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**Impact of low intensity summer rainfall on *E. coli*-discharge event dynamics with reference to sample acquisition and storage**

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37 **Abstract**

38 Understanding the role of different rainfall scenarios on faecal indicator organism (FIO)  
39 dynamics under variable field conditions is important to strengthen the evidence-base on  
40 which regulators and land managers can base informed decisions regarding diffuse  
41 microbial pollution risks. We sought to investigate the impact of low intensity summer rainfall  
42 on *E. coli* – discharge (Q) patterns observed at the headwater catchment scale in order to  
43 provide new empirical data on FIO concentrations observed during base-flow conditions. In  
44 addition, we evaluated the potential impact of using automatic samplers to collect and store  
45 freshwater samples for subsequent microbial analysis during summer storm sampling  
46 campaigns. The temporal variation of *E. coli* concentrations with Q was captured during six  
47 events throughout a relatively dry summer in central Scotland. The relationship between *E.*  
48 *coli* concentration and Q was complex with no discernible patterns of cell emergence with Q  
49 that were repeated across all events. On several occasions an order of magnitude increase  
50 in *E. coli* concentrations occurred even with slight increases in Q, but responses were not  
51 consistent and highlighted the challenges of attempting to characterise temporal responses  
52 of *E. coli* concentrations relative to Q during low intensity rainfall. Cross-comparison of *E.*  
53 *coli* concentrations determined in water samples using simultaneous manual grab and  
54 automated sample collection was undertaken with no difference in concentrations observed  
55 between methods. However, the duration of sample storage within the autosampler unit was  
56 found to be more problematic in terms of impacting on the representativeness of microbial  
57 water quality, with unrefrigerated autosamplers exhibiting significantly different  
58 concentrations of *E. coli* relative to initial samples after 12 hours storage. The findings from  
59 this study provide important empirical contributions to the growing evidence-base in the field  
60 of catchment microbial dynamics.

61

62 **Keywords:** autosampler; climate change; diffuse pollution; faecal indicator organism; storm  
63 event; water quality

64

65        **1. Introduction**

66            Recognition of the implications of diffuse water pollution from agriculture on the  
67 freshwater environment has improved significantly over the last few decades. However, the  
68 spatial and temporal complexity of pollutant losses from land to water continues to challenge  
69 our understanding of contaminant transfer processes across a range of spatial and temporal  
70 scales (Harris & Heathwaite, 2012; Haygarth *et al.*, 2012). The evidence-base that underpins  
71 current understanding is more developed for some pollutants than for others, for example,  
72 our knowledge of diffuse pollution is more advanced for nutrients than for microbial  
73 pollutants, such as pathogens, often interpreted through analysis of faecal indicator  
74 organisms (FIOs) (Oliver *et al.*, 2010; Kay *et al.*, 2008). Regulatory monitoring of FIOs is  
75 undertaken throughout the world to ensure water quality complies with health-related  
76 standards and associated legislation. Understanding how agriculture impacts microbial water  
77 quality when coupled with contrasting climatic and environmental conditions is critical in  
78 order to design better mitigation strategies to protect surface waters and further improve  
79 microbial water quality (Fish *et al.*, 2014).

80            Observations have shown that over 90% of the catchment input of microbial  
81 contamination occurs after rainfall-runoff, usually following storm events (McKergow and  
82 Davies-Colley, 2010; Kay *et al.*, 2007; Kay *et al.*, 1999), with at least an order of magnitude  
83 difference in FIO concentrations between base and storm flows commonly reported (Kay *et*  
84 *al.*, 2010). However, there has been comparatively little work exploring the role of low  
85 intensity rainfall (e.g.  $<4\text{mm hr}^{-1}$ ; MET Office, 2009), and the impact these events may have  
86 on microbial concentrations in freshwater when interspersed during prolonged dry weather  
87 spells. The influence and timing of smaller rainfall events on in-stream FIO concentrations  
88 could be significant during a drier summer season given the potential for bacterial transfer  
89 through and across cracking and crusted soils coupled with high FIO source loading on  
90 pasture from direct defecation by grazing livestock and increased manure and slurry  
91 applications to land (Oliver *et al.*, 2005a). Summertime also represents a key sampling

92 period given seasonally important policy drivers, e.g. the EU Bathing Waters Directive (CEC,  
93 2006). Furthermore, the typical base-flow conditions in streams and rivers during summer  
94 periods reduce the opportunity for dilution of FIOs entering waterbodies following summer  
95 rainfall. This may be problematic at the local scale (e.g. cattle drinking from streams and  
96 opportunities for within-herd pathogen cycling), but when scaling up to the larger catchment  
97 network the overall FIO load will be reduced because of low discharge (Q). However, the  
98 lack of empirical observations to confirm or refute the importance of these 'minor' rainfall  
99 events in changing *E. coli*-discharge dynamics during dominantly dry weather warrants  
100 further attention; particularly as such occurrences may become more common across parts  
101 of the UK and Northern Europe under a changing climate (Arnell *et al.*, 2015).

