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Chemiluminescence- and qPCR-based detection of public health-related bacteria in water

Annette Søndergaard Bukh

Preface

This thesis is submitted in partial fulfillment of the requirements for obtaining the degree of Doctor of Philosophy. The thesis consists of an extended summary of results and related literature and four supporting papers.

The work was carried out at the Section of Environmental Engineering at the Department of Biotechnology, Chemistry, and Environmental Engineering, Aalborg University, Denmark from January 2008 to December 2010 under the supervision of Associate Professor Peter Roslev.

Aalborg, December 2011

Annette Søndergaard Bukh

Acknowledgements

Abstract

Detection of fecal and opportunistic pathogenic microorganisms is of great importance in the control of water quality. Fecal contaminations in water are often assessed by detecting the fecal indicator bacteria (FIB) *E. coli* and enterococci. Standard cultivation-based methods for detection of FIB in water are associated with long analysis times (≥21 h) that impedes the possibility of rapid intervention in case of contamination situations. Furthermore, no information of the presence of waterborne opportunistic pathogens of non-enteric origin is provided. Detection of opportunistic pathogens in water using cultivation-based methods is often associated with very long analysis times (e.g., 7 days for *Legionella* spp.) Hence, more rapid methods not solely based on cultivation for determining the microbial water quality are needed.

The objectives of this Ph.D. study were to develop a luminescence-based principle for rapid detection of viable indicator bacteria in water, and to establish molecular-based assays (qPCR) for rapid and sensitive detection of FIB and the opportunistic pathogens *Legionella* spp. and the *Mycobacterium avium* complex (MAC) in domestic water.

Sensitive detection principles for viable *E. coli* ("ColiLight II") and enterococci ("EntLight") based on enzymatic cleavage of chemiluminescent 1,2-dioxetanes specific for the enzymes β-D-galactosidase, β-D-glucuronidase, and β-D-glucosidase, respectively, have been characterized and validated. ColiLight II and EntLight were capable of detecting low concentrations of stressed *E. coli* in potable water (<30 CFU 100 mL^{-1}) and of stressed enterococci in water (62 CFU). The assays can be applied for rapid and simple detection of viable FIB in water contaminated with fecal matter and has the potential to be used as a method for early warning (1-7 h) of fecal pollutions in different water types.

The ColiLight II assay was, together with qPCR, applied to study the survival and persistence of four clinical *E. coli* strains in water and biofilms on stainless steel (SS) and polyethylene (PE) surfaces in a flow-through reactor with non-disinfected drinking water. It was found that PE surfaces supported more drinking water biofilm formation than SS surfaces and contained more culturable *E. coli*. Strain specific variation can significantly affect detection and persistence of *E. coli* in drinking water matrices, and some clinical *E. coli* strains may persist longer in drinking water than culture collection strains.

The opportunistic pathogens *Legionella* spp. and MAC are known to be present in domestic hot water systems. However, this has never been reported for Danish hot water systems. Hence, the occurrence of MAC and *Legionella* spp. in hot water systems of nine public day care centers in Denmark was examined using cultivation-based methods and qPCR to determine whether these systems may serve as potential reservoirs for these opportunistic pathogens. *Legionella* spp. were detected in all day care centers either in water or in biofilms, whereas *L. pneumophila* was detected in water or biofilm in four systems. MAC was detected in all systems. In addition, the persistence of *M. avium* cells and naked DNA in hot water was studied in cross-linked polyethylene (PEX)

microcosms to evaluate the fate of cells and nucleic acids in water and biofilms. Naked DNA and DNA in dead *M. avium* cells persisted for at least 28 days. The net abundance of live cells in the planktonic phase and in biofilm indicated a growth potential of *M. avium* at 44°C in PEX pipes. The results implied that water and biofilm in day care hot water systems can potentially serve as reservoirs of MAC, and these systems may support persistence and growth.

Collectively, in this Ph.D. study, novel rapid detection methods for FIB and the opportunistic pathogens *Legionella* spp. and MAC were established, characterized, and validated. The methods were successfully applied for detection of FIB and selected opportunistic pathogens in various aquatic matrices and biofilms. Based on these findings, new knowledge about survival, occurrence, and persistence of these bacteria in water was obtained.

Resumé (in Danish)

Detektion af fækale og opportunistiske patogene mikroorganismer er vigtig i bestemmelsen af vandkvalitet. Fækale forureninger i vand bestemmes ofte ved at undersøge tilstedeværelsen af de fækale indikatorbakterier (FIB) *E. coli* og enterokokker. FIB i vand påvises ved brug af standard, dyrknings-baserede metoder. Disse metoder har lange analysetider (≥21 timer), som umuliggør en hurtig indsats i kontamineringssituationer. Derudover angiver disse metoder ikke noget om tilstedeværelsen af non-fækale, vandbårne, opportunistiske patogener. Dyrkningsbaseret detektion af opportunistiske patogener i vand er ofte forbundet med meget lange analysetider (f.eks. 7 dage for *Legionella* spp.), og for nogle patogener eksisterer der ikke dyrkningsmetoder. Der er således behov for hurtigere metoder, der ikke udelukkende er baseret på dyrkning, til bestemmelse af den mikrobielle vandkvalitet.

Formålet med dette Ph.D.-projekt var at udvikle et princip baseret på luminescens til hurtig detektion af levende indikatorbakterier i vand samt at opsætte hurtige og følsomme molekylære assays (qPCR) til detektion af fækale indikator bakterier (FIB) og udvalgte opportunistiske patogener i vand.

Følsomme detektionsprincipper for levende *E. coli* ("ColiLight II") og enterokokker ("EntLight") baseret på enzymatisk hydrolyse af chemiluminescerende 1,2-dioxetaner specifikke for enzymerne β-D-galactosidase, β-D-glucuronidase og β-D-glucosidase er blevet karakteriseret og valideret. ColiLight II og EntLight kunne detektere lave koncentrationer af stressede *E. coli* i drikkevand (<30 CFU 100 ml^{-1}) og af stressede enterokokker i vand (62 CFU). Metoderne kan anvendes til hurtig og simpel detektion af levende FIB i vand forurenet med fækalier og har potentiale for at kunne bruges som en metode til at give en hurtig advarsel (1-7 timer) om fækale forureninger i forskellige vandtyper.

Detektionsprincippet ColiLight II og qPCR blev anvendt til at undersøge overlevelsen af fire kliniske stammer af *E. coli* i vand og biofilm på overflader af rustfrit stål og polyethylen (PE) i en flow-through reaktor med drikkevand. Der blev dannet mere biofilm og fundet flere levende *E. coli* på PE overfladerne sammenlignet med overfladerne af rustfrit stål. Stamme-specifik variation kan påvirke detektionen og overlevelsen af *E. coli* i drikkevandsmatricer signifikant, og nogle kliniske *E. coli* stammer kan muligvis overleve længere tid i drikkevand sammenlignet med referencestammer.

Forekomst af de opportunistiske patogener *Legionella* spp. og MAC i varmtvandssystemer er tidligere blevet påvist, dog ikke i danske varmtvandssystemer. Derfor blev tilstedeværelsen af MAC og *Legionella* spp. i varmtvandssystemer i ni offentlige børnehaver i Danmark undersøgt ved brug af dyrkningsbaserede metoder og qPCR for at bestemme, om disse varmtvandssystemer indeholder MAC og *Legionella* spp.. *Legionella* spp. blev fundet i alle ni systemer enten i vand og/eller biofilm, mens *L. pneumophila* blev fundet i vand og/eller biofilm i fire af de ni systemer. MAC var til stede i alle ni systemer.

Overlevelsen af *M. avium* celler og holdbarheden af frit DNA i varmt vand blev undersøgt i batchsystemer af krydsbundent PE (PEX) ved brug af dyrkningsbaserede metoder og qPCR for at evaluere, hvor længe DNA fra celler og frie nukleinsyrer kan detekteres i vand og biofilm. Frit DNA og DNA i døde *M. avium* celler kunne påvises i mindst 28 dage. Mængden af levende *M. avium* celler i den vandige fase og i biofilm steg, hvilket indikerer at *M. avium* kan formere sig ved 44˚C i PEX rør. Resultaterne indikerede, at vand og biofilm i varmtvandssystemerne i børnehaver er potentielle kilder til MAC og *Legionella* spp., og at disse systemer muligvis bidrager til, at disse grupper af bakterier kan overleve i varmtvandssystemerne.

I dette Ph.D. studium blev nye metoder til hurtig detektion af FIB og de opportunistiske patogener *Legionella* spp. og MAC etableret, karakteriseret og valideret. Metoderne blev anvendt til detektion af FIB og de opportunistiske patogener i forskellige vandige matricer og biofilm. Med disse resultater er der opnået ny viden omkring forekomst og overlevelse af disse bakterier i vand.

Abbreviations

ATCC	American Type Culture Collection
BB	Bead beating
BCYE	Buffered charcoal yeast extract
bp	Base pairs
CAB	Community-acquired bacteremia
CCA	Chromocult Coliform Agar
CFU	Colony forming unit
CI	Confidence interval
CIEEL	Chemically initiated electron exchange luminescence
CPS	Counts per second
C_T	Threshold cycle
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
ssDNA	Single stranded DNA
DS	Danish standard
DVC-FISH	Direct viable count – fluorescence in situ hybridization
dxs	D-1-deoxyxylulose 5-phosphate synthase gene
E	PCR efficiency
mEI	membrane-*Enterococcus* indoxyl-D-glucoside
EMA	Ethidium monoazide
FIB	Fecal indicator bacteria
FISH	Fluorescence in situ hybridization
β-GAL	β-D-galactosidase
β-GLU	β-D-glucosidase
β-GUS	β-D-glucuronidase
GVPC	Glycine, vancomycin, polymyxin B, cycloheximide
HPC	Heterotrophic plate count
IPTG	Isopropyl β-D-1-thiogalactopyranoside
ISO	International Organization for Standardization
LLOQ	Lower limit of quantification
LOD	Limit of detection
MAC	*Mycobacterium avium* complex
MAP	*Mycobacterium avium* subsp. *paratuberculosis*
MetGlu	Methyl-β-D-glucuronide
MF	Membrane filtration
mip	Macrophage infectivity potentiator gene
MPN	Most probable number
MUD	4-methylumbelliferyl-β-D-glucoside
MUGal	4-methylumbelliferyl-β-D-galactoside
MUGlu	4-methylumbelliferyl-β-D-glucuronide

ND	Not determined
ONPG	Ortho-nitrophenyl-β-galactoside
P/A	Presence/absence
PC	Polycarbonate
PCI	Phenol:Chloroform:Isoamyl alcohol
PCR	Polymerase chain reaction
qPCR	Quantitative real-time PCR
PEX	Cross-linked polyethylene
PMA	Propidium monoazide
PVC	Polyvinyl chloride
cPVC	Chlorinated PVC
r	Correlation coefficient
R^2	Coefficient of determination
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SE	Standard error
S/N	Signal-to-noise ratio
SS	Stainless steel
TBX	Tryptone Bile X-glucuronide
TC	Total coliforms
TR	Triggering reagent
VBNC	Viable but non-culturable
UTI	Urinary tract infection

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Objectives

The objectives of this thesis were:

- To develop luminescence-based sensor principles for rapid detection of fecal bacterial contamination in water. The luminescence-based principles should have the potential of being transferred to an automated (or semi-automated) sampling and analysis platform as part of an early warning system for fecal indicator bacteria (FIB).

- To establish and validate qPCR-based assays for detection and quantification of FIB in water. Together with traditional cultivation-based methods, qPCR assays should be able to validate results obtained with the rapid luminescence-based detection principles.

- To establish and validate qPCR-based assays for detection and quantification of *Legionella* spp. and the *Mycobacterium avium* complex (MAC) in water and biofilm. These opportunistic, waterborne pathogens were included for two reasons: (1) Similar to FIB, traditional cultivation-based detection is time consuming and prohibits early warning, and (2) unlike FIB, *Legionella* spp. and MAC likely occur more frequently in domestic water systems, and more directly impact public health.

List of supporting papers

Paper I   Bukh, A. S., Roslev, P. (2010) Characterization and validation of a chemiluminescent assay based on 1,2-dioxetanes for rapid detection of viable *Escherichia coli*. Appl Microbiol Biotechnol **86:**1947-57.

Paper II   Bukh, A. S., Hansen, N. E., Roslev, P. Detection and persistence of clinical *Escherichia coli* in drinking water evaluated by a rapid enzyme assay and qPCR. Accepted for publication in Advances in Microbiology.

Paper III   Bukh, A. S., Roslev, P. Characterization and validation of a chemiluminescent assay based on a 1,2-dioxetane for rapid detection of enterococci in contaminated water and comparison with standard methods and qPCR. J Appl Microbiol **111:**407-416.

Paper IV   Bukh, A. S., Roslev, P. Occurrence of the *Mycobacterium avium* complex and *Legionella* in hot water systems of public day care centers, and growth potential of *M. avium* in PEX water pipes. Manuscript.

Supporting papers will be referred to by their roman numbers e.g., Paper I.

Supplementary papers (not included in the thesis)

Bukh, A. S., Schønheyder, H. C., Emmersen, J. M. G., Søgaard, M. Bastholm, S., Roslev, P. (2009) *Escherichia coli* phylogenetic groups are associated with site of infection and level of antibiotic resistance in community-acquired bacteraemia: a 10 year population-based study in Denmark. J Antimicrob Chemother **64:**163-68.

Roslev, P., Bukh, A. S., Iversen, I. L., Sønderbo, H. L., Iversen, N. (2010) Application of mussels as biosamplers for characterization of faecal pollution in coastal recreational waters. Water Sci Technol **62:**586-93.

Roslev, P. and Bukh, A. S. (2011) State of the art molecular markers for fecal pollution source tracking in water. Appl Microbiol Biotechnol **89:**1341-1355.

Roslev, P., de Evgrafov, M. R., Bukh, A. S. Detection, survival, and regrowth potential of intestinal and environmental enterococci in seawater. Manuscript.

1 Microbial drinking water quality

1.1 Fecal indicator bacteria

Fecal indicator bacteria (FIB) are non-pathogenic bacteria found in the feces of all warm-blooded animals, and their presence in water indicates a fecal contamination and hereby the possibility that enteric pathogens also are present in the water (Gerba 2009). The criteria for an ideal indicator organism are given below (Table 1).

Table 1 Criteria for an ideal indicator organism (modified from Gerba 2009)

- The organism should be useful for all types of water
- The organism should be present whenever enteric pathogens are present
- The organism should have a reasonable longer survival time than the hardiest enteric pathogen
- The organism should not grow in water
- The testing method should be easy to perform
- The density of the indicator organism should have some direct relationship to the degree of fecal pollution
- The organism should be a member of the intestinal microflora of warm-blooded animals

Total coliforms (TCs), *E. coli* and enterococci are widely used as indicators of fecal contaminations of water (Rompré et al. 2002, Gerba 2009). The group of TCs includes the genera *Escherichia*, *Citrobacter*, *Enterobacter*, and *Klebsiella*, and members of this group are aerobic or facultative anaerobic, Gram-negative, non-spore forming, rod-shaped bacteria that produce gas upon lactose fermentation in predescribed culture media within 48 hours at 35˚C. TCs possess β-D-galactosidase (β-GAL) activity (Gerba 2009, Carrero-Colón et al. 2011, Edge and Boehm 2011). However, some of the organisms in the TC group are not limited to fecal sources, and hence the use of *Escherichia coli* has been suggested for indication of fecal contaminations (Gerba 2009, Carrero-Colón et al. 2011). *E. coli* produces indole from tryptophan, is able to grow at 44˚C to 45˚C, and possesses β-GAL and β-D-glucuronidase (β-GUS) activity (Gerba 2009, Carrero-Colón et al. 2011, Edge and Boehm 2011).

