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Poikilothermic animals as a previously unrecognized source of fecal indicator bacteria in a backwater ecosystem of a large river

Christina Frick[a,b], Julia Vierheilig[b,c,‡]*, Rita Linke [c,‡], Domenico Savio[d,‡], Horst Zornig[e], Roswitha Antensteiner[f], Christian Baumgartner[g], Christian Bucher[h,i], Alfred P. Blaschke[j,‡], Julia Derx[j,‡], Alexander K.T. Kirschner[d,k‡], Gabriela Ryzinska-Paier[a,c], René Mayer[c,‡], Dagmar Seidl[a], Theodossia Nadiotis-Tsaka[a], Regina Sommer[k,‡], Andreas H. Farnleitner[c,d,‡]#

Vienna City Administration, Municipal Department 39, Vienna, Austria[a]; Centre for Water Resource Systems (CWRS), Vienna University of Technology, Vienna, Austria[b]; Institute of Chemical Engineering, Research Group Environmental Microbiology and Molecular Ecology, Vienna University of Technology, Vienna, Austria[c]; Karl Landsteiner University of Health Sciences, Krems, Austria[d]; Pro Fisch OG, Vienna, Austria[e]; Vienna, Austria[f]; Nationalpark Donau-Auen GmbH, Orth/Donau, Austria[g]; Institute of Hydraulic Engineering and Water Resources Management, Vienna University of Technology, Vienna, Austria[h]; Institute of Building Construction and Technology, Vienna University of Technology, Austria[i]; Institute of Hydraulic Engineering and Water Resources Management, Vienna University of Technology, Austria[j]; Unit of Water Hygiene, Institute for Hygiene and Applied Immunology, Medical University of Vienna, Vienna, Austria[k]

Running Head: Poikilothermic animals as a source of fecal indicators

Address correspondence to Andreas H. Farnleitner, andreas.farnleitner@kl.ac.at
*Present address: Department of Microbiology and Ecosystem Science, Division of Microbial Ecology, University of Vienna, Austria

‡ Interuniversity Cooperation Centre for Water and Health www.waterandhealth.at.

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, birds) and poikilothermic animals (earthworms, gastropods, frogs, and fish) 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 1-4 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 *E. coli* and enterococci loads further supported the importance of poikilotherms as potential pollution sources. 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 sub-tropical and tropical habitats and water resources.

52 **Importance of the Study**

53 The current fecal indicator concept is based on the assumption that the standard fecal
54 indicator bacteria (SFIB) *Escherichia coli*, intestinal enterococci and *Clostridium*
55 *perfringens* only multiply in the guts of humans and other homeothermic animals and can
56 therefore indicate fecal pollution and the potential presence of pathogens from those
57 groups. The findings of the present study showed that SFIB can also occur in high
58 concentrations in poikilothermic animals (i.e., animals with body temperatures that vary
59 with the ambient environmental temperature, such as fish, frogs and snails) in an alluvial
60 backwater area in a temperate region, indicating that a reconsideration of this long-
61 standing indicator paradigm is needed. This study suggests that poikilotherms must be
62 considered to be potential primary sources of SFIB in future studies.

63

64 **Introduction**

65 Microbiological water quality monitoring is strongly dependent on investigations of
66 standard fecal indicator bacteria (SFIB). *Escherichia coli* (*E. coli*) and intestinal enterococci
67 have been considered the most important SFIB for more than 100 years (1, 2), since the
68 introduction of the fecal indicator concept (3). Furthermore, *Clostridium perfringens* (*C.*
69 *perfringens*) has also been used as a fecal indicator since the beginning of water quality
70 testing (1, 4). SFIB are considered sensitive indicators of the extent of fecal contamination
71 in water resources, and the monitoring of SFIB is an essential tool for water safety
72 management. SFIB can easily be detected by standardized cultivation-based methods,
73 e.g., ISO 16649-2 (5) for *E. coli*, ISO 7899-2 (6) for intestinal enterococci and ISO 14189
74 (7) for *C. perfringens*. Their occurrence at high concentrations in the excreta of humans
75 and other homeothermic animals and their inability to replicate in the non-intestinal
76 environment are the most basic requirements for microbial fecal indicators. However, the
77 usefulness of SFIB as fecal indicators has been increasingly questioned following the

78 discovery of potential long-term persistence and re-growth of SFIB in the environment (8,
79 9) and so-called “naturalized populations” (10-12), which are thought to persist and
80 proliferate in non-intestinal environments. The potential of poikilothermic vertebrates (i.e.,
81 animals whose body temperature varies with the ambient environmental temperature) to
82 serve as primary habitats of SFIB may further interfere with the traditional fecal indicator
83 concept. However, quantitative investigations on the occurrence of SFIB in poikilothermic
84 vertebrates are scarce. Furthermore, there is little available knowledge regarding the
85 occurrence of SFIB in invertebrates, such as snails or slugs. For a better understanding of
86 the importance of alternative sources of SFIB in the environment, comparative
87 investigations are needed, including all suspected non-biotic and biotic compartments.
88
89 Existing studies on the quantitative occurrence of SFIB in alternative animal sources give a
90 very limited picture that is based on fragmentary information from various habitats with
91 differing environmental conditions. Until the current study, *E. coli* and enterococci had not
92 been detected in earthworm casts (13), although other studies observed a positive
93 significant correlation between earthworm abundance and *E. coli* occurrence in soil (14). In
94 another study, *Enterococcus casseliflavus* was identified as a dominant species in the
95 feces of the garden snail (*Cornu aspersum*) at concentrations of up to 9.0 $\log_{10}$ colony
96 forming units (CFU) g^{-1} feces (15). Investigations of edible snails (*C. aspersum* and *Helix*
97 *lucorum*) revealed that *E. coli* and enterococci counts varied from 4.0 to 5.5 and 5.0 to 6.0
98 $\log_{10}$ CFU g^{-1} feces, respectively (16). In another study, two pooled samples from slugs
99 (*Limax* spp.) had *E. coli* concentrations of 4.9 and 6.0 $\log_{10}$ CFU g^{-1}. The *E. coli*
100 concentration in the organs and tissues of fish increased with an increase in the bacterial
101 load of the water body, with intestinal tract concentrations of *E. coli* ranging from 2.0 to 5.0
102 $\log_{10}$ MPN g^{-1} in investigated species (17). An investigation of the occurrence of *E. coli* in
103 grass carp (*Ctenopharyngodon idella*), silver carp (*Hypophthalmichthys molitrix*) and rohu