102         While year-on-year variability in hydrological responses in catchments (e.g. Meays *et*  
103 *al.*, 2006) and seasonal variations in stream Q (e.g. Wilkes *et al.*, 2009; Kay *et al.*, 2008) can  
104 impact on water quality, interpretation of the microbial signature in aquatic samples may also  
105 be influenced by monitoring strategy, e.g. choice of sampling frequency or method. The  
106 monitoring of pollutant flux dynamics within catchment systems tends to generate a time-  
107 series in which the sampling interval determines the quality of capture of storm events.  
108 Logistically, the intensive capture of samples throughout a storm hydrograph is made easier  
109 through the use of an automatic sampler. Approaches to water quality monitoring are guided  
110 by cost constraints and availability of resources. For microbial parameters, the aseptic grab  
111 sampling method is unequivocal for providing a water sample suitable for FIO quantification.  
112 Compared with automated alternatives this approach is demanding in terms of staff  
113 resource, particularly during high frequency sampling, e.g. during storm events. Water  
114 collected by an autosampler allows the acquisition of representative samples for subsequent  
115 analysis of many physical and chemical parameters such as suspended sediment and most  
116 nutrient fractions (e.g. Owen *et al.*, 2012; Granger *et al.*, 2011; Bilotta *et al.*, 2010). However,  
117 the use of autosamplers is perhaps more contested when collecting samples for microbial  
118 water quality analysis, with a degree of scepticism associated with the quality of data

119 resulting from samples that have been held in stasis for prolonged periods, or cannot be  
120 guaranteed to have been collected aseptically (Hathaway et al., 2014). This is because: 1)  
121 the reception bottle in an autosampler unit will be non-sterile at the point of sample  
122 collection, 2) there is an opportunity for microbial cross-contamination between samples  
123 during collection via the inlet hose, and 3) some microbial die-off will be likely depending on  
124 sample storage times in the autosampler unit.

125         Despite these limitations a number of studies have used autosamplers (equipped  
126 with and without refrigeration units for sample storage) for microbial water quality  
127 assessment across a range of temperature conditions (e.g. Guber *et al.* 2011; Wilkinson *et*  
128 *al.* 2011; Vinten *et al.* 2008; Oliver *et al.* 2005b; Solo-Gabriele *et al.* 2000). Ghazaleh *et al.*  
129 (2014) evaluated the effect of storage time on FIOs in estuarine water held in an  
130 autosampler with a view that little data exists on 'bottle-effects' during the first 24 hours on  
131 containment. Ferguson (1994) used a refrigerated autosampler to specifically investigate  
132 differences in FIOs from manually versus automatically derived water samples, and  
133 concluded that concentrations of FIOs in samples taken from autosamplers differed from  
134 those taken manually, but that the size of the difference was negligible for the purpose of  
135 environmental monitoring. Importantly however, this study was based on samples collected  
136 during dry weather days only. Therefore, we still lack an understanding of the role of different  
137 rainfall scenarios on FIO dynamics under variable field conditions, which is vital for  
138 strengthening the evidence-base on which regulators and land managers can base informed  
139 decisions. The role of low intensity rainfall could be significant for localised in-stream FIO  
140 concentrations particularly during the warmer, drier summers that are becoming more  
141 commonplace in the UK (Arnell et al., 2015). Thus, the aim of this study was to: (i)  
142 investigate the temporal patterns of *E. coli* emergence with Q from a small headwater  
143 catchment throughout an dry summer in central Scotland; and (ii) evaluate the impact of  
144 different methods of sample acquisition and storage on *E. coli* concentrations.

145

146 **2. Materials and methods**

147 *2.1. Study catchment*

148 This study investigated microbial water quality in a stream draining from a 0.37km<sup>2</sup>  
149 headwater catchment located in Stirlingshire, Central Scotland (Figure 1). The catchment  
150 area is characterised by low density livestock and arable farming with a small amount of  
151 mixed woodland. Specifically, land use is categorised as 50.0% improved grassland, 25.2%  
152 arable, 16.6% rough grazing and 8.2% woodland. A number of fields adjacent to the  
153 monitoring point were grazed by ca. 20 sheep, and a field at the source of the stream was  
154 grazed by 12 dairy cows throughout the monitoring period. All livestock had direct access to  
155 the watercourse for drinking. The bedrock at this site is described as sandstone with  
156 superficial deposits of Devensian Diamicton with raised tidal flat deposits of silt and clay also  
157 present. The soil type is typical of brown forest soils with gleying and is made up of the  
158 Oglegarth, Balvorist and Lenniaston soil units, which represent noncalcareous gley, peaty  
159 gley and humus-iron podzol, respectively (Soil Survey of Scotland Staff, 1970-1987). The  
160 slope from the point of maximum elevation to the catchment outlet represents a gradient of  
161 3.4%.

162

163 **INSERT FIGURE 1 HERE**

164

165 *2.2. In situ hydrological monitoring*

166 A V-notch weir was installed at the designated catchment outlet to provide monitoring  
167 infrastructure for continuous Q measurements and associated water quality parameters, e.g.  
168 turbidity. The gauging station contained a CR800 datalogger connected to an ARG100 rain  
169 gauge, OBS 3 turbidity meter, SOP18X solar panel and a PDCR1830 pressure transducer  
170 (all Campbell Scientific, Loughborough, UK). The rain gauge provided measurement of daily  
171 rainfall and rainfall intensity; the turbidity meter provided a continuous record of in-stream

172 turbidity and the pressure transducer, built into a stilling well, recorded water depth for later  
173 conversion to stream Q. Stage height was converted to Q using an established rating curve  
174 for the site. The two-year mean discharge at the site is 140 Ls<sup>-1</sup>. The Campbell datalogging  
175 equipment was also linked to an unrefrigerated automatic ISCO 3700 water sampler  
176 (Teledyne Isco Inc., Lincoln, USA) for capture of storm-related water samples.

