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***Escherichia coli* and enterococci are sensitive and reliable indicators for human, livestock and wildlife faecal pollution in alpine mountainous water resources**

A.H. Farnleitner^{1,*}, G. Ryzinska-Paier¹, G.H. Reischer¹, M.M. Burtscher¹, S. Knetsch², A.K.T. Kirschner², T. Dirnböck³, G. Kuschnig⁴, R.L. Mach¹, and R. Sommer²

¹Institute for Chemical Engineering, Research Area Applied Biochemistry and Gene Technology, Research Group Environmental Microbiology and Molecular Ecology, Vienna University of Technology, Vienna, Austria.

²Institute of Hygiene and Applied Immunology, Department for Water Hygiene, Medical University of Vienna, Vienna, Austria.

³Prigglitz 66, 2640 Gloggnitz, Lower Austria, Austria.

⁴Vienna Waterworks, Vienna, Austria.

Abstract

Aims—This study evaluated the applicability of standard faecal indicator bacteria (SFIB) for alpine mountainous water resources monitoring.

Methods and Results—*Escherichia coli*, enterococci (ENTC) and *Clostridium perfringens* were investigated by standard or frequently applied methods in a broad range of animal and human faecal sources in a large alpine mountainous area. *C. perfringens* occurred only in human, livestock and carnivorous source groups in relevant average concentrations (log 4.7–7.0 CFU per g) but not in herbivorous wildlife sources. *E. coli* proved to be distributed in all faecal source groups with remarkably balanced average concentrations (log 7.0–8.4 CFU per g). Except for single faecal samples from the cattle source group, prevalence rates for ENTC source groups were generally > 90% with average concentrations of log 5.3–7.7 CFU per g. In order to test the faecal indication capacity in the environment, faecal prevalence data were comparatively analysed with results from the concurrently performed multi-parametric microbial source tracking effort on karst spring water quality from the investigated alpine mountainous catchment (Reischer et al. 2008; *Environ. Microbiol.* 10:2598–2608).

Conclusion—*E. coli* and enterococci are reliable faecal indicators for alpine mountainous water resources monitoring, although *E. coli* is the more sensitive one. *C. perfringens* did not prove an indicator of general faecal pollution but is suggested a conservative microbial source tracking marker for anthropogenic faecal influence.

Significance and Impact of Study—Applicability of SFIB is currently hotly debated. This is the first study providing comprehensive information on the applicability of SFIB at alpine mountainous habitats.

*corresponding author: Institute for Chemical Engineering, Research Area Applied Biochemistry and Gene Technology, Research Group Environmental Microbiology and Molecular Ecology, Vienna University of Technology, Getreidemarkt, 1060 Vienna, Austria
A.Farnleitner@aon.at.

Keywords

Escherichia coli; *Clostridium perfringens*; enterococci; general faecal pollution indicators; microbial source tracking marker; water quality monitoring

Introduction

As much as 20% to 25% of the global population depends largely or entirely on karst water with a significant part originating from mountainous area (Ford and Williams, 2007). The Austrian water supply is covered by approximately 50% from such type of alpine mountainous karst water resource. Spring water issuing from aquifer areas with prevailing matrix-flow conditions reveals excellent raw water quality (Wilhartitz et al., 2009). However, during unfavourable conditions, e.g. caused by storm-flow runoff through large, temporarily activated karstic conduits (Ford and Williams, 2007), microbial water quality at the spring outlet can decrease rapidly (Stadler et al., 2008). With respect to the given hydro-geological situation efficient diagnostic tools for the detection and analysis of faecal pollution are needed to guide microbial system assessment, enable internal microbial water quality monitoring, or allow regulatory surveillance activities (WHO, 2004, Farnleitner et al., 2008).

Selective cultivation of standard faecal indicator bacteria (SFIB) is still the methodological and regulatory basis for faecal pollution analysis of water resources (WHO, 2004). Nonetheless, application of SFIB needs basic knowledge on their ecology in context to the primary (i.e. intestinal systems, faecal materials) and the secondary habitats (i.e. soil, sediment and water, cf. discussion for details). The use of SFIB for microbiological water quality monitoring has increasingly been debated during the last decade (Ishii and Sadowsky, 2008, Boehm et al., 2009). Unfortunately, the availability of comprehensive studies is very limited in order to judge the indication performance of SFIB on sound scientific criteria. Furthermore, performance characteristics have to be evaluated for well specified aquatic habitats and defined monitoring applications.

