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Clostridium perfringens Is Not Suitable for the Indication of Fecal Pollution from Ruminant Wildlife but Is Associated with Excreta from Nonherbivorous Animals and Human Sewage

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During a 3-year study, *Clostridium perfringens* was investigated in defined fecal sources from a temperate alluvial backwater area of a large river system. The results reveal that using *C. perfringens* as a conservative water quality indicator for total fecal pollution monitoring is no longer justified but suggest that it can be used as a tracer for excreta from nonherbivorous wildlife and human sewage.

The reliable detection of fecal pollution in water is of high importance, as fecal material often contains a significant number of intestinal pathogens. The standard fecal indicator bacteria (SFIB), *Escherichia coli* and intestinal enterococci, have been used to monitor total fecal pollution for over a century. However, the suitability of the SFIB as general indicators has been increasingly questioned because these bacteria can establish "naturalized" populations in nonintestinal environments (1, 2). Genetic marker assays for total fecal pollution have also recently been developed (3), but there is still debate about their specificity, and there is still potential for further methodical improvements (4, 5). *Clostridium perfringens* has been suggested as an alternative to SFIB almost since the advent of the indicator concept (6). It has frequently been applied to various water resources on several continents (7–14). Despite the widespread application of *C. perfringens* as a general fecal indicator, recent studies of its basic molecular biology actually contradict the idea that it universally occurs in fecal pollution sources from different hosts. This contradiction is based on a genomic analysis that uncovered *C. perfringens* as an anaerobic, fastidious, pathogenic, "flesh-eating" organism, with the essential requirement of various amino acids (15). Based on this knowledge, *C. perfringens* is expected to occur in the fecal excreta from mixed-diet and carnivorous organisms, where its particular nutritional requirements are met by the food selection of the respective host.

The aim of this research was to investigate the quantitative occurrence of *C. perfringens* over a 3-year study period in well-defined wildlife and human-associated fecal sources that appeared in a typical backwater area of a large river system in the temperate zone. The hypothesis was that *C. perfringens* does not frequently occur in ruminant wildlife but is mainly associated with nonherbivorous animal excreta and human sewage. To date, there have been extremely limited systematic investigations on the quantitative occurrence of *C. perfringens* in wildlife fecal excrements (16–18). This paucity of data is surprising given the importance of *C. perfringens* in veterinary issues. However, there are numerous investigations available on the various clinically important *C. perfringens* pathotypes. These studies focus almost exclusively on qualitative characteristics (see, e.g., references 19 and 20). To the best of our knowledge, this report represents the first rigorous effort to quantitatively test the occurrence of *C. perfringens* in herbivorous and nonherbivorous animal excreta and in human sewage. To generate a robust data set, a multiyear longitudinal study was designed, and a defined, postmortem fecal sampling strategy was applied.

Investigation area, fecal sources, sampling, and bacteriological analysis. The Porous Groundwater Well Aquifer (PGWA) backwater area is a riverine wetland located on the north side of the Danube River at the southeastern borderline of Vienna, Austria. The PGWA is a national park and an important water resource. Throughout an area of approximately 12 km^2, fecal samples from freshly hunted animals ($n = 73$), which represent the dominant animal fecal emission sources in the area, were collected between 2010 and 2012. The hunted herbivorous ruminants included *Cervus elaphus* (red deer), *Capreolus capreolus* (roe deer), *Ovis orientalis musimon* (European mouflon), and *Dama dama dama* (European fallow deer). Additionally, *Sus scrofa* (wild boar) was included as a hunted, mixed-diet animal. The numbers of the hunted species included in this study reflect the relative abundances of the respective populations in this area. During the officially declared hunting season, fecal material was retrieved from the rectum immediately after the animals were shot. In addition to postmortem sampling, avian fecal droppings ($n = 25$) from the carnivorous *Phalacrocorax carbo sinensis* (great cormorant) and other mixed-diet avian species were collected (hunting of avian populations was not allowed). For domesticated animals ($n = 20$),

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TABLE 1 Prevalence and abundance of *Clostridium perfringens* (or presumptive *C. perfringens*) in ruminant and nonruminant fecal sources from an alluvial backwater system (2010 to 2012)[a]