177

### 178 *2.3. Water sample collection during rainfall events*

179 During rainfall events water samples were collected for microbial analysis using an  
180 automatic sampler. Bottles used in the autosampler were sterilised by autoclaving (20 min  
181 121 °C, 1.5 bar) and were deployed in the field as close to a storm event as possible to  
182 minimise contamination. Field technicians were notified of any autosampler activity through  
183 an SMS message sent via a modem connected to the datalogging equipment on-site.  
184 Samples were therefore retrieved with minimal delay and all samples returned to the  
185 laboratory in a cool-box and analysed within 12 hours of their collection.

186 In total, six events were analysed to determine the concentration of *E. coli*  
187 concentrations in response to stream-flow. The ISCO autosampler was programmed to  
188 respond to Q thresholds that, when exceeded, triggered the sampler on a time-proportional  
189 basis. The stage height at which the sampler was triggered was variable and pre-defined to  
190 ensure that coverage of a range of events was achieved for different antecedent flow  
191 conditions. On occasion the autosampler was triggered manually in anticipation of a forecast  
192 rainfall event. Once triggered, water samples were collected on a time-proportional basis  
193 appropriate to the forecasted 'storm' event. This strategy was flexible meaning that obtaining  
194 samples was not solely reliant on flow exceedance and thresholds were manipulated to take  
195 account of changing base levels and lack of Q response due to low rainfall. In total, three  
196 events were triggered by flow exceedance and three triggered manually.

197

### 198 *2.4. Microbiological analysis*

199 Standard UK Environment Agency methods of membrane filtration were used to  
200 determine bacterial concentrations (EA, 2009). Each water sample was vacuum-filtered with  
201 20 mL of phosphate buffered saline (PBS) through a 0.45 µm cellulose acetate membrane  
202 (Sartorius Stedim Biotech., Goettingen, Germany). The membrane was then aseptically  
203 transferred to the surface of a plate containing Membrane Lactose Glucuronide Agar  
204 (MLGA) (CM1031, Oxoid, Basingstoke, UK), inverted and incubated at 37°C (±0.2°C) for 18–  
205 24 h for the determination of presumptive *E. coli* colonies. For each analysis, 100mL, 10mL,  
206 1mL of sample were filtered, with further serial 1:10 dilutions made as appropriate to ensure  
207 capture of between 20 to 200 colony forming units (CFU). Method blanks were regularly  
208 used to assess aseptic technique and to evaluate sterilisation efficiency between samples.  
209 All sample analysis was performed in duplicate.

210

#### 211 *2.5. Autosampler versus grab sampling*

212 A ‘grab versus autosampler’ comparative study was also conducted to establish  
213 whether the autosampler unit impacted on the microbial parameters being enumerated (e.g.  
214 carry-over contamination in sample inlet hose or reduced *E. coli* numbers through  
215 competition with other bacteria). On 20 occasions, under different flow conditions, the auto-  
216 sampler was triggered for sample collection and an equivalent grab sample taken from the  
217 same point in the stream. Samples were not stored in the autosampler but instead removed  
218 immediately to enable a determination of the role of carry-over contamination as opposed to  
219 die-off (see Section 2.6). In parallel, an additional 22 comparative autosampler and grab  
220 samples were collected from a second headwater catchment site in Lancashire, England, in  
221 order to augment the data and provide a cross comparison to samples obtained from a  
222 stream under much higher flows during wetter weather. These 22 samples were collected  
223 from across multiple flow conditions during 7 different monitored events.

224

#### 225 *2.6. E. coli die-off dynamics during storage in autosampler units*

226 The impact of storage conditions, such as temperature and duration, on the microbial  
227 quality of samples held within autosamplers was investigated to complement the 'grab  
228 versus autosampler' comparative study. We investigated the die-off of *E. coli* concentrations  
229 in stored samples held under both ambient and refrigerated (4°C) autosampler conditions in  
230 July. Our approach was to mimic the collection of water samples that had been heavily  
231 contaminated with faecal material and therefore to inoculate bottles with sufficiently high *E.*  
232 *coli* starting concentrations to enable determination of a die-off profile over time but also  
233 reflect realistic field conditions. In total, 8 litres of stream water was artificially contaminated  
234 with ~1kg of fresh ovine faeces, mixed, and then 900mL distributed to each replicate sterile  
235 autosampler bottle before being sealed and placed within the autosampler unit. Four  
236 replicate bottles were used in both the ambient (standard ISCO 3700 stored outside) and  
237 refrigerated (ISCO bottles kept within a coldroom at 4°C) treatments. To determine the  
238 temperature profile within the ambient treatment we installed a DS1921G ThermoChron i-  
239 button temperature logger (iButtonLink, WI, USA) within the body of the autosampler unit,  
240 where the water samples were stored. Bottles were shaken briefly prior to sampling and a 20  
241 mL volume was sampled from the bottles after 0, 5, 24, 48, 72, 96, 120, 144, 192 and 241  
242 hours and the water analysed for *E. coli* as described above.

243

## 244 2.7 Statistical analysis

245 All *E. coli* counts underwent  $\log_{10}$  transformation prior to statistical analysis. To  
246 determine whether there was any difference in the CFUs reported using autosampler versus  
247 grab sampling methods we used the Altman-Bland graphical method coupled with a follow-  
248 up correlation and paired *t*-test (Altman & Bland, 1983). For analysis of die-off curves,  
249 different phases of cell population dynamics were identified from a visual inspection of the  
250 curves and categorised as: 1) slow die-off and 2) rapid die-off. Linear least squares  
251 regression was used to find the rate of change for replicates within each phase of population  
252 change. A Wilcoxon signed rank test was used to determine whether there was a significant

253 difference in the rate of change of cell numbers between treatments. All statistical tests were  
254 performed in the statistical package 'R' v 2.15.2 (2012).