Enterococci are catalase-negative, Gram-positive cocci that are able to grow on media containing bile salts and the selective reagent sodium azide, to hydrolyse esculin, and possess β-D-glucosidase (β-GLU) activity (Devriese et al. 1993, Facklam et al. 2002, Fisher and Phillips 2009, Carrero-Colón et al. 2011).

1.2 Traditional determination of the microbial drinking water quality

In Denmark, the microbial quality of drinking water is determined by routinely assessment of the levels of heterotrophic bacteria and FIB, and according to the European Council Directive, *E. coli* and enterococci must not be detected in 100 mL of drinking water (European Council Directive 1998).

The levels of FIB are determined using random sampling and standard, cultivation-based analysis methods (Table 2). In these methods, the enzymes β-GAL, β-GUS, and β-GLU are accepted for detection of TCs, *E. coli*, and enterococci, respectively (Manafi 2000, Rompré et al. 2002, Orenga et al. 2009).

Table 2 Standard methods for determining the microbial drinking water quality in Denmark

Detection method	Target bacteria	Principle	Analysis time
DS/EN ISO 6222-1 (2002) Water quality – Enumeration of culturable microorganisms – Colony count by inoculation in a nutrient agar culture medium	Heterotrophs	Cultivation in tryptone yeast extract agar at 22˚C and 36˚C, respectively	68 h at 22˚C, 44 h at 36˚C
DS/EN ISO 9308-1 (2002) Water quality – Detection and enumeration of *Escherichia coli* and coliform bacteria – Part 1: Membrane filtration method	TCs and *E. coli*	MF followed by cultivation on Lactose TTC agar with Tergitol 7 at 36˚C + confirmation	21 h + 21 h for confirmation
DS/EN ISO 7899-2 (2000) Water quality – Detection and enumeration of intestinal enterococci – Part 2: Membrane filtration method	Enterococci	Cultivation on Slanetz-Bartley agar at 36˚C + confirmation on Bile Esculin Azide agar at 44˚C	44 h + 2 h for confirmation

Microbial contaminations of drinking water are most often rapid episodes of low intensity, and the probability of detecting a fecal contamination depends on several parameters including the intensity of the contamination (Figure 1A), the duration and distribution of the contamination, the sampling frequency (Figure 1B), the time interval between sampling and resampling, and the sensitivity of the analysis method (Boe-Hansen et al. 2003). In Denmark, the frequency of sampling depends on the size of the water distribution system, and for small systems (i.e. <350,000 m^3 water per year), the sampling frequency can be less than four times a year (Danish EPA 2011).
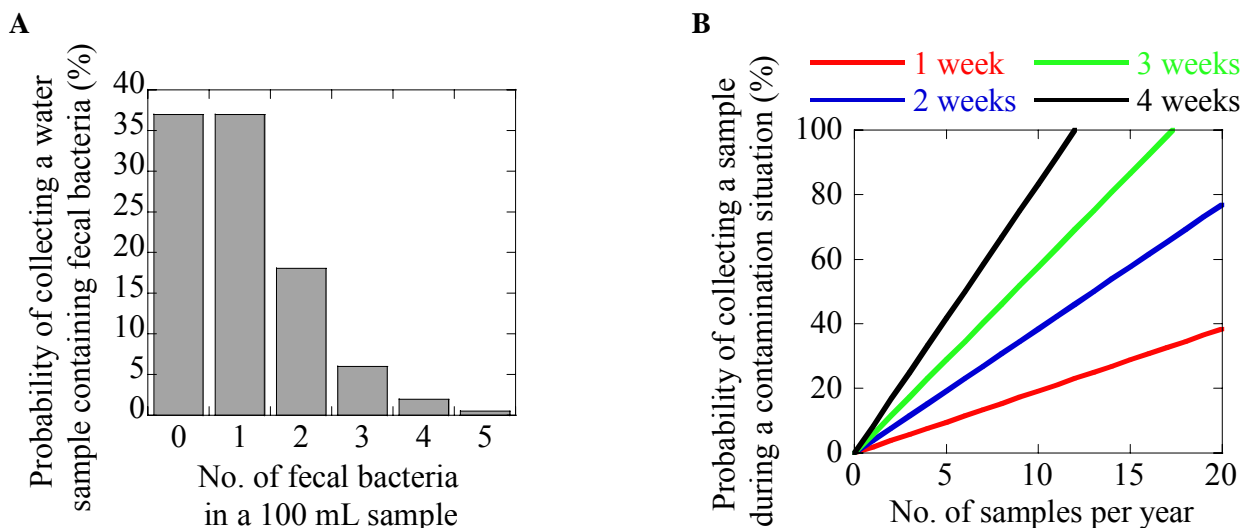
Figure 1 (A) The probability of collecting a water sample containing a certain amount of fecal bacteria in a 100 mL water sample collected from a water distribution system with a contamination of 1 fecal bacterium per 100 mL. **(B)** The probability that a sample is collected during a contamination situation is given by the product of the duration of the contamination and the sampling frequency. This probability is illustrated for contamination situations of 1, 2, 3, and 4 weeks (modified from Boe-Hansen et al. 2003).

Traditional, cultivation-based methods for assessing the microbial water quality often have a low analytical limit of detection (LOD) (e.g., Wohlsen et al. 2006, Al-Turki and El-Ziney 2009), but are associated with long analysis times (≥ 21 h) that preclude rapid intervention in case of contamination events (Table 2). Another problem combined with cultivation-dependent methods is the metabolic state of the target bacteria in environmental waters. Many bacteria are able to survive in aquatic matrices for long periods of time by starvation and/or by entering a viable but non-culturable (VBNC) state (Roszak and Colwell 1987, Colwell 2000, Bjergbæk and Roslev 2005, Oliver 2005, Oliver 2010). These bacteria may be difficult or even impossible to resuscitate and then grow in culture media. Hence, they will not be detected using cultivation-dependent standard methods leading to an underestimation of the abundance of the target bacteria.

2 Rapid enzyme-based detection of fecal indicator bacteria in water

Application of enzyme-based detection principles may facilitate a more rapid response of fecal contaminations compared to standard cultivation-based methods. Enzyme-based detection does not require the formation of microcolonies as is the case of plate spread techniques as e.g., the ISO standard methods 9308-1 and 7899-2 for detection of *E. coli* and enterococci in drinking water, respectively (Table 2, ISO 9308-1 2000, ISO 7899-2 2000). The enzyme-based methods detect viable and active cells, and moreover, a relatively non-complex sample processing provides a potential of these methods for being transferred to sensor-based platforms to provide automatic on-line monitoring within the drinking water distribution net or elsewhere.

Here, the term 'rapid' is used for methods that provide results within one working day, i.e. within 6-8 h, including the sample collection and preparation time.

Several studies have focused on fluorescent-based enzyme methods for rapid detection of β-GUS activity in *E. coli*. The methods are based on hydrolysis of the fluorogenic substrate 4-methylumbelliferone-glucuronide (MUGlu), and have been applied to detect *E. coli* in different waters such as tap water (Berg and Fiksdal 1988), coastal waters (Fiksdal et al. 1994, Lebaron et al. 2005), freshwater (George et al. 2000, Petit et al. 2000, Garcia-Armisen et al. 2005), wastewater (George et al. 2001) and river water (Wildeboer et al. 2010). Less effort has been made for setting up rapid enzyme-based detection principles for enterococci even though the analysis time in the cultivation-based standard method for assessing the abundance of these bacteria in water are significantly higher compared to that of *E. coli* (Table 2).

2.1 Chemiluminescence-based detection

Chemiluminescence-based detection is reported as being more sensitive compared to fluorescence- and chromogenic-based detection (Olesen et al. 2000, Van Dyke and Woodfork 2002). In addition, it has been shown that the chemiluminescent substrates 1,2-dioxetanes specific for β-GAL and β-GUS are 20-fold more sensitive than the fluorogenic substrate MUGlu and a 100-fold more sensitive than the chromogenic substrate ortho-nitrophenyl-β-galactoside (ONPG) (Van Poucke and Nelis 1995).

2.1.1 Chemiluminescent 1,2-dioxetanes as enzyme substrates

Bronstein et al. (1989) described 1,2-dioxetane chemiluminescent substrates specific for alkaline phosphatase and β-GAL. These substrates were later used in e.g., reporter gene assays and immunoassays (Jain and Magrath 1991, Bronstein et al. 1996, Olesen et al. 1997, Olesen et al. 2000, Dungchai et al. 2007). The 1,2-dioxetanes are high-energy compounds consisting of a four-membered cyclic peroxide (Figure 2). An adamantyl core, a fluorescent chromophore, and an enzyme cleavable group are attached to the four-membered ring in order to provide stability, emissive properties, and chemiluminescent activation, respectively (Bronstein 1997, Giri 2002).
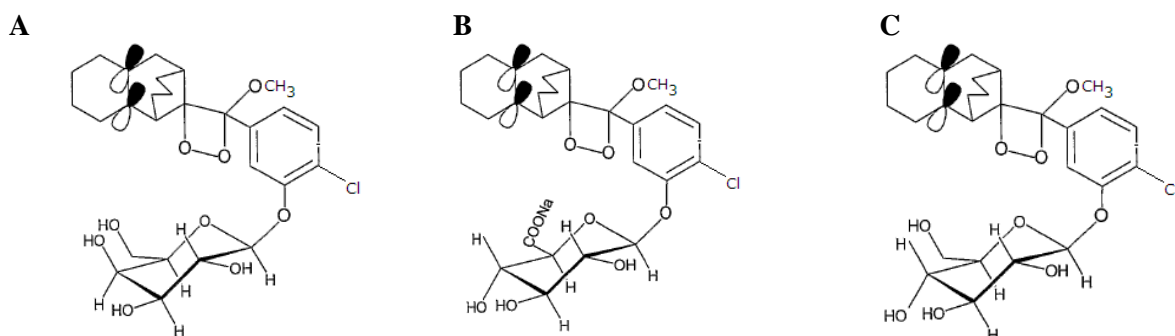
Figure 2 Structure of the 1,2-dioxetanes **(A)** [(4-methoxy-4(3-β-D-galactose-4-chlorophenyl)] spiro [1,2-dioxetane-3-1,3-tricyclo[7.3.1.0^{2,7}]tridec-2,7-ene], **(B)** sodium [4-methoxy-4(3-β-D-glucuronic acid-4-chlorophenyl)] spiro [1,2-dioxetane-3-1,3-tricyclo[7.3.1.0^{2,7}]tridec-2,7-ene], and **(C)** [(4-methoxy-4(3-β-D-glucoside-4-chlorophenyl)] spiro [1,2-dioxetane-3-1,3-tricyclo[7.3.1.0^{2,7}]tridec-2,7-ene] (modified from Giri et al. 2010).

The enzyme-removable protecting group is specific for a given enzyme. When the 1,2-dioxetane is hydrolyzed by the enzyme (Figure 3), an electron-rich dioxetane phenolate anion with a half-life of 2-30 min is formed (Bronstein et al. 1989, Olesen et al. 2000).

Figure 3 Enzymatic decomposition of a 1,2-dioxetane **(1)**. The 1,2-dioxetane is hydrolyzed by β-GAL, and a meta-stable anion **(2)** is formed. Via a CIEEL mechanism, the cyclic peroxide decomposes into two carbonyl fragments **(3 and 5)** of which one is in the electronically excited state **(3)**. When the excited state molecule returns to its ground state, a photon **(4)** is emitted (modified from Giri et al. 2010).
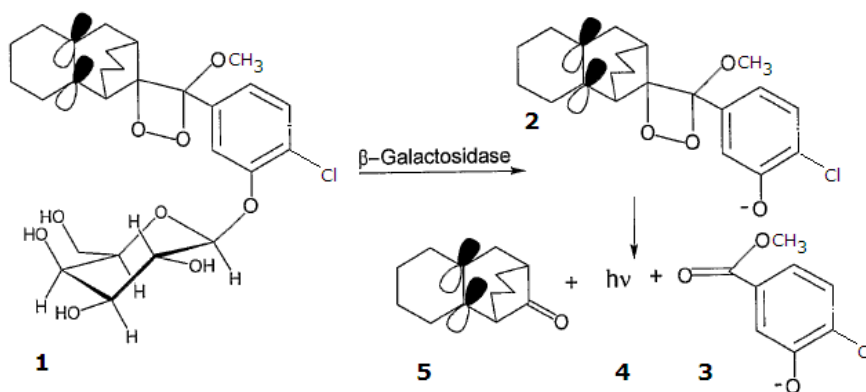
Decomposition of this meta-stable anion can be described using the chemically initiated electron exchange luminescence (CIEEL) mechanism (Schuster 1979, Beck and Köster 1990, Hummelen et al. 1991). Upon hydrolysis, energy is transferred from the phenolate anion to the dioxetane ring causing cleavage of two bonds in the cyclic peroxide. The released energy excites one of the resulting carbonyl fragments which emits chemiluminescent light as it converts back to its ground state (Bronstein et al. 1989, Olesen et al. 2000).

Enzyme reactions leading to decomposition of 1,2-dioxetanes are performed in aqueous solutions where proton transfer occurs. Hence, the excited state charge-transfer is easily quenched resulting in low chemiluminescence intensity. To overcome this problem, a water-soluble polymeric quaternary ammonium compound can be introduced after the hydrolysis reaction (Figure 4). This compound enhances the luminescence intensity by introducing a hydrophobic environment surrounding the excited molecules and hereby eliminates or severely reduces quenching (Giri 2007).

Figure 4 (A) Reduction of quenching where the water-soluble polymeric quaternary ammonium compound **(1)** encapsulates the excited state molecule **(2)** (modified from Giri 2007), and **(B)** the effect on the luminescence intensity given in counts per second (CPS) upon addition of a water-soluble polymeric quaternary ammonium compound, Triggering Reagent, TR (Bukh, unpublished results).

