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Quantitative PCR Profiling of *Escherichia coli* in Livestock Feces Reveals Increased Population Resilience Relative to Culturable Counts under Temperature Extremes

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ABSTRACT: The relationship between culturable counts (CFU) and quantitative PCR (qPCR) cell equivalent counts of *Escherichia coli* in dairy feces exposed to different environmental conditions and temperature extremes was investigated. Fecal samples were collected in summer and winter from dairy cowpats held under two treatments: field-exposed versus polytunnel-protected. A significant correlation in quantified *E. coli* was recorded between the qPCR and culture-based methods ($r = 0.82$). Evaluation of the persistence profiles of *E. coli* over time revealed no significant difference in the *E. coli* numbers determined as either CFU or gene copies during the summer for the field-exposed cowpats, whereas significantly higher counts were observed by qPCR for the polytunnel-protected cowpats, which were exposed to higher ambient temperatures. In winter, the qPCR returned significantly higher counts of *E. coli* for the field-exposed cowpats, thus representing a reversal of the findings from the summer sampling campaign. Results from this study suggest that with increasing time post-defecation and with the onset of challenging environmental conditions, such as extremes in temperature, culture-based counts begin to underestimate the true resilience of viable *E. coli* populations in livestock feces. This is important not only in the long term as the Earth changes in response to climate-change drivers but also in the short term during spells of extremely cold or hot weather.
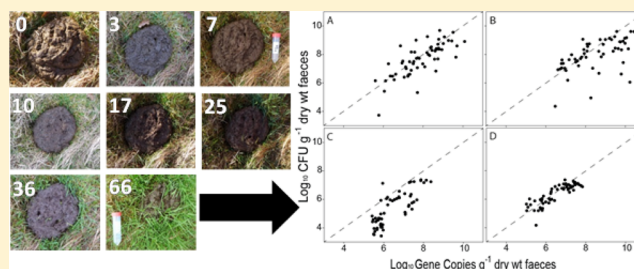
1. INTRODUCTION

Agricultural practices contribute a significant burden of fecal material onto pasture via direct defecation by grazing livestock and through applications of solid and liquid manures.[1] Managing the spatial and temporal input of this fecal loading to pasture is important to minimize the proportion of fecal indicator organisms (FIOs), e.g., *Escherichia coli* and intestinal enterococci, that may be mobilized from fecal sources and delivered to surface waters following rainfall events.[2,3] Contributions of FIOs to the aquatic environment can also occur via point-source inputs, such as sewage outfalls and leaking septic tanks, and via direct defecation attributed to wildlife populations. Environmental monitoring of FIOs is, therefore, undertaken throughout the world to ensure that water quality complies with health-related standards and associated legislation and to guide and prioritize catchment management efforts.[4] However, shifts in temperature regimes and in the intensity and patterns of rainfall associated with climate change can influence pollutant fate and transfer in the environment,[5] including the behavior of FIOs, thus further challenging the environmental regulation of complex catchment systems.

The conventional and widely accepted approach for quantifying FIO concentration in waters and other environmental matrices is through the culturing of cells using nutrient-rich media. The application of molecular methods, principally quantitative polymerase chain reaction (qPCR), has recently gained attention in the field of catchment microbial dynamics; however, the deployment of qPCR for quantifying FIOs in environmental matrices for regulatory purposes is the subject of much debate.[6,7] Nevertheless, qPCR eliminates the requirement for sample incubation and quantifies DNA, allowing for a much-quicker reporting of the quantity of target microorganisms in samples, i.e., 2−4 h versus 24 h for culturing. There is also recognition that culture-based approaches may underestimate concentrations of target cells in environmental samples if, for example, cells are stressed by hostile environmental conditions and enter a viable-but-nonculturable (VBNC) state.[8−11]

In agricultural environments, livestock feces represent a particularly important land-based reservoir of FIOs and potential zoonotic pathogens such as *E. coli* O157:H7, *Campylobacter* spp., and *Salmonella* spp. This is because excretions by livestock undergo no microbial treatment phase other than natural die-off, and so the microbiological content of feces deposited directly to pasture is often high. This contrasts with livestock manures that, by their very nature, are a managed

resource and may therefore be treated during on-farm storage, e.g., composted, aerated, batch-stored, etc. Numbers of FIOs present in feces vary with livestock type, diet, and season,[12] and current estimates of *E. coli* accumulation and depletion on pasture are informed or modeled using studies that are mainly reliant on culture-based approaches (e.g., refs 13−17). It is probable that current culture-based estimates of *E. coli* loading to agricultural land, and their subsequent persistence patterns, are not truly reflective of FIO population dynamics given the potential for *E. coli* to become stressed or injured.[18] This is especially true when considering that cells will have transitioned from a favorable, warm, nutrient-rich livestock gut environment into a less-favorable, cool, terrestrial environment. Thus, survival curves of *E. coli* in fecal material, informed by culturable counts, are more likely to reflect a combination of true "die-off" coupled with fluctuating proportions of *E. coli* entering a VBNC state, and their application in environmental models may potentially underestimate environmental risks that are routinely indexed using FIOs. However, given that health risks (and protective regulatory limits) have been indexed to culturable measures of FIOs, it is unclear whether or not the failure to include VBNCs actually constitutes a measurable additional health threat.

