



Poikilothermic Animals as a Previously Unrecognized Source of Fecal Indicator Bacteria in a Backwater Ecosystem of a Large River

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ABSTRACT Quantitative information regarding the presence of *Escherichia coli*, intestinal enterococci, and *Clostridium perfringens* in poikilotherms is notably scarce. Therefore, this study was designed to allow a systematic comparison of the occurrence of these standard fecal indicator bacteria (SFIB) in the excreta of wild homeothermic (ruminants, boars, carnivores, and birds) and poikilothermic (earthworms, gastropods, frogs, and fish) animals inhabiting an alluvial backwater area in eastern Austria. With the exception of earthworms, the average concentrations of *E. coli* and enterococci in the excreta of poikilotherms were equal to or only slightly lower than those observed in homeothermic excreta and were 1 to 4 orders of magnitude higher than the levels observed in the ambient soils and sediments. Enterococci reached extraordinarily high concentrations in gastropods. Additional estimates of the daily excreted SFIB (*E. coli* and enterococcus) loads (DESL) further supported the importance of poikilotherms as potential pollution sources. The newly established DESL metric also allowed comparison to the standing stock of SFIB in the sediment and soil of the investigated area. In agreement with its biological characteristics, the highest concentrations of *C. perfringens* were observed in carnivores. In conclusion, the long-standing hypothesis that only humans and homeothermic animals are primary sources of SFIB is challenged by the results of this study. It may be necessary to extend the fecal indicator concept by additionally considering poikilotherms as potential important primary habitats of SFIB. Further studies in other geographical areas are needed to evaluate the general significance of our results. We hypothesize that the importance of poikilotherms as sources of SFIB is strongly correlated with the ambient temperature and would therefore be of increased significance in subtropical and tropical habitats and water resources.

IMPORTANCE The current fecal indicator concept is based on the assumption that the standard fecal indicator bacteria (SFIB) *Escherichia coli*, intestinal enterococci, and

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Clostridium perfringens multiply significantly only in the guts of humans and other homeothermic animals and can therefore indicate fecal pollution and the potential presence of pathogens from those groups. The findings of the present study showed that SFIB can also occur in high concentrations in poikilothermic animals (i.e., animals with body temperatures that vary with the ambient environmental temperature, such as fish, frogs, and snails) in an alluvial backwater area in a temperate region, indicating that a reconsideration of this long-standing indicator paradigm is needed. This study suggests that poikilotherms must be considered to be potential primary sources of SFIB in future studies.

KEYWORDS *Clostridium perfringens*, *Escherichia coli*, alluvial, earthworm, enterococci, excreta, fishes, frog, mollusk, poikilothermic

Microbiological water quality monitoring is strongly dependent on investigations of standard fecal indicator bacteria (SFIB). *Escherichia coli* and intestinal enterococci have been considered the most important SFIB for more than 100 years (1, 2), since the introduction of the fecal indicator concept (3). Furthermore, *Clostridium perfringens* has also been used as a fecal indicator since the beginning of water quality testing (1, 4). SFIB are considered sensitive indicators of the extent of fecal contamination in water resources, and the monitoring of SFIB is an essential tool for water safety management. SFIB can easily be detected by standardized cultivation-based methods, e.g., ISO 16649-2 (5) for *E. coli*, ISO 7899-2 (6) for intestinal enterococci, and ISO 14189 (7) for *C. perfringens*. Their occurrence at high concentrations in the excreta of humans and other homeothermic animals and their inability to replicate in the nonintestinal environment are the most basic requirements for microbial fecal indicators. However, the usefulness of SFIB as fecal indicators has been increasingly questioned following the discovery of potential long-term persistence and regrowth of SFIB in the environment (8, 9) and of so-called “naturalized populations” (10–12), which are thought to persist and proliferate in nonintestinal environments. The potential of poikilothermic vertebrates (i.e., animals whose body temperature varies with the ambient environmental temperature) to serve as primary habitats of SFIB may further interfere with the traditional fecal indicator concept. However, quantitative investigations on the occurrence of SFIB in poikilothermic vertebrates are scarce. Furthermore, there is little available knowledge regarding the occurrence of SFIB in invertebrates, such as snails or slugs. For a better understanding of the importance of alternative sources of SFIB in the environment, comparative investigations are needed, including of all suspected nonbiotic and biotic compartments.

Existing studies on the quantitative occurrence of SFIB in alternative animal sources give a very limited picture that is based on fragmentary information from various habitats with differing environmental conditions. Until the current study, *E. coli* and enterococci had not been detected in earthworm casts (13), although other studies observed a significant positive correlation between earthworm abundance and *E. coli* occurrence in soil (14). In another study, *Enterococcus casseliflavus* was identified as a dominant species in the feces of the garden snail (*Cornu aspersum*), at concentrations of up to $9.0 \log_{10}$ CFU · g⁻¹ feces (15). Investigations of edible snails (*C. aspersum* and *Helix lucorum*) revealed that *E. coli* and enterococcus counts varied from 4.0 to 5.5 and 5.0 to 6.0 \log_{10} CFU · g⁻¹ feces, respectively (16). In another study, two pooled samples from slugs (*Limax* spp.) had *E. coli* concentrations of 4.9 and 6.0 \log_{10} CFU · g⁻¹. The *E. coli* concentration in the organs and tissues of fish increased with an increase in the bacterial load of the water body, with intestinal tract concentrations of *E. coli* at a most probable number (MPN) of 2.0 to 5.0 \log_{10} per g⁻¹ in investigated species (17). An investigation of the occurrence of *E. coli* in grass carp (*Ctenopharyngodon idella*), silver carp (*Hypophthalmichthys molitrix*), and rohu (*Labeo rohita*) from aquaculture facilities in which animal manure was directly discharged into fish ponds revealed mean intestinal tract *E. coli* concentrations of $5.0 \pm 0.5 \log_{10}$ CFU · g⁻¹ feces, compared to $3.0 \pm 0.7 \log_{10}$ CFU · g⁻¹ feces from control ponds without manure (18). In Japanese tree frogs (*Hyla japonica*) maintained in a laboratory, the observed concentrations

TABLE 1 Occurrence and abundance of the standard fecal indicator bacterium *Escherichia coli* in diverse animal groups from an alluvial backwater (2010 to 2013)

Fecal source	n	Occurrence (%)	Abundance (\log_{10} CFU \cdot g ⁻¹ feces) ^a				
			Mean	Median	Percentile		
					5th	95th	Max
Poikilothermic invertebrates							
Earthworm	26	0	ND	ND	ND	ND	ND
Gastropod	26	77	4.2	4.2	3.0	5.5	6.8
Total	52	38	4.2	4.2	3.0	5.5	6.8
Poikilothermic vertebrates							
Frog	19	68	5.2	5.0	3.2	8.3	8.5
Fish	27	85	4.6	4.6	3.0	6.8	8.1
Total	46	78	4.8	4.7	3.0	8.1	8.5
Homeothermic vertebrates							
Bird	15	73	5.0	4.8	2.3	8.5	9.2
Ruminant	43	93	5.0	4.6	2.7	7.4	9.1
Wild boar	16	100	6.6	6.2	5.2	8.4	9.0
Carnivore	17	100	7.0	7.0	4.6	9.4	9.5
Total	91	91	5.7	5.9	2.7	8.9	9.5

