Assessment of source tracking methods for application in spring water

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ABSTRACT

For discriminating between human and animal faecal contamination in water, microbial source tracking (MST) approaches using different indicators have been employed. In the current study, a range of 10 such MST indicators described in the scientific literature were comparatively assessed. Bacteriophages infecting host strains of *Bacteroides* (GA-17, GB-124 and ARABA 84) as well as sorbitol-fermenting bifidobacteria proved useful for indicating human faecal contamination while *Rhodococcus coprophilus* was associated with animal-derived faecal contamination. These potential source indicators were present in samples of faecal origin, i.e. either in human wastewater or animal waste, from many different regions in Switzerland and therefore showed a geographic stability. In addition, the MST indicators were abundant in surface water and were even sensitive enough to detect faecal contamination in spring water from two study areas in Switzerland. This is the first study that has compared and successfully applied MST methods in spring water.

Key words | contamination, indicator, pollution, source tracking, spring water

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INTRODUCTION

Faecal water contamination is of concern for public health, because a broad range of pathogens may cause human diseases by the faecal-oral route of transmission. Water supplies using water from vulnerable springs in rural areas may also be affected by such contaminations. Human faecal contamination is considered to be more important in terms of human health risk than animal pollution, although in some instances, contamination of animal origin can also have a significant impact on microbial water quality (WHO 2003). This was shown in a recent study, where gastrointestinal illness risks associated with exposure to recreational waters impacted by human and by fresh cattle faeces were similar, but risks associated with exposure to water contaminated with gull, pig and chicken faeces were shown to be lower (Soller *et al.* 2010). The need for discrimination between human and animal sources of surface water contamination has thus been recognised.

Indicator microorganisms such as *Escherichia coli* and enterococci are commonly used for quality assessment of surface and drinking water. Since these indicator bacteria are part of the human as well as the animal intestinal flora, standardised microbiological procedures for their detection in water (ISO 9308-1:2000 and ISO 7899-2:2000) are not suitable to distinguish between human and animal sources of contamination. This question has to be addressed by microbial source tracking (MST) approaches. Many different MST studies have been published, which focused on faecal samples and recreational waters (e.g. reviewed in Sinton *et al.* (1998); Scott *et al.* (2002); Meays *et al.* (2004); Savichtcheva & Okabe (2006); Stoeckel & Harwood (2007)), but only a few studies have dealt with the comparative assessment of different MST tools (Stoeckel *et al.* 2004; Blanch *et al.* 2006; Field & Samadpour 2007; Balleste *et al.* 2010).

Spring water is an important natural drinking water resource throughout the world. In Switzerland, it covers 40% of the drinking water demand. Another 40% of the drinking water derives from groundwater and 20% from surface water. In Switzerland, 38% of the drinking water (22%

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spring water and 16% groundwater) is not treated before consumption (SVGW 2008; BAFU 2013). Around 15–20% of the springs are located in regions with karst geology. The groundwater flow velocity within karst aquifers is dependent on meteorological and hydrogeological conditions and the retention time. During periods of dry weather, when slow to intermediate flow systems are dominant, only low levels of contamination occur (Auckenthaler 2004). After heavy rainfall events, when fast flow systems are present, faecal contamination occurs frequently in karst springs.

To date, only a small number of studies have considered methods that could be used for the discrimination of faecal contamination in spring water (Reischer *et al.* 2006, 2007, 2008; Wicki 2011). MST indicators applied to wastewater or surface water may also be useful in spring water investigations, provided that the sensitivity of methods is high enough for lower contamination levels prevailing in spring water. We thus selected a range of organisms, previously used in MST, to determine their occurrence in surface and spring water from two study areas in Switzerland, as well as in human wastewater, liquid manure and slaughterhouse wastewater. Sorbitol-fermenting bifidobacteria, phages infecting different *Bacteroides* host strains (GA-17, GB-124, ARABA 84 and ARABA 19) and *Streptococcus agalactiae* were used as indicators of human faecal contamination. Bacteriophages infecting *Bacteroides* host strains (RBA 63, 64 and KBA 60) and *Rhodococcus coprophilus* were applied as indicators of animal contamination. Sorbitol-fermenting bifidobacteria, phages infecting the host strains GA-17 and GB-124 and *R. coprophilus* have previously been described in the scientific literature and have been shown to perform well in source-specific detection of faecal contamination in water (Long *et al.* 2003; Blanch *et al.* 2006; Ebdon *et al.* 2007). The other indicators were previously described to be useful for MST in Switzerland (Wicki *et al.* 2011). In addition, faecal contamination in general, without discriminating the source, was measured using the most common indicator bacteria *E. coli* and enterococci as well as bacteriophages of the two host strains *E. coli* WG5 (somatic coliphages) and *Bacteroides fragilis* RYC 2056. Heterotrophic plate count (HPC), used as an additional quality parameter of drinking water in Switzerland, was included for spring water analysis.

The goal of the present study was to comparatively assess a range of 10 MST indicators described in the scientific literature in different environmental samples and to identify MST tools with a potential for application not only in surface but also in spring water in Switzerland.

METHODS

An overview of all microorganisms, test volumes and media used in this study is shown in Table 1.

Microbiological analysis of universal indicator organisms

Universal bacterial indicators

Escherichia coli, enterococci and HPC were analysed with methods that are used in official food control in Switzerland and are based on international standards (*E. coli:* ISO 9308-1:2000 ISO 16649-1:2001; enterococci: ISO 7899-2:2000; HPC: ISO 4833-1&2:2013). For analysing *E. coli* in wastewater samples, the spread-plate method was applied, whereas for detection of *E. coli* and enterococci in surface and spring water samples, the membrane filter technique was used (0.45 μm Microsart CN-Filter 11406Z-50 SC).

Somatic coliphages

Faecal contamination in general, without source attribution, was determined by enumeration of somatic coliphages using the bacterial host strain *E. coli* WG5 (provided by the Department of Microbiology, University of Barcelona, Spain). Wastewater samples were serially diluted before analysis. Surface and spring water samples were used directly or 1 mL of the eluate was used after filtration. Filtration and resuspension were carried out following a method previously described (Mendez *et al.* 2004). Further analysis of somatic coliphages followed the standard protocol for detection and enumeration of somatic coliphages (ISO 10705-2:2001).

