Joint Research Programme
BTO 2024.028 | Februari 2024

Validation of a rapid RT-PCR method for intestinal enterococci (10 species) in distributed drinking water

Version 2024

Joint Research Programme

KWR

Bridging Science to Practice

Report

Validation of a rapid RT-PCR method for intestinal enterococci (10 species) in distributed drinking water (Version 2024)

Revision

BTO 2024.028| Februari 2024

This research is part of the Joint Research Programme of KWR, the water utilities and Vewin.

Project number
402045/249

Project manager
Michiel Hootsmans

Client
BTO - Bedrijfsonderzoek

Authors
Leo Heijnen in collaboration with the Expertgroup RT-PCR from the drinking water laboratories: Adrie Atsma, Katrien de Maeyer, Gabi van Pelt, Eline Stroobach, Bea Timmermans, Jerom Vranken and Rik de Vries

Quality Assurance
Gertjan Medema

Sent to
This report is distributed to BTO-participants, it is not a public report.

Keywords

RT-PCR, molecular, DNA, RNA, enterococci, fecal indicator, rapid method, detection

Year of publishing
2024

More information
Leo Heijnen
T +316069743
E leo.heijnen@kwrwater.nl

PO Box 1072
3430 BB Nieuwegein
The Netherlands

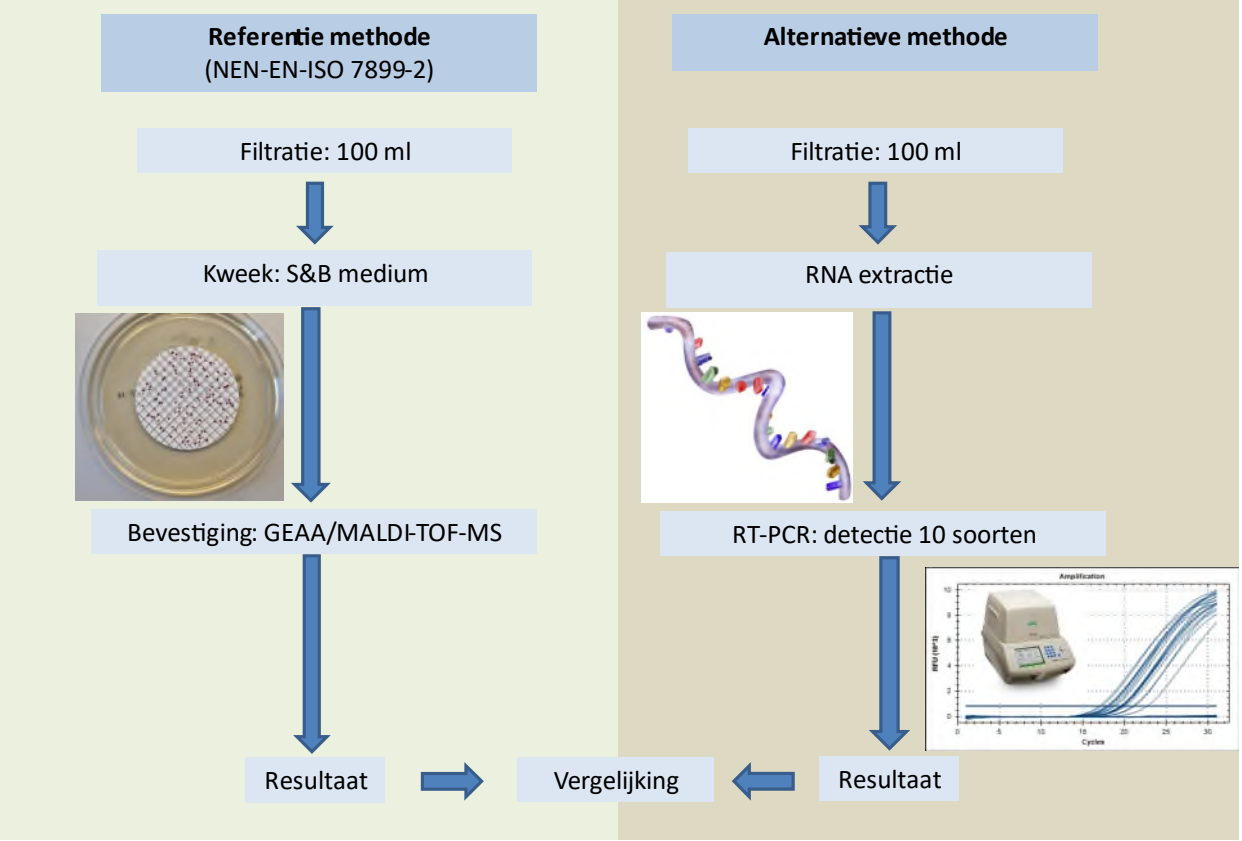
T +31 (0)30 60 69 511
F +31 (0)30 60 61 165
E info@kwrwater.nl
I www.kwrwater.nl

Managementsamenvatting

RT-PCR methode voor 10 soorten intestinale enterococcen gevalideerd en gevoeliger dan kweekmethode

Auteur: Leo Heijnen

Sinds 2018 is het gebruik van de RT-PCR-methode voor detectie van *E. coli* door de Inspectie Leefomgeving en Transport (ILT) toegestaan als snel alternatief voor de kweekmethode. Door ook voor intestinale enterococcen een snelle (en gevalideerde) RT-PCR-methode in te zetten, net als voor het andere fecale indicatororganisme *E. coli*, is het mogelijk om binnen één dag te beoordelen of drinkwater hygiënisch betrouwbaar is. Dit maakt snelle reactie na calamiteiten mogelijk waardoor gezondheidsrisico's en overlast voor de consument (kookadvies) worden beperkt. Deze methode is in 2019 ontwikkeld. Na een traject van optimalisatie van de procedure en aanpassing van de uitvoering van de gevoeligheidsstudie voldoet de methode aan ISO16140-2:2016 en blijkt uit een vergelijkingsstudie met de kweekmethode dat met de RT-PCR-methode selectieve detectie van intestinale enterococcen mogelijk is. De resultaten verkregen met RT-PCR zijn zeer vergelijkbaar met de resultaten verkregen met de standaard kweekmethode. Vanwege hogere gevoeligheid van RT-PCR en het detecteren van enterococcen die niet (meer) kweekbaar zijn worden deze indicatorbacteriën met RT-PCR frequenter aangetoond. Dit rapport is een revisie van het eerder verschenen rapport (BTO 2021.005) waarin de resultaten zijn uitgebreid met de vergelijkingsstudie en waarin de resultaten van de onderdelen "interlaboratorium studie" en de "gevoeligheidsstudie" zijn vervangen door nieuwe resultaten. In vervolgstappen zal worden gestreefd naar acceptatie van deze RT-PCR methode als snel alternatief voor de standaard kweekmethode door Inspectie Leefomgeving en Transport (ILT).



Schematische weergave van de opzet van het onderzoek

Belang: enterococcen snel aantonen voor hygiënische betrouwbaarheid drinkwater

Voor het vaststellen van de hygiënische betrouwbaarheid van drinkwater zijn metingen van de fecale indicatororganismen *E. coli* en intestinale enterococcen wettelijk voorgeschreven. Vooral voor het efficiënt managen van calamiteiten en de hygiënische betrouwbaarheid na ingrepen in het leidingnet is de beschikbaarheid van snelle detectiemethoden, waarmee deze indicatororganismen kunnen worden aangetoond, van belang. Met snelle detectie kunnen gezondheidsrisico's en overlast voor de consument worden beperkt. Voor snelle detectie van *E. coli* is sinds 2018 een snelle en wettelijk geaccepteerde real-time RT-PCR (RT-PCR) methode beschikbaar (KWR 2017.098). Daarna is ook een RT-PCR methode ontwikkeld voor de meest relevante intestinale enterococcen (minimaal 10 soorten, BTO 2019.209(s)), maar die was nog niet gevalideerd.

Aanpak: validatie van een nieuwe methode

In 2020 is in samenwerking met alle Nederlandse drinkwaterlaboratoria en de Vlaamse drinkwaterlaboratoria van De Watergroep en Pidpa validatieonderzoek uitgevoerd volgens NEN-EN-ISO16140-2: 2016. De methode bleek echter op de onderdelen "interlaboratorium studie" en de "gevoeligheidsstudie" niet volledig aan de criteria te voldoen (BTO 2021.005). Na een traject van optimalisatie van de procedure en aanpassing van de uitvoering van de gevoeligheidsstudie zijn deze onderdelen herhaald. Daarnaast is een vergelijkingsstudie uitgevoerd waarbinnen de drinkwaterlaboratoria de RT-PCR methode hebben toegepast op reguliere drinkwatermonsters en de resultaten hebben vergeleken met de standaard kweekmethode.

Resultaten: RT-PCR voor intestinale enterococcen kan kweekmethode vervangen

Bij dit onderzoek voldeed de RT-PCR methode voor enterococcen aan alle criteria van ISO16140-2: 2016. De vergelijkingsstudie liet zien dat met kweek volgens NEN-EN-ISO7899-1 en RT-PCR vergelijkbare resultaten worden verkregen in het overgrote deel (91,8%) van de onderzochte praktijkmonsters. Watermonsters waarin met kweek wel intestinale enterococcen worden aangetoond en met RT-PCR niet zijn zeer zeldzaam (0,5%). Het komt vaker voor dat monsters alleen in de RT-PCR intestinale enterococcen laten zien (7,7%). De oorzaak hiervoor is waarschijnlijk dat de RT-PCR gevoeliger is en/of ook enterococcen detecteert die niet (meer) kweekbaar zijn.

Toepassing: methode is beschikbaar en geïmplementeerd

Dit onderzoek heeft gezorgd voor validatie en implementatie van een snelle methode voor detectie van intestinale enterococcen bij de deelnemende drinkwaterlaboratoria. Vanwege de beperkte verschillen tussen RT-PCR en kweek zijn de experts van deze werkgroep van mening dat deze methode ingezet kan worden voor snelle screening van Enterococcen. Met name in situaties waarbij een snelle uitspraak voor aanwezigheid van deze indicator organismen wenselijk is, zoals bij calamiteiten en reparatiewerkzaamheden in de drinkwaterdistributie.

Rapport

Dit onderzoek is beschreven in het rapport *Validation of a rapid RT-PCR method for intestinal enterococci (10 species) in distributed drinking water (Revision)* (BTO-BTO 2023.073). Dit rapport is een revisie van het eerder verschenen rapport (BTO 2021.005) waarin de resultaten zijn uitgebreid met de vergelijkingsstudie en de resultaten van de onderdelen "interlaboratorium studie" en de "gevoeligheidsstudie" zijn vervangen door nieuwe resultaten.

Meer informatie

- KWR 2017.098: Validation of an RT-PCR method for rapid detection of E. coli in distributed drinking water
- BTO 2019.209(s): Ontwikkeling van een RT-PCR voor snelle detectie van enterococcen
- BTO 2021.005: "Validation of a real-time RT-PCR method for rapid detection of 10 intestinal enterococci species in distributed drinking water

Contents

1 Preface

This report is a partial revision of the report previously published in 2021 “Validation of a real-time RT-PCR method for rapid detection of 10 intestinal enterococci species in distributed drinking water (BTO 2021.005)”. This previous report described the validation of an RT-PCR method for the detection of 10 intestinal enterococci species according to ISO 16140-2:2016. The validation study at that time did not meet the criteria of the following two validation components described in: the sensitivity study and the interlaboratory study.

The sensitivity study in 2021 was performed using artificially contaminated drinking water samples prepared by mixing surface water from different locations with drinking water. However, some of these mixed samples caused inhibition of RT-PCR reactions (Heijnen 2021). Inhibition of RT-PCR means that the RT-PCR is not working efficiently due to presence of PCR inhibiting substances, which results in false negative results, these inhibitors were present from surface water. This is why the sensitivity study was repeated with the use of surface water samples, containing relatively high concentrations of intestinal enterococci, from other locations. These high concentrations minimize the volume of surface water needed to compose artificially contaminated samples and avoid issues with inhibition of RT-PCR reactions. The experiences with inhibition of RT-PCR also emphasized the need to use an internal control to monitor the presence of inhibitors to avoid the generation of false negative results. This is why the drinking water laboratories always use proper controls (spiked samples) to determine the possible presence of inhibitors and an internal control assay was subsequently developed to simplify monitoring of inhibition.
The interlaboratory study was performed in 2021 in a time period where part of the participating laboratories were relatively unexperienced in performing this newly developed RT-PCR assay, resulting in data of insufficient quality. This issue was solved by gaining more laboratory experience in molecular biology techniques and subsequent in-house validation of the method by each participating laboratory before repeating the interlaboratory study.

The sensitivity and interlaboratory studies which were repeated and are reported here. This report is, to a great extent, identical to the previous report (BTO 2021.005) with the exception of the following chapters: Chapter 4.1.2 describing the sensitivity study, Chapter 4.4 where the data of the interlaboratory study has been replaced by more recent data, and Chapter 6 “Discussion, conclusions and recommendations” was modified in accordance with the new data included.

In addition to the validation study, a comparison study between culture and this RT-PCR method was conducted. In this comparison study the RT-PCR was used by the collaborating drinking water laboratories on samples from practical situations after which the results were compared with the standard culture method. The results of this comparison study are summarized in Appendix VII.

The previous version of this report has been reviewed by the RIVM and was subsequently edited to include their remarks, resulting in the current version of this report.

2 Background

Detection of the fecal indicator bacteria *E. coli* and intestinal enterococci plays a key role to determine hygienic safety of distributed drinking water. An alternative rapid method for detection of *E. coli* using "Real-time Reverse Transcription Polymerase Chain Reaction" (RT-PCR) was validated in 2017 (Heijnen 2017) and confirmed to provide results that are equivalent with the results obtained with the culture based reference method. In addition to a rapid method for detection of *E. coli,* a rapid method to detect intestinal enterococci is required to be able to obtain insight in the presence of fecal contamination and therefore the potential presence of fecally transmitted pathogens in (distributed) drinking water. This is why an RT-PCR method was developed for rapid detection of intestinal enterococci for the same purpose. The following steps were taken to select the most relevant intestinal enterococci species that should be included in the newly developed RT-PCR method: 1) literature research, 2) feces research (Taucer-Kapteijn, Hoogenboezem et al. 2017) and 3) an inventory of the intestinal enterococci species observed in drinking water in the Netherlands and Belgium using the regular culture method (according to NEN-EN-ISO7899-2) and species identification using MALDI-TOF-MS. A summary of this inventory was made previously (by Maja Taucer from "Het Waterlaboratorium", currently working at PWN) and is attached to this report in Appendix I. Based on this inventory, a selection of ten relevant intestinal enterococci species was made and collaborative research between "Het Waterlaboratorium" and KWR was performed to develop a new RT-PCR method for rapid detection of at least these ten selected intestinal enterococci species (Heijnen 2019).

Table 1. The ten selected intestinal enterococci species

	Selected species
1	*Enterococcus faecium*
2	*Enterococcus faecalis*
3	*Enterococcus durans*
4	*Enterococcus hirae*
5	*Enterococcus casseliflavus*
6	*Enterococcus mundtii*
7	*Enterococcus gallinarum*
8	*Enterococcus moraviensis*
9	*Enterococcus haemoperoxidus*
10	*Enterococcus avium*

A validation procedure, according to ISO 16140-2:2016, was performed to conclude whether this new RT-PCR provides the results that are comparable with the results of the standard culture method (NEN-EN-ISO7899-2) and therefor can be used as an alternative method. This validation study was performed by KWR (as expert laboratory) in close collaboration with the drinking water laboratories in the Netherlands and Belgium (Table 2). This report summarizes the results previously described (Heijnen 2021) and the results of the sensitivity and interlaboratory study which were replaced with data from newly performed studies.
The current intended use of this rapid method is 1) the analysis of samples after repair or replacement of drinking water distribution pipes and 2) to manage calamities, where this method can be used to rapidly monitor the spread of a contamination and the effect of measures taken.

Table 2. Collaborating laboratories and their affiliated drinking water companies

Drinking water-laboratory (DWL)	Abbreviation	Location	Persons involved	Drinkwater company (DWC)
Aqualab Zuid	AqZ	Werkendam, NL	Gabi van Pelt	Brabant Water Evides WML Limburgs drinkwater
De Watergroep	De Watergroep	Gent, B	Jerom Vranken Bea Timmermans	De Watergroep
Het Waterlaboratorium	HWL	Haarlem, NL	Eline Stroobach	Dunea Waternet PWN
Pidpa	Pidpa	Antwerp, B	Katrien de Maeyer	Pidpa
Vitens laboratorium	Vitens	Leeuwarden, NL	Adrie Atsma	Vitens Oasen
WLN	WLN	Glimmen, NL	Rik de Vries	WMD Drinkwater Waterbedrijf Groningen

3 Scope

The scope of this report is the validation of an alternative molecular method for rapid detection of intestinal enterococci in distributed drinking water, according to ISO16140-2 (Anonymous 2016). The alternative method is based on detection of a fragment of 16S ribosomal RNA from intestinal enterococci using Real time Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). The reference method which is used for this validation is the culture method as described in NEN-EN-ISO 7899-2 (Anonymous 2000). When using the reference culture method for water quality monitoring, the criterion “absence of indicator organisms in 100 ml” is used in the decision-making process; no further use is made of the quantitative information of the sample. Therefore, the alternative and reference methods were evaluated as qualitative methods in this validation study. The aim of this study was to determine the performance characteristics of the RT-PCR for detection of intestinal enterococci and to generate support for acceptance for the use of the real-time RT-PCR as an alternative for the legally prescribed culture method (NEN-EN-ISO 7899-2) for detection of intestinal enterococci in distributed drinking water.

4 Description of the methods

4.1 Reference method (culture)

For this study the membrane filtration method (NEN-EN-ISO 7899-2) for detection of intestinal enterococci was used as reference method (Anonymous 2000). This method is based on membrane filtration and subsequent culturing on Slanetz and Bartley (S&B) selective agar medium followed by a confirmation of the presumptive intestinal enterococci to hydrolyze Aesculine on Bile Esculine Azide Agar at 44 °C and, for the sensitivity study, species identification of intestinal enterococci colonies was performed using MALDI-TOF-MS.

4.2 Alternative method (real-time RT-PCR)

A rapid RT-PCR method specifically targeting 16S ribosomal RNA from intestinal enterococci is in this validation study the alternative method. A detailed “Standard Operating Procedure” (SOP) is provided in Appendix I. The organizing laboratory (KWR) and the laboratories participating in this study used this SOP. In short, the RT-PCR involves four distinct steps:

1. Sample processing
   - *Filtration*
     A volume of 100 ml water is filtered through a polycarbonate membrane filter.
   - *Lysozyme treatment*
     The filter is transferred to a solution containing Lysozyme and incubated at 37°C to degrade the bacterial cell wall.
   - *RNA extraction using the Biomerieux Nuclisens Kit*
     After lysozyme treatment, the membrane filter is transferred to a lysis buffer. The detergents in this lysis buffer promote lysis of the bacterial cell membrane and releases the cell content. RNA purification subsequently takes place using magnetic silica beads to which the nucleic acids bind. The beads are then transferred to a series of wash buffers. Eventually the RNA is detached from the beads in an elution buffer.

2. Controls
   Two types of controls are carried out: analysis of a blank and a positive control sample.
   - For a blank sample, 100 ml of DNA and RNA-free water is filtered and subjected to RNA extraction and real-time RT-PCR analysis.
   - For a positive control sample, 100 ml of DNA and RNA-free water, with addition of approximately 50 CFU intestinal enterococci is filtered, treated with Lysozyme and subjected to RNA extraction and real-time RT-PCR analysis.

3. Real-time RT-PCR
   For each sample, duplicate RT-PCR reactions are conducted. Commercially available products containing reagents to perform the reverse transcription reaction and subsequently the PCR reaction in one mixture (one-step RT-PCR); a cocktail of six specific primers and a probe. During the one-step RT-PCR reaction, the RNA (extracted from the sample) is first converted into cDNA (copy DNA) during a Reverse Transcription (RT) reaction followed by a PCR reaction which specifically amplifies and detects a fragment of intestinal enterococci 16S rRNA. For every sample the number of PCR cycles required to generate a detectable signal (Ct-value, also known as Cq or Cp value), is used to determine the presence or absence of intestinal enterococci.