255

### 256 **3. Results**

#### 257 *3.1 E. coli - Q relationships*

258 This study captured the temporal response of *E. coli* concentrations with Q from a  
259 small headwater catchment during six rain events during the relatively dry summer of 2013  
260 in central Scotland (Fig 2 and Fig 4a-f). The corresponding ambient temperature profile of  
261 the monitoring period is shown in Figure 3. These six events accommodated a range of peak  
262 Q with the smallest event reaching a maximum Q of 0.03 Ls<sup>-1</sup> (event 2; 15<sup>th</sup> June) and the  
263 largest event reaching a maximum Q of 1.04 Ls<sup>-1</sup> (event 1; 27<sup>th</sup> May). All peak Q values  
264 recorded were therefore low and approximately two orders of magnitude lower than the  
265 mean Q at this site over a typical hydrological year (140 Ls<sup>-1</sup>), with rain events failing to  
266 generate substantial stream flow and little hydrological response from the catchment during  
267 the summer monitoring period. Table 1 provides summary characteristics for each of the six  
268 events. The rainfall associated with event 1 resulted in a classic storm hydrograph response,  
269 with a steep rising limb and a gentle falling limb; although the peak Q was low at just over 1  
270 Ls<sup>-1</sup>, this was not unusual for a small headwater stream such as this during summer  
271 baseflow conditions. Hydrological activity was minimal over the course of the next 18 days  
272 and the peak Q of event 2 provided a contrasting and poorly defined hydrograph and  
273 pollutograph response, whilst hydrographs of the remaining storm events that were  
274 monitored had only marginally improved definition. The event associated with the highest  
275 peak concentration of *E. coli* occurred in July (event 4; 2855 CFU/100mL) despite the event  
276 generating a peak Q of only 0.087 Ls<sup>-1</sup>. The lowest peak concentration of *E. coli* (118  
277 CFU/100mL) was associated with the event that generated the largest peak Q (event 1). The  
278 two events captured in July occurred in close succession only two days apart and this  
279 general period of elevated hydrological activity appeared to generate much higher

280 concentrations of *E. coli* in water exported from the catchment. Concentrations recorded  
281 during events 4 and 5 were an order of magnitude greater than previous events although the  
282 microbial signatures did not follow a clear pattern with Q and no correlation was observed  
283 between Q and *E. coli* during these events. The peak instantaneous load for each event was  
284 also calculated to take into account the low flow impact on *E. coli* export from the headwater  
285 catchment (see Table 1). If the contributing area of the catchment is taken into account then  
286 the maximum instantaneous load observed over all six events was 182 CFU s<sup>-1</sup> ha<sup>-1</sup>.

287

288 **INSERT FIGURE 2, 3 & 4 HERE**

289 **INSERT TABLE 1 HERE**

290

291

292 *In-situ* turbidity readings for the six sampling dates varied from as low as 1 NTU  
293 through to 132 NTU (Table 1) and overall a relatively weak (but significant) correlation was  
294 observed between *E. coli* and turbidity observed across all events ( $r = 0.36$ ;  $P < 0.001$ ).  
295 Event 1 (lowest *E. coli* peak and highest Q) recorded the lowest turbidity values throughout  
296 the event. The highest turbidity values were associated with event 5 which registered the 2<sup>nd</sup>  
297 largest peak of *E. coli* (2350 CFU/100mL). No difference ( $P > 0.05$ ) was evident in *E. coli*  
298 concentrations determined during the rising limb versus the falling limb of storm  
299 hydrographs. The relationship between *E. coli* concentration and Q was explored across  
300 these six events but appeared complex with no consistent discernable patterns of cell  
301 emergence with Q and no clear trends in hysteresis observed.

302

### 303 *3.2 Autosampler vs Grab sampling*

304 A total of 42 comparative samples were collected simultaneously via aseptic grab  
305 sampling and using an autosampler collection hose connected to an ISCO 3700 automatic  
306 sampler. The 42 samples were collected over the course of multiple events from two  
307 different sites in the UK. Results of this cross comparison study are presented as a scatter

308 plot in Figure 5. In order to test for differences between the two methods it was necessary to  
309 first plot the difference between the CFUs obtained via the two different methods (e.g.  $CFU_1$   
310 -  $CFU_2$ ) versus the average of the CFUs produced using both methods (e.g.  $[CFU_1 + CFU_2] /$   
311 2) (Fig 6), and to then determine, through correlation, whether we can assume  
312 independence of the between-method differences and the size of the measurements (Altman  
313 & Bland, 1983). The correlation coefficient of the data presented in Figure 6 was found to be  
314  $-0.1$  ( $P > 0.05$ ) suggesting no significant association linking between-method differences  
315 and the size of the measurements. With independence confirmed, a paired  $t$ -test confirmed  
316 that there was no significant difference ( $P > 0.05$ ) between the CFUs observed by the two  
317 alternative methods of sample acquisition.