In relation to the primary intestinal habitat two fundamental requirements of SFIB are, i) presence in significant numbers in faeces in order to achieve reasonable detection limits, and ii) a balanced distribution of concentrations between animal and human faecal pollution sources to ensure un-discriminative detectability of total faecal pollution levels. Since the implementation of the faecal indicator concept more than 100 years ago, *Escherichia coli* - as the most reliable representative of the coliform group - intestinal enterococci (ENTC), and *Clostridium perfringens* have most frequently been used as SFIB (Slanetz and Bartley, 1957; Bonde, 1966; Geldreich et al., 1968; Geldreich and Kenner, 1969; Bisson and Cabelli, 1980; Leclerc et al., 2001). Much knowledge has been gained on the prevalence and abundance of *E. coli*, ENTC, and *C. perfringens* in human, sewage and animal faecal sources (e.g. Maki and Picard, 1965; Smith, 1965; Finegold et al., 1974; Yamagishi et al., 1976; Hausschild et al., 1979; Crane et al., 1983; Ferguson et al., 2009). However, a great deal of diverse cultivation techniques have been used for quantifying SFIB in faecal sources making comparisons with standard methods used for water quality monitoring difficult (e.g. ISO based protocols). Furthermore, investigations were mainly focused on human and livestock faecal sources neglecting abundant wildlife populations in rural areas irrespective of their potential relevance as zoonotic reservoirs (for review see Cotruvo et al., 2004). These gaps of knowledge appear very surprising given the importance of SFIB for quality monitoring and water resources management on a worldwide scale.

The aim of this research was i) to establish basic knowledge on the multiple prevalence and abundance of *E. coli*, *ENTC* and *C. perfringens* in human, livestock and wildlife faecal pollution sources in fresh to medium aged faecal material (< 3 weeks) as occurring in a large and representative alpine mountainous area in the Northern Calcareous Alps of Austria and, ii) to evaluate its faecal indication capacity in spring water of the corresponding catchment. For this purpose, a comprehensive set of faecal samples from dominating wildlife populations (chamois, red deer, roe deer), abundant livestock (cattle kept on summer pastures), human faecal sources (individual, sewage and cesspit samples from alpine huts and tourism activities) and less abundant populations (herbivorous and carnivorous wildlife) were investigated. Occurrence of SFIB was also concurrently determined in spring water from the respective alpine mountainous catchment together with a multi-parametric set of alternative microbial and source tracking parameters. Water quality analysis was paralleled by on-line hydrological spring water characterisation covering discharge at base and event flow situations. To our knowledge, this is the first comprehensive study on the quantitative occurrence of several SFIB including also wildlife pollution sources.

Materials and methods

Study area and file collection of specimens

The investigated alpine mountainous area is located in the Northern Calcareous Alps in Austria reaching altitudes up to approx. 2300 m with a total investigated area of approx. 100 km². Vegetation comprises summer pastures, natural calcareous alpine swards with open krummholz and forests. The limestone karst aquifer spring 2 (LKAS2) catchment is part of the investigated area (catchment area approx. 70 km²) with a mean altitude of 1380 m above sea level and with LKAS2 accessible in the respective valley at 650 m. Since this area has been the focus of several detailed studies (geology, hydrogeology, vegetation, spring water quality) further background information can be found in the respective publications (references found in Farnleitner et al., 2005; Reischer et al., 2008). Two routes (A, B) were selected in order to cover all different alpine mountainous habitats available in the considered catchment. 14 and 15 field sampling tours were realised along sampling route A (total length 32 km) and B (total length 35 km), respectively, from 2004 to 2006. Sampling design was based on single faecal samples (SFS) and pooled faecal samples (PFS); PFS included 10 single samples of equal volume from closely located areas within a corresponding habitat type. About 5-8 g of faecal material was aseptically collected from faecal droplets or cow pats for SFS or PFS in sterile faecal sampling plastic containers (Greiner Bio-One, Kremsmuenster, Austria). A representative set of cesspit and sewage samples were also collected at each field trip from 10 different mountain huts scattered over the investigation area. In total, 162 SFS and 171 PFS from 1760 individual faecal specimens and 65 cesspit/sewage samples were collected. Additionally, 46 faecal samples from human individuals living in the eastern Austrian area (representing potential visiting tourists) were recovered. As the investigation was focusing on the occurrence of SFIB in fresh to medium aged faeces, still deformable animal faecal droplets, not yet completely dried, were collected. The age of collected faecal droplets was limited to a maximum of 3 weeks as the sampling frequency of field trips were performed every 2-3 weeks during summer time allowing to distinguish fresh/medium aged faeces from older deposits (> 3 weeks). Collected faecal droplets were transported under dark conditions and ambient temperatures in insulated sampling boxes to the laboratory, stored at 4°C and investigated within 72 h.

