Microbial source tracking in highly vulnerable karst drinking water resources

D. Diston, R. Robbi, A. Baumgartner and R. Felleisen

ABSTRACT

Water resources situated in areas with underlying karst geology are particularly vulnerable to fecal pollution. In such vulnerable systems, microbial source tracking (MST) methods are useful tools to elucidate the pathways of both animal and human fecal pollution, leading to more accurate water use risk assessments. Here, we describe the application of a MST toolbox using both culture-dependent bacteriophage and molecular-dependent 16S rRNA assays at spring and well sites in the karstic St Imier Valley, Switzerland. Culture-dependent and molecular-dependent marker performance varied significantly, with the 16S rRNA assays displaying greater sensitivity than their phage counterpart; HF183 was the best performing human wastewater-associated marker while Rum2Bac was the best performing ruminant marker. Differences were observed in pollution regimes between the well and spring sampling sites, with the spring water being more degraded than the well site. Our results inform the choice of marker selection for MST studies and highlight differences in microbial water quality between well and spring karst sites.

Key words | 16S rRNA, bacteriophages, karst, water pollution

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INTRODUCTION

Karst areas are an important source of drinking water throughout the world, with Switzerland drawing 80% of its drinking water from groundwater aquifers (Tripet 2005). Fractures, sinkholes, and other conduits which are characteristic of karst systems, facilitate the rapid movement of water from the terrestrial surface to receiving subterranean waterbodies, with rainfall-driven influx of microbial pollution a particular problem (Pronk et al. 2009; Sinreich et al. 2014; Diston et al. 2015). This rainfall-driven overland flow may transport a range of human and animal pollutants to water resources in karst systems and as karst systems have short water retention times, in the order of hours as opposed to days (Pronk et al. 2009), little reduction of this terrestrial-derived microbial load occurs between system input and output.

Contamination of important drinking water resources by fecal pollution may lead to outbreaks of illness and economic degradation (Beaudeau et al. 2008), with human fecal pollution representing a greater risk to water users than animal fecal pollution (Soller et al. 2010). Traditional indicators of non-specific fecal pollution such as indicator bacteria, are a useful first step in identifying incidences of aquatic fecal contamination. However, as their discriminatory ability is limited and they offer no indication of pollutant source, opportunities to rectify pollution problems and undertake water use risk assessments are limited. Microbial source tracking (MST) techniques are powerful tools, able to identify the sources of pollution in catchments

using either library-dependent or library-independent methods. Application of MST techniques allows targeted remediation strategies to be employed, saving time and money (see review by Ahmed *et al.* (2016) for a discussion of current MST tools).

Here, we describe the application of a MST toolbox employing both culture- and molecular-dependent parameters within two highly vulnerable karst water resources. Previous work has shown that karst systems within Switzerland are vulnerable to rainfall-driven microbial contamination (Diston *et al.* 2015) and therefore we undertook more intense sampling than has been previously conducted in previous Swiss MST studies. The aims of this paper are: (i) to compare the sensitivity of culture- and molecular-dependent MST indicators in two highly vulnerable karst catchments (one spring and one well subject to similar land use); and (ii) to trial 16S rRNA Bacteroidales indicators not previously used in Switzerland.

MATERIALS AND METHODS

Sample locations and collection of water samples

Historic data provided by the Cantonal Laboratory, Bern allowed the identification of 14 sites within the St. Imier valley (Bern, Switzerland) where thermotolerant *Escherichia coli* were frequently recorded in groundwater samples (data not shown). To assess the magnitude of fecal pollution, preliminary sampling was undertaken at all 14 sites on two occasions using fecal coliforms (FC), intestinal enterococci (ENT), and the human wastewater-associated marker HF183 (Seurinck *et al.* 2005). Two sites, Sonceboz-Sombeval and Tavannes, were found to consistently have higher concentrations of all three parameters and were selected for detailed study (Figure 1).

The sites at Sonceboz and Tavannes were sampled 13 times between June and December 2014 at approximately 2-week intervals. Both sites are related to karst aquifers and are presumed to respond rapidly to rainfall. The sampling point at Sonceboz was in a pumping house, which extracts water from the aquifer via a well, whereas the sampling point at Tavannes was a small reservoir receiving water directly from the aquifer. Although the sampling

sites are located only 4 km apart, they belong to different drainage basins. Land use at both sites is similar, with a mixture of extensive cattle grazing, meadows, forest, and small towns of less than 5,000 residents (2015 GIS data provided by Canton Bern Department of Economics).

