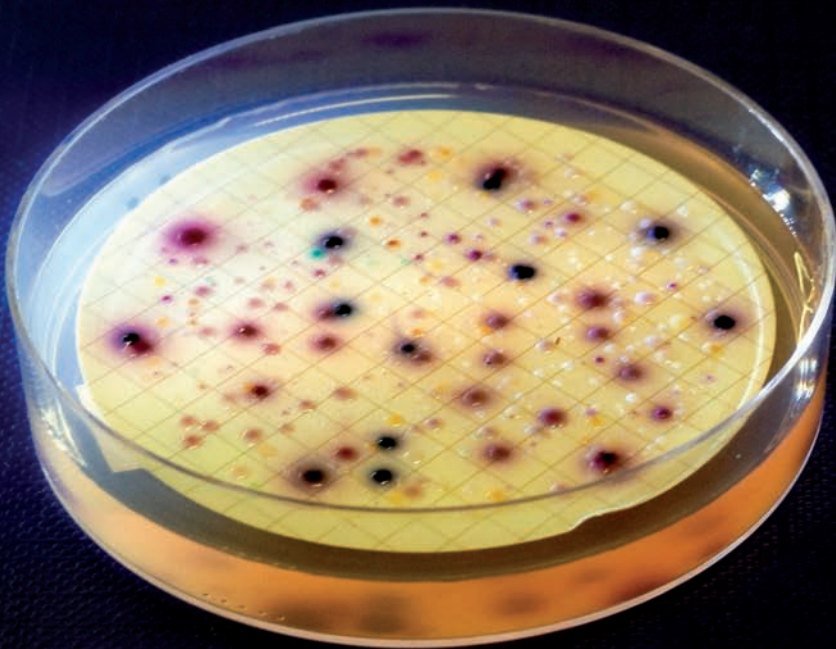




Tarja Pitkänen

Studies on the Detection Methods of *Campylobacter* and Faecal Indicator Bacteria in Drinking Water



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ACADEMIC DISSERTATION

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To Irene and Mikael,
from whom I learnt how to
work hard and who brought
out my interest on
microbiology

Abstract

Tarja Pitkänen. Studies on the Detection Methods of *Campylobacter* and Faecal Indicator Bacteria in Drinking Water. National Institute for Health and Welfare (THL), Research 39. 183 pages. Helsinki, Finland 2010.

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Safe drinking water is essential for human health. Globally, faecal contamination of drinking water and subsequent waterborne gastrointestinal infection outbreaks are a major public health concern. Drinking water safety is based on the prevention of access of faecal material to water, and purification and disinfection treatments at the waterworks. In spite of these measures, waterborne outbreaks occur in even industrialized countries with centralized water supplies and sanitation. The majority of such outbreaks take place at relatively small groundwater abstraction plants utilizing minimal water treatment, and the source of contamination often remains unclear. There has been an evident need for more specific knowledge of the environmental hazards that endanger water safety and cause contaminations. The detection and source tracking of the microbial hazards has been compromised by unsatisfactory methods needed for accurate and rapid resolution of the cases.

As the first part of this study, a survey was carried out where microbiological and chemical hazards compromising water quality at twenty community water supplies in central Finland were identified in 2002-2004. In addition, the source of faecal contamination was resolved during a contamination case of a municipal drinking water distribution in August 2004 in eastern Finland.

As the second part, methods to detect indicators of microbial water quality, *Escherichia coli* and coliform bacteria, as well as novel methods to detect a major zoonotic pathogen, *Campylobacter jejuni*, from drinking water were developed and tested. The methods were based on cultivation and on quantitative real-time PCR detection and were selected with the aim of finding more efficient, less laborious and less time-consuming practices with adequate specificities and sensitivities.

The results of the survey showed that faecal indicator bacteria, *Escherichia coli*, intestinal enterococci or *Clostridium perfringens*, could be detected in 10% of the groundwater samples originating from five water supply plants, all of them serving less than 250 consumers. With respect to the other water quality hazards, coliform bacteria were detected in 40% of samples using enhanced methods, 54% of samples were coliphage positive and 16% of samples contained more than 0.10 mg/l nitrite. The on-site hazard identification procedure revealed that multiple hazardous characteristics were often associated with the water supply wells, e.g. the possibility of surface water run-offs as well as bank filtration and an insufficient depth of the protective soil layer. However, at three out of five water supplies, where faecal indicator bacteria were present the given risk values were relatively low (19, 24 and 25 out of 70).

A multiparameter approach was applied in solving and source tracking of a faecal contamination case of a municipal drinking water distribution system. The contamination was detected employing faecal indicator bacteria and *C. jejuni* was also isolated. Pulsed-field gel electrophoresis and DNA fingerprinting were successfully employed together with on-site technical investigations and spatial and temporal analysis to track and remove the faecal contamination source. It was also shown that the water contamination was associated with three *Campylobacter* infected patients found in the community.

Five alternative cultivation methods for detection and counting of *Escherichia coli* and coliform bacteria from non-disinfected waters were compared to the reference method ISO 9308-1 (LTTC). Three of them: Colilert®-18, Chromocult® coliform agar and chromogenic *E. coli*/coliform medium achieved equal or higher coliform bacteria counts than LTTC, but the alternative media seemed to produce lower *E. coli* counts than LTTC. Colilert®-18, Chromocult® coliform agar and chromogenic *E. coli*/coliform medium can be used as potential alternative media once the specific problems related to false positive and false negative results can be eliminated.

For enhanced enrichment of *Campylobacter*, the performance of a novel portable microbial enrichment unit (PMEU) was compared to that of conventional static incubation. In PMEU enrichment of inoculated tap water, *C. jejuni* counts were higher ($p < 0.01$) than with static enrichment, resulting in more rapid detection of growth. After three days' storage of the water sample, the growth in PMEU was observed after 16 h in comparison to 40 h with static enrichment. The realtime PCR detection after enrichment reduced the analysis time even more: a specific *Campylobacter* result was obtained within five hours whereas it required several days by culturing. Rapid *Campylobacter* detection could be achieved also by a fluorescent in-situ hybridization (FISH) method and the FISH results indicated that cultivation-based techniques may have seriously underestimated the actual *Campylobacter* counts.

Keywords: *Campylobacter*, drinking water safety, *Escherichia coli*, equivalence, faecal contamination, microbiological methods, water quality monitoring.

Tiivistelmä (Abstract in Finnish)

Tarja Pitkänen. Studies on the Detection Methods of *Campylobacter* and Faecal Indicator Bacteria in Drinking Water. [Tutkimuksia kamylobakteerien ja suolistoperäisten indikaattoribakteerien havaitsemismenetelmistä talousvedessä]. Terveyden ja hyvinvoinnin laitos (THL), Tutkimus 39. 183 sivua. Helsinki 2010. ISBN 978-952-245-319-8 (printed), ISBN 978-952-245-320-4 (pdf)

Talousveden suolistoperäinen saastuminen ja siitä johtuvat vatsatautiepidemiat ovat merkittävä maailmanlaajuinen kansanterveysongelma. Talousveden laatu pyritään turvaamaan estämällä suolistoperäisen aineksen pääsy pinta- ja pohjavesilaitosten raakavesiin ja vedenjakeluverkostoon, sekä veden puhdistus- ja desinfiointikäsitelyillä. Maissa, joissa on keskitetty vesihuolto, vesiepidemioita havaitaan pääosin pienillä pohjavedenottoilla, joilla veden puhdistustoimet ovat vähäisiä.

Tutkimuksen ensimmäisessä osassa selvitettiin veden laatua uhkaavia tekijöitä 20 pohjavesilaitoksella Keski-Suomessa vuosina 2002-2004. Lisäksi selvitettiin suolistoperäisen saastumisen lähde elokuussa 2004 havaitussa talousvesiverkoston saastumistapauksessa.

Tutkimuksen toisessa osassa kehitettiin ja testattiin uusia menetelmiä sekä veden mikrobiologisen laadun indikaattoreiden, *Escherichia coli* -bakteerin ja koliformisten bakteerien, että ihmisillä mm. saastuneen veden välityksellä suolistoinfektioita aiheuttavan *Campylobacter jejuni* -bakteerin, havaitsemiseksi talousvesinäytteistä. Bakteriviljelyyn ja reaaliaikaiseen PCR-detektioon perustuvien menetelmien valinnassa tavoitteena oli löytää mikrobiologisen veden laadun analysointiin entistä tehokkaampia, spesifisempiä ja vähemmän aikaavieviä menetelmiä.

Tutkituista pohjavesinäytteistä 10 % sisälsi suolistoperäisiä indikaattoribakteereita: *Escherichia coli* -bakteereita, suolistoperäisiä enterokokkeja tai *Clostridium perfringens* -bakteereita. Löydöksiä oli viideltä eri vedenottomolta, jotka kaikki olivat pieniä, alle 250 veden käyttäjää palvelevia laitoksia. Myös muita vedenlaatuongelmia havaittiin: 40 % tutkituista näytteistä sisälsi herkistetyillä menetelmillä tutkittuina koliformisia bakteereita, 54 % sisälsi kolifaageja ja 16 % näytteistä sisälsi nitriittiä yli 0.10 mg/l. Vedenottoilla tehdyissä kartoituksissa vesiturvallisuutta vaarantaviksi tekijöiksi havaittiin pintavesivaluntojen ja rantaimetyymisen mahdollisuus sekä liian ohut pohjaveden pintaa suojaavan maakerroksen paksuus. Havaituista uhkista huolimatta kolmella niistä viidestä laitoksesta, joiden vedessä havaittiin suolistoperäisiä indikaattoribakteereita, arvioitu riskiluku oli suhteellisen alhainen (riskiluku 19, 24 ja 24, maksimin ollessa 70).

Vesimikrobiologisia menetelmiä hyödynnettiin laajasti selvitettäessä kunnallisessa talousveden jakeluverkostossa tapahtunutta suolistoperäistä saastumistapausta. Saastuminen havaittiin indikaattoribakteerin avulla ja myös *C. jejuni* pystyttiin eristämään vesijärjestelmästä. Mikrobiologiset tyyppitysmenetelmät, pulssikenttägeelielektroforeesi ja DNA-sormenjälkitekniikka, yhdessä paikan päällä tapahtu-

neiden teknisten tutkimusten, sekä ajallisen ja paikallisen yhteensopivuuden kanssa mahdollistivat saastelähteen paikantamisen ja poistamisen. Lisäksi osoitettiin veden saastumisen olevan yhteydessä kolmeen todettuun kampylobakteeri-infektioon kunnassa.

Vaihtoehtoisista viljelymenetelmistä *E. coli* -bakteerin ja koliformisten bakteerien havaitsemiseksi ja laskemiseksi desinfiomattomista vesistä Colilert®-18, Chromocult® coliform agar ja kromogeeninen *E. coli*/coliform kasvualusta antoivat yhtä suuria tai korkeampia koliformisten bakteerien pesäkelukumääriä kuin referenssimenetelmä ISO 9308-1 (LTTC), mutta LTTC antoi korkeampia *E. coli* pesäkelukumääriä kuin mikään testatuista viidestä vaihtoehtoisesta menetelmästä. Colilert®-18, Chromocult® coliform agar ja kromogeeninen *E. coli*/coliform kasvualusta ovat potentiaalisia vaihtoehtoisia menetelmiä, mikäli havaitut virhepositiivisiin ja virhenegatiivisiin tuloksiin liittyvät ongelmat saadaan poistettua.

Kampylobakteerin rikastaminen tehostui uuden kannettavan mikrobien rikastusyksikön (portable microbial enrichment unit, PMEU) avulla. Siirrostetun ja kolme päivää ennen analyysiä kylmässä säilytetyn talousveden PMEU-rikastuksessa kasvu havaittiin jo 16 h kuluttua rikastuksen aloittamisesta ja *C. jejuni* lukumäärät olivat korkeampia ($p < 0.01$) kuin perinteisessä staattisessa rikastamisessa, jossa kasvu havaittiin vasta 40 h kuluttua. Rikastuksen jälkeen käytetty reaaliaikainen PCR-menetelmä lyhensi analyysiäikää entisestään: lajispesifinen kampylobakteeritulos saatiin viidessä tunnissa, kun tuloksen saanti viljelymenetelmällä kestää useita päiviä. FISH-menetelmä (fluorescent in-situ hybridization) osoittautui myös nopeaksi kampylobakteerin havaitsemismenetelmäksi. Hybridisaatiotulokset osoittivat, että viljelymenetelmillä saadut tulokset voivat vakavasti aliarvioida kampylobakteerien lukumääriä.

Avainsanat: *Escherichia coli*, kampylobakteeri, mikrobiologiset menetelmät, suolistoperäinen saastuminen, talousveden turvallisuus, vastaavuus, veden laadun monitorointi.

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Abstract

Tiivistelmä (Abstract in Finnish)

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Original publications

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- I Tarja Pitkänen, Päivi Karinen, Ilkka T. Miettinen, Heidi Lettojärvi, Annika Heikkilä, Reetta Maunula, Vesa Aula, Henry Kuronen, Asko Vepsäläinen, Liina-Lotta Nousiainen, Sinikka Pelkonen and Helvi Heinonen-Tanski. Microbial contamination of groundwater at small community water supplies in Finland. (Submitted)
- II Tarja Pitkänen, Ilkka T. Miettinen, Ulla-Maija Nakari, Johanna Takkinen, Kalle Nieminen, Anja Siitonen, Markku Kuusi, Arja Holopainen and Marja-Liisa Hänninen. (2008) Faecal contamination of a municipal drinking water distribution system in association with *Campylobacter jejuni* infections. Journal of Water and Health 6(3): 365-376. doi: 10.2166/wh.2008.050.
- III Tarja Pitkänen, Piia Paakkari, Ilkka T. Miettinen, Helvi Heinonen-Tanski, Lars Paulin and Marja-Liisa Hänninen. (2007) Comparison of media for enumeration of coliform bacteria and *Escherichia coli* in non-disinfected water. Journal of Microbiological Methods 68: 522-529. doi:10.1016/j.mimet.2006.10.007
- IV Tarja Pitkänen, Juliane Bräcker, Ilkka T. Miettinen, Anneli Heitto, Jouni Pesola and Elias Hakalehto. (2009) Enhanced enrichment and detection of thermotolerant *Campylobacter* species from water using the Portable Microbe Enrichment Unit and real-time PCR. Canadian Journal of Microbiology 55: 849–858. doi:10.1139/W09-040.
- V Markku J. Lehtola, Tarja Pitkänen, Lea Miebach and Ilkka T. Miettinen. (2006) Survival of *Campylobacter jejuni* in potable water biofilms: a comparative study with different detection methods. Water Science & Technology 54(3):57-61. doi: 10.2166/wst.2006.448.

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The author's contribution

- I Tarja Pitkänen participated in the collection of the samples, performed a part of the bacteriological analyses, participated in reporting and interpreting of the results and was mainly responsible for writing the paper.
- II Tarja Pitkänen participated in the solving of the contamination case, planned and partly performed the analyses of *Campylobacter* and indicator bacteria from the water samples and also DNA fingerprinting of coliform bacteria. Tarja Pitkänen coordinated the genotyping, participated in the interpreting of the results and wrote the paper.
- III Tarja Pitkänen selected the methods to be compared, planned the experiments, participated in sampling and laboratory analyses, calculated and interpreted the results and wrote the paper.
- IV Tarja Pitkänen planned and supervised the laboratory experiments, participated in the interpreting of the results and wrote the main parts of the paper.
- V Tarja Pitkänen supervised the cultivation and PCR analyses of *Campylobacter jejuni* and participated in writing of the paper.

Abbreviations

AFLP	Amplified fragment length polymorphism
AODC	Acridine orange direct count
ATCC	American Type Culture Collection
CC	Chromocult® coliform agar
CFU	Colony forming unit
DAPI	4'6-diamidino-2-phenylindole
DVC	Direct viable count
EHEC	Enterohaemorrhagic <i>Escherichia coli</i>
EPA	Environmental Protection Agency (US)
ERIC	Enterobacterial repetitive intergenic consensus
ESP	Extracellular polymeric substances
FISH	Fluorescent in situ hybridization
HPC	Heterotrophic plate count
HRM	High-resolution melt analysis
ISO	International Organization for Standardization
LTTC	Lactose triphenyl tetrazolium chloride
mCCDA	Modified charcoal cefoperazone desoxycholate agar
MAR	Multiple antibiotic resistance
MF	Membrane filtration
MLSA	Membrane Lauryl Sulfate agar
MPN	Most probable number
MST	Microbial source tracking
MTF	Multiple-tube fermentation
NRC	National Research Council of the National Academies
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PMEU	Portable microbe enrichment unit
PNA	Peptide nucleic acid
R2A	Reasoner's 2 agar
REA	Restriction enzyme analysis
REP	Repetitive extragenic palindromic
RFLP	Restriction fragment length polymorphism
rRNA	Ribosomal ribonucleic acid
TSA	Tryptone soya agar
UV	Ultraviolet
VBNC	Viable but not culturable
WHO	World Health Organization
WSP	Water safety plan
YEA	Yeast extract agar

1 Introduction

Safe drinking water is essential for human health: contaminated drinking water has the potential to exert serious health concerns (WHO, 2004). The association between water quality and disease has been recognized for more than a hundred years but still today the transmission of waterborne diseases is a major public health concern (Hrudey and Hrudey, 2007; NRC, 2004; Theron and Cloete, 2002). Availability of clean water is taken for granted by the populations of the industrialized nations but the need for safeguarding the wholesome good quality of potable water requires continuous vigilance from water supply companies and this is regulated by public health officers (Geldreich, 1990; Percival, 2000). Historically *Vibrio cholerae* and *Salmonella* spp. were the principal waterborne pathogens of concern causing cholera and typhoid fever, respectively (NRC, 2004). These infections still occur in many parts of the developing world and may re-emerge in the developed countries in cases where there are disruptions to the public water supply during natural disasters such as floods or earthquakes (Szewzyk *et al.*, 2000). The introduction of water treatment processes and sanitation has dramatically reduced the incidence of these historically important waterborne bacterial infections and thus the relative significance of waterborne viral and protozoan infections has increased (Schoenen, 2002).

Microbial contamination of water can be traced primarily to the presence of animal or human faeces that may originate from untreated sewage entering the distribution system, from animal waste being carried by rain runoff or by melting snow, or from failure or breakdown in the water treatment process (Geldreich, 1990; Percival, 2000). Faecal contamination of drinking water is a reality in many developing countries (Pedley and Howard, 1997; WHO, 2004) but the incidents of faecal contamination occur also in industrialized countries (Neumann *et al.*, 2005). In fact, many outbreaks of waterborne gastrointestinal illnesses have been traced to water supplies in developed nations where the drinking water is usually assumed to be of good quality (Hrudey and Hrudey, 2007). The contamination may take place at the water source (reservoir), at the water treatment plant or within the distribution system. Human or animal faeces may contain pathogenic microbes (bacteria, viruses, protozoans, helminths) and waterborne infections are highly probable in cases when there are failures to prevent faecal contamination of drinking water. Since the drinking water distribution networks usually serve a large number of individuals, faecal contamination of water may lead to a serious outbreak of disease.

As water is a basic human right, and life and development require water of suitable quantity and quality, the World Health Organization has provided guidelines for drinking water quality and safety management (WHO, 2004). In the prevention of waterborne human diseases, it is important to have accurate and reliable methods for determining the microbial water quality. Monitoring of the hygienic quality of drinking water is based on the indicator organism approach, since many of the known waterborne pathogens are difficult to detect and it is not feasible to issue

regulations requiring the authorities to monitor the complete spectrum of microorganisms (NRC, 2004). Some enteric bacteria are used to indicate the possible faecal contamination of drinking water and the detection methods for these indicators are standardized.

However, the survival, transport, removal and inactivation of waterborne pathogens, especially certain viruses and protozoa, differ substantially from the survival of conventional indicator bacteria in the aqueous environment and this decreases the predictive value of indicator bacteria for guaranteeing the microbiological safety of water. The prevention of future outbreaks has to be based on a clear understanding of the causes of waterborne disease outbreaks, i.e. learning from past mistakes, including problems encountered in pathogen detection, and adopting preventive actions at the most vulnerable water supply systems (Hrudey and Hrudey, 2007; Hunter *et al.*, 2003). Regulatory water quality requirements in the future should promote microbial hazard identification, determination of the acceptable level of risk and adoption of risk management practices (WHO, 2004).

The microbiological methods currently applied in water quality monitoring are often laborious and time-consuming. Especially at small water supply plants, the sampling and analysis are performed only infrequently. In the present study, the hazards to the microbial safety of drinking water were estimated at small community water supplies in central Finland. The aim of this thesis was also to test and further improve the less time-consuming but still sufficiently specific and sensitive methods for detection and typing of *Campylobacter* and faecal indicator bacteria in drinking water quality assessment. Several detection methods were compared for coliform bacteria and *Escherichia coli* to be used as quality indicators of non-disinfected waters and methods for detecting *Campylobacter* in water were developed. The detection methods of coliform bacteria and *Campylobacter* spp. were tested in the investigation of a drinking water contamination case.

2 Review of the literature

2.1 Drinking water supply: water treatment and distribution

Surface waters and groundwater serve as sources of potable water for communities. In Finland, 37 % of the drinking water supplied by waterworks originates from surface water, 52 % is groundwater and 11 % is artificially recharged groundwater (Isomäki *et al.*, 2008). The selection of potable water source depends on the local circumstances, local source water quality, and the availability of water from different sources.

Surface water supplies

Waterworks which use surface water as their raw water source usually need to resort to more extensive and more advanced treatment process than waterworks using groundwater which is considered to be protected better from contaminants than surface waters. The surrounding environment of raw water sources, such as lakes, ponds and rivers affects readily on the surface water quality, resulting in large temporal variations (Geldreich, 1990; Percival, 2000). Most of waterborne pathogens of public health concern are introduced from either a point source (e.g. sewage discharge) or a non-point source (e.g. agriculture, rainfall) to the surface waters (NRC, 2004).

Water treatment at waterworks using surface water has several steps including pretreatment, coagulation, filtration and disinfection (Percival, 2000; WHO, 2004). In theory, water treatment technology can successfully remove the impurities from the source water. There is a serious concern that a momentary break in the treatment chain may allow the contaminants to gain access to the finished water and for this reason there are multiple barriers to protect water from pollution (Geldreich, 1990). Risk-based performance targets are measures of \log_{10} reduction of pathogens based on source water quality and may be employed to determine the microbial reduction necessary to ensure water safety (WHO, 2004). In large-scale waterworks which use surface water as their raw water source, it has been reported that a 6 \log_{10} coliform reduction (99.9999 %) can be readily attained but fortunately relatively few waterworks are forced to treat such badly polluted water (Geldreich, 1990).