An advantage of the 1,2-dioxetane-derived chemiluminescence is a long lifetime of the excited state molecule. This eases the detection, as the glow-type light emission will persist for several minutes compared to flash-type reactions (Figure 5).
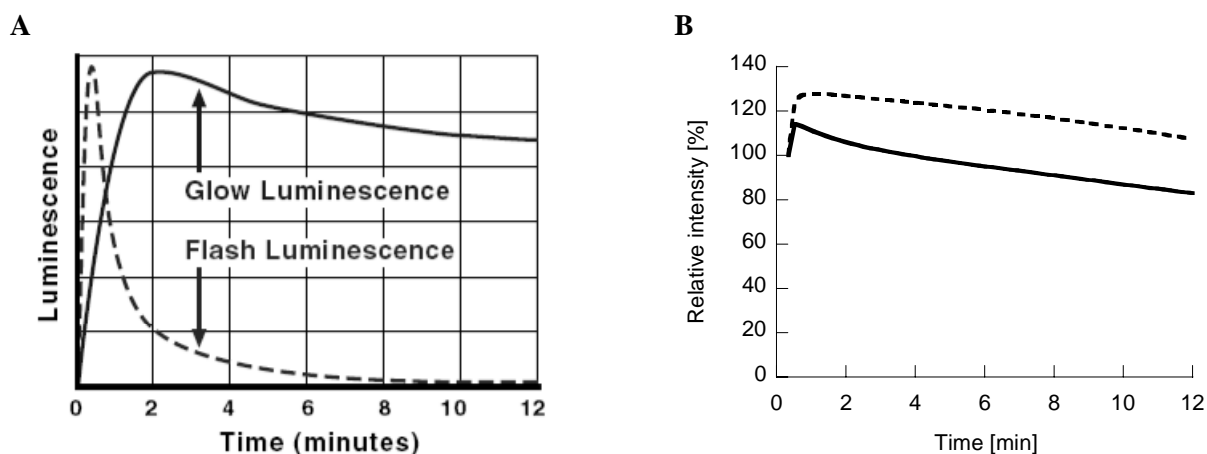
**Figure 5 (A)** Kinetics of flash- and glow-type luminescence reactions (modified from Van Dyke and Woodfork 2002), and **(B)** of light emission as relative intensity (%) for the 1,2-dioxetanes [(4-methoxy-4(3-β-D-galactose-4-chlorophenyl)] spiro [1,2-dioxetane-3-1,3-tricyclo[7.3.1.0^{2,7}]tridec-2,7-ene] and sodium [4-methoxy-4(3-β-D-glucuronic acid-4-chlorophenyl)] spiro [1,2-dioxetane-3-1,3-tricyclo[7.3.1.0^{2,7}]tridec-2,7-ene] activated with β-GAL (solid line) and β-GUS (dashed line), respectively (modified from Paper I).

Furthermore, no excitation source is needed in chemiluminescence as is the case for fluorescence which simplifies requirements for the detection devise. Background luminescence (i.e. noise, N) will also be reduced as many naturally occurring compounds that are fluorescent due to the presence of aromatic compounds (e.g., some amino acids), will not be excited and hence will not interfere with the light emission.

Chemiluminescent 1,2-dioxetanes are very stable compounds, and no significant contribution to the measured chemiluminescence intensity by autohydrolysis of the substrate at room temperature are seen (Student's *t*-test; $P > 0.095$, n=3) (Figure 6A). An experiment examining the stability of the 1,2-dioxetane sodium [4-methoxy-4(3-β-D-glucuronic acid-4-chlorophenyl)] spiro [1,2-dioxetane-3-1,3-tricyclo[7.3.1.0^{2,7}]tridec-2,7-ene] at 10˚C over time has shown a long shelf life of this substrate

(Figure 6B). The measured signal-to-noise (S/N) ratio varied over time, but the substrate did not degrade. The long shelf life is important as on-line monitoring of the microbial water quality may take place at-line in connection with the water distribution system, and it would be favorable that the system can run autonomously for 1-2 month without any maintenance.

Figure 6 Stability of the 1,2-dioxetane 1,2-dioxetane sodium [4-methoxy-4(3-β-D-glucuronic acid-4-chlorophenyl)] spiro [1,2-dioxetane-3-1,3-tricyclo[$7.3.1.0^{2,7}$]tridec-2,7-ene] specific for β-GUS. **(A)** Autohydrolysis over time measured at room temperature (mean counts per second (CPS) ±95% CI; n=3), and **(B)** shelf life at 10˚C of the undiluted substrate (mean signal-to-noise ratio (S/N) ±95% CI; n=3) (Bukh, unpublished results).
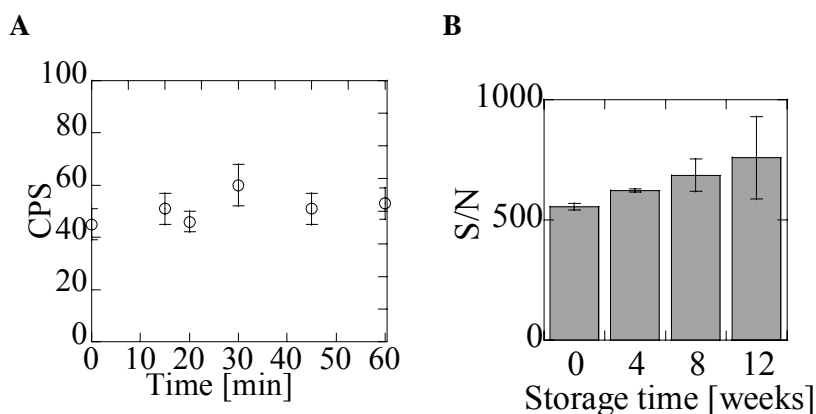
2.1.2 Detection of fecal indicator bacteria using chemiluminescent 1,2-dioxetanes

A detection principle for β-GAL based on a chemiluminescent 1,2-dioxetane was introduced by Van Poucke and Nelis for detection of TCs in sterile water within 7-10 hours (Van Poucke and Nelis 1995). After 6 hours of pre-incubation, 1 CFU of *E. coli* per 100 mL of sterile water, and after 9 hours, 1 CFU of *K. pneumoniae* per 100 mL of sterile water could be detected. Later, the detection principle by Van Poucke and Nelis (1995) was evaluated for detection of microcolonies on membrane filters of waterborne coliforms and *E. coli* based on β-GAL and β-GUS activity after pre-enrichment of samples for 5-9 hours on Colicult agar supplied with cefsulodin for suppression of *Aeromonas* spp. (Van Poucke and Nelis 2000). It was possible to detect pure cultures of *E. coli* after 5.5-6.5 hours of incubation, but when applied to natural samples, approximately 20% of *E. coli* did not form microcolonies and could not be detected using a CCD-camera or X-ray film within this time. Using solid phase cytometry, it was possible to detect microcolonies and non-dividing single cells based on hydrolysis of fluorescein-labelled substrates within 3.5 hours (Van Poucke and Nelis 2000). However, this technique requires trained technical skills as well as acquisition of expensive equipment.

Novel, chemiluminescent detection principles for rapid detection of β-GAL in TCs, β-GUS in *E. coli*, and β-GLU in enterococci have recently been optimized, characterized, and evaluated regarding their potential for rapid assessment of the microbial water quality (Paper I, Paper II, Paper III). The chemiluminescent 1,2-dioxetane specific for β-GUS was used in an assay, ColiLight II, for rapid detection of viable *E. coli* spiked into non-disinfected drinking water (Paper I). In this study, <30 CFU of stressed *E. coli* per 100 mL of water were detected within 6 hours. In another study, the method for detection of enterococci, EntLight, was used to detect 62 CFU of stressed *Ent. faecalis* cells in water after a pre-enrichment for 6 hours in selective broth (Paper III).

2.2 Challenges of applying enzyme-based methods

Application of enzyme-based methods for detection of fecal contaminations in water is associated with several challenges, and some are summarized (Table 5) and discussed below.

Table 5 Challenges in application of rapid, enzyme-based methods for detection of fecal contaminations in water (Van Poucke and Nelis 1997, Tryland and Fiksdal 1998, Rompré et al. 2002, Fiksdal and Tryland 2008)

- The target bacteria are often present in low abundance
- The physiological status of the target bacteria (the target bacteria may be dormant)
- Suppression of activity in target bacteria by high concentrations of heterotrophic bacteria
- Target bacteria lack expression of target enzymes (false-negatives)
- Presence of non-target organisms expressing the target enzyme (false-positives)

2.2.1 Abundance of target bacteria

Since target bacteria often are of low abundance in the water, samples needs to be concentrated in order to obtain detectable levels of enzyme activity. Using membrane filtration (MF) for sample concentration followed by incubation of the membrane filter on selective agar medium, it is possible to quantify the number of targets since only target bacteria will grow and form microcolonies on the selective agar medium. However, it takes time for the target bacteria to form microcolonies, and stressed and injured cells may not be able to do so resulting in underestimating of the abundance of target bacteria. An advantage of using MF is to remove extracellular target enzymes from non-target species that would have interfered with the specificity of the enzyme method. Additionally, MF is a fast way to concentrate samples if the waters are of relatively low turbidity such as drinking water. Methods for time- and flow integrated sampling are emerging, and this sampling strategy shows promising as more knowledge about the microbial water quality will be gained. Furthermore, the use of larger sample volumes indirectly lowers the LOD of the analysis method, and thus results in more sensitive analyses.

The ColiLight II assay (Paper I, Paper II) has been used for detection of TCs in drinking water samples collected using flow-integrated sampling at a waterworks in North Jutland, Denmark, during a contamination situation in the winter of 2009 (Table 6). The data obtained from the samples suggest that the ColiLight II assay in combination with flow-integrated sampling is applicable to detect stressed TCs in real drinking water samples. Furthermore, the data confirm the gain in representativeness of the microbial water quality when samples are concentrated as the CCA plates were positive for TCs (1 L) in contrast to Colilert-18 (100 mL). The negative detection using ColiLight II in sample 2 may be explained by strain-associated variability in enzyme activity (Tryland and Fiksdal 1998, Paper II) in combination with a level of TCs in the 10-L samples around the analytical LOD of the ColiLight II assay (Paper I). This problem could be overcome by increasing the sample volume.

Table 6 Detection of TCs using ColiLight II in combination with flow-integrated sampling of drinking water collected at a waterworks during a contamination situation in the winter of 2009 (mean±SE; n=3). HPC was performed at 22°C according to DS 6222-1 (DS 6222-1 2002). TC abundance was determined using Colilert-18 (100-mL samples) and Chromocult Coliform Agar (CCA) (1-L samples). ND: Not determined (Bukh, unpublished results)

Sample	DS 6222-1 (22°C; 68±4 h)	Colilert-18 (18 h)	CCA (24 h)	ColiLight II (6.5 h)
	[CFU/mL]	[MPN/100 mL]	[CFU/100 mL]	P/A in 10 L
1	ND	0.3-5.6	ND*	Positive
2	2.5±0.5	<0.46	9.1	Negative
3	185±5	<0.46	2.0	Positive

* Using ISO 9308-1 on 10-L water samples, confluating growth of TCs was observed.

One of the drawbacks of MF in combination with enzyme methods is that the accumulated deposit on the membrane may inhibit the activity of the target bacteria as the higher concentrations of heterotrophic bacteria may suppress the activity in the target bacteria comparable to the inhibition of growth of target bacteria in standard plate spreading.

2.2.2 Physiological state of target bacteria

Another issue to be addressed is the physiological status of the target bacteria. It is well-known that when fecal bacteria and pathogens enter oligotrophic environments they may enter a VBNC state as a survival strategy. This state may be induced by several stress factors such as nutrient starvation, exposure to antimicrobial agents, and temperature changes (Roszak and Colwell 1987, Colwell 2000, Oliver 2005, Oliver 2010). Survival experiments in freshwater microcosms have suggested that β-GUS activity was retained in VBNC *E. coli* cells, and that a measured decrease in β-GUS activity followed the loss of genetic integrity by means of DNA stability (Petit et al. 2000). Using enzyme-based methods for fluorescent-based detection of TCs and fecal coliforms in wastewater (George et al. 2001), viable *E. coli* in river water and wastewater (Garcia-Armisen et al. 2005), and viable *E. coli* in coastal seawater (Lebaron et al. 2005), and using chemiluminescent-based detection of *E. coli* in drinking water (Paper II), and enterococci in water contaminated with raw municipal wastewater (Paper III) have resulted in significant linear correlations (r=0.72-0.96) between the outcome of the enzyme-based methods and standard methods for determining the microbial water quality (e.g., ISO 9308-1 2000, ISO 9308-3 1999). The findings were similar: The slopes of the log-log linear regression lines obtained between activity of the target enzyme and the abundance determined with standard method were below 1 (0.44-0.83) indicating that the enzyme activity per culturable target cell decreased as the number of culturable target cells increased. By some authors, this was explained by an underestimation of culturable cells in less contaminated waters due to the presence of VBNC cells with retained enzyme activity (George et al. 2000, Petit et al. 2000). This suggestion was later supported by results showing that the ratio between β-GUS activity and viable *E. coli* detected by DVC-FISH in slightly to highly contaminated freshwater samples were nearly 1:1 (Garcia-Armisen et al. 2005).

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In media used for pre-enrichment, the addition of selective agents, that suppress the growth of non-target cells, may suppress the enzyme activity within stressed and injured target cells as these cells may have increased susceptibility towards the selective agents. However, it has been shown that rapid, enzyme assays can detect low concentrations of stressed *E. coli* and enterococci after pre-enrichment in a selective medium (Paper I, Paper III).

2.2.3 Interference from false-positive organisms

β-GAL is expressed in Gram-negative bacteria such as *Enterobacteriaceae*, *Vibrionaceae*, *Pseudomonadaceae*, and *Neisseriaceae*, in several Gram-positive bacteria, yeasts, protozoa, and fungi (Tryland and Fiksdal 1998), and β-GUS is produced in most *E. coli* strains (Kilian and Bülow 1976, Edberg et al. 1986, Rice et al. 1990, Martins et al. 1993), some *Shigella* and *Salmonella* strains, a few *Yersina*, *Citrobacter*, *Edwardia*, and *Hafnia* strains, and in other bacteria such as *Flavobacterium* spp., *Bacteroides* spp., *Staphylococcus* spp., *Streptococcus* spp., anaerobic corynebacteria, and *Clostridium* spp. (Kilian and and Bülow 1976, Tryland and Fiksdal 1998). All enterococci express β-GLU (Devriese et al. 1993, Facklam et al. 2002, Fisher and Phillips 2009), but the enzyme is also expressed in various bacteria and fungi (e.g., Woodward and Wiseman 1982). That is, the target enzymes used in standard reference methods as well as in rapid enzyme methods are not specific for the target bacteria. Several sources may contribute to target enzyme activity as illustrated below (Figure 7).

