Short-term microbial release during rain events from on-site sewers and cattle in a surface water source

Johan Åström, Thomas J. R. Pettersson, Georg H. Reischer and Malte Hermansson

ABSTRACT

The protection of drinking water from pathogens such as *Cryptosporidium* and *Giardia* requires an understanding of the short-term microbial release from faecal contamination sources in the catchment. Flow-weighted samples were collected during two rainfall events in a stream draining an area with on-site sewers and during two rainfall events in surface runoff from a bovine cattle pasture. Samples were analysed for human (BacH) and ruminant (BacR) *Bacteroidales* genetic markers through quantitative polymerase chain reaction (qPCR) and for sorbitol-fermenting bifidobacteria through culturing as a complement to traditional faecal indicator bacteria, somatic coliphages and the parasitic protozoa *Cryptosporidium* spp. and *Giardia* spp. analysed by standard methods. Significant positive correlations were observed between BacH, *Escherichia coli*, intestinal enterococci, sulphite-reducing *Clostridia*, turbidity, conductivity and UV254 in the stream contaminated by on-site sewers. For the cattle pasture, no correlation was found between any of the genetic markers and the other parameters. Although parasitic protozoa were not detected, the analysis for genetic markers provided baseline data on the short-term faecal contamination due to these potential sources of parasites. Background levels of BacH and BacR makers in soil emphasise the need to including soil reference samples in qPCR-based analyses for *Bacteroidales* genetic markers.

Key words | *Bacteroidales*, bifidobacteria, flow-weighted sampling, microbial risk analysis, rainfall events, water quality

Johan Åström (corresponding author)
Tyréns AB, Lilla Badhusgatan 2,
SE-411 21 Gothenburg,
Sweden
E-mail: *johan.astrom@tyrens.se*

Johan Åström
Thomas J. R. Pettersson
Water Environment Technology,
Chalmers University of Technology,
SE-412 96 Gothenburg,
Sweden

Georg H. Reischer
Research Group Environmental Microbiology and Molecular Ecology,
Institute of Chemical Engineering,
Vienna University of Technology
and
Interuniversity Cooperation Center Water and Health, Gumpendorfer Straße 1a/166,
A-1060 Vienna,
Austria

Malte Hermansson
Department of Chemistry and Molecular Biology, Microbiology, University of Gothenburg,
SE-405 30 Gothenburg,
Sweden

INTRODUCTION

Peak concentrations of pathogens in a drinking water source due to short-term microbial release represent a risk for waterborne infections among drinking-water consumers. Sources for human pathogens include on-site sewers and municipal sewage systems in urbanised areas such as treated wastewater effluent and sewer overflows. Zoonotic pathogens from animals may be distributed as a diffuse leakage from cattle pastures and agricultural activities in the catchment. Compared with human faeces, the risk related with animal faeces is considered to be low (Field & Samadpour 2007), but waterborne outbreaks have been linked to animal pathogens such as *Campylobacter* spp. and the parasites *Giardia* spp. and *Cryptosporidium* spp. (Craun *et al.* 2004; Hrudey & Hrudey 2004). In the period 2006–2010, the number of reported cases in Sweden of human cryptosporidiosis was in the range of 100–390 and, for giardiasis, 1,210–1,530 persons per year (SMI 2011). The zoonotic potential of animal pathogens such as *Cryptosporidium* and *Giardia* is highly dependent upon genotype (Cotruvo *et al.* 2004), while standard methods for the analysis of parasites only provide a total number of (oo)cysts in a certain volume (ISO 2006). The detection and quantification of faecal contamination from various hosts and sources in the catchment support the prioritisation of mitigation measures in the catchment to reduce the shedding of pathogens in a drinking water source.

doi: 10.2166/wh.2013.226

Several microbial faecal source tracking (MST) methods have been developed to overcome the limitation in traditional faecal indicators to separate faecal matter from different hosts, which may be utilised to characterise microbial events. MST methods include the detection of host-specific markers by quantitative polymerase chain reaction (qPCR). Strictly anaerobic members of the order *Bacteroidales* have been increasingly employed (Kildare *et al.* 2007; Stapleton *et al.* 2009; Reischer *et al.* 2011; Sokolova *et al.* 2012a). Before MST methods can be generally used, the applicability of these methods in different geographic areas needs to be validated since environmental factors may cause local bacterial adaptation and evolution, implying DNA sequence divergence and erroneous qPCR results (Hagerdorn *et al.* 2011). Santo Domingo *et al.* (2007) encourage the application of MST as a complement to traditional faecal indicators and physico-chemical parameters and the comparison with hydrological characteristics in the catchment.

Surface runoff of pathogens from agricultural land to water is influenced by a variety of factors, such as rainfall intensity, vegetation type, soil type and land slope (Ferguson *et al.* 2003). Rainfall simulators have been used to determine the transport of animal pathogens on vegetated and non-vegetated soil in chambers (Atwill *et al.* 2002; Trask *et al.* 2004) and in field studies (Collins *et al.* 2005; Ferguson *et al.* 2007). Ferguson *et al.* (2007) assessed the transport of *Cryptosporidium parvum* oocysts, *Escherichia coli* and PRD1 bacteriophage seeded into artificial bovine faecal pats during simulated rainfall events. It was reported that rainfall events mobilised 0.5–0.9% of the *C. parvum* oocysts from faecal pats and transported them a distance of 10 m across the bare soil sub-plot, while the transport at vegetated sub-plots was substantially lower. Field measurements during rainfall events are needed to characterise the mobilisation of pathogens under natural condition from cattle pastures as well as from on-site sewers. In the context of microbial events, MST analysis using host-specific genetic markers of the order *Bacteroidales* may be used as a complement to traditional microbial analyses, providing additional support for ruminant- or human-specific release of faecal matter into the water source.

From Lake Rådasjön on the Swedish west coast, drinking water is provided to potentially more than half a million people. The parasites *Cryptosporidium* spp. and *Giardia* spp. have been detected at low levels in regular raw water monitoring, indicating a potential risk for infection particularly in the case of suboptimal drinking water treatment. Point sources include on-site sewers and the municipal sewer systems that overflow during heavy rainfalls, while diffuse sources include nearby cattle and horse pastures with surface runoff to the lake. Recently, a microbiological module coupled to a three-dimensional hydrodynamic model was used to assess the transport of *Bacteroidales* genetic markers BacH and BacR and potential pathogens from selected contamination sources to the raw water intakes (Sokolova *et al.* 2012a, 2012b). While hydrodynamic modelling accounts for the dynamic change in, for example, wind, water temperature and flows into the lake, the rapid water quality changes at single contamination sources due to rainfall events needs to be addressed.