104 (*Labeo rohita*) from aquaculture facilities in which animal manure was directly discharged
105 into fish ponds revealed mean intestinal tract *E. coli* concentrations of 5.0 ± 0.5 $\log_{10}$ CFU
106 g^{-1} feces, compared to 3.0 ± 0.7 $\log_{10}$ CFU g^{-1} feces from control ponds without manure
107 (18). In Japanese tree frogs (*Hyla japonica*) maintained in a laboratory, the observed
108 concentrations of *E. coli*, enterococci and *Clostridium* spp. were 8.3 to 9.9 $\log_{10}$ CFU g^{-1},
109 6.9 ± 1.3 $\log_{10}$ CFU g^{-1}, and 6.1 to 7.1 $\log_{10}$ CFU g^{-1} wet intestinal content, respectively
110 (19). The concentration of *E. coli* in bullfrogs (*Rana catesbeiana*) maintained in a
111 laboratory was 7.1 to 8.4 $\log_{10}$ CFU g^{-1} feces (20). Except for the abovementioned studies
112 on individual species, comparative studies on the quantitative occurrence of SFIB in
113 poikilothermic and invertebrate animals within or across habitats were lacking until the
114 current study.
115
116 The aim of this study was to assess the abundance of SFIB in the excreta of various wild
117 animals living in a typical Central European riverine wetland located on the north side of
118 the Danube River at the south-eastern border of Vienna, Austria to support quantitative
119 cross-comparisons of potential sources of SFIB. Groups of animals that can reach high
120 biomass, including homeothermic vertebrates (deer, wild boars, carnivores, and birds),
121 poikilothermic vertebrates (fish and amphibians), and invertebrates (lumbricid fauna and
122 mollusks) were considered in this study. Standardized ISO enumeration methods were
123 chosen to investigate the abundances of *E. coli*, intestinal enterococci and *C. perfringens*
124 in excreta of the examined animal groups and in soil and sediment samples of the 12 km^2
125 wide study area (porous aquifer backwater area = PA area). To further support an
126 interpretation of the results, SFIB concentrations in the excreta of the evaluated animal
127 groups were converted into estimated daily excreted SFIB loads (DESL). The groups´
128 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 groups´ 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 was evaluated in 98 and 91 fecal samples from poikilothermic and homoeothermic animals, respectively (Table 1a and 1b). *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 rate of *E. coli* was similar and ranged from 77 to 93% (Table 1a). The occurrence of enterococci in frogs and fish was 68 and 85%, respectively. The high occurrence of enterococci in gastropods (96%) was comparable to that 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 vs. mean values for *E. coli* and enterococci concentrations revealed a high level of agreement for all the groups of fecal samples (Table 1a and 1b). Mean *E. coli* concentrations ranged from 4.2. to 4.6 and from 5.0 to 5.2 $\log_{10}$ CFU g^{-1} feces in gastropod and fish samples and in bird, ruminant, and frog samples, respectively (Table 1a). The mean enterococci concentrations ranged from 3.3 to 4.7 $\log_{10}$ CFU g^{-1} feces in the frog, fish and ruminant samples (Table 1b). The mean concentration of enterococci in gastropod fecal samples (5.1 $\log_{10}$ CFU g^{-1}) was comparable to those observed in samples from wild boar and carnivores (5.0 and 5.1 $\log_{10}$ CFU g^{-1}, respectively) (Table 1b). The average *E. coli* concentrations were highest in the wild boar and carnivore fecal samples, with 6.6 to 7.0 $\log_{10}$ CFU g^{-1} feces observed, whereas the highest enterococci concentrations were found in bird fecal samples with 6.1 $\log_{10}$ CFU g^{-1} feces. The variation in the observed *E. coli* and enterococci concentrations in fecal samples was extremely high for both groups of animals, spanning many orders of

155 magnitude. In this respect, the distances of the 95th vs. the 5th percentiles for the
156 poikilothermic and homoeothermic animal samples were 5.3 (8.3 - 3.0) and 7.1 (9.4 - 2.3)
157 $\log_{10}$ CFU g^{-1} feces for *E. coli*, and 5.1 (7.1 - 2.0) and 6.7 (9.0 - 2.3) $\log_{10}$ CFU g^{-1} feces for
158 enterococci, respectively (Table 1a and 1b). The highest *E. coli* concentrations measured
159 in the excreta of the poikilothermic and homeothermic animals evaluated in this study were
160 observed for frogs (8.5 $\log_{10}$ CFU g^{-1} feces) and carnivores (9.5 $\log_{10}$ CFU g^{-1} feces),
161 respectively (Table 1a). The highest enterococci concentrations were found in the excreta
162 of gastropods (7.4 $\log_{10}$ CFU g^{-1} feces) and birds (9.2 $\log_{10}$ CFU g^{-1} feces) (Table 1b).
163
164 **Occurrence and abundance of *Clostridium perfringens* in animal feces.** The number
165 of fecal samples analyzed for *C. perfringens* included 98 poikilothermic and 91
166 homeothermic animal samples (Table 1c). The occurrence of *C. perfringens* in fecal
167 material ranged from 39 to 54% in poikilotherms and from 50 to 60% in birds, wild boars
168 and carnivores (Table 1c), whereas only 9% of ruminant fecal samples contained *C.*
169 *perfringens*. As was observed for *E. coli* and enterococci, the median and mean values for
170 *C. perfringens* concentrations exhibited a high level of agreement for all examined animal
171 groups (Table 1c). Mean concentrations ranged from 2.6 to 2.9 and from 3.4 to 3.7 $\log_{10}$
172 CFU g^{-1} feces in the earthworm, gastropod and fish samples and in the frog, bird,
173 ruminant, and wild boar samples, respectively (Table 1c). The average concentrations
174 were highest in the carnivore fecal samples (5.6 $\log_{10}$ CFU g^{-1} feces). The variation in *C.*
175 *perfringens* concentrations in fecal samples was lower in poikilotherms compared to
176 homeothermic animals. The distances of the 95th vs. the 5th percentiles were 3.5 (5.5 –
177 2.0) and 5.4 (7.4 - 2.0) $\log_{10}$ CFU g^{-1} feces for poikilothermic and homoeothermic animals,
178 respectively (Table 1c). The highest *C. perfringens* concentrations in poikilotherms were
179 observed for frogs (6.1 $\log_{10}$ CFU g^{-1} feces) (Table 1c). 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 g^{-1} feces, respectively) (Table 1c).