4. Interpretation of the data
   Different criteria are used to interpret the results:

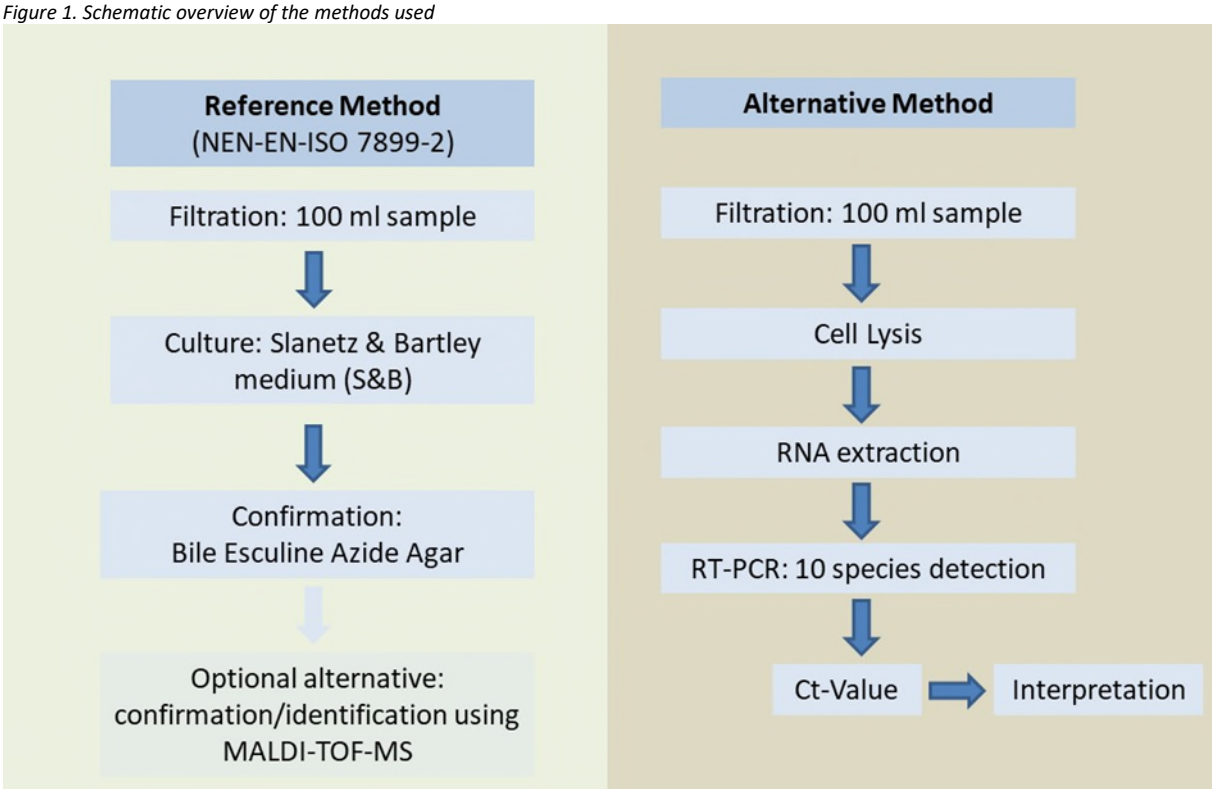
- A maximum Ct value is used as criterion to determine the presence of intestinal enterococci in the tested sample.
  - In this study samples are considered to contain intestinal enterococci if the average Ct value of duplicate RT-PCR reactions is < Ct 36.
- Criteria for the results of the analyses of negative and positive control samples are used to determine the integrity of the analysis.
  - In this study analysis results are considered unusable with negative control samples giving average Ct values <38 or one of the two duplicate giving a Ct value <36
  - Positive control samples are considered positive with average Ct values <36.

The criteria are based on practical experiences and can vary between different laboratories due to differing reagents and real-time PCR hardware they use. These criteria should therefore be verified by each individual laboratory and internal verification should be performed (according to ISO16140-3: 2021) to guarantee the correctness of the PCR outcome and prevent the occurrence of diverging results between different laboratories.

4.3 Schematic overview of the methods

A schematic overview of the reference and alternative methods applied in this validation study is shown in Figure 1.

Figure 1. Schematic overview of the methods used



5 Validation protocol

The validation protocol according to NEN-EN-ISO 16140-2:2016 included two phases:
- A method comparison study of the alternative method against the reference method carried out in the organizing laboratory (KWR).
- An interlaboratory study that compares the alternative method against the reference method carried out in different laboratories (organized by KWR).

5.1 Method comparison study

The method comparison study consisted of three parts:
- Sensitivity study: comparing results of the reference method to results of the alternative method in contaminated samples.
- Relative level of detection (RLOD) study: a comparative study to determine the RLOD in artificially contaminated samples.
- Inclusivity/exclusivity study: the ability for detecting different strains of target organisms is tested (Inclusivity), as well as the reliability with which non-target organisms are not detected (Exclusivity).

5.1.1 Paired or unpaired study (5.1.2 in ISO 16140-2:2016)

Since there is no shared initial (enrichment) step for both the reference and alternative methods, it is impossible to perform the reference method and the alternative method on exactly the same sample. In this validation study different test portions of the same sample batch are used for the two methods. This makes the resulting data “unpaired”.

5.1.2 Sensitivity study (5.1.3 in ISO 16140-2:2016)

The ability to detect the target organisms in distributed drinking water contaminated with intestinal enterococci and the absence of signal in distributed water containing no contaminating intestinal enterococci is tested in this part of the method comparison study. Analysis of naturally contaminated samples is preferred for this part. However, this option is not feasible for distributed drinking water samples, since samples naturally contaminated with intestinal enterococci are very rare and for this reason very difficult to collect. Therefore, the sensitivity study is performed on samples of drinking water which are artificially contaminated with surface water naturally containing intestinal enterococci. The tested drinking water samples and the contaminating surface water samples originated from different locations in the Netherlands to mimic a broad natural variety of bacterial background from drinking water and broad intestinal enterococci strain composition. MALDI-TOF-MS analysis of the intestinal enterococci strains from surface water used to contaminate drinking water demonstrates the presence of at least seven RT-PCR targeted species (*E. faecium, E. faecalis, E. hirae, E. mundtii, E. durans, E. casseliflavus* and *E. moraviensis*) in these surface water samples (Table 4), whereas three species are not detected (*E. avium, E. haemoperoxidus* and *E. gallinarum*) and two additional species (*E. termitis* and *E. sulfureus*) are detected in these samples.

5.1.2.1 Selection of categories and sample types to be used (5.1.3.1 in ISO 16140-2:2016)

The scope of this validation is the detection of intestinal enterococci in distributed drinking water. Hence, the only category and type in this validation study is distributed drinking water (without disinfectant residual).

5.1.2.2 Samples artificially contaminated by mixture with surface water

Artificially contaminated drinking water samples were composed by mixing drinking water with surface water or diluted sewage (containing intestinal enterococci). Drinking water samples (n=42), surface water samples (n=6) and two sewage samples from the sewage treatment plant from the city of Utrecht were randomly collected by the Drinking Water Laboratories of HWL, Vitens and WLN in the Netherlands. Aliquots of 100 ml of the 42 drinking water samples were analysed with RT-PCR and culture and 100 ml aliquots of 39 of these 42 drinking water samples were artificially contaminated with surface water and subsequently analysed with RT-PCR and culture. This results in a total of 81 samples for the sensitivity study (42 drinking water samples and 39 artificially contaminated samples). The concentration of intestinal enterococci in the surface water samples was determined using the reference method on the same day that the samples were collected and transported to KWR. Additionally, the species of a random selection of intestinal enterococci colonies, detected in these surface water samples, were identified using MALDI-TOF-MS and reported (Table 4). The determined intestinal enterococci concentrations in the surface water samples were used to compose 39 artificially contaminated water samples by mixing surface water with drinking water to a concentration of approximately 5 CFU Intestinal enterococci/100 ml. Analyses were performed on the 39 artificially contaminated drinking water samples and the 42 drinking water samples without contamination with surface water. A summary of the distributed drinking water samples is shown in Table 3, a summary of the contaminated surface water samples used to create mixtures of surface water with drinking water is shown in Table 4 and a summary of the composed mixtures of surface water with drinking water is shown in Table 5.

Table 3. Drinking water samples used for the sensitivity study.

No.	Name	Date	Lab	Location Code
1	DW-1.1	27-06-2022	HWL	PMO-DW
2	DW-1.2	27-06-2022	HWL	PSC-DW
3	DW-1.3	27-06-2022	HWL	PKW-DW
4	DW-1.4	27-06-2022	HWL	PAN-PO-RW
5	DW-1.5	27-06-2022	HWL	PBG-PO-RW
6	DW-1.6	27-06-2022	HWL	PLA-PO-RW
7	DW-1.7	27-06-2022	HWL	PWM-PO-RW
8	DW-1.8	27-06-2022	HWL	PLD-RW-001
9	DW-1.9	27-06-2022	HWL	PLD-RW-004
10	DW-1.10	27-06-2022	HWL	PWK-PO-001
11	DW-1.11	27-06-2022	HWL	PWK-PO-002
12	DW-1.12	27-06-2022	WLN	1365588
13	DW-1.13	27-06-2022	WLN	1365599
14	DW-1.14	27-06-2022	WLN	1365597
15	DW-1.15	27-06-2022	WLN	1365595
16	DW-1.16	27-06-2022	WLN	1365596
17	DW-1.17	27-06-2022	WLN	1365589
18	DW-1.18	27-06-2022	WLN	1365591
19	DW-1.19	27-06-2022	WLN	1365594
20	DW-1.22	27-06-2022	WLN	1365598
21	DW-2.1	04-07-2022	AQZ	5988815 - PBHK80HDL1+2
22	DW-2.2	04-07-2022	AQZ	5988816 - PBPL80HD2-2
23	DW-2.3	04-07-2022	AQZ	5988817 - PKRL80HD01
24	DW-2.4	04-07-2022	AQZ	5988818 - POUD80UITG
25	DW-2.5	04-07-2022	AQZ	5988819 - PBRA80UITG
26	DW-2.6	04-07-2022	AQZ	5988820 - PHAA80UITG
27	DW-2.7	04-07-2022	AQZ	5988821 - POSS80UITG
28	DW-2.8	04-07-2022	AQZ	5988822 - PHUY80UITG
29	DW-2.9	04-07-2022	AQZ	5988823 - PHAL80UITG
30	DW-2.10	04-07-2022	Vitens	V2206086434 - G1119952027067

31	DW-2.11	04-07-2022	Vitens	V2206086447 - G1119952021955
32	DW-2.12	04-07-2022	Vitens	V2206086626 - G1119952026811
33	DW-2.13	04-07-2022	Vitens	V2206086654 - G1119952026810
34	DW-2.14	04-07-2022	Vitens	V2206086727 - G1119952026905
35	DW-2.15	04-07-2022	Vitens	V2206086789 - G1119952027066
36	DW-2.16	04-07-2022	Vitens	V2206086800 - G1119952025962
37	DW-2.17	04-07-2022	Vitens	V2206086938 - G1119952023236
38	DW-2.18	04-07-2022	Vitens	V2206087008 - G1119952024545
39	DW-2.19	04-07-2022	Vitens	V2206087036 - G1119952025751
40	DW-2.20	04-07-2022	Vitens	V2206087058 - G1119952026992
41	DW-2.21	04-07-2022	Vitens	V2206098514 - G1119952026809
42	DW-KWR	27-06-2023	KWR	Drinking water KWR laboratory

Drinking water samples collected from different locations in The Netherlands used for the sensitivity study. The laboratory collecting the samples (Lab), the sample code provided by the laboratory (code), the location where the sample was collected (Location).

Table 4. Surface water samples used to compose artificially contaminated drinking water samples

Name	Date	Lab	Code	Concentration (CFU/100 ml)	Detected species (MALDI-TOF-MS)
OW-1.3	27-06-2022	WLN	Lewenborg Lijzijde	2.71E+03	*E. faecium (4); E. faecalis (5); E. hirae (1)*
OW-1.9	27-06-2022	WLN	Boezem (Winschoterkade)	4.65E+02	*E. faecium (10)*
OW-1.11	27-06-2022	WLN	Molukkenvijver	1.13E+03	*E. mundtii (3); E. hirae (2); E. faecium (3); E. durans (1); E. faecalis (1)*
STP-1	04-07-2022	KWR	Sewage Utrecht	1.94E+06	*No data*
OW-2.6	04-07-2022	Vitens	Dalfsen (location 1)	2.60E+02	*E. hirae (2), E. sulfureus (1), E. mundtii (2)*
OW-2.7	04-07-2022	Vitens	Dalfsen (location 2)	1.76E+02	*E. faecalis (1), E. durans (1), E. sulfureus (1), E. casseliflavus (1), E. moraviensis (1)*
OW-2.10	04-07-2022	Vitens	Vollenhove	1.70E+02	*E. faecalis (1), E. faecium (1), E. durans (1), E. casseliflavus (1), E. termitis (1)*
STP-2	04-07-2022	KWR	Sewage Utrecht	1,97E+06	*No data*

Surface water samples collected from different locations in The Netherlands and used for the sensitivity study. The laboratory collecting the samples (Lab), the sample code provided by the laboratory (code) and/or the location where the sample was collected (Location). The concentration of Intestinal enterococci and the species identification of 10 (WLN) or 5 (Vitens) colonies are provided.

Table 5. Composition of 100 ml samples containing mixtures of surface water with drinking water

No.		Drinking water (Table 3)	Surface water (Table 4)	Volume surface (ml) water in 100 ml sample
43	Mix1.1	DW-1.1	STP-1	0.36
44	Mix-1.2	DW-1.2	OW-1.3	0.26
45	Mix-1.3	DW-1.3	OW-1.9	1.51
46	Mix-1.4	DW-1.4	OW-1.11	0.62
47	Mix-1.5	DW-1.5	STP-1	0.36
48	Mix-1.6	DW-1.6	OW-1.9	1.51
49	Mix-1.7	DW-1.7	OW-1.11	0.62
50	Mix-1.8	DW-1.8	OW-1.11	0.62
51	Mix-1.9	DW-1.9	STP-1	0.36
52	Mix-1.10	DW-1.10	OW-1.9	1.51
53	Mix-1.11	DW-1.11	OW-1.11	0.62
54	Mix-1.12	DW-1.12	STP-1	0.36
55	Mix-1.15	DW-1.14	OW-1.9	1.51
56	Mix-1.16	DW-1.16	OW-1.11	0.62

57	Mix-1.17	DW-1.17	STP-1	0.36
58	Mix-1.18	DW-1.18	OW-1.9	1.51
59	Mix-1.19	DW-1.19	OW-1.11	0.62
60	Mix-1.20	DW-1.20	STP-1	0.36
61	Mix-2.1	DW-2.1	STP-2	2.78
62	Mix-2.2	DW-2.2	OW-2.6	2.69
63	Mix-2.3	DW-2.3	OW-2.7	3.98
64	Mix-2.4	DW-2.4	OW-2.10	4.12
65	Mix-2.5	DW-2.5	STP-2	2.78
66	Mix-2.6	DW-2.6	OW-2.6	2.69
67	Mix-2.7	DW-2.7	OW-2.7	3.98
68	Mix-2.8	DW-2.8	OW-2.10	4.12
69	Mix-2.9	DW-2.9	STP-2	2.78
70	Mix-2.10	DW-2.10	STP-2	2.78
71	Mix-2.11	DW-2.11	OW-2.4	0.15
72	Mix-2.12	DW-2.12	OW-2.6	2.69
73	Mix-2.13	DW-2.13	OW-2.7	3.98
74	Mix-2.14	DW-2.14	OW-2.10	4.12
75	Mix-2.15	DW-2.15	STP-2	2.78
76	Mix-2.16	DW-2.16	OW-2.6	2.69
77	Mix-2.17	DW-2.17	OW-2.7	3.98
78	Mix-2.18	DW-2.18	OW-2.10	4.12
79	Mix-2.19	DW-2.19	STP-2	2.78
80	Mix-2.20	DW-2.20	OW-2.6	2.69
81	Mix-2.21	DW-2.21	OW-2.7	3.98

5.2 Relative level of detection study (5.1.4 in ISO 16140-2:2016)

Analyses were carried out with the alternative and reference method on drinking water samples which are experimentally contaminated with intestinal enterococci, to determine the relative detection level. Intestinal enterococci were added at five contamination levels to drinking water from the city of Nieuwegein (Utrecht, The Netherlands).

5.2.1 RLOD of different intestinal enterococci species

The method to be validated is designed to detect at least the ten different intestinal enterococci species displayed in Table 1 using a mixture of four primer pairs (Heijnen, Timmers and Elsinga 2019). This can potentially result in different RLOD's for the different intestinal enterococci species targeted by the primer pairs. However, due to the high level of genetic relationship between the targeted species, each of the four primer pairs amplify identical 16S rRNA fragments from the species they target. This makes it highly unlikely that differences in sensitivity occur between species targeted with one primer pair and it is therefore very likely that the RLOD is only influenced by the primer pair used to amplify a 16S rRNA fragment. Therefore, the RLOD is determined by using one intestinal enterococci species for each primer pair used in the RT-PCR. The selected reference strains used to determine the RLOD are shown in Table 6.

Table 6. Reference strains used to determine the RLOD.

	Primerpair	Species	Referencestrain
1	*Ent_A1*	*E. casseliflavus*	*DSM20680*
2	*Ent_BC1*	*E. faecium*	*ATCC19434 (Vitroids)*
3	*Ent_F3*	*E. faecalis*	*ATCC19433 (Vitroids)*
4	*Ent_E2*	*E. moraviensis*	*DSM15919*

5.2.2 Protocol used to determine the RLOD's

The following protocol was used to make samples contaminated with known concentrations of intestinal enterococci:

Quantified intestinal enterococci cultures

- *Enterococcus faecium* and *Enterococcus faecalis:* commercially available, accurately quantified, bacterial reference suspensions (Vitroids, Sigma Aldrich) were used to make contaminated samples. .
    - The number of culturable intestinal enterococci in the used Vitroid batches, provided by the manufacturer, was first confirmed by the analyses of four vials from every Vitroid batch. The average concentration of these four vials was used to generate water samples supplemented with accurately quantified levels of *E. faecium* and *E. faecalis*.
- *E. casseliflavus* or *E. moraviensis*: since there are no vitroids or other quantified reference materials available containing these species, the following approach was used to make quantified intestinal enterococci suspensions:
    - Fresh cultures were grown overnight in non-selective liquid Lab-lemco broth.
    - The concentration in these cultures was determined (in 4-fold) using the standard culture method. The average concentration was used to contaminate drinking water with known contamination levels after which the concentration was verified on the samples used to determine the RLOD (Appendix IV).

Contaminated samples

- Dilutions prepared:
    - Using drinking water from the city of Nieuwegein
    - Large volume batches containing different concentrations were prepared and used on the day of preparation. Multiple analyses were performed on 100 ml portions with the alternative method and the reference method.
- Contamination levels:
    - 1st level: no Intestinal 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)
    - 5th level: 10 CFU/100 ml (5 samples)

All samples were analyzed using RT-PCR and the reference culture method, the intestinal enterococci concentrations were also determined using the culture method.

5.3 Inclusivity and exclusivity (5.1.5 in ISO 16140-2:2016)

5.3.1 Inclusivity

The inclusivity of the RT-PCR method was tested on a range of well-characterized bacterial strains belonging to the ten targeted intestinal enterococci species (Table 1). At least five strains were selected for each of the ten species, these five strains were preferably consisting of:

- Two reference strains obtained from internationally recognized culture collections (like ATCC or DSMZ). Only one reference strain was available for the species *E. durans, E. hirae, E. moraviensis* and *E. haemoperoxidus*.
- At least three strains isolated by the collaborating drinking water laboratories by culture. These strains are isolated from water samples (ground-, drinking- or surface-water) obtained from regular water quality screening or feces samples obtained from animals living in the vicinity of abstraction wells in the dune area in the Netherlands (Taucer-Kapteijn, Hoogenboezem et al. 2017). These strains are identified to the species level using MALDI-TOF-MS and sequence analysis of the 16S rRNA gene.

The results of species identification using MALDI-TOF-MS was confirmed with 16S rRNA gene species identification 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. haemoperoxidus* using MALDI-TOF-MS were identified as different but closely related species using 16S rRNA sequencing (using primers 785F and 907R). The minor differences between the 16S rRNA genes of *E. moraviensis* and *E. gallinarum* were previously described (Ryu, Henson et al. 2012) and this makes it difficult to discriminate these species. Some strains identified as *E. moraviensis* or *E. haemoperoxidus* 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 which was described previously (Švec, Vancanneyt et al. 2006).

A summarized description of the strains used for this inclusivity study is shown in Table 7.

The RT-PCR analyses were conducted on RNA in which the concentration was normalized to a concentration equivalent to 1-20 CFU intestinal enterococci.
This was carried out as follows:

- Colonies of the bacterial strains were grown on S&B agar medium for a maximum time of 48 hours.
- A homogeneous cell suspension was created from a freshly isolated colony in sterile PBS (1 mM KH_2PO_4, 155 mM NaCl, 3 mM Na_2HPO_4 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/100µl.
- Volumes of 100µl were analyzed in duplicate on S&B plates (in duplicate) to determine the concentration of culturable cells in the suspensions.
- A volume of 100µl of this suspension was used to isolate RNA.
- RT-PCR reactions were performed in duplicate on diluted RNA. Dilutions were composed in such a way that RT-PCR reactions were performed on RNA isolated from the equivalent of 1-20 CFU intestinal enterococci (Appendix V).