The aim of this study was to characterise the short-term microbial impact from on-site sewers and a waterfront cattle pasture during rainfall events, using flow-weighted sampling. The samples collected during the events were analysed for human and ruminant *Bacteroidales* genetic markers (BacH and BacR) and sorbitol-fermenting bifidobacteria (SFB), in addition to faecal indicator bacteria, somatic coliphages and physico-chemical parameters. Samples were also analysed for the parasites *Giardia* spp. and *Cryptosporidium* spp. Furthermore, sewage, soil, manure and sediment were analysed as reference samples to determine levels of BacH and BacR in faecal matrices and potential background in non-faecal environmental matrices.

MATERIALS AND METHODS

Study area

Lake Rådasjön has a surface area of close to 2 km^2 and a catchment area of 14.9 km^2, being part of the larger catchment area of the river system Mölndalsån (268 km^2). Further downstream, Mölndalsån joins the river Göta älv in Gothenburg before entering the Kattegat Sea. The area surrounding the lake was established as a nature reserve in 2006, allowing pasturing cattle and horses around the lake to improve the value of the ecosystem. Several households with on-site sewers are located north of the lake. A general

description of the lake including a map is provided in Sokolova *et al.* (2012a).

Sampling

Event samplings

Event-based samplings during rainfalls was performed in a stream (site 3, 12°3′52,391″ E 57°40′11,28″ N), located 100 m upstream the lake shoreline and draining a catchment of 0.6 km^2 including horse pastures and several on-site sewers, on 15–17 June (event 3:I) and 15–16 October (event 3:II) 2008. Event-based samples were also taken at a bovine cattle pasture (site 17, 12°5′25,485″ E 57°39′49,94″ N), on 9 November (event 17:I) and 11–13 November (event 17:II) 2008. Surface runoff from the cattle area, with a catchment of 0.0045 km^2, was collected using perforated drainage pipes (ø 120 mm) with half the diameter submerged in the ground. To take representative samples, three drainage pipes with lengths of 10, 6.5 and 3.5 m, respectively, positioned in three directions (about 150°), were used to collect surface runoff into a conduit from where samples were collected. Six young cattle were kept in the studied area during the summer season until one month prior to the sampling occasions. Satellite imageries of the catchment areas are provided in Figure 1.

An ISCO 6712 sampler (Teledyne Isco™, Inc., NE, USA) equipped with 24 bottles was used for automated flow-weighted sampling. The sampler was programmed to sample when exceeding a base flow that was determined on-site before each rain event. The sampler was programmed to collect water samples representing the runoff volume expected in the forecasted rain, as guided by expected duration and intensity. Subsamples were automatically collected into composite samples (see Table 1 for details) until all composite bottles were filled. One sample of 10 L was prepared for parasite analysis, with representative volumes from the composite samples taken during the event. Water flow was registered with an ISCO 750 Area Velocity Flow module (site 3) and an ISCO 730 Bubbler Flow module (site 17). Rain depth was measured with a tipping bucket ISCO 674 Rain Gauge (Teledyne Isco™, Inc.). A WAVECOM Modem serviced by a FLOWLINK data retrieval package (Teledyne Isco™, Inc.) enabled remote data collection from the sampler.

The sampling bottles (1 L polypropylene bottles) and the tubes connecting to the sampler were thoroughly washed before use at 80 °C for 10 min. Samples were kept below 4 °C by filling the bottom of the sampler unit with ice prior to each event. The temperature inside and outside the sampler was monitored by loggers (Thermistors, type 10 k NTC, Gemini Data Loggers Ltd, UK). During all

Figure 1 | Satellite imageries of the sampling areas with sampling point, streams and height curves indicated.

Table 1 | Catchment areas and description of rainfall event sampling performed in 2008

	Site 3 (Stream)		Site 17 (Cattle pasture)	
	Event 3:I	Event 3:II	Event 17:I	Event 17:II
First sample fill	June 15 06:14	Oct. 15 15:22	Nov. 9 13:07	Nov. 11 12:40
Last sample fill	June 17 06:11	Oct. 16 05:12	Nov. 9 17:17	Nov. 12 19:03
Number of composite samples[a]	7[b] (8)	8 (8)	8 (8)	8 (8)
Volume between each subsample (m^3)	7.5	15	0.09	1.0
Precipitation (mm)	21	7	6	9
Antecedent dry period (days)	43	2	1	0

[a]Numbers in parenthesis denote number of subsamples in each composite sample.
[b]Composite samples nos. 4 and 5 from this event were pooled prior to analysis.

events, the sampling tube was rinsed three times prior to each subsample collection to minimise contamination between the sampling cycles. To evaluate the extent of carry-over in the sampling, a series of sterile deionised water, undertreated sewage and sterile deionised water were sampled and analysed for total coliforms, *E. coli* and the human genetic marker BacH (see below). The carryover due to contamination in the sampling tubes from this test was on average 0.3% for one sampling cycle.

Reference samplings for sewage, manure, soil and sediment

Samples of untreated sewage, representing dry-weather flow during a single day ($N = 4$), were collected at the inlet of a sewer pumping station south of the lake using sterile polyethylene bottles. A composite sample, consisting of 500 mL from each of these sampling occasions (2 L in total) was prepared for analysis of parasites. Fresh bovine manure samples from ten animals of different age and sex, from the same herd as was represented at site 17, was collected, pooled and diluted (60 g manure in 300 mL sterile deionized water) prior to further analysis ($N = 3$). Pristine soil samples were collected from three sites selected at hillocks around the lake at two different occasions. A core of soil at 1–5 cm depth was sampled in duplicate at each site (25 g soil, 12 samples in total). Five composite sediment samples, each consisting of 15 subsamples from an area of about 0.5 m^2, were collected along the stream upstream site 3, at distances 0, 120, 320, 330 and 520 m from the lake shoreline (Figure 1). For each composite sample, top sediment layers were collected with a sterile 50 mL syringe into one sterile glass bottle. All samples were kept at +4 °C during transport to the laboratory.