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% (cf. supplemental material, Table S3a). 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 g^{-1}) than those observed in the backwater (1.2 to 1.5 $\log_{10}$ CFU g^{-1}). The highest concentrations were observed in the upper layer of the backwater (3.1 $\log_{10}$ CFU g^{-1}) and in the upper layer of the side ditches (3.2 $\log_{10}$ CFU 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 g^{-1} and maximum values ranging from 0.7 to 2.7 $\log_{10}$ CFU g^{-1} (cf. supplemental material, Table S3a).

In 18 to 61% of the investigated sediment samples intestinal enterococci were observed, and the occurrence decreased in the deeper sediment layers (cf. supplemental material, Table S3b). The mean enterococci concentrations in the three layers ranged from 1.1 to 1.6 $\log_{10}$ CFU g^{-1} in the backwater and from 1.4 to 2.0 $\log_{10}$ CFU g^{-1} in the side ditches. The highest concentrations were detected in the two upper layers of the backwater (2.3 $\log_{10}$ CFU g^{-1}) and in the upper layer of the side ditches (3.7 $\log_{10}$ CFU 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 g^{-1} (cf. supplemental material, Table S3b). The highest concentration measured in soil was 2.2 $\log_{10}$ CFU g^{-1}.

The occurrence of *C. perfringens* in all three investigated sediment layers was high and ranged from 78 to 100% (cf. supplemental material, Table S3c). The mean *C. perfringens* concentrations in the three sediment layers of the backwater ranged from 1.7 to 2.0 $\log_{10}$ CFU g^{-1} and from 2.0 to 2.1 $\log_{10}$ CFU g^{-1} in the side ditches. The highest values observed

in the backwater and side ditches were 2.7 and 3.1 $\log_{10}$ 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 $\log_{10}$ CFU g^{-1}, and the highest observed value was 2.7 $\log_{10}$ CFU g^{-1} (cf. supplemental material, 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 as 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 (cf. Table 1) were also reflected in the 5^{th} and 95^{th} percentiles of the DESL simulations (Table 2). For the simulated 95^{th} percentile values (the 95^{th} 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 ≥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 2 and Figure 1). Gastropods, birds and ruminants were identified as potentially important sources for enterococci for the simulated 95^{th} percentile values (potential contribution to total DESL ≥36%). Surprisingly, poikilotherms (primarily gastropods) potentially contributed an average of 22.2% of the daily excreted intestinal enterococci load, which was higher than that from ruminants and wild boars (Table 2 and Figure 1). The main producers of *C. perfringens* were clearly birds, which contributed an

232 estimated daily average of 70.7% of these SFIB, followed by carnivores (14.8%) and wild
233 boars (6.1%). The potential importance of poikilotherms as sources for *C. perfringens* was
234 low compared to homeothermic animals (Table 2 and Figure 1). Humans did not play a
235 significant role as potential sources of SFIB within the considered area.
236
237 **Comparison of daily SFIB loads from excreta with the standing stock in sediments**
238 **and soils.** The total estimated standing stock of *E. coli* in the soil and sediment for the
239 whole PA area ranged from 12.5 to 14.1 $\log_{10}$ CFU (5 to 95% percentiles) (Figure 1).
240 Interestingly, the estimates for the daily excreted *E. coli* loads for the sum of all animal
241 fecal sources was in the same range as the total sediment and soil stock (Figure 1). For
242 enterococci, the situation was comparable to *E. coli*, except that the 5 to 95% range of the
243 estimated standing stock was somewhat higher (11.9 to 14.7 $\log_{10}$ CFU). In contrast to *E.*
244 *coli* and enterococci, the daily load estimate for C*. perfringens* for the sum of all animal
245 excreta was, on average, more than two orders of magnitude lower than the standing *C.*
246 *perfringens* stock in the sediment and soil of the PA area (Figure 1, cf. supplemental
247 material table S4)
248
249 **Discussion**
250 **High potential of poikilothermic animals to serve as a primary habitat for *E. coli.*** The
251 results of the presented study provide evidence that *E. coli* is a natural inhabitant of a large
252 fraction of the investigated poikilothermic animals. The high occurrence (i.e., 68 - 85%,
253 Table 1a) and abundance of *E. coli* in the investigated fecal excreta from the PA study
254 area, which was comparable to homeothermic species, contradict previous findings and
255 conclusions that gastropods (21), fish (22-24) and frogs are only vectors that shed *E. coli*
256 after ingesting contaminated food, soil or sediment. The observed *E. coli* concentrations in
257 the fecal material of poikilotherms (4.2 to 5.2 $\log_{10}$ CFU g^{-1}, Table 1) were at least 2 to 4

