

A 16S rRNA reverse transcriptase-PCR method for rapid detection of intestinal enterococci in distributed drinking water

Leo Heijnen^{1,*}, Eline Stroobach², Adrie Atsma³, Gabi van Pelt⁴, Hendrik Jan de Vries⁵, Katrien De Maeyer⁶, Bea Timmermans⁷, Liesbeth Visser⁸, Maja Taucer-Kapteijn⁹, Gertjan Medema^{1,10}

¹KWR Water Research Institute, Water Microbiology, 3433 PE, Nieuwegein, the Netherlands

²Het Waterlaboratorium, 2031 BE, Haarlem, the Netherlands

³Vitens, 8912 AA, Leeuwarden, the Netherlands

⁴Aqualab Zuid, 4251 NN, Werkendam, the Netherlands

⁵WLN, 9756 AD, Glimmen, the Netherlands

⁶Pidpa, 2018, Antwerpen, Belgium

⁷De Watergroep, 3001, Leuven, Belgium

⁸Brabant Water, 5223 MA, 's-Hertogenbosch, the Netherlands

⁹PWN, 1991 AS, Velsbroek, the Netherlands

¹⁰Delft University of Technology, 2628 CD, Delft, the Netherlands

*Corresponding author. KWR, Water Research Institute, Groningenvaart 9, 3433 PE Nieuwegein. E-mail: Leo.heijnen@kwrwater.nl

Abstract

Aim: To develop and validate a real-time reverse transcription PCR (RT-PCR) method for the rapid and sensitive detection of intestinal enterococci in drinking water. Conventional culture methods require 24–48 h, limiting rapid response to faecal contamination events.

Methods: An RT-PCR assay targeting 16S rRNA was developed to detect ten *Enterococcus* species, selected based on their prevalence in human and animal faeces and drinking water samples. Drinking water samples (100 ml) were filtered, treated with lysozyme, followed by RNA extraction and one-step real-time RT-PCR. Validation followed ISO16140-2:2016 with ISO7899-2:2000 as reference method and an interlaboratory study, using artificially contaminated samples and uncontaminated drinking water samples.

Results: The RT-PCR method demonstrated a sensitivity of 94.6%, slightly higher than the reference culture method (89.2%). The combined RLOD value (0.851) indicated comparable detection capability between methods, with slightly improved sensitivity of the RT-PCR for *Enterococcus faecium*. Inclusivity testing confirmed detection of all targeted species, while exclusivity testing showed no cross-reactivity with non-target bacteria. Interlaboratory validation confirmed consistent performance across laboratories. Application in drinking water monitoring showed 91.8% agreement between RT-PCR and culture results in samples collected after distribution network repairs, with RT-PCR detecting enterococci more frequently than the reference method.

Conclusions: The validated RT-PCR method provides reliable detection of intestinal enterococci in drinking water with sensitivity comparable to the reference method, but with a much shorter time-to-result (3–4 h). This rapid detection capability enables faster responses to faecal contamination events and supports improved public health protection and operational efficiency for drinking water utilities.

Impact Statement

This study presents a rapid, sensitive RT-PCR method for detecting intestinal enterococci in drinking water with a time-to-result of only 3–4 h, compared with 24–48 h for conventional culture methods. Faster detection enables water utilities to respond more quickly to potential faecal contamination events and to verify the hygienic safety of drinking water after repair works. Implementation of this method was conducted in the Netherlands, improving public health protection, while reducing operational delays in drinking water supply systems.

Keywords rapid detection, faecal contamination, faecal indicator bacteria, water quality

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Introduction

The faecal indicator bacteria (FIB) *Escherichia coli* (*E. coli*) and intestinal enterococci are the cornerstone of monitoring the microbial safety of drinking water. The EU Drinking Water Directive (EC 2020), as well as other drinking water regulations, require the monitoring of *E. coli* and intestinal enterococci in drinking water. Presence of these FIB in drinking water serves as an indication that the water is contaminated with human or animal faeces and hence the potential presence of waterborne pathogens. Culture-based methods are commonly used for the monitoring of these faecal indicators in distributed drinking water and are also embedded in the EU Drinking Water Directive as reference methods. A major drawback of these culture-based methods is the long incubation period which is required to produce a result (typically 24–48 h). This hampers rapid detection and response to faecal contamination incidents to prevent outbreaks of waterborne disease. Also, rapid methods would allow water utilities to rapidly confirm the hygienic quality of drinking water after repair works, or after corrective actions have been taken as response to a contamination event. The ability to return normal water supply as soon as possible improves utility efficiency and reduces impact on drinking water users. Recognizing this need, we recently developed and validated a rapid Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) method targeting 16S ribosomal RNA to qualitatively detect *E. coli* in drinking water at a sensitivity equivalent of 1 colony forming unit (CFU) per 100 ml (Heijnen *et al.* 2024) reducing the time-to-result from 18–24 h to 3–4 h. 16S rRNA was selected as a target to achieve sufficient sensitivity, as it can reach over 50% of the total RNA produced in rapidly growing *E. coli* cells (Lu *et al.* 2009) and the 16S rRNA abundance is associated to the vitality of the cells (Poulsen *et al.* 1993, Lahtinen *et al.* 2008). Thus, high copy numbers of 16S rRNA are present in viable cells making it possible to achieve a high detection sensitivity. Indeed, RT-PCR methods targeting 16S rRNA from faecal indicator bacteria and source tracking markers in environmental waters were more sensitive than qPCR methods targeting DNA from 16S ribosomal RNA genes of these bacteria (Pitkänen *et al.* 2013).

A PCR method for the detection of enterococci in recreational waters using the large subunit rRNA gene of enterococci has been developed and shown to correlate well, both with culturable intestinal enterococci detected by standard culture methods (Haugland *et al.* 2005, Siefing *et al.* 2008, Whitman *et al.* 2010, Converse *et al.* 2012), as well as with the gastrointestinal illness occurrence in swimmers (Wade *et al.* 2006, Wade *et al.* 2008, Wade *et al.* 2010, Colford *et al.* 2012). This relationship was used to recommend bathing water quality criteria based on PCR data in the 2012 USEPA Recreational Water Quality Criteria that can be used to issue water quality advisories on a daily basis (USEPA 2012) and a standardized method was published (USEPA 2015, Sivaganesan *et al.* 2019). Several studies demonstrated the benefits of this rapid detection method for enterococci (Dorevitch *et al.* 2017, Saleem *et al.* 2023). This method was shown to be sensitive enough for recreational waters, but not sensitive enough for drinking water testing to confirm absence of intestinal enterococci in 100 ml (Frahm and Obst 2003, Maheux *et al.* 2011) that is required under the EU Drinking Water Directive (EC 2020). The objectives of the present study were:

- to develop a method for the rapid detection of intestinal enterococci in drinking water samples with a sensitivity equivalent to 1 CFU per 100 ml,
- to validate it against the ISO 7899–2:2000 (ISO-Standard 2000) reference method from the EU Drinking Water Directive, according to ISO 16140–2 (ISO-Standard 2016);
- to evaluate its application for monitoring of drinking water in (unchlorinated) distribution networks in situations that require a rapid result: after distribution network repairs or suspected/confirmed faecal contamination events.

