Occurrence of thermotolerant *Campylobacter* spp. and adenoviruses in Finnish bathing waters and purified sewage effluents

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ABSTRACT

A total of 50 Finnish bathing water samples and 34 sewage effluent samples originating from 17 locations were studied in the summers of 2006 and 2007. *Campylobacter* were present in 58% and adenoviruses in 12% of all bathing water samples; 53% of all sewage effluent samples were positive for *Campylobacter* spp. and 59% for adenoviruses. *C. jejuni* was the most common *Campylobacter* species found and human adenovirus serotype 41 was the most common identified adenovirus type. Bathing water temperature displayed a significant negative relationship with the occurrence of *Campylobacter*. One location had identical pulsed-field gel electrophoresis patterns of *C. coli* isolates in the bathing water and in sewage effluent, suggesting that sewage effluent was the source of *C. coli* at this bathing site. The counts of faecal indicator bacteria were not able to predict the presence of *Campylobacter* spp. or adenoviruses in the bathing waters. Thus the observed common presence of these pathogens in Finnish sewage effluents and bathing waters may represent a public health risk. The low water temperature in Finland may enhance the prevalence of *Campylobacter* in bathing waters. More attention needs to be paid to minimizing the concentrations of intestinal pathogens in bathing waters.

Key words | adenoviruses, bathing water, *Campylobacter* spp., faecal indicator bacteria, microbiological quality, sewage effluent

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INTRODUCTION

Faecal pathogenic bacteria, viruses and protozoa occurring in surface waters can pose a risk to public health, not only because surface waters are widely used in the production of drinking water but also because they are used for recreational purposes, such as swimming and diving. Many pathogens known to be able to cause gastrointestinal illness are found in surface waters and recreation has been associated with waterborne infections all around the world (Pond 2005).

Finland is uniquely rich in surface water, with its 10,000 lakes and ponds and rivers stretching over 20,000 km. Finnish surface waters are typically shallow and contain considerable amounts of humus. Because of their shallow depth, low discharges of rivers and rather cold climate with a long period of ice cover, surface waters are vulnerable to pollution. The Baltic Sea is also vulnerable to microbial pollution because of its shallowness and because it is really a closed, brackish basin (Finnish Environment Institute 2008).

Campylobacter species have been found commonly in many surface waters (Martikainen *et al.* 1990; Obiri-Danso & Jones 1999a; Sails *et al.* 2002; Eyles *et al..* 2003; Rechenburg & Kistemann 2009; Van Dyke *et al.* 2010; Jokinen *et al.* 2011). *Campylobacter* spp. in surface waters may originate from the faeces of birds and other animals, sewage discharges or as agricultural runoff (Atabay & Corry 1998;

Jones 2001; Broman *et al.* 2002; Rechenburg & Kistemann 2009). Schönberg-Norio *et al.* (2004) and Denno *et al.* (2009) have reported that water recreation is one risk-factor for *Campylobacter* infections. The highest risk is in children aged 5 or under (Schönberg-Norio *et al.* 2006).

Adenoviruses are divided into subgroups A to F based on phylogenetic analyses of the component proteins (Bailey & Mautner 1994). Currently there are 51 recognized human-infecting adenovirus serotypes (often referred to as human serotypes within species of HAdV-A to HAdV-F). Different species of adenoviruses cause various symptoms, such as infections of the eyes and upper respiratory tract and diarrhoea. Traditional categorization of adenoviruses suggests that there are human carriers for those serotypes which cause infections in humans. However, human-species-related serotypes have been found also in non-human animals (Enriquez 2002; Rodriguez-Lazaro *et al.* 2012) and recent sequencing-studies of animal adenoviruses (Roy *et al.* 2009) suggest that cross-species transmission is possible.

Studies investigating the occurrence of adenoviruses in surface waters have been initiated only recently as more efficient analytical methods have become available. Adenovirus isolation has previously been reported from, for example, river, creeks and lakes, including recreational beaches and samples collected downstream from a sewage plant (Jiang & Chu 2004; Pusch *et al.* 2005; Xagoraraki *et al.* 2007; Mia-gostovich *et al.* 2008; Verheyen *et al.* 2009). Adenoviruses are thought to be good indicators for faecal contamination of water (Pina *et al.* 1998). Thus adenoviruses may serve as indicators of noroviruses, which cause the majority of cases of waterborne illness in Finland (Zacheus & Miettinen 2011), but the indicator value is limited due to the fact that human populations do not necessarily excrete both viruses at the same time.

Not only untreated but also treated sewage is an important source of faecal contamination deteriorating the microbiological quality of natural waters around the world. Sewage treatment plants are usually intended to remove nutrients, such as phosphorus and nitrogen, and organic compounds but the removal of microbes is not considered as being so important (Koivunen *et al.* 2003). In Finland, microbiological quality of sewage effluent is not controlled after purification of municipal sewage (Anonymous 2006).

Heavy rain or melting waters may interfere with the operation of sewage plants and sometimes even untreated sewage has to be piped directly into surface waters, which usually results in a heavy load of microbes, including pathogens, gaining access to the surface waters. Both *Campylobacter* spp. and adenoviruses have been found in untreated and treated sewage (Jones 2001; Rechenburg & Kistemann 2009; Symonds *et al.* 2009; Fong *et al.* 2010; Schlindwein *et al.* 2010; Hellein *et al.* 2011).