258 orders of magnitude higher than the mean *E. coli* concentrations in ambient sediments and
259 soils (ranging from 0.5 to 1.8 $\log_{10}$ CFU g^{-1}, Table S3, Mann-Whitney U test, $p < 0.001$ and
260 $n = 110$). These huge differences in detected concentrations clearly falsify the hypothesis
261 of a vector-based spread of *E. coli* from sediments or soils in the PA area by poikilothermic
262 animals. Recently performed 16S rRNA gene sequencing of intestinal microbiota also
263 supports these findings, for example, the fish gut microbiota much more closely resembled
264 the gut of mammals than that of environmental communities (25), and the gut microbiota of
265 frogs consisted of a community that was more similar to communities of terrestrial
266 vertebrates than to fish (26). It should be mentioned that extremely large variations of *E.*
267 *coli* concentrations in the excreta were observed (from not detectable to 8.5 CFU $\log_{10}$
268 CFU g^{-1} feces), indicating that *E. coli* was not a constant member of the microbiota of
269 poikilotherms in the PA area. The occurrence and abundance of *E. coli* in poikilothermic
270 animals probably depended on many factors, likely including the type and status of the
271 host species, the availability and range of food resources, as well as the season and
272 temperature conditions (21-23, 27-29). One remarkable exception were earthworms, as *E.*
273 *coli* was not detected in the recovered casts of these poikilotherms (Table 1). This finding
274 is in agreement with previous studies (13, 30). Moreover, there is some evidence for a
275 selective reduction of coliform bacteria (including *E. coli*) and intestinal enterococci in
276 earthworms (31, 32).
277
278 ***E. coli* occurrence in the excreta of homeothermic animals agrees with previous**
279 **findings.** The results of this study confirm that *E. coli* is an abundant member in a very
280 large portion of the investigated homeothermic animals (Table 1a), that was even
281 ubiquitously present in the wild boars and carnivores tested throughout the investigation.
282 The extremely large variation in *E. coli* concentrations observed in the excreta was
283 comparable with that observed for poikilothermic animals (Table 1a). The average *E. coli*

284 concentrations in birds from the study area were comparable to reported values for geese
285 (33, 34). Other studies observed slightly lower (3.6 CFU g^{-1} to 4.4 log_{10} MPN g^{-1})
286 concentrations in geese and cranes (35, 36). Higher average values were also reported for
287 geese (6.9 CFU log_{10} g^{-1}) and other bird species (up to 8.1 log_{10} CFU g^{-1} in ducks, gulls,
288 and swan) by several studies (34, 35, 37, 38). The mean *E. coli* concentrations in
289 ruminants from an Austrian alpine region and from French deer were two and one log
290 higher compared to the results of the present study, respectively (39, 40). The mean *E.*
291 *coli* concentration in deer excreta was 5.7 log_{10} CFU g^{-1} feces (calculated from 5.06 log_{10}
292 CFU 100 ml^{-1} slurry, containing 21.8 mg 100 ml^{-1} fecal material, on average) (41). *E. coli*
293 concentrations of 10^5 to 10^8 CFU g^{-1} were observed in domesticated ruminants (beef) (42,
294 43), higher than those obtained from the current study site. In wild boar from the study
295 area, the mean *E. coli* concentration in stool was comparable to values reported from a
296 French study (7.09 log_{10} CFU g^{-1}) (40) and values in swine (7.1 CFU log_{10} g^{-1}) (44). A
297 mean *E. coli* concentration of 7.0 log_{10} CFU g^{-1} was reported for dogs (calculated from 6.31
298 log_{10} CFU 100 ml^{-1} slurry, containing 19.8 mg 100 ml^{-1} fecal material, on average) (41),
299 which is comparable to the results from the PA area. Other studies detected lower mean
300 *E. coli* concentrations of 4.4 (45) and 5.4 log_{10} CFU g^{-1} (46).
301
302 **Gastropods qualify as primary habitats for intestinal enterococci.** The occurrence
303 (96%) and abundance (median of 5.7 log_{10} CFU g^{-1} excreta) of intestinal enterococci in
304 gastropods were comparable to the levels observed in homeothermic feces in the PA area
305 (Table 1b). These results also agree with previous reports of extraordinarily high and
306 permanent levels of *Enterococcus* in the gastropod *C. aspersum* (15). Both results provide
307 strong evidence that gastropods must be considered as a primary habitat for intestinal
308 enterococci. Intestinal enterococci were also present in a large fraction of frogs and fish
309 (68-85%), with observed concentrations of at least 1 to 3 orders of magnitude higher than

310 those measured in ambient soil and sediment samples (Table 2 and S3, cf. vector
311 hypothesis as discussed above, Mann-Whitney U test, $p < 0.001$ and $n = 110$). The results
312 of the occurrence of enterococci in frogs and fish also largely agreed with former studies
313 on individual populations from different habitats (19, 22, 47). As already highlighted for *E.*
314 *coli*, an extremely large variation in the concentration of enterococci was observed in the
315 excreta of poikilotherms (from not detectable to 6.9 $\log_{10}$ CFU g^{-1} feces from fish),
316 indicating that intestinal enterococci, with the notable exception of gastropods, were not a
317 constant member of the microbiota of poikilotherms in the PA area but showed a distinct
318 distribution and pronounced population dynamics. Further investigations are needed to
319 understand the factors that affect the occurrence and dynamics of intestinal enterococci in
320 poikilothermic animals (see also discussion for *E. coli* above).
321 For earthworms, our results contradicted those of a previous study. Picon et al. (48)
322 detected *Enterococcus* sp. in the intestinal content of earthworms and considered it to be
323 endogenous because it could not be detected in the surrounding soil. In the PA area,
324 enterococci were detected in only one earthworm sample, but were absent in the rest of
325 the casts of the worms assayed (i.e., 96%, Table 1b).
326
327 **Enterococci concentrations in feces of homeothermic animals support existing**
328 **knowledge.** The concentrations of enterococci in feces observed in this study strongly
329 indicate that intestinal enterococci are ubiquitous members of the microbiota of
330 homoeothermic animals (93-100%, Table 1). Mean enterococci concentrations for excreta
331 of geese and other species were previously reported to be somewhat lower (2.7 to 5.5
332 $\log_{10}$ CFU g^{-1}) (34, 35, 49) than those observed in this study, and average values in duck,
333 gull and crane were reported as being between 6.7 and 8.0 $\log_{10}$ CFU g^{-1} (34-36, 38). The
334 mean concentrations of enterococci observed in the excreta of ruminants from an Austrian
335 alpine region were slightly higher (6.0 to 6.4 $\log_{10}$ CFU g^{-1} in individual samples) (39)