318

319 **INSERT FIGURE 5 and 6 HERE**

320

### 321 *3.3 Effect of autosampler storage on E. coli die-off*

322 Three distinct phases of *E. coli* population dynamics were observed within samples  
323 stored under both ambient and refrigerated conditions inside an autosampler unit (regrowth;  
324 slow die-off; rapid die-off). However, a 'growth rate' for the treatments is not presented  
325 because of the limited availability of sampling points during this phase. This initial population  
326 increase prior to two-stage 1<sup>st</sup>-order decline (Figure 7) was more pronounced for *E. coli* kept  
327 under ambient conditions (24 h) compared to those kept under refrigerated conditions (5 h).  
328 The magnitude of increase under ambient temperature conditions was equivalent to 0.33  
329  $\log_{10}$  *E. coli*, whereas for the refrigerated treatment the magnitude of increase measured  
330 0.14  $\log_{10}$  *E. coli* (see Fig 7). Table 2 shows the average rate of change for each of the two  
331 die-off phases of the two temperature treatments and the results of a Mann-Whitney-  
332 Wilcoxon signed rank test used to determine whether these rates of change differed across  
333 treatments. The rate of die-off accelerated in both treatments after 120 h, with die-off rate  
334 occurring more rapidly in the refrigerated treatment during the final die-off phase ( $P < 0.05$ ).  
335 Differences between *E. coli* counts at each time point relative to the initial concentration

336 were also investigated for both temperature treatments. Under refrigerated conditions a  
337 significant difference ( $P < 0.05$ ) in *E. coli* counts was only observed after 120 hours of  
338 storage (though at 96 hours  $P = 0.06$ ). Concentrations of *E. coli* stored under ambient  
339 conditions showed no significant difference over the first 5 hours of storage relative to the  
340 initial sample, but following 12 hours *E. coli* concentration had become significantly higher ( $P$   
341  $< 0.05$ ) than the initial input.

342

343 **INSERT FIGURE 7 HERE**

344 **INSERT TABLE 2 HERE**

345

#### 346 **4. Discussion**

##### 347 *4.1 E. coli concentrations in response to minor rainfall events*

348 Large storm events are known to mobilise and transfer diffuse microbial pollutants  
349 from agricultural land to water, although the extent of this is dependent upon catchment  
350 characteristics such as land use, topography and soil type, together with rainfall patterns and  
351 antecedent soil moisture (McKergow & Davies-Colley, 2010). Our knowledge of how these  
352 factors interact to affect diffuse microbial pollution is limited because of the complexity and  
353 heterogeneity of catchment systems (Winter *et al.*, 2011; Fish *et al.*, 2009). The impact of  
354 relatively small but persistent rainfall events on microbial water quality during warmer and  
355 typically drier summer periods is one such scenario that has evaded investigation. Our  
356 results have highlighted a number of general observations about the subtleties of microbial  
357 pollution during intermittent rainfall throughout dry weather periods, and have provided some  
358 insight into how contrasting event characteristics across a typical mixed land use area can  
359 regulate *E. coli* dynamics. While rainfall did occur during the study period, the accompanying  
360 increase in Q was minor compared to studies focusing on the monitoring of large storm  
361 driven pulses of microbial pollution through catchment systems (e.g. Wyer *et al.*, 2010).

362 Data from the six monitored events suggest that in the water column of a small  
363 agricultural stream, even very small increases in Q can give rise to elevated *E. coli*

364 concentrations. Previous reports have demonstrated that levels of FIOs can increase by at  
365 least an order of magnitude during 'event' conditions (Kay *et al.*, 2010). Importantly, our  
366 results, e.g. 'event 1', support the scalability of this 'rule' from large catchments and major  
367 intense storms down to much smaller headwater catchments and events driven by more  
368 modest rainfall. Although the hydrograph for 'event 2' accommodated a much reduced peak  
369 Q this is not surprising given the consistently low baseflow conditions prior to this event  
370 despite the antecedent rainfall being actually higher than for the previous event. Little, if any,  
371 of that rainfall however, generated any noticeable impact on the baseflow Q of the stream,  
372 probably due to the lower intensity precipitation distributed over a longer timeframe resulting  
373 in little external hydrological input being successfully delivered to the stream. Despite 'event  
374 2' converting to a weak hydrograph signature, the increase in *E. coli* concentration was  
375 around five times higher than during 'event 1'. The slight increase in flow from a very low  
376 baseflow condition would probably have been insufficient to resuspend the uppermost layer  
377 of streambed sediment which can, if conditions allow, provide a source of higher *E. coli*  
378 concentrations relative to the water column (Pachepsky & Shelton, 2011; Muirhead *et al.*,  
379 2004). Given the scale of this 'event' it is also unlikely that carriage of bacterial cells from the  
380 surrounding land contributed to this increase. Thus, the increase in *E. coli* for 'event 2' most  
381 likely reflects the deposition of fresh faecal material into the stream either by cattle further  
382 upstream or by sheep grazing in fields adjacent to the monitoring point. Furthermore, the  
383 frequency of animal activity in and around the watercourse is likely to have increased during  
384 the warm weather (see increasing temperatures throughout the study period in Fig 3) leading  
385 to more defecation in close proximity to the stream, or directly into the water (White *et al.*,  
386 2001).