Bathing water quality is monitored in European member states, including Finland, according to Bathing Water Directive 2006/7/EC (European Union 2006). The current directive lists only two microbiological indicator variables, *Escherichia coli* and intestinal enterococci, instead of the 19 physical, chemical and microbiological variables mentioned in the previous directive 76/160/EEC (European Union 1975). The values of these two indicator variables and the results of surveillance studies made during four bathing seasons are the basis for the classification of bathing waters into four categories; excellent, good, sufficient and poor. Directive 2006/7/EY (European Union 2006) obliges the Commission to report on scientific, analytical and epidemiological developments relevant to the parameters assessed in bathing water quality, and this obligation extends to detection of viruses.

In this study, the occurrence and contamination levels of *Campylobacter* spp. and adenoviruses in Finnish bathing waters and sewage effluents were investigated. The aim was to gather information about the possible role of sewage treatment plants in bathing water contamination in Finland. We also tested the functionality of indicator bacteria (*E. coli* and intestinal enterococci) listed in the bathing water directive (European Union 2006) to be able to predict the presence of *Campylobacter* and adenoviruses in Finnish bathing sites.

MATERIALS AND METHODS

Sampling and study locations

A total of 50 bathing water samples and 34 sewage effluent samples were collected at 17 different locations scattered around Central and Southern Finland (Figure 1). The
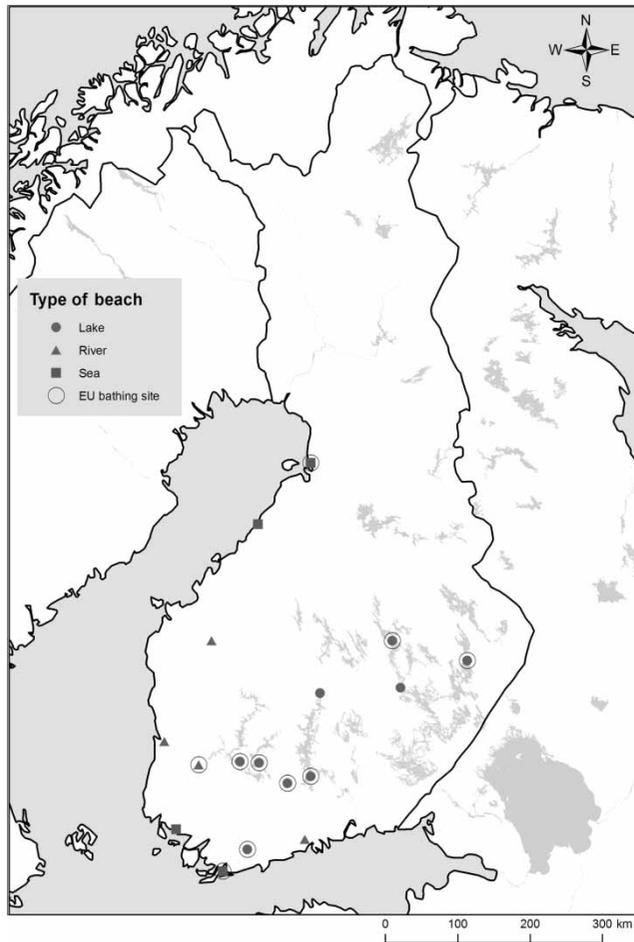
Figure 1 │ Study locations included 10 beaches which are regulated under the European Bathing Directive 2007/7/EY and seven smaller beaches. The bathing waters studied originated from lakes (*n* = 9), rivers (*n* = 4) or from the Baltic Sea (*n* = 4). The distances between bathing sites and sewage discharge sites ranged from 270 m up to 20 km. In 16 out of 17 locations, the distance was less than 5 km.

sampling locations were selected randomly, and the Geographic Information System was utilized in order to determine the distance between bathing site and the nearest sewage discharge site in the same drainage basin. Type of beach and distance between bathing site and sewage discharge site in a straight line is shown in the Table 1. Samples were collected during two consecutive summers, 2006 and 2007, during the best swimming season in Finland.

Six-litre bathing water samples were taken from every location (*n* = 17) in June and August 2006 and in June 2007. In the summer of 2007, 16 out of 17 bathing water samples were re-evaluated. Two-litre sewage effluent samples were taken in June in both years from every

location. The samples were immediately transported to the laboratory, stored at $3 \pm 2\,°C$ and analysed as soon as possible, preferably within 24 h.

Analysis of *Esherichia coli* and intestinal enterococci

E. coli and intestinal enterococci counts were analysed by filtering 10 and 100 ml aliquots of bathing water samples through 0.45-μm pore size membrane (Millipore, Bedford, USA) and from serial dilutions of sewage effluent samples on Chromocult® Coliform Enhanced Selectivity (CC-ES) agar (Merck, Darmstadt, Germany) and on Chromocult® Enterococci (CE) agar (Merck). Media were incubated at $36 \pm 2\,°C$ for 21 ± 3 h. Dark blue to violet colonies in CC-ES medium were counted as *E. coli* and red colonies in CE medium were considered as intestinal enterococci.

