

## Categorical performance characteristics of method ISO 7899-2 and indicator value of intestinal enterococci for bathing water quality monitoring

Ananda Tiwari, Anna-Maria Hokajärvi, Jorge W. Santo Domingo, Ari Kauppinen, Michael Elk, Hodon Ryu, Balamuralikrishna Jayaprakash and Tarja Pitkänen

### ABSTRACT

Intestinal enterococci indicate the fecal contamination of bathing waters. This study defines the performance characteristics of the reference method ISO 7899-2:2000 with water samples collected from inland and coastal bathing areas in Finland. From a total of 341 bacterial isolates grown on Slanetz and Bartley medium, 63.6% were confirmed as intestinal enterococci on bile aesculin agar. The partial 16S rRNA gene sequences showed that *Enterococcus faecium* and *Enterococcus faecalis* clades accounted for 93.1% of the confirmed isolates. The range of the false positive and false negative rate of the ISO 7899-2 was 0.0–18.5% and 5.6–57.1%, respectively, being affected by the presumptive colony count on the membrane. The analysis of multiple sample volumes is proposed to reach 10–100 colonies per membrane when 47 mm diameter membranes are used to prevent overestimation of low counts and underestimation of the high counts.

**Key words** | bathing water quality monitoring, *Enterococcus* spp., partial 16S rRNA sequencing, Slanetz and Bartley medium, standard method ISO 7899-2

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### INTRODUCTION

Intestinal enterococci are used as fecal indicator bacteria (FIB) for bathing water quality monitoring in European Union (EU) member countries (European Bathing Water Directive; 2006/7/EC). The presence of intestinal enterococci is considered as a sign of fecal contamination in environmental waters since they are released through feces of warm-blooded animals, including human beings (Wheeler *et al.* 2002). The species distribution of enterococci released from feces of different host animals varies and is thought to be similar between the individuals of the same host species

having similar feeding habits (Layton *et al.* 2010). The prevalence of certain *Enterococcus* species has been proposed to provide useful information of the fecal contamination sources (Moore *et al.* 2006). Specifically, *Enterococcus faecium* and *Enterococcus faecalis* are considered the most prevalent *Enterococcus* species in human feces (Moore *et al.* 2006; Layton *et al.* 2010). Pourcher *et al.* (1991) and Wheeler *et al.* (2002) reported that the host range of *E. faecalis* is limited to dogs, humans, and chickens. However, the *Enterococcus* genus is a large group of bacteria and not all species originate from feces. For example, *Enterococcus mundtii*, *Enterococcus casseliflavus*, *Enterococcus aquimarinus*, and *Enterococcus sulfureus* are often associated with vegetation (Muller *et al.* 2001; Moore *et al.* 2006).

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Monitoring regulations for bathing water are based on the FIB counts which indicate the extent, but not the source, of fecal contamination. Different contamination sources can cause varying levels of waterborne infection risk for bathers, sometimes without a clear correlation to FIB counts. Some human pathogens such as enteric viruses are host-specific and originate from human feces (Colford *et al.* 2007). For such agents, contamination of bathing water with human fecal material represents a higher infection risk than contamination from other sources (Colford *et al.* 2007). However, non-human fecal contamination can also contribute to infection risks at bathing areas due to the possible occurrence of zoonotic pathogens such as *Escherichia coli* O157:H7, *Campylobacter*, *Salmonella*, *Leptospira*, *Cryptosporidium*, and *Giardia* (as reviewed in USEPA 2009). Thus, identifying sources of contamination is important to accurately estimate the human health risk and eliminate it.

The international standard method ISO 7899-2 (2000) is the reference method for enumeration of intestinal enterococci according to the European Bathing Water Directive (2006/7/EC), focusing on four intestinal *Enterococcus* species: *E. faecalis*, *E. faecium*, *Enterococcus durans*, and *Enterococcus hirae*. However, the ISO 7899-2 standard does not include specifications of the performance characteristics of the method or specify the reliable counting range for the enterococci enumeration. The present study focused on characterizing the *Enterococcus* species isolated using the ISO 7899-2 method from the bathing water samples and evaluated the categorical performance characteristics (i.e., sensitivity, specificity, selectivity, false positive rate, false negative rate, and efficiency) of the method. In addition, we applied a combination of fecal source tracking and regulatory bathing water quality monitoring to support the interpretation of FIB count results.

## MATERIALS AND METHODS

### Bathing water samples

A total of 21 water samples were collected from five bathing areas in the central and western part of Finland during the bathing season of 2013 with the help of local health

authorities (Table 1). Collected samples were transported to the laboratory in ice coolers, stored at  $3 \pm 2$  °C and analyzed within 24 h.

### Enterococci enumeration

Intestinal enterococci were enumerated using the ISO 7899-2 method as described by Pitkänen *et al.* (2013). In brief, after filtration of water volumes, membranes (GN6, Pall Life Sciences, Michigan, USA) were incubated on Slanetz and Bartley medium (S&B, Oxoid Ltd, Basingstoke, UK) at  $36 \pm 2$  °C for  $44 \pm 4$  h. The sample volumes 1, 10, 100 and/or 1,000 mL were used with the aim to produce 10–100 presumptive colonies per membrane to follow the principles specified in the standard ISO 8199 (2005). With a few exceptions, the total colony counts ranged from 1 to 149 colonies per membrane. All colonies detected were raised as with red, maroon, or pink color and were considered as presumptive enterococci, even when the color was only light or the colony size was very small. After counting the presumptive enterococci, the membranes containing presumptive enterococci colonies were transferred on preheated bile aesculin azide medium (BEA, Scharlau, Barcelona, Spain) and incubated at  $44 \pm 0.5$  °C for 2 h. Black or brown color formation on the BEA medium confirmed the presumptive colony was intestinal enterococci. When no color formation on BEA was observed, the presumptive colony was defined as unconfirmed. All or at least 10 confirmed and 10 unconfirmed colonies per each analyzed sample were sub-cultured on non-selective tryptone soya agar medium (TSA, Oxoid Ltd) and incubated at  $36 \pm 2$  °C for 2 days. The isolates were stored at  $-75$  °C in nutrient broth containing 15% glycerol.

### Partial 16S rRNA sequencing of the bacteria isolated from Slanetz and Bartley medium

To prepare the total genomic DNA of the isolates for the further analysis, sterile inoculating loops were used to transfer bacterial biomass into 0.1 mL of sterile deionized water and stored at  $-18$  °C. Subsequently, the bacterial suspensions were heat-treated at 95 °C for 10 minutes. The *Enterococcus* spp. specific quantitative polymerase chain reaction (qPCR) assay targeting the 23S rRNA gene

**Table 1** | Public bathing areas sampled in the summer of 2013 in central and western parts of Finland

Sampling site	Type of bathing area	Estimated number of bathers/day during the bathing season	Bathing water classification <sup>a</sup>	Identified risks for water quality in the bathing water profile	Beach facilities	Number of samples
A	Inland EU bathing area	>100	Excellent	Runoffs through a nearby stream, waterfowl	Sandy beach, toilets, waste bins	3
B	Inland EU bathing area	Up to 400	Sufficient	Sewage overflow from a pumping station through rain water pumping station, waterfowl	Sandy beach, toilets, park around the beach	4
C	Inland EU bathing area	Up to 1,350	Good	Cyanobacteria due to the eutrophication, sewage overflows from pumping stations, sewage effluents, runoff from rain water system	Two sandy beaches, toilet, wide parks, playground, tennis court	3
D	Coastal EU bathing area	>100	Poor	Waterfowl (gulls), scattered loading, sewage overflows from pumping stations	Sandy beach, toilets, waste bins, camping area beside, different activities for users	6
E	Coastal small national bathing area	Not available	Not available	Profile not available. Dense aquatic vegetation in a shallow bay reported, runoffs from nearby streams, waterfowl and sewage discharges	Not available	5

Characteristics of the bathing area are reported by the local health protection authorities (Valvira 2015).