Microbial analyses

Water samples were analysed for indicator bacteria within 6 h from sampling using standardised methods. For total coliforms and *E. coli* the Colilert™ Quantitray method was used (IDEXX Laboratories, Inc., ME, USA). Analysis for intestinal enterococci and sulphite-reducing *Clostridia* were through the standard membrane filtration methods (ISO 1986, 2000a), but without preheating in the latter. Somatic coliphages were analysed by a plaque assay within 24 h from sampling using *E. coli* ATCC 13706 as the host strain (ISO 2000b). *Cryptosporidium* spp. and *Giardia* spp. (oo)cysts were analysed within 4 days from sampling by a standard method (ISO 2006) including membrane filtration, immunomagnetic separation (IMS), staining and enumeration with microscopy.

For the analysis of bifidobacteria, water was filtered through 0.45 μm filters (Sartorius, France) within 24 h from sampling, followed by anaerobic incubation at 37 °C for 44 h on Human Bifid Sorbitol (HBSA) agar (Mara & Oragui 1983). Yellow colonies, due to the fermentation of sorbitol, were classified as presumptive SFB (ø > 0.5 mm). In the further verification, isolates that did not grow on reinforced clostridial agar (RCA, incubated aerobically at 37 °C for 24 h) were classified as SFB.

DNA extraction

Water, sewage, sediment and diluted manure and soil were filtered through Isopore 0.2 μm polycarbonate membrane filters (Millipore™, Bedford, MA, USA) within 24 h of sampling. As large a volume as possible during 2 h was filtered (up to 500 mL for each sample), and the remaining unfiltered volume was decanted by a sterile pipette. The filters were immediately frozen and stored up to one month at −20 °C and at −70 °C for longer periods until DNA extraction. The extraction was performed as described by Griffiths *et al.* (2000), with the following exceptions. Isopropanol was used for DNA precipitation and centrifugation at each step was performed at 13,000 rpm for 5 min. The washed and air-dried pelleted DNA was resuspended in 50 μL 10 mM Tris–EDTA buffer (pH 8.0). DNA extraction of the solid soil samples was undertaken using the PowerSoil™ DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA) following the standard procedure. Negative controls for each round of extraction were performed to exclude contamination of DNA during the processing. Extracted samples were stored at −70 °C until qPCR analyses.

qPCR procedures

The qPCR analyses were performed as previously described for human (BacH) and ruminant (BacR) *Bacteroidales* genetic markers (Sokolova *et al.* 2012a), using the same primers and probes as in the original description (Reischer *et al.* 2006, 2007). Briefly, the qPCR analyses were performed on an iCycler iQ 5 (Bio-Rad, Hercules, CA, USA). The annealing temperatures used were 61 °C for BacH and 60 °C for BacR. Plasmid standards for the qPCR analyses of BacH and BacR were diluted ten-fold (minimum five dilution steps) in an unspecific DNA background (5 ng/μL poly d(I-C) solution) and used for the quantification in all qPCR runs. As the *Bacteroidales* cells carrying these markers are not cultivated and copy numbers cannot be directly related to cell numbers, marker equivalents (ME) values were used, which signify the copy number after possible DNA losses during extraction. Care was taken to treat all samples equally and all reactions were performed in duplicate and on several dilutions of each sample. For each sample the dilution that resulted in the highest qPCR result was selected for quantification (Reischer *et al.* 2006, 2007).

Physico-chemical analyses

Samples from the events were analysed for physico-chemical parameters, including turbidity (ISO 1999), conductivity (ISO 1994) and UV254 following standard procedures.

Statistical analyses

Data from microbial analyses were presented on a logarithmic scale ($\log_{10}$) in the results. To illustrate the variation over time in the flow-weighted samples, data for the faecal indicators and physico-chemical parameters were plotted against runoff volume. Site-specific correlation coefficients were calculated for parameters analysed during the rainfall events after grouping data from both events at each location. Due to the non-linear nature of the parameters, the Spearman rank correlation coefficients were calculated, and statistical significance was assessed at the 95% confidence level (Spearman's rho, two-tailed, $\alpha = 0.05$).

RESULTS

Rainfall event sampling

The first rainfall event (3:I), sampled from the stream at site 3, was preceded by a 1.5 month dry weather period (Table 1). As illustrated in the hydrograph, a rapid runoff followed this intensive rainfall (21 mm) with a maximum flow of 15 L/s (Figure 2(a)). Event 3:II on the other hand, with a smaller rain depth (7 mm), followed a rainy period, with one dry weather day preceding the event. A smooth increase of the stream flow was observed, with a peak flow of 17 L/s towards the end of the event (Figure 2(b)). The levels of faecal indicators during the events 3:I and 3:II are presented in Figure 2(c) and (d). The highest levels in 3:I were observed for the BacH (6.3×10^7 ME/100 mL), followed by *E. coli*, intestinal enterococci, somatic coliphages, the BacR marker and sulphite-reducing *Clostridia*. The highest concentration of *E. coli* (3.3×10^4 most probable number (MPN)/100 mL) was observed at the beginning of the event, while *E. coli*

