Assessment of the Decay Rates of Microbial Source Tracking Molecular Markers and Fecal Indicator Bacteria from Different Sources

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Assessment of the Decay Rates of Microbial Source Tracking Molecular Markers and Fecal Indicator Bacteria from Different Sources

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Running Head: Decay of fecal indicators of different sources

Keywords: Fecal pollution; Environmental persistence; Fecal indicators; Microbial Source Tracking; Inactivation

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ABSTRACT

Aims

Evaluate the T_{90} and compare the decay of different fecal indicator bacteria (FIB) and molecular microbial source tracking (MST) markers of human and animal sources during summer and winter.

Methods and Results

The persistence of *Escherichia coli* and enterococci and several MST molecular markers targeting host-specific *Bifidobacterium* and *Bacteroidales* species (BifHM, BifCW, BifPL, HF183/BFD, Rum2Bac and Pig2Bac) was assessed at the same time using mesocosms. Dialysis bags filled with diluted wastewater from different sources were kept in an outdoor water tank and monitored regularly to assess the inactivation rates. The T_{90} values of *E. coli* by culture methods ranged from 1.52 to 5.69 days in summer and 2.06 to 6.19 days in winter, whereas with qPCR 2.29-4.23 days in summer and 4.17-8.09 days in winter. T_{90} values for enterocci ranged from 1.15 to 3.10 days in summer and from 3.01 to 5.46 days in winter. Significant differences were observed between fecal sources for both markers. For the MST makers similar T_{90} values were obtained in summer (1.05 to 1.91 days), whereas higher variability was observed in winter (2.90 to 6.12 days).

Conclusions

Different inactivation kinetics were observed for the FIB from the different sources, especially for *E. coli* in ruminant samples. A higher variability among T_{90} values of the different MST markers in winter was observed, whereas similar T_{90} values were

detected in summer highlighting the stronger effect of environmental parameters during this season.

Significance and Impact of the study

The diverse inactivation rates observed in bacteria from different fecal sources have implications when these rates are used to model microbial pollution in water. The use of FIB T_{90} of different sources is essential to develop reliable predictive models. Since different inactivation of *E. coli* regarding the source of pollution has been observed, the source of the pollution has to be considered for modeling approaches.

Keywords: Fecal pollution; Environmental persistence; Fecal indicators; Microbial Source Tracking; Inactivation; Decay rates

INTRODUCTION

Besides their ecological and ecosystem functions, freshwater systems are vital to human life. Consequently, the fecal pollution of waterbodies poses a threat for human health through the transmission of waterborne pathogens; the organic matter, nutrients and pollutants that are discharged impair the environment and lead to oxygen depletion, eutrophication and the disruption of biotic communities and functions, and have an economic impact when recreational and shellfish harvesting areas are affected (Colford *et al.*, 2007; Gourmelon *et al.*, 2010). Monitoring fecal indicator bacteria (FIB) such as *E. coli* (EC) and enterococci (ENT) has led to vast improvements in public health protection over the past century (Ashbolt *et al.*, 2001). However, some epidemiological studies have failed to detect a correlation between FIB and pathogens and human health when pollution does not originate from a point source (Colford *et al.*, 2007). This fact can be attributed to the increased health risk following exposure to human waste and the

widespread distribution of FIB in different animal species, thus making it impossible to indicate the source of the pollution (Kelty *et al.*, 2012; Harwood *et al.*, 2014). Many microbial source tracking (MST) markers have been described to address this issue in recent years (Bernhard and Field, 2000; Reischer *et al.*, 2006; Mieszkin *et al.*, 2009; Gómez-Doñate *et al.*, 2012; Layton *et al.*, 2013; Green *et al.*, 2014) and have been tested successfully throughout the world (Gawler *et al.*, 2007; Ahmed *et al.*, 2009; Shanks *et al.*, 2010; Boehm *et al.*, 2013; Reischer *et al.*, 2013). Such tools make it possible to achieve a more accurate quantitative microbial risk assessment and to manage the affected area more effectively (Reischer *et al.*, 2008; Gourmelon *et al.*, 2010; Wyer *et al.*, 2010; McQuaig *et al.*, 2012; Wilkes *et al.,* 2014;Verhougstraete *et al.*, 2015).

Modeling strategies are currently used for catchment management and "ecological forecast", and involve scientists, policymakers and water stakeholders. Models use mainly hydrology and physicochemical parameters, but the number of studies to use fecal bacteria as a proxy for microbial pathogens is increasing significantly (Hipsey *et al.*, 2008; Coffey *et al.*, 2013; de Brauwere *et al.*, 2014). The incorporation of accurate inactivation values for bacteria is therefore crucial to developing better explanatory and anticipatory prediction models. However, the inactivation of bacteria is affected by multiple environmental, physicochemical and biological factors such as temperature (Bertrand *et al.*, 2012; Blaustein *et al.*, 2013), sunlight (Sinton *et al.*, 2002; Nguyen *et al.*, 2015), salinity (Schulz and Childers, 2011), predation (Gonzalez *et al.*, 1992; Menon *et al.*, 2003; Bell *et al.*, 2009) and suspended solids (Kay *et al.*, 2005). Furthermore, diverse survival rates of certain EC and ENT strains from different pollution sources due to intrinsic characteristics have been reported (Anderson *et al.*, 2005). In order to isolate the influence of specific factors, a multiple microcosm or

mesocosm approach under the same conditions is required. Despite this, the interaction between them is often the factor that enhances or decreases the inactivation rate, e.g. predation is enhanced by temperature (Barcina *et al*., 1997; Hipsey *et al*., 2008) and sunlight increases oxygen reactive species in the presence of organic matter, thereby reinforcing inactivation (Appiani and McNeill, 2015; Maraccini *et al.*, 2012). Microcosms/mesocosms in laboratory conditions that focus on single factors have therefore been found to differ from on-site experiments (Balleste and Blanch, 2010; Green *et al*., 2011; Bae and Wuertz, 2015; He *et al*., 2015). In this study, an on-site approach was used in order to assess and compare the inactivation rates of certain fecal indicator bacteria (EC and ENT) from different sources (human, porcine, bovine and poultry) and detect potential differences between them. In parallel, the inactivation of several microbial source tracking molecular markers targeting *Bifidobacterium* (BifHM, BifCW, BifPL) and host-specific *Bacteroidales* species (HF183/BFD, Rum2Bac, Pig2Bac) was analyzed in order to explore differences and assess their potential to be included in models to determine pollution sources in freshwater bodies.

MATERIALS AND METHODS

Experimental design

The inactivation of FIB and MST molecular markers was explored through an on-site experimental approach, as previously described (Balleste and Blanch, 2010; Casanovas-Massana and Blanch, 2013). To this end, dialysis bags filled with different water matrices (prepared as described below) were kept in a 60 m^3 well water-fed concrete deposit, located on the grounds of the university and used for experimental purposes. Experiments were performed in two different seasons: summer (mean experiment temperature of 24.9ºC) and winter (mean experiment temperature of 14.5ºC).