<sup>a</sup>The classification excellent > good > sufficient > poor is based on based on quality results in 2009–2012.

(Ludwig & Schleifer 2000) was used to confirm the identity of the isolates. The heat-treated suspensions were shipped on dry ice to the laboratory of the United States Environmental Protection Agency (Cincinnati, OH) for 16S rRNA gene sequencing. The partial 16S rRNA gene sequences were obtained by using universal bacterial primers 8F (AGAG TTTGATCCTGGCTCAG) and 787R (CGACTACAGGGTATCT AAT), as described by Ryu et al. (2013).

Unique phylogenetic contigs were selected on the basis of sequence homology with bioinformatics software CD-hits (98% cutoff value) (Li & Godzik 2006). The representative contigs were aligned with reference sequences collected from the National Center for Biotechnology Information (NCBI) GenBank. A neighbor-joining phylogenetic tree was constructed with molecular evolutionary genetics analysis (MEGA) software version 6 with 1,000 bootstrap value and muscle sequence aligner (Tamura et al. 2013). The species identification of the isolates was initially made on the basis of phylogenetic clades, and was cross-validated *in silico* with cluster sequence aligner in MEGA-6 with

*Enterococcus* genus specific primer Ent1 targeting to 16S rRNA sequence as additional *in silico* confirmation. Species-specific primers Faecium1 (*E. faecium*), Faecalis1 (*E. faecalis*), and Casseli1 (*E. casseliflavus*) were also tested *in silico* as described in Ryu et al. (2013) (Supplementary material, Table S1, available with the online version of this paper). The species identification was also confirmed with RDP seqmatch (Cole et al. 2013).

### Performance analysis of the ISO 7899-2 method for enterococci monitoring

The performance of the standard method ISO 7899-2 for intestinal enterococci enumeration from bathing water samples was tested by calculating false positive rate, false negative rate, sensitivity, specificity, selectivity, and efficiency as defined in the standard method ISO 13843 (2017; previous version published as technical report ISO/TR 13843 in 2000) using Equations (1)–(6). True positive isolates were defined as isolates that were confirmed as

intestinal enterococci in the BEA confirmation test (i.e., the primary confirmatory test; ISO 13843:2017) and which belong to either *E. faecalis* or *E. faecium* clade using phylogenetic analysis (i.e., the secondary identification test; ISO 13843:2017). The unconfirmed isolates which belong to *E. faecalis* or *E. faecium* clades were defined as false negative isolates. The isolates confirmed as intestinal enterococci, but did not belong to *E. faecalis* or *E. faecium* clades, were defined as false positive isolates. True negative isolates were defined as unconfirmed isolates that did not belong to *E. faecalis* or *E. faecium* clades.

#### False positive rate

$$= \frac{\text{Number of false positive isolates}}{\text{Number of true positive isolates} + \text{Number of false positive isolates}} \quad (1)$$

#### False negative rate

$$= \frac{\text{Number of false negative isolates}}{\text{Number of false negative isolates} + \text{Number of true negative isolates}} \quad (2)$$

#### Sensitivity

$$= \frac{\text{Number of true positive isolates}}{\text{Number of true positive isolates} + \text{Number of false negative isolates}} \quad (3)$$

$$\text{Specificity} = \frac{\text{Number of true negative isolates}}{\text{Number of false positive isolates} + \text{Number of true negative isolates}} \quad (4)$$

$$\text{Selectivity} = \frac{\text{Number of true positive isolates}}{\text{Number of total studied isolates}} \quad (5)$$

$$\text{Efficiency} = \frac{\text{Number of true positive isolates} + \text{Number of true negative isolates}}{\text{Number of total studied isolates}} \quad (6)$$

### Quantification of the other fecal microbes and source tracking identifiers

*Escherichia coli* was enumerated using Colilert Quanti-Tray method in the local water laboratories near the bathing water sampling locations according to standard method ISO 9308-2 (2012). All the other microbiological analyses were carried out in the laboratory of the National Institute

for Health and Welfare (Kuopio, Finland). The primers and probes used in the qPCR and reverse transcriptase (RT)-qPCR assays of this study are listed in the Supplementary material (Table S1).

Noroviruses and adenoviruses were concentrated from 800 to 2,000 mL water samples as previously described (Jalava et al. 2014), and using glass fiber pre-filters (Millipore). Viral nucleic acids were extracted and detected using previously described RT-qPCR and qPCR methods (Kauppinen et al. 2012, 2014), except that Taqman Environmental Master Mix 2.0 (Life Technologies) was used in the adenovirus qPCR assay.

Thermotolerant *Campylobacter* spp. (*C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis*) were analyzed semi-quantitatively as previously described (Hokajärvi et al. 2013) and following the principles in the standard ISO 17995 (2005). In brief, sample volumes of 10, 100, and 1,000 mL were concentrated using membranes with 0.45 µm pore size (GN6, Pall Life Sciences, Michigan, USA) and enriched in Bolton and Preston broths. Typical *Campylobacter* growth on complete modified-charcoal cefoperazone desoxycholate medium (mCCDA, Oxoid Ltd) was confirmed by Gram staining and motility, the absence of aerobic growth, oxidase and catalase tests. The species identification of thermotolerant *Campylobacter* isolates from water was achieved by a real-time qPCR method coupled with restriction fragment analysis as previously described by Pitkänen et al. (2008).

*Bacteroidetes* spp. and source tracking markers of human, gull, pig, and ruminant feces were quantified using DNA-based qPCR assays and RNA-based RT-qPCR assays as described earlier by Pitkänen et al. (2013). In brief, a volume of up to 300 mL water was filtered through a polycarbonate membrane with the pore size 0.4 µm (Nuclepore Polycarbonate, Whatman, Kent, UK). The membranes were stored at -75 °C prior to the nucleic acid extraction, cDNA synthesis, and the target gene quantification.