^aAbundance data (i.e., median, mean, 5th and 95th percentiles, max) were calculated, excluding nondetectable data. All results are given in CFU \cdot g⁻¹ feces (wet weight). Detection limits for earthworms, 1.5 to 3.0 \log_{10} CFU \cdot g⁻¹; for snails, 1.9 to 3.0 \log_{10} CFU \cdot g⁻¹; for frogs, 1.8 to 3.0 \log_{10} CFU \cdot g⁻¹; for fish, 0.8 to 2.4 \log_{10} CFU \cdot g⁻¹; for birds, 1.8 to 2.2 \log_{10} CFU \cdot g⁻¹; for ruminants, 1.7 to 2.0 \log_{10} CFU \cdot g⁻¹; for boar, 1.7 to 2.0 \log_{10} CFU \cdot g⁻¹; and for carnivores, 1.9 to 2.0 \log_{10} CFU \cdot g⁻¹. Mean, arithmetic mean; max, maximum; ND, not detectable.

of *E. coli*, enterococci, and *Clostridium* spp. were 8.3 to 9.9 \log_{10} CFU \cdot g⁻¹, 6.9 ± 1.3 \log_{10} CFU \cdot g⁻¹, and 6.1 to 7.1 \log_{10} CFU \cdot g⁻¹ wet intestinal content, respectively (19). The concentration of *E. coli* in bullfrogs (*Rana catesbeiana*) maintained in a laboratory was 7.1 to 8.4 \log_{10} CFU \cdot g⁻¹ feces (20). Except for the above-mentioned studies on individual species, comparative studies on the quantitative occurrence of SFIB in poikilothermic and invertebrate animals within or across habitats were lacking until the current study.

The aim of this study was to assess the abundance of SFIB in the excreta of various wild animals living in a typical Central European riverine wetland located on the north side of the Danube River at the southeastern border of Vienna, Austria, to support quantitative cross-comparisons of potential sources of SFIB. Groups of animals that can reach high biomass, including homeothermic vertebrates (deer, wild boars, carnivores, and birds), poikilothermic vertebrates (fish and amphibians), and invertebrates (lumbricid fauna and mollusks) were considered in this study. Standardized ISO enumeration methods were chosen to investigate the abundances of *E. coli*, intestinal enterococci, and *C. perfringens* in excreta of the examined animal groups and in soil and sediment samples of the 12-km²-wide study area (porous aquifer [PA] backwater area). To further support an interpretation of the results, SFIB concentrations in the excreta of the evaluated animal groups were converted into estimated daily excreted SFIB loads (DESL). The groups' DESL values were compared to each other and to the standing stock of SFIB in the sediment and soil from the investigated area. This facilitated an estimation of each group's contribution to the total SFIB load in the study area.

RESULTS

Occurrence and abundance of *Escherichia coli* and intestinal enterococci in animal feces and excreta. The occurrence and abundance of *E. coli* and intestinal enterococci were evaluated in 98 and 91 fecal samples from poikilothermic and homeothermic animals, respectively (Tables 1 and 2). *E. coli* and enterococci (except one sample) were not detected in any of the earthworm samples. In the gastropod, frog, fish, bird, and ruminant fecal samples, the occurrence rates of *E. coli* were similar and ranged from 77 to 93% (Table 1). The occurrence rates of enterococci in frogs and fish were 68 and 85%, respectively. The high occurrence of enterococci in gastropods

TABLE 2 Occurrence and abundance of standard fecal indicator intestinal enterococci in diverse animal groups from an alluvial backwater (2010 to 2013)

Fecal source	n	Occurrence (%)	Abundance (\log_{10} CFU \cdot g $^{-1}$ feces) ^a				
			Mean	Median	Percentile		
					5th	95th	Max
Poikilothermic invertebrates							
Earthworm	26	4	3.3 ^b	3.3 ^b			3.3 ^b
Gastropod	26	96	5.1	5.7	2.8	7.1	7.4
Total	52	50	5.1	5.6	2.8	7.1	7.4
Poikilothermic vertebrates							
Frog	19	68	4.7	4.4	3.5	6.6	6.6
Fish	27	85	3.3	3.3	2.0	5.4	6.9
Total	46	78	3.8	3.6	2.0	6.5	6.9
Homeothermic vertebrates							
Bird	15	93	6.1	6.4	2.8	9.0	9.2
Ruminant	43	97	4.6	4.5	2.6	6.4	8.3
Wild boar	16	100	5.0	4.9	3.6	6.7	7.3
Carnivore	17	100	5.1	4.6	2.3	8.9	8.9
Total	91	97	5.0	4.6	2.4	8.8	9.2

^aAbundance data (i.e., median, mean, 5th and 95th percentiles, max) were calculated, excluding nondetectable data. All results are given in CFU \cdot g $^{-1}$ feces (wet weight). Detection limits for earthworms, 1.5 to 3.0 \log_{10} CFU \cdot g $^{-1}$; for snails, 1.9 to 3.0 \log_{10} CFU \cdot g $^{-1}$; for frogs, 1.8 to 3.0 \log_{10} CFU \cdot g $^{-1}$; for fish, 0.8 to 2.4 \log_{10} CFU \cdot g $^{-1}$; for birds, 1.8 to 2.2 \log_{10} CFU \cdot g $^{-1}$; for ruminants, 1.7 to 2.0 \log_{10} CFU \cdot g $^{-1}$; for boar, 1.7 to 2.0 \log_{10} CFU \cdot g $^{-1}$; and for carnivores, 1.9 to 2.0 \log_{10} CFU \cdot g $^{-1}$. Mean, arithmetic mean; max, maximum.

^bOnly one positive result.