Table 7. Intestinal Enterococcus strains used for the inclusivity study

Nr	Species	16S sequence	Strain ID	Origin	Supplier
1	*E. faecalis*	*E. faecalis*	*DSM20478*	*Reference strain*	*DSMZ*
2	*E. faecalis*	*E. faecalis*	*ATCC19433*	*Reference strain*	*Sigma (Vitroids)*
3	*E. faecalis**	*E. faecalis*	*Vitens 2*	*Surface water*	*Vitens/HWL*

4	*E. faecalis**	*E. faecalis*	*Vitens 7*	*Drinking water*	*Vitens/HWL*
5	*E. faecalis**	*E. faecalis*	*Mens 19/1*	*Human Feces*	*Vitens/HWL*
6	*E. faecalis**	*E. faecalis*	*Gans 1/1*	*Goose feces*	*Vitens/HWL*
7	*E. faecium*	*E. faecium*	*DSM20477*	*Reference strain*	*ATCC*
8	*E. faecium*	*E. faecium*	*ATCC19434*	*Reference strain*	*Sigma (Vitroids)*
9	*E. faecium**	*E. faecium*	*Vitens 35*	*Ground water*	*Vitens/HWL*
10	*E. faecium**	*E. faecium*	*Vitens 14*	*Surface water*	*Vitens/HWL*
11	*E. faecium**	*E. faecium*	*Vitens 26*	*Surface water*	*Vitens/HWL*
12	*E. faecium**	*E. faecium*	*Mens 20/1*	*Human Feces*	*Vitens/HWL*
13	*E. durans*	*E. durans*	*DSM20633*	*Reference strain*	*DSMZ*
14	*E. durans**	*E. durans*	*Vitens 20*	*Surface water*	*Vitens/HWL*
15	*E. durans**	*E. durans*	*Vos 13/3*	*Fox feces*	*Vitens/HWL*
16	*E. durans**	*E. durans*	*Vitens 18-1*	*Surface water*	*Vitens/HWL*
17	*E. durans**	*E. durans*	*Mens 1/2*	*Human Feces*	*Vitens/HWL*
18	*E. hirae*	*E. hirae*	*DSM28619*	*Reference strain*	*DSMZ*
19	*E. hirae**	*E. hirae*	*Vitens 19*	*Surface water*	*Vitens/HWL*
20	*E. hirae**	*E. hirae*	*Vitens 98*	*Surface water*	*HWL*
21	*E. hirae**	*E. hirae*	*Vitens 97*	*Surface water*	*HWL*
22	*E. hirae**	*E. hirae*	*Mens 10/7*	*Human Feces*	*HWL*
23	*E. casseliflavus*	*E. casseliflavus*	*DSM20680*	*Reference strain*	*DSMZ*
24	*E. casselliflavus*	*E. casseliflavus*	*DSM20382*	*Reference strain*	*DSMZ*
25	*E. casselliflavus**	*E. gallinarum*	*MCOC92*	*Surface water*	*Vitens*
26	*E. casselliflavus**	*E. gallinarum*	*MCOC48*	*Water (unknown)*	*Vitens*
27	*E. casselliflavus**	*E. gallinarum*	*MCOC14*	*Drinking water*	*Vitens*
28	*E. casselliflavus**	*E. gallinarum*	*MCOC32*	*Drinking water*	*Vitens*
29	*E. mundtii*	*E. mundtii*	*DSM4838*	*Reference strain*	*DSMZ*
30	*E. mundtii*	*E. mundtii*	*DSM4839*	*Reference strain*	*DSMZ*
31	*E. mundtii**	*E. mundtii*	*MCOC30*	*Drinking water*	*Vitens*
32	*E. mundtii**	*E. mundtii*	*MCOC33*	*Ground water*	*Vitens*
33	*E. mundtii**	*E. mundtii*	*MCOC34*	*Ground water*	*Vitens*
34	*E. mundtii**	*E. mundtii*	*MCOC43*	*Surface water*	*Vitens*
35	*E. gallinarum*	*E. gallinarum*	*DSM24841*	*Reference strain*	*DSMZ*
36	*E. gallinarum*	*E. gallinarum*	*DSM20628*	*Reference strain*	*DSMZ*
37	*E. gallinarum**	*E. gallinarum*	*MCOC66*	*Drinking water*	*Vitens*
38	*E. gallinarum**	*E. gallinarum*	*MCOC91*	*Drinking water*	*Vitens*
39	*E. gallinarum**	*E. gallinarum*	*MCOC126*	*Drinking water*	*Vitens*

40	*E. moraviensis*	*E. moraviensi/E. silesiacus*	*DSM15919*	*Reference strain*	*DSMZ*
41	*E. moraviensis**	*E. silesiacus*	*MCOC123*	*Rinsing water*	*Vitens*
42	*E. moraviensis**	*E. rotai/E. moraviensis*	*MCOC124*	*Surface water*	*Vitens*
43	*E. moraviensis**	*E. rotai/E. moraviensis*	*MCOC127*	*Drinking water*	*Vitens*
44	*E. moraviensis**	*E. rotai/E. moraviensis*	*MCOC118*	*Surface water*	*Vitens*
45	*E. haemoperoxidus*	*E. haemoperoxidus*	*DSM15920*	*Reference strain*	*DSMZ*
46	*E. haemoperoxidus**	*E. haemoperoxidus*	*MCOC105*	*Drinking water*	*Vitens*
47	*E. haemoperoxidus**	*E. silesiacus*	*MCOC108*	*Drinking water*	*Vitens*
48	*E. haemoperoxidus**	*E. silesiacus*	*MCOC111*	*Drinking water*	*Vitens*
49	*E. haemoperoxidus**	*E. moraviensis*	*MCOC115*	*Drinking water*	*Vitens*
50	*E. haemoperoxidus**	*E. silesiacus*	*MCOC120*	*Surface water*	*Vitens*
51	*E. avium*	*E. avium*	*DSM20679*	*Reference strain*	*DSMZ*
52	*E. avium*	*E. avium*	*DSM20063*	*Reference strain*	*DSMZ*
53	*E. avium**	*E. avium*	*MCOC56*	*Surface water*	*Vitens*
54	*E. avium**	*E. avium*	*Mens 2*	*Human Feces*	*HWL*
55	*E. avium**	*E. avium*	*Mens 16*	*Human Feces*	*HWL*
	** species identification based on MALDI-TOF-MS*				

5.3.2 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 intestinal enterococci like *Streptococci*, *Staphylococci* and *Aerococci*
- Three intestinal enterococci species not belonging to the ten selected target species of Table 1.

The collection of tested species are shown in Table 8.

The real-time RT-PCR analyses were conducted on RNA isolated from bacterial suspensions containing 73-660 CFU (average 250 CFU).

This was carried out as follows:

- Colonies of most bacterial species were grown on LL agar medium for a maximum time of 48 hours. *Legionella* species were cultured on BCYE medium for 72 hours
- 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 cell counts, dilutions were made (in PBS) to a concentration of 200 cells/100µl.
- Volumes of 100µl were analyzed in duplicate on LLA (or BCYE for *Legionella*) plates (in duplicate) to determine the concentration of culturable cells in the suspensions.
- A volume of 100µl of this suspension was used to isolate RNA.
- RT-PCR reactions were performed in duplicate on RNA isolated from suspensions containing 73-660 CFU. (Appendix V).

Table 8. Strains used for the exclusivity study

Nr	Species	Strain ID
1	*Enterobacter aerogenes*	EPA202 (ATCC 13048)

2	*Enterobacter cloacae*	WR3
3	*Staphylococcus warneri*	WR51
4	*Staphylococcus aureus*	WR10
5	*Aerococcus viridans*	DSM 20311
6	*Aerococcus suis*	DSM 21500
7	*Streptococcus anginosus*	DSM 20563
8	*Hydrogenophaga pseudoflava*	DSM 1034
9	*Aquabacterium commune*	DSM 11901
10	*Aquabacterium parvum*	DSM 11968
11	*Citrobacter freundii*	DSM 30039
12	*Escherichia coli*	WR1
13	*Klebsiella oxytoca*	DSM 5175
14	*Serratia marcescens*	DSM 30121
15	*Kluyvera ascorbata*	DSM 4611
16	*Pantoea agglomerans*	DSM 3493
17	*Cronobacter sakazakii*	DSM 4485
18	*Klebsiella pneumoniae*	DSM30104
19	*Aeromonas hydrophila*	DSM6173
20	*Pseudomonas fluorescens*	P17 (ATCC 49642)
21	*Aquaspirillum sp.*	NOX (ATCC 49643)
22	*Salmonella panama*	SP5
23	*Flavobacterium johnsoniae*	A3
24	*Escherichia fergusonii*	DSM13698
25	*Aeromonas veronii*	ATCC 35624
26	*Staphylococcus epidermidis*	*DSM1798*
27	*Streptococcus equinus*	*DSM20062*
28	*Streptococcus salivarius*	*DSM20067*
29	*Legionella pneumophila*	*ATCC33152*
30	*Legionella anisa*	ATCC 35291
	Other intestinal enterococci species (not part of the 10 selected species)	
31	*Enterococcus termitis*	*DSM22803*
32	*Enterococcus malodoratus*	*DSM20681*
33	*Enterococcus villorum*	*DSM15688*

5.4 Interlaboratory study

All Dutch drinking water laboratories and two drinking water laboratories in Belgium were participating 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 which must be tested in this collaboration study was reached by organizing two interlaboratory studies, offering a total of 24 samples for every collaborator. Three contamination levels were

prepared (0, 1 and 5 CFU/100 ml) and divided in one study analysing samples containing 0 (n=4) and 5 CFU/100 ml (n=8) and one study analysing samples containing 0 (n=4) and 1 CFU/100 ml (n=8). Samples were received by the collaborators within 24 hours after preparation and processed within 6 hours after delivery. The collaborating laboratories of AqZ, De Watergroep, HWL, WLN and Vitens delivered two individual laboratory technicians to perform the analysis of the interlaboratory studies independently. KWR and Pidpa delivered one Lab technician, adding up to the generation of 12 datasets. In case of two collaborators from one laboratory the processing and analysis occurred by two separate persons on two independent sets of samples. The collaborator from KWR was not involved in the preparation of the samples. Every collaborator was performing the reference method and the alternative method on all samples.

For every interlaboratory study homogenous samples were prepared in large batches (containing the 3 contamination levels). Due to practical reasons, one enterococcus species (*E. faecalis*) was used to contaminate drinking water. Based on the limited variation between the RLOD's of the different species (Table 15), it is not expected that large differences will occur between interlaboratory studies using different Intestinal Enterococci species. Vitroids® (Sigma-Aldrich containing *Enterococcus faecalis* WDCM 00009) were used to contaminate drinking water, originating from the city of Nieuwegein, at the described levels. Individual samples with volumes of 250 ml were aliquoted from the batches and sent in a cooling box partially filled with ice to the collaborators. For every interlaboratory study the mean *Enterococcus faecalis* concentration in the prepared sample batches was confirmed using the reference method.

5.5 Statistical analysis

All statistical analyses are performed as described in ISO 16140-2:2016

6 Results

6.1 Method comparison study

6.1.1 Sensitivity study

The sensitivity study was performed on 42 distributed drinking water samples (Table 3) and 39 drinking water samples contaminated with surface water containing intestinal enterococci at an average concentration of 4,3 CFU/100 ml (range: 0-14 CFU/100 ml,

Table 9 and Appendix III) derived from different locations (Table 4). A detailed overview of the results is shown in Appendix III and a summary of the results is shown in

Table 9. The sensitivity characteristics of the RT-PCR method, as calculated from results of the sensitivity study are shown in Table 10.

Table 9. Overview of the RT-PCR and culture results to determine the sensitivity of both methods.

	Drinking water					Contaminated drinking water			
		Culture		RT-PCR			Culture		RT-PCR
Nr	Name	CFU/100 ml	Pos/Neg	Pos/Neg	Nr	Name	CFU/100 ml	Pos/Neg	Pos/Neg
1	DW-1.1	0	Neg	Neg	43	Mix-1.1	1	Pos	Pos
2	DW-1.2	0	Neg	Neg	44	Mix-1.2	1	Pos	Pos
3	DW-1.3	0	Neg	Neg	45	Mix-1.3	8	Pos	Pos
4	DW-1.4	0	Neg	Neg	46	Mix-1.4	6	Pos	Pos
5	DW-1.5	0	Neg	Neg	47	Mix-1.5	5	Pos	Pos
6	DW-1.6	0	Neg	Neg	48	Mix-1.6	8	Pos	Pos
7	DW-1.7	0	Neg	Neg	49	Mix-1.7	6	Pos	Pos
8	DW-1.8	0	Neg	Neg	50	Mix-1.8	9	Pos	Pos
9	DW-1.9	0	Neg	Neg	51	Mix-1.9	5	Pos	Pos
10	DW-1.10	0	Neg	Neg	52	Mix-1.10	14	Pos	Pos
11	DW-1.11	0	Neg	Neg	53	Mix-1.11	8	Pos	Pos
12	DW-1.12	0	Neg	Neg	54	Mix-1.12	1	Pos	Pos
13	DW-1.13	0	Neg	Neg	55	Mix-1.15	11	Pos	Pos
14	DW-1.14	0	Neg	Neg	56	Mix-1.16	4	Pos	Pos
15	DW-1.15	0	Neg	Neg	57	Mix-1.17	3	Pos	Pos
16	DW-1.16	0	Neg	Neg	58	Mix-1.18	14	Pos	Pos
17	DW-1.17	0	Neg	Neg	59	Mix-1.19	6	Pos	Pos
18	DW-1.18	0	Neg	Neg	60	Mix-1.20	4	Pos	Pos
19	DW-1.19	0	Neg	Neg	61	Mix-2.1	5	Pos	Pos
20	DW-1.20	0	Neg	Neg	62	Mix-2.2	0	Neg	Neg
21	DW-2.1	0	Neg	Pos	63	Mix-2.3	5	Pos	Pos
22	DW-2.2	0	Neg	Neg	64	Mix-2.4	3	Pos	Pos
23	DW-2.3	0	Neg	Neg	65	Mix-2.5	5	Pos	Pos
24	DW-2.4	0	Neg	Neg	66	Mix-2.6	0	Neg	Neg
25	DW-2.5	0	Neg	Neg	67	Mix-2.7	1	Pos	Pos
26	DW-2.6	0	Neg	Neg	68	Mix-2.8	2	Pos	Pos
27	DW-2.7	0	Neg	Neg	69	Mix-2.9	4	Pos	Pos
28	DW-2.8	0	Neg	Neg	70	Mix-2.10	5	Pos	Pos
29	DW-2.9	0	Neg	Neg	71	Mix-2.11	0	Neg	Neg
30	DW-2.10	0	Neg	Neg	72	Mix-2.12	1	Pos	Neg
31	DW-2.11	0	Neg	Neg	73	Mix-2.13	2	Pos	Pos
32	DW-2.12	0	Neg	Neg	74	Mix-2.14	4	Pos	Pos
33	DW-2.13	0	Neg	Neg	75	Mix-2.15	9	Pos	Pos
34	DW-2.14	0	Neg	Neg	76	Mix-2.16	0	Neg	Neg
35	DW-2.15	0	Neg	Neg	77	Mix-2.17	1	Pos	Pos
36	DW-2.16	0	Neg	Neg	78	Mix-2.18	4	Pos	Neg
37	DW-2.17	0	Neg	Neg	79	Mix-2.19	2	Pos	Pos
38	DW-2.18	0	Neg	Pos	80	Mix-2.20	0	Neg	Pos
39	DW-2.19	0	Neg	Neg	81	Mix-2.21	0	Neg	Neg
40	DW-2.20	0	Neg	Neg					
41	DW-2.21	0	Neg	Pos					
42	DW KWR	0	Neg	Neg					

Table 10. Summary of results found with the reference method and the alternative method in the sensitivity study

	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**

Sensitivity for the alternative method:

$$SE_{alt} = \frac{PA + PD}{PA + ND + PD} \cdot 100\% = \frac{31 + 4}{31 + 2 + 4} \cdot 100\% = 94.6\%$$

Sensitivity for the reference method:

$$SE_{ref} = \frac{PA + ND}{PA + ND + PD} \cdot 100\% = \frac{31 + 2}{31 + 2 + 4} \cdot 100\% = 89.2\%$$

Relative trueness/sensitivity

$$AC = \frac{PA + NA}{N} \cdot 100\% = \frac{31 + 44}{81} \cdot 100\% = 92.6\%$$

False positive ratio for the alternative method:

$$FPR = \frac{FP}{NA} \cdot 100\%$$

FP (false positive) is a positive result by the alternative-method which has not been confirmed by the reference method. Since there is no possibility to confirm the RT-PCR results no confirmation is carried out. This value is therefore not defined in this case.
Three positive results were observed in 42 drinking water samples (7.1%) to which no Intestinal enterococci from surface water were added.

The acceptability limit for an unpaired study consisting of 1 category is: $\mathrm{ND} - \mathrm{PD} = 3$. The actually observed value is: $\mathrm{ND} - \mathrm{PD} = -2$. The acceptability limit has not been exceeded demonstrating that the sensitivity of the RT-PCR method meets the criterium of ISO 16140-2:2016.

6.1.2 Relative level of detection study (RLOD)

The RLOD for this RT-PCR method was determined by carrying out analyses with both the culture and real-time RT-PCR method on drinking water samples which were artificially contaminated with four different contamination levels:

- 1st level: 0 CFU/100 ml (n=5)
- 2nd level: 0.7 CFU/100 ml (n=20)
- 3rd level: 1.5 CFU/100 ml (n=10)
- 4th level: 5 CFU/100 ml (n=5)

The raw data of the analysed samples is displayed in Appendix III and the results are summarised in Table 11 (E. casseliflavus), Table 12 (E. moraviensis), Table 13 (E. faecium) and Table 14 (E. faecalis).

Table 11. Summarized results on different contamination levels to determine the RLOD for detection of E. casseliflavus

Contamination level	Positive	Negative
Level 1: 0 CFU/100 ml		
Reference method (n=5)	0	5
Alternative method (n=5)	0	5
Average concentration measured (CFU/100 ml)	0	
Level 2: 0,7 CFU/100 ml		
Reference method (n=20)	16	4
Alternative method (n=20)	20	0
Average concentration measured (CFU/100 ml)	1.5 (SD=1.3)	
Level 3: 1.5 CFU/100 ml		
Reference method (n=10)	10	0
Alternative method (n=10)	10	0
Average concentration measured (CFU/100 ml)	3.1 (SD=1.6)	
Level 4: 5 CFU/100 ml		
Reference method (n=5)	5	0
Alternative method (n=5)	5	0
Average concentration measured (CFU/100 ml)	8.6 (SD=2.4)	

Table 12. Summarized results on different contamination levels to determine the RLOD for detection of E. moraviensis

Contamination level	Positive	Negative
Level 1: 0 CFU/100 ml		
Reference method (n=5)	0	5
Alternative method (n=5)	0	5
Average concentration measured (CFU/100 ml)	0	
Level 2: 0,7 CFU/100 ml		
Reference method (n=20)	7	13
Alternative method (n=20)	8	12
Average concentration measured (CFU/100 ml)	0.4 (SD=0.6)	
Level 3: 1,5 CFU/100 ml		
Reference method (n=10)	6	4
Alternative method (n=10)	9	1
Average concentration measured (CFU/100 ml)	1.0 (SD=1.1)	
Level 4: 5 CFU/100 ml		
Reference method (n=5)	5	0
Alternative method (n=5)	5	0
Average concentration measured (CFU/100 ml)	3.6 (SD=2.7)	

Table 13. Summarized results on different contamination levels to determine the RLOD for detection of E. faecium

Contamination level	Positive	Negative
Level 1: 0 CFU/100 ml		
Reference method (n=5)	0	5
Alternative method (n=5)	0	5
Average concentration measured (CFU/100 ml)	0	
Level 2: 0,7 CFU/100 ml		
Reference method (n=20)	17	3
Alternative method (n=20)	13	7
Average concentration measured (CFU/100 ml)	1.0 (SD=0.8)	
Level 3: 1.5 CFU/100 ml		
Reference method (n=10)	7	3
Alternative method (n=10)	10	0
Average concentration measured (CFU/100 ml)	1.3 (SD=1.3)	
Level 4: 5 CFU/100 ml		
Reference method (n=5)	5	0
Alternative method (n=5)	5	0
Average concentration measured (CFU/100 ml)	5,4 (SD=2.1)	

Table 14. Summarized results on different contamination levels to determine the RLOD for detection of E. faecalis

Contamination level	Positive	Negative
Level 1: 0 CFU/100 ml		
Reference method (n=5)	0	5
Alternative method (n=5)	0	5
Average concentration measured (CFU/100 ml)	0	
Level 2: 0,7 CFU/100 ml		
Reference method (n=20)	13	7
Alternative method (n=20)	14	6
Average concentration measured (CFU/100 ml)	0,7 (SD=0.8)	
Level 3: 1.5 CFU/100 ml		
Reference method (n=10)	8	2
Alternative method (n=10)	7	3
Average concentration measured (CFU/100 ml)	1.6 (SD=1.2)	
Level 4: 5 CFU/100 ml		
Reference method (n=5)	5	0
Alternative method (n=5)	5	0
Average concentration measured (CFU/100 ml)	6,2 (SD=1.9)	

Calculation of the RLOD has been carried out with calculation 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 results are summarized in Table 15.

Table 15. RLOD calculations

Name	RLOD	RLODL	RLODU	b=ln(RLOD)	sd(b)	z-Test statistic	p-value
		95% confidence					
E. casseliflavus	0.54	0.23	1.26	-0.62	0.43	1.45	1.85
E. moraviensis	0.60	0.28	1.31	-0.51	0.39	1.31	1.81
E. faecium	0.45	0.23	0.89	-0.81	0.34	2.34	1.98
E. faecalis	0.78	0.40	1.54	-0.24	0.34	0.72	1.53
Combined	0.66	0.47	0.92	-0.42	0.17	2.50	1.99

RLODL=lower limit of 95% confidence interval for RLOD; RLODU=upper limit of 95% confidence interval for RLOD; b=ln(RLOD)=logarithm of the RLOD value; sd(b)=standard deviation of b; z-Test statistic=absolute value of the z-test with the null hypothesis H0: b=0; p-value= p-value of the z-test.