Table 1 | Microorganisms, volumes and appropriate media used in this study

		Volume analysed		
Microorganisms	**Method[a]**	**Wastewater**	**Surface water (mL)**	**Spring water (mL)**
Sorbitol-fermenting *Bifidobacteria*	Culture on HBSA	0.1 mL of a serial dilution 10^{-1}–10^{-4}	1, 10, 100	10, 100, 1,000
Phages infecting *Bacteroides* host strains[b]	Plaque assay on BPRMA	1 mL	500	1,000
Rhodococcus coprophilus	Culture on modified MM3	10 mL	100	
	LightCycler PCR	10 mL	100	200
Streptococcus agalactiae	LightCycler PCR after enrichment in Todd–Hewitt broth	10 mL	500	1,000
Escherichia coli	Culture on TSA and TBX	0.1 mL of a serial dilution 10^{-1}–10^{-6}	1, 10, 100	1, 10, 100
Enterococci	Culture on m-enterococcus agar and BEA	NA	1, 10, 100	1, 10, 100
Heterotrophic plate count	Culture on plate count agar	NA	NA	0.1, 1
Somatic coliphages	Plaque assay on MSA	1 mL of a serial dilution 10^{-1}–10^{-6}	1, 10, 100	1, 10, 100

[a]Abbreviations of media: BPRMA: *Bacteroides* phage recovery medium agar; HBSA: human bifid sorbitol agar; TSA: tryptone soya agar; TBX: tryptone bile glucuronid agar; BEA: bile esculin agar; MSA: modified Scholtens' agar.
[b]*Bacteroides* host strains: GA-17, GB-124, ARABA 84, ARABA 19, RBA 63, RBA 64, KBA 60, RYC 2056.
PCR, polymerase chain reaction; NA, not analysed.

Bacteriophages of *B. fragilis* RYC 2056

Faecal contamination was determined using the bacterial host strains *B. fragilis* RYC 2056 (provided by the Department of Microbiology, University of Barcelona, Spain). Analysis was performed as previously described (Wicki *et al.* 2011). Samples were filtered through a membrane filter of 0.22 μm pore size and 47 mm diameter (GSWP04700, Millipore) to concentrate surface and spring water samples before analysis. Filtration and resuspension were carried out following a method previously described (Mendez *et al.* 2004). Enumeration of plaque-forming units (PFU) followed the standard protocol for detection and enumeration of *Bacteroides* bacteriophages (ISO 10705-4:2001) with the following modification for surface and spring water samples: 5 mL elution buffer containing bacteriophages was mixed with 5 mL of the host strain and 12.5 mL semisolid *Bacteroides* phage recovery medium (BPRM) agar. The mixture was poured onto a BPRM agar plate with a diameter of 145 mm. Plates were incubated for 20–22 h at 37 °C under anaerobic conditions using AnaeroGen (Oxoid).

Analysis of human-specific MST indicator organisms

Sorbitol-fermenting bifidobacteria

Wastewater, slaughterhouse and liquid manure samples were serially diluted, and 0.1 mL of each dilution was spread-plated on Human Bifid Sorbitol Agar (HBSA) (Mara & Oragui 1983). Surface and spring water samples, as shown in Table 1, were filtered through membranes with a pore size of 0.45 μm (Microsart CN-Filter 11406Z-50 SC). Filters were placed on HBSA and plates were incubated for 3 days at 37 °C under anaerobic conditions (AnaeroGen, Oxoid). Presumptive colonies of sorbitol-fermenting *Bifidobacteria*, appearing as yellow to brown colonies, were confirmed by light microscopy, and isolates from surface and spring water samples were further analysed with the Api20A test (BioMérieux). In addition, Gram staining was performed and growth was tested under strict anaerobic conditions. As positive controls, the reference strains *Bifidobacterium adolescentis* (DSM 20083) and *Bifidobacterium breve* (DSM 20213) were inoculated directly on HBSA, and

as a negative control, 100 mL distilled sterile water was filtered.

Bacteroides host strains detecting human-specific bacteriophages

A range of different human-specific *Bacteroides* host strains was used for the detection of bacteriophages. The host strains GA-17, GB-124 (provided by the Department of Microbiology, University of Barcelona, Spain), ARABA 84 and ARABA 19 (previously isolated in our laboratories) were used as host strains for detecting bacteriophages of human faecal origin (Payan *et al.* 2005; Wicki *et al.* 2011). Samples were processed as described above for bacteriophages of *B. fragilis* RYC 2056. If two types of plaques were present, both were counted except for host ARABA 84, where only clear plaques were counted in wastewater samples.

Streptococcus agalactiae

Samples were filtered through membranes with a pore size of 0.45 μm (Microsart CN-Filter 11406Z-50 SC). Subsequently, DNA was extracted as described for *R. coprophilus*. A LightCycler PCR (polymerase chain reaction) assay was performed to detect *S. agalactiae* using a LightCycler 1.1 Instrument (Roche, Switzerland) (Wicki 2011). Primers and probes were previously described to be useful for analysis of water samples (Ke *et al.* 2000; Wicki 2011). The following reagents and concentrations were used: 2 μL of LightCycler FastStart DNA MasterHybProbe (10×), 0.8 μL of the forward primer Sag59-F (5′-TTT CAC CAG CTG TAT TAG AAG TA-3′) (10 pmol/μL), 0.8 μL of the reverse primer Sag190-R (5′-GTT CCC TGA ACA TTA TCT TTG AT-3′) (10 pmol/μL), 0.4 μL of each probe (STB-F: 5′-AAGCCCAGCAAATGGCTCAAA-FL-3′ and STB-C: 5′-LC640-GCTTGATCAAGATAGCATTCAGTTGA-PH-3′) (10 pmol/μL), 9.4 μL of H_20, 2 μL of $MgCl_2$ solution (25 mM) and 5 μL of template DNA. The standard amplification protocol was 95 °C for 15 min followed by 45 cycles of amplification (95 °C for 1 s, 55 °C for 14 s and 72 °C for 5 s). Each run contained a positive (DSM 2134 strain) and negative (H_2O) control, which were extracted together with the samples analysed.