Figure 7 Potential contributions to measured enzyme activity in rapid enzyme assays for detection of fecal contaminations in water. Green box: Specific enzyme activity is often significantly correlated to the number of culturable bacteria. Yellow boxes: Contribution of VBNC target bacteria as well as non-target bacteria from fecal origin can be acceptable in enzyme assays for detection of fecal contaminations in water. Red boxes: Interference from enzyme activity of dead target bacteria and of non-fecal origin should be limited due to its lack of association with a potential health risk (modified from Fiksdal and Tryland 2008).
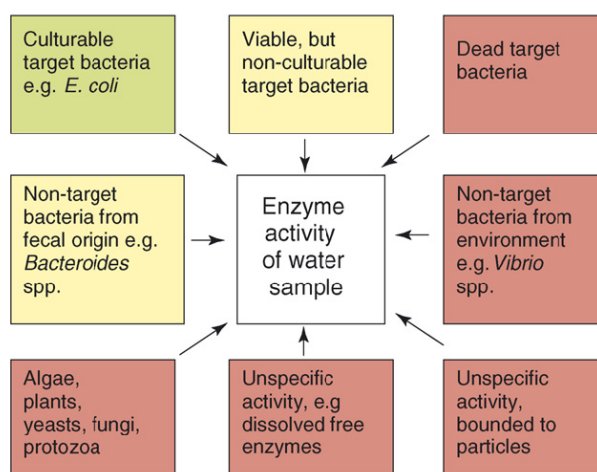
Hence, there is a need to introduce a selective step to increase the specificity of the enzyme-based detection methods. However, this will compromise the rapidity of the methods. When Van Poucke and Nelis validated their detection principle using natural waters including surface and tap waters (Van Poucke and Nelis 1995, Van Poucke and Nelis 1997), they found that the rapidity of the sensitive detection principle compromised the specificity of the method as the enzyme activity in non-target bacteria (mainly in *Aeromonas* spp., *Staphylococcus* spp., *Bacillus* spp., *Flavobacterium* spp., and *Pseudomonas* spp.) was not completely eliminated during the applied pre-incubation step in Colicult medium (Van Poucke and Nelis 1997). Furthermore, they observed that low initial abundance of target bacteria (<10 TCs per 100 mL) resulted in false-negative responses using the

enzyme-based method as the applied pre-incubation step did not result in predominance of target bacteria over an excess of other non-target bacteria that also expressed the target enzyme.

Later, Tryland and Fiksdal (1998) evaluated the potential influence of false-positive bacteria in rapid enzyme-based assays for detection of TCs and *E. coli* in water. They found that the degree of interference depends on the initial level of non-targets relative to the level of target bacteria and the individual cell enzyme activity. Furthermore, they observed a lower level of induction of β-GAL activity by IPTG and of β-GUS activity by MetGlu in the non-target environmental isolates included in the study (e.g., *Sphingomonas* spp., *Pseudomonas* spp., and *Staphylococcus* spp.) compared to in TCs and *E. coli* (Tryland and Fiksdal 1998). These findings suggest that many of the false-positive bacteria must be present in much higher levels (2-3 log units) compared to the target bacteria to interfere with the rapid enzyme assays. Other studies have suggested that introducing pre-enrichment in selective broth supplied with enzyme inducers can eliminate or severely reduce the interference of non-target bacteria (Paper I, Paper II, Paper III).

2.3 Performance of enzyme-based detection principles

Qualitative methods can be used as screening tools to determine whether there is a fecal contamination present or not. If a contamination is detected using the qualitative method, quantification can subsequently be performed with the routine methods if necessary. Prior to the application of a new, qualitative method, the analytical requirements such as incubation time and – temperature etc. and the performance of the method such as the sensitivity, specificity, LOD, robustness, and representativeness must be addressed in order to understand the quality of the method outcome (Trullols et al. 2004, Sartory 2005, Paper I). The sensitivity of a method is defined as the ability of the method to detect truly positive samples as positive, and the specificity of the method is defined as the ability of the method to detect truly negative samples as negative. Below, several enzyme-based detection methods used for detection of TCs, *E. coli*, and enterococci in different types of water are compared regarding specificity, sensitivity, and analysis time (Table 7).

Table 7 Comparison of performance criteria and analysis time for enzyme-based detection methods. n: number of samples; Spec.: specificity; Sens.: sensitivity; Time: analysis time; P/A: presence/absence; ND: not determined

Analysis	Matrix	Enzyme/substrate	n	Spec. (%)	Sens. (%)	Time [hours]	References
Colilert-18 (MPN)	Freshwater	β-GUS/MUGlu, β-GAL/ONPG	432 wells, 590 wells	92.6 90.4	96.5 93.7	18	Chao et al. (2004)
Colisure (MPN)	Chlorinated sewage effluent	β-GUS/MUGlu, β-GAL/?	526 tubes, 523 tubes	96.0 96.7	97.6 100	24	McFeters et al. (1997)
Colifast (P/A)	Raw water, in-process water, and wastewater	β-GUS/MUGlu	522 vials	97	98	8-14	Lesne et al. (2001)
Enterolert (MPN)	Marine and freshwater	β-GLU/MUD	138	94.9	99.6	24	Budnick et al. (1996)
(P/A)	Pure cultures of isolates from sewage, river and coastal water	β-GUS/MUGlu, β-GAL/MUGal	-	ND	ND	0.5	Fiksdal et al. (1994), Tryland and Fiksdal (1998)
(P/A)	Pond and river water, coastal seawater, raw sewage	β-GUS/MUGlu, β-GAL/MUGal	-	ND	ND	0.5	George et al. (2000), George et al. (2001), Garcia-Armisen et al. (2005), Lebaron et al. (2005)
(P/A)	River water	β-GUS/MUGlu	-	ND	ND	0.5	Wildeboer et al. (2010)
(P/A)	Surface, distribution, tap, and well water	β-GAL/1,2-dioxetane	80	40.3	81.9	6-7	Van Poucke and Nelis (1995), Van Poucke and Nelis (1997)
ColiLight I (P/A)	Drinking water	β-Gal/GP*- luciferin	-	ND	ND	7-8	Bastholm et al. (2008)
ColiLight II (P/A)	Pure culture and drinking water	β-GUS/1,2-dioxetane β-GAL/1,2-dioxetane	30 strains	94 60	100 100	4.5	(Paper I, Paper II)
EntLight (P/A)	Pure culture and seawater	β-GLU/1,2-dioxetane	42 strains	94	100	6.5	(Paper III)

*GP: β-galactopyranosyl

From Table 7 we see that the ColiLight II and EntLight assays performed similar to the methods based on defined substrate technologies regarding specificity and sensitivity, with an exception for the rather low specificity of the ColiLight II assay for detection of β-GAL. The other P/A tests with analysis times of 0.5-8 hours have not been evaluated regarding their specificity and sensitivity (Table 7). However, significant correlations have been reported between these P/A methods and

standard reference methods as was also the case for ColiLight II and EntLight (George et al. 2000, George et al. 2001, Garcia-Armisen et al. 2005, Lebaron et al. 2005, Wildeboer et al. 2010, Paper II, Paper III). The analysis time for ColiLight II and EntLight was significantly lower compared to the Colilert-18, Colisure, and Enterolert analyses, and a few hours lower than Colifast.

A relatively high analytical LOD of 10^2-10^3 cells of the ColiLight II and EntLight assays have been reported (Paper I, Paper III). Hence, a single FIB cannot be detected directly in a 100-mL water sample (the sample size used when testing the microbial drinking water quality in Denmark). P/A methods cannot determine the magnitude of bacteria concentrations. However, in drinking water, the maximum permissible value is <1 CFU per 100 mL and hence LOD of the P/A method is not necessarily the limiting factor. When analyzing large samples of a known volume, the LOD of the analysis method will be of less importance. That is, the limit of <1 CFU per 100 mL equals <1,000 CFU per 100 L which are possible to detect using the proposed detection principles, ColiLight II and EntLight, in combination with time- and flow-integrated sampling. Furthermore, the pre-enrichment included in the methods further enhances the sensitivity.

3 Selected opportunistic pathogens in water distribution systems

Legionella spp. and the MAC are opportunistic human pathogens. Both groups of bacteria are naturally found in freshwaters and humid environments, but also in domestic systems such as drinking water distribution systems, hot tubs, spas, and hot water systems (Falkinham 2004, Vaerewijck et al. 2005, Burnsed et al. 2007, Falkinham 2009, Feazel et al. 2009, Nishiuchi et al. 2009, Marciano-Cabral et al. 2010, Krøjgaard et al. 2011, Paper IV).

3.1 *Legionella* spp.

Legionella spp. are Gram-negative, aerobe, catalase-positive coccobacillus with more than 50 known species of which 19 have been associated with human infection (Joseph and Ricketts 2008, Gomez-Valero et al. 2009). It has been shown that *L. pneumophila*, the causative agent of Legionnaires' disease, can parasitize and multiply intracellularly in human monocytes (Horwitz and Silverstein 1980), human alveolar macrophages (Nash et al. 1984), and amoebae such as *Naegleria* and *Acanthamoeba*, which are ubiquitous in soil and water (Rowbotham 1980). *Legionella* spp. thrive at temperatures between 20-45˚C and may survive at even higher temperatures.

Danish hot water systems are mainly supplied by groundwater that naturally contains protozoa. In such systems, *Legionella* spp. can survive and proliferate by parasitizing protozoan hosts (Mampel et al. 2006, Declerck et al. 2009), and persist in biofilms for longer periods of time, even for years (Storey et al. 2004, van der Kooij et al. 2005). A recent study including 708 healthy Danish blood donors showed that 22.9% of all the donors had antibodies against *Legionella*, and these findings suggest that *Legionella* spp. are widespread in the environment, and that exposure to these bacteria is common (Rudbeck et al. 2008). The annual disease prevalence of *Legionella* pneumonia in Denmark has been more or less constant since 1995 with about 120 reported cases per year of which approximately 15% are lethal (Joseph and Ricketts 2008, SSI 2010).

Traditional cultivation-based methods for detection of *Legionella* spp. exist (ISO 11731 1998). However, *Legionella* spp. are fastidious when grown in the laboratory and require long analysis time (7-10 days) and even longer for confirmation steps (additional two or more days). More rapid methods for detection and quantification of *Legionella* spp. would be favorable.

3.2 MAC

MAC includes the *M. avium* subspecies *avium*, *paratuberculosis* (MAP), and *silvaticum* and *M. intracellulare* (Frothingham and Wilson 1993, Inderlied et al. 1993, Nichols et al. 2004). MAC strains are aerobic, non-motile, acid-fast Gram-positive, slowly growing bacilli that may produce a yellow pigment in the absence of light (Inderlied et al. 1993). In humans, MAC can cause pulmonary infections, hypersensitive pneumonitis, lymphadenitis, non-pulmonary lesions, and disseminated disease (Vaerewijck et al. 2005). MAC infections have mostly been associated with disseminated infections in HIV/AIDS patients, other immunodepressed patients, or elderly (Inderlied et al. 1993, Karakousis et al. 2004, Hernández-Garduño and Elwood 2010).

MAC is ubiquitous in nature and can be isolated from natural sources of water, pools, soils, dusts, and domestic water systems (Falkinham 2004, Falkinham 2009, Feazel et al. 2009, Nishiuchi et al.

2009, Paper IV), and MAC thrive at high temperatures and may survive at 50-55˚C (Schulze-Röbbecke and Buchholtz 1992). A widespread occurrence of the opportunistic pathogen MAC in domestic water systems has been reported (Table 3 and reviewed by e.g., Vaerewijck et al. 2005 and Falkinham 2009). Until recently, however, the occurrence of MAC in Danish domestic water systems has never been reported. In a recent study, water and biofilm samples collected from Danish hot water systems in public day care centers were examined for the presence of MAC, and the results indicated that MAC is widespread in both water and biofilms in these systems as well (Table 3, Paper IV).

Table 3 Non-exhaustive overview of the occurrence of MAC in water and biofilm in domestic water systems

Water system	Samples	Detection	Location	Number of MAC positive samples (number of samples)	References
Piped water supply systems	Water from wells, hot and cold municipal water supplies, showers, and standpipes	Culture	USA Finland Zaire Kenya	21% (14) 45% (11) 20% (5) 0% (14)	von Reyn et al. (1993)
Drinking water distribution systems	Water and biofilm from pipe materials or water meters	Culture	Eight locations throughout the USA	Water (528): 3% positive for *M. avium*, and 1% positive for *M. intracellulare* Biofilm (55): 5% positive for *M. avium*, and 24% positive for *M. intracellulare*	Falkinham et al. (2001)
Municipal hot water systems	Water and biofilm samples from showerheads	16S rRNA gene sequencing, qPCR	45 sites in nine cities in the USA	Water (12): 16S: 3.6%, qPCR: 92.9% Biofilm (89): 16S: 15.6%, qPCR: 78.1%	Feazel et al. (2009)
Municipal hot water systems	Water from shower and bathtub, and biofilm from showerheads, bathtub inlets, and bathroom drain	Culture	29 sites, Japan	Water (55): 15% Biofilm (104): 24%	Nishiuchi et al. (2009)
Drinking water	Cold water (pre- and post flush) and biofilm from faucets	qPCR	Two metropolitan areas in the Midwest, USA	Water (33): 81% of pre-flush and 88% of post-flush samples positive for MAP Biofilm (33): 76% positive for MAP	Beumer et al. (2010)
Municipal drinking water system	Cold water (post flush)	Culture	One site in North-central USA	Water (2): 100%	Marciano-Cabral et al. (2010)
Municipal hot water systems	Hot water (pre- and post flush) and biofilm from showerheads, showerhead tubes, and faucets	qPCR	Nine sites in Aalborg, Denmark	Water (18): 78% of pre-flush and 78% of post-flush samples Biofilm (22): 67%	Paper IV

It has been shown that MAC can form biofilm on various surface materials including those widely applied in domestic water systems such as brass, stainless steel (SS), and different types of plastics such as PVC and PEX (Table 4).

Table 4 Non-exhaustive overview of levels of MAC in biofilms on various surface materials in different water matrices

Water type	Detection	Temperature	Surface material and level of biofilm	References
Drinking water distribution systems	Culture		Brass, plastic (av. 600 CFU of *M. intracellulare* cm^{-2} and 0.3 CFU of *M. avium* cm^{-2}	Falkinham et al. (2001)
Tap water	Culture, FISH	7, 15, 20°C	PVC: 7°C ~ 10^3 CFU cm^{-2}, $5 \cdot 10^5$ cells cm^{-2} 15°C ~ 50 CFU cm^{-2}, $4 \cdot 10^4$ cells cm^{-2} 20°C ~ $5 \cdot 10^3$ CFU cm^{-2}, $5 \cdot 10^5$ cells cm^{-2}	Lehtola et al. (2007) Torvinen et al. (2007)
Autoclaved potable water	Culture, microscopy	35°C	SS: 10^4-$5 \cdot 10^4$ CFU cm^{-2} PC: 10^4-10^5 CFU cm^{-2}	Williams et al. (2009)
Deionized water	Culture	24°C	cPVC and copper: ~10^3 CFU cm^{-2} Galvanized and iron: ~$3 \cdot 10^4$ CFU cm^{-2}	Norton et al. (2004)
Municipal hot water systems	qPCR	~44°C	Brass: av. $2 \cdot 10^3$ genome copies cm^{-2} PVC: ~10^3 genome copies cm^{-2}	Paper IV
Hot tap water	Culture, qPCR	44°C	PEX: 10^4-10^5 CFU cm^{-2}, 10^4 genome copies cm^{-2}	Paper IV

As is the case with *Legionella* spp., MAC can parasitize protozoa and proliferate intracellularly in these hosts (Cirillo et al. 1997, Vaerewijck et al. 2005). MAC is considered as an important human pathogen, and a tendency of increasing MAC infections has been reported (Cassidy et al. 2009, Lai et al. 2010, Moore et al. 2010, Thomson 2010, Winthrop et al. 2010). In Denmark, little is known about the disease prevalence of MAC.