336 compared to the current study area. The mean enterococci concentration for deer was 4.3
337 $\log_{10}$ CFU g^{-1} (calculated from 3.56 $\log_{10}$ CFU 100 ml^{-1} slurry, containing 21.8 mg 100 ml^{-1}
338 fecal material on average) (41), which was comparable to results from the PA area. The
339 concentration of enterococci and lactobacilli in swine was previously reported as
340 approximately 8.0 $\log_{10}$ CFU g^{-1} (50) and 5.5 $\log_{10}$ CFU g^{-1} (51), respectively, somewhat
341 higher than what was observed in the present study area. In addition, the enterococci
342 concentration in dogs was assessed in multiple studies, and was reported to be 6.7 $\log_{10}$
343 CFU g^{-1} (49), 6.9 $\log_{10}$ CFU g^{-1} (calculated from a slurry containing 19.8 mg 100 ml^{-1}) (41)
344 and 4.05 $\log_{10}$ CFU g^{-1} (52). The reported enterococci concentration in cats (5.6 $\log_{10}$ CFU
345 g^{-1}) was comparable to the mean value determined for carnivores in the present study
346 (53).
347
348 ***Clostridium perfringens* exhibited a very distinct distribution in animal excreta**.
349 Genomic analysis predicts *C. perfringens* as an anaerobic, fastidious, pathogenic
350 organism, with the essential requirement of various amino acids satisfied by active
351 degradation and import of various materials from tissues, coupled with the ability to
352 produce very persistent spores (54). Based on this information, the primary intestinal
353 habitats with actively reproducing *C. perfringens* are expected to especially occur in
354 carnivores but also in mixed-diet animals, where its particular nutritional requirements are
355 met (55). Additionally, the long-term persistence of *C. perfringens* spores is expected to
356 support its distribution in the environment, contributing to a specific background level of
357 spores in soils and sediments. Both theoretical expectations were met by the *C.*
358 *perfringens* data set from the PA area (Table 1c and S3). The highest *C. perfringens*
359 concentrations were observed in carnivores (mean of 5.6 $\log_{10}$ CFU g^{-1} feces), which were
360 two orders of magnitude higher than those observed in mixed-diet animals (wild boars)
361 (Table 1c). Also in line with expectations, concentrations of *C. perfringens* in poikilothermic

362 animals (including earthworms) were not significantly different than those observed in
363 ambient sediments (Mann-Whitney U test, p=0.044 and n=136) and soils (Mann-Whitney
364 U test, p=0.835 and n=136). The detection of *C. perfringens* or members of the genus
365 *Clostridium* has already been reported from gastropods (56-58) and diverse fish and frog
366 species (19, 20, 47, 59) and do not contradict the results from this study. Earthworms
367 apparently take up spores during food consumption and shed them with the casts,
368 because their abundance is not reduced during the gut passage (31). These reported
369 results are in good agreement with our findings, where 54% of the investigated casts
370 contained detectable concentrations of *C. perfringens* (mean concentrations of 2.8 $\log_{10}$
371 CFU g^{-1} excreta).
372
373 **Are poikilotherms relevant sources of *E. coli* and enterococci in the PA area?**
374 Determinations of the occurrence of SFIB in the excreta of animals do not necessarily
375 inform on their relevance as potential pollution sources. To investigate the potential
376 relevance of the studied animal groups to pollute the PA area, we followed a new strategy
377 by estimating the DESL. Estimates on the DESL provided clear evidence that both
378 homeothermic and poikilothermic animals must be regarded as potential sources of *E. coli*
379 and intestinal enterococci in the studied area (Table 2). In addition, the estimated DESL for
380 *E. coli* and enterococci accounted for the determined background concentrations within a
381 period of a single day on average (Table 3). However, it must be stated that the DESL
382 metric does not provide any information with respect to the actual level of water pollution.
383 Such estimates would need to consider additional information, such as the transport and
384 persistence of SFIB in the catchment area. The DESL estimate provides a novel metric to
385 evaluate the capacity of a group of animals to contribute to the overall amount of SFIB
386 produced within a defined area and time, it does not predict the actual SFIB load for a
387 specific single day.