Analysis of animal-specific MST indicator organisms

Rhodococcus coprophilus

Two methods were used for detecting *R. coprophilus*. A culture-based method on modified MM3 agar was carried out as previously described (Long *et al.* 2003). In addition, a new LightCycler PCR assay was performed (Wicki *et al.* 2012). Defined sample volumes, as shown in Table 1, were filtrated through 0.2 μm GTTP hydrophobic filters (Millipore Isopore™ membrane filters). The filters were placed in 5 mL pyrophosphate Bennett's broth and vortexed to release the cells from the membrane. Heat treatment for 6 min at 55 °C in a water bath followed to kill background bacteria. After this step, 100 μL of the resuspension was spread-plated 10 times on modified MM3. For the PCR assay, 1 mL of the resuspended bacteria were further processed. DNA was extracted from all samples using the DNeasy blood and tissue kit (Qiagen, Switzerland). After centrifugation of defined volumes (specified below), DNA was isolated following the manufacturers' protocol employing a pretreatment for Gram-positive bacteria. All samples were eluted in buffer AE (provided in the kit) in a final volume of 200 μL. PCR was performed using a LightCycler 1.1 Instrument (Roche) in a 20 μL reaction volume consisting of the following reagents and concentrations: 4 μL LightCycler FastStart DNA Master^{Plus} HybProbe (5×), 1 μL of each primer (CL1.1 F: 5′- TGG GCG GAT TAG TGG CGA A -3′; CL9 R: 5′- GTT AGC CGG TGC TTC TTC TG -3′) (10 pmol/μL), 0.8 μL of each probe (RC_3′FL: 5′- ACT GGG TCT AAT ACC GGA TAT GAC CAT- FL -3′; RC_5′LC640: 5′- LC640-ATG CAT GTC CTG TGG TGG AAA GGT TTA CTG- PH -3′) (5 pmol/μL), 7.4 μL H_2O and 5 μL template DNA. The amplification protocol was 95 °C for 10 min followed by 45 cycles of amplification (95 °C for 15 s, 60 °C for 20 s and 72 °C for 25 s). Each run contained a positive (DSM 43347 strain) and a negative (H_2O) control, which were extracted together with the analysed samples.

Bacteroides host strains detecting animal-specific bacteriophages

The host strains RBA 63, 64 and KBA 60 were used as host strains for bacteriophages of animal faecal origin

(Wicki *et al.* 2011). Samples were processed as described above for bacteriophages of *B. fragilis* RYC 2056.

Turbidity

Turbidity was recorded every 10 min with a WTM 500 online turbidity meter (SIGRIST-Photometer AG, Ennetbürgen, Switzerland) in all springs during the entire monitoring period. The WTM 500 measures light of 880 nm scattered at a 90° angle according to ISO 7027. The measuring range was from 0.001 to 500 FNU (Formazin Nephelometric Units). Sample flow rate was from 3.2 to 4.0 L/min.

Analysis of human wastewater and samples from animal origin

Untreated human wastewater samples were collected from 10 different Swiss wastewater treatment plants (WTPs), each processing sewage from more than 100,000 inhabitants. One sample was collected from each WTP. A further three wastewater samples were collected on different days from a smaller WTP. Seven wastewater samples from two large abattoirs slaughtering cows, calves, bulls and pigs were collected on different dates. In addition, four liquid manure samples from two Swiss farms keeping 20–30 cows were analysed. Figure 1 shows the location of the sampling sites.

Study areas with spring and surface water sampling sites

Two study areas, termed 'Röschenz' and 'Oberdorf', located in the northwestern part of Switzerland in the Jura Mountains showing typical karst geology, were selected for investigation (Figure 2). Both regions are situated in rural territories characterised by intense dairy farming and animal husbandry.

In the 'Röschenz' study area, the two springs 'Kächbrunnenquelle' (KQ) and 'Lützelquelle' (LQ), and in the 'Oberdorf' area, the 'Z'Hof' (ZQ) spring were examined. All three karst springs considerably contribute to the public water supply of the respective area. In both study areas, the karst springs are located close to a stream, the Lützel stream in the 'Röschenz' area and the Weigist stream in the 'Oberdorf' area, that receive runoff from livestock. All springs are located downstream of the effluent discharge point from WTPs. Surface water sampling sites included sites upstream and downstream from the wastewater effluent, and in the 'Oberdorf' area additionally at a tributary stream called 'Heimsten'.

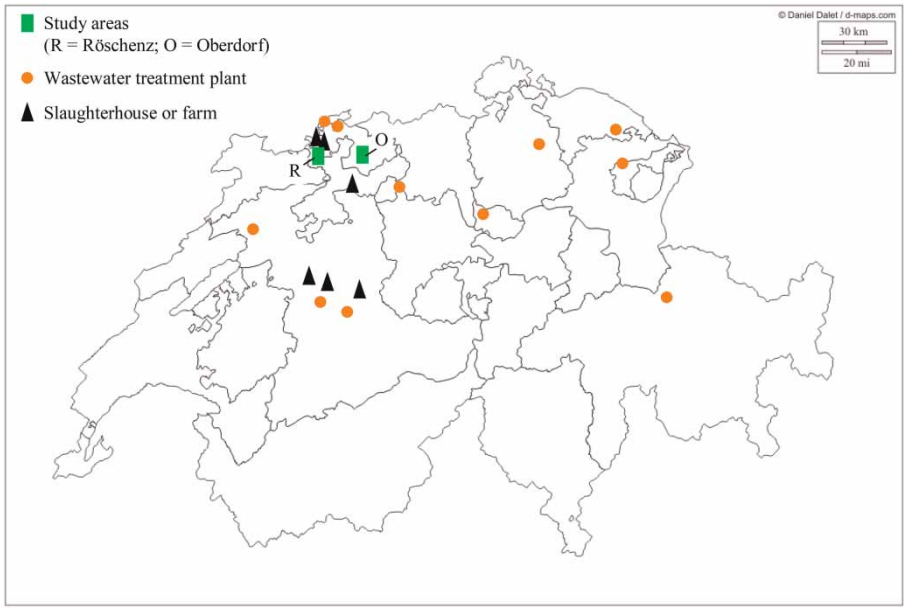

Figure 1 | Distribution of study areas and further sampling sites in Switzerland. The original map of Switzerland is available from http://d-maps.com/carte.php?lib=switzerland_map&num_car=4055&lang=en.