Although a number of studies have reported on the relationship between culturable and qPCR-based counts of FIOs in recreational waters,[19−21] little research has explored the cross-comparison of culture versus qPCR counts of FIOs over time in livestock feces. The aim of this study was to explore the temporal relationship between culturable counts and qPCR cell equivalent counts of *E. coli* in dairy feces exposed to different environmental conditions (e.g., contrasting temperature fluctuations and rainfall wetting−drying−rewetting cycles) and over contrasting seasons. This was undertaken in the context of improving knowledge of terrestrial pollution sources and associated risk to soil, water quality, and human health. The experiments were designed to test the hypothesis that qPCR profiling of *E. coli* in livestock feces would reveal increased population resilience (inferred by cells entering a VBNC state) when exposed to unfavorable environmental conditions over time, relative to culturable counts.

2. MATERIALS AND METHODS

2.1. Sample Collection from Fecal Source. A total of 12 fresh dairy cowpats were collected on two sampling occasions in 2013 from a conventional 165 ha dairy farm in Stirlingshire, Scotland. The fresh cowpats varied in mass from ∼1 to 2 kg. The cowpats were collected in June and December and represented fresh feces excreted at the start of the summer and winter seasons in the northern hemisphere. The cowpats served as replicate samples and were collected within 30 min of excretion from 12 different cows on each sampling occasion. The dairy herd consisted of 80 head of Holstein Friesian cattle, with cows normally housed from October through to the end of March and pasture grazing typical from April to September. Fresh cowpats were collected from a covered holding-barn that was used during the transfer of dairy cows to the parlor for morning milking. A mechanical scraping system cleaned the barn floor twice daily, and so all cowpats collected were assured to be fresh deposits.

A total of 10 of the 12 cowpats were sampled and analyzed for *E. coli* and dry-matter (DM) content. The other two cowpats served as indicators of internal cowpat temperature and had a DS1921G Thermochron i-button temperature logger

(iButtonLink; Whitewater, WI) placed within the core of the fecal matrix. The 10 experimental cowpats were randomly divided into two treatments ($n = 5$) representing field-exposed and polytunnel-protected cowpats and transferred to these treatment locations within 20 min of collection. The field-exposed cowpats were placed on flat grassland, with zero history of grazing, at the University of Stirling. Cowpats were set out at 1 m apart in one adjacent row to ensure that each seasonal experiment was undertaken on grassland previously unoccupied by fecal material. The placement of the cowpats was such that it prevented any wash-off of *E. coli* from feces being able to contaminate another cowpat following heavy rainfall. No manure-spreading activity occurred in the vicinity of the experiment, thus eliminating any potential for aerial transfer of *E. coli* onto the grassland plot. The field-exposed cowpats were subjected to seasonal outdoor temperature and rainfall conditions. In contrast, the polytunnel-protected cowpats experienced zero rewetting from rainfall and warmer ambient air temperatures. In summer, therefore, the polytunnel environment mimicked a drought and warming treatment, while during winter, it represented a more mild winter treatment than that experienced in the field.

Each cowpat was sampled upon collection and then repeatedly on 11 further occasions during each of the seasonal sampling programmes (i.e., 12 samples per seasonal experiment), with higher-frequency sampling in the immediate period following excretion. This resulted in samples being collected on days 0, 0.5, 1, 3, 4, 7, 8, 10, and 14 before a lower-frequency sampling was adopted. On every occasion, approximately 15 g of feces (3 × 5 g subsamples) was randomly sampled from each cowpat using a sterile spatula (70% IMS, rinsed with sterile water) and placed into sterile 50 mL collection tubes. The 15 g of feces sampled on each occasion represented approximately 0.75−1.5% of the total wet weight of the feces, thus not greatly diminishing the mass of the fecal reservoir and so maintaining protection for the population of *E. coli* remaining within the feces. Fecal samples were extracted from the core of the cowpats to avoid sampling surface crust. Samples were assumed to be well-mixed and homogeneous following fecal passage through the ruminant digestive system and gut.[9] Microbial analysis was initiated within 1 h of the samples being collected.

2.2. Culturing of *E. coli*. A total of 2 g of fresh feces was used for microbial analysis, and the remainder (∼13 g) was used to determine the gravimetric water content by drying at 105 °C for 24 h (until constant mass) and weighing the residual. For culture-based analysis, 1 g of feces was transferred to 9 mL of sterile phosphate buffered saline (PBS) and then thoroughly mixed on an orbital shaker (160 rpm for 60 min at ambient temperature) to disperse microbial cells from the fecal matrix. Further serial 1:10 dilutions were then made as appropriate to ensure the capture of between 20 to 200 colony forming units (CFU) once the sample had been transferred to an agar growth medium.