The Drinking Water Directive and reference method refer to ‘intestinal enterococci’. Unlike *E. coli*, intestinal enterococci is not a taxonomic denominator. Traditionally, four *Enterococcus* species are regarded as intestinal enterococci: *E. faecalis*, *E. faecium*, *E. durans*, and *E. hirae* (WHO 2021). However, other *Enterococcus* species have been detected in faeces of humans and animals, such as *E. casseliflavus* in cattle faeces and *E. moraviensis* and *E. haemoperoxidus* in geese droppings (Taučer-Kapteijn *et al.* 2017). Therefore, the prevalence of different *Enterococcus* species in human and animal faeces as well as in drinking water in the Netherlands and Flanders were combined to determine the *Enterococcus* species to be included in the RT-PCR method and to select the corresponding PCR primers.

Materials and methods

Selection of enterococcus species

The study of Taučer-Kapteijn *et al.* (2017) determined the presence of different *Enterococcus* species in animal (including human) droppings that had been deposited in the abstraction area of a phreatic groundwater abstraction site for drinking water production (Table 1).

These data were combined with data from *Enterococcus* species that had been detected in drinking water samples during routine and event monitoring using the ISO 7899–2:2000 method and typed using MALDI-TOF (Table 2). These data showed that the four ‘traditional’ intestinal enterococci (*E. faecium*, *E. faecalis*, *E. mundtii*, and *E. hirae*) were commonly detected, but also other species, including *E. casseliflavus*, *E. moraviensis*, *E. durans*, and *E. gallinarum* were frequently found. *Enterococcus termitis*, *E. gilvus*, and *E. silesiacus* were occasionally found in situations with enterococci contaminations in Dutch and Flemish distribution systems. The species that were most frequently detected in mammalian and avian droppings and in drinking water were selected to be included in the RT-PCR method. This resulted in a selection of ten species to be included in the RT-PCR assay (Table 2).

Primer and probe design

A similarity matrix of the sequences was generated for the 16S rRNA gene of the ten selected *Enterococcus* species, using type strains present in the SILVA NR database (SI). Species with a similarity of 98.7% or more were clustered and primers, which were specific for the selected *Enterococcus* species and would yield amplicons of less than 160 base pairs to ensure efficient PCR were selected using ARB software (Ludwig *et al.* 2004). Annealing temperature optimum of the primer pairs was tested in a range from

Table 1 Relative (%) presence of enterococcus species in faeces of animals as determined by culture on Slanetz and Bartley agar and MALDI-TOF typing (24).

<i>Enterococcus</i> spp.	Red fox <i>Vulpes vulpes</i> (20; 384) ^a	Rabbit <i>Oryctolagus cuniculus</i> (9; 108) ^a	Sheep <i>Ovis aries</i> (11; 126) ^a	Highland cattle <i>Bos taurus</i> (11; 51) ^a	Dog <i>Canis lupus familiaris</i> (10; 144) ^a	Human <i>Homo sapiens</i> (20; 342) ^a	Goose <i>Anser anser</i> (20; 231) ^a
<i>E. faecalis</i>	39.6	0.9		35.3	54.2	27.2	29.4
<i>E. faecium</i>	23.7			5.9	11.8	35.1	7.8
<i>E. hirae</i>	25.5		69.0	5.9	18.1	23	
<i>E. durans</i>	4.9		2.4		8.3	3.8	
<i>E. casseliflavus</i>	1.0		5.6	52.9	2.1	2.6	5.2
<i>E. gallinarum</i>		98.1	9.5		1.4	2.0	3.9
<i>E. mundtii</i>	2.9		13.5		0.7	5.8	8.7
<i>E. moraviensis</i>	0.3	0.9					23.8
<i>E. haemoperoxidus</i>							11.3
<i>E. avium</i>					2.8	18.7	
<i>E. gilvus</i>	0.3						4.3
<i>E. termitis</i>							3.5
<i>E. saccharolyticus</i>						2.3	
<i>E. silesiacus</i>							2.2
<i>E. aquimarinus</i>	1.0						
<i>E. thailandicus</i>					0.7		
<i>E. malodoratus</i>	0.5						
<i>E. sulfurens</i>	0.3						

^aNumbers represent the number of samples analysed; the number of colonies analysed.

Table 2 Enterococcus species identified in drinking water distribution network samples in the Netherlands and Belgium between June 2017 and July 2018.

<i>Enterococcus</i> species	Samples detected	(%)	Concurrent detection of <i>E. coli</i>	Inclusion in PCR
<i>E. casseliflavus</i>	139	34.1	+	+
<i>E. faecium</i>	78	19.1	+	+
<i>E. mundtii</i>	69	16.9	+	+
<i>E. faecalis</i>	66	16.2	+	+
<i>E. hirae</i>	24	5.9	+	+
<i>E. gallinarum</i>	8	2.0	+	+
<i>E. haemoperoxidus</i>	6	1.5	—	+
<i>E. durans</i>	5	1.2	—	+
<i>E. termitis</i>	4	1.0	—	—
<i>E. moraviensis</i>	2	0.5	—	+
<i>E. malodoratus</i>	2	0.5	—	—
<i>E. gilvus</i>	2	0.5	—	—
<i>E. avium</i>	2	0.5	—	+
<i>E. villorum</i>	1	0.2—	—	—
Enterococcus (total)	408	100		

Enterococcus was detected using the ISO 7899–2:2000 method and identified using MALDI-TOF.

53 to 62°C. This resulted in 4 primer pairs (SI, Table S2) at an annealing temperature of 60°C that captured at least the 10 Enterococcus species that were most frequently detected in mammalian and avian droppings in abstraction areas and in drinking water with sufficient (*in silico*) specificity. The RT-PCR would theoretically detect 97.8% of the culturable enterococci detected in the drinking water samples. Given the close genetic relationship

between Enterococcus species, it is likely that the RT-PCR will detect more Enterococcus species. The primers and the probe were purchased from IDT (Leuven, Belgium). The probe was labelled with carboxyfluorescein and quenched with a double Zen/Iowa Black™ FQ quencher.