Pretreatment of surface water at waterworks may include coarse and fine screens, sedimentation in storage reservoirs and aeration procedures. The pretreatment may have only a minimal effect on the bacterial and viral concentrations in water, but the sedimentation is thought to result in a variable reduction of protozoan cysts and oocysts (Betancourt and Rose, 2004). In coagulation, particles and turbidity from water is reduced by sedimentation after formation of flocs (flocculation). The surface waters in Finland are typically humus-rich having a high content of organic carbon (Miettinen *et al.*, 1994; Vartiainen *et al.*, 1987), and the

coagulation/flocculation process is commonly employed to remove natural organic substances. Simultaneously, the microbial removal from the water is achieved: the removal efficiency of coagulation/flocculation can be below 1 log₁₀ (Stetler *et al.*, 1984) but rise to as much as 4 log₁₀ (99,99%) especially for protozoan cysts and oocysts (Lee *et al.*, 2007; WHO, 2004) if the process parameters such as coagulant dosages and pH are suitably optimized (Logsdon, 1990; WHO, 2004).

Filtration can be achieved in many ways e.g. through rapid or slow sand filters, activated charcoal filters or anthracite filters. The efficiency of the filtration on microbial removal is highly dependent on the filter type, appropriate re-establishments/backwashing of the filter and the retention time of water in the filter (Betancourt and Rose, 2004; Hijnen *et al.*, 2010; WHO, 2004). The slow sand filtration has been shown to be capable for 3-4 log₁₀ removal of bacteria, viruses and protozoan cysts (Logsdon, 1990). For correct operation of rapid sand filters in the treatment of turbid surface waters, effective prior coagulation is essential (Logsdon, 1990).

Disinfection techniques

Disinfection represents the final protective barrier of the water treatment before water distribution and it is intended to inactivate any remaining pathogenic microbes that have possibly escaped removal in the previous treatment steps (Schoenen, 2002). The resistance of microbes to different disinfectants and disinfection techniques differs significantly (Hoff and Akin, 1986). Traditional bacterial water quality indicators as well as bacterial pathogens of faecal origin are more sensitive to all disinfection means than many enteric viruses and protozoan cysts and oocysts (Sobsey, 1989; WHO, 2004). The disinfection techniques commonly employed in potable water production include chlorination, chloramination, treatment with chlorine dioxide, ozonation and ultraviolet light radiation (Percival, 2000; White, 1986).

Disinfection based on chlorine chemicals is relatively inexpensive and easy to use and is therefore the most commonly applied disinfection technique by water companies (Farland and Gibb, 1993; White, 1986). Chlorine chemicals are preferred also since they have a long residence time in water which has a low organic matter content and have thus a residual effect against regrowth of bacteria within the distribution systems. However, in the presence of organic matter, the chlorine demand is high and mutagenic and carcinogenic disinfection by-products can be formed (Bellar *et al.*, 1974; Boorman *et al.*, 1999; Rook, 1974). This has been considered as a threat to public health in previous decades when prechlorination in early stages of surface water treatment were used (Geldreich, 1990; Vartiainen and Liimatainen, 1986; White, 1986). Furthermore, the high concentrations of chlorine can be tasted, decreasing the palatability of the water.

For the reasons above, chlorine has been applied in disinfection at as low concentrations as possible. Therefore, inefficient disinfectant concentrations may

occur in stagnant parts of the distribution systems if there are no booster stations situated throughout the distribution system (van der Wende and Characklis, 1990). With the concentrations and contact times applied generally, chlorination is ineffective against protozoan cysts and oocysts and thus also physical removal such as filtration needs to be used for removal of these contaminants (WHO, 2004). In addition, microbes that are sheltered inside invertebrates (Levy, 1990) or are associated with distribution system biofilms or soft deposits may be protected against low chlorine concentrations (van der Wende and Characklis, 1990).

Of the disinfection techniques which leave no residual effect, ozonation has been proved to be effective against many microbes including protozoan cysts (Peeters *et al.*, 1989). Ultraviolet disinfection, if properly installed and maintained (Sommer *et al.*, 2008), is efficient in killing all microbial pathogens including enteric viruses (Hijnen *et al.*, 2006). In many cases, ozonation and/or UV disinfection are employed together with chlorine in order to have proper protection not only against bacteria but also against enteric viruses and protozoa.

Abstraction of groundwater

The need of multi-barrier advanced water treatment process has contributed to the fact that waterworks using surface water are usually relatively large units with efficient water quality monitoring programs. In contrast, a large part of waterworks using groundwater as their raw water are small community water supplies utilizing only minimal water treatment. The selection of the treatment process depends on the soil characteristics in the aquifer. Throughout the world, the treatment process may most commonly be limited to hardness reduction. Aeration to remove iron and manganese is also common, and with non-alkaline waters as is the case in Finland, pH adjustment is performed. In its pristine state, the quality of groundwater from deep aquifers is good. On the contrary, groundwater from shallow wells is vulnerable to surface contamination, particularly during heavy rainfall or floods if the protection of an abstraction point in the close neighborhood of a well is inadequate, enabling surface runoff (Kay *et al.*, 2007; Richardson *et al.*, 2009).

Improper source protection and inadequate treatment have led to poor water quality in the water supplied to small communities (Geldreich, 1990; Lahti and Hiisvirta, 1995; Miettinen *et al.*, 2001; Pedley and Howard, 1997). Increased risk of contamination of groundwater can occur at the point of recharge if the soil barrier protection is lost due to human activities and faecal material is spread onto land (Geldreich, 1990; Pedley and Howard, 1997). Leaking septic tanks, sewers, landfill and mining sites and unintentional bank filtration (infiltration of polluted surface streams) are known hazards that may lead to the contamination of a groundwater source (Geldreich, 1990; Isomäki *et al.*, 2008; Percival, 2000). An increasing number of groundwater intake plants have recently installed ultraviolet (UV) disinfection systems in Finland to decrease the consequences of possible microbial contamination (Hirvonen, 2009; Jääskeläinen, 2007; Orava *et al.*, 2003).

Distribution

The integrity of water distribution system is essential if one wishes to maintain water safety. The distribution system is a complex consisting of a network of pumping stations, service reservoirs, water mains, and service pipes constructed out of a variety of materials (Percival, 2000). Water quality degradation events can therefore be caused by several factors within the system (Besner *et al.*, 2001). The known causes for breaks in distribution system integrity are maintenance or repair works, leakages from cracks and joints and pressure drops that can lead to seepage of contaminated water into the underground pipes (Jakopanec *et al.*, 2008; LeChevallier *et al.*, 2003). In addition, service reservoirs that have allowed leaching of water, or even small animals like rodents, squirrels and birds, to enter the reservoir have led to potable water contamination (Miettinen *et al.*, 2006; Palmer *et al.*, 1983; Richardson *et al.*, 2007). Moreover, cross connections of water main and sewers (Engberg *et al.*, 1998; Kuusi *et al.*, 2005) and back siphonage from incorrectly installed domestic (e.g. washing machines) or industrial appliances may pose a risk of contamination (Pipes, 1990; Risebro *et al.*, 2007).

2.1.1 Biofilms of distribution systems

Biofilms growing on the inner surfaces of the distribution pipelines harbour most of the bacteria present in drinking water systems (Costerton *et al.*, 1995; Percival, 2000). In fact, the majority of bacteria in nature exist attached to surfaces (Watnick and Kolter, 2000) and these surface-associated bacteria have different phenotypic characteristics from planktonic bacterial cells (O'Toole *et al.*, 2000). Biofilm consists of living and dead microorganisms, microbial derived extracellular polymeric substances (EPS), and organic and inorganic substances as organized structure (Davey and O'Toole, 2000; Percival *et al.*, 1998; Tolker-Nielsen and Molin, 2000) having channels allowing exchange of nutrients and gases between water phase and biofilms (Costerton *et al.*, 1995).

There can be a huge diversity of microorganisms existing in potable water systems (Block *et al.*, 1993; Block *et al.*, 1997; van der Wende and Characklis, 1990). Microbial community in biofilms consists mostly of gram-negative bacteria (Keinänen *et al.*, 2003; Lehtola *et al.*, 2004; Norton and LeChevallier, 2000; Smith *et al.*, 2000). Particularly the presence of *Proteobacteria* species e.g. *Acidovorax*, *Acinetobacter*, *Afipia*, *Caulobacter*, *Hydrogenophaga*, *Polaromonas*, *Pseudomonas* and *Sphingomonas*, and *Bacteroidetes* species e.g. *Flavobacterium* and *Sphingobacterium* has been reported from drinking water systems and biofilms (Magic-Knezev *et al.*, 2009; Martiny *et al.*, 2005; Penna *et al.*, 2002; Percival *et al.*, 1998; Pitkänen *et al.*, 2008; Tokajian *et al.*, 2005). The predominant part of the microbial community in biofilms, however, consists of uncultured species (Keinanen-Toivola *et al.*, 2006). Moreover, the use of chlorine disinfection may lead to the development of chlorine resistant species (van der Wende and Characklis, 1990).

Bacteria are not the only biota present in biofilms: there are also fungi, yeasts, protozoa, free amoebae (Block *et al.*, 1993), invertebrates (Levy, 1990) and viruses (Skraber *et al.*, 2005). The presence of biofilms in potable water distribution systems is responsible for chemical, physical and biological effects (Block and Melo, 1992; Percival, 2000): they can harbor indicator bacteria (coliforms), increase heterotrophic plate counts (Lehtola *et al.*, 2007), support the growth of opportunistic pathogens and invertebrates, increase chlorine demands, cause discolouration, taste and odour in water, and accelerate corrosion of pipes.

The control of biofilms is one of the major goals in water distribution management practice (Block and Melo, 1992; Percival, 2000). In addition to supporting the growth of autochthonous microbes in the distribution system, biofilms may protect faecal indicator microbes and pathogenic microbes against disinfectants after contamination events (Szewzyk *et al.*, 2000; Trachoo *et al.*, 2002). The efficacy of monochloramine has been reported to be superior to free chlorine for controlling biofilm formation (Lechevallier *et al.*, 1988; Momba and Binda, 2002). In general, disinfection alone is not enough to prevent biofilm development; low concentrations of microbially available nutrients in water may reduce biofilm formation (Lehtola *et al.*, 2002) and regular mechanical cleaning of inner surfaces of pipes is also recommended (Zacheus *et al.*, 2001).

2.2 Monitoring of microbiological drinking water quality

2.2.1 Water quality guidelines and legislation

Drinking water should not contain any substances in numbers or concentrations that could potentially be hazardous to human health (European Union, 1998). The World Health Organization (WHO) has listed the important waterborne bacterial, viral and protozoan pathogens and provided criteria for acceptability of drinking water (WHO, 2004). The WHO's fact sheets include information on 19 bacterial pathogen species, eight viral pathogens, 11 protozoan pathogens, three helminths pathogens, toxic cyanobacteria and eight groups of indicator and index organisms (WHO, 2004). In addition, facts on more than a hundred chemicals are given. The WHO guidelines represent basis for drinking water legislation worldwide both in developed and developing countries and the supporting material includes information on microbiological, chemical, radiological and acceptability aspects.

Routine water quality monitoring is conducted to protect public health and ensures compliance with regulations. Bacterial indicators are used to determine if the drinking water sources are microbiologically safe, if the treatment of the drinking water has been adequate and if the integrity of drinking water distribution system has been maintained (NRC, 2004). The goals of monitoring include the detection of the ingress of possible faecal contamination and the assessment of effectiveness of disinfection (Percival, 2000; Pipes, 1990). A single monitoring result is not enough to guarantee achievement of these goals and thus regular monitoring programs need

to be arranged (Maul *et al.*, 1990). A zero tolerance for faecal indicator bacteria is employed and usually 100 ml volumes of water are tested. The sampling frequency depends on the size of the population served or on the volume of the water to be distributed (European Union, 1998; NRC, 2004).

In Europe, council directive 98/83/EC on the quality of water intended for human consumption lists the requirements for drinking water quality monitoring for regulatory purposes in Member States (European Union, 1998). These regulations have been adopted in Finland according to the decree 461/2000 of Ministry of Social Affairs and Health. For water quality monitoring, the decree states that as a mandatory microbial water quality requirement no *Escherichia coli* or intestinal enterococci should be detected in 100 ml of a potable water sample, and also 29 mandatory concentration limits for chemical parameters are listed (STM, 2000). In addition, the decree includes 19 other recommended water quality parameters of which three are microbial: it is recommended that *Clostridium perfringens* and coliform bacteria are absent in 100 ml potable water sample and that there are no unusual changes in heterotrophic plate count at 22 °C. In addition to the above parameters, the water quality requirements for water sold in bottles or containers include also testing for *Pseudomonas aeruginosa* and a heterotrophic plate count at 37 °C.

It is essential that immediate actions are taken following a presumptive positive indicator bacteria result from a treated water sample, whether from the treatment works, service reservoirs or consumers tap (Percival, 2000; Pipes, 1990; Valvira, 2009a). In cases when faecal contamination of drinking water is evident, full emergency level actions need to be initiated, including a water avoidance or a boil water advisory (Hunter *et al.*, 2003). At any level of contamination, further samples from the same site and from related locations have to be taken immediately. The examination should be extended from the most commonly monitored coliform bacteria to intestinal enterococci and *C. perfringens* and also a prolonged incubation time may be used if the first cultivation analysis results are negative. Simultaneously, the confirmation of presumptive results should be organized. Control of adequate sampling, confirmation of successful and uninterrupted treatment process and checking the integrity of the distribution system all need to be done.

There are problems with basic water quality determinations; especially at small water supplies the sampling may be infrequent and there is a time lag after the sampling before the result is available (Edberg *et al.*, 1997). There are also reports of waterborne outbreaks and associations of water with gastrointestinal illness even though no *E. coli* were detected in the water (Engberg *et al.*, 1998, Hänninen *et al.*, 2003; Melby *et al.*, 1991; Payment, 1997; Vogt *et al.*, 1982). Therefore this kind of water quality testing alone is insufficient to protect public health. In order to minimize the chance of failure, water companies are encouraged to initiate and implement management plans called “water safety plans” (WSP) for controlling water quality (WHO, 2004). This preventive management framework for safe

drinking water covers abstraction, treatment and delivery of drinking water; it is a combination of control and management measures for protection of water sources, control of treatment processes and management of distribution. Currently, the European Commission is preparing a revision of the drinking water directive (European Commission, 2010). The aim of the revision is to adopt progress in science and technology into the new directive, and to include the concept of risk assessment as well as small water supply issues in the directive.

2.2.2 Indicator bacteria in microbiological quality monitoring

Indicator bacteria of water quality are employed in the assessment of potential hazards, exposure assessment, contamination source identification and evaluating the effectiveness of risk reduction actions. However, no single indicator or method or set of indicators or methods is appropriate for all applications (Godfree *et al.*, 1997). The selection of a suitable indicator may be dependent on the intended use of information and also on the water supply type. A proposed three-level phased monitoring framework (NRC, 2004) is presented in Table 1. For early warning of potential microbial contamination, a rapid, simple and broadly applicable technique would be appropriate. For confirmation of health risk and for contamination source identification, more efforts are needed. The concentration of an ideal water quality indicator should be quantitatively related to the degree of public health risk and the specificity and speed of the detection and enumeration method are also essential attributes of an ideal indicator (Gerba, 2000; NRC, 2004).

Bacterial indicator organisms are required for drinking water monitoring purposes. Coliform bacteria, *Escherichia coli* (or thermotolerant coliform bacteria), intestinal enterococci (previously faecal streptococci), *Clostridium perfringens* (or sulphite reducing clostridia) and heterotrophic plate counts (HPC) are utilized around the world as indicator organisms. Of these, *E. coli*, intestinal enterococci and *C. perfringens* are considered to be of faecal origin and can serve as index organisms (Mossel, 1982) of faecal pathogens, while coliform bacteria and especially HPC indicate the general cleanliness of water (APHA *et al.*, 1998). Standardization of detection and enumeration methods for these parameters is essential if one wishes to reduce the variation in results.

Table 1. Phases of water quality investigations and the most desirable attributes of the water quality indicators in each phase. Modified from The U.S. National Research Council (NRC, 2004).

Level of investigation	Indicators of waterborne pathogens should preferably have:	Desirable attributes of indicator methods include:
A. Screening, routine monitoring	<ul style="list-style-type: none"> • Correlation to health risk • Presence in greater numbers than pathogens • Simple detection and enumeration methods available 	<ul style="list-style-type: none"> • Broad applicability • Adequate sensitivity • Rapidity of results • Logistical feasibility
B. Investigation to confirm health risk	<ul style="list-style-type: none"> • Presence whenever the pathogens are present • No significant proliferation in aqueous environment 	<ul style="list-style-type: none"> • Specificity to desired target organism • Possibility for quantification
C. Detailed investigations: source identification and mitigation	<ul style="list-style-type: none"> • Similar (or greater) survival and transport to pathogens • Specificity to a faecal source of origin 	<ul style="list-style-type: none"> • Provides information on viability or infectivity

Escherichia coli

Escherichia coli is the best bacterial indicator for a fresh faecal pollution (Edberg *et al.*, 2000; Tallon *et al.*, 2005). *E. coli* is a member of the coliform and faecal coliform groups (the latter being referred to also as thermotolerant coliform group) (Pipes, 1990). These groups were used as indirect indicators of faecal pollution before the introduction of improved methods for direct *E. coli* detection. *E. coli* are indigenous microbiota living in the intestinal track of humans and warm-blooded

animals and they are found in faeces in larger numbers than other coliform bacteria. Only certain *E. coli* strains are animal or human pathogens. For example, *E. coli* O157:H7 (Enterohaemorrhagic *E. coli*, EHEC) is an important waterborne pathogen (Chalmers *et al.*, 2000; Leclerc *et al.*, 2004).

Coliform organisms other than *E. coli* suffer from several deficiencies as indicators of faecal contamination that restrict their indicator value (Pipes, 1990). In particular, their original source is not the intestinal tract but soil or water and thus they grow in aquatic environments (Tallon *et al.*, 2005) as well as in distribution system biofilms (LeChevallier *et al.*, 1996). However, coliform bacteria may serve as a sign of any change in water quality; indicate the efficiency of water treatment and the adequacy of disinfectant residual in distribution systems. This is supported by a finding that high heterotrophic colony counts were associated with a higher incidence of β -galactosidase positive coliform species consisting of non-faecal (saprophytic) enterobacteria such as *Klebsiella trevisanii*, *Klebsiella terrigena*, *Buttiauxella agrestis*, *Serratia fonticola* and *Enterobacter agglomerans* (Gavini *et al.*, 1985).

The survival of *E. coli* in water has been shown to be at least as good as the survival of enteric bacterial pathogens such as *Campylobacter jejuni* that do not grow in aquatic environments (Edberg *et al.*, 2000; Korhonen and Martikainen, 1991b) even though the possible differences in the culturability of these bacteria may complicate the exact comparison (Lund, 1996). On the contrary, the survival of cultivable *E. coli* is negligible compared to certain other microbes e.g. to survival *Cryptosporidium* ssp., *Giardia* sp. and enteric viruses; lessening the indicator value of *E. coli* detection using cultivation methods for these groups of micro-organisms (Geldreich, 1997; Leclerc *et al.*, 2001; Theron and Cloete, 2004).

Other indicator microbes and physico-chemical parameters

It has been recommended that microbial indicators more resistant than *E. coli* in environmental conditions or physico-chemical parameters may be helpful when used in conjunction with *E. coli* to supplement the water quality assessment (Cronin *et al.*, 2006; Tallon *et al.*, 2005). Intestinal enterococci and *Clostridium perfringens* are indicators for faecal pollution (Edberg *et al.*, 2000) but are present in raw sewage and in natural waters though at less frequent densities than *E. coli* (Geldreich, 1997; Gerba, 2000; Godfree *et al.*, 1997).

The resistance of intestinal enterococci (faecal streptococci) to disinfection processes is approximately twice that of *E. coli* (Havelaar, 1987; Hijnen *et al.*, 2006; Nieuwstad *et al.*, 1991) enabling better survival in the water environment. Currently in the EU legislation, intestinal enterococci are used as a second stage microbial indicator of drinking water quality (European Union, 1998). Intestinal enterococci are considered especially valuable in situations when coliform bacteria but not *E. coli* have been detected from water (Edberg *et al.*, 1997).

Spores of *C. perfringens* are extremely persistent in the environment (Davies *et al.*, 1995; Marcheggiani *et al.*, 2008; Schijven *et al.*, 2003) and are also resistant to water disinfection processes (Havelaar, 1987; Hijnen *et al.*, 2006; Nieuwstad *et al.*, 1991). Therefore their indicator value has been questioned: clostridial spores could either be natural environmental inhabitants or represent a historical source of faecal contamination (Edberg *et al.*, 1997; Edberg *et al.*, 2000; NRC, 2004). This exceptionally long survival may result in over-protection or be non-discriminatory. However, *C. perfringens* may be indicative of remote or intermittent faecal pollution (Sartory, 1986).

In addition, the use of *Bacillus subtilis* endospores (aerobic spore-forming bacteria) has been proposed to serve as an indicator of changes in water quality and an index of water treatment efficiency (Locas *et al.*, 2008; Mazoua and Chauveheid, 2005). *B. subtilis* spores have been reported to be more resistant to ozone than *Cryptosporidium* oocysts and *Giardia* cysts (Owens *et al.*, 2000) and that they have a similar resistance to UV disinfection as *C. perfringens* (Hijnen *et al.*, 2006).

Characterization of bacterial composition of faeces has revealed that obligate anaerobes, such as species of genera *Bacteroides*, *Eubacterium* and *Clostridium*, are present at high concentrations in faecal material (Finegold *et al.*, 1974; Hayashi *et al.*, 2005; Holdeman *et al.*, 1976; Rigottier-Gois *et al.*, 2003; Wilson and Blitchington, 1996). Since many of these microbial species are uncultivable, their detection from water has been challenging (Fiksdal *et al.*, 1985) before the recent development of molecular methods (Dick and Field, 2004).

None of bacterial indicator microbes are optimal for indicating the presence of human enteric viruses (Grabow *et al.*, 2001; Lucena *et al.*, 2006). Therefore, the applications of viral indicators; somatic coliphages, F-specific RNA bacteriophages and *Bacteroides fragilis* phages, as well as direct enteric virus detection have been utilized in certain situations (Blanch *et al.*, 2006; Costan-Longares *et al.*, 2008; Espinosa *et al.*, 2009; Havelaar, 1987; Pourcher *et al.*, 2007; Savichtcheva and Okabe, 2006; Schijven *et al.*, 2003; Skraber *et al.*, 2004; Stetler, 1984).

A variety of basic physico-chemical parameters, such as pH, conductivity and temperature can be utilized to analyse water quality (Edberg *et al.*, 1997). These system parameters might be useful in the detection of water quality changes, especially when continuous online measurements can be employed. In the detection of bacterial contamination of groundwater attributable to surface water, it is recommended that there should be online monitoring of parameters such as turbidity, UV-extinction and increase in discharge (Auckenthaler *et al.*, 2002). Moreover, online measurements of organic carbon (Pronk *et al.*, 2006) and particle-size distribution have been postulated to be even better indicators for faecal bacteria contamination than turbidity (Pronk *et al.*, 2007).