To get to this stage, 1 mL of each serially diluted sample was filtered with ∼20 mL of sterile PBS through cellulose acetate membranes of 0.45 μm pore size (Sartorius Stedim Biotech; Goettingen, Germany) using a filtration unit (Sartorius). The filters were aseptically transferred to membrane lactose glucuronide agar (MLGA) (CM1031, Oxoid; Basingstoke, UK) and incubated inverted at 37 °C (±0.2 °C) for 18−24 h for the determination of presumptive *E. coli*. Equipment was flame-sterilized between samples, and method blanks (i.e., sterile PBS) were used to confirm aseptic technique and the

sterilization procedure between samples. The limit of detection was 100 CFU per g of fresh (wet) weight feces. All sample analysis was performed in duplicate.

2.3. Extraction and Purification of DNA from Feces. Total DNA was extracted and purified using the Powerfecal DNA Isolation Kit (MO BIO Laboratories, Inc.; Carlsbad, CA). Fecal material (0.1 g) was obtained from the same 50 mL collection tubes as those that were sampled simultaneously for the MLGA plate counts. The samples were added to MO BIO Bead Tubes containing 750 μL of bead solution and then vortexed for 1 min before storage at −30 °C until onward processing. Extraction and purification of DNA was performed using a PowerVac vacuum manifold (MO BIO Laboratories, Inc.) with a minor modification to the supplier's standard protocol. As recommended by the manufacturer, the high-salt solution C4 included with the kit was diluted 1:1 (v:v) with 100% ethanol to maximize DNA yields.

An internal sample processing control (SPC) step was included in the standard purification protocol to estimate the efficiency of DNA recovery following cell lysis (see below). A known quantity (1×10^4 DNA copies) of a linearized plasmid construct was added to each clarified fecal lysate prior to loading the silica spin columns supplied with the kit. This recombinant plasmid (pPE7335) was constructed in-house in the vector pCR2.1-TOPO (Invitrogen; Carlsbad, CA) and harbors an internal fragment of *cpeA* (encoding the α subunit of the light-harvesting biliprotein, phycoerythrin) from the cyanobacterium *Synechococcus* sp. PCC 7335. The SPC source organism was selected in the knowledge that it was highly unlikely that this marine photoautotroph (or its genomic DNA) would be found naturally in land-based fecal material.

2.3.1. Detection and Enumeration of E. coli by qPCR. The number of *E. coli* cells within the fecal samples was estimated by a probe-based qPCR protocol targeting the signature gene, *uidA* (encoding β-D-glucuronidase, the enzyme activity reported by MLGA selective medium), as first suggested by Frahm and Obst.[22]

2.3.2. Production of uidA Standard DNA. A ~1.8 kb DNA fragment of *uidA* was amplified from wild type *E. coli* genomic DNA by the PCR using the primer pair uidAF (5′-TCCTGTAGAAACCCCAACC-3′) and uidAR (5′-CCRAAGTTCATGCCAGTCC-3′). The primers target nucleotides 9−27 and 1763−1781, respectively, of the 1812 bp long *uidA* coding region. After an initial denaturation at 95 °C for 2 min, the reaction was cycled 30 times at 95 °C for 30 s, 55 °C for 15 s, and 72 °C for 1 min 30 s, followed by a 5 min final extension period at 72 °C.

The PCR product was resolved through a 1% agarose gel and purified with the Wizard SV Gel and PCR Clean-Up System (Promega; Madison, WI). The fragment was ligated in pCR2.1-TOPO and transformed into TOP10 *E. coli* cells (Invitrogen). Several putative recombinant clones were screened for an insert of the expected size by colony PCR using the same cycling conditions. The identity of the insert found in one of these clones (named puidA2) was confirmed by DNA sequencing on both strands and found to be a 100% nucleotide match to *uidA* from *E. coli*.

2.3.3. Optimization of the qPCR for the Enumeration of uidA and the SPC Assay. A 10-fold dilution series (1×10^2−1×10^8 copies) of puidA2 was prepared for the optimization of the qPCR cycling conditions. The assays were performed with a Stratagene MX3000p instrument using Quantitect Probe PCR kit reagents (Qiagen; Hilden, Germany) and the primer pair of uidAQF and uidAQR with the probe uidAQP.[23] The probe DNA was labeled at the 5′- end with the reporter dye FAM and at the 3′- end with the quencher BHQ-1. Following empirical optimization, all further reactions (including those performed with the fecal DNA samples) were carried out in volumes of 25 μL containing 1× Quantitect Probe PCR master mix, 0.4 μM of each primer, 80 nM probe, and 1 μL of sample DNA. The HotStarTaq DNA polymerase included in the kit was activated at 95 °C for 15 min, and the reactions were then cycled 40 times through a two-step protocol. Reactions were denatured at 95 °C for 10s at each cycle followed by a combined annealing and extension step at 61 °C for 10s.

Apart from the primers and the probe used, identical qPCR reactions and cycling conditions were adopted for the SPC assay. A 107 bp long fragment of *cpeA* from *Synechococcus* PCC 7335 was targeted with the primer pair 7335cpeAF (5′-TAGAAGCCGCTGAGAAGCTC-3′) and 7335cpeAR (5′-GGCTGCTTCACCTTCGTTAG-3′) and reported with a FAM/BHQ1-labeled probe (5′-TCTAGACCAGGTTGCT-CAAGAAGCTTATGATG-3′). The primers bind at nucleotide positons 113−132 and 200−219 within the 495 bp long *cpeA* coding sequence (GenBank accession number: WP_006455006.1), respectively, and the probe binds the noncoding strand at nucleotides 141−172.