Four different water matrices were prepared, one for each fecal source: human (HM), pig (PG), ruminant (CW) and poultry (PL). To that end, sewage collected from a wastewater treatment plant that serves a population equivalent of 300,000 was used to prepare the human source matrix, while wastewater collected from three different abattoirs (for slaughtering pigs (8,000 animals per day), ruminants (400 animals per day) and poultry (96,000 animals per day)) was used to prepare the animal water matrices. Wastewater samples were diluted 1:10 in unfiltered groundwater collected from the outdoor deposit and used to fill the dialysis bags. The aim was to evaluate the T_{90} and compare the decay of the different MST markers and FIB of different sources. The experiment was performed in duplicate using dialysis bags with a porous cut-off of 14 KDa (Medicell dialysis tubing; Visking, London, UK), prepared in advance in accordance with the manufacturer's instructions. Two dialysis bags were filled per sampling day and fecal source with 50-100 ml of each water matrix. They were then placed at a depth of 10-25 cm from the water surface in the outdoor tank. Two dialysis bags per fecal source were collected at different time intervals during the sampling period: samples were collected every 24h at the beginning of the experiment and every 48-72h towards the end to cover a wider range of inactivation. The sampling periods were run for two weeks in summer (water temperature between 25-28ºC and pH 8-8.7) and for three weeks in winter (water temperature between 8.5-14.5ºC and pH 7.6-8). Prolonged incubation was avoided due to degradation of the membranes, which is faster in high temperatures. Samples were collected at around 10 a.m. every day. Turbidity (NTU) was measured in water from the dialysis bags using a HACH Ratio Turbidimeter 18900 (HACH Company, Düsseldorf, Germany).

Water temperature and pH were measured daily at sampling time and continuous environmental data (temperature, solar radiation, etc.) were obtained from local

meteorological services based in the same university facilities [http://www.infomet.am.ub.es/].

Enumeration of fecal indicator bacteria by culture media

The enumeration of EC and ENT fecal indicator bacteria was performed using the membrane filtration technique. Different volumes regarding the bacterial concentration were analyzed by filtration through a filter with a pore size of 0.45 µm (EZ-PAK, Millipore, Darmstadt, Germany). EC were enumerated in accordance with ISO standard 16649-1:2001 (ISO, 2001) and filters were placed on Mineral Modified Glutamate Agar (MMGA) (Scharlab, Barcelona, Spain) for 4 h at 37ºC to allow bacterial resuscitation to take place. After the enrichment, filters were transferred to chromogenic Tryptone Bile Glucuronic Agar medium (TBX) (Scharlab) and incubated for 18 h at 44ºC. ENT were enumerated in accordance with ISO standard 7899-2:2000 (ISO, 2000) and filters were placed in Enterococcus Agar medium (Difco, Madrid, Spain) and incubated for 48 h at 37ºC, before being confirmed with Bile Esculin Agar (Difco) for 4 h at 44ºC.

Nucleic acid extraction

Ten milliliters from each sample were concentrated by filtration through a filter with a pore size of 0.22 µm (SO-PAK, Millipore, Darmstadt, Germany), from which DNA was extracted. Filters were placed in 0.5 ml of GITC buffer (5 M guanidine thiocyanate, 100 mM EDTA [pH 8.0], 0.5% sarkosyl) and frozen at -20ºC in lysis buffer until DNA extraction. The DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany) with some modifications, as previously reported in the bibliography (Gourmelon *et al*., 2007): the proteinase K step was omitted, and 700 µl of QIAGEN AL buffer was added to the filters kept in GITC buffer. Filtration and DNA extraction controls were run together with the samples.

158 **MST marker quantification by real-time quantitative PCR**

159 The different MST markers described in the bibliography (Table S1) were analyzed by
160 real-time qPCR to target the 16S rRNA gene from host specific *Bifidobacterium*
161 species: BifHM, BifCW and BifPL (Gómez-Doñate *et al*., 2012), and host specific
162 *Bacteroidales* species: HF183 (Haugland *et al*., 2010), Rum2Bac (Mieszkin *et al*.,
163 2010) and Pig2Bac (Mieszkin *et al*., 2009). BifHM and HF183 markers have been
164 linked to human sources, BifCW and Rum2Bac to ruminant sources, Pig2Bac to porcine
165 sources and BifPL to poultry sources. The primers, probes and protocols described in
166 the bibliography were used, unless otherwise indicated in Table S1. As an alternative to
167 the culture methods, the EC 16S rRNA gene was enumerated by qPCR as an indirect
168 measure of total EC genomes using a TaqMan assay with the probe and primers
169 previously described (Huijsdens *et al*., 2002).

170 Amplification was performed in a 20 µl reaction mixture using TaqMan Environmental
171 Master Mix 2.0 (Applied Biosystems, Foster City, CA, USA) in a StepOne Real-Time
172 PCR System (Applied Biosystems). The mixture contained 900 nM of each primer, 250
173 nM of the corresponding probe and 5 µL of the DNA sample. With regard to the
174 quantification of HF183, the mixture contained 1 mM of each primer, 80 nM of 6-
175 carboxyfluorescein (FAM)-labeled TaqMan probe (Applied Biosystems) and 5 µL of
176 DNA. The thermal-cycler conditions were 10 min at 95°C, 40 denaturation cycles of 15
177 s at 95°C, and 1 min of annealing and extension at 60°C.

178 All samples, negative controls, and extraction and filtration blanks were run in
179 duplicate, and analyses were repeated when discordance between duplicates was
180 detected. Ten fold dilutions of the wastewater samples were assayed to detect inhibition
181 problems. Results were expressed as gene copies/100 ml (GC 100 ml^{-1}). Five points on

the standard curves were included in duplicate for each run and were generated from different 10-fold serial dilutions of the linearized plasmid containing the target gene. pGEM®-T Easy Vector (Promega, Madison, WI, USA) was used for HMBif, PLBif, CWBif and EC, linearized using ScaI restriction digestion; pBluescript II SK (Agilent Technologies, Santa Clara, CA, USA) was used for HF183, linearized using NotI restriction digestion; and pCR2.1 was used for Rum2Bac and Pig2Bac (kindly supplied by Professor M. Gourmelon, IFREMER, Brest, France), linearized using NotI restriction digestion. The quantification limits for Pig2Bac, Rum2Bac and HF183 were 70, 4 and 3 gene copies (GC) per reaction, respectively, the quantification limits for *Bifidobacterium* markers were 45, 40 and 25 GC per reaction for the human, poultry and ruminant assays, respectively and the quantification limit for EC was 16 GC per reaction.

Data analysis

The results obtained from the culture medium analyses (expressed as colony forming units per 100 ml (CFU 100 ml^{-1})) and the qPCR analyses (expressed as gene copies per 100 ml (GC 100 ml^{-1})) were used to calculate the inactivation kinetics for EC, ENT and the six MST molecular markers analyzed in each experiment. Measurements were $\log_{10}$ transformed and daily reductions were used to calculate the first-order decay models proposed by Chick and co-workers (Chick, 1908). The following equations were used to calculate the decay rates (K_s) and the time required to achieve a 90% reduction in the initial population (T_{90} values [days]):

$$N_t/N_0 = 10^{-k_s t}$$

$$T_{90} = 1/k_s$$

where N_t is the CFU 100 ml^{-1} or 16S rRNA GC 100 ml^{-1} at time t, and N_0 is the CFU 100 ml^{-1} or 16S rRNA gene GC 100 ml^{-1} at time 0.