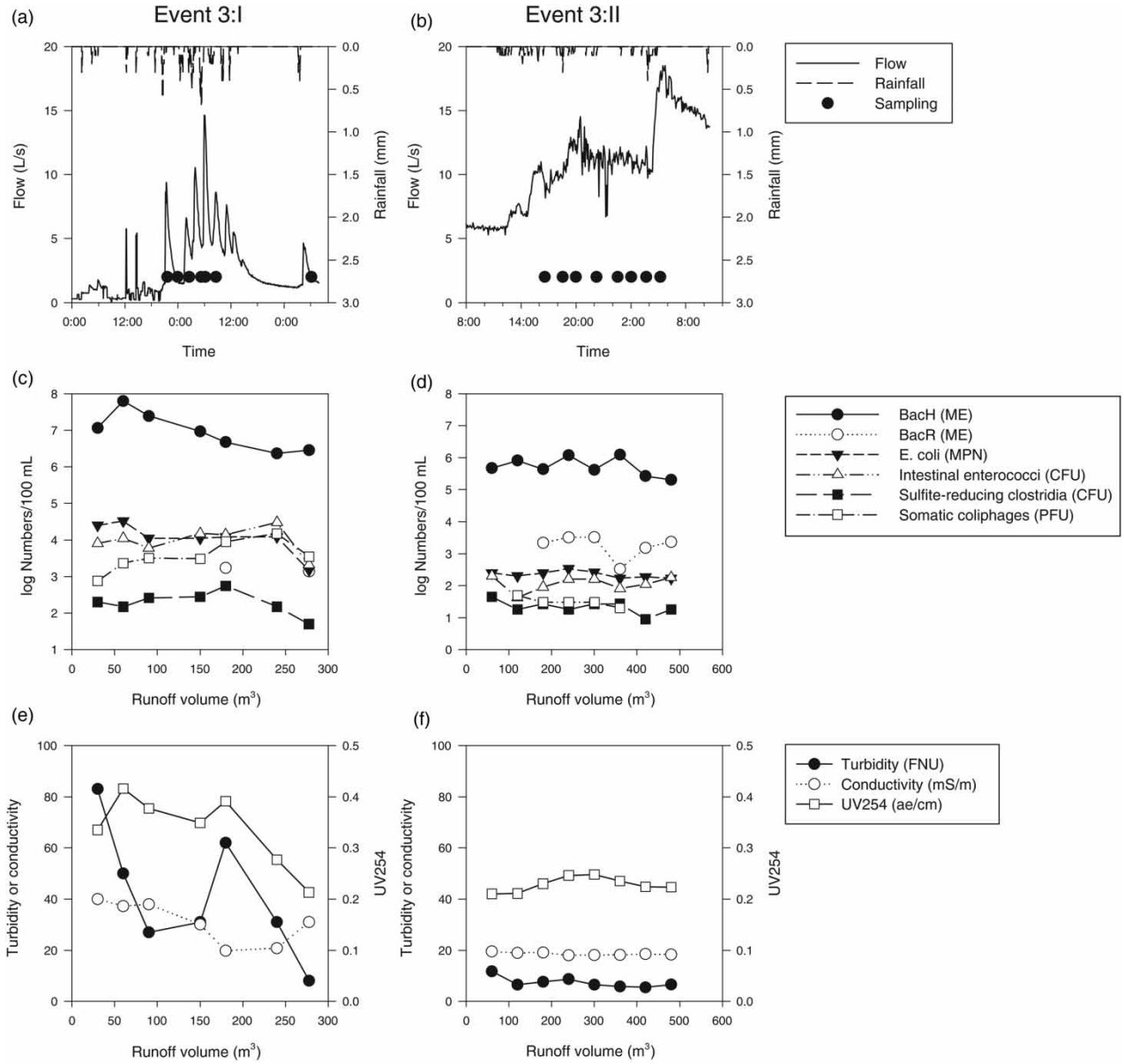

Figure 2 | Rainfall events with hydrological response and collection of composite samples during event 3:I (a) and 3:II (b) with data for faecal indicators ((c) and (d)) and physico-chemical parameters ((e) and (f)) for the two events. ME = marker equivalents.

was in the same range as intestinal enterococci in the later samples. For the somatic coliphages, increasing concentrations were similarly observed at the end of the event. In event 3:II, the highest levels were observed for BacH, followed by BacR and *E. coli* with a maximum of 340 MPN/100 mL. High and varying levels were observed for all physico-chemical parameters in 3:I, declining during the event with the exception of turbidity, while low and stable levels were observed during 3:II (Figure 2(e) and (f)). SFB were detected in only one of the fifteen samples from site 3 and at the level 2.2×10^3 CFU/100 mL.

During event 17:I, with a rainfall of 6 mm after a week with sporadic and low-intensity rainfalls (Table 1), a maximum flow of 0.7 L/s was registered (Figure 3). For 17:I, the sampling represented the beginning of the runoff hydrograph, with five samples collected before the peak and three