Faecal sample processing, SFIB enumeration and characterisation

Approximately 1 g of homogenised wet faecal sample, was weighed, suspended in 100 ml buffered peptone saline and diluted in ten-fold dilutions from 10⁻² to 10⁻⁶, resulting in a detection limit ranging from 17 to 110 CFU per g of faeces (depending on the exact mass).

Determination of *E. coli* was performed by surface plating technique using Trypton-Bile-X-Glucuronide (TBX) medium (Oxoid, UK) and incubation at $44\pm 0.5^{\circ}\text{C}$ for 44 ± 4 h. Representative colonies ($n = 98$) were further isolated for genotypic characterisation. Genotypic identification was done by a species-specific PCR using the *uidA* gene region (*uidA*_PCR) as previously described (Bej et al., 1990). ENTIC was performed by membrane filtration and subsequent dry heat incubation at $44\pm 0.5^{\circ}\text{C}$ for 44 ± 4 h using Slanetz-Bartley medium (SB, Oxoid, UK). Further characterisation was done by transferring membranes to Bile-Esculin agar (BEA, Oxoid, UK) for 2 h. Presumptive *C. perfringens* were enumerated by surface plating technique and incubation at $44\pm 1^{\circ}\text{C}$ for 21 ± 3 h in anaerobic jars using Tryptose-Sulphite-Cycloserine medium (TSC, Scharlau, Spain). Species-specific PCR identification for representative isolates was based on the phospholipase C gene (*pC*_PCR) as described previously (Fach and Popoff, 1997).

Hydrological characterisation, water quality monitoring and statistics

A detailed description of the, i) selected sampling design including a three years basic monitoring program (2004-2006), ii) analysis of two late summer events (2005 and 2006), and iii) the hydrological and physicochemical LKAS2 characteristics was published in detail already by Reischer et al. (2008). In this study, which was conducted concurrently with the herein described work, a multi-parametric set of indicators was successfully applied at the LKAS2 for faecal source tracking. Basic data on *E. coli*, ENTIC and *C. perfringens* are referenced from the work of Reischer et al. (2008) making a comparison on the occurrence of SFIB in faecal sources versus spring water possible. All statistical procedures were performed with SPSS version 17.0.

Results

Methodical performance characteristics

Genotypic identification was only performed for selected *E. coli* and *C. perfringens* colonies as presumptive ENTIC were confirmed by the additional Bile-Esculin test (c.f. methods). No inhibition effects were discernable for the *uidA*_PCR and *pC*_PCR under the applied test conditions. A total amount of 96 out of 98 *E. coli* isolates (i.e. 98%), randomly chosen from TBX plates covering all faecal sources, were *uidA*_PCR positive. Because of the excellent specificity of the TBX method, recovered *E. coli* concentrations from faecal and sewage were handled as confirmed results for further analysis and data interpretation. In the case of *C. perfringens*, 41 out of 47 presumptive isolates (i.e. 87%) from human, cattle and carnivorous faecal sources could be identified as *C. perfringens* by *pC*_PCR. However, *pC*_PCR confirmed only 14 out of 34 presumptive isolates (i.e. 40%) from wildlife sources (chamois, red deer, roe deer) as *C. perfringens*. Presumptive *C. perfringens* concentrations from wildlife were divided by a factor of 2.5 (i.e. 40 positives among 100 tested) in order to convert to the anticipated level of confirmed results and to compensate for the false positives from TSC agar.

Median bulk densities of randomly selected faecal specimens did not differ between samples from human individuals (median 1.02 g per cm^3 , range 0.75 – 1.15 g per cm^3 , $n = 34$), red deer (median 1.04 g per cm^3 , range 0.82 – 1.17 g per cm^3 , $n = 45$), chamois (median 1.04 g per cm^3 , range 0.78 – 1.14 g per cm^3 , $n = 72$) and cattle (median 1.03 g per cm^3 , range 0.90 – 1.36 g per cm^3 , $n = 70$). No correlations between concentrations of SFIB versus bulk density of respective faecal samples were discernable, irrespective whether values below the detection thresholds were included or not (range of Spearman rho = |0.00 – 0.20|, $n = 34 - 187$).