The RLOD's for detection of all tested enterococci species are below 1.0; which means that these species can be detected with at least the same sensitivity using the RT-PCR in comparison with the reference culture method. Based on the 95% confidence interval shown for *E. faecium* it is expected that this species can be detected at a significantly higher sensitivity than the other tested species. The combined RLOD for all four tested species is moderately below 1.0 (0.66 with 95% confidence interval: 0.47-0.92) suggesting that RT-PCR is at least as sensitive as the reference method for detection of intestinal enterococci.

The acceptability limit (AL) for this unpaired dataset is: 2.5. All four RLOD's meet this AL.

6.1.3 Inclusivity and exclusivity study (5.1.5 in ISO 16140-2:2016)

The inclusivity was tested on a collection of 55 intestinal enterococci strains consisting of at least five strains for every of the ten selected species (Table 7). Exclusivity was tested on 30 non-enterococci strains and three enterococci strains that do not belong to the 10 selected species (Table 8).

Inclusivity

The results of the inclusivity tests are summarized shown in Table 16 and raw data of these tests are shown in Appendix V.

Table 16. Summarized results of the inclusivity tests

Nr	Strain		RT-PCR Result
1	*E. faecalis*	DSM20478	Pos
2	*E. faecalis*	ATCC19433	Pos
3	*E. faecalis*	Vitens 2	Pos
4	*E. faecalis*	Vitens 7	Pos
5	*E. faecalis*	Mens 19/1	Pos
6	*E. faecalis*	Gans 1/1	Pos
6	*E. faecium*	DSM20477	Pos
7	*E. faecium*	ATCC19434	Pos
8	*E. faecium*	Vitens 35	Pos
9	*E. faecium*	Vitens 14	Pos
10	*E. faecium*	Vitens 26	Pos
11	*E. faecium*	Mens 20/1	Pos
12	*E. durans*	DSM20633	Pos
13	*E. durans*	DSM 100907	Pos
14	*E. durans*	Vitens 20	Pos
15	*E. durans*	Vos 13/3	Pos
16	*E. durans*	Vitens 18-1	Pos
17	*E. durans*	Mens 1/2	Pos
18	*E. hirae*	DSM28619	Pos
19	*E. hirae*	DSM20160	Pos
20	*E. hirae*	Vitens 19	Pos
21	*E. hirae*	Vitens 98	Pos
22	*E. hirae*	Vitens 97	Pos
23	*E. hirae*	Mens 10/7	Pos
24	*E. casseliflavus*	DSM20680	Pos
25	*E. casselliflavus*	DSM20382	Pos
26	*E. casselliflavus*	MCOC56	Pos
27	*E. casselliflavus*	MCOC5	Pos
28	*E. casselliflavus*	MCOC14	Pos
29	*E. casselliflavus*	MCOC32	Pos
30	*E. mundtii*	DSM4838	Pos
31	*E. mundtii*	DSM4839	Pos
32	*E. mundtii*	MCOC30	Pos
33	*E. mundtii*	MCOC33	Pos
34	*E. mundtii*	MCOC34	Pos
35	*E. mundtii*	MCOC43	Pos
36	*E. gallinarum*	DSM24841	Pos
37	*E. gallinarum*	DSM20628	Pos
38	*E. gallinarum*	MCOC66	Pos
39	*E. gallinarum*	MCOC91	Pos
40	*E. gallinarum*	MCOC126	Pos
41	*E. moraviensis*	DSM15919	Pos
42	*E. moraviensis*	MCOC121	Pos
43	*E. moraviensis*	MCOC123	Pos
44	*E. moraviensis*	MCOC124	Pos
45	*E. moraviensis*	MCOC127	Pos
46	*E. moraviensis*	MCOC118	Pos
47	*E. haemoperoxidus*	DSM15920	Pos
48	*E. haemoperoxidus*	MCOC105	Pos
49	*E. haemoperoxidus*	MCOC108	Pos
50	*E. haemoperoxidus*	MCOC111	Pos
51	*E. haemoperoxidus*	MCOC115	Pos
52	*E. haemoperoxidus*	MCOC120	Pos
53	*E. avium*	DSM20679	Pos
54	*E. avium*	DSM20063	Pos
55	*E. avium*	MCOC56	Pos

These results show that all (100%) tested intestinal enterococci strains were positive using the RT-PCR method. One isolate from surface water (nr 3, Vitens 2) was tested negative in the validation study performed in 2021

(Heijnen 2021). However, on basis of the 16S rRNA sequence of this *Enterococcus faecalis* strain, it was concluded that the primers used in this RT-PCR assay match perfectly on this 16S rRNA sequence. Therefore, this strain was re-cultured, RNA was re-isolated and RT-PCR was performed again. The RT-PCR test confirmed that this strain was also positive, resulting in a 100% inclusivity.

Exclusivity

The results of the exclusivity tests are shown in Table 17 and raw data of these tests are summarized in Appendix V.

Table 17. Summarized results of the exclusivity tests

	Strain		RT-PCR result
1	E. faecalis	DSM 20478	Pos
2	E. faecalis	ATCC19433	Pos
3	E. faecalis	Vitens 2	Neg
4	E. faecalis	Vitens 7	Pos
5	E. faecalis	Mens 19/1	Pos
6	E. faecalis	Gans 1/1	Pos
7	E. faecium	DSM 20477	Pos
8	E. faecium	ATCC19434	Pos
9	E. faecium	Vitens 35	Pos
10	E. faecium	Vitens 14	Pos
11	E. faecium	Vitens 26	Pos
12	E. faecium	Mens 20/1	Pos
13	E. durans	DSM 20633	Pos
14	E. durans	Vitens 20	Pos
15	E. durans	Vos 13/3	Pos
16	E. durans	Vitens 18-1	Pos
17	E. durans	Mens 1/2	Pos
18	E. hirae	DSM 28619	Pos
19	E. hirae	Vitens 19	Pos
20	E. hirae	Vitens 98	Pos
21	E. hirae	Vitens 97	Pos
22	E. hirae	Mens 10/7	Pos
23	E. casseliflavus	DSM20680	Pos
24	E. casselliflavus	DSM20382	Pos
25	E. casselliflavus	MCOC92	Pos
26	E. casselliflavus	MCOC48	Pos
27	E. casselliflavus	MCOC14	Pos
28	E. casselliflavus	MCOC32	Pos
29	E. mundtii	DSM4838	Pos
30	E. mundtii	DSM4839	Pos
31	E. mundtii	MCOC30	Pos
32	E. mundtii	MCOC33	Pos
33	E. mundtii	MCOC34	Pos
34	E. mundtii	MCOC43	Pos
35	E. gallinarum	DSM24841	Pos
36	E. gallinarum	DSM20628	Pos
37	E. gallinarum	MCOC66	Pos
38	E. gallinarum	MCOC91	Pos
39	E. gallinarum	MCOC126	Pos
40	E. moraviensis	DSM15919	Pos
41	E. moraviensis	MCOC123	Pos
42	E. moraviensis	MCOC124	Pos
43	E. moraviensis	MCOC127	Pos
44	E. moraviensis	MCOC118	Pos
45	E. haemoperoxidus	DSM15920	Pos
46	E. haemoperoxidus	MCOC105	Pos
47	E. haemoperoxidus	MCOC108	Pos
48	E. haemoperoxidus	MCOC111	Pos
49	E. haemoperoxidus	MCOC115	Pos
50	E. haemoperoxidus	MCOC120	Pos
51	E. avium	DSM20679	Pos
52	E. avium	DSM20063	Pos
53	E. avium	MCOC56	Pos
54	E. avium	Mens 2	Pos
55	E. avium	Mens 16	Pos

The results of the exclusivity tests show that detection of the other bacterial genera was not observed. Also, RNA isolated from species of closely related genera like *Streptococcus*, *Aerococcus* or *Staphylococcus* was not detected in the RT-PCR assay. Testing RNA from the *Enterococcus* species *Enterococcus malodoratus* and

Enterococcus villorum did not show a signal in the RT-PCR assay. Based on the 16S rRNA sequence of *E. malodoratus* and *E. villorum* it was predicted, using an in silico analysis on the ARB database (www.arb-silva.de) (Yilmaz, Parfrey et al. 2013) that the primer sequences would not bind to the 16s rRNA sequences of these Intestinal enterococci species, making it unlikely that these species produce a RT-PCR signal. The exclusivity study shows that *Enterococcus termitis* is an *Enterococcus* species that can be detected using this RT-PCR assay. Detection of *Enterococcus termitis* is confirmed "in-silico" by the 16S rRNA sequence which is very related (99%) to the 16S rRNA sequences of *Enterococcus moraviensis* and *Enterococcus haemoperoxidus* containing identical sequences at the primer binding sites. Based on this "in-silico" analysis it was also concluded that the sequences of the primer binding sites of the Enterococci species *E. flavescens, E. saccharolyticus, E. pseudoavium, E. thailandicus, E. gilvus, E. lactis, E. caccae, E. silesiacus, E. raffinosus, E. quebecensis, E. ureasiticus* and *E. ureilyticus* perfectly matched the used primers making it likely that also these species can be detected using this RT-PCR method. Hence, besides the initially ten target Enterococci, other Enterococci will be detected as well using this RT-PCR method, which will result in more positive results than previously anticipated. But it is expected that the additional Enterococci will be detected using the reference method as well.

6.2 Interlaboratory study

An interlaboratory study was performed with 12 collaborators from seven different laboratories. In this interlaboratory study 24 drinking water samples were analyzed by every collaborator. These 24 samples were contaminated at three contamination levels (0, 1 and 5 CFU/100ml) with eight samples at every contamination level.

6.2.1 Calculations and summary of data

The results obtained by the individual collaborators in the interlaboratory study are shown in Table 18, the positive results for the reference method are summarized in Table 19 and the positive results for the alternative method are summarized in Table 20. Detailed information about the results, including Ct values and culture values, is shown in Appendix VI. Data from samples 13-24 from participant 5 was not available due to technical problems with the device used for this experiment.

Table 18. Summarized results of RT-PCR and culture method on 24 samples by 12 collaborators

		Aqualab Zuid				HWL				Vitens				WLN				De Watergroep				KWR		Pidpa	
Sample	Concentration	Participant 1		Participant 2		Participant 3		Participant 4		Participant 5		Participant 6		Participant 7		Participant 8		Participant 9		Participant 10		Participant 11		Participant 12	
	(CFU/100 ml)	RT-PCR	Culture	RT-PCR	Culture	RT-PCR	Culture	RT-PCR	Culture	RT-PCR	Culture	RT-PCR	Culture	RT-PCR	Culture	RT-PCR	Culture	RT-PCR	Culture	RT-PCR	Culture	RT-PCR	Culture	RT-PCR	Culture
1	5	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
2	0	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
3	5	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
4	5	Positive	Positive	Negative	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
5	5	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
6	0	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
7	0	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Positive	Negative
8	5	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
9	5	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
10	0	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
11	5	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
12	5	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
13	1	Positive	Positive	Positive	Positive	Positive	Negative	Negative	Positive	No data due to technical problems with RT-PCR system		Positive	Positive	Positive	Negative	Positive	Positive	Negative	Negative	Negative	Positive	Positive	Negative	Positive	Positive
14	0	Negative	Negative	Negative	Negative	Negative	Negative	Positive	Negative			Negative	Negative	Negative	Negative	Positive	Negative	Negative	Negative	Positive	Negative	Negative	Negative	Negative	Negative
15	1	Positive	Positive	Positive	Negative	Positive	Negative	Negative	Negative			Positive	Positive	Positive	Negative	Positive	Negative	Negative	Negative	Negative	Negative	Positive	Positive	Negative	Negative
16	1	Positive	Negative	Positive	Positive	Negative	Positive	Positive	Positive			Positive	Positive	Positive	Negative	Negative	Positive	Negative	Positive	Positive	Positive	Positive	Positive	Positive	Positive
17	0	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative			Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
18	1	Positive	Positive	Negative	Positive	Positive	Negative	Positive	Positive			Positive	Negative	Negative	Negative	Positive	Negative	Positive	Negative	Negative	Positive	Positive	Negative	Negative	Positive
19	1	Positive	Positive	Positive	Positive	Negative	Positive	Negative	Positive			Positive	Positive	Negative	Positive	Positive	Positive	Positive	Negative	Positive	Negative	Negative	Positive	Positive	Positive
20	0	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative			Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
21	1	Negative	Negative	Positive	Positive	Positive	Positive	Negative	Negative			Negative	Positive	Negative	Positive	Positive	Positive	Positive	Positive	Negative	Negative	Negative	Negative	Positive	Positive
22	1	Negative	Negative	Positive	Negative	Negative	Negative	Negative	Positive			Negative	Positive	Positive	Positive	Negative	Negative	Positive	Positive	Positive	Negative	Positive	Negative	Negative	Negative
23	0	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative			Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
24	1	Positive	Positive	Positive	Positive	Negative	Positive	Negative	Positive			Positive	Negative	Positive	Negative	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Negative

Results are obtained from samples send out at April 06 and May 09 2023.

Table 19. Positive results obtained with the reference method.

		Contamination Level		
Collaborator		L_0 [a]	L_1 [b]	L_2 [c]
AqZ	1	0/8	5/8	8/8
	2	0/8	6/8	8/8
HWL	3	0/8	4/8	8/8
	4	0/8	6/8	8/8
Vitens	5	0/4		8/8
	6	0/8	6/8	8/8
WLN	7	0/8	3/8	8/8
	8	0/8	5/8	8/8
De Watergroep	9	0/8	4/8	8/8
	10	0/8	4/8	8/8
KWR	11	0/8	4/8	8/8
Pidpa	12	0/8	5/8	8/8
Total		**0/92**	**52/88**	**96/96**
		P_0	P_1	P_2

[a] Number of positive reference-method results at contamination level 0 (0 CFU/100ml)

[b] Number of positive reference-method results at contamination level 1 (1 CFU/100ml)

[c] Number of positive reference-method results at contamination level 2 (5 CFU/100ml)

Table 20. Positive results obtained with the Alternative method

		Contamination Level		
Collaborators		L_0 [a]	L_1 [b]	L_2 [c]
AqZ	1	0/8	6/8	8/8
	2	0/8	7/8	7/8
HWL	3	0/8	4/8	8/8
	4	1/8	2/8	8/8
Vitens	5	0/4		8/8
	6	0/8	6/8	8/8
WLN	7	0/8	5/8	8/8
	8	1/8	6/8	8/8
De Watergroep	9	0/8	5/8	8/8
	10	1/8	4/8	8/8
KWR	11	0/8	6/8	8/8
Pidpa	12	1/8	5/8	8/8
Total		**4/92**	**56/88**	**95/96**
		P_0	P_1	P_2

[a] Number of positive alternative-method results at contamination level 0 (0 CFU/100ml)

[b] Number of positive alternative-method results at contamination level 1 (1 CFU/100ml)

[c] Number of positive alternative-method results at contamination level 2 (5 CFU/100ml)

The specificity (SP) for the reference method:

$$SP_{ref} = \left(1 - \frac{P_0}{N_-}\right) \cdot 100\% = \left(1 - \frac{0}{92}\right) \cdot 100\% = 100\%$$

The specificity for the alternative method is calculated:

$$SP_{alt} = \left(1 - \frac{P_0}{N_-}\right) \cdot 100\% = \left(1 - \frac{4}{92}\right) \cdot 100\% = 95{,}7\%$$

The results obtained in level L1 of this unpaired study (Table 21) were evaluated for both methods by all collaborators in Table 22.

Table 21. Summary of the results all collaborators obtained with the reference and alternative methods for level L_1 (1 CFU/100 ml).

	Reference method positive (R+)	Reference method negative (R-)
Alternative method positive (A+)	Positive Agreement (PA) 33	Positive Deviation (PD) 23
Alternative method negative (A-)	Negative Deviation (ND) 18	Negative Agreement (NA) 14

Sensitivity for the alternative method:

$$SE_{alt} = \frac{PA + PD}{PA + ND + PD} \cdot 100\% = \frac{33 + 23}{33 + 18 + 23} \cdot 100\% = 75{,}6\%$$

Sensitivity for the reference method:

$$SE_{ref} = \frac{PA + ND}{PA + ND + PD} \cdot 100\% = \frac{33 + 18}{33 + 18 + 23} \cdot 100\% = 68{,}9\%$$

Relative trueness/sensitivity:

$$AC = \frac{PA + NA}{N} \cdot 100\% = \frac{33 + 14}{88} \cdot 100\% = 53{,}4\%$$

False positive ratio for the alternative method:

$$FPR = \frac{FP}{NA} \cdot 100\%$$

FP (false positive) is a positive result by the alternative-method, which was not confirmed as positive by a confirmation method. Since no confirmation can be carried out after real-time RT-PCR, this value is not defined in this case.
However, four positive results were observed in 92 analysed drinking water samples to which no Intestinal enterococci were added. A false positive ratio based on these false positives would be 4/92*100% = 4.3%.

Evaluation of the interlaboratory study results is carried out using the formulas of clause 5.2.4.2 (NEN-EN-ISO 16140-2).

Table 22. Evaluation of the results of the interlaboratory study

	L_0	L_1	L_2
Reference P_x	0	52	96
Reference N_x	92	88	96
$(P+)_{ref} = \frac{P_x}{N_x} =$	0,0%	59,1%	100%
Alternative P_x	4	56	95
Alternative N_x	92	88	96
$(P+)_{alt} = \frac{P_x}{N_x} =$	4,3%	63,6%	99,0%
$AL = (ND-PD)_{max} =$	3	11	2
PA	0	33	94
NA	88	14	0
ND	0	18	1
PD	4	23	1
$ND - PD =$	-4	-5	0

P_x: number of samples with a positive result obtained with the reference method al level *x* (L_0, L_1 or L_2)

The acceptability limit AL is calculated as follows:

$$(ND-PD)_{max} = \sqrt{3N_x \cdot \left((P+)_{ref} + (P+)_{alt} - 2 \cdot (P+)_{ref} \cdot (P+)_{alt}\right)}$$

It is concluded that $ND - PD \leq AL$ at all levels: L_0 (0 CFU/100 ml: ND-PD=-4, AL=3), L_1 (1 CFU/100 ml: ND-PD=-5, AL=11) and L_2 (5 CFU/100 ml: ND-PD=0, AL=0).

The positive deviation (PD) at level 1 is higher than the negative deviation (ND) demonstrating that low concentrations (in the 1 CFU/100 ml range) are detected more frequently with the alternative method than with the reference method.

7 Discussion, conclusions and recommendations

ISO16140-2:2016 was used to perform this validation study, the study comprises two phases:

- A method comparison study
    - Sensitivity study
    - RLOD study
    - Inclusivity/Exclusivity study
- An interlaboratory study

7.1 Method comparison study

7.1.1 Sensitivity study

The results of the previously described sensitivity study (Heijnen 2021) were disturbed by the usage of surface water with low concentrations of intestinal enterococci to compose artificially contaminated drinking water samples. This presumably resulted in PCR-assays suffering from inhibition caused by the presence of RT-PCR-inhibiting substances due to the addition of relatively large volumes of surface water. The contamination of drinking water with large volumes of untreated surface water does not mimic situations that are expected to happen in practical situations and are therefore avoided in this study. The sensitivity study was repeated using surface water samples and sewage water, with high concentrations of intestinal enterococci, to compose artificially contaminated drinking water samples. This study was executed on drinking water samples (n=42) and artificially contaminated drinking water samples (n=39) with a mean concentration of 4.3 CFU/100 ml (range: 0-14 CFU/100 ml). Both drinking water and surface water samples were obtained from different geographic locations in the Netherlands to mimic natural variation in the bacterial composition in drinking water and variation in the contaminating surface water and the Intestinal enterococci species present in these surface waters.

A slightly higher but statistically insignificant (Chi^2 test, $p>0.05$) sensitivity to detect intestinal enterococci was found for the RT-PCR method (94.6%) compared to the reference culture method (89.2%). An AL value of 3, as described in ISO16140-2:2016) for the Acceptable Limit (AL: Negative Deviation (ND) – Positive Deviation (PD)), was amply met in this study (ND-PD = -2). This suggests that intestinal enterococci can be detected using RT-PCR in drinking water with the same sensitivity as with the culture method. In the previous validation study of 2021 (Heijnen 2021) a comparable sensitivity was found for the reference method (92.9%), but the sensitivity for the alternative method was only 62.8% and ND-PD was 12. These differences appear to be the result of inhibition of RT-PCR in the validation study performed in 2021, due to the usage of large volumes of surface water. This emphasises the need for the usage of an internal control to monitor the execution of the procedure from sample to result and the potential presence of RT-PCR inhibiting substances. This is why the collaborating water laboratories use additional spiked samples as controls in their routine practice and why an internal control (IC) was developed by WLN. This IC consists of the addition of bacterial cells from *Thermus aquaticus* to the sample and the specific detection of this bacterial species using RT-PCR. The daily use of this procedure to check water quality is currently evaluated by the collaborating laboratories and appears to be promising. The observation that contaminants, present in surface water, may lead to inhibition of RT-PCR also emphasises the need for optimisation of extraction procedures to remove inhibitors and/or the use of alternative inhibitor-resistant reagents to perform RT-PCR reactions.