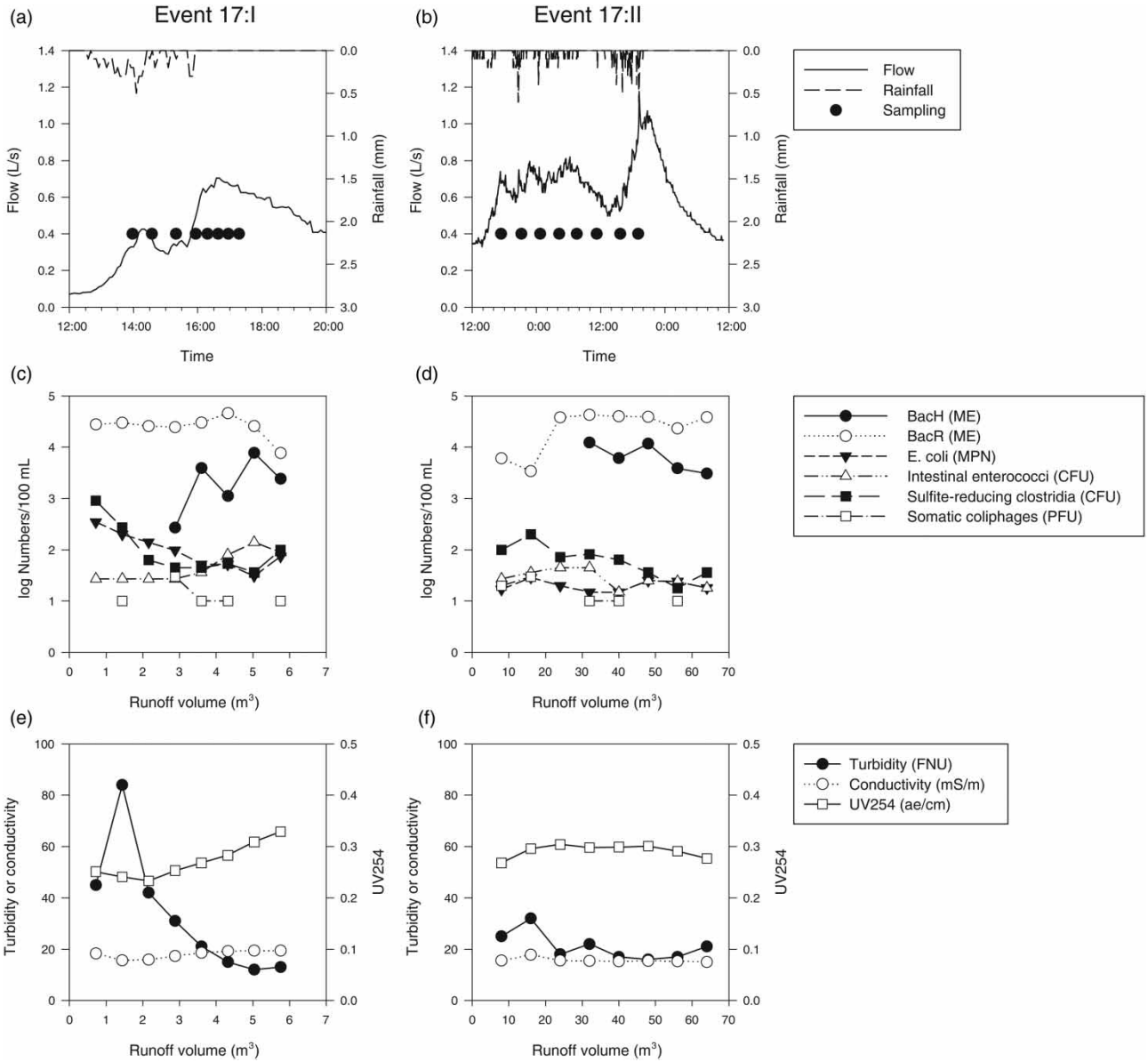

Figure 3 | Rainfall events with hydrological response and collection of composite samples during event 17:I (a) and 17:II (b) with data for faecal indicators ((c) and (d)) and physico-chemical parameters ((e) and (f)) for the two events. ME = marker equivalents.

samples representing the peak and the receding limb (Figure 3(a)). Event 17:II followed a wet period with 9 mm rainfall on 10–11 November, 2 days after 17:I. For 17:II, representing a rainfall of 16 mm, a hydrograph consisting of four separate maxima was measured, where the highest flow (1.2 L/s) was observed as the final peak (Figure 3(b)).

For event 17:I, the highest microbial concentrations were observed at the beginning of the event followed by a decay, except for the BacR marker, which was detected at high levels throughout the event. Higher initial levels were found for sulphite-reducing *Clostridia* (910 CFU/100 mL) compared with *E. coli* (350 MPN/100 mL). For 17:II, an increase was observed for the BacR marker, while the other indicators were detected at low levels comparable to those determined for 17:I. For 17:I, the turbidity decayed after the second sample, while the

levels of UV254 increased during the event. For 17:II, the physico-chemical parameters were stable throughout the event (Figure 3(f)). SFB were not detected in 17:I or 17:II.

Correlations analyses from event samplings

Positive and strong correlations were observed for the stream (events 3:I and 3:II) between *E. coli* and all parameters, except BacR (Table 2). Turbidity was well correlated with all parameters, except BacR. Generally, the BacH was positively correlated to the other measured parameters, except to the total coliforms, BacR and somatic coliphages. The BacR marker was not significantly correlated to any other parameter. For the cattle pasture events (17:I and 17:II) on the other hand, only a few correlations were significant and several were negative, such as between *E. coli* and the BacH. A few positive correlations were found between the indicator bacteria and the physico-chemical parameters, such as between intestinal enterococci and UV254.

Giardia and *Cryptosporidium*

The parasites were not detected in any of the sampled events or in the sewage.

Table 2 | Spearman's correlation coefficients for site 3 (events 3:I and 3:II) and 17 (17:I and 17:II). Significant correlations ($p < 0.05$) are marked in bold

		Indicator bacteria[a]				Indicator virus[b]	Genetic markers[c]		Physico-chemical parameters[d]		
		TC	EC	IE	SRC	SC	BacH	BacR	Turb	Cond	UV254
Site 3											
	TC		**0.89**	**0.65**	0.62	**0.82**	0.60	0.29	**0.86**	0.56	–0.01
	EC			**0.82**	**0.80**	**0.68**	**0.82**	0.13	**0.90**	**0.74**	**0.77**
	IE				**0.83**	**0.79**	**0.65**	0.18	**0.86**	**0.68**	**0.65**
	SRC					**0.71**	**0.82**	–0.26	**0.84**	**0.77**	**0.70**
	SC						0.56	0.03	**0.64**	**0.60**	0.44
	BacH							–0.45	**0.77**	**0.79**	**0.72**
	BacR								0.21	–0.55	0.38
	Turb									**0.74**	**0.67**
	Cond										0.45
	UV254										
Site 17											
	TC		**0.78**	–0.16	0.25	–0.11	–0.24	–0.10	0.26	**0.56**	–0.47
	EC			–0.23	0.24	0.10	**–0.67**	–0.27	0.35	**0.66**	**–0.50**
	IE				–0.08	–0.32	0.20	0.33	**–0.68**	0.46	**0.75**
	SRC					0.19	–0.07	–0.20	**0.57**	0.24	–0.22
	SC						–0.61	–0.62	0.59	0.08	–0.29
	BacH							0.35	–0.07	–0.18	0.47
	BacR								–0.22	–0.30	0.09
	Turb									–0.13	**–0.81**
	Cond										0.04
	UV254										

[a]Total coliforms (TC), *E. coli* (EC), intestinal enterococci (IE) and sulphite-reducing *Clostridia* (SRC).
[b]Somatic coliphages (SC).
[c]Human (BacH) and ruminant (BacR) *Bacteroidales* genetic markers.
[d]Turbidity (Turb), conductivity (Cond) and UV absorbance at 254 nm (UV254).

Reference samples

The BacH marker was detected in sewage and once in bovine manure, while the BacR marker was detected in bovine manure but not in sewage (Table 3). The reference samples of soil were positive for the BacH and BacR markers, detected at similar levels (median 5.3 and 5.5 log ME/g dry-weight matter; Table 3). In the analysis for *E. coli* that was performed on the soil samples collected at the second sampling occasion ($N = 6$), all were negative (<23 MPN/g dry-weight). In the sediment samples from the stream at site 3, both BacH and BacR were detected with BacH at about 2 log-units higher levels (Table 3). *E. coli* were detected in all sediment samples (median 5.6 MPN/g dry-weight).