The qPCR reactions were performed in duplicate for both *uidA* and the SPC assay. Copy number was quantified with reference to the appropriate standards (1×10^2−1×10^8 target copies) run alongside each set of samples. Linear regression of the standard curves indicated that PCR efficiencies were within the range 95−110% with r^2 values close to unity for most cases. Those experiments in which the PCR efficiencies reported for the standard DNAs deviated beyond these limits were repeated until within range. Duplicate "no-template controls" (NTC; all reaction components apart from DNA) were included with each set of samples analyzed but were never significantly above background (DNA sample buffer only).

2.3.4. Amplification Control for the Detection of qPCR Inhibitors and the Final Determination of E. coli Cell Numbers. To assess whether the purified fecal DNA samples contained residual PCR inhibitors, a third set of experimental reactions was performed in duplicate before the final quantitation of *uidA* copy number. The amplification controls (ACs) were performed using the 7335cpeA primers and probe under identical experimental conditions as the SPC assays, with the exception that the 25 μL reactions were spiked with 1×10^6 copies of the linearized plasmid pPE7335 prior to the qPCR. The degree of any potential PCR inhibition was determined from the quotient of observed to expected (O/E; i.e., cpeA copy number detected/(1×10^6)). Where the AC quotient was below unity, the results obtained for the corresponding fecal sample *uidA* and SPC assays were adjusted accordingly. Very few samples (3/237) had an AC of less than 0.5 and required adjustment beyond the inherent resolution of the qPCR (±1 cycle). The mean AC value obtained for the remaining samples was 0.8, and so in practice, only minor upward adjustments to the *uidA* (and SPC assay) copy numbers were made when required.

Once adjusted for the AC, the SPC (observed *cpeA* copy number/(1×10^4)) was applied to correct for the DNA recovery efficiency. The number of *uidA* copies in the 0.1 g fecal sample was then corrected for the volume (100 μL) used to elute DNA samples from the silica spin columns, as summarized below:

$$uidA \text{ copy number} = \left(\frac{Q_{uidA}}{AC}\right) \times \left(\frac{SPC}{AC}\right) \times \frac{100}{1}$$

where Q_{uidA} is the copy number reported for the uncorrected qPCR.

Finally, for each time point ($n = 12$), the average number of *E. coli* cells in the experimental duplicate was normalized to 1 g of dry excreta and expressed as the sample mean $\pm$ SE ($n = 5$). For all samples, the limit of detection was <100 gene copies per sample.

2.4. Statistical Analysis. All *E. coli* counts underwent $\log_{10}$ transformation prior to statistical analysis, and distributions of *E. coli* were log normally distributed as determined using the Kolmogorov−Smirnov goodness of fit test. Differences at the $p < 0.05$ level (95% confidence interval) were considered statistically significant. The Pearson product−moment correlation coefficient (r) was used to compare concentrations of *E. coli* in samples determined using culture- versus qPCR-based methods. A two-way analysis of variance (ANOVA) and a Tukey multiple comparison test were used to test for differences in *E. coli* numbers determined over time and between methods and to test for any interactions between these factors (Minitab 16.0 software, Minitab Inc.; State College, PA).

3. RESULTS AND DISCUSSION

All culture-method blanks were negative for *E. coli*, indicating that no cross-contamination occurred during sample processing. A total of 237 comparative samples were analyzed simultaneously via culture- and qPCR-based methods. The samples were collected over the course of two contrasting seasons and from differing temperature scenarios. With all 237 samples combined, correlation analysis yielded a significant relationship between the two methods ($r = 0.82$; $P < 0.001$, Figure 1). When the samples were separated by treatment and
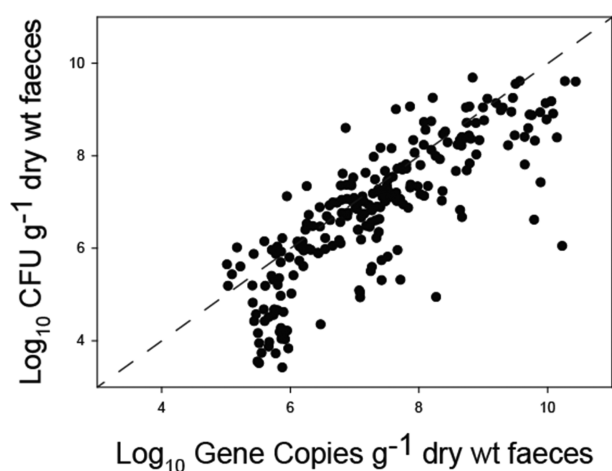
Figure 1. Comparison of *E. coli* concentrations ($\log_{10}$ CFU vs $\log_{10}$ gene copies) for all samples, as determined using culture- and qPCR-based approaches.

season, all correlations remained significant ($P < 0.001$), with r values of 0.76, 0.55, 0.79, and 0.86 for the summer field-exposed and polytunnel-protected cowpats and the winter field-exposed and polytunnel-protected cowpats, respectively (Figure 2). In a U.S. study concerning bovine feces, a pair-wise comparison between culturable counts of *E. coli* and counts

inferred using their corresponding genomic marker (EPA-EC23S), which targets a section of the 23S rRNA gene (using multicopy gene targets) rather than *uidA* (a single-copy gene assay), returned a correlation coefficient of 0.57.[24] However, it should be noted that the culture-based method of *E. coli* enumeration used in this U.S. study relied on a most-probable-number approach rather than membrane filtration.