Main conclusion for the sensitivity study

- The sensitivity of RT-PCR is comparable to the sensitivity of the culture method for the detection of intestinal enterococci in distributed drinking water

7.1.2 RLOD study

Analyses were carried out on drinking water contaminated with four different intestinal enterococci species (*E. casseliflavus, E. moraviensis, E. faecium* and *E. faecalis*) at four concentration levels (5; 1.5; 0.7 and 0 CFU/100 ml) to determine the relative level of detection (RLOD) of the alternative method to the reference method. The identical sequences of the amplified 16S rRNA for the species within each group targeted with one primer pair (Table 2, Appendix II) pair makes it highly unlikely that different *Enterococcus* species targeted by the same primer pair will result in different RLOD's . This makes that the four species, mentioned above, are considered as representative for all the 10 species targeted in the RT-PCR method. The RLOD for detection of the tested species are lower than the required AL of 2.5 but close to 1.0 (for *E. casseliflavus*, *E. moraviensis* and *E. faecalis*), meaning that the alternative method is likely to detect intestinal enterococci with comparable sensitivity as the reference method. The RLOD to detect *E. faecium* is 0.45 and the complete 95% confidence interval is lower than 1.0 (0.23-0.89) suggesting that this species can be detected with higher sensitivity compared to the culture method.

Main conclusion of the RLOD study

- The RLOD study demonstrates a comparable limit of detection between RT-PCR and the culture method.
    - A lower RLOD is observed for *Enterococcus faecium*

7.1.3 Inclusivity/exclusivity study

The inclusivity was tested on 55 *Enterococcus* strains (Table 7) and exclusivity was tested on 30 non-*Enterococcus* (Table 8) strains and three *Enterococcus* strains not belonging to the 10 selected target species (Table 1). All *Enterococcus* strains were detected. One strain was not detected in the validation study in 2021, however repeating the culturing, RNA extraction and RT-PCR of this strain resulted in a clear RT-PCR signal. No signals were obtained with RT-PCR using the tested non-enterococci, including strains from bacterial genera closely related to *Enterococcus* (*Enterobacter, Staphylococcus* and *Streptococcus*). From the three tested *Enterococcus* species that do not belong to the 10 selected *Enterococcus* species, only *Enterococcus termitis*, a species closely related to *Enterococcus moraviensis,* was detected. *Enterococcus termitis* is also observed sporadically in distributed drinking water (Table 3, Appendix I) and in fecal samples from Geese (Table 4, Appendix I). In-silico analysis of primer sequences demonstrates that detection of intestinal enterococci may not be limited to the ten selected *Enterococcus* species but suggests that also *E. termitis*, *E. flavescens, E. saccharolyticus, E. pseudoavium, E. thailandicus, E. gilvus, E. lactis, E. caccae, E. silesiacus, E. raffinosus, E. quebecensis, E. ureasiticus* and *E. ureilyticus* can be detected with RT-PCR. The inventory (Appendix I) demonstrates that part of these additional enterococci species (*E. termitis, E. thailandicus, E. gilvus* and *E. saccharolyticus*) can also be present in fecal material or have been detected in water previously suggesting that the detection of these species does not result in discrepancies between culture and RT-PCR. However, from the other species it is currently unclear if these species can be present in water, if they are cultured and recognized on S&B culture plates and if they are identified to these species using MALDI-TOF-MS. Further research should give insight into the implications of detection of these species using RT-PCR.

Main conclusion of the Inclusivity/exclusivity study

- The study shows that RT-PCR can be used to detect all tested intestinal enterococci species and no other bacterial species are tested positive using this method.
    - Detection of additional intestinal enterococci is demonstrated for *E. termitis* and, based on in-silico analyses expected for a limited number of other *Enterococcus* species.

7.2 Interlaboratory study

The collaborating laboratories have performed in house validation of the intestinal enterococci RT-PCR at their respective laboratories and the interlaboratory study was subsequently performed. Three contamination levels (0, 1 and 5 CFU/100 ml) were analyzed by 11 participants.
The results at level L_2 (5 CFU/100 ml) show that RT-PCR and culture results are comparable, all samples were positive using culture and only one sample was negative using RT-PCR (100% culture and 98.9% RT-PCR). At contamination level L_1 (1 CFU/100ml), 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%). At level L_0 four samples (4.3%) found positive using RT-PCR whereas no positives were found using the culture method (0%). The criteria, described in ISO16140-2: 2016, were met at all contamination levels.

Main conclusion interlaboratory study

- The interlaboratory study demonstrates that RT-PCR results are comparable with results obtained with the culture method on samples analyzed by different participants from different laboratories.

7.3 Overall conclusion

This validation study demonstrates that results using this RT-PCR for the detection of intestinal enterococci are comparable with results obtained with the reference culture method according to the criteria of ISO16140-2:2016.

8 Literature

Anonymous (2000). "ISO 7899-2:2000(en) Water quality -Detection and enumeration of intestinal enterococci - ." ISO standard.
Anonymous (2000). "Water quality - Detection and enumeration of intestinal enterococci - Part 2: Membrane filtration method." NEN-EN-ISO 7899-2
Anonymous (2016). "Microbiology of the food chain - Method validation - Part 2: Protocol for the validation of alternative (proprietary) methods against a reference method (ISO 16140-2:2016)." NEN-EN-ISO.
Heijnen, L. (2017). "Validation of a real-time RT-PCR method for rapid detection of E. coli in distributed drinking water." KWR report 2017.098
Heijnen, L. (2021). "Validation of a real-time RT-PCR method for rapid detection of 10 intestinal enterococci species in distributed drinking water." BTO report **402045/172**.
Heijnen, L., P. Timmers and G. Elsinga (2019). "Ontwikkeling van een RT-PCR voor snelle detectie van enterococcen." BTO report **BTO 2019.209(s)**
Heijnen, L., Timmers, P., Elsinga, G. (2019). "Ontwikkeling van een RT-PCR voor snelle detectie van enterococcen." BTO rapport **2019.209(s)**
Ryu, H., M. Henson, M. Elk, C. Toledo-Hernández, J. Griffith, D. Blackwood, R. Noble, M. Gourmelon, S. Glassmeyer and J. S. S. Domingo (2012). "Development of Quantitative PCR Assays Targeting the 16S rRNA Genes of Enterococcus spp. and Their Application to the Identification of Enterococcus Species in Environmental Samples." Applied and Environmental Microbiology **79**: 196 - 204.
Švec, P., M. Vancanneyt, I. Sedláček, S. M. Naser, C. Snauwaert, K. Lefebvre, B. Hoste and J. Swings (2006). "Enterococcus silesiacus sp. nov. and Enterococcus termitis sp. nov." Int J Syst Evol Microbiol **56**(Pt 3): 577-581.
Taucer-Kapteijn, M., W. Hoogenboezem, R. Hoogenboezem, S. de Haas and G. Medema (2017). "Source tracking of Enterococcus moraviensis and E. haemoperoxidus." J Water Health **15**(1): 41-49.
Yilmaz, P., L. W. Parfrey, P. Yarza, J. Gerken, E. Pruesse, C. Quast, T. Schweer, J. Peplies, W. Ludwig and F. O. Glöckner (2013). "The SILVA and "All-species Living Tree Project (LTP)" taxonomic frameworks." Nucleic Acids Research **42**(D1): D643-D648.

I Appendix. Onderbouwing van de keuze voor 10 soorten

Memo opgesteld door Maja Taucer (Het Waterlaboratorium) i.s.m. de Nederlandse en Vlaamse drinkwaterlaboratoria (zoals genoemd in tabel 1).

Ontwikkelen van een enterokokken RT-PCR methode als snel alternatief voor de NEN-EN-ISO 7899-2:2000

Inleiding

Een snelle en accurate controle van het drinkwater op aanwezigheid van fecale indicator organismen helpt de drinkwaterbedrijven een goede volksgezondheid te bewaken. In 2018 is de snelle methode Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) voor detectie van de afwezigheid van *E. coli* RNA door IL&T geaccepteerd. Naast de snelle RT-PCR methode voor detectie van het RNA van *E. coli*, wensen drinkwaterbedrijven ook een snelle methode voor detectie van enterokokken in drinkwater als alternatief voor de NEN-EN-ISO 7899-2:2000 methode. De drinkwaterlaboratoria willen daarom een enterokokken RT-PCR ontwikkelen, gevolgd door een validatietraject met als doel ook voor deze methode wettelijke acceptatie te krijgen.

De WHO richtlijn beschrijft vier enterokokkensoorten waarvan de relatie met fecale bronnen duidelijk en geaccepteerd is: *E. faecalis, E. faecium, E. durans* en *E. hirae*. Naast deze soorten kunnen op basis van feces-, literatuur- en MALDI-TOF onderzoek van drinkwater in Nederland en België, zes andere enterokokkensoorten eveneens in verband worden gebracht met fecale origine (bijlage 1). Deze zes soorten (*E. casseliflavus, E. mundtii, E. gallinarum, E. moraviensis, E. haemoperoxidus* en *E. avium*) en de vier door WHO beschreven soorten zijn geselecteerd voor de ontwikkeling van de RT-PCR.

De voorgestelde opzet voor het de ontwikkeling en valdiatie van de methode wordt beschreven in het plan van aanpak (*23 oktober verzonden naar RIVM)*. In deze memo wordt uiteengezet waarom de keuze voor tien enterokokkensoorten als target van de RT-PCR volgens de Nederlandse en Belgische drinkwaterlaboratoria (Tabel 1) de beste keuze is. Het is wenselijk als RIVM deze uiteenzetting wil onderschrijven of verwerpen voordat gestart wordt met praktijkonderzoek.

Tabel 1. Samenwerkende drinkwaterlaboratoria en de aan hen gelieerde drinkwaterbedrijven

Drinkwaterlaboratorium (DWL)	Locatie	Drinkwaterbedrijf (DWB)
Aqualab Zuid	Werkendam, NL	• Brabant Water • Evides • Watermaatschappij Limburg
De Watergroep	Gent, B	• De Watergroep
Het Waterlaboratorium	Haarlem, NL	• Dunea • Waternet • PWN
Pidpa	Antwerpen, B	• Pidpa
Vitens laboratorium	Leeuwarden, NL	• Vitens N.V. • OASEN
Waterlab Noord	Glimmen, NL	• Water Maatschappij Drenthe • Waterbedrijf Groningen
Water-link	Antwerpen, B	• Water-link

Detectie van enterokokkensoorten met behulp van NEN-EN-ISO 7899-2:2000 en bevestiging met MALDI-TOF

Dankzij de bevestiging van kolonies met behulp van MALDI-TOF, als vervanging voor de klassieke bevestiging volgens de ISO 7899-2:2000 methode, kunnen Nederlandse en Belgische drinkwaterlaboratoria de enterokokkensoorten snel identificeren. Het op naam brengen van bacterie-isolaten geeft een voortschrijdend inzicht in het voorkomen van enterkokkensoorten in drinkwater in de praktijk.

Uit een inventarisatie van het voorkomen van enterokokkensoorten in Nederland en Vlaanderen blijkt dat de keuze voor deze tien soorten een goed uitganspunt is voor de ontwikkeling van een RT-PCR methode (Tabel 2 en 3), die in potentie gelijkwaardig is aan de NEN-EN-ISO 7899-2:2000 methode. De meest voorkomende soorten zijn *E. casseliflavus E. faecalis, E. mundtii, E. faecium, E. hirae, E. durans, E. gallinarum* en *E. moraviensis.*

Tabel 2. Totaal aantal kve per soort geïsoleerd uit distributienetten van verschillende drinkwaterbedrijven in Nederland en België tussen 2014 en 2017.

	HWL	AQZ	Vitens	De Watergroep	Totaal
E. casseliflavus	15	65	11	45	136
E. faecalis	4	44	19	57	124
E. mundtii	23	36	22		81
E. faecium	5	20		45	70
E. moraviensis	38		8		46
E. hirae	1			21	22
E. durans		1		13	14
E. gallinarum		5		5	10
E. termitis	5				5
E. gilvus	1				1
E. silesiacus	1				1
Totaal	**93**	**171**	**60**	**186**	**510**

Tabel 3. Relatieve (%) verdeling van enterokokkensoorten in DW monsters (data AQZ, HWL, WLN, Vitens, De Watergroep, Pidpa en Water-Link) voor de periode tussen juni 2017 en juli 2018.

Targetorganisme van RT-PCR van tien soorten	Soortnaam	Voorkomen in # monsters	(%)	Gelijktijdig voorkomen van *E. coli*
x	*E. casseliflavus*	139	34,1	+
x	*E. mundtii*	69	16,9	+
x	*E. faecium*	78	19,1	+
x	*E. faecalis*	66	16,2	+
x	*E. hirae*	24	5,9	+
x	*E. gallinarum*	8	2,0	+
x	*E. haemoperoxidus*	6	1,5	
x	*E. durans*	5	1,2	
	E. termitis	4	1,0	
x	*E. moraviensis*	2	0,5	
	E. malodoratus	2	0,5	
	E. gilvus	2	0,5	
x	*E. avium*	2	0,5	
	E. vilorum	1	0,2	
		408	100,0	

Op basis van de verzamelde dataset zou minimaal 97,8% van de drinkwatermonsters waarin met kweek enterokokken werden aangetroffen, ook met gebruik van de te ontwikkelen RT-PCR methode gericht op tien enterokokkensoorten positief zijn.
Vanwege de genetische verwantschap tussen enterokokkensoorten is het zeer aannemelijk, dat de RT-PCR methode meer dan tien enterokokkensoorten zal detecteren. Gezien de specificiteit van de RT-PCR niet beperkt wordt tot deze tien soorten, zal een hoger percentage dan 97,8% overeenkomstigheid met de kweekmethode worden aangetroffen. Daarnaast wordt verwacht dat met de RT-PCR methode vaker *E. durans* en *E. avium* worden aangetroffen. Dat zijn de soorten die met de bevestiging volgens de ISO 7899-2:2000 methode mogelijk worden gemist, maar duidelijk geassocieerd zijn met de fecale bronnen (zie Bijlage 1). Dit zal een verbetering van de detectie van de aanwezigheid van Enterokokken betekenen ten opzichte van de huidige kweekmethode. Hierbij wordt voorgesteld om een RT-PCR methode te ontwerpen gericht op de tien enterokokkensoorten, waarna deze methode getoetst wordt in een validatiestudie. De hypothese van de validatiestudie is de gelijkwaardigheid van de RT-PCR methode gericht op de beschreven tien enterokokkensoorten aan kweek volgens NEN-EN-ISO 7899-2:2000. We verzoeken het RIVM aan de hand van deze memo aan te geven of zij het gekozen traject kunnen onderschrijven.

Bijlage 1: Het voorkomen van enterokokkensoorten in de darm van warmbloedige dieren.

Onderzoek waarbij feces van meerdere gastheren werd onderzocht op de samenstelling van enterokokkensoorten laat zien dat sommige soorten enterokokken (zoals *E. moraviensis* en *E. haemoperoxidus*) gastheerspecifiek kunnen zijn en dat de verdeling van de soorten per gastheer kan variëren (Tabel 4). *E. moraviensis*, *E. haemoperoxidus* en *E. termitis* zijn genetisch zeer verwant en kwamen in dit onderzoek ook in dezelfde gastheer voor (ganzen). Ganzen zijn bekende dragers van mens pathogene organismen (Graczyk et al., 1998; Zhou et al., 2004; Feare et al., 1999; Moriarty et al., 2011). Enterokokkensoorten die wijzen op ganzen origine zijn daardoor in drinkwater onderzoek relevant. Tevens blijkt dat *E. avium* naast *E. faecium* en *E. faecalis* een veel voorkomende enterokok is in menselijke feces (Tabel 4 en (Carvalho et al., 2006; Layton et al., 2010)). Dat maakt dat ook deze soort relevant is voor de detectie van fecale besmetting in drinkwater, ondanks het feit dat deze soort niet vaak in drinkwatermonsters werd gedetecteerd met kweek volgens NEN-EN-ISO 7899-2:2000 gevolgd door bevestiging met MALDI-TOF.

Tabel 4. Relatieve (%) verdeling van enterokokkensoorten in feces van geselecteerde gastheren met behulp van de SBA+MALDI-TOF (Taucer-Kapteijn et al., 2016).

Enterococcus spp.	red fox	rabbit	sheep	highland cattle	dog	human	goose
E. faecalis	39.6	0.9		35.3	54.2	27.2	29.4
E. faecium	23.7			5.9	11.8	35.1	7.8
E. hirae	25.5		69.0	5.9	18.1	2.3	
E. durans	4.9		2.4		8.3	3.8	
E. casseliflavus	1.0		5.6	52.9	2.1	2.6	5.2
E. gallinarum		98.1	9.5		1.4	2.0	3.9
E. mundtii	2.9		13.5		0.7	5.8	8.7
E. moraviensis	0.3	0.9					23.8
E. haemoperoxidus							11.3
E. avium					2.8	18.7	
E. gilvus	0.3						4.3
E. termitis							3.5
E. saccharolyticus						2.3	
E. silesiacus							2.2
E. aqamarinus	1.0						
E. thailandicus					0.7		
E. malodoratus	0.5						
E. sulfurens	0.3						

II SOP RT-PCR for detection of intestinal enterococci

The SOP for detection of intestinal enterococci using real-time RT-PCR (RT-PCR) is divided in two parts. The first part describes the procedure to concentrate the drinking water sample and isolate nucleic acids from the concentrate. The second part describes the procedure for specific detection of RNA from intestinal enterococci. It is expected that multiple commercially available kits and "home made" extraction procedures can be used for extraction of RNA with sufficient yield and limited inhibition problems, reverse transcription of RNA to DNA and specific amplification and detection using real-time PCR.

II.I Isolation of nucleic acid from water samples for targeted detection of specific bacteria using real-time RT-PCR

1. *Subject*

This protocol describes a non-proprietary method for the isolation of RNA from drinking water.

2. *Application*

This protocol is applied for the isolation of nucleic acid from drinking water.

3. *Principle*

This method detects 16S ribosomal RNA from intestinal enterococci. Detection of 16S rRNA makes detection of low concentrations of intestinal enterococci possible due to high concentrations of this RNA-species in bacterial cells.

4. *Definitions*

DNA sequence

A DNA-strand is made up of separate nucleotides. Each nucleotide consists of a phosphate group, a sugar (deoxyribose) and a base. A DNA-strand is built from a sequence of four possible nucleotides (guanine, cytosine, adenine and thymine). This sequence determines the genetic information (Fig. 1). The nucleotide sequence of a DNA-strand is called DNA-sequence.

RNA-sequence

An RNA strand is made up of separate nucleotides. Each nucleotide consists of a phosphate group, a sugar (ribose) and a base. A RNA-strand is built from a sequence of four possible nucleotides (guanine, cytosine, adenine and uracil). This sequence determines the genetic information (Fig. 1). The nucleotide sequence of an RNA strand is called RNA-sequence. RNA is a single stranded copy of the DNA and is used as a code for protein synthesis.

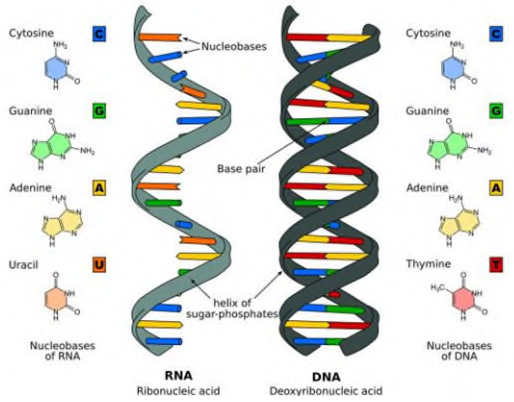


Fig. 1. (Sponk / Wikimedia Commons / CC BY-SA 3.0) Double-stranded DNA consisting of nucleotides whose sequence determines the genetic code and which serve as a template in DNA amplification. A single-stranded copy of DNA, RNA, is made in the cell. RNA moves freely in the cell and is used as a template for protein synthesis.

RNA-template

An RNA-template is a specific RNA-fragment that serves as a template for DNA amplification during Reverse Transcriptase (as, for example, in the protocol "Analysis of intestinal enterococci based on real-time Reverse Transcriptase PCR (RT-PCR)").

5. Reagents

5.1 DNA/RNA/DNase/RNAse-free water
5.2 Positive control of the target organism
Add 20-100 colony forming units (CFU) of a control strain to 100 ml of DNA/RNA/DNase/RNAse-free water (5.1). A suspension of a control can be prepared from a fresh quantified bacterial culture or purchased reference material can be used (for example: Bioballs© (Biomerieux) or Vitroids© (Sigma))
5.3 TE-Buffer, pH 8.0
5.4 Lysozyme 10 mg/ml
5.5 Lysis buffer, e.g. NucliSENS Biomerieux
5.6 Extraction of reagents, e.g. NucliSENS Magnetic Extraction Reagents Biomerieux:
- Magnetic Silica beads
- Wash Buffer 1
- Wash Buffer 2
- Wash Buffer 3
- Elution Buffer

6. Equipment and tools

6.1. Laboratory gloves, suitable for use in moleculair biological analyses
6.2. Calibrated micropipettes of various volumes
6.3. Sterile DNase/RNAse-free barriertips of various volumes
6.4. PCR tubes / 96-well plates and sealing
6.5. Micro-centrifuge
6.6. Thermomixer
6.7. Vortex
6.8. UV-cabinet, DNA-free
6.9. UV-cabinet in which DNA-material from samples is processed
6.10. Centrifuge
6.11. Real-time PCR device
6.12. Safe-lock tubes (Eppendorf tubes)
6.13. Filtration funnel with a polycarbonate membrane (Ø47mm and 0.2-0.4µm pore size)
6.14. Membrane filtration setup
6.15. Magnetic rack or magnetic pen
6.16. Inoculation loops

6.17. Optional: DNA extraction robot, e.g. KingFisher mL (Thermo). This protocol describes how to perform the isolation semi-automatically using the KingFisher mL.