The data were analyzed using the statistical software R version 3.2.2 (R Core Team, 2016) through the RStudio interface (RStudio Team, 2015), and the "PMCMR" (Pohlert, 2016). Two-way analysis of variance (ANOVA) with Tukey's honest significant difference (HSD) post-hoc multiple-comparison test was performed to detect significant differences between markers, seasons and fecal sources. Significant differences in means were determined at an alpha level of 0.05. The Pearson's correlation coefficient was used to determine any relationship between cultured-EC and qPCR-EC measurements. SigmaPlot 10.0 (Systat Software Inc.) was used to plot the inactivation graphs.

RESULTS

Initial and environmental conditions

During the summer assay, the water temperature ranged from 25 ºC to 28ºC, the average environmental temperature ranged from 21.7 ºC to 28.3ºC, with a mean of 24.9ºC, and the maximum global solar radiation ranged from 784 W/m^2 to 1100 W/m^2 (Table S2). In winter, the water temperature ranged from 8.5ºC to 11ºC, with a peak of 14.5ºC on the last sampling day, the average environmental temperature ranged from 11.2ºC to 18.2ºC, with a mean of 12.9ºC, and maximum global solar radiation values ranged from 273 W/m^2 to 871 W/m^2 (Table S2). The groundwater used to prepare the different water matrices presented conductivity of around 2.0-3.5 mS during both seasons, with a pH of 8-8.7 in summer and 7.6-8 in winter at sample collection times. Thus, assays run in summer were subjected to higher temperatures, higher solar irradiation, higher alkalinity

due to an increase in photosynthesis, and greater expected predation rates, as reported elsewhere (Barcina *et al*., 1997), than in winter.

Low levels of EC and ENT were detected in the groundwater used as a matrix before the wastewater was added (EC <10 CFU 100 ml^{-1} and ENT < 5 CFU 100 ml^{-1}). The MST molecular markers were not detected, with the exception of BifPL, which presented values of 3.1 x 10^3 and 6.6 x 10^3 GC 100 ml^{-1} (Table S3). However, the different MST markers were detected in great abundance in the original wastewater samples used (Table S3) and remained above the limit of detection of the qPCR methods after being diluted 1:10 to prepare the different water matrices. Turbidity differed between experiments, with lower values in the HM and PL samples (17-27 and 20-34 NTU) than in the PG and CW samples (35-113 and 40-120 NTU). Turbidity increased over the course of the experiment due to aggregation of the suspended organic matter (OM) during both seasons.

Persistence of fecal indicator bacteria

In summer, the inactivation of EC and ENT by culture methods (culture-EC) followed a first-order exponential model, whereas a "shoulder" pattern was mostly observed in winter (Fig. 1). However, in these cases, a linear decay followed the one to two days of the no-decay period (lag). All points were therefore used for the analysis considering simplest model. For EC measured by qPCR (qPCR-EC), similar trends were observed (Fig. 1).

The K_s and T_{90} were measured for every source, indicator, season and replicate (Table 1, Table S4). Different T_{90} values for FIB were observed (EC and ENT) with respect to the pollution sources analyzed. In this study, FIB from PG and CW sources showed generally higher persistence rates than those observed for HM and PL sources (Table 1).

These differences were significant for culture-EC, qPCR-EC and ENT when the seasonal effects were considered (P-value < 0.001). However, the T_{90} values of ENT showed higher levels of similarity between sources (1.24-3.12 in summer and 3.00-5.54 in winter) than those observed for EC, in particular for culture-EC (1.54-5.73 in summer and 1.64-6.35 in winter), which presented wider variability between sources.

Seasonal differences between the T_{90} values were also analyzed. In this case, ENT and qPCR-EC showed higher inactivation rates in summer than in winter, and lasted around twice as long at low temperatures (P-value < 0.001 and P-value = 0.006, respectively). Although not statistically significant, a higher inactivation rate in summer was generally observed by culture-EC, with the exception of PL samples, for which no differences were reported between the seasons.

At T_0, the abundance of EC detected by qPCR was 1.8–7.9 times higher than that detected by culture methods. This difference was greater in winter (4.2-7.95) than in summer (1.8–4.5), thus suggesting that there were more dead or viable but non-culturable bacteria at cold temperatures. The differences among the two detection methods increased with time, and were 250-1100 times greater by the end of experiment. When a general comparison of culture-EC and qPCR-EC methods was carried out, a higher T_{90} was observed with qPCR methods, but similar T_{90} values were reported in two assays (PL summer and CW winter) and higher T_{90} values were observed through the culture technique in CW summer. When bovine EC T_{90}s were disregarded, a correlation between the two techniques was observed (Pearson's r = 0.88).

When the persistence of the FIBs from the same fecal source was compared by taking the seasons into account, there were generally no significant differences between the T_{90}

values of culture-EC and those of ENT. Meanwhile, the persistence rate of both differed from the T_{90} of qPCR-EC, thus revealing that the DNA showed a longer detection time, as expected (P-value < 0.001 for culture-EC and ENT in HM and PG, P-value = 0.005 for culture-EC and 0.018 for ENT in PL). The only exception was in the ruminant experiment, in which the T_{90} values observed for culture-EC and qPCR-EC were similar, but differed from those of ENT (P-value < 0.001).

Persistence of MST markers by qPCR

The persistence of the markers BifHM, BifCW, BifPL targeting human, bovine and poultry-specific *Bifidobacterium* species and the markers HF183, Pig2Bac and Rum2Bac targeting human, porcine and bovine-related *Bacteroidales* group species was analyzed by qPCR. The two human markers (BifHM and HF183) were analyzed in the prepared sewage matrix. At the start of the experiment (T_0), both markers showed similar concentrations ($4.1 \cdot 10^6$ $\pm 0.9 \cdot 10^6$ and $2.0 \cdot 10^6$ $\pm 1.0 \cdot 10^6$ GC 100 ml^{-1}, respectively). They were detected for just 2–3 days in summer when T_{90} values of 2.20 and 1.05 days, respectively, were obtained. However, they were detected during the 10 sampling days in winter (Fig. 2), when higher T_{90} value differences were reported between them: 6.12 and 3.26 days, respectively (Table 2, Table S5).

The levels of the BifCW marker were much higher than those of Rum2Bac at the start of the experiment ($4.7 \cdot 10^6 \pm 4.2 \cdot 10^6$ GC 100 ml^{-1} and $8.3 \cdot 10^4 \pm 6.5 \cdot 10^4$ GC 100 ml^{-1}, respectively). Despite this, both markers were detected for just 3 days in summer and showed similar T_{90} values (1.62 and 1.48 days, respectively). In winter, however, BifCW was detected for 15 days and Rum2Bac for 9 days, with respective T_{90}s of 5.17 and 4.64 days (Table 2, Table S5).