E. coli concentrations (as CFU or gene copies per gram of dry-weight feces) were examined over time under different treatments of open-field exposure or polytunnel protection (Figures 3 and 4). Daily rainfall distribution over the sampling period was recorded for the field-exposed cowpats to provide environmental context alongside the daily average internal temperature of the cowpat environment (Figures 3 and 4). Summary meteorological characteristics associated with the summer and winter sampling campaigns are shown in Table 1 for context.

It has been suggested that most molecular estimations of *E. coli* load in bovine feces have struggled to achieve the same degree of quantitative sensitivity as that delivered by culture-based counts.[25] Evaluation of the die-off dynamics of *E. coli* informed by either culture or qPCR revealed no significant difference ($P > 0.05$) in the *E. coli* numbers determined as either CFU or gene copies during the summer for the field-exposed cowpats. In contrast, a significant difference ($P < 0.001$) was observed between the counts determined from the two methods for the polytunnel-protected cowpats. The results informed by qPCR revealed higher counts for the summer polytunnel samples than for those determined by culture. Furthermore, a significant interaction between the time of sampling and the method was recorded ($P < 0.05$), with the numerical differences between the two methods becoming more pronounced as the feces aged over time.

In winter, qPCR was again found to record significantly higher *E. coli* counts relative to culturable counts ($P < 0.001$) in one of the two treatments. Interestingly, for this season, the discrepancy was observed for the field-exposed rather than the polytunnel-protected cowpats, a reversal of the findings from the summer sampling campaign. During the winter sampling, however, there was no significant interaction ($P > 0.05$) between the time since excretion and the enumeration method. The counts were consistently higher throughout the winter sampling of field-exposed cowpats when reported by the qPCR. Conversely, the