The validation study methods are as reported in (Heijnen *et al.* 2024) and described in the following paragraphs.

Reference method (culture)

For this study, the membrane filtration method (ISO 7899–2:2000) for the detection of intestinal enterococci was used as reference method (ISO-Standard 2000). This method is based on membrane filtration of 100 ml of drinking water and subsequent culturing at $36 \pm 2^\circ\text{C}$ for 44 ± 4 h on Slanetz and Bartley (S&B) selective agar medium, followed by a confirmation of colonies with typical colour (red, maroon, or pink) using MALDI-TOF-MS or a confirmation reaction where the ability of the presumptive enterococci to hydrolyze Aesculin on Bile Esculin Azide Agar at $44 \pm 0.5^\circ\text{C}$ in 2 h is tested.

Alternative method

The 16S rRNA RT-PCR method with the four primer sets to capture the 10 *Enterococcus* species was validated as the alternative method.

Filtration

A volume of 100 ml drinking water was filtered through a track-edged polycarbonate membrane filter (Sartorius, Germany) with a diameter of 47 mm and a pore size of 0.2 or 0.4 μm (depending on laboratory) to concentrate the microorganisms.

The filter was folded and then transferred to a 1.5 ml microcentrifuge tube containing 1 ml of PCR-grade water and lysozyme (at a final concentration of 10 mg/ml), which, after insertion of the filter, was incubated at 37°C for 30 min to degrade the bacterial cell wall. All liquid was transferred to a centrifuge tube with 2 ml Nuclisens lysis buffer (Biomérieux, Amersfoort, The Netherlands). After incubation for 15 min at room temperature. EasyMAG magnetic silica beads (50 μl) were added and incubated again for 15 min at room temperature, after which the beads were washed semi-automatically using a Kingfisher ML (Thermo Scientific, Bleiswijk, The Netherlands), using three consecutive EasyMAG wash buffers (nr. 1, 2, and 3), as indicated by the supplier. Washed beads were resuspended in 50 μl Nuclisens elution buffer, incubated for 5 min at 60°C and 1400 rpm in a thermomixer. Magnetic beads were separated from the eluate using a Magnasphere magnetic separation stand (Promega, Leiden, The Netherlands).

Real-time RT-PCR

For each sample, duplicate real-time RT-PCR reactions were conducted using Evoscript RNA master mix (Roche chemicals) to perform the reverse transcription reaction and the subsequent PCR in one mixture (one-step RT-PCR) with a cocktail of four specific primer pairs and one probe. RT-PCR was performed in a total volume of 20 μl per reaction containing 5 μl sample RNA. The final concentration of primers and probe in the reactions are shown in the SI (Table S2). RT-PCR reactions were performed on a BioRad CFX Opus 96 real-time PCR device using the following temperature profile: 15 min at 60°C (reverse transcription), 10 min at 95°C polymerase activation followed by 45 cycles of 10 seconds at 95°C , 30 seconds at 60°C , followed by 30 seconds at 40°C . For every sample the number of PCR cycles required to generate a detectable signal (Ct-value) was used to determine the presence or absence of enterococci (see criteria below).

Controls

Two types of controls were included in each batch of analysis: analysis of a blank and a positive control sample. The blank sample consisted of 100 ml of DNA and RNA-free water filtered and subjected to RNA extraction and real-time RT-PCR analysis. For a positive control sample, approximately 50 CFU *Enterococcus faecalis* were added to 100 ml of DNA- and RNA-free water, filtered, and subjected to RNA extraction and real-time RT-PCR analysis.

Interpretation of the data

The criteria used to interpret the results:

1. Samples were considered to contain enterococci if the average Ct value of duplicate RT-PCR reactions was ≤ 35 , and the blank samples were negative and the positive control sample was positive.
2. Analysis results were considered unusable when both technical replicate blank samples yielded average Ct values < 38 .
3. Positive control samples were considered positive if their average Ct values ≤ 35 . The obtained Ct value was also compared to earlier RT-PCR batches to check for inhibition of the extraction/RT/PCR.

Criteria can vary between different laboratories due to the reagents and real-time PCR hardware they use. These criteria should therefore be determined by each laboratory individually. In this study, each laboratory ran a calibration curve of the PCR with low concentrations of *Enterococcus* DNA at each sample batch they tested and determined the Ct threshold. In their practice, the calibration curve and corresponding threshold Ct values were stable and close to 35.

Validation study

A validation of the RT-PCR method was performed following the steps described in ISO 16140–2: 2016 (ISO-Standard 2016), comparing the performance to a reference method: the Slanetz and Bartley culture method, followed by an interlaboratory study. As the requirement in the EU Drinking Water Directive is absence of enterococci in 100 ml of drinking water, both the alternative and reference methods were compared as qualitative methods. The validation protocol included two phases:

A method comparison study to assess the sensitivity, relative level of detection, inclusivity, and exclusivity of the alternative method against the reference method (carried out by KWR).

An interlaboratory study in which 6 water laboratories compared the alternative method against the reference method (organized by KWR).

Method comparison study

The method comparison study consisted of four parts:

Sensitivity assessment: comparing results of the reference method to results of the alternative method in contaminated samples.

Relative level of detection (RLOD) assessment: a comparative study to determine the RLOD in artificially contaminated samples.

Inclusivity assessment: the ability for detecting different strains of target organisms.

Exclusivity assessment: the reliability with which non-target organisms are not detected.

Unpaired study

Since there is no shared initial (enrichment) step for both the reference and alternative method, it is impossible to perform the reference method and the alternative method on the same exact sample. In this validation study different aliquots of the same sample were used for the two methods. This made the resulting data 'unpaired'.

Sensitivity

The sensitivity study evaluated both methods by comparing their results for uncontaminated and faecally contaminated drinking water. Analysis of naturally contaminated samples is preferred. However, this option was not feasible since distributed drinking water samples are rarely contaminated with enterococci. Therefore, drinking water was artificially contaminated with surface water or with diluted sewage containing enterococci. The drinking water samples and the surface water samples originated from different locations in the Netherlands to include a broad variety of *Enterococcus* and non-*Enterococcus* strains from (drinking) water environments. Drinking water samples ($n = 42$), surface water samples ($n = 6$) and two sewage samples from the sewage treatment plant in the city of Utrecht were collected, and the concentration of enterococci in the surface water and sewage samples was determined using the reference method on the day of collection. The enterococci concentrations in the surface water or sewage samples were determined to subsequently prepare 39 artificially contaminated drinking water samples with a target concentration of approximately 5 CFU enterococci per 100 ml. Analyses were performed on the 39 artificially spiked drinking water samples and the 42 uncontaminated drinking water samples. An overview of the drinking water samples, surface water samples, sewage samples and details on the spiking of the drinking water samples is present in the SI (Table S4-6). The formulas described in ISO 16140-2:2016 (ISO-Standard 2016), were used to calculate the sensitivity of the alternative method and of the reference method and the relative trueness. In these formulas, PA is the Positive Agreement (both methods yield a positive result), NA is the Negative Agreement (both methods yield a negative result), PD is the Positive Deviation (alternative method yields positive result, while the reference method yields a negative result) and ND is the Negative Deviation (alternative method yields a negative result while the reference method yields a positive result).