The concentrations of nitrogen compounds and phosphorus may also reflect water contamination, since they are present in wastewater at high concentrations (Katz *et*

al., 2009; Postma *et al.*, 1992). In addition, some chemical compounds, such as boron and faecal sterols have been utilized as tracers of human wastewater discharges (Cronin *et al.*, 2006; Glassmeyer *et al.*, 2005; Moreno *et al.*, 2008; Savichtcheva and Okabe, 2006).

2.2.3 Detection methods of indicator bacteria

In the conventional cultivation methods for the detection and enumeration of faecal indicator bacteria from water samples, differential and selective media and specific incubation conditions are used (NRC, 2004; Rompre *et al.*, 2002). The solid and liquid cultivation media utilized contain the substances necessary to support the growth of the target bacteria, selective components to inhibit the growth of non-target bacteria and the media may also contain specific growth factors and differential components (Atlas, 1995; NRC, 2004).

The most commonly used concentration technique of water prior to cultivation of bacteria is membrane filtration (MF) (ISO 8199, 2005; Pepper *et al.*, 2000). In the MF methods, microbes present in water samples are concentrated by filtration on membranes usually with a pore-size of 0.45 µm. The sensitivity of the method can be improved by increasing the volume of water samples to be filtered (Hijnen *et al.*, 2000; Payment *et al.*, 1989). After filtration, the filter is transferred to a selective solid nutrient medium, which usually contains chemical compounds causing a characteristic indicator reaction upon the growth of target bacteria during the incubation. Bacterial colonies can then be differentially enumerated and the density of target bacteria calculated as the colony count per volume of water filtered (Pipes, 1990).

Alternatively, the filter can be placed into a liquid broth cultivation medium, and the MF may be followed by a subsequent elution of the concentrate from the filter prior to further processing; this step is more common in pathogen analyses than in indicator bacteria methods. Different liquid broth enrichment methods can be employed in water microbiology also without the filtration step (ISO 8199, 2005) and may be valuable when samples of non-filterable water need to be tested. The liquid methods are quantitative if utilized in multiple-tube formats (Most probable number, MPN) and automated MPN approaches have been developed to ease the work load. If only one liquid broth tube is used, a qualitative presence/absence result is obtained (Gerba, 2000).

Cultivation methods for detection of Escherichia coli and coliform bacteria

The definitions of microbial indicators are historically tied to the methods used for their measurement (NRC, 2004). After the introduction of novel methods that are more specific in their taxonomic grouping, it may be necessary to modify the definitions of the indicator groups. Coliform bacteria are currently defined as facultatively anaerobic, gram-negative, non spore-forming, oxidase negative, rod-shaped bacteria that ferment lactose to acid and gas within 48 h at 35 °C and are β-

galactosidase positive members of *Enterobacteriaceae* (APHA *et al.*, 1998; ISO 9308-1, 2000).

Thermotolerant coliform bacteria are coliform bacteria able to grow and produce gas at 44 °C. *E. coli* are (thermotolerant) coliform bacteria that possess the enzyme β -glucuronidase and they are able to produce indole from tryptophan within 24 h at the elevated incubation temperature of 44 °C (ISO 9308-1, 2000). Thermotolerant coliform bacteria other than *E. coli* are primarily species belonging to the genera *Klebsiella*, and also strains of genera *Citrobacter* and *Enterobacter* may express characteristics typical of thermotolerant coliform bacteria (Alonso *et al.*, 1999).

The era of use coliforms in water pollution determinations began in 1891, when T. Smith published in 13th Annual Report of the New York State Board of Health, a presumptive *E. coli* test that utilized lactose-based fermentation tubes (NRC, 2004). Subsequently after the widespread acceptance of the coliform test, it was discovered that not all coliform bacteria are of faecal origin and a faecal coliform test utilizing a higher incubation temperature (thermolorant coliforms) was used to overcome this problem (Gerba, 2000; Pipes, 1990). Even today, these traditional methods, relying on lactose fermentation with the production of acid and gas, which require further confirmation of the presumptive counts, are widely used despite the fact that they are labour intensive, slow and non-specific. The failure of traditional methods to exhibit selectivity has led to false-negative results when water containing interfering background microbiota has been tested (Evans *et al.*, 1981; Gerba, 2000).

The addition of enzymatic reactions: β -D-galactosidase splitting lactose disaccharides and β -D-glucuronidase catalyzing the hydrolysis of β -D-glucopyranosiduronic acids, to traditional methods has significantly improved the selectivity of these procedures (Manafi, 1996; Rompre *et al.*, 2002; Tallon *et al.*, 2005). Tests of β -D-galactosidase and β -D-glucuronidase activities has been developed for specific detection of coliform organisms and *E. coli*, respectively. Especially the β -glucuronidase test (Edberg *et al.*, 1988) has enhanced considerably the rapidity, specificity and sensitivity of the assays and reduced the interest in studying thermotolerant coliforms (Tallon *et al.*, 2005).

Currently, various cultivation methods are available to detect coliforms and *E. coli* from drinking water and a selection of these methods is presented in Table 2. Most of the coliform and *E. coli* methods employed are quantitative, but sometimes a simple presence/absence measurement will suffice. The wide range of modified media reflects the situation that there is no universal medium optimal for each application (Rompre *et al.*, 2002). In Finland, conventional mEndo Agar LES (Les Endo) medium is currently the most commonly used method for *E. coli* and coliform detection from drinking water. However, the Colilert® Quantitray is increasingly being employed by the water industry and also in Finnish regulatory monitoring after its acceptance for that purpose; initially for bathing water monitoring (STM, 2008) but later also for drinking water monitoring (Valvira, 2009b).

Table 2. A selection of cultivation-based methods employed for detection of coliform bacteria, faecal coliform bacteria and/or *Escherichia coli* from drinking water. Modified from Manafi, 2000.

Method	Reference/manufacturer
<i>Solid media in conventional MF methods</i>	
• mEndo Agar LES (LesEndo) with or without m-FC medium	(APHA <i>et al.</i> , 1998; SFS 3016, 2001; SFS 4088, 2001)
• Lactose Tergitol TTC agar (LTTC) with or without <i>E. coli</i> direct plating on TSA/TBA	(ISO 9308-1, 2000; Schets and Havelaar, 1991)
• Laurylsulphate Agar (LSA)	(NEN 6571, 1982; SCA, 2009; Schets <i>et al.</i> , 2002)
• m-T7 medium	(Lechevallier <i>et al.</i> , 1983)
• Membrane Lauryl Sulfate agar (MLSA)	(SCA, 2009)
<i>Solid media based on enzymatic activity detection in MF methods</i>	
• Membrane lactose glucuronide agar (MLGA)	(Sartory and Howard, 1992; SCA, 2009)
• MI agar	(Brenner <i>et al.</i> , 1993)
• Chromocult® Coliform agar	Merck KgaA, Germany
• ColiScan MF	Micrology Laboratories, U.S.
• CHROMagar ECC	Chromagar, France
• Rapid <i>E. coli</i> 2™	Biorad Laboratories, France
• Chromogenic <i>E.coli</i> /coliform	Oxoid, UK
<i>Liquid media in conventional MTF methods (MPN and P/A)</i>	
• Lactose broth	(Atlas, 1995)
• Lauryl tryptose broth (LTB)	(APHA <i>et al.</i> , 1998; SCA, 2009)
<i>Liquid media based on enzymatic activity detection in MPN and P/A methods</i>	
• Colilert®-18 or Colilert® together with QuantiTray®	IDEXX Laboratories, U.S.
• Colisure®	IDEXX Laboratories, U.S.
• Colitag™	CDI International, The Netherlands
• ColiComplete® discs used with Lauryl Sulfate Tryptose Broth (LST) tubes	BioControl Systems, Inc., U.S.
• Readycult® Coliforms	Merck KgaA, Germany

MF, Membrane filtration; MTF, Multiple-tube fermentation; MPN, Most Probable Number; P/A, Presence-Absence.

Confirmation of the presumptive counts is essential when conventional MF methods are used. A cytochrome-oxidase test is required to exclude oxidase-positive bacteria such as lactose fermenting *Aeromonas* or *Pseudomonas* genera from the confirmed

coliform count (Rompere *et al.*, 2002). The indole test is utilized for differentiation of *E. coli* from other coliform bacteria. However, these conventional confirmation tests based on metabolic reactions are not totally specific and may require further testing (Perez *et al.*, 1986); for example a coliform bacteria species *Klebsiella oxytoca* is indole positive. In order to prevent false-positive *E. coli* results, the testing of gas production from lactose at 44.0-44.5 °C has been employed together with the indole test (Niemi *et al.*, 2003). The testing of β -D-glucuronidase enzyme activity, which is mostly limited to *E. coli* (Kilian and Bulow, 1976; Perez *et al.*, 1986), has been utilized increasingly and this might replace the previously used indole and gas tests.

In enzymatic cultivation methods, during cultivation the chromogenic or fluorogenic substrate is hydrolyzed by a specific enzyme, yielding chromo- or fluorochromes that can be readily detected (Manafi, 1996). The chromogenic substrates are common in solid and liquid media whereas rapidly diffusing fluorescence of fluorogenic substrates are mostly utilized in liquid cultures. There are numerous substrates which can be added to the cultivation media for detection of β -D-galactosidase activity, for example chromogenic *o*-nitrophenyl- β -D-galactopyranoside (ONPG), 6-chloro-3-indolyl- β -D-galactopyranoside (Salmon-Gal), 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (XGal) and fluorescent 4-methylumbelliferyl- β -D-galactoside (MUGal) (Manafi, 1996; Manafi, 2000; Rompere *et al.*, 2002).

For detection of β -D-glucuronidase activity, for example fluorogenic 4-methylumbelliferyl- β -D-glucuronide (MUGlu) and chromogenic 5-bromo-4-chloro-3-indolyl- β -D-glucuronide (XGLUC), 5-bromo-6-chloro-3-indolyl- β -D-glucuronide (magenta-glc) and 6-chloro-3-indolyl- β -D-glucuronide (salmon-glc) are utilized (Manafi, 1996; Rompere *et al.*, 2002). In several commercially available media, two substrates are added, providing the possibility for simultaneous detection of coliform bacteria and *E. coli*. These media allow more rapid, easier and more specific detection and enumeration of coliform bacteria and *E. coli* than the conventional lactose-based media.

Cultivation methods for faecal indicator bacteria other than E. coli

Faecal enterococci and *Clostridium perfringens* have been used as alternatives or additional indicators to coliforms and *E. coli*. Faecal enterococci (faecal streptococci) are presently named as “intestinal enterococci” (Godfree *et al.*, 1997). The protocols for enumeration of intestinal enterococci have been based on the MPN approach in a liquid enrichment broth that contains azide (APHA *et al.*, 1998) as well as on the more widely utilized membrane filtration method using Slanetz and Bartley agar (also known as m-enterococcus agar) (ISO 7899-2, 2000; Slanetz and Bartley, 1957).

Several other enumeration media for intestinal enterococci are also used, such as KF streptococcus agar and kanamycin-esculinazide agar (Audicana *et al.*, 1995) and a MPN-method utilizing 4-methylumbelliferyl- β -D-glucoside (ISO 7899-1, 1998;

Niemi and Ahtiainen, 1995). Intestinal enterococci have the ability to grow under a wide range of conditions: from 10 °C up to 45 °C, at a pH of 9.6 and in the presence of 6.5% sodium chloride and they even can survive for at least 30 min at 60 °C (Godfree *et al.*, 1997). Intestinal enterococci are gram-positive, catalase-negative cocci possessing the Lancefield Group D antigen (ISO 7899-2, 2000). The international standard methods exhibit the best recoveries for the intestinal enterococci species i.e. *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus durans* and *Enterococcus hirae* (ISO 7899-2, 2000; ISO 9308-1, 2000).

Clostridium perfringens is gram-positive, spore-forming, non-motile and strictly anaerobic rod-shaped bacterium (Payment *et al.*, 2002). Its detection method includes cultivation of a group of sulphite reducing anaerobes (clostridia) (ISO 6461-2, 1986) from which *C. perfringens* is separated by confirmation tests. It has been claimed that it might be difficult to simultaneously recover both vegetative cells of clostridia and clostridial spores since some spores might be unable to germinate without heat activation but vegetative cells will not survive heat treatment (Payment *et al.*, 2002).

The detection method for sulphite-reducing clostridia most commonly utilized in Europe is a membrane filtration method employing tryptose-sulphite-cycloserine (TSC) medium or a membrane *Clostridium perfringens* medium (mCP) (Armon and Payment, 1988; Bisson and Cabelli, 1979; European Union, 1998; ISO 6461-2, 1986; Sartory, 1986). There have been unresolved issues related to the storage conditions of the agar plates and to the different recoveries between the media (Payment *et al.*, 2002; Sartory *et al.*, 1998). In addition, the conventional confirmation tests of *C. perfringens* have been laborious (Sartory *et al.*, 1998) and these have restricted the use of this indicator bacterium. However, the recent development of a confirmation test is said to substantially simplify the analysis (Sartory *et al.*, 2006).

Equivalence of quantitative microbiological methods

A wide range of cultivation media are employed in water quality monitoring. Typically, when a new medium is introduced, its performance is generally validated against other media that are already being employed, e.g. see Brenner *et al.* (1993). As coliform bacteria and *E. coli* are the most common water quality indicators, a large number of coliform method comparisons have been done so far. The comparisons have been made between the classical cultivation methods based on lactose fermentation and these conventional media have also been compared against various commercial tests utilizing chromogenic and fluorogenic substrates (Alonso *et al.*, 1999; Alonso *et al.*, 1998; Brenner *et al.*, 1993; Buckalew *et al.*, 2006; Clark and Elshaarawi, 1993; Eckner, 1998; Hamilton *et al.*, 2005; McFeters *et al.*, 1997; Hörman and Hänninen, 2006; Rompre *et al.*, 2002; Schets *et al.*, 1993). Moreover, the performance of different chromogenic and fluorogenic media have been compared to each other (Fricker *et al.*, 2008; Fricker *et al.*, 1997; Maheux *et al.*, 2008; Olstadt *et al.*, 2007).

Since several methods are often available for the same purpose, the selection of methods varies in different countries and even in different laboratories within a single country. When large databases of water quality results are being evaluated, a comparison of the results obtained using different media in different laboratories may be needed. This has created a need to quantitatively compare the recoveries of cultivation media to each other. The interest of equivalence has focused on methods for indicator bacteria enumeration since most of the methods for pathogens have so far been only qualitative or semi-quantitative. The international standard method ISO 17994 lists the criteria for establishing equivalence between two quantitative microbiological cultivation media (ISO 17994, 2004) and in recent years this standard has streamlined the various procedures used in comparison trials (Sartory, 2005).

In Europe, the European drinking water directive lists reference methods that must be used in microbial water quality monitoring for regulatory purposes (European Union, 1998). However, the directive states that alternative methods may be used if the Member State is able to demonstrate that the results are at least as reliable as those produced by the reference method. This statement has led to numerous comparison trials especially for detection methods of coliform bacteria and *E. coli* in Europe since the selectivity of the stated reference method (ISO 9308-1, 2000) is claimed to be inadequate (Bernasconi *et al.*, 2006; Mavridou *et al.*, 2010; Niemelä *et al.*, 2003; Niemi *et al.*, 2001; Schets *et al.*, 2002).

Molecular methods for detection of indicator bacteria

Molecular biology techniques provide new opportunities for indicator detection. To achieve the specific detection of coliform group of organisms, primers targeted to *lacZ* gene coding β -D-galactosidase have been utilized (Bej *et al.*, 1990) together with primers for *uidA* gene coding β -D-glucuronidase for *E. coli* detection (Bej *et al.*, 1991; Fricker and Fricker, 1994; Iqbal *et al.*, 1997). For specific *E. coli* detection, in addition to *uidA* gene (Juck *et al.*, 1996), *wecA* gene coding enterobacterial common antigen (Bayardelle and Zafarullah, 2002), *phoE* gene coding outer membrane protein (Spierings *et al.*, 1993), and elongation factor *tuf* gene (Paradis *et al.*, 2005) that was reported to multiply also *Escherichia fergusonii* (Maheux *et al.*, 2009), have been tested. In addition, *lacY* and *cyd* genes encoding lactose permease and cytochrome bd complex, respectively, have been utilized in a multiplex PCR assay (Horakova *et al.*, 2008).

Recently there have been improvements in quantitative real-time PCR applications in *E. coli* detection, but these techniques still require extensive laboratory work and expensive equipment (Rompre *et al.*, 2002). The detection of *E. coli* at low concentrations which are required in drinking water testing may also be limited due to endogenous DNA contamination of PCR reagents (Silkie *et al.*, 2008). Real-time PCR has been utilized for *uidA* gene (Frahm and Obst, 2003) and also for 16S-ITS-23S gene region of *E. coli* (Khan *et al.*, 2007). Specific molecular beacon probe

methods (Sandhya *et al.*, 2008) have been developed to detect the amplified mRNA fragment of *E. coli* during the real-time 3-4 hour nucleic acid sequence based amplification (NASBA) assay (Heijnen and Medema, 2009).

Oligonucleotide probes targeted to 16S ribosomal ribonucleic acid have been tested for specifically visualizing *E. coli* cells with in situ hybridization and epifluorescence microscopy (Regnault *et al.*, 2000) using peptide oligonucleotide (PNA) probes (Prescott and Fricker, 1999). The FISH technique (Amann and Fuchs, 2008) is promising since the time requirement of detection is fairly short (result obtained during a working day) (Rompre *et al.*, 2002), but the counting of bacterial cells by epifluorescence microscopy is a demanding task and may suffer from insufficient sensitivity. A direct viable count (DVC) method has been employed to increase the size of the target cells (Garcia-Armisen and Servais, 2004; Juhna *et al.*, 2007) and also *in situ* PCR application for *E. coli* detection has been developed (Tani *et al.*, 1998).

2.3 *Campylobacter* spp.

The family *Campylobacteraceae* contains gram-negative bacilli that have a curved or spiral S-shape, microaerobic growth requirements and a low G+C content in their relatively small genome (Ketley, 1997; Nuijten *et al.*, 1990). Genera *Campylobacter*, *Arcobacter* and *Helicobacter*, and in addition, misclassified species *Bacteroides urealyticus* and genus *Sulfurospirillum* are included into the family (Debruyne *et al.*, 2008). *Campylobacter* is the type genus of the *Campylobacteraceae*.

Campylobacter spp. cells are small rods that are 0.2 to 0.8 µm wide and 0.5 to 5 µm long and have a polar flagellum at one or both ends of the cell that enables the display of rapid corkscrew-like darting and spinning motions (Butzler, 2004; Debruyne *et al.*, 2008). *Campylobacter* do not utilize carbohydrates: the energy is taken from amino acids or from tricarboxylic acid cycle intermediates (Debruyne *et al.*, 2008). In old cultures in the stationary phase of growth or in unfavorable environments, the cells may form coccoid bodies (Ketley, 1997). Oxidase activity is present in most *Campylobacter* species.

There are 17 validly named species in the genus *Campylobacter*; the type species of the genus is *Campylobacter fetus* (Debruyne *et al.*, 2008). Fourteen out of the 17 *Campylobacter* species have been so far isolated from humans (Lastovica and Allos, 2008). *Campylobacter jejuni* subsp. *jejuni* (later abbreviated as *C. jejuni*) and *Campylobacter coli* are responsible for more than 95 % of diagnosed human *Campylobacter* infections (Butzler, 2004) and with *C. jejuni* being substantially more abundant than *C. coli* (Gillespie *et al.*, 2002). However, the predominance of *C. jejuni* and *C. coli* may be biased by the selective cultivation media employed (Lastovica and Allos, 2008).

The other species of *Campylobacteraceae* isolated from human patients with diarrhea include *Campylobacter upsaliensis*, *Campylobacter lari*, *Campylobacter fetus* subsp. *fetus*, *Campylobacter jejuni* subsp. *doylei* and *Campylobacter concisus*, but these isolations have required refinements in both isolation and identification methods (Butzler, 2004). These kinds of refinements can include application of a microaerobic atmosphere containing hydrogen, that is also required for isolation of species that have unknown pathogenicity such as *Campylobacter hyointestinalis* and *Campylobacter mucosalis* (Debruyne *et al.*, 2008).

Thermotolerant campylobacteria are able to grow at 42 °C but not below 30 °C, and this group, also referred to as thermophilic campylobacteria, includes the species *C. jejuni*, *C. coli*, *C. lari* and *C. upsaliensis* (Snelling *et al.*, 2005a; Thomas *et al.*, 1999a). *C. lari* strains, often isolated from wild birds and surface water, have been differentiated from *C. jejuni* and *C. coli* due to their resistance to nalidixic acid (Debruyne *et al.*, 2008). Heterogeneity within the *C. lari* species has been reported, as nalidixic acid-susceptible and urease-producing strains have been described (Endtz *et al.*, 1997; Matsuda and Moore, 2004). A recently named new species *Campylobacter insulaenigrae* isolated from marine mammals as well as the species *Campylobacter helveticus* isolated from dogs are also thermotolerant, as they grow at 42 °C (Debruyne *et al.*, 2008; Foster *et al.*, 2004; Stoddard *et al.*, 2007).

2.3.1 Infections and waterborne outbreaks

Campylobacter jejuni is a zoonotic pathogen only recognized in the 1970s after its isolation method from faeces was developed (Dekeyser *et al.*, 1972; Skirrow, 1977). Subsequently *Campylobacter jejuni* has proved to be a major cause of human infections transmitted by the faecal-oral route through contaminated food and water (Allos, 2001; Butzler, 2004). *Campylobacter* infection is an acute self-limited gastrointestinal illness with an incubation period that is longer than most other intestinal infections: the mean incubation period is 3.2 days ranging approximately from 1 to 7 days (Blaser and Engberg, 2008; Ketley, 1997). Infection with campylobacteria is characterized by diarrhea lasting about 3 days, accompanied by fever and abdominal cramps (Allos, 2001; Butzler, 2004). Headache, myalgia, vomiting and blood in faeces may occur as well as prolonged abdominal pain and subsequent complications, such as reactive arthritis or more serious Guillain-Barré syndrome (GBS) (Blaser and Engberg, 2008; Butzler, 2004).

There is a great variation in the duration of the illness and it is known that not all infected persons become ill (Allos, 2001; Black *et al.*, 1992). Milder degrees of illness, that do not require medical attention, are common (Blaser and Engberg, 2008) and thus the majority of self-limiting gastrointestinal illnesses often go unreported (Olson *et al.*, 2008). It has been estimated that for every reported *Campylobacter* case, ~40 persons experience symptoms (Mead *et al.*, 1999). The faeces remain *Campylobacter* positive for about 2-7 weeks after the illness if no antibiotic treatment is given (Blaser and Engberg, 2008; Butzler, 2004) and

antibodies to *Campylobacter* antigens are present for several months conferring a short-term immunity to the homologous strain (Black *et al.*, 1992).