At each site, 2×1 gallon (4.55 L) grab samples of untreated drinking water were collected in sterile polypropylene carboys (Nalgene, USA), with sample containers being pre-rinsed *in situ* three times before sample collection. Water samples were kept on ice blocks in a polystyrene cool box and transported back to the Federal Food Safety and Veterinary Office laboratory (Köniz, Bern, Switzerland) within 2 hours. All filtrations were conducted within 6 hours of sample collection.

MST indicators and fecal indicator bacteria

A range of culture- and molecular-dependent, source-specific and general MST parameters were assessed in this study. Three bacteriophage groups were assayed: bacteriophages infecting human-specific host strains *Bacteroides fragilis* GB-124 (Payan *et al.* 2005; Ebdon *et al.* 2007) and *B. thetaiotaomicron* ARABA-84 (Wicki *et al.* 2011; Diston & Wicki 2015), and also bacteriophages infecting the general host strain *E. coli* WG-5 (somatic coliphage (Anon 2001a, 2001b)). Phage infecting *Bacteroides* strains have been reported as being human specific, with GB-124 being one of the best performing and widely used host strains, while ARABA-84 is a phage host strain local to Switzerland. Somatic coliphages were used as a reference point to indicate the general magnitude of fecal contamination.

For each water sample, triplicate 1 L filtrations were carried out. As phage numbers within groundwater samples are typically intermittent or low, 500 mL samples were concentrated using the optimized method of Mendez *et al.* (2004). Water samples were first amended using $MgCL_2$ to a final concentration of 0.05 M and filtered at a flow rate of 2 L per hour through a 47 mm 0.22 μm nitrocellulose membrane (Millipore, Ireland). Membranes were then cut into eight fragments and transferred to an Erlenmeyer flask containing 5 mL of eluting solution (3% (w/v) beef extract, 3% (v/v) Tween 80, and 0.5 M NaCl, pH 9.0). The 5 mL eluate was placed in an ultrasonic bath for 4 minutes, the membrane subsequently removed and the whole eluate assayed according to
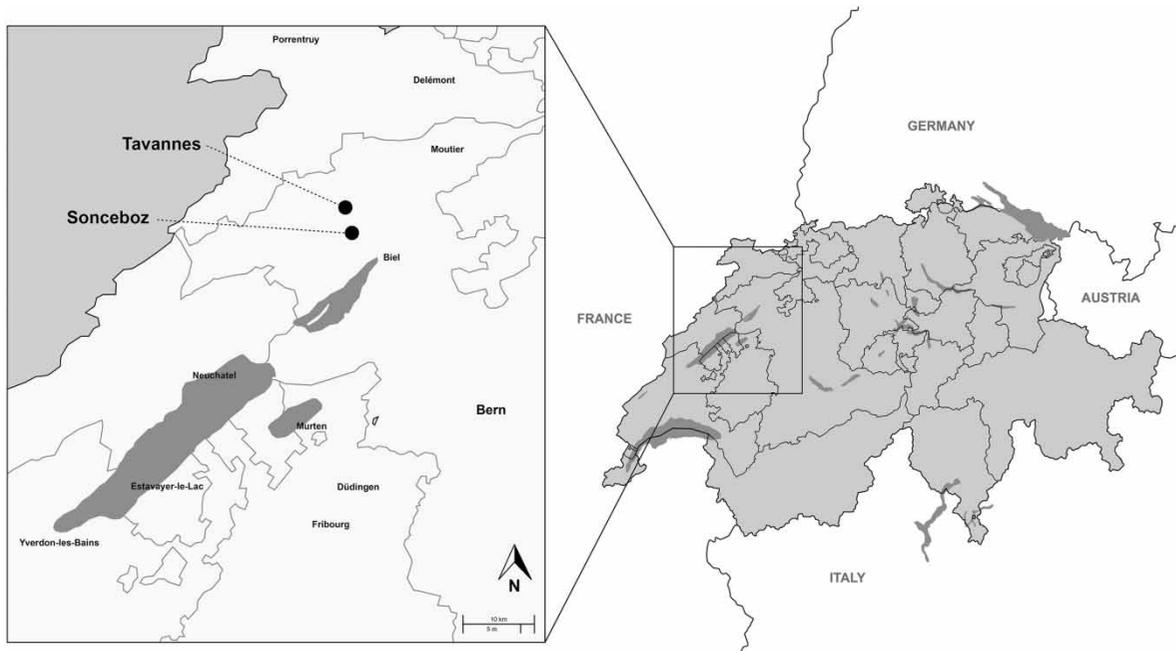
Figure 1 │ Map showing the location of the sampling sites.

the International Standard method for the respective phage group (Anon 2001a, 2001b). The fragments of the filtration membrane were placed face down on a host monolayer to enumerate any phage which remained attached to the membrane and incubated at 37 °C for 18 hours. Positive filtrations using sterile Elga Purelab Ultra water spiked with appropriate reference phage and blank filtrations using only sterile Elga Purelab Ultra water were conducted to ensure the filtration process was functioning. Positive assays containing a defined number of plaque forming units (PFU) and blank assays were also conducted in order to ensure double-agar assay functioned as expected.