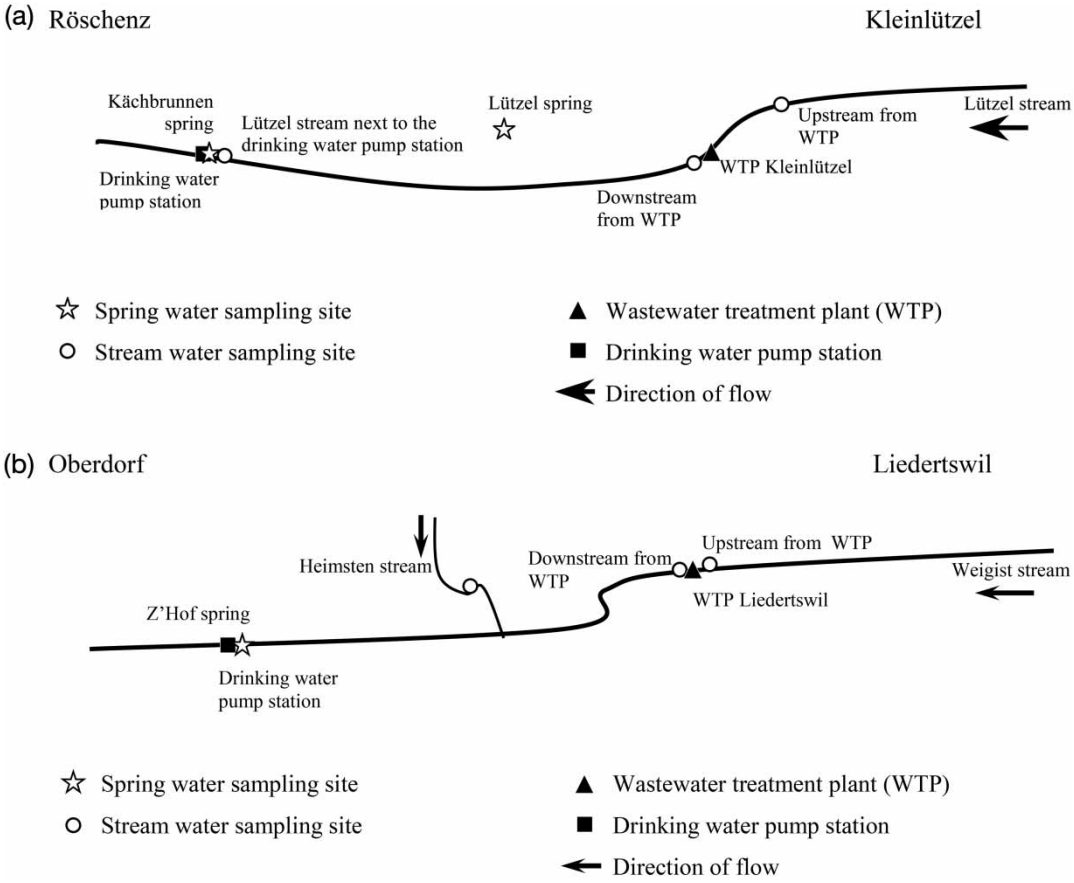

Figure 2 | (a) Study area Röschenz; (b) study area Oberdorf.

A schematic representation of the study areas and sampling sites is shown in Figures 1, 2(a) and 2(b). Based on previous findings (Schudel 2000; Auckenthaler 2004; Wicki 2011), both human and animal faecal contamination was to be expected in surface and spring water from the areas in question.

Surface and spring water sample collection

Surface water samples were taken at a depth of approximately 15–20 cm and away from the banks. Spring and surface water samples were collected in sterilised Nalgene polypropylene bottles (VWR, Switzerland). A total volume of approximately 10 L was collected per sampling from each site.

Dry weather sampling

Over a period of 7 months, from March to September 2009, five series of sampling were performed. Spring and surface water samples from the same study area were collected on the same day. In the Röschenz area, samples were collected on 21st April, 5th May, 26th May, 18th August and 22nd September. Additional samples were collected from the LQ spring on 17th February, 24th March and 7th April, and from the KQ spring on 24th March and 7th April. Samples in the Oberdorf area were from 31st March, 14th April, 28th April, 12th May and 29th September.

Sampling after rainfall events

Additional spring water samples were taken after three rainfall events. Such a rainfall event was defined as rain of more than 10–20 mm/h according to local weather radar. Samples were collected approximately 72 h after rainfall events on 25th May, 14th July and 8th August. After the rainfall event of 14th July 2009, samples were taken not only on 17th July

but also on 21st July, because additional rainfall (<10 mm/h) was recorded on 18th July.

Statistics

Statistical tests were performed by application of the SYSTAT statistical analysis and graphics software, version 13.0.

RESULTS

Occurrence of universal and MST indicators in human and slaughterhouse wastewater and in liquid manure

Concentrations of universal faecal indicators measured in samples obtained from human and animal origin are shown in Table 2. With the exception of one slaughterhouse wastewater sample, which was negative for *E. coli* and one liquid manure sample negative for somatic coliphages, all samples were positive for these two universal indicators. In samples of human and animal origin, higher counts of *E. coli* were found than of somatic coliphages. The highest concentration of universal faecal indicators (*E. coli* and somatic coliphages) was detected in liquid manure, followed by human wastewater and slaughterhouse samples. Bacteriophages infecting host *B. fragilis* RYC 2056 were much more prevalent in human than slaughterhouse wastewater and were never detected in liquid manure (Table 2).