297 The porcine MST marker Pig2Bac presented a high concentration ($3.5 \cdot 10^8 \pm 2.5 \cdot 10^8$ GC
298 100 ml^{-1}) at the start of the experiment, which made the detection time longer: 8 days in
299 summer and 15 days in winter (Table 2). Nonetheless, low T_{90} values were reported
300 (1.60 days in summer and 2.90 days in winter). The BifPL levels were similar to the
301 other molecular markers ($2.9 \pm 1.5 \cdot 10^6$ GC 100 ml^{-1}). This MST marker was detected
302 for 7 days in summer, with a T_{90} value of 1.91 days, and for 12 days in winter, with a
303 T_{90} value of 3.77 days.

304 The T_{90} values observed in summer for the six molecular markers showed very similar
305 values; they ranged from 1.05 days for HF183 to 2.20 days for BifHM, and non-
306 significant differences were observed between the six. However, Tukey's HSD post-hoc
307 test showed significant differences between BifHM and HF183 (P-value = 0.0415).
308 Moreover, differences between the T_{90} values of molecular markers were observed in
309 winter (P-value < 0.001), and these ranged from the shortest T_{90} of 2.90 days for
310 Pig2Bac to the longest of 6.12 days for BifHM (Table 2, Table S5). Tukey's HSD post-
311 hoc test showed no differences between Rum2Bac and BifCW or between Pig2Bac,
312 HF183 and BifPL. When the seasonal differences for each molecular marker were
313 analyzed, all persisted longer in winter than in summer, but the differences were non-
314 significant for Rum2Bac and BifPL (Table 2). Additionally, since three of the markers
315 target *Bifidobacterium* species and the other three species are from the *Bacteroidales*
316 group, the differences between the two groups were analyzed. In general,
317 *Bifidobacterium* markers persisted longer than *Bacteroidales* in both seasonal assays (P-
318 value: 0.020).

319 The T_{90} of MST molecular markers and FIB were compared for each fecal source.
320 Similar inactivation rates of HF183 were observed with culture-EC and ENT, whereas
321 the inactivation rate of BifHM (especially in winter) was significantly higher than the

rest of the markers (P-value < 0.001 for HF183, culture-EC, ENT and 0.002 for qPCR-EC). For the ruminant wastewater experiments, a similar inactivation rate was observed for Rum2Bac, CWBif and ENT. In the pig wastewater experiments, Pig2Bac persisted for significantly less time than FIB (P-value = 0.001 for culture-EC and P-value < 0.001 for ENT and qPCR-EC), and for the poultry experiment, BifPL showed a similar persistence rate to the FIB markers.

DISCUSSION

In this study, an on-site experimental approach was used to compare the inactivation rates of FIB and several MST molecular markers in water from different fecal pollution sources. The approach used dialysis bags to ensure that the water and solutes diffused into the environment and were exposed to multiple environmental parameters. Therefore, the system responded to the physical, chemical and environmental changes of the surroundings. The first-order decay function was used in order to be able to compare all decays using the simplest model. However, if this data would be used for modeling purposes a delayed Chick-Watson model should be explored for some winter assays when greater initial resistance to inactivation has been detected (Brooks and Field, 2017).

Several studies have analyzed the persistence of EC and ENT, generally coming from a unique pollution source; however they do not question the fact that there may be differences among different pollution sources (Marti *et al*., 2011; Solecki *et al*., 2011; Blaustein *et al*., 2013; Ahmed *et al*., 2014). This aspect is in particular relevant for modeling, when different inactivation rates regarding the source have to be considered to predict the duration of a specific pollution event. In this study different inactivation rates were observed between the FIB depending on the source of the fecal pollution; this

346 was particularly evident for EC in pig and ruminant assays. With respect to the ruminant
347 experiments, high T_{90} values were obtained for FIB compared to the other assays. They
348 also showed smaller differences between the seasons, thus suggesting that other factors
349 play a key role in the inactivation of the markers and have to be considered. For
350 example, other studies reported differences between strains of EC and ENT of various
351 animal sources (McLellan *et al*., 2003; Ahmed *et al*., 2006). Furthermore, certain strains
352 isolated from diverse sources showed different adaptations to the environment, with
353 contrasting persistence times (Anderson *et al*., 2005; He *et al*., 2015). For example, FIB
354 from cattle manure have been reported to have a higher persistence rate than sewage
355 (Korajkic *et al*., 2013). This variability has been attributed to the different intrinsic
356 properties of FIB from particular fecal sources or to the characteristics of waste
357 composition and microbial community structures in the original sample. In this study,
358 the water collected from the ruminant and porcine abattoirs contained more organic
359 matter (OM) (e.g. blood) than HM and PL samples. The OM aggregates throughout the
360 experiment probably acted as a shelter for live bacteria to protect them from sunlight
361 and predators. Furthermore, dissolved and particulate OM has been reported to act as a
362 photosensitizer. When it absorbs solar irradiation, it produces oxygen reactive species
363 such as singlet oxygen ($^1O_2$), superoxide and hydroxyl radicals (Appiani and McNeill,
364 2015). A lower inactivation rate of EC than ENT in a wetland system has been
365 associated with the null effect of singlet oxygen on the former (Nguyen *et al*., 2015).
366 The difference was attributed to cell membrane characteristics: gram-negative bacteria
367 have an extra outer membrane that may limit the uptake of exogenous photosensitizers,
368 thereby reducing cell damage. This hypothesis could also explain the greater persistence
369 rate of EC compared to ENT in the ruminant experiment. It is important to notice that
370 this accumulation of OM may be an artifact of performing the experiment inside

dialysis bags, in real environmental conditions the aggregates maybe dissolved or deposited.

Different fecal sources contain diverse bacterial strains with different levels of resistance to environmental processes. This can have implications if this parameter is used to model microbial populations in water and can reduce the effectiveness of water quality protection standards. For example, if the T_{90} obtained from sewage in this study had been used in the model, the presence of EC may have been underestimated when the pollution originated from ruminant sources. A recent meta-analysis using first-order decay rates, based on previously published data, show a large variation in the data leading to wide credible intervals ranging for example from a T_{90} of 1.6 to 55.7 in case of *E. coli* (Brooks and Field, 2016). This study establishes a mean decay rate constant with credible variation intervals. The majority of FIB results in our study fit within these intervals except ruminant EC and ENT in both seasons and swine ENT in summer showing higher T_{90} values. Since the source of the pollution may introduce differences in the decay, incorporating MST molecular markers can result in additional information about the catchment area, by helping identify not only land uses, but also proper FIB kinetics.