Standard procedures for isolation of MAC from water samples do not exist. However, recently an optimal procedure for processing drinking water samples for the isolation of MAC has been proposed (Thomson et al. 2008). The author suggested concentration by membrane filtration compared to centrifugation, and incubation for three weeks at 32-35°C on Middlebrook Agar or in Lowenstein-Jensen slants showed no difference in recovery. Introduction of a decontamination step, as is also included in the standard method for isolation of *Legionella* spp., has been suggested (e.g., Stinear et al. 2004, Thomson et al. 2008), whereas others have suggested not doing so, at least not in water holding low levels of microorganisms, due to a very low recovery rate (Falkinham et al. 2001, Falkinham 2004). Cultivation-based detection of MAC is tedious and has a very long analysis time (~3 weeks). Hence, more rapid methods for detection and quantification of MAC are needed.

4 Detection of fecal indicator bacteria and opportunistic pathogens using qPCR

Molecular methods based on amplification of nucleic acids for detection and quantification of microorganisms in water are emerging, and these methods provide more rapid detection together with high specificity and analytical sensitivity compared to traditional cultivation-based methods. Molecular amplification-based methods include conventional PCR and quantitative real-time PCR (qPCR). For some pathogens, no culture-based detection systems exist and thus molecular techniques are the only methods for detection (Girones et al. 2010). Due to high sensitivity and specificity, qPCR techniques are widely used in microbial ecology, and show great promise for future use for determination of microbial water quality.

qPCR is now widely used for detection and quantification of FIB in different water types. In 2003, Frahm and Obst established probe-based qPCR assays for detection and quantification of enterococci and *E. coli* in drinking water (Frahm and Obst 2003). Primers for enterococci targeted a part of the 23S rRNA gene, and *E. coli* primers targeted a part of the *uidA* gene encoding β-GUS. Using natural water samples, an agreement between the qPCR assays and cultivation-based methods of 96% and 98% was found for enterococci and *E. coli*, respectively. Later, these primers and probe designed for *E. coli* were used for evaluation of the survival and persistence of *E. coli* in filter-sterilized groundwater at 4°C (Cook and Bolster 2007). In another study, a SYBR Green-based qPCR assay targeting the D-1-deoxyxylulose 5-phosphate synthase gene (*dxs*) was used for detection and quantification of *E. coli* in drinking water and biofilms (Paper II). The assay could quantify down to ~23 genome copies in a given sample. Other studies have focused on detection and quantification of *E. coli* pathotypes such as enterotoxigenic *E. coli* in surface water, seawater and freshwater (Lothigius et al. 2010, Ram et al. 2010), and enterohaemorragic *E. coli* e.g., in surface water and watershed samples (Mull and Hill 2009, Park et al. 2011). A probe-based qPCR assay has been described for detection of enterococci targeting a part of the 23S rRNA gene. The assay could detect 27 cells in freshwater samples in less than three hours (Haugland et al. 2005). Using these primers and probe, He and Jiang (2005) detected enterococci in various water types including sewage, brackish coastal waters, and creek samples (He and Jiang 2005), and later, Walters et al. (2009) evaluated the persistence of enterococci and of naked DNA from *Ent. faecium* in seawater microcosms (Walters et al. 2009). In another study, a SYBR Green-based qPCR assay targeting the 16S rRNA gene was applied to detect and quantify enterococci in water contaminated with raw municipal wastewater (Paper III).

qPCR has also been applied for detection and quantification of the opportunistic pathogens *Legionella* spp. including the causative agent of Legionnaires' disease *L. pneumophila* in hot water samples (e.g., Joly et al. 2006, Yaradou et al. 2007, Chang et al. 2009, Delgado-Viscogliosi et al. 2009, Chen and Chang 2010, Krøjgaard et al. 2011, Paper IV) and biofilm samples collected from municipal hot water systems (e.g., Feazel et al. 2009, Chen and Chang 2010, Paper IV). Only a few studies have focused on detection and quantification of MAC using qPCR in water and biofilm from municipal hot water systems (Feazel et al. 2009, Paper IV).

4.1 SYBR Green-based detection and quantification of PCR products

SYBR Green is an intercalating dye that binds to the minor groove of dsDNA (Figure 8A). The dye is excited at ~480 nm and has emission maximum 520 nm. In the bound state, SYBR Green is a thousand fold more fluorescent compared to the unbound state. SYBR Green-based quantification of PCR products is based on the increase in fluorescence from bound dye as the amount of dsDNA (PCR products) in the sample increases. During denaturation, DNA becomes single-stranded (ssDNA), and SYBR Green is free in solution (Figure 8B). During annealing, primers hybridize to the amplicon, and the dye will bind to the dsDNA. During the extension step, more DNA becomes double-stranded, and more SYBR Green will bind resulting in an increase in fluorescence (Wilhelm and Pingoud 2003).
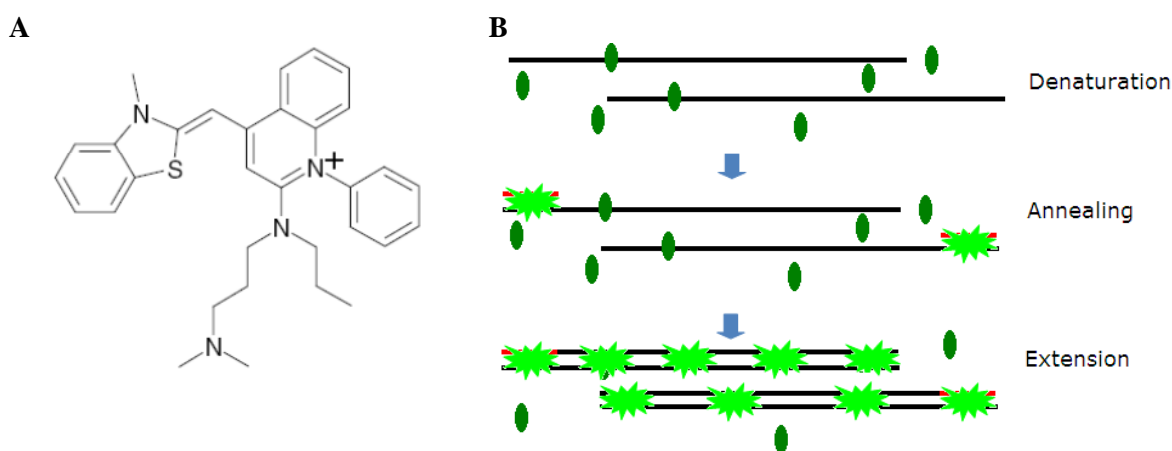

Figure 8 (A) SYBR Green dye free in solution (Kubista et al. 2006), and **(B)** the SYBR Green detection mechanism during the PCR reaction.

This increase in fluorescence depends on the initial amount of target DNA present in the amplification reaction. This is utilized for quantification in the linear amplification stage (above the cycle threshold, C_T) where the amount of amplified target is proportional to the initial amount of target DNA (Smith and Osborn 2009). However, the SYBR Green dye binds to all dsDNA so the primers must be highly specific for the target amplicon to avoid non-specific binding, and the amplification specificity of the qPCR assay should be evaluated.

Absolute quantification is based on standard curves based on C_T values plotted against known concentrations of serial dilutions of templates as illustrated below (Figure 9). From standard curves, the parameters PCR efficiency (E), regression equations, and correlation coefficients (R^2) describing the analytical performance of the qPCR reaction can be achieved. In addition, the minimum number of copies in a sample that can be measured accurately can be extracted from a standard curve. This value is determined the lower limit of quantification (LLOQ). Below LLOQ, quantitative data cannot be produced, but it is still possible to detect targets. The lowest concentration at which the target still can be detected is determined LOD (Peters and Maurer 2001).
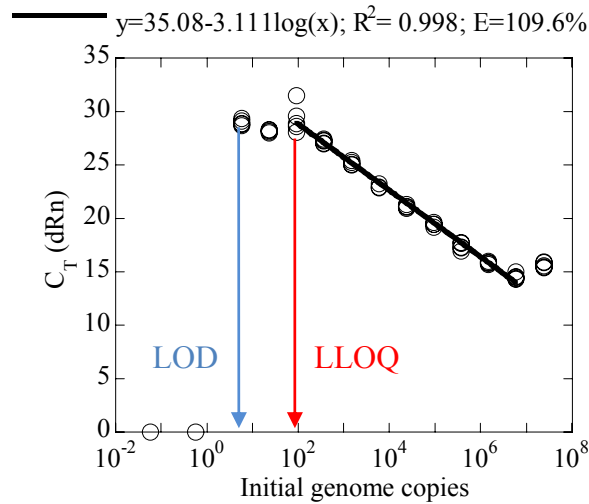
y=35.08-3.111log(x); R^2= 0.998; E=109.6%

Figure 9 Standard curve for quantification of *E. coli* based on the amplification of *dxs*. LOD determines the lower limit of detection, and LLOQ determines the lower limit of quantification (modified from Paper II).

The accuracy of the quantification of PCR products depends on the accuracy of the quantification of the DNA standards used to generate the standard curve. The outcome varies with the applied methods for measuring the DNA concentration as illustrated below (Figure 10).
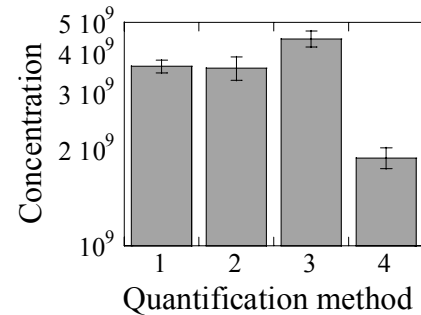

Figure 10 Example of variation in the outcome of different methods used for quantification of DNA standards of *E. coli* ED1a (mean±SE; n=3). **(1)** McFarland [CFU mL^{-1}]; **(2)** plate counts (TBX agar) [CFU mL^{-1}]; **(3)** UV/VIS spectrophotometry (Nanodrop) [genome copies mL^{-1}]; and **(4)** total cell counts (microscopy) [cells mL^{-1}] (Bukh, unpublished results).

Additional, DNA standards can be quantified using the Invitrogen PicoGreen dsDNA quantification kit. The advantages using this method over UV/VIS spectrophotometry (A$_{260\ nm}$), which is widely applied for quantification of DNA standards used in qPCR, are that PicoGreen is very sensitive with a LOD of down to 5 ng of dsDNA mL^{-1} (Batchelor et al. 2003), and highly specific as the dye only binds to dsDNA in contrast to spectrophotometry, where RNA, ssDNA, and nucleotides will interfere with the measured DNA concentration as will proteins and phenols if these substances were not completely removed during the DNA extraction procedure.

4.2 Challenges in application of qPCR assays for water samples

4.2.1 Abundance of target bacteria

The low abundance of FIB and bacterial pathogens in waters necessitates concentration of target bacteria prior to sample analysis in order to be able to detect these targets. Methods such as filtration, size-fractionation, centrifugation, immunomagnetic separation or combinations hereof are often used (Noble and Weisberg 2005, Jofre and Blanch 2010). The effect of the different sample

processing methods can have a great influence on the outcome of the following qPCR analysis as illustrated below for membrane filtration vs. centrifugation (Figure 11).

Figure 11 (A) Effect of different sample processing methods on the outcome of qPCR (mean±SE; n=2), and **(B)** the recovery of amplified genome copy numbers (mean±SE; n=2) based on MPN enumerations. DNA was extracted from 100-mL synthetic seawater samples spiked with $1.57 \cdot 10^6$ MPN mL^{-1} of *Ent. faecalis* ATCC 19433. Sample processing methods were MF using **(1)** 0.45 μm 47 mm nylon membrane filter with 10 sec of sonication and 10 sec of vortexing in the presence of 0.05% Tween-80 (x3); **(2)** 0.45 μm 47 mm nylon membrane filter with 1 min of vortexing; **(3)** 0.45 μm 47 mm nylon membrane filter, and **(4)** 0.45 μm 47 mm mixed cellulose ester membrane filter, and **(5)** centrifugation (Bukh, unpublished results).

4.2.2 Inhibitors

Another parameter affecting the outcome of the qPCR analysis is the composition of the sample matrix. Upon concentrating targets in water samples, substances that may inhibit the amplification reaction are also likely to be concentrated. These inhibitors include humic acids, metals such as iron, and biomass of non-target organisms (Noble and Weisberg 2005, Smith and Osborn 2009, Girones et al. 2010, Jofre and Blanch 2010). Hence, the choice of the method used for nucleic acid extraction is crucial to the quantification. This is illustrated below (Figure 12).
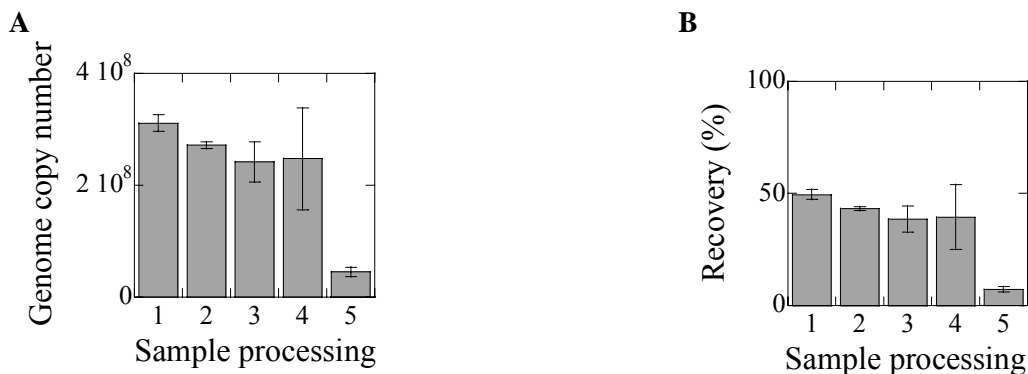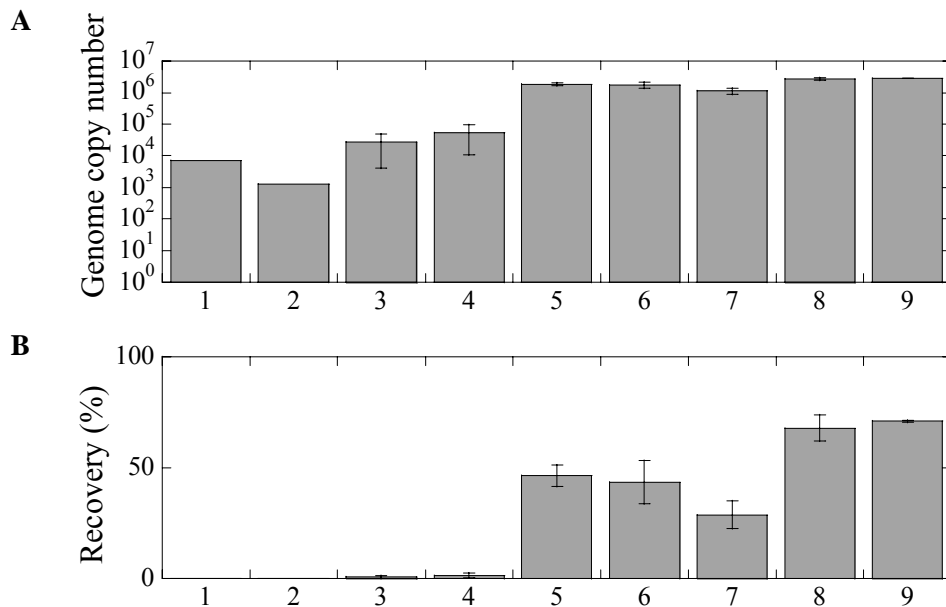
A

B

Figure 12 (**A**) Effect of different DNA extraction methods on the outcome of qPCR (mean±SE; n=2), and (**B**) the recovery of amplified genome copy numbers (mean±SE; n=2) based on MPN enumerations. DNA was extracted from 1-mL drinking water samples spiked with *E. coli* ED1a using (**1**) UltraClean Fecal DNA Isolation Kit (MoBio); (**2**) UltraClean Microbial DNA Isolation Kit (MoBio); (**3**) PowerWater DNA Isolation Kit (MoBio); (**4**) RapidWater DNA Isolation Kit (MoBio); (**5**) Bead beating (BB) using Garnet beads (0.7 mm) followed by extraction using Phenol:Chloroform:Isoamyl alcohol (PCI); (**6**) BB Quartz + PCI; (**7**) BB Glass 0.5 mm + PCI; (**8**) BB Glass 0.1 mm + PCI; (**9**) PCI (Bukh, unpublished results).