## RESULTS

### Fecal microbes and fecal source identifiers

Bathing areas with prior indication of microbiological quality problems or existing water quality hazards were selected

**Table 2** | Comparison of the molecular source identifier copy numbers generated by RNA-based RT-qPCR and DNA-based qPCR methods using *Enterococcus* spp. (Entero1), *E. coli* (EC23S857), *Bacteroidetes* spp. (GenBac3), human-specific *Bacteroidetes* (HF183), and gull-specific *Catellibacoccus marimammalium* (Gull4) assays

Assay	RNA-based signal higher	Equal or non-detect	DNA-based signal higher
Entero1	6/19 (32%)	12/19 (63%)	1/19 (5%)
EC23S857	10/19 (53%)	0/19 (0%)	9/19 (47%)
GenBac3	11/19 (58%)	0/19 (0%)	8/19 (42%)
HF183	5/19 (26%)	12/19 (63%)	2/19 (11%)
Gull4	9/19 (47%)	5/19 (26%)	5/19 (26%)

for this study. During the sampling campaign, two coastal bathing areas exceeded the water quality standards (Supplementary material, Table S2, available with the online version of this paper). The national threshold value for a single sample (STM 2008a, 2008b) was exceeded in a total of five samples for intestinal enterococci and in two samples for *E. coli* from the coastal sampling sites D and E. The confirmation rates for intestinal enterococci varied from 8% to 100%, being exceptionally high at site E (small coastal bathing area). In the sampling site B (inland bathing area), the counts of enterococci and *E. coli* did not exceed the quality standards for the inland waters but the RNA-based qPCR signals of *Enterococcus* spp. and *E. coli* specific genetic markers peaked in August (Table S2).

RNA-based RT-qPCR and DNA-based qPCR assays were used to detect the molecular source identifiers (Table S2). The GenBac3 marker of *Bacteroidetes* spp. was detected in all water samples. Based on the HF183 genetic marker, human-specific fecal contamination was prevalent at bathing sites B and C but remained absent from other areas. The gull-specific Gull4 marker was detected at least once in all of the bathing areas with site D showing the highest relative abundance. Swine and ruminant specific contamination (Pig-2-Bac and Rum-2-Bac markers) were not detected in any of the samples. RNA-based RT-qPCR resulted in higher marker copy numbers more often than the DNA-based qPCR (Table 2).

While noroviruses were not detected in any of the samples, four samples collected from the inland bathing areas were positive for adenoviruses (three out of four detections were below the quantification limit of the method) (Table S2). *Campylobacter* was abundant in all eight studied samples taken from the inland bathing areas, but only in two

of the seven studied samples in coastal bathing areas (Table S2). *C. jejuni* and *C. coli* were found only from inland bathing areas, *C. jejuni* being more common. *C. lari* was found from inland bathing areas A and C and also from coastal bathing area D.

### Species distribution of bacteria isolated using the ISO 7899-2 method

A partial 16S rRNA sequence was successfully obtained for a total of 341 bacterial colonies grown on Slanetz and Bartley medium, among them, 217 were confirmed as intestinal enterococci on BEA agar, and 124 of the isolates remained unconfirmed (Table 3). The colonies were collected from a total of 27 membrane filters from two to nine membranes on BEA medium from each bathing water site (A–E). Based on phylogenetic analysis, the confirmed isolates were grouped into three bacterial genera: *Enterococcus* spp., *Pediococcus* spp., and *Lactobacillus* spp. (Figure 1). Among the unconfirmed bacterial isolates, eight different genera were identified: *Enterococcus* spp., *Lactococcus* spp., *Staphylococcus* spp., *Lactobacillus* spp., *Staphylococcus* spp., *Pediococcus* spp., *Leuconostoc* spp., and *Macroccoccus* spp. (Figure 1). Intestinal enterococci clades (*E. faecium* and *E. faecalis* clades) represented 67.7% of the isolates (231 out of 341). Altogether, 88.0% (300 out of 341) of the bacteria isolated from the colonies grown on the S&B medium in the bathing water analysis belonged to the genus *Enterococcus* spp. The rest of the isolates (12.0%,  $n = 41$ ) were non-enterococci Gram-positive bacteria.

Out of the 217 isolates confirmed as intestinal enterococci in BEA agar, 202 isolates (93.1%) were classified to *E. faecalis* and *E. faecium* clades and were considered as true positives (Table 3, Figure 2(a)). The other confirmed isolates ( $n = 15$ ) were classified as *Enterococcus gallinarum*, *Pediococcus* spp., and *Lactobacillus* spp. and were considered as false positive results. Among the 124 unconfirmed isolates, 29 were *E. faecalis* and *E. faecium* and were considered as false negative results (Table 3, Figure 2(a)). A total of 76.6% of the unconfirmed isolates were considered as true negatives as they belonged to clades associated with environmental enterococci and non-enterococci species. The true negatives identified (i.e., isolates being able to grow on Slanetz and Bartley



**Table 3** | Distribution of confirmed and unconfirmed enterococci isolates of the ISO 7899-2 method in the intestinal enterococci, environmental enterococci, and non-enterococci clades based on the phylogenetic analysis of the partial 16S rRNA sequences

Site	Intestinal enterococci		Environmental enterococci			Non-enterococci				Total
	<i>E. faecium</i> clade	<i>E. faecalis</i> clade	<i>E. avium</i> clade	<i>E. gallinarum</i> clade	Other	<i>Macrococcus</i> spp. and <i>Staphylococcus</i> spp. clade	<i>Streptococcus</i> spp. and <i>Lactococcus</i> spp. clade	<i>Lactobacillus</i> spp. and <i>Pediococcus</i> spp. clade	<i>Leuconostoc</i> spp. clade	
Number of confirmed isolates										
A	12	8	0	2	0	0	0	5	0	27
B	31	6	0	0	0	0	0	7	0	44
C	13	7	0	0	0	0	0	0	0	20
D	47	22	0	0	0	0	0	1	0	70
E	2	54	0	0	0	0	0	0	0	56
Total (conf.)	105	97	0	2	0	0	0	13	0	217
Number of unconfirmed isolates										
A	1	0	0	3	0	0	11	0	0	15
B	4	2	8	4	10	1	1	4	1	35
C	4	1	2	13	0	7	0	0	0	27
D	10	5	1	20	4	0	2	1	0	43
E	0	2	0	2	0	0	0	0	0	4
Total (unconf.)	19	10	11	42	14	8	14	6	1	124
Total (all)	124	107	11	44	14	8	14	18	1	341