As expected, the detection of EC by qPCR showed a longer inactivation rate and was generally twice as long as when detected by culture methods. A few exceptions were reported in summer, when a higher inactivation rate was observed, thereby suggesting that both the decay rate of microorganisms and the total degradation of the DNA were affected. However, the numbers of EC enumerated by qPCR and culture methods diverged throughout the experiment and were greater by the end. This suggests that there was less recovery of culturable EC than DNA detection over time. This fact has to

be seriously considered when considering changing the detection of EC for water quality analysis by culture to molecular methods (Heijnen and Medema, 2009).

In summer, a more homogenous decay rate was observed between the different MST markers analyzed in this study compared with the winter decay rates. This similarity may be associated with the high number of factors that inactivated the bacteria and degraded the DNA (e.g. temperature, predation and irradiation). In winter, meanwhile, more diverse decay trends were observed between the markers. When there is less sunlight, lower temperatures and less predation activity, persistence and inactivation rates are probably linked to the intrinsic characteristics of bacteria (cell resilience, membrane structure, physiological characteristics, etc.) and show a more diverse pattern, as also revealed by the biphasic behavior in some instances. Thus, in summer, the similar behavior can be assigned to the different MST molecular markers, whereas in winter, it is necessary to consider the different inactivation rates of the markers, as already reported for mtDNA and *Bacteroidales* markers (Green *et al*., 2011; He *et al*., 2015). In this study, a longer persistence time was detected for host-specific *Bifidobacterium* species compared to *Bacteroidales*. A longer persistence time for *Bif. adolescentis* compared to HF183 has previously been reported in other studies that show similar T_{90} values (when temperature differences are taken into account) (Jeanneau *et al*., 2012; He *et al*., 2016). Since both are anaerobic genera, the different decay rates could be attributed to different oxygen tolerance times (Rolfe *et al*., 1978; Andriantsoanirina *et al*., 2013) or different predation rates, depending on the cell wall characteristics (gram positive vs. negative) (Barcina *et al*., 1997; Pernthaler, 2005).

Several studies have been conducted regarding the inactivation of FIB and MST molecular markers; some rely on microcosms or laboratory conditions, whereas others

rely on on-site assays that take environmental parameter fluctuations into account (Ahmed *et al*., 2014). Moreover, they use different methods to report the results (e.g. the use of linear or bi-phasic models to calculate the inactivation rate; the use of common or natural logarithms to calculate the inactivation constant; and reporting of the T_{90} or T_{99}) and different parameters throughout the experiments (sunlight, predation, temperature, etc.), thereby making it difficult to compare the data. However, the decay constant measured at different temperatures can be adjusted to the same temperature by means of the Arrhenius equation (Van Kessel *et al*., 2007; Bae and Wuertz, 2015). A recent publication compiled some studies and standardized the results so that they could be compared (natural log and T_{99}). The data obtained in this study have been compared with this approach, and similar inactivation rates for HF183 and other *Bacteroidales* molecular markers have shown to be consistent with other studies (K_s ranging between -0.48 and -1.1) (Bae and Wuertz, 2009; Dick *et al*., 2010; Jeanneau *et al*., 2012; Liang *et al*., 2012; Bae and Wuertz, 2015; He *et al*., 2016) and fall inside the credible interval established by Brooks (Brooks and Field, 2016). Other ruminant and porcine *Bacteroidales* molecular markers showed similar inactivation rates to those obtained in this study for Rum2Bac and Pig2Bac markers (Marti *et al*., 2011; Solecki *et al*., 2011; Sokolova *et al*., 2012; Brooks and Field 2016). However, other experiments have shown shorter or longer persistence rates that could be attributed to the different experimental designs, which are usually performed in microcosms under controlled conditions (darkness, filtered water, etc.) (Okabe and Shimazu, 2007; Bae and Wuertz, 2015). Even though the initial concentrations of these molecular markers were higher than those of FIB, the MST molecular markers studied were detected for only 2-3 days in summer, with the exception of Pig2Bac and BifPL. Although Pig2Bac showed a lower persistence rate, its longer detection time can be explained by the high concentrations in

444 the initial sample (mean $5 \cdot 10^9$ GC 100 ml^{-1}). A slightly higher T_{90} value with a smaller
445 detection limit for BifPL made the detection time of this marker longer. Thus, the
446 marker concentrations at the beginning of the assays (point source in environmental
447 situations), together with the limit of detection of the molecular method, are important
448 factors that should be considered when the MST molecular markers are selected.

449 The use of reliable inactivation values is essential for water quality management
450 strategies when developing explanatory and anticipatory prediction models.Many
451 studies have used FIB, mainly EC, as a proxy for microbial pathogens (Hispey *et al*.,
452 2008; Coffey *et al*., 2013; de Brauwere *et al*., 2014; Jonsson and Agerberg 2015).
453 However, in this study a different inactivation rate has been showed for FIB from
454 different fecal pollution sources showing, what can have implications when using this
455 parameter for modeling the microbial populations in water, and can reduce efficacy of
456 regulatory standards for protection of water quality. For example, if the T_{90} obtained in
457 this study from sewage would be used to model, the presence of EC may be
458 underestimated when the pollution is originated from ruminant sources. Therefore, the
459 inclusion of MST markers in such models can provide complementary information of
460 the catchment not only by helping to identify land uses but also to select the proper FIB
461 kinetics and obtain more accurate models.

462 In conclusion, reporting and understanding bacterial inactivation rates and
463 systematizing the method used by studies may facilitate data collection and help select
464 the ideal parameters for the geographical area or climatic conditions. The inactivation of
465 FIB and MST molecular markers is affected by: a) the season, which affects
466 temperature, sunlight, predation rates and alkalinity; b) different intrinsic characteristics
467 of the indicator/marker strains, which are more important in winter, when the

environmental parameters have less impact; c) the source of the pollution and therefore bacteria with different levels of resistance to the environmental conditions; and/or d) the role of the OM associated with the water matrix as a shelter for microorganisms. The different decay trends for FIB observed in human and animal fecal pollution sources is a key issue when natural inactivation rates are used to model the kinetics of the fecal pollution in a water catchment. Thus, incorporating the MST molecular markers would facilitate microbial fecal pollution modeling.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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719 Figure 1. Persistence of *E. coli* measured by culture (A, D) and qPCR methods (B, E)
720 and enterococci (C, F) for each experimental approach, including four fecal pollution
721 sources: human (HM, filled circle), ruminant (CW, triangle), porcine (PG, filled
722 triangle) and poultry (PL, circle) and two seasons: summer (A, B and C) and winter (D,
723 E and F). X-axis is time expressed in days.

724

725 Figure 2. Inactivation of host-specific *Bifidobacterium* and *Bacteroidales* proposed as
726 MST molecular markers for human (BifHM (circle) and HF183 (triangle)), ruminant
727 (Rum2Bac (square), BifCW (rhombus)), porcine (Pig2Bac (filled triangle)) and poultry
728 (BifPL (hexagon)) analyzed in this study in summer (A) and winter (B).

729

730 Table 1. T_{90} and standard deviation (SD) of *E. coli* measured by culture and qPCR
731 methods (culture-EC, qPCR-EC) and enterococci (ENT) for each fecal source: human
732 (HM), ruminant (CW), porcine (PG) and poultry (PL)), and season (summer and
733 winter). Values are the average of two independent experiments.