4.2.3 Quantification

Due to the relatively long persistence of DNA from dead cells and naked DNA, methods based on DNA amplification tend to overestimate the cell abundance in a given sample. This interference affects the applicability of using qPCR for a direct measurement of the microbial water quality. Hence, it is important to gain knowledge about the persistence of molecular markers in environmental waters (Roslev and Bukh 2011). The persistence of naked DNA has been examined for *Bacteroides vulgatus* in non-disinfected drinking water and in sterile drinking water (Saunders et al. 2009). In sterile drinking water at 10˚C, naked DNA was found to persist for at least 200 days whereas in non-sterile drinking water, the naked DNA was found to persist for about 150 days. Hence, the persistence of DNA from non-viable sources in non-disinfected drinking water is affected by predation by aquatic microorganisms. In hot water, it has been shown that DNA from dead cells and naked DNA of *M. avium* can persist for at least 28 days at 44˚C in the water phase of hot tap water microcosms (Paper IV). To abate or severely reduce this interference, it has been suggested that DNA intercalating dyes with an azide group such as ethidium monoazide (EMA) and propidium monoazide (PMA) can be used for selective discrimination of live cells, dead cells and naked DNA (Nocker et al. 2006, Bae and Wuertz 2009, Chang et al. 2009, Delgado-Viscogliosi et al. 2009, Gedalanga and Olson 2009, Chen and Chang 2010). The dye penetrates permeable, dead cells and binds to DNA by intercalation. The sample is exposed to bright visible light for a few minutes allowing the azide group to covalently bind to DNA leaving the DNA insoluble and as a result, naked DNA and DNA in the dead cells will be removed during DNA extraction (Nocker et

al. 2006). Using this technique, it is assumed that DNA in viable cells is protected from the dye by intact cell membranes. PMA has been favored for EMA as the latter can penetrate live cells of some bacterial species (Nocker et al. 2006, Flekna et al. 2007). When optimizing the PMA treatment to the sample matrix and the applied PCR method, this technique is a promising tool to discriminate between live and dead cells in microbial ecology. However, the direct use of PMA in qPCR assays where target amplicons are of small sizes has recently been questioned (Luo et al. 2010). Luo et al. (2010) showed that PMA did not suppress PCR amplification when the target was a 190 bp region of the 16S rRNA gene, but was able to suppress amplification when nearly the whole gene was amplified. The authors suggested to apply a two-step nested PCR in order to overcome the problem of amplifying a short DNA fragment where the first step is amplification of a large fragment (here nearly the whole 16S rRNA gene) in order to discriminate between live and dead cells, and subsequently a step using the PCR products as templates to amplify the short region (here the 190 bp region) (Luo et al. 2010). However, this will make the technique incapable of being used together with qPCR assays where the goal is to quantify targets in a given sample.

4.3 Determination of the microbial water quality by qPCR

The future potential of applying qPCR assays for assessing the microbial water quality is promising, but the outcome of these methods is different compared to the traditional cultivation-based methods used today. Hence, it is important to evaluate the outcome of the analyses using standard reference methods such as MF and MPN-based methods. Some qPCR assays have been evaluated in such a way. In freshwater samples collected at two recreational beaches (n=103), Haugland et al. (2005) determined a positive correlation between log-transformed results obtained using qPCR and the MF-method followed by incubation on mEI agar (r=0.68). The qPCR assay for detection and quantification of enterococci was well-described with a LLOQ of 27 cells (Haugland et al. 2005). In addition, positive correlations between log-transformed measurements obtained using the same primers and probe and the MF-method followed by incubation on mEI agar for enterococci were obtained for wastewater samples (r=0.76) and lake water samples (r=0.74-0.84), but not for stormwater samples (r=0.03) (Lavender and Kinzelman 2009). In the same study, the correlation between a qPCR assay based on *E. coli* Smartbeads and the Colilert-18 method was tested. Positive correlations were observed between the outcome of the two methods in wastewater samples (r=0.99) and in lake water samples (r=0.69-0.75) (Lavender and Kinzelman 2009). In another study using Smartbeads for detection and quantification of FIB in recreational waters (Noble et al. 2010), significant positive linear correlations were determined between the outcome of qPCR analyses and of the MF-method with incubation on mEI agar (r=0.86), Enterolert (r=0.82), and Colilert-18 (r=0.84). A qPCR assay for detection and quantification of *E. coli* in two different types of drinking water spiked with a reference strain and three clinical isolates, respectively, was evaluated using a culture-based MPN method (Figure 13A). A positive correlation between the outcome of the two methods (r=0.82) was reported (Paper II). A qPCR assay for detection of enterococci was evaluated using water samples contaminated with raw municipal wastewater using two ISO standard methods for detection of enterococci (ISO 7899-2 2000, ISO 7899-1 1999) (Figure 13B). Significant positive correlations between log-transformed results obtained using qPCR and the MPN-based ISO method

78991-1 (r=0.95; P<0.001), and between qPCR and the MF-based ISO method 7899-2 (r=0.97; P<0.001) were found (Paper III).
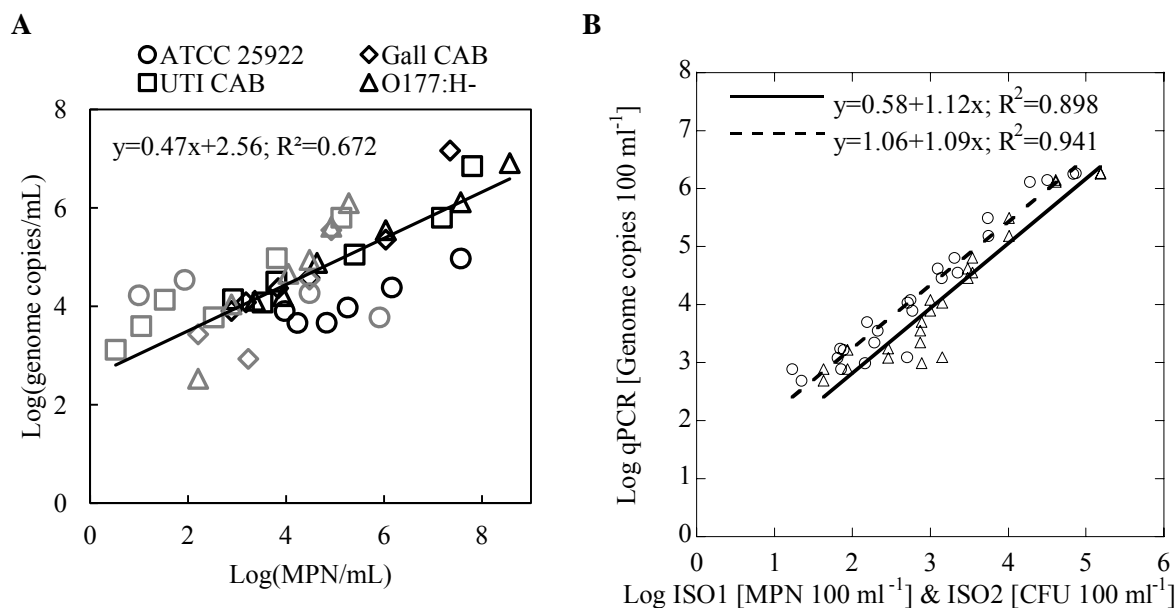

Figure 13 Correlation between (**A**) a culture-based MPN method and a qPCR assay for *E. coli* ATCC 25922 and three clinical strains *E. coli* UTI CAB, *E. coli* Gall CAB, and *E. coli* O177:H- after incubation for 24 hours at 10°C in two different types of drinking water collected in Aalborg (black) and Aarhus (grey) municipalities, respectively (Paper II), and (**B**) between ISO standard methods (—Δ—: ISO 7899-1, - -O- -: ISO 7899-2) and a qPCR assay for enterococci in sewer-contaminated water (Paper III).

Recently, Maheux et al. (2011) proposed real time PCR assays in combination with a whole genome amplification (WGA) step for rapid and sensitive detection (4.5 CFU per 100 mL of water sample in less than five hours) of enterococci and the fecal-specific species *Ent. faecalis* and *Ent. faecium* in potable water (Maheux et al. 2011). The WGA step was comprised of a 3-hours non-specific amplification reaction of total gDNA in the sample. The method still needs to be validated using culture-based methods to evaluate its usefulness in monitoring the microbial water quality, but it shows great potential as a rapid and sensitive real time PCR assay for this purpose. However, the WGA step compromises the ability of the method to quantify the target bacteria, and the method is hence qualitative.

In municipal hot water samples (n=92) collected from a French hospital, positive linear correlations between the results obtained using cultivation on BCYE/GVPC agar and qPCR assays targeting the 16S rRNA gene in *Legionella* spp. (r=0.73) and the macrophage infectivity potentiator gene (*mip*) in *L. pneumophila* (r=0.85) have been reported (Joly et al. 2006). In another French study, a similar correlation (r=0.86) in water samples (n=136) collected from municipal hot water systems was reported for a "ready-to-use" qPCR assay from GeneSystems targeting *L. pneumophila* (Yaradou et al. 2007). An Italian study found a lower correlation (r=0.63) between the results obtained using a qPCR assay targeting *mip* in *L. pneumophila* and cultivation on BCYE/GVPC agar in hot water samples (n=76) collected from hotels (Bonetta et al. 2010). Positive linear correlations between the outcome of a cultivation-based method using Middlebrook Agar and a qPCR assay targeting the

16S rRNA gene in MAC in hot water (r=0.69) and filter-sterilized hot water (r=0.83) have also been described (Paper IV).

Positive linear correlations between qPCR results and results obtained using traditional culture-based methods for consecutive samples indicate that qPCR can be used as important supplements for detection of FIB and opportunistic pathogens in water, and qPCR allows for rapid, sensitive, and highly specific detection and quantification of target bacteria within few hours of collecting the samples. This makes qPCR a promising tool for providing early warning of bacterial pollutions in waters of health-related concerns such as drinking water, recreational waters, and municipal hot water systems.

5 Conclusions

The objectives of this Ph.D. study were to develop rapid luminescence-based detection principles for fecal bacterial contamination in water, and to establish molecular-based assays for detection of FIB and selected opportunistic pathogens in water. The following conclusions are based on the experimental work presented in the four papers included in this thesis.

Novel, rapid chemiluminescent methods for detection of TCs and *E. coli* (ColiLight II) and enterococci (EntLight) have been established and thoroughly characterized regarding incubation time, temperature, pH, luminescence enhancer, enzyme inducers, substrate affinity, sensitivity, and specificity. The enzyme-based detection methods were more rapid (6-8 hours) compared to traditional cultivation-based methods (≥18 hours) for assessing the microbial water quality. The methods proved to be robust and highly sensitive assays for detection of β-GAL, β-GUS, and β-GLU activity within the target bacteria.

It was shown that ColiLight II based on β-GUS activity could be applied to detect low concentrations of stressed *E. coli* in drinking water (<30 CFU per 100 mL). ColiLight II was suitable for detection of different strains of *E. coli* in drinking water and drinking water biofilms. It was observed that the enzyme activity within various strains affected the detection of *E. coli* in these drinking water matrices. The results suggested that clinical strains of *E. coli* may persist longer in drinking water compared to culture collection strains.

EntLight could successfully be applied for specific detection of enterococci in water contaminated with raw municipal wastewater within 6-8 hours. Furthermore, EntLight showed a potential for providing early warnings of fecal pollutions in water within 1 hour of sampling.

qPCR assays were optimized, characterized, and validated for detection and quantification of *E. coli*, enterococci, *Legionella* spp., *L. pneumophila*, MAC, and eubacteria in water and biofilms. Sample processing and DNA extraction were optimized to minimize variations in sample preparation and eliminate PCR inhibitors to achieve robust results. The assays were successfully applied for detection and quantification of target bacteria in real water systems including drinking water, water contaminated with raw municipal wastewater, municipal hot water, and biofilms.

The persistence of four different strains of *E. coli* in drinking water and biofilm on SS and PE surfaces was examined in a flow-through reactor. More culturable *E. coli* cells were associated with biofilm on PE surfaces compared to SS surfaces. Some of the strains showed prolonged survival in the drinking water flow-through system with a 3 to 3.4 times longer persistence compared to the theoretical washout. The results indicated a strain-differentiated removal of *E. coli* from both water and PE and SS surfaces in the lab-scale drinking water reactor system due to biofilm formation.

The survival and persistence of the opportunistic pathogen MAC in hot water and biofilm was assessed using PEX microcosms. MAC remained culturable for at least 28 days in both the water

phase and in biofilm. A 3 $\log_{10}$-fold decay in concentration of both culturable MAC cells and total MAC genome copies was observed in the water phase during the initial two weeks. During the last two weeks of incubation, a 2 $\log_{10}$-fold increase was observed using both cultivation and qPCR. This, together with an observed increase in concentration of culturable and total MAC in biofilm, indicated net growth in the hot water and/or biofilm.

Investigations of the microbial quality in water and biofilm in hot water systems collected from nine public day care centers showed a widespread occurrence of the opportunistic pathogens *Legionella* spp. and MAC within these systems. These observations lead to the conclusion that the examined hot water systems serve as reservoirs for opportunistic pathogens, and may impact public health by serving as potential sources of infection in susceptible individuals.

Positive linear correlations were determined between the results using the novel enzyme-based methods, the qPCR assays, and traditional culture-based methods. This indicated that the rapid methods ColiLight II, EntLight, and qPCR can be used as important supplements to traditional cultivation-dependent methods for assessing the microbial water quality or at least serve as tools for providing early warnings of public health-related bacterial contaminations in water.

6 Perspectives

In this Ph.D. study, novel rapid methods for assessing the microbial water quality were established, characterized, and validated. The methods were successfully applied for detection of selected FIB and opportunistic pathogens in various water matrices and biofilms. Based on these findings, new knowledge about survival and persistence of these bacteria in various water matrices was obtained.

The simplicity of the detection principle of the novel enzyme-based methods provides a potential of transferring the detection principles to a sensor-based platform. This could provide a basis for more frequent analysis of the microbial water quality. Alternatively, the proposed methods could be available as a kit allowing for early warnings of fecal contaminations within one work day (6-8 hours).