Human MST indicators (sorbitol-fermenting bifidobacteria, bacteriophages infecting GB-124, ARABA 84, ARABA 19 and *S. agalactiae*) were restricted to human wastewater samples and were never detected in slaughterhouse wastewater or liquid manure (Table 2). In contrast, human MST indicators, with one exception for phages infecting ARABA 19, were found in all human wastewater

Table 2 | Universal and specific MST indicators in human and animal wastewater and in liquid manure

	Human wastewater			Slaughterhouse wastewater			Liquid manure		
	Nr. pos	Median[a]	Range[a]	Nr. pos	Median[a]	Range[a]	Nr. pos	Median[a]	Range[a]
Universal indicator									
E. coli	13/13	4.7	4.0–5.6	6/7	3.6	0–5.3	4/4	6.4	5.0–6.9
Somatic coliphages	13/13	3.7	2.7–4.8	7/7	3.2	2.4–4.4	3/4	4.2	0–4.9
Phages of RYC 2056	13/13	2.0	1.1–3.8	3/7	0	0–0.9	0/4	n/a	n/a
Human MST indicator									
Sorbitol-fermenting *Bifidobacteria*	13/13	4.5	4.0–5.2	0/7	n/a	n/a	0/4	n/a	n/a
Phages of GA-17	13/13	1.7	0.9–2.6	1/7	0	0–0.5	0/4	n/a	n/a
Phages of GB-124	13/13	1.8	0.6–2.7	0/7	n/a	n/a	0/4	n/a	n/a
Phages of ARABA 84[b]	10/10	1.6	0.9–2.1	0/3	n/a	n/a	0/2	n/a	n/a
Phages of ARABA 19[b]	9/10	1.0	0–1.4	0/3	n/a	n/a	0/2	n/a	n/a
S. agalactiae[b]	10/10	1.5	0.6–1.8	0/5	n/a	n/a	0/2	n/a	n/a
Animal MST indicator									
R. coprophilus (culture)	1/13	0	0–3.0	5/7	3.0	0–3.7	3/3	6.9	3.6–6.9
R. coprophilus (PCR)[b]	1/10	0	0–3.6	5/7	0.3	0–1.8	2/2	7.5	3.9–7.8
Phages of KBA 60[b]	0/10	n/a	n/a	4/4	0.3	0–0.8	0/1	n/a	n/a
Phages of RBA 63[b]	0/10	n/a	n/a	3/4	0	0–0.3	0/1	n/a	n/a
Phages of RBA 64[b]	0/10	n/a	n/a	3/4	0	0–0.7	0/1	n/a	n/a

Nr. pos: number of positives of tested samples (analysed volumes according to Table 1).
n/a: not applicable.
[a]Log colony-forming units (CFU) or PFU/mL.
[b]Data were processed in previous studies (Wicki 2011; Wicki *et al.* 2011; Wicki *et al.* 2012).

samples analysed. The highest concentration was found for sorbitol-fermenting bifidobacteria with a median log concentration of 4.51 CFU/mL and a range between 3.99 and 5.19 CFU/mL, thus being similar to that of *E. coli*. Bacteriophages infecting GA-17 were also abundant in human wastewater and could furthermore be detected in one slaughterhouse wastewater sample, but the measured counts were much lower than in human samples (Table 2). The median log concentrations of different human-derived bacteriophages and *S. agalactiae* were similar, ranging between 1.04 and 1.76 CFU/mL and about 3 log below the concentration of sorbitol-fermenting bifidobacteria (Table 2).

Animal MST indicators were more abundant in samples of animal origin than in human wastewater. As shown in Table 2, *R. coprophilus* was present in all liquid manure samples and in five out of seven slaughterhouse wastewater samples. In addition, one human wastewater sample tested positive for this MST indicator. Concentrations determined with the molecular approach (real-time PCR) were slightly higher than those obtained with the culture-based method for detecting *R. coprophilus*, except for slaughterhouse samples where a lower median log concentration was found. Bacteriophages infecting the three host strains included in this study were exclusively detected in samples from slaughterhouses, but in low numbers only.

Occurrence of universal and MST indicators in surface water

The universal faecal indicators *E. coli*, enterococci and somatic coliphages were detected in all surface water samples from five out of six sampling sites. The lowest contamination was detected in the tributary stream 'Heimsten' of the Oberdorf study area: median concentrations were low with 6 CFU/100 mL for *E. coli* and 10 CFU/100 mL for enterococci as well as somatic coliphages, and in one sample, only 5 CFU/100 mL enterococci and no *E. coli* or somatic coliphages were detected (data not shown).

As shown in Table 3, most of the universal indicators and human MST indicators were present in significantly higher concentrations downstream than upstream from WTPs (one-sided p-values < 0.05 for *E. coli*, enterococci and somatic coliphages and for sorbitol-fermenting *Bifidobacteria*, phages of GA-17 and *S. agalactiae* in the Wilcoxon–Mann–Whitney test with data stratified into two groups based on the location of the treatment plant). In the Röschenz study area, additional samples were collected approximately 1 km further downstream from the effluent discharge point of the WTP. Compared to the other two sampling sites in the stream, median concentrations of both universal and human faecal indicators were lower (data not shown). As shown in Table 3, in samples collected upstream from WTPs, concentrations of somatic coliphages were higher than that for *E. coli* and vice versa in samples collected downstream.

The animal faecal indicator *R. coprophilus* was present in all samples from all six surface water sampling sites after analyses with the culture-based approach (Table 3 and two additional sampling sites of which data are not shown). With the molecular approach, only 5 out of 30 surface water samples were positive. Culture-based detection of *R. coprophilus* revealed higher median concentrations upstream than downstream from WTPs of both study areas (Table 3). In the Lützel stream, the concentration increased again approximately 1 km further downstream of the effluent discharge point with a median value of 151 CFU/500 mL and a range of 45–290 CFU/500 mL (data not shown). Bacteriophages infecting different host strains were present in only low numbers, and real differences were not observed among different sampling sites.

Spring water investigation

During periods of dry weather, universal faecal indicator bacteria were frequently detected in spring water samples. In six out of eight samples from the LQ spring, in six out of seven samples from the KQ spring and in four samples from the ZQ spring, either *E. coli* or enterococci were detected or more than 100 CFU/mL of HPC was found. As shown in Table 4, median concentrations were low for universal indicator organisms and MST indicators. One exception was observed for culture-based detection of *R. coprophilus* where median concentrations exceeded the values of universal indicator organisms *E. coli*, enterococci, somatic coliphages and phages infecting *B. fragilis* RYC 2056.