7. *Method*

7.1. Control samples

- *Method blank*

  Each analysis run includes a method blank to check for contamination with nucleic acid from intestinal enterococci. The sample consists of 100 ml of DNA/RNA/DNase/RNAse-free water (5.1) that will be analyzed exactly as the other samples.
- *Positive control*

For each analysis run a positive control sample (5.2) is analyzed in exactly the same manner as the other samples.

7.2 RNA isolation:

There are many different DNA/RNA extraction kits available. When selecting a kit, it is important to test whether the RNA yield is adequate and there is no contamination of RNA from the organism of interest present in the products of the kit.
The protocol described here uses the NucliSENS extraction kit (Biomerieux) and is based on the procedure described by the supplier.

7.2.1 Lysis

- Vacuum filter 100 ml sample, using a filtration funnel, through a polycarbonate filter with a pore size of 0.2-0.4 µm.
- Fold the filter as shown in figure 2.

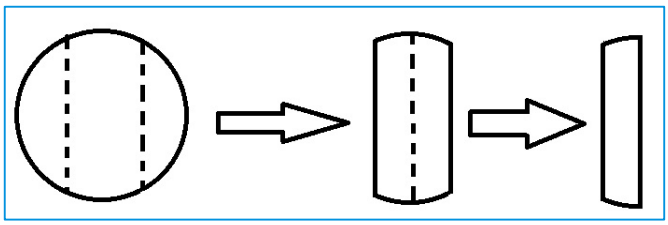


Figure 2: Folding the filter.

- Transfer the filter into an Safe lock tube (6.12)*.
- Add 1 ml of sample to the filter. For the control samples, add 1 ml of DNA/RNA/DNase/RNAse-free water (5.1) to the filter (6.12)*.
- Add 10 µl of lysozyme (10mg/mL) (5.4) to each tube*.
- Vortex for 10 seconds*.
- Place the tubes at 37˚C for 30 minutes to hydrolyse the bacterial cell wall*.
- For each sample and control, centrifuge the lysis tube at 2700 rpm for 30 seconds to remove the solution from the cap.
- Open the safe lock tube and transfer the entire contents to the lysis tube of the Biomerieux extraction Kit*.

The steps marked with an asterix (*) are essential for RNA isolation of gram-positive bacteria (like intestinal enterococci) and optional for extraction of nucleic acids from gram negative bacteria (like *E. coli*). When this option is not used, the folded filter is placed directly in the lysis tube.

- Vortex for 30 seconds.
- Incubate for 15 minutes at room temperature.
- Vortex for 30 seconds.
- Fix the filter between the lid and the tube, using an inoculation loop.
- Centrifuge the tubes at 1500g for 30 seconds.
- Remove and dispose the filter.

7.2.2 Binding

- Vortex the silica suspension.
- Add 50 µl of magnetic silica suspension to each sample.
- Keep the silica in a homogeneous suspension by vortexing it briefly before each addition.
- Incubate for 15 minutes at room temperature.
- Centrifuge the tubes at 1500 g for 2 minutes.
- Remove the lysis buffer carefully (be sure not to disturb the silica pellet!).
- Resuspend the silica pellet carefully in 350 µl Wash Buffer 1 (Biomerieux Kit).

7.2.3 Washing using the KingFisher mL

- For each sample, take a Kingfisher mL Tube Strip and prepare with the following buffers.
  - Tube B: 350 µl Wash Buffer 1
  - Tube C: 500 µl Wash Buffer 2
  - Tube D: 500 µl Wash Buffer 2
  - Tube E: 500 µl Wash Buffer 3
- Transfer the contents of the tube (magnetic silica in 350 µl Wash buffer 1) to tube A of the Kingfisher tube strip. Place the tube strip in the rack and the tube-rack in the KingFisher.
- Place the Kingfisher mL Tip Comb in the Kingfisher to protect the magnetic rods.
- Start the Kingfisher program. The KingFisher mL mixes beads with Wash Buffer and subsequently transfers the magnetic silicabeads to the following Wash Buffer. This process of mixing and magnetic transfer of beads continues for five washing steps until the magnetic beads are placed in the last tube of the tubestrip (figure 3).

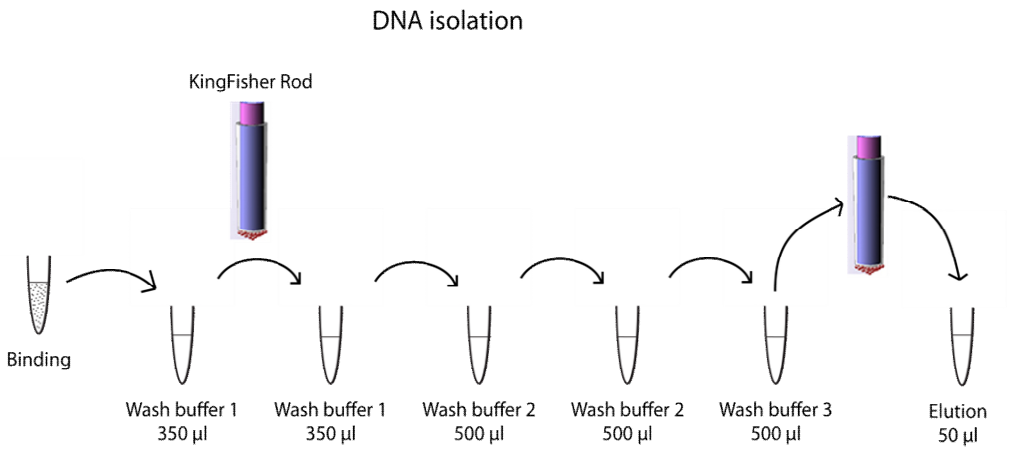


Figure 3: Nucleic acid isolation by means of magnetic beads and use of KingFisher mL.

- Meanwhile, prepare a new KingFisher mL Tube Strip containing 50 µl elution buffer in Tube A for each sample. During the break of the Kingfisher protocol, change the strips and press start to resume the protocol and the silica is transferred to the elution buffer in tube A.
- Transfer the magnetic silica beads (in elution buffer) to a clean safe lock tube (6.12)

7.2.4 Elution

- Incubate for 5 minutes at 60 °C and 1400rpm in the thermomixer (6.6).
- Place the tubes in a magnetic tuberack.
- Pipette the eluate, without silica, into a clean Safe lock tube (6.12).

The DNA/RNA isolate in a buffered solution, is used for consecutive molecular biological analysis, such as real-time-RT-PCR. It can be stored in a freezer (-70 ± 10) °C.

II.II Real-time RT-PCR for specific detection of intestinal enterococci

1. *Subject*

This protocol describes a non-proprietary method for detection of the presence or absence of intestinal enterococci in water using Reverse Transcriptase real-time Polymerase Chain Reaction (RT-PCR).

2. *Application*

This method applies to the determination of the presence or absence of intestinal enterococcal ribosomal RNA in water. The lower analysis limit is 1 CFU/100 ml, unless the nature or the volume of disruptive components in the matrix interfere with the determination (PCR efficiency).

3. *Principle*

Ribosomal RNA is ideally used for the detection of low concentrations of specific micro-organisms. In the analysis described here, Reverse Transcriptase is used to convert enterococcal RNA into cDNA. Secondly, the real-time PCR analysis of intestinal enterococci detects this cDNA. This method is aimed at determining the absence or presence of intestinal enterococci in 100 ml.

4. *Terms and definitions*

Intestinal enterococci

The genus of enterococci consists of a large genus of lactic acid bacteria of the phylum *Firmicutes*. Intestinal enterococci are gram-positive cocci that often occur in pairs (diplococci) or short chains, and are difficult to distinguish from streptococci on physical characteristics alone. Different intestinal enterococci species are common commensal organisms in the intestines of humans and other warm blooded animals.
The RT-PCR method targets ten, most relevant, intestinal enterococci species. The selection of these ten species (table 1) is based on: 1) an inventory, performed by the drinking water laboratories in the Netherlands and Belgium, of the species encountered in distributed drinking water and 2) the potential relation with the fecal origin of the species (Bijlage I, (Taucer-Kapteijn, Hoogenboezem et al. 2017)).

Table 1: Intestinal enterococci targeted in the RT-PCR

	Selected species
1	*Enterococcus faecium*
2	*Enterococcus faecalis*
3	*Enterococcus durans*
4	*Enterococcus hirae*
5	*Enterococcus casseliflavus*
6	*Enterococcus mundtii*
7	*Enterococcus gallinarum*
8	*Enterococcus moraviensis*
9	*Enterococcus haemoperoxidus*
10	*Enterococcus avium*

DNA sequence

A DNA-strand is made up of separate nucleotides. Each nucleotide consists of a phosphate group, a sugar (deoxyribose) and a base. A DNA-strand is built from a sequence of four possible nucleotides (guanine, cytosine, adenine and thymine). This sequence determines the genetic information (Fig. 1). The nucleotide sequence of a DNA-strand is called DNA-sequence.

RNA-sequence

An RNA strand is made up of separate nucleotides. Each nucleotide consists of a phosphate group, a sugar (ribose) and a base. A RNA-strand is built from a sequence of four possible nucleotides (guanine, cytosine, adenine and uracil). This sequence determines the genetic information (Fig. 1). The nucleotide sequence of an RNA strand is called RNA-sequence. RNA is a single stranded copy of the DNA and is used as a code for protein synthesis.

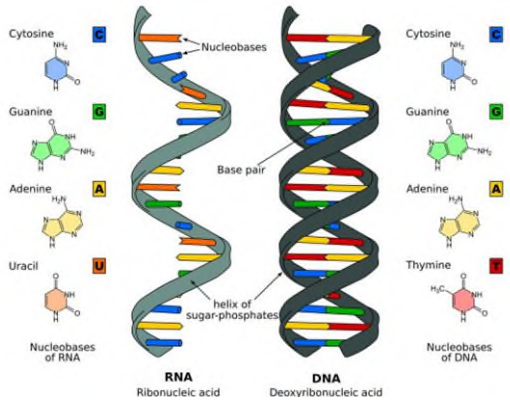


Fig. 1. (Sponk / Wikimedia Commons / CC BY-SA 3.0) Double-stranded DNA consisting of nucleotides whose sequence determines the genetic code and which, according to the key-lock principle, serve as a template in DNA amplification. A single-stranded copy of DNA, RNA, is made in the cell. RNA moves freely in the cell and is used as a template for protein synthesis.

Reverse Transcription

In Reverse Transcription, RNA is converted into complementary DNA (cDNA). cDNA is more stable than RNA and can be used in consecutive molecular biological analyses, such as real-time PCR. Reverse Transcriptase is the enzyme that converts target RNA into cDNA.

PCR cycle

In a single PCR cycle, the DNA template of interest is doubled. Each cycle covers three successive phases: denaturation (double-stranded DNA is split into single-stranded DNA), annealing (primer and probe annealing) and elongation (formation of new DNA strands).

Real-time Reverse Transcriptase PCR (RT-PCR)

PCR or polymerase chain reaction is a technique which amplifies a fragment of DNA, specific to a particular organism (DNA template). In Reverse Transcriptase PCR, prior to conventional PCR amplification, all RNA present is converted into cDNA, which then serves as a target for subsequent PCR amplification. The addition 'real-time' refers to the way of obtaining the result of the PCR reaction, i.e. the multiplication of the specific DNA fragment can be followed during the analysis on a computer screen of the PCR measuring device.

Taq-polymerase

The specific multiplication of the DNA-template takes place at relatively high temperatures. Therefore, Taq-polymerase is used, which is a naturally thermostable enzyme originally isolated from *Thermus aquaticus*, a bacterium naturally living in hot water sources and geysers. The EVOscript RNA Master mix used here contains Taq-polymerase.

Primers (reverse and forward)

Single-stranded DNA fragments (oligonucleotides) which serve as a starting point for reverse transcription and DNA amplification. The sequence of the primers determines which DNA template is produced. The length of a primer varies between 15 and 30 nucleotides. The reverse and forward primers each attach to one of the two complementary (matching) DNA or RNA strands.

Probe

A probe is a single-stranded DNA fragment, labelled with a fluorescent dye. Additionally, each probe has a molecule attached to it that extinguishes the fluorescent signal: the quencher. Taq-polymerase is

able to degrade a probe so that the dye and quencher disconnect and the fluorescent signal can be detected with the aid of a real-time PCR device.

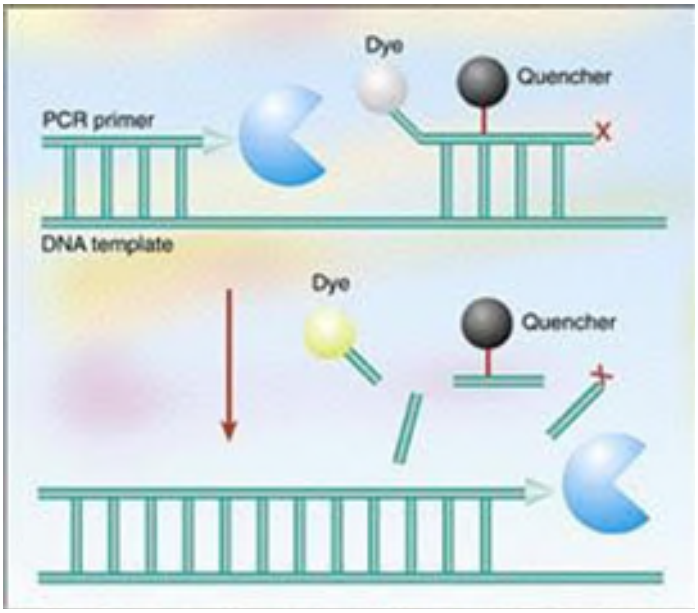


Fig. 2. Schematic representation of a fluorescent probe in action.

RNA-template

An RNA template is a specific RNA-fragment that serves as a template for DNA amplification during real-time PCR. The target-RNA, which is used to detect intestinal enterococci, is located on the 16S ribosomal RNA-gene.

Cq-value

The quantification cycle (Cq) is the number of PCR cycles (multiplying the DNA exponentially) needed to generate a fluorescent signal that exceeds the detection limit (threshold). The threshold line represents the detection limit above which the fluorescent signal is detected. The Cq-value is the intersection of the graph representing the number of fluorescence units with the detection limit (Fig. 4). The threshold line is placed at the point where the S-curves rise fastest. Cq-value can also be expressed as Ct-value, depending on the supplier of the real-time PCR.

One-step real-time RT-PCR amplification mix

An RT-PCR amplification mix contains all the components necessary to convert target RNA into cDNA and trigger the subsequent PCR reaction. A onestep RT-PCR amplification mix consists of:

- master mix;
- primers (forward and reverse);
- probe;
- elution buffer.

5. Controls

5.1 Positive control

A positive control sample is measured in duplicate in every PCR-plate by adding 2.0 µl RNA isolate derived from an intestinal enterococci strain to a well with reaction mix.

Note

If the result does not meet the quality requirements, identify the cause and determine the consequences for the analyzed samples of the entire PCR plate.

5.2 Blank control

A blank control is measured in duplicate in every PCR plate by adding 2.0 µl RNA isolate from PCR water to the reaction mixture. The blank control must have a Cq-value greater than or equal to 40 cycles.

Note

If the blank control result does not meet the requirement, identify the cause and determine the consequences for the analyzed samples of the entire PCR plate.

5.3 Second-line control

To be defined later.

5.4 Third-line control

Interlaboratory studies will be used as third line controls

6. Reagents

RNA is very sensitive to environmental enzymatic degradation. Only use RNA- and PCR-grade certified reagents and chemicals unless otherwise stated.

6.1 RNAse away or bleach solution

RNAse away or other bleach solutions should be used to remove RNA degrading enzymes (RNAse) and DNA from surfaces and pipettes. It is also used to clean UV cabinets, worktops, pipettes and filtration manifolds before RNA isolation.

6.2 RT-PCR grade water: ultrapure, RNAse-free, molecular grade

6.3 Elution Buffer (Nuclisens, Biomerieux)

6.4 Primers and Probe

The sequences of the primers are shown in table 2. The stock solution of primers and probes (100 µM) is stored at (-18 ± 3) °C for 2 years. Before use, the stock solution should be diluted with PCR water to a concentration of 10 µM. The working solution is stored in a refrigerator at (5 ± 3) °C for 3 months.

Table 2. Names, sequences and specificity of the primers

	Primerpair	Primers	Sequences	Specificity
1	*Ent_A1*	Ent_BC1F	5'-GTGGGTAACCTGCCCATC-3'	*E. casseliflavus*
		Ent_A4R	5'-TTCTTCCATGCGGAAAATAGTG -3'	*E. gallinarum*
2	*Ent_BC1*	Ent_BC1F	5'-GTGGGTAACCTGCCCATC-3'	*E. avium*
		Ent_C2R	5'-AACCAWGCGGTTTCGATTG-3'	*E. durans*
				E. faecium
				E. hirae
				E. mundtii
3	*Ent_F3*	Ent_BC1F	5'-GTGGGTAACCTGCCCATC-3'	*E. faecalis*
		ENTCm-Rev4-v2	5'- CCATGCGGCATAAACTGTTA -3'	
4	*Ent_E2*	Ent_DE1F	5'-CGTGGGTAACCTACCCATC -3'	*E. haemoperoxidus*
		Ent_E2R	5'-CCATGCGGTGTCGACTATTA -3'	*E. moraviensis*
	Probe	Ent_P1	5'-CTTGGAAACAGGTGCTAATACCG-3'	*E.* spp

The probe should have a fluorophore (e.g. FAM) on the 5' end and a suitable quencher on the 3' end (e.g. BHQ1).

Note:
Probes should be protected from light as much as possible. Light may affect the fluorescent dye attached to the probe.

6.5 Onestep RT-PCR mix

In paragraph 6.5.1 an example are given of how to prepare the amplification mix using a specific One-step RT-PCR mixture (Evoscript RNA Master mix, Roche diagnostics). However, the user is free to use other one-step RT-PCR mixes or separate cDNA synthesis and PCR mixes.

6.5.1 One-step RT-PCR: Roche Evoscript RNA master mix

The mixture contains the following components:

Component	Per reaction	Concentration
Evoscript RNA Master mix (5X)	4 µl	1X
Primer: Ent_BC1F	0,4 µl	200 nM
Primer: Ent_A4R	1,4 µl	700 nM
Primer: Ent_C2R	1,4 µl	700 nM
Primer: ENTCm-Rev4-v2	1,4 µl	700 nM
Primer: Ent_DE1F	0,4 µl	200 nM
Primer: Ent_E2R	1,4 µl	700 nM
Probe: Ent_P1	0,4 µl	200 nM
RT-PCR grade H2O	4,2 µl	
RNA sample (in elution buffer)	5 µl	
Total	**20 µl**	

7. Equipment and tools

7.1 Refrigerator at a temperature of 5 (± 3) °C
7.2 Freezer at a temperature of -18 (± 3) °C
7.3 Positive control:

A standardized and quantified suspension of an enterococcus control strain. A volume of 20-100 µl containing 20-100 CFU is used as a positive control.

7.4 Laboratory gloves suitable for molecular biological use.
7.5 Calibrated micropipettes with various volumes.
7.6 Sterile DNAse/RNAse-free barrier tips with various volumes.
7.7 96-well plates and sealing
7.8 Microcentrifuge
7.9 Vortex
7.10 UV cabinet for operations without free DNA/RNA material.
7.11 UV cabinet for operations with free DNA/RNA material.
7.12 Real-Time PCR Device
7.13 Safe-lock tubes

8. RT-PCR program

8.1 For the One-Step real-time RT-PCR using Evoscript RNA master mix (Roche chemicals), use the following program:

- 60 °C 15 minutes followed by 95 °C 10:00 minutes Polymerase activation
- 45 cycles: (95 °C/00:10s and 60 °C/00:30s + Plate read)
- 40 °C/00:30s

When using another brand of One-step RT-PCR mix or a separate cDNA synthesis and PCR mix, adjust the PCR programs accordingly.

9. Sample analysis

9.1 Procedure for the preparation of the PCR plate.

Prepare the One-step RT-PCR or cDNA synthesis/amplification mixes shortly before use. Take a 96-well plate and pipette the required amount of one-step RT-PCR amplification mix (6.5.1) for each sample into a well. Add the necessary amount of RNA-solution, centrifuge the plate at 2000 rpm for 30 seconds and run the One-step RT-PCR program in the PCR-machine as described by the manufacturer (see paragraph 8).

9.2 Interpretation of RT-PCR results

Assess the amplification plots of the method blank and the positive control sample and use the table below to interpret the results.