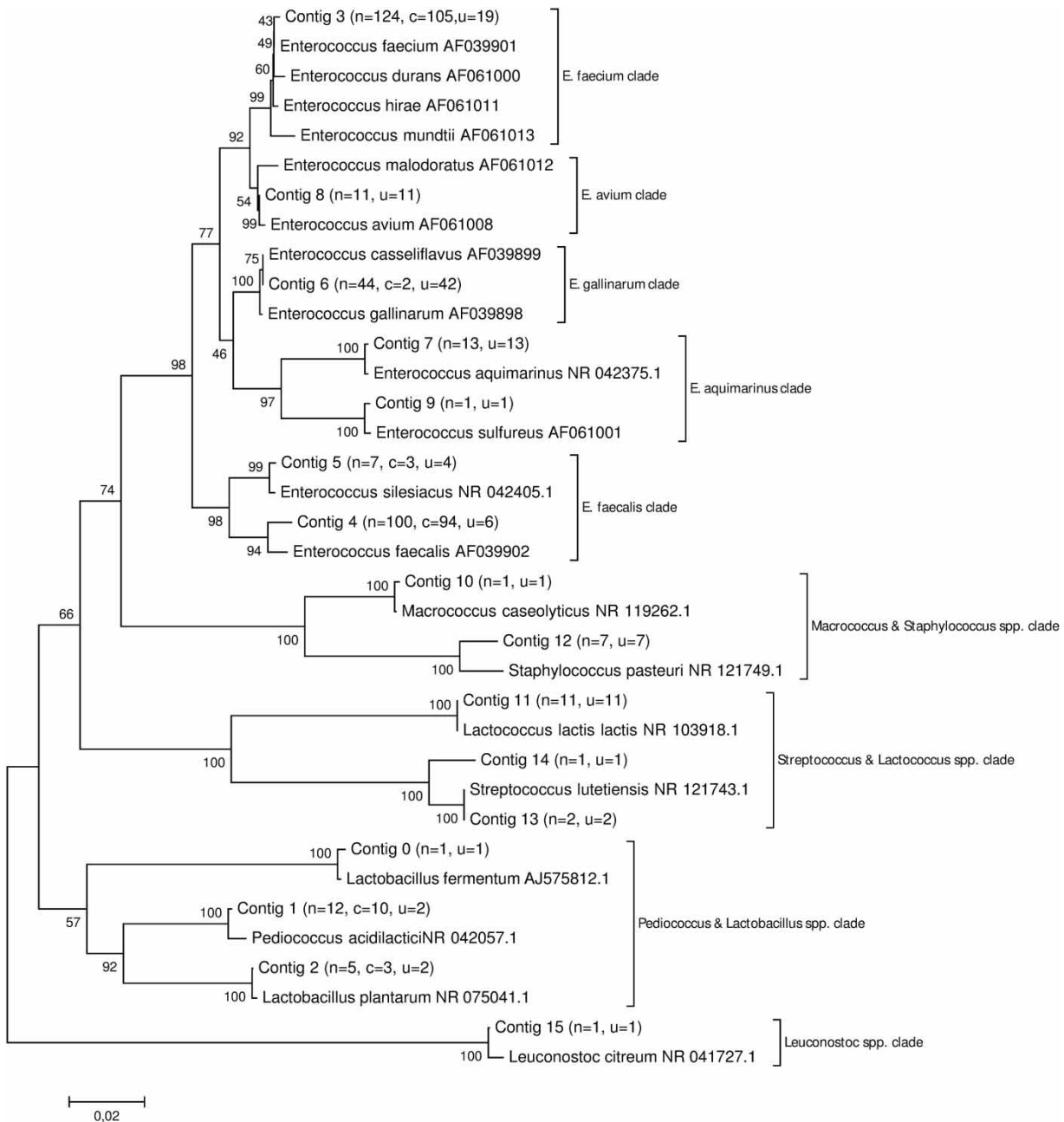
medium, but remaining unconfirmed on BEA medium) included environmental enterococci species *Enterococcus avium*, *E. casseliflavus*, *E. aquimarinus*, and *E. sulfureus* and other genera like *Lactococcus*, *Staphylococcus*, *Leuconostoc*, and *Macrococcus* (Figure 1).

The isolates identified as false positives ( $n = 15$ ) or false negatives ( $n = 29$ ) were re-streaked from the storage stock and sub-cultured on TSA medium (at  $36 \pm 2^\circ\text{C}$  for 2 days) prior to streaking on the BEA medium. The repetition of the BEA confirmation from the pure cultures showed that only three isolates out of 15 false positives (two *Lactobacillus* spp. isolates and one *Pediococcus* spp. isolate) were truly able to produce blackish color on the BEA medium. All of the unconfirmed 29 isolates identified as false negatives according to method ISO 7899-2 were positive and confirmed to be intestinal enterococci when re-streaked from the pure culture on the BEA.

To further confirm the phylogenetic clustering, the isolates were challenged against 23S rRNA-based *Enterococcus* spp. specific qPCR assay Entero1 (Ludwig & Schleifer 2000) and the corresponding partial 16S rRNA sequence was tested

*in silico* against 16S rRNA-based *Enterococcus* spp. specific Ent1 primer (Ryu et al. 2013). All the isolates classified as *Enterococcus* species in the phylogenetic analysis were amplified in the Entero1 assay and their corresponding partial 16S rRNA sequences aligned (100%) with the Ent1 primer *in silico*. Of the non-enterococci isolates, three out of 13 false positive *Pediococcus* spp. and *Lactobacillus* spp. isolates amplified with the Entero1 assay. However, none of the non-enterococci partial 16S rRNA sequences aligned with the Ent1 primer *in silico*.

Furthermore, the partial 16S rRNA sequences of the isolates classified to the *E. faecium*, *E. faecalis*, and *E. gallinarum* clades were tested *in silico* against the species-specific primers Faecium1, Faecalis1, and Casseli1 (Ryu et al. 2013), respectively. Faecium1 primer aligned with the sequences associated with the *E. faecium* clade ( $n = 124$ ) except for one sequence that was associated with *Enterococcus mundtii* when further analyzed with RDP seqmatch. Faecalis1 and Casseli1 primers aligned with the sequences associated with the *E. faecalis* and *E. gallinarum* clades, respectively ( $n = 107$  and  $n = 44$ ). In addition, Casseli1 primer aligned with the isolates classified into the *E. avium* clade ( $n = 11$ ).