In order to give a general indication of the hygienic quality of the studied water sources, FC and intestinal ENT were assayed (together termed FIB). FIB were enumerated following the International Standard methods (Anon 2000a, 2000b) using membrane filtration on a 47 mm 0.45 μm mixed cellulose ester membrane (Millipore). 100 mL and 1 mL volumes of groundwater were filtered in triplicate and membranes placed on agars. FC were incubated for 24 hours at 44 °C on mFC agar (Difco, BD, France) while ENT were incubated for 48 hours at 36 °C on mENT agar (Difco, BD). Positive (sterile Elga Purelab Ultra water spiked with reference bacteria) and blank

filtrations (sterile Elga Purelab Ultra water only) were conducted in parallel.

In contrast to the time-consuming phage-based indicators, quantitative polymerase chain reaction (qPCR) assays targeting Bacteroidales 16S rRNA gene sequences were used to indicate ruminant, general, and human wastewater-associated contamination. Six markers were assayed: human wastewater-associated markers HF183 (Seurinck *et al.* 2005) and HuBac (Layton *et al.* 2006); ruminant-specific markers BacR (Reischer *et al.* 2006, 2013) and Rum2Bac (Mieszkin *et al.* 2009); and general markers GenBac (Dick & Field 2004) and AllBac (Layton *et al.* 2006). These markers have been widely researched and used in numerous studies globally (Staley *et al.* 2012, 2015). Primer and probe sequence of these markers are shown in Table 1.

500 mL of water sample was filtered on a 47 mm 0.2 μm cyclopore track etched membrane (Whatman, USA). The filter was folded and transferred into a 15 mL DNA-free, screw-top test tube containing 0.5 mL of GITC buffer (5 M guanidine isothiocyanate, 100 mM EDTA (pH 8.0), 0.5% Sarkosyl), inverted and stored at −20 °C until DNA extraction. QIAmp DNA mini kit (Qiagen, USA) was used to extract DNA from thawed filters. 3 μL DNA aliquots, and dilutions thereof, were used in qPCR assays and were run on an ABI

Table 1 | Details of qPCR assays used

Target	Function	Primer sequence 5′–3′	Reference
AllBac (16S rRNA target)	Forward primer Reverse primer Probe	GAGAGGAAGGTCCCCCAC CGCTACTTGGCTGGTTCAG (FAM)CCATTGACCAATATTCCTCACTGCTGCCT(BHQ-1)	Layton *et al.* (2006)
GenBac (16S rRNA target)	Forward primer Reverse primer Probe	AACGCTAGCTACAGGCTTAACA ACGCTACTTGGCTGGTTCA (FAM)CAATATTCCTCACTGCTGCCTCCCGTA(BHQ-1)	Dick & Field (2004)
HF183 (16S rRNA target)	Forward primer Reverse primer	TACCCCGCCTACTATCTAATG TACCCCGCCTACTATCTAATG	Seurinck *et al.* (2005)
HuBac (16S rRNA target)	Forward primer Reverse primer Probe	GGGTTTAAAGGGAGCGTAGG CTACACCACGAATTCCGCCT (FAM)TAAGTCAGTTGTGAAAGTTTGCGGCTC(BHQ-1)	Layton *et al.* (2006)
BacR (16S rRNA target)	Forward primer Reverse primer Probe	GCGTATCCAACCTTCCCG CATCCCCATCCGTTACCG (FAM)CTTCCGAAAGGGAGATT(BHQ-1)	Reischer *et al.* (2006)
Rum2Bac	Forward primer Reverse primer Probe	ACAGCCCGCGATTGATACTGGTAA CAATCGGAGTTCTTCGTGAT (FAM)ATGAGGTGGATGGAATTCGTGGTGT(BHQ-1)	Mieszkin *et al.* (2009)
Human enterovirus	Forward primer Reverse primer Probe	GGCCCCTGAATGCGGCTAAT CACCGGATGGCCAATCCAA (FAM)CGGACACCCAAAGTAGTCGGTTCCG(BHQ-1)	Donaldson *et al.* (2002)
Norovirus genogroup I	Forward primer Reverse primer Probe 1 Probe 2	CGYTGGATGCGNTTYCATGA CTTAGACGCCATCATCATTYAC (FAM)AGATYGCGATCYCCTGTCCA(BHQ-1) (FAM)AGATCGCGGTCTCCTGTCCA(BHQ-1)	Kageyama *et al.* (2003)
Norovirus genogroup II	Forward primer Reverse primer Probe	CAAGAGTCAATGTTTAGGTGGATGAG TCGACGCCATCTTCATTCACA (FAM)TGGGAGGGCGATCGCAATCT(BHQ-1)	Jothikumar *et al.* (2005) Kageyama *et al.* (2003)
Human rotavirus (group A)	Forward primer Reverse primer Probe	CAGTGGTTGATGCTCAAGATGGA TCATTGTAATCATATTGAATACCCA (FAM)ACAACTGCAGCTTCAAAAGAAGWGT(BHQ-1)	Jothikumar *et al.* (2009)
MS2 bacteriophage	Forward primer Reverse primer Probe	CCTCAGCAATCGCAGCAAA GGAAGATCAATACATAAAGAGTTGAACTTC (FAM)CAAACATGAGGATTACCCATGTCGAAGACA(BHQ-1)	O'Connell *et al.* (2006)