388 Cleary, the results of this study are restricted to backwater environments in the Central
389 European region. Additionally, the investigation period spanned the warm season, from
390 March to November. For such regions, it seems likely that poikilothermic animals play only
391 a minor role during the cold period of the year (from November to February). However, an
392 investigation of the whole seasonal cycle was beyond the aim of this study. Because
393 bacterial growth conditions in poikilothermic animals strongly depend on the temperature,
394 it seems likely that Mediterranean, sub-tropical and tropical climates may support SFIB
395 production in poikilotherms far better than the PA area. We speculate that temperature
396 effects are stronger in the intestine of these animals as compared to the ambient soil,
397 because the digestive tract functions like a “bio-reactor” with increased nutrient availability
398 due to mechanical maceration and digestive processes. Further studies are needed to
399 examine this hypothesis. It would also be interesting to elucidate whether a relationship
400 between previously reported “naturalized” SFIB populations in soils or sediments (8, 11,
401 65) correlate with the abundance and activity of poikilothermic animals, especially when
402 the biomass of poikilotherms is high.
403
404 **Is there a need to re-define the fecal indicator paradigm for *E. coli* and intestinal**
405 **enterococci?** *E. coli* and intestinal enterococci have been thought to indicate fecal
406 pollution from homeothermic mammals and birds and therefore signal the potential
407 occurrence of pathogens from these groups of animals (60). The results of this study
408 strongly indicate that these fecal indicators also occur commonly in poikilothermic
409 invertebrates and vertebrates at the PA area and have the capacity to contribute to fecal
410 pollution levels. It is clear that further investigations in other areas are needed to
411 substantiate these findings. If so, there would be a need to re-evaluate the current fecal
412 indicator paradigm. Depending on the biotic and abiotic characteristics of the habitat, we
413 hypothesize that *E. coli* and intestinal enterococci may originate, to a variable extent, from

414 animals other than homeothermic animals living in and around water resources, soils and
415 sediments. These results do not suggest that *E. coli* and intestinal enterococci should not
416 be used as indicators for fecal pollution. However, our results suggest that interpretation of
417 these data, especially at low contamination levels, is more complex than previously
418 believed, and strategies to properly apply and interpret the results of these water quality
419 monitoring tools must be adapted accordingly.
420
421 **Materials and Methods**
422 **Investigation area**. The investigated porous aquifer (PA) backwater area is a typical
423 Central European riverine wetland located on the north side of the Danube River at the
424 south-eastern border of Vienna, Austria, covering an area of approximately 12 km². The
425 PA area is an important resource for drinking water and is also part of a national park. The
426 Viennese national park area plays a strategic role as a wilderness and recreation area
427 (61). Forestry and sports fishing are of minor importance due to national park regulations
428 (62). Within the PA area, the City of Vienna has designated hunting grounds that are
429 managed by the Forestry Administration Office. Detailed information on the limnologic and
430 hydrological characteristics of the PA area is available elsewhere (63, 64).
431
432 **Sampling strategy.** Fecal samples were collected directly from the investigation area
433 between 2010 and 2013 from homeothermic animals (cats, dogs, deer, wild boars and
434 birds), poikilothermic vertebrates (fish and amphibians) and invertebrates (lumbricid fauna,
435 mollusks). The species or groups of species were chosen on the basis of their occurrence
436 at the area and because they present the genera with the highest abundances and
437 biomasses. Detailed knowledge on the species distribution is available for the considered
438 national park area (65). Samples were recovered as individual fecal samples from
439 individual animals. The only exception to this sampling strategy was a fraction of the fish

fecal samples, which had to be pooled because of the very low accessible fecal material per animal to enable microbiological analysis. To ensure that sampling was representative, samples for each group were taken on several dates within a two to three 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 ± 3°C in the dark until analysis. Sampling permission had been granted according to national park regulations (MA22-229/2011, MA22-13854/2013).

Vierheilig et al. (55) previously reported on *C. perfringens* concentrations in wild homeothermic vertebrates partially using the same ruminant, carnivore, birds and wild boar fecal samples. To facilitate comparisons between the study of Vierheilig et al. (Copyright © American Society for Microbiology, Applied and Environmental Microbiology, volume 79(16), 2013, pp 5089-92, doi: 10.1128/AEM.01396-13) with the present study, all samples where a full SFIB dataset 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

466 mouflon) and *Dama dama dama* (European fallow deer). *Sus scrofa* (wild boar, n = 16, all
467 from the PA area) was included as a mixed-diet animal. Sample collection from
468 vertebrates is described in detail by Vierheilig et al. (55). Avian fecal matter from the
469 piscivorous *Phalacrocorax carbo sinensis* (great cormorant, n = 2) was collected in the PA
470 area. Samples from the other avian species (*Anas platyrhynchos* (wild duck) and other
471 *Anatidae* (n = 6), *Sterna hirundo* (common tern, n = 3), and *Charadriiformes* (waders, n =
472 4), were obtained from the closely associated Neusiedler See – Seewinkel national park
473 and an alluvial forest in Lower Austria (Neubach). Sampling in the PA area had to be
474 waived for avian species to minimize the disturbance within this area. For domesticated
475 animals (n = 17), feces from dogs (*Canis lupus familiaris*) and cats (*Felis catus*) were
476 collected by pet owners or from trails where individuals walk their dogs. The abundance of
477 small vertebrates (mice) was negligible for the experimental period (see supplemental
478 material).
479
480 **Investigated poikilothermic vertebrates and poikilothermic invertebrates**. The total
481 number of recovered fecal samples from poikilothermic vertebrates and poikilothermic
482 invertebrates was 98. The fish species *Esox lucius* (pike, n = 2), *Silurus glanis* (wels
483 catfish, n = 1), *Sander lucioperca* (pikeperch, n = 1), *Abramis brama* (bream, n = 8),
484 *Aspius aspius* (asp, n = 1), *Cyprinus carpio morpha hungaricus* (carp, n = 4), *Perca*
485 *fluviatilis* (redfin perch, n = 6), *Rutilus rutilus* (roach, n = 4), *Carassius gibelio* (Prussian
486 carp, n = 1), *Abramis ballerus* (blue bream, n = 3), *Lepomis gibbosus* (pumpkin seed, n =
487 1) and *Scardinius erythrophthalmus* (rudd, n = 1) were directly trapped by electrical fishing
488 at the PA area. The fecal material was primarily investigated as individual samples (n =
489 14). Only in cases where the accessible amount of fecal material per fish was lower than
490 0.25 g we pooled 2 to 4 samples (n = 6). Because fishermen routinely plant fish from a fish
491 farm in Lower Austria into the PA area, fish fecal samples were also obtained from that fish