(96%) was comparable to those observed in birds and ruminants (93 and 97%, respectively). *E. coli* and enterococci were detected in 100% of samples from wild boar and carnivores. Median versus mean values for *E. coli* and enterococcus concentrations revealed a high level of agreement for all the groups of fecal samples (Tables 1 and 2). Mean *E. coli* concentrations ranged from 4.2 to 4.6 and from 5.0 to 5.2 \log_{10} CFU \cdot g $^{-1}$ feces in gastropod and fish samples and in bird, ruminant, and frog samples, respectively (Table 1). The mean enterococcus concentrations ranged from 3.3 to 4.7 \log_{10} CFU \cdot g $^{-1}$ feces in the frog, fish, and ruminant samples (Table 2). The mean concentration of enterococci in gastropod fecal samples (5.1 \log_{10} CFU \cdot g $^{-1}$) was comparable to those observed in samples from wild boar and carnivores (5.0 and 5.1 \log_{10} CFU \cdot g $^{-1}$, respectively) (Table 2). The average *E. coli* concentrations were highest in the wild boar and carnivore fecal samples, with 6.6 to 7.0 \log_{10} CFU \cdot g $^{-1}$ feces observed, whereas the highest enterococcus concentrations were found in bird fecal samples, with 6.1 \log_{10} CFU \cdot g $^{-1}$ feces. The variation in the observed *E. coli* and enterococcus concentrations in fecal samples was extremely high for both groups of animals, spanning many orders of magnitude. In this respect, the differences between the 95th and 5th percentiles for the poikilothermic and homeothermic animal samples were 5.3 (8.3 to 3.0) and 7.1 (9.4 to 2.3) \log_{10} CFU \cdot g $^{-1}$ feces for *E. coli*, and 5.1 (7.1 to 2.0) and 6.7 (9.0 to 2.3) \log_{10} CFU \cdot g $^{-1}$ feces for enterococci, respectively (Tables 1 and 2). The highest *E. coli* concentrations measured in the excreta of the poikilothermic and homeothermic animals evaluated in this study were observed for frogs (8.5 \log_{10} CFU \cdot g $^{-1}$ feces) and carnivores (9.5 \log_{10} CFU \cdot g $^{-1}$ feces), respectively (Table 1). The highest enterococcus concentrations were found in the excreta of gastropods (7.4 \log_{10} CFU \cdot g $^{-1}$ feces) and birds (9.2 \log_{10} CFU \cdot g $^{-1}$ feces) (Table 2).

Occurrence and abundance of *Clostridium perfringens* in animal feces. The number of fecal samples analyzed for *C. perfringens* included 98 poikilothermic and 91 homeothermic animal samples (Table 3). The occurrence of *C. perfringens* in fecal material ranged from 39 to 54% in poikilotherms and from 50 to 60% in birds, wild boars, and carnivores (Table 3), whereas only 9% of ruminant fecal samples contained *C. perfringens*. As was observed for *E. coli* and enterococcus concentrations, the median and mean values for *C. perfringens* concentrations exhibited a high level of agreement

TABLE 3 Occurrence and abundance of the standard fecal indicator bacterium *Clostridium perfringens* in diverse animal groups from an alluvial backwater (2010 to 2013)

Fecal source	n	Occurrence (%)	Abundance (\log_{10} CFU \cdot g ⁻¹ feces) ^a				
			Mean	Median	Percentile		
					5th	95th	Max
Poikilothermic invertebrates							
Earthworm	26	54	2.8	2.8	2.1	3.5	4.0
Gastropod	26	39	2.6	2.7	2.0	3.2	3.3
Total	52	46	2.7	2.7	1.9	3.3	4.0
Poikilothermic vertebrates							
Frog	19	42	3.6	3.5	2.1	5.5	6.1
Fish	27	41	2.9	2.8	2.1	4.2	4.5
Total	46	41	3.2	3.0	2.0	4.6	6.1
Homeothermic vertebrates							
Bird	15	60	3.4	3.1	2.0	6.1	7.5
Ruminant	43	9	3.5	3.5	2.2	5.1	5.3
Wild boar	16	50	3.7	3.6	2.5	5.1	5.7
Carnivore	17	59	5.6	5.3	4.4	7.4	7.4
Total	91	34	4.2	3.8	1.9	7.4	7.5

^aAbundance data (i.e., median, mean, 5th and 95th percentiles, max) were calculated, excluding nondetectable data. All results are given in CFU \cdot g⁻¹ feces (wet weight). Detection limits for earthworms, 1.5 to 3.0 \log_{10} CFU \cdot g⁻¹; for snails, 1.9 to 3.0 \log_{10} CFU \cdot g⁻¹; for frogs, 1.8 to 3.0 \log_{10} CFU \cdot g⁻¹; for fish, 0.8 to 2.4 \log_{10} CFU \cdot g⁻¹; for birds, 1.8 to 2.2 \log_{10} CFU \cdot g⁻¹; for ruminants, 1.7 to 2.0 \log_{10} CFU \cdot g⁻¹; for boar, 1.7 to 2.0 \log_{10} CFU \cdot g⁻¹; and for carnivores, 1.9 to 2.0 \log_{10} CFU \cdot g⁻¹. Mean, arithmetic mean; max, maximum.

for all examined animal groups (Table 3). Mean concentrations ranged from 2.6 to 2.9 and from 3.4 to 3.7 \log_{10} CFU \cdot g⁻¹ feces in the earthworm, gastropod, and fish samples and in the frog, bird, ruminant, and wild boar samples, respectively (Table 3). The average concentrations were highest in the carnivore fecal samples (5.6 \log_{10} CFU \cdot g⁻¹ feces). The variation in *C. perfringens* concentrations in fecal samples was lower in poikilotherms compared to that in homeothermic animals. The differences between the 95th and 5th percentiles were 3.5 (5.5 to 2.0) and 5.4 (7.4 to 2.0) \log_{10} CFU \cdot g⁻¹ feces for poikilothermic and homeothermic animals, respectively (Table 3). The highest *C. perfringens* concentrations in poikilotherms were observed for frogs (6.1 \log_{10} CFU \cdot g⁻¹ feces) (Table 3). Among the homeothermic animals assayed, the highest concentrations of *C. perfringens* were detected in fecal samples of birds and carnivores (7.5 and 7.4 \log_{10} CFU \cdot g⁻¹ feces, respectively) (Table 3).

Occurrence and abundance of SFIB in soils and sediments. The occurrence of *E. coli* in sediment from the three investigated layers ranged from 32 to 94% (see Table S3a in the supplemental material). The mean *E. coli* concentrations in the three investigated sediment layers of the side ditches were slightly higher (1.5 to 1.8 \log_{10} CFU \cdot g⁻¹) than those observed in the backwater (1.2 to 1.5 \log_{10} CFU \cdot g⁻¹). The highest concentrations were observed in the upper layer of the backwater (3.1 \log_{10} CFU \cdot g⁻¹) and in the upper layer of the side ditches (3.2 \log_{10} CFU \cdot g⁻¹). *E. coli* was present in 14 to 57% of soil samples from the four different porous aquifer backwater area (PA area) sampling sites, with values ranging from 0.5 to 1.8 \log_{10} CFU \cdot g⁻¹ and maximum values ranging from 0.7 to 2.7 \log_{10} CFU \cdot g⁻¹ (Table S3a).

In 18 to 61% of the investigated sediment samples, intestinal enterococci were observed, and the occurrence decreased in the deeper sediment layers (see Table S3b in the supplemental material). The mean enterococcus concentrations in the three layers ranged from 1.1 to 1.6 \log_{10} CFU \cdot g⁻¹ in the backwater and from 1.4 to 2.0 \log_{10} CFU \cdot g⁻¹ in the side ditches. The highest concentrations were detected in the two upper layers of the backwater (2.3 \log_{10} CFU \cdot g⁻¹) and in the upper layer of the side ditches (3.7 \log_{10} CFU \cdot g⁻¹). The occurrence of enterococci in soil samples at the four investigated areas varied from 38 to 60%, with mean concentrations ranging from 1.2 to 1.6 \log_{10} CFU \cdot g⁻¹ (Table S3b). The highest concentration measured in soil was 2.2 \log_{10} CFU \cdot g⁻¹.