DISCUSSION

We have assessed the variability of microbial impact during rainfalls based on flow-weighted event samples collected at two potential sources for *Cryptosporidium* spp. and *Giardia* spp. around Lake Rådasjön. The lake is comparable to many other surface waters in Northern Europe in that the continuous faecal impact of point and diffuse sources is assumed to be low, but where sudden peak concentrations historically have resulted in waterborne outbreaks (Hrudey & Hrudey 2004). The infection risk due to faecal impact in water is determined by the prevalence of infection in humans and animals. In a study on 265 persons in Sweden infected by cryptosporidiosis, *C. parvum* was the dominant form followed by *C. hominis* and *C. meleagridis* (Insulander *et al.* 2013). For the cattle herd represented at site 17, 1 out of 10 animals was positive for *Cryptosporidium* spp. in fresh faecal pats when occasionally tested in August 2010 (unpublished data). Although other investigations in Sweden indicate that the non-zoonotic form *C. bovis* dominated among dairy herds (Silverlås *et al.* 2010), cattle around Lake Rådasjön may represent a potential risk for the drinking water, especially during rainfall.

The fact that none of the composite water samples tested positive for *Cryptosporidium* or *Giardia* demonstrates the difficulties in retrieving and quantifying these parasites in water samples. The recovery efficiency for these parasites has been reported to be highly variable and may be lower than 50%, resulting in falsely negative water samples (Hansen & Ongerth 1991; Petterson *et al.* 2007). Furthermore, the low prevalence of the parasites among humans during endemic rates of infection result in a low probability of detecting parasites from a few on-site sewers, which may explain the non-detects at site 3 and in sewage. For the microbial risk assessment, indicators such as BacH and BacR may provide baseline host-specific data on faecal contamination which do not suffer from the difficulties of detecting low levels of parasites in water. Based on the ratio of *Bacteroidales* genetic markers and model pathogens, Sokolova *et al.* (2012b) demonstrated that human faecal contamination at site 3 may significantly contribute to the pathogen levels at the raw water intake in Lake Rådasjön. Given the higher endemic prevalence of *Cryptosporidium* spp. in cattle compared

Table 3 | Human and ruminant markers and sorbitol-fermenting bifidobacteria in reference samples

	Human marker, BacH		Ruminant marker, BacR		Sorbitol-fermenting bifidobacteria, SFB	
Reference sample	**Positive (Total)**	**Median (Max)**	**Positive (Total)**	**Median (Max)**	**Positive (Total)**	**Median (Max)**
Sewage	4 (4)	8.9 (9.1)[a]	0 (4)	–	4 (4)	6.6 (6.7)[c]
Manure	1 (3)	0 (5.0)[a]	3 (3)	10.6 (11.6)[a]	NA	NA
Soil[d]	12 (12)	5.3 (5.9)[b]	12 (12)	5.5 (6.2)[b]	NA	NA
Sediment[e]	5 (5)	7.5 (8.5)[b]	2 (5)	5.3 (5.6)[b]	3 (5)	5.8 (6.5)[f]

[a]log ME/100 mL.
[b]log ME/g dry-weight matter.
[c]log CFU/100 mL.
[d]From pristine sites.
[e]From upstream site 3.
[f]log CFU/g dry-weight matter.
NA – not analysed.

with humans, site 17 was considered to be an important source contributing to the levels of parasites at the raw water intakes.

The faecal contamination and microbial impact during the rainfall events varied between the different events at both sampling sites, with a duration from a few hours (17:I, Figure 3) to 2 days (3:I, Figure 2). The positive correlation between the BacH marker, faecal indicator bacteria and physico-chemical parameters measured in the stream receiving effluent from on-site sewers (site 3) strengthen the evidence that human faecal matter is an important source at this site. Furthermore, the BacH marker and *E. coli* were observed at high levels in the sediment samples collected from this stream. For the bovine pasture (site 17), on the other hand, the BacR marker was detected at higher levels compared with BacH, and this finding was confirmed by cow pats observed adjacent to the sampling point.

Correlations between levels of faecal indicators and pathogens and *Bacteroidales* genetic markers detected with conventional and qPCR analyses have been published in previous MST studies (Savichtcheva *et al.* 2007; Reischer *et al.* 2008; Fremaux *et al.* 2009; Stapleton *et al.* 2009). Reischer *et al.* (2008) showed that 80% of the variation of faecal indicator levels during events could be explained by ruminant-specific marker levels, successfully indicating the dominance of ruminant faecal sources in that catchment. Stapleton *et al.* (2009) included qPCR analyses for the same ruminant marker (BacR) as in the present study, while another human-specific marker (HF8 cluster) was used for detection of human faecal matter. They observed a transition from human faecal influence to mixed sources followed by a ruminant dominance, the latter explained by ruminants present in the upper catchment.

Results from the present study clearly show that the correlation between the genetic markers and faecal indicators are site-specific, in that several significant correlations with faecal indicators and physico-chemical correlations were observed at site 3 but not at site 17. Sampling at site 17 mainly reflected the manure runoff from a small cattle pasture and where the runoff of the faecal matter from cow pats, accumulated during the previous grazing season, can be expected to decrease during the rainfall events. Site 3 reflects a larger catchment area with faecal impact from a number of on-site sewers, where the variable transport time to the downstream sampling site can be expected to provide a smoothing effect in the microbiological levels. Somatic coliphages, considered as an index for human enteric viruses in terms of composition, morphology and structure, were positively correlated to the indicator bacteria, turbidity and conductivity at site 3. However, the somatic coliphages were not significantly correlated to the genetic markers BacH or BacR at any site.

Event samples taken at site 3 exhibited generally high concentrations of indicator bacteria and somatic coliphages compared with site 17, indicating a continuous release of faecal matter into this stream from the on-site sewers. An extended dry period preceded event 3:1 and the rainfall during this event may have triggered a resuspension of sediment accumulated during several weeks. Resuspension has been reported to carry significant loads of microorganisms into the water (Muirhead *et al.* 2004). For the small cattle pasture (site 17), the microbial release from bovine manure was most evident at the beginning of the runoff (17:1) and considerably lower in the second event (17:II). This is in agreement with the study by Ferguson *et al.* (2007) where subsequent rainfall was reported to mobilise less than 1% of the original *Cryptosporidium* oocyst load and up to 15% of the original *E. coli* load from bovine faecal pats.

