IDENTIFYING HUMAN AND ANIMAL FAECAL CONTAMINATION IN SURFACE AND DRINKING WATER: NEW CONCEPTS AND APPROACHES

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ABBREVIATIONS

DNA	Desoxyribonucleic acid
CFU	Colony-forming unit
FOEN	Federal Office for the Environment
FOPH	Federal Office of Public Health
ISO	International organisation for standardization
MST	Microbial source tracking
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
PFU	Plaque forming unit
SLMB	Schweizerisches Lebensmittelbuch
Swiss TPH	Swiss Tropical and Public Health Institute
UV	Ultraviolet
WHO	World health organisation
WTP	wastewater treatment plant

SUMMARY

Waterborne disease outbreaks caused by various pathogens have been reported from all over the world. They also play an important role in industrialized countries, despite good sanitation and high standards of hygiene. In Switzerland, waterborne disease outbreaks are uncommon but faecal contamination of springs occurs quite frequently in some areas. As a consequence, microbiological quality of drinking water is not constantly sufficient and there is potential for improvement. Based on the hygiene ordinance, cantonal authorities contribute with control actions to the supply of safe water to consumers. Microbiological quality assessment based on current drinking water guidelines do not discriminate between human and animal sources of contamination. However, methods for discriminating between human and animal faecal contamination are needed to identify sources of contamination and to provide specific scientific data as a basis for risk management measures and for the implementation of remedial interventions.

The main goal of this thesis was to establish approaches for the identification of human and animal faecal contamination in surface and drinking water and to develop concepts for future investigations. A number of methods can be used to help identify sources of faecal contamination in water. The general concept is referred to as microbial source tracking (MST). Different methodologies for MST were previously used mainly for analysis of faecal samples and recreational waters such as streams, lakes and beaches. Prior to this study, MST was not applied in Switzerland.

In the present thesis, source tracking approaches never before used in MST as well as methods previously described in scientific literature were tested for their potential to indicate human or animal faecal contamination in Switzerland.

Two bacterial strains (*Bacteroides thetaiotaomicron* ARABA 84 and *B. fragilis* ARABA 19) specific for bacteriophages present in human faecal contamination and three strains (*B. caccae* RBA 63 and RBA 64 as well as *B. fragilis* KBA 60) specific for bacteriophages indicating animal faecal contamination were isolated from human wastewater and animal faecal specimens. Thereafter they were used to determine the source of surface and spring water faecal contamination.

In addition, the potential of *Streptococcus agalactiae* was tested as a new human MST indicator. Different methods of detecting these bacteria in domestic and slaughterhouse wastewater were compared. Three DNA extraction methods and five polymerase chain reaction (PCR) assays were tested to identify the most suitable combination. The most sensitive detection method, a LightCycler real-time PCR assay, detected *S. agalactiae* in human wastewater but not in animal samples and showed that this bacteria is potentially useful as human MST indicator.

Rhodococcus coprophilus was previously used in MST to indicate animal faecal contamination in water. For the detection of this organism, a culture-dependent method and a conventional as well as a TaqMan real-time PCR assay were previously published. The evaluation of these existing approaches, however, did not reveal any satisfactory result. A novel LightCycler real-time PCR assay was therefore designed and validated in the course of the present thesis. Compared with previously used assays, this new molecular approach showed advantages such as improved sensitivity and specificity and was much faster than the culture-based method.

Sorbitol-fermenting bifidobacteria and phages infecting the *Bacteroides* host strains GA-17 and GB-124 were previously described to indicate human faecal contamination. Together with the newly developed and validated approaches, these established MST indicators were included in a comparative study and their potential to indicate human or animal faecal contamination in Swiss surface and spring water was tested. Based upon this assessment, sorbitol-fermenting bifidobacteria and phages of the human host strains *B. thetaiotaomicron* ARABA 84 and *B. ovatus* GB-124 can be recommended for detecting human faecal contamination in Swiss surface and drinking water.

The above-mentioned approaches give evidence of the faecal input sites of contamination. To localize possible faecal input sites, they can be followed by an additional approach described in this thesis. This approach is based on the screening of multiresistant *Escherichia coli* and on the characterisation of selected isolates by antibiotic resistance profiles and pulsed field gel electrophoresis in order to identify identical strains. Thereby, a well equipped toolbox could be provided not only to discriminate sources of faecal contamination but also to localize possible faecal input sites.

By offering this toolbox, the present thesis provides the basis to bridge the gap from innovation to application: Recommendations based upon the concepts and protocols for analysis of MST indictors developed in this study will be elaborated and distributed to water suppliers and enforcement authorities by the Federal Office of Public Health.

ZUSAMMENFASSUNG

Pathogene Mikroorganismen, die durch Wasser übertragen werden, führen weltweit häufig zu Krankheitsausbrüchen. Auch in Industrieländern, welche über gute sanitäre Anlagen verfügen und hohe Hygienestandards aufweisen, sind solche Übertragungswege von Bedeutung. In der Schweiz treten durch Trinkwasser verursachte Krankheitsausbrüche selten auf, dennoch sind in einigen Gegenden mikrobiologische Verunreinigungen durch fäkale Indikator Bakterien relativ häufig. Dies hat zur Folge, dass die mikrobiologische Qualität des Trinkwassers nicht immer einwandfrei ist und es besteht Handlungsbedarf, um diesbezüglich Verbesserungen zu erreichen.

Basierend auf den Richtlinien aus der Hygieneverordnung, führen Kantonale Laboratorien Untersuchungen zur Kontrolle des Trinkwassers durch, womit sie einen wichtigen Beitrag für sicheres Trinkwasser leisten. Eine Unterscheidung von menschlichen und tierischen fäkalen Verunreinigungen ist jedoch anhand der gesetzlich vorgeschriebenen mikrobiologischen Kriterien nicht möglich. Methoden, die eine Unterscheidung von menschlichen und tierischen Verunreinigungen erlauben, wären jedoch notwendig, um eine wissenschaftlich fundierte Datengrundlage für ein verbessertes Risiko Management und für die Umsetzung von Sanierungsmassnahmen zu erhalten.

Ziel der vorliegenden Arbeit war, Verfahren zu etablieren, die eine Unterscheidung von menschlichen und tierischen fäkalen Kontaminationen in Oberflächengewässern und Trinkwasser ermöglichen. Zudem sollten Konzepte für zukünftige Anwendungen der Methoden entwickelt werden. Das Konzept des „Microbial Source Tracking“ (MST) umfasst verschieden Ansätze, zur Identifizierung des Ursprungs fäkaler Kontamination. Verschiedene MST Methoden wurden bereits beschrieben, die bislang hauptsächlich für die Untersuchung

fäkaler Proben oder zur Untersuchung von Wasserproben aus Badegewässern wie Flüsse, Strände oder Seen verwendet wurden. Im Rahmen der vorliegenden Arbeit wurde das Konzept des MST zum ersten Mal in der Schweiz verwendet. Dabei wurden neue, selbst entwickelte Ansätze sowie bereits früher beschriebene MST Methoden im Hinblick auf ihr Potential für die Detektion von menschlichen oder tierischen Kontaminationen in der Schweiz getestet.

Zwei Bakterienstämme (*Bacteroides thetaiotaomicron* ARABA 84 und *B. fragilis* ARABA 19), die spezifisch Bakteriophagen menschlichen Ursprungs detektieren, und drei Stämme (*B. caccae* RBA 63 und RBA 64 sowie *B. fragilis* KBA 60), die spezifisch Phagen von tierischer Herkunft erkennen, wurden aus menschlichem Abwasser und tierischen Fäzes isoliert und im Folgenden verwendet, um die Herkunft fäkaler Verunreinigungen in einem Oberflächengewässern und Quellwasser zu bestimmen.

Zudem wurde das Potential von *Streptococcus agalactiae* als neuer, menschlicher MST Indikator untersucht, indem verschiedene Methoden zum Nachweis dieser Bakterien in menschlichem und tierischem Abwasser getestet wurden. Drei Extraktionsmethoden und fünf PCR-Assays wurden verglichen, um die bestmögliche Kombination zu finden. Mit der sensitivsten Methode, einer LightCycler real-time PCR, konnte *S. agalactiae* in menschlichem Abwasser, aber nicht in tierischen Proben nachgewiesen und so die Eignung dieser Bakterien als menschlicher MST Indikator gezeigt werden.

Rhodococcus coprophilus wurde bereits früher als tierischer MST Indikator verwendet. Zur Detektion dieser Bakterien wurde in früheren Publikationen ein kultureller Nachweis, eine konventionelle PCR-Methode sowie eine TaqMan PCR beschrieben. Die Verwendung dieser

Ansätze ergab jedoch keine zufriedenstellenden Resultate. Deshalb wurde im Rahmen dieser Dissertation eine neue LightCycler real-time PCR entwickelt und validiert, welche im Vergleich zu früher beschriebenen Methoden sensitiver und spezifischer und zudem viel schneller ist als der kulturelle Nachweis.

Sorbitol fermentierende Bifidobakterien und Phagen, welche die Wirtsstämme *B. thetaiotaomicron* GA-17 und *B. ovatus* GB-124 infizieren, wurden als menschliche MST Indikatoren beschrieben. Zusammen mit den neu entwickelten und validierten Ansätzen wurden diese MST Indikatoren in einer Vergleichsstudie untersucht und ihr Potential zur spezifischen Identifizierung von menschlichen oder tierischen fäkalen Verunreinigungen in Schweizer Oberflächengewässern und Quellwasser getestet. Auf Grund der im Rahmen der Arbeit durchgeführten umfassenden Untersuchungen können Sorbitol fermentierende Bifidobakterien, die Wirtsstämme *B. thetaiotaomicron* ARABA 84 und *B. ovatus* GB-124 für einen Nachweis von menschlichen Kontaminationen im Schweizer Oberflächengewässern und Quellwasser empfohlen werden.

Die oben erwähnten MST Indikatoren geben Hinweise auf den Ursprung fäkaler Kontaminationen. Um den Ort des Eintrags zu lokalisieren, kann in der Folge ein weiterer Ansatz, der in dieser Arbeit beschrieben wird, verwendet werden. Dieser Ansatz basiert auf dem selektiven Nachweis multiresistenter *Escherichia coli* und deren weiteren Charakterisierung durch Antibiogramme und Pulsfeldgelelektrophorese um genetisch identische Stämme zu finden.

In der vorliegenden Arbeit wurde ein Methodenset ausgearbeitet, mit dessen Hilfe einerseits eine Unterscheidung menschlicher und tierischer fäkaler Kontamination möglich ist und

anderseits potentielle Orte des Eintrags lokalisiert werden können. Dieses Methodenset bildet die Grundlage, um zukünftig die Kluft zwischen Innovation und praktischer Anwendung zu überbrücken. Denn basierend auf den Konzepten und den Methoden zum Nachweis der MST Indikatoren, die in dieser Arbeit beschrieben sind, werden Empfehlungen ausgearbeitet, welche das Bundesamt für Gesundheit den Verantwortlichen für Wasserversorgungen und den Vollzugsbehörden zur Verfügung stellen wird.

1. INTODUCTION

1.1 The need to discriminate faecal contamination in Swiss surface and drinking water

Faecally contaminated drinking water can cause waterborne diseases. Consequently, safe production and distribution of drinking water is an important public health issue. Water quality assessment in Switzerland, and in most other countries, is based on using bacterial indicators – *Escherichia coli* and enterococci – which are characteristic human and animal intestinal flora. Standardised microbiological procedures for detecting these bacteria in water do not distinguish between human and animal sources of contamination (ISO 9308-1: 2000; ISO 7899-2: 2000). Therefore, different methods have been devised for detecting the source of contamination and used for a variety of purposes: to improve water quality by identifying problematic sources and determining the effect of implemented remedial solutions (Scott et al., 2002; Simpson et al., 2002), for more cost-effective and efficient management and remediation (Savichtcheva and Okabe, 2006; Simpson et al., 2002), to measure the proportional contribution of each source of faecal contamination (Field and Samadpour, 2007) and to assess health risks associated with the origin of faecal contamination (Scott et al., 2002).

A number of methods can be used to help identify sources of faecal contamination in water. The general concept is referred to microbial source tracking (MST) (Scott et al., 2002) and is a field of intensive research worldwide (Meays et al., 2004; Savichtcheva and Okabe, 2006; Scott et al., 2002; Simpson et al., 2002; Sinton et al., 1998; Stoeckel and Harwood, 2007). Methods to specifically detect human and animal faecal contamination were frequently used to identify specific sources in surface water from the United States,

Canada, New Zealand and Europe. The results of such studies showed that no single organism alone can be used for discrimination everywhere, but no consensus has been reached so far on a set or concept to be applied worldwide or in specific regions. As MST methods were not used in Switzerland before, and the utility of particular methods showed geographical differences, both previously described methods and new approaches had to be tested for application. In addition, the focus of research in Switzerland was set differently than in other countries with the major target being drinking water faecal contamination.

Identifying the source of contamination could help cantonal authorities and water suppliers to assess contamination-associated health risks (Scott et al., 2002). In addition, enforcement authorities would have specific scientific data as a basis for the implementation of remedial interventions. Methods for detecting human and animal faecal contamination would also help to define protection sites of drinking water supplies.

1.2 Drinking-water quality in Switzerland

Beside non-pathogenic microorganisms, a wide range of pathogens, including viruses, bacteria and protozoa, can be present in contaminated water and may cause disease if the water is then used for drinking, recreation, food production or personal hygiene. Surveillance of all possible pathogens would be expensive and time-consuming. For this reason indicator organisms which are naturally found in human or animal faeces are assessed to confirm the presence of contamination and show that pathogens potentially be present. Contamination may originate from a number of sources, such as sewage effluents, farming activity, livestock, wildlife and domestic animals (WHO 2003). An approach based on indicator organisms was first used in the 19th century. In 1893, Robert Koch recommended a maximum of 100 cells per millilitre of filtered water for prevention of

human disease and thus contributed to current tolerance values for water quality control. He showed that a level of waterborne bacteria below 100 colonies per millilitre could prevent an outbreak of disease (Exner et al., 2003).

In Switzerland, a value of 100 CFU/ml of heterotrophic mesophilic microbes (also known as heterotrophic plate count, HPC) is still stipulated in the Swiss Hygiene Ordinance and used to determine water quality. However, HPC does not correlate directly with faecal contamination; and HPCs are used mainly to measure the efficiency of water-treatment processes (tolerance value: 20 CFU/ml) or to indicate bacterial regrowth in water supplies (tolerance value: 300 CFU/ml). Today, the quality of Swiss drinking water is assured by tolerance values of the faecal indicator organisms *E. coli* and enterococci as well as the number of HPCs that are present (see Table 1).

According to the Swiss Food Law (LMG, Art. 23), water suppliers are responsible for self-monitoring the water quality. In addition, cantonal authorities contribute to the supply of safe water to consumers through controls based on the Hygiene Ordinance (HyV, SR 817.024.1). Together with other controls, such as management of wastewater, different treatment procedures for the production of safe drinking water and the protection of water resources, legal regulations contribute to the good quality of drinking water within many European countries, including Switzerland. Even so, quality assessments by cantonal authorities in the years 1982-1991 showed that 13% of drinking water samples tested did not meet the required quality standards as stated in the Hygiene Ordinance (Baumgartner, 1994). Estimated numbers of unreported contaminations may be even higher since quality testing is not performed in the aftermath of environmental events such as heavy rains or floods.

Table 1 Tolerance value of indicator organisms and HPCs in water[1]

Indicator organisms	Tolerance value (CFU[2]/volume)	Product
E. coli	Not detectable/100 ml	Untreated and treated drinking water, mineral water, ice
Enterococci	Not detectable/100 ml	Untreated and treated drinking water, mineral water, ice
Heterotrophic mesophylic microbes	100/ml	Source of raw drinking water
	300/ml	Drinking water in the water supply
	20/ml	Treated drinking water

[1] Source: Hygiene Ordinance (HyV, SR 817.051)
[2] Colony-forming unit

1.3 Waterborne pathogens and disease outbreaks

The understanding of the transmission of waterborne pathogens was a cornerstone in water management and public health. Since John Snows' (1813-1858) research activities in the early 19th century, it is well known that water polluted with human sewage can lead to waterborne outbreaks. He described various modes of transmission and showed that cholera was a waterborne disease before Robert Koch (1843-1910) finally cultivated the bacteria *Vibrio cholerae* (Merrill and Timmreck, 2006). *V. cholerae* was – and, in some parts of the world, still is – frequently transmitted through water. Major waterborne outbreaks, including large numbers of deaths, were caused by this bacterium worldwide (Griffith et al., 2006). With increasing standards for water quality, sanitation and personal hygiene, the risk of waterborne diseases decreased, and other pathogens became much more relevant. Several bacteria, protozoa and viruses can be transmitted through water and may cause waterborne diseases. Important pathogens which are abundant in surface and

drinking water are shown in Table 2. Pathogens which are ubiquitous in water, resistant to environmental factors (including treatment procedures) and which have small infectious doses are of special concern. Among these, viruses (in particular noroviruses), and two protozoan parasites, *Giradia lamblia* and *Cryptosporidium parvum,* are significant pathogens and have the potential to become endemic in the future (LeClerc et al., 2002). Waterborne disease outbreaks caused by these pathogens have frequently been reported worldwide (Karanis et al., 2007; Kvitsand and Fiksdal, 2010; Lysen et al., 2009; Smith et al., 2006). In Switzerland, noroviruses are often involved in disease outbreaks. Most cases involve person-to-person transmissions, but waterborne transmission has also been reported (see Table 3). Although the two protozoan parasites *G. lamblia* and *C. parvum* are highly abundant in Swiss surface water, and *Cryptosporidium* spp. is also present in spring water, no waterborne outbreaks have been reported (Wicki et al., 2009; Ruchti, 1999). By the same token, pathogens such as pathogenic *E. coli*, *Shigella sonnei* and *Campylobacter jejuni* have repeatedly been associated with waterborne outbreaks and consequently might also be of importance in the future (see Table 3). These three pathogens, and others, are subject to mandatory reporting. In September 1987, reporting of certain communicable diseases to the Federal Office of Public Health (FOPH) was required by law along with listed pathogens. The current ordinance on the reporting requirements of communicable diseases also requires that the FOPH be notified of unusual increases in diseases (SR 818.141.1). Systematic lists of outbreaks reported to the FOPH are available back to 1988. Table 3 shows only two significant waterborne outbreaks prior to this date, most likely reflecting a lack of data before registration rather than a real increase in outbreaks.

Table 2 Common water-transmissible pathogens abundant in ground and drinking water

	Pathogen	Infective dose
Bacterial pathogens	*Campylobacter jejuni*	10-500
	Salmonella Typhi	10^3
	Shigella spp.	10-100
	Vibrio cholerae	10^6-10^8
Protozoa	*Cryptosporidium parvum*	1-30
	Giardia lamblia	1-10
	Entamoeba histolytica	10^3
Viral pathogens	Adenoviruses	not specified
	Rotaviruses	10-100
	Norovirus	< 100
	Hepatits A/E	1-10
	Astroviruses	1-10
	Enteroviruses	5-10

References: (Auckenthaler et al., 2003; Baumgartner and Schmid, 1998; Parashar et al., 2001)

Although waterborne disease outbreaks are uncommon in Switzerland, they do happen. As summarised in Table 3, some outbreaks have led to severe health consequences and even deaths. One important outbreak with more than a thousand infected persons occurred in La Neuveville (Maurer and Stürchler, 2000). In-depth epidemiological investigations of outbreaks, like that of Maurer and Stürchler (2000), is the exception in Switzerland. A wide range of different pathogens can be transmitted through water. Most typically, they provoke flulike symptoms, including diarrhoea (see Table 2). If diarrhoeal patients are not systematically screened for various pathogens and if diseases are not completely recognised through surveillance, outbreaks can remain undiscovered. This underscores the need for comprehensive and effective surveillance systems and systematic epidemiological outbreak investigations. Although the number of outbreaks reported in different industrialised countries is likely to be diverse, they reflect to a certain extent, the efficiency of surveillance systems. Consequently, inadequate disease surveillance might also account

for the rather low number of waterborne diseases in Switzerland. The potential for waterborne diseases is estimated by the presence of pathogens in Swiss raw water. An ongoing study by the Federal Office for the Environment (FOEN), assessing the microbiological quality of Swiss groundwater, confirms frequent contamination of groundwater resources and the presence of pathogens. Initial results from this study show frequent contamination in samples from karstified and fissured aquifer types (unpublished data, FOEN).

Table 3 Possible waterborne disease outbreaks caused by different pathogens reported in Switzerland

Year	Location	Supposed pathogen	Number of infections	Comments	Ref.
1945	Glion	*Salmonella* Typhi	101 (16 deaths)		1
1963	Zermatt	*Salmonella* Typhi	437 (3 deaths)	Failure in treatment combined with faecal contamination of a steam	1
1988	Val d'Hérens, VS	Unknown	16	Surface water	2
1991	Obersaxen, GR	*Escherichia coli*	25	Drinking water	2
1991	Oensingen, SO	*Escherichia coli*	40	Drinking water	2
1992	Colombier, NE	*Escherichia coli*	60	Drinking water	2
1994	St. Gallen	Unknown	16	Surface water suspected	2
1995	Diemtigen, BE	Unknown	20	Water	2
1995	Bern	HPC	29	Drinking water	2
1995	Kehrsatz, BE	*Campylobacter jejuni*	21	Drinking water	2
1995	Wengen, BE	*Campylobacter jejuni*	40-100	Drinking water	2, 3
1996	Realp, UR	Unknown	16	Drinking water suspected	2
1997	Basel	*Campylobacter jejuni*	Unknown	Public swimming pool suspected	2
1997	Glatt, SG	*Salmonella* spp.	19	Surface water suspected	2
1997	Steinach, SG	Unknown	5	Surface water suspected	2
1997	Bern	Echovirus	15		3
1997	La Neuveville, BE	Unknown	30		3
1998	La Neuveville, BE	*Campylobacter jejuni* *Shigella sonnei* EPEC Norovirus[a]	1431-1607	Wastewater entered into drinking water following a pump failure.	2, 3
1999	Küblis, GR	Norovirus[a]	30-60	Groundwater used to prepare drinks.	2
1999	Fribourg	Norovirus[a]	29	Drinking water	2
2001	Zurich	Norovirus[a]	650	Drinking water suggested. Failure in treatment. No bacteria detected in the water.	4
2001	St. Gallen	Unknown	30 (10 hospitalised)	Drinking water suggested. Bacteria detected in the water.	2
2002	Thyon-Les Collons, VS	Norovirus[a]	70-90	Bacteria present in drinking water. Possible person-to-person transmission.	2
2002	Arth-Goldau, SZ	*Campylobacter jejuni*	38	No bacteria detected in the water	2
2003	Aarau, AG	*Campylobacter jejuni*	9	Stream water consummation	2
2005	Bern	*Campylobacter jejuni*	> 20	Transmission through drinking water suspected. No bacteria detected in the water.	2
2005	Haut-Intyamon, FR	EHEC/EPEC	4	Heavy rainfall	2
2007	Aargau	Unknown	30	Heavy rainfall. Failure in treatment. Bacteria detected in the water.	5
2007	Aarau, AG	*Shigella sonnei*	7	Failure in treatment. No bacteria detected in the water.	2
2008	Adliswil, ZH	*Campylobacter jejuni* VTEC ETEC Norovirus[a]	185	Wastewater entered into drinking water following an incident during refurbishment of a plant.	6

Ref.: Reference

[a] Also known as small round structured virus (SRSV) or Norwalk-like virus (NLV)

EHEC: enterohaemorrhagic *E. coli*; EPEC: enteropathogenic *E. coli*; VTEC: verocytotoxin-producing *E. coli*; ETEC: enterotoxigenic *E. coli*

Sources: 1: (Bernard, 1965), 2: unpublished data FOPH, 3: (Maurer and Stürchler, 2000), 4: (Auckenthaler et al., 2003), 5: Annual report, cantonal laboratory, AG, 6: Annual report, cantonal laboratory, ZH

Faecal contamination leading to human or waterborne disease outbreaks is influenced by several factors, such as inconsistent or inadequate treatment or treatment failure, rainfall and insufficient filter capacity of the ground. In this context, it is important to note that water can be free of indicator organisms and still contain pathogens. The effect of disinfectants on microorganisms depends on the type of disinfectant, the physiology of the microorganism, the reactor used and the water quality (pH, temperature and turbidity) (Auckenthaler et al., 2003). There is growing awareness that water treatment and disinfection are not always adequate to ensure the supply of a safe end product. Viruses, bacterial spores and particularly protozoan parasites like *G. lamblia* and *C. parvum* require specific treatment for elimination from drinking water (Auckenthaler et al., 2003; Betancourt and Rose, 2004). Chlorine is the most frequently used disinfectant worldwide (Auckenthaler et al., 2003) and also commonly in Switzerland. Inactivation of pathogens depends on chlorine concentration and contact time. Because *E. coli* and enterococci are sensitive to chlorine, they can be quickly inactivated, while certain pathogens persist. As shown in Table 3, waterborne outbreaks are frequently associated with failure of treatment. In other countries, too, inadequate, interrupted or intermittent treatment has repeatedly been associated with disease outbreaks (Reynolds et al., 2008).

1.4 Vulnerability of karstified aquifers

Drinking water in Switzerland is obtained from three natural resources: 40% from spring water, another 40% from groundwater and 20% from surface water. As shown in Figure 1, karstified aquifers are common in Switzerland, and 15-20% of spring water derives from regions with karst geology. Karstified aquifers are characterized by fluctuations in water quality parameters. Hydraulic processes are complex; but for purposes of simplicity, they can be divided into slow- and fast-flow systems. The flow velocity of karstified aquifers

and the transport of microorganisms depend on meteorological and hydrogeological conditions. During periods of dry weather, slow- to intermediate-flow systems are dominant and low levels of contamination occur. After heavy rainfall, flow velocities increase and springs become vulnerable to faecal contamination (Auckenthaler, 2004). This is due to thin or absent covering layers and, therefore, little or no attenuation of contaminants. In addition, water can flow rapidly through dissolution-enlarged fissures or sinkholes (Auckenthaler et al., 2003).

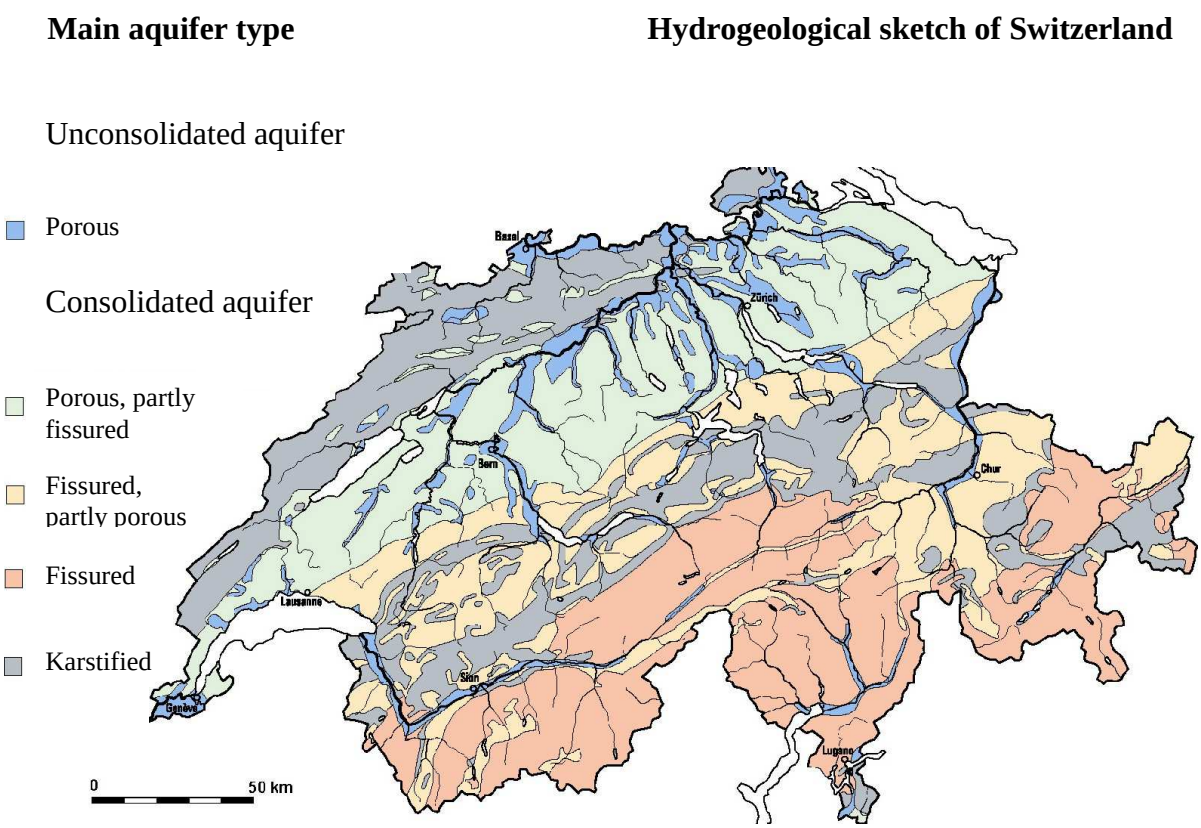

Figure 1 Predominant aquifer types in Switzerland (Source: Modified from FOEN)

Worldwide, waterborne disease outbreaks have been associated with heavy rainfall and flooding (Curriero et al., 2001; Griffith et al., 2006; Rose et al., 2001). A combination of heavy rainfall and karstified geology increases the risk of contamination with faecal bacteria including pathogens and, consequently, the risk of human disease.