TABLE 4 Daily production of *Escherichia coli*, intestinal enterococci, and *Clostridium perfringens* in the study area^a

Fecal source	<i>E. coli</i>				Enterococci				<i>C. perfringens</i>			
	Median	Mean	Percentile		Median	Mean	Percentile		Median	Mean	Percentile	
			5th	95th			5th	95th			5th	95th
Gastropod	0.8	2.4	<0.1	5.8	13.6	22.2	0.2	81.5	0.1	0.7	<0.1	2.6
Fish	4.5	9.8	0.1	41.6	0.4	2.4	<0.1	5.6	0.4	2.1	<0.1	9.9
Frog	0.8	1.3	<0.1	4.8	<0.1	0.2	<0.1	1.2	0.8	2.6	<0.1	13.9
Bird	15.6	24.3	0.5	88.7	60.2	57.4	3.4	99.3	85.0	70.7	10.4	99.5
Ruminant	21.9	25.7	1.1	62.0	7.0	11.3	0.2	36.5	0.8	2.4	<0.1	8.0
Boar	20.7	28.6	0.8	86.5	0.8	4.2	<0.1	19.2	1.6	6.1	<0.1	40.3
Carnivore	2.3	7.6	0.1	43.5	0.8	2.6	<0.1	13.1	4.0	14.8	0.1	76.5
Human	0.3	0.3	<0.1	0.7	<0.1	<0.1	<0.1	0.1	0.6	1.2	<0.1	5.2

^aThe relative distributions (including the median and 5th and 95th percentiles) of shed fecal indicator bacteria were estimated by a Monte Carlo simulation and are given as percentages.

The occurrence of *C. perfringens* in all three investigated sediment layers was high and ranged from 78 to 100% (see Table S3c in the supplemental material). The mean *C. perfringens* concentrations in the three sediment layers of the backwater ranged from 1.7 to 2.0 log₁₀ CFU · g⁻¹ and from 2.0 to 2.1 log₁₀ CFU · g⁻¹ in the side ditches. The highest values observed in the backwater and side ditches were 2.7 and 3.1 log₁₀ CFU · g⁻¹, respectively. *C. perfringens* was detected in 47 to 100% of soil samples, with mean concentrations ranging from 1.3 to 1.8 log₁₀ CFU · g⁻¹, and the highest observed value was 2.7 log₁₀ CFU · g⁻¹ (Table S3c).

E. coli concentrations correlated well with that of enterococci ($n = 110$, $r = 0.639$, and $P < 0.01$) and moderately with that of *C. perfringens* ($n = 110$, $r = 0.412$, and $P < 0.01$) in sediment, whereas in soil no significant correlations of *E. coli* to enterococci ($n = 37$, $r = 0.042$, and $P = 0.804$) and *C. perfringens* ($n = 37$, $r = 0.242$, and $P = 0.149$) were observed.

Estimated daily SFIB loads excreted by the evaluated animal groups. Load estimations were made as an additional metric to support evaluations of animal groups' potential as sources of SFIB in the defined study area. The extremely high variations in SFIB concentrations observed in the fecal material of the investigated animals (see Tables 1 to 3) were also reflected in the 5th and 95th percentiles of the DESL simulations (Table 4). For the simulated 95th percentile values (the 95th percentile can be interpreted as a value reflecting the concurrence of high animal abundance, high fecal excretion rate, and high SFIB concentrations in excreta for an evaluated animal group), fish, birds, ruminants, and carnivores qualified as *E. coli* sources with potential significance for the PA area (potential contribution to total DESL, $\geq 42\%$). For the average and median values for simulated cases, the groups of birds, ruminants, and boars were indicated as potentially important sources of *E. coli* (cf. Table 4 and Fig. 1). Gastropods, birds, and ruminants were identified as potentially important sources for enterococci for the simulated 95th percentile values (potential contribution to total DESL, $\geq 36\%$). Surprisingly, poikilotherms (primarily gastropods) potentially contributed an average of 22.2% of the daily excreted intestinal enterococcal load, which was higher than that from ruminants and wild boars (Table 4 and Fig. 1). The main producers of *C. perfringens* were clearly birds, which contributed an estimated daily average of 70.7% of these SFIB, followed by carnivores (14.8%) and wild boars (6.1%). The potential importance of poikilotherms as sources for *C. perfringens* was low compared to that of homeothermic animals (Table 4 and Fig. 1). Humans did not play a significant role as potential sources of SFIB within the considered area.

Comparison of daily SFIB loads from excreta with the standing stock in sediments and soils. The total estimated standing stock of *E. coli* in the soil and sediment for the whole PA area ranged from 12.5 to 14.1 log₁₀ CFU (5th to 95th percentiles) (Fig. 1). Interestingly, the estimates for the daily excreted *E. coli* loads for the sum of all animal fecal sources was in the same range as the total sediment and soil stock (Fig. 1). For enterococci, the situation was comparable to that for *E. coli*, except that the range of the 5th to 95th

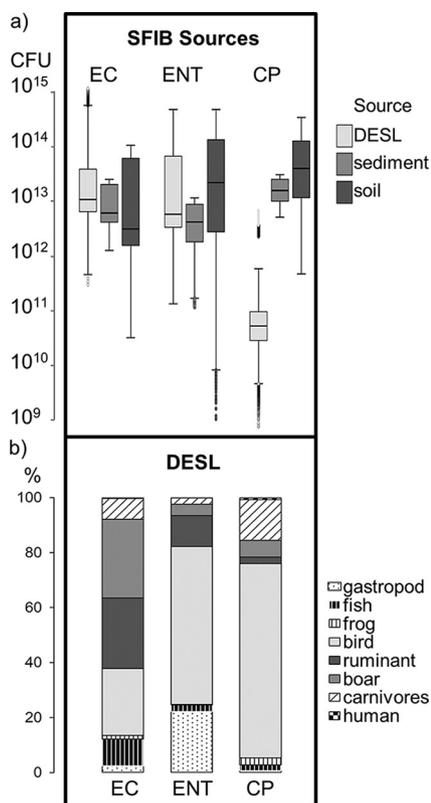


FIG 1 (a) Daily SFIB production (DESL) of animals in an alluvial backwater area compared to the standing stock of *Escherichia coli* (EC), intestinal enterococci (ENT) and *Clostridium perfringens* (CP) in sediment and soil of the investigation area. Values are given in CFU for the whole study area. Box plots indicate the median, the 25th and 75th percentiles (box), minimum and maximum values (whiskers), and outliers (dots). (b) Relative distribution of animal sources for the mean DESL.

percentile values of the estimated standing stock was somewhat higher (11.9 to 14.7 log₁₀ CFU). In contrast to *E. coli* and enterococci, the daily load estimate for *C. perfringens* for the sum of all animal excreta was, on average, more than two orders of magnitude lower than the standing *C. perfringens* stock in the sediment and soil of the PA area (Fig. 1; see also Table S4 in the supplemental material).