Abundance of SFIB in faecal excrements

Except for two SFS (i.e. 0.5%), *E. coli* occurred ubiquitously in all investigated wildlife, livestock, and human individual faecal samples. In cesspits and sewage *E. coli* revealed somewhat lower prevalence rates of 88% and 93%, explainable by partial treatment with chlorine (Table 1).

For chamois, red deer and cattle populations as well as human individuals average *E. coli* concentrations in positive samples showed remarkably low variations ranging from $\log_{10}$ 7.0 CFU to $\log_{10}$ 8.0 CFU per g faeces, irrespective of whether SFS or PFS were considered (Table 1). In contrast, a pronounced variation of the results from single determinations was evident. In this respect, ratios of the 95% to 5% percentiles varied up to $\log_{10}$ 4.1 for PFS and up to $\log_{10}$ 4.6 for SFS.

A high ENTIC prevalence rate of 87% - 92% (SFS) and 95% - 100% (PFS) was discernable for the investigated wildlife populations (chamois, red-deer) as well as the human individual samples (96%, Table 2). ENTIC were found in 92% and 100% of the cesspit and sewage samples. In cattle populations ENTIC showed a decreased prevalence of 69% (SFS) and 91% (PFS), respectively. All carnivores and other herbivores shed ENTIC.

For chamois, red deer and cattle populations as well as human individuals average ENTIC concentrations in positive samples ranged from $\log_{10}$ 5.3 CFU to $\log_{10}$ 7.3 CFU per g faeces, with lowest average concentrations for cattle populations and highest for human chamois PFS (Table 2). For these sources the ratios of the 95% to 5% percentiles varied up to $\log_{10}$ 4.3 (PFS) and $\log_{10}$ 5.8 (SFS).

Prevalence of *C. perfringens* in faecal droplets of chamois and red deer populations was low to very low (4 - 28%). In contrast, *C. perfringens* revealed a markedly increased prevalence in faecal pats of ruminant livestock populations (63 - 88%) and for human individual (83%), cesspit (96%) and sewage (98%) pollution sources (Table 3).

In agreement with prevalence rates, average concentrations of *C. perfringens* for positive samples showed also low average values for chamois and red deer populations ranging from $\log_{10}$ 1.7 - 2.8 CFU (SFS) and $\log_{10}$ 2.1 - 3.4 CFU (PFS) per g faeces, whereas significantly increased average values (Mann Whitney U, $p < 0.05$) for cattle and human faecal sources ($\log_{10}$ 4.7 - 5.8 CFU per g faeces) were obvious. For these sources, the ratios of the 95% to 5% percentiles varied up to $\log_{10}$ 2.9 (PFS) and $\log_{10}$ 4.1 (SFS). Interestingly, carnivorous faecal sources resulted in highest average *C. perfringens* concentrations of $\log_{10}$ 7.0 CFU per g faeces (Table 3).

Low to very low correlations were found between the concentrations of *E. coli*, ENTIC and *C. perfringens* in faecal excrements and cesspits (Fig 1a-c). Slightly higher correlations were only observed for *E. coli* vs. ENTIC concentrations and ENTIC vs. *C. perfringens* concentrations from sewage and cesspit samples, respectively (Fig. 1a, 1c).

The median *E. coli* to ENTIC ratios for faecal excreta including wildlife, livestock and individual human sources ranged from $\log$ 1.1 to $\log$ 2.3. Sewage and cesspit sources showed reduced median ratios of $\log$ -0.2 to $\log$ -0.3 (Table 4a). As a consequence of the low correlations between the different groups of SFIB, variation of single ratios was quite pronounced ranging over 6 orders of magnitude. Reasonable *E. coli* to *C. perfringens* ratios could only be calculated for human and cattle sources because of *C. perfringens* being absent in large parts of wildlife sources (Table 4b).

Occurrence of SFIB in spring water

Results on the detection rate and on the abundance of SFIB as recovered from the investigation of the karstic spring LKAS2 remarkably reflected the general prevalence and abundance of SFIB as found in the faecal sources investigated in the catchment area (Table 5). For the seasonal spring monitoring approach, *E. coli* and ENTC could be detected in 66% of the investigated water samples with a trend towards higher *E. coli* concentrations. *C. perfringens* could only be detected in 24% of the investigated samples. Results from flood event investigations in the spring further supported this picture (Tab 5). *E. coli* and ENTC detection rates approached 100%, whereas *C. perfringens* was detected only in 20% of the investigated samples. The 95% percentiles amounted to 1490 CFU per litre, 391 CFU per litre, and 4 CFU per litre for *E. coli*, ENTC and *C. perfringens*, respectively (see discussion for details on the evaluation of the indication capacity at the spring water).