Analysis of thermotolerant *Campylobacter*

Thermotolerant *Campylobacter* spp. (*C. jejuni*, *C. coli*, *C. lari* and *C. upsaliensis*) were analysed semi-quantitatively following the principles in the standard ISO 17995:2005 (Anonymous 2005). The analysed bathing water sample volumes were 10, 100 and 1,000 ml, and for the sewage effluent samples volumes were 0.1, 1 and 10 ml (Pitkänen *et al.* 2009). The samples were filtered through membranes with 0.45 μm pore size (Millipore, Bedford, USA), which were then placed into the enrichment broths. In case of volumes less than 10 ml, samples were inoculated directly into the broths. The enrichment for bathing water samples was performed in Bolton and Preston broths at $37 \pm 1\,°C$ for 44 ± 4 h and sewage effluent samples were incubated in Preston broth at a higher temperature: at $41.5 \pm 1\,°C$ for 21 ± 3 h (Koenraad *et al.* 1995). In the case of typical *Campylobacter* growth on complete modified-charcoal cefoperazone deso-xycholate medium (Oxoid, Hampshire, UK), after incubation at $41.5 \pm 1\,°C$ for 44 ± 4 h, the cultures were confirmed to be *Campylobacter* spp. by gram staining and motility, the absence of aerobic growth, oxidase and catalase tests. The hippurate hydrolysis test was used for preliminary differentiation of *C. jejuni* from other thermotolerant *Campylobacter* spp. The *Campylobacter* spp. isolates were stored at $-70\,°C$ in nutrient broth containing 15% glycerol.

Table 1 │ Descriptive data of studied bathing sites and studied bathing water samples; type of beach, distance between bathing site and sewage discharge site as the crow flies, semiquanti-tative results of *Campylobacter* spp., concentration and detected serotypes of adenoviruses, the counts of *E. coli* and intestinal enterococci, water turbidity and temperature

Sampling location	Type of beach	Bathing site – sewage discharge site, distance (m)	Sampling time[a]	*Campylobacteria* (cfu/l)	Adenoviruses (AdV viral genomic copies/ml)	*E. coli* (cfu/100 ml)	Intestinal enterococci (cfu/100 ml)	Turbidity (NTU)	Temperature (°C)
1	Lake	270	1	1–10	ND	41	2	1.87	–
			2	1–10	ND	7	55	3.44	19.0
			3	1–10	ND	15	1	1.74	17.3
2	Lake	560	1	1–10	ND	5	8	8.67	20.0
			2	1–10	ND	15	30	5.25	20.7
			3	ND	ND	43	2	4.15	17.8
3	Lake	560	1	10–100	ND	44	30	2.15	15.7
			2	ND	ND	180	41	3.31	18.8
			3	10–100	ND	62	55	1.10	17.7
4	Lake	590	1	1–10	ND	3	2	4.62	20.7
			2	1–10	ND	76	15	6.61	19.0
			3	ND	ND	18	2	4.38	19.7
5	Lake	690	1	>100	ND	15	<1	0.81	18.9
			2	ND	ND	11	3	1.32	21.2
			3	–	–	–	–	–	–
6	Lake	690	1	ND	ND	2	110	2.24	21.2
			2	ND	8.8×10^3/AdV 40[c]	2	<1	2.26	21.0
			3	ND	ND	8	68	0.98	20.5
7	Lake	900	1	1–10	ND	5	20	1.37	20.5
			2	1–10	1.3×10^4	35	5	1.12	18.0
			3	ND	ND	17	4	1.89	20.0
8	Lake	1,400	1	1–10	ND	<1	10	1.90	15.3
			2	ND	ND	<1	<1	2.34	20.5
			3	1–10	ND	10	3	1.69	17.0
9	Lake	3,200	1	ND	ND	7	7	1.51	19.3
			2	ND	ND	55	20	1.32	21.0
			3	ND	ND	10	10	1.23	15.8
10	Sea	620	1	ND	ND	2	<1	5.07	18.0
			2	ND	ND	2	1	4.45	22.0
			3	ND	ND	5	5	1.75	20.0
11	Sea	2,100	1	1–10	6.9×10^3	10	10	10.00	17.5
			2	10–100	ND	150	51	1.61	16.0
			3	ND	ND	20	2	1.39	16.0
12	Sea	3,300	1	1–10	ND	20	50	25.34	11.0
			2	ND	ND	350	16	10.77	21.0
			3	1–10	ND	156	15	25.02	15.0
13	Sea	4,400	1	10–100	1.3×10^4/ AdV 41[c]	4,280	960	6.20	17.0

(*continued*)