DISCUSSION

High potential of poikilothermic animals to serve as a primary habitat for *E. coli*. The results of the present study provide evidence that *E. coli* is a natural inhabitant of a large fraction of the investigated poikilothermic animals. The high occurrence (i.e., 68 to 85%; Table 1) and abundance of *E. coli* in the investigated fecal excreta from the PA study area, which were comparable to those of homeothermic species, contradict previous findings and conclusions that gastropods (21), fish (22–24), and frogs are only vectors that shed *E. coli* after ingesting contaminated food, soil, or sediment. The observed *E. coli* concentrations in the fecal material of poikilotherms (4.2 to 5.2 log₁₀ CFU · g⁻¹; Table 1) were at least 2 to 4 orders of magnitude higher than the mean *E. coli* concentrations in ambient sediments and soils (ranging from 0.5 to 1.8 log₁₀ CFU · g⁻¹; Mann-Whitney U test, *P* < 0.001 and *n* = 110; see Table S3a in the supplemental material). These huge differences in detected concentrations clearly disprove the hypothesis of a vector-based spread of *E. coli* by poikilothermic animals from sediments or soils in the PA area. Recently performed 16S rRNA gene sequencing of intestinal microbiota also supports these findings; for example, the fish gut microbiota much more closely resembled that in the gut of mammals than that of environmental communities (25), and the gut microbiota of frogs consisted of a community that was more

similar to those in communities of terrestrial vertebrates than in fish (26). It should be mentioned that extremely large variations of *E. coli* concentrations in the excreta were observed (from not detectable to 8.5 CFU \log_{10} CFU \cdot g⁻¹ feces), indicating that *E. coli* was not a constant member of the microbiota of poikilotherms in the PA area. The occurrence and abundance of *E. coli* in poikilothermic animals probably depended on many factors, likely including the type and status of the host species and the availability and range of food resources, as well as the season and temperature conditions (21–23, 27–29). One remarkable exception was earthworms, as *E. coli* was not detected in the recovered casts of these poikilotherms (Table 1). This finding is in agreement with those of previous studies (13, 30). Moreover, there is some evidence for a selective reduction of coliform bacteria (including *E. coli*) and intestinal enterococci in earthworms (31, 32).

***E. coli* occurrence in the excreta of homeothermic animals agrees with previous findings.** The results of this study confirm that *E. coli* is an abundant member in a very large portion of the investigated homeothermic animals (Table 1), which was even ubiquitously present in the wild boars and carnivores tested throughout the investigation. The extremely large variation in *E. coli* concentrations observed in the excreta was comparable with that observed for poikilothermic animals (Table 1). The average *E. coli* concentrations in birds from the study area were comparable to reported values for geese (33, 34). Other studies observed slightly lower (3.6 CFU \cdot g⁻¹ to 4.4 \log_{10} MPN \cdot g⁻¹) concentrations in geese and cranes (35, 36). Higher average values were also reported for geese (6.9 CFU \log_{10} \cdot g⁻¹) and other bird species (up to 8.1 \log_{10} CFU \cdot g⁻¹ in ducks, gulls, and swan) by several studies (34, 35, 37, 38). The mean *E. coli* concentrations in ruminants from an Austrian alpine region and from French deer were two and one log higher than the results of the present study, respectively (39, 40). The mean *E. coli* concentration in deer excreta was 5.7 \log_{10} CFU \cdot g⁻¹ feces (calculated from 5.06 \log_{10} CFU \cdot 100 ml⁻¹ slurry, containing 21.8 mg \cdot 100 ml⁻¹ fecal material, on average) (41). *E. coli* concentrations of 10⁵ to 10⁸ CFU \cdot g⁻¹ were observed in domesticated ruminants (beef cattle) (42, 43), which were higher than those obtained from the current study site. In wild boar stool from the study area, the mean *E. coli* concentration was comparable to values reported from a French study (7.09 \log_{10} CFU \cdot g⁻¹) (40) and values for swine (7.1 CFU \log_{10} \cdot g⁻¹) (44). A mean *E. coli* concentration of 7.0 \log_{10} CFU \cdot g⁻¹ was reported for dogs (calculated from 6.31 \log_{10} CFU \cdot 100 ml⁻¹ slurry, containing 19.8 mg \cdot 100 ml⁻¹ fecal material, on average) (41), which is comparable to the results from the PA area. Other studies detected lower mean *E. coli* concentrations of 4.4 (45) and 5.4 \log_{10} CFU \cdot g⁻¹ (46).

Gastropods qualify as primary habitats for intestinal enterococci. The occurrence (96%) and abundance (median of 5.7 \log_{10} CFU \cdot g⁻¹ excreta) of intestinal enterococci in gastropods were comparable to the levels observed in feces of homeothermic animals in the PA area (Table 2). These results also agree with previous reports of extraordinarily high and permanent levels of *Enterococcus* in the gastropod *C. aspersum* (15). Both results provide strong evidence that gastropods must be considered a primary habitat for intestinal enterococci. Intestinal enterococci were also present in a large fraction of frogs and fish (68 to 85%), with observed concentrations at least 1 to 3 orders of magnitude higher than those measured in ambient soil and sediment samples (see Table S3b, cf. vector hypothesis as discussed above; Mann-Whitney U test, $P < 0.001$ and $n = 110$). The results of the occurrence of enterococci in frogs and fish also largely agreed with those of former studies on individual populations from different habitats (19, 22, 47). As already highlighted for *E. coli*, an extremely large variation in the concentration of enterococci was observed in the excreta of poikilotherms (from not detectable to 6.9 \log_{10} CFU \cdot g⁻¹ feces from fish), indicating that intestinal enterococci were not a constant member of the microbiota of poikilotherms in the PA area, with the notable exception of gastropods, but showed a distinct distribution and pronounced population dynamics. Further investigations are

needed to understand the factors that affect the occurrence and dynamics of intestinal enterococci in poikilothermic animals (see also the discussion for *E. coli* above).

For earthworms, our results contradicted those of a previous study. Picon and Teisaire (48) detected *Enterococcus* in the intestinal content of earthworms and considered it to be endogenous because it could not be detected in the surrounding soil. In the PA area, enterococci were detected in only one earthworm sample, but were absent in the rest of the casts of the worms assayed (i.e., 96%; Table 2).