Campylobacter enteritis affects most frequently children aged < 4 years and the infection rate is higher in boys and men than in girls and women (Allos, 2001; Olson *et al.*, 2008). The infection frequency displays a strong seasonal variation in temperate climates with infections being more common in summer than in winter (Butzler, 2004; Olson *et al.*, 2008; Samuel *et al.*, 2004; Thomas *et al.*, 1999a). The reason for the summer peak of human infections remain unknown but it may be affected by the higher prevalence of sporadic foodborne infections together with higher probability of waterborne outbreaks in conjunction with summer activities, such as outdoor food handling practices and the use of non-community water supplies (Olson *et al.*, 2008).

Based on human volunteer studies, exposure to less than 800 cells of *Campylobacter jejuni* may lead to infection and illness but there are remarkable differences between the strains in their infectivities (Black *et al.*, 1992). A quantitative description of the dose-response relation for *C. jejuni* is available as the existing experimental data of infection has been adequately fitted with a Beta-Poisson model and this has established the relatively high infectivity of *C. jejuni* (Medema *et al.*, 1996). Inter-human transmission is infrequent and has only been described in young children (Butzler, 2004), and thus acquired immunity due to repeated exposure after the first years of life may have an important role in protecting individuals from infection (Olson *et al.*, 2008). In developing countries, where there may be close contact with live chickens inside the home and without proper sanitation, *Campylobacter* infections are hyper-endemic, and the repeated exposure leads to the development of immunity early in life (Blaser and Engberg, 2008; Butzler, 2004).

The main sources of *Campylobacter* infection are foods of wild and domestic animal origin, wild birds, and drinking of untreated water (Jacobs-Reitsma *et al.*, 2008; Thomas *et al.*, 1999a). The foodborne *Campylobacter* infections are mainly sporadic (Ketley, 1997; Miller and Mandrell, 2005; Olson *et al.*, 2008) and are most frequently associated with handling and consumption of poultry meat (Jacobs-Reitsma *et al.*, 2008; Vellinga and Van Loock, 2002).

The fact that the number of persons infected during foodborne *Campylobacter* outbreaks is low in comparison to the number of sporadically infected persons may be due to the limitations in the current surveillance and strain typing systems (Frost, 2001; Wassenaar and Newell, 2000). The foodborne *Campylobacter* outbreaks are typically caused by unpasteurized milk or by undercooked meat (Frost, 2001; Frost *et al.*, 2002). The difficulty in detecting an outbreak may also be attributed to the relatively long incubation period of *Campylobacter* infection (On *et al.*, 2008).

Waterborne outbreaks

The majority of the reported *Campylobacter* outbreaks are waterborne (Frost, 2001) and a waterborne *Campylobacter* outbreak may affect thousands of individuals via the drinking water distribution (Miller and Mandrell, 2005). In contrast to the foodborne outbreaks that include usually a single pathogen, in waterborne outbreaks, multiple infectious agents might be present (Olson *et al.*, 2008) principally since sewage is the most likely contamination source (Maurer and Sturchler, 2000; Vestergaard *et al.*, 2007). *Campylobacter* species, most commonly *C. jejuni*, are the causative agents in waterborne gastrointestinal illness outbreaks all over the world (Miettinen *et al.*, 2005; Said *et al.*, 2003; Schuster *et al.*, 2005). The other etiological agents in the waterborne outbreaks in the developed countries include norovirus, *Cryptosporidium*, *Giardia* and pathogenic *E. coli* (Hrudey and Hrudey, 2007). Waterborne outbreaks caused by rotavirus, *Shigella*, hepatitis A, *Salmonella* and *Toxoplasma* have also been reported, but to a lesser extent (Hrudey and Hrudey, 2007).

Surprisingly, many developed countries do not have an adequate surveillance system for detecting waterborne diseases (NRC, 2004). In Europe, the UK and the Nordic countries including Finland are notable exceptions (Miettinen, 2009). Reported waterborne outbreaks may represent only a fraction of the total that actually occur because many cases are not detected or reported (Neumann *et al.*, 2005). Investigation of a waterborne outbreak involves several steps requiring cooperation from a wide range of parties in the community (Risebro and Hunter, 2007). The small number of waterborne infection cases may be difficult to differentiate from the general background incidence of infections. In practice, a waterborne outbreak is detectable when the infection cases clustered by region and time exceed the normal incidence (Hunter *et al.*, 2003).

The first reported waterborne *Campylobacter* outbreak took place at 1978 in Bennington, Vermont, US (Vogt *et al.*, 1982). Subsequently numerous reports on waterborne *Campylobacter* outbreaks have been published, also in recent years (Table 3.) (for a review of outbreaks occurring in 1978-2000 see Kuusi (2004)). The majority of the *Campylobacter* outbreaks have been linked to non-community water supplies often groundwater supplies without adequate disinfection (Kuusi *et al.*, 2004; Kvitsand and Fiksdal, 2010; Olson *et al.*, 2008). However, disinfection does not necessarily protect against a waterborne outbreak if the contamination occurs in the water distribution system (Miettinen *et al.*, 2006; Richardson *et al.*, 2007). Unusual weather events with heavy precipitation are often associated with the contamination of the distribution system and subsequent cases of illness (Auld *et al.*, 2004; Curriero *et al.*, 2001; Thomas *et al.*, 2006).

Table 3. The characteristics of some reported drinking waterborne *Campylobacter* outbreaks in 1998-2007.

Country (Year)	Water supply (number of consumers)	Microbiological water analysis	Probable cause of the water contamination	Reference
Finland (1998)	Municipal groundwater supply (15 000)	Coliforms and <i>Campylobacter</i> not detected	Sewage flooding during water mains repair work	Kuusi <i>et al.</i> , 2005
Switzerland (1998)	Naturally filtrated groundwater (3 400)	High concentrations of faecal indicators, <i>Campylobacter</i> not analysed	A pump failure producing a spill of sewage into the groundwater	Maurer and Sturchler, 2000
UK (2000)	Community supply of 442 properties	<i>E. coli</i> detected, <i>Campylobacter</i> not analysed	Crack in the wall of a submerged water reservoir and heavy rainfall	Richardson <i>et al.</i> , 2007
Finland (2000)	Municipal groundwater supply (5 500)	<i>C. jejuni</i> and <i>E. coli</i> isolated from large volumes of water	Heavy rainfall together with surface water runoff or soil infiltration	Hänninen <i>et al.</i> , 2003; Kuusi <i>et al.</i> , 2004
France (2000)	Community groundwater supply (2 600)	High concentrations of faecal indicators, but not <i>Campylobacter</i>	Agricultural runoff together with failure in chlorination system	Gallay <i>et al.</i> , 2006
Finland (2001)	Municipal groundwater supply (600-800)	<i>E. coli</i> detected from large volumes, but not <i>Campylobacter</i>	Lake infiltration after a high demand of potable water	Hänninen <i>et al.</i> , 2003
Finland (2001)	Community ground-water supply (18 000)	<i>C. jejuni</i> , <i>C. coli</i> and <i>E. coli</i> detected from large volumes	Surface water runoff by a ditch from a nearby farm	Hänninen <i>et al.</i> , 2003
Norway (2007)	Municipal groundwater supply (3 600)	Coliforms and high colony counts detected, but not <i>Campylobacter</i>	Pressure falls and leakages in the water distribution, possibility of birds gain access to the water reservoir	Jakopanec <i>et al.</i> , 2008
Denmark (2007)	Untreated groundwater (5800)	High concentrations of faecal indicators, <i>Campylobacter</i> not reported	Backflow from the sewage treatment plant into the drinking water system	Vestergaard <i>et al.</i> , 2007

Water may contribute to the burden of sporadic *Campylobacter* infections more than realized, particularly when private wells and surface water is used as drinking water without any treatment (Kapperud *et al.*, 2003; Olson *et al.*, 2008). In addition, bathing in natural waters has been identified as a risk factor of *Campylobacter* infection (Koenraad *et al.*, 1997; Schönberg-Norio *et al.*, 2004). Water also cause *Campylobacter* colonization in farm animals (Jacobs-Reitsma *et al.*, 2008; Korhonen, 1993) and the use of treated water has been reported to protect against transmission *Campylobacter* in broiler flocks (Guerin *et al.*, 2007).

2.3.2 Occurrence and survival in water

The normal habitat of *Campylobacter* is the intestine of warm-blooded animals and birds (Abulreesh *et al.*, 2006; Ketley, 1997). *Campylobacter* are frequently isolated from aquatic environments (Hörman *et al.*, 2004; Jones, 2001; Koenraad *et al.*, 1997; Korhonen, 1993; Savill *et al.*, 2001; Thomas *et al.*, 1999a) and are likely to originate from recent faecal contamination of water by wild birds such as waterfowls, domestic animals, community sewage effluents and agricultural runoffs (Abulreesh *et al.*, 2006; Koenraad *et al.*, 1994; Obiri-Danso and Jones, 1999b).

The sources of contamination and the environmental conditions affect the diversity *Campylobacter* in aquatic environments (Abulreesh *et al.*, 2006). *C. jejuni* is the species identified most frequently from surface waters (Thomas *et al.*, 1999a), and its occurrence is associated with sewage discharges (Bolton *et al.*, 1987). The isolation of other thermotolerant species from aquatic sources, particularly *C. coli* and *C. lari*, is also common (Thomas *et al.*, 1999a), though these associate more with the presence of agricultural runoffs and large flocks of waterfowl rather than with sewage (Bolton *et al.*, 1987; Obiri-Danso and Jones, 1999a).

Mechanisms that enable *Campylobacter* to survive in the environment are poorly understood. The mechanisms evidently differ from those for other enteric bacteria (Murphy *et al.*, 2006). *C. jejuni* enter into a viable but non-culturable (VBNC) state under unfavorable conditions. Under stress, the size of the bacterial cells is often reduced; the shape of cells is transformed from rod to cocci (Jones, 2001; Oliver, 2005; Rollins and Colwell, 1986).

The key factors affecting the survival of *Campylobacter* in aquatic environments include temperature, light, and biotic interactions but also oxygen and nutrient concentrations have an impact (Thomas *et al.*, 1999a). The survival of *Campylobacter* is favoured by a low temperature, absence of sunlight and low numbers of autochthonous microbiota but there are differences in the survival capacity between various *Campylobacter* species (Korhonen and Martikainen, 1991a; Korhonen and Martikainen, 1991b; Obiri-Danso and Jones, 1999b; Thomas *et al.*, 1999b). It has been reported that *C. lari* can survive better in aquatic environments than *C. jejuni* and *C. coli* (Obiri-Danso *et al.*, 2001).

The viability of *Campylobacter* in the distribution systems is favoured by biofilms (Svensson *et al.*, 2008) and by free-living amoebae harboring bacteria intracellularly (Snelling *et al.*, 2005b). The presence of protozoan grazers such as *Acanthamoeba* spp. has been reported to increase the survival of *C. jejuni* in water (Axelsson-Olsson *et al.*, 2005) and it is known that the presence of this protozoan organism allows proliferation and protection of the bacteria from disinfection (Harb *et al.*, 2000; Winiacka-Krusnell and Linder, 1999).

2.3.3 Detection methods

Campylobacter species are fastidious bacteria that are oxygen sensitive and are grown in a microaerobic atmosphere containing 3-10 % (v/v) oxygen and 5-10 % (v/v) carbon dioxide (Kelly, 2001). An early *Campylobacter* isolation method from faeces was based on the small size of the *Campylobacter* cells (Butzler, 2004). In this differential filtration technique, *Campylobacter* were separated from other faecal bacteria utilizing a 0.65 µm pore-size membrane filter through which faecal suspension including the *Campylobacter* cells passed while other bacteria were retained on the filter (Skirrow, 1977). Subsequently, more selective isolation media obviated the need for differential filtration (Bolton *et al.*, 1984; Karmali *et al.*, 1986). Even today, modifications of the filtration technique, often exploiting *Campylobacter* motility (Goossens *et al.*, 1986), are employed in the detection of *Campylobacter* species other than *C. jejuni* and *C. coli* (Butzler, 2004).

For diagnosis of *Campylobacter* enteritis, microscopic examination of fresh faeces, antigen tests and PCR methods are available. The isolation of *C. jejuni* and *C. coli* from faeces is relatively easily conducted by employing microaerobic incubation for at least 48 h at 42 °C after plating on selective media (Butzler, 2004). For detection of non-*jejuni*, non-*coli* *Campylobacter* species, method refinements such as special atmospheric and temperature conditions, prolonged incubation or the use of cultivation media with modified antibiotic contents or the use of non-selective media, are required (Lastovica and Allos, 2008).

The media used for the isolation of *Campylobacter* from food and water are derived from the abovementioned media originally designed for *Campylobacter* detection from human stool samples (Jacobs-Reitsma *et al.*, 2008). However, the direct plating on the solid media used for faecal samples that usually contain a large number of viable *Campylobacter* cells, is often unsuitable for the recovery of low numbers of *Campylobacter* present in environmental samples (Rollins and Colwell, 1986). There are several liquid enrichment media available to be used before plating on solid selective media to promote the recovery of low numbers of *Campylobacter* cells that might be damaged by various environmental stresses and thus have a low cultivability (Jacobs-Reitsma *et al.*, 2008). The selective agents most often used include cefoperazone, amphotericin B, trimethoprim and vancomycin, and the media also contain sterile sheep's or horse's blood, or charcoal to neutralize the toxic effects of oxygen and light (Jacobs-Reitsma *et al.*, 2008). During enrichment, a lower incubation

temperature (37 °C) maybe used, but the isolation on a solid medium is usually performed at 41.5 °C.

The international standard method (ISO 17995, 2005) for *Campylobacter* detection from water entails the use of Bolton and Preston enrichment broths and plating on modified charcoal cefoperazone desoxycholate agar (mCCDA) after enrichment. According to the standard procedure, the water sample, preferably of several liters, is concentrated through a membrane filter and the filter is placed into the enrichment broth. According to the standard, a 0.45 µm pore-size of membrane filter is employed, but also membranes with a 0.20 µm pore-size can be utilized in *Campylobacter* analysis from water (SCA, 2002). After static incubation at 37 °C for 48 h, the mCCDA plates are incubated at 41.5 °C for 48 h. Accordingly, it takes a total of 4 days to confirm a negative *Campylobacter* result.

In case of a presumptive positive result, further confirmation is required that may require several days. For confirmation of the isolates, at least typical colony morphology, absence of aerobic growth, Gram-negativity, motility and oxidase-positivity are required (ISO 17995, 2005). Additional biochemical tests such as hippurate hydrolysis test for differentiation of *C. jejuni* may be performed, but the test results often are confusing (Debruyne *et al.*, 2008). In recent years, a wide range of nucleic acid-based methods, particularly PCR methods, have become available, to be used mainly as additional qualitative confirmation tests. PCR methods targeted to *hipO* gene may provide a more accurate differentiation between *C. jejuni* and *C. coli* than the phenotypic hippurate test (Abu-Halaweh *et al.*, 2005; Abulreesh *et al.*, 2006; Jensen *et al.*, 2005).

Molecular biology techniques provide new opportunities for microbial detection. The reliance on the conventional cultivation detection alone for *Campylobacter* detection from water is not practical, since VBNC *Campylobacter* cells do occur (Abulreesh *et al.*, 2006; Tholozan *et al.*, 1999). Various molecular *Campylobacter* detection methods are available, most of which are based on PCR technology with or without real-time application though they usually require a prior enrichment step (Hernandez *et al.*, 1995; Jacobs-Reitsma *et al.*, 2008; Moore *et al.*, 2001; Nam *et al.*, 2005; Sails *et al.*, 2003; Waage *et al.*, 1999). However, there have been rapid developments in sample preparation techniques (cell concentration and DNA purification) and recently, also applications of direct quantitative realtime PCR detection from water samples have been published (Van Dyke *et al.*, 2010). In addition to the culturable cells, the methods without any enrichment steps might also detect viable but not culturable cells (VBNC) but theoretically also dead cells and this difference must be clarified before these new techniques can replace the conventional cultivation methods (Birch *et al.*, 2001; Nogva *et al.*, 2000).

Molecular primers and probes are often designed to detect simultaneously the thermotolerant *Campylobacter* species, *C. jejuni*, *C. coli* and *C. lari* and often also *C. upsaliensis* by a single assay. The published *Campylobacter* specific primers and probes have been targeted to 16S rRNA (Bang *et al.*, 2002; Josefsen *et al.*, 2004;

Moreno *et al.*, 2003) or 23S rRNA (Fermer and Engvall, 1999) ribosomal genes, or to flagellin genes *flaA* and *flab* (Moore *et al.*, 2001; Waage *et al.*, 1999). In addition, applications of the use of chromosomal *glyA* (Jensen *et al.*, 2005), *gafF* and *ceuE* (Nayak *et al.*, 2005) genes and primers towards ORF-C target sequence (Sails *et al.*, 2003) have been reported. In addition to PCR methods, also nucleic acid sequence-based amplification (NASBA) assays (Cook, 2003; Cools *et al.*, 2006) and the fluorescent in situ hybridization (FISH) techniques towards ribosomal sequences (Buswell *et al.*, 1998; Lehtola *et al.*, 2005; Moreno *et al.*, 2003) have been exploited for *Campylobacter* detection.

2.3.4 Typing methods

After the *Campylobacter* detection, there are several techniques for strain typing. The subtyping methods are applied to study *Campylobacter* epidemiology and can be employed in several ways e.g. species identification, studies of transmission routes and source tracking in outbreak investigations (On *et al.*, 2008). The subtyping methods include phenotyping methods, serotyping, biotyping, phage typing, and a number of genotyping methods, such as pulsed-field gel electrophoresis (Butzler, 2004). All these currently available strain typing techniques require a culturable isolate: only minor genotypic information is achievable from sequencing of the PCR-products of direct molecular detection. The use of serotyping has decreased, and today the molecular typing methods are employed in many laboratories (On *et al.*, 2008; Wassenaar and Newell, 2000).

The typing of *Campylobacter* flagellin genes *flaA* and *flaB* has been used in outbreak investigations (Clark *et al.*, 2003), employing several techniques based on either restriction fragment length polymorphism (*fla*-RFLP) (Wassenaar and Newell, 2000) or direct sequencing (On *et al.*, 2008). In the comparison of large numbers of strains, irrespective of their geographical or chronological origin, pulsed-field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP) and multilocus sequence typing analysis techniques can be employed (On *et al.*, 2008).

PFGE is a technically demanding method, consisting of digestion of the whole genome with rare-cutting restriction enzymes and separation of fragments in a coordinated pulsed electric field (Wassenaar and Newell, 2000). PFGE is currently performed worldwide according to the PulseNet protocol and has exhibited a high discriminatory power (On *et al.*, 2008). In AFLP profiling, the digestion of DNA is followed by oligonucleotide adaptor ligation, PCR amplification with a labeled primer, and finally electrophoresis (Wassenaar and Newell, 2000). Multilocus sequence typing involves sequencing of fragments of many household genes and it enables worldwide data comparisons (On *et al.*, 2008).

2.4 Microbial tracking of the faecal contamination source

As public health practitioners focus on preventing the contamination of water supplies (NRC, 2004), it is clear that the best way to avoid future waterborne outbreaks involves learning from past mistakes (Hrudey and Hrudey, 2007). During a waterborne outbreak investigation, the cause of the contamination needs to be identified, before it can be eliminated (Tallon *et al.*, 2005). Outbreak investigations include spatial and temporal analysis of the events, technical investigations and usually also an epidemiological study and microbiological strain typing in order to establish the common source of the strains isolated from patients and from the contaminated water (Miller and Mandrell, 2005). In conjunction with the other investigations, microbial source tracking (MST) of the faecal contamination source in water may take place.

Waters contaminated with human faeces are generally regarded as a greater risk to human health than waters contaminated with faeces of animal origin (Scott *et al.*, 2002). Initially, the concept of indicator density relationship, namely faecal coliforms:faecal streptococci ratio (FC/FS) was developed to differentiate between human and animal sources (Geldreich and Kenner, 1969) and though this ratio was employed it met with little success (Godfree *et al.*, 1997). Another transformation of indicator bacteria results, the use of the atypical:typical coliform ratio (AC/TC) has been proposed as a way to estimate the amount of time that faecal material has been in the environment (Black *et al.*, 2007).

In previous years, MST studies have focused on genotypic bacterial source typing methods. With DNA fingerprinting patterns, these MST studies have been utilized to more accurately achieve the goal of differentiation between human and non-human sources (Tallon *et al.*, 2005). MST is usually library dependent and the analysis of a large collection of micro-organisms from different sources is required before the method can be put into practice (McLellan *et al.*, 2003; Simpson *et al.*, 2002).

Various methods have been exploited for source tracking of faecal bacteria, primarily *E. coli* isolates, in water. These include ribotyping, pulsed-field gel electrophoresis (PFGE), denaturing-gradient gel electrophoresis, repetitive DNA sequences (rep-PCR); methods employing repetitive extragenic palindromic (REP) sequences, enterobacterial repetitive intergenic consensus (ERIC) sequences, or BOX element, and amplified fragment length polymorphism (AFLP), host-specific 16S rDNA genetic markers, analysis of the sequences of the 16S rRNA genes and multiple-antibiotic-resistance (MAR) profiles (Carson *et al.*, 2003; Dombek *et al.*, 2000; Guan *et al.*, 2002; Leung *et al.*, 2004; Meays *et al.*, 2004; Simpson *et al.*, 2002).

Currently, an increasing number of MST studies involve host-specific molecular markers such as human-specific 16S rRNA *Bacteroidetes* gene markers, specific detection of *Bacteroides* and *Bifidobacterium* species and host-specific mitochondrial DNA analysed by quantitative realtime PCR or by conventional multiplex or nested PCR methods (Ahmed *et al.*, 2009; Ballesté *et al.*, 2010; Reischer *et al.*, 2007; Silkie

and Nelson, 2009). Also the subgroups of F-specific RNA phages (Muniesa *et al.*, 2009) and phages infecting selected strains of *Bacteroides* (Payan *et al.*, 2005) have been explored and favoured partly due to the less expensive nature of their analytical methods as ways to identify the origin of faecal pollution. It is however noteworthy that on its own, a single MST indicator is not able to determine the source of faecal pollution or to differentiate between human and non-human sources (Blanch *et al.*, 2006).