Discussion

Occurrence of SFIB in their primary intestinal habitat

To our knowledge, this is the first available report on a comparative quantitative investigation of *E. coli* and alternative SFIB in faecal sources including herbivorous wildlife populations. Furthermore standard- or frequently applied cultivation methods for bacterial water quality investigations were used in order to ensure comparability of results. *E. coli* was found to occur at remarkably balanced average concentrations regardless of whether human, livestock, or wildlife sources were investigated. The pronounced variability of *E. coli* concentrations between SFS has been reported for decades (e.g. Geldreich, 1978; Donnison et al., 2008). This variability can simply be explained by the natural microbial populations dynamics as observed in the intestinal system (Williams and Crabb, 1961). Since faecal droppings were up to three weeks old, die-off and re-growth of SFIB in the faecal material might also have shifted populations (Sinton et al., 2007). The observed variability of *E. coli* concentrations between SFS is not considered a practical limitation for water quality monitoring, since rigorous mixing processes during mobilisation and transfer in the catchment are expected. This effect was already anticipated in the application of the PFS approach leading to a significant decrease in variability as compared to SFS. Furthermore, a remarkably decreased variability in *E. coli* to ENTC ratios was also observed for spring water samples when compared to ratios in faecal samples again supporting the postulated mechanism (Fig. 2).

It is currently not clear why approx. 1/3 of the investigated cow pats (SFS) did not carry ENTC. A detailed investigation on ENTC isolates from corresponding cattle faecal droplets at the LKAS2 catchment area during 2004 revealed that *S. bovis*, *E. faecium*, *E. hirae* and *E. durans* were the dominating isolates (Burtscher et al., 2006). Hence, a rapid decrease of ENTC in faecal droplets due to fast dying *S. bovis* populations (Geldreich and Kenner, 1969; Feachem, 1975) alone cannot explain the lower prevalence rate. Average ENTC concentrations were reasonably high and quite balanced for the rest of the investigated source groups (human, herbivorous wildlife, carnivorous wildlife). ENTC thus fulfils the basic criteria as a total faecal pollution indicator, although less excellently than *E. coli*.

Quite surprisingly, *C. perfringens* could only be detected in relevant concentrations in human, livestock and carnivorous faecal sources. For these source groups concentrations were in the range of previously reported values for *C. perfringens* (Bonde, 1963; Yamagishi et al., 1976; Cabelli, 1977) but below concentrations observed for *E. coli* and ENTC. Interestingly, highest concentrations of *C. perfringens* were found for carnivorous faecal sources. Analysis of the genome suggests *C. perfringens* to be an anaerobic fastidious pathogenic flesh eating organism (Shimizu et al., 2002). Occurrence of *C. perfringens* in

faeces of carnivorous animals and also humans (feeding on protein rich meat, fish or cheese) in higher numbers may thus be a logic consequence of its ecology. The very low prevalence and almost neglectable abundance in herbivorous wildlife severely limits *C. perfringens* as an indicator of total faecal pollution. *C. perfringens* has been suggested by several researchers as an indicator of stream or surface water quality (Bonde, 1963; Bisson and Cabelli, 1980; Fujioka and Shizumura, 1985; Sartory, 1988; Sorensen et al., 1989). As those studies focused mainly on the detection of sewage influence our results do not necessarily contradict this previous work. Because *C. perfringens* was found in humans as well as in herbivorous livestock but not in herbivorous wildlife, we suggest *C. perfringens* as a conservative microbial source tracking marker for faecal pollution from anthropogenic sources. Preliminary data on the occurrence of *C. perfringens* in faeces of native roe deer populations versus populations held in fenced areas and fed on silage in the LKAS2 area further supported this hypothesis. Only faecal material from populations held in fenced areas contained significant *C. perfringens* concentrations (data not shown). It appears unlikely that the suggested indication capacity for anthropogenic faecal pollution might be impaired by faecal droplets from carnivorous wildlife sources because of the comparatively low abundance of such animals. However, further work needs to be done in other environments to corroborate this hypothesis.