cross-comparison between the results for protected feces monitored within the polytunnel environments during the winter revealed no systematic difference ($P > 0.05$) between the *E. coli* counts determined by either method. Clearly, our study focused on relationships between culture and qPCR results using source material with high *E. coli* loading. From a water-monitoring perspective, observed *E. coli* concentrations are likely to be much lower than those recorded here. It is important to stress, therefore, that our results provide a useful step in understanding differences in patterns of enumeration of *E. coli* in fecal sources on land but do not inform on the reliability of comparisons between culture and qPCR approaches for routine water quality monitoring. Nevertheless, it is useful to develop an understanding of the environmental scenarios that might lead to these two methods returning different concentrations of indicator bacteria for the same environmental sample, especially with some environmental regulators (e.g., the U.S. Environmental Protection Agency) offering qPCR as an approved enumeration method, albeit in the water environment.[26,27]
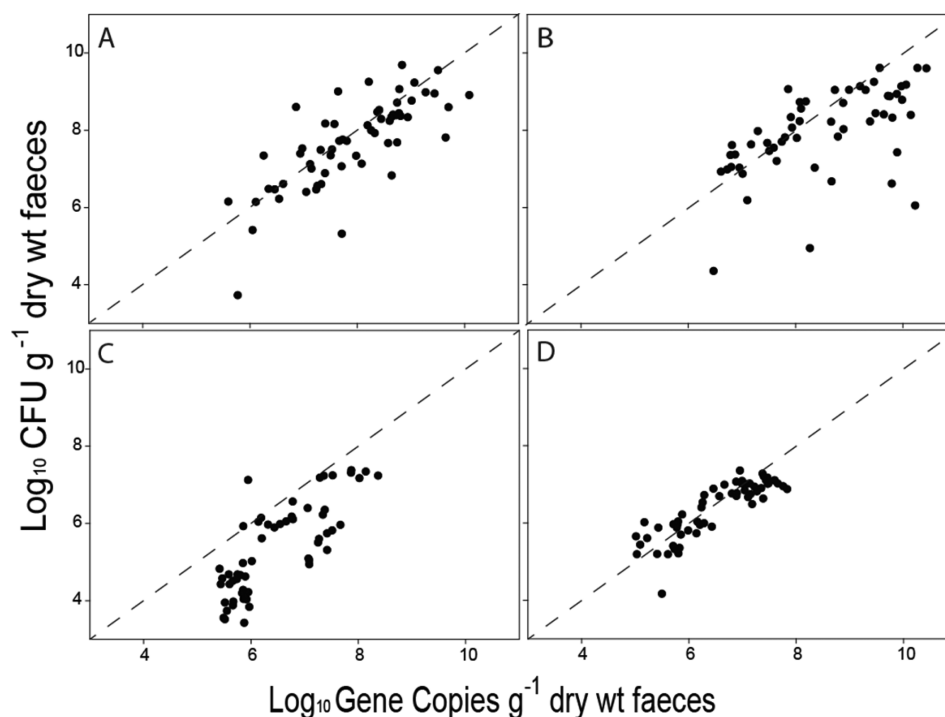
Figure 2. Comparison of *E. coli* concentrations (log$_{10}$ CFU vs log$_{10}$ gene copies) across seasons and treatments, as determined using culture- and qPCR-based approaches, respectively. A: summer field-exposed; B: summer polytunnel-protected; C: winter field-exposed; and D: winter polytunnel-protected.
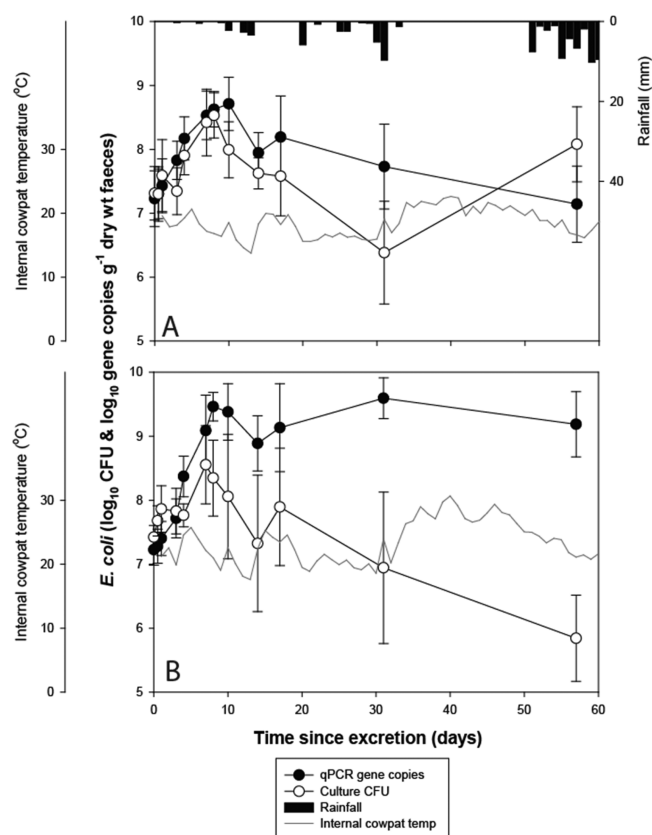
Figure 3. *E. coli* population numbers over time during summer as quantified by culture (CFU) and qPCR (gene copies). A: Field-exposed cowpats; B: polytunnel-protected cowpat (no rainfall). Data points are the mean of five replicates ± the standard error.
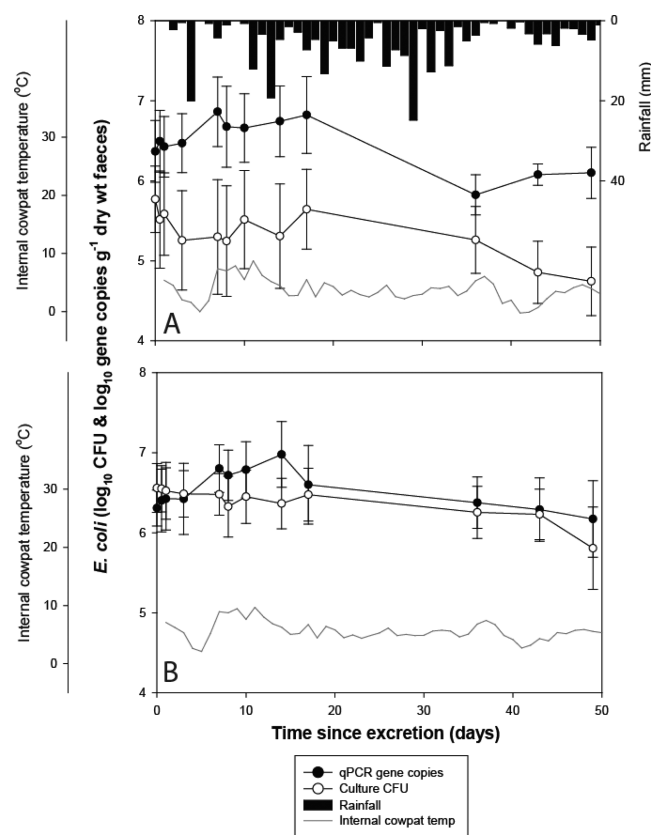
Figure 4. *E. coli* population numbers over time during winter as quantified by culture (CFU) and qPCR (gene copies). A: Field-exposed cowpats; B: polytunnel-protected cowpat (no rainfall). Data points are the mean of five replicates ± the standard error.