7500 Real Time PCR System (Applied Biosystems) with either Roche FastStart Universal Probe Mastermix (for AllBac, GenBac, Rum2Bac, BacR, and HuBac) or Roche SYBR green Fast Start Probe Mastermix (for HF183). Positive amplicons were inserted into plasmids (TOPO TA Cloning® Kit, *E. coli* DH5α-T1^R, Invitrogen, Germany), the DNA extracted (PureLink® Quick Plasmid Miniprep Kit, Invitrogen) and quantified using a Nanodrop spectrophotometer. Standard curves using extracted plasmid DNA from 1.0×10^5 to 1 copy/reaction were created and run in the qPCR assays. The concentrations of the primers and probes in the qPCR systems remained unchanged from the original papers. Blank

filtrations using sterile Elga Purelab Ultra water were conducted in parallel. Where samples returned a negative result, they were re-tested using further dilutions to investigate possible inhibition.

Human enteric viruses

Norovirus genogroups I and II, human enterovirus, and human rotavirus group A were assayed using qPCR. Samples were concentrated using the method of Katayama *et al.* (2002) and although this method uses only 1 L of water, it has been used successfully in many environmental

studies (e.g., Fong *et al.* 2005, 2010; Gentry *et al.* 2009). MgCl$_2$ was added to 500 mL groundwater (it was not possible to filter 1 L owing to filter clogging) achieving a final concentration of 25 mM and amended samples were then spiked with 5,000 PFU of process control phage MS2. 10% (v/v) C$_2$H$_4$O$_2$ was used to acidify samples to pH 3.75 and sample was then filtered through a 47 mm 0.45 μm HA negatively charged membrane (Millipore). The membrane was washed using 100 mL of 0.5 mM H$_2$SO$_4$ and 10 mL of 1 mM NaOH poured onto the membrane to elute virions. Eluate was recovered in a tube containing 0.1 mL of 50 mM H$_2$SO$_4$ and 0.1 mL of 100X Tris-EDTA (TE) buffer. Further purification was carried out using Centriprep YM-50 concentrator columns (Millipore), achieving a final volume of 280 μL. RNA extraction was conducted using QIAamp Viral RNA Mini Kit (Qiagen, USA) using 280 μL of the concentrate with a final elution volume of 60 μL. High Capacity cDNA Archive Kit (Applied Biosystems, USA) was used to synthesize cDNA, which was then stored at −20 °C until required.

The viral qPCR assays remained unchanged from the original publication, with the exception of substituting TAMRA for BHQ-1 in all systems (Table 1). Assays conducted were: norovirus genogroup I (Kageyama *et al.* 2003), norovirus genogroup II (Jothikumar *et al.* 2005), human enterovirus (Donaldson *et al.* 2002), and group A human rotavirus (Jothikumar *et al.* 2009). In order to monitor the filtration, elution, RNA extraction, and reverse transcription procedure, spiked MS2 phages, as described above, were used as a process control (O'Connell *et al.* 2006). Viral assays contained 3 μL DNA (and dilutions thereof), equating to 25 mL equivalent volume of groundwater with a sensitivity of 40 virions per liter, and were run on an ABI 7500 Real Time PCR System (Applied Biosystems) using Roche FastStart Universal Probe Mastermix throughout. Standard curves using purified qPCR product from 1.0×10^5 to 1 copy/reaction were included in the qPCR assays. Positive (RNA extracted from viral positive stool) and negative controls (sterile Elga Purelab Ultra water) were assayed in parallel.

