Vierheilig J, Frick C, Mayer RE, Kirschner AKT, Reischer GH, Derx J, Mach RL, Sommer R, and Farnleitner AH. 2013. *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. Appl. Environ. Microbiol.

SUPPLEMENTAL MATERIAL:

Study area. The Porous Groundwater Well Aquifer (PGWA) area is a floodplain of the Danube River at the southeastern border of the city of Vienna, Austria, which extends from river kilometer 1908 to 1917 and has a width of 2-3 km. As one of the most important wetlands in Austria and Europe, it was designated a biosphere reserve by UNESCO in 1977. It is also a Ramsar Site, part of the Natura 2000 Network, and the Danube Alluvial Zone National Park. It serves a wide range of socio-economic and ecological functions, including recreational space, the capacity for flood retention, a groundwater recharge, and as a drinking water supply for the metropolitan area of Vienna (1). It covers wetlands, woods, and dry meadows, and contains a diverse fauna and flora (for more details, see 2).

Investigated fecal sources. Within the PGWA area, the city of Vienna has designated hunting grounds, which cover an area of 11.58 km^2. Populations of wild boars and herbivorous ruminants inhabit the area and are managed by the Forestry Administration Office (Vienna Municipal Department of Forestry and Urban Agriculture, MA 49). The most recent population size estimates were available for 2010 and resulted in the following numbers of individuals for the respective species: 176 red deer, 44 roe deer, 17 fallow deer, 20 European mouflon, and 150 wild boars (G. Walzer, personal communication). The age of the animal populations ranged between 0-14 years for red deer, 0-8 years for mouflon, and 0-6 years for roe deer, fallow deer, and wild boars.

Except for red and fallow deer, the wildlife demonstrates rather territorial behavior. Within their habitat, they naturally feed on a diverse menu including different grasses, herbs, buds, twigs, diverse field crops, acorns, chestnuts, and wild fruits. Additionally, they were fed roughage (hay) and succulent feed (grass and corn silage, beets). At the bait site, they were mainly offered corn and peas (G. Walzer, personal communication). After the animals were shot, transported, broken down, and washed (a duration of approximately 1 hour), approximately 10-20 g of fecal samples were taken out of the last 10 cm of the colon. Avian fecal droppings included samples (each approximately 0.25-5 g) from *Phalacrocorax carbo sinensis* (great cormorant, n = 6), *Sterna hirundo* (common tern, n = 3), *Anas platyrhynchos* (mallard, n = 2), and unspecified members of *Anatinae* (duck, n = 2), *Cygninae* (swan, n = 2), *Anatidae* (wildfowl, n = 2), *Charadriiformes* (shore birds, n = 4), and *Aves* (bird, n = 4).

All four investigated wastewater treatment plants (WWTPs) apply activated sludge treatment. WWTP1 is designed for a population equivalent of 4.0 million, and the current load corresponds to a population equivalent of 3.0 million. WWTP2 is a smaller facility designed for a population equivalent of 40,000 and is currently overloaded. The two other facilities, WWTP3 and WWTP4,

were included in the persistence study to achieve a more representative sample setup and to account for the fluctuating water quality of the WWTPs. WWTP3 and WWTP4 are designed for 23,000 and 116,000 population equivalents, respectively.

Bacteriological analysis. The samples were stored at 5 ± 3°C and analyzed within 8 hours (sewage), or a maximum of 5 days (feces) for presumptive *Clostridium perfringens*, *C. perfringens*, *E. coli,* and intestinal enterococci, using the membrane filtration method according to ISO standards as previously described in detail (3). Presumptive *C. perfringens* were defined as black or grey colonies on tryptose-sulfite-cycloserine agar (No 1–178, Scharlau, Spain, and supplement cycloserine, No SR0088E, Oxoid, UK) after anaerobic incubation at 44°C for 21 h. To confirm the presence of *C. perfringens*, the acid phosphatase test was applied according to ISO standard 14189 (4, 5). An amount of homogenized fecal material ranging from 0.25 to 2 g was weighed (the exact wet weight was registered) in 100 ml of sterile peptone-saline solution (No 64544, Bio-Rad, Hercules, CA, USA), thoroughly shaken, suspended for 30 min, and shaken again. After the suspensions were allowed to settle for 30 min, tenfold serial dilutions were prepared and analyzed (10^{-2}-10^{-6}), resulting in a detection limit ranging from $\log_{10}$ 1.7 to $\log_{10}$ 2.0 CFU per g feces for most samples. The detection limit was only higher for two samples ($\log_{10}$ 2.5 and $\log_{10}$ 2.6 CFU per g feces) because of the small amount of material available.

SUPPLEMENTAL TABLE S1: Temporal distribution of the prevalence and abundance data (*Clostridium perfringens*) presented in Table 1 between the years 2010-2012.

Fecal source	Sample year	n	Prevalence [%]	Abundance [$\log_{10}$ CFU g^{-1}] or [$\log_{10}$ CFU 100 ml^{-1}]			
				Median	Mean	Min	Max
Ruminants	2010	15	13	2.3	2.3	2.3	2.3
	2011	21	5	3.2	3.2	3.2	3.2
	2012	17	6	1.8	1.8	1.8	1.8
Mixed diet	2010	8	75	4.2	5.7	2.2	6.4
	2011	22	45	3.5	4.7	2.0	5.7
	2012	9	56	3.1	3.5	1.8	4.0
WWTPs	2010	6	100	3.5	3.6	3.1	4.0
	2011	25	100	3.5	3.7	2.4	4.4
	2012	19	100	3.4	3.5	2.6	3.9

CP, *Clostridium perfringens*; (p.CP), presumptive *C. perfringens*, n, replicate number; WWTP, Wastewater Treatment Plant.