Table 1. Summary Meteorological Characteristics of the Summer and Winter Sampling Campaigns

	mean/max ambient field temperature (°C)	mean/max ambient polytunnel temperature (°C)	mean field humidity (%)	mean field UV index	mean field wind speed (ms^{-1})
summer sampling (June 3–July 30 2013)	16.3/30.1	23.3/56.0	75.0	0.5	0.3
winter sampling (Dec 2 2013–Jan 20 2014)	5.4/12.8	5.0/15.5	89.5	0.0	0.9

Average daily internal cowpat temperatures and rainfall measurements taken over the two seasons provide useful contextual data to help explain the observed correlations between the methods and the persistence profiles of *E. coli* over time. The strongest seasonal correlation between qPCR- and culture-based counts was recorded in winter in the polytunnel environment. Environmental conditions recorded for this treatment were the least variable overall, with daily average internal cowpat temperatures accommodating a range of 7.6 °C during the period of study. In contrast, a broader temperature range of some 13 °C was recorded in the cowpats exposed to the polytunnel treatment during summer, when the correlation between qPCR- and culture-based counts was weakest. Neither the summer nor winter polytunnel treatments experienced any environmental variability due to seasonal differences in wetting–drying–rewetting cycles from rainfall. The weak correlation between methods observed in summer within the polytunnel would suggest that the temperature regime was less favorable (e.g., internal cowpat temperatures exceeding 15 °C and accommodating a greater degree of day-to-day fluctuation) and that this impacted on the *E. coli* population, with cells failing to grow on nutrient-rich media but still readily enumerated by the qPCR (though nondiscriminately as VBNC, lysed cells, or both).

Initially, however, each method revealed that the summer warming environment appears to have promoted a period of approximately 10 days of *E. coli* regrowth in the fecal matrix in both treatments immediately post-defecation. The potential for *E. coli* regrowth in livestock feces under field conditions during the period immediately post-defecation has been reported from different countries around the world.[16,28−31] All of these studies estimated *E. coli* regrowth using culturable cell count data. Of particular interest in the present study, therefore, is that the regrowth phase of the *E. coli* population observed through an increase in cell counts during early summer was mirrored also in the qPCR data set. This suggests that the phenomenon of *E. coli* regrowth in livestock feces post-defecation is not an artifact of the culturing methodology but equates to an actual population size increase. Others who have reported on the persistence profiles of FIOs using both culturable counts of *E. coli* and its genomic marker (EPA-EC23S) also observed significant increases in CFU and gene-copy counts post-defecation, but only for the first 2−5 days after excretion.[24] In their study, however, very little information was reported on the temperature regime to which the cowpats were exposed. Additionally, all cowpats were protected from rainfall, thus limiting any detailed comparison with the findings from the different temperature treatments considered here.

In this study, the pronounced warming effect experienced in the polytunnel enclosure over time (mean hourly air temperature of 23.3 °C; maximum temperature of 56.0 °C) led to internal cowpat temperatures often in excess of 20 °C and, in turn, a decline in culturable cell counts. Furthermore, Table 2 shows that the percent of dry-matter content changes over time across the different treatments and highlights considerable

Table 2. Changes in Mean Percent of Dry-Matter Content of Cowpats over Time and across Different Treatments

	mean dry-matter content of feces (%)			
	summer		winter	
day	field-exposed	polytunnel	field-exposed	polytunnel
0	12.34	11.36	15.21	14.30
0.5	12.34	11.71	14.15	13.98
1	12.87	11.33	14.38	14.22
3	14.26	15.18	13.58	13.17
4	15.02	19.30	nd^a	nd
7	16.93	31.03	14.91	13.38
8	17.31	20.69	14.41	13.61
10	18.25	30.24	13.96	13.30
14	19.78	36.20	13.44	13.50
17	28.46	34.56	13.62	13.52
31	19.92	46.18	nd	nd
36	nd	nd	13.36	14.75
43	nd	nd	14.04	15.54
49	nd	nd	14.12	15.03
57	38.05	90.41	nd	nd

^{a}nd: no data.

desiccation associated with the cowpats held under the polytunnel treatment, particularly during the summer. Interestingly, these temperature and dry-matter effects appear not to have compromised the *E. coli* counts, as determined by qPCR. It is likely, therefore, that the environmental conditions within the polytunnel were such that they led to shifts in cell metabolic stages promoting the development of dormancy and a VBNC state in some of the *E. coli* population,[18] in addition to the qPCR also reflecting a proportion of dead cells. Importantly, Figures 3 and 4 both show some evidence of a decline in qPCR counts over time occurring in parallel with declines in CFU. The observation that the qPCR signal does decline on some occasions over time thus provides evidence to suggest that when culture and qPCR counts deviate, a proportion of the qPCR signal is likely to represent VBNC *E. coli* and not just dead cells.

During the same period, the cowpats that were exposed to field conditions experienced a much lower maximum hourly air temperature of 30.1 °C and a mean hourly temperature of 16.3 °C, some 7 °C cooler than that in the polytunnel enclosure. In addition, the cowpats received a total 95.7 mm of rainfall over the duration of the experiment, with this rainfall delivering moisture and, therefore, rehydration to the fecal matrix, potentially sustaining the viability of the *E. coli* population within the cowpats.[32] As a result, the deviation in the persistence profiles of *E. coli*, as determined by the two contrasting quantification methods, appears to have been minimized.

The summer polytunnel treatment provided a means of assessing the sensitivity of *E. coli* population response to climate change variables in the form of higher temperatures and drought conditions and also enables the transferability of

findings to areas of the world where summer temperatures frequently exceed 30 °C. Our results suggest that, under some scenarios, these traditional methods may underestimate the true burden of *E. coli* populations in fecal sources on pasture relative to qPCR, although it is difficult to ascertain the proportion of dead cells versus those that are truly VBNC using the latter method. The use of qPCR combined with ethidium monoazide (EMA) or propidium monoazide (PMA) in future studies may remove the lack of differentiation between viable and nonviable bacterial cells.[33] Furthermore, more targeted research is needed to investigate the nuances of *E. coli* sensitivity to incremental increases in temperature and its possible transition to (and resuscitation from) a VBNC state within a range of environmental matrices.