1.5 Microbial source tracking: State of the science

A number of methods can be used to help identify sources of faecal contamination in water. The general concept is referred to variably as microbial source tracking (MST) (Scott et al., 2002), bacterial source tracking (BST), faecal source tracking or simply source tracking (Field and Samadpour, 2007). The goal of MST is to determine the source responsible for faecal pollution, targeting a specific classification depending on the question to be answered (U.S. Environmental Protection Agency, 2005):

- humans vs. all other sources
- species-specific results (e.g. humans vs. cows vs. horses vs. deer)
- group comparisons (humans vs. livestock vs. wildlife)
- specific individual hosts (e.g. cows from a certain farm vs. other farms vs. other livestock on farms vs. human)

Simply discriminating human from nonhuman contamination would be the first choice of water quality managers (U.S. Environmental Protection Agency, 2005). The majority of studies have used MST methods to analyse faeces and recreational waters such as streams, lakes and beaches. Findings are likely to be different for spring and surface water. Nevertheless, previous MST investigations could aid future research.

The MST methods can be classified as follows:

- Genotypic or phenotypic methods (library-dependent)
- Chemical tests to differentiate sources of contamination
- Comparison of two or more bacterial population ratios
- Detection of biological targets indicative for the source

Genotypic or phenotypic methods

Table 4 shows the many genotypic methods that have been described. The application of these methods in MST requires prior construction of libraries of individual isolates, which is time-consuming and expensive. Phenotypic methods such as antibiotic resistance, carbon use profiles and fatty acid methyl ester (FAME) profiling are also library-dependent (Field and Samadpour, 2007; Simpson et al., 2002). Methods based on libraries are often geographically restricted to areas from which isolates were characterised for library construction. The minimum number of isolates needed to construct libraries for reliable classification has not yet been defined. However, libraries need to contain hundreds of isolates per potential source impacting a specific watershed. Some authors suggested that using the genetically diverse *E. coli* for source tracking may require a library size of up to 40,000 isolates (Ahmed et al., 2005). Another disadvantage of library-dependant methods is their temporal instability. Population compositions in the intestine as well as in the environment are dynamic, and genetic characteristics of individual strains change as well (Anderson et al., 2006; Foley et al., 2009; Hartel et al., 2002; Jenkins et al., 2003; Kelsey et al., 2008; Lu et al., 2004; Scott et al., 2003). The time and effort required to establish these libraries as well as the spatial and temporal restrictions limits the practical use of these methods. Library-dependent methods have mainly been used for characterising *E. coli* isolates and will be further discussed below (New potential methods for microbial source tracking).

Table 4 Advantages and disadvantages of genotypic methods

METHOD	ADVANTAGES	DISADVANTAGES
rep-PCR (PCR analysis of repetitive DNA)	• Highly reproducible • Rapid; easy to perform • Requires limited training • May be useful to differentiate host source	• Requires reference library • Requires cultivation of target organism • Libraries may be geographically specific • Libraries may be temporally specific
RAPD (Random amplification of polymorphic DNA)	• Rapid; easy to perform • May be useful to differentiate host source	• Requires reference library • Requires cultivation of target organism • Libraries may be geographically specific • Libraries may be temporally specific • Has not been used extensively for source tracking
AFLP (Amplified fragment-length polymorphism)	• Highly reproducible • May be useful to differentiate host source	• Labour-intensive • Requires cultivation of target organism • Requires reference library • Requires specialised training of personnel • Libraries may be geographically specific • Libraries may be temporally specific • Variations in methods used in different studies
PFGE (Pulsed-field gel electrophoresis)	• Highly reproducible • May be useful to differentiate host source	• Labour-intensive • Requires cultivation of target organism • Requires specialised training of personnel • Requires reference library • Libraries may be geographically specific • Libraries may be temporally specific
Ribotyping	• Highly reproducible • Can be automated • May be useful to differentiate host source	• Labour-intensive (unless automated system used) • Requires cultivation of target organism • Requires reference library • Requires specialised training of personnel • Libraries may be geographically specific • Libraries may be temporally specific

Reference: U.S. Environmental Protection Agency (2005)

Chemical tests to differentiate sources of contamination

Chemicals can be used for source tracking if they are associated with faecal discharges. Sterols, stanols, fluorescent whitening agents (FWAs), sodium tripolyphosphate (STP), long-chain alkylbenzenes (LABs), caffeine, fragrances, bile acids and pesticides have been used to detect human and non-human faecal contamination (Field and Samadpour, 2007; Simpson et al., 2002; Sinton et al., 1998). Feacal sterols have been widely used to indicate the source of faecal contamination. Coprostanol is a sterol which was found in high amounts in human faecal discharges (Blanch et al., 2004). It was shown that coprostanol cannot be used to distinguish between human and pig faecal contamination because it occurs in similar concentrations in faeces of both species (Sundram et al., 2006). The analysis of four faecal sterols (coprostanol, 24-ethylcoprostanol, epicoprostanol and cholestanol) showed no direct relation to humans or a specific animal species (Blanch and Jofre, 2004). However, two of them (24- ethylcoprostanol and epicoprostanol) were later identified as useful tools for MST when combined with other parameters in predictive models (Blanch et al., 2006). FWAs and STP, compounds included in washing powder, are found in faecal discharges and are therefore potential indicators of human-derived contamination (Sinton et al., 1998). LABs are used in the production of a widely applied surfactant in detergent. The restriction of LABs to industrial and domestic discharges makes it a potential tool for MST (Sinton et al., 1998). Caffeine, which is present in coffee and tea, has also been proposed as an indicator for human-derived contamination. A correlation was found between a combination of caffeine and fragrance materials and human wastewater (Standley et al., 2000). There are some problems associated with the use of chemicals as MST indicators. Based on chemical methods, low concentrations can be detected in water, and chemicals persisting over long time periods may indicate old or remote contamination. Some chemicals can be stored in sediments and recovered years later. For example, stanols and bile acids were successfully used to identify the origin of

faecal inputs in archaeological sites (Bull et al., 2002). Detection of persistent chemicals is therefore difficult to interpret, as they may not correlate with indicator organisms and pathogens.

Comparison of two or more bacterial population ratios

The use of bacterial population ratios for source tracking has been proposed by several authors. However, it is difficult to use the ratio of two different species to discriminate between human and animal contamination in surface and spring water. The ratios are altered when the ecological and survival characteristics of different species vary under environmental conditions. Mara and Oragui (1983) showed that the ratio of *E. coli* to total number of *Bifidobacteria* found in human faeces was different from that found in human wastewater. The ratio between *E. coli* and the total number of *Bifidobacteria* is higher in wastewater and freshwater than that found in fresh samples of faeces (Mara and Oragui, 1983). The variability of the ratio of *E. coli* to total number of *Bifidobacteria* probably differs due to different survival times in water. The potential of bacterial population ratios for discriminating faecal contamination depends on the characteristics of the different species under diverse environmental conditions. Provided that two species have equal ecological and survival characteristics, they could be used as MST tools.

Detection of biological targets indicative for the source

Library-independent detection of species or strains which specifically indicate human or animal contamination is advantageous. These organisms could be used ubiquitously without preliminary experiments for characterising isolates needed to create libraries for reliable classification. Several organisms and markers indicative for human or animal sources of contamination have been proposed, and a broad range of different detection

methods are available in the literature (Field and Samadpour, 2007). Culture-based and molecular methods have been used to detect host-specific bifidobacteria and *Rhodococcus coprophilus* in faeces and environmental waters (Blanch et al., 2006; Bonjoch et al., 2004; Long et al., 2003; Mara and Oragui, 1981; Savill et al., 2001). Molecular methods for detecting human and animal-specific bifidobacterium species have been published (Bonjoch et al., 2004; Nebra et al., 2003). Sorbitol-fermenting bifidobacteria were found to be potentially useful MST indicators in a comparative study investigating several MST parameters (Blanch et al., 2006). Methods using bacteriophages were also found to be useful to distinguish human and animal sources of contamination. Bacteriophages of the host strain *Bacteroides thetaotaomicron* GA-17 and certain genotypes of F-RNA phages were shown to be promising (Blanch et al., 2006). F-RNA phages can be divided into four subgroups. Subgroup II and III are dominant in human faeces, whereas subgroups I and IV are dominant in animal faeces. Problems associated with the use of F-RNA-coliphage subgroups as source indicators could be their low concentrations in spring water. F-RNA-coliphages were detected in only 60% of surface water samples analysed (Cole et al., 2003). Another promising host strain, GB-124, was isolated in England (Payan et al., 2005). Bacteriophages infecting this strain were found in human wastewater but never in animal faeces, and they were successfully applied in surface water (Ebdon et al., 2007).

Other potential methods based on subtractive hybridization, microarray analyses, polymerase chain reaction (PCR) assays targeting toxin, virulence genes, mitochondrial genes and direct monitoring of viral pathogens have recently been described (Field and Samadpour, 2007). The majority of MST studies used currently are based on molecular methods identifying host-specific markers from sequences of the phylum *Bacteroidetes*. Specific PCR primers have been designed that are able to discriminate human, ruminant,

pig and horse faecal contamination (Bernhard and Field, 2000; Dick et al., 2005; Layton et al., 2006; Okabe et al., 2007; Reischer et al., 2006; Reischer et al., 2007). Markers have been shown to be geographically stable and methods to be sensitive, with low detection limits (Field and Samadpour, 2007; Savichtcheva and Okabe, 2006). However, a rapid decrease in PCR-based detection of bacteroides was observed during summer months, and these methods might be limited in time as markers potentially move from one species to another (Field and Samadpour, 2007; Savichtcheva and Okabe, 2006).

Scientists agree that a conclusive result cannot be achieved through the application of any single method. Different MST tools have therefore been combined and predictive models have been designed using multiple markers to identify primary sources of pollution (Balleste et al., 2010; Blanch et al., 2006). However, different matrices show different responses, and thus far no model for spring water has been reported.

2. METHODOLOGY

Prior to this study, MST was not applied in Switzerland. The first step of this thesis was therefore a literature search to define potential concepts for identifying human and animal contamination in Swiss surface and drinking water. Potential source tracking tools available from the literature were selected for direct application or for improvement. Additionally, methods which were never before used in MST were taken into consideration. The selected toolbox was then tested in an assessment of several stages, including direct testing of wastewater as well as field studies. Comparative studies were performed to test individual methods against all other methods from the toolbox.

2.1 Selection of potential source tracking tools

The potential utility of MST indicators depends on the same criteria as for faecal indicators in general. The ideal indicator organism should fulfil the following criteria (Auckenthaler et al., 2003):

- The indicator should be present if pathogens are present
- The indicator should persist in the environment as long as pathogens persist
- The indicator should be more resistant than pathogens to treatment procedure
- Detection of the indicator should be fast and easy
- The indicator should be found in higher concentrations than pathogens

In addition to these criteria, the MST indicator used to discriminate between human and animal faecal contamination should be restricted to either human or animal faeces. A target fulfilling all these criteria is difficult to find. Based on a search of the literature, the following MST indicators (which successfully identified human or animal sources of

contamination in faecal samples and surface water) were chosen for further research in Switzerland. Sorbitol-fermenting bifidobacteria, *R. coprophilus* and phages infecting different *Bacteroides* host strains were selected for assessment. Methods available from the literature and new approaches were tested.

Sorbitol-fermenting bifidobacteria

Bifidobacteria have potential both as general faecal indicators and MST indicators. Sorbitol-fermenting bifidobacteria were shown to be human-specific, whereas strains grown on trypticase phytone yeast broth (TPYB) at 45°C were mainly derived from animal faeces (Sinton 1998). In 1983, sorbitol-fermenting bifidobacteria were shown to be specific indicators for human faecal contamination, and human bifid sorbitol agar (HBSA) was developed as selective medium for these bacteria (Mara and Oragui, 1983). Sorbitol-fermenting bifidobacteria are present in high concentrations in human faeces and wastewater. In comparison with *E. coli*, concentrations of sorbitol-fermenting bifidobacteria were higher in human faeces and comparable in raw and treated sewage (Mara and Oragui, 1983). Environmental water presents a different picture. Gram-positive, anaerobic bifidobacteria are not able to grow in water, and they do not survive for a long time in the environment. Jagals et al. (Jagals et al., 1995) demonstrated an increase in the faecal coliform-to-bifidobacteria ratio with distance from sources of human faecal contamination. Although sorbitol-fermenting bifidobacteria were found in animal faeces and mainly in faeces from pigs, they were successfully applied to indicate human faecal contamination in sewage and surface water (Blanch et al., 2006; Long et al., 2005; Mara and Oragui, 1983). Molecular methods for detecting *B. dentium* and *B. adolescentis* performed well in indicating human faecal contamination (Bonjoch et al., 2004; Nebra et al., 2003). Both culture-based detection of sorbitol-fermenting bifidobacteria and

molecular methods for detecting *B. dentium* and *B. adolescentis* were included in a comparative study assessing several MST parameters in Europe (Blanch et al., 2006). Sorbitol-fermenting bifidobacteria were found to be potential useful MST indicators, but molecular approaches were not (Blanch et al., 2006). Therefore, the culture-based method was selected for use of these bacteria as an indicator for recent human faecal pollution.

Rhodococcus coprophilus

Rhodococcus coprophilus is an aerobic, Gram-positive bacterium able to form a fungus-like mycelium. It occurs naturally in the intestines of herbivores. First described and classified by Rowbotham and Cross (1977a and b), *R. coprophilus* was one of the first bacteria used in MST. Due to the fact that it was never isolated from human faeces, it is a promising indicator for animal faecal contamination. The bacterium is present in faeces from a broad spectrum of different animal species, including cattle, sheep, pigs, horses, ducks, geese and hens, and has also been found in water contaminated with animal faeces (Jagals et al., 1995; Mara and Oragui, 1981; Oragui and Mara, 1983; Savill et al., 2001). When *R. coprophilus*-contaminated grass and hay is ingested by herbivores, the bacterium survives passage through the digestive system, and recontaminates voided dung (Savill *et al.*, 2001). Both culture-based and molecular methods for detecting *R. coprophilus* have been described (Mara and Oragui, 1981; Savill et al., 2001). The culture-based detection procedure is time-consuming, requiring more than 21 days of incubation (Mara and Oragui, 1981; Oragui and Mara, 1983; Jagals et al., 1995). In addition, complete inhibition of contaminating bacteria was not achieved on selective media (Mara and Oragui, 1981). Sinton et al. (Sinton et al., 1998) described the long survival of *R. coprophilus* in environmental waters and therefore concluded that the organism cannot be used to indicate recent pollution. In order to identify faecal contamination of spring water, the long

persistence of the target microorganism in water may be beneficial. Although it is important to note that results cannot give any indication about the time of faecal pollution, they are useful in determining animal-derived faecal contamination. In the present study, culture-based and molecular methods that are available in the literature as well as a new molecular approach were used to detect *R. coprophilus*.

Phages of *Bacteroides* host strains

Bacteroides phages infect defined host strains of Gram-negative, anaerobic bacteria *Bacteroides* spp., which are naturally found in the intestinal tract. For use as an indicator organism, detection of the bacteriophages is superior to host detection because phages are more persistent in the environment (Savichtcheva and Okabe, 2006). Prior to this thesis, bacteriophages of the host strains *B. fragilis* (HSP40), *B. thetaotaomicron* GA-17 and *B. ovatus* GB-124 were shown to indicate human faecal pollution (Blanch et al., 2006; Ebdon et al., 2007; Payan et al., 2005; Scott et al., 2002). The restriction of these organisms to human faeces makes them a promising indicator for human contamination. Using the host strain *B. thetaotaomicron* GA-17 for detecting bacteriophages was considered to be a very reliable approach in a comparative study (Blanch et al., 2006). Bacteriophages of the host strain GB-124, not included in the comparative study by Blanch et al. (2006), were never detected in animal samples but were abundant in human wastewater and in surface water (Ebdon et al., 2007; Payan et al., 2005). However, *Bacteroides* bacteriophages were shown to be restricted to geographic area. Consequently, the specificity and sensitivity of bacteriophage methods must be tested before use. The *Bacteroides* host strains GA-17 and GB-124 were selected for assessment in Switzerland. In addition, new potential host strains were isolated and tested.

2.2 New potential methods for microbial source tracking

Multiresistant *Escherichia coli*

Escherichia coli has been used for nearly a hundred years as a faecal indicator in food and water control. Due to absence of urease and β-glucuronidase, *E. coli* can be differentiated from other *Enterobacteriaceae* and culture is fast and easy to perform. In addition, this bacterium is molecularly well characterised. It is therefore tempting to use *E. coli* to discriminate between human and animal faecal contamination, and a broad range of phenotypic and genotypic approaches have been tried (Ahmed et al., 2005; Carson et al., 2001; Duran et al., 2009; Kon et al., 2009; Moussa and Massengale, 2008; Myoda et al., 2003; Parveen et al., 2001; Stoeckel et al., 2004; Wiggins et al., 1999). The majority of these methods rely on huge databases which are limited to specific areas (Ahmed et al., 2005). The high genetic diversity of *E. coli* populations, differences in diversity among individuals of the same host species and temporal variability all indicate a complex population dynamics that limits the potential of *E. coli* as an indicator for discriminating between human and animal faecal contamination (Anderson et al., 2006; Stoeckel et al., 2004).

Given the established variability within *E. coli* populations, a new approach for characterising microbial contaminant sites was investigated in the context of this thesis. A selective plate was used to screen for multiresistant *E. coli* strains in order to reduce the number of isolates, which were then further analysed by antibiotic resistance profiles and molecular fingerprinting techniques (PFGE).

Streptococcus agalactiae

Streptococcus agalactiae is an aerotolerant, Gram-positive bacterium which causes mastitis in cattle and severe invasive disease in humans. For a long time *S. agalactiae* was the most important agent of bovine mastitis (Fink, 2002). Today, the bacteria have become very rare on Swiss farms, and the epidemiological impact of *S. agalactiae* for mastitis is now negligible in Switzerland (Busato et al., 2000; Ivemeyer et al., 2009; Roesch et al., 2007). *Streptococcus agalactiae* has been cultivated from samples of other animals but infrequently detected in animal faeces (Amborski et al., 1983; Bishop et al., 2007; Fink, 2002; Pereira et al., 2010; Yildirim et al., 2002). Therefore, *S. agalactiae* of animal origin is supposed to be of little significance for water contamination. In contrast, human *S. agalactiae* isolates prevalent in humans are excreted with human urine and faeces, and it is reasonable to assume that they might also be found in environmental waters (Manning et al., 2004). *S. agalactiae* was not previously used in MST, and its potential as faecal source indicator was investigated for the first time in the context of this thesis.

2.3 Assessing methods for application in Switzerland

The assessment of all methods tested within this PhD thesis comprised several stages of testing.

Stage 1: Proof of concept

The first stage of assessment as a proof of concept was restricted to laboratory testing. For this purpose, spiked samples of pure cell cultures, human and animal wastewater or liquid manure were analysed. Limits of detection were defined in different matrices of interest, e.g. wastewater and environmental raw water. In addition, false positive and false negative

rates were defined using liquid manure, human and slaughterhouse wastewater to assess the sensitivity and specificity of the methods. To further validate the techniques, standard procedures were defined to specify sample collection, filtration, materials, equipment and data analysis. Methods that failed in this first step of validation (e.g. growth of animal bifidobateria on TPYB agar or culture-based detection of *S. agalactiae*) were not considered for further research and are therefore not described in this study.

Step 2: Geographic stability

To test for the geographic stability of the selected methods, human wastewater, liquid manure and slaughterhouse wastewater were collected from different parts of Switzerland (see Figure 2).

Step 3: Field validation

Two well-characterised study areas were selected, and all methods were tested in the field with surface and spring water samples where human and animal faecal contamination was expected to occur. A method was considered to be feasible when it was sensitive enough to detect the target in environmental samples.

Prior to this thesis, no MST studies had been done to investigate whether the selected organisms occur in spring water. Field tests were performed to test the feasibility of the methods to detect target organisms in spring water under different weather conditions. Samples were analysed after dry periods and after rainfall, when faecal contamination was expected.

Step 4: Comparative analysis

A comparative analysis of all methods included in the selected toolbox was performed. Based on results obtained from analysis of human and animal wastewater, surface water and spring water, the appropriate combination of indicators for reliable source tracking in Switzerland was proposed.

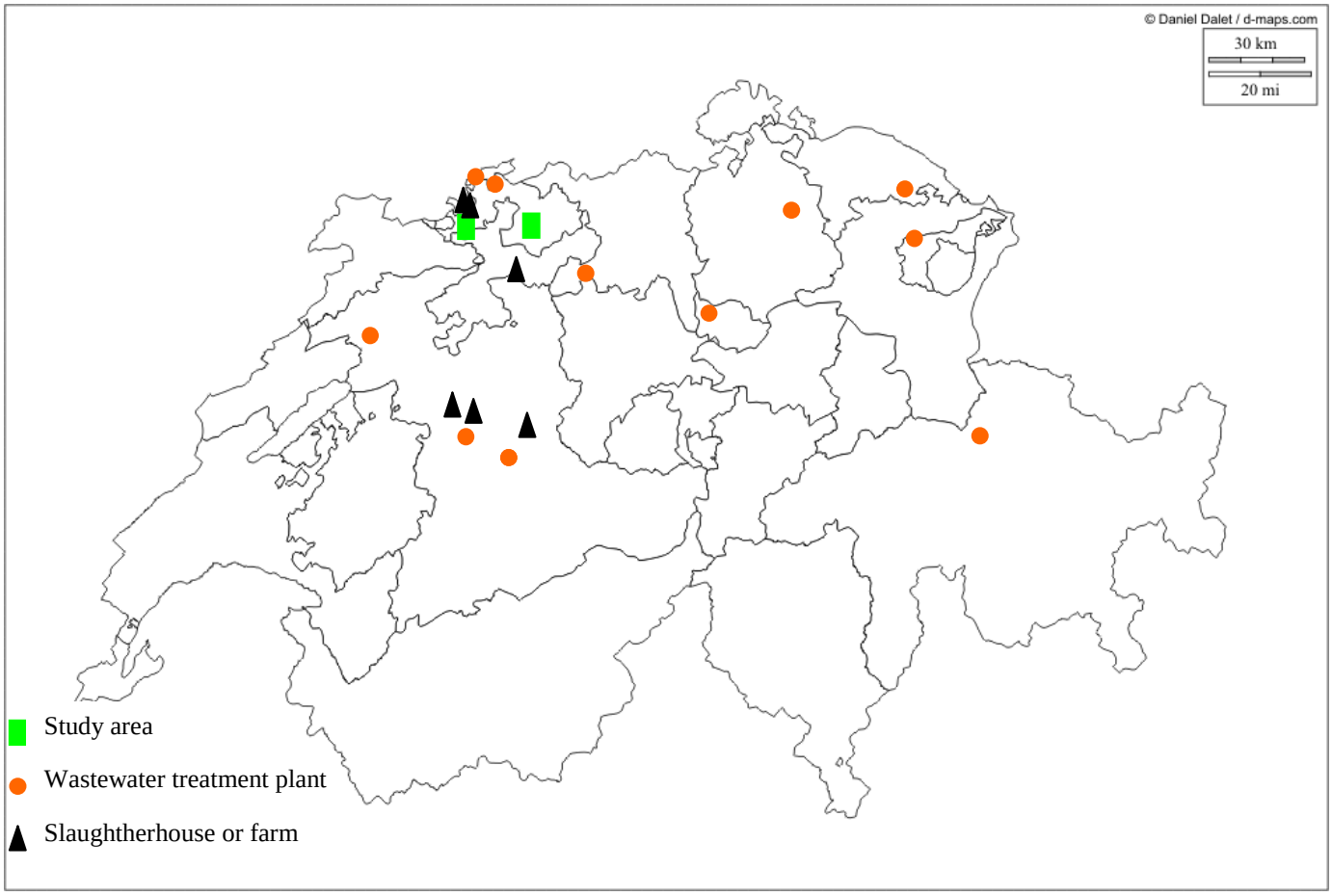

Figure 2 Distribution of sampling sites in the study area of Switzerland
The original map is available under the following URL: http://d-maps.com/conditions.php?lang=en

2.4 Study areas

Two study areas were selected for investigation in the context of the thesis. Both areas are located in the north-western part of Switzerland, with karst as the main aquifer type (see Figure 1). The springs located in these study areas frequently showed increased concentrations of faecal indicator organisms. *Escherichia coli* concentrations of more than 1000 CFU per 100 ml were repeatedly reported. The areas are located in rural territories characterised by intense dairy farming and animal husbandry. Therefore, animal faeces are assumed to contaminate spring water. In addition, human faecal contamination is also expected because all springs are located downstream of the effluent discharge point of wastewater treatment plants.

The Röschenz study area is located in the Lützel valley in canton Basel-Landschaft. The water supply is mainly derived from the two karst springs: Kächbrunnenquelle (KQ) and Lützelquelle (LQ). The KQ spring is located on the east side of the Lützel stream and the LQ spring on the west side. The catchment areas of the two springs are different. Previous hydrogeological studies showed that the Lützel stream infiltrates somewhat into the KQ spring (Auckenthaler, 2004) and is fed by a region at the eastern side of the Lützel valley. The stream receives runoff from agricultural animals, mainly cows, and from a wastewater treatment plant. The LQ spring is fed by infiltrating rainwater on a plateau south-west of the spring (Auckenthaler, 2004). The Niederhuggerwald hamlet, located on that plateau, is characterised by intense dairy farming and animal husbandry. The households in the hamlet are not connected to a wastewater treatment plant. Domestic wastewater is collected together with liquid manure in a reservoir and used as fertilizer in the spring and summer months. Previous hydrogeological tracer experiments demonstrated a hydrogeological connection from a meadow of this hamlet to the LQ spring (Auckenthaler,

2004). The spring water is treated in a six-step procedure (flocculation, sedimentation, rapid sand filtration, ozonisation, charcoal filtration and chemical disinfection). Contamination is of special concern if the treatment process fails.

The Oberdorf study area is located in the Waldenburger valley, in canton Basel-Landschaft. The “Z’Hof” (ZQ) spring is an important source of the water supply of Niederdorf and occasionally supplies water to the Waldenburger valley. After heavy rainfall, faecal contamination has been observed in spring water. The catchment area of the ZQ spring is complex, and the spring is fed by several inflows. Hydrogeological investigations have shown a connection from the Weigistbach stream to the ZQ spring. Diffuse contamination from the agricultural catchment area has rarely been demonstrated. The authors conclude that faecal contamination is mainly of human origin. Wastewater treatment plants have a stormwater overflow to release untreated wastewater after rainfall (Schudel, 2005). Although the stream receives runoff from agricultural animals, mainly cows and horses, human sewage from wastewater treatment plants may be dominant. Infiltration is not expected during dry periods because the stream infiltrates completely into the ground around 500 metres upstream of the ZQ spring. The spring water is treated by chemical disinfection, and contamination with pathogens resistant to this treatment is of special concern.

3. OBJECTIVES

The main goal of this thesis was to find applicable methods and strategies to identify the source of faecal contamination in drinking water supplies by discriminating human and animal pollution. Recommendations for application of microbial source tracking by cantonal control authorities will be defined based on the results obtained through this work. To achieve this goal, the project comprised the following four stages:

1. Establishment of detection methods and investigation of sewage and surface water

Main question:

- Are the proposed detection methods useful for discriminating human and animal faecal contamination in Switzerland?

2. Improvement of detection methods and validation under natural conditions

3. Spring water investigation

Main questions:

- Do the indicator organisms proposed to discriminate between human and animal faecal contamination occur in detectable concentrations in spring water?
- Is there a temporal persistence of multiresistant resistant *E. coli* populations in spring water?
- What is the main source of contamination in the two study areas?
- Does rainfall enhance human faecal contamination?

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4. PUBLICATIONS

Paper 1:

Novel *Bacteroides* host strains for detection of human and animal specific bacteriophages in water

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Paper1:

Novel *Bacteroides* host strains for detection of human and animal specific bacteriophages in water

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Abstract

Bacteriophages active against specific *Bacteroides* host strains were shown to be suitable for detection of human faecal pollution. However, the practical application of this finding is limited because some specific host strains were restricted to certain geographic regions. In this study, novel *Bacteroides* host strains were isolated that discriminate human and animal faecal pollution in Switzerland. Two strains specific for bacteriophages present in human faecal contamination and three strains specific for bacteriophages indicating animal faecal contamination were evaluated. Bacteriophages infecting human strains were exclusively found in human wastewater, whereas animal strains detected bacteriophages only in animal waste. The newly isolated host strains could be used to determine the source of surface and spring water faecal contamination in field situations. Applying the newly isolated host *B. thetaiotaomicron* ARABA 84 for detection of bacteriophages allowed the detection of human faecal contamination in spring water.

Introduction

Outbreaks of waterborne diseases as a result of faecal contamination of water resources are caused by various bacteria, viruses and protozoan pathogens. Pathogens which impact human health are more frequently transmitted through faecal pollution of human than of animal origin. Human pathogens reaching drinking water resources pose a hazard to public health and may cause economic losses due to temporary closure and decontamination of the water supply. In order to differentiate between human and animal contamination of drinking water and to trace the origin of faecal pollution, effective and easy to handle methods should therefore be established.

Bacteriophages infecting specific *Bacteroides* host strains were shown to be applicable in microbial source tracking (Sinton *et al.* 1998; Scott *et al.* 2002). Several host strains have been isolated and evaluated for their specificity. *B. fragilis* HSP40 was described as a host strain for detection of human specific bacteriophages (Tartera & Jofre 1987; Tartera *et al.* 1989). Due to low sensitivity of *B. fragilis* HSP40, additional human host strains, *B. ovatus* GB−124 and *B. thetaiotaomicron* GA−17, were isolated (Payan *et al.* 2005). However, differences in geographic distribution of these strains were observed. While *B. fragilis* HSP40 and *B. thetaiotaomicron* GA−17 performed well in certain areas, bacteriophages were absent in others or formed turbid plaques which were difficult to interpret (Puig *et al.* 1999; Duran *et al.* 2002; Payan *et al.* 2005). Therefore, the aim of the present study was to identify new host strains suitable to detect either human or animal faecal pollution for the socioecological area of Switzerland and to assess the occurrence and concentration of bacteriophages infecting these host strains in wastewater, surface water and springs.

Materials and Methods

Isolation of *Bacteroides* host strains

Isolation of novel *Bacteroides* host strains for detection of human or animal specific bacteriophages was based on a method for isolation of *Bacteroides* bacteriophage host strains suitable for tracking sources of faecal pollution in water (Payan *et al.* 2005).