Table 1 | continued

Sampling location	Type of beach	Bathing site – sewage discharge site, distance (m)	Sampling time[a]	Campylobacteria (cfu/l)	Adenoviruses (AdV viral genomic copies/ml)	E. coli (cfu/100 ml)	Intestinal enterococci (cfu/100 ml)	Turbidity (NTU)	Temperature (°C)
			2	10–100	ND	2,370	1,860	1.19	18.0
			3	10–100	ND	360	320	8.59	13.0
14	River	520	1	10–100	ND	14	20	3.99	19.0
			2	1–10	ND	2	<1	5.09	21.8
			3	1–10	ND	13	64	2.69	20.0
15	River	5,000	1	10–100	3.4×10^4/ AdV 41[b]	30	410	6.00	20.5
			2	ND	7.7×10^3	70	24	4.65	21.0
			3	ND	ND	8	9	4.64	18.0
16	River	900	1	1–10	ND	93	560	17.52	20.0
			2	ND	ND	7	36	6.66	21.4
			3	1–10	ND	6	17	24.56	21.2
17	River	20,000	1	1–10	ND	9	4	9.42	19.5
			2	ND	ND	9	17	11.20	21.5
			3	1–10	ND	8	2	5.05	17.4

ND = not detected, NTU = nephelometric turbidity unit.
[a]1 = June 2006, 2 = August 2006, 3 = June 2007.
[b]Similarity > 93%.
[c]Similarity > 96%.
– = not available.

The species identification of thermotolerant *Campylobacter* isolates from water and of DNA extracts from enrichment broth cultures was achieved by a real-time quantitative polymerase chain reaction (qPCR) coupled with restriction enzyme analysis as previously described by Pitkänen *et al.* (2008). In brief, THERM1 and THERM4 primers (Fermer & Engvall 1999) and restriction enzyme *Alu*I (New England Biolabs Inc., Beverly, USA) were used.

The comparison of *Campylobacter* isolates originating from bathing water and sewage effluent was done by pulsed-field gel electrophoresis (PFGE) using restriction enzyme *Sma*I (Roche Diagnostics, Basel, Switzerland). The result was confirmed by typing the strains which had indistinguishable *Sma*I profiles with enzyme *Kpn*I. The preparation of genomic DNA and running conditions were done according to the PulseNet standardized protocol for molecular subtyping of *C. jejuni* (www.cdc.gov/pulsenet/protocols/campy_protocol.pdf). *Campylobacter* isolates analysed with PFGE originated from four locations (4, 13, 3 and

14) in summer 2006 and from four locations in summer 2007 (8, 13, 16 and 17). In total, 51 bathing water isolates and 51 sewage effluent isolates were studied with PFGE.

Analysis of adenoviruses

Adenoviruses were analysed from DNA extracted from water samples with adenovirus specific qPCR (Jothikumar *et al.* 2005). Adenoviruses were concentrated from 500-ml bathing water samples following the principles of the method described by Gilgen *et al.* (1997) and Kukkula *et al.* (1999). For the sewage effluent samples, adenoviruses were concentrated from 600 ml with the two-phase separation method (World Health Organization 2003). Sewage effluents were mixed with two polymers; after the polymers had separated into layers, viruses were obtained by dropwise collection from the lower layer and interface, where they had accumulated. After concentration, DNA was extracted from 200-μl samples using DNA High Pure Viral Nucleic

Acid kit (Roche Oy, Finland) according to the manufacturer's instructions. The PCR-reaction mix (volume of 20 µl) included 2x TaqMan universal PCR master mix (Applied Biosystems), forward (JTVXF, GGACGCCTCG-GAGTACCTGAG) and reverse (JTVXR, ACIGTGGGGTTTCTGAACTTGTT) primers and probe (JTVXP, CTGGTGCAGTTCGCCCGTGCCA) (Jothikumar et al. 2005) and nuclease-free water (Promega). Finally 5 µl of template DNA was added. PCR conditions were the same as used by Jothikumar et al. (2005). Quantification of each group was calculated against a standard curve using a 10-fold diluted standard series with DNA of ATCC human adenovirus serotype 41 strain Dugan (VR-931) as a control. The detection limit of qPCR assays was determined from a dilution series of positive-control DNA (10^6 to 10^2 target molecules per reaction). A minimum sensitivity of 10^2 target molecules per reaction was achieved for each assay. The slope value of the adenovirus quantification curve, which also describes the PCR efficiency of the method (value $3.33 \approx 100\%$ PCR efficiency), was 3.502 and the regression coefficient was 0.97. These values were similar to those reported previously (Jothikumar et al. 2005).

The qPCR fragments obtained were sequenced with the primers JTVXF and JTVXFR using BigDye v. 3.1 terminator chemistry and analysed on an ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Sequencing was performed with the same qPCR fragment from two to five times. The sequences were compared with the adenovirus hexon gene sequences in the EMBL database with BLAST (basic local alignment search tool).

Physicochemical analysis

The temperature of bathing water samples was measured at the time of sampling. Turbidity was measured in the laboratory with Turb 555IR (WTW).

Statistical analysis

Microsoft Excel 2003 software was used for recording all individual results. Statistical analyses were performed with the Statistical Package for Social Sciences (SPSS) 15.0 for Windows. Qualitative data for the pathogen variables were used in all statistical analysis. Log10 conversions of the indicator bacteria variables were used. For results below the detection limit, a value of half of the detection limit was used.

Binary logistic regression was used to study the association between the concentration of faecal indicator bacteria and the occurrence of pathogens in the bathing waters and also association of physicochemical results and occurrence of Campylobacter spp. Differences in occurrence of Campylobacter spp. between different types of beach was studied with binary logistic regression. Sampling time was taken into account as a categorical covariate in every case and the non-parametric Mann–Whitney U test was used to support the results. Crosstabs with Fischer's exact test were used to compare Campylobacter spp. results between sampling times.