Intestinal Enterococci	Blanc Sample Blanc RT-PCR	Positive control	Result
Neg	Neg	Pos	Negative
Neg	Neg	Neg	Failed experiment
Pos	Pos	Pos	Failed experiment
Pos	Pos	Neg	Failed experiment
Pos	Neg	Pos	Positive
Pos	Neg	Neg	Positive
Neg	Pos	Neg	Failed experiment
Neg	Pos	Pos	Failed experiment

The following criteria are used to score the results:
Positive: Ct values<36 (average of duplicate reactions)
Negative: Ct values>36 (average of duplicate reactions)

10. Waste processing

Dispose the microbiological waste and PCR plates in the appropriate waste.

11. Literature

- NPR 6268: Algemene principes bij kwaliteitsborging van bacteriologisch onderzoek van water.
- Nederlandse Vereniging voor Microbiologie, Veilig werken met micro-organismen, parasieten en cellen in laboratoria en andere werkruimten 4e druk, 2010.
- NPR 7394 Water - Algemene principes bij kwaliteitsborging van moleculairbiologisch onderzoek.

III Sensitivity study

				Culture		RT-PCR								Culture		RT-PCR			
Nr	Name	DW	Contaminant	CFU/100 ml	Pos/Neg	Ct_1	Ct_2	$Ct_{average}$	Pos/Neg	Nr	Name	DW	Contaminant	CFU/100 ml	Pos/Neg	Ct_1	Ct_2	$Ct_{average}$	Pos/Neg
1	DW-1.1	DW-1.1	N/A	0	Neg	N/A	N/A	N/A	Neg	43	Mix-1.1	DW-1.1	STP-1	1	Pos	28.7	28.6	28.7	Pos
2	DW-1.2	DW-1.2	N/A	0	Neg	N/A	N/A	N/A	Neg	44	Mix-1.2	DW-1.2	OW-1.3	1	Pos	31.0	30.9	31.0	Pos
3	DW-1.3	DW-1.3	N/A	0	Neg	N/A	N/A	N/A	Neg	45	Mix-1.3	DW-1.3	OW-1.9	8	Pos	30.4	29.9	30.2	Pos
4	DW-1.4	DW-1.4	N/A	0	Neg	N/A	N/A	N/A	Neg	46	Mix-1.4	DW-1.4	OW-1.11	6	Pos	31.7	31.5	31.6	Pos
5	DW-1.5	DW-1.5	N/A	0	Neg	N/A	N/A	N/A	Neg	47	Mix-1.5	DW-1.5	STP-1	5	Pos	30.0	29.8	29.9	Pos
6	DW-1.6	DW-1.6	N/A	0	Neg	N/A	N/A	N/A	Neg	48	Mix-1.6	DW-1.6	OW-1.9	8	Pos	30.0	29.8	29.9	Pos
7	DW-1.7	DW-1.7	N/A	0	Neg	N/A	N/A	N/A	Neg	49	Mix-1.7	DW-1.7	OW-1.11	6	Pos	29.9	30.0	29.9	Pos
8	DW-1.8	DW-1.8	N/A	0	Neg	N/A	N/A	N/A	Neg	50	Mix-1.8	DW-1.8	OW-1.11	9	Pos	29.6	29.5	29.5	Pos
9	DW-1.9	DW-1.9	N/A	0	Neg	N/A	N/A	N/A	Neg	51	Mix-1.9	DW-1.9	STP-1	5	Pos	30.4	30.4	30.4	Pos
10	DW-1.10	DW-1.10	N/A	0	Neg	N/A	N/A	N/A	Neg	52	Mix-1.10	DW-1.10	OW-1.9	14	Pos	30.0	29.8	29.9	Pos
11	DW-1.11	DW-1.11	N/A	0	Neg	N/A	N/A	N/A	Neg	53	Mix-1.11	DW-1.11	OW-1.11	8	Pos	30.7	30.5	30.6	Pos
12	DW-1.12	DW-1.12	N/A	0	Neg	N/A	N/A	N/A	Neg	54	Mix-1.12	DW-1.12	STP-1	1	Pos	28.8	28.8	28.8	Pos
13	DW-1.13	DW-1.13	N/A	0	Neg	N/A	N/A	N/A	Neg	55	Mix-1.15	DW-1.15	OW-1.9	11	Pos	31.1	31.1	31.1	Pos
14	DW-1.14	DW-1.14	N/A	0	Neg	N/A	N/A	N/A	Neg	56	Mix-1.16	DW-1.16	OW-1.11	4	Pos	30.4	30.2	30.3	Pos
15	DW-1.15	DW-1.15	N/A	0	Neg	N/A	N/A	N/A	Neg	57	Mix-1.17	DW-1.17	RWZI-1	3	Pos	31.1	30.8	31.0	Pos
16	DW-1.16	DW-1.16	N/A	0	Neg	N/A	N/A	N/A	Neg	58	Mix-1.18	DW-1.18	OW-1.9	14	Pos	30.9	30.7	30.8	Pos
17	DW-1.17	DW-1.17	N/A	0	Neg	N/A	N/A	N/A	Neg	59	Mix-1.19	DW-1.19	OW-1.11	6	Pos	30.2	30.2	30.2	Pos
18	DW-1.18	DW-1.18	N/A	0	Neg	N/A	N/A	N/A	Neg	60	Mix-1.20	DW-1.20	STP-1	4	Pos	29.9	29.7	29.8	Pos
19	DW-1.19	DW-1.19	N/A	0	Neg	N/A	N/A	N/A	Neg	61	Mix-2.1	DW-2.1	STP-2	5	Pos	28.9	29.0	28.9	Pos
20	DW-1.20	DW-1.20	N/A	0	Neg	N/A	N/A	N/A	Neg	62	Mix-2.2	DW-2.2	OW-2.6	0	Neg	N/A	N/A	N/A	Neg
21	DW-2.1	DW-2.1	N/A	0	Neg	28.7	28.7	28.7	Pos	63	Mix-2.3	DW-2.3	OW-2.7	5	Pos	32.3	32.6	32.4	Pos
22	DW-2.2	DW-2.2	N/A	0	Neg	N/A	N/A	N/A	Neg	64	Mix-2.4	DW-2.4	OW-2.10	3	Pos	34.5	35.0	34.7	Pos
23	DW-2.3	DW-2.3	N/A	0	Neg	N/A	N/A	N/A	Neg	65	Mix-2.5	DW-2.5	STP-2	5	Pos	29.6	29.6	29.6	Pos
24	DW-2.4	DW-2.4	N/A	0	Neg	N/A	N/A	N/A	Neg	66	Mix-2.6	DW-2.6	OW-2.6	0	Neg	39.0	35.9	37.4	Neg
25	DW-2.5	DW-2.5	N/A	0	Neg	N/A	N/A	N/A	Neg	67	Mix-2.7	DW-2.7	OW-2.7	1	Pos	34.1	33.8	33.9	Pos
26	DW-2.6	DW-2.6	N/A	0	Neg	N/A	N/A	N/A	Neg	68	Mix-2.8	DW-2.8	OW-2.10	2	Pos	34.5	33.6	34.0	Pos
27	DW-2.7	DW-2.7	N/A	0	Neg	N/A	N/A	N/A	Neg	69	Mix-2.9	DW-2.9	STP-2	4	Pos	31.7	31.7	31.7	Pos
28	DW-2.8	DW-2.8	N/A	0	Neg	N/A	N/A	N/A	Neg	70	Mix-2.10	DW-2.10	STP-2	5	Pos	29.8	30.0	29.9	Pos
29	DW-2.9	DW-2.9	N/A	0	Neg	N/A	N/A	N/A	Neg	71	Mix-2.11	DW-2.11	OW-2.4	0	Neg	N/A	N/A	N/A	Neg
30	DW-2.10	DW-2.10	N/A	0	Neg	42.4	34.7	38.6	Neg	72	Mix-2.12	DW-2.12	OW-2.6	1	Pos	41.9	36.1	39.0	Neg
31	DW-2.11	DW-2.11	N/A	0	Neg	N/A	N/A	N/A	Neg	73	Mix-2.13	DW-2.13	OW-2.7	2	Pos	33.7	32.7	33.2	Pos
32	DW-2.12	DW-2.12	N/A	0	Neg	N/A	N/A	N/A	Neg	74	Mix-2.14	DW-2.14	OW-2.10	4	Pos	31.3	31.2	31.3	Pos
33	DW-2.13	DW-2.13	N/A	0	Neg	N/A	N/A	N/A	Neg	75	Mix-2.15	DW-2.15	STP-2	9	Pos	30.5	30.6	30.6	Pos
34	DW-2.14	DW-2.14	N/A	0	Neg	N/A	N/A	N/A	Neg	76	Mix-2.16	DW-2.16	OW-2.6	0	Neg	N/A	N/A	N/A	Neg
35	DW-2.15	DW-2.15	N/A	0	Neg	N/A	N/A	N/A	Neg	77	Mix-2.17	DW-2.17	OW-2.7	1	Pos	35.7	36.0	35.8	Pos
36	DW-2.16	DW-2.16	N/A	0	Neg	N/A	N/A	N/A	Neg	78	Mix-2.18	DW-2.18	OW-2.10	4	Pos	39.9	37.3	38.6	Neg
37	DW-2.17	DW-2.17	N/A	0	Neg	N/A	N/A	N/A	Neg	79	Mix-2.19	DW-2.19	STP-2	2	Pos	30.7	30.4	30.6	Pos
38	DW-2.18	DW-2.18	N/A	0	Neg	31.3	31.6	31.5	Pos	80	Mix-2.20	DW-2.20	OW-2.6	0	Neg	35.1	35.0	35.1	Pos
39	DW-2.19	DW-2.19	N/A	0	Neg	N/A	N/A	N/A	Neg	81	Mix-2.21	DW-2.21	OW-2.7	0	Neg	38.1	42.5	40.3	Neg
40	DW-2.20	DW-2.20	N/A	0	Neg	N/A	N/A	N/A	Neg										
41	DW-2.21	DW-2.21	N/A	0	Neg	31.6	31.5	31.5	Pos										
42	DW KWR			0	Neg	N/A	N/A	N/A	Neg										

IV RLOD study

	E. casseliflavus					E. moraviensis					E. faecium					E. faecalis				
	Culture	rRT-PCR				Culture	rRT-PCR				Culture	RT- PCR				Culture	RT- PCR			
CFU/100ml	Colonies/100ml	Ct_1	Ct_2	Ct_{av}	Result	Colonies/100ml	Ct_1	Ct_2	Ct_{av}	Result	Colonies/100ml	Ct_1	Ct_2	Ct_{av}	Result	Colonies/100ml	Ct_1	Ct_2	Ct_{av}	Result
0	0	N/A	N/A	N/A	Neg	0	N/A	N/A	N/A	Neg	0	N/A	N/A	N/A	Neg	0	N/A	N/A	N/A	Neg
0	0	N/A	N/A	N/A	Neg	0	N/A	N/A	N/A	Neg	0	N/A	N/A	N/A	Neg	0	N/A	N/A	N/A	Neg
0	0	N/A	N/A	N/A	Neg	0	N/A	N/A	N/A	Neg	0	N/A	N/A	N/A	Neg	0	N/A	N/A	N/A	Neg
0	0	N/A	N/A	N/A	Neg	0	N/A	N/A	N/A	Neg	0	N/A	N/A	N/A	Neg	0	N/A	N/A	N/A	Neg
0	0	N/A	N/A	N/A	Neg	0	N/A	N/A	N/A	Neg	0	N/A	N/A	N/A	Neg	0	N/A	N/A	N/A	Neg
0,7	0	26,3	26,3	26,3	Pos	0	N/A	N/A	N/A	Neg	0	32,6	32,8	32,7	Pos	0	36,1	35,4	35,7	Pos
0,7	0	NA	NA	N/A	Neg	0	N/A	N/A	N/A	Neg	0	31,2	31,7	31,5	Pos	0	38,1	36,3	37,2	Neg
0,7	0	37,2	36,6	36,9	Pos	0	32,4	32,5	32,4	Pos	0	30,6	30,8	30,7	Pos	2	N/A	N/A	N/A	Neg
0,7	0	NA	NA	N/A	Neg	0	N/A	N/A	N/A	Neg	0	29,7	34,3	32,0	Pos	1	34,0	33,8	33,9	Pos
0,7	0	NA	NA	N/A	Neg	0	N/A	N/A	N/A	Neg	0	31,4	32,1	31,7	Pos	1	N/A	N/A	N/A	Neg
0,7	1	27,1	26,8	26,9	Pos	0	N/A	N/A	N/A	Neg	0	N/A	N/A	N/A	Neg	0	32,8	32,1	32,5	Pos
0,7	1	N/A	N/A	N/A	Neg	0	N/A	N/A	N/A	Neg	0	31,1	32,1	31,6	Pos	1	32,2	32,0	32,1	Pos
0,7	1	N/A	N/A	N/A	Neg	0	29,0	29,0	29,0	Pos	1	30,7	29,8	30,3	Pos	0	33,4	33,3	33,4	Pos
0,7	1	N/A	N/A	N/A	Neg	0	N/A	N/A	N/A	Neg	1	31,9	30,8	31,4	Pos	0	N/A	N/A	N/A	Neg
0,7	1	N/A	N/A	N/A	Neg	0	32,7	32,7	32,7	Pos	1	30,7	30,8	30,7	Pos	0	33,5	34,8	34,2	Pos
0,7	1	32,5	34,2	33,4	Pos	0	29,5	29,7	29,6	Pos	1	33,9	34,5	34,2	Pos	1	N/A	N/A	N/A	Neg
0,7	1	27,1	27,0	27,0	Pos	0	N/A	N/A	N/A	Neg	1	33,5	33,6	33,6	Pos	1	36,2	34,3	35,2	Pos
0,7	1	N/A	N/A	N/A	Neg	0	30,7	30,7	30,7	Pos	1	39,3	37,6	38,5	Neg	0	36,2	34,7	35,4	Pos
0,7	1	N/A	N/A	N/A	Neg	1	34,7	35,9	35,3	Pos	1	32,8	33,1	33,0	Pos	2	30,4	30,1	30,3	Pos
0,7	2	N/A	N/A	N/A	Neg	1	30,7	30,5	30,6	Pos	2	34,6	34,2	34,4	Pos	2	31,9	31,7	31,8	Pos
0,7	2	28,2	28,3	28,2	Pos	1	N/A	N/A	N/A	Neg	2	29,8	30,0	29,9	Pos	1	36,0	35,6	35,8	Pos
0,7	2	29,0	29,1	29,0	Pos	1	N/A	N/A	N/A	Neg	2	N/A	N/A	N/A	Neg	0	N/A	N/A	N/A	Neg
0,7	2	36,7	36,5	36,6	Pos	1	N/A	N/A	N/A	Neg	2	34,5	33,2	33,8	Pos	2	25,9	25,9	25,9	Pos
0,7	2	27,3	27,1	27,2	Pos	1	34,3	34,3	34,3	Pos	2	34,5	33,1	33,8	Pos	0	31,2	31,6	31,4	Pos
0,7	2	27,6	27,7	27,6	Pos	2	32,7	34,8	33,7	Pos	2	30,9	32,4	31,6	Pos	0	N/A	N/A	N/A	Neg
Average colonies/100ml	1,1					0,4					1,0					0,7				
Positives	15				10	7				8	13				17	13				14
Negatives	5				10	13				12	7				3	7				6
2	1	28,7	28,2	28,5	Pos	0	29,4	29,4	29,4	Pos	1	31,8	32,8	32,3	Pos	1	N/A	N/A	N/A	Neg
2	1	29,4	29,2	29,3	Pos	0	28,8	28,6	28,7	Pos	1	31,2	31,4	31,3	Pos	0	31,2	31,6	31,4	Pos
2	1	29,2	29,6	29,4	Pos	0	33,0	32,7	32,9	Pos	1	29,7	30,0	29,9	Pos	1	33,3	33,7	33,5	Pos
2	3	28,6	28,7	28,7	Pos	0	27,8	27,7	27,7	Pos	1	30,5	31,4	30,9	Pos	4	29,0	29,1	29,1	Pos
2	3	31,6	32,3	31,9	Pos	1	N/A	N/A	N/A	Neg	2	29,3	29,5	29,4	Pos	0	32,2	32,7	32,4	Pos
2	4	28,1	28,2	28,2	Pos	1	28,4	28,1	28,2	Pos	3	31,2	31,5	31,3	Pos	2	N/A	N/A	N/A	Neg
2	4	33,6	33,1	33,3	Pos	1	29,8	29,4	29,6	Pos	4	30,9	30,8	30,8	Pos	2	N/A	N/A	N/A	Neg
2	4	32,8	32,7	32,7	Pos	2	31,0	30,9	31,0	Pos	0	31,1	31,1	31,1	Pos	2	29,5	29,4	29,4	Pos
2	5	30,9	30,7	30,8	Pos	2	27,0	27,0	27,0	Pos	0	30,8	31,7	31,2	Pos	2	32,0	32,4	32,2	Pos
2	5	28,4	28,3	28,3	Pos	3	29,8	30,1	29,9	Pos	0	31,3	31,2	31,2	Pos	2	31,2	31,2	31,2	Pos
Average colonies/100ml	3,3					1,9					2,3					2,2				
Positives	10				10	6				9	7				10	8				7
Negatives	0				5	4				1	3				0	2				3
5	11	28,7	28,5	28,6	Pos	2	26,4	26,1	26,2	Pos	4	29,3	29,0	29,1	Pos	9	29,6	29,7	29,7	Pos
5	6	28,4	28,2	28,3	Pos	2	26,8	26,2	26,5	Pos	4	29,7	28,9	29,3	Pos	6	29,7	29,8	29,7	Pos
5	6	29,0	28,6	28,8	Pos	1	28,0	27,8	27,9	Pos	5	30,0	29,7	29,9	Pos	7	31,8	31,6	31,7	Pos
5	10	29,0	28,7	28,8	Pos	6	28,1	27,5	27,8	Pos	5	29,9	29,8	29,8	Pos	5	29,9	29,9	29,9	Pos
5	10	30,1	30,2	30,1	Pos	7	27,2	27,7	27,5	Pos	9	29,7	28,9	29,3	Pos	4	29,1	29,0	29,1	Pos
Average colonies/100ml	8,6					3,6					5,4					6,2				
Positives	5				5	5				5	5				5	5				5
Negatives	0				0	0				0	0				0	0				0

V Inclusivity and exclusivity

Raw data inclusivity study

Nr	Species	Strain ID	Culture S&B (CFU/test sample)			RT-PCR			
			A	B	ge	CT_1	CT_2	CT_{av}	Result
1	*E. faecalis*	DSM20478	4	9	6.5	32.6	32.4	32.5	Pos
2	*E. faecalis*	ATCC19433				RLOD study			Pos
3	*E. faecalis*	Vitens 2	10	9	9.5	31.5	31.4	31.5	Pos
4	*E. faecalis*	Vitens 7	2	3	2.5	31.8	31.6	31.7	Pos
5	*E. faecalis*	Mens 19/1	4	3	3.5	35.4	36.6	36.0	Pos
6	*E. faecalis*	Gans 1/1	7	20	13.5	35.1	35.8	35.5	Pos
7	*E. faecium*	DSM20477	4	4	4.0	31.5	31.5	31.5	Pos
8	*E. faecium*	ATCC19434				RLOD study			Pos
9	*E. faecium*	Vitens 35	1	2	1.5	29.9	29.8	29.9	Pos
10	*E. faecium*	Vitens 14	4	4	4.0	31.8	31.4	31.6	Pos
11	*E. faecium*	Vitens 26	5	5	5.0	30.7	31.1	30.9	Pos
12	*E. faecium*	Mens 20/1	1	2	1.5	33.1	32.7	32.9	Pos
13	*E. durans*	DSM20633	5	8	6.5	31.3	31.5	31.4	Pos
14	*E. durans*	Vitens 20	2	3	2.5	29.1	29.2	29.2	Pos
15	*E. durans*	Vos 13/3	5	6	5.5	28.9	28.7	28.8	Pos
16	*E. durans*	Vitens 18-1	2	4	3.0	31.2	31.5	31.4	Pos
17	*E. durans*	Mens 1/2	3	4	3.5	28.0	27.7	27.9	Pos
18	*E. hirae*	DSM28619	11	17	14.0	31.4	31.7	31.6	Pos
19	*E. hirae*	Vitens 19	6	18	12.0	28.3	28.4	28.4	Pos
20	*E. hirae*	Vitens 98	1	1	1.0	33.6	33.9	33.8	Pos
21	*E. hirae*	Vitens 97	4	2	3.0	29.5	29.7	29.6	Pos
22	*E. hirae*	Mens 10/7	5	3	4.0	29.3	29.1	29.2	Pos
23	*E. casseliflavus*	DSM20680	1	1	1.0	29.3	29.5	29.4	Pos
24	*E. casselliflavus*	DSM20382	6	11	8.5	29.3	29.2	29.3	Pos
25	*E. casselliflavus*	MCOC56	4	3	3.5	31.8	31.8	31.8	Pos
26	*E. casselliflavus*	MCOC48	2	3	2.5	33.1	32.4	32.8	Pos
27	*E. casselliflavus*	MCOC14	8	9	8.5	33.5	33.9	33.7	Pos
28	*E. casselliflavus*	MCOC32	5	5	5.0	29.3	29.7	29.5	Pos
29	*E. mundtii*	DSM4838	7	5	6.0	31.2	29.7	30.5	Pos
30	*E. mundtii*	DSM4839	2	6	4.0	32.2	32.8	32.5	Pos
31	*E. mundtii*	MCOC30	5	5	5.0	29.4	29.2	29.3	Pos
32	*E. mundtii*	MCOC33	5	5	5.0	30.4	28.5	29.5	Pos
33	*E. mundtii*	MCOC34	5	6	5.5	29.5	29.1	29.3	Pos
34	*E. mundtii*	MCOC43	7	8	7.5	27.6	27.7	27.7	Pos
35	*E. gallinarum*	DSM24841	1	2	1.5	30.6	30.2	30.4	Pos
36	*E. gallinarum*	DSM20628	1	3	2.0	28.4	28.5	28.5	Pos
37	*E. gallinarum*	MCOC66	2	2	2.0	34.6	24.6	29.6	Pos
38	*E. gallinarum*	MCOC91	2	1	1.5	31.5	32.2	31.9	Pos
39	*E. gallinarum*	MCOC126	2	2	2.0	29.0	29.2	29.1	Pos
40	*E. moraviensis*	DSM15919	2	2	2.0	32.5	32.2	32.4	Pos
41	*E. moraviensis*	MCOC123	3	3	3.0	33.3	35.2	34.3	Pos
42	*E. moraviensis*	MCOC124	3	3	3.0	33.0	32.9	33.0	Pos
43	*E. moraviensis*	MCOC127	1	1	1.0	34.8	34.1	34.5	Pos
44	*E. moraviensis*	MCOC118	2	2	2.0	31.7	32.2	32.0	Pos
45	*E. haemoperoxidus*	DSM15920	18	24	21.0	30.9	30.0	30.5	Pos
46	*E. haemoperoxidus*	MCOC105	2	3	2.5	32.5	32.7	32.6	Pos
47	*E. haemoperoxidus*	MCOC108	1	1	1.0	32.5	32.7	32.6	Pos
48	*E. haemoperoxidus*	MCOC111	5	3	4.0	33.8	33.6	33.7	Pos
49	*E. haemoperoxidus*	MCOC115	2	1	1.5	33.8	33.6	33.7	Pos
50	*E. haemoperoxidus*	MCOC120	3	3	3.0	32.5	32.2	32.4	Pos
51	*E. avium*	DSM20679	13	17	15.0	33.1	33.0	33.1	Pos
52	*E. avium*	DSM20063	8	9	8.5	33.9	32.7	33.3	Pos
53	*E. avium*	MCOC56	4	4	4.0	30.1	29.4	29.8	Pos
54	*E. avium*	Mens 2	1	2	1.5	32.2	32.2	32.2	Pos
55	*E. avium*	Mens 16	4	9	6.5	32.1	32.1	32.1	Pos