In two independent trials, two human wastewater samples were examined for new *Bacteroides* host strains specific for bacteriophages of human origin. Isolation of presumptive *Bacteroides* strains, preparation of phage suspensions and screening of specific strains over three levels (level 1 to level 3) were undertaken according to a previously described method (Payan *et al.* 2005). Gram-negative rods growing under strict anaerobic conditions were classified as level 1 isolates. Level 1 isolates were further classified into level 2 isolates when

they proliferated well in BPRM broth (OD620 > 1) and when they detected bacteriophages in phage suspensions prepared from samples of their origin. Level 2 isolates were further classified as level 3 isolates showing specificity for their origin. For classification of human level 3 isolates, one slaughterhouse wastewater sample was used to prepare the phage suspension and for classification of animal level 3 isolates phage suspension was prepared from two human wastewater samples.

Animal *Bacteroides* host strains were isolated from wastewater of two slaughterhouses, six samples of pooled cow manure (from one to approximately ten cows) and eight samples of horse faeces. Isolation of animal strains from slaughterhouse wastewater was performed in the same way as for human host strains. Animal faeces were analysed within 2 hours after collection by plating them onto *Bacteroides* bile esculine (BBE) agar (Livingston *et al.* 1978). To prepare phage suspensions, faecal samples were suspended in peptone saline solution in a ratio of 1:10 (wt/vol) and filtered with MillexGP filters (Millipore). The suspensions were frozen with 10% glycerol (85%) at -70°C or else used immediately for analysis. Further screening and classification over the three levels of the animal *Bacteroides* isolates was done according to Payan *et al.* (2005). For classification of animal level 2 isolates, phage suspensions were prepared from animal samples (slaughterhouse, cow and horse faeces). For classification of animal level 3 isolates the human phage suspension, previously prepared to test human isolates, was used.

Specific isolates of human and animal origin were phenotypically characterized with API rapid ID 32 A (Biomérieux) or by sequencing of the 16sRNA gene (IMD, Zuerich, Switzerland) and used for further analysis during the field validation.

During the whole study, *B. fragilis* RYC 2056 (provided by the Department of Microbiology, University of Barcelona, Spain) was used as reference strain. Enumeration of plaque forming units (PFU) was done following the standard protocol for detection and enumeration of bacteriophages (ISO 10705-4, 2001). According to the standard method, *Bacteroides* host strains which are used for detection of bacteriophages were utilised in the exponential growth phase after inoculation in a ratio of 1:10 (wt/vol) in *Bacteroides* phage recovery medium (BPRM) (ISO 10705-4, 2001). *B. fragilis* RYC 2056 was incubated for 3 h and all novel *Bacteroides* host strains for 5 h under anaerobic condition. For this purpose, plates were incubated in an anaerobic jar and anaerobic condition was generated with AnaeroGen (Oxoid).

Field validation of new *Bacteroides* host strains

Human wastewater Ten waste water treatment plants were randomly selected from all Swiss wastewater treatment plants, each processing sewage from more than 100'000 inhabitants. The selected plants were distributed all over the country. Waste water samples from these plants were filtered through a hydrophilic polyethersulfone membrane with 0.22 µm pore size (MillexGP, Millipore) to remove bacteria and analysed within 24 h or frozen with 10% Glycerol (85%) at -70°C. From each sample, serial dilutions were analysed in pairs. All samples were analysed with the finally selected new host strains (*B. thetaiotaomicron* ARABA 84, *B. fragilis* ARABA 19, *B. fragilis* KBA 60, *B. caccae* RBA 63 and *B. caccae* RBA 64). Enumeration of PFU was carried out according to a standard method for detection and enumeration of bacteriophages (ISO 10705-4, 2001).

Samples of animal origin Liquid manure from four farms and three wastewater samples obtained from two slaughterhouses were analysed. The farms with 20 to 30 cows or horses were located in different regions of Switzerland. Slaughterhouses represented wastewater

from cows, calves, bulls and swine. Animals were delivered daily from all over the country. Animal phage suspensions were prepared as described above. The standard method for detection and enumeration of bacteriophages was used for enumeration of PFU in dilution series of each sample (ISO 10705-4, 2001).

Study area Two study areas in the North-Western part of Switzerland were chosen for field studies. They are located in karst areas which are vulnerable to contamination and where the land is used for agricultural purposes. In both study areas, a wastewater treatment plant is located along the stream to be investigated. Because the effluents are being discharged into the stream sampling sites were chosen upstream and downstream of the effluent discharge point.

In the Roeschenz study area, two karst springs, termed “Lützelquelle” (LQ) and “Kächbrunnenquelle” (KQ), were investigated. Both springs are very vulnerable to contamination (Auckenthaler 2004). They are located close to the Lützel stream and downstream of the wastewater treatment plant. Previous hydrogeological studies have shown that the Lützel stream infiltrates to some extend into the KQ spring. The LQ spring is fed by infiltrating rainwater from a plateau south of the spring on which the hamlet of Huggerwald is located (Auckenthaler 2004).

In the second study area, Oberdorf, the karst spring “Z´Hof” (ZQ) was also studied, as it is vulnerable to contamination being infiltrated by the “Weigistbach” stream (Schudel *et al.* 2000). The wastewater treatment plant discharges sewage upstream of the spring. During dry periods all the water from the stream infiltrates the ground about 500 m upstream from the ZQ spring.

Surface and spring water During a period of 6 months, from March to August 2009, four samples from all seven sampling sites were analysed during dry weather conditions.

During the period of investigation, 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. After the second rainfall event, on the 14th of July 2009, samples were taken not only on the 17th of July but also on 21st of July because additional rainfall (< 10mm/ h) was recorded on the 18th of July.

One litre of spring water and 0.5 litre of river water were filtered through a membrane filter of 0.22 µm pore size and 47 mm diameter (GSWP04700, Millipore) to concentrate the water samples before analysis. Filtration and re-suspension were done following a method previously described (Mendez *et al.* 2004). Samples were analysed within 48 h according to the standard method ISO 10705-4 (2001) with the following modification: 5 ml elution buffer containing bacteriophages was mixed with 5 ml of the host strain and 12.5 ml semisolid BPRM agar. The mixture was poured onto a BPRM agar plate with a diameter of 145 mm. Plaque forming units were counted after 21 h of anaerobic incubation.

To determine non-specific faecal contamination, somatic coliphages were quantified using the bacterial host strain *E. coli* WG5 (provided by the Department of Microbiology, University of Barcelona, Spain). Analysis was according to the standard procedure for enumeration of somatic coliphages (ISO 10705-2, 2001).

Statistical analysis

Statistical tests were performed with the software package, SPSS 14.0.

Results

Isolation of *Bacteroides* host strains

Presumptive *Bacteroides* strains were isolated from wastewater and animal faeces. *Bacteroides* strains grow as typical black colonies on BBE agar. The occurrence of such presumptive isolates was much higher in human waste water than in horse or cow faeces. While both human wastewater samples were positive for *Bacteroides* strains, five of ten cow samples (manure and slaughterhouse waste water) were negative for *Bacteroides* strains after analysis on selective BBE agar. Therefore, more animal samples than human samples were analysed to get an appropriate number of isolates for further examination (Table 1).

During the screening of specific host strains, isolates were classified into three levels (Table 1). From the isolates obtained by analysis of human wastewater, 81% were identified as strictly anaerobic, Gram- negative rods and therefore classified as level 1 isolates. 90% of isolates from bovine samples and 76% of isolates from horse faeces could be classified as level 1 isolates.

Twenty-four (25%) isolates obtained from human waste water, 16 (23%) isolates from bovine samples and 16 (18%) isolates from horse samples had an optical density at 620 nm of > 1 after incubation under anaerobic conditions for 18-23 h in BPRM broth (ISO 10705– 4). These isolates were classified as level 2 isolates.

Two human host strains (termed ARABA 19 and ARABA 84) were classified as level 3 isolates because they detected no bacteriophages in animal phage suspension (0 PFU/ml). One isolate from cow faeces (KBA 60) and two isolates from horse faeces (RBA 63 and RBA 64)

did not detect bacteriophages in human phage solution. Hence they were specific for bacteriophages of animal origin.

The five host strains specific for detection of bacteriophages of either human or animal origin were confirmed to belong to the *Bacteroides* species by either API or DNA sequencing. The two human host strains, showing specificity for bacteriophages of human origin, were identified as *B. thetaiotaomicron* (ARABA 84) and *B. fragilis* (ARABA 19). KBA 60, isolated from cow faeces, belongs to the species *B. fragilis* and the host strains RBA 63 and RBA 64, isolated from horse faeces, were identified as *B. caccae.*

Table 1 *Bacteroides* isolates obtained from wastewater of human bovine and horse origin in Switzerland

Host origin	No. of samples	No. of isolates	Level 1 isolates	Level 2 isolates	Level 3 isolates	Strain designation
Human	2	96	78 (81%)	24 (25%)	2 (2%)	*B. fragilis* ARABA 19 *B. thetaiotaomicron* ARABA 84
Bovine	10	69	62 (90%)	16 (23%)	1 (2%)	*B. fragilis* KBA 60
Horse	8	89	68 (76%)	16 (18%)	2 (2%)	*B. caccae* RBA 63 *B. caccae* RBA 64

Level 1: gram-negative rods growing under strict anaerobic conditions.
Level 2: level 1 isolates with good growth in BPRM broth (OD620 > 1) and positive phage counts testing phage suspension of the same origin as the isolate.
Level 3: level 2 isolates showing specificity for their origin.
The percentages of positive isolates given in parentheses were calculated from the initial number of isolates.

Field validation of new *Bacteroides* host strains

Human wastewater The new *Bacteroides* isolates were used as host strains to test for phage suspensions from human wastewater collected from ten different treatment plants. The human host strain ARABA 84 detected bacteriophages in 100% of human wastewater samples tested.

When the host ARABA 19 was used for detection of phages, only one out of ten samples was negative. The median concentration of bacteriophages detected by the host strain ARABA 84 and ARABA 19 was 39 and 9.5 PFU/ml, respectively. *B. fragilis* RYC 2056 which was used as reference strain detected a median of 85 PFU/ml in the same human wastewater samples.

Bacteriophages infecting the animal host strains KBA 60, RBA 63 or RBA 64 were absent in human waste water.

Samples of animal origin Bacteriophages detected by the reference strain RYC 2056 were not found in liquid manure. Therefore, only one out of four liquid manure samples was analysed with host strains ARABA 19, ARABA 84, KBA 60, RBA 63 and RBA 64. The sample tested negative with all host strains.

Bacteriophages which were active against the animal host strains KBA 60, RBA 63 and RBA 64 were present in 67% of the slaughterhouse wastewater samples. The median concentration of bacteriophages detected was one PFU/ml for phages infecting RBA 63 and two PFU/ml for bacteriophages active against RBA 64 and KBA 60. The median of bacteriophages infecting the host RYC 2056 was one PFU/ml and therefore comparable to the concentrations detected by animal hosts.

Bacteriophages active against human strains ARABA 84 and ARABA 19 were not detected in any of the animal samples.

Table 2 Detection of bacteriophages active against novel *Bacteroides* host strains in water samples from two streams in Switzerland

	Phages of ARABA 84		**Phages of ARABA 19**		**Phages of RBA 63**		**Phages of RBA 64**		**Phages of KBA 60**	
Sampling site	% positive	Median[a]	% positive	Median[a]	% positive	Median[a]	% positive	Median[a]	% positive	Median[a]
Lützel upstream WTP	100	102	25	0	0	0	0	0	50	1
Lützel downstream WTP	100	140	33[b]	0[b]	0	0	25	0	50	0.5
Weigistbach upstream WTP	25	0	0	0	25	0	25	0	75	1
Weigistbach downstream WTP	100	9.5	0	0	25	0	50	0.5	25	0

WTP= wastewater treatment plant
[a] Median values are in PFU per 0.5 litre
Four samples per sampling site were analysed except for [b]
[b] Only 3 samples were analysed
"Lützel" and "Weigistbach" refer to names of the streams in the study area of Röschenz and Oberdorf respectively.

Surface water samples For field validation, 16 surface water samples were analysed. The occurrence and median concentration of bacteriophages active against novel host strains are summarized in Table 2. In both study areas, bacteriophages reacting with the human host strain ARABA 84 were present in all samples taken downstream from the wastewater treatment plants. There was a significant difference between bacteriophages detected by host ARABA 84 in samples collected upstream and downstream of the effluent discharge point in the Weigistbach, Oberdorf (Mann− Whitney U= 0, n_1= n_2= 4, p = 0.017 two-tailed). The concentration of bacteriophages infecting any other host strain did not increase in samples collected downstream of the wastewater treatment plant (Mann− Whitney U test, n_1= n_2= 4; p> 0.05). In addition, no significant differences in phage counts were observed between samples collected upstream and downstream of the effluent discharge point in the Roeschenz study area (Mann− Whitney U test, n= 4; p> 0.05).

Spring water The concentration of bacteriophages detected by the novel host strains ARABA 19, ARABA 84, KBA 60, RBA 63 and RBA 64 was determined during different weather conditions. The median values of bacteriophages detected in the three springs during dry weather conditions and after rainfall are shown in Table 3. As shown, bacteriophages detected by the host strains ARABA 19, ARABA 84, KBA 60 and RBA 63 were present in the investigated spring water samples after rainfall. However, the new host strains never detected bacteriophages during dry weather conditions.

In total, 24 spring water samples were analysed: Twelve samples were taken during dry weather conditions and representing low water flow conditions and additional 12 samples were taken after rainfall. The general faecal contamination was measured with somatic coliphages detected by *E. coli* WG5. The median concentration of somatic coliphages in

spring water increased significantly from 4 PFU per 100 ml during dry weather conditions to 675 PFU per 100 ml after rainfall (Mann− Whitney U= 11, n_1= n_2= 12, $p < 0.05$ two-tailed). Beside the demonstrated increase of general faecal contamination, an increase of human faecal contamination was detected. The concentration of bacteriophages detected by ARABA 84 increased significantly after rainfall (Mann− Whitney U= 12, n_1= 12, n_2= 11, $p < 0.05$ two-tailed). The concentration of bacteriophages reacting with any other host strain did not increase after rainfall (Mann− Whitney U test, n_1= n_2= 12; $p > 0.05$).

Table 3 Bacteriophage concentrations detected by novel host strains in spring water analysed before and after rainfall events in the study area in Switzerland

Isolate	Weather	Spring water sampling sites					
		LQ		KQ		ZQ	
		Median[a]	% positive	Median[a]	% positive	Median[a]	% positive
ARABA 84	dry	0	0	0	0	0	0
	rainfall	6.5	100	4[b]	67[b]	2.5	75
ARABA 19	dry	0	0	0	0	0	0
	rainfall	1.5	50	0	25	0	0
RBA 63	dry	0	0	0	0	0	0
	rainfall	0	25	0	25	0	25
RBA 64	dry	0	0	0	0	0	0
	rainfall	0	0	0	0	0	0
KBA 60	dry	0	0	0	0	0	0
	rainfall	0[b]	0[b]	0	0	0	25

[a] Median value of bacteriophages in PFU per litre
Four samples were analysed collected at four different time points for each type of weather condition
[b] only three samples analysed
LQ: Spring Lützelquelle, KQ: Spring Kächbrunnenquelle, ZQ: Spring Z´Hof

The load of faecal contamination was different after the three rainfall events. The highest concentration of somatic coliphages was found after the second rainfall event (median 25’600 PFU and 1’020 PFU per 100 ml). A lower median of 14 PFU per 100 ml and 330 PFU per 100 ml was found after the first and third rainfall event. As shown in Table 4, novel host strains indicating the highest faecal contamination were obtained following the second rainfall event.

Table 4 Presence of bacteriophages detected by novel *Bacteroides* host strains in spring water analysed after rainfall in the study area in Switzerland

	Rainfall event 1			**Rainfall event 2**			**Rainfall event 3**		
Isolate	**LQ**	**KQ**	**ZQ**	**LQ**	**KQ**	**ZQ**	**LQ**	**KQ**	**ZQ**
ARABA 84	+	n.d.	+	+	+	+	+	+	+
ARABA 19	-	-	-	+	-	-	+	+	-
RBA 63	-	-	-	+	+	+	-	-	-
RBA 64	-	-	-	-	-	-	-	-	-
KBA 60	-	-	-	-	-	-	-	-	+

LQ: Spring Lützelquelle, KQ: Spring Kächbrunnenquelle, ZQ: Spring Z´Hof
n.d.= not determined

After the first rainfall event, bacteriophages active against the human host strain ARABA 84 were present in two springs (LQ and ZQ). The result for the third spring (KQ) is missing because the agar plate which was used for analysis did not solidify. Specific bacteriophages active against other host strains were not detected in the three springs after the first rainfall event.

Higher phage counts were present after the second rainfall event in both samples taken from each spring. Bacteriophages active against ARABA 84 and RBA 63 were detected in all three springs. Moreover, one sample from the LQ springs tested positive for bacteriophages

detected by ARABA 19. Specific bacteriophages infecting other host strains were not detected after the second rainfall event.

Using the two human host strains, ARABA 84 and ARABA 19, for detection of bacteriophages after the third rainfall event, all water samples from the LQ springs and KQ were positive. In the ZQ spring, bacteriophages reacting with the *Bacteroides* hosts ARABA 84 and KBA 60 were present. However, bacteriophages detected by any other host strain could not be found in analysed spring water samples after this rainfall event.

While somatic coliphages detected by *E. coli* WG5 were present after all three rainfall events, bacteriophages infecting the reference strain RYC 2056 could be detected in only one spring water sample after the second rainfall event.

Discussion

Previous studies documented the variation in detection of *Bacteroides* host strains in different geographic regions. Therefore, different host strains need to be tested for use in particular regions. Moreover, the isolation of new specific host strains and their validation in the region of interest is recommended (Payan *et al.* 2005).

In this study, five *Bacteroides* host strains with specificity for bacteriophages of either human or animal origin were isolated. With human host strains higher numbers of bacteriophages were detected than with animal host strains. However, the five strains successfully differentiated between human and animal wastewater. The occurrence and concentration of bacteriophages infecting the five host strains was similar to the values obtained using the reference host *B. fragilis* RYC 2056 in wastewater, surface water and spring water. The

suitability of individual strains varied according to whether they would be applied in testing for contamination in wastewater, surface and spring water (see below).

Human host strains There was a clear difference between the two human strains ARABA 84 and ARABA 19. In human wastewater and surface water samples, plaque counts were always higher when using the host ARABA 84 compared to the host ARABA 19. Higher sensitivity of the host strain ARABA 84 was also observed in spring water samples. When bacteriophages active against ARABA 19 were present in spring water samples, ARABA 84 always detected bacteriophages in the same sample as well. Moreover, bacteriophages active against ARABA 84 were present in both study areas while ARABA 19 never detected bacteriophages in the Oberdorf study area.

Treating wastewater reduces its bacterial population. Nevertheless, treated sewage is still heavily loaded with faecal bacteria (Vilanova *et al.* 2004; Vilanova & Blanch 2005). Therefore, human faecal contamination might be present in surface water downstream of the effluent discharge point from wastewater treatment plants. The increase of bacteriophages detected by ARABA 84 downstream from the treatment plant in the Weigistbach confirmed this hypothesis. However, a similar increase was not detected in the Roeschenz study area which might be due to additional wastewater effluent discharge further upstream.

Animal host strains There is a difference between *Bacteroides* isolates found in human and animal intestine. *Bacteroides* isolates are found in very low numbers in animal faeces which made an isolation of new animal host strains difficult. Bacteriophages active against *B. fragilis* RYC 2056 were not detected in liquid manure. Therefore, only one out of four liquid manure samples was analysed for bacteriophages active against novel host strains. No bacteriophages were detected in the specimen. *B. fragilis* RYC 2056 is recommended as a

reference strain to determine the concentrations of phage suspensions (Payan *et al.* 2005). In this study, it was shown that phages infecting host *B. fragilis* RYC 2056 were absent or found in low concentrations in animal waste. Other studies, in which wastewater of animal origin was analysed, showed the same trend (Puig *et al.* 1999; Blanch *et al.* 2004; Payan *et al.* 2005).

For the first time we describe here host strains with specificity for phages of animal origin. The occurrence and detection of bacteriophages was comparable for the three animal host strains in waste water. In surface and spring water, a comparison of the three strains was difficult. The occurrence of bacteriophages detected by KBA 60 was higher compared to bacteriophages infecting the two other hosts in surface water. While in spring water the most phages were detected by RBA 63. The reason for the different patterns of occurrence of bacteriophages in surface and spring water samples might be due to differences in persistence of the host strains. Such a phenomenon was recently shown for F− specific RNA phage subgroups (Muniesa *et al*. 2009).

Bacteriophages were present in very low numbers in liquid manure and slaughterhouse wastewater. The field validation was thus undertaken to get a better knowledge of the potential of the newly isolated animal host strains for use in microbial source tracking. Bacteriophages detected by all three animal host strains were present in surface water. Moreover, RBA 63 and KBA 60 detected bacteriophages in spring water after rainfall events. However, in most of the analysed water samples bacteriophages active against animal host strains were not detected or present only in low concentrations. The practicability of using animal host strains for microbial source tracking should be evaluated in further studies by including additional indicators of animal pollution.

Increased faecal contamination after rainfall In Switzerland, spring water from karst aquifers are important sources of drinking water but are vulnerable to contamination including with faecal bacteria (Auckenthaler *et al.* 2003). In this study, somatic coliphages were used to test the microbiological quality of spring water and revealed considerable faecal contamination after rainfall. This finding was in accordance with previous studies which detected an increase of faecal indicator organisms, *E. coli* and enterococci after rainfall (Schudel *et al.* 2000; Auckenthaler 2004). In both study areas, previous hydrogeological analysis indicated that faecal contamination derives mainly from agriculture although human contamination is probable (Schudel *et al.* 2000; Auckenthaler 2004). To evaluate the potential of the newly isolated host strains, these karst aquifers were considered to be ideal study areas.

The differentiation of the origin of various types of contamination in spring water is crucial for public health. In this study, specific *Bacteroides* host strains were tested to assess spring water contamination. A significant increase of human faecal contamination after rainfall was detected when using the human host strain ARABA 84. Detection of somatic coliphages confirmed an increase of faecal pollution after rainfall. Therefore, it can be concluded that human faecal contamination could successfully be detected using the host strain ARABA 84, especially after rainfall events where high faecal contamination is present.

Prior to this study, no human specific faecal indicator has been used for field investigations in Switzerland. Bacteriophages were detected by human host strain ARABA 84, indicating the presence of human faecal pollution in samples collected from all three springs after rainfall events. Besides the human contamination detected in spring water, additional contamination from agriculture was expected (Schudel *et al.* 2000; Auckenthaler 2004). However, the abundance of bacteriophages indicating animal faecal pollution in water samples was rather

low and animal faecal contamination was detected in only a small number of environmental water samples.

Geographical distribution and host restriction A good indicator of human or animal contamination would be easy detectable, geographically widespread, restricted to the host organism and would be prevalent and abundant in the host organism as well as in environmental samples.

The suitability of previously isolated *Bacteroides* host strains such as *B. thetaiotaomicron* GA−17 varied in different geographical areas (Payan *et al.* 2005). Such variations might be related to regional distinctions in diets which lead to different species composition of the faecal flora. Leser *et al.* (2000) showed that the bacterial species composition in the large intestine of pigs changed with different experimental diets; some profiles obtained by genetic fingerprinting techniques were stable while others varied in distribution and were related to specific diets. Geographical differences might also be due to spatial and temporal differences as previously reported for the composition of *E. coli* isolates within and between host species (human versus individual animals) (Gordon 2001; Stoeckel *et al.* 2004). In this study, bacteriophages active against human host strains were abundant in human waste water obtained from plants distributed all over Switzerland. In addition, animal host strains detected bacteriophages in samples from slaughterhouses representing wastewater from animals from all over the country. This suggests that these bacteriophages of novel host strains are widespread in Switzerland. In addition, the field studies in two areas in the North-Western part of Switzerland showed that the host strains detected bacteriophages in Swiss surface and spring water samples. Therefore, we conclude that novel bacteroides host strains were useful to indicate human and animal faecal contamination in Switzerland. Further international

comparative studies would be desirable in order to evaluate the applicability and performance of these novel indicators in other geographic regions.

In this study, the analysis of human wastewater, liquid manure and slaughterhouse wastewater indicated host restriction of bacteriophages of the isolated human and animal host strains. Although bacteria of the genus *Bacteroides* and corresponding phages are present in the human and animal faecal flora, it was shown that bacteriophages of particular strains such as *B. fragilis* HSP40, *B. ovatus* GB−124 and *B. thetaiotaomicron* GA−17 were almost restricted to samples from human origin (Tartera & Jofre 1987; Tartera *et al.* 1989; Payan *et al.* 2005). This might be due to the co-evolution of phages, bacteria and the host (human or animal).

Conclusion

The human host strain ARABA 84 was shown to perform well in detecting wastewater, surface water and even spring water faecal contamination. The high specificity of the strain detecting bacteriophages of human origin could be shown in wastewater analysis. No plaque forming units were detected in animal samples. Moreover, the sensitivity of the strain was demonstrated through spring water analysis. After rainfall, human contamination could be identified by use of ARABA 84 host strain in three springs used as a source for drinking water. In conclusion, *Bacteroides thetaiotaomicron* ARABA 84 is a promising new tool for microbial source tracking.

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***Paper 2*:**

Comparative test of methods for detecting *Streptococcus agalactiae* in wastewater and environmental water samples

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Paper 2:

Comparative test of methods for detecting *Streptococcus agalactiae* in wastewater and environmental water samples

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Abstract

This study compares different methods of detecting *Streptococcus agalactiae* in domestic and slaughterhouse wastewater. Three DNA extraction methods and five polymerase chain reaction (PCR) assays targeting four different genes of *S. agalactiae* were tested to find the most suitable combination. The limit of detection of these methods was assessed using pure cultures of different *S. agalactiae* strains and after spiking *S. agalactiae* into human wastewater and liquid manure. The most sensitive detection method consisted of DNA extraction with the DNeasy Blood and Tissue kit from Qiagen and a LightCycler PCR assay based on the *cfb* gene. This method detected *S. agalactiae* in all human wastewater samples originating from 10 different plants situated all over Switzerland with a median concentration of 3×10^2 CFU/10 ml. In contrast, quantitative analysis of slaughterhouse wastewater was negative. The method was further validated and applied in field investigations in two study areas. Analysis of a total of 34 surface water samples showed *S. agalactiae* to be present in 10 samples which were all contaminated with treated sewage from wastewater treatment plants. The results showed that detection of *S. agalactiae* was associated with human faecal contamination and that *S. agalactiae* appears to have a potential as a new indicator for human faecal pollution in water.

Introduction

Streptococcus agalactiae is a Gram-positive bacterium possessing a group B Lancefield antigen and therefore also known as group B streptococcus. In both human and veterinary medicine the bacteria is recognized as a pathogenic agent. Whereas the term *S. agalactiae* is used for isolates of human or animal origin, B streptococcus (GBS) usually refers to human isolates.

Research activities in veterinary medicine have focused mainly on the epidemiological importance of *S. agalactiae* as a pathogen of bovine mastitis. The obligate intramammary pathogen can be transmitted from infected cows serving as reservoirs to non-infected cows in situations where hygienic practises are inadequate (Keefe, 1997). For a long time *S. agalactiae* was the most important agent of bovine mastitis (Fink, 2002). Today, other pathogens are much more relevant, and the epidemiological impact of *S. agalactiae* for mastitis has become negligible in many European countries (Busato et al., 2000; Ivemeyer et al., 2009; Piepers et al., 2007; Roesch et al., 2007; Sampimon et al., 2009). *S. agalactiae* could also be cultivated from samples obtained from mammals, birds, fish, amphibians and reptiles (Fink, 2002). In addition, *S. agalactiae* was sporadically isolated from animals living in an aquatic environment. In such studies, isolates were usually obtained from visceral organs (Amborski et al., 1983; Bishop et al., 2007; Pereira et al., 2010; Yildirim et al., 2002).

The epidemiological understanding of human diseases caused by *S. agalactiae* has changed over time. Until the late 1930s, *S. agalactiae* was only isolated from healthy persons and therefore considered to be unimportant for human health. Later, *S. agalactiae* was shown to be linked to neonatal infections (Fink, 2002). *S. agalactiae* is currently a leading cause of morbidity and mortality in neonates and pregnant women, and an increase in invasive disease in humans has been reported (Fink, 2002). In many industrialised countries, pregnant women are tested for *S. agalactiae* colonisation to prevent transmission from mother to child during birth (Sendi et al., 2008). The prevalence in healthy humans is around 20% to 30% (Manning et al., 2004). Human *S. agalactiae* isolates are excreted with human urine and faeces, and it is reasonable to assume that they might also be found in environmental waters. However, little is known about the abundance of *S. agalactiae* in wastewater and whether it occurs in environmental waters. The aims of this study were (i) to compare different DNA extraction methods and (ii) PCR assays to find a sensitive method for detecting *S. agalactiae*, and (iii) to

determine the occurrence of *S. agalactiae* in human wastewater, slaughterhouse wastewater and environmental water samples.

Materials and Methods

Preparation of bacterial stock suspensions for comparison of different DNA extraction methods and PCR assays

Stock suspensions were prepared with three different *S. agalactiae* strains: DSM 2134 (also known as ATCC 13813, NCTC 8181), DSM 6784 (ATCC 27956) and NCTC 9993 in saline solution (0.85%). The bacterial concentration of the suspension was determined by tenfold serial dilution in sterile H_2O and plate counts on Columbia blood agar plates (Biomérieux, Switzerland). Aliquots (100 µl) of the suspension, containing 10^9 CFU/ml, were stored at – 70°C.

DNA extraction methods

Serial dilutions of three *S. agalactiae* strains (DSM 2134, DSM 6784 and NCTC 9993) in tap water were used to evaluate different extraction methods. One millilitre of each dilution containing from 10^2 to 10^7 CFU/ml was used for DNA extraction. The DSM 2134 and DSM 6784 strains were used to compare all three of the DNA extraction methods listed below, and NCTC 9993 was used for extraction in methods 1 and 3.