Fecal source		**culture-EC**		**qPCR-EC**		**ENT**	
		T_{90}	SD	T_{90}	SD	T_{90}	SD
HM	Summer	1.52	0.08	2.62	0.16	1.15	0.17
	Winter	2.06	0.01	4.26	0.39	3.01	0.11
CW	Summer	5.69	0.13	3.87	0.58	2.19	0.22
	Winter	6.19	0.16	6.14	0.64	4.44	0.01
PG	Summer	2.71	0.10	4.23	0.73	3.10	0.25
	Winter	4.55	0.30	8.09	0.38	5.46	0.18
PL	Summer	2.34	0.17	2.29	0.11	1.39	0.05
	Winter	2.21	0.07	4.17	0.41	3.52	0.19

734

735

736 Table 2. T_{90} of the six MST molecular markers analyzed in the inactivation experiments
737 (BifHM, BifCW, BifPL, HF183, Rum2Bac and Pig2Bac), standard deviation (SD) and
738 days during they were detected throughout the experiment. Values are the average of
739 two independent experiments.

	Summer			Winter			Diff. seasons P-value
	T_{90}	SD	Days detected	T_{90}	SD	Days detected	
BifHM	2.20	0.27	3	6.12	0.03	10	**0.0282**
HF183	1.05	0.19	2	3.26	0.23	10	**0.0099**
BifCW	1.62	0.12	3	5.17	0.22	15	**0.0073**
Rum2Bac	1.48	0.02	3	4.64	0.44	9	0.0616
BifPL	1.91	0.58	7	3.77	0.01	12	0.1377
Pig2Bac	1.60	0.01	8	2.90	0.10	15	**0.0311**

740

741 **SUPPORTING INFORMATION**

742 Table S1. List of primers and probes used for quantitative real time PCR to detect the
743 16S rRNA gene of host-specific microbial molecular markers and *E. coli*.

744

745 Table S2. Climatic data including minimum, maximum and average environmental
746 temperature (Tmin, Tmax, Tmean), maximum and average global radiation (Max GR,
747 Mean GR) and daily cumulative global solar radiation (DCGSR) for all the sampling
748 dates.

749

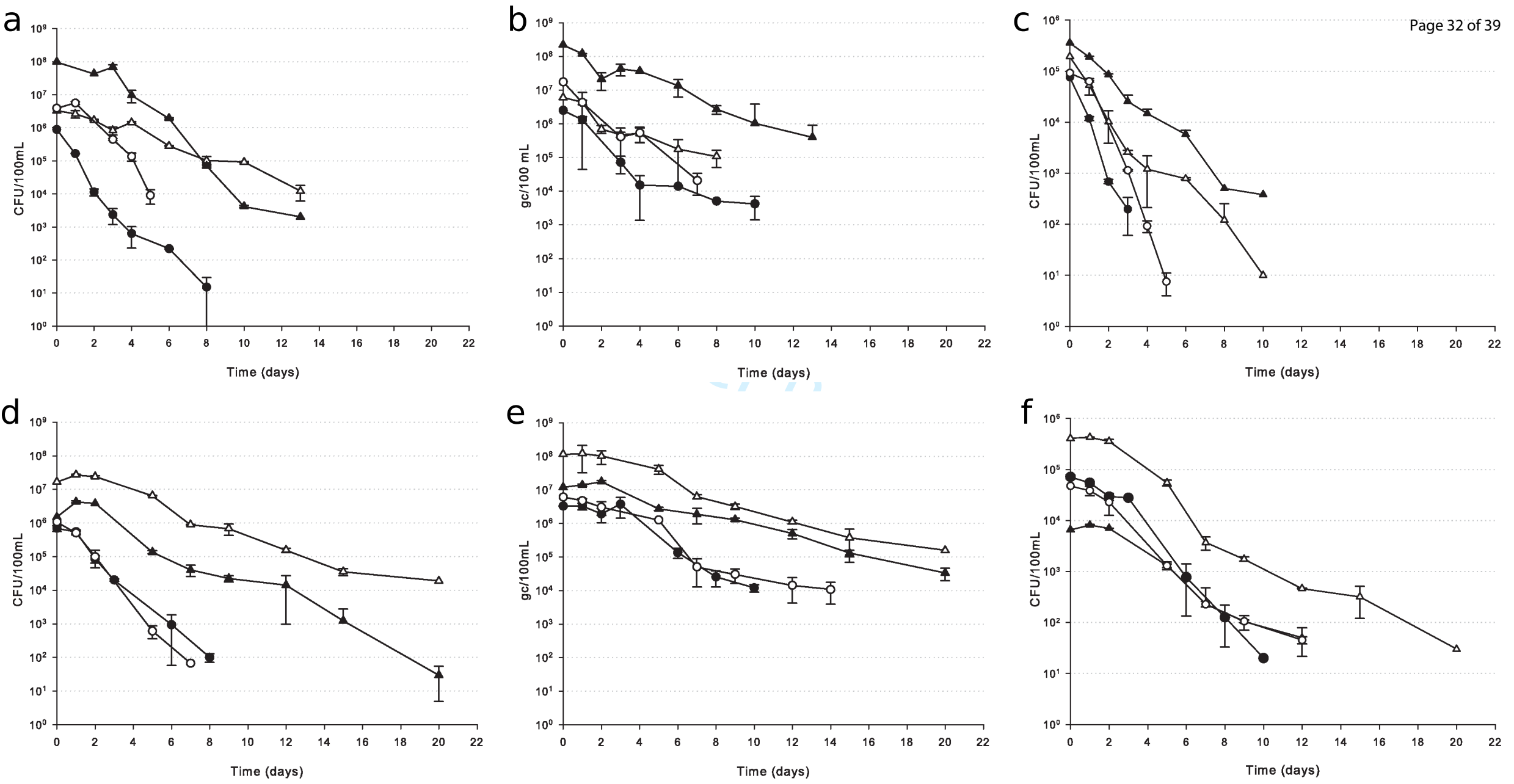
750 Table S3. Enumeration of fecal indicator bacteria (*E. coli* and Enterococci (CFU 100
751 ml^{-1}) and microbial source tracking molecular markers (BifHM, HF183, BifCW,
752 Rum2Bac, Pig2Bac, BifPL (gc 100 ml^{-1}) at source samples (groundwater and
753 wastewater samples from human (HM), ruminant (CW), porcine (PG) and poultry (PL)
754 sources) used to prepare the different experiments. ND: Not detected; NA: Not available

755

756 Table S4. Inactivation data (K_s, R^2, and T_{90}) of *E. coli* measured by culture and qPCR
757 methods (culture-EC, qPCR-EC) and enterococci (ENT) for each experiment: human
758 sewage (HM), cow wastewater (CW), porcine wastewater (PG) and poultry wastewater
759 (PL)) performed during two seasons (summer and winter).