387 'Event 3' resulted in a similar, though slightly more pronounced, hydrograph and in  
388 turn a more defined increase in *E. coli* concentrations relative to 'event 2'. This repeated  
389 pattern could suggest that an in-stream store of *E. coli*, possibly held within a faecal deposit,  
390 was being eroded over time with increases in Q. However, more controlled laboratory-based

391 mobilisation experiments (e.g. Hodgson *et al.*, 2009) and flume studies (e.g. McDaniel *et al.*,  
392 2013) would be needed to determine critical thresholds of *E. coli* release both from  
393 sediment, and also from submerged faecal deposits. The exact reasons for the elevated  
394 microbial counts recorded during events 4 and 5 are unclear but certainly the rainfall  
395 distribution between event 3 and 4 had increased, which resulted in an increased baseflow  
396 Q. Elevated turbidity would provide a useful surrogate to indicate any direct faecal pollution;  
397 however, while turbidity was relatively high for events 4 and 5 other events also exhibited  
398 high turbidity but did not show the same response in *E. coli* concentration. This adds further  
399 evidence to suggest that while turbidity can, under certain circumstances, serve as a useful  
400 proxy for microbial water quality it is perhaps not as robust a surrogate as sometimes  
401 assumed via anecdotal accounts of diffuse microbial pollution. Others have raised similar  
402 concerns of the usefulness of turbidity as a surrogate for *E. coli* presence given that spatially  
403 distinct sources of *E. coli* and turbidity can exist in catchment systems (McKergow & Davies  
404 Colley, 2010), though this is often more of an issue at larger catchment scales.

405         The calculation of peak instantaneous loads is crucial for considering the overall  
406 impact of varying storm typologies on microbial water quality. For example, the combination  
407 of Q and *E. coli* concentrations observed during event 5 resulted in the highest recorded  
408 peak instantaneous *E. coli* load at this site (6744 CFU s<sup>-1</sup>, equivalent to 182 CFU s<sup>-1</sup> ha<sup>-1</sup>).  
409 This relatively small microbial load was associated with the highest rainfall rates observed  
410 over the study period but still represented a relatively minor rainfall event during low flow  
411 stream conditions. In comparison, *E. coli* load from grazed grassland following a more  
412 intense rainfall event, with daily rainfall in excess of 20mm day<sup>-1</sup>, resulted in 1.25 x 10<sup>6</sup> CFU  
413 s<sup>-1</sup> ha<sup>-1</sup> (Oliver *et al.*, 2005b).

414

#### 415 *4.2 Evaluating the role of autosamplers for microbial water quality assessment*

416         There are reported differences in microbial concentrations determined in samples  
417 collected manually versus those obtained using autosamplers, although these differences

418 were considered too small to be of practical significance (Ferguson, 1994). Likewise, our  
419 analysis also showed no significant difference between autosampler-determined water  
420 quality and duplicate samples collected using aseptic grab sampling. However, while  
421 autosamplers can reduce the resources needed for continual monitoring, maintaining the  
422 integrity of microbial populations in aquatic samples is essential for accurate and  
423 reproducible environmental monitoring. The results of our die-off experiment clearly  
424 demonstrated the advantage of refrigeration in maintaining concentrations of *E. coli* at levels  
425 close to their original magnitude at the point of sample collection. Up to 96 hours after  
426 collection the concentrations of *E. coli* did not differ significantly from concentrations at time  
427 0. This finding complements the results reported by Ferguson (1994) whereby faecal  
428 coliform levels did not change throughout the 18 hour duration of monitoring in a refrigerated  
429 autosampler.

430         Concentrations of *E. coli* under ambient conditions changed more quickly relative to  
431 the refrigerated samples and differed from the initial concentration within only 12 hours of  
432 sample collection, but the difference related to an increase in cell numbers over time rather  
433 than an expected decline. This may be due to the high faecal matter content of the inoculum  
434 applied to each replicate bottle at the onset of the experiment which represented a heavily  
435 polluted water sample typical of stream water contaminated by faeces from direct defecation  
436 by grazing livestock. The high loading with organic matter coupled with the warm  
437 temperatures at times in excess of 20°C, and protection from UV radiation, could have  
438 provided conditions conducive for supporting high numbers of *E. coli* and their subsequent  
439 replication. Growth of *E. coli*, including the pathogenic strain *E. coli* O157, in sterile  
440 freshwater with natural nutrients at low concentrations has been reported (Vital *et al.*, 2008;  
441 Williams *et al.*, 2012). However, while our study was carried out over a period of very warm  
442 weather in Scotland the average temperature over the first 24 hours was only 15°C  
443 compared with previous studies using temperatures more conducive for *E. coli* growth, e.g.  
444 30°C (Vital *et al.*, 2008). The high faecal matter content and associated protective habitat  
445 and supply of nutrients could have provided conditions that enabled cell replication despite

446 the suboptimal temperatures for cell growth (Shelton *et al.*, 2014). Data reported by others  
447 suggests that bottle-effects from short term (3 - 9 h) or extended short term (3 - 24 h) holding  
448 in an autosampler under ambient conditions do not impact significantly on culturable  
449 *Enterococcus* spp. counts (Ghazaleh *et al.*, 2014). The extended short-term results contrast  
450 with our finding for another FIO, *E. coli*, whereby significant differences from  $T_0$   
451 concentrations were observed after only 12 hours. This difference may relate to the different  
452 indicator organism under investigation, contrasting properties of the estuarine versus fresh  
453 water sources or could have been driven by variable temperature profiles associated with  
454 the two studies, though temperatures are not reported explicitly by Ghazaleh *et al.* (2014).