1. Qiagen kit: One millilitre of the sample was pre-treated with enzymatic lysis buffer and DNA was isolated with the DNeasy Blood and Tissue kit (Qiagen, Switzerland) following the protocol for DNA extraction from Gram- positive bacteria. The sample was eluted in Buffer AE in a final volume of 200 µl.

2. Lysozyme/proteinase K: One millilitre of the sample was centrifuged at 6000 × g for 10 min, and 840 µl of the supernatant was discarded. DNA extraction was performed as previously described: The remaining sample was pre-treated with 20 µl of lysozyme (Merck, Switzerland) at a concentration of 10 mg/ml and incubated at room temperature for 15 min. 20 µl of proteinase K (2 mg/ml) was added for digestion at 60°C for 30 min. Finally, the sample was boiled for 10 min. Lysates were tested directly without further purification of DNA (Furrer et al., 1991; Sukhnanand et al., 2005).

3. Roche MagNA Lyser: One millilitre of the sample was centrifuged for 10 min at 20,000 × g, and 800 µl of the supernatant was discarded. The pellet was resuspended in 255 µl of MagNA Pure Bacteria Lysis Buffer (Roche, Switzerland) and 5 µl of lysozyme (10 mg/ml in 10 mm Tris HCl, pH 8) and incubated for 15 min at 37°C. Thereafter, 40 µl of proteinase K (20 mg/ml, Roche, Switzerland) was added to the sample and further incubated for 10 min at 70°C. The sample (500 µl) was transferred into a MagNA Lyser Green Beads tube (Roche, Switzerland) and disrupted with a MagNA Lyser Instrument (Roche, Switzerland) for 30 s at 3800 × g. After cooling for 1 min and centrifuging for 5 min at 17,000 × g, 490 µl of the sample was transferred to a new Eppendorf tube. A High Pure Template Preparation kit (Roche, Switzerland) was used for DNA extraction. The extraction was done according to the manufacturer's protocol with the following modifications: 400 µl of binding buffer and 200 µl of isopropanol was added to the sample and loaded onto the column twice. Finally, the sample was eluted in 200 µl of elution buffer.

PCR assays

Five different PCR assays were compared using the limit of detection. To determine the limit of detection in CFU per volume, serial dilutions of three different *S. agalactiae* strains were used. Serial dilutions containing from 10^2 to 10^7 CFU/ml were prepared as described above

under DNA extraction methods. The DNA concentration in three samples (10^7 CFU/ml) of diffferent *S. agalactiae* strains was determined using NanoDrop ND 1000 Spectrophotometer.

In addition, following DNA extraction tenfold serial dilution of individual samples (10^7 CFU/ml) of three *S. agalactiae* strains was carried out. Seven dilutions were analysed with the LightCycler PCR assay and semi-nested PCR.

The PCR assays compared were based on previously published primers and probes. Table 1 lists the primer sequences, target genes and their accession numbers. The primers and probes were synthesised by Microsynth (Switzerland) and TIB Molbiol (Germany), respectively.

The LightCycler PCR assay was performed using a LightCycler 1.1 instrument (Roche, Switzerland) in a 20 µl reaction volume consisting of the following reagents and concentrations: 2 µl of LightCycler FastStart DNA MasterHybProbe (10×), 0.8 µl of the forward primer Sag59-F (10 pmol/µl), 0.8 µl of the reverse primer Sag190-R (10 pmol/µl), 0.4 µl of each probe (STB-F and STB-C) (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 and negative control, which were extracted together with the samples analysed. Moreover, a serial dilution over 7 log was included in each PCR run to generate the standard curve used to control the efficiency and to quantify the PCR product. Data analysis was performed using the second-derivative maximum method of the LightCycler software (version 4.1.1.21).

Table 1 Background information on different primer pairs and probes used in the study.

PCR assay	Target gene	GenBank accession number	Labelling	Primer and probe sequences	Reference
LightCycler PCR	*cfb* gene	X72754	Sag59-F: Sag190-R: STB-F: STB-C:	5'-TTT CAC CAG CTG TAT TAG AAG TA-3' 5'-GTT CCC TGA ACA TTA TCT TTG AT-3' 5'-AAGCCCAGCAAATGGCTCAAA-FL-3' 5'-LC640-GCTTGATCAAGATAGCATTCAGTTGA-PH-3'	(Ke et al., 2000)
Semi-nested PCR	16S rRNA gene	DQ232512	DSF1: DSF2: DSR1:	5'-TGC TAG GTG TTA GGC CCT TT-3' 5'-GGC CTA GAG ATA GGC TTT CT-3' 5'-CTT GCG ACT CGT TGT ACC AA-3'	(Ahmet et al., 1999)
Conventional PCR 1	*hylB* gene	Y15903	Sa-hylF: Sa-hylR:	5'-CAT ACC TTA ACA AAG ATA TAT AAC CCA AA-3' 5'-AGA TTT TTT AGA GAA TGA GAA GTT TTT T-3'	(Sukhnanand et al., 2005)
Conventional PCR 2	*sodA* gene	Y12224.1	SodA-F: SodA-R:	5'-GCC CTA ATT AAA GAA ACA AAA GAG T-3' 5'-CTG TTA TCA GCT TGC TTA CCT TAA A-3'	(Sukhnanand et al., 2005)
Conventional PCR 3	*sodA* gene	Y12224.1	SodA-F2: SodA-R2:	5'-GAT TTA GCC TTA TTA AAA ATT GCT CAT-3' 5'-ATT CGC TTA ATT GAA GAT AAT AGG ACT GT-3'	(Sukhnanand et al., 2005)

Semi-nested PCR was performed using a 9800 Fast Thermal Cycler (Applied Biosystems). The first amplification reaction was carried out in a 25 µl reaction volume consisting of the following reagents and concentrations: 0.2 µl of AmpliTaq DNA polymerase LD (5 U/µl), 2.5 µl of GeneAmp 10× PCR Buffer II, 1.5 µl of $MgCl_2$ solution (25 mM), 2 µl of dNTPs (2.5 mM), 2.5 µl of the forward primer DSF1 (10 pmol/µl), 2.5 µl of the reverse primer DSR1 (10 pmol/µl), 8.3 µl of H_20 and 5 µl of template DNA. The standard amplification protocol for the first PCR run was 94°C for 4 min followed by 30 cycles of amplification (94°C for 1 min, 67°C for 1 min and 72°C for 1 min) and one cycle at 72°C for 10 min. The second amplification was performed using the same reagents and concentrations except that DSF2 was used instead of DSF1 and the annealing step was carried out at 67°C for 25 s. Furthermore, 2.8 µl of the PCR product was used as template for the second reaction (Ahmet et al., 1999). In each run positive (*S. agalactiae* DSM 2134) and negative control (H_2O) controls were included and were extracted together with the samples analysed. The PCR products were analysed by gel electophoresis using a 2% agarose gel. As a marker, 500 ng per well of a 100-base-pair (bp) DNA ladder (New England BioLabs from Bio Concept, Switzerland) was run on every gel. After electrophoresis, gels were stained in a solution of ethidium bromide at a concentration of 1.1 mg/ml for 20 min. DNA was visualised under UV illumination (320 nm) using AlphaImager software (version 4.1.0). The PCR reaction amplified a 450-bp fragment after the first PCR run and a 265-bp fragment after the second PCR run.

Three conventional PCR assays were performed using a 9800 Fast Thermal Cycler (Applied Biosystems). All amplifications were performed in a 25 µl reaction volume consisting of the following reagents and concentrations: 0.2 µl of AmpliTaq DNA polymerase LD (5 U/µl), 2.5 µl of GeneAmp 10× PCR Buffer II, 1.5 µl of $MgCl_2$ solution (25 mM), 2 µl of dNTPs (2.5 mM), 2.5 µl of the forward primer (10 pmol/µl), reverse primer Sa-hylR (10 pmol/µl), 8.3 µl of H_20 and 5 µl of template DNA. Different primer pairs were used for the three assays:

Sa-hylF/Sa-hylR to amplify a 950-bp fragment in PCR 1, SodA-F/SodA-R to amplify a 911-bp fragment in PCR 2, and SodA-F2/SodA-R2 to amplify a 849-bp fragment in PCR 3. The amplification protocol was performed as previously described (Sukhnanand et al., 2005): 95°C for 5 min followed by 30 cycles of amplification (95°C for 30 s, 55°C for 30 s and 72°C for 30 s) and one cycle at 72°C for 10 min. Each run contained a positive (*S. agalactiae* DSM 2134) and negative control (H_2O), which were extracted together with the samples analysed. The PCR products were examined by gel electrophoresis using a 2% agarose gel. On every gel, 500 ng per well of a 100-bp ladder (New England biolabs from Bio Concept, Switzerland) was run as a marker. After electrophoresis, gels were stained in a solution of ethidium bromide at a concentration of 1.1 mg/ml for 20 min. The DNA was visualised under UV illumination (320 nm) using AlphaImager software (version 4.1.0).

Spiking in human wastewater and liquid manure

To determine the most suitable combination of DNA extraction and amplification methods for detecting *S. agalactiae*, spiking experiments were performed. Bacterial cells from a dilution series were spiked into human and animal wastewater to analyse the susceptibility of the evaluated PCR assays to inhibitors.

S. agalactiae DSM 1234 was grown on Columbia blood agar plates for 12 h at 37°C. Cells were suspended in peptone saline solution to reach a density corresponding with a McFarland 2 standard. A serial dilution of *S. agalactiae* was performed in peptone saline solution, and the concentration of the suspension was determined by plate counts on Columbia blood agar. These suspensions were then used for spiking experiments. Seven samples with 9 ml of human wastewater were prepared and spiked with 1 ml of each dilution containing 9.15×10^2 CFU/ml to 9.15×10^8 CFU/ml. The amount of *S. agalactiae* cells present in the human wastewater before spiking was determined quantitatively using the LightCycler 1.1

instrument. To calculate absolute limits of detection, the initial concentration (100 CFU/10 ml) was added to the amount of cells spiked. From each dilution containing 2.3×10^2 CFU/ml to 2.3×10^8 CFU/ml, 1 ml was spiked into 9 ml of liquid manure (diluted in a ratio of 1:100 (wt/vol) in peptone saline solution). Spiked human and animal samples were filtered through a Microsart CN filter with 0.45 µm pore size (Sartorius from Vitaris, Switzerland). For enrichment, the filter was incubated in 9 ml of Todd Hewitt Broth for 12 h at 37°C. One millilitre from each sample was used for DNA extraction with the Qiagen kit, and another millilitre of the sample was used for extraction with the Roche MagNA Lyser kit. The samples were analysed using the five PCR assays, as described above.

Samples of human and animal origin

Ten wastewater treatment plants were randomly selected from all Swiss wastewater treatment plants, each processing sewage from more than 100,000 inhabitants. The selected plants were distributed throughout the country. One wastewater sample was collected from the influent at each plant.

In addition, liquid manure samples from two farms with 20–30 cows and five wastewater samples from slaughterhouses were analysed. Slaughterhouses represented wastewater from cows, calves, bulls and swine. Animals are brought daily from all over the country to be slaughtered.

Each sample of human or animal origin was analysed in duplicate, using a quantitative and a qualitative (presence-absence) method. For analysis, 4 × 10 ml from each sample was filtered through a Microsart CN filter with 0.45 µm pore size (Sartorius from Vitaris, Switzerland).

For the quantitative analysis, the filter was transferred into a glass vial with a height of 10 cm and diameter of 12 mm containing 5 ml of elution buffer (6 ml of Tween 80, 2 g of Lab-lemco (Oxoid), 5.844 g of NaCl, 200 ml of dd H_2O) (Mendez et al., 2004). Bacterial cells were resuspended by sonification at 25°C for 4 min. The suspension was transferred into a 15 ml polypropylene tube and centrifuged for 10 min at 6000 × g. The DNA was extracted using the Qiagen kit and analysed using the LightCycler PCR assay. Samples with crossing points (C_P) < 40 were counted positive.

For the qualitative method, the filter was immersed in 9 ml of Todd Hewitt Broth and incubated for 12 h at 37°C for enrichment. One millilitre from each sample was used for DNA extraction using the Qiagen kit. The DNA was amplified using both the LightCycler PCR assay and the three conventional PCR assays. When the results of duplicates were once negative and once positive, the sample was considered to be negative.

Study area and sampling sites

Two sites in the north-western part of Switzerland were chosen for the field validation. They are located in karst areas which are vulnerable to faecal contamination over long distances and across many layers. In both study areas, a wastewater treatment plant is located along the stream under study. Because the effluents are discharged into the stream, sampling sites were chosen upstream and downstream of the effluents.

In the study area of Röschenz, two karst springs, named “Lützelquelle” (LQ) and “Kächbrunnenquelle” (KQ), were investigated. Both springs are known to be very vulnerable to contamination (Auckenthaler, 2004). They are located close to the “Lützel” stream and downstream of a wastewater treatment plant. Previous hydrogeological studies showed that the Lützel stream infiltrates to some extent into the KQ spring, whereas the LQ spring is fed

by infiltrating rainwater on a plateau south of the spring (Auckenthaler, 2004). In the study area of Röschenz, three sampling sites were located in the Lützel stream. One site was located upstream and two sites downstream from the wastewater treatment plant. One site downstream from the wastewater treatment plant was close to flowing effluent from the wastewater treatment plant and the other site was 1 km further downstream.

In the second study area of Oberdorf, we examined the "Z´Hof" (ZQ) karst spring, which is also vulnerable to contamination. The "Weigistbach" stream infiltrates into the spring to some extent (Schudel et al., 2000). Upstream, sewage from the wastewater treatment plant enters into the Weigistbach. During dry periods the stream infiltrates completely into the ground around 500 m upstream of the ZQ spring. Water samples were taken from the Weigistbach both upstream and downstream of the wastewater treatment plant. In addition, samples were collected at an additional sampling site at a tributary stream called "Heimsten".

In both study areas, the land is used for agricultural purposes. Previous tracer experiments showed that the stream sampling sites chosen for this study receive run-off from animals, mainly cows.

Collection and processing of environmental water samples

During a period of 8 months, from February to September 2009, 34 stream water samples were analysed. Five to seven samples were collected from each of the six sampling sites.

During the period of investigation, 20 spring water samples were taken during dry weather conditions. In addition, 12 (4 × 3) spring water samples were taken ~72 h after rainfall of >10–20 mm/h according to local weather radar.

Surface and spring water samples were analysed using a qualitative method. One litre of spring or surface water was filtered through a Microsart CN filter with 0.45 µm pore size (Sartorius from Vitaris, Switzerland) to concentrate the water samples before analysis. The filter was immersed in 9 ml of Todd Hewitt Broth and incubated for 12 h at 37°C for enrichment. One millilitre from each sample was used for DNA extraction using the Qiagen kit. DNA was amplified using LightCycler PCR as described above. Samples with exponential amplification and C_P values < 40 were counted positive. For confirmation, the extraction and amplification were repeated for all samples with positive results. Whenever one negative and one positive result were obtained from duplicates, the sample was considered to be negative.

Results

Comparison of different DNA extraction methods

The extraction method using the Qiagen DNeasy Blood and Tissue kit proved the most suitable. As shown in Table 2, the limit of detection was always lower than after extraction with lysozyme and proteinase K. Furthermore, compared with the Roche MagNa Lyser kit, extraction with the Qiagen kit resulted in a lower or equal detection limit. The DNA extraction methods were further compared in spiking experiments. As shown in Table 3, using the Qiagen kit for extraction resulted in lower values than using the Roche kit. The only exception was analysis using semi-nested PCR, where the Roche kit performed better.

Comparison of different PCR assays

Analysis of pure bacterial cell cultures with LightCycler PCR and semi-nested PCR showed comparable limits of detection (Table 2). However, amplification using semi-nested PCR produced several visible bands on the gel, indicating unspecific amplification in addition to

the expected amplicon. The limits of detection of the three conventional PCR assays were at least one log higher compared with the other two assays. Using differing strains for amplification revealed differences. For example, amplification of *S. agalactiae* DSM 6784 at a concentration of < 25 CFU per reaction was not observed with the LightCycler PCR assay. With the two other reference strains the limit of detection was lower. The DNA concentration did not explain this variation. In samples with 10^7 CFU/ml the DNA concentration was 2.6 ng/µl for DSM 2134 strain, 2.4 ng/µl for DSM 6784 and 1.54 ng/µl for NCTC 9993 strain.

Performing serial dilution after DNA extraction with the Qiagen kit resulted in a lower limit of detection, equivalent to 10–1000 CFU/ml or 0.25–25 CFU per reaction using LightCycler PCR. Using the semi-nested PCR assay with 1–100 CFU/ml and 0.025–2.5 CFU per reaction also resulted in a lower limit of detection.

The utility of the different PCR assays was further compared after spiking *S. agalactiae* into wastewater and liquid manure. As shown in Table 3, great differences in susceptibility to PCR inhibitors were observed using different PCR assays for analysis. When *S. agalactiae* was spiked into liquid manure, low concentrations could only be amplified by the LightCycler PCR assay. The human wastewater samples used for spiking already contained ~100 CFU/10 ml of *S. agalactiae*. This background was considered in calculating the limits of detection of the five different PCR assays (Table 3). Using LightCycler PCR, all spiked samples as well as human wastewater not spiked with *S. agalactiae* cells turned out to be positive after extraction by both kits. The limit of detection was thus $< 1 \times 10^2$ CFU/10 ml using this assay.

Table 2 Limit of detection of different *S. agalactiae* strains using combinations of different DNA extraction methods and PCR assays

DNA extraction method	Strain [a]	PCR assay				
		LightCycler PCR	Semi-nested PCR	Conventional PCR 1	Conventional PCR 2	Conventional PCR 3
Qiagen kit	1	2.5	2.5	2.5×10^2	2.5×10^2	2.5×10^1
	2	2.5×10^1	2.5	2.5×10^3	2.5×10^2	2.5×10^2
	3	2.5	2.5	2.5×10^1	2.5×10^1	2.5×10^1
Lysozyme /proteinase K	1	2.5×10^1	2.5×10^2	2.5×10^5	2.5×10^3	2.5×10^2
	2	2.5×10^2	2.5×10^1	NA [b]	2.5×10^5	2.5×10^4
Roche MagNaLyser	1	2.5	2.5	2.5×10^3	2.5×10^3	2.5×10^3
	2	2.5×10^1	2.5	2.5×10^3	2.5×10^3	2.5×10^3
	3	2.5	2.5	2.5×10^2	2.5×10^2	2.5×10^2

The limit of detection in CFU/reaction was determined using different *S. agalactiae* strains:
[a] 1 = DSM 2134; 2= DSM 6784; 3 = NCTC 9993
[b] NA: not amplified

Table 3 The limits of detection of different PCR assays after spiking *S. agalactiae* into human wastewater and liquid manure.

PCR assay	DNA extraction	Limit of detection (CFU/10 ml)	
		Wastewater	Liquid manure
LightCycler PCR	R	$< 1 \times 10^{2}$[a]	2.3×10^{2}
	Q	$< 1 \times 10^{2}$[a]	2.3×10^{2}
Conventional PCR 1	R	$> 9.2 \times 10^{8}$[b]	$> 2.3 \times 10^{8}$[b]
	Q	$< 1 \times 10^{2}$[a]	2.3×10^{7}
Conventional PCR 2	R	$> 9.2 \times 10^{8}$[b]	$> 2.3 \times 10^{8}$[b]
	Q	1×10^{3}	2.3×10^{7}
Conventional PCR 3	R	$> 9.2 \times 10^{8}$[b]	$> 2.3 \times 10^{8}$[b]
	Q	$< 1 \times 10^{2}$[a]	2.3×10^{7}
Semi-nested PCR	R	9.3×10^{3}	2.3×10^{5}
	Q	9.2×10^{5}	2.3×10^{4}

R = Extraction with Roche kit
Q = Extraction with Qiagen kit
[a] The wastewater was positive without addition of spiked cells.
[b] No amplification of samples with the highest concentration spiked

As shown in Table 3, the same limit of detection of $< 1 \times 10^{2}$ CFU/10 ml was obtained using conventional PCR assays 1 and 3 after extraction with the Qiagen kit; the limit of detection of conventional PCR assay 2 was 1×10^{3}. However, no amplification was observed with the three conventional PCR assays when the same samples were extracted with the Roche kit, even with the highest spiked concentration of 9.2×10^{8}.

When the semi-nested PCR assay was used to analyse spiked samples, the limit of detection was 9.3×10^3 CFU/10 ml after DNA extraction with the Roche kit and 9.2×10^5 CFU/10 ml after extraction with the Qiagen kit.

When *S. agalactiae* was spiked into liquid manure, the lowest detection limit (2.3×10^2 CFU/10 ml) was obtained with LightCycler PCR after the extraction using both kits.

Occurrence of *S. agalactiae* in samples of human and animal origin

S. agalactiae was detected in duplicates in all human wastewater samples. The median concentration quantified by real-time PCR on the LightCycler was 3×10^2 CFU/10 ml with a range between 8 CFU/10 ml and 2.4×10^5 CFU/10 ml. At 94% (slope = –3.49), the efficiency of the PCR run was good. When the three conventional PCR assays were used to analyse the 10 human wastewater samples, 7 samples with the primer pairs Sa-hylF/ Sa-hylR and SodA-F2/ SodA-R2 and 8 samples with the primer pair SodA-F/ SodA-R were positive. Two human wastewater samples were negative following analysis with the three conventional PCR assays.

Although all human wastewater samples were positive, *S. agalactiae* was never detected in samples of animal origin after analysis with quantitative LightCycler PCR. After enrichment, only one slaughterhouse wastewater sample was found to be positive. Because of the enrichment step, the initial concentration could not be quantified. But since this sample was negative without enrichment, the initial concentration of *S. agalactiae* can be considered to be very low. Analysis by the three conventional PCR assays also showed all samples of animal origin to be negative.

Occurence of *S. agalactiae* in surface and spring water

A presence-absence test, including enrichment, was used to analyse 34 surface water samples from six sites located in two study areas. In 29.4% of these samples *S. agalactiae* could be amplified by LightCycler PCR after enrichment. As shown in Table 4, *S.* agalactiae was detected in 10 out of 34 surface water samples. All the positive samples were taken at sampling sites downstream from wastewater treatment plants. The 10 samples collected downstream and close to the effluent discharge point of the treatment plants were all positive (Table 4). *S.* agalactiae could not be detected in surface water samples taken 1 km further downstream from the wastewater treatment plant in the Röschenz study area nor upstream from the two plants. Moreover, samples taken from the Heimsten tributary stream in the Oberdorf study area were negative. In other words, in both study areas we observed a significant difference between stream sampling sites receiving human wastewater from plants and any other stream sampling site receiving run-off from agricultural animals.

In 32 water samples taken from three different springs, *S. agalactiae* could not be detected. Consequently, there was no difference between the samples taken during dry weather conditions and those taken after rainfall (Table 4).

Table 4 Occurrence of *S. agalactiae* in surface and spring water.

Sampling site	No. of samples analysed	% positive
Springs		
LQ	12	0
KQ	11	0
ZQ	9	0
Along the streams		
Lützel upstream WTP	7	0
Lützel downstream WTP	5	100
Lützel close to KQ	7	0
Weigistbach upstream WTP	5	0
Weigistbach downstream WTP	5	100
Heimsten	5	0

LQ: Lützelquelle, KQ: Kächbrunnenquelle, ZQ: Z´Hof
WTP: Wastewater treatment plant
"Lützel", "Heimsten" and "Weigistbach" refer to names of the streams in the study area of Röschenz and Oberdorf, respectively.

Discussion

To provide better insight into the occurrence of *S. agalactiae* in human and animal wastewater and environmental water samples, different detection methods were compared and further evaluated and validated in field studies.

Comparison of different detection methods

Field investigations rely on a sensitive, effective and easy-to-handle detection method. Accordingly, we analysed different DNA extraction methods and PCR assays to find the most sensitive combination of extraction method and PCR assay to detect *S. agalactiae*.

Experimental comparison of three different DNA extraction methods showed that the Qiagen DNeasy Blood and Tissue kit performed best. Its detection limits were mostly lower than the other two methods.

The PCR assays were based on primers and probes that are available in the literature (Table 1). The performance characteristics (sensitivity, specificity and reproducibility) of the semi-nested PCR and LightCycler PCR assays are well described (Ahmet et al., 1999; Ke et al., 2000). The values for the limit of detection calculated in our study were consistent with previously published studies that reported values ranging from 1 to 3 CFU and 10 to 24 CFU per reaction for LightCycler PCR and 6 CFU/ml for semi-nested PCR (Ahmet et al., 1999; Ke et al., 2000). Spiking experiments in human and animal wastewater revealed the robustness of the LightCycler PCR assay and showed that the other assays were susceptible to inhibitors. Robustness was further demonstrated through performance of the assay on two different instruments (LightCycler 1.1 and 480) with comparable C_P values and efficiency (data not shown).

The conventional PCR assays evaluated in this study were previously used to discriminate between *S. agalactiae* isolates of human and animal origin through further sequencing of the PCR product (Sukhnanand et al., 2005). Considering the high detection limit of the three assays and the fact that it was not possible to detect *S. agalactiae* in animal samples, we did not undertake such discrimination in our study and the assays were not considered further for the field investigations.

Taken together, the Qiagen kit for extraction followed by amplification with LightCycler PCR turned out to be the most suitable combination of DNA extraction and amplification methods for detecting *S. agalactiae*. Consequently, we used this combination for field evaluation and validation. Analysis of surface and spring water samples was qualitative only. Because of the rather low concentration of *S. agalactiae* detected in wastewater (median 3×10^2 CFU/10 ml), we filtrated one litre and then performed an enrichment step prior to DNA extraction.

Occurrence in wastewater and environmental water samples

Little is known about the presence of *S. agalactiae* in human and animal wastewater and whether it can lead to contamination of environmental waters. Contamination of water derived from humans as well as animals was previously shown in the two study areas (Auckenthaler, 2004; Schudel et al., 2000). In addition to human contamination, mainly owing to sewage discharged from wastewater treatment plants, sampling locations receive run-off from agricultural sites (Auckenthaler, 2004; Schudel et al., 2000). Liquid manure from cows and slaughterhouse wastewater represents wastewater from animals that are important to animal husbandry in both study areas.

The investigation of human wastewater and samples of animal origin showed that the prevalence of *S. agalactiae* is much higher in human populations than in animal ones. The

negative results of samples of animal origin confirmed previous findings that the bacteria have become very rare on Swiss farms (Busato et al., 2000; Ivemeyer et al., 2009; Roesch et al., 2007). Our results, together with the fact that *S. agalactiae* is an obligate intramammary pathogen in cows and has nearly been eradicated from Swiss farms, suggest that *S. agalactiae* from animal origin is of little significance for surface water contamination. The negative results of samples from stream water sampling sites receiving run-off from agricultural animals further support this hypothesis. Moreover, *S. agalactiae* originating from aquatic animals has not been detected in surface water (Amborski et al., 1983; Bishop et al., 2007; Pereira et al., 2010). Because *S. agalactiae* was only detected in surface water, samples collected downstream of the effluent discharge point and repeatedly over time in all 10 samples collected near the effluent discharge point, we suggest that our positive results indicate high human faecal contamination.

Host specificity has an important influence on the debate over transmission of *S. agalactiae* from animals to humans or vice versa. Different animals can be infected with *S. agalactiae* sporadically, and they may act as zoonotic reservoirs. According to the current state of research, zoonotic transmission is rare. Moreover, there is substantial evidence that human and bovine *S. agalactiae* isolates represent distinct populations. Different ribotypes and serotypes have been found (Dogan et al., 2005; Sukhnanand et al., 2005). There are differences in the sequence of genes for resistance to erythromycin and tetracycline, the housekeeping gene *sodA* and the virulence gene *hylB* (Dogan et al., 2005; Sukhnanand et al., 2005). Moreover, the capsular polysaccharide (*cps*) gene cluster and insertion sequences (IS elements) differ in human and animal *S. agalactiae* isolates (Shakleina et al., 2004; Zhao et al., 2006). However, human *S. agalactiae* isolates were able to cause diseases in animals, including of aquatic origin (Evans et al., 2009; Pereira et al., 2010; Van den Heever and Giesecke, 1980). Our results show that *S. agalactiae* is rarely detected in surface water.

Conseqeuently, indirect transmission of *S. agalactiae* through water is unlikely and, if it exists, of little epidemiological significance.

Conclusion

Detecting *S. agalactiae* in human waste water and slaughterhouse wastewater is best accomplished by a combination of DNA extraction using the DNeasy Blood and Tissue kit from Qiagen and the LightCycler PCR assay. This method was used successfully to detect *S. agalactiae* in all human wastewater samples. The occurrence of *S. agalactiae* was restricted to environmental samples with human contamination in the north-western part of Switzerland. Consequently, *S. agalactiae* appears to have a potential as a new indicator for human faecal pollution in water and further validation is ongoing. However, *S. agalactiae* in human wastewater and environmental water samples was rather low.

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Paper 3:

Improved detection of *Rhodococcus coprophilus* with a new real-time PCR assay

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Abstract

Agricultural practices such as spreading liquid manure or the utilisation of land as animal pastures can result in faecal contamination of water resources. *Rhodococcus coprophilus* is used in microbial source tracking to indicate animal faecal contamination in water. The existing culture-dependent method is time-consuming. Processing time for culturing *R. coprophilus* is ~20 days which limits the use of this method for detecting *R. coprophilus*. In the present study, we designed and validated a real-time polymerase chain reaction (PCR) assay based on the *R. coprophilus* 16S rRNA gene. The PCR was highly specific and sensitive for DNA from target and non-target host species as well as human and animal wastewater and had a low detection limit of between 2.1 and 29 CFU/ml. The method, including a filtration step, was further validated in a field investigation in Switzerland. Our work demonstrated that the method is sufficiently sensitive and robust to detect *R. coprophilus* in surface and spring water. Furthermore, compared with PCR assays that are available in the literature or to the culture-dependent method, the new molecular approach improves the detection of *R. coprophilus*.