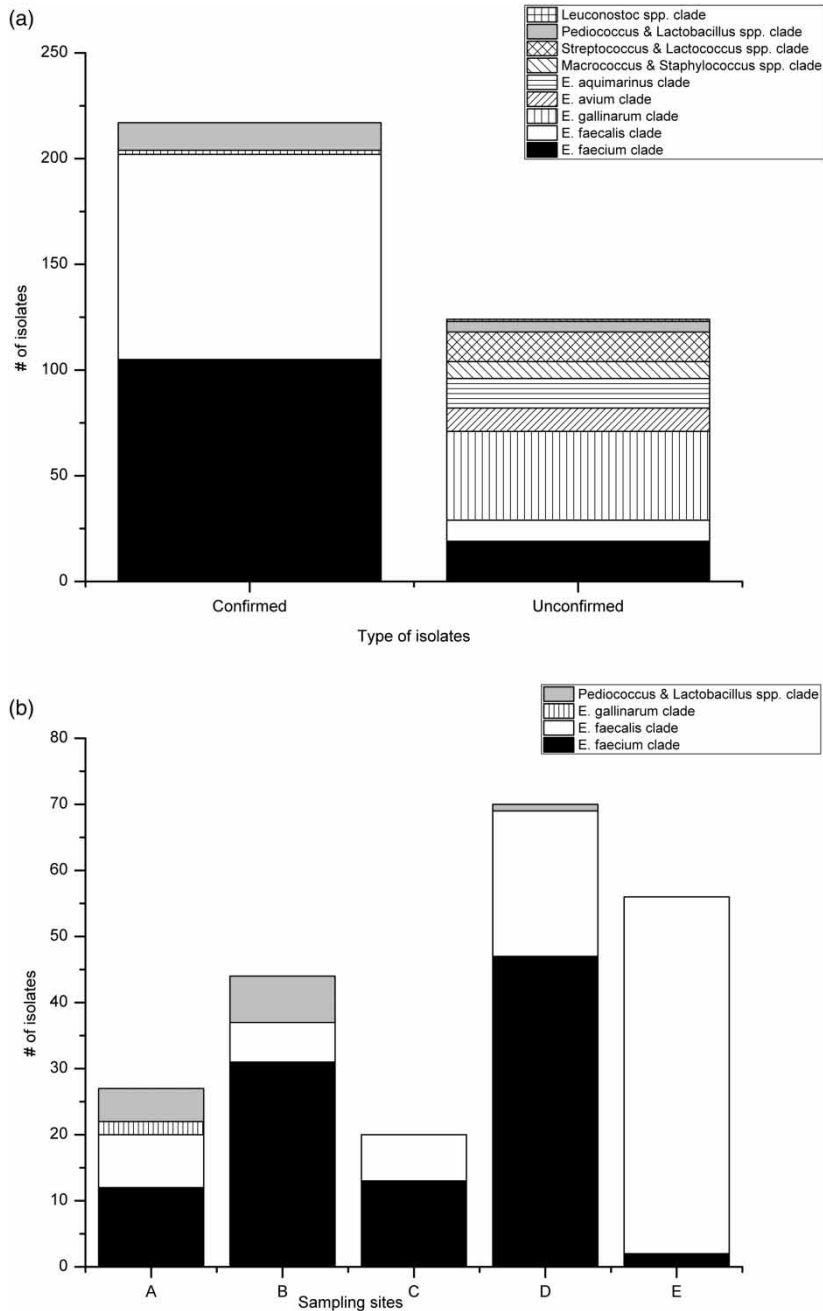


**Figure 1** | Unrooted neighbor-joining tree of bacterial 16S rRNA gene sequences obtained during bathing water monitoring using ISO 7899-2 method. The figure and the selection of reference sequences are adapted from Ryu et al. (2013). n, the total number of isolates in each contig; c, the total number of confirmed isolates; u, the total number of unconfirmed isolates.

### Categorical performance characteristics of the ISO 7899-2 method

The categorical performance characteristics of the ISO 7899-2 method are presented in Table 4. Overall, the results

indicate that the method is sufficiently sensitive, specific, selective, and efficient for the enumeration of intestinal enterococci. The confirmed isolates belonging to the *E. gallinarum* clade and genera *Pediococcus* and *Lactobacillus* created false positive results for the ISO 7899-2



**Figure 2** | Distribution of (a) the total confirmed and unconfirmed isolates in the bacterial clades, and the bacterial clade identification of (b) the confirmed isolates and (c) the unconfirmed isolates from the study sites (A–E). (Continued.)

method with a rate of 18.5% when less than 10 presumptive colonies per membrane were counted and with a rate of 7.0% when the presumptive colony counts ranged from 12 to 62 per membrane (Table 4). When the colony count exceeded 100 per membrane, the false positives were no

longer detected. The false positive intestinal enterococci findings originated from bathing areas of the sites A, B, and D (Figure 2(b)).

False negative findings were detected using the ISO 7899-2 method from all bathing areas. The number of *E. faecalis*



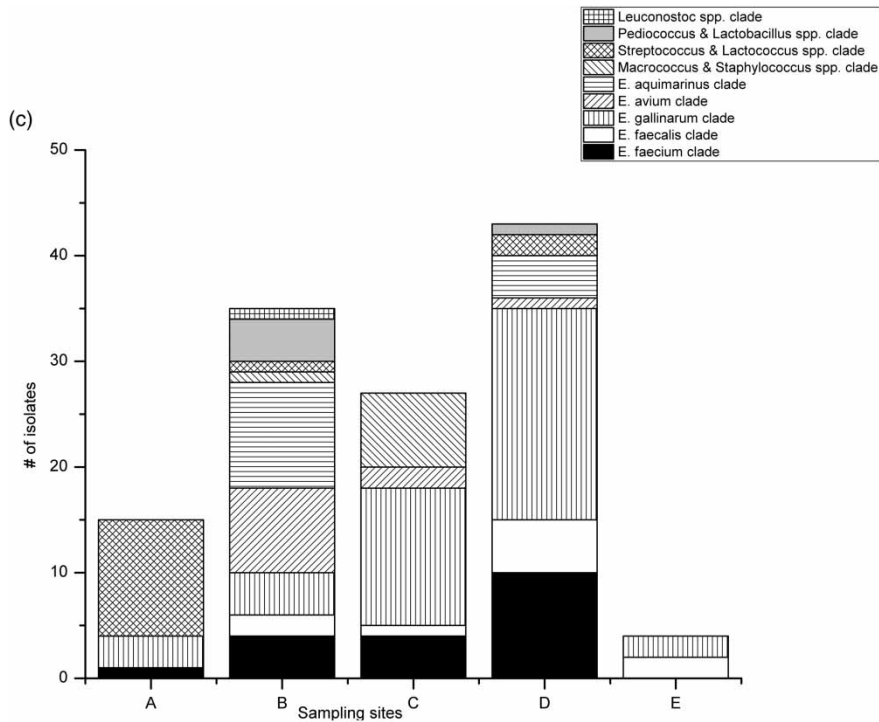


Figure 2 | Continued.

and *E. faecium* isolates that remained unconfirmed was the highest at bathing area D (Figure 2(c)), the site where the FIB counts exceeded the water quality standards. When taking into account the number of presumptive colonies on the membrane filter of the ISO 7899-2 method, it was seen that the presumptive colony count affected the false negative rates of the method (Table 4). The false negative rate was as high as 57.1% when analyzing isolates from membranes exceeding the limit of reliable counting, being more than 100 colonies per membrane. When the presumptive colony counts ranged from 12 to 62 per membrane, the false negative rate was still relatively high, being 20.9%.

## DISCUSSION

This study confirmed that ISO 7899-2 method is a reliable method and can be applied for the enumeration of intestinal enterococci from bathing water. Most of the confirmed intestinal enterococci isolates from bathing water in Finland (i.e., 93.1%) were identified as members of *E. faecalis* or *E. faecium* clades based on their partial 16S rRNA gene sequences. *E. gallinarum*, *Pediococcus* spp., and *Lactobacillus* spp. isolates were false positives on the BEA medium used for *Enterococcus* confirmation in the ISO 7899-2 method. The false positive rate was as high as 18.5% when

Table 4 | Performance of ISO 7899-2 method for bathing water monitoring

Total number of all colonies/membrane	Median (min–max) of presumptive colony count/membrane	Number of isolates	False positive rate (%)	False negative rate (%)	Sensitivity (%)	Specificity (%)	Selectivity (%)	Efficiency (%)
<10 colonies	4.5 (1–8)	45	18.5	5.6	95.7	77.3	48.9	86.7
10–100	42.0 (12–62)	234	7.0	20.9	87.5	87.5	56.8	87.6
>100 colonies	137.0 (106–149)	42	0.0	57.1	77.8	100.0	66.7	81.0
Unknown	NA	20	NA	NA	NA	NA	NA	NA

NA; not available.

the presumptive colony count on Slanetz and Bartley medium membranes was less than 10 CFU, suggesting that interpreting the blackening of the BEA medium is prone to subjective errors. Even small traces of color were mistakenly interpreted as a positive result when only a few colonies were present. However, this does not explain all the false positive findings as only two *Lactobacillus* spp. isolates and one *Pediococcus* spp. isolate were able to produce blackish color on the BEA medium when BEA confirmation was repeated with the pure cultures.