The perspectives for further research on the molecular-based methods may concern the application of standardized guidelines for sample processing methods and qPCR assays for achieving rapid standard methods.

7 References

Al-Turki A, El-Ziney MG (2009) Evaluation of Commercial Colilert18-Quantitray® Method by ISO Techniques for Enumeration and Quantification of Total Coliforms and *Escherichia coli* in Drinking-Water of Buraidah, Saudi Arabia. J Appl Sci 9: 3357-3363

Bae S, Wuertz S (2009) Rapid decay of host-specific fecal *Bacteroidales* cells in seawater as measured by quantitative PCR with propidium monoazide. Water Res 43: 4850-4859

Bastholm S, Bjergbæk LA, Wahlstrøm L, and Roslev P (2008) A simple bioluminescence assay for early warning detection of coliform bacteria in drinking water. W J Microbiol Biotechnol 24: 2323-2330.

Batchelor R, Hagen D, Johnson I, Beechem J (2003) A fluorescent high-throughput assay for double-stranded DNA: the RediPlate PicoGreen assay. Comb Chem High Throughput Screen 6: 287-291

Beck K, Köster H (1990) Applications of Dioxetane Chemiluminescent Probes to Molecular Biology. Anal Chem 62: 2258-2270

Berg JD, Fiksdal L (1988) Rapid detection of total and fecal coliforms in water by enzymatic hydrolysis of 4-methylumbelliferone-beta-D-galactoside. Appl Environ Microbiol 54: 2118-2122

Beumer A, King D, Donohue M, Mistry J, Covert T, Pfaller S (2010) Detection of *Mycobacterium avium* subsp. *paratuberculosis* in Drinking Water and Biofilms by Quantitative PCR. Appl Environ Microbiol 76: 7367-7370

Bjergbæk LA, Roslev P (2005) Formation of nonculturable *Escherichia coli* in drinking water. J Appl Microbiol 99: 1090-1098

Boe-Hansen R, Albrechtsen H-J, Arvin E, Spliid H (2003) Mikrobielle forureninger - Vi ser kun toppen af isbjerget. danskVAND 71: 86-90

Bonetta S, Bonetta S, Ferretti E, Balocco F, Carraro E (2010) Evaluation of *Legionella pneumophila* contamination in Italian hotel water systems by quantitative real-time PCR and culture methods. J Appl Microbiol 108: 1576-1583

Bronstein I, Edwards B, Voyta JC (1989) 1,2-dioxetanes: novel chemiluminescent enzyme substrates. Applications to immunoassays. J Biolumin Chemilumin 4: 99-111

Bronstein I, Martin CS, Fortin JJ, Olesen CEM, Voyta JC (1996) Chemiluminescence: sensitive detection technology for reporter gene assays. Clin Chem 42: 1542-1546

Bronstein IY (1997) Assays using chemiluminescent, enzymatically cleavable substituted 1,2-dioxetanes and kits therefore. United States Patent 5605795, 02/25/1997

Budnick GE, Howard RT, and Mayo DR (1996) Evaluation of Enterolert for Enumeration of Enterococci in Recreational Waters. Appl Environ Microbiol 62: 3881-3884

Burnsed LJ, Hicks LA, Smithee LMK, Fields BS, Bradley KK, Pascoe N, Richards SM, Mallonee S, Littrell L, Benson RF, Moore MR, Legionellosis Outbreak Investigation Team (2007) A Large, Travel-Associated Outbreak of Legionellosis among Hotel Guests: Utility of the Urine Antigen Assay in Confirming Pontiac Fever. Clinical Infectious Diseases 44: 222-228

Carrero-Colón M, Wickham GS, and Turco RF (2011) Taxonomy, phylogeny, and physiology of fecal indicator bacteria. In: Sadowsky MJ and and Whitman RL (eds) The Fecal Bacteria. ASM Press, Washington, DC, USA, pp 23-38

Cassidy PM, Hedberg K, Saulson A, McNelly E, Winthrop KL (2009) Nontuberculous Mycobacterial Disease Prevalence and Risk Factors: A Changing Epidemiology. Clin Infect Dis 49: e124-e129

Chang B, Sugiyama K, Taguri T, Amemura-Maekawa J, Kura F, Watanabe H (2009) Specific detection of viable *Legionella* cells by combined use of photoactivated ethidium monoazide and PCR/real-time PCR. Appl Environ Microbiol 75: 147-153

Chao K, Chao C, and Chao W (2004) Evaluation of Colilert-18 for Detection of Coliforms and *Eschericha coli* in Subtropical Freshwater. Appl Environ Microbiol 70: 1242-1244

Chen N, Chang C (2010) Rapid quantification of viable legionellae in water and biofilm using ethidium monoazide coupled with real-time quantitative PCR. J Appl Microbiol 109: 623-634

Cirillo J, Falkow S, Tompkins L, Bermudez L (1997) Interaction of *Mycobacterium avium* with environmental amoebae enhances virulence. Infect Immun 65: 3759-3767

Colwell RR (2000) Viable but nonculturable bacteria: a survival strategy. J Infect Chemother 6: 121-125

Cook KL, Bolster CH (2007) Survival of *Campylobacter jejuni* and *Escherichia coli* in groundwater during prolonged starvation at low temperatures. J Appl Microbiol 103: 573-583

Danish EPA (2011) Bekendtgørelse om vandkvalitet og tilsyn med vandforsyningsanlæg. BEK no. 1024 af 31.10.2011.

Declerck P, Behets J, Margineanu A, van Hoef V, De Keersmaecker B, Ollevier F (2009) Replication of *Legionella pneumophila* in biofilms of water distribution pipes. Microbiol Res 164: 593-603

Delgado-Viscogliosi P, Solignac L, Delattre J (2009) Viability PCR, a Culture-Independent Method for Rapid and Selective Quantification of Viable *Legionella pneumophila* Cells in Environmental Water Samples. Appl Environ Microbiol 75: 3502-3512

Devriese LA, Pot B, Collins MD (1993) Phenotypic identification of the genus *Enterococcus* and the differentiation of phylogenetically distinct enterococcal species and species groups. J Appl Bacteriol 75: 399-408

DS 6222-1 (2002) Water quality - Enumeration of culturable micro-organisms - Colony count by inoculation in a nutrient agar culture medium. Danish Standards Association, Copenhagen, Denmark

Dungchai W, Siangproh W, Lin J-, Chailapakul O, Lin S, Ying X (2007) Development of a sensitive micro-magnetic chemiluminescence enzyme immunoassay for the determination of carcinoembryonic antigen. Anal Bioanal Chem 387: 1965-1971

Edberg SC, Piscitelli V, and Cartter M (1986) Phenotypic Characteristics of Coliform and Noncoliform Bacteria from a Public Water Supply Compared with Regional and NationalClinical Species. Appl Environ Microbiol 52: 474-478

Edge TA and Boehm AB (2011) Classical and molecular methods to measure fecal bacteria. In: Sadowsky MJ and and Whitman RL (eds) The Fecal Bacteria. ASM Press, Washington, DC, USA, pp 241-273

European Council Directive (1998) European Counsil Directive 98/83/EC of 3 November 1998 on the quality of water intended for human consumption. 31998L0083

Facklam RR, Carvalho MGS, Teixeira LM (2002) History, Taxonomy, Biochemical Characteristics, and Antibiotic Susceptibility Testing of Enterococci. In: Gilmore MS, Clewell DB, Courvalin P, Dunny G, Murray BE and Rice LB (eds) The Enterococci - Pathogenesis, Molecular Biology, and Antibiotic Resistance. ASM Press, Washington, DC, USA, pp 1-54

Falkinham III JO (2004) Environmental sources of *Mycobacterium avium* linked to routes of exposure. In: Pedley S, Bartram J, Rees G, Dufour A and Cotruvo JA (eds) Pathogenic Mycobacteria in Water. IWA Publishing, London, UK, pp 26-38

Falkinham III JO (2009) Surrounded by mycobacteria: nontuberculous mycobacteria in the human environment. J Appl Microbiol 107: 356-367

Falkinham III JO, Norton CD, LeChevallier MW (2001) Factors Influencing Numbers of *Mycobacterium avium, Mycobacterium intracellulare*, and Other Mycobacteria in Drinking Water Distribution Systems. Appl Environ Microbiol 67: 1225-1231

Feazel LM, Baumgartner LK, Peterson KL, Frank DN, Harris JK, Pace NR (2009) Opportunistic pathogens enriched in showerhead biofilms. PNAS 106: 16393-16399

Fiksdal L, Pommepuy M, Caprais MP, and Midttun I (1994) Monitoring of fecal pollution in coastal waters by use of rapid enzymatic techniques. Appl Environ Microbiol 60: 1581-1584

Fiksdal L, Tryland I (2008) Application of rapid enzyme assay techniques for monitoring of microbial water quality. Curr Opin Biotechnol 19: 289-294

Fisher K, Phillips C (2009) The ecology, epidemiology and virulence of *Enterococcus*. Microbiology 155: 1749-1757

Flekna G, Štefanič P, Wagner M, Smulders FJM, Možina SS, Hein I (2007) Insufficient differentiation of live and dead *Campylobacter jejuni* and *Listeria monocytogenes* cells by ethidium monoazide (EMA) compromises EMA/real-time PCR. Res Microbiol 158: 405-412

Frahm E, Obst U (2003) Application of the fluorogenic probe technique (TaqMan PCR) to the detection of *Enterococcus* spp. and *Escherichia coli* in water samples. J Microbiol Methods 52: 123-131

Frothingham R, Wilson KH (1993) Sequence-based differentiation of strains in the *Mycobacterium avium* complex. J Bacteriol 175: 2818-2825

Garcia-Armisen T, Lebaron P, Servais P (2005) β-d-glucuronidase activity assay to assess viable *Escherichia coli* abundance in freshwaters. Lett Appl Microbiol 40: 278-282

Gedalanga PB, Olson BH (2009) Development of a quantitative PCR method to differentiate between viable and nonviable bacteria in environmental water samples. Appl Microbiol Biotechnol 82: 587-596

George I, Crop P, Servais P (2001) Use of β-D-galactosidase and β-D-glucuronidase activities for quantitative detection of total and fecal coliforms in wastewater. Can J Microbiol 47: 670-675

George I, Petit M, Servais P (2000) Use of enzymatic methods for rapid enumeration of coliforms in freshwaters. J Appl Microbiol 88: 404-413

Gerba CP (2009) Indicator microorganisms. In: Maier RM, Pepper IL and Gerba CP (eds) Environmental microbiology, 2nd edn. Academic Press, NY, USA, pp 485-499

Giri BP (2007) Polymeric ammonium or phosphonium salts having added π-electrons and higher molecular weight as enhancers for chemiluminescent systems. United States Patent 7,300,766 B2, Nov. 27

Giri BP (2002) Chemiluminescent 1,2-dioxetanes. United States Patent 6,461,876 B1, Oct. 8

Giri BP, Dagli D, Singh P (2010) Ultra-sensitive chemiluminescent substrates for enzymes and their conjugates. United States Patent 7,781,229, Aug. 24

Girones R, Ferrús MA, Alonso JL, Rodriguez-Manzano J, Calgua B, de Abreu Corrêa A, Hundesa A, Carratala A, Bofill-Mas S (2010) Molecular detection of pathogens in water - The pros and cons of molecular techniques. Water Res 44: 4325-4339

Gomez-Valero L, Rusniok C, Buchrieser C (2009) *Legionella pneumophila*: Population genetics, phylogeny and genomics. Infect Gen Evol 9: 727-739

Haugland RA, Siefring SC, Wymer LJ, Brenner KP, Dufour AP (2005) Comparison of *Enterococcus* measurements in freshwater at two recreational beaches by quantitative polymerase chain reaction and membrane filter culture analysis. Water Res 39: 559-568

He J, Jiang S (2005) Quantification of Enterococci and Human Adenoviruses in Environmental Samples by Real-Time PCR. Appl Environ Microbiol 71: 2250-2255

Hernández-Garduño E, Elwood RK (2010) Demographic risk factors of pulmonary colonization by non-tuberculous mycobacteria. Int J Tuberc Lung Dis 14: 106-112

Horwitz MA, and Silverstein SC (1980) Legionnaires' Disease Bacterium (*Legionella pneumophila*) Multiplies Intracellularly in Human Monocytes. J Clin Invest 66: 441-450

Hummelen JC, Luider TM, Oudman D, Koek JN, Wynberg H (1991) 1,2-Dioxetanes: Luminescent and Nonluminescent Decomposition, Chemistry, and Potential Applications. In: Baeyens WRG, Keukeleire DD and Korkidis (eds) Luminescence Techniques In Chemical And Biochemical Analysis. Marcel Dekker Inc., NY, USA, pp 567-598

Inderlied CB, Kemper CA, Bermudez LE (1993) The *Mycobacterium avium* complex. Clin Microbiol Rev 6: 266-310

ISO 11731 (1998) Water quality - Detection and enumeration of *Legionella*. International Organization for Standardization. Geneve, Switzerland

ISO 7899-1 (1999) Water quality - Detection and enumeration of intestinal enterococci in surface and waste water -Part 1: Miniaturized method (Most Probable Number) by inoculation in liquid medium. International Organization for Standardization. Geneve, Switzerland

ISO 7899-2 (2000) Water quality - Detection and enumeration of intestinal enterococci - Part 2: Membrane filtration method. International Organization for Standardization. Geneve, Switzerland

ISO 9308-1 (2000) Water quality - Detection and enumeration of *Escherichia coli* and coliform bacteria - Part 1: Membrane filtration method. International Organization for Standardization. Geneve, Switzerland

ISO 9308-3 (1999) Water quality - Detection and enumeration of *Escherichia coli* and coliform bacteria - Part 3: Miniaturized method (Most Probable Number) for the detection and enumeration of *E. coli* in surface and waste water. International Organization for Standardization. Geneve, Switzerland

Jain V, Magrath IT (1991) A Chemiluminescent Assay for Quantification of β-Galactosidase in the Femtogram Range: Application to Quantification of β-Galactosidase in *lacZ*-Transfected Cells. Anal Biochem 199: 119-124

Jofre J, Blanch AR (2010) Feasibility of methods based on nucleic acid amplification techniques to fulfill the requirements for microbiological analysis of water quality. J Appl Microbiol 109: 1853-1867

Joly P, Falconnet P, Andre J, Weill N, Reyrolle M, Vandenesch F, Maurin M, Etienne J, Jarraud S (2006) Quantitative Real-Time *Legionella* PCR for Environmental Water Samples: Data Interpretation. Appl Environ Microbiol 72: 2801-2808

Joseph C, Ricketts K (2008) The epidemiology of Legionnaires' disease. In: Heuner K and Swanson M (eds) *Legionella*: Molecular Microbiology. Caister Academic Press, Norfolk, UK, pp 35-54

Karakousis PC, Moore RD, Chaisson RE (2004) *Mycobacterium avium* complex in patients with HIV infection in the era of highly active antiretroviral therapy. Lancet Infect Dis 4: 557-565

Kilian M, and Bülow P (1976) Rapid diagnosis of *Enterobacteriaceae*. I. Detection of bacterial glycosidases. Acta path microbiol scand Sect B 84: 245-251