Sensitivity for the alternative (RT-PCR) method:

Sensitivity for the reference method:

Relative trueness/sensitivity

The false positive ratio could not be determined since confirmation of results was not possible with the use of the alternative method.

Determination of the RLOD

Analyses were carried out with the alternative and reference method on drinking water samples that were artificially contaminated with enterococci to determine the relative detection level.

To determine the RLOD, enterococci were added at four contamination levels, ranging from 0 CFU/100 ml to 5 CFU/100 ml, to (unchlorinated) drinking water from the city of Nieuwegein (Utrecht, The Netherlands).

The RT-PCR method is designed to detect at least ten different enterococci species using the mixture of four primer pairs (SI, Table S2). This can potentially result in different RLOD's for the different enterococci species targeted by the primer sets. However, due to the high similarity between the 16S rRNA genes of the target enterococci species and the RT-PCR target sequences, it is expected that the RLOD is primarily influenced by the primer pair used for amplification. Therefore, the RLOD is determined by using one *Enterococcus* species for each of the four primer pairs (SI, Table S3). Commercially available, accurately quantified, bacterial reference suspensions (Vitroids, Sigma Aldrich) were used to make samples contaminated with *E. faecium* and *E. faecalis*. The number of culturable enterococci in the vitroids batches was confirmed by the analyses of 4 vials from every used Vitroids batch. The average concentration of these 4 vials was used to make water samples supplemented with accurately quantified *E. faecium* and *E. faecalis*. There are no Vitroids or other quantified reference materials available containing *E. casseliflavus* or *E. moraviensis*. For these species, fresh cultures were grown overnight in non-selective liquid Lab-Lemco broth. The concentration in these cultures was determined (in 4-fold) using the reference method. The average concentration was used to contaminate drinking water with known contamination levels. Large volume batches were prepared at 4 contamination levels and multiple analyses were performed on 100 ml portions with the alternative method and the reference method on the same day the batches were prepared.

Four contamination levels and replications were applied and analysed:

1st level: no enterococci, 5 samples

2nd level: 0.7 CFU/100 ml, 20 samples

3rd level: 2 CFU/100 ml, 10 samples

4th level: 5 CFU/100 ml, 5 samples

Inclusivity

The inclusivity of the RT-PCR method was tested on a range of well-characterized bacterial strains belonging to the ten targeted enterococci species. At least five strains were selected for each of the ten species, from internationally recognized culture collections (ATCC or DSMZ) and strains isolated by the collaborating drinking water laboratories. The strains from the drinking water laboratories have been isolated from water samples (ground-, drinking-, or surface water) or faecal samples obtained from animals living in the vicinity of abstraction wells in the dune area in the Netherlands and identified using MALDI-TOF-MS (Taučer-Kapteijn *et al.* 2017). The results of species identification using MALDI-TOF-MS were subsequently confirmed by sequencing of the 16S rRNA gene for all used strains from seven species (*E. faecalis*, *E. faecium*, *E. durans*, *E. hirae*, *E. mundtii*, *E. gallinarum*, and *E. avium*). Some strains identified as *E. casseliflavus*, *E. moraviensis*, or *E. haemoperoides* by the MALDI-TOF-MS were identified as different, but closely related, *Enterococcus* species using 16S rRNA sequencing (using primers 785F and 907R). The minor differences between the 16S rRNA genes of *E. moraviensis* and *E. gallinarum*, which make it difficult to discriminate these species, as previously described (Ryu *et al.* 2012). Some strains identified as *E. moraviensis* or *E. haemoper-*

oxidus with MALDI-TOF-MS were identified as *E. silesiacus* by 16S gene sequencing. This can be explained by the close genetic relationship between these species (Švec *et al.* 2006). The 55 strains used for this inclusivity study are listed in the SI (Table S7).

The RT-PCR analyses were conducted in duplicate on RNA, of which the concentration was normalized to a concentration equivalent to 1–20 CFU enterococci. Colonies of the bacterial strains were grown on S&B agar for 48 h. A homogeneous cell suspension was created from a freshly isolated colony in sterile PBS (1 mM KH₂PO₄, 155 mM NaCl, 3 mM Na₂HPO₄, pH 7.4). The quantity of cells in the suspension was estimated by flow cytometry using SYBR green staining of the cells. Based on these estimated cell counts, dilutions were made (in PBS) to a concentration of 20–100 cells/100 µl. Volumes of 100 µl were analysed in duplicate on S&B agar (in duplicate) to determine the concentration of culturable cells in the suspensions. A volume of 100 µl of this suspension was used to isolate RNA.

Exclusivity

The exclusivity of the RT-PCR method was tested on a collection of 33 bacterial strains. This collection consisted of bacterial species that can be present in water, bacterial species related to enterococci such as *Streptococcus*, *Staphylococcus*, and *Aerococcus*, and three *Enterococcus* species outside of the ten selected target species. The collection of species tested is given in the SI (Table S8). The real-time RT-PCR analyses were conducted on RNA isolated from bacterial suspensions containing 73–660 CFU (average 250 CFU). Colonies of most bacterial species were grown on LabLemco agar (LLA) medium for a maximum of 48 h. *Legionella* species were cultured on Buffered Charcoal Yeast Extract (BCYE) medium for 72 h. A homogeneous cell suspension was created from a freshly isolated colony in sterile PBS. The quantity of cells in the suspension was estimated by flow cytometry using SYBR green staining of the cells. Based on these cell counts, dilutions were made (in sterile PBS) to a concentration of 200 cells/100 µl. Volumes of 100 µl were analysed in duplicate on LLA (or BCYE for *Legionella*) plates (in duplicate) to determine the concentration of culturable cells in the suspensions. A volume of 100 µl of this suspension was used to isolate RNA. RT-PCR reactions were performed in duplicate on RNA isolated from suspensions containing 73–660 CFU.