As the presumptive colony count affects the method performance, it is important to determine the reliable working range of the colony counting methods. In the ISO 7899-2 method, membrane filters (usually 47 mm in diameter) with the presumptive enterococci colonies are transferred from the Slanetz and Bartley medium to the pre-heated BEA medium for confirmation. In our study, it was observed that membranes having more than 100 presumptive colonies had a false negative rate as high as 57.1%. This is because when the colonies are too dense it is difficult to see the blackening on the BEA medium correctly. In our data, presumptive colony counts ranging from 10 to 62 colonies per membrane were considered reliable for enumeration based on the recommendations presented in the standard ISO 8199 (2005). As compared to other water microbiology methods based on membrane filtration, in ISO 9308-1 (2014) for coliform bacteria and *E. coli* enumeration, the range of quantitative determination (colonies per 47-mm membrane filter) was defined as 10–100 (Lange *et al.* 2013) and in ISO 14189 (2013) for enumeration of *Clostridium perfringens* the validated range was 10–80.

Different media have unique ingredients for selective growth of targeted species and means to inhibit growth of non-targeted species (Ferguson *et al.* 2005). *Enterococcus* species are known to reduce triphenyltetrazolium chloride of Slanetz and Bartley medium to formazan to form red colored bacterial colonies (Slanetz & Bartley 1957). In ISO 7899-2 method, the change in colony color is used for the presumptive enterococci identification. However, Slanetz and Bartley medium does not have any inhibitor for Gram-positive bacteria. Previously, Svec & Sedlacek (1999) have reported that other bacteria than enterococci are able to grow on the Slanetz and Bartley medium. The growth of Gram-positive bacteria other than enterococci was clearly seen in our study.

In addition to the *Enterococcus* species, we identified *Pediococcus* spp., *Lactobacillus* spp., *Lactococcus* spp., *Staphylococcus* spp., *Leuconostoc* spp., and *Macrococcus* spp. from the colonies grown on the Slanetz and Bartley medium.

At a bathing area, the measured enterococci counts can be accumulated from multiple sources like urban runoff, wastewater treatment plants, wildlife defecation, and even from environmental sources (as reviewed in Byappanahalli *et al.* 2012). In our study, both intestinal and environmental species of the enterococci were detected from all studied bathing areas, although the number of environmental enterococci isolates was very low and consisted solely of *E. gallinarum* clade members at study sites A and E.

As noted before (Savichtcheva & Okabe 2006; Hoka-järvi *et al.* 2013), the FIB counts did not show relation to the presence of adenoviruses or *Campylobacter* spp. In addition, a recent study describes the poor indicator value of FIB in bathing waterborne norovirus outbreaks (Kauppinen *et al.* 2017). In our study, the exceedances of the quality standards were seen without detection of these pathogens and vice versa (i.e., these pathogens were detected when the water quality standards were not exceeded). It has been proposed that adenoviruses could be used as bathing water quality indicators, potentially indicating the presence of enteric viruses that are infectious to humans (Wyn-Jones *et al.* 2011). We found adenoviruses from sites B and C from where human-specific source identifier HF183 was found and adenoviruses remained absent at sites D and E where HF183 was not detected. However, site A was the exception; adenoviruses were detected but not HF183.

Besides the detection of *Campylobacter* species causing campylobacteriosis in humans (*C. jejuni* and *C. coli*), the third *Campylobacter* species identified was *C. lari*, which is associated with waterfowl feces such as seagulls (Ryu *et al.* 2014). *C. lari* and the Gull4 marker specific for gull feces were detected at sites A, C, and D. The highest Gull4 copy number (6.17 log<sub>10</sub>/100 mL) was recorded at the beginning of July from site D using the RNA-based RT-qPCR. The higher target copy numbers generated using RNA-based RT-qPCR compared to the DNA-based qPCR is in agreement with the previous studies, and the rRNA:rDNA ratio may indicate the overall activity levels of the targets (Pitkänen *et al.* 2013; Kapoor *et al.* 2014). The simultaneous exceedance of the intestinal enterococci quality standard

and detection of *C. lari*/Gull4 indicate fresh gull feces as a source to bathing water quality deterioration at sites A, C, and D. Indeed, gull feces are a potential source of *E. faecalis*, as Pourcher et al. (1991) reported that 70% of the total *Enterococcus* isolates from seagulls were *E. faecalis*. However, many other enterococci species like *E. avium*, *E. gallinarum*, *E. durans*, *E. hirae*, and *E. casseliflavus* have been reported from gull feces (Fogarty et al. 2003).

The origin of the extremely high *E. faecalis* counts on the small national bathing area (coastal site E) suffering repeatedly from peaking enterococci levels remained unidentified. At this site, the high counts of enterococci were detected at the end of the bathing season (in July and August). In our investigation, the *E. coli* counts were absent or very low and no human fecal contamination or waterborne pathogens were detected. The local environmental health authorities have had to close the bathing area due to the high counts, even though the true human health risk was questioned. In one sample, a low number of Gull4 marker specific for gull feces was seen using the DNA-based qPCR assay. The absence of Gull4 marker in the RNA-based RT-qPCR may indicate the absence of metabolically active target cells and that the contamination did not originate from fresh feces. The traces of DNA may originate from dormant, inactive, and dead cells or naked DNA occurring in the surface waters (Keer & Birch 2003). Further, the absence of *E. coli* at site E supports the avian contamination source as the enterococci count per gram feces have been reported to be high on feces of wild birds while *E. coli* was not detected or the counts were low (Moriarty et al. 2008). However, although the presence of intestinal enterococci in the environmental water is considered as a sign of fecal contamination, environmental sources of *E. faecalis* and *E. faecium* like soil, sediments, and vegetation have been reported (Badgley et al. 2011; Byappanahalli et al. 2012). Instead of fecal contamination, the blooming of aquatic plants may provide an alternative explanation for the increased enterococci counts. Indeed, green algae has been suggested to play a role in enterococci ecology as *Cladophora* was a source and sink of enterococci in coastal waters in the United States (Whitman et al. 2003; Verhoughstraete et al. 2010). At site E of our study, the abundance of aquatic plants such as *Myriophyllum* spp. was noted. The invasion of common watermilfoil *Myriophyllum sibiricum* Komarov 1914 (Magnoliophyta:

Haloragaceae) has been previously identified as a problem in shallow Åland lakes in southwestern Finland (Lindholm et al. 2008). The growth was reported as a new environmental problem resembling an underwater jungle with the surface water pH exceeding 10 without any simple solution to lake restoration. The impacts of this vegetation include oxygen deprivation due to the accumulation of decaying plant material, but its link to the growth of environmental enterococci remains unknown.