760

761 Table S5. Inactivation data for each experiment (K_s, R^2, and T_{90}) of the six microbial
762 source tracking molecular markers (MST) evaluated in this study.


a
CFU/100mL
Time (days)
b
gc/100 mL
Time (days)
c
CFU/100mL
Time (days)
d
CFU/100mL
Time (days)
e
gc/100mL
Time (days)
f
CFU/100mL
Time (days)

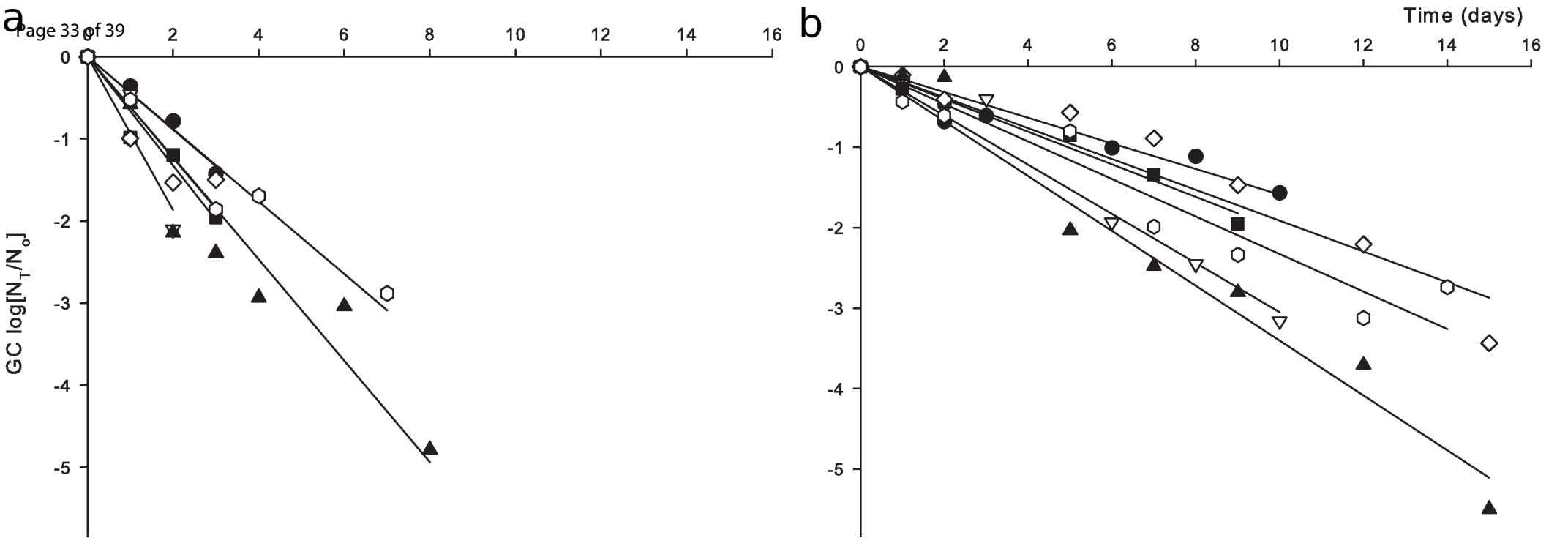

a
b
Time (days)
GC log[N_T/N_o]
0
2
4
6
8
10
12
14
16
-1
-2
-3
-4
-5

Table S1. List of primers and probes used for quantitative real time PCR to detect the 16S rRNA gene of host-specific microbial molecular markers and *E. coli*.

Oligonucleotide	Sequence (5' - 3')	Reference
EC-For	CATGCCGCGTGTATGAAGAA	(1)
EC-Rev	CGGGTAACGTCAATGAGCAAA	
EC-Probe	TATTAACTTTACTCCCTTCCTCCCCGCTGAA	
HF183	ATCATGAGTTCACATGTCCG	(2)
BFDRev	CGTAGGAGTTTGGACCGTGT	
BFDFAM	FAM-CTGAGAGGAAGGTCCCCCACATTGGA-BHQ1	
BacFw	GGCGCACGGGTGAGTAAC	Gomez-Donate, personal communication
BacRev	TGTGGGGGACCTTCCTCTC	
Pig2Bac	6-FAM-TCCACGGGATAGCC-MGB-NFQ	(3)
BacB2-590F	ACAGCCCGCGATTGATACTG	Modified from (4)
Bac708Rm	CAATCGGAGTTCTTCGTGATATCTAA	
Rum-2-Bac	FAM-ATGAGGTGGATGGAATT-MGB-NFQ	
Bif-F	TTCGGGTTGTAAACCGCTTTT	(5)
Bif-R	TACGTATTACCGCGGCTGCT	
HMBif	FAM-TCGGGGTGAGTGTACCT-MGB-NFQ	
CWBif	FAM-TTCGGCCGTGTTGAGT-MGB-NFQ	
PLBif	FAM-GAGAGTGAGTGTACCCGTT-MGB-NFQ	
PGBif	FAM-CGCAAGTGAGTGTACCTT-MGB-NFQ	

Table S2. Climatic data including minimum, maximum and average environmental temperature (Tmin, Tmax, Tmean), maximum and average global radiation (Max GR, Mean GR) and daily cumulative global solar radiation (DCGSR) for all the sampling dates.

	Tmin	Tmax	Tmean	Max GR W/m2	Mean GR W/m2	DCGSR W/m2
08/07/14	18.2	25.9	21.7	1030	335	47968
09/07/14	19.9	26.2	21.8	1033	271	38872
10/07/14	19.6	28.7	23.3	974	353	50513
11/07/14	19.3	28.6	23.4	966	348	49876
12/07/14	19.7	24.9	22.7	1100	309	44235
13/07/14	19.7	28.5	24.1	1017	338	48395
14/07/14	22.4	26.1	23.6	1076	197	28271
15/07/14	21.8	28.2	25.0	784	216	30889
16/07/14	22.6	30.9	27.1	956	339	48606
17/07/14	24.7	31.1	28.3	947	334	47857
18/07/14	24.2	32.1	27.7	975	285	40765
19/07/14	24.7	29.8	27.0	1009	269	38578
20/07/14	22.7	32.5	27.7	1001	291	41649
21/07/14	22.1	29.8	25.1	980	345	49477
Mean	**21.54**	**28.81**	**24.89**	**989.14**	**302.14**	**43282.21**
10/02/15	6.9	18.1	12.0	620	162	23396
11/02/15	8.1	16.3	11.8	635	127	18273
12/02/15	8.9	15.4	11.8	605	116	16680
13/02/15	11.2	17.2	13.3	610	151	21741
14/02/15	9.4	16	13.1	273	67	9672
15/02/15	10.1	15.4	12.4	491	77	11159
16/02/15	8.8	17.4	12.8	646	152	21837
17/02/15	11	16.7	13.4	269	53	7700
18/02/15	10.5	16.2	13.0	696	144	20798
19/02/15	8.8	14.6	11.6	647	169	24326
20/02/15	7.9	15.8	11.3	636	164	23596
21/02/15	8.9	13.3	11.2	583	66	9460
22/02/15	7.7	17	11.8	708	196	28192
23/02/15	7.4	20.3	13.3	631	128	18510
24/02/15	8.6	14.8	11.3	705	195	28118
25/02/15	9	20.1	13.2	503	131	18876
26/02/15	10.6	17.5	13.5	619	88	12703
27/02/15	10	17.1	13.3	871	153	22102
28/02/15	7.6	14.9	11.2	716	164	23581
01/03/15	11.5	19.3	15.2	694	150	21510
02/03/15	14.5	23.9	18.2	729	129	18447
03/03/15	13.1	18.1	15.1	733	181	25860
04/03/15	8.5	14.5	11.6	319	31	4435
05/03/15	7.6	20.3	13.7	787	224	31990
Mean	**9.4**	**17.1**	**12.9**	**613.6**	**134.1**	**19290.1**