The reversal of experimental findings observed in winter is intriguing, with the field-exposed cowpats now accommodating a significant lowering in *E. coli* counts as determined by the two methods of enumeration. The field-exposed cowpats received no protection from frost and freezing conditions, and the minimum internal cowpat temperature recorded was −0.5 °C. In comparison, the internal cowpat temperature of the polytunnel treatment did not drop below freezing, with a lowest recorded temperature of 0.5 °C. The subfreezing temperatures probably played a key role in encouraging a proportion of the exposed *E. coli* population to enter a VBNC state,[34] although, as mentioned previously, it is difficult to discriminate between free DNA, dead cells, and VBNC cells. Others have reported that stressful conditions, such as low temperature, have induced *E. coli* O157 into a VBNC state.[35] Similar observations concerning the detrimental impact of sustained (week-long) subfreezing temperatures on culturable *E. coli* counts in sheep and dairy feces on pasture across a headwater catchment in England have been reported and support our findings.[15]

In the present study, the 312.8 mm of rainfall that fell during the winter period would have rehydrated the cowpats frequently (see the rainfall distribution pattern in Figure 4) but was probably unwelcome when combined with low temperatures and the freezing of the rehydrated fecal material.[36] The more unfavorable conditions for the monitored *E. coli* populations during the winter phase of our study, therefore, were experienced by the field-exposed cowpats, whereas the cowpats within the polytunnel treatment experienced a more stable and comfortable environment, with internal cowpat temperatures evidently higher than those under field-relevant conditions (see Figure 4). The protection from cold weather within the polytunnel appears to have sustained culturable *E. coli* counts at levels directly comparable to those returned using qPCR. In contrast, the field-exposed, frost-exposed cowpats would have represented a less conducive habitat to support cell maintenance and likely promoted a more rapid decline of *E. coli*, either through cell lysis or the development of a VBNC population of *E. coli*, which would explain the significant difference observed between quantification methods.

The results of this study are important for developing a better understanding of how and when culture and qPCR enumeration of FIOs may differ, but they also draw attention to the complexity of *E. coli* persistence patterns in environmental systems. Our study focused on generic *E. coli*, an indicator organism used widely by environmental regulators to infer fecal contamination of soil and water; however, pathogenic strains of *E. coli*, e.g., *E. coli* O157:H7, have been reported to respond, with respect to their metabolic state, in a similar manner to

temperature extremes.[37] Research in New Zealand hypothesized that higher *E. coli* counts associated with the feces of grazing cattle, relative to housed cattle, may reflect a continuous ingestion of FIOs from fecally contaminated pasture.[38] This raises questions over whether such gut−sward−gut recycling could provide the necessary optimal conditions to resuscitate VBNC *E. coli*, including pathogenic strains, during a secondary passage through the gut environment. An interesting area of future research would be to explore whether grazing livestock activity does indeed facilitate a longer-lasting legacy of the terrestrial *E. coli* reservoir, with the ingestion of cells prolonging *E. coli* viability. If that holds true for *E. coli* O157, it would suggest a mechanism by which the cells could maintain membrane integrity and viability over extended periods, increasing the opportunity for their eventual transfer from land to aquatic receptors when re-excreted, thus posing longer-term potential risks to the health of downstream water users. Although the prevalence of *E. coli* O157:H7 in the environment is low relative to nonpathogenic *E. coli*, the health and economic impacts associated with human infection are significant.[39]

In conclusion, the deployment of qPCR- and culture-based methods to determine *E. coli* concentrations in fecal matrices correlate reasonably well, as supported by the statistical analysis of the 237 comparative samples used in this study. The comparability between methods was most promising when conditions were relatively stable and generally favorable for the persistence of *E. coli* in the environment. With increasing time post-defecation, and with the onset of challenging environmental conditions (particularly extremes in temperature), it is likely that culture-based counts begin to underestimate the true resilience of viable *E. coli* populations in livestock feces, although more research is needed to discriminate between VBNC and lysed cells. The qPCR analysis has demonstrated that under sublethal, challenging environmental conditions, *E. coli* is likely to persist in fecal sources in higher concentrations than in those informed by culture-based methods but in a VBNC state (representing a proportion of the difference between culturable and qPCR-based counts). The ability to integrate new molecular strategies into ongoing catchment monitoring and management is limited by our understanding of how new molecular targets behave relative to traditional culture-based targets that have been used to set regulatory standards. Our findings provide a critical first step in understanding key differences in patterns of detection and enumeration of *E. coli* in fecal sources on agricultural land and have important implications for informing environmental monitoring campaigns that target enumeration of FIOs and microbial compliance parameters. This is especially true given that increased attention is now being devoted to the development of new approaches to quantify the risk that climate change poses to water quality and human health.[40]

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Notes

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■ ABBREVIATIONS

FIO   fecal indicator organism

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