Rainfall data

Rainfall data were sourced from the Swiss Rainfall Bulletin (Federal Office of Meteorology and Climatology

MeteoSwiss) and 1–5 day cumulative totals were calculated. The nearest weather station to the sampled sites was Courtelary, approximately 9 and 12 km from Sonceboz and Tavannes, respectively.

Statistical methods

As the data were non-normally distributed (Shapiro–Wilk test) and log transformation did not produce normally distributed data, non-parametric statistical methods were used throughout. Microsoft Excel 2013 for Windows was used to compute normality tests, descriptive statistics, Spearman rank correlations, and Mann–Whitney U tests. The interpretation of Spearman rank ρ was as follows: 0.9 to 1 = very strong, 0.7 to 0.89 = strong, 0.5 to 0.69 = moderate, 0.3 to 0.49 = low, 0.16 to 0.29 = weak, and <0.16 = not correlated. In all statistical tests, *P* values <0.05 were used to indicate statistical significance.

RESULTS AND DISCUSSION

Water characteristics

Temperature of the samples varied throughout the monitoring period (typically between 5 °C and 12 °C at both sites) and there were no significant relationships between temperature and parameter concentrations observed at either site.

General indicators of fecal pollution

Across both sites, FIB were detected in 100% of samples ($n = 26$), with FC always present at a higher concentration that ENT (median = 182 and 50 CFU/100 mL, respectively; Table 2). In conjunction, somatic coliphage were also detected in 100% of samples ($n = 26$), with a median value of 567 PFU/500 mL. Interestingly, AllBac was present in only 77% of samples (median 6.74×10^5 copies/100 mL; $n = 20$) and no inhibition of these samples was observed. Based on the ubiquitous presence of FIB and somatic coliphage at both sites, it is surprising that AllBac was absent from six samples. A previous study has also reported AllBac being absent from samples where FIB were present,

Table 2 | The overall microbial characteristics of the study sites

	FC (CFU/ 100 mL)	ENT (CFU/ 100 mL)	WG-5 phage (PFU/ 500 mL)	ARABA-84 phage (PFU/ 500 mL)	GB-124 phage (PFU/ 500 mL)	AllBac (cn/100 mL)	GenBac (cn/100 mL)	HF183 (cn/ 100 mL)	HuBac (cn/100 mL)	Rum2Bac (cn/100 mL)	BacR (cn/ 100 mL)	Norovirus genogroup I (cn/ 100 mL)	Norovirus genogroup II (cn/ 100 mL)	Human enterovirus (cn/100 mL)	Rotavirus Group A (cn/ 100 mL)
n	26	26	26	26	26	26	26	26	26	26	26	26	26	26	26
Min	34	3	42	5	3	18	22	14	1.0×10^3	320	3,426	–	–	167	–
Median	182	50	567	27	18	6.74×10^5	3.69×10^5	3.20×10^3	5.21×10^2	5.93×10^3	7.05×10^4	–	–	167	–
Max	3,700	258	3,040	46	35	6.70×10^6	3.81×10^6	6.84×10^4	4.39×10^8	2.50×10^5	2.75×10^5	–	–	167	–
% detected	100	100	100	62	46	77	54	100	46	54	35	0	0	4	0

again with no sample inhibition (Diston et al. 2015). GenBac, the alternative 16S rRNA indicator used in the study had both a lower detection rate (54%, $n = 14$) and median concentration value of 3.69×10^5 copies/100 mL than AllBac. These data suggest that AllBac is more suitable for identifying general fecal pollution in Switzerland than GenBac. The data also highlight the usefulness of cost-effective FIB when compared to 16S rRNA assays for indicating the presence of non-specific fecal pollution in karst catchments.

Human MST indicators

While FIB and the general indicators discussed above highlight the presence of fecal contamination, MST indicators allow insight into contributing sources and as mentioned in the 'Introduction', the presence of human indicators is of particular importance owing to the elevated risk from human fecal material. Of the four human-specific indicators tested, HF183 was the best performing, detected in every sample (median 3.20×10^3 copies/100 mL). The Swiss host strain, ARABA-84, was the next best performing indicator, detecting phage in 62% of samples (median = 27 PFU/ 500 mL), and finally phage infecting GB-124 and the 16S rRNA indicator HuBac were detected in 46% of samples each (median = 18 PFU/500 mL and 5.21×10^2 copies/ 100 mL, respectively). For culture-dependent phage indicators, our data agree with previously published studies showing the predominance of phage able to infect the local host strain, ARABA-84, over a host strain developed in another geographical region, GB-124 (Diston et al. 2015; Diston & Wicki 2015). Phage infecting ARABA-84 were more frequently detected and in higher concentrations than phage infecting GB-124, showing the marginal advantages of using a native host strain.