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## CONCLUSION

ISO 7899-2 method can be considered as a reliable method for the intestinal enterococci enumeration from bathing waters. The use of multiple sample volumes is recommended to reach the reliable counting range even in cases of peaking enterococci counts. However, intestinal enterococci counts alone were insufficient for water quality assessments. Even on the occasions of low intestinal enterococci counts, the presence of fecal pathogens was observed in some inland samples of the study. Therefore, identifying of the presence of fecal pathogens and the contaminant source at the bathing area is important for developing microbial infection risk estimates, preventing contamination events, and protecting public health.

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## REFERENCES

- Badgley, B. D., Thomas, F. I. & Harwood, V. J. 2011 [Quantifying environmental reservoirs of fecal indicator bacteria associated with sediment and submerged aquatic vegetation](#). *Environ. Microbiol.* **13**, 932–942.
- Byappanahalli, M. N., Nevers, M. B., Korajkic, A., Staley, Z. R. & Harwood, V. J. 2012 [Enterococci in the environment](#). *Microbiol. Mol. Biol. Rev.* **76** (4), 685–706.
- Cole, J. R., Wang, Q., Fish, J. A., Chai, B., McGarrell, D. M., Sun, Y., Brown, T., Porras-Alfaro, A., Kuske, C. R. & Tiedje, J. M. 2013 [Ribosomal database project: data and tools for high throughput rRNA analysis](#). *Nucl. Acids Res.* **42**, D633–D642.
- Colford Jr., J. M., Wade, T. J., Schiff, K. C., Wright, C. C., Griffith, J. F., Sandhu, S. K., Burns, S., Sobsey, M., Lovelace, G. & Weisberg, S. B. 2007 [Water quality indicators and the risk of illness at beaches with nonpoint sources of fecal contamination](#). *Epidemiology* **18**, 27–35.
- Directive 2006/7/EC of the European Parliament and of the Council of 15 February 2006 concerning the management of bathing water quality and repealing directive 76/160/EEC. *Official Journal of the European Union, L*. **64**, 37–51.
- Ferguson, D. M., Moore, D. F., Getrich, M. A. & Zhouwandai, M. H. 2005 [Enumeration and speciation of enterococci found in marine and intertidal sediments and coastal water in southern California](#). *J. Appl. Microbiol.* **99**, 598–608.
- Fogarty, L. R., Haack, S. K., Wolcott, M. J. & Whitman, R. L. 2003 [Abundance and characteristics of the recreational water quality indicator bacteria \*Escherichia coli\* and enterococci in gull faeces](#). *J. Appl. Microbiol.* **94** (5), 865–878.
- Hokajärvi, A.-M., Pitkänen, T., Siljanen, H., Nakari, U.-M., Torvinen, E., Siitonen, A. & Miettinen, I. 2013 [Occurrence of thermotolerant \*Campylobacter\* spp. and adenoviruses in Finnish bathing waters and purified sewage effluents](#). *J. Water Health* **11** (1), 120–134.
- ISO 7899-2 2000 [Water Quality – Detection and Enumeration of Intestinal Enterococci – Part 2: Membrane Filtration Method](#). International Organization for Standardization, Geneva, Switzerland.
- ISO 17995 2005 [Water Quality – Detection and Enumeration of Thermotolerant \*Campylobacter\* Species](#). International Organization for Standardization, Geneva, Switzerland.
- ISO 8199 2005 [Water Quality – General Guidance on the Enumeration of Micro-Organisms by Culture](#). International Organization for Standardization, Geneva, Switzerland.
- ISO 9308-2 2012 [Water Quality – Enumeration of \*Escherichia Coli\* and Coliform Bacteria, Part 2: Most Probable Number Method](#). International Organization for Standardization, Geneva, Switzerland.
- ISO 14189 2013 [Water Quality – Enumeration of \*Clostridium perfringens\* – Method using membrane filtration](#). International Organization for Standardization, Geneva, Switzerland.
- ISO 9308-1 2014 [Water Quality – Enumeration of \*Escherichia coli\* and Coliform Bacteria. Part 1: Membrane Filtration Method for Waters with Low Bacterial Background Flora](#). International Organization for Standardization, Geneva, Switzerland.
- ISO 13843 2017 [Water Quality – Requirements for Establishing Performance Characteristics of Quantitative Microbiological Methods](#). International Organization for Standardization, Geneva, Switzerland.
- ISO/TR 13843 2000 [Water Quality – Guidance on Validation of Microbiological Methods](#). International Organization for Standardization, Geneva, Switzerland.
- Jalava, K., Rintala, H., Ollgren, J., Maunula, L., Gomez-Alvarez, V., Revez, J., Palander, M., Antikainen, J., Kauppinen, A., Räsänen, P., Siponen, S., Nyholm, O., Kyyhkynen, A., Hakkarainen, S., Merentie, J., Pärnänen, M., Loginov, R., Ryu, H., Kuusi, M., Siitonen, A., Miettinen, I., Santo Domingo, J. W., Hänninen, M. L. & Pitkänen, T. 2014 [Novel microbiological and spatial statistical methods to improve strength of epidemiological evidence in a community-wide waterborne outbreak](#). *PLoS One* **9** (8), e104713.
- Kapoor, V., Pitkänen, T., Ryu, H., Elk, M., Wendell, D. & Santo Domingo, J. W. 2014 [Distribution of human-specific \*Bacteroidales\* and fecal indicator bacteria in an urban watershed impacted by sewage pollution distributed using RNA- and DNA-based quantitative PCR assays](#). *Appl. Environ. Microbiol.* **81**, 91–99.
- Kauppinen, A., Ikonen, J., Pursiainen, A., Pitkänen, T. & Miettinen, I. T. 2012 [Decontamination of a drinking water pipeline system contaminated with adenovirus and \*Escherichia coli\* utilizing peracetic acid and chlorine](#). *J. Water Health* **10** (3), 406–418.
- Kauppinen, A., Martikainen, K., Matikka, V., Veijalainen, A.-M., Pitkänen, T., Heinonen-Tanski, H. & Miettinen, I. T. 2014 [Sand filters for removal of microbes and nutrients from wastewater during a one-year pilot study in a cold temperate climate](#). *J. Environ. Manage.* **133**, 206–213.
- Kauppinen, A., Al-Hello, H., Zacheus, O., Kilponen, J., Maunula, L., Huusko, S., Lappalainen, M., Miettinen, I., Blomqvist, S. & Rimhanen-Finne, R. 2017 [Increase in outbreaks linked to bathing water in the summer of 2014 in Finland](#). *Euro Surveill.* **22** (8), pii = 30470.
- Keer, J. T. & Birch, L. 2003 [Molecular methods for the assessment of bacterial viability](#). *J. Microbiol. Methods* **53**, 175–183.
- Lange, B., Strathmann, M. & Ossmer, R. 2013 [Performance validation of chromogenic coliform agar for the enumeration of \*Escherichia coli\* and coliform bacteria](#). *Lett. Appl. Microbiol.* **57**, 547–553.
- Layton, B. A., Walters, S. P., Lam, L. H. & Boehm, A. B. 2010 [Enterococcus species distribution among human and](#)