Table S3. Enumeration of fecal indicator bacteria (*E. coli* and Enterococci (CFU 100 ml^{-1}) and microbial source tracking molecular markers (BifHM, HF183, BifCW, Rum2Bac, Pig2Bac, BifPL (gc 100 ml^{-1}) at source samples (groundwater and wastewater samples from human (HM), ruminant (CW), porcine (PG) and poultry (PL) sources) used to prepare the different experiments. ND: Not detected; NA: Not available

	Groundwater		**HM**		**CW**		**PG**		**PL**	
	Summer	**Winter**	**Summer**	**Winter**	**Summer**	**Winter**	**Summer**	**Winter**	**Summer**	**Winter**
E. coli	$8.0 \cdot 10^{0}$	$1.5 \cdot 10^{0}$	$7.09 \cdot 10^{6}$	$8.14 \cdot 10^{6}$	$2.25 \cdot 10^{7}$	$1.50 \cdot 10^{7}$	$9.95 \cdot 10^{8}$	$1.07 \cdot 10^{7}$	$2.86 \cdot 10^{7}$	$1.07 \cdot 10^{7}$
ENT	$3.5 \cdot 10^{0}$	$0.5 \cdot 10^{0}$	$7.95 \cdot 10^{5}$	$7.45 \cdot 10^{5}$	$2.05 \cdot 10^{6}$	$1.95 \cdot 10^{6}$	$2.65 \cdot 10^{6}$	$5.50 \cdot 10^{4}$	$9.34 \cdot 10^{5}$	$5.18 \cdot 10^{5}$
BifHM	ND	ND	**$9.96 \cdot 10^{7}$**	**$5.40 \cdot 10^{7}$**	NA	NA	NA	NA	NA	NA
HF183	ND	ND	**$1.14 \cdot 10^{8}$**	**$2.64 \cdot 10^{6}$**	NA	NA	NA	NA	NA	NA
BifCW	ND	ND	NA	NA	**$3.85 \cdot 10^{7}$**	**$7.90 \cdot 10^{7}$**	NA	NA	NA	NA
Rum2Bac	ND	ND	NA	NA	**$8.84 \cdot 10^{6}$**	**$3.79 \cdot 10^{5}$**	NA	NA	NA	NA
Pig2Bac	ND	ND	NA	NA	NA	NA	**$4.19 \cdot 10^{9}$**	**$7.06 \cdot 10^{8}$**	NA	NA
BifPL	$6.63 \cdot 10^{3}$	$3.12 \cdot 10^{3}$	NA	NA	NA	NA	NA	NA	**$2.14 \cdot 10^{7}$**	**$1.65 \cdot 10^{7}$**

Table S4. Inactivation data (K_s, R^2, and T_{90}) of *E. coli* measured by culture and qPCR methods (culture-EC, qPCR-EC) and enterococci (ENT) for each experiment: human sewage (HM), cow wastewater (CW), porcine wastewater (PG) and poultry wastewater (PL)) performed during two seasons (summer and winter).

Source	**Season**		**culture-EC**		**qPCR-EC**		**ENT**	
			1	**2**	**1**	**2**	**1**	**2**
HM	Summer	K_s	-0.684	-0.639	-0.398	-0.366	-0.787	-0.969
		R^2	0.942	0.884	0.782	0.755	0.951	0.989
		T_{90}	1.46	1.57	2.51	2.73	1.27	1.03
	Winter	K_s	-0.486	-0.483	-0.251	-0.221	-0.340	-0.324
		R^2	0.973	0.982	0.931	0.861	0.949	0.905
		T_{90}	2.06	2.07	3.979	4.53	2.94	3.09
CW	Summer	K_s	-0.179	-0.173	-0.289	-0.234	-0.426	-0.490
		R^2	0.962	0.955	0.859	0.615	0.899	0.922
		T_{90}	5.59	5.78	3.46	4.28	2.35	2.04
	Winter	K_s	-0.159	-0.164	-0.152	-0.176	-0.226	-0.225
		R^2	0.938	0.924	0.944	0.968	0.953	0.936
		T_{90}	6.31	6.08	6.59	5.69	4.43	4.46
PG	Summer	K_s	-0.332	-0.370	-0.270	-0.211	-0.305	-0.342
		R^2	0.808	0.772	0.968	0.861	0.976	0.989
		T_{90}	2.72	2.70	3.71	4.74	3.27	2.93
	Winter	K_s	-0.210	-0.231	-0.120	-0.128	-0.179	-0.188
		R^2	0.935	0.903	0.954	0.961	0.924	0.936
		T_{90}	4.76	4.33	8.36	7.82	5.58	5.33
PL	Summer	K_s	-0.450	-0.407	-0.421	-0.452	-0.734	-0.701
		R^2	0.844	0.859	0.843	0.995	0.958	0.939
		T_{90}	2.22	2.46	2.37	2.21	1.36	1.43
	Winter	K_s	-0.461	-0.442	-0.224	-0.257	-0.295	-0.273
		R^2	0.903	0.908	0.902	0.991	0.893	0.976
		T_{90}	2.17	2.26	4.46	3.88	3.39	3.66

Table S5. Inactivation data for each experiment (K_s, R^2, and T_{90}) of the six microbial source tracking molecular markers (MST) evaluated in this study.

		BifHM		HF183		BifCW		Rum2Bac		Pig2Bac		BifPL	
		1	**2**	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**
Summer	K_s	-0.498	-0.418	-1.091	-0.848	-0.654	-0.587	-0.684	-0.669	-0.627	-0.623	-0.431	-0.666
	R^2	0.972	0.927	0.870	0.894	0.718	0.741	0.866	0.973	0.894	0.752	0.953	0.996
	T_{90}	2.01	2.39	0.916	1.18	1.53	1.71	1.46	1.49	1.60	1.61	2.32	1.50
Winter	K_s	-0.163	-0.164	-0.293	-0.323	-0.188	-0.200	-0.231	-0.202	-0.354	-0.338	-0.265	-0.249
	R^2	0.802	0.839	0.966	0.967	0.919	0.920	0.961	0.979	0.974	0.960	0.953	0.955
	T_{90}	6.15	6.10	3.42	3.10	5.33	5.01	4.33	4.95	2.83	2.96	3.78	3.77

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