Krøjgaard LH, Krogfelt KA, Albrechtsen H-, Uldum SA (2011) Cluster of Legionnaires' disease in a newly built block of flats, Denmark, December 2008 - January 2009. Euro Surveill 16: 19759

Kubista M, Andrade JM, Bengtsson M, Forootan A, Jonák J, Lind K, Sindelka R, Sjöback R, Sjögreen B, Strömbom L, Ståhlberg A, Zoric N (2006) The real-time polymerase chain reaction. Mol Aspects Med 27: 95-125

Lai C, Tan C, Chou C, Hsu H, Liao C, Huang Y, Yang P, Luh K, and Hsueh P (2010) Increasing Incidence of Nontuberculous Mycobacteria, Taiwan, 2000-2008. Emerg Infect Dis 16: 294-296

Lavender JS, Kinzelman JL (2009) A cross comparison of QPCR to agar-based or defined substrate test methods for the determination of *Escherichia coli* and enterococci in municipal water quality monitoring programs. Water Res 43: 4967-4979

Lebaron P, Henry A, Lepeuple A, Pena G, Servais P (2005) An operational method for the real-time monitoring of *E. coli* numbers in bathing waters. Mar Pollut Bull 50: 652-659

Lehtola MJ, Torvinen E, Kusnetsov J, Pitkanen T, Maunula L, von Bonsdorff C, Martikainen PJ, Wilks SA, Keevil CW, Miettinen IT (2007) Survival of *Mycobacterium avium, Legionella pneumophila, Escherichia coli*, and Caliciviruses in Drinking Water-Associated Biofilms Grown under High-Shear Turbulent Flow. Appl Environ Microbiol 73: 2854-2859

Lesne J, Bonadonna L, Holt D, Prescott A (2001) Def Final Report. http://docs.google.com/viewer?a=v&q=cache:t0kl5pAdd8J:www.colifast.no/Def%2520%2520FINAL%2520REPORT%2520rev2.doc rev. 2: 1-22

Lothigius Å, Sjöling Å, Svennerholm A-, Bölin I (2010) Survival and gene expression of enterotoxigenic *Escherichia coli* during long-term incubation in sea water and freshwater. J Appl Microbiol 108: 1441-1449

Luo J, Lin W, Guo Y (2010) Method to detect only viable cells in microbial ecology. Appl Microbiol Biotechnol 86: 377-384

Maheux AF, Bissonnette L, Boissinot M, Bernier JT, Huppé V, Bérubé È, Boudreau DK, Picard FJ, Huletsky A, Bergeron MG (2011) Method for rapid and sensitive detection of *Enterococcus* sp. and *Enterococcus faecalis/faecium* cells in potable water samples. Water Res 45: 2342-2354

Mampel J, Spirig T, Weber SS, Haagensen JAJ, Molin S, Hilbi H (2006) Planktonic Replication Is Essential for Biofilm Formation by *Legionella pneumophila* in a Complex Medium under Static and Dynamic Flow Conditions. Appl Environ Microbiol 72: 2885-2895

Manafi M (2000) New developments in chromogenic and fluorogenic culture media. Int J Food Microbiol 60: 205-218

Marciano-Cabral F, Jamerson M, Kaneshiro ES (2010) Free-living amoebae, *Legionella* and *Mycobacterium* in tap water supplied by a municipal drinking water utility in the USA. J Water Health 8: 71-82

Martins MT, Rivera IG, Clark DL, Stewart MH, Wolfe RL, and Olson BH (1993) Distribution of *uidA* gene sequences in *Escherichia coli* isolates in water sources and comparison with the expression of beta-glucuronidase activity in 4-methylumbelliferyl-beta-D-glucuronide media. Appl Environ Microbiol 59: 2271-2276

McFeters GA, Broadaway SC, Pyle BH, Pickett M, and Egozy Y (1997) Comparative performance of Colisure. J Am Water Works Assoc 89: 112-120

Moore J, Kruijshaar M, Ormerod L, Drobniewski F, Abubakar I (2010) Increasing reports of non-tuberculous mycobacteria in England, Wales and Northern Ireland, 1995-2006. BMC Public Health 10: 612

Mull B, Hill VR (2009) Recovery and Detection of *Escherichia coli* O157:H7 in Surface Water, Using Ultrafiltration and Real-Time PCR. Appl Environ Microbiol 75: 3593-3597

Nash TW, Libby DM, Horwitz MA (1984) Interaction between the legionnaires' disease bacterium (*Legionella pneumophila*) and human alveolar macrophages. Influence of antibody, lymphokines, and hydrocortisone. J Clin Invest 74: 771-782

Nichols G, Ford T, Bartram J, Dufour A, Portaels F (2004) Introduction. In: Pedley S, Bartram J, Rees G, Dufour A and Cotruvo JA (eds) Pathogenic Mycobacteria in Water - A Guide to Public Health Consequences, Monitoring and Management. WHO, IWA Publishing, London, UK, pp 1-14

Nishiuchi Y, Tamura A, Kitada S, Taguri T, Matsumoto S, Tateishi Y, Yoshimura M, Ozeki Y, Matsumura N, Ogura H, Maekura R (2009) *Mycobacterium avium* complex organisms predominantly colonize in the bathtub inlets of patients' bathrooms. Jpn J Infect Dis 62: 182-186

Noble RT, Weisberg SB (2005) A review of technologies for rapid detection of bacteria in recreational waters. J Water Health 3: 381-392

Noble RT, Blackwood AD, Griffith JF, McGee CD, Weisberg SB (2010) Comparison of Rapid Quantitative PCR-Based and Conventional Culture-Based Methods for Enumeration of *Enterococcus* spp. and *Escherichia coli* in Recreational Waters. Appl Environ Microbiol 76: 7437-7443

Nocker A, Cheung C, Camper AK (2006) Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. J Microbiol Methods 67: 310-320

Norton CD, LeChevallier MW, Falkinham III JO (2004) Survival of *Mycobacterium avium* in a model distribution system. Water Res 38: 1457-1466

Olesen CEM, Fortin JJ, Voyta JC, Bronstein I (1997) Detection of β-galactosidase and β-glucuronidase using chemiluminescent reporter gene assays. Recombinant Protein Protocols 63: 61-70

Olesen CEM, Yan Y, Liu B, Martin D, D'Eon B, Judware R, Martin C, Voyta JC, Bronstein I (2000) Novel methods for chemiluminescent detection of reporter enzymes. Methods Enzymol 326: 175-202

Oliver JD (2005) The Viable but Nonculturable State in Bacteria. J Microbiol 43 Spec No: 93-100

Oliver JD (2010) Recent findings on the viable but nonculturable state in pathogenic bacteria. FEMS Microbiol Rev 34: 415-425

Orenga S, James AL, Manafi M, Perry JD, Pincus DH (2009) Enzymatic substrates in microbiology. J Microbiol Methods 79: 139-155

Park SH, Hanning I, Jarquin R, Moore P, Donoghue DJ, Donoghue AM, Ricke SC (2011) Multiplex PCR assay for the detection and quantification of *Campylobacter* spp., *Escherichia coli* O157:H7, and *Salmonella* serotypes in water samples. FEMS Microbiol Lett 316: 7-15

Peters FT, Maurer HH (2001) Bioanalytical method validation - How, how much and why? Toxichem Krimtech 68: 116

Petit M, George I, Servais P (2000) Survival of *Escherichia coli* in freshwater: β-D-glucuronidase activity measurements and characterization of cellular states. Can J Microbiol 46: 679-684

Ram S, Vajpayee P, Shanker R (2010) Enterotoxigenic *Escherichia coli* in sewage-impacted waters and aquatic weeds: quantitative PCR for culture-independent enumeration. J Appl Microbiol 108: 1007-1014

Rice EW, Allen MJ, and Edberg SC (1990) Efficacy of β-D-Glucuronidase Assay for Identification of *Escherichia coli* by the Defined-Substrate Technology. Appl Environ Microbiol 56: 1203-1205

Rompré A, Servais P, Baudart J, de-Roubin M, Laurent P (2002) Detection and enumeration of coliforms in drinking water: current methods and emerging approaches. J Microbiol Methods 49: 31-54

Roslev P, Bukh AS (2011) State of the art molecular markers for fecal pollution source tracking in water. Appl Microbiol Biotechnol 89: 1341-1355

Roszak DB, and Colwell RR (1987) Survival Strategies of Bacteria in the Natural Environment. Microbiol Rev 51: 365-379

Rowbotham TJ (1980) Preliminary report on the pathogenicity of *Legionella pneumophila* for freshwater and soil amoebae. J Clin Pathol 33: 1179-1183

Rudbeck M, Mølbak K, Uldum S (2008) High prevalence of antibodies to *Legionella* spp. in Danish blood donors. A study in areas with high and average incidence of Legionnaires' disease. Epidemiol Infect 136: 257-262

Sartory DP (2005) Validation, verification and comparison: Adopting new methods in water microbiology. Water SA 31: 393-396

Saunders AM, Kristiansen A, Lund MB, Revsbech NP, Schramm A (2009) Detection and persistence of fecal *Bacteroides vulgatus* as water quality indicators in unchlorinated drinking water. System Appl Microbiol 32: 362-370.

Schulze-Röbbecke R, and Buchholtz K (1992) Heat Susceptibility of Aquatic Mycobacteria. Appl Environ Microbiol 58: 1869-1873

Schuster GB (1979) Chemiluminescence of Organic Peroxides. Conversion of Ground-State Reactants to Excited-State Products by the Chemically Initiated Electron-Exchange Luminescence Mechanism. Acc Chem Res 12: 366-373

Smith CJ, Osborn AM (2009) Advantages and limitations of quantitative PCR (Q-PCR)-based approaches in microbial ecology. FEMS Microbiol Ecol 67: 6-20

SSI (2010) LEGIONELLA-PNEUMONI 2009 *In Danish*. EPI-NYT Week 41: 1-2

Stinear T, Ford T, and Vincent V (2004) Analytical methods for the detection of waterborne and environmental pathogenic mycobacteria. In: Pedley S, Bartram J, Rees G, Dufour A and Cotruvo JA (eds) Pathogenic Mycobacteria in Water. IWA Publishing, London, UK, pp 55-73

Storey MV, Långmark J, Ashbolt NJ, and Stenström TA (2004) The fate of legionellae within distribution pipe biofilms: measurement of their persistence, inactivation and detachment. Water Sci Technol 49: 269-275

Thomson RM (2010) Changing Epidemiology of Pulmonary Nontuberculous Mycobacteria Infections. Emerg Infect Dis 16: 1576-1583

Thomson R, Carter R, Gilpin C, Coulter C, Hargreaves M (2008) Comparison of Methods for Processing Drinking Water Samples for the Isolation of *Mycobacterium avium* and *Mycobacterium intracellulare*. Appl Environ Microbiol 74: 3094-3098

Torvinen E, Lehtola MJ, Martikainen PJ, Miettinen IT (2007) Survival of *Mycobacterium avium* in Drinking Water Biofilms as Affected by Water Flow Velocity, Availability of Phosphorus, and Temperature. Appl Environ Microbiol 73: 6201-6207

Trullols E, Ruisánchez I, Rius FX (2004) Validation of qualitative analytical methods. Trends Anal Chem 23: 137-145

Tryland I, Fiksdal L (1998) Enzyme characteristics of β-D-galactosidase- and β-D-glucuronidase-positive bacteria and their interference in rapid methods for detection of waterborne coliforms and *Escherichia coli*. Appl Environ Microbiol 64: 1018-1023

Vaerewijck MJM, Huys G, Palomino JC, Swings J, Portaels F (2005) Mycobacteria in drinking water distribution systems: ecology and significance for human health. FEMS Microbiol Rev 29: 911-934

van der Kooij D, Veenendaal HR, Scheffer WJH (2005) Biofilm formation and multiplication of *Legionella* in a model warm water system with pipes of copper, stainless steel and cross-linked polyethylene. Water Res 39: 2789-2798

Van Dyke K, Woodfork K (2002) Light probes. In: Van Dyke K, Van Dyke C and Woodfork K (eds) Luminescence biotechnology: instruments and applications. CRC Press LLC, Boca Raton, FL, USA, pp 3-27

Van Poucke SO, Nelis HJ (2000) Rapid detection of fluorescent and chemiluminescent total coliforms and *Escherichia coli* on membrane filters. J Microbiol Methods 42: 233-244

Van Poucke SO, Nelis HJ (1997) Limitations of Highly Sensitive Enzymatic Presence-Absence Tests for Detection of Waterborne Coliforms and *Escherichia coli*. Appl Environ Microbiol 63: 771-774

Van Poucke SO, Nelis HJ (1995) Development of a sensitive chemiluminometric assay for the detection of β-galactosidase in permeabilized coliform bacteria and comparison with fluorometry and colorimetry. Appl Environ Microbiol 61: 4505-4509

von Reyn CF, Waddell RD, Eaton T, Arbeit RD, Maslow JN, Barber TW, Brindle RJ, Gilks CF, Lumio J, Lahdevirta J (1993) Isolation of *Mycobacterium avium* complex from water in the United States, Finland, Zaire, and Kenya. J Clin Microbiol 31: 3227-3230

Walters SP, Yamahara KM, Boehm AB (2009) Persistence of nucleic acid markers of health-relevant organisms in seawater microcosms: Implications for their use in assessing risk in recreational waters. Water Res 43: 4929-4939

Wildeboer D, Amirat L, Price RG, Abuknesha RA (2010) Rapid detection of *Escherichia coli* in water using a hand-held fluorescence detector. Water Res 44: 2621-2628

Wilhelm J, Pingoud A (2003) Real-Time Polymerase Chain Reaction. ChemBioChem 4: 1120-1128

Williams MM, Yakrus MA, Arduino MJ, Cooksey RC, Crane CB, Banerjee SN, Hilborn ED, Donlan RM (2009) Structural Analysis of Biofilm Formation by Rapidly and Slowly Growing Nontuberculous Mycobacteria. Appl Environ Microbiol 75: 2091-2098

Wilson DA, Yen-Lieberman B, Reischi U, Gordon SM, Procop GW (2003) Detection of *Legionella pneumophila* by real-time PCR for the *mip* gene. J Clin Microbiol 41: 3327-3330

Winthrop KL, McNelley E, Kendall B, Marshall-Olson A, Morris C, Cassidy M, Saulson A, Hedberg K (2010) Pulmonary Nontuberculous Mycobacterial Disease Prevalence and Clinical Features: An Emerging Public Health Disease. Am J Respir Crit Care Med 182: 977-982

Wohlsen T, Bates J, Vesey G, Robinson WA, and Katouli M (2006) Evaluation of the methods for enumerating coliform bacteria from water samples using precise reference standards. Lett Appl Microbiol 42: 350-356

Woodward J, Wiseman A (1982) Fungal and other β-D-glucosidases - their properties and applications Enzyme Microb Technol 4: 73-79

Yaradou DF, Hallier-Soulier S, Moreau S, Poty F, Hillion Y, Reyrolle M, Andre J, Festoc G, Delabre K, Vandenesch F, Etienne J, Jarraud S (2007) Integrated Real-Time PCR for

Detection and Monitoring of *Legionella pneumophila* in Water Systems. Appl Environ Microbiol 73: 1452-1456