Enterococcus concentrations in feces of homeothermic animals support existing knowledge. The concentrations of enterococci in feces observed in this study strongly indicate that intestinal enterococci are ubiquitous members of the microbiota of homeothermic animals (93 to 100%; Table 2). Mean enterococcus concentrations for excreta of geese and other species were previously reported to be somewhat lower (2.7 to 5.5 \log_{10} CFU \cdot g⁻¹) (34, 35, 49) than those observed in this study, and average values in ducks, gulls, and cranes were reported as being between 6.7 and 8.0 \log_{10} CFU \cdot g⁻¹ (34–36, 38). The mean concentrations of enterococci observed in the excreta of ruminants from an Austrian alpine region were slightly higher (6.0 to 6.4 \log_{10} CFU \cdot g⁻¹ in individual samples) (39) compared to those in the current study area. The mean enterococcus concentration for deer was 4.3 \log_{10} CFU \cdot g⁻¹ (calculated from 3.56 \log_{10} CFU \cdot 100 ml⁻¹ slurry, containing 21.8 mg \cdot 100 ml⁻¹ fecal material on average) (41), which was comparable to results from the PA area. Concentrations of enterococci/lactobacilli and enterococci in swine have been previously reported as approximately 8.0 \log_{10} CFU \cdot g⁻¹ (50) and 5.5 \log_{10} CFU \cdot g⁻¹ (51), respectively, somewhat higher than what was observed in the present study area. In addition, the enterococcus concentration in dogs was assessed in multiple studies, and the reported values were 6.7 \log_{10} CFU \cdot g⁻¹ (49), 6.9 \log_{10} CFU \cdot g⁻¹ (calculated from a slurry containing 19.8 mg \cdot 100 ml⁻¹) (41), and 4.05 \log_{10} CFU \cdot g⁻¹ (52). The reported enterococcus concentration in cats (5.6 \log_{10} CFU \cdot g⁻¹) was comparable to the mean value determined for carnivores in the present study (53).

***Clostridium perfringens* exhibited a very distinct distribution in animal excreta.** Genomic analysis predicts *C. perfringens* as an anaerobic, fastidious, pathogenic organism, with the essential requirement of various amino acids satisfied by active degradation and import of various materials from tissues, coupled with the ability to produce very persistent spores (54). Based on this information, the primary intestinal habitats with actively reproducing *C. perfringens* are expected to occur especially in carnivores but also in mixed-diet animals, where its particular nutritional requirements are met (55). Additionally, the long-term persistence of *C. perfringens* spores is expected to support its distribution in the environment, contributing to a specific background level of spores in soils and sediments. Both theoretical expectations were met by the *C. perfringens* data set from the PA area (Table 3; see also Table S3c in the supplemental material). The highest *C. perfringens* concentrations were observed in carnivores (mean of 5.6 \log_{10} CFU \cdot g⁻¹ feces), which were two orders of magnitude higher than those observed in mixed-diet animals (wild boars) (Table 3). Also in line with expectations, concentrations of *C. perfringens* in poikilothermic animals (including earthworms) were not significantly different than those observed in ambient sediments (Mann-Whitney U test, $P = 0.044$ and $n = 136$) and soils (Mann-Whitney U test, $P = 0.835$ and $n = 136$). The detection of *C. perfringens* or members of the genus *Clostridium* has already been reported from gastropods (56–58) and diverse fish and frog species (19, 20, 47, 59) and do not contradict the results from this study. Earthworms apparently take up spores during food consumption and shed them with the casts, because spore abundance is not reduced during the gut passage (31). These reported results are in good agreement with our findings, where 54% of the investigated casts contained detectable concentrations of *C. perfringens* (mean concentrations of 2.8 \log_{10} CFU \cdot g⁻¹ excreta).

Are poikilotherms relevant sources of *E. coli* and enterococci in the PA area? Determinations of the occurrence of SFIB in the excreta of animals do not necessarily inform on their relevance as potential pollution sources. To investigate the potential relevance of the studied animal groups to pollution of the PA area, we followed a new strategy by estimating the DESL. Estimates of the DESL provided clear evidence that

both homeothermic and poikilothermic animals must be regarded as potential sources of *E. coli* and intestinal enterococci in the studied area (Table 4). In addition, the estimated DESL for *E. coli* and enterococci accounted for the determined background concentrations in sediment and soil within a period of a single day on average (Fig. 1). However, it must be stated that the DESL metric does not provide any information with respect to the actual level of water pollution. Such estimates would need to consider additional information, such as the transport and persistence of SFIB in the catchment area. The DESL estimate provides a novel metric to evaluate the capacity of a group of animals to contribute to the overall amount of SFIB produced within a defined area and time.

Clearly, the results of this study are restricted to backwater environments in the Central European region. Additionally, the investigation period spanned the warm season, from March to November. For such regions, it seems likely that poikilothermic animals play only a minor role during the cold period of the year (from November to February). However, an investigation of the whole seasonal cycle was beyond the aim of this study. Because bacterial growth conditions in poikilothermic animals strongly depend on the temperature, it seems likely that Mediterranean, subtropical, and tropical climates may support SFIB production in poikilotherms far better than the PA area. We speculate that temperature effects are stronger in the intestines of these animals compared to the ambient soil because the digestive tract functions like a "bioreactor" with increased nutrient availability due to mechanical maceration and digestive processes. Further studies are needed to examine this hypothesis. It would also be interesting to elucidate whether a relationship between previously reported "naturalized" SFIB populations in soils or sediments (8, 11, 76) correlates with the abundance and activity of poikilothermic animals, especially when the biomass of poikilotherms is high.

Is there a need to redefine the fecal indicator paradigm for *E. coli* and intestinal enterococci? *E. coli* and intestinal enterococci have been thought to indicate fecal pollution from homeothermic mammals and birds and therefore signal the potential occurrence of pathogens from these groups of animals (61). The results of this study strongly indicate that these fecal indicators also occur commonly in poikilothermic invertebrates and vertebrates in the PA area and have the capacity to contribute to fecal pollution levels. It is clear that further investigations in other areas are needed to substantiate these findings. If so, there would be a need to reevaluate the current fecal indicator paradigm. Depending on the biotic and abiotic characteristics of the habitat, we hypothesize that *E. coli* and intestinal enterococci may originate, to a variable extent, from animals other than homeothermic animals living in and around water resources, soils, and sediments. These results do not suggest that *E. coli* and intestinal enterococci should not be used as indicators for fecal pollution. However, our results suggest that interpretation of these data, especially at low contamination levels, is more complex than previously believed, and strategies to properly apply and interpret the results of these water quality monitoring tools must be adapted accordingly.

MATERIALS AND METHODS

Investigation area. The investigated porous aquifer (PA) backwater area is a typical Central European riverine wetland located on the north side of the Danube River at the south-eastern border of Vienna, Austria, covering an area of approximately 12 km². The PA area is an important resource for drinking water and is also part of a national park. The Viennese national park area plays a strategic role as a wilderness and recreation area (62). Forestry and sport fishing are of minor importance due to national park regulations (63). Within the PA area, the City of Vienna has designated hunting grounds that are managed by the Forestry Administration Office. Detailed information on the limnological and hydrological characteristics of the PA area is available elsewhere (64, 65).

Sampling strategy. Fecal samples were collected directly from the investigation area between 2010 and 2013 from homeothermic animals (cats, dogs, deer, wild boars, and birds) and poikilothermic vertebrates (fish and amphibians) and invertebrates (lumbricid fauna and mollusks). The species or groups of species were chosen on the basis of their occurrence in the area and because they present the genera with the highest abundances and biomasses. Detailed knowledge of the species distribution is available for the considered national park area (60). Samples were recovered as individual fecal samples from individual animals. The only exception to this sampling strategy was a fraction of the fish fecal

samples, which had to be pooled to enable microbiological analysis because of the very small amount of accessible fecal material per animal. To ensure that sampling was representative, samples for each group were taken on several dates within a 2- to 3-year time frame. As poikilotherms are only active during warm, frost-free periods, the investigation and sampling was limited to the frost-free season of the year (March to November). Fecal samples were taken directly from each individual. The intestinal content was obtained by softly squeezing the collected animals (earthworms and fish), briefly trapping individuals and collecting the droppings (birds, mollusks, and some of the frogs), or from the intestines of dissected animals (frogs, ruminants, and wild boars). Cormorant samples were taken directly beneath trees in which animals were asleep, where identification of the excreta was assured. All samples were aseptically collected in sterile plastic vials and stored at $5 \pm 3^\circ\text{C}$ in the dark until analysis. Sampling permission had been granted according to national park regulations (permits MA22-229/2011 and MA22-13854/2013).