Raw data exclusivity study

Nr	Strain		Culture	rRT-PCR		
	Species	Strain ID	CFU	Ct1	Ct2	Result
1	*Enterobacter aerogenes*	EPA202 (ATCC 13048)	250	N/A	N/A	Neg
2	*Enterobacter cloacae*	WR3	540	N/A	N/A	Neg
3	*Staphylococcus warneri*	WR51	129	N/A	N/A	Neg
4	*Staphylococcus aureus*	WR10	169	N/A	N/A	Neg
5	*Aerococcus viridans*	DSM 20311	73	N/A	N/A	Neg
6	*Aerococcus suis*	DSM 21500	120	N/A	N/A	Neg
7	*Streptococcus anginosus*	DSM 20563	90	N/A	N/A	Neg
8	*Hydrogenophaga pseudoflava*	DSM 1034	206	N/A	N/A	Neg
9	*Aquabacterium commune*	DSM 11901	89	N/A	N/A	Neg
10	*Aquabacterium parvum*	DSM 11968	74	N/A	N/A	Neg
11	*Citrobacter freundii*	DSM 30039	660	N/A	N/A	Neg
12	*Escherichia coli*	WR1	524	N/A	N/A	Neg
13	*Klebsiella oxytoca*	DSM 5175	335	N/A	N/A	Neg
14	*Serratia marcescens*	DSM 30121	320	N/A	N/A	Neg
15	*Kluyvera ascorbata*	DSM 4611	460	N/A	N/A	Neg
16	*Pantoea agglomerans*	DSM 3493	148	N/A	N/A	Neg
17	*Cronobacter sakazakii*	DSM 4485	622	N/A	N/A	Neg
18	*Klebsiella pneumoniae*	DSM30104	80	N/A	N/A	Neg
19	*Aeromonas hydrophila*	DSM6173	198	N/A	N/A	Neg
20	*Pseudomonas fluorescence*	P17 (ATCC 49642)	520	N/A	N/A	Neg
21	*Aquaspirillum sp.*	NOX (ATCC 49643)	186	N/A	N/A	Neg
22	*Salmonella panama*	SP5	80	N/A	N/A	Neg
23	*Flavobacterium johnsoniae*	A3	189	N/A	N/A	Neg
24	*Escherichia fergusonii*	DSM13698	208	N/A	N/A	Neg
25	*Aeromonas veronii*	ATCC 35624	160	N/A	N/A	Neg
26	*Staphylococcus epidermidis*	*DSM1798*	177	N/A	N/A	Neg
27	*Streptococcus equinus*	*DSM20062*	300	N/A	N/A	Neg
28	*Streptococcus salivarius*	*DSM20067*	96	N/A	N/A	Neg
29	*Legionella pneumophila*	*ATCC33152*	412*	N/A	N/A	Neg
30	*Legionella anisa*	ATCC 35291	519*	N/A	N/A	Neg
	Other enterococci species (not part of the 10 species)					Neg
31	*Enterococcus termitis*	*DSM22803*	19	27	27	Pos
32	*Enterococcus malodoratus*	*DSM20681*	71	N/A	N/A	Neg
33	*Enterococcus villorum*	*DSM15688*	408	N/A	N/A	Neg
			* Legionella species were cultured on BCYE			

VI Interlaboratory study

April 06 2023		Aqualab Zuid								HWL							
Sample	Concentration	Participant 1				Participant 2				Participant 3				Participant 4			
		RT-PCR				RT-PCR				RT-PCR				RT-PCR			
	(CFU/100 ml)	Ct_1	Ct_2	Ct_{av}	Culture	Ct_1	Ct_2	Ct_{av}	Culture	Ct_1	Ct_2	Ct_{av}	Culture	Ct_1	Ct_2	Ct_{av}	Culture
1	5	28.1	28.0	28.1	6	28.1	28.2	28.1	3	32.4	32.8	32.6	7	31.3	31.2	31.3	7
2	0	ND	ND	ND	0	ND	ND	ND	0	ND	ND	ND	0	35.9	39.0	37.4	0
3	5	29.8	29.9	29.8	8	29.0	28.9	28.9	6	33.2	34.1	33.6	4	31.3	31.2	31.3	8
4	5	30.1	27.5	28.8	9	40.9	ND	43.0	2	32.3	32.4	32.4	6	30.1	30.1	30.1	5
5	5	30.2	30.5	30.4	5	29.0	28.6	28.8	5	32.1	32.2	32.2	5	31.7	31.6	31.6	1
6	0	ND	40.1	42.5	0	40.2	ND	42.6	0	ND	ND	ND	0	ND	ND	ND	0
7	0	ND	ND	ND	0	37.4	39.3	38.3	0	37.1	35.0	36.0	0	ND	ND	ND	0
8	5	28.7	28.8	28.8	7	27.0	27.5	27.2	7	30.8	30.3	30.5	7	32.8	32.7	32.7	4
9	5	29.8	30.2	30.0	3	30.7	30.9	30.8	4	34.6	34.3	34.4	6	32.4	32.6	32.5	9
10	0	ND	ND	ND	0	44.3	ND	44.7	0	ND	ND	ND	0	ND	ND	ND	0
11	5	27.9	27.9	27.9	8	28.8	28.4	28.6	5	31.1	31.2	31.2	9	31.1	31.0	31.1	5
12	5	32.9	29.0	31.0	2	31.0	31.0	31.0	2	31.2	31.8	31.5	4	30.1	29.9	30.0	6
Culture: average per participant (CFU/100ml)					6.0				4.3				6.0				5.6
Culture: average of all (CFU/100 ml)					6.1												

April 06 2023		Vitens								WLN							
Sample	Concentration	Participant 5				Participant 6				Participant 7				Participant 8			
		RT-PCR				RT-PCR				RT-PCR				RT-PCR			
	(CFU/100 ml)	Ct_1	Ct_2	Ct_{av}	Culture	Ct_1	Ct_2	Ct_{av}	Culture	Ct_1	Ct_2	Ct_{av}	Culture	Ct_1	Ct_2	Ct_{av}	Culture
1	5	30.1	29.8	29.9	6	30.5	30.6	30.5	5	28.0	27.8	27.9	2	28.1	28.1	28.1	7
2	0	ND	ND	ND	0	ND	ND	ND	0	41.0	37.1	39.1	0	ND	38.2	ND	0
3	5	30.6	30.6	30.6	5	31.2	31.2	31.2	12	28.6	28.0	28.3	4	28.8	29.1	29.0	4
4	5	29.3	29.9	29.6	10	31.9	31.9	31.9	5	27.3	27.1	27.2	6	28.2	28.2	28.2	10
5	5	31.0	31.0	31.0	7	31.4	31.5	31.5	6	28.6	27.6	28.1	7	28.1	28.1	28.1	5
6	0	ND	ND	ND	0	ND	ND	ND	0	ND	ND		0	41.7	ND	ND	0
7	0	ND	ND	ND	0	44.0	ND	44.0	0	ND	ND		0	ND	ND	ND	0
8	5	31.0	30.6	30.8	4	30.5	30.4	30.4	7	30.2	30.2	30.2	6	29.5	29.5	29.5	7
9	5	29.1	29.1	29.1	11	31.8	31.7	31.8	3	28.3	28.2	28.3	13	30.4	30.3	30.4	7
10	0	ND	ND	ND	0	41.7	ND	41.7	0	ND	ND		0	35.4	33.9	34.7	0
11	5	29.2	29.1	29.1	8	35.2	33.7	34.5	11	26.9	26.4	26.6	10	27.7	27.9	27.8	7
12	5	30.3	30.2	30.2	4	29.5	29.6	29.5	10	28.7	28.8	28.8	7	28.4	28.1	28.2	7
Culture: average per participant (CFU/100ml)					6.9				7.4				6.9				6.8

April 06 2023		De Watergroep								KWR				Pidpa			
Sample	Concentration	Participant 9				Participant 10				Participant 11				Participant 12			
		RT-PCR				RT-PCR				RT-PCR				RT-PCR			
	(CFU/100 ml)	Ct_1	Ct_2	Ct_{av}	Culture	Ct_1	Ct_2	Ct_{av}	Culture	Ct_1	Ct_2	Ct_{av}	Culture	Ct_1	Ct_2	Ct_{av}	Culture
1	5	32.6	31.0	31.8	1	29.9	29.4	29.7	6	30.4	30.4	30.4	4	31.1	32.2	31.6	6
2	0	ND	ND	ND	0	ND	ND	ND	0	ND	ND	ND	0	ND	ND	ND	0
3	5	31.9	33.2	ND	2	30.4	30.1	30.3	3	32.7	33.0	32.8	4	31.5	31.2	31.4	7
4	5	31.5	31.4	31.4	8	31.5	31.3	31.4	10	30.4	30.1	30.3	2	31.7	31.6	31.7	5
5	5	31.3	30.7	31.0	9	30.5	30.3	30.4	10	30.7	30.6	30.7	4	32.2	31.8	32.0	4
6	0	ND	ND	ND	0	ND	ND	ND	0	ND	ND	ND	0	ND	ND	ND	0
7	0	ND	ND	ND	0	ND	ND	ND	0	ND	ND	ND	0	33.2	32.6	32.9	0
8	5	32.0	31.7	31.8	11	30.7	30.9	30.8	9	31.5	31.9	31.7	14	32.1	31.9	32.0	5
9	5	33.1	32.3	32.7	3	29.7	30.0	29.8	4	30.7	30.5	30.6	5	31.7	30.9	31.3	8
10	0	ND	ND	ND	0	ND	ND	ND	0	ND	ND	ND	0	ND	ND	ND	0
11	5	30.1	30.1	30.1	6	30.4	30.5	30.4	2	30.0	29.8	29.9	6	33.4	33.1	33.2	4
12	5	30.2	31.1	30.6	6	30.2	30.2	30.2	11	29.1	29.1	29.1	6	30.8	30.6	30.7	8
Culture: average per participant (CFU/100ml)					5.8				6.9				5.6				5.9

May 09 2023		Aqualab Zuid								HWL							
Sample	Concentration	Participant 1				Participant 2				Participant 3				Participant 4			
		RT-PCR				RT-PCR				RT-PCR				RT-PCR			
	(CFU/100 ml)	Ct_1	Ct_2	Ct_{av}	Culture	Ct_1	Ct_2	Ct_{av}	Culture	Ct_1	Ct_2	Ct_{av}	Culture	Ct_1	Ct_2	Ct_{av}	Culture
13	1	29.7	29.5	29.6	1	31.0	30.7	30.8	2	31.8	31.6	31.7	0	ND	ND	ND	3
14	0	45.0	44.6	44.8	0	35.4	37.6	36.5	0	ND	ND	ND	0	34.5	34.6	34.6	0
15	1	32.9	32.3	32.6	2	30.2	30.0	30.1	0	34.9	34.6	34.7	0	38.5	36.0	37.2	0
16	1	31.0	32.5	31.8	0	30.9	31.1	31.0	1	ND	ND	ND	2	29.7	29.8	29.7	3
17	0	45.0	42.3	43.7	0	35.9	ND	ND	0	ND	ND	ND	0	ND	ND	ND	0
18	1	31.1	31.3	31.2	2	35.3	40.7	38.0	2	31.8	32.1	31.9	0	32.7	33.6	33.2	1
19	1	32.7	32.2	32.5	1	29.1	28.7	28.9	1	ND	40.4	ND	1	ND	ND	ND	2
20	0	ND	ND	ND	0	36.3	36.9	36.6	0	ND	35.2	ND	0	ND	ND	ND	0
21	1	ND	ND	ND	0	36.2	34.5	35.3	2	30.2	30.1	30.1	2	40.1	ND	ND	0
22	1	ND	ND	ND	0	35.0	36.4	35.7	0	ND	ND	ND	0	36.5	ND	ND	1
23	0	ND	ND	ND	0	42.6	ND	43.8	0	38.6	ND	38.6	0	ND	ND	ND	0
24	1	31.8	31.6	31.7	3	30.5	30.3	30.4	1	ND	41.8	41.8	1	ND	39.8	39.8	1
Culture: average per participant (CFU/100ml)					1.1				1.1				0.8				1.4
Culture: average of all (CFU/100 ml)					1.0												

May 09 2023		Vitens								WLN							
Sample	Concentration	Participant 5				Participant 6				Participant 7				Participant 8			
		RT-PCR				RT-PCR				RT-PCR				RT-PCR			
	(CFU/100 ml)	Ct_1	Ct_2	Ct_{av}	Culture	Ct_1	Ct_2	Ct_{av}	Culture	Ct_1	Ct_2	Ct_{av}	Culture	Ct_1	Ct_2	Ct_{av}	Culture
13	1	No data due to Technical problems with RT-PCR device				30.3	30.1	30.2	2			29.9	0			31.1	3
14	0					ND	ND	ND	0			ND	0			30.8	0
15	1					30.5	30.4	30.4	1			31.5	0			31.3	0
16	1					30.7	30.8	30.7	1			29.5	0			ND	1
17	0					ND	ND	ND	0			ND	0			ND	0
18	1					30.5	30.4	30.4	0			ND	0			30	0
19	1					29.0	28.9	28.9	1			ND	2			32	1
20	0					ND	ND	ND	0			ND	0			ND	0
21	1					ND	ND	ND	1			ND	1			30.3	2
22	1					ND	ND	ND	5			29.3	2			ND	0
23	0					ND	ND	ND	0			ND	0			ND	0
24	1					31.0	30.6	30.8	0			30.3	0			30.1	2
Culture: average per participant (CFU/100ml)					1.6				1.4				0.6				1.1

May 09 2023		De Watergroep								KWR				Pidpa			
Sample	Concentration	Participant 9				Participant 10				Participant 11				Participant 12			
		RT-PCR				RT-PCR				RT-PCR				RT-PCR			
	(CFU/100 ml)	Ct_1	Ct_2	Ct_{av}	Culture	Ct_1	Ct_2	Ct_{av}	Culture	Ct_1	Ct_2	Ct_{av}	Culture	Ct_1	Ct_2	Ct_{av}	Culture
13	1	41.3	ND	41.3	0	38.0	35.2	36.6	1	32.4	31.8	32.1	0	32.2	31.9	32.0	1
14	0	ND	ND	ND	0	34.2	35.8	35.0	0	ND	ND	ND	0	ND	ND	ND	0
15	1	ND	ND	ND	0	ND	ND	ND	0	36.8	35.3	36.0	3	ND	ND	ND	0
16	1	ND	ND	ND	1	32.9	32.0	32.5	2	31.3	31.1	31.2	3	32.7	32.5	32.6	2
17	0	ND	ND	ND	0	ND	ND	ND	0	ND	ND	ND	0	41.7	ND	41.7	0
18	1	34.8	34.2	34.5	0	ND	ND	ND	2	33.3	33.0	33.2	0	38.5	ND	38.5	1
19	1	34.8	35.6	35.2	0	32.8	33.1	32.9	0	ND	ND	ND	1	31.7	31.5	31.6	1
20	0	ND	ND	ND	0	ND	ND	ND	0	ND	ND	ND	0	39.8	40.2	40.0	0
21	1	33.5	34.6	34.1	1	ND	ND	ND	0	ND	ND	ND	0	34.2	34.4	34.3	1
22	1	32.1	32.4	32.3	2	32.6	33.1	32.9	0	30.4	30.4	30.4	0	ND	ND	ND	0
23	0	ND	ND	ND	0	ND	ND	ND	0	ND	ND	ND	0	ND	ND	ND	0
24	1	36.2	35.2	35.7	1	36.5	38.7	37.6	2	32.1	32.9	32.5	1	32.7	32.7	32.7	0
Culture: average per participant (CFU/100ml)					0.6				0.9				1.0				0.8

VII Application of RT-PCR in practice

The RT-PCR was used by the collaborating drinking water laboratories in practical situations and the results were compared with the culture method to study its application to monitor the hygienic quality of distributed drinking water. The dataset (Table 23) comprises data from samples (collected by Vitens, AqZ, WLN and HWL) after repair or replacement of distribution pipes.

Table 23. Results of RT-PCR and culture analyses on samples collected after repair or replacement of distribution pipes

	Total				
	All 4 labs	**Vitens**	**AqZ**	**WLN**	**HWL**
Culture_positive/RT-PCR_positive (n)	**10**	3	3	3	1
(%)	**0.5**	0.9	1.5	1.7	0.1
Culture_positive/RT-PCR_negative (n)	**10**	1*	2*	4*	3*
(%)	**0.5**	0.3	1.0	2.3	0.3
Culture_negative/RT-PCR_positive (n)	**141**	46	15	9	71
(%)	**7.7**	13.1	7.6	5.2	6.3
Culture_negative/RT-PCR_negative (n)	**1681**	301	178	156	1046
(%)	**91.3**	85.8	89.9	90.7	93.3
Total nr. of samples	**1842**	351	198	172	1121
* concentrations in Culture+/RT-PCR- samples (CFU/100ml):		1	1/1	1/1/1/1	1/1/1

** all cultured intestinal enterococci are typed (using MALDI-TOF) as species belonging to the 10 species targeted by the RT-PCR method.*

The data demonstrates that the results of the culture method match with RT-PCR for the vast majority of samples (91.8%). Most samples (91.3%) were tested negative using RT-PCR and culture whereas only a small fraction was tested positive with both (0.5%) methods. Only 0.5% of the samples was tested positive with the culture method and negative with RT-PCR, all these samples contained intestinal enterococci at a concentration of 1 CFU/100 ml. This means that Culture_positive/RT-PCR_negative discrepancies are likely to be the result of statistical distribution of intestinal enterococci cells in these samples resulting in absence or presence in 100 ml aliquots. Of course, statistical distribution also plays a role in the Culture_negative/RT-PCR_positive fraction of samples. Culture_negative/RT-PCR_positive is the second largest category (7.7%), a small part (presumably 0.5%) of this category can be explained by statistical distribution due to low concentrations in these samples. However, the relatively high percentage suggests a higher sensitivity for RT-PCR or detection of intestinal enterococci which are not culturable with the reference method. A comparable fraction of drinking water samples (7.1%) was also Culture_negative/RT-PCR_positive in the sensitivity study (5.1.1) confirming these results.

Conclusions: application in practice

Based on this dataset with results from samples after repair or replacement of distribution pipes, the following conclusions can be drawn:

- The RT-PCR method delivers the same results as the culture method in 91.3% of all tested samples
- Only 0.5% of the samples are tested Culture_positive/RT-PCR_negative. These discrepancies are likely to be the result of statistical distribution of intestinal enterococci cells in these samples.
- 7.7% of the samples are tested Culture_negative/RT-PCR_Positive meaning that more positive samples are expected when RT-PCR is used to test the hygienic water quality of distributed drinking water.