				Abundance (log_{10} CFU g^{-1} or log_{10} CFU 100 ml^{-1})[b]							
		Prevalence (%)		Median		Mean		Min		Max	
Fecal source	*n*	CP	(p.CP)	CP	(p.CP)	CP	(p.CP)	CP	(p.CP)	CP	(p.CP)
Red deer	40	8	(33)	2.3	(2.6)	2.2	(3.1)	1.8	(2.0)	2.3	(3.8)
Roe deer	8	0		n. d.		n. d.		n. d.		n. d.	
Fallow deer	3	33		3.2		3.2		3.2		3.2	
Mouflon	2	0		n. d.		n. d.		n. d.		n. d.	
Σ Ruminants	53	8	(26)	2.3	(2.6)	2.7	(3.1)	1.8	(2.0)	3.2	(3.8)
Wild boar	20	60	(85)	3.6	(4.0)	4.7		2.0		5.7	
Avian[c]	19	47	(58)	2.8	(3.3)	5.5	(6.1)	1.8	(2.1)	6.4	(7.1)
Σ Mixed diet	39	54	(72)	3.4	(3.7)	5.2	(5.7)	1.8	(2.0)	6.4	(7.1)
Cormorant	6	100		7.2		7.3		3.5		7.7	
Dog	12	67	(100)	5.8		6.9	(6.8)	4.4	(3.0)	7.4	
Cat	8	63	(75)	5.9	(7.0)	7.0	(7.1)	4.5		7.4	
Σ Carnivores	26	73	(92)	5.9		7.1	(7.0)	3.5	(3.0)	7.7	
WWTP1	25	100		3.1		3.2		2.4	(2.6)	3.6	
WWTP2	25	100		3.8		3.9		3.0		4.4	
Σ WWTPs	50	100		3.5		3.6	(3.7)	2.4	(2.6)	4.4	

[a] CP, *Clostridium perfringens*; (p.CP), presumptive *C. perfringens*; *n*, replicate number; WWTP, wastewater treatment plant. Presumptive *C. perfringens* results are shown explicitly only when they differ from those of *C. perfringens*.
[b] Abundance data (i.e., median, mean, min, max) were calculated excluding nondetectable data and log transformed after the addition of a value of 1. CFU g^{-1} or 100 ml^{-1}, CFU per g feces (wet weight) or per 100 ml of sewage effluent from WWTPs; Mean, arithmetic mean; Min, minimum; Max, maximum; n. d., not detectable. Detection limit = log_{10} 1.7 to log_{10} 2.0 CFU g^{-1} feces (except for 2 samples, log_{10} 2.5 and log_{10} 2.6 CFU g^{-1} feces) or log_{10} 1.0 CFU 100 ml^{-1} sewage effluent (WWTPs).
[c] Birds other than cormorants (see the supplemental material for more details).

feces from dogs and cats, which may occasionally enter the area, were collected nearby. Two representative wastewater treatment plants (WWTP1 and WWTP2) that discharged into the Danube River were also analyzed during this time period ($n = 2 \times 25$). Direct sewage transfer from the Danube River to the investigated backwater happens frequently during flood events (21).

All samples were aseptically collected in sterile 50-ml plastic vials (feces) or 1,000-ml glass bottles (WWTP effluent) and stored at 5 ± 3°C in the dark until analysis. Bacteriological analysis included *C. perfringens*, presumptive *C. perfringens*, *E. coli*, and intestinal enterococci, according to established ISO standards (for a detailed description of the microbiological methods and definition of parameters, refer to the supplemental material). To ensure direct comparability with the standardized water quality testing procedure, samples for clostridia analysis were not pasteurized. The European Drinking Water Directive stipulates this approach for *C. perfringens* enumeration, which includes vegetative cells and spores (22). However, a preliminary comparison of *C. perfringens* concentrations from pasteurized versus unpasteurized samples did not reveal significantly different results (data not shown), which supported our previous investigations on the pasteurization effect from samples of human origin (23). Selected WWTP influent and effluent samples were also used to demonstrate the differential persistence between *C. perfringens* and *E. coli* in microcosm experiments, spanning a minimum of 163 h. The data analysis was performed with SigmaPlot, version 11.0 (Systat Software, Inc., San Jose, CA), applying the nonparametric Mann-Whitney U test to analyze for pairwise differences. For further details on area and fecal sources, please refer to the supplemental material.

Prevalence and abundance of *C. perfringens*. *C. perfringens* showed very low prevalence and abundance in fecal samples from ruminant herbivores (Table 1). *C. perfringens* could be detected in only 8% of the ruminant samples ($n = 53$) and at a low average concentration of log_{10} 2.7 CFU per g feces for the positive fecal samples (Table 1). Consistently low prevalence and abundance of *C. perfringens* in ruminant wildlife excreta were also evident in comparisons of the results from the investigated years (see Table S1 in the supplemental material). The mixed-diet fauna, including wild boars and birds, had a prevalence rate of 54%, with a significantly increased average abundance of log_{10} 5.2 CFU per g feces, compared to the samples from the herbivores (Table 1, $P < 0.001$). Remarkably, the highest abundance of *C. perfringens* in avian excreta was found in the piscivorous cormorants, reaching concentrations of up to log_{10} 7.7 CFU per g feces. The examination of dog and cat feces showed *C. perfringens* prevalence rates of 67% and 63%, respectively, and the average concentrations were more than 4 orders of magnitude higher than those observed in the herbivore fecal samples. The unbalanced distribution of *C. perfringens* also became clear when the concentrations were compared to the abundances of *E. coli* and enterococci in the feces of herbivorous wildlife in the backwater area (Fig. 1). The recovered results are in agreement with the preliminary findings at an alpine watershed, where *C. perfringens* rarely occurred in herbivorous fecal sources (16). These results relate to a very specific environ-