Non-parametric Kruskal–Wallis (Monte Carlo significance) and Kruskal–Wallis multicomparison test (Conover 1980) were used to study associations between the type of beach and the concentrations of indicator bacteria and physicochemical parameters of bathing water. For turbidity, the log10-conversion of values was used. Univariate analysis of variance was used to determine if sampling time had any effect on results. Kruskal–Wallis was also used to compare indicator bacteria and physicochemical results between sampling times. The association of physicochemical results of bathing water and the concentrations of indicator bacteria were analysed with a linear regression model.

RESULTS

Physicochemical results

Temperature and turbidity data for bathing sites are shown in Table 1 and the the physicochemical data for different bathing water temperature ranges are shown in Table 2. In the most of the bathing water samples, the temperature was between 17 and 20 °C.

The Kruskal–Wallis test showed a significant difference in the bathing water temperature and turbidity between different types of beaches. The temperature was lower in samples taken from the sea than samples taken from rivers ($p < 0.05$), and lake samples were intermediate in temperature between sea and river samples. Bathing water turbidity was significantly lower in samples taken from

Table 2 │ The presence of *Campylobacter* spp. and adenoviruses, counts of *E. coli* and intestinal enterococci and physicochemical results in different bathing water temperature ranges

Water temperature ranges (˚C)	No. of samples	Sampling time[a]	Water temperature, median and range (˚C)	Turbidity, median and range (NTU)[b]	*E. coli*, median and range (cfu/100 ml)	Intestinal enterococci, median and range (cfu/100 ml)	*Campylobacter* spp. (% of samples positive)	Adenoviruses (% of samples positive)
10–16	8	1, 2, 3	15.5 (11.0–16.0)	2.0 (1.2–25.3)	32 (<1–360)	23 (2–320)	75	0
17–20	24	1, 2, 3	18.9 (17.0–20.0)	3.7 (0.8–17.5)	15 (2–4,280)	8 (<1–1,860)	66	13
>20	17	1, 2, 3	21.0 (20.5–22.0)	4.6 (1.0–24.5)	7 (<1–350)	17 (<1–410)	35	18

[a]1 = June 2006, 2 = August 2006, 3 = June 2007.
[b]NTU = Nephelometric turbidity unit.

lakes than in those taken from rivers ($p < 0.05$). Samples taken from the sea were more similar to the samples taken from rivers than from lakes. Univariate analysis did not indicate sampling time to have an effect on results.

The Kruskal–Wallis test also revealed that the bathing water temperature was significantly higher at the end of the summer 2006 (August) than in the early summer 2006 (June) and in the early summer 2007 (June) ($p < 0.05$). There was no significant difference between early summers 2006 and 2007.

Campylobacter spp.

Fifty-eight per cent of all bathing water samples (29 out of 50 samples) and 53% of all sewage effluent samples (18 out of 34 samples) were positive for *Campylobacter* spp. (Tables 1–4). The lowest semi-quantitative count in bathing waters was 1–10 colony forming unit (CFU)/l and the highest count was over 100 CFU/l, with a median value of 1–10 CFU/l. In sewage effluent samples, the lowest semi-quantitative count was 100–1,000 CFU/l and the highest count was over 10,000 CFU/l, with median 100–1,000 CFU/l. *C. jejuni* was the most common species found in both bathing waters and sewage effluent samples. Samples also contained *C. coli*, *C. lari* and unidentified *Campylobacter* spp. isolates.

Bathing water samples taken from three locations (locations 1, 13, 14) and sewage effluent samples taken from four locations (locations 1, 3, 8, 13) were found to be positive for *Campylobacter* spp. at every sampling time. Bathing water samples taken from three locations (locations 6, 9, 10) and sewage effluent samples taken from three locations (locations 7, 10, 12) were found to be negative for *Campylobacter* spp. at every sampling time.

Binary logistic regression revealed that bathing water temperature significantly influenced the occurrence of *Campylobacter* spp. ($p < 0.05$). *Campylobacter*-positive sites had a lower bathing water temperature than *Campylobacter*-negative sites. There was no significant association between occurrence of *Campylobacter spp.* and bathing water turbidity. The Mann–Whitney U test supported previous results. Binary logistic regression did not show the type of beach (lake, river, sea) to have an effect on the occurrence of *Campylobacter* spp. in the bathing water samples.

The *SmaI* profiles of the *C. coli* strains isolated from bathing water and sewage effluent in the beginning of summer 2006 were indistinguishable in sampling location 4 (Figure 2). In addition, the *KpnI* profiles of the strains appeared indistinguishable. A total of 22 different *SmaI* profiles were found in all the bathing water and sewage effluent samples. Nine isolates were not restricted by *SmaI*, and therefore, no PFGE result was obtained.

Crosstabs with Fischer's exact test indicated significant differences between sampling times in the occurrence of *Campylobacter* spp. in bathing waters ($p < 0.05$). The result of early summer of 2006 (14 positive sites out of 17 sites) deviated from the results of the late summer of 2006 (seven positive sites out of 17 sites) and from the early summer of 2007 (eight positive sites out of 16 sites).