Interlaboratory study

All Dutch drinking water laboratories and two drinking water laboratories in Belgium participated in the interlaboratory study organized by KWR. Due to logistical reasons, it was not possible for the participating laboratories to process 24 samples in one interlaboratory study, as described in ISO 16140–2:2016. Therefore, the required number of samples to be tested in this collaboration study was reached by organizing two interlaboratory studies, offering a total of 24 samples for every collaborator. For every interlaboratory study, homogenous samples were freshly prepared in large batches. Vitroids® (Sigma-Aldrich containing *Enterococcus faecalis* WDCM 00009) were used to contaminate drinking water, originating from the city of Nieuwegein, at predefined concentrations. Individual samples with volumes of 250 ml were aliquoted from the batches and sent to the participating laboratories on the same day in a cooling box partially filled with ice. For every interlaboratory study the mean *Enterococcus faecalis* concentration in

the prepared sample batches was confirmed using the reference method by KWR. The first interlaboratory study consisted of samples containing 0 ($n = 4$) and 5 CFU/100 ml ($n = 8$), while the second study consisted of samples containing 0 ($n = 4$) and 1 CFU/100 ml ($n = 8$) enterococci in drinking water.

Samples were received by the collaborators within 24 h after preparation and processed within 6 h after delivery. The participating laboratories of WLN, HWL, AQZ, Vitens, and De Watergroep deployed two individual laboratory technicians to perform the analysis of the interlaboratory studies independently to reach the 10 participants required for the interlaboratory study. KWR and Pidpa deployed one technician, which led to the generation of 12 datasets. In case of two collaborators from one laboratory, the processing and analysis occurred by two separate technicians on two independent sets of samples. The collaborator from KWR was not involved in the preparation of the samples. Every collaborator performed both the reference method and the alternative method on all samples.

The specificity and sensitivity for both the reference and the alternative method, as well as the relative sensitivity, were determined using the formulas described in ISO 16140–2:2016 (ISO-Standard 2016).

Application in practice

The RT-PCR method was applied in drinking water practice and compared to the reference method, in two situations in which rapid assessment of the absence or presence of enterococci was relevant:

1. After drinking water distribution pipe repair or replacement, where the water utility wanted rapid evaluation of the hygienic quality of the works to determine if the pipe could be put back in service safely. A total of 1842 samples were collected between 2020 and 2023 in the Netherlands, where no residual chlorine is present in the network). These samples were analysed by the drinking water laboratories of Vitens (Leeuwarden, The Netherlands), Het Waterlaboratorium (Haarlem, The Netherlands), WLN (Glimmen, The Netherlands), and AQZ (Werkendam, The Netherlands).
2. Drinking water samples taken as follow-up of a positive enterococci detection by culture from fire hydrants in the laboratory of De Watergroep (Brussels, Belgium, June–September 2023) or a faecal contamination event in a drinking water distribution network in the laboratory of PIDPA (Antwerpen, Belgium, September 2022).

These comparisons in practice were analysed by describing the culturable $\pm$ and RT-PCR $\pm$ of the samples in the two-by-two matrix.

Results

Method comparison study

Sensitivity

The sensitivity was assessed with 42 distributed drinking water samples (SI, Table S4). A subsample of 39 of these drinking water samples was contaminated with surface or sewage water to contain enterococci at an average concentration of 4.3 CFU/100

Table 3 Overview of data obtained with reference and alternative analyses of all samples, partly contaminated with surface water.

	Reference method positive (R+)	Reference method negative (R-)
Alternative method positive (A+)	+/+ Positive agreement (PA) 31	-/+ Positive deviation (PD) 4
Alternative method negative (A-)	± Negative deviation (ND) 2	-/- Negative agreement (NA) 44

Table 4 Relative level of detection calculations for the four *Enterococcus* species (RLOD: relative level of detection; RLODL and RLODU: lower and upper 95% confidence levels of the RLOD; ln: natural logarithm; sd: standard deviation).

Name	RLOD	RLODL	RLODU	b = ln (RLOD)	sd(b)	z-test statistic	P-value
95% confidence							
<i>E. casseliflavus</i>	1.64	0.84	3.22	0.50	0.34	1.47	0.14
<i>E. moraviensis</i>	0.59	0.28	1.25	-0.52	0.37	1.40	1.84
<i>E. faecium</i>	0.41	0.20	0.82	-0.90	0.35	2.56	1.99
<i>E. faecalis</i>	1.07	0.53	2.16	0.07	0.35	0.19	0.85
Combined	0.85	0.60	1.21	-0.16	0.74	0.93	1.65

ml (range: 0–14 CFU/100 ml) (SI, Table S5). A detailed overview of the results is shown in the SI (Table S9). The summarizing two-by-two matrix with the outcomes of the sensitivity study is shown in Table 3.

Based on these outcomes, the sensitivity for the reference and alternative method was calculated, as well as the relative true-ness/sensitivity using the formulas from ISO 16140-2:2016:

Sensitivity for the RT-PCR method:

Sensitivity for the reference method:

Relative trueness/sensitivity

There were three positive results observed using the alternative method in the 42 uncontaminated drinking water samples (7.1%).

The acceptability limit for an unpaired study consisting of 1 category is: the observed value is: the acceptability limit has not been exceeded by the observed value, demonstrating that the sensitivity of the RT-PCR method meets the criterion of ISO 16140-2 (2016).

RLOD

The results of the detection of enterococci in the drinking water samples that were spiked with low levels of the four *Enterococcus* species are given in the SI (Table S9-12). These results were used to calculate the RLOD using the program RLOD_MCS_clause_5-1-4-2_V3_2015-08-15 as published on the ISO website (https://standards.iso.org/iso/16140/-5/ed-1/en/RLOD_MCS_clause_5-1-4-2_V3_2015-08-15.xlsm). The calculated RLOD values are given in Table 4.

The RLOD for detection of *E. faecium* as well as the upper 95% confidence limit is below 1.0, which means that this species can be detected with somewhat higher sensitivity using the RT-PCR in comparison with the reference culture method. The RLOD's for detection of *E. casseliflavus*, *E. moraviensis*, and *E. faecalis* do not significantly differ from 1.0, suggesting that the sensitivity of detection of these *Enterococcus* species is comparable to the reference method. The combined RLOD for all four tested species is moderately below 1.0 (0.851), suggesting that the RLOD for the RT-PCR

is comparable to the reference method. The acceptability limit for this unpaired dataset is: 2.5. All four RLOD's meet this criterion.