Table 3 | Occurrence of universal and specific MST indicators in surface water

	Upstream from wastewater treatment plants				Downstream from wastewater treatment plants			
	Lützel stream		Weigistbach stream		Lützel stream		Weigistbach stream	
	Nr. pos	Median (range)[a]	Nr. pos	Median (range)[a]	Nr. pos	Median (range)[a]	Nr. pos	Median (range)[a]
Universal indicator								
E. coli	5/5	1.7×10^3 ($825–1\times10^5$)	5/5	245 ($35–7.2\times10^4$)	5/5	2.8×10^4 ($1\times10^4–1\times10^5$)	5/5	2.4×10^4 ($705–3\times10^5$)
Enterococci	5/5	1.5×10^3 ($410–1.2\times10^5$)	5/5	470 ($25–4.9\times10^4$)	5/5	5×10^3 ($3.2\times10^3–1\times10^5$)	5/5	1×10^4 ($1.5\times10^3–7.9\times10^4$)
Somatic coliphages	5/5	3.3×10^3 ($525–2.1\times10^4$)	5/5	470 ($40–4\times10^4$)	5/5	1.8×10^4 ($4.9\times10^3–2.9\times10^5$)	5/5	1.2×10^3 ($70–4.1\times10^4$)
Phages of RYC 2056	5/5	6 (2–85)	0/5	n/a	4/5	25 (0–39)	2/4	4 (0–179)
Human-specific MST indicator								
Sorbitol-fermenting *Bifidobacteria*[b]	1/5	0 (0–5)	0/5	n/a	5/5	700 (30–875)	4/5	140 (0–37,000)
Phages of GA-17	5/5	5 (2–32)	0/5	n/a	5/5	19 (3–25)	3/5	1 (1–77)
Phages of GB-124	5/5	13 (2–224)	1/5	0 (0–4)	4/5	15 (0–169)	1/5	0 (0–7)
Phages of ARABA 84	5/5	36 (35–1,196)	2/5	0 (0–7)	5/5	113 (17–384)	4/5	9 (0–216)
Phages of ARABA 19	1/5	0 (0–1)	0/5	n/a	1/4	0 (0–1)	0/4	n/a
S. agalactiae	0/5	n/a	0/5	n/a	5/5	n.q.	5/5	n.q.
Animal-specific MST indicator								
R. coprophilus (culture)	5/5	120 (40–430)	5/5	70 (15–250)	5/5	60 (18–205)	5/5	55 (10–320)
R. coprophilus (PCR)[c]	0/5	n/a	2/5	0 ($0–4.3\times10^4$)	0/5	n/a	2/5	0 ($0–1.5\times10^5$)
Phages of KBA 60	2/5	0 (0–3)	3/5	1 (0–3)	2/5	0 (0–5)	1/5	0 (0–1)
Phages of RBA 63	0/5	n/a	1/5	0 (0–4)	0/5	n/a	1/5	0 (0–1)
Phages of RBA 64	0/5	n/a	1/5	0 (0–5)	1/5	0 (0–2)	2/5	0 (0–1)

Nr. pos: number of positives of tested samples (analysed volumes according to Table 1).
n.q.: not quantified; n/a: not applicable.
[a]CFU/PFU/500 mL.
[b]Values were extrapolated and zero values refer to an initial volume of 100 mL.
[c]The sample volume analysed per PCR reaction is in accordance with an initial sample volume of 2.5 mL.

Table 4 | Presence of universal and specific MST indicators in spring water samples during periods of dry weather

	LQ spring		KQ spring		ZQ spring	
Universal indicator	**Nr. pos**	**Median (range)**	**Nr. pos**	**Median (range)**	**Nr. pos**	**Median (range)**
E. coli (CFU/100 mL)	5/8	2 (0–20)	6/7	3 (0–36)	1/4	0 (0–2)
Enterococci (CFU/100 mL)	5/8	2 (0–276)	6/7	2 (0–143)	1/4	0 (0–4)
HPC (CFU/mL)	8/8	52 (11–2.1×10^3)	7/7	26 (24–235)	4/4	154 (0–1×10^3)
Somatic coliphages (CFU/100 mL)	6/8	3 (0–98)	5/7	4 (0–245)	1/5	0 (0–15)
Phages of RYC 2056 (PFU/L)	2/8	0 (0–2)	0/7	n/a	0/5	n/a
Human-specific MST indicator[a]						
Sorbitol-fermenting *Bifidobacteria*	1/8	0 (0–11)	3/7	0 (0–35)	0/5	n/a
Phages of GA-17	0/8	n/a	1/7	0 (0–3)	0/5	n/a
Phages of GB-124	2/8	0 (0–1)	0/7	n/a	0/5	n/a
Phages of ARABA 84	2/8	0 (0–3)	0/7	n/a	0/5	n/a
Phages of ARABA 19	0/8	n/a	0/7	n/a	0/5	n/a
S. agalactiae	0/8	n/a	0/7	n/a	0/5	n/a
Animal-specific MST indicator[a]						
R. coprophilus (culture)	7/7	135 (20–400)	6/7	154 (0–380)[b]	3/5	30 (0–350)[b]
R. coprophilus (PCR)[c]	0/8	n/a	0/7	n/a	1/5	0 (0–715)
Phages of KBA 60	1/8	0 (0–1)	0/7	n/a	0/5	n/a
Phages of RBA 63	0/8	n/a	1/7	0 (0–1)	0/4	n/a
Phages of RBA 64	0/8	n/a	0/7	n/a	0/5	n/a

Nr. pos: number of positives of tested samples (analysed volumes according to Table 1).
n/a: not applicable.
[a]CFU/PFU/L.
[b]Absent in 200 mL.
[c]The sample volume analysed per PCR reaction is in accordance with an initial sample volume of 5 mL.

After three rainfall events, universal faecal indicators were detected in all samples except for phages infecting *B. fragilis* RYC 2056 (Table 5). The highest faecal contamination was observed in all spring water samples collected on 17th July 2009 after the second rainfall event. An increase of turbidity was recorded after the second rainfall event in all three springs and after the third rainfall event in the LQ spring and KQ springs only. However, no increase of turbidity was observed after the first rainfall event in all springs (data not shown).

LQ spring

In the LQ spring, human faecal contamination was indicated in two samples collected during dry periods, by the presence of bacteriophages infecting the host strains GB-124 and ARABA 84 (Table 4). In one sample, sorbitol-fermenting bifidobacteria were also found. After rainfall, human faecal contamination was detected in all samples collected from the LQ spring, as indicated by the presence of human-specific bacteriophages (Table 5). However, sorbitol-fermenting bifidobacteria, bacteriophages infecting the host GB-124 and *S. agalactiae* were never detected after rainfall in the LQ spring. During periods of dry weather and after rainfall, animal faecal contamination was constantly indicated with the culture-based method detecting *R. coprophilus*. During periods of dry weather, phages infecting the host KBA 60 were also found in one sample, and bacteriophages infecting the host RBA 63 were observed in one sample collected after the second rainfall event. Other animal faecal indicators were not detected in the LQ spring.