Importantly, both the BacH and BacR markers were detected in soil samples (Table 3). Six of the twelve soil samples were also tested for *E. coli* and all samples were negative. Although it is not likely that these samples were recently exposed to human or bovine faecal matter, we cannot completely rule out this possibility. Based on these results, we suggest that further investigations of presence of faecal contamination by wild animals, or an indigenous soil *Bacteriodales* community that might cause cross-reactions with BacH and BacR markers in soil, is warranted (see below). The BacR marker concentrations in 3:I (Figure 2) and in upstream sediment samples (Table 3) were several orders of magnitude lower than the BacH markers. The lack of ruminant faecal sources upstream suggests that the BacR detected in events 3:I and 3:II may result from background concentrations in soil and sediment, as has previously been reported for a karst spring catchment (Reischer *et al.* 2011). For the bovine pasture (site 17), soil may potentially be the source for the BacH markers and contributes to

the BacR marker levels. The fact that neither the BacR at site 3 nor the BacR or BacH at site 17 were correlated to *E. coli* during the events further suggests soil as a likely source. A background contribution of BacH and BacR from soil at low levels should not be disregarded, which emphasises that: (i) reference samples on soil should be included when applying any *Bacteroidales* qPCR assay for MST in new catchments; (ii) findings of *Bacteroidales* genetic markers in the absence of or uncorrelated to other indicator bacteria should be interpreted with caution; and (iii) further research is needed to develop MST assays that are not impacted by background concentrations from non-faecal matter.

With one exception, culturable SFB were not detected in any of the event samples but were found in high levels in sewage and sediment (Table 3). Although reported to be a strong method in Europe for distinguishing human from animal faeces (Blanch *et al.* 2006), the SFB analysis in the present study was complicated by a high presence of atypical colonies, implying a doubtful basis for the selection of colonies for verification (Mara & Oragui 1983). Together with the inability to follow the dynamics during the events, as a result of non-detectable levels, analysis for SFB was of low value compared with *Bacteroidales* qPCR for human MST.

CONCLUSIONS

Significant positive correlations between the host-specific human *Bacteroidales* genetic marker (BacH) and culturable faecal indicator bacteria in event water samples collected from the site affected by on-site sewers provide support for the impact of fresh faecal matter from humans. A pasture cattle site showed higher ruminant *Bacteroidales* marker (BacR) levels, but no correlations with other indicators were found. The rapid short-term variations observed under rainfall events provide baseline data for the potential event release of pathogens from streams with on-site sewers and from cattle pastures, which may be considered in microbial risk analyses for the drinking water supply. Analysis of host-specific genetic markers was found to be a valuable complement to traditional faecal indicators and can provide baseline host-specific data on faecal contamination, avoiding the difficulties of detecting low levels of parasites in water. Results from the microbial source tracking suggest that mitigation measures should be directed at the on-site sewers in the area, preferably by connecting the households to the municipal sewer system, while additional investigations are needed to characterise the risk due to zoonotic pathogens from cattle as well as wildlife and birds. The presence of BacH and BacR in pristine soil samples emphasises that when analysing for *Bacteroidales* genetic markers in new catchments, soil and sediment from the area should be investigated as reference samples.

ACKNOWLEDGEMENTS

The research presented above was funded jointly by the Swedish Water & Wastewater Association (SWWA) and the TECHNEAU project funded by the European Commission (contract 018320). Samplings and analyses were partly funded by the city of Gothenburg (bacterial indicators) and the municipality of Mölndal (parasites). We are grateful for assistance during the field sampling from Pär Olausson (municipality of Härryda) and Lars-Ove Sörman (Chalmers University of Technology).

REFERENCES

Atwill, E. R., Hou, L., Karle, B. M., Harter, T., Tate, K. W. & Dahlgren, R. A. 2002 Transport of *Cryptosporidium parvum* oocysts through vegetated buffer strips and estimated filtration efficiency. *Appl. Environ. Microbiol.* **68** (11), 5517–5527.

Blanch, A. R., Belanche-Munoz, L., Bonjoch, X., Ebdon, J., Gantzer, C., Lucena, F., Ottoson, J., Kourtis, C., Iversen, A., Kuhn, I., Moce, L., Muniesa, M., Schwartzbrod, J., Skraber, S., Papageorgiou, G. T., Taylor, H., Wallis, J. & Jofre, J. 2006 Integrated analysis of established and novel microbial and chemical methods for microbial source tracking. *Appl. Environ. Microbiol.* **72** (9), 5915–5926.

Collins, R., Elliott, S. & Adams, R. 2005 Overland flow delivery of faecal bacteria to a headwater pastoral stream. *J. Appl. Microbiol.* **99** (1), 126–132.

Cotruvo, J. A., Dufour, A., Rees, G., Bartram, J., Carr, R., Cliver, D. O., Craun, G. F., Fayer, R. & Gannon, V. P. J. 2004 *Waterborne Zoonoses. Identification, Causes and Control.* IWA Publishing, London.

Craun, G. F., Calderon, R. L. & Craun, M. F. 2004 Waterborne outbreaks caused by zoonotic pathogens in the USA. In: *Waterborne Zoonoses. Identification, Causes and Control*

(J. A. Cotruvo, A. Dufour, G. Rees, J. Bartram, R. Carr, D. O. Cliver, G. F. Craun, R. Fayer & V. P. J. Gannon, eds). World Health Organization, Geneva.

Ferguson, C. M., Davies, C. M., Kaucner, C., Ashbolt, N., Krogh, M., Rodehutskors, J. & Deere, D. 2007 Field scale quantification of microbial transport from bovine faeces under simulated rainfall events. *J. Water Health* **5** (1), 83–95.

Ferguson, C., De Roda Husman, A. M., Altavilla, N., Deere, D. & Ashbolt, N. 2003 Fate and transport of surface water pathogens in watersheds. *Crit. Rev. Environ. Sci. Technol.* **33** (3), 299–361.

Field, K. G. & Samadpour, M. 2007 Fecal source tracking, the indicator paradigm, and managing water quality. *Water Res.* **41** (16), 3517–3538.

Fremaux, B., Gritzfeld, J., Boa, T. & Yost, C. K. 2009 Evaluation of host-specific *Bacteroidales* 16S rRNA gene markers as a complementary tool for detecting fecal pollution in a prairie watershed. *Water Res.* **43** (19), 4838–4849.

Griffiths, R. I., Whiteley, A. S., O'Donnell, A. G. & Bailey, M. J. 2000 Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA- and rRNA-based microbial community composition. *Appl. Environ. Microbiol.* **66** (12), 5488–5491.

Hagerdorn, C., Blanch, A. R. & Harwood, V. J. 2011 *Microbial Source Tracking: Methods, Applications, and Case Studies*. Springer, New York.