Inclusivity and exclusivity

The inclusivity was tested on a collection of 55 *Enterococcus* strains consisting of at least five strains for each of the ten selected species. All 55 strains of the target *Enterococcus* species were detected with the RT-PCR method (SI, Table S13). The results of the exclusivity tests (SI, Table S14) show that no detection of the other bacterial genera was observed. RNA from closely related genera, such as *Streptococcus*, *Aerococcus*, and *Staphylococcus* did not yield a positive result. RNA from the *Enterococcus* species *E. malodoratus* and *E. villorum* did also not yield a positive result using the RT-PCR assay. Based on the 16S rRNA sequence of *E. malodoratus* and *E. villorum*, it was predicted (*in silico*) that the primer sequences would not bind to the 16S rRNA sequence of these enterococci species, making it unlikely that these species produce a RT-PCR signal. The exclusivity study shows that *E. termitis* is an *Enterococcus* species that can be detected using this RT-PCR assay. The detection of *E. termitis* was confirmed *in silico* based on its 16S rRNA sequence, which shares 99% similarity with the 16S rRNA sequences of *E. moraviensis* and *E. haemoperoxidus*, including identical sequences at the primer binding sites.

Interlaboratory study

An interlaboratory study was performed with 12 collaborators from seven different laboratories, in which 24 drinking water samples were analysed by every collaborator. These samples were either not contaminated (0 CFU/100 ml) or contaminated at two contamination levels (1 and 5 CFU/100 ml), yielding eight samples at every level. The results and the data analysis as prescribed in ISO16140 are summarized in Table 5. Data from samples 13–24 analysed by participant 5 was not available due to a technical malfunction.

Four samples (4.3%) were found positive using RT-PCR at contamination level 0 CFU/100 ml, whereas all samples were negative at this level with the reference method, resulting in a specificity of 95.7% for RT-PCR and 100% for the culture method. At all

Table 5 Number of positive results of the analyses of 24 samples by 12 collaborators using the reference and rt-pcR method.

	L_1	L_2	L_3
Set level organising lab	0 CFU 100 ml ⁻¹	1 CFU 100 ml ⁻¹	5 CFU 100 ml ⁻¹
Mean level participants	0 CFU 100 ml ⁻¹	1.3 CFU 100 ml ⁻¹	4.2 CFU 100 ml ⁻¹
(P+) _{ref}	0.0% (0/92)	59.1% (52/88)	100% (96/96)
(P+) _{alt}	4.3% (4/92)	63.6% (56/88)	99.0% (95/96)
AL	3	11	2
ND – PD =	-4	-5	0
	Specificity		
SP _{ref}		100%	
SP _{alt}		95.7%	
	Sensitivity		
SE _{ref}		68.9%	
SE _{alt}		75.6%	

L_1 , L_2 , and L_3 : contamination levels as determined by the reference method.

(P+)_{ref}: percentage positive reactions found by collaborators with the reference method.

(P+)_{alt}: percentage positive reactions found by collaborators with the alternative method.

AL: acceptability limit.

ND-PD: Negative deviation—Positive Deviation (should be < AL).

SE_{alt}: Sensitivity for the alternative method.

SE_{ref}: Sensitivity for the reference method.

contamination levels, the ND-PD was lower than the acceptability limit, confirming that results in this interlaboratory study met this criterion. It should be noted that the number of positive deviations (PD) is larger than the number of negative deviations (ND) at L_1 (0 CFU/100 ml) and L_2 (1 CFU/100 ml), indicating a lower detection limit for the alternative method, which is in agreement with the RLOD results. The results at level L_3 (5 CFU/100 ml) show that all samples were positive with the culture method and one sample was negative using RT-PCR (100% culture and 99.0% RT-PCR). At contamination level L_2 (1 CFU/100 ml), the use of the alternative method resulted in a slightly higher percentage of positive samples (63.6%) than with the use of the reference method (59.1%). This demonstrates that low concentrations of enterococci are detected more frequently with the RT-PCR method than with the use of the culture method.

Application in drinking water practice

The application of the RT-PCR method in practical situations was demonstrated in two case studies. The first case was a method comparison study comparing the results obtained using RT-PCR and culture on samples from different drinking water companies taken to evaluate the microbiological safety of the drinking water after mains repair. In the second case study, follow-up samples were taken after a faecal contamination incident was indicated by the detection of enterococci by culture.

Case study 1: method comparison on samples after mains repair

A method comparison study was performed on drinking water samples ($n = 1842$) from distribution networks, taken after pipe repairs. These situations called for rapid availability of information about the microbiological safety, to determine if there was a health risk to drinking water users or if the repair was performed hygienically and the water was safe to drink. These samples were analysed using both RT-PCR and culture (Table 6).

This comparison study demonstrates that results obtained by the RT-PCR method in these drinking water samples taken after mains repair agree with the results obtained with the culture method, in 91.8% of samples in all laboratories (range: 86.7%–93.4%). There were 10 samples (0.5%) where enterococci were detected with the culture method and not detected by RT-PCR. The concentrations of culturable enterococci were 1 CFU/100 ml in 9 of these samples, so the discrepancy could be attributable to the Poisson probability of 1 *Enterococcus* bacterium being present or absent in the 100 ml that was analysed by either method (Haas and Heller 1988, Koyama *et al.* 2016). MALDI-TOF typing of each of these *Enterococcus* colonies confirmed they were one of the ten *Enterococcus* species for which the RT-PCR method was developed. In 7.7% of the samples, RT-PCR indicated the presence of enterococci, while the culture method did not yield any enterococci. This suggests either a higher sensitivity for RT-PCR or the detection of non-culturable enterococci with the RT-PCR method. A comparable fraction of drinking water samples (7.1%) was also culture-/RT-PCR + in the sensitivity study, in accordance with these results from the application in practice.

Case study 2: Method comparison on follow-up samples after a suspected contamination event

One water utility detected intestinal enterococci by culture in a 100 ml water sample from a distribution network (taken at a household faucet) during routine water quality monitoring. They took follow-up samples from the same and neighbouring households and analysed these with both the reference culture method and the RT-PCR method for enterococci. Another water utility detected intestinal enterococci by culture in a 100 ml sample taken from a fire hydrant and followed this up with sampling the same fire hydrant and analyse these 100 ml samples with both the culture and RT-PCR method for enterococci. As expected, in these situations where the presence of culturable intestinal enterococci had been established with the reference culture method (Table 7),

Table 6 Number (and percentage) of drinking water samples taken after mains repair in which enterococci were detected with culture and/or RT-PCR.