Faecal indication capacity of SFIB at the alpine mountainous spring habitat

Observed loads of SFIB at karst springs are expected to be a result of faecal material input, microbial transfer efficiency and die-off, assuming that extra-intestinal reservoirs of SFIB do not exist or do not actively contribute to environmental fluxes of SFIB (e.g. soils with interfering autochthonous populations). Under such a situation, prevalence rates and concentration ratios in spring water should correspond to patterns of SFIB as found in the contributing faecal sources. Indeed, results on the detection rate and on the abundance of SFIB in the LKAS2 spring remarkably reflected the general prevalence and abundance of SFIB as found in the faecal sources. Furthermore, the observed median *E. coli* to ENTC ratios from LKAS2 (log 0.3 and 0.6 for monitoring and flood event data, respectively) were also in correspondence to the median *E. coli* to ENTC ratios from animal faecal sources (log 1.1 to 2.3). Due to a higher persistence of ENTC in the environment a decreasing *E. coli* to ENTC ratio with time of environmental exposure is expected (Feachem, 1975). In contrast, *E. coli* to ENTC ratios from LKAS2 were remarkably higher than ratios in waste water and cesspits (log -0.2 to -0.3). These results are in perfect agreement with the concurrently performed source tracking study identifying ruminants as the dominating faecal pollution sources in the LKAS2 catchment (Reischer et al., 2008). In this respect it should be highlighted, that *E. coli* to ENTC ratios do not contain any information on the origin of faecal contamination as their ratios in animal and human sources were not different at all (cf. Table 4a). However, supporting information for improved data interpretation - as done in our case - can be derived from such differential persistence kinetics of SFIB (Feachem, R., 1975).

Most importantly, the good faecal indication capability of *E. coli* and enterococci in the LKAS2 was strongly supported by a very tight coupling to the observed ruminant-specific faecal pollution component being clearly distinguishable from non-intestinal parameters (Reischer et al., 2008). Multi-parametric analysis (n = 12 microbial and physiochemical parameter) revealed the highest correlations between *E. coli* and ENTC concentrations for the monitoring setup (r = 0.84). For the flood events, the highest correlations were observed for the “inter-related” faecal parameter group including *E. coli*, ENTC and ruminant-specific *Bacteroidetes* marker BacR (r = 0.81 - 0.88). The tightly correlated faecal indicator group revealed lower correlations with other microbial and physicochemical indicators such as turbidity (r = 0.24 - 0.60) or aerobic spore formers (r = 0.17 - 0.67) - a potential indicator for

soil influence - strongly pointing to the fact that the origin of *E. coli* and ENTIC was from faecal pollution sources and not from soil habitats (cf. Reischer et al., 2008 for details). No signs of any indication bias for *E.coli* and enterococci could thus be found by the multi-parametric catchment and spring aquifer study.

The detection frequency of *C. perfringens* in the LKAS2 was very low and in line with its occurrence in the investigated faecal sources. Only during periods of strongest faecal pollution, such as during the peak phases of floods, a good correlation to other SFIB was discernible (data not shown).

Recent results from other geographical regions indicated that *E. coli* populations may become “naturalised” by sustaining autochthonous populations not only in tropical but also in some temperate environments (Ishii and Sadowsky, 2008). Almost no information exists for ENTIC on this subject but the situation is likely to be similar (Fujioka et al., 1999). Due to the fastidious nature of *C. perfringens* (Shimizu et al., 2002), significant growth in the secondary environment seems not very likely (Konishi et al., 1981; Zaiss et al., 1983). The primary habitats are diseased or dead bodies of animals (i.e. flesh as a basic growth substrate), large amounts of *C. perfringens* cells and spores are probably produced in carcasses especially during periods of higher temperatures. However, *E. coli* could recently be detected in the top soil layer of alpine pasture grassland up to concentration levels of 10^4 MPN per gram soil (Texier et al., 2008). In contrast, *E. coli* could not be isolated in soil from areas not frequented by cattle (Texier et al., 2008). Own investigations in the LKAS2 catchment support these results showing faecal coliforms up to 2.6×10^3 CFU per g of soil from pasture areas as in contrast to non-pasture areas revealing not detectable- or very low concentrations (i.e. < 2 CFU per g of soil; Burtscher, 2002). Soil layers below areas with high faecal exposure, such as soil below cattle pastures, are thus likely to receive, store, or even support the existence of naturalised populations in low to moderate concentrations as compared to their primary, intestinal habitat. The occurrence of non-faecal derived *E. coli* or ENTIC, especially at very low concentrations, can thus not be excluded in spring water sources. A relevant faecal indication bias is only expected when strong erosive run-off events from catchments with dominating pasturing activities would happen. Enteric pathogenic organisms - likely to be stored or sustained under intensively pastured areas - may be mobilised as well under such situations (Fremaux et al., 2010). However, in the course of the performed multi-parametric source tracking investigation no signs of any significant indication bias from *E.coli* or enterococci from non-intestinal compartments could be found. In contrast, these investigations proved the excellent applicability of these SFIB for water quality monitoring at alpine mountainous water resources.