455         Results from the autosampler evaluation phase of this study reinforce some  
456 important issues regarding the collection of samples for microbial water quality sampling. If  
457 care is taken to sterilise autosampler bottles immediately before they are deployed then they  
458 can offer an effective method of sample acquisition, particularly in remote field locations  
459 during storm sampling campaigns. Others have shown that appropriate steps need to be  
460 taken to reduce residual FIO accumulation within autosampler inlet hoses (Hathaway *et al.*,  
461 2014). However, sample storage time in the autosampler unit needs careful consideration  
462 depending on the anticipated length of a sampling campaign. Storage beyond 12 hours  
463 inside a standard autosampler unit is likely to impact on FIO numbers in freshwater samples,  
464 reinforcing the importance of ensuring that field technicians are alerted via  
465 telecommunications (e.g. SMS) when an autosampler routine is initiated. Clearly, a key  
466 benefit of refrigeration is to shorten the length of the growth phase making this a more  
467 accurate method for sample collection if using an autosampler unit. Previous research has  
468 reported FIO concentrations from samples stored in an unrefrigerated autosampler unit for  
469 up to a week by applying a correction factor to account for the expected die-off rate of the  
470 target population (Vinten *et al.*, 2008). By using this back calculation the authors retraced  
471 die-off curves to obtain the initial FIO concentration held in the sample collection bottle at  $T_0$ .  
472 While the rationale for such an approach may appear logical the opportunity for erroneously  
473 estimating FIO population change under field-relevant conditions is large. The results of our

474 study urge caution on the use of such an approach, especially if samples are obtained in  
475 summer where ambient temperatures in bottles could reach in excess of 20°C as part of a  
476 diurnal cycle.

477

## 478 **Conclusion**

479         Low intensity (<4mm hr<sup>-1</sup>) rainfall events observed at headwater scales during  
480 summer months can increase FIO concentrations in small streams by an order of magnitude.  
481 While the absolute concentrations recorded in this study were low, this finding is important  
482 for demonstrating the transferability of rules of FIO behaviour whereby an increase in Q  
483 observed in well-defined hydrographs moving from relatively 'low' to 'high' flow carries a  
484 signature of increasing *E. coli* concentrations. However, further research is needed to tease  
485 out the subtleties of *E. coli*-Q event dynamics across a breadth of different storm typologies  
486 while also disentangling any interference in microbial water quality signatures of large FIO  
487 sources (e.g. direct deposition) on concentration-Q responses, which is clearly a challenge  
488 in summer grazing seasons. The overall microbial load exported during low intensity rainfall  
489 events is much reduced (by up to four orders of magnitude, if not more) compared with high  
490 intensity rainfall events and particularly those that occur during periods of wetter weather  
491 and so the impact of these events is perhaps spatially constrained. Sampling methods can  
492 also affect the reporting of microbial water quality if storage of samples within autosampler  
493 units is not given proper consideration. Our study provides some assurance of minimal  
494 deterioration of sample quality when water is collected using an automatic sampler for  
495 subsequent microbiological analysis provided that samples are collected in a prompt fashion  
496 for return to the laboratory.

497

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504

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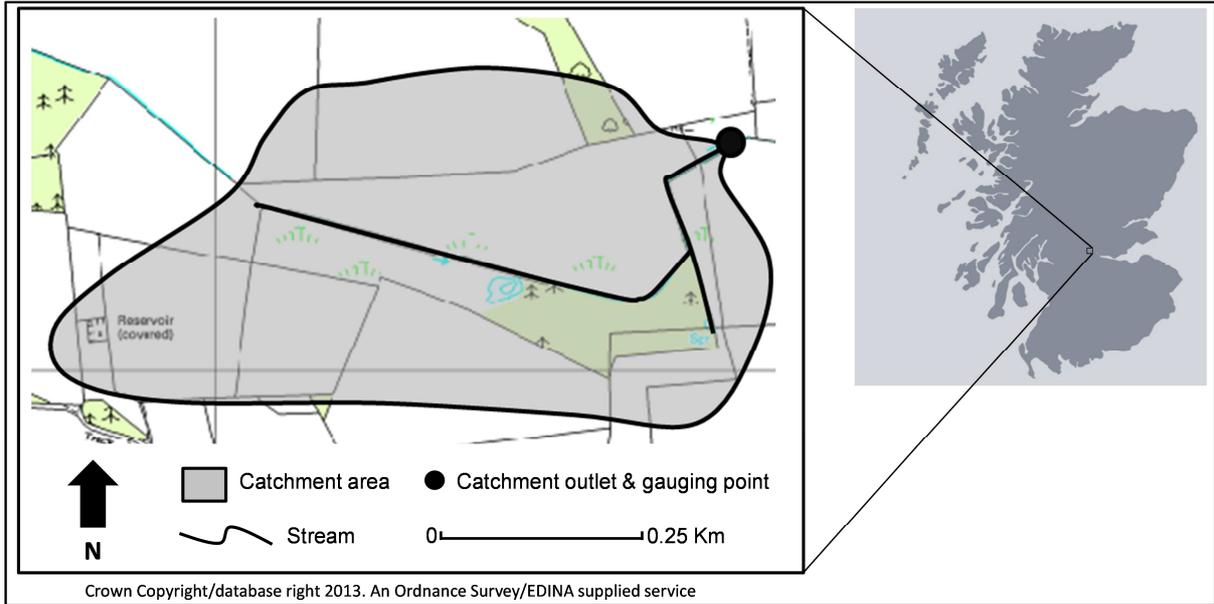
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684 **Figure 1:** Location and area of the study catchment