3 Aims of the study

The overall goal of the study was to provide better tools to improve public health protection by studying detection methods for waterborne pathogens and indicator bacteria. The rationale for investigating *Campylobacter* detection from drinking water came from the outbreak surveillance system data indicating that in many epidemiologically confirmed waterborne *Campylobacter* outbreaks, there had been failures in the detection and isolation of *Campylobacter* from water samples. Moreover, hazards of water safety were studied at small community water supplies that have been associated with waterborne outbreaks. During the study, a set of microbiological cultivation and molecular methods were compared for their abilities to detect waterborne health risks and then they were also utilized to identify the contamination source. The specific aims in the study were:

1. to identify the most important hazards compromising microbial drinking water safety at small and medium scale community water supplies in central Finland,
2. to validate detection and source tracking methods of faecal contamination during contamination of a communal drinking water distribution system and to evaluate if the water contamination was associated with cases of gastrointestinal illness in the community,
3. to compare performance of alternative cultivation methods on the enumeration of *Escherichia coli* and coliform bacteria from non-disinfected water with values obtained with a reference method,
4. to study the performance of a novel portable microbial enrichment device and real-time PCR detection coupled with restriction fragment analysis on fast and species-specific *Campylobacter* detection from a water sample,
5. to evaluate the survival of *Campylobacter jejuni* in drinking water biofilms based either on microbial cultivation or on a fluorescent *in situ* hybridization technique, which detects also viable but not cultivable cells.

4 Materials and methods

4.1 Bacterial isolates and their cultivation for challenge studies (III, IV, V)

For equivalence studies of *E. coli* and coliform bacteria media (III), pure cultures of *E. coli* (strain ATCC 8739) and *Enterobacter aerogenes* (strain ATCC 13048^T) were used for spiking according to the manufacturers' (EZ-FPC™ Microorganisms, Microbiologics Inc., St. Cloud, MN 56303, USA) instructions. As positive and negative controls in the confirmation tests, the strains of *E. coli* (ATCC 8739), *Enterobacter cloacae* (ATCC 13047^T), *Enterococcus faecium* (ATCC 35667) and *Pseudomonas fluorescens* (ATCC 49642) were used. The strains were cultivated aerobically at 37±1 °C on tryptone soya agar (TSA), except for *P. fluorescens* which was incubated at 22 °C.

In *Campylobacter* detection studies (IV and V), two strains were used during the tests: the environmental strain of *Campylobacter jejuni* isolated in 2004 from water associated with fecal contamination of a municipal drinking water system (II), and the environmental strain of *Campylobacter coli* isolated from eastern Finland in 1987 (Laboratory of Environmental Microbiology, National Public Health Institute, Kuopio, Finland). The strains were stored in nutrient broth containing 15% glycerol at -70 °C or lower. *Campylobacter* strains were cultivated under microaerobic conditions (CampyGen; Oxoid, Basingstoke, UK) on mCCDA medium (Oxoid) at 41.5 °C. In the experiments, colonies were transferred from the medium to a tube with a sterile 10 µm loop, weighed, and then suspended in sterile deionized water. The absorbance of the suspension at 420 nm was adjusted to a predetermined value to ensure reproducibility between the experiments. The CFU of the suspension was counted on mCCDA medium.

In *Campylobacter* PCR, *Campylobacter jejuni* (ATCC 33291) was used as a positive control (II, IV, V). In the DNA fingerprinting of coliform bacteria, *Escherichia coli* (ATCC 8739) and *Enterobacter cloacae* (ATCC 13047) were employed as control strains (II).

4.2 Microbiological analysis (I-V)

In the analysis of indicator bacteria and bacterial pathogens, water samples were concentrated by filtering them through a 0.45 µm, 47 mm, mixed cellulose ester membrane filter (Millipore Corporation, Bedford, USA) (I-V). The majority of analyses were performed in the Laboratory of Environmental Microbiology, Department of Environmental Health, National Public Health Institute (KTL), in Kuopio, Finland. In addition, in study I, Department of Environmental Sciences,

University of Kuopio and Finnish Food Safety Authority, Kuopio Research Unit participated and in study II, Savolab, Mikkeli, Finland was involved.

4.2.1 Enumeration of *Escherichia coli* and coliform bacteria (I-III)

Escherichia coli and coliform bacteria were analysed with the membrane filtration method using Chromocult® Coliform Agar (CC) (Merck, Darmstadt, Germany) (I-III). For a part of the samples, also the international standard method ISO 9308-1 (LTTC) (2000) and the MPN method Colilert®-18 with 51-well Quanti-tray® (Colilert) (IDEXX Laboratories, Inc., Maine, USA) (in studies I and III) or membrane filtration with LES Endo and mFC media (SFS 3016, 2001; SFS 4088, 2001) (in study II) were used. All plates and trays were counted after 21 ± 3 h incubation at 36 ± 2 °C (except mFC medium at 44.0 ± 0.5 °C). For the groundwater samples, the incubation time was extended to 44 ± 4 h for plates and trays that did not show any typical growth after 21 ± 3 h as stated in the note of the reference method (ISO 9308-1, 2000) (I,III).

The presumptive coliform colonies were confirmed and all or at least ten colonies or wells from each plate and tray were chosen for confirmation. The selected colonies were subcultivated on Tryptone Soy Agar (TSA, Oxoid Ltd, Basingstoke, Hampshire, England) and incubated overnight at 37 ± 1 °C. From the selected Colilert/Quantitray-wells, a drop of each well's contents was subcultivated on CC before subculturing on TSA. The oxidase test was performed for all selected colonies. All oxidase negative cultures were tested for production of indole and gas formation in Lactose Tryptone Lauryl Sulphate Broth at 44.0 – 44.5 °C (LTL SB, Oxoid Ltd, Basingstoke, Hampshire, England). Furthermore, gram staining of the cultures was performed as an additional confirmation and the species identification of a part of the coliform isolates was performed by API 20 E test kit (Biomérieux, Marcy l'Etoile, France).

Oxidase negative cultures were considered to be confirmed coliform bacteria. For confirmed *E. coli* counts, in addition to oxidase negativity, a positive reaction in the indole test was required (I-III).

In study III, some of the conventionally confirmed (indole, gas) *E. coli* cultures exhibited a non-typical *E. coli* appearance on chromogenic or fluorogenic medium, and these isolates were taken for additional analyses. At first, the presence of *lacZ* and *uidA* genes specific for coliform bacteria and *E. coli* was tested by the PCR method (Bej *et al.*, 1991; Fricker *et al.*, 1994). In addition, partial 16S rDNA gene sequences were produced and similarity searches of the sequences against a bacteria database were performed using blastn (NCBI, <http://www.ncbi.nlm.nih.gov/blast/>).

4.2.2 Detection of *Campylobacter* spp. (I, II, IV, V)

The cultivation and identification of thermophilic campylobacteria was performed following the principles of the draft standard ISO/DIS 17995:2003 which was

finalized and published later (ISO 17995, 2005) (I, II, IV, V). Briefly, the water samples were membrane filtered and enriched in microaerobic conditions (Campygen, Oxoid) in Bolton and/or Preston broths before plating on mCCDA medium (Oxoid, Hampshire, UK) (I, IV,V). Alternatively, half of a sample was concentrated on a filter which was enriched for 48 h in complete Bolton broth (LabM, Lancashire, UK) and the other half was concentrated on a filter which was initially enriched in Bolton broth for 4–12 h before the addition of the selective supplement (II). In the case of typical *Campylobacter* growth on mCCDA medium, the cultures were confirmed to be campylobacteria by gram staining and testing for motility, aerobic growth, oxidase, catalase and hippurate hydrolysis.

The species identification of *Campylobacter* isolates was done by polymerase chain reaction – restriction enzyme analysis, PCR–REA (Engvall *et al.*, 2002; Fermer and Engvall, 1999). The method differentiates between *C. jejuni*, *C. coli*, *C. upsaliensis* and *C. lari* with the primers THERM1 (Eyers *et al.*, 1993) and THERM4 (Fermer and Engvall, 1999) targeting the 23S rRNA gene of thermophilic campylobacteria using the restriction enzyme, *AluI* (New England Biolabs Inc., Beverly, Massachusetts).

PCR detection after enrichment (II,IV,V)

In addition to plate counts, conventional PCR detection (II,V) and quantitative real-time PCR detection and enumeration (IV) were performed to detect *Campylobacter* after enrichment. The DNA from the enrichment broths was extracted from 1 mL subsamples employing Microbial DNA isolation kit (UltraClean, Mo Bio Laboratories, Inc., Carlsbad, California). The PCR protocol was the same as used for species identification of isolates.

The conventional PCR was conducted using thermal cyclers T1 and Tgradient (Biometra, Goettingen, Germany). A quantitative real-time PCR method was build employing the hot start DyNAmo HS SYBR Green qPCR kit and the Rotorgene 3000 (Corbett Research, Sydney, Australia) with the melting point analysis. The quantification of the real-time PCR detection was done with Rotor-Gene 6 software (Corbett Research) by comparing the results from the samples to a standard curve made from serial dilutions of DNA extracted from a known concentration of *C. jejuni* ATCC 33291. In conventional PCR, and in real-time PCR in case of an unspecific melting peak, the correct size (491 bp) and purity of the PCR product were verified using agarose gel electrophoresis.

Fluorescent in situ hybridization (FISH) (V)

For the FISH analyses (V), 10mL sonicated biofilm suspension and 50mL outlet water were filtered (0.2mm, Anodisc, Whatman). Bacteria on the filter were hybridised with CJE195 PNA as described by Lehtola *et al.* (2005). The numbers of hybridised bacteria were counted by epifluorescence microscopy (Olympus BX51, Japan).

4.2.3 Microbial source tracking (II)

The microbiological proof of the water as a cause of the *Campylobacter* infections associated with the contamination case of a municipal drinking water distribution system (II) was sought utilizing microbial source tracking (MST).

The similarity of the cultivated *C. jejuni* isolates from patients, drinking water samples and from the suspected contamination source was analysed from the pulsed-field gel electrophoresis (PFGE) patterns according to the PulseNet standardized protocol (www.cdc.gov/pulsenet/protocols.htm).

Moreover, a genomic fingerprinting method, rep-PCR (Rademaker *et al.*, 1998), was employed to study the similarity of a total of 47 *E. coli* isolates and nine other coliform bacteria isolates from tap water sampling points taken during the drinking water contamination. Repetitive BOX and ERIC elements were amplified employing two single primers, BOXA1R (Versalovic *et al.*, 1994) and ERIC2 (Versalovic *et al.*, 1991), respectively. The cell suspensions from colonies grown on TSA stored at -20 °C in sterile distilled water prior to the analysis were used as BOX-PCR and ERIC-PCR templates. After PCR amplification, the PCR products were electrophoresed in 2% agarose gels containing ethidiumbromide, then visualized and preserved digitally. The DNA fingerprint patterns were compared using Bionumerics 4.1 software (Applied Maths, Sint-Martens-Latem, Belgium).

4.2.4 Other microbiological water analyses (I,II,V)

Intestinal enterococci were determined (I,II) on Slanetz & Bartley medium (Oxoid, Basingstoke, UK) using the standard method ISO 7899-2 (2000) and in cases when no visible colonies were detected after 48 hours of incubation, the incubation was continued up to 72 hours.

Clostridium perfringens was tested (in study I) on mCP medium (Oxoid, Hampshire, UK) according to European Union Drinking Water Directive (European Union, 1998).

The heterotrophic plate count (HPC) was determined (I,II,V) at 22 °C by spread-plating on R2A medium (Difco, Sparks, Md., U.S.A.) (Reasoner and Geldreich, 1985) according to the standard methods for heterotrophic plate count, 9215 (APHA *et al.*, 1998). Additionally HPC was analysed using yeast extract agar (YEA, Oxoid, Basingstoke, UK or LabM, Lancashire, UK) with the pour-plate technique according to the standard ISO 6222 (1999) (II) and by using membrane filtration of 1, 10, 100 and 1000 ml (I). Total cell counts were counted with an Olympus BH-2 epifluorescence microscope (Olympus Optical Co., Tokyo, Japan) as acridine orange direct counts (AODC) (studies I and V) (Hobbie *et al.*, 1977) or by DAPI staining (V) (Porter and Feig, 1980).

The DNA-coliphages and male-specific coliphages were determined (I) from 3 x 500 ml water samples using the EPA two-step enrichment procedure (EPA, 2001) with hosts *E. coli* ATCC 13706 and ATCC 700609 for somatic phages as well as ATCC 15597 and ATCC 700891 for male-specific coliphages. Nalidixic acid was used with the host ATCC 700609 and if the host ATCC 700891 was used, ampicillin was added.

Noroviruses were determined (I,II) from a water sample that was concentrated without prefiltration using a positively charged membrane and then the filters were eluted using beef extract solution (Gilgen *et al.*, 1997; Kukkula *et al.*, 1999). RNA was extracted from microconcentrated eluates, and noroviruses were analysed using the primers and a reverse transcriptase (RT)-PCR protocol as described by Vinjé and Koopmans (1996) and Kukkula *et al.* (1999) (I).

The determination of enterohaemorrhagic *E. coli* (EHEC) was achieved (I) by incubating the water sample concentrate on a filter overnight in 50 ml of MTSB with 20 mg/l novobiocin (Lab M). A loopful of the enrichment broth was transferred onto SMAC agar (Oxoid) and incubated overnight at 37 °C. From the area of confluent growth, a loopful of bacteria was suspended into sterile water to yield a density of one MacFarland. Two µl of this suspension was used as a template in the PCR to detect *stx1* and *stx2* genes (Paton and Paton, 2002). In addition to the enrichment method, *E. coli* isolates from analyses of *E. coli* and coliform bacteria (in the study I) were tested accordingly.

For detection of *Listeria* spp. (I), the filter was incubated in 50 ml of UVM I enrichment broth (Oxoid) at 30 °C for 24 h. Then 0.5 ml of broth was transferred into 50 ml of UVM II broth and incubated at 30 °C for 24 h. Finally 0.2 ml of this broth was transferred to Palcam (Oxoid) and blood agar plates and incubated in 5% CO₂ at 37 °C for 48 h.

For detection of *Yersinia* spp. (I), the filter was incubated in 50 ml of tryptone soya (TS) broth at 20 °C for 18 h. *Yersinia* were detected in TS broth by PCR, direct cultivation and cold enrichment methods. DNA was isolated from 0.1 ml of TS broth using Instagene (Biorad, California, USA) and analysed for *Yersinia* by *yadA* (Kapperud *et al.*, 1993) and *ail* PCRs (Lambertz *et al.*, 1996). A total of 10 µl of TS broth was streaked directly to CIN agar (Oxoid) plate and incubated at 30 °C for 18 h. In the cold enrichment, TS-broth was diluted 1:10 in TS broth and kept at 4 °C for 21 days and then 10 µl of broth was plated onto a CIN agar plate and incubated at 30 °C for 18 h.

Salmonella was determined (I) using RVS enrichment method NMKL 71 (1999). The filter was incubated in 50 ml BPW broth (Oxoid, Hampshire, UK) at 37°C for 18 h. One ml of BPW was transferred into 9 ml of RVS broth (Oxoid), and incubated at 41.5 °C for 18 h. Ten µl of RVS broth was streaked to XLD (LabM, Lancashire, UK) and Önöz (Merck, Darmstadt, Germany) agar plates and incubated at 37°C for 18 h before the possible serological analyses.

4.3 Physico-chemical water analysis (I, III, IV)

During the groundwater sampling (I), portable equipment using pH 340 and L F-30 probes (WTW, Weilheim, Germany) were used to analyse the temperature, pH and electrical conductance of the sampled waters. Nitrate, nitrite, chloride and sulphate were measured with standard methods (ISO 10304-1, 1992) as well as the chemical oxygen demand (SFS 3036, 1981). The molecular size distribution of natural organic matter (humic fractions) was analysed using a high-pressure size exclusion chromatography method (HPSEC) (Vartiainen *et al.*, 1987).

The nutrient concentrations of the groundwater samples (I) and of the inlet water of biofilm reactor (V) were analysed. Total phosphorus was measured using a spectrophotometric ascorbic acid method (SFS-EN 1189, 1997) and total non-purgeable organic carbon was assayed by a high temperature combustion method with a Shimadzu 5000 TOC analyser (Kyoto, Japan) (SFS-EN 1484, 1997).

Inlet water of biofilm reactor (V) was studied also for microbially available nutrients. Microbially available phosphorus (MAP) was analysed by a bioassay (Lehtola *et al.*, 1999). Assimilable organic carbon (AOC) was analysed (van der Kooij *et al.*, 1982) by a modified method (Miettinen *et al.*, 1999). The modification included addition of inorganic nutrients, i.e. AOC was measured as AOC_{potential} (Miettinen *et al.*, 1999).

Free chlorine residual in water was analysed (in studies IV and V) with Palintest® Micro 1000 Chlorometer (England).

4.4 Experimental set-up and samples

4.4.1 Small community water supplies (I)

Twenty small and medium scale waterworks in northern Savo, Central Finland were sampled in autumn 2002, spring 2003, autumn 2003 and spring 2004 (Table 4.). The four consecutive sampling rounds were concentrated in seasons when it was believed that the risk of contamination of the groundwater would be at its highest. The selected study sites supplied drinking water for a relatively small number of consumers and many of these suppliers did not employ professional personnel to monitor the safety of their drinking water.

A sample was 40 liters of water was taken into sterile bottles and plastic containers from raw water, before any treatment (potential pH adjustment and disinfection). After the sampling point, the distributed water of eight out of 20 suppliers was disinfected (at seven waterworks with UV and at one waterworks with chlorine, Table 4.). The other 12 waterworks out of 20 did not utilize disinfection or any other water treatment except for pH adjustment after the sampling point. The water

samples were kept in a cool box and transported immediately to the laboratory, where the microbiological analyses were started on the same day or no more than 24 hours after sampling. Water samples for physico-chemical analyses were frozen at -20 °C and analysed within the next three months.

Table 4. The groundwater supplies included in the study.

Water supply	Number of persons served ¹	Availability of water (m ³ /d)	Ownership ²	Disinfection after the sampling point
1	120	100	CO	-
2	4500	3000	MU	UV
3	350	250	CO	-
4	140	5900	CO	-
5	70	50	CO	-
6	150	250	CO	-
7	220	110	CO	Cl ₂
8	22800	17000	MU	-
9	450	500	MU	UV
10	3000	950	MU	-
11	5400	900	CO	-
12	850	600	MU	-
13	2450	800	MU	-
14	3220	5700	MU	UV
15	2970	5300	MU	UV
16	50	300	CO	-
17	770	1800	MU	UV
18	2430	2200	MU	UV
19	430	1300	MU	UV
20	90	50	CO	-

¹ In two cases only the sum of the two water supply plants (water supplies 14-15 and water supplies 17-18) was known and the number/water supply was estimated from the amount of the produced water.

² CO = water supply administered by local water cooperative, MU = municipal water supply plant.

The counts of faecal indicator bacteria (*Escherichia coli*, intestinal enterococci, *Clostridium perfringens*) were analysed from 100 ml and 1000 ml water samples of small community water supplies (I). Also the other indicator microbes (coliform bacteria, heterotrophic plate count, total cell count, DNA coliphages and male specific coliphages) were measured. In addition, the presence of selected pathogenic microbes (noroviruses, thermotolerant *Campylobacter* spp., enterohaemorrhagic *E. coli* (EHEC), *Listeria* spp., *Yersinia* spp. and *Salmonella* spp.) was tested from those

groundwater samples. The analysed sample volume was 3000 ml for *Campylobacter* and 1000 ml for the other pathogens.

On-site hazard identification was conducted at each water supply by visiting the plant in conjunction with the samplings together with the person in charge of the water supply maintenance. A questionnaire form consisting of 25 questions with multiple-choice answers was completed. The on-site hazard identification was supplemented with a review of related background material such as the maps of the sites, previous compliance monitoring results and evaluation of the overall state of the construction and maintenance of the water works.

The sum of risk estimate values was calculated for each water supply from numerical risk estimate values that were related to the probability of the hazard occurrence and to the severity of water safety reduction. The selected principal hazards included: topography near the water supply well, possibility for surface water runoffs or uncontrolled river or lake bank filtration, occurrence of sand and gravel mining sites, roads, agricultural activities, sewerage, inhabitation with sewage treatment activities or ditches nearby, and an insufficient depth of protective layers above the water table.

4.4.2 Investigation of the contaminated drinking water system (II)

Contamination of drinking water occurred in a rural municipality with a population of 5,100 in eastern Finland in August 2004 (II). The drinking water used by the community was non-disinfected ground water distributed by the local municipal waterworks. Before distribution, the pH was adjusted and, if necessary, the water was stored in a large storage tower.

Faecal contamination was detected in routine water quality monitoring samples taken on the previous day. Chlorination of water was initiated, extended water sampling was started and a boil-water notice was issued to consumers as soon as possible, all within 24 hours after the detection of contamination. Several sampling points including the raw water at the waterworks, water in the storage tower and four tap water points along the distribution line were sampled. In addition, the analyses were performed from a sample taken from the suspected contamination source: the well which collected rainwater from the roof of the water storage tower.

During this faecal contamination case of a municipal drinking water distribution system, *Escherichia coli*, coliform bacteria and heterotrophic bacteria counts were analysed in the local laboratory, using their routine methods. After the faecal contamination was detected, the testing for thermophilic campylobacteria, *Salmonella*, noroviruses and intestinal enterococci was also performed.

Patients with microbiologically confirmed *Campylobacter* infection who lived in the community where the drinking water was contaminated and who had experienced symptoms between 31 July and 20 August 2004 were contacted by phone and

interviewed. The onset date, description and duration of symptoms of *Campylobacter* infection were determined and the exposure including the other potential sources of *Campylobacter* infection was assessed for a 2-week period before the infection. Stool specimens from the patients were analysed for bacterial pathogens and the patient isolates were stored at -70 °C.

4.4.3 Equivalence testing of *E. coli* and coliform bacteria methods (III)

Non-disinfected water samples were collected for equivalence testing purposes (III). The samples contained naturally *E. coli* and/or coliform bacteria or were artificially contaminated after sampling (spiked samples). A total of 110 samples included 41 ground water samples from small community water supplies, 25 bathing water samples from eight freshwater bathing areas in North Savo, 24 spiked sterile deionized water samples, 13 spiked tap water samples and seven spiked well water samples. Sterile deionized water and unchlorinated tap water were spiked with the quantitative bacterial pellets of the coliform strains. Spiking of well water samples was done with effluent from the wastewater treatment plant of the city of Kuopio, Finland.

For equivalence testing of *E. coli* and coliform bacteria methods, membrane filtration method ISO 9308-1 (2000) based on lactose fermentation on LTTC agar (LTTC) (Tergitol-7 Agar, including 0.125% TTC Solution, Oxoid Ltd, Basingstoke, Hampshire, England) was used as the reference method. The alternative media tested were:

- 1) LES Endo Agar (LES Endo) (Merck KgaA, Darmstadt, Germany), the Finnish national standard method SFS 3016 (SFS 3016, 2001) and,
- 2) the MPN method Colilert®-18 with 51-well Quanti-tray® (Colilert) (IDEXX Laboratories, Inc., Maine, USA),
- 3) Chromocult® COLIFORM Agar (CC) (Merck KgaA, Darmstadt, Germany),
- 4) Harlequin™ *E. coli*/Coliform Medium (HECM) (LAB M™, International Diagnostics Group plc, Bury, Lancashire, UK) and
- 5) Chromogenic *E. coli*/coliform medium (CECM) (Oxoid Ltd, Basingstoke, Hampshire, England).