Adenoviruses

In all, 12% of bathing water samples (6 out of 50 samples) and 59% of sewage effluent samples (20 out of 34 samples) were positive for adenoviruses (Tables 1–4). Early in the summer of 2006, three locations (locations 11, 13, 15) had

Table 3 | Semiquantitative results of *Campylobacter* spp., concentration and detected serotypes of adenoviruses and the counts of *E. coli* and intestinal enterococci in sewage effluent samples

Sampling location	Sampling time[a]	*Campylobacteria* (cfu/10 ml)	Adenoviruses (AdV viral genomic copies/ml)	*E. coli* (cfu/ml)	Intestinal enterococci (cfu/ml)
1	1	1–10	ND	150	90
	2	1–10	2.1×10^7|AdV 41[e]	60	20
2	1	1–10	ND	270	180
	2	ND	1.3×10^7|AdV 41[e]	570	210
3	1	1–10	ND	<10	40
	2	1–10	ND	40	100
4	1	>100	ND	40	30
	2	ND	2.3×10^6|AdV 41[c]/AdV 40[c]	80	120
5	1	1–10	ND	260	250
	2	ND	3.2×10^6	430	500
6	1	ND	ND	270	172
	2	1–10	ND	<10	<10
7	1	ND	ND	<10	20
	2	ND	2.8×10^7|AdV 41[d]	110	430
8	1	1–10	1.4×10^6/AdV 41[e]	70	70
	2	1–10	ND	270	100
9	1	1–10	1.4×10^6	280	33
	2	ND	ND	4,460	1,160
10	1	ND	3.0×10^5	<10	<10
	2	ND	5.7×10^8|AdV 41[c]	<10	20
11	1	1–10	3.7×10^6|AdV 41[c]	<10	10
	2	ND	2.2×10^6	1,090	370
12	1	ND	ND	70	36
	2	ND	1.4×10^7|AdV 40[e]	30	50
13	1	>100	2.5×10^7|AdV 41[c]	63,000	22,000
	2	> 100	1.2×10^7|AdV 41[c]	3,190	2,030
14	1	10–100	8.6×10^6|AdV 41[e]	800	340
	2	ND	1.0×10^7|AdV 41[c]	390	130
15	1	1–10	9.6×10^5|AdV 41[c]	670	150
	2	ND	7.3×10^6|AdV 40/AdV 41[b]	240	50
16	1	ND	ND	2,200	2,100
	2	1–10	1.6×10^7|AdV 41[b]	4,400	4,200
17	1	ND	ND	<10	50
	2	1–10	1.2×10^5	210	130

[a]1 = June 2006, 2 = June 2007.
[b]Similarity < 93%.
[c]Similarity > 93%.
[d]Similarity > 96%.
[e]Similarity > 99%.

Table 4 | Summary of the occurrence of *Campylobacter,* adenoviruses, *E. coli* and intestinal enterococci in sewage effluent samples

	Campylobacter spp, (% of samples positive)	Adenoviruses, (% of samples positive)	*E. coli,* median and range (cfu/ml)	Intestinal enterococci, median and range (cfu/ml)
June 2006 ($n = 17$)	65	41	150 (<10–63,000)	70 (<10–22,000)
June 2007 ($n = 17$)	41	77	240 (<10–4,460)	130 (<10–4,200)

adenoviruses in both the bathing water and sewage effluent samples. In the summer of 2007 all bathing water samples were negative. The number of adenoviruses in positive bathing water samples varied from 10^3 up to 10^4 viral genomic copies/ml and in sewage effluent samples from level 10^5 up to 10^8 viral genomic copies/ml (Tables 1 and 3). In general, the numbers detected in sewage effluents in the summer 2007 were higher than in the previous year and also higher in sewage effluent samples than in bathing water samples. Sewage effluent samples taken from five locations (locations 10, 11, 13, 14, 15) were positive at both sampling times. Bathing water samples taken from 12 locations (locations 1, 2, 3, 4, 5, 8, 9, 10, 12, 14, 16, 17) and sewage effluent samples taken from two locations (locations 3, 6) were negative for adenoviruses at every sampling time.

Sequencing of the amplified PCR fragments detected human adenoviruses serotypes 40 and 41 (Tables 1 and 3). The adenovirus serotype 41 was detected in most (62%) of the positive samples. In locations 13, 14 and 15, the same serotype occurred in both years in the sewage effluents. In locations 13 and 15, the same serotype was detected in 2006 in both bathing water and sewage effluent.

Faecal indicator bacteria

There were variations in faecal indicator bacteria results between study locations (Tables 1 and 3). Linear regression model results indicated that those bathing waters where *E. coli* counts were higher, had a lower temperature ($B = -0.107$, $p < 0.05$). This was not seen with intestinal enterococci. Bathing water turbidity values were not associated with faecal indicator bacteria results. The Kruskal–Wallis test did not show any differences in the counts of faecal indicator bacteria between different types of beaches and between sampling times.