Table 5 | Number of universal and specific MST indicators in spring water samples collected after three rainfall events

	LQ spring			KQ spring			ZQ spring		
Sampling after rainfall events	**1 28.5.09**	**2 17/21.7.09**	**3 11.8.09**	**1 28.5.09**	**2 17/21.7.09**	**3 11.8.09**	**1 28.5.09**	**2 17/21.7.09**	**3 11.8.09**
Universal indicator									
E. coli (CFU/100 mL)	130	1.3×10^3/142	358	17	7.6×10^3/250	1.4×10^3	9	206/13	28
Enterococci (CFU/100 mL)	290	1×10^3/292	1.2×10^3	80	9.8×10^3/520	2.2×10^3	12	115/11	1.2×10^2
HPC (CFU/mL)	1×10^4	3.4×10^4/4.5×10^3	7.3×10^3	296	4.3×10^4/2.7×10^3	1.6×10^3	84	2×10^3/106	2.9×10^3
Somatic coliphages (CFU/100 mL)	168	2.6×10^4/1×10^3	330	14	5.2×10^4/5.5×10^3	2.5×10^4	6	1.6×10^4/20	53
Phages of RYC 2056 (PFU/L)	0	3/0	0	0	0/0	0	0	0/0	0
Human-specific MST indicator (CFU/PFU/L)									
Sorbitol-fermenting *Bifidobacteria*	0	0/0[a]	0	0	0/0[a]	29	0	100/0	24
Phages of GA-17	1	15/0	3	0	0/0	1	0	0/0	0
Phages of GB-124	0	0/0	0	0	10/1	0	0	2/0	0
Phages of ARABA 84[c]	8	5/5	70	n.a.	0/5	4	4	4/0	1
Phages of ARABA 19[c]	0	4/0	3	0	0/0	1	0	0/0	0
S. agalactiae	0	0/0	0	0	0/0	0	0	0/0	0
Animal-specific MST indicator (CFU/PFU/L)									
R. coprophilus (culture)	42	5/5	90	390	105	110	0[b]	10/110	465
R. coprophilus (PCR)[d]	0	0/0	0	0	0/0	0	0	0/0	0
Phages of KBA 60[c]	n.a.	0/0	0	0	0/0	0	0	0/0	0
Phages of RBA 63[c]	0	2/0	0	0	4/0	0	0	3/0	0
Phages of RBA 64[c]	0	0/0	0	0	0/0	0	0	0/0	0

n.a. = not analysed.
[a]Absent in 500 mL.
[b]Absent in 200 mL.
[c]Data were shown in a previous study (Wicki *et al.* 2011).
[d]Absent in 5 mL.

KQ spring

In four out of seven samples collected during periods of dry weather from the KQ spring, human faecal contamination could be demonstrated either by detection of sorbitol-fermenting bifidobacteria or by the presence of bacteriophages infecting the host GA-17 (Table 4). As shown in Table 5, human faecal contamination also occurred after the second and the third rainfall event. Bacteriophages infecting the host strain GB-124 and ARABA 84 were present after the second rainfall event, and after the third rainfall event, sorbitol-fermenting bifidobacteria and phages infecting the host strains GA-17, ARABA 84 and ARABA 19 were found. Animal faecal contamination was observed in six out of seven samples collected during periods of dry weather and in all samples collected after rainfall. In all these samples, *R. coprophilus* was detected with the culture-based detection method. In addition, the host strain RBA 63 detected phages in one sample collected during periods of dry weather and in another sample collected after rainfall. Other animal faecal indicators were not found in the KQ spring.

ZQ spring

During periods of dry weather, human MST indicators were not detected in samples from the ZQ spring in the Oberdorf study area (Table 4). As shown in Table 5, human faecal contamination occurred after rainfall. Bacteriophages infecting the host ARABA 84 were found after all three rainfall events, sorbitol-fermenting bifidobacteria after two and phages infecting the host GB-124 after the second event. From the two samples collected after the second rainfall event, human MST indicators were only detected in the sample collected on 17th September (Table 5). As shown in Table 4, animal faecal contamination was indicated only by *R. coprophilus* during periods of dry weather in the ZQ spring. After the first rainfall event, universal faecal contamination was low and animal faecal contamination could not be demonstrated (Table 5). However, an increase of animal faecal contamination was observed after the second and the third rainfall event where *R. coprophilus* was detected with the culture-based method. In one sample, the host strain RBA 63 also detected phages and in another sample KBA 60 bacteriophages were found.

DISCUSSION

During the present study, MST indicators such as sorbitol-fermenting bifidobacteria bacteriophages infecting the host strains GA-17 and GB-124 have been used for the first time in Switzerland. Therefore, human and slaughterhouse wastewater as well as liquid manure samples were analysed in order to determine the occurrence of these MST indicators and to compare them with recently described MST indicator bacteria. Human and animal samples were therefore collected from many different regions in Switzerland (Figure 2).

The occurrence of the human faecal indicator sorbitol-fermenting bifidobacteria was restricted to human wastewater. Its log median concentration (4.51 CFU/100 mL) was slightly lower than in a study carried out in Spain (6.41 CFU/100 mL) (Blanch *et al.* 2006). Bacteriophages of the host strain GA-17 were also present in all human wastewater samples and only once in a sample of animal origin. Based on a questionnaire sent to the slaughterhouses, a human faecal input is unlikely in slaughterhouse wastewaters. Previously, for these phages of the host strain GA-17, a concentration of 4.17 log PFU/100 mL in wastewater was reported (Blanch *et al.* 2006), the extrapolated median concentration observed in our study of 3.72 log PFU/100 mL was in the same range. The bacteriophages of the host strain GB-124 were found at slightly lower concentrations (mean = 104 PFU/mL) than values reported for samples from Spain with an average concentration of 500 PFU/mL and slightly higher than the average of 25 PFU/mL of samples from England (Blanch *et al.* 2006). Consequently, counts of bacteriophages infecting the host strain GB-124 were higher in samples from other European countries than in samples from England.