The components of the compared solid media are presented in Table 5. The components of the MPN method were not known.

The membrane filtration, incubation and confirmations were performed as described in 4.2. The membrane filtered sample volumes were 10, 50, 100 or 1000 ml. Colilert analyses were performed with 100 ml samples: sterile deionized water was added to 10 and 50 ml samples until a volume of 100 ml was reached.

Table 5. The contents of compared agar plates (g/l).

LTTC		LES Endo		CC		HECM		CECM	
Peptone	10,0	Peptone		Peptones	3,0	Tryptone	20,0	Peptone	5,0
Meat extract	5,0	from meat	3,7						
		Casein							
		hydrolysate	3,7						
		Tryptose	7,5						
Yeast extract	6,0	Yeast extract	1,2					Yeast extract	3,0
Lactose	20,0	Lactose	9,4	Sorbitol	1,0			Lactose	2,5
		K ₂ HPO ₄	3,3	Na ₂ HPO ₄	2,2			K ₂ HPO ₄	3,5
		KH ₂ PO ₄	1,0	NaH ₂ PO ₄	2,7			KH ₂ PO ₄	1,5
		NaCl	3,7	NaCl	5,0			NaCl	5,0
				Sodium pyruvate	1,0				
Tergitol -7	0,01	Sodium deoxy-cholate	0,1	Tergitol-7	0,15	Bile Salts No. 3	1,5		
TTC Solution	0,125%	Na-Laurylsulfate	0,05						
Bromo-thymol blue	0,05	Para-rosaniline (fuchsin)	0,8	Chromo-genic mixture	0,4	X-glucuronide	0,075	Chromo-genic mix	20,3
						Magenta-gal	0,1	Neutral red	0,03
Agar	13,0	Agar	15,0	Agar	10,0	Agar	15,0	Agar	15,0

CC = Chromocult Coliform agar, HECM = Harlequin *E. coli*/coliform medium, CECM = Chromogenic *E. coli*/coliform medium.

Typical and atypical colonies on LTTC and LES Endo agar plates were counted separately. On LTTC agar plates yellow colonies with halo were considered as typical and red colonies as atypical growth. On LES Endo agar plates red colonies with metallic sheen were considered as typical and red colonies without any sheen as being atypical. On chromogenic media (Colilert, CC, HECM and CECM) *E. coli* and other coliforms were counted separately according to the manufacturer's instructions. The amount of background growth was estimated from all media. Colony forming units (CFU) per analyzed sample volume on LTTC and LES Endo were counted after confirmation. From the chromogenic and fluorogenic media, both unconfirmed and confirmed counts were counted. With Colilert, counts were finally converted to MPN results.

4.4.4 Enhanced enrichment of *Campylobacter* from water (IV)

The Portable Microbe Enrichment Unit (PMEU) is a portable incubator, where the microbes are enriched in specific syringes containing the samples in the enrichment broth (IV, Fig. 1). To the bottom of each syringe, a desired gas mixture was funneled through a sterile filter and a needle. The gas was bubbled through the broth, agitating the broth and the bacterial cells. For testing of the performance of PMEU in *Campylobacter* enrichment, parallel microaerobic enrichments were

conducted using the PMEU and conventional enrichment (STATIC) following the principles of the international standard ISO 17995 (2005).

Campylobacter enrichment using the PMEU was conducted with a gas mixture of 85% NO₂, 5% O₂, and 10% CO₂ (Specialty Gases, Oy Aga Ab, Espoo, Finland). In the conventional STATIC enrichment, the microaerobic conditions were obtained using gas-generating pouches (CampyGen; Oxoid) in incubation jars. The volume of the enrichment broths was 30 mL and the enrichment temperature was 37 °C. The total enrichment time was 48 h, and 2 mL subsamples from the broths were taken several times during the incubation. The *Campylobacter* viable counts from broth subsamples were enumerated by plating the broths and serial dilutions onto mCCDA medium microaerobically for 48 h at 41.5 °C.

Prior to parallel tests with PMEU and STATIC, optimization of the PMEU procedure for *Campylobacter* enrichment was done. Optimization tests consisted of

- (i) the comparison between microaerobic and aerobic incubations,
- (ii) the optimization of antibiotic concentrations in the PMEU,
- (iii) the testing of the suitability of antifoam drops for the PMEU enrichment,
- (iv) the testing of optimal subsampling times.

The optimization of the antibiotic concentrations was needed, since in the PMEU enrichment, in addition to nutrients, selective substances may also be more readily available for microbes than in the STATIC enrichment case. The bubbling of broths in the PMEU caused foaming, especially in the Preston broth. The foam blocked the gas flow and bubbling of the PMEU syringe. In an attempt to solve this problem, antifoam drops (IDEXX Laboratories, Westbrook, Maine) were used in the Preston broth enrichments in the PMEU. Additionally, the growth curve of the *C. jejuni* strain was compared with that of *C. coli*.

The performance of *Campylobacter* enrichment and detection methods in water analyses and the comparison between PMEU and STATIC enrichments were done using drinking water and bathing water samples inoculated with an environmental strain of *C. jejuni*. Bolton broth (LabM, Bury, UK) was used for drinking water samples and Preston broth (Oxoid) for bathing water samples (ISO 17995, 2005). The drinking water used in the tests was tap water from the city of Kuopio, Finland that contained 0.18 mg/L chlorine, which was later on deactivated with 0.02 mol/L sodium thiosulfate (at a concentration of 50 µL / 100 mL). A bathing water sample was taken from a recreational site on a lake in eastern Finland.

Triplicate 1000 mL drinking water samples (multiple samples from the same canister) and triplicate 500 mL aliquots of bathing water were taken immediately after spiking and concentrated onto membrane filters that were subjected to the PMEU and STATIC enrichments. Additionally, the drinking water canisters were stored in the dark, at 4 °C, and sampled again after 3 and 7 days of storage. The broths were sampled, after the predetermined enrichments time prior to

Campylobacter detection, which was conducted with both plate counting and real-time PCR.

4.4.5 Survival of *Campylobacter jejuni* in biofilms (V)

The loss of bacteria during the hybridisation and washing steps of FISH procedure was tested with several water samples containing different amounts of *C. jejuni*. Sterile milli-Q-water was spiked with *C. jejuni* and water was filtered through two parallel 0.2mm membranes (Anodisc). One membrane was analysed with FISH, the other with AODC. Bacterial numbers on filters were counted by epifluorescence microscopy and by comparing the numbers of bacteria on membranes; the loss of bacteria during FISH was calculated.

The drinking water biofilms were grown in a Propella® reactor, which was run with tap water from the city of Kuopio, Finland for 4 weeks before spiking (V). Water flow through the reactor was 186 mL/min, the retention time of the water in the reactor was 12.6 h and the water temperature was 15 °C. The Reynolds number for water flow was 15,000; i.e. water flow was turbulent and water was mixed effectively in the reactor. Biofilms growing in the reactor were analysed from removable PVC coupons. Biofilms were detached from the coupons into 25 mL sterile water by sonication. The formation of biofilms on the coupons of Propella® reactor was analysed with plate counting (HPC; R2A agar, Difco) and by direct counting of total cell counts (DAPI staining).

The reactor was spiked with water containing an environmental strain of *C. jejuni*. The number of *C. jejuni* in biofilms was followed for 1 week and in outlet water of the reactor for 3 weeks employing the FISH method and cultivation. The presence of *C. jejuni* in water and biofilms was analysed from sample volumes that were modified from 0.1mL to 1,000mL for water samples and from 0.1mL to 10mL for biofilm samples.

4.5 Statistical analysis (I, III, IV)

SPSS for Windows, versions 12.0.1, 14.0.1 and 15.0.1 (SPSS Inc., Chicago, USA) and SAS statistical package version 8.2 (SAS OnlineDoc®, SAS Institute Inc., Cary, NC, USA) were used for the statistical analyses. Before the statistical analyses, the counts were converted to their natural logarithms and geometric means and geometric standard deviations were used. The differences were evaluated statistically significant in cases when p -value ≤ 0.05 .

With the data of small community water supplies (I), prior to the analyses, results below the detection limit were converted to the half of the detection limit. Spearman rank correlation was used to analyze correlations between water supply characteristics and water quality parameters. Differences in the occurrence of

dichotomous variables between water supply characteristics and water quality parameters were examined with Chi-square test and Fisher's exact test. Difference in coliphage positivity between sampling times was analyzed with Kruskal-Wallis test.

The concentrations of coliform bacteria (I) were compared between analytical procedures using Wilcoxon signed-rank test. Wilcoxon rank-sum test was used to compare differences in total cell counts in spring and autumn samples. McNemar test was used to evaluate the difference between paired proportions of male specific coliphages and somatic coliphages. The average concentrations of heterotrophic bacteria and physicochemical parameters were reported as geometric means (GM) and geometric standard deviations (GSD). The associations between the observed hazards to water quality, water supply characteristics and different water quality parameters was analysed using crosstabs and regression models. Logistic regression models were used to calculate odds ratios (OR) for faecal contamination and for the presence of coliform bacteria. Linear regression was used for the calculation of coefficients of determination for heterotrophic plate count and for nitrite concentration after logarithmic transformation.

In the statistical analyses of equivalence testing (III), the difference between sample types was analyzed with one-way ANOVA with Tukey's post hoc test. In cases of unequal variances, the non-parametric test of Kruskal-Wallis with Monte-Carlo significance was utilized. The differences in confirmation rates between media were examined with logistic regression and between confirmation tests with Wilcoxon signed ranks test. Generalized estimating equations in the GENMOD procedure in SAS were used to account for the dependencies in the data.

Comparisons between the media (III) were carried out following the principles of ISO 17994 (2004). All samples having a zero confirmed count with the trial or reference media and samples having confirmed counts higher than the predetermined higher limit of precise enumeration were excluded from the comparisons. In each comparison, counts from the same sample and the same analysed volume obtained on the trial medium were paired to counts obtained on the reference medium. Both cfus and MPN results were rounded to the nearest whole number before calculations. The relative difference between compared media in each sample (ISO 17994, 2004; Sartory, 2005) was calculated from natural logarithm transformed paired count data (a_i , b_i):

$$\chi_i = 100 \cdot [\ln(a_i) - \ln(b_i)]\%$$

The mean relative difference was also determined:

$$\bar{x} = \frac{\sum x_i}{n}$$

where n = number of samples,

as well as expanded uncertainty:

$$U = 2s_x = \frac{2s}{\sqrt{n}}$$

where s = standard deviation.

A value of 10% was used as the maximum acceptable deviation from zero (D) in a two-sided evaluation (III).

The significance of the difference between the PMEU and STATIC enrichments (IV) was evaluated utilizing univariate analysis of variance including a time factor. One-way analysis of variance was performed in the comparison between real-time PCR results and colony counts.

5 Results

5.1 Water quality hazards at small community water supplies (I, II)

During the water quality monitoring at twenty community water supplies in central Finland (I), the weather in autumn 2002 and spring 2003 had low precipitation and the mean temperature was higher than the long-term average resulting in high evaporation. Furthermore, the snow cover in winter 2002-2003 was only 10-30 mm, limiting the extent of flooding in spring 2003. During these two first sampling rounds, no faecal indicator microbes were detected.

In autumn 2003 and in spring 2004, precipitation and temperature were average in comparison with the long-term records. There were no floods or heavy rains recorded in the study area during the sampling periods. During these two latter samplings, faecal indicator microbes (*Escherichia coli*, intestinal enterococci and/or *C. perfringens*) were found in eight of the total 80 groundwater samples originating from five different water supply plants (water supplies 1, 5, 6, 7 and 20). In one sample from the water supply 7 in spring 2004 *Yersinia enterocolitica* serotype O6 was detected. None of the other analysed pathogens were detected from any of the samples.

The presence of coliform bacteria and coliphages was common in the raw waters of the small community water supplies, 40 % and 54 % of the samples were positive, respectively. There was a difference in the frequency of coliphage positive samples related to the sampling time: the positive results were more frequent in autumn 2002 and spring 2003 than during the latter two samplings. Male specific coliphages were found more often than somatic coliphages (29 % and 5.1 %, respectively; $p = 0.003$). Coliform bacteria other than *E. coli* were present together with faecal indicator bacteria in the groundwater. The coliform bacteria species identified from the groundwater samples were *Serratia* sp., *Rahnella* sp., *Enterobacter* sp., *Kluyvera* sp. and also *Yersinia* sp. strains were isolated and identified from solid coliform media. Coliphages were also present together with faecal indicator bacteria, except in spring 2004, in the absence of coliphages, there were *E. coli* present in water supply 1 and enterococci in water supply 20.

The numbers of colony forming units of heterotrophic bacteria, total cell counts and physico-chemical properties of the studied groundwater samples are shown in Table 6. There was no seasonal difference in the numbers of culturable heterotrophs on the R2A medium or on the YEA medium. The total cell counts (AODC) were higher in autumn than in spring ($p = 0.021$). The culturability (heterotrophic plate count divided by the total cell count) was higher with the R2A than with the YEA medium ($p < 0.001$) even though the geometric means of culturability rates with both media were very low, 0.213 % and 0.003 %, respectively. However, the maximum

culturability differed from the geometric mean. It was obtained in a single sample from water supply 12 and was 56.4 % on the R2A and 0.28 % on the YEA.

The European Union (1998) parametric value for nitrite in raw water (0.10 mg/l) was exceeded in 16 % of the samples and this accounted for most of the non-compliance of the physicochemical water quality observed in the study. Most of the non-compliance of the nitrite concentrations occurred during the sampling conducted in the autumn of 2003 and even the higher limit value for nitrite in tap water (0.50 mg/l) was exceeded once (Table 6.). Moreover, the organic matter content measured as COD_{Mn} once exceeded the parametric value.

Table 6. Heterotrophic plate counts (cfu/ml) on R2A medium and on yeast extract agar medium (YEA), acridine orange direct counts (AODC, cells/ml) detected using epifluorescent microscope, and physicochemical properties of 20 Finnish groundwater work raw waters with the corresponding recommended or mandatory limit value in drinking water.

	GM (GSD) (n=80)	Range	Limit value
Heterotrophic plate count (R2A)	114 (5)	<10-21400	no value
Heterotrophic plate count (YEA)	1.48 (8.14)	<1-108	no abnormal change
Total cell count (AODC)	53500 (2)	11300-387000	no value
Chloride mg/l	2.5 (3.7)	0.1-19.3	< 250 [§]
COD _{Mn} O ₂ mg/l [*]	0.8 (0.7)	0.0-6.4 [‡]	< 5.0
Electrical conductance µS/cm	102 (2)	21-371	< 2500
Nitrate (NO ₃) mg/l	0.7 (6.0)	0.0-32.5	< 50
Nitrite (NO ₂) mg/l	0.05 (0.09)	0.00-0.59 [‡]	< 0.50 and 0.10 ^{**}
pH	6.7 (1.1)	5.9-8.0 [‡]	6.5-9.5
Phosphate-P µg/l	5.1 (2.1)	0.4-16.0	no value
Sulphate mg/l	7.0 (2.4)	1.0-44.9	< 250 ^{††}
Temperature °C	6.7 (1.3)	2.8-12.0	no value
Total organic carbon mg/l [†]	0.9 (1.4)	0.5-2.2	no abnormal change

* n=60, † n=79, ‡ Limit value exceeded, § National limit value in Finland for water works producing less than 10 m³ a day or to fewer than 50 persons is 100 mg/l. In addition, the national recommendation intended to prevent corrosion of pipeline materials is 25 mg/l, ** Limit value for water leaving the water supply plant, †† National recommendation in order to prevent corrosion of pipeline materials is 150 mg/l.

Table 7. summarizes the water quality results of the study that were considered as threats to water safety and lists the principal hazardous characteristics identified at each supply (I). The highest scores of hazardous characteristics (the sum of risk estimate values was 44 or 45 out of 70) were obtained from water supplies 1 and 7, where also faecal indicator bacteria were present. Water supplies 9 and 10 had the highest sum of the risk estimate values, but there was no failure in the microbiological or physico-chemical quality of their water. Surprisingly, the lowest

sum of the risk estimate values (19 out of 70) were reached with suppliers 5 and 18 even though at supply 5 intestinal enterococci were present at once, and at supply 18 coliform bacteria were constantly present during the study.

The most common hazard identified and classified as reducing water quality was poor well construction permitting contamination of wells with surface water in conjunction with rainfall or flooding. The second most common identified hazard was an insufficient depth of protective soil layer above the water table. The depth of the protective soil layer above the water table was less than 5 meters in the majority of the water supplies. An insufficient soil depth was associated with findings of *E. coli*, intestinal enterococci and coliform bacteria (I, Table 2.).

The overall state of the water works construction and maintenance had a significant association with high counts of coliform and heterotrophic bacteria. All of the samples with faecal indicator bacteria originated from water supplies serving less than 250 consumers. In addition to the water supply size, faecal contamination was associated with the concentrations of nitrite, total organic carbon, total phosphorus, with presence of coliform bacteria and heterotrophic plate counts, and with sampling time (I, Table 2.).

Table 7. Summary of observed water quality hazards considered as threats for water safety by increasing the risk for water contamination at studied water supplies.

Water supply	Faecal indicator bacteria	Other microbes	Physicochemical quality	The risk estimate*	Principal hazardous characteristics
1	<i>E. coli</i> in 100 ml, enterococci in 1000 ml	Coliforms present in each sample	High nitrite and phosphorus concentrations	45	Surface water runoffs, insufficient depth of the protective layer
2	-	High total cell count	-	36	Bank filtration, sand and gravel mining
3	-	-	-	36	Surface water runoffs, agricultural activities
4	-	-	High phosphorus concentration	24	-
5	Enterococci in 1000 ml, after 72 h	The highest total cell count	Highest concentration of organic matter	19	Bank filtration
6	<i>E. coli</i> in 1000 ml	High total cell count	-	24	Surface water runoffs
7	<i>E. coli</i> in 100 ml, enterococci and <i>C. perfringens</i> in 1000 ml	Frequently positive for phages	High nitrite and phosphorus concentrations	44	Surface water runoffs
8	-	-	-	38	Surface water runoffs, sand and gravel mining
9	-	-	-	45	Surface water runoffs, bank filtration
10	-	-	-	45	Surface water runoffs, bank filtration
11	-	Frequently positive for phages	-	31	Agricultural activities

12	-	Coliforms present in each sample, highest HPC count	-	32	Surface water runoffs, sand and gravel mining
13	-	-	High phosphorus concentration	40	Agricultural activities, sand and gravel mining
14	-	-	-	31	Surface water runoffs, bank filtration
15	-	-	-	25	Surface water runoffs
16	-	-	High nitrite concentration	37	Surface water runoffs
17	-	-	-	39	Surface water runoffs, sand and gravel mining, municipal sewerage networks
18	-	Coliforms present in each sample	-	19	-
19	-	-	-	34	Agricultural activities
20	Enterococci in 100 ml	-	-	25	Surface water runoffs

- = Not observed. * The risk estimate calculated based on the presence of hazardous activities (not on the analysed water quality parameters). The theoretical minimum score of risk estimate values for the supply was 5 (no hazardous characteristics identified) and the theoretical maximum was 70 (all studied hazardous characteristics present at the highest level).

Contamination of a municipal drinking water distribution system was noticed at the beginning of August 2004 in eastern Finland as *E. coli* and coliform bacteria were found from the distribution (II). The indicator bacteria counts declined rapidly after corrective actions (chlorination and flushing) took place at the waterworks (Figure 1.). One week after the detection of faecal contamination, a few sporadic coliform bacteria and *E. coli* were present in distant parts of the distribution line where there were low chlorine concentrations (below 0.5 mg/l). The counts of intestinal enterococci were not analysed from all samples. The highest intestinal enterococci count was approx. 200 cfu/100 ml which was measured from a sample taken on 4 August.

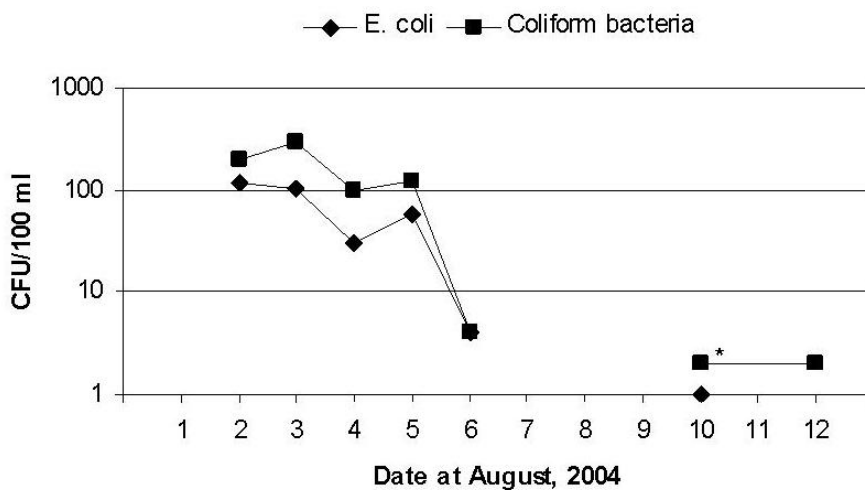


Figure 1. *The highest counts of E. coli and coliform bacteria per day detected from the contaminated municipal drinking water distribution system. *, detected from a sample volume of 250 ml.*

In the earlier monitoring samplings of this distribution system (II), no microbial contamination had been detected. The raw water entering the waterworks remained uncontaminated: all microbial findings were from the distribution system. It was known that a week before the contamination event, a considerable amount of precipitation had occurred (II, Figure 1.). Further on-site technical investigations revealed that the roof of the water storage tower was almost fully carpeted with bird faeces. It was found that a rainwater gutter of the roof had been left in an incorrect position during the previous cleaning and maintenance of the water storage tower. This mistake had the consequence that the gutter channelled the rainwater from the roof directly into the drinking water in the water tower instead of into the rainwater drain.

DNA fingerprint analysis of indicator bacteria isolated from the contaminated distribution resulted in seven clusters in a total of 56 coliform isolates from the water samples (II). The BOX and ERIC consensus tree clustered *E. coli* isolates in their own three groups and the other coliform isolates in their own four groups (II, Figure 4). The *E. coli* isolates in cluster A were identified in all three distribution sampling points (2, 3 and 4) and also the three coliform isolates in cluster C which originated from sampling points 2, 3 and 4.

5.2 *Campylobacter* in drinking water distribution systems (II, V)

In a subsequent analysis of the contamination case of communal drinking water distribution (II), *C. jejuni* was found in three distribution system sampling points as well as in a sample taken from the well collecting rainwater which had flushed from the roof of the water storage tower. Noroviruses or *Salmonella* spp. were not detected in the analysed water samples.