- animal hosts using multiplex PCR. *J. Appl. Microbiol.* **109**, 539–547.
- Li, W. & Godzik, A. 2006 Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. *Bioinformatics* **22**, 1658–1659.
- Lindholm, T., Rönholm, E. & Häggqvist, K. 2008 Change due to invasion of *Myriophyllum sibiricum* in shallow lake in Åland, SW Finland. *Aquatic Invasions* **3**, 10–13.
- Ludwig, W. & Schleifer, K. 2000 How quantitative is quantitative PCR with respect to cell counts? *Syst. Appl. Microbiol.* **23**, 556–562.
- Moore, D. F., Zhouwandai, M. H., Ferguson, D. M., McGee, C., Mott, J. B. & Stewart, J. C. 2006 Comparison of 16S rRNA sequencing with conventional and commercial phenotypic techniques for identification of enterococci from the marine environment. *J. Appl. Microbiol.* **100**, 1272–1281.
- Moriarty, E., Nourozi, F., Robson, B., Wood, D. & Gilpin, B. 2008 Evidence for growth of enterococci in municipal oxidation ponds, obtained using antibiotic resistance analysis. *Appl. Environ. Microbiol.* **74**, 7204–7210.
- Muller, T., Ulrich, A., Ott, E. M. & Muller, M. 2001 Identification of plant-associated enterococci. *J. Appl. Microbiol.* **91**, 268–278.
- Pitkänen, T., Miettinen, I. T., Nakari, U. M., Takkinen, J., Nieminen, K., Siitonen, A., Kuusi, M., Holopainen, A. & Hanninen, M. L. 2008 Faecal contamination of a municipal drinking water distribution system in association with *Campylobacter jejuni* infections. *J. Water Health* **6**, 365–376.
- Pitkänen, T., Ryu, H., Elk, M., Hokajärvi, A.-M., Siponen, S., Vepsäläinen, A., Räsänen, P. & Santo Domingo, J. W. 2013 Detection of fecal bacteria and source tracking identifiers in environmental waters using rRNA-based RT-qPCR and rDNA-based qPCR assays. *Environ. Sci. Technol.* **47**, 13611–13620.
- Pourcher, A. M., Devriese, L. A., Hernandez, J. F. & Delattre, J. M. 1991 Enumeration by a miniaturized method of *Escherichia coli*, *Streptococcus bovis* and enterococci as indicators of the origin of fecal pollution of waters. *J. Appl. Bacteriol.* **70**, 525–530.
- Ryu, H., Henson, M., Elk, M., Toledo-Hernandez, C., Griffith, J., Blackwood, D., Noble, R., Gourmelon, M., Glassmeyer, S. & Santo Domingo, J. W. 2013 Development of quantitative PCR assays targeting 16S rRNA gene of *Enterococcus* spp. and their application to the identification of *Enterococcus* species in environmental samples. *Appl. Environ. Microbiol.* **79**, 196–204.
- Ryu, H., Grond, K., Verheijen, B., Elk, M., Buehler, D. M. & Santo Domingo, J. W. 2014 Intestinal microbiota and species diversity of *Campylobacter* and *Helicobacter* spp. in migrating shorebirds in Delaware Bay. *Appl. Environ. Microbiol.* **80** (6), 1838–1847. doi:10.1128/AEM.03793-13.
- Savichtcheva, O. & Okabe, S. 2006 Alternative indicators of fecal pollution: relations with pathogens and conventional indicators, current methodologies for direct pathogen monitoring and future application perspectives. *Water Res.* **40** (13), 2463–2476. <http://dx.doi.org/10.1016/j.watres.2006.04.040>.
- Slanetz, L. W. & Bartley, C. H. 1957 Numbers of enterococci in water, sewage, and feces determined by the membrane filter technique with an improved medium. *J. Bacteriol.* **74**, 591–595.
- STM 2008a Decree of the Ministry of Social Affairs and Health Concerning the Quality Requirements and Surveillance of Bathing Water in Public Bathing Areas (177/2008). Available at: <http://www.finlex.fi/fi/laki/alkup/2008/20080177> (accessed 29 March 2016).
- STM 2008b Decree of the Ministry of Social Affairs and Health Concerning the Quality Requirements and Surveillance of Bathing Water in Small Public Bathing Areas (354/2008). Available at: <http://www.finlex.fi/fi/laki/alkup/2008/20080354> (accessed 29 March 2016).
- Svec, P. & Sedlacek, I. 1999 Occurrence of *Enterococcus* spp. in water. *Folia Microbiologica* **44** (1), 3–10.
- Tamura, K., Stecher, G., Peterson, D., Filipinski, A. & Kumar, S. 2013 MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* **30** (12), 2725–2729.
- USEPA 2009 United States Environmental Protection Agency, February 2009; Review of Zoonotic Pathogens in Ambient Waters. USEPA Office of Water, Health and Ecological Criteria Division.
- Valvira 2015 Identified Bathing Waters in Finland in 2015. Available at: <http://www.valvira.fi/ymparistoterveys/terveydensuojelu/uimavesi> (accessed 29 March 2016).
- Verhougstraete, M. P., Byappanahalli, M. N., Rose, J. B. & Whitman, R. L. 2010 *Cladophora* in the Great Lakes: impacts on beach water quality and human health. *Water Sci Technol.* **62** (1), 68–76. doi: 10.2166/wst.2010.230.
- Wheeler, A. L., Hartel, P. G., Godfrey, D. G., Hill, J. L. & Segars, W. I. 2002 Potential of *Enterococcus faecalis* as a human fecal indicator for microbial source tracking. *J. Environ. Qual.* **31** (4), 1286–1293.
- Whitman, R. L., Shively, D. A., Pawlik, H., Nevers, M. B. & Byappanahalli, M. N. 2003 Occurrence of *Escherichia coli* and Enterococci in *Cladophora* (Chlorophyta) in nearshore water and beach sand of Lake Michigan. *Appl. Environ. Microbiol.* **69** (8), 4714–4719. <http://doi.org/10.1128/AEM.69.8.4714-4719.2003>.
- Wyn-Jones, A. P., Carducci, A., Cook, N., D'Agostino, M., Divizia, M., Fleischer, J., Gantzer, C., Gawler, A., Girones, R., Holler, C., Husman, A. M. D., Kay, D., Kozyra, I., Lopez-Pila, J., Muscillo, M., Nascimento, M. S., Papageorgiou, G., Rutjes, S., Sellwood, J., Szewzyk, R. & Wyer, M. 2011 Surveillance of adenoviruses and noroviruses in European recreational waters. *Water Res.* **45**, 1025–1038.

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