The poor performance of HuBac (in terms of low detection frequency and median values), especially when compared to HF183 and phage infecting ARABA-84, is of note and suggests that it should not be developed for use in Switzerland. As has been shown previously, molecular-dependent qPCR assays are usually more sensitive than culture-dependent phage assays (Boehm et al. 2013; Harwood et al. 2013; Diston et al. 2015) and the poor performance of HuBac was initially surprising. However, recent work conducted by Ahmed et al. (2016), published after the design

and sampling campaign of our study, shows that HuBac has poor specificity for human wastewater, 64.6%, whereas HF183 has a sensitivity of 94.6%. These data explain why HuBac performed poorly in our study. It is advisable to assess the specificity of HF183 within Switzerland if further studies using these markers are to be conducted.

Ruminant MST indicators

Previous work found that BacR performed poorly in Switzerland (Diston et al. 2015) and it was for this reason that we included an alternative ruminant indicator in the study. Rum2Bac was detected more frequently than BacR, 54% and 35%, respectively, although the median BacR concentration was higher, 7.05×10^4 cn/100 mL compared to 5.93×10^3 cn/100 mL for Rum2Bac. Specificities of between 99 and 100% have been reported for BacR (Ohad et al. 2015; Reischer et al. 2006) and 100% for Rum2Bac (Mieszkin et al. 2009); and although far from conclusive, our data appear to show that Rum2Bac may perform better than BacR in Switzerland and could be developed further. If these ruminant markers are to be used more widely in Switzerland, it is recommended that region-specific specificity and sensitivity assessments are conducted.

Viral pathogens

Viral pathogens were largely absent from water samples, with norovirus GI and GII, group A rotavirus not being detected while enterovirus was found in only one sample

(two other samples gave positive results that were on or below the limit of detection (LOD) of 40 virions per liter). In this positive sample, FIB, somatic coliphage, HF183, and HuBac were co-occurring, whereas human-specific phage indicators were absent in this sample.

Comparison of sites

It is clear from both the FIB and the MST data that Tavannes experienced a greater level of fecal pollution than Sonceboz during the monitoring period and, as FIB and general fecal markers were present on all sampling occasions, the data indicate that both sites have a consistent problem with fecal contamination (Tables 3 and 4 and Figure 2). As identified by Mann–Whitney tests (data not shown), differences in all parameters excluding ENT and somatic coliphage were statistically significant between sites, highlighting site-specific water quality.

With the exception of ENT, Tavannes recorded consistently higher concentrations of general fecal indicators, MST parameters, and the only occasion of enterovirus detection. Although FIB were detected in 100% of samples from both sites, indicating ubiquitous fecal contamination, AllBac was only detected in 54% of samples at Sonceboz (this situation was mirrored to a lesser extent by GenBac). Moreover, both ruminant and human wastewater-associated 16S rRNA markers also followed this pattern; HuBac, BacR, and Rum2Bac were detected on a single occasion at Sonceboz whereas at Tavannes they were present at 85%, 62%, and 100%, respectively. As indicated by the GIS data, predominant land use patterns are very similar, suggesting something

Table 3 | The culture-dependent microbial characteristics of the study sites

	FC (CFU/100 mL)		ENT (CFU/100 mL)		WG-5 phage (PFU/500 mL)		ARABA-84 phage (PFU/500 mL)		GB-124 phage (PFU/500 mL)	
	S	T	S	T	S	T	S	T	S	T
n	13	13	13	13	13	13	13	13	13	13
Min	34	47	3	5	42	76	5	17	6	3
Median	116	247	57	49	414	615	18	33	8	20
Max	2,033	3,700	149	258	3,040	1,845	34	46	21	35
% detected	100	100	100	100	100	100	46	77	31	62

S = Sonceboz, T = Tavannes.

Table 4 | The molecular-dependent microbial characteristics of the study sites

	AllBac (cn/100 mL)		GenBac (cn/100 mL)		HF183 (cn/100 mL)		HuBac (cn/100 mL)		Rum2Bac (cn/100 mL)		BacR (cn/100 mL)		Norovirus genogroup I (cn/100 mL)		Norovirus genogroup II (cn/100 mL)		Human enterovirus (cn/100 mL)		Rotavirus Group A (cn/100 mL)	
	S	T	S	T	S	T	S	T	S	T	S	T	S	T	S	T	S	T	S	T
n	13	13	13	13	13	13	13	13	13	13	13	13	13	13	13	13	13	13	13	13
Min	18	3.23×10^5	22	2.46×10^5	14	2.50×10^5	1,008	2.3	320	9.07×10^2	3,426	2.03×10^4	<LOD	<LOD	<LOD	<LOD	<LOD	167	<LOD	<LOD
Median	6,829	1.52×10^6	3,833	9.13×10^5	509	8.64×10^3	1,008	1.40×10^2	320	7.10×10^3	3,426	8.81×10^4	<LOD	<LOD	<LOD	<LOD	<LOD	167	<LOD	<LOD
Max	4.51×10^5	6.70×10^6	9.44×10^5	3.81×10^6	5,188	6.48×10^4	1,008	4.39×10^8	320	2.5×10^5	3,426	2.75×10^5	<LOD	<LOD	<LOD	<LOD	<LOD	167	<LOD	<LOD
% detected	54	100	46	62	100	100	8	85	8	100	8	62	0	0	0	0	0	8	0	0