Introduction

Agricultural practices, such as spreading liquid manure or the use of land as animal pastures, can result in faecal contamination of water resources. In order to maintain high water quality, water resources should be protected from faecal pollution by the detection and remediation of faecal input sites.

Different microbial source tracking (MST) approaches have been proposed in order to detect the source of faecal contamination (Meays et al., 2004; Savichtcheva and Okabe, 2006; Scott et al., 2002; Sinton et al., 1998). *R. coprophilus,* first described and classified by Rowbotham

and Cross (1977 a and b), was one of the first bacteria used in MST. It was shown that this actinomycete is present at high levels in faeces of different animal species, such as cattle, sheep, pigs, horses, ducks, geese and hens (Mara and Oragui, 1981; Savill et al., 2001).

The absence in human faecal specimens qualifies *R. coprophilus* as a good indicator for animal pollution but its use is limited by inadequate detection methods. The selectivity of M3 agar developed by Rowbotham and Cross (1977 a and b) was found to be inadequate for the detection of *R. coprophilus* in sewage. Consequently, the M3 agar was modified by adding naladixic acid and sodium azide as supplements to increase selectivity, but complete inhibition of contaminating bacteria was still not achieved (Mara and Oragui, 1981). The major drawback of the detection procedure on this modified M3 (MM3) agar, however, was the long incubation time (Jagals et al., 1995; Mara and Oragui, 1981; Oragui and Mara, 1983). To overcome these restrictions, a conventional as well as a TaqMan real-time PCR assay were developed by the group of Savill et al. (2001). Our evaluation of these approaches did not reveal any satisfactory result. Therefore, the aim of this study was to design a new LightCycler real-time PCR assay with high sensitivity and specificity, and to compare the novel procedure with the previously published PCR assays.

Materials and Methods

Isolation of presumptive colonies

One liquid manure sample was analysed with a culture-based detection method. A serial dilution was performed in peptone saline solution and 100 µl of each dilution was plated in duplicate on modified MM3 agar (Mara and Oragui, 1983). The agar was prepared by dissolving 0.466 g of KH_2PO_4, 0.732 g of Na_2HPO_4, 0.1 g of KNO_3, 0.29 g of NaCl, 0.1 g of $MgSO_4$ $7H_2O$, 0.02 g of $CaCO_3$, 0.2 g of propionic acid sodium salt, 200 µl of $FeSO_4$ $7H_2O$ (1 mg/ml), 180 µl of $ZnSO_4$ $7H_2O$ (1 mg/ml), 20 µl of $MnSO_4$ $4H_2O$ (1mg/ml), 200 µl of

nalidixic acid (25 mg/ml), 18 g of granulated Agar-agar (Merck, Switzerland) and 3.5mg of sodium azide in 1l of distilled water. The agar was mixed under heating. After sterilisation by autoclaving at 121 °C for 15 min and cooling to 50 °C, 1 ml of amphotericin B (2.5 mg/ml) and 1 ml of thiamine hydrochloride (4 mg/ml) were added. With a final pH of 7.0 ± 0.1, the liquid medium (30 ml) was poured into petri dishes (90 mm diameter). Inoculated plates were incubated at 30 °C for 14 days followed by a 7-day exposure to sunlight at room temperature. All presumptive, reddish colonies were inoculated on Tryptone Soya Broth (TSB) including 14 g agar prepared as recommended by the German Collection of Microorganisms and Cell Cultures (http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium535.pdf) and incubated at 30 °C for 4 days for further confirmation. Based on colony morphology observed on TSB agar, isolates were classified into two groups (typical or untypical). Isolates which grew as dry, reddish and irregular colonies with a bumpy surface were considered to be typical. The Microbank® preservation system (Pro-lab Diagnostics) was used to store all isolates at -70 °C.

Isolation of DNA

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 protocol pretreatment for Gram-positive bacteria. All samples were eluted in buffer AE (provided in the kit) in a final volume of 200 µl.

Characterisation of strains

The 16S rRNA gene of the reference strains *R. coprophilus* DSM 43347, DSM 43591, DSM 44751 and DSM 43447 and three typical and six untypical strains isolated from liquid manure were characterised by 16S rRNA sequencing. The DNA was extracted as described under

„Isolation of DNA“ before sequencing by the Institut für Medizinische und molekulare Diagnostik AG (IMD) (Switzerland).

LightCycler PCR development

For primer design, the publicly available database (NCBI) was searched for *R. coprophilus* sequences. Multiple sequence alignments were performed with clustalw (http://www.ebi.ac.uk/Tools/clustalw2/) and included all 16S rRNA sequences of *R. coprophilus* as well as sequences obtained from newly isolated and sequenced strains (for details see “Isolation of presumptive colonies” and “Characterisation of strains”). Five primers were designed on the 16S rRNA gene sequence (accession: X80626). The chosen primers were placed in regions identified as being conserved among the *R. coprophilus* strains. Four primers were designed on a sequence of the gene for the DNA gyrase B subunit (accession: AB014271). For primer design, the software Genefisher (http://bibiserv.techfak.uni-bielefeld.de/genefisher2/) was used. Analysis included BLAST searches to ensure that primers were specific to *R. coprophilus.* Fifteen different primer combinations, including previously described primers, RhodoF and RhodoR (see Table 1), were used to analyse serial dilutions of two *R. coprophilus* reference strains (DSM 43347 and DSM 43591). Eleven primer combinations targeted the sequence of the 16S rRNA gene and four combinations the sequence on the gene for the DNA gyrase B subunit. DNA from one ml samples of serial dilutions was extracted as described under “Isolation of DNA”. A conventional PCR approach 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 (10 pmol/µl), 9 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). The PCR products were loaded on a 2% agarose gel. On every gel, 500 ng per well of a 100-bp ladder

(New England biolabs) was run as a molecular size-marker. After electrophoresis, gels were stained in a solution of ethidium bromide at a concentration of 1.1 mg/ml for 20 min. The DNA was visualised under UV illumination (320 nm) using the AlphaImager software version 4.1.0. Primer combinations with a low detection limit were further used to test specificity with typical and untypical isolates from liquid manure.

Hybridisation probes (RC_3'FL and RC_5'LC640) were designed in conserved regions from the 16S rRNA gene sequence of *R. coprophilus*. The sequence of the LightCycler probe RC_5'LC640 was identical with that of the TaqMan probe RhodoPr. The combinations of forward primer CL1.1F, reverse primer CL9R and the probes RC_3'FL and RC_5'LC640 were further validated. The sequences of primers and probes are shown in Table 1. The PCR procedure described above was slightly modified to a real-time PCR setup: 0.8 µl of each probe (RC_3'FL and RC_5'LC640) (5 pmol/µl) and 7.4 µl H_2O were used. Each run contained a positive (DSM 43347 strain) and a negative (H_2O) control, which were extracted together with the analysed samples. Moreover, DNA from a serial dilution of *R. coprophilus* DSM 43591 or DSM 43347 over 5 logs was included in each PCR run to generate a standard curve used to control the efficiency and to quantify the PCR product. The data analysis was performed using the second derivative maximum method of the LightCycler software (version 4.1.1.21). Samples with quantification cycle (Cq) < 40 were counted as positive (Bustin et al., 2009).

Table 1 Background information on different primer pairs and probes used in the study.

PCR assay	Target gene	GenBank accession number	Labelling	Primer and probe sequences	Reference
LightCycler PCR	16S rRNA gene	X80626	CL1.1F: CL9 R: RC_3'FL: RC_5'LC640:	5'- TGG GCG GAT TAG TGG CGA A -3' 5'- GTT AGC CGG TGC TTC TTC TG -3' 5'- ACT GGG TCT AAT ACC GGA TAT GAC CAT- FL -3' 5'- LC640-ATG CAT GTC CTG TGG TGG AAA GGT TTA CTG- PH -3'	This study
TaqMan PCR	16S rRNA gene	X80626	RhodoF: RhodoR: RhodoPr:	5'-GGG TCT AAT ACC GGA TAT GAC CAT -3' 5'-GCA GTT GAG CTG CGG GAT TTC ACA C -3' 6FAM-ATG CAT GTC CTG TGG TGG AAA GGT TTA CTG-TAMRA	(Savill et al., 2001)
Conventional PCR	16S rRNA gene	X80626	RhodoF: RhodoR:	5'-GGG TCT AAT ACC GGA TAT GAC CAT -3' 5'-GCA GTT GAG CTG CGG GAT TTC ACA C -3'	(Savill et al., 2001)

LightCycler PCR validation

DNA from *R. coprophilus* reference strains (DSM 43347, DSM 43591, and DSM 44751) and typical as well as untypical isolates were used to validate the LightCycler PCR assay. The strains were grown on TSB agar plates for 4 days at 30 °C. Cells were suspended in 10 ml phosphate buffered saline (PBS) solution containing 2% bovine serum albumin (BSA) to reach a high density corresponding with a McFarland 2-4 standard. The tubes were kept at room temperature for at least one hour to obtain a homogeneous solution with loose, single cells. Five ml of the suspension was transferred to a new glass vial and vortexed. Serial dilutions of strains were performed in PBS solution containing 2% BSA and the concentration of the suspension was determined by plate counts on TSB agar. One ml of each dilution was extracted as described under „Isolation of DNA“. The validation was based on a guideline from the Swiss Accreditation Service (Swiss Accreditation Service, 2006).

Sensitivity refers to how often the assay returns a positive result when a target is present and specificity refers to how often it is negative in the absence of the target. Both values were determined by analysis of all presumptive colonies isolated from one liquid manure sample. Seven typical and 28 untypical isolates were analysed. In addition, human and animal wastewaters were analysed (as described under “Samples of human and animal origin”) containing a wide range of bacterial species originating from the intestinal tract. Repeatability was determined with a sample containing 2×10^3 CFU/ml of *R. coprophilus* (DSM 43347) measured in ten separate runs on the LightCycler Instrument 1.1. For the determination of the detection limit in CFU per volume, serial dilutions of three different *R. coprophilus* reference strains were used. DNA was analysed from serial dilutions containing 2.1×10^0 CFU/ml to 2.1×10^8 CFU/ml for strain DSM 43347, 7.5×10^{-1} CFU/ml to 7.5×10^7 CFU/ml for strains DSM 43591 and 2.9×10^0 CFU/ml to 7.5×10^8 CFU/ml for strain DSM 44751. The

amplification was repeated in several (>10 times) runs to determine whether the results were reproducible.

Two serial dilutions from two different strains were prepared for the determination of the recovery rate. DNA from a serial dilution of DSM 43347 strain (1.7×10^4 CFU/ml to 1.7×10^8 CFU/ml) and a serial dilution of DSM 43591 strain (7.5×10^2 CFU/ml to 7.5×10^6 CFU/ml) were analysed in pairs. For quantification, the serial dilution of the other strain was used to generate the standard curve.

In addition, the recovery rate of the method including a filtration and re-suspension step was evaluated. Seven samples with 9 ml of human wastewater were prepared and spiked with one ml of each dilution from a serial dilution containing 1.7×10^2 CFU/ml to 1.7×10^8 CFU/ml of *R. coprophilus* DSM 43347. These samples were filtered through a Microsart CN-filter with 0.45 µm pore size (Sartorius). Subsequently, the filter was immersed in 5 ml of elution buffer (6 ml Tween 80, 2 g Lab-lemco (Oxoid), 5.844g NaCl, 200 ml dd H_2O) (Mendez et al., 2004) and cells were re-suspended by sonification at 25 °C for 4 min. After centrifugation, DNA was extracted and analysed with the LightCycler PCR assay as described above. DNA was extracted from a serial dilution (7.5×10^1 CFU/ml to 7.5×10^6 CFU/ml) of *R. coprophilus* DSM 43591 to generate the standard curve for quantification of spiked samples and to determine the recovery rate.

Comparison of three different PCR assays

The LightCycler PCR assay was compared to two assays that were based on primers and probes that are available from the literature. The primer sequences, target genes and their accession numbers are shown in Table 1. DNA from all samples used for comparison was extracted as described under „Isolation of DNA“. The TaqMan PCR was performed as

described by Savill et al. (2001). Samples were amplified during 50 cycles and results with quantification cycles (Cq) < 40 were counted as positive. For the conventional PCR assay, DNA was amplified using the TaqMan PCR assay. The PCR products were analysed by means of gel electrophoresis as described above under 2.4.

In order to compare the three PCR assays, the same DNA was amplified as described above for the determination of the detection limit for the LightCycler PCR. For further comparison, presumptive isolates, including typical and untypical isolates, from liquid manure and human and animal wastewater were analysed.

Samples of human and animal origin

Human and animal wastewater samples were analysed in order to further validate the LightCycler PCR assay and in order to compare the three assays. Human wastewater samples were collected from the influent at ten different Swiss treatment plants that each processed sewage from more than 100,000 inhabitants. Five wastewater samples from two big slaughterhouses were analysed. Slaughterhouses provided wastewater from cows, calves, bulls and pigs. In addition, two liquid manure samples from two Swiss farms having 20-30 cows were analysed. Liquid manure samples were diluted 1: 100 in peptone saline solution (1 g peptone, 8.5 g NaCl, 1000 ml dd H_2O).

From each sample with human or animal origin 10 ml was filtered through a Microsart CN-filter with 0.45 µm pore size (Sartorius) followed by a washing step with 9 ml peptone saline solution. Subsequently, the filter was transferred into a glass vial with a height of 10 cm and 12 mm in diameter containing 5 ml elution buffer (6 ml Tween 80, 2 g Lab-lemco (Oxoid), 5.844 g NaCl, 200 ml dd H_2O) (Mendez et al., 2004). Bacterial cells were re-suspended by sonification at 25 °C for 4 min. The suspension was transferred into a 15 ml polypropylene tube followed by centrifugation for 10 min at 6’000 × g. The pellet was frozen at -70 °C prior

to DNA extraction. Finally, DNA was extracted (see „Isolation of DNA“.) and analysed with the three PCR assays as described above. As an inhibition control, another 10 ml from each sample was spiked with *R. coprophilus* (DSM 43347). Peptone saline solution was used as the negative control. In order to quantify the initial concentration of *R. coprophilus* in wastewater, DNA from a serial dilution of 7.5×10^1 CFU/ml to 7.5×10^6 CFU/ml of *R. coprophilus* DSM 43591 was used to generate the standard curve.

Surface and spring water samples

From April to September 2009, twenty-eight spring water samples and thirty surface water samples were analysed for the validation of the PCR assays under natural conditions. Samples were collected from three springs and three different streams located in the northwestern part of Switzerland. One litre of spring water and 500 ml of surface water were filtered through 0.2 µm PVP-free GTTP membranes (Millipore) and the bacteria were re-suspended in 5 ml Bennett’s broth as previously described (Long et al., 2003). One ml per sample was used for DNA extraction (see „Isolation of DNA“). After centrifugation, pellets were stored at -70 °C before further extraction and amplification. As a positive control, an additional surface water sample was taken downstream of the effluent of the treatment plant and was spiked with *R. coprophilus* (DSM 43347). 100 ml of sterile water was used as the negative control. Positive and negative controls were treated in the same way as the samples.

Analysis

Statistical tests were performed with the software package, SPSS 13.0.

Results

Confirmation of presumptive colonies

From the liquid manure sample grown on modified MM3 agar, 35 presumptive colonies with reddish pigmentation were selected. All colonies were very small in diameter and therefore the only criterion taken into consideration for selection was a reddish pigmentation. After inoculation on TSB agar, these presumptive colonies were classified into 7 (20%) typical and 28 (80%) untypical colonies. The concentration of *R. coprophilus* in the liquid manure sample was 8×10^6 CFU/ml.

Sequencing results of the 16S rRNA gene showed that three sequences of the reference strains (DSM 43347, DSM 44751 and DSM 43591) had high similarity with a *R. coprophilus* sequence from NCBI database (accession: X80626.1) sequence. However, the 16S rRNA gene sequence from DSM 43591 strain showed additional similarity with *R. zopfii* (accession: AF191343.1). The strain showed an identity of 99% to both sequences (accession: X80626.1 and AF191343.1). Based on our classification into typical and untypical strains, we would have classified the fourth reference strain DSM 43447 as untypical based on its colony morphology. The sequencing of this strain showed high similarity to the *R. yunnanesis* sequence (accession: AY602219.2) and could not be confirmed as being *R. coprophilus*. The sequencing of strains from liquid manure confirmed our classification into typical and untypical isolates. Sequences of the three typical isolates were highly similar to *R. coprophilus* (accession: X80626.1) and sequences of the six untypical isolates showed high homology with *Dietzia* sp.. The untypical isolates from liquid manure identified as *Dietzia* sp. produced false positive results on selective modified MM3 agar plates.

Design and validation of the new *R. coprophilus* PCR assay

From the eleven different combinations targeting the 16S rRNA gene and the four combinations targeting the sequence on the gene for DNA gyrase B subunit, only the described primer pair (CL1.1F and CL9R) was specific after combination with the hybridisation probes (RC_3'FL and RC_5'LC640) (Table 1). The primer and probes were therefore selected for further validation.

While all seven typical isolates gave positive signals using the LightCycler PCR assay, no amplification was observed with DNA from any of the 28 untypical isolates. Therefore, both sensitivity and specificity of the LightCycler PCR assay were high (100%). High specificity was also shown with the analysis of human wastewater representing a mixture of microorganisms naturally present in faeces, where nine out of ten human wastewater samples were negative

The sensitivity was slightly lower in samples of animal origin. From seven analysed samples, five were positive. The repeatability was 0.2 (Cq) and consistent with the standard deviation of ten repeated measurements. As shown in Table 2, the detection limit ranged between 2.1 and 29 CFU/ml. The values shown in Table 2 represent results of one experiment. The repeated analysis of the same samples showed reproducibility of the LightCyler PCR assay with equivalent Cq values.

The median recovery rate of strain DSM 43347 in non-filtered samples was 132% (n= 5; 71.2%–262%) when the DSM 43591 strain was used as the standard to generate the standard curve and 46.3% (n= 5; 14.4%–297%) for DSM 43591 strain when DSM 43347 strain was used as the standard. In human wastewater, the median recovery rate of DSM 43347 strain was 70.6% (n= 5; 24.1%–165%) when the DSM 43591 strain was used as the standard and 47.4% (n= 5; 14.3%–92.9%) when the standard DSM 43347 strain was used to generate the standard curve.

Table 2 Detection limit of the LightCycler PCR, the TaqMan PCR and the conventional PCR assays.

Strain	CFU/ ml	DNA (CFU/ reaction)[a]	LightCycler PCR		TaqMan PCR		Conventional PCR
			Cq	Efficiency	Cq	Efficiency	Band[b]
DSM 43347	2.1×10^8	5'250'000	11.73	100%	19.06	76%	+
	2.1×10^7	525'000	15.00		20.90		+
	2.1×10^6	52'500	17.48		23.71		+
	2.1×10^5	5'250	20.70		27.60		+
	2.1×10^4	525	24.05		32.71		+
	2.1×10^3	52.5	27.42		35.84		+
	2.1×10^2	5.25	32.64		(41.53)		+
	2.1×10^1	0.5	33.82		(47.11)		-
	2.1×10^0	0.05	37.09		n.a.		-
DSM 43591	7.5×10^7	1'875'000	15.71	100%	n.a.	80%	+
	7.5×10^6	187'500	18.56		32.20		+
	7.5×10^5	18'750	20.96		34.02		+
	7.5×10^4	1'875	24.17		38.13		+
	7.5×10^3	187.5	28.46		(43.07)		+
	7.5×10^2	18.8	31.44		(47.36)		+
	7.5×10^1	1.88	35.47		n.a.		-
	7.5×10^0	0.18	36.22		n.a.		-
	7.5×10^{-1}	0.02	n.a.		n.a.		-
DSM 44751	2.9×10^8	7'250'000	12.41	98%	22.94	75%	+
	2.9×10^7	725'000	15.41		n.a.		+
	2.9×10^6	72'500	18.14		28.42		+
	2.9×10^5	7'250	21.24		34.0		+
	2.9×10^4	725	24.91		37.58		+
	2.9×10^3	72.5	29.74		(44.61)		+
	2.9×10^2	7.25	32.01		(45.80)		+
	2.9×10^1	0.7	34.54		n.a.		-
	2.9×10^0	0.07	n.a.		n.a.		-

[a] The CFU/reaction was calculated on the initial CFU/ml and is therefore an approximate value
n.a. = no amplification
[b] + visible band, - no band detected

Comparison of different PCR assays

Analysis of pure bacterial cell cultures revealed differences in the detection limits of the three different PCR assays (Table 2). The detection limits of the TaqMan PCR assay (between 2.1×10^3 and 7.5×10^4 CFU/ml) and the conventional PCR assays (between 2.1×10^2 and 7.5×10^2 CFU/ml) were higher in comparison with the LightCycler PCR assay (2.1 and 29 CFU/ml). Strong distinctions were observed in Cq values of the two real-time PCR assays. A Wilcoxon signed rank test was performed and a significant difference ($p < 0.01$) was observed between Cq values of the two real-time PCR assays. As shown in Table 2, differences between Cq values were higher when DNA from *R. coprophilus* DSM 43591 or DSM44751 was amplified than after amplification of DNA from strain DSM 43347.

While both sensitivity and specificity of the LightCycler PCR assay were high, similar estimations were more difficult to establish for the other two assays. In Table 3, the findings for typical and untypical isolates are shown and represent the data of a single experiment. While amplifications with the LightCycler were reproducible, results were inconsistant with the two other assays. Using the TaqMan PCR assay, only 3-5 of 7 typical isolates could be amplified and from the 28 untypical isolates, only 2-4 strains were detected (data not shown). As shown in Table 3, some typical strains could not be amplified using the TaqMan PCR and were confirmed to be *R. coprophilus* through sequencing. In addition, stains with high sequence homology to *Dietzia* sp. produced false positive results in the TaqMan PCR assay.

Table 3 Typical and untypical *R. coprophilus* strains analysed with two real-time PCR assays.

Strain identification	LightCycler Cq	TaqMan Cq	Classification	Highest similarity with sequence of:
RCK 36	13.10	n.a.	Typical	Not sequenced
RCK 47b	10.8	n.a.	Typical	Not sequenced
RCK 53	14.7	n.a.	Typical	*R. coprophilus* (X80626.1)
RCK 56	15.61	n.a.	Typical	*R. coprophilus* (X80626.1)
RCK 57	13.25	16.99	Typical	Not sequenced
RCK 66	16.87	19.69	Typical	*R. coprophilus* (X80626.1)
RCK 69	18.19	19.62	Typical	Not sequenced
RCK 54	n.a.	41.94	Untypical	*Dietzia* sp. (DQ337507.1)
RCK 55	n.a.	39.14	Untypical	*Dietzia* sp. (DQ337507.1)
RCK 67	n.a.	43.54	Untypical	*Dietzia* sp. (AB376626.1)
RCK 68	n.a.	36.86	Untypical	*Dietzia* sp. (DQ060380.1)

n.a. = no amplification

R. coprophilus in samples of human and animal origin

As shown in Table 4, *R. coprophilus* was detected with the two real-time PCR assays in 1 of 10 (10%) human wastewater samples and twice (20%) with the conventional PCR assay. The concentration quantified by the real-time PCR on the LightCycler was 4.3×10^4 CFU/10ml. All spiked human wastewater samples used as positive controls were positive in the analyses with the three PCR assays. Samples of animal origin contained a lot of PCR inhibitors. All seven undiluted animal samples were negative after analysis with the three PCR assays. However, more samples could be amplified after a 1:10 dilution. From 7 samples, 5 (71.4%) were positive with the LightCycler PCR assay and the initial concentrations were determined

to be 8×10^4 CFU/10ml and 7×10^8 CFU/10ml in the two liquid manure samples and between 20 and 6.5×10^2 CFU/10ml in the three positively tested slaughterhouse wastewater samples. The inhibition controls of the two samples that were negative after analysis with the LightCycler PCR assay were negative, indicating the presence of a strong PCR inhibitor in the samples. The same number of samples (5/7, 71.4%) was positive using the conventional PCR. With the TaqMan PCR assay, all seven samples from animal origin (liquid manure and slaughterhouse wastewater) were found to be negative. From the seven inhibition controls (samples spiked with *R. coprophilus*) 5 (71.4%) were positive after analysis with the LightCycler PCR assay, 4 (57%) with the TaqMan PCR assay and 7 (100%) with the conventional PCR assay.

Table 4 Detection of *R. coprophilus* after analysis of water samples with three different PCR assays.

		Positive samples obtained with different PCR assays		
Sample origin	No.	LightCycler PCR	TaqMan PCR	Conventional PCR
Human[a]	10	1 (10%)	1 (10%)	2 (20%)
Human[a] inhibition control	10	10 (100%)	10 (100%)	10 (100%)
Animal[b]	7	0	0	0
Animal[b] inhibition control	7	1 (14.3%)	2 (28.6%)	4 (57.1%)
Animal[b] 1:10 diluted	7	5 (71.4%)	0	5 (71.4%)
Animal[b] inhibition control 1: 10 diluted	7	5 (71.4%)	4 (57%)	7 (100%)
Surface Water	30	5 (16.7%)	0	0
Spring Water	28	1 (3.6%)	0	0

No. = Number of samples
[a] samples from 10 different wastewater treatment plants
[b] samples were obtained from two slaughterhouses and two farms

Analysis of surface and spring water samples

As shown in Table 4, *R. coprophilus* could be amplified with the LightCycler PCR assay in 5 (16.7%) of 30 analysed surface water samples. The numbers ranged from 80 to 3×10^4 CFU per 100 ml with a median value of 8.6×10^3. From 28 spring water samples, one positive result was obtained with 715 CFU/l. All samples were negative when the conventional PCR assay or the TaqMan PCR assay was used for analysis.

Discussion

We developed a new LightCycler PCR assay detecting *R. coprophilus*, to overcome the drawbacks of detection methods used prior to this study. Compared with PCR assays that are available from the literature and with the culture-based method, the new molecular approach showed considerable advantages.

The design of a new PCR assay for detection of *R. coprophilus* is challenging. There are only nine nucleotide sequence entries available in the public databases, seven of them targeting the 16S rRNA gene, one the gene for DNA gyrase B subunit and one the phthalate dioxygenase large subunit gene (available: < http://www.ncbi.nlm.nih.gov/sites/entrez?db=nuccore&cmd=search&term=Rhodococcus%20coprophilus >, accessed July 2010). From 15 different combinations of primers tested in this study, only the described primer pair (CL1.1F and CL9R) was specific after combination with hybridisation probes. In general, the specificity should not be based on the probes of a PCR assay but on the primers, so as to avoid competitive reaction within the same PCR run. However, analysis of liquid manure, slaughterhouse wastewater and human wastewater suggested that the method is reliable for detecting *R. coprophilus* in environmental samples. Beside good general performance such as a low detection limit, reproducibility and good

efficiency of the new assay, some disadvantages have been observed. The transfer of the PCR assay to the LightCycler® 480 Instrument was not satisfactory (data not shown). In addition, low fluorescence signals were observed. This might be due to the location of the probes close to the 5' end which could result in insufficient time for fluorescence measurement because of a rapid displacement by Taq polymerase. As shown in Table 2-4, the new LightCycler PCR assay compared to the PCR assays previously described by Savill et al. (2001), had advantages including higher specificity and sensitivity, lower detection limit and better general performance. The detection limit of the conventional PCR assay obtained in this study was smaller than in a previously published study that reported a value of 60 CFU per PCR (Savill et al., 2001). On the other hand, values for the detection limit of the TaqMan PCR assay were higher in our study when compared to the values described by Savill et al .(2001). Previous studies did not include any other strain for validation except for DSM 43347 (equal to ATCC 29080) (Savill et al., 2001). In this study, higher detection limits were observed for other strains using the TaqMan PCR assay for analysis (Table 2).

Using the culture-based method on selective agar plates (Jagals et al., 1995; Mara and Oragui, 1981; Oragui and Mara, 1983), *R. coprophilus* was found in animal faeces and in water contaminated with animal faeces. In our study, slightly more bacteria were found in liquid manure than the numbers stated by Mara and Oragui (1981) from *R. coprophilus* 3.9 to 2.5×10^6 CFU/g in animal faecal specimens or the values obtained by Savill et al. (2001) from 3.3×10^5 to 3.6×10^6 CFU/g in cow faeces. The abundance of *R. coprophilus* in surface water was similar to values found previously with the range of 10 to 1×10^4 CFU/100 ml (Long et al., 2003). In comparison with the time-consuming culture-based method, the LightCycler PCR assay showed several advantages. We showed that confirmation of presumptive colonies was necessary in order to distinguish typical from untypical isolates because only 20% of presumptive colonies were *R. coprophilus*. The subculture increased the

specificity of the culture-based method although resulted in an even more time-consuming procedure. In contrast, detecting *R. coprophilus* with the culture-independent molecular approach is easy, rapid and reliable.

Beside *R. coprophilus*, *Streptococcus bovis* (Mara and Oragui, 1981; Oragui and Mara, 1983), thermophilic bifidobacteria (Gavini et al., 1991), F-RNA phage subgroup I (Havelaar et al., 1990) and different molecular methods targeting the phylum bacteroidetes (Layton et al., 2006; Reischer et al., 2006; Shanks et al., 2006; Shanks et al., 2008; Shanks et al., 2010) were described to indicate animal contamination. We selected *R. coprophilus* as the indicator of animal faecal pollution based on its high abundance in environmental water, including watersheds from which raw drinking water was obtained (Long et al., 2003; Mara and Oragui, 1981; Oragui and Mara, 1983). The goal was to establish and validate a reliable method to investigate the occurrence of *R. coprophilus* in environmental water samples, including spring water. Our work demonstrated that the method, with a low detection limit, can be applied for that purpose and that the method is sufficiently sensitive and robust to detect *R. coprophilus* in spring water. However, the number (16.7%) of positive surface water samples was rather small and might be increased by testing larger sample volumes of water. PCR inhibitors are concentrated when larger volumes are analysed. It is therefore important to include positive controls to test for PCR-inhibition. Further validation should also assess filtration losses resulting from larger volumes. Sinton et al. (1998) described the long-term survival of *R. coprophilus* in environmental waters and therefore concluded that the organism cannot be used to give an indication of recent pollution. For an application of the method for analysis of spring water, the long persistence of the target microorganism in water may be beneficial and because bacteria from both remote and recent pollution are present contributes to the higher probability of detecting this organism. Although it is important to be aware that results cannot

give any indication on the specific time of faecal pollution, they are useful to determine animal-derived faecal contamination.