492 farm (n = 7, *Cyprinus carpio morpha hungaricus*). Amphibians were caught using a hand
493 net (n = 15, *Bombina bombina* and *Pelophylax ridibundus,* all from the PA area) and were
494 briefly caged or decapitated. In addition, freshly killed amphibians from streets were also
495 collected (n = 4, *Bufo bufo,* from Lower Austria). Fecal samples from gastropods (n = 26,
496 *Arion* sp., *Helix pomatia*, *Lymnaea stagnalis*, and *Viviparus* sp.*,* all from the PA area) were
497 retrieved from living, briefly caged individuals. Earthworms (n = 26, *Allolobophora rosea*
498 *rosea*, *Helodrilus deficiens*, *Lumbricus rubellus*, *Octolasion lacteum*, *Octodrilus*
499 *transpadanus*, *Proctodrilus tuberculatus*, *Octodrilus* sp., and *Lumbricus* sp., all from the PA
500 area) were collected by digging (66), and species were identified in the lab by comparisons
501 made with formalin-preserved individuals. Reptiles were omitted from the study due to
502 their low abundance.
503
504 **Investigated soil and sediment samples**. To support comparisons of SFIB
505 concentrations in fecal samples with those in the ambient environment, soil and sediment
506 samples were analyzed from July 2010 to May 2011 (monthly, except from December to
507 February). The PA investigation area (12 km²) was categorized into the water area (1.4
508 km^2) and the different terrestrial habitat types (alluvial forest protected by a dam 7.3 km^2,
509 alluvial forest outside of the dam-protected area 0.1 km^2, bank and reef 2.3 km^2, marsh
510 0.09 km^2, and “Heißlände” 1.7 km^2, as described elsewhere) (67) The water area was
511 further categorized into several sections depending on the hydrologic conditions
512 (backwater and side ditches) (63). Seven representative locations in the PA area were
513 chosen for sediment sampling. Three sampling sites with different connectivity to the river
514 were located at the primary backwater (n = 65) as well as four sites at side ditches and
515 small ponds (n = 45). Three of the latter sampling sites were chosen due to the expected
516 high frequency (high abundance) and fecal contamination potential of ruminants and wild
517 boars at the sites, as determined from the tracks of the animals and the presence of a

518 nearby feeding area for game. Sediments were sampled in the backwaters at a water
519 depth of approximately 20 to 100 cm with a sediment corer. Each sample contained three
520 subsamples taken within 10 m², and the materials (separated into three layers: from the
521 upper first centimeter, the layer from 1 to 5 cm, and the layer from 5 to 10 cm) were
522 thoroughly mixed (68). Seven locations were chosen for soil sampling, one representing
523 the bank and reef zone (n=8), four representing alluvial forest soil (n=22), one representing
524 the so called “Heißlände”, a dry, sandy and brush-covered habitat that is not connected to
525 the groundwater (n=4), and one for a marsh zone at a small side ditch (n=7). Soil samples
526 (three subsamples within a defined 10 m² area marked by stakes) were taken from the
527 upper 10 cm (one layer) with a corer. Subsamples were thoroughly mixed and examined
528 as previously described (68). One milliliter of all fresh sediment and soil samples was
529 weighted to allow the results to be converted from CFU g^{-1} to CFU ml^{-1} (equal to cm^3).
530
531 **Microbiological analysis**. Bacteriological analysis of fecal samples was performed as
532 previously described (39), including counts of *C. perfringens*, *E. coli* and intestinal
533 enterococci according to established ISO standards. Cultivation-based ISO standard
534 methods were chosen to ensure comparability and interpretation of the results with respect
535 to routine water quality monitoring programs. In brief, *E. coli* was quantified with TBX agar
536 (44°C, 48 h) according to ISO 16649-2 (5). Enterococci were enumerated on Slanetz and
537 Bartley agar (36°C, 48 h) following ISO 7899-2 (6). *C. perfringens* was quantified in
538 accordance with ISO 14189 (7) on TSC agar (44°C, 24 h). In the fecal samples, vegetative
539 cells and spores were investigated (without pasteurization of the sample), whereas soil
540 and sediment samples were pasteurized (15 min, 60°C) such that only spores were
541 detected. For quality control, the following type strains were used: *E. coli*, NCTC 9001;
542 *Enterococcus faecalis*, NCTC 775; and *Clostridium perfringens*, NCTC 8237. Exactly
543 weighed fecal samples (approx. 1 g or less if fecal material was limited) were suspended

544 in 100 ml (or less if fecal material was limited) peptone saline diluent (250 ml distilled
545 water, 2.5 g peptone, 1.25 g NaCl, 0.87 g di-sodium hydrogen phosphate, and 0.37 g di-
546 potassium hydrogen phosphate) as described previously (39). After 30 min of suspension
547 time, samples were shaken and allowed to settle for 15 min. Sediment and soil samples
548 were prepared by mixing approximately 1 g of sample in 100 ml peptone saline diluent and
549 slowly shaking for 30 min on a shaker (Lab Tec MS30A), after which the suspension was
550 allowed to settle for 1 h (69, 70).
551 One milliliter aliquots of suspensions and prepared dilutions (10^{-2} up to 10^{-6}) were
552 analyzed by the membrane filtration method (using 0.45-µm cellulose-nitrate membrane
553 filters). The detection limit (DL) depends on the mass of sample material used and is
554 calculated by the following formula