S = Sonceboz, T = Tavannes.

unique about the Sonceboz source, which may inactivate/ remove microbial markers from the system. One hypothesis is that microbial communities may be degraded owing to the nature of the pipe network leading to the sampling point. At Sonceboz, water is pumped through a series of pipes, with the water often remaining stationary for a number of hours. When compared to Tavannes, which has no piped network, retention time is longer and may lead to stagnation and microbial inactivation. It is evident from the data that both sites receive human and ruminant fecal pollution, with the magnitude of both sources being greater at Tavannes. Based on the presence of enterovirus in conjunction with the overall larger load of human wastewater-associated parameters, it can be assumed that the water at Tavannes is of a greater health risk to humans than that at Sonceboz.

Between the sites, only one statistically significant correlation was observed (GenBac, $\rho = 0.96$). However, as there was usually 1 day between sampling at both sites, and as karst systems respond quickly to rainfall, this is not unexpected. At both sites, no relationship between rainfall (1–5 day cumulative) and any parameter was observed.

Differences in hydrological characteristics of the two sampling sites may, in part, account for some of the variation in observed microbial communities. The monitoring point at Tavannes is a reservoir receiving water directly from a spring, whereas the monitoring point at Sonceboz is a pumping station draining a well. The well water at Sonceboz comes from a smaller drainage area and is subject to a smaller variety of fecal inputs. In contrast, Tavannes is a natural spring draining a larger area, which may contain more heterogeneous microbial inputs (Zheng *et al.* 2013). This may explain why, despite having similar land use, the microbial contamination problem at Tavannes is worse than that at Sonceboz.

Within-site correlations

At both sites, a number of statistically significant correlations were recorded. At Sonceboz, very strong positive linear correlations between AllBac and FC ($\rho = 0.99$), GenBac and FC ($\rho = 1.00$), AllBac and GenBac ($\rho = 1.00$) were observed. Strong linear correlations were found between GB-124 and
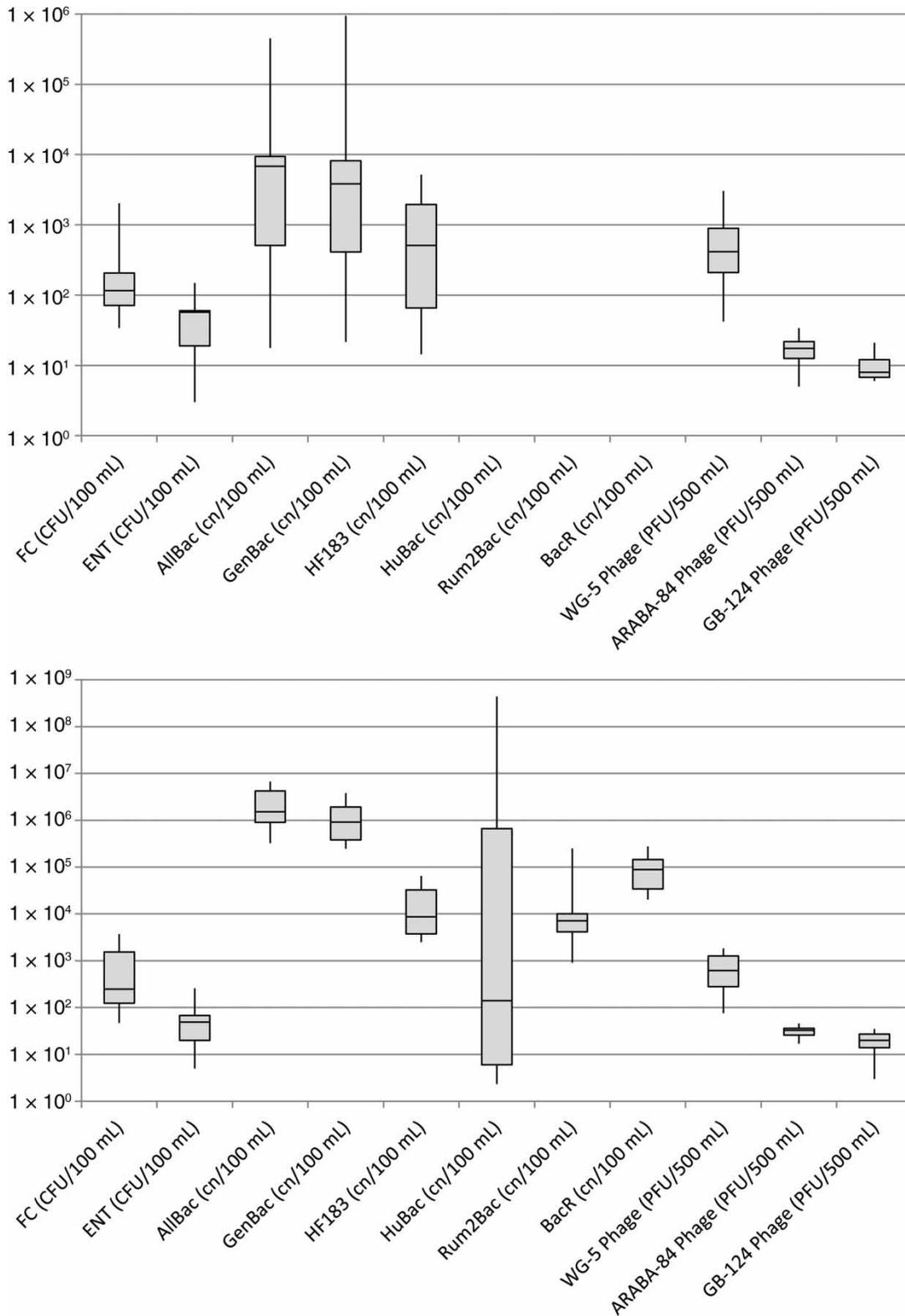
Figure 2 | MST and water quality indicator concentrations at Sonceboz (above) and Tavannes (below) sampling sites. Within-box solid lines represent the median, upper and lower edges of the box represent third and first quartiles, and upper and lower whiskers represent maximum and minimum values, respectively.