In conclusion, this study impressively demonstrates that *E. coli* and enterococci occur ubiquitously in faeces from human and animal sources irrespectively whether livestock or wildlife is regarded. Although investigations were limited to wildlife as occurring in mountainous areas the result of the study is likely being applicable also to other herbivorous wildlife populations with comparable diet and physiology. *C. perfringens* did not proof to indicate general faecal pollution but it is suggested as a conservative source tracking marker for anthropogenic faecal pollution. *C. perfringens* occurred in all faecal sources associated with human activities. Further studies in other geographical areas are needed in order to verify this hypothesis. In any case, higher volumes of water have to be sampled in order to compensate for the lower abundance of *C. perfringens* in faeces as compared to *E. coli* or ENTIC (cf. Table 4a/4b). Combined analysis of faecal prevalence data (i.e. primary habitat) with results from the concurrently performed multi-parametric microbial source tracking effort on the respective karst spring water quality (i.e. secondary habitat) revealed a very good capacity for *E. coli* and ENTIC to indicate microbial faecal pollution. *E.coli* and enterococci thus represent very reliable and useful general faecal indicators for water

resource monitoring at alpine mountainous areas. However, these results are probably very habitat specific and must not be transferred to other locations, such as to lower altitude regions, without verification. It is the opinion of the authors that the current debate on the applicability of SFIB can only be solved if monitoring applications are precisely defined and discussed in a distinguished way.

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Figure 1a: *E. coli* versus ENTC Figure 1b: *E. coli* versus *C. perfringens* Figure 1c: ENT versus *C. perfringens* **Fig. 1.**

Correlation analysis of log *E. coli* versus log enterococci (ENTC) concentrations (a), log *E. coli* versus log *Clostridium perfringens* concentrations (b), and log enterococci versus log *Clostridium perfringens* concentrations (c) as found in wildlife, livestock and human pollution sources in the investigated catchment. Spearman correlation coefficients (ρ) were calculated. The diagonal shows the 1:1 ratio of the respective couples of indicators.

Fig. 2. Correlation plot of log *E. coli* versus log enterococci (ENTC) concentrations as found in animal and human faecal pollution sources and in the respective spring water of the LKAS2. The diagonal shows the 1:1 ratio. Indicator concentrations from spring water are given in log CFU per 10 m³ of spring water.

Table 1

Prevalence and abundance of *Escherichia coli* in faeces and sewage

Source		Prevalence		Abundance			
		n	% positiv	log ₁₀ median [CFU/g] ⁵	log ₁₀ mean ¹ [CFU/g]	log ₁₀ percentile [CFU/g]	
Chamois	SFS	52	98	6.2	7.2	3.6	8.1
	PFS	62	100	6.8	7.8	4.5	8.5
Red Deer	SFS	36	100	6.1	7.0	3.4	7.8
	PFS	36	100	6.9	7.5	4.1	8.2
Cattle	SFS	48	100	6.4	7.0	4.1	7.8
	PFS	60	100	6.6	7.0	5.1	7.6
Human	individual	46	100	6.4	8.0	4.3	8.9
	cesspit	25	88	7.1	7.8	4.1	8.8
	sewage	40	93	4.2	5.7	2.5	6.7
Roe Deer	SFS	12	100	5.3	7.1	2.7	8.1
Camivore ²	SFS	12	100	8.3	8.4	6.5	8.9
Sheep/Goat	SFS ³	2	100	8.4	8.4	5.8	8.7
	PFS ⁴	3	100	7.1	7.6	6.2	8.1

¹ arithmetic

² samples were taken from 12 carnivores including martens, foxes, 1 cat and 1 dog

