filters, which represents a key conclusion of this joint preparatory study. The kind of filter to be used for RNA isolation was discussed with the participating laboratories after the first interlaboratory study. As a result of the discussion the JRC carried out a comparison study using polycarbonate and mixed cellulose filters for RNA isolation. The specificity of the alternative method was 100 % in both cases while the sensitivity was lower using mixed cellulose filters (81.8 %) compared to polycarbonate filters (100 %). The selection of filters for bacterial culture and molecular biology is based on the filter material. Cellulose filters are structured in a matrix into which cells are captured; when put on an agar medium, the capillary flow of the agar pushes the liquid into the membrane where the growth starts. For the molecular techniques, the purpose is to wash out the cells from the membrane. This process is longer when using cellulose filters. There is a consensus that polycarbonate and similar non-absorptive materials are ideal for capturing cells for the detection of intracellular DNA (iDNA). In contrast, absorbent materials like cellulose are preferred for membranes used to capture extracellular, dissolved, cell-free DNA (eDNA) (Bairoliya et al., 2022). For detecting viable *E. coli* in drinking water, the goal is to capture cells only, excluding extracellular RNA, and confirm their viability via 16S rRNA. Therefore, polycarbonate was the appropriate choice.

Regarding the extraction procedure, the key step seemed to be the cell lysis which was optimal using a high-speed bead beater or vortex for 20 min at maximum speed. The SensiFAST™ Probe kits come in three variations: high ROX, low ROX, and no ROX. The use of adequate master mix ROX concentration for each real time PCR instrument was critical. ROX is a passive reference dye used in real-time PCR to normalise fluorescence signals and reduce variability between technical replicates Using the appropriate ROX concentration for each specific real-time PCR instrument ensures accurate and reliable data normalisation (Wang et al., 2007).

The outcome of the first interlaboratory study was used to adapt the alternative method's SOP, which was tested in a second interlaboratory study. The second study was on sample filtrates and generated 15 valid datasets that were used for comparing the performance of the alternative method to the reference method. The estimated specificity and sensitivity of the alternative method obtained in the second interlaboratory study were very close to the ones obtained by Heijnen et al. (2024) in the validation study. The specificity of the alternative method reached 97.7 %, very close to the specificity obtained in the previous validation study (97.9 %). The sensitivity of the alternative method increased in respect to the first interlaboratory study reaching 91.1 %, indicating a slightly lower performance when compared to the reference method. Its score, however, is fully compatible with the results described in the Dutch and Flemish recent validation study. The highest sensitivity of the reference method in this study could be explained in part because all samples were analysed only by the organising laboratory. The alternative method involves more steps than the reference method and practical experience is crucial to reach the highest sensitivity. In this study, the sensitivity of the alternative method was slightly lower than that of the reference method. Nevertheless, the shorter time to yield results (4–6 h) makes the alternative method a valuable option for monitoring microbial drinking water quality in situations requiring quick assessment, such as massive social gatherings, post-disaster scenarios and contamination events. Fast detection of microbial contamination is essential for decision-makers to enable early warning and quick reactions, using appropriate interventions, to prevent waterborne outbreaks and protect human, animal and environmental health.

Even after the SOP was improved following the first interlaboratory study, four laboratories had to be excluded from the specificity and sensitivity analyses because they did not fulfil the validity criteria used in our study. In this specific case, two laboratories produced blank samples with Ct values lower than 35 suggesting a possible contamination and two laboratories had high Ct values for control B indicating a potential problem during cDNA synthesis.

The equipment and reagents used by the different laboratories may influence the criteria for determining positive and negative samples. These criteria must be confirmed by each laboratory. In this study, some laboratories familiar with the method used a cut-off value of 35, whilst for one laboratory, a $\Delta Ct > 2$ was sufficient to identify a positive sample. To ensure accurate and comparable results, it is essential for each laboratory to determine their own RLOD, to decide which Ct value represents a positive result. Consequently, further studies or individual meetings are recommended to verify and harmonise the criteria used by each laboratory.

Moreover, adequate training, including a period of practical experience, can be considered an important factor improving the performance of the method and it is highly recommended for laboratories willing to implement the alternative method as a routine practice, as also suggested by Aw et al. (2019). The alternative method involves more steps than the reference method; particularly the RNA extraction step was problematic for some participating laboratories. Automation could provide another solution to prevent incorrect RNA extractions. Against this background, the Dutch normalisation institute (NEN) has made a standard of this method, in collaboration with Dutch and Flemish experts (NEN-Norm, 2023).

Additional interlaboratory studies using naturally contaminated

drinking water would be preferable, but not really feasible. However, a next step to analysing water samples is very important. Samples contaminated with reference materials or with trace amounts of wastewaters would be a subsequent stage. Special care should be taken to avoid delay during shipment of the samples or substances that stabilise nucleic acids in the bacterial cells could be added to the samples to minimise this effect.

Samples collected by the participating laboratories would be desirable to confirm the applicability of this method in actual drinking water management actions. Concentrated drinking water samples in filter membranes from each sampling location could be used by the different participant laboratories to compare the reference and alternative methods on site, while a membrane filter sample could be shipped to the organising laboratory for verification of results. A similar approach was adopted by Haugland et al. (2021) to study the detection of *E. coli* in recreational waters, in which the participants collected the samples and analysed them by the reference and alternative method. In this case, a set of samples collected in filter membranes were stored and sent to the organising laboratory for additional analysis.

Finally, other promising techniques such as the digital PCR (dPCR) should be further investigated for *E. coli* detection and quantification in environmental samples (Moinet et al., 2024) and drinking water (Tiwari et al., 2022). Although processing time and analysis costs for dPCR are typically higher than those for real-time PCR, studies focused on surface water have shown comparable results for both techniques, highlighting improved sensitivity and lower inhibition for dPCR (Tiwari et al., 2022). A major advantage of dPCR is that it does not require a standard curve for quantification and, as reported by many studies, has a lower limit of quantification (LOQ) compared to real-time PCR, enabling the detection of trace levels of pathogens. An application of dPCR in water quality assessment has been reported in a study where this technique was used to differentiate enteric *E. coli* from benign environmental *Escherichia*, whose presence could lead to erroneous public health risk notifications (Moinet et al., 2024).

5. Conclusions

This study demonstrated for the first time the applicability of a real-time RT-PCR as an alternative qualitative method for *E. coli* detection in drinking water at an EU-representative scale, in laboratories with different levels of expertise. The sensitivity of the alternative method aligns with the existing reference method, and its use for *E. coli* detection in drinking water could reduce significantly the time to results from 21-24 h to 4–6 h, enabling public authorities to issue early warnings and respond quickly with appropriate interventions. Our research identified the most critical steps of the alternative method, emphasising the importance of training and practical experience to achieve the highest analysis sensitivity, and highlighted the need for further development of these studies to ensure the comparability of fast methods for early *E. coli* detection before their incorporation in a legal framework. The use of quantitative alternative methods like dPCR for monitoring drinking water microbial quality would provide highly sensitive and accurate detection of microbial contaminants, ensuring reliable assessment of water safety. Additionally, international harmonisation and standardisation of molecular methods for early recognition of faecal contamination in distributed drinking water are essential to provide a consistent and reliable approach across different laboratories for water quality management and public health.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2025.121786.

Data availability

Data will be made available on request.

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