Conclusion

In conclusion, the present study improves the analysis of *R. coprophilus* in wastewater, surface and spring water. Compared with all assays that are available in the literature, the new molecular approach showed advantages such as improved sensitivity and specificity and a much lower detection limit. Consequently, there is evidence to suggest that the new molecular approach is a useful tool to identify animal sources of faecal pollution in water. However, larger volumes of environmental water samples should be analysed to further validate and improve the method in respect of the amount of positive water samples.

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Paper 4:

Assessment of library independent microbial source tracking methods for application in Switzerland

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Paper 4:

Assessment of library independent microbial source tracking methods for application in Switzerland

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Abstract

For discriminating between human and animal faecal contamination, different microbial source tracking (MST) approaches have been described. In the current study, a range of MST indicators were used that are described in the scientific literature. From a total of eleven specific MST indicators, bacteriophages infecting the host strains *Bacteroides thetaiotaomicron* GA-17, *Bacteroides ovatus* GB-124, *Bacteroides thetaiotaomicron* ARABA 84 as well as sorbitol-fermenting bifidobacteria proved useful for indicating human faecal contamination and *Rhodococcus coprophilus* indicated animal-derived faecal contamination in Swiss surface and drinking water. These potential source indicators were present in samples of faecal origin (human or animal) from all over Switzerland and therefore showed a geographic stability. In addition, they were abundant in surface water and were sensitive enough to detect faecal contamination in spring water from two study areas in Switzerland.

Introduction

Spring water is an important natural water resource throughout the world. In Switzerland, it covers 40% of the drinking water demand. Around 15%-20% of the springs are located in regions with karst geology. The flow velocity of karst aquifers is dependent on meteorological and hydrogeological conditions. 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 flow velocities increase, these springs are vulnerable to faecal contamination. A broad range of pathogens, present in human or animal faeces, can enter water resources and may cause human diseases. As the retention time of karst waters in the soil varies, these water resources have thus to be carefully monitored.

Microbiological indicator organisms such as *Escherichia coli* and enterococci are commonly used for quality assessment of drinking water. Both *E. coli* and enterococci are part of the human and animal intestinal flora. Standardised microbiological procedures for detecting these bacteria in water do not distinguish between human and animal sources of contamination (ISO 9308-1: 2000, ISO 7899-2: 2000). However, the need for such discrimination has been recognised and different microbial source tracking (MST) approaches have been described (Meays et al., 2004; Savichtcheva and Okabe, 2006; Scott et al., 2002; Sinton et al., 1998; Stoeckel and Harwood, 2007). The majority of MST studies focused on faecal samples and recreational waters, and a comparative assessment of different MST tools was made only in a few studies (Balleste et al., 2010; Blanch et al., 2006; Field and Samadpour, 2007; Stoeckel et al., 2004).

Only a small number of studies considered methods that can be used for the discrimination of faecal spring water contamination. MST indicators applied to wastewater or surface water may also be useful in spring water investigations. However, the sensitivity of the methods has to be high enough for this purpose. In addition, it was shown that the distribution of MST indicators in different geographic regions is an important subject that should be considered prior to planning MST studies. Therefore, an objective of this study was to assess the abundance of human and animal MST indicators in faecal samples obtained from many different regions spread all over Switzerland.

Studies using human or animal faecal indicators have either not been performed in Switzerland or were investicated in the context of this extensive assessment (Wicki et al., 2010a,b,c). We selected a range of organisms, previously used in MST, to determine their occurrence in human wastewater, liquid manure and slaughterhouse wastewater, as well as in surface and spring water, from two study areas in Switzerland. Sorbitol-fermenting

bifidobacteria, *Streptococcus agalactiae* and phages infecting the host strains *Bacteroides thetaiotaomicron* GA-17, *B. ovatus* GB-124, *B. thetaiotaomicron* ARABA 84 and *B. fragilis* ARABA 19 were used as indicators of human faecal contamination, and bacteriophages infecting host strains *B. caccae* RBA 63, *B. caccae* 64 and *B. fragilis* KBA 60 and *R. coprophilus* as indicators of animal contamination. All selected MST indicators have previously been described in the scientific literature and have been shown to perform well in detecting source-specific faecal contamination in water (Blanch et al., 2006; Ebdon et al., 2007; Long et al., 2003; Wicki et al., 2010 a,b,c).

The objectives of the study were (i) to determine the occurrence of the MST indicators in human and slaughterhouse wastewater as well as liquid manure from Switzerland, (ii) to test their potential for an application in Swiss surface and spring water and (iii) to identify the origin of faecal contamination in three selected karst springs.

Materials and Methods

Microbiological analysis of MST and unspecific indicator organisms

Bacteriophages infecting Bacteroides host strains:

A range of different *Bacteroides* host strains was used for the detection of bacteriophages. *Bacteroides thetaiotaomicron* GA-17, *B. ovatus* GB-124 (provided by the Department of Microbiology, University of Barcelona, Spain), *B. thetaiotaomicron* ARABA 84 and *B. fragilis* ARABA 19 (previously isolated in our laboratories) were used as host strains for detecting bacteriophages of human faecal origin. *B. caccae* RBA 63, *B. caccae* 64 and *B. fragilis* KBA 60 were used as host strains for bacteriophages of animal faecal origin. As a reference host strain *B. fragilis* RYC 2056 was included in the study. Enumeration of plaque

forming units (PFU) followed the standard protocol for detection and enumeration of bacteriophages (ISO 10705-4, 2001). Plates were incubated for 20-22h at 37 ºC under anaerobic conditions using AnaeroGen (Oxoid). Sample dilution or filtration was performed as previously described (Wicki et al., 2010a). If two types of plaques were present, both were counted except for host ARABA 84, where only clear plaques were counted in wastewater samples.

Sorbitol-fermenting bifidobacteria:

Sorbitol-fermenting bifidobacteria were analysed on Human Bifid Sorbitol Agar (HBSA) (Mara and Oragui, 1983) and plates were incubated for three days at 37 ºC under anaerobic conditions (AnaeroGen, Oxoid). Presumptive colonies of sorbitol-fermenting *Bifidobacteria* appeared as yellow to brown colonies. Bacteria were confirmed by light microscope. Isolates from surface and spring water samples were analysed with the Api20A test (BioMérieux). Additionally, Gram staining was performed and growth tested under strict anaerobic conditions. All human wastewater samples were serially diluted and analysed in pairs. Different volumes of surface and spring water samples were analysed: 1 ml, 10 ml and 100 ml of surface water and 10 ml, 100 ml and 1000 ml of spring water. Samples were filtered through membranes with a pore size of 0.45µm (Microsart CN-Filter 11406Z-50 SC) and filters were placed on HBSA. All plates were incubated as described above. As positive controls, the reference strains *B. adolescensis* (DSM 20083) and *B. breve* (DSM 20213) were inoculated directly on HBSA and as a negative control, 100 ml distilled sterile water was filtered.

***Rhodococcus coprophilus*:**

A culture-based method on modified MM3 agar and a LightCycler PCR assay were used for detecting *R. coprophilus.* Sample analyses and processing was done as previously described (Long et al., 2003; Wicki et al., 2010b).

***Streptococcus agalactiae*:**

A molecular approach was used for detecting *S. agalactiae.* Filtration of samples, DNA extraction and analysis were performed as previously described (Wicki et al., 2010c)

Unspecific indicators:

E. coli, enterococci and heterotrophic plate counts were analysed with membrane filtration methods used for official food control in Switzerland (Federal Office of Public Health, 2000). Surface and spring water samples were analysed by the cantonal laboratory, Basel-Landschaft.

In addition, unspecific faecal contamination 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). Analysis was performed according to the standard procedure for enumeration of somatic coliphages (ISO 10705-2, 2001).

Analysis of human wastewater and samples from animal origin

MST indicators such as sorbitol-fermenting bifidobacteria, bacteriophages infecting *B. thetaiotaomicron* GA-17 and *B. ovatus* GB-124 as well as the animal faecal indicator *R. coprophilus* were not used previously for source tracking in Switzerland. Human and slaughterhouse wastewater as well as liquid manure samples were analysed in order to determine the occurrence of MST indicators and to compare them with recently described

MST indicator bacteria. In addition, the concentrations of unspecific faecal indicators *E. coli*, somatic coliphages and phages infecting the host RYC 2056 were investigated. Untreated human wastewater samples were collected from ten different Swiss wastewater treatment plants (WTP), 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. Furthermore, seven wastewater samples from two big abattoirs slaughtering cows, calves, bulls and pigs were analysed. In addition, four liquid manure samples from two Swiss farms keeping 20-30 cows were analysed.

Study areas with spring and surface water sampling sites

Two study areas in the northwestern part of Switzerland located in the Jura Mountains showing typical karst geology were selected for investigation. Both regions are situated in rural territories characterised by intense dairy farming and animal husbandry. The karst springs from both study areas are located close to a stream that receives run-off from agricultural animals. All springs are located downstream of the effluent discharge point from wastewater treatment plants. Based on previous findings described below, both human and animal faecal contamination was to be expected in surface and spring water from the areas in question.

In the “Röschenz” study area the water supply is mainly derived from the two karst springs of Kächbrunnenquelle (KQ) and Lützelquelle (LQ). The KQ spring is located to the east of the Lützel stream and the LQ spring is on the west side. The catchment areas of the two springs are different. Previous hydrogeological studies showed that the Lützel stream infiltrates to some extent into the KQ spring (Auckenthaler, 2004) and is fed by a region at the eastern side of the Lützel valley, whereas the LQ spring is fed by infiltrating rainwater on a plateau south-west of the spring (Auckenthaler, 2004). The hamlet Niederhuggerwald located on that

plateau is characterised by intense dairy farming and animal husbandry. The households in the hamlet are not connected to a wastewater treatment plant. The domestic wastewater is collected together with liquid manure in a slurry basin in the hamlet, and is used in the spring and summer months as fertilizer. Besides the LQ and KQ springs, three surface water-sampling sites along the Lützel stream were investigated. Surface water-sampling sites included one site upstream and two sites downstream from the wastewater effluent. The sampling sites downstream from the effluent included one sampling site, which was very close to the effluent, and another sampling site, which was approximately one km further downstream from the effluent and very close to the KQ spring.

In the second study area of Oberdorf, we examined the "Z´Hof" (ZQ) karst spring. The spring is one of the important sources of the water supply of Niederdorf and occasionally provides the water supply of the Waldenburger valley (Schudel, 2005). The "Weigistbach" stream infiltrates into the spring to some extent (Schudel et al., 2000). Upstream, sewage from the wastewater treatment plant enters into the Weigistbach. During dry periods the stream infiltrates completely into the ground around 500 m upstream of the ZQ spring. Surface water samples were taken from the Weigistbach both upstream and downstream of the wastewater treatment plant. In addition, surface water samples were collected at an additional sampling site at a tributary stream called "Heimsten".

Surface and spring water sample collection

Over a period of seven months, from March to September 2009, five samples from all six surface water-sampling sites were analysed. Samples from the same study area were collected at the same day and samples were taken at a depth of approximately 15-20 cm and away from the banks.

During periods of dry weather, eight samples were collected from the LQ spring, seven from the KQ spring and five from the ZQ spring. Spring and surface water samples were collected in sterilised Nalgene polypropylene bottles (VWR, Switzerland).

During the period of investigation, 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. After the second rainfall event, on the 14th of July 2009, samples were taken not only on the 17th of July but also on 21st of July because additional rainfall (< 10mm/ h) was recorded on the 18th of July.

All MST indicators, unspecific faecal indicators *E. coli*, enterococci, somatic coliphages and phages infecting the host RYC 2056 were investigated in surface and spring water samples. Additionally, HPC was determined in spring water. Data from MST indicators previously investigated in Switzerland were included for comparison and are marked as such in the Tables.

Results

Occurrence of unspecific faecal and MST indicators in human and slaughterhouse wastewater as well as in liquid manure

Numbers of unspecific faecal indicators were measured in samples obtained from human and animal origin. As shown in Table 1, most of the samples were positive for *E. coli* and somatic coliphages. One slaughterhouse wastewater sample was negative for *E. coli* and a liquid manure sample for somatic coliphages. In samples of human and animal origin more *E. coli* where found than somatic coliphages. The highest faecal contamination was detected in liquid manure with a median log concentration of 6.38 CFU/ml (range: 5.02-6.86 CFU/ml) for

E. coli and 4.23 PFU/ml (range: 0-4.87 PFU/ml) for somatic coliphages followed by human wastewater samples and samples obtained from slaughterhouses. Bacteriophages infecting host RYC 2056, listed under unspecific organisms, were much more prevalent in human than animal faeces and never detected in slaughterhouse wastewater.

Table 1 Unspecific and MST indicators in human and animal wastewater as well as 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]
Unspecific indicator									
E. coli	13/13	4.67	3.96-5.61	6/7	3.63	0-5.27	4/4	6.38	5.02-6.86
Somatic coliphages	13/13	3.69	2.72-4.84	7/7	3.22	2.41-4.40	3/4	4.23	0-4.87
Phages of RYC 2056	13/13	2.05	1.10-3.84	3/7	0	0-0.88	0/4	neg	neg
Human MST indicator									
Sorbitol-fermenting Bifidobacteria	13/13	4.51	3.99-5.19	0/7	neg	neg	0/4	neg	neg
Phages of GA-17	13/13	1.72	0.88-2.62	1/7	0	0-0.48	0/4	neg	neg
Phages of GB-124	13/13	1.76	0.65-2.66	0/7	neg	neg	0/4	neg	neg
Phages of ARABA 84[b]	10/10	1.59	0.95-2.06	0/3	neg	neg	0/2	neg	neg
Phages of ARABA 19[b]	9/10	1.04	0-1.40	0/3	neg	neg	0/2	neg	neg
S. agalactiae[b]	10/10	1.48	0.62-1.8	0/5	neg	neg	0/2	neg	neg
Animal MST indicator									
R. coprophilus (culture)	1/13	0	0-3.00	5/7	3.00	0-3.70	3/3	6.90	3.60-6.95
R. coprophilus (PCR)[b]	1/10	0	0-3.63	5/7	0.30	0-1.81	2/2	7.54	3.90-7.85
Phages of KBA 60[b]	0/10	neg	neg	4/4	0.3	0-0.78	0/1	neg	neg
Phages of RBA 63[b]	0/10	neg	neg	3/4	0	0-0.30	0/1	neg	neg
Phages of RBA 64[b]	0/10	neg	neg	3/4	0	0-0.70	0/1	neg	neg

[a] log CFU or PFU/ml
[b]data was processed in a previous study (Wicki et al., 2010a,b,c)

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 1). In addition, human MST indicators, with one exception for phages infecting ARABA 19, were found in all human wastewater 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 CFU/ml and 5.19 CFU/ml. As shown in Table 1, the concentration of sorbitol-fermenting bifidobacteria in human wastewater was similar to that of *E. coli.* Bacteriophages infecting GA-17 were also abundant in human wastewater. However, the host strain GA-17 detected bacteriophages in one slaughterhouse wastewater sample. Median log concentrations of different human-derived bacteriophages and *S. agalactiae* were similar and between 1.04 and 1.72 CFU/ml and about 3 log below the concentration of sorbitol-fermenting bifidobacteria.

Animal MST indicators were more abundant in samples of animal origin than in human wastewater. As shown in Table 1, *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. Findings of bacteriophages infecting the three host strains included in this study were only detected in samples from slaughterhouses. However, bacteriophages were detected in low numbers.

Table 2 Occurrence of unspecific 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]
Unspecific indicator								
E. coli	5/5	1.7×10^3 ($825\text{-}1\times10^5$)	5/5	245 ($35\text{-}7.2\times10^4$)	5/5	2.8×10^4 ($1\times10^4\text{-}1\times10^5$)	5/5	2.4×10^4 ($705\text{-}3\times10^5$)
Enterococci	5/5	1.5×10^3 ($410\text{-}1.2\times10^5$)	5/5	470 ($25\text{-}4.9\times10^4$)	5/5	5×10^3 ($3.2\times10^3\text{-}1\times10^5$)	5/5	1×10^4 ($1.5\times10^3\text{-}7.9\times10^4$)
Somatic coliphages	5/5	3.3×10^3 ($525\text{-}2.1\times10^4$)	5/5	470 ($40\text{-}4\times10^4$)	5/5	1.8×10^4 ($4.9\times10^3\text{-}2.9\times10^5$)	5/5	1.2×10^3 ($70\text{-}4.1\times10^4$)
Phages of RYC 2056	5/5	6 (2-85)	0/5	0	4/5	25 (0-39)	2/4	4 (0-179)
Human MST indicator								
Sorbitol-fermenting Bifidobacteria [c]	1/5	0 (0-5)	0/5	0	5/5	700 (30-875)	4/5	140 (0-37000)
Phages of GA-17	5/5	5 (2-32)	0/5	0	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 (35-1196)	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	0	1/4	0 (0-1)	0/4	0
S. agalactiae	0/5	0	0/5	0	5/5	n.q.	5/5	n.q.

Animal 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)[d]	0/5	0	2/5	0 (0-4.3×10^4)	0/5	0	2/5	0 (-1.5×10^5)
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	0	1/5	0 (0-4)	0/5	0	1/5	0 (0-1)
Phages of RBA 64	0/5	0	1/5	0 (0-5)	1/5	0 (0-2)	2/5	0 (0-1)

[a] CFU/PFU per 500 ml

[c] Values were extrapolated and zero values refer to an initial volume of 100 ml

[d] the sample volume analysed per PCR reaction is in accordance with an initial sample volume of 2.5 ml

n.q. not quantified

Occurrence of unspecific and MST indicators in surface water

The unspecific 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 of the Oberdorf study area (data not shown). Median concentrations were low with 6 CFU/100 ml for *E. coli* and 10 CFU/100 ml for enterococci as well as somatic coliphages. 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 2, unspecific indicators and human MST indicators were present in higher concentrations downstream than upstream from wastewater treatment plants. Only enterococci concentrations in the Lützel stream and phages infecting the host ARABA 19 did not increase downstream from the wastewater treatment plants. In the Röschenz study area, additional samples were collected about one km further downstream from the effluent discharge point of the wastewater treatment plant. Compared to the other two sampling sites in the stream, median concentrations of both unspecific and human faecal indicators were lower (data not shown). As shown in Table 2, in samples collected upstream from wastewater treatment plants, 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 (data not shown). With the molecular approach, only five out of 30 surface water samples were positive which is probably due to the low sample volume analysed. Culture-based detection of *R. coprophilus* revealed higher median concentrations upstream than downstream from wastewater treatment plants of both study areas (Table 2). In the Lüzel stream, the concentration increased again about one 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. Bacteriophages infecting different host

strains were present in only low numbers and real differences were not observed among different sampling sites.

Spring water investigation

The quality of Swiss drinking water is regulated by tolerance values stated in the Swiss Hygiene Ordinance (HyV, SR 817.024.1) for *E. coli* and enterococci as well as the number of HPC that are present. During periods of dry weather, about 84% of samples and after rainfall 100% of samples exceeded one or several of the decreed tolerance values. During periods of dry weather, unspecific faecal indicator bacteria were frequently detected in spring water samples but at low concentrations. In six out of eight samples from the LQ spring, in six out of seven samples from the KQ spring and in all four samples from the ZQ spring either *E. coli* or enterococci were detected or more than 100 CFU/ml of HPC were found. As shown in Table 3, median concentrations were low for unspecific indicator organisms and MST indicators. One exception was observed for culture-based detection of *R. coprophilus* where median concentrations exceeded the values of unspecific indicator organisms *E. coli*, enterococci, somatic coliphages and phages infecting RYC 2056.

After three rainfall events, unspecific faecal indicators were detected in all samples except for phages infecting RYC 2056 (Table 4). The highest faecal contamination was observed in all spring water samples collected on 17th of July 2009 after the second rainfall event.

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 3). In one sample, sorbitol-fermenting bifidobacteria were also found. After rainfall, human faecal contamination was detected in all samples collected from the LQ spring. Human faecal contamination was indicated by the presence of human specific bacteriophages (Table 4). However, sorbitol-fermenting bifidobateria, bacteriophages

infecting the host GB-124 and *S. agalactiae* were not 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 detected 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.

In four out of seven samples collected during periods of dry weather from the KQ spring, human faecal contamination was detected either by sorbitol-fermenting bifidobacteria or by the presence of bacteriophages infecting the host GA-17 (Table 3). As shown in Table 4, human faecal contamination was also detected 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 sorbitol-fermenting bifidobacteria, and phages infecting the host strains GA-17, ARABA 84 and ARABA 19 were present after the third rainfall event. 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 detected in the KQ spring.

Table 3 Presence of unspecific and specific MST indicators in spring water samples during periods of dry weather

	LQ spring		KQ spring		ZQ spring	
Unspecific 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	0	0/5	0
Human MST indicator [a]						
Sorbitol-fermenting Bifidobacteria	1/8	0 (0-11)	3/7	0 (0-35)	0/5	0
Phages of GA-17	0/8	0	1/7	0 (0-3)	0/5	0
Phages of GB-124	2/8	0 (0-1)	0/7	0	0/5	0
Phages of ARABA 84	2/8	0 (0-3)	0/7	0	0/5	0
Phages of ARABA 19	0/8	0	0/7	0	0/5	0
S. agalactiae	0/8	0	0/7	0	0/5	0
Animal MST indicator [a]						
R. coprophilus (culture)	7/7	135 (20-400)	6/7	154 (0[b]-380)	3/5	30 (0[b]-350)
R. coprophilus (PCR) [c]	0/8	0	0/7	0	1/5	0 (0-715)
Phages of KBA 60	1/8	0 (0-1)	0/7	0	0/5	0
Phages of RBA 63	0/8	0	1/7	0 (0-1)	0/4	0
Phages of RBA 64	0/8	0	0/7	0	0/5	0

[a] CFU/PFU per litre

[b] absent in 200 ml

[c] the sample volume analysed per PCR reaction is in accordance with an initial sample volume of 5 ml

Table 4 Number of unspecific and 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
Unspecific 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 MST indicator [a]									
Sorbitol-fermenting Bifidobacteria	0	0 [b]/ 0	0	0	0 [b]/ 0	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[d]	8	5/5	70	-	0/5	4	4	4/0	1
Phages of ARABA 19[d]	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 MST indicator [a]									
R. coprophilus (culture)	42	5/5	90	390	10/5	110	0[c]	10/110	465
R. coprophilus (PCR) [e]	0	0/0	0	0	0/0	0	0	0/0	0
Phages of KBA 60[d]	-	0/0	0	0	0/0	0	0	0/0	1
Phages of RBA 63[d]	0	2/0	0	0	4/0	0	0	3/0	0
Phages of RBA 64[d]	0	0/0	0	0	0/0	0	0	0/0	0

[a] CFU/PFU per litre

[b] absent in 500 ml

[c] absent in 200 ml

[d] Data was processed in a previous study (Wicki et al., 2010a)

[e] the sample volume analysed per PCR reaction is in accordance with an initial sample volume of 5 ml

During periods of dry weather, human MST indicators were not detected in samples from the ZQ spring in the Oberdorf study area (Table 3). As shown in Table 4, human faecal contamination occurred after rainfall. Bacteriophages infecting the host ARABA 84 were detected after all three rainfall events, sorbitol-fermenting bifidoacteria 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 once (Table 4). As shown in Table 3, animal faecal contamination was indicated only by *R. coprophilus* during periods of dry weather in the ZQ spring. After the first rainfall event, unspecific faecal contamination was low and animal faecal contamination was not detected (Table 4). 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 detection method. In one sample, the host strain RBA 63 also detected phages and in another sample KBA 60 bacteriophages were found.

Selection of potential MST indicators

Human MST indicators, such as sorbitol-fermenting bifidobacteria and phages infecting the host strains *B. thetaiotaomicron* GA-17, *B. ovatus* GB-124 and *B. thetaiotaomicron* ARABA 84, were found in all human wastewater samples (Table 1), and were present in higher median concentrations downstream than upstream of wastewater treatment plants (Table 2). These four human MST indicators were also detected in spring water during periods of dry weather and after rainfall events (Table 3 and 4). The remaining two human MST indicators successfully indicated human faecal contamination but were found in low numbers in either surface or spring water.

The animal faecal indicator *R. coprophilus* was detected in most of the samples of animal origin and only once in human wastewater. Surface water samples were always positive with

the culture-based detection method and concentrations were higher upstream than downstream from wastewater treatment plants in both study areas. Bacteriophages indicating animal faecal contamination were detected only at low concentrations. The host strains *B. fragilis* KBA 60 and *B. caccae* RBA 63 detected phages in spring water samples.

Discussion

Methods to discriminate human and animal faecal contamination have rarely been applied in Switzerland, and the majority of the methods were assessed for the first time in this study (Wicki et al. 2010a,b,c). In previous studies, geographical differences in the abundance of certain MST indicators were observed (Payan et al., 2005). It was therefore important to test the selected MST indicators before use in Switzerland. Human and animal samples were collected from many different regions spread all over Switzerland and were therefore applicable to assess the geographic distribution of investigated MST indicators.

Human MST indicators, sorbitol-fermenting bifidobacteria, phages infecting the host strains GA-17, GB-124, ARABA 84 and *S. agalactiae* were found in all human wastewater samples from different regions in Switzerland. Bacteriophages infecting the human host strain ARABA 19 were also frequently detected (9/10). With the exception of phages infecting the host GA-17, human MST indicators were never detected in samples of animal origin. However, the indicators were detected at different concentrations. The human faecal indicator sorbitol-fermenting bifidobacteria were restricted to human wastewater and the log median concentration was slightly higher than the previously reported value of 6.41 PFU/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. An extrapolation of phages to a log median concentration of 3.72 PFU/100 ml was slightly lower than the previously

reported value of 4.17 PFU/100 ml (Blanch et al., 2006). The bacteriophages of the host strain GB-124 were found at slightly lower concentrations than values reported from Spanish samples with an average concentration of 500 PFU/ml and slightly higher than the average of 25 PFU/ml from English samples (Blanch et al., 2006). With one exception (phages of ARABA 19), the concentration of all human MST indicators increased downstream from wastewater treatment plants. The ratio of sorbitol-fermenting bifidobacteria to other human faecal indicators altered from wastewater to surface water. This might be due to the persistence of the anaerobic bacteria in environmental water. It was shown that they do not survive for a long time and therefore they do only indicate recent faecal contamination (Scott et al., 2002). As human MST indicators such as sorbitol-fermenting bifidobacteria and phages of the host strains *B. thetaiotaomicron* GA-17, *B. ovatus* GB-124 and *B. thetaiotaomicron* ARABA 84 were additionally found in spring water, they are potentially useful for MST in Switzerland.

Bacteriophages of animal host strains were detected in only low concentrations in all samples analysed. From four animal MST indicators investigated in this study, *R. coprophilus* was superior. High concentrations of *R. coprophilus* were detected with the two methods (culture and real-time PCR) in animal samples from different regions of Switzerland. In addition, they were detected once in human wastewater. In our study, slightly more *R. coprophilus* were found in liquid manure (culture: 4×10^3–9×10^6 CFU/g; PCR: 8×10^3–9×10^7 CFU/g) than the numbers stated by Mara and Oragui (1981) from *R. coprophilus* 3.9 to 2.5×10^6 CFU/g in animal faecal specimens or the values obtained by Savill et al. (2001) from 3.3×10^5 to 3.6×10^6 CFU/g in cow faeces. Higher concentrations of *R. coprophilus* were found upstream than downstream from wastewater treatment plants and the bacteria was also detected in spring water. Therefore, *R. coprophilus*, is potentially useful for MST in Switzerland including spring water investigations. However, the culture-based method is time-consuming. A more

rapid molecular approach was therefore also included in this study but because the assessed sample volumes were not optimally chosen, they might have accounted for the low number of positive results. This demonstrates that not only an ideal target organism is needed but also that the method (culture versus real-time PCR) has a high influence on the result.

In all springs, human and animal faecal contamination was detected. Specific MST indicators were found after rainfall events and in the LQ and the KQ spring also during periods of dry weather. As expected, an increase of faecal contamination was observed after rainfall and the percentage of positive samples was higher than during periods of dry weather. This shows the vulnerability of the investigated karst springs. Our results confirmed previous hydrogeological investigations of the selected springs which suggested the presence of human and animal faecal contamination (Auckenthaler, 2004; Schudel, 2005). A study of particle transport in karst aquifers showed that transport of microorganisms, chemical pollutants, turbidity as well as 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 were present after 35-116h after rainfall (Auckenthaler, 2004). Samples from all springs were therefore collected approximately 72h after rainfall and hydrogeological parameters such as turbidity were measured in all springs (data not shown). Data of turbidity was accessible only after analysis of rainfall events. No increase of turbidity was observed after the first rainfall event but an increase was observed after the second rainfall event in all three springs. After the third rainfall event, increased values of turbidity were observed in the LQ spring and KQ spring. 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.

Conclusion

The MST indicators selected for our study were all specific to indicate the origin of faecal contamination and they were all abundant in either human or animal samples (slaughterhouse wastewater or liquid manure) from Switzerland. We conclude that sorbitol-fermenting bifidobacteria and phages of the host strains *B. thetaiotaomicron* GA-17, *B. ovatus* GB-124 and *B. thetaiotaomicron* ARABA 84 are potentially useful for identifying human faecal contamination in surface and spring water. In addition, *R. coprophilus* turned out to be promising as an indicator of animal faecal contamination in spring water. However, it was shown that the method used to target this bacterium has an influence on the results and should therefore be further optimised. For future studies, a sampling strategy after rainfall is recommended and more than a single sample should be collected to characterise sources of faecal contamination.