ARABA-84 ($\rho = 0.72$) and HF183 and ARABA-84 ($\rho = 0.83$) and moderate linear correlations between GB-124 and ENT ($\rho = 0.62$) and GenBac and GB-124 ($\rho = 0.61$).

At Tavannes, fewer significant positive linear correlations were observed; HF183 and AllBac ($\rho = 0.86$), BacR and Rum2Bac ($\rho = 0.74$). Moderate positive linear correlations were observed between WG-5 and ENT ($\rho = 0.69$), GB-124 and FC ($\rho = 0.69$), GB-124 and Rum2Bac ($\rho = 0.60$), FC and GenBac ($\rho = 0.60$), BacR and HF183 ($\rho = 0.59$), BacR and GenBac ($\rho = 0.57$), and HuBac and GB-124 ($\rho = 0.60$).

It is of note that there were more significantly statistical/stronger positive linear correlations at Sonceboz than Tavannes. As the water at Sonceboz is likely to be sourced from a small hydrological area, few pollutant sources are likely to contribute to the microbial load, leading to increased parameter correlation. The hydrological situation at Tavannes is more complex, with the spring draining a larger area than Sonceboz. Therefore, more pollutant sources with varying degrees of hydraulic conductivity contribute to the microbial fingerprint of the site, causing poor correlation of the parameters.

CONCLUSIONS

We analyzed two karst water sources for a range of microbiological parameters, and for the first time trialled new molecular-dependent MST indicators in Switzerland. This study has added to the understanding of how both culture- and molecular-dependent MST indicators relate to each other in karst systems, and the geographical limitation of indicators. The primary conclusions from this study may be summarized as follows:

- Molecular indicators appear to offer a more sensitive assessment of human wastewater-associated pollution in karst systems.
- Despite being geographically close and having similar land use, drinking water sourced from springs (Tavannes) may be of worse microbial quality than that of wells (Sonceboz).
- Disparities in group-specific indicators were observed; Rum2Bac appears to be more sensitive than BacR, HF183 is more sensitive than HuBac and AllBac is more sensitive than GenBac.

In order to increase understanding of the geographical limitations in terms of sensitivity and specificity, the proposed MST indicators should be evaluated in both karst and non-karst catchments in Switzerland. Further work could include studies using greater volumes of groundwater, thereby enhancing viral detection, and in conjunction with viral infectivity assays, could better inform microbial risk assessments by improving selection of the most appropriate MST toolbox.

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