Three cases of *Campylobacter* infections matching spatially and temporally to the communal drinking water contamination were identified in studies conducted by the National Public Health Institute, despite the fact that there was no increase in the number of patients experiencing gastrointestinal symptoms according to local reports. The *Campylobacter* isolates from the patients and from the water samples were compared utilizing 23S rRNA PCR-REA pattern analysis and *Sma*I and *Kpn*I PFGE profiling. The patient isolates were found to be identical to the isolate taken from the rainwater well and another rainwater well isolate was identical with the distribution system isolates (II, Figures 2. and 3.).

The survival of *Campylobacter* in drinking water distribution system biofilms was followed in experimental laboratory-scale contamination of the Propella® reactor (V). It was possible to follow the presence of *Campylobacter* for 1 week in biofilms and for 3 weeks in outlet water of the reactor. *Campylobacter* cells were present in biofilms throughout the study period and in the outlet water for 2.5 weeks as analysed by fluorescent in-situ hybridization (FISH).

5.3 Testing and development of microbiological methods for drinking water analysis (I, III, IV, V)

5.3.1 Counts of indicator bacteria on different media (I, III)

When groundwater samples were analysed (I), the R2A medium gave higher numbers of colony forming units of heterotrophic bacteria than the YEA medium, the geometric means being 114 CFU/ml and 1 CFU/ml, respectively (Table 6.). The Spearman correlation between R2A and YEA media was 0.674 ($p < 0.001$) and R2A resulted in counts above the detection limit of the method (for R2A 10 cfu/ml and for YEA 1 cfu/ml) more often than YEA ($p = 0.037$).

Both the ten-fold increase of sampling volume and doubling the incubation time significantly increased the counts of coliform bacteria detected from groundwater samples (I). The Chromocult® Coliform Agar medium gave more positive results than the standard Lactose TTC medium (Table 8.).

Table 8. *Coliform bacteria results obtained using different volumes and incubation times on ISO 9308-1 medium (LTTC) and on Chromocult Coliform Agar medium (CC).*

Variable		Total number of positive findings	Number of analyses	Correlation (Spearman)	Sig.*
Volume (ml)	100	29	199	0.756 ($p < 0.001$)	$p = 0.031$
	1000	39			
Incubation (h)	21±3	12	183	0.450 ($p < 0.001$)	$p < 0.001$
	44±4	56			
Medium	LTTC	27	204	0.687 ($p < 0.001$)	$p < 0.001$
	CC	44			

*McNemar's test was used for presence-absence data.

Of the total of 110 samples, only samples with numerical results were included in the *E. coli* and coliform bacteria media comparisons (III). Depending on the comparison, the total number of the samples included varied from 25 to 59, being lower for *E. coli* than for coliforms. The numbers of the samples included in the comparisons are shown in Table 9.

Table 9. Samples in the *Escherichia coli* and coliform bacteria media comparison.

	LES Endo		Colilert		CC		HECM		CECM	
	LTTC		LTTC		LTTC		LTTC		LTTC	
	EC	TC	EC	TC	EC	TC	EC	TC	EC	TC
Bathing water	6	15	7	19	8	12	9	15	8	14
Ground water	6	17	3	6	6	20	3	13	6	16
Spiked sterile water	13	13	6	7	14	15	12	12	15	16
Spiked tap water	4	4	2	2	5	5	5	5	5	5
Spiked well water	7	7	7	7	5	7	7	7	7	7
Total	36	56	25	41	38	59	36	52	41	58

LTTC = Lactose TTC Tergitol-7 medium, CC = Chromocult Coliform agar, HECM = Harlequin *E. coli*/coliform medium, CECM = Chromogenic *E. coli*/coliform medium, EC = *Escherichia coli*, TC = coliform bacteria.

When chromogenic and fluorogenic media are employed according to the instructions of the manufacturers', no confirmations of presumptive counts are performed. In this trial, a partial confirmation of colonies and wells was performed for all media tested. By using chromogenic and fluorogenic media, presumptive *E. coli* colonies/wells can be differentiated from presumptive non-*E. coli* coliform colonies/wells. This kind of preliminary differentiation is not possible when conventional LTTC and Les Endo methods are used, and in these cases confirmation procedures are essential.

Counts of coliform bacteria

The mean coliform confirmation rates of the trial media varied between 65 and 100 %, being lower with bathing water samples than with other samples (III, Table 1. and Figure 1.). The lowest confirmation rates were obtained from non-*E. coli* coliform colonies grown on CC and CECM. On LTTC and CC, in addition to the oxidase positive presumptive non-*E. coli* coliform colonies, and also gram-positive bacteria were detected exhibiting the typical appearance of the colonies.

According to the standard methods ISO 9308-1 and SFS 3016, the colonies grown on the standard media (LTTC and Les Endo) but not exhibiting lactose fermentation, are considered atypical and are not counted. In this study, the majority of colonies both on LTTC and on LES Endo were atypical (Table 10.). Additional experiments were conducted to confirm the coliform identifications, not only for the typical colonies, but also for these atypical types. The confirmation rate of atypical colonies on LTTC was high (54 %) compared to that on Les Endo (only 10 %).

Table 10. Percentages of presumptive typical and atypical colonies (z), colonies taken into confirmation (n) and confirmation rates (k) of coliform bacteria on LTTC and LES Endo media.

Medium	Colony morphology	z (%)	n (%)	k (%)
LTTC	Typical	18	23	91
	Atypical	82	6	54
LES Endo	Typical	27	32	94
	Atypical	73	10	10

In the coliform comparison, the uncertainties of the mean relative difference were high due to the restricted number of samples and it was not possible to prove equivalence between any of the trial media as compared to the reference medium. The equivalence would have required that the upper and lower limits ($2 \times \text{S.E.M.}$) of mean relative difference would not have crossed the asymmetric maximum deviation limits ($-D$ and $+D$) that are commonly set to a value of 10 in equivalence studies (Figure 2.). The closest equivalents to LTTC were confirmed counts on CC and CECM media. On the contrary, it was possible to demonstrate non-equivalence: the coliform counts on Colilert were significantly higher than coliform counts on LTTC when bathing water samples were included and the coliform counts on Les Endo and HECM were significantly lower than the coliform counts on LTTC.

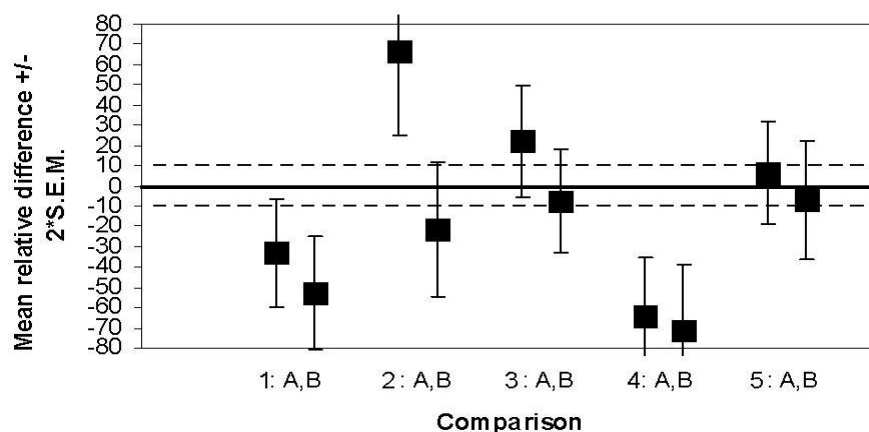


Figure 2. The mean relative differences of confirmed counts in coliform comparison. Comparisons: 1 = LE-LTTC, 2 = Colilert-LTTC, 3 = CC-LTTC, 4 = HECM-LTTC, 5 = CECM-LTTC. A = All samples included, B = Bathing water samples excluded. The dashed line shows the maximum acceptable deviation limits (D).

E. coli counts

The mean *E. coli* confirmation rates were high (more than 80 %) on Les Endo, LTTC and on presumptive *E. coli* colonies/wells of chromogenic/fluorogenic media (III, Table 3.). The additional gas production test at 44.0–44.5 °C had little or no effect on the confirmation rates. A part of the presumptive non-*E. coli* coliform colonies on CC, HECM and CECM and of Colilert wells gave positive *E. coli* confirmation results: especially in Colilert yellow wells, of which approximately 30 % contained oxidase negative, indole positive bacteria. The further identification of these isolates showed that only half of them were truly *E. coli*, the other half were possibly mixed cultures of coliform bacteria other than *E. coli*.

In *E. coli* comparison, all alternative media tested gave significantly lower *E. coli* counts than reference medium LTTC (III, Table 4.). When the confirmed counts were evaluated, the mean relative difference between Colilert and LTTC became inconclusive (Figure 3.). This change was due the above mentioned high proportion of confirmed *E. coli* in presumptive non-*E. coli* coliform (yellow) wells.

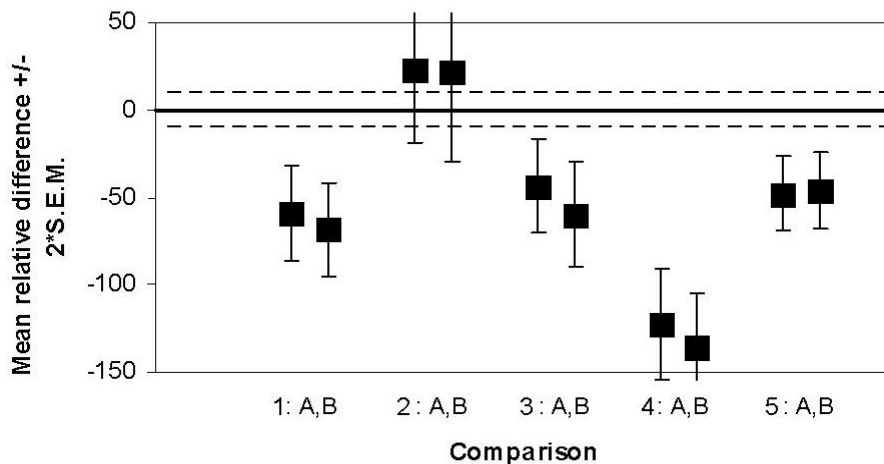


Figure 3. The mean relative differences of confirmed counts in *E. coli* comparison. Comparisons: 1 = LE-LTTC, 2 = Colilert-LTTC, 3 = CC-LTTC, 4 = HECM-LTTC, 5 = CECM-LTTC. A = All samples included, B = Bathing water samples excluded. The dashed line shows the maximum acceptable deviation limits (D).

5.3.2 *Campylobacter* enrichment and PCR detection (IV)

During the optimization of the PMEU procedure for *Campylobacter* enrichment, it was seen that the microaerobic bubbling in PMEU and also microaerobic conditions during STATIC incubation were essential for growth of *Campylobacter*. The

optimization of antibiotic concentrations in the PMEU gave an indication that a lowered concentration might favor the *Campylobacter* growth in Bolton broth (IV, Table 1.). However, the full concentration was used in subsequent tests in order to provide comparability against the STATIC method. The presence of antifoam drops did not have any effect on *Campylobacter* counts and they were used in the following Preston enrichments in PMEU. It was concluded that the growth of *C. jejuni* gained more than the growth of *C. coli* from PMEU enrichment (IV, Figure 2.). Subsampling times of 0, 16, 24, 40, and 48 h were chosen to be used in subsequent tests.

From first dechlorinated and then inoculated tap water sample, *C. jejuni* counts were significantly higher ($p < 0.01$) in PMEU enrichment than in STATIC enrichment (IV, Figure 3.). Especially after 3 days storage of the inoculated tap water, with the PMEU enrichment, the growth was observed sooner (after 16 h) than with the STATIC enrichment (real growth not until 40 h). The bathing water inoculation experiment where Preston enrichment was used did not reveal any difference ($p = 0.26$) between the PMEU and STATIC enrichments (IV, Figure 4.).

The time requirement for *Campylobacter* detection after enrichment (IV) shortened considerably by using real-time PCR detection (~5 h) compared to colony count detection (48 h). The real-time PCR was quantified against colony counts obtained from the control strain dilutions. The PCR results originating from samples of PMEU and STATIC enrichments did not differ from the colony count results.

5.3.3 FISH detection of *Campylobacter* (V)

The mean heterotrophic plate count of the Propella® reactor biofilms was 86 000 CFU/cm² and the mean total cell count of the biofilms was 1 700 000 cells/cm² before inoculation of the reactor with *C. jejuni*. The mean concentrations (µg/l) of total organic carbon, AOC, total phosphorus and MAP in the inlet water of the reactor were 3 400, 69, 1.4, and 0.25, respectively. The inlet water contained free chlorine 0.06 mg/l.

After the inoculation of the Propella® reactor, culturable *Campylobacter* were detected 2 h after the inoculation in biofilms, but not after that time point. Using the FISH detection, *Campylobacter* cells were present for at least for one week in biofilms (V, Figure 1.). In the outlet water, the last detection of culturable *Campylobacter* was achieved one day after the inoculation. The FISH results indicated, however, that *Campylobacter* were present in the outlet water for 2.5 weeks.

The loss of bacteria during the FISH hybridisation and washing steps was estimated to be as high as 40 – 90 %. Therefore these FISH results, even though higher than the cultivation results, may still underestimate the real numbers of *Campylobacter* cells in the samples.

6 Discussion

The aim of the microbiological water quality analysis is to identify if harmful microbes are present and subsequently to minimize the public health risk from consuming water intended for drinking. Assessment of health effects should document and quantify the health risks resulting from microbes in water (NRC, 2004; WHO, 2004). The risk estimates are created by linking epidemiologic evidence for disease to measured concentrations of either pathogens or indicators in water (Szewzyk *et al.*, 2000). Despite the observed correlations between the indicator organisms and pathogens, the presence of all enteric pathogens in water cannot be predicted by employing a single indicator organism (Savichtcheva and Okabe, 2006). Ideally a zero concentration of microbial indicators in drinking water means a zero health risk, but it is difficult to determine a quantitative relationship between detected indicator concentration and the degree of public health risk (Hrudey and Hrudey, 2004; NRC, 2004; Payment, 1997). For example, many inputs of quantitative microbial risk assessment (QMRA, Medema and Ashbolt, 2006) such as microbial concentrations, indicator-to-pathogen ratios, dose responses and water consumption are uncertain (Smeets *et al.*, 2008; Szewzyk *et al.*, 2000).

6.1 Factors affecting faecal contamination of ground water supplies (I, II)

Our data from Finland suggests that the smallest water supply plants, often managed by local water cooperatives, are more likely to encounter problems (both microbiological and physicochemical) in their water quality than municipal water supply plants, which on average are larger water production units. The five community water supplies where the faecal indicator microbes were detected were all small, serving less than 250 consumers. It has been recognized also worldwide that small water supply plants, not only in developing countries but also in the developed ones, suffer serious limitations of funding, personnel and knowledge (Anonymous, 1997; Hulsmann, 2005; Pedley and Howard, 1997; WHO, 1997). Consequently, deteriorations of water quality can occur at small water supplies and there are potential health risks present (e.g. Gaut *et al.*, 2008; Hamsch *et al.*, 2007; Malakauskas *et al.*, 2007; Richardson *et al.*, 2009).

In this study, the main hazard identified to reduce water safety was insufficient protection against the influence of surface water. The identified characteristics enabling the impact of surface water to groundwater quality were:

- a poor well construction and maintenance enabling surface water runoffs directly into raw water wells,
- an insufficient depth of the protective soil layer above the groundwater table,
- the possibility of uncontrolled river or lake bank infiltration.

Agricultural activities and sand and gravel mining sites considered beforehand to have a major impact on the water quality (Isomäki *et al.*, 2008) did not lead to the presence of faecal microbes in the water at the present study. Later on, the studies on Finnish small community water supplies continued and then, in addition to the findings of faecal indicator bacteria from some samples, *Giardia intestinalis* was detected for the first time from groundwater supplies in Finland (Juselius *et al.*, 2010). In that study, the main contaminating hazard identified was sewage treatment activities (household septic tanks) located in close proximity to the wells.

The high risk estimate does not mean that at the moment of sampling, water is contaminated and a low risk estimate does not completely guarantee safety from contamination. In this study, risks were identified to be present at all of the studied 20 community water supplies; the given risk estimate values varied from 19 to 45. Correspondingly, there were also more or less findings of microbial or chemical water quality indicators in the raw waters; coliphages, if nothing else, were detected also from the cleanest water supplies.

The findings of faecal indicator microbes were associated with elevated concentrations of nitrite, total organic carbon and total phosphorus. Research findings supporting a link between nutrients and faecal contamination have been previously reported elsewhere e.g. from karst springs where total organic carbon and nitrate entered the groundwater at the same time with *E. coli* after rainfall events (Pronk *et al.*, 2007) and from Spanish spring and river waters where the levels of phosphate correlated with faecal indicators (Fernandez-Molina *et al.*, 2004). The association between the occurrence of faecal microbes and nitrogen compounds is not surprising since intensive farming produces large amounts of faecal material and agricultural activities are also known to release nitrate and nitrite into the groundwater (Maticic, 1999; Saarijärvi *et al.*, 2004).

The highest risk estimate value (44 or 45) was given for four community water supplies. At two of these supplies, no indication of fresh faecal contamination was detected, but coliforms, coliphages or nitrite were present in the raw water. At the other two, the raw water was proved to be faecally contaminated; there were faecal indicator bacteria, coliforms, coliphages, and nitrite present. At three other water supplies examined in this study, where faecal indicator bacteria were present, the given risk estimate values were relatively low: 19, 24 and 25 out of 70. This indicates that the presence of even a single risk, if it is serious enough such as insufficient well construction or uncontrolled bank filtration, might lead to contamination.

The present study shows that the preparedness to suppress the adverse health effects in a case of contamination by applying disinfection before distributing the water is not fully in line with the identifiable water quality hazards. All seven water supplies with UV disinfection equipment were municipally owned and had no findings of faecal indicators in their raw waters before disinfection during the study, but one had

the highest risk estimate value. At a supply applying chlorination operated by a water cooperative, there was a real need for disinfection since faecal indicator bacteria and also *Yersinia enterocolitica* were detected in raw water used by that supply. The other four supplies with faecal indicator findings did not utilize disinfection.

In this study, the sampling time was associated with the presence of faecal contamination that was detected by the presence of faecal indicator bacteria. The representativeness of the sampling is one of the main factors affecting the detection or non-detection of faecal contamination in a water supply (Hrudey and Hrudey, 2004). The variability of the water quality over time and space means that multiple samples taken over an extended period of time are needed if one wishes to have a certain degree of reliability of the measurement; only limited information can be gathered from a single sample (Maul *et al.*, 1990; NRC, 2004). It has been estimated that there is no more than about 5 % probability to detect faecal contamination by standard water quality monitoring programmes (van Lieverloo *et al.*, 2007). It has been stated that in compliance monitoring, the 100 ml volumes may be sufficient and a large number of small-volume samples is preferable to a smaller number of large-volume samples (Haas, 1993; Locas *et al.*, 2008). For hazard identification purposes and in cases where there is a suspicion of waterborne illness transmission, however, larger volumes may be essential (Hänninen *et al.*, 2003; Hargy *et al.*, 2010). In this present study, only a small number of samples were analysed and the detection of faecal indicator bacteria was promoted by using ten-fold volume of samples and prolonged incubation times.

Rapid and simple indicator bacteria techniques are employed in water quality monitoring for early warning of potential microbial contamination (Gerba, 2000; NRC, 2004; Theron and Cloete, 2004). In the contamination case reported in this study, the faecal contamination of tap water was detected by cultivation of *E. coli* from samples taken shortly after a heavy rainfall during a conventional monitoring programme of the hygienic quality of drinking water. Before the water quality of the system had been in compliance with regulations as it has been monitored six times per year with samples being collected from the municipal drinking water distribution. It is known that there may be an increased risk of microbiological contamination of drinking water after heavy rainfall (Curriero *et al.*, 2001; Thomas *et al.*, 2006; WHO, 2004). The systematic timing of the regular monitoring of drinking water quality in association with heavy downpours or other extreme weather events may enhance the detection of water contamination and subsequently prevent waterborne disease outbreaks.

One of the key components of a water safety plan is the water supply system assessment including hazard identification and upgrading of the system in case that the capacity to meet health based targets is not even theoretically possible (WHO, 2004). Based on water quality data and on waterborne outbreaks, it seems that the upgrading of the system would be extremely desirable in many of the small community water supplies. The limitations of financial resources should not hinder

the initiation of improvements since the benefits from prevention of waterborne illness would certainly outweigh the costs of improvements (Hunter *et al.*, 2009). The majority of waterborne *Campylobacter* outbreaks have been associated with non-community water supplies without constant disinfection (Olson *et al.*, 2008). In the United States, in order to prevent of these kinds of events, new regulations were laid down in 2006 that require disinfection at both ground and surface water before distribution. In the future it will become clear if this has had any effect to decrease the numbers of waterborne *Campylobacter* outbreaks.

6.2 Combination of microbiological methods for waterborne outbreak investigations (II)

More sophisticated microbial methods are needed for confirmation of the waterborne health risk and for contamination source identification if there is contamination of drinking water with microbes detected by the indicator screening or by epidemiological evidence (NRC, 2004; Theron and Cloete, 2004). In this present case, the actions to prevent further infections followed immediately after the presumptive *E. coli* finding and included further water sampling and analysis, the initiation of water chlorination and finally after confirmation of the *E. coli* finding also an advisory to boil water before consumption. The expanded sampling and direct measurement of pathogens from water samples revealed the involvement of the pathogen *C. jejuni* in addition to the bacterial indicators of faecal contamination, *E. coli* and intestinal enterococci.

The multiparameter approach utilizing the water microbiological methods to solve this contamination case clearly demonstrated that the whole distribution system had been microbiologically contaminated. Tracking of the drinking water contamination source revealed evidence to indicate that faecal material from wild birds on the roof of the water tower was the source of the problem. This was confirmed by on-site technical investigations that identified the flaws in the maintenance of rainwater gutters in the water storage tower and the findings were supported with the local precipitation data. Subsequently, genotyping of *C. jejuni* isolates from tap water and from the rainwater well showed identical PFGE patterns. The fact that the faecal material originated from wild birds which are commonly known to excrete *Campylobacter* in their stools (Broman *et al.*, 2002; Kapperud and Rosef, 1983; Waldenström *et al.*, 2002) supported these conclusions. In addition, rep-PCR fingerprinting data indicated that some *E. coli* and other coliform bacterial isolates isolated from different points in the distribution system may have had the same origin.

Rep-PCR fingerprinting proved to be very suitable for the rapid analysis of large numbers of *E. coli* and coliform isolates as previously proposed (Rademaker *et al.*, 1998) and our results suggested that BOX fingerprinting is more discriminatory than ERIC fingerprinting. The fingerprinting method was found to be useful in evaluating whether isolates from different parts of the drinking water distribution network and

from different sampling times may have one common origin within one contamination incident. In previously reported analyses using rep-PCR, a large pattern library was used, as the goal of studies had been to discriminate between different possible host sources (Carson *et al.*, 2003; Dombek *et al.*, 2000; Leung *et al.*, 2004; McLellan *et al.*, 2003). In this present work, the rep-PCR was applied without a large library of analysed isolates, since it was not the aim to distinguish between possible hosts. If there had been an existing library, however, this would have allowed further interpretation of the results.