The association between the microbiological parameters in bathing water

The association between the occurrence of *Campylobacter* spp. and adenoviruses and the counts of *E. coli* and intestinal enterococci in bathing water samples depended on some unknown factor related to sampling time when studied with binary logistic regression. However, Mann–Whitney statistical analyses indicated that counts of *E. coli* and intestinal enterococci were higher in bathing water samples where *Campylobacter* spp. or adenoviruses were present than in samples where they were absent, although results did not reach statistical significance. Moreover, Mann–Whitney statistical analyses revealed that *E. coli* and intestinal enterococci counts were higher also in samples where any of the studied pathogens were present, but similarly this result did not reach statistical significance.

DISCUSSION

The present study confirms that the pathogens studied, *Campylobacter* spp. and adenoviruses, can be isolated from bathing waters and sewage effluents in circumstances typical in Northern Europe. The counts of indicator bacteria mentioned in Bathing Water Directive 2006/7/EC (European Union 2006), *E. coli* and intestinal enterococci, did not have any significant relationship with the presence of the pathogens studied. It should be noted that only a few individual samples were taken from bathing sites and sewage discharge sites and samples were not taken at the same time from every study location. Exceptional situations like sewage overflows were not studied. For example, in sewage overflows situation the pathogen load to the reserving waters may be significantly higher than it would be in the purified sewage effluents.

Figure 2 │ *Sma*I PFGE profiles of *Campylobacter* strains isolated from bathing water and sewage effluent samples from sampling site 4 in summer 2006. Two isolates from bathing water in August 2006 were not typeable by *Sma*I. There were identical *C. coli* isolates in bathing water samples and sewage effluent samples in June 2006 (*Sma*I profile C). The rest of isolates were *C. jejuni*. Standard = *Salmonella* Braenderup H9812.

Our results support previous findings that *Campylobacter* spp. are frequently isolated from aquatic environments (Koenraad *et al.* 1997; Jones 2001; Savill *et al.* 2001; Hörman *et al.* 2004; Van Dyke *et al.* 2010; Jokinen *et al.* 2011). In our study, 58% of the analysed bathing water samples contained *Campylobacter*. The normal habitat of *Campylobacter* is the intestine of warm-blooded animals and birds (Ketley 1997; Abulreesh *et al.* 2006) and faecal contamination of water may originate not only from wild birds and animals but also from domestic animals, sewage effluents and agricultural runoffs (Koenraad *et al.* 1994; Atabay & Corry 1998; Obiri-Danso & Jones 1999b; Jones 2001; Broman *et al.* 2002; Abulreesh *et al.* 2006; Rechenburg & Kistemann 2009). All bathing water samples and sewage effluent samples taken from locations 1 and 13 were positive for *Campylobacter*. It is known that in the seawater at location 13, there is a problem with gulls, which commonly carry *Campylobacter* spp. in their faeces (Pitkänen *et al.* 2008). The direct distance between beach (<100 users per day) and sewage treatment plant (receiving sewage from ca. 6,500 inhabitants) was about 4,500 m. At the lake water site 1, the distance between beach (<100 users per day) and sewage treatment plant (serves ca. 130,000 inhabitants) was only about 300 m in a straight line. The shorter distance may pose a greater risk of sewage contamination.

In our study we investigated only sewage effluent as a possible source of *Campylobacter* in bathing water. Only one location (location 4) had identical *C. coli* PFGE patterns in the isolates from bathing water and sewage effluent samples taken at the beginning of summer 2006. This result highlights the fact that *Campylobacter* spp. have a wide range of host animals in addition to humans and there are many different genotypes circulating in aquatic environments (Koenraad *et al.* 1994; Ketley 1997; Obiri-Danso & Jones 1999b; Abulreesh *et al.* 2006; Mullner *et al.* 2010).

Key factors affecting the survival of *Campylobacter* in aquatic environments include temperature, light, biotic interactions, nutrient concentrations and oxygen (Thomas *et al.* 1999). Since samples in our study were taken only in the summer, values in different seasons cannot be compared. The reports from Thomas *et al.* (1999), Rollins & Colwell (1986) and Wilkes *et al.* (2011) support our finding that the occurrence of *Campylobacter* is higher when the

water temperature is lower. Rodríguez & Araujo (2012) have also reported that an increase in water temperature and sunlight accelerates Campylobacter inactivation. Moreover, Nakari et al. (2010) have reported that the number of domestically acquired Campylobacter infections is much higher in summer than in winter. Although the occurrence of Campylobacter is lower in warmer bathing waters, there are more people swimming and thus exposed. In addition to low temperature, the survival of Campylobacter in aquatic environments is favoured by the absence of sunlight, i.e. protection offered by the ice cover during winter against, for example, UV-radiation (Korhonen 1993) and low numbers of autochthonous microbiota (Thomas et al. 1999). However, there are also differences in the survival capabilities of the different Campylobacter species (Korhonen & Martikainen 1991a, 1991b; Obiri-Danso & Jones 1999a). Obiri-Danso et al. (2001) have reported that C. lari survives better in aquatic environments than C. jejuni and C. coli. In our study, however, the most common species found in the bathing water and sewage effluent samples was C. jejuni, which is the most common cause of Campylobacter infections (Blaser & Engberg 2008). C. jejuni is also the species identified most frequently from surface waters (Bolton et al. 1987; Koenraad et al. 1997; Thomas et al. 1999). The occurrence of C. jejuni in water has also been associated with sewage discharges (Bolton et al. 1987).