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Paper 5:

Identification of faecal input sites in spring water by the use of multiresistant *Escherichia coli*

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Abstract

The localisation of faecal input sites is important for water management. For this purpose we developed a new approach which is based on the screening of multiresistant *Escherichia coli* using selective agar plates followed by the characterisation of selected isolates by antibiotic resistance profiles and molecular fingerprinting techniques (pulsed field gel electrophoresis, PFGE). This approach was successfully applied under field conditions in a study area located in the north-western part of Switzerland. *E. coli* isolates from spring water and surface water samples collected in this area were screened with selective agar plates. In this way 24 different groups consisting of strains with the same pattern of antibiotic resistances were found. Of the total, four groups were further analysed with PFGE. Strains with identical PFGE profiles were repeatedly detected, demonstrating the suitability of this method for the localisation of faecal input sites over an extended period of time. Identical PFGE patterns of strains detected in water from two springs were also found in the stream flowing through the study area. These results demonstrated the applicability of the new approach for the examination of incidents of faecal contamination in drinking water.

Introduction

Escherichia coli is commonly used for the microbiological quality assessment of drinking water (Edberg et al., 2000). Its characteristics of being easy detectable, normally not being pathogenic to humans and being present in higher concentrations than pathogens makes this organism a good candidate as a general indicator of faecal pollution (Scott et al., 2002). *E. coli* is found in the intestines of warm-blooded animals and has been used in different microbial source tracking (MST) approaches to discriminate between human and animal faecal contamination. Different phenotyping methods like biochemical tests, carbon utilization profiles, fatty acid methyl ester and antibiotic resistance analysis have been used in

MST studies employing *E. coli* as an indicator (Ahmed et al., 2005; Moussa et al., 2008; Duran et al., 2009; Wiggins et al., 1999). Analyses of *E. coli* using genotypic methods, such as ribotyping, various PCR methods, pulsed field gel electrophoresis and O-serogrouping were used as well for MST purposes (Carson et al., 2001; Kon et al., 2009; Stoeckel et al., 2004; Myoda et al., 2003; Parveen et al., 2001). Most of these methods are library dependent, requiring a host origin database. The genetic heterogeneity and temporal and spatial variability of *E. coli* populations have been assessed in different studies (Scott et al., 2002; Anderson et al., 2006; Hartel et al., 2002; Jenkins et al., 2003; Kelsey et al., 2008). These approaches, however, may imply the need for large databases which thus limit the use of *E. coli* as an MST indicator (Jenkins et al., 2003, Lu et al., 2005).

Besides discrimination of human and animal faecal pollution, the localization of the faecal input site is of great interest and would contribute to more cost and time-effective remediation measures.

In the present study, we investigated a study area in Switzerland with two springs known to be vulnerable to faecal contamination and displaying a high level of pollution especially after rainfall (Auckenthaler, 2004). The objective was to localise the faecal input site in environmental samples from the study area. For this purpose, we made use of the variability within *E. coli* described above and screened for particular strains in spring and surface water in order to trace back the origin of faecal pollution. An approach using a selective plate for the screening for multiresistant *E. coli* strains was used in order to reduce the number of isolates which were subsequently further analyzed by antibiotic resistance profiles and molecular fingerprinting techniques (PFGE).

Materials and Methods

Study area and sampling sites

The study area was situated in the Lützel valley in the north-western part of Switzerland, as shown in Fig. 1. Two karst springs named “Lützelquelle” (LQ) and “Kächbrunnenquelle” (KQ) were investigated. The springs are located close to the Lützel stream and downstream from a wastewater treatment plant (WTP) with outlets flowing into the stream. Previous studies showed that both springs are vulnerable to faecal contamination, especially after rainfall (Auckenthaler, 2004). The Lützel stream is known to infiltrate to some extent into the KQ spring. In contrast, the LQ spring is fed by infiltrating rainwater from a hamlet named Niederhuggerwald. The hamlet is characterised by intense dairy farming and animal husbandry and is located on a plateau to the south-west of the valley (Auckenthaler, 2004).

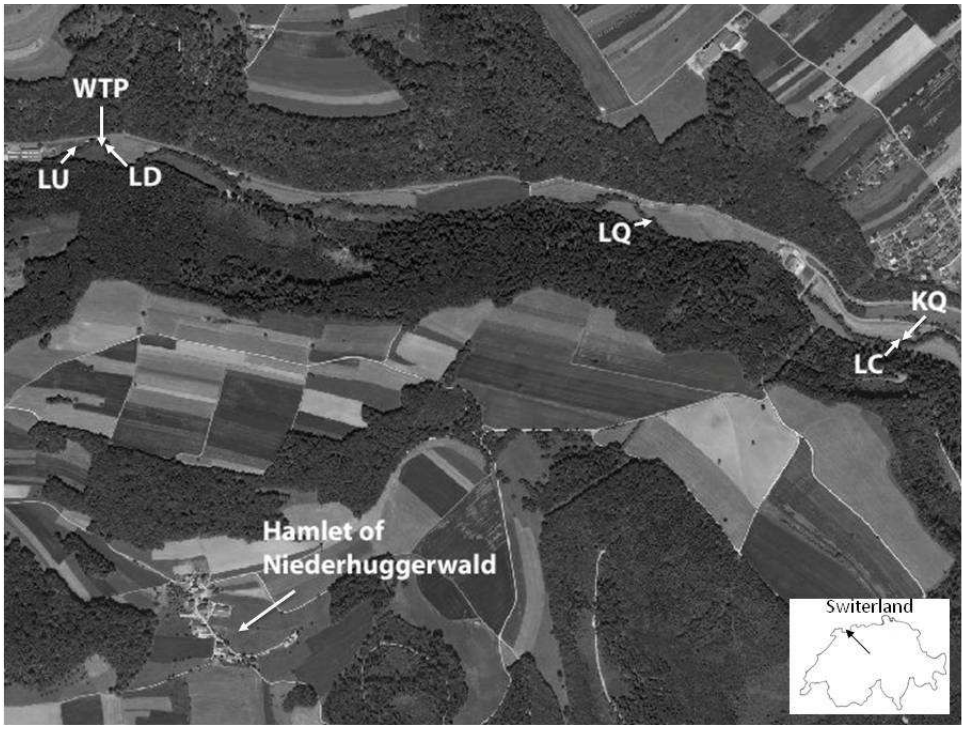

Fig. 1 Study area and sampling sites; LQ: LQ spring, KQ: KQ spring, WTP: Waste water treatment plant, LU: Lützel upstream from WTP, LD: Lützel downstream from WTP, LC: Lützel close to KQ spring. The image was reproduced by permission of swisstopo (BA11001).

Besides the LQ and KQ springs, three surface water sampling sites along the Lützel stream were investigated. Surface water sampling sites included one site upstream (LU) and one site downstream from the WTP close to a flowing WTP outlet (LD). The third site (LC) was approximately one km further downstream from the WTP and very close to the KQ spring.

Preparation of selective antibiotic plates

A selective antibiotic plate known as TSA4 was generated to screen for a multiresistant *E. coli* in the LQ spring TSA4 consisting of Tryptone Soya Agar (TSA) (Oxoid AG, Pratteln, Switzerland), supplemented by the following four antibiotic additives: streptomycin (25 µg/mL), sulfamethoxazole (150 µg/mL), tetracycline (20 µg/mL) and trimethoprim (10 µg/mL). An additional selective plate known as TSA7 was also prepared. TSA7 contained the same antibiotics as TSA4 as well as ciprofloxacin (5 µg/mL), kanamycin sulphate (25 µg/mL) and nalidixic acid (30 µg/mL) as three additional antibiotics. All antimicrobials were purchased from Sigma Aldrich GmbH (Buchs, Switzerland).

Sample collection and processing

Water samples were taken over a period of seven months, namely from March to September 2009. Eight dry weather samples from the two spring water sampling sites were analyzed and three rainfall events were included in the study. A rainfall event was defined as rain of more than 10-20 mm/hour according to local weather radar. Spring water samples were collected approximately 72 hours after rainfall. In addition, surface water samples were analysed during dry weather conditions. Six samples were collected at sampling sites upstream (LU) and one km downstream (LC) from the WTP and another four samples were taken close to a flowing WTP outlet (LD).

Samples were collected in sterilised Nalgene polypropylene bottles (VWR, Dietikon, Switzerland) and were processed within 24 hours after collection. Surface water samples were taken at a depth of approximately 15-20 cm and away from the bank.

Defined water volumes (1000 mL, 100 mL, 10 mL for spring water and 100 mL, 10 mL, 1 mL for surface water) were filtrated through Microsart CN-Filter membranes with a pore size of 0.45 µm (Sartorius Stedim Biotech GmbH, Göttingen, Germany). The filters were first incubated on TSA (Oxoid AG, Pratteln, Switzerland) for two hours at 37 ºC and then transferred onto the selective TSA4 plate. As a negative control, 100 mL of distilled sterile water was filtrated and processed the same way as the water samples. Moreover, susceptible *E. coli* isolates (DSM 1103 / ATCC 25922 / NCIB 12210), were inoculated on the plates as a negative growth control. The plates were incubated for 16-20 hours at 37 ºC and all colonies obtained from spring water samples and three to ten colonies from surface water samples were further confirmed by inoculation on the chromogenous agar T.B.X. (Oxoid AG, Pratteln, Switzerland) and incubation for 16-20 hours at 44 ºC.

The selective TSA7agar plate was used in addition to TSA4 for samples of one dry weather and two rainfall events. The samples were processed as described above.

Antibiogram

A total of 173 *E. coli* isolated from TSA4 were grown on Columbia Blood Sheep Agar (bioMérieux, Geneva, Switzerland) overnight at 37 ºC. Three single colonies of each isolate were then suspended in one ml of Mueller Hinton Broth (Oxoid AG, Pratteln, Switzerland) and incubated for six hours at 37 ºC in a water bath. The turbidity of the suspension was adjusted for comparative purposes with 0.5 Mc Farland (bioMérieux, Geneva, Switzerland) in one mL 0.9% NaCl. The isolates were streaked equally onto Mueller Hinton Agar plates (Oxoid AG, Pratteln, Switzerland).

Twelve commercially available antibiotic test discs, namely amoxicillin/clavulanic acid (20/10 µg), ampicillin (10 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), kanamycin (30 µg), nalidixic acid (30 µg), polymyxin B (300 IU), streptomycin (10 µg), sulphonamide (300 µg), tetracycline (30 µg), trimethoprim (5 µg) trimethoprim/sulfamethoxazole (1.25/23.75 µg), were stamped onto the plates and incubated for 18 hours at 37 ºC. Antibiotics were chosen that are in common use in human and veterinary medicine. Sulphonamide test discs were obtained from Oxoid AG (Pratteln, Switzerland) and the remaining discs from Becton Dickinson (Allschwil, Switzerland). Inhibition zones were assessed according to international standards (CLSI / NCCLS, 2005). *E. coli* (DSM 1103 / ATCC 25922 / NCIB 12210) was used as a negative control strain.

PFGE

A total of 81 *E. coli* isolates comprising four groups (I-IV) of resistance profiles were further typed with PFGE. PFGE was performed in accordance with Baumgartner et al. (2007), with the following modifications: Agar plugs were incubated on a thermomixer (Eppendorf, Hamburg, Germany) and washed in a Rotamix shaker (Heto Lab Equipment A/S, Alerod, Denmark). Purified DNA was digested with restriction enzymes for five hours. Electrophoresis conditions were improved to a final switch time of 60 seconds and a run time of 16 hours.

Electronic processing of data

The data was statistically analysed with the SPSS Statistics 16.0 software package with a five per cent significance level. The analysis of PFGE patterns was conducted with the software BioNumerics version 3.5.

Results

Occurrence of multiresistant *E. coli*

Cell counts of multiresistant *E. coli* are shown in Table 1. During dry weather conditions, multiresistant *E. coli* were found in the LQ spring in three out of eight (37.5%) samples, with cell counts ranging between 0-10 CFU/L and a median of 0 CFU/L. In the KQ spring, five of eight (62.5%) samples showed the presence of multiresistant *E. coli*, with cell counts between 0-3 CFU/L a median of 1 CFU/L.

Table 1 Occurrence of multiresistant *E. coli* at different sampling sites

Sampling site	**Number of positive samples**	***E. coli* counts**[a]	
		Median	**Range**
Spring water			
Spring Lützelquelle (LQ)			
Dry weather samples	3/8	0	0-10
Rainfall event samples	3/3	13	8-24
Spring Kächbrunnenquelle (KQ)			
Dry weather samples	5/8	1	0-3
Rainfall event samples	3/3	4	0-15
Surface water			
Lützel upstream from WTP (LU)	6/6	7.5	1-210
Lützel downstream from WTP (LD)	4/4	155	27-260
Lützel close to KQ spring (LC)	6/6	9.5	2-440

WTP: Wastewater treatment plant
a CFU/L for spring water and CFU/100 mL for surface water

After rainfall events, multiresistant *E. coli* were present in all three samples collected from both springs. In the LQ spring the cell counts ranged between 8-24 CFU/L, with a median of 13 CFU/L. In the KQ spring the cell counts ranged between 0-15 CFU/L, with a median of 4 CFU/L. The effect of rainfall events on the increase of multiresistant *E. coli* was statistically significant in the LQ spring but not in the KQ spring (Mann-Whitney-U-test, $n_1 = 8$, $n_2 = 3$, $p = 0.024$ and $p = 0.28$)..

As shown in Table 1, multiresistant *E. coli* were detected in all surface water samples. The occurrence of multiresistant *E. coli* differed between the three surface water sampling sites. In surface water samples collected upstream from the WTP (LU), multiresistant *E. coli* were detected between 1 to 210 CFU/100 mL, with a median of 7.5 CFU/100mL. The concentration was higher in samples collected close to the effluent (LD), with a range between 27-260 CFU/100 mL and a median of 155 CFU/100 mL. Similarly, samples collected one kilometer further downstream (LC) showed multiresistant *E. coli* counts between 2-440 CFU/100 mL and a median of 9.5 CFU/100 mL. However, no significant differences were observed between the individual sampling sites (Mann-Whitney-U-test $p > 0.05$).

Typing of isolates

After typing 173 multiresistant *E. coli* isolates with antibiogram they could be subdivided into 21 groups with different resistance patterns. The characteristics of four groups (Groups I-IV) further typed with PFGE are shown in Table 2. Group I was composed of seven isolates with resistance to streptomycin, sulfamethoxazole, sulphonamide, tetracycline and trimethoprim. Typing with PFGE revealed six different PFGE patterns within this group. Five isolates had different genotypes and the remaining two isolates shared the same genotype. Group II was composed of 32 isolates with resistance to ampicillin, streptomycin, sulfamethoxazole,

sulphonamide, tetracycline and trimethoprim. Again, PFGE analysis highlighted the heterogeneity within the group with 21 different patterns. From the total of 32 isolates, 16 represented individual genotypes and the other 16 isolates together revealed five different genetic fingerprints.

Group III comprised 39 isolates with resistance to ampicillin, chloramphenicol, ciprofloxacin, kanamycin, nalidixic acid, streptomycin, sulfamethoxazole, sulphonamide, tetracycline and trimethoprim. After PFGE analysis, six different genotypes could be discriminated, of which four were represented by individual isolates. The remaining two shared several isolates with identical PFGE patterns.

Finally, group IV consisted of four isolates with resistance to amoxicillin/clavulanic acid, ampicillin, ciprofloxacin, kanamycin, nalidixic acid, streptomycin, sulfamethoxazole, sulphonamide, tetracycline and trimethoprim. Two different genotypes were detected with PFGE. Three out of the four isolates were clones and one isolate revealed a different PFGE pattern.

Table 2 Characteristics of groups consisting of *E. coli* isolates from surface and spring water from the study area in Switzerland

	Groups differing in the number of resistances			
	I	II	III	IV
Number of isolates	7	32	39	4
Number of antibiotic resistances	5	6	10	10
Number of PFGE patterns	6	21	6	2
Identical isolates in spring and surface water	-	-	+	+

Temporal and spatial distribution of PFGE patterns

Identical PFGE patterns of isolates originating from samples collected at different time points indicated the repeated detection of the same multiresistant *E. coli* strain over time. The same PFGE pattern of six multiresistant *E. coli* isolates, referred to as II (A) in Fig. 2, revealed that a particular strain was detected repeatedly in the Lützel stream over six weeks. The strain was detected on the 24th of March 2009 and on the 7th of April 2009 in the Lützel stream upstream (LU) and downstream from the WTP (LC), on the 21st of April 2009 in the Lützel stream downstream from the WTP (LC) and on the 5th of May 2009 in the Lützel stream upstream from the WTP (LU). The identical PFGE pattern of two strains in Fig. 2, referred to as II (B), reveals a multiresistant *E. coli* strain which was detected repeatedly in the KQ spring over a period of four months. This strain was detected on the 21st of April 2009 and on the 21st of July 2009 in the KQ spring.

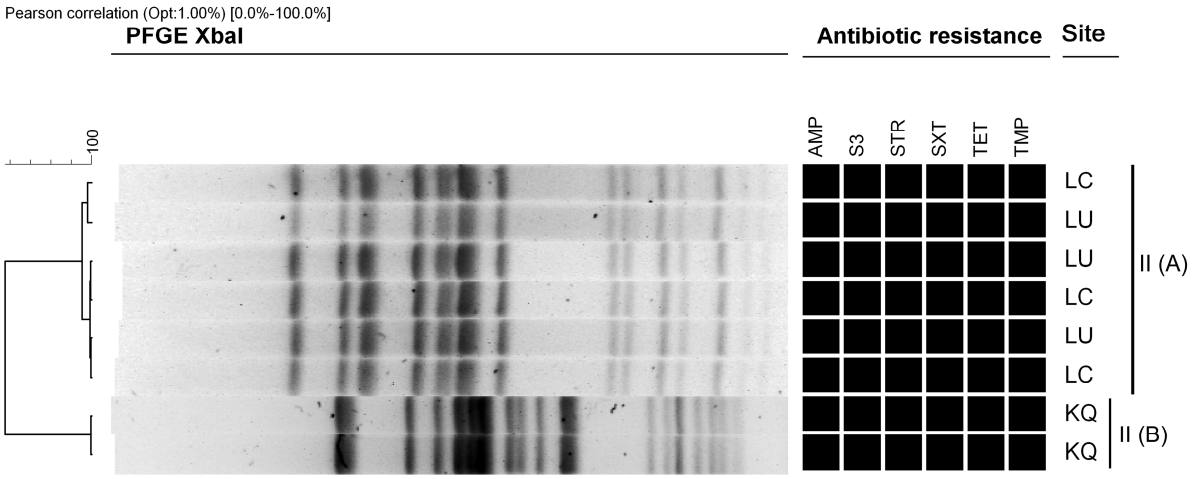

Fig. 2 Identical PFGE patterns of two multiresistant *E. coli* strains. One genotype (II (A)) was present over six weeks in surface water and another genotype (II (B)) over four months in spring water; Antibiotics: AMP: Ampicillin, S3: Sulphonamide, STR: Streptomycin, SXT: Trimethoprim / Sulfamethoxazole, TET: Tetracycline TMP: Trimethoprim; Sampling sites: LC: Lützel close to KQ, LU: Lützel upstream from wastewater treatment plant, KQ: KQ spring.

The spatial distribution of PFGE patterns was demonstrated by the detection of identical strains at spring and surface water sampling sites. As shown in Fig. 3, a particular multiresistant *E. coli* strain IV (D) was detected twice in the LQ spring on the 17th of July 2009 and on the 21st of July 2009. This strain was also detected in the Lützel stream downstream from the WTP on the 28th of July 2009 and close to the KQ spring on the 28th of July 2009 and on the 18th of August 2009.

Another strain found in the KQ spring was also detected at different surface water sampling sites. The pattern, referred to as III (C) in Fig. 3, shows that this strain was found on the 24th of March 2009 in the Lützel stream upstream from the WTP, in the Lützel stream close to the KQ spring as well as in the KQ spring itself.

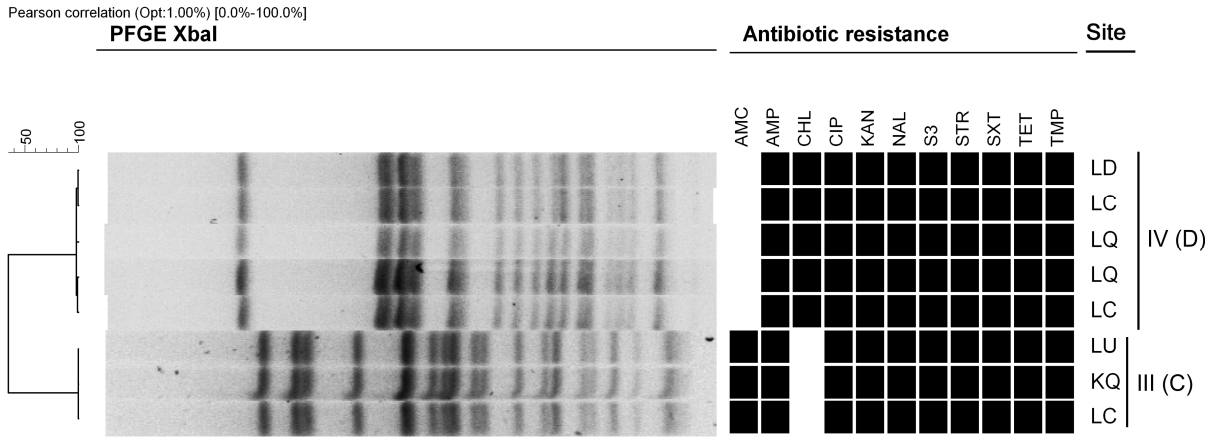

Fig. 3 Identical PFGE patterns of multiresistant *E. coli* strains in surface and spring water; A strain found in the KQ spring was also detected at different surface water sampling sites (III (C)) and a particular multiresistant *E. coli* strain (IV (D)) was detected twice in the LQ spring and in the Lützel stream. Antibiotics: AMC: Amoxicillin / Clavulanic, AMP: Ampicillin, CHL: Chloramphenicol, CIP: Ciprofloxacin, KAN: Kanamycin, NAL: Nalidixic acid, S3: Sulphonamide, STR: Streptomycin, SXT: Trimethoprim / Sulfamethoxazole, TET: Tetracycline TMP: Trimethoprim; Sampling sites: LD: Lützel downstream from wastewater treatment plant, LC: Lützel close to KQ, LQ: LQ spring, LU: Lützel upstream from wastewater treatment plant, KQ: KQ spring.

Selectivity of the antibiotic plate used

Nine water samples were analyzed simultaneously with the selective TSA4 and TSA7 plates. As shown in Table 3, fewer cell counts were detected with TSA7 in both spring and surface water samples. With the selective TSA4 plate cell counts ranged between 0-40 CFU/L, with a median of 14.5 CFU/L for the spring water and between 670-1320 CFU/L with a median of 980 CFU/L for the surface water. By comparison, using the selective TSA7 plate cell counts ranged between 0-3 CFU/L, with a median of 0.5 CFU/L for the spring water, and between 10-100 CFU/L, with a median of 60 CFU/L for the surface water. The difference in cell counts between the plates was statistically significant (Wilcoxon signed-rank test, n = 9, p = 0.012).

Table 3 Comparison of multiresistant *E. coli* cell counts in water samples analyzed with two selective agar plates

	Spring water samples			**Surface water samples**		
	Number	*E. coli* counts[a]		Number	*E. coli* counts[a]	
		Median	Range		Median	Range
Selective plate						
TSA4 (Four antibiotics)	6	14.5	0-40	3	980	670-1320
TSA7 (Seven antibiotics)	6	0.5	0-3	3	60	10-100

a CFU/L

With the increase of antibiotics used to prepare the second selective plate the number of different resistance profiles was reduced. A total of 23 isolates grown on TSA7 agar and typed with antibiogram turned out to have the same resistance profile (data not shown). A comparison of Group II and III in Table 2, also reveals that resistance against a higher number of antibiotics leads to a reduction of isolates with identical PFGE patterns.

Discussion

A novel approach

The localisation of precise input sites of faecal contamination in water resources is of great importance for water management. In the present study we tested an approach based on the screening for multiresistant *E. coli* and the further characterization of isolates by PFGE for the identification of microorganisms with identical genetic fingerprints in springs and suspected faecal input sites.

E. coli is a widespread bacterium which is well established as a general indicator for the microbiological quality of drinking water (Scott et al., 2003). Consequently, it was also proposed as an indicator organism for use in MST. The use of library dependent methods for the characterization of *E. coli* isolates, however, such as antibiotic resistance analysis (ARA) or PFGE patterns for MST, have been questioned (Stoeckel et al., 2004; USEPA, 2005). The source allocation of *E. coli* isolates was described as difficult due to the changes of community composition during the transition of *E. coli* from host to the environment, the high genetic diversity and the excessive number of isolates required for the development of accurate libraries (Lasalde et al., 2005; Parveen et al., 2001; Carson et al., 2003; Gordon, 2001). In the present study, we present an approach designed to circumvent the described

variability and to reduce the number of isolates to be analyzed by relying solely on multiresistant *E. coli* in order to trace back the origin of faecal contamination.

Resistance to antibiotics is widely spread in *E. coli* all over the world and an increase in its prevalence has been observed in recent years (Erb et al., 2007). It was therefore possible to select two particular multiresistant *E. coli* strains isolated from the LQ spring and to generate selective plates (TSA4 and TSA7) based on their resistance profiles. Antibiotics active against Gram-negative bacteria that are important for human or veterinary medicine in Switzerland were chosen for screening. The concept of detecting the same microorganism in the water source and at the suspected faecal input site by antibiogram and PFGE had previously been shown to be possible (Dolejska et al., 2009). These authors tested a series of bacterial isolates from both surface water and avian faeces directly using the two methods in order to detect identical strains. By contrast, in our approach selective agar plates with various antibiotics were used to screen for multiresistant *E. coli* prior to further comparative analyses. The advantage of this procedure was that the number of isolates was greatly reduced and larger sample volumes could be screened for particular multiresistant strains. This makes our method very sensitive since it is based on a filtration technique that allows the analysis of large volumes of water for particular *E. coli* strains.

The results obtained by molecular fingerprinting with PFGE revealed that testing for antibiotic resistance alone was not accurate enough to detect particular multiresistant *E. coli* clones. Isolates with the same resistance profiles displayed differences when further typed with PFGE. The 173 isolates detected with TSA4 were classified to form 21 groups consisting of isolates with the same resistance profile. These groups were further subdivided after analysis of the isolates by PFGE. In order to find identical isolates, a number of different water samples were collected and all isolates had to be characterized; this proved to be a

rather time-consuming approach. The comparison of the two selective agar plates (TSA4 and TSA7) used for screening, revealed that plates with a higher selectivity (e.g. more antibiotics included) resulted in less variability among the isolates. From this we conclude that in further studies plates with more than four antibiotics could be useful and reduce time and effort by reducing the number of isolated strains. In addition, the temporal persistence of PFGE patterns is limited in time. A study by Lu et al. (2004) showed that the PFGE patterns of a particular strain changed after eight weeks while patterns of other strains remained stable. Our results revealed the persistence of particular genotypes over a certain period of time, which confirms the suitability of the method over a given time frame. On the other hand, it also shows the discriminatory power of this molecular fingerprinting technique. Isolates that differ by a single genetic event show 2-3 band differences (Foley et al., 2009). The reliability of this new approach is proven by the confirmation of strains using PFGE typing. The striking discriminatory power and reproducibility of PFGE has made it a widely acceptable method in epidemiology (Foley et al., 2009). Our approach can be used to link spring water faecal contamination to the source of bacterial input sites. The combination of three criteria, screening of multiresistant strains and typing with both antibiogram and PFGE, makes this approach sensitive and reliable. However, the approach does not indicate direct hydraulic connectivity. For this purpose, a combination with widely used chemical tracers may be useful (Käss, 1998; Bombach et al., 2010). While hydrogeological tracer tests indicate hydrogeological connections from one point to another, our new approach can be used on a broader scale for the examination of incidents of faecal contamination in drinking water. It is important to keep in mind the complexity of particular water systems especially in areas with karst geology. Therefore, other criteria such as geological characteristics and the land use of study areas should be considered. By combining our approach with hydrogeological measurements, incidents in springs can be extensively investigated.

Detection of faecal input sites

By the screening of multiresistant *E. coli* and further PFGE-based molecular typing it was shown that infiltration of the stream into the KQ spring is most likely and there was an indication that there might be an additional input site from the stream or another location with a hydraulic connection to the stream and the LQ spring.

A particular multiresistant *E. coli* strain found in the KQ spring and at sampling sites upstream and downstream from the wastewater treatment plant showed that there is a possible hydraulic connectivity from the surface water to the spring. This finding confirmed prior chemical tracer experiments (Auckenthaler, 2004).

The present study provided indications for an additional infiltration into the LQ spring. The same multiresistant *E. coli* strain that was present over four days in the LQ spring could be detected in the stream next to the spring as well as further upstream. Such an interaction has been previously investigated in an extensive investigation (Auckenthaler, 2004). In view of the local geological formation, an infiltration of the Lützel stream into the LQ spring that was initially considered to be possible was not detected. Based on these previously measured hydrogeological parameters (turbidity, conductivity and discharge) and tracer tests, a connection between the stream and the LQ spring was considered to be unlikely (Auckenthaler, 2004). The multiresistant strain detected in the LQ spring was detected downstream from the WTP but not upstream and could therefore originate from the plant. As this strain was detected after a rainfall event, weather conditions probably play an important role in the interaction of the stream and the LQ spring. Previously conducted hydrogeological investigations were performed during low flow conditions (Auckenthaler, 2004). However, in order to further investigate possible connections between the stream and the spring, tracer tests after a rainfall event are needed.

Beside an infiltration from the stream into the LQ spring, another input site was considered to be responsible for the contamination of both sampling sites. Previous hydrogeological studies showed that the LQ spring is fed by infiltrating rainwater on a plateau south-west of the spring (Auckenthaler et al., 2002). In our study, this connection was not found. Suspected faecal input sites around the hamlet of Niederhuggerwald, which is situated on this plateau, showed the presence of *E. coli* in high concentrations but no multiresistant *E. coli* were detected (results not shown). Samples were collected only once at the hamlet. It is therefore important to remember that this finding does not constitute proof of the absence of an interaction between both sites.