Hansen, J. S. & Ongerth, J. E. 1991 Effects of time and watershed characteristics on the concentration of *Cryptosporidium* oocysts in river water. *Appl. Environ. Microbiol.* **57** (10), 2790–2795.

Hrudey, S. E. & Hrudey, E. J. 2004 *Safe Drinking Water*. Lessons from Recent Outbreaks in Affluent Nations. IWA Publishing, London.

Insulander, M., Silverlas, C., Lebbad, M., Karlsson, L., Mattsson, J. G. & Svenungsson, B. 2013 Molecular epidemiology and clinical manifestations of human cryptosporidiosis in Sweden. *Epidemiol. Infect.* **141** (5), 1009–1020.

ISO 1986 *ISO 6461-1, Water quality – Detection and Enumeration of the Spores of Sulfite-reducing Anaerobes (*Clostridia*) – Part 1: Method by Enrichment in a Liquid Medium*. International Organization for Standardization, Geneva.

ISO 1994 *ISO 7888:1985: Water Quality – Determination of Electrical Conductivity*. International Organization for Standardization, Geneva.

ISO 1999 *ISO 7027, Water Quality – Determination of Turbidity*. International Organization for Standardization, Geneva.

ISO 2000a *ISO 7899-2, Water Quality – Detection and Enumeration of Intestinal Enterococci – Part 2: Membrane Filtration Method*. International Organization for Standardization, Geneva.

ISO 2000b *ISO 10705-2, Water Quality – Detection and Enumeration of Bacteriophages – Part 2: Enumeration of Somatic Coliphages*. International Organization for Standardization, Geneva.

ISO 2006 *ISO 15553, Water Quality – Isolation and Identification of Cryptosporidium Oocysts and Giardia Cysts from Water*. International Organization for Standardization, Geneva.

Kildare, B. J., Leutenegger, C. M., McSwain, B. S., Bambic, D. G., Rajal, V. B. & Wuertz, S. 2007 16S rRNA-based assays for quantitative detection of universal, human-, cow-, and dog-specific fecal *Bacteroidales*: a Bayesian approach. *Water Res.* **41** (16), 3701–3715.

Mara, D. D. & Oragui, J. I. 1983 Sorbitol-fermenting bifidobacteria as specific indicators of human faecal pollution. *J. Appl. Bacteriol.* **55** (2), 349–357.

Muirhead, R. W., Davies-Colley, R. J., Donnison, A. M. & Nagels, J. W. 2004 Faecal bacteria yields in artificial flood events: quantifying in-stream stores. *Water Res.* **38** (5), 1215–1224.

Petterson, S. R., Signor, R. S. & Ashbolt, N. J. 2007 Incorporating method recovery uncertainties in stochastic estimates of raw water protozoan concentrations for QMRA. *J. Water Health* **5**(Suppl. 1), 51–65.

Reischer, G. H., Haider, J. M., Sommer, R., Stadler, H., Keiblinger, K. M., Hornek, R., Zerobin, W., Mach, R. L. & Farnleitner, A. H. 2008 Quantitative microbial faecal source tracking with sampling guided by hydrological catchment dynamics. *Environ. Microbiol.* **10** (10), 2598–2608.

Reischer, G. H., Kasper, D. C., Steinborn, R., Farnleitner, A. H. & Mach, R. L. 2007 A quantitative real-time PCR assay for the highly sensitive and specific detection of human faecal influence in spring water from a large alpine catchment area. *Lett. Appl. Microbiol.* **44** (4), 351–356.

Reischer, G. H., Kasper, D. C., Steinborn, R., Mach, R. L. & Farnleitner, A. H. 2006 Quantitative PCR method for sensitive detection of ruminant fecal pollution in freshwater and evaluation of this method in alpine karstic regions. *Appl. Environ. Microbiol.* **72** (8), 5610–5614.

Reischer, G. H., Kollanur, D., Vierheilig, J., Wehrspaun, C., Mach, R. L., Sommer, R., Stadler, H. & Farnleitner, A. H. 2011 Hypothesis-driven approach for the identification of fecal pollution sources in water resources. *Environ. Sci. Technol.* **45** (9), 4038–4045.

Santo Domingo, J. W., Bambic, D. G., Edge, T. A. & Wuertz, S. 2007 Quo vadis source tracking? Towards a strategic framework for environmental monitoring of fecal pollution. *Water Res.* **41** (16), 3539–3552.

Savichtcheva, O., Okayama, N. & Okabe, S. 2007 Relationships between *Bacteroides* 16S rRNA genetic markers and presence of bacterial enteric pathogens and conventional fecal indicators. *Water Res.* **41** (16), 3615–3628.

Silverlås, C., de Verdier, K., Emanuelson, U., Mattsson, J. G. & Björkman, C. 2010 *Cryptosporidium* infection in herds with and without calf diarrhoeal problems. *Parasitol. Res.* **107** (6), 1435–1444.

SMI 2011 Statistics for Infectious Diseases (in Swedish). Available from: www.smittskyddsinstitutet.se/statistik/. Last accessed: 2011-02-07. Smittskyddsinstitutet/Swedish Institute for Communicable Disease Control, Stockholm.

Sokolova, E., Åström, J., Pettersson, T. J. R., Bergstedt, O. & Hermansson, M. 2012a Decay of *Bacteroidales* genetic markers in relation to traditional fecal indicators for water quality modeling of drinking water sources. *Environ. Sci. Technol.* **46** (2), 892–900.

Sokolova, E., Åström, J., Pettersson, T. J. R., Bergstedt, O. & Hermansson, M. 2012b Estimation of pathogen concentrations in a drinking water source using hydrodynamic modelling and microbial source tracking. *J. Water Health* **10** (3), 358–370.

Stapleton, C. M., Kay, D., Wyer, M. D., Davies, C., Watkins, J., Kay, C., McDonald, A. T., Porter, J. & Gawler, A. 2009 Evaluating the operational utility of a Bacteroidales quantitative PCR-based MST approach in determining the source of faecal indicator organisms at a UK bathing water. *Water Res.* **43** (19), 4888–4899.

Trask, J. R., Kalita, P. K., Kuhlenschmidt, M. S., Smith, R. D. & Funk, T. L. 2004 Overland and near-surface transport of *Cryptosporidium parvum* from vegetated and nonvegetated surfaces. *J. Environ. Qual.* **33** (3), 984–993.

First received 28 November 2012; accepted in revised form 6 May 2013. Available online 3 June 2013