Vierheilig et al. (55) previously reported on *C. perfringens* concentrations in wild homeothermic vertebrates, partially using the same ruminant, carnivore, bird, and wild boar fecal samples. To facilitate comparison between the study of Vierheilig et al. (55) and the present study, all samples where a full SFIB data set was available were also included in the present analysis. No fecal samples from livestock were included, since such animal groups are not allowed in the PA national park area. The wildlife in the PA environment can be considered representative of wildlife in riverine backwater environments.

Investigated homeothermic vertebrates. The total number of recovered vertebrate samples was 91. Ruminant samples ($n = 43$, all from the PA area) included *Cervus elaphus* (red deer), *Capreolus capreolus* (roe deer), *Ovis orientalis musimon* (European mouflon), and *Dama dama dama* (European fallow deer). *Sus scrofa* (wild boar, $n = 16$, all from the PA area) was included as a mixed-diet animal. Sample collection from vertebrates is described in detail by Vierheilig et al. (55). Avian fecal matter from the piscivorous species *Phalacrocorax carbo sinensis* (great cormorant, $n = 2$) was collected in the PA area. Samples from other avian species (*Anas platyrhynchos* [wild duck] and other *Anatidae* [$n = 6$], *Sterna hirundo* [common tern, $n = 3$], and Charadriiformes [waders; $n = 4$]) were obtained from the closely associated Neusiedler See–Seewinkel national park and an alluvial forest in Lower Austria (Neubach). Sampling in the PA area had to be waived for avian species to minimize the disturbance within this area. For domesticated animals ($n = 17$), feces from dogs (*Canis lupus familiaris*) and cats (*Felis catus*) were collected by pet owners or from trails where individuals walk their dogs. The abundance of small vertebrates (mice) was negligible for the experimental period (see supplemental material).

Investigated poikilothermic vertebrates and poikilothermic invertebrates. The total number of recovered fecal samples from poikilothermic vertebrates and poikilothermic invertebrates was 98. The fish species *Esox lucius* (pike, $n = 2$), *Silurus glanis* (wels catfish, $n = 1$), *Sander lucioperca* (pikeperch, $n = 1$), *Abramis brama* (bream, $n = 8$), *Aspius aspius* (asp, $n = 1$), *Cyprinus carpio morpha hungaricus* (carp, $n = 4$), *Perca fluviatilis* (redfin perch, $n = 6$), *Rutilus rutilus* (roach, $n = 4$), *Carassius gibelio* (Prussian carp, $n = 1$), *Abramis ballerus* (blue bream, $n = 3$), *Lepomis gibbosus* (pumpkin seed, $n = 1$), and *Scardinius erythrophthalmus* (rudd, $n = 1$) were directly trapped by electrical fishing at the PA area. The fecal material was primarily investigated as individual samples ($n = 14$). Only in cases where the accessible amount of fecal material per fish was less than 0.25 g did we pool 2 to 4 samples ($n = 6$). Because fishermen routinely plant fish from a fish farm in Lower Austria into the PA area, fish fecal samples were also obtained from that fish farm ($n = 7$, *Cyprinus carpio morpha hungaricus*). Amphibians were caught using a hand net ($n = 15$, *Bombina bombina* and *Pelophylax ridibundus*, all from the PA area) and were briefly caged or decapitated. In addition, freshly killed amphibians from streets were also collected ($n = 4$, *Bufo*, from Lower Austria). Fecal samples from gastropods ($n = 26$, *Arion* sp., *Helix pomatia*, *Lymnaea stagnalis*, and *Viviparus* sp., all from the PA area) were retrieved from living, briefly caged individuals. Earthworms ($n = 26$, *Allolobophora rosea rosea*, *Helodrilus deficiens*, *Lumbricus rubellus*, *Octolasion lacteum*, *Octodrilus transpadanus*, *Proctodrilus tuberculatus*, *Octodrilus* sp., and *Lumbricus* sp., all from the PA area) were collected by digging (66), and species were identified in the lab by comparisons made with formalin-preserved individuals. Reptiles were omitted from the study due to their low abundance.

Investigated soil and sediment samples. To support comparisons of SFIB concentrations in fecal samples with those in the ambient environment, soil and sediment samples were analyzed from July 2010 to May 2011 (monthly, except from December to February). The PA investigation area (12 km²) was categorized into the water area (1.4 km²) and the different terrestrial habitat types (alluvial forest protected by a dam, 7.3 km²; alluvial forest outside the dam-protected area, 0.1 km²; bank and reef, 2.3 km²; marsh, 0.09 km²; and "Heilnde," 1.7 km²; as described elsewhere) (67). The water area was further categorized into several sections depending on the hydrologic conditions (backwater and side ditches) (64). Seven representative locations in the PA area were chosen for sediment sampling. Three sampling sites with different connectivity to the river were located at the primary backwater ($n = 65$), as well as four sites at side ditches and small ponds ($n = 45$). Three of the latter sampling sites were chosen due to the expected high frequency (high abundance) and fecal contamination potential of ruminants and wild boars at the sites, as determined from the tracks of the animals and the presence of a nearby feeding area for game. Sediments were sampled in the backwaters with a sediment corer at a water depth of approximately 20 to 100 cm. Each sample contained three subsamples taken within 10 m², and the materials (separated into three layers: from the upper first centimeter, the layer from 1 to 5 cm, and the layer from 5 to 10 cm) were thoroughly mixed (68). Seven locations were chosen for soil sampling, one representing the bank and reef zone ($n = 8$), four representing alluvial forest soil ($n = 22$), one representing the so-called "Heilnde," a dry, sandy, brush-covered habitat that is not connected to the groundwater ($n = 4$), and one for a marsh zone at a small side ditch ($n = 7$). Soil samples (three subsamples within a defined 10-m² area marked by stakes) were taken from the upper 10 cm (one layer)

with a corer. Subsamples were thoroughly mixed and examined as previously described (68). One milliliter of all fresh sediment and soil samples was weighted to allow the results to be converted from $\text{CFU} \cdot \text{g}^{-1}$ to $\text{CFU} \cdot \text{ml}^{-1}$ (equal to cm^3).