On the one hand, our results support the findings of previous hydrogeological tracer tests that the Lützel stream infiltrates into the KQ spring and, on the other hand, give some indication that there might be an additional input site from the stream or another location with a hydraulic connection with the stream and the LQ spring. Because the second connectivity was not consistent with previous studies we conclude that further tracer experiments should be performed after a rainfall event in order to confirm our findings and, if necessary, other possible input sites are be considered. Our results indicate that spring water contamination in the study area originates to a certain extent from surface water. Although the multiresistant strain detected in the KQ spring did not originate from the wastewater treatment plant, human pathogens could also infiltrate the springs if surface water infiltrates downstream from the wastewater treatment plant. The water supply of Röschenz is mainly derived from the two springs. The impact on human health as a result of spring water contamination can be considered to be low as the water is treated in a six-step procedure (flocculation, sedimentation, rapid sand filtration, ozonisation, charcoal filtration and chemical disinfection) but is of special concern if the treatment process fails. The results provide evidence that the

treatment procedure is essential in the study area and should be carefully monitored in order to prevent disease transmission in the future.

Conclusion

The following conclusions can be drawn from our study:

- The method applied was based on the screening of multiresistant *E. coli* in water samples. Comparative analysis of two selective plates showed that the number of isolates can be reduced by increasing the number of antibiotics included in selective plates. With the more selective TSA7 plate, considerably fewer strains were isolated compared to the less selective TSA4 plate. Thus, the plates used must be generated to be as selective as possible.
- Antibiotic resistance analysis and PFGE were used in order to type multiresistant isolates. The results showed that PFGE discriminated between isolates which were unrecognized by antibiotic resistance analysis. Therefore, PFGE is needed in order to detect identical isolates.
- The approach used, based on the screening of multiresistant *E. coli*, was able to identify the same strain both in spring and surface water and confirmed previous evidence of infiltration. The results also indicate that there might be an additional input site for one spring.
- Multiresistant *E. coli* can serve as an environmental tracer which is helpful in localizing possible input sites for contaminants. In combination with classical hydrogeological tracers it therefore constitutes an advantage in the characterization of microbial contaminant sites.
- The contamination of spring water in the study area provides evidence that a treatment procedure is essential in the study area and should be carefully monitored in order to prevent waterborne diseases.

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5. DISCUSSION AND CONCLUSION

5.1 General considerations

The idea of using microbes as faecal source indicators was already suggested in the early 1900s (Sinton 1998) and constantly gained popularity since the 1980s. Today, microbial source tracking (MST) is a field of intensive research worldwide (Meays et al., 2004; Savichtcheva and Okabe, 2006; Scott et al., 2002; Simpson et al., 2002; Sinton et al., 1998; Stoeckel and Harwood, 2007), in Switzerland, however, it was applied for the first time in the context of this thesis.

The objective for the use of MST in Switzerland was different than in other countries because the major target was drinking water faecal contamination. In other countries however, different priorities were set. In the United States e.g., MST was frequently used to establish total maximum daily loads (TMDL) for impaired waters. A TMDL establishes the maximum pollutant load that a water body can receive and still meet water quality standards. MST was therefore used as an approach to determine the sources of faecal contamination affecting a water body in the U.S. (Simpson et al., 2002).

A research programme of the European Union focused on source tracking of faecal pollution in surface water with the aim to find MST methods applicable in different geographic regions in Europe (Blanch et al., 2004). Predictive models were then performed to distinguish human and non-human sources (Blanch et al., 2006). Also in New Zealand and Canada, different MST methods were used to detect specific sources in surface water (Devane et al., 2007; Edge and Schaefer 2006; Gilpin et al., 2002).

Only a few studies applied MST in spring or tap water (Plummer and Long, 2007; Reischer et al., 2007; van der Wielen and Medema, 2010). As faecal contamination in drinking water is of special concern for public health, the goal of the present study was to identify concepts and approaches to detect and discriminate contaminations from human and animal sources in water including spring water. Approaches which are useful for an application in Switzerland are described and, for the first time, data from an extensive comparative study of different MST indicators in spring water are presented.

5.2 Methodology and sampling strategies

Design of the assessment

Potential source tracking tools already described in the scientific literature and as well new MST methods were tested and improved when necessary. As described in the methodology part of this thesis, the assessment was performed in four consecutive steps:

1. a proof of concept restricted to laboratory testing,
2. a test of the geographical stability with human and animal wastewater,
3. an evaluation of the selected methods in the field, and finally
4. a comparative study in which all methods potentially useful in the other stages were included.

Several reviews have been published which describe appropriate assessment and design of MST studies (Field and Samadpour, 2007; Santo Domingo et al., 2007; Stoeckel and Harwood, 2007). Although the experimental design of the present study was defined before these studies were published, the critical steps are in accordance with these concepts. Field

and Samadpour (2007) described an approach consisting of 6 stages including two stages that where not assessed in the present study: testing with blind samples and measurement of the resulting improvement in water quality. Survival and persistence of MST indicators in the environment and correlation with pathogens are criteria which should be further assessed (Field and Samadpour, 2007). It is known that sorbitol-fermenting bifidobacteria survive only for a short time in the environment and that *R. coprophilus* persist in water for more than 17 weeks (Arango, 2000; Bonjoch et al., 2009; Jagals et al., 1995; Oragui and Mara, 1983). However, the persistence of new MST indicators in environmental water is not known and should be further investigated.

More than 20 different targets were tested to identify either human or animal faecal sources. In addition, an approach was developed in the present study to localize faecal input sites of contamination based on the screening of multiresistant *Escherichia coli* followed by the characterisation of selected isolates by antibiotic resistance profiles and molecular fingerprinting techniques (PFGE). Both antibiotic resistance profiles and PFGE were used in MST before (Myoda et al., 2003; Simpson et al., 2002; Stoeckel et al., 2004; Wiggins et al., 1999) but never in combination to identify a particular multiresistant clone of *E. coli*.

The method used to target MST indicators

The method applied to detect an organism has an influence on the result because sensitivity, specificity and robustness differ from one method to another. In the course of this thesis, different methods to detect one single organism were compared and the results of this comparison were summarized in two manuscripts. Major differences were observed when different methods were used for analysis of paired samples. For the detection of *S. agalactiae*, three DNA extraction methods and five PCR assays targeting four different genes were tested to find the most suitable combination. These methods revealed considerable differences in

sensitivity and robustness (Paper 2). In a second manuscript (Paper 3), four assays were compared for detecting *R. coprophilus*. Again, major differences were observed in sensitivity, specificity, robustness and duration of the compared assays. These results showed that not only ideal indicator organisms have to be selected but also the most suitable methods for their detection have to be applied as a prerequisite for the success of MST studies.

Samples used for the assessment of different MST indicators

Human wastewater, slaughterhouse wastewater and liquid manure were analysed for testing the proof of concept and to test for geographic stability. These samples were collected from many different regions spread all over Switzerland and were therefore applicable to assess the geographic distribution of investigated MST indicators. In addition, the pooled wastewater samples consisted of faecal matter from many humans or animals. Therefore, the specificity of the tested methods could be well determined. The sensitivity (including numbers of false negative results) of the tested methods might be different if individual faecal samples are tested.

The two study areas investigated for field validation of different MST indicators were selected because they were previously well characterized based on unspecific faecal indicators and hydrogeological parameters (Auckenthaler, 2004; Auckenthaler et al., 2002; Raso, 2000; Ruchti, 1999; Schudel et al., 2000). In addition, human and animal faecal contamination could be expected in surface water and in the investigated springs (Auckenthaler, 2004; Schudel et al., 2000). Consequently, the study areas were well chosen for a first validation of MST indicators in spring water and to create an appropriate toolbox which is now available to investigate further study areas.

Time point of sampling

Stoeckel and Harwood (2007) described the importance of representative sampling because faecal contamination is not distributed evenly over time. Among other things, transport pathways, growth or inactivation of organisms, and diurnal and seasonal patterns have an influence on MST indicators and should also be considered for spring water analysis. An increase of faecal contamination is not observed immediately after rainfall. The appropriate time-point for sampling might therefore be important to measure the impact of rainfall on spring water quality. Hydrological parameters (e.g. turbidity) can be used to describe pollution dynamics and the appearance of faecal contamination (Auckenthaler, 2004). Turbidity was constantly monitored in the investigated springs but did not increase after every rainfall event (Paper 4). Nevertheless, an increase of faecal contamination was observed in all three springs and specific MST indicators were found more frequently after heavy rains. A sampling strategy after rainfall was important and is therefore also recommended for future studies. In addition, more than a single sample should be collected to characterise sources of faecal contamination because specific MST indicators were not detected in all samples. Moreover, data 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.

5.3 The ideal MST indicator

The ideal indicator of human or animal contamination would be easy detectable, geographically widespread, restricted to the host organism, prevalent and abundant in the host organism and should correlate with human pathogens. The host restriction leads to specificity and the prevalence and abundance in the host organism and in environmental waters has an influence on the sensitivity of a method applied to detect a particular MST indicator. Some

organisms are more sensitive to UV or oxygen and therefore they die off more quickly than others. Consequently, they indicate recent pollution. Organisms that are resistant to environmental factors and persist or even multiply in the environment, do not indicate the time point of faecal contamination. Characteristics of investigated MST indicators that successful passed the first two stages of assessment (see "Methodology") are discussed below.

Indicators of human faecal contamination

Sorbitol-fermenting bifidobacteria:

Sorbitol-fermenting bifidobacteria were among the first human specific indicators used for MST. They were described as specific indicators of human faecal contamination and human bifid sorbitol agar (HBSA) was developed as selective medium to detect these bacteria (Mara and Oragui, 1983). Currently, the detection of these bacteria based on that selective media is still frequently used in MST (Blanch et al., 2006; Blanch and Jofre, 2004; Bonjoch et al., 2009; Bonjoch et al., 2005; Long et al., 2003; Mushi et al., 2010; Ottoson, 2009). Blanch et al. (2006) performed a comparative study by investigating several MST indicators and concluded that sorbitol-fermenting bifidobacteria are potentially useful (Blanch et al., 2006). In the present study, they were found in high numbers in all human wastewater samples and at lower levels in surface water (Paper 4), which was in accordance with previous findings (Jagals et al., 1995; Long et al., 2003; Mara and Oragui, 1983). In previous studies, an increase of the faecal coliform/bifidobacteria ratio with the distance from sources of human faecal contamination was observed (Jagals et al., 1995). In our investigations, sorbitol fermenting bifidobactera were not detected in any samples of animal origin. This result was in accordance with several studies (Mara and Oragui, 1983; Mushi et al., 2010) but in contrast to others (Bonjoch et al., 2005; Ottoson, 2009). Presumptive yellow colonies typical for sorbitol-

fermenting bifidobacteria were also detected after analysis of animal samples. However, these colonies were either built by cocci or bacteria missing the typical V/Y structures and could thus not be confirmed to be bifidobacteria. These results showed that there is a need for confirmation of presumptive isolates as previously recommended (Mara and Oragui, 1983; Mushi et al., 2010). Sorbitol-fermenting bifidobacteria seem to have a widespread geographical distribution as they were found in all human wastewater samples from Switzerland and additionally in samples from many other countries (Blanch et al., 2006; Long et al., 2003; Mushi et al., 2010). Survival and persistence were previously assessed and sorbitol-fermenting bifidobacteria were described to be good indicators of recent faecal pollution. (Jagals et al., 1995; Mushi et al., 2010; Oragui and Mara, 1983). However, the use of these indicator might be limited as seasonal variations were observed in environmental water (Field and Samadpour, 2007). In the context of this thesis, sorbitol-fermenting bifidobacteria were investigated for the first time in spring water and were successfully detected in three springs from two study areas in Switzerland. During periods of dry weather, four out of twenty and after rainfall three out of ten spring water samples with human contamination were positive (Paper 4).

Sorbitol-fermenting bifidobacteria were restricted to human samples, abundant in Swiss human wastewater and detected in surface and spring water. It can therefore be concluded that sorbitol-fermenting bifidobacteria are potentially useful to indicate recent human faecal contamination in environmental water from Switzerland including also spring water.

Bacteriophages of the host strains *Bacteroides thetaiotaomicron* GA-17 and *B. ovatus* GB-124:

The two human host strains *B. thetaotaomicron* GA-17 and *B. ovatus* GB-124 were previously isolated in Spain and England (Payan et al., 2005) and were shown to indicate

human faecal pollution (Blanch et al., 2006; Ebdon et al., 2007; Payan et al., 2005; Scott et al., 2002). In addition, the use of the host strain *B. thetaotaomicron* GA-17 for detecting bacteriophages was considered to be a very reliable approach in a comparative study (Blanch et al., 2006). Bacteriophages of the host strain GB-124, not included in the comparative study by Blanch et al. (2006), were never detected in animal samples but were abundant in human wastewater and in surface water (Ebdon et al., 2007; Payan et al., 2005). Therefore, these human MST indicators were selected for assessment in Switzerland. *Bacteroides* bacteriophages were shown to be restricted to particular geographic areas or formed turbid plaques which are difficult to interpret (Duran et al., 2002; Payan et al., 2005; Puig et al., 1999). Nevertheless, they were present in all human wastewater samples from Switzerland. Concentrations of bacteriophages infecting the host strain GA-17 (log median: 3.72 PFU/100 ml) were slightly lower than the previously reported value of 4.17 PFU/100 ml (Blanch et al., 2006). The bacteriophages of the host strain GB-124 were found at slightly lower concentrations than values reported for Spanish samples with an average concentration of 500 PFU/ml and with a slightly higher concentration than the average of 25 PFU/ml from English samples (Blanch et al., 2006). The host strains GA-17 and GB-124 were also successfully detected in surface and spring water. The numbers of positive samples were similar and various counts of phages were detected by individual host strains in different samples but were not constantly higher for one or the other host strain. However, with the host GA-17 very small and turbid plaques which were difficult to interpret were detected and phages were found in one slaughterhouse wastewater sample. Therefore, the use of the human host strain GB-124 is recommended for future detection of human faecal contamination in Switzerland.

New *Bacteroides* host strains ARABA 84 and ARABA 19 detecting human derived bacteriophages:

The two *Bacteroides* host strains ARABA 84 and ARABA 19 were selected from 98 isolates obtained from two human wastewater samples and specifically detect bacteriophages of human origin. There was a clear difference between the two host strains. In human wastewater and surface water samples, plaque counts were always higher when using the host strain ARABA 84 compared to the host strain ARABA 19. A higher sensitivity of the host strain ARABA 84 was also observed for spring water samples. When bacteriophages active against ARABA 19 were present in spring water samples, ARABA 84 always detected bacteriophages in the same sample (Paper 1). As other MST indicators were more frequently detected in surface and spring water, ARABA 19 is not recommended for further research.

Bacteriophages of the host ARABA 84 were present in all human wastewater samples and never detected in slaughterhouse wastewater or liquid manure. Concentrations of bacteriophages infecting this host strain were slightly lower than bacteriophages detected by the host strains GB-124 and GA-17 in human wastewater. In surface and spring water samples, the host ARABA 84 detected more bacteriophges than the other two host strains. Human faecal contamination of springs was most frequently detected by human specific phages infective for *B. thetaiotaomicron* ARABA 84. During dry weather, sorbitol-fermenting bifidobacteria were present in three samples where phages infecting the host strain ARABA 84 were not detected. After rainfall, phages infecting *B. ovatus* GB-124 were found in only one positive sample, but there were no phages active against the host ARABA 84 (Paper 4).

The human host strain ARABA 84 detected bacteriophages restricted to human samples and phages were detected in all Swiss human wastewater samples and frequently in surface water.

Phages infecting the host strain ARABA 84 were more frequently detected in spring water than any other human MST indicator. Therefore, it can be concluded that phages of the host ARABA 84 are potentially useful to indicate human faecal contamination in surface and spring water from Switzerland. However, further international comparative studies would be desirable in order to evaluate the applicability and performance of this new MST indicator in other geographic regions

***Streptococcus agalactiae*:**

In this study, the potential of *S. agalactiae* for the use in microbial source tracking was evaluated for the first time. A method was therefore needed with the potential to detect these bacteria in environmental water. Different methods were tested which revealed considerable differences in sensitivity and robustness. Detecting *S. agalactiae* was best accomplished by a combination of DNA extraction using the DNeasy Blood and Tissue kit from Qiagen and a LightCycler PCR assay using primers and probes that were available in the literature (Paper 2) (Ke et al., 2000). With this combination, *S. agalactiae* was detected in all human wastewater samples and never in samples from animal origin. Consequently, the stage 1 and 2 assessment was successfully passed. For the field testing, an initial filtration step as well as an enrichment step, was included in the detection method. The results showed that the occurrence of *S. agalactiae* was restricted to surface water samples with human contamination. In addition, *S. agalactiae* was constantly detected over time in samples collected downstream from effluent discharge points of wastewater treatment plants from two study areas in Switzerland. Therefore, it can be concluded that *S. agalactiae* successfully indicated human pollution in surface water. However, the comparative study showed that the method could not be used to indicate human faecal contamination in spring water and methods detecting other MST indicators were more sensitive (Paper 4). In order to determine the occurrence of *S. agalactiae* in environmental samples, an initial volume of one litre was

filtered followed by an enrichment step. The sensitivity of the method might be increased by testing larger sample volumes of water. However, further validation should then be performed to test for PCR inhibitors which are also concentrated when larger volumes are analysed (Santo Domingo et al., 2007). In addition, it has to be considered that the potential of *S. agalactiae* for MST is directly linked to the prevalence of these organisms in humans and animals. Although the epidemiological impact of *S. agalactiae* for mastitis has become negligible in many European countries (Busato et al., 2000; Ivemeyer et al., 2009; Piepers et al., 2007; Roesch et al., 2007; Sampimon et al., 2009), during application of this MST indicator the occurrence of *S. agalactiae* in farms needs to be monitored.

Considering these points, it is concluded that other methods assessed in this study were superior and further research is needed before this MST indicator could be used in practice.

Indicators of animal faecal contamination

New *Bacteroides* host strains RBA 63, 64 and KBA 60 detecting animal derived phages:

Out of a total of 158 presumptive *Bacteroides* isolates obtained from ten bovine and eight horse faecal specimens, three *Bacteroides* host strains *B. caccae* RBA 63, *B. caccae* 64 and *B. fragilis* KBA 60 were selected because they specifically indicated animal contamination (Paper 1). Bacteriophages of these host strains were not found in all animal samples and only low numbers were detected. Nevertheless, these animal MST indicators were included in the comparative assessment. They were successfully detected in surface water and bacteriophages of the host strains RBA 63 and KBA 60 also in spring water. However, they were found in only a small number of samples and at low concentrations. In order to increase the number of positive samples and the sensitivity, either higher sample volumes could be analysed or an

enrichment step could be performed. In conclusion, further research is needed before using these animal MST indicators.

***Rhodococcus coprophilus*:**

Already in 1977, when *Rhodococcus coprophilus* was described for the first time, it was suggested that the ratio of these bacteria to other actinomycetes may indicate the presence of dairy farm effluents in surface water (Rowbotham and Cross, 1977a; Rowbotham and Cross, 1977b). *Rhodococcus coprophilus* was detected at high levels in faeces of different animal species such as cattle, sheep, pigs, horses, ducks, geese and hens (Mara and Oragui, 1981; Savill et al., 2001). Because of this wide distribution in animal faeces, *R. coprophilus* was used for assessment in Switzerland. Different methods were tested which revealed considerable differences in sensitivity, specificity and robustness (Paper 3). A LightCycler real-time PCR assay and a culture-based method detected high numbers of *R. coprophilus* in slaughterhouse wastewater and liquid manure (Paper 4). The cell counts obtained for *R. coprophilus* in liquid manure (culture: 4×10^3–9×10^6 CFU/g; PCR: 8×10^3–9×10^7 CFU/g) were slightly higher than the numbers stated by Mara and Oragui (1981) ranging from 3.9 to 2.5×10^6 CFU/g in animal faecal specimens or the values obtained by Savill et al. (2001) ranging from 3.3×10^5 to 3.6×10^6 CFU/g in cow faeces. The two methods performed well in the first two stages of assessment and were therefore used for the comparative study. The results of surface and spring water analysis with the two methods were different and higher cell counts were detected with the culture-based method. This might be due to a decrease of the sample volume analysed with the real-time PCR assay. Through sample processing (filtration, resuspension and DNA extraction), the volume was decreased and a volume of 5 µl analysed per PCR reaction was consistent with an initial sample volume of only 2.5 ml surface water and 5 ml spring water (Paper 3). The developed LightCycler real-time PCR must therefore be further improved to increase sensitivity. With the culture-based

detection method, analysis was much more time-consuming but animal faecal contamination was detected in all surface water samples and frequently in spring water. *Rhodococcus coprophilus* is known to be resistant against unfavourable environmental influence such as dry and cold periods (Arango, 2000) and was shown to survive for 17 weeks at different temperatures. *Rhodococcus coprophilus* thus indicates remote but not recent pollution of farm animal origin (Oragui and Mara, 1983). This might account for the rather high numbers of *R. coprophilus* detected in environmental water samples.

In comparison to other animal MST indicators, detecting of *R. coprophilus* with the culture-based method was superior because bacteria were found in high numbers in animal samples and they were detected frequently in surface and spring water. As the culture-based method is time-consuming, an optimisation of the new real-time PCR assay is recommended. In addition, other methods should be evaluated to detect animal sources of contamination.

5.4 Springs under investigation

Prior to this study, no specific faecal indicator has been used to characterise spring faecal contamination in Switzerland. Spring water from karst aquifers are important sources of drinking water but are vulnerable to faecal contamination (Auckenthaler et al., 2003). The quality of Swiss drinking water is assured by tolerance values of *E. coli* and enterococci as well as the number of HPC that are present. The results of this thesis showed that during periods of dry weather about 84% of samples and after rainfall 100% of samples exceeded the tolerance values stated in the Swiss Hygiene Ordinance (HyV, SR 817.024.1). However, this water is treated before distribution to costumers and therefore not defined as drinking water. Previous studies in the Röschenz and Oberdorf study areas, investigating the three selected karst springs, showed that considerable faecal contaminations are present after rainfall events,

are likely to derive from both human and animal origin (Auckenthaler, 2004; Schudel et al., 2000). Therefore, these study areas were used for the field validation of MST indicators in the comparative assessment.

The presence of human faecal contamination could be confirmed for the first time based on approaches specifically targeting organisms derived from human sources. Human and animal faecal contamination was detected in all three springs. As human pathogens are frequently detected in human waste, human faecal contamination is of special concern. In fact, human pathogens were detected in the LQ spring (Auckenthaler et al., 2002). Water from the selected springs is treated before consumption and a public health risk is of concern if treatment fails. As only a chemical treatment is performed in the Oberdorf study area, pathogens that survive this treatment might cause human infections.

The results of this study confirmed prior experiments that suggested human faecal contamination in the investigated springs. Solutions should therefore be found to reduce this negative influence. Possible interventions would be an adequate treatment of several steps in the ZQ spring, discard of water or remediation of treatment plants to reduce the hazard for public health.

5.5 From innovation to application

Detection of human and animal faecal contamination in surface and drinking water

Based upon the results of the present study, sorbitol-fermenting bifidobacteria and phages of the human host strains *B. thetaiotaomicron* ARABA 84 and *B. ovatus* GB-124 can be recommended for detecting human faecal contamination in Swiss surface and drinking water. In contrast, the selection of a MST indicator indicative for animal faecal contamination turned

out to be more difficult. *Rhodococcus coprophilus* was superior for an application in surface and spring water than other animal MST indicators and should be taken into consideration for future research. In particular, the detection of this indicator based on the new PCR assay described in this thesis should be improved and compared with other methods for identification of animal sources such as for example an assay previously described to detect ruminant faecal pollution (Reischer et al., 2006).

For identification of source specific faecal contamination in spring water, a repeated sampling after rainfall is recommended. In future investigations, a combination of source specific MST indicators described in the present work and unspecific indicators should be assessed. Based on this toolbox and sampling strategy, human faecal contamination could be detected in springs. For identification of animal contamination, a two-step approach is recommended. A first step of identification of animal sources based on the detection of *R. coprophilus* should be followed by a second step to identify specific faecal input sites based on the screening of multiresistant *E. coli* and further characterisation of selected isolates by antibiotic resistance profiles and PFGE.

Determination of human health risks

The present study describes approaches to detect sources of faecal contamination and to specifically identify faecal input sites. Human and animal specific MST indicators help to estimate health risks associated with the origin of faecal contamination (Scott et al., 2002). However, determination of the source of a contamination as of human or animal origin does not directly quantify the associated risk. Some critical aspects have to be considered to further assess the impact of faecal contamination for public health. Beside the undisputable health threat linked to human faecal contamination, animal faeces pose a potential hazard as well. However, only little information on the human health risks of non-human faecal contamination is available (Field and Samadpour, 2007). A recent study showed that

gastrointestinal illness risks associated to exposure to recreational waters impacted by human sources may not substantially differ from risks associated with water impacted by fresh cattle faeces. However, risks associated to exposure to gull, pig, and chicken faecal contaminated water were shown to be lower (Soller et al., 2010). In fact, waterborne disease outbreaks were associated with zoonotic pathogens abundant in water such as *E. coli* O157:H7 or *Campylobacter jejuni* (Furtado et al., 1998; Money et al., 2010; Rangel et al., 2005; Said et al., 2003). Concepts and approaches described in this study to discriminate human and animal faecal pollution can be used in combination with epidemiological studies to assess health risks associated with the origin of faecal contamination.

Different methods to detect pathogens were shown to be useful to evaluate the microbiological quality of water (Girones et al., 2010), but routine monitoring of all possible pathogens is expensive and time-consuming. Methods described in this study should be further used to investigate a correlation between MST indicators and pathogens. In addition, detection of so called pathogens does not always indicate pathogenicity for humans. Molecular subtyping of the waterborne pathogens *Giardia* spp. and *Cryptosporidium* spp. showed that most species and genotypes are host adapted and that some genotypes involved in human disease are human specific (Appelbee et al., 2005; Xiao, 2010; Xiao and Fayer, 2008). In addition, many viruses are host-specific, too (Field and Samadpour, 2007; Fong and Lipp, 2005; Oliver et al., 2003). Therefore, methods targeting only human pathogens or genetic characterisation of pathogens should be used to define their correlation with new MST indicators.

Prevention of faecal contamination by remediation

In daily practice, when water suppliers are faced with the presence of faecal bacteria, they react with actions such as temporal closure of pumping stations, separation of the

contaminated reservoir from drinking water network and treatment to remove microorganisms in the system. If faecal contamination is repeatedly present, then remediation might be needed to prevent waterborne diseases. New concepts and approaches described in this thesis may contribute to protection and prevention of faecal contaminations of springs by providing responsible authorities with sound scientific data as a basis for risk management measures. As a consequence, faecal contamination of springs might be reduced and the number of waterborne outbreaks, which were frequently associated with a failure in treatment, would thus be decreased. Responsible authorities receive well-grounded data to implement remedial interventions and to take appropriate action.

New concepts described in this thesis can be applied in combination with currently used hydrogeological methods to define protection sites for drinking water supplies. Approaches to discriminate sources of faecal contamination give evidence of faecal input sites and can then be followed by an approach to localize faecal input sites based on screening of multiresistant *E. coli* and by the characterisation of selected isolates by antibiotic resistance profiles and PFGE.

Faecal contamination is often associated with high costs. Factors such as closure of beaches or shellfish harvesting areas, previously described to cause economic losses (Scott et al., 2002), are not of importance in Switzerland. However, faecal spring water contamination can cause economic damage due to temporal closure of pumping stations, separation of the contaminated reservoir from drinking water network and chlorination and flushing to remove microorganisms in the system. In addition, the affected community has to purchase drinking water from other water suppliers. Such costs would also be reduced by remedial measures to prevent further faecal contamination of drinking water supplies.

6. FINAL CONCLUSIONS

- From more than 20 different methods targeting potential source specific indicators, 11 were included in a comparative study of wastewater, surface water and spring water. From these methods, a set of methods was identified representing a “toolbox” of useful MST indicators for further practical application.

- Several human MST indicators were successfully applied in all matrixes: Sorbitol-fermenting bifidobacteria and phages of the host strains *B. ovatus* GB-124 and *B. thetaiotaomicron* ARABA 84 were found in all human wastewater samples. They were present in higher concentrations downstream than upstream wastewater treatment plants and were detected in spring water during periods of dry weather and after rainfall events.

- The selection of an animal MST indicator turned out to be a more difficult task. *Rhodococcus coprophilus* was detected in most of the samples of animal origin and only once in human wastewater. Surface water samples were always positive with the culture-based detection method and concentrations were higher upstream than downstream from wastewater treatment plants in both study areas. Based on a culture-based method, *R. coprophilus* was detected in spring water during periods of dry weather and after rainfall events. However, further research is needed to optimize the detection method for this indicator organism.

- For identification of human faecal contamination in spring water, repeated sampling after rainfall is recommended based on several specific and unspecific indicators.

- The methods described to specifically identify human or animal faecal contamination are simple and can be performed at relatively low cost. The present thesis provides the basis to bridge the gap from innovation to application: Recommendations based upon the concepts and protocols for analysis of MST indictors developed in this study will be elaborated by the Federal Office of Public Health and distributed to water suppliers and enforcement authorities.

- Comparison of different methods targeting the same MST indicator showed different results. Therefore, not only an appropriate target is needed but also an efficient and sensitive method for detection. Comparative analysis was useful to find the best detection method.

- The described toolbox is useful to indicate human faecal contamination and can be applied in combination with epidemiological data to assess the health risk.

- Based on methods from the toolbox, human and animal faecal contamination can be identified and evidence may be obtained for faecal input sites. The approach based on the screening of multiresistant *E. coli*, can be used to identify identical strains and help locate possible input sites for contaminants. In combination with classical hydrogeological tracers it therefore constitutes a progress in the characterization of microbial contaminant sites and helps to define new protection sites.

- The present thesis is the first MST study performed in Switzerland and describes approaches which are useful to distinguish between human and animal sources of contamination in Swiss surface and spring water. In addition, this is the first study

worldwide where different MST indicators were included in a comparative study for spring water analysis.

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