Laboratory	Lab 1	Lab 2	Lab 3	Lab 4	Total
Culture pos./RT-PCR pos.	1 (0.1%)	3 (0.9%)	3 (1.5%)	3 (1.7%)	10 (0.5%)
Culture neg./RT-PCR neg.	1046 (93.3%)	301 (85.8%)	178 (89.9%)	156 (90.7%)	1681 (91.3%)
Culture neg./RT-PCR pos.	71 (6.3%)	46 (13.1%)	15 (7.6%)	9 (5.2%)	141 (7.7%)
Culture pos./RT-PCR neg.	3 ^a (0.3%)	1 ^a (0.3%)	2 ^a (1.0%)	4 ^a (2.3%)	10 (0.5%)
Culture/RT-PCR agreement	1047 (93.4%)	304 (86.7%)	181 (91.4%)	159 (92.4%)	1691 (91.8%)
Total samples (n)	1121	351	198	172	1842

^aIn the culture positive/RT-PCR negative samples, 1 CFU was found by culture in each of these samples. MALDI-TOF typing showed that each of these isolates was one of the 10 *Enterococcus* species for which the RT-PCR was developed.

Table 7 Number (and percentage) of follow-up drinking water samples taken in response to enterococci detection in a drinking water distribution network, in which enterococci were detected with culture and/or RT-PCR.

Laboratory	Utility 1	Utility 2	Total
Culture pos./RT-PCR pos.	25 (34%)	12 (50%)	37 (38%)
Culture neg./RT-PCR neg.	21 (28%)	3 (13%)	24 (24%)
Culture neg./RT-PCR pos.	26 (35%)	6 (25%)	32 (33%)
Culture pos./RT-PCR neg.	2* (3%)	3* (13%)	5 (5%)
Culture/RT-PCR agreement	46 (64%)	15 (63%)	61 (62%)
Total samples (n)	74	24	98
*concentration (CFU 100 ml ⁻¹)	1, 12	1, 1, 2	

the percentage of positive samples is much higher than in the samples after repair, both with culture and with RT-PCR (Table 6). Enterococci were detected in 78%–87% of the follow-up samples, either by culture, by RT-PCR or both. There was agreement between the result of the RT-PCR and the reference method in 62%–64% of the samples. Many more samples (33%) were positive with RT-PCR whilst negative with the culture method than in the repair samples. The 5% culture+/RT-PCR- samples contained low numbers of enterococci (1–2 CFU per 100 ml), except for one sample that contained 12 CFU per 100 ml. While the Poisson probability of low concentrations described above can explain the 5% culture+/RT-PCR—results, it cannot for the 12 CFU per 100 ml and it is currently not clear why the RT-PCR did not detect the enterococci in this sample.

Discussion

The study describes the validation of a new method for the rapid detection of intestinal enterococci using real-time RT-PCR detecting *Enterococcus*-specific 16S rRNA of ten *Enterococcus* species that were selected based on their presence in human and animal faecal samples and drinking water samples in the Netherlands and Belgium. The validation study used the ISO 16140-2:2016 methodology (ISO-Standard 2016). The main conclusion of this study is that the alternative RT-PCR method is comparable to the reference culture method (ISO 7899-2:2000). The validation data (sensitivity, RLOD, interlaboratory data) as well as the data from application in practice indicate that the sensitivity of detecting intestinal enterococci in drinking water samples with the RT-PCR method is, at least, the same as with the culture method. The positive extraction and RT-PCR control that was used in this study also did not

indicate extraction, RT or PCR inhibition. The application of this RT-PCR method in surface waters suffered from inhibition of the PCR (data not shown). This underscores the need for an internal control to monitor potential inhibition in the steps of the procedure from sample to result. An internal control of *Thermus aquaticus* cells added to the sample and a specific 16S rRNA RT-PCR is currently under evaluation by the collaborating laboratories. The results of the validation study did not indicate inhibition played a role in drinking water samples, so the current RT-PCR procedure is applicable to drinking water, but application to surface water may need further optimization of extraction procedures to remove inhibitors and/or the usage of alternative inhibitor-resistant reagents.

The RLOD was established for a representative species for each of the primer/probe sets: *E. casseliflavus*, *E. moraviensis*, and *E. faecalis*, and was not significantly different from 1.0. The RLOD for *E. faecium* was significantly lower than 1 (0.4), suggesting that this species can be detected with higher sensitivity than the other tested species. The observed RLOD met the threshold in the ISO16140-2:2016, and indicate that the alternative method is likely to detect intestinal enterococci with comparable or higher sensitivity than the reference method.

The inclusivity/exclusivity test on 58 enterococci strains and on 30 non-enterococci strains showed all targeted *Enterococcus* species were detected. From the three *Enterococcus* species that do not belong to the 10 selected species, only *Enterococcus termitis*, a species closely related to *Enterococcus moraviensis*, was detected. *Enterococcus termitis* is also observed sporadically in distributed drinking water (Table 2) and in faecal samples from geese (Table 1) making it beneficial that also this species is detected in the RT-PCR. The non-enterococci strains, including strains from

bacterial genera closely related to *Enterococcus* (*Enterobacter*, *Staphylococcus*, and *Streptococcus*), yielded no RT-PCR signals.

There are more *Enterococcus* species reported in than the ten species that are selected for the RT-PCR. The ten species that were selected for the development of the *Enterococcus* RT-PCR in drinking water were the species that were most abundant/prevalent in the local context of the Netherlands and Belgium, both in drinking water samples and in human and mammalian or avian faeces, using the reference culture method (ISO7899) and are included in the MALDI-TOF reference database. Extrapolation to other settings requires knowledge about the prevalence of *Enterococcus* species. Nevertheless, studies from different settings have shown the same species are prevalent in humans and (farm) animals in Japan (Tamai and Suzuki 2023), wild birds from Poland (Stępień-Pyśniak *et al.* 2017), and faecally contaminated seawater of beaches in Brazil (Christ *et al.* 2017). The Polish study also showed 100% agreement between MALDI-TOF species assignment and sequencing of the *rpoA* gene. Similar high agreement was found in Dutch drinking water samples (Taučer-Kapteijn *et al.* 2013). In the Brazilian study, 16S rRNA gene sequencing yielded a different *Enterococcus* species than the MALDI-TOF in 7/40 isolates. Also, with clinical *Enterococcus faecium* and *E. faecalis* isolates, the MALDI-TOF misidentified 12/16 *E. faecalis* isolates for *E. faecium*, as established by whole genome sequencing (Garza-González *et al.* 2020). Species assignment via the MALDI-TOF for *Enterococcus* species isolated from dairy samples (*E. faecalis* ($n = 9$), *E. faecium* ($n = 5$), *E. durans* ($n = 6$), *E. hirae* ($n = 18$) and *E. gallinarum* ($n = 1$)) was identical to 16S rRNA gene sequencing for 38 of the 39 isolates (97%) (Kim *et al.* 2023). The reported differences in species identification between MALDI-TOF and genome sequencing were between closely related species and did not yield other *Enterococcus* species than the ten species that were selected for the RT-PCR development, so we feel confident that the selected primer/probe sets are robust. Obviously, the RT-PCR design is biased towards *Enterococcus* species/strains that are culturable by the reference method but may also detect enterococci that are not or difficult to culture using the reference method.