Microbiological analysis. Bacteriological analysis of fecal samples was performed as previously described (39), including counts of *C. perfringens*, *E. coli*, and intestinal enterococci according to established ISO standards. Cultivation-based ISO standard methods were chosen to ensure comparability and interpretation of the results with respect to routine water quality monitoring programs. In brief, *E. coli* was quantified with tryptone bile X-glucuronide (TBX) agar (44°C, 48 h) according to ISO 16649-2 (5). Enterococci were enumerated on Slanetz and Bartley agar (36°C, 48 h) following ISO 7899-2 (6). *C. perfringens* was quantified in accordance with ISO 14189 (7) on tryptose sulfite cycloserine (TSC) agar (44°C, 24 h). In the fecal samples, vegetative cells and spores were investigated (without pasteurization of the sample), whereas soil and sediment samples were pasteurized (15 min, 60°C) so that only spores were detected. For quality control, the following type strains were used: *E. coli* NCTC 9001, *Enterococcus faecalis* NCTC 775, and *Clostridium perfringens* NCTC 8237. Exactly weighed fecal samples (approximately 1 g or less if fecal material was limited) were suspended in 100 ml (or less, if fecal material was limited) peptone saline diluent (250 ml distilled water, 2.5 g peptone, 1.25 g NaCl, 0.87 g di-sodium hydrogen phosphate, and 0.37 g di-potassium hydrogen phosphate), as described previously (39). After 30 min of suspension time, samples were shaken and allowed to settle for 15 min. Sediment and soil samples were prepared by mixing approximately 1 g of sample in 100 ml peptone saline diluent and slowly shaking for 30 min on a shaker (Lab Tec MS30A), after which the suspension was allowed to settle for 1 h (69, 70).

One-milliliter aliquots of suspensions and prepared dilutions (10^{-2} up to 10^{-6}) were analyzed by the membrane filtration method (using 0.45- μm cellulose-nitrate membrane filters). The detection limit (DL) depends on the mass of sample material used and is calculated by the formula $\text{DL} = V/G$, where DL is the limit of detection of target bacteria (given in CFU per g sample), V is the volume of diluent (in ml) used for the suspension of the sample material, and G is the mass of sample material in grams (55). For most of the fecal samples (84%), the detection limit was lower than $120 \text{ CFU} \cdot \text{g}^{-1}$. For 8% of samples, the detection limit was between 120 and $499 \text{ CFU} \cdot \text{g}^{-1}$. For a few samples (7%), the detection limit was between 500 and $1,000 \text{ CFU} \cdot \text{g}^{-1}$ (in cases where very little material was available). The detection limits for soil and sediment were as high as $10 \text{ CFU} \cdot \text{g}^{-1}$ fresh material. The results are given in $\log_{10} \text{ CFU} \cdot \text{g}^{-1}$ wet material unless otherwise specified.

Estimating daily SFIB loads excreted by the evaluated groups of animals. Although the primary focus of the study was to establish quantitative data on the occurrence of SFIB in the feces of homeothermic and poikilothermic animals, the determined concentrations were also converted into estimates of SFIB loads from the daily excreted animal fecal emissions. Load estimates were made to further evaluate the significance of the evaluated groups of animals as potential sources of SFIB and to compare them with the standing stock of SFIB in the soil and sediment of the PA area. The load estimation was based on the pollution source profile (PSP) method, which was previously established and applied for an alpine karstic watershed in the Northern Calcareous Alps of Austria (71). The PSP method described in Farnleitner et al. (72) was extended with a Monte Carlo simulation. Briefly, the PSP principle is based on two steps, as follows: (i) the estimation of expected fecal emission rates of the animal groups selected (i.e., the amount of fecal mass excreted per area over a given time) and (ii) multiplication of the determined fecal emission rates by the determined SFIB concentrations in the excreta (72). The estimated loads of SFIB for the considered groups of animals were expressed per the 12-km² PA area and per day. A detailed description of the study area (specification of surface and water volume) is given as supplemental material (see Section S1.1 in the supplemental material). Finally, to support comparisons, the estimated daily excreted SFIB loads (DESL) were expressed as percentages with respect to the total DESL (sum of all partial animal loads). Expected fecal emission rates for the animal groups (animal fecal masses produced per day and PA area) were determined by the best available data on animal population sizes or animal standing stocks (given as biomasses or individual numbers in the study area) multiplied by the specific excretion rate of an animal group (given as the expected amount of fecal material produced per considered type of organism and day; see reference 72). All multiplications were performed using the SPSS Monte Carlo simulation tool to estimate average, median, and 5th and 95th percentile values. Estimated population sizes or standing stock numbers were obtained from literature on the PA area and from information provided by local national park authorities. Specific fecal excretion rate estimates (i.e., the mass of feces excreted per animal or animal biomass per day) were obtained from the literature (if available) or estimated by expert judgment. A detailed overview of the types and ranges of values used and the corresponding information sources is given in the supplemental material (see Section S1.2 and Table S1 in the supplemental material). It should be mentioned that hibernation and reduced activity due to cold temperatures were not considered, as the investigation was restricted to the warm season (see sampling design). Thus, the established estimates represent conditions of active poikilotherms during warm and humid periods (cf. sampling design). Human visitors of the national park area were also included as potential fecal sources in the comparisons (cf. supplemental material).

Estimating the standing stock of SFIB in sediment and soil. For this estimation, the 12 km² of the PA area was categorized into the water area and the different terrestrial habitat types as described above. Corresponding volumes of the bottom sediment (i.e., 4 selected layers: 0 to 1, 1 to 5, 5 to 10, and below 10 cm) and soil (i.e., 2 selected layers: 0 to 10 cm and below 10 cm) were calculated from a digital terrain model (5 m \times 5 m grids) as described elsewhere (67), including the complete sediment or soil layer

above the gravel layer (see Table S2 in the supplemental material). Standing stock values for SFIB (i.e., SFIB numbers per PA area) were estimated by multiplying the calculated volumes of sediment or soil with the SFIB concentrations observed in sediment and soil samples from the corresponding sections of the study area (cf. Table S2). For the sediment and soil layer below 10 cm, no measured SFIB data were available from the study area. To calculate the standing stock in this bottom layer, the SFIB concentration from the layers above were used but were reduced by one log order. This assumption is based on literature which reports a strong decrease in SFIB concentrations with increased depth in riverine soils and sediments (73–75). As all SFIB concentrations in samples from Heißblände ($n = 4$) were below the detection limit, the area for Heißblände was not considered for the calculation. All multiplications were made using the SPSS Monte Carlo simulation tool to estimate average, median, and 5th and 95th percentile standing stock values.

Statistical analysis. The analysis of SFIB data was performed using Microsoft Excel 2010 and IBM SPSS statistics (version 23). Microbiological data were $\log_{10}(x + 1)$ transformed for presentations in tables and figures. For the comparison of group means, the Mann-Whitney U test was used (nonparametric). Correlation analysis was performed with the nonparametric Spearman's correlation. For the applied Monte Carlo simulations with SPSS (cf. SFIB fecal loads and standing stock estimates; see above paragraph) the number of simulated cases of random multiplications was set to 100,000 with a stop criterion (confidence interval of the mean was within 1%) and using individual values for simulations. A sensitivity analysis for the DESL estimation is presented as supplemental material.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.00715-18>.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

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