Identical *SmaI/KpnI* PFGE genotypes of *C. jejuni* isolates were found from all three identified patients and one isolate from the well of a rainwater drain. The genotyping results combined with spatial and temporal analysis of the event and patient interview data indicated that most probably the patients had been infected by drinking contaminated tap water.

The resolution of this contamination case and identifying the association between water contamination and infected patients required multidisciplinary cooperation between the experts from public health, drinking water and research laboratory sectors. It is common that individuals will not seek medical help if their gastrointestinal symptoms are mild (Blaser and Engberg, 2008). Therefore it is probable that a substantial proportion of waterborne disease outbreaks are never detected despite the existence of outbreak notification systems (Hunter *et al.*, 2003; Neumann *et al.*, 2005). The definite water contamination detected in the present study did not result in higher than normal number of patients with gastroenteric symptoms requiring medical care in the community. Nonetheless, the molecular techniques applied in this study for revealing the contamination route and the association of patient isolates with water contamination appeared to be very useful epidemiological tools.

6.3 Choice of the optimal medium for indicator bacteria analysis from water (I, III)

There are many important requirements for an ideal indicator method (see Table 1). Adequate sensitivity relates to the ability to detect the target organisms even when they are present only at low numbers, are stressed or injured, or are accompanied by non-target micro-organisms (Rompre *et al.*, 2002; Tallon *et al.*, 2005). Method specificity on the other hand prevents false positive alarms. Differential and selective media contain inhibitory agents intended to suppress the growth of non-target bacteria (Atlas, 1995) and also certain cultivation conditions, such as elevated incubation temperature, are adopted to improve the specificity (NRC, 2004). These agents and conditions may also suppress the growth of injured or stressed target bacteria and supportive agents promoting the growth of target bacteria have been incorporated into modified media (Atlas, 1995; Rompre *et al.*, 2002).

The choice of the one single medium for coliform bacteria monitoring is a challenge since the level of background growth can vary greatly (Schets *et al.*, 2002), especially during the contamination episodes in comparison to the normal situation. The currently available solid cultivation media employed in membrane filtration of *E. coli* and coliform bacteria do not seem to maintain maximum sensitivity without lowering the selectivity (this study; Geissler *et al.*, 2000; Niemi *et al.*, 2001) and therefore it might be impossible to achieve ideal broad applicability of the method. Instead, for waters with high background microbiota, a less sensitive but an adequately selective medium should be used and for clean waters, such as disinfected tap waters, another, more sensitive medium with a lower selectivity would be the best choice (ISO/WD 9308-1, 2009).

Of the solid media tested in this study, the current reference medium (LTTC) and also a chromogenic alternative medium (CC) suffered from extensive background growth making these media unsuitable for testing of bathing waters or other water with high counts of background microbiota. On both LTTC and CC, the maximum sensitivity was reached, but at the cost of lack of specificity. In this present study, the growth of oxidase-positive bacteria which possessed β -D-galactosidase activity was not fully inhibited and also gram-positive cocci exhibiting a colony appearance similar to that of coliform bacteria were found on LTTC and CC, indicating the need to include oxidase-test and gram-staining as confirmation tests with these methods. Fortunately the color reactions obtained on CC made counting on that medium much easier than on LTTC meaning that CC may represent a feasible alternative. In this present study, the liquid medium Colilert had no visible problems attributable to background microbiota. The exception was bathing water samples where 23% of yellow Colilert wells contained oxidase positive bacteria; this result that may be related to the shelf-life of the Colilert reagent.

As anticipated, higher coliform bacteria counts were obtained on chromogenic media than on lactose based media (Alonso *et al.*, 1996; Geissler *et al.*, 2000; Landre *et al.*, 1998) also in the present study. It has been conventional wisdom to state that the higher counts obtained using β -D-galactosidase test are due to a broader group of coliform organisms with this technique than can be detected by the traditional lactose fermentation test (Schets *et al.*, 2002). One recent publication, however, reported data that there was no difference in the coliform genera diversity detected by the different tests (Fricker and Eldred, 2009).

There has been also an on-going debate on the prevalence of β -D-glucuronidase-negative *E. coli* in water. In a Dutch study, it was reported that as much as 14 % of the studied *E. coli* strains were β -D-glucuronidase-negative at 44 °C (Schets and Havelaar, 1991), but this might have been due to the diffusion of fluorescence on the solid medium that was employed. Surprisingly, in the present study, approximately 30% of yellow, non-fluorescing Colilert wells were conventionally confirmed to actually contain *E. coli*. These kinds of false-negative results may well lead to an underestimation of the microbiological risks as the presence of coliform bacteria but not *E. coli* may not initiate the immediate full emergency level actions (Hunter *et*

al., 2003) needed to suppress the possible adverse health effects of the faecal contamination. Schets *et al.* (2002) also reported a similar observation, in their data 11% of yellow Colilert wells contained *E. coli*. Moreover, in a study of *E. coli* pure cultures, it was found that in Colilert, approximately 20 % fewer *E. coli* strains exhibited β -D-glucuronidase production than the situation with the other three media when analysed using the same enzymatic principles (Maheux *et al.*, 2008). In a recent report, it was stated if the results are counted in daylight then this may lead to an underestimation of the *E. coli* counts (Pitkänen *et al.*, 2009). In order to prevent the false-negative *E. coli* results in the water analysis, it is essential to perform the reading of the Colilert fluorescence in a special dark chamber or in a darkened room and even the slightest fluorescence must be interpreted as a positive reaction.

Currently, one of the main drawbacks to the use of traditional indicator methods is the time delay before warnings can be issued (Edberg *et al.*, 1997). This is due to the long laboratory sample processing time and thus there is a clear need for more rapid, sensitive and specific methods (NRC, 2004; Rompre *et al.*, 2002; Tallon *et al.*, 2005). An automated analyzer utilizing enzymatic properties of coliforms and fluorometry has already been developed, and is able to provide coliform presence/absence results in a reduced time (Berg and Fiksdal, 1988; Tryland *et al.*, 2001; Tryland *et al.*, 2002). However, the comparability of the results between the laboratories and previous results is often needed and this may slow down the introduction of new methods.

As a tool to assist the introduction of new methods, criteria for establishing equivalence between microbiological methods have been written into an international standard (ISO 17994, 2004). This rather new standard however might need revision in the near future since it fails to provide some key information about the selection of sample types and information on statistical groupings. The selection of representative samples for the trials is essential for achieving reliable (true) results. Currently, if one were to use only out of scope sample types with extensive background growth, then it might be possible to establish equivalence of more selective alternative media in comparison to the current reference medium that suffers from a lack of selectivity. It is also the case that the essential number of samples compared prior to a conclusive evaluation expands rapidly if the difference between the recoveries is small, as due to the variability bacterial counts in the samples, the 95% confidence interval might become too wide (Sartory, 2005), increasing the work load of comparison.

As a consequence of the abovementioned reasons, the parallel use of a more selective standard method based on lactose fermentation (LES Endo) and a more sensitive, new chromogenic method (CC) was validated in our laboratory. In the future, the use of LES Endo might be replaced by the CC medium with added antibiotics to suppress the background microbiota (Alonso *et al.*, 1996; Geissler *et al.*, 2000), though this would require further validation.

The majority of the published water microbiological cultivation method equivalence studies have dealt with the enumeration of *E. coli* and coliform bacteria. To a lesser extent, also alternative methods for other common water quality indicator bacteria, such as intestinal enterococci, have been proposed (Eckner, 1998). In the detection of heterotrophic plate counts, the use of YEA medium according to international standard ISO 6222 (1999) is obligatory in compliance monitoring of water, according to European Union regulations (European Union, 1998). In the present study, the R2A medium yielded significantly higher heterotrophic plate counts than the YEA medium confirming the earlier results of Gibbs and Hayes (1988). The result suggests that R2A medium is more suitable than water plate count medium for microbial monitoring of raw groundwater with low nutrient levels. The better suitability of the R2A medium results from the fact that numerical results can be obtained more often and the more convenient spread-plating technique may be used.

However, the change of the method in HPC analysis needs to be done with caution as the indicator value of HPC is based on a long series of analysis (APHA *et al.*, 1998). However, it is probable that the R2A method would give at least as reliable or better results as the YEA method and thus the introduction of R2A method as an alternative method for drinking water compliance monitoring of water with low nutrient levels would be possible. The acceptance of alternative method for regulatory testing will however currently require an equivalence study performed in European Member States (EMAG/EGM, 2005; European Union, 1998; Sartory, 2005). In the future, the preparation of an international standard for R2A method should be recommended as it would enable the inclusion of the method into the future regulations.

6.4 Improvements in *Campylobacter* detection from water (IV, V)

It has often proved impossible to isolate *Campylobacter* in investigations of waterborne *Campylobacter* outbreaks mainly due to the insufficient and delayed water sampling and analysis (Bopp *et al.*, 2003; Hänninen *et al.*, 2003; Jakopanec *et al.*, 2008). In an outbreak investigation, it is common that the research activity and efforts are concentrated on epidemiology and on clinical samples rather than on water sampling (NRC, 2004). Moreover, due to the relatively long incubation time of *Campylobacter* infection (Blaser and Engberg, 2008; Ketley, 1997), it is difficult to collect water quickly enough so that the sample would be representative of the water quality that was likely responsible for the outbreak.

However, some of the isolation failures have possibly been associated with the deficiencies in the *Campylobacter* detection methods from water (Jacobs-Reitsma *et al.*, 2008; Rollins and Colwell, 1986) i.e. they have not been sufficiently sensitive and rapid (Miller and Mandrell, 2005). In this study, an enhanced enrichment in a portable microbial enrichment unit (PMEU) and real-time PCR detection was shown to be efficient in enriching stressed *Campylobacter* cells and in rapid detection of

enriched cells, respectively. Moreover, it was shown by employing the FISH peptide nucleic acid probe method that the conventional cultivation method might underestimate the actual numbers of *Campylobacter* in water and biofilms.

In the isolation of *Campylobacter* from raw milk, microaerobic gas bubbling through Teflon tubing directly to the enrichment broth was utilized already in 1983 (Lovett *et al.*, 1983). There is also a publication (Heisick *et al.*, 1984), where a constant gas flow into the *C. jejuni* enrichment broths was studied in comparison with an evacuation-replacement method. Even though the incubation *Campylobacter* enrichments is still commonly conducted statically in jars with a modified atmosphere, in its online Bacteriological Analytical Manual the U.S. Food and Drug Administration recommends shaking of the *Campylobacter* enrichment in combination with a bubbler system to achieve a continuous gas flow (Hunt *et al.*, 2001). In this present study, the microaerobic enrichment of *Campylobacter* from water samples with constant gas flow was achieved using PMEU technology. This present data is in agreement with these earlier findings, i.e., the bubbling of the broths resulted in higher recoveries than static enrichment. In addition, the portability of the PMEU enables the immediate start of incubation after concentration of a water sample, even at the sampling site.

The quantity and quality of background microbiota differs between the water types. Therefore, modifications of the *Campylobacter* cultivation procedure may be helpful depending on the pollution level of the water under examination (ISO 17995, 2005). For clean waters, where the number of *Campylobacter* cells present in the water at the time of sampling can be very low, in addition to the abovementioned enhanced enrichment, the sensitivity of the method may be increased also by concentration of a large quantity of water (this study, Hänninen *et al.*, 2003; Hunt *et al.*, 2001). The increased sample volume may require the use of larger diameter filters than the conventional 47 mm, especially if the water under examination is turbid. For water with abundant microbiota, the antagonistic effects of background growth in enrichment broths may out-compete the growth of *Campylobacter* during the enrichment process (Abulreesh *et al.*, 2005). The selectivity of the enrichment needs to be increased by selective agents of broths, or by incubating the broths at the elevated temperature of 41.5 °C instead of 37 °C (Koenraad *et al.*, 1995) and also the time of the enrichment may be shortened.

Ideally quantitative real-time PCR for *Campylobacter* detection would be applied directly to the water samples (Nam *et al.*, 2005; VanDyke *et al.*, 2010; Yang *et al.*, 2003), but in previous years direct PCR detection has suffered from the low sensitivity of the methods as well as the existence of PCR inhibitors (Abulreesh *et al.*, 2006). Moreover, the cultivation methods have been essential to detect solely viable *Campylobacter* and the isolation of the strains is also needed prior to the strain typing such as pulsed-field gel electrophoresis which are employed in outbreak investigations. Consequently, an enrichment step prior to real-time PCR was applied in this study, similarly to the procedure used in a Swedish outbreak investigation (Andersson *et al.*, 1997). The PCR detection after enrichment

substantially shortened the time requirement for detection and confirmation of *Campylobacter* i.e. from two or more days down to five hours which is much shorter than the conventional cultivation method. The advantages of the PCR detection were highlighted especially in the analysis of water samples with high background growth since for PCR confirmation, pure cultures are not needed.

In this study, detection and species-level identification of thermotolerant *Campylobacter* species was achieved rapidly using the SYBR Green real-time PCR and restriction fragment analysis (REA) of a 23S rRNA gene fragment. It is possible to achieve even faster *Campylobacter* detection by using probe-based real-time PCR, but then a second assay may be required for species identification (Abu-Halaweh *et al.*, 2005). In the future, a recently developed high-resolution melt (HRM) analysis technique may speed up the *Campylobacter* species differentiation and replace the restriction fragment analysis. In HRM, a PCR product is denatured slowly increasing the temperature until an intercalating DNA dye, such as SYBR green is released and the fluorescence signal is simultaneously assessed using a real-time PCR detector (On *et al.*, 2008).

C. jejuni may display increased survival within biofilms of aquatic environments. This study with a non-cultivation-based method is in agreement with the previous studies (Buswell *et al.*, 1998; Svensson *et al.*, 2008; Trachoo *et al.*, 2002) that the presence of VBNC organisms within the biofilms may cause a dramatic underestimation of the number of surviving bacteria by cultivation methods. There was a widespread difference between cultivation and FISH methods despite the fact that a 40-90 % loss of bacteria during FISH was detected in the present study when the filters were immersed into the washing buffer. Later, the immersing in washing buffer has been replaced in FISH procedures by rinsing with deionized water through the membrane (Mezule *et al.*, 2009) that should minimize the loss of cells during hybridization.

The FISH method alone does not provide a straightforward answer to the question of viability or infectivity of bacteria and therefore the coupling of FISH with direct viable count (DVC) method has been utilized previously in an *E. coli* study (Juhna *et al.*, 2007). This application was not applied in the present study but it might be useful also in the *Campylobacter* studies. It is possible that DVC-FISH would detect at least a part of cells that are not culturable when conventional cultivation methods are employed (Garcia-Armisen and Servais, 2004). Furthermore, *Campylobacter* cells are known to form coccoid or viable but not culturable (VBNC) bodies when there is environmental stress (Ketley, 1997; Jones, 2001; Oliver, 2005; Rollins and Colwell, 1986). It has been reported that coccoid forms may not hybridise as well as the spiral forms (Lehtola *et al.*, 2005) which complicates the interpretation of the FISH results as coccoid cells might sometimes be culturable and VBNC cells may have a spiral form (Svensson *et al.*, 2008).

6.5 Future research needs

Faecal indicator bacteria provide an indirect estimation of the presence and quantity of faecal pathogens in the water (NRC, 2004; Tallon *et al.*, 2005). The presence of faecal indicator bacteria is a sign of faecal contamination, but the absence of the faecal indicators cannot be taken as an assurance of non-contamination, particularly of the absence of enteric viruses (Baggi *et al.*, 2001; Grabow *et al.*, 2001). The concentrations of microbial indicators and pathogens decline at different rates upon leaving the intestinal tract due to their varying sensitivities to disinfectants and to environmental stress. Furthermore, microbial transport in soil can vary between the groups of microbes (Davies *et al.*, 1995; Owens *et al.*, 2000; Pourcher *et al.*, 2007; Schijven *et al.*, 2003). The limitations of the conventional microbiological detection methods based on cultivation further complicate the assessment of the relationship (Theron and Cloete, 2004) as viable but not cultivable forms of faecal bacteria are common (Oliver, 2005; Szewzyk *et al.*, 2000).

It is possible to achieve the required specificity and also to decrease the time requirement for microbial detection by employing DNA-based methods, most promisingly by utilizing PCR techniques (Tallon *et al.*, 2005; Theron and Cloete, 2004). The problem of detecting of non-viable cells in addition to the viable bacteria has so far restricted applications of the PCR techniques. Recently, PCR pre-treatment techniques using DNA-intercalating agents and differentiating living and dead cells have been vigorously studied (Cenciarini-Borde *et al.*, 2009) and may well provide novel tools for rapid and accurate detection of viable microbes (Cawthorn and Witthuhn, 2008; Inoue *et al.*, 2008; Nocker *et al.*, 2006; Nocker *et al.*, 2009; Nocker *et al.*, 2007a; Nocker *et al.*, 2007b).

The correct timing and adequate extent of water sampling prior to microbiological analysis clearly affects the reliability of the microbiological results achieved. The availability and utilization of easy-to-use, on-site microbial concentrators has been restricted so far and separate concentrations have been conducted for each microbial group. The cost-effectiveness and utility of the techniques might be increased by employing concentrations techniques to simultaneously recover viruses, bacteria and parasites for downstream applications (Polaczyk *et al.*, 2007).

Microbial water quality measurements are always retrospective involving time consuming laboratory methods (Hrudey and Hrudey, 2004). It is a reality that the current water quality monitoring systems are testing water that people have already drunk and will be drinking during the sample processing time. Thus there is a lag time before mitigation actions will be taken, as demonstrated during the contamination case in this study. To achieve the best possible public health protection, proactive risk management, such as Water Safety Plans (WHO, 2004) and prediction based warning systems are needed in the future (NRC, 2004). So that the public can be warned before exposure occurs, models that predict future water quality conditions, based on factors such as rainfall (Maier and Dandy, 2000), and on-line sensors that collect data on water treatment processes (Smeets *et al.*, 2010)

and about water quality (Hall *et al.*, 2007; Skadsen *et al.*, 2008) should be further studied and employed. A special emphasis should be placed on defining of the relationships between water quality changes and adverse health effects and also on ways to handle and process on-line data (Henderson *et al.*, 2009).

7 Practical implications

Hazard identification conducted at community water supplies should raise awareness of the possible health risks caused by the use of vulnerable groundwater aquifers for drinking water purposes without water purification treatments or disinfection. In microbial hazard identification, a multiple parameter approach should be recommended, especially in small community water supplies where the water quality may be monitored only infrequently. There should be an attempt to create a knowledge base about which characteristics increase the hazards to water quality, and in addition to setting up appropriate risk assessment procedures, disinfection should be encouraged at small community water supplies.

For adequate identification and quantification of the microbial hazards, a wide set of indicators such as *E. coli*, enterococci, coliform bacteria, *C. perfringens* and coliphages should be analysed from sample volumes of 1000 ml or larger volumes during an intensive sampling period. In order to detect the worst possible situation for water quality, the sampling should be conducted during or immediately after heavy precipitation events.

The methods for detection and enumeration of waterborne pathogens and microbial indicators are constantly been developed. As the specificity and speed of the detection and enumeration of indicator bacteria are essential, it can be predicted that in the next couple of years, utilization of the cultivation methods based on enzymatic reactions for *E. coli* and coliform bacteria enumeration from water will replace conventional lactose-based methods. This change is being supported by the International Organization for Standardization which is preparing revisions of the conventional standard methods, including the revision of the *E. coli* and coliform bacteria reference method (ISO 9308-1, 2000).

Despite the several limitations associated with the use of bacterial indicators for waterborne pathogens, their use in day-to-day monitoring of water quality is well justified and will remain so in the future. There has been significant methodological progress in pathogen analysis but many of the pathogen detection methods are still time consuming and expensive, and pathogens are likely to be present only at low numbers in large volumes of drinking water. Pathogenic bacteria, protozoa and viruses should be analysed, however, if tap water is believed to be the source of an outbreak of enteric disease.

Campylobacter have rather rarely been detected directly from water samples in waterborne outbreak investigations. The isolation of *Campylobacter* was successful only in two out of the six outbreaks where the presence of *Campylobacter* in water was analysed out of a total of nine waterborne *Campylobacter* outbreaks as reviewed in this study. Even though the non-detection might be related to the long lag between exposure and initiation of microbiological studies or to complete lack of

direct pathogen analysis, there is a need for simple concentration techniques for large quantities of water, and for more sensitive, more specific and quicker *Campylobacter* detection methods. In this study, a portable microbial enrichment unit for *Campylobacter* cultivation was tested. In the future, even greater efforts should be expended to the development of inexpensive and rapid methods and of portable instruments for testing of microbial water quality that can survive repeated transport and use in the field.

8 Conclusions

Faecal indicator bacteria were detected in 10% of the groundwater samples analysed in the study. The main on-site hazards to water safety at small community water supplies included inadequate well construction and maintenance, an insufficient depth of the protective soil layer and bank filtration. The identified hazards did not mean that water was contaminated at the moment of sampling. Improved site specific identification of hazards causing potential health risks is needed, but a low risk estimate does not completely guarantee water safety. As a preventive measure, the upgrading of the water treatment processes and utilization of disinfection at small Finnish groundwater supplies are recommended.

Cultivation and molecular detection and typing methods together with on-site investigations identified the faecal contamination source of a municipal drinking water distribution system. In addition, *C. jejuni* infections in three patients were found to be associated with the contaminated water. The cause was a maintenance failure on the roof of the water storage tower together with rainfall that leached faecal material from wild birds into the water. The experience of multidisciplinary cooperation gained during the resolution of the case will be applicable to other faecal contamination cases of drinking water that might occur in the future.

More efficient and specific and less time-consuming methods for enumeration and typing of *E. coli* and coliform bacteria from non-disinfected water as well as for detection of *Campylobacter* were found in the study. These improvements in methodology for analysis of the faecal bacteria from water might promote public health protection as they could be anticipated to result in very important time savings and improve tracking of faecal contamination source in waterborne outbreak investigations.

The coliform bacteria counts on Colilert®, Chromocult® coliform agar and chromogenic *E.coli*/coliform medium were on average higher, ranging from -21 % to +85 % when compared to the reference medium. The amount of background microbiota affected the results, increasing the need for additional confirmation tests. The alternative media had, on average, lower *E. coli* counts than the reference medium, but this was probably due to false positive results occurring on reference medium and false negative results on alternative media.

An efficient *Campylobacter* enrichment and rapid five hour detection and species identification was achieved with the portable microbial enrichment unit (PMEU) in combination with real-time PCR detection. Moreover, the fluorescent *in-situ* hybridization (FISH) method offered a tool for the detection of *Campylobacter* in water and biofilms. The FISH analyses revealed that *C. jejuni* can survive in biofilms for weeks and that the cultivation method seriously underestimated the numbers of *Campylobacter* present in the system.

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