The infective dose of Campylobacter is known to be low, for example, 500 C. jejuni cells for unpasteurized milk (Robinson 1981). Since it is estimated that adults swallow about 16 ml and children about 37 ml of water while swimming in a swimming pool (Dufour et al. 2006), the semi-quantitative results found in our study seem to point to a relatively low risk of Campylobacter infection. If the semi-quantitative result is 1,000 cfu/l, ingestion of about 0.5 litres would be needed in order to get an infectious dose. However, it should be recalled that semi-quantitative results based on ISO 17995:2005 (Anonymous 2005) are only rough estimates and culture methods probably underestimate the real number of Campylobacteria in water because of viable but not culturable cells (Lehtola et al. 2006). In addition, sampling errors have to be considered; samples were taken from only one single point on the beach at one time point.

Adenoviruses were found in some of the analysed bathing and sewage samples. Adenoviruses are thought to be good indicators for faecal contamination of water (Pina et al. 1998). In our study, this assumption is supported by the Campylobacter findings in bathing waters from the same locations as the adenovirus findings. Since the adenovirus serotypes detected in bathing water and sewage effluent were identical, it can be assumed that sewage effluent may be a contamination source for adenoviruses in the waters in the recreational bathing sites close to sewage discharge sites.

There are 51 identified human adenovirus serotypes. Health outcomes attributed to adenovirus infection include both enteric-related illnesses and respiratory system and eye infections. Serotypes 40 and 41 form a unique subgroup, which has been associated with gastroenteritis especially in children (Fong & Lipp 2005; Jiang 2006). The first waterborne enteric disease outbreak in Finland attributed to adenoviruses was reported in a Finnish municipality in April 1994. Some 1,500–3,000 people had symptomatic acute gastroenteritis (Kukkula et al. 1997). So far there have not been any reported adenovirus outbreaks related to recreational waters in Finland. The infective dose of adenoviruses is low; only 1–100 virus particles (WHO 2007). Some studies have shown that adenovirus serotypes 40 and 41 survive longer than faecal indicator bacteria in sewage and the environment (Enriquez et al. 1995). Adenoviruses also seem to be more persistent and present in greater numbers than enteroviruses, for example, in sewage (Fong & Lipp 2005) and that is why they might be good indicators of human contamination. Adenoviruses have been detected in a wide variety of aqueous environments including bathing waters and sewage (Pusch et al. 2005; Xagoraraki et al. 2007; Miagostovich et al. 2008; Symonds et al. 2009). The occurrence and more specifically the abundance of enteric viruses in freshwaters can vary extensively, one factor being the source of the contamination (Pusch et al. 2005; Xagoraraki et al. 2007). Among other influences, host excretion, water temperature, susceptibility to sunlight inactivation and virus attachment to suspended solids control occurrence and survival of adenoviruses (Fong & Lipp 2005).

The adenovirus hexon gene is variable across the area detected by qPCR and the different adenovirus serotypes share sequence homology of between 81 and 92%. Since adenovirus human serotypes 40 and 41 (F species) shares

a similarity of 92% in the hexon gene, a 93% similarity cut-off in BLAST searches for the hexon gene can be used as a threshold for the identification of serotype.

In most of the bathing waters analysed, the concentrations of *E. coli* and intestinal enterococci were low. However, there were some locations where the concentrations were high indicating that hygienic quality of the bathing water on those sites was poor. Despite the fact that high concentrations of indicator bacteria were associated with the occurrence of the pathogens analysed, thermotolerant *Campylobacter* species and adenoviruses were also found in some of the sites where the concentrations of indicator bacteria were low. The evaluation of the results is hindered because there were very few adenovirus-positive samples. However, the non-significant relationship between indicator bacteria and faecal pathogens seems to be a typical finding (Savichtcheva & Okabe 2006).

As mentioned earlier, conditions in water are important in survival of microbes, for example, temperature for Campylobacter survival (Thomas *et al.* 1999). Although, water temperature and turbidity were different depending on the type of beach, there was no consistent relation between the type of beach (lake, river, sea) and presence of studied microbes.

CONCLUSIONS

The present study provides further evidence for the fact that *Campylobacter* can frequently be isolated from bathing waters and sewage effluent. Sewage effluent may be one source of *Campylobacter* spp. in bathing waters in addition to many other possible sources. Adenoviruses seem to be more common in sewage effluent than in bathing waters, which highlights their value as indicator for human faecal contamination of water. The presence of the traditionally employed faecal indicators, *E. coli* and intestinal entero-cocci, did not exhibit any significant relationship with the presence of *Campylobacter* spp. and adenoviruses. The variation in pathogen results between the two years may indicate that factors such as variability of the environmental conditions or the variability of pathogen load and the treatment efficiency of sewage purification process together affect the pathogen concentrations and subsequently how

large the public health risk caused by the pathogens might be. More attention needs to be paid to minimizing the concentrations of intestinal pathogens in bathing waters and in the most important contamination sources such as sewage effluents. If one wishes to obtain better understanding of the association between indicator and pathogenic microbes in water, then more data need to be collected on the occurrence, sources and survival of microbes in natural aqueous environments.

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