³ samples were taken from 2 sheeps

⁴ samples were taken from 2 sheeps and 1 goat

⁵ CFU/g: colony forming units per g

Table 2

Prevalence and abundance of enterococci (ENTC) in faeces and sewage

Source	Prevalence		Abundance			
	n	% positiv	log ₁₀ median [CFU/g] ⁵	log ₁₀ mean ¹ [CFU/g]	log ₁₀ percentile [CFU/g]	5% 95%
Chamois	SFS	52	87	4.5	6.8	2.0 7.8
	PFS	58	95	6.3	7.3	4.2 8.3
Red Deer	SFS	36	92	4.9	6.0	2.5 7.1
	PFS	35	100	5.3	6.7	3.4 7.8
Cattle	SFS	48	69	4.1	5.5	2.2 6.3
	PFS	55	91	4.2	5.3	2.0 6.3
Human	individual	46	96	4.9	6.4	2.5 7.4
	cesspit	25	92	6.9	7.7	4.3 8.7
	sewage	40	100	4.6	5.3	3.2 5.8
Roe Deer	SFS	12	92	4.0	6.4	2.6 7.2
Camivore ²	SFS	12	92	7.1	7.7	5.3 8.6
Sheep/Goat	SFS ³	2	100	7.3	7.3	7.3 7.3
	PFS ⁴	3	100	7.5	7.3	5.9 7.5

¹ arithmetic² samples were taken from 12 carnivores including martens, foxes, 1 cat and 1 dog³ samples were taken from 2 sheeps⁴ samples were taken from 2 sheeps and 1 goat⁵ CFU/g: colony forming units per g

Table 3

Prevalence and abundance of *C. perfringens* in faeces and sewage

Source	Prevalence		Abundance			
	n	% positiv	log ₁₀ median [CFU/g] ⁵	log ₁₀ mean ¹ [CFU/g]	log ₁₀ percentile [CFU/g]	5% 95%
Chamois	SFS	52	4	1.7	1.7	1.7
	PFS	62	15	1.8	2.1	2.6
Red Deer	SFS	36	28	2.2	2.8	3.3
	PFS	36	25	2.3	3.4	4.2
Cattle	SFS	48	63	3.8	4.9	5.8
	PFS	60	88	4.1	4.7	5.4
Human	individual	46	83	4.0	5.8	6.3
	cesspit	25	96	4.7	5.5	6.2
	sewage	40	98	3.8	4.2	4.9
Roe Deer	SFS	12	25	3.1	5.2	5.7
Camivore ²	SFS	12	83	6.9	7.0	7.3
Sheep/Goat	SFS ³	2	100	2.6	2.6	2.7
	PFS ⁴	3	67	2.9	2.9	2.9

¹ arithmetic² samples were taken from 12 carnivores including martens, foxes, 1 cat and 1 dog³ samples were taken from 2 sheeps⁴ samples were taken from 2 sheeps and 1 goat⁵ CFU/g: colony forming units per g

Table 4a*E. coli* to enterococci ratios

Source	n	log ₁₀ median	log ₁₀ percentile	
			5%	95%
Chamois	98	1.1	-1.5	3.8
Red Deer	68	1.5	-1.6	4.3
Cattle	83	2.3	-0.1	4.2
Human	44	1.7	-1.3	3.4
cesspit	22	-0.3	-2.3	2.3
sewage	37	-0.2	-1.9	2.3

n = replicate number

Table 4b*E. coli* to *C. perfringens* ratios

Source	n	log ₁₀ median	log ₁₀ percentile 5%	log ₁₀ percentile 95%
Chamois		not possible to calculate		
Red Deer		not possible to calculate		
Cattle	83	2.3	0.7	5.4
Human	38	2.0	-4.6	5.3
	22	2.1	-1.1	4.2
cesspit				
sewage	36	1.0	-1.6	3.0

n = replicate number

Table 5
Detectable *E. coli*, enterococci (ENTC) and *C. perfringens* concentrations in spring water (LKAS2) during 2004-2006¹

E. coli																	
Prevalence		Abundance		Prevalence		Abundance		Prevalence		Abundance		Prevalence		Abundance			
%		[CFU/l]		%		[CFU/l]		%		[CFU/l]		%		[CFU/l]			
n	pos.	median	5%	95%	n	pos.	median	5%	95%	n	pos.	median	5%	95%			
Monitoring	53	66	3	0	420	53	66	2	0	141	51	24	0	0	3		
Event	70	100	177	32	1490	70	100	50	6	391	70	20	0	0	4		

Abbreviations: CFU/l: colony forming units per litre of spring water; range of 5% to 95% percentile; n = replicate number; pos.: number of positive detections.

¹Raw data set originating from Reischer et al. 2008 but analysed and presented in a different way to support the herein given work.