With one exception (phages of ARABA 19), the concentration of all human MST indicators increased downstream from WTPs. Such an increase was expected, because microorganisms are not completely removed during the process of wastewater treatment. The ratio of sorbitol-fermenting bifidobacteria to other human faecal indicators decreased

from wastewater to surface water. This might be due to the short persistence of the anaerobic bacteria in environmental water. It was shown that they do not survive for a long time and therefore only indicate recent faecal contamination (Scott *et al.* 2002).

This was the first study investigating and comparing several MST indicators in spring water. The presence of human faecal contamination could be confirmed for the first time in Swiss spring water based on the assessed human MST indicators. Spring water from karst aquifers is an important source of drinking water but is vulnerable to faecal contamination, in particular after rainfall events (Schudel *et al.* 2000; Auckenthaler 2004). As expected, an increase of faecal contamination was observed after rainfall. This shows the vulnerability of the investigated karst springs. Although an increase of human-specific MST indicators was not observed, sorbitol-fermenting bifidobacteria and phages of the host strains GB-124 and ARABA 84 were found at least once in all springs. Therefore, they are generally of potential use for MST in water resources with low faecal contamination.

Even though the investigated springs are located in regions where the land is predominantly used for agricultural purposes, human faecal contamination was frequently detected. This shows that WTPs may impact the water quality of springs in these rural regions. As human pathogens occur frequently in human waste, human faecal contamination is of special concern and it is particularly important to detect it. In fact, human pathogens were found in the LQ spring in a previous study (Auckenthaler *et al.* 2002). The quality of Swiss drinking water is assured by tolerance values stated in the Ordinance on Hygiene (Federal Department of Home Affairs 2014) of zero *E. coli* and enterococci per 100 mL and 300 HPC/mL. During periods of dry weather in our study, about 84%, and after rainfall even 100%, of spring water samples exceeded the tolerance values for drinking water. The spring water from springs selected in this study is vulnerable to contamination and is therefore treated before distribution. Although water from the selected springs is treated before consumption, a public health risk may arise if treatment fails. In the Oberdorf study area today, only a turbidity measurement with a chemical disinfection is performed. This one-step treatment might not be sufficient to eliminate all pathogens, such as, for example, *Cryptosporidium parvum*, and might cause human infections. Possible interventions would be a treatment including an additional filtration step in the ZQ spring, discard of water after rainfall or remediation of treatment plants to reduce the hazard for public health.

From four animal MST indicators investigated in this study, *R. coprophilus* was superior. Bacteriophages of animal host strains were detected in only low concentrations in all samples analysed. High concentrations of *R. coprophilus* were detected with both methods applied (culture and real-time PCR) in animal samples from different regions of Switzerland. For the first time, *R. coprophilus* was detected in one human wastewater sample. This observation may be explained by low amounts of animal faeces present in the human wastewater. Based on questionnaires sent to WTPs, animal impact could not be excluded from 7 out of 10 plants. In our study, slightly more *R. coprophilus* was found in liquid manure (culture: 4×10^3–9×10^6 CFU/g; PCR: 8×10^3–9×10^7 CFU/g) than by Mara & Oragui (1981) in animal faecal specimens (3.9×10^3–2.5×10^6 CFU/g) or by Savill *et al.* (2001) in cow faeces (3.3×10^5–3.6×10^6 CFU/g). Higher concentrations of *R. coprophilus* were found upstream than downstream from WTPs, and the bacterium was also detected in spring water. Therefore, *R. coprophilus* is potentially useful for MST in Switzerland, including spring water investigations. However, not only ideal target organisms but also efficient and easy-to-handle detection methods are needed for MST studies. The culture-based method is time-consuming and a rapid molecular approach was therefore included in this study and the drawbacks of the culture-based methods could thus be overcome. Although sensitivity of the molecular approach is basically high, the number of positive results obtained was lower, presumably because the assessed sample volumes were not optimally chosen and should be increased in future experiments. Consequently, for detecting animal faecal contamination in spring water, the assessed methods need further improvement and even additional animal MST indicators might be evaluated for further MST studies in Switzerland.

A study of particle transport in karst aquifers showed that transport of microorganisms, chemical pollutants

and turbidity, as well as an increase in discharge, are strongly related to precipitation (Auckenthaler *et al.* 2002). Hydrological parameters were used to describe pollution dynamics and the increase of faecal contamination in spring water. Previous studies in the LQ spring showed that microorganisms and an increase of turbidity were present after 35–116 h after rainfall (Auckenthaler 2004). Samples from all springs were therefore collected approximately 72 h after rainfall. Although only one sample was collected during a peak of turbidity from the ZQ spring, human faecal contamination was present after all rainfall events but not during periods of dry weather. Information about the correlation of MST indicators with hydrogeological parameters would provide valuable information about the optimal time-point for sampling and should therefore be further investigated.

Furthermore, future studies should tackle the question of the quantitative relationship between the occurrence of the MST indicators and the presence of human pathogens. Such investigation would need key research, fundamental to a more confident assessment of a potential exposure risk from contaminated water and to management of water quality (Roslev & Bukh 2011).

CONCLUSIONS

Based upon the results of the present study, we conclude that a combination of sorbitol-fermenting bifidobacteria and phages infecting the host strains GB-124 and ARABA 84 are promising for detecting human faecal contamination in Swiss surface and spring water, because they were found in all human wastewater samples and were never detected in samples of animal origin (Table 2), they were present in higher median concentrations downstream than upstream of WTPs (Table 2) and these three human MST indicators were also detected in spring water during periods of dry weather and after rainfall events (Tables 4 and 5). Host strain GA-17 showed a similar performance with surface and spring water and is thus promising for detecting human faecal contamination, but the fact that it was also found in one slaughterhouse sample might imply further evaluation of its specificity.

The selection of an MST tool indicative for animal faecal contamination turned out to be difficult. *R. coprophilus* was found in higher concentrations in surface and spring water than other animal MST indicators used for comparison in this study. In future research, detection of *R. coprophilus* should be further improved and compared with other methods for identification of animal sources.

Since based upon our results, low levels of the indicators in question have to be expected in spring water, we recommend using more than just one MST indicator, and several samples should be taken covering different meteorological conditions.

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