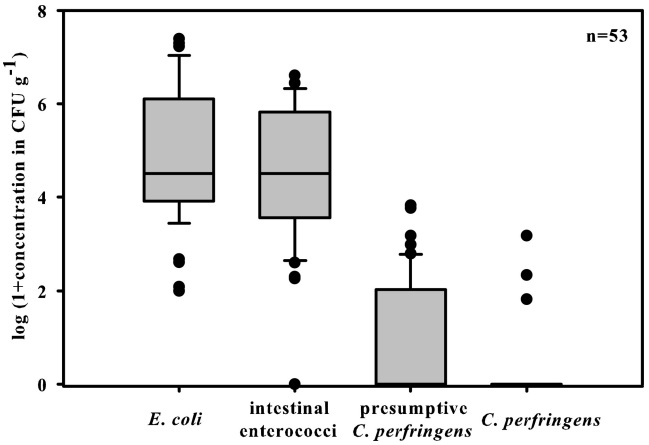

FIG 1 Observed concentrations of fecal indicators (*Escherichia coli*, intestinal enterococci, presumptive *Clostridium perfringens*, and *C. perfringens*) in fecal samples from herbivorous ruminants. CFU g^{-1}, CFU per g feces (wet weight); boxes, 25th and 75th percentiles; lines within the boxes, medians; whiskers, 10th and 90th percentiles. Data are log transformed after the addition of a value of 1.

ment (e.g., high altitude, little vegetation and food availability, long periods of cold temperatures). It was not possible to draw any substantial conclusions on the occurrence of *C. perfringens* in the fecal excreta from nonalpine areas based on this investigation (16). The recovered data also appear to be in agreement with those from a study of an Australian watershed, where a set of native and feral wildlife fecal samples ($n = 72$) was investigated (17). Unfortunately, the investigations were limited to two single sampling events and did not give information on the temporal variability of *C. perfringens* or the consistency of the results for the investigated sources.

C. perfringens could be detected in 100% of the effluent WWTP samples, with concentrations in the expected range of $\log_{10}$ 2.4 to $\log_{10}$ 4.4 CFU per 100 ml wastewater (Table 1). Assuming a moderate dilution of human feces in the sewer channel (i.e., $\log_{10}$ 1.0 dilution) and conservative treatment efficacy in the WWTP (i.e., $\log_{10}$ 1.0 reduction), the *C. perfringens* concentrations would be approximately $\log_{10}$ 4.4 to $\log_{10}$ 6.4 CFU per g feces. These concentrations fit well with the previously reported median and average concentrations of $\log_{10}$ 4.0 and $\log_{10}$ 5.8 CFU per g feces from the investigated Viennese population (16).

Interestingly, the distinct patterns of *C. perfringens* concentrations between the considered fecal source groups were also reflected by the presumptive *C. perfringens* concentrations (Table 1, values in brackets). However, a clear trend toward higher presumptive *C. perfringens* concentrations was evident, most likely due to a broader taxonomic composition.

Implications of the results on the indicator capacity of *C. perfringens*. The results of this longitudinal study strongly indicate that defining *C. perfringens* as a conservative indicator for total fecal pollution monitoring in water is no longer justified (24, 25). Instead, the results illustrate that *C. perfringens* is a conservative indicator for fecal excreta from nonherbivorous wildlife and human-associated sewage. In this respect, it is not surprising that *C. perfringens* proved to be an excellent indicator for point source emissions from wastewater treatment plants in rivers and other lotic systems (9, 11, 12) and as a tracer for sewage sludge pollution (13, 14). Although the feces of carnivorous wildlife can contain very high concentrations of *C. perfringens*, it seems unlikely that this type of feces plays a significant role in water pollution, as the abundance of predators is usually very low in comparison to that of the remaining animals. Depending on the situation, dogs and cats may play a substantial role in *C. perfringens* emission, especially in urban areas. As livestock are not found in the investigated backwater area, this type of fecal source was not investigated. However, a previous study in an alpine area indicated that herbivorous livestock may carry significant *C. perfringens* concentrations, which were most likely promoted by special feeding practices (16).

Recent studies indicated that the concentrations of *C. perfringens* are correlated with certain pathogens (*Cryptosporidium* spp.) and with infection risk from recreational activities (26, 27). Because of this finding, *C. perfringens* was suggested as a potential alternative indicator to SFIB for recreational water quality monitoring (28). The implicit rationale behind this suggestion is based on its wastewater-associated nature. It is important that *C. perfringens* is a spore-forming organism that can be extremely resistant to various environmental factors such as heat, low water availability, radiation, or disinfection procedures (8, 29, 30). *C. perfringens* does not reproduce in aquatic systems (31, 32). The spores may be detected long after a pollution event has occurred and far from the source. To demonstrate the persistent nature of *C. perfringens*, we performed microcosm experiments using various raw and treated wastewater effluents and the intrinsic *C. perfringens* and *E. coli* contaminants (see Fig. S1 in the supplemental material). Remarkable differential levels of persistence of *C. perfringens* and *E. coli* were evident, which highlighted the conservative nature of *C. perfringens* populations. Finally, as *C. perfringens* occurs at lower numbers than *E. coli* or enterococci (16, 17), larger sampling volumes have to be used for water quality monitoring and risk management.

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