Overall, the application of both the RT-PCR method and the reference method by the drinking water utilities, for samples taken after drinking water mains repairs, showed that both methods yield the same results in 91.8% of the 1842 samples. In only a small fraction (0.5%) of these samples, intestinal enterococci were only detected with culture and not with RT-PCR. These discrepancies could be attributable the likelihood of an *Enterococcus* bacterium being present in the 100 ml sample at very low (around 1) concentrations of intestinal enterococci in most of these 100 ml samples. The reverse, detection of intestinal enterococci with RT-PCR and not with culture was much more prevalent in these samples (7.7% samples). A comparable percentage (7.1%) was found in the sensitivity study where 42 random drinking water samples were analysed. And also the uncontaminated samples for the interlaboratory study yielded 4.3% positives with the RT-PCR method, but none with the culture method. The sensitivity of the RT-PCR method was comparable to the culture method, except for *E. faecium*, where the RT-PCR method was slightly more sensitive (RLOD = 0.4). This is not so much more sensitive that it could explain the 7%–8% RT-PCR positive/culture-negative samples. Our results show the specificity of the RT-PCR method is very high, rendering the likelihood that the RT-PCR would yield false-positive signals as a result of other microbial DNA cross-reacting with the

primer/probe sets unlikely. To exclude this, the whole of the 16S rRNA gene should be sequenced, but the concentration of target RNA in our samples is generally too low for 16S rRNA gene sequencing. The RT-PCR method could detect *Enterococcus* species in drinking water samples that do not grow (well) on Slanetz and Bartley agar or are not confirmed by the MALDI-TOF method. The PCR products were considered not to contain sufficient sequence variability for species differentiation, so sequencing was not performed. Further studies on samples that are RT-PCR positive and culture negative are needed to determine the origin of the RT-PCR positive signal. The RT-PCR method could also detect *Enterococcus* species or strains that are viable, but non-culturable in the reference method. As we did not find this discrepancy for *E. coli* in similar samples (Heijnen *et al.* 2024), and *E. coli* in these drinking water samples would come from similar contamination sources as enterococci, we find this hypothesis less likely. Also, all these samples were from non-chlorinated drinking water systems, so the discrepancies cannot be attributed to loss of culturability, but not RT-PCR signal, by chlorine oxidation (Heijnen *et al.* 2024). For the samples in case study 2, these were from locations where enterococci were already detected using the culture method and re-sampling was performed immediately after enterococci detection. This resulted in a high percentage of positive samples with both methods. Also, residual chlorine was present in the distribution network where these samples were taken, which may at least partially, explain the higher percentage of RT-PCR positives (70.5%) than culture positives (42.9%) in these samples. Overall, the more frequent detection of enterococci in mains repair samples with the RT-PCR method than with the reference method will result in more frequent non-compliance of drinking water after repairs. But in 91.8% of the repairs, the RT-PCR will be able to rapidly confirm that the repair works were conducted hygienically, and the drinking water is safe and water supply can be restored. This does benefit drinking water consumers and water utilities alike, as repairs do not need to result in prolonged periods of cut-off from normal water supply or boil water advisories, roadblocks can be removed, and repair crews can be devoted to new activities. In situations where drinking water gets contaminated, the use of RT-PCR can provide information to issue public warnings and initiate corrective actions much quicker than the reference method, improving public health protection of drinking water supply. If the 7%–8% RT-PCR positive/culture negative samples after repair are the result of detection of viable, non-culturable enterococci or enterococci that do not grow well on culture media, and the RT-PCR detects a situation that is associated with a public health risk, while the culture method does not, is subject to further investigation.

While this study was done mostly in networks and repair situations without residual disinfectant present in the drinking water, rapid methods for faecal indicator bacteria are beneficial also in networks with residual disinfectant. Rapid detection of breaches in network integrity and loss of disinfectant residual may help to prevent or limit outbreaks. Also, rapid methods would allow water utilities to rapidly confirm the hygienic quality of drinking water after repair works, or after corrective actions have been taken as response to a contamination event, also in the presence of a disinfectant residual. Therefore, rapid methods provide advantages in drinking water (network) monitoring, regardless of the use of disinfectant residuals.

The RT-PCR method for the ten selected *Enterococcus* species was validated according to ISO 16140-2:2016, against ISO 7899–

2 as the reference culture method from the EU Drinking Water Directive. The alternative and reference methods were evaluated as qualitative methods in this validation study. The criterion in the EU Drinking Water Directive for enterococci is absence in 100 ml. Detection of intestinal enterococci in 100 ml of drinking water means non-compliance, regardless of the amount of enterococci detected. The results of this validation study were used to obtain formal approval from the Netherlands Drinking Water Inspectorate for the use of the RT-PCR method as alternative method for drinking water monitoring. Water utilities now have the possibility to harness the short time-to-result of the RT-PCR method in situations where a rapid result is beneficial for public health protection and for consumer comfort and utility efficiency. The collaborating water utilities are continuing to deploy the RT-PCR in such situations and document the collective experiences with the application of the RT-PCR method for enterococci and for *E. coli* (Heijnen *et al.* 2024).

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Author contributions

Leo Heijnen (Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Visualization, Writing – original draft), Eline Stroobach (Data curation, Investigation, Methodology, Writing – review & editing), Adrie Atsma (Data curation, Investigation, Methodology, Writing – review & editing), Gabi van Pelt (Data curation, Investigation, Methodology, Writing – review & editing), Hendrik Jan de Vries (Data curation, Investigation, Methodology, Writing – review & editing), Katrien De Maeyer (Data curation, Investigation, Methodology, Writing – review & editing), Bea Timmermans (Data curation, Investigation, Methodology, Writing – review & editing), Liesbeth Visser (Data curation, Investigation, Methodology, Writing – review & editing), Maja Taucer-Kapteijn (Conceptualization, Methodology, Writing – review & editing), Gertjan Medema (Validation, Writing – original draft)

Supplementary material

Supplementary material is available at *Journal of Applied Microbiology* online.

Conflicts of interest

None declared.

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Data availability

Most data underlying this article are available in the article and in its online Supplementary material. Raw data underlying this article will be shared on reasonable request to the corresponding author. Raw data from the water laboratories participating in this research will be shared on request to the corresponding author with permission of the water laboratories.

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