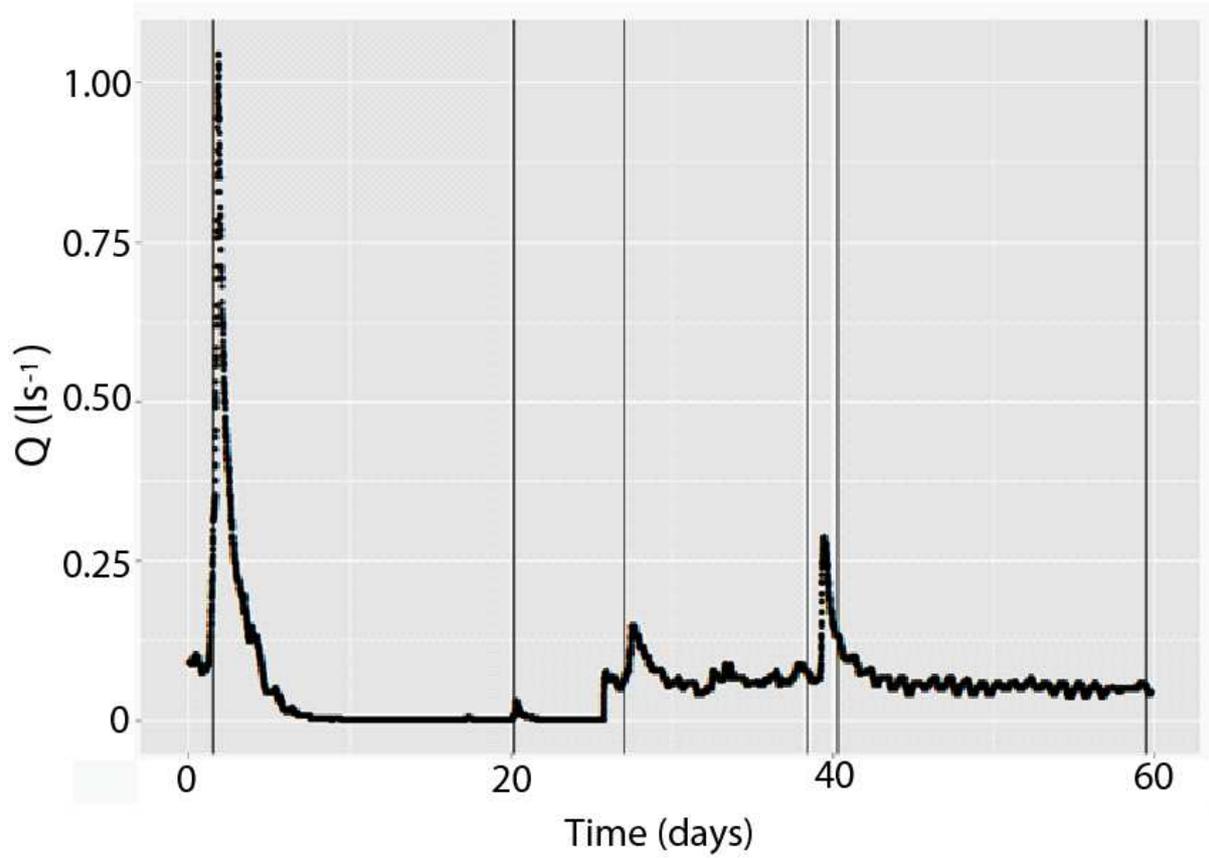
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691 **Figure 2:** Hydrograph of the entire study period with vertical lines indicating when the first  
 692 sample of each event was captured. Events 1-6 are sequential in their occurrence.

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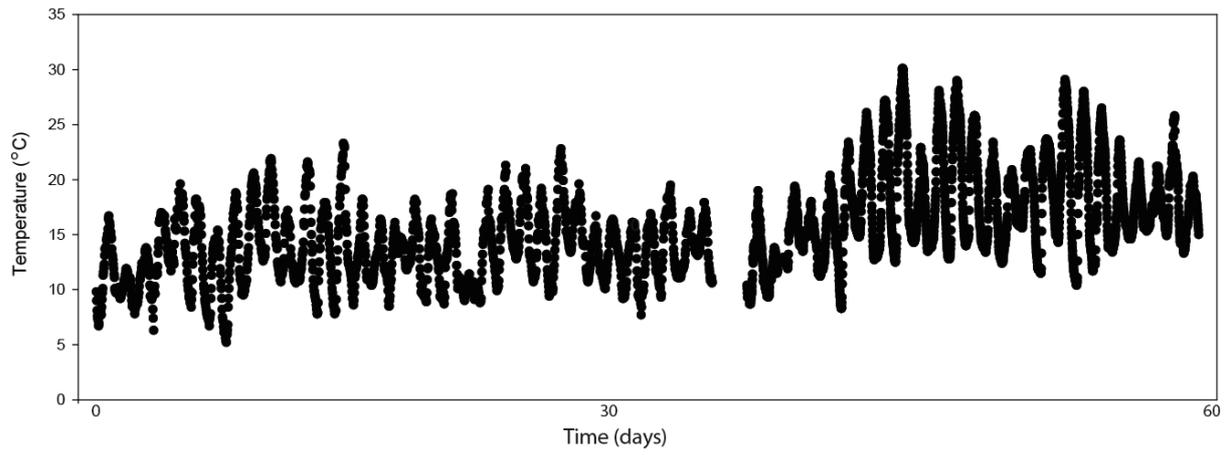
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702 **Figure 3:** Air temperature profile of the study period

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