[a] 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}, colony-forming units per g feces (wet weight) or per 100 ml of sewage effluent (WWTPs); Mean, arithmetic mean; Min, minimum; Max, maximum. 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).

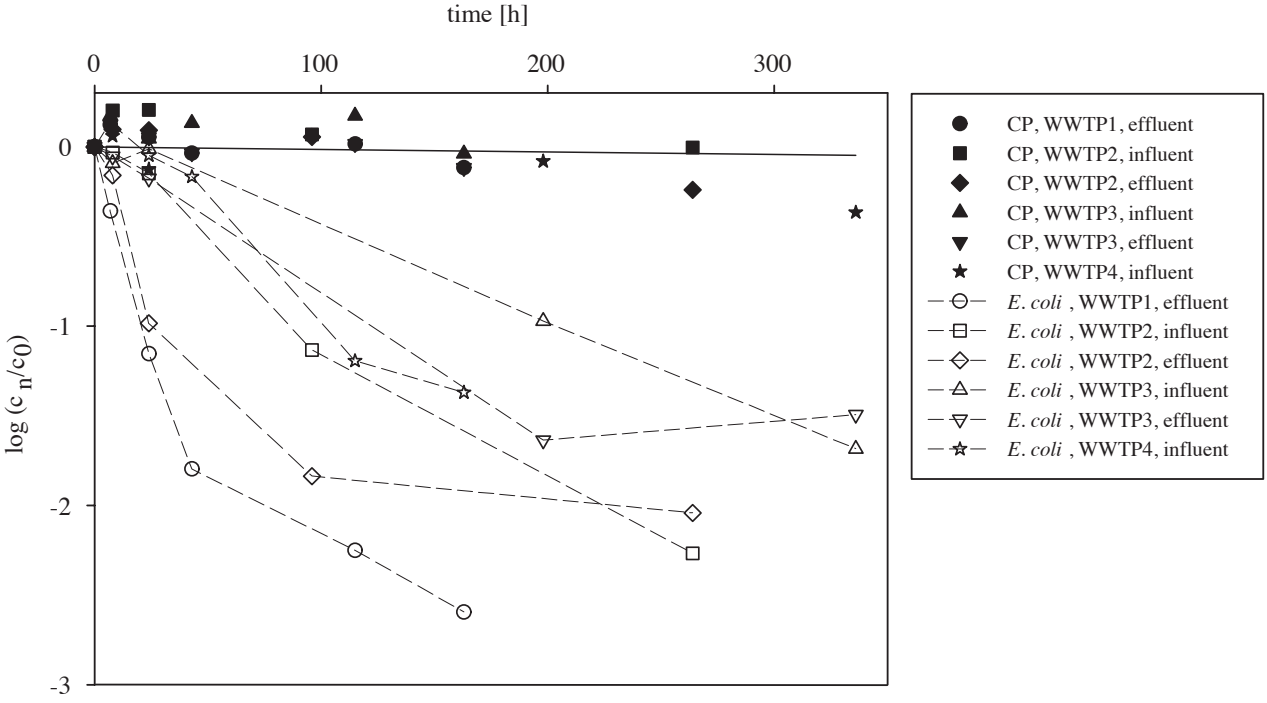
[c] Birds other than cormorants (see the supplemental material for more details).

To investigate the persistence of *E. coli* and *C. perfringens*, the collected WWTP influent and effluent samples were equilibrated and incubated at 22 ± 3°C for batch culture experiments. Fractions of 70 ml were recovered from the WWTP batch cultures after defined time periods, homogenized by sonication and analyzed. For the sewage influent samples, tenfold serial dilutions were prepared, 10^{-1}-10-^{4} for *C. perfringens* and 10^{-2}-10^{-5} for *E. coli*, resulting in detection limits of $\log_{10}$ 3.0 CFU and $\log_{10}$ 4.0 CFU per 100 ml sewage, respectively. For the sewage effluent samples from all the investigated WWTPs, volumes of 10 ml, 1 ml, and 0.1 ml were filtrated and analyzed, resulting in a detection limit of $\log_{10}$ 1.0 CFU per 100 ml sewage effluent.

Laboratory quality control samples, such as positive controls for media control and sterile water as the negative control, were processed for every sample series.

The term "prevalence" is used to represent the occurrence of (presumptive) *C. perfringens* throughout the sample setup in this publication (i.e., percentage of positive samples). The abundance data are expressed as logarithmic values for better illustration, as usual in microbiological studies. All of the calculations were performed using the original data, and the log transformation was only applied as a final step after the addition of a value of 1.

SUPPLEMENTAL FIGURE S1: Differential persistence of the fecal indicators *Escherichia coli* and *Clostridium perfringens* in wastewater over time at 22°C. Persistence is shown as a logarithm of the quotient c_t/c_0, where c_t is concentration at time t and c_0 is concentration at time 0. CP, *Clostridium perfringens*; WWTP, Wastewater Treatment Plant.



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