$$DL = \frac{V}{G}$$

555
556 where DL is the limit of detection of target bacteria (given in CFU per g sample), V is the
557 volume of diluent (in ml) used for the suspension of the sample material, and G is the
558 mass of sample material in g (55). For most of the fecal samples (84%), the detection limit
559 was lower than 120 CFU g^{-1}. For 8% of samples, the detection limit was between 120 and
560 499 CFU g^{-1}. For a few samples (7%), the detection limit was between 500 and 1,000 CFU
561 g^{-1} (in cases where very little material was available). The detection limits for soil and
562 sediment were as high as 10 CFU g^{-1} fresh material. The results are given in $\log_{10}$ colony-
563 forming units (CFU) g^{-1} wet material unless otherwise specified.
564
565 **Estimating daily SFIB loads excreted by the evaluated groups of animals**. Although
566 the primary focus of the study was to establish quantitative data on the occurrence of SFIB
567 in the feces of homoeothermic 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, 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: 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 (73). 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 (section 1.1). 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 (73)). All multiplications were performed by the SPSS Monte Carlo simulation tool to estimate average, median, 5% and 95% 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 (section 1.2. and table S1). 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: 0-1, 1-5, 5-10, and below 10 cm) and soil (i.e., 2 selected layers: 0-10 cm and below 10 cm) were calculated from a digital terrain model (5 m × 5 m grids) as described elsewhere (67), including the complete sediment or soil layer above the gravel layer (cf. supplemental material, Table S2). 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. supplemental material, 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 (74-76). As all SFIB concentrations in samples from “Heißlände” (n=4) were below the detection limit, the area for “Heißlände” was not considered for the calculation. All multiplications were made using the SPSS Monte Carlo simulation tool to estimate average, median, 5% and 95% 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 paragraph above) 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.

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655

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887 TABLE 1 Occurrence (%) and abundance (log_{10} CFU g^{-1} feces) of the standard fecal
888 indicator bacteria *Escherichia coli* (a), intestinal enterococci (b) and *Clostridium*
889 *perfringens* (c) in diverse animal groups from an alluvial backwater (2010- 2013).
890
891 TABLE 2 Daily production of *Escherichia coli* , intestinal enterococci and *Clostridium*
892 *perfringens* at the study area. The relative distributions (including the median, 5^{th} and 95^{th}
893 percentiles) of shed fecal indicator bacteria were estimated by a Monte Carlo simulation
894 and are given as percentages.
895
896 FIGURE 1 a) Daily SFIB production (DESL) of animals in an alluvial backwater area
897 compared to the standing stock of *Escherichia coli* (EC), intestinal enterococci (ENT) and
898 *Clostridium perfringens* (CP) in sediment and soil of the investigation area. Values are
899 given in CFU for the whole study area. Box plots indicate the median, the 25% and 75%-
900 percentile (box), minimum and maximum values (whiskers), outliers (dots) and extreme
901 values (stars). b) Relative distribution of animal sources for the mean DESL.

Table 1

a) *E. coli*

Fecal source	N	occur-rence	abundance[a] mean	median	5%	95%	max
earthworm	26	0	n.d.	n.d.	n.d.	n.d.	n.d.
gastropod	26	77	4.2	4.2	3.0	5.5	6.8
Σ poikilothermic invertebrates	**52**	**38**	**4.2**	**4.2**	**3.0**	**5.5**	**6.8**
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
Σ poikilothermic vertebrates	**46**	**78**	**4.8**	**4.7**	**3.0**	**8.1**	**8.5**
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
Σ homeothermic vertebrates	**91**	**91**	**5.7**	**5.9**	**2.7**	**8.9**	**9.5**

902

b) enterococci

Fecal source	N	occur-rence	abundance[a] mean	abundance[a] median	percentiles 5%	percentiles 95%	max
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
Σ poikilothermic invertebrates	**52**	**50**	**5.1**	**5.6**	**2.8**	**7.1**	**7.4**
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
Σ poikilothermic vertebrates	**46**	**78**	**3.8**	**3.6**	**2.0**	**6.5**	**6.9**
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
Σ homeothermic vertebrates	**91**	**97**	**5.0**	**4.6**	**2.4**	**8.8**	**9.2**

903

c) *C. perfringens*

Fecal source	N	occur-rence	abundance[a] mean	median	percentiles 5%	95%	max
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
Σ poikilothermic invertebrates	**52**	**46**	**2.7**	**2.7**	**1.9**	**3.3**	**4.0**
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
Σ poikilothermic vertebrates	**46**	**41**	**3.2**	**3.0**	**2.0**	**4.6**	**6.1**
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
Σ homeothermic vertebrates	**91**	**34**	**4.2**	**3.8**	**1.9**	**7.4**	**7.5**

[a] Abundance data (i.e., median, mean, 5% and 95% percentiles, max) were calculated excluding non-detectable data. All results are given in CFU g^{-1} feces (wet weight); Mean, arithmetic mean; Max, maximum; n.d., not detectable. Detection limits for earthworms 1.5 to 3.0 $\log_{10}$ CFU g^{-1}, for snails $\log_{10}$ 1.9 to 3.0 CFU g^{-1}, for frogs $\log_{10}$ 1.8 to 3.0 CFU g^{-1}, for fish 0.8 to 2.4 $\log_{10}$ CFU g^{-1}, for birds 1.8 to 2.2 $\log_{10}$ CFU g^{-1}, for ruminants $\log_{10}$ 1.7 to 2.0 CFU g^{-1}, for boar $\log_{10}$ 1.7 to 2.0 CFU g^{-1}, and for carnivores $\log_{10}$ 1.9 to 2.0 CFU g^{-1}.

[b] Only one positive result.

904

905 Table 2

906

	E. coli				Enterococci				*C. perfringens*			
			Percentiles				Percentiles				Percentiles	
	Median	Mean	5%	95%	Median	Mean	5%	95%	Median	Mean	5%	95%
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	0.0	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	0.0	0.0	< 0.1	0.1	0.6	1.2	< 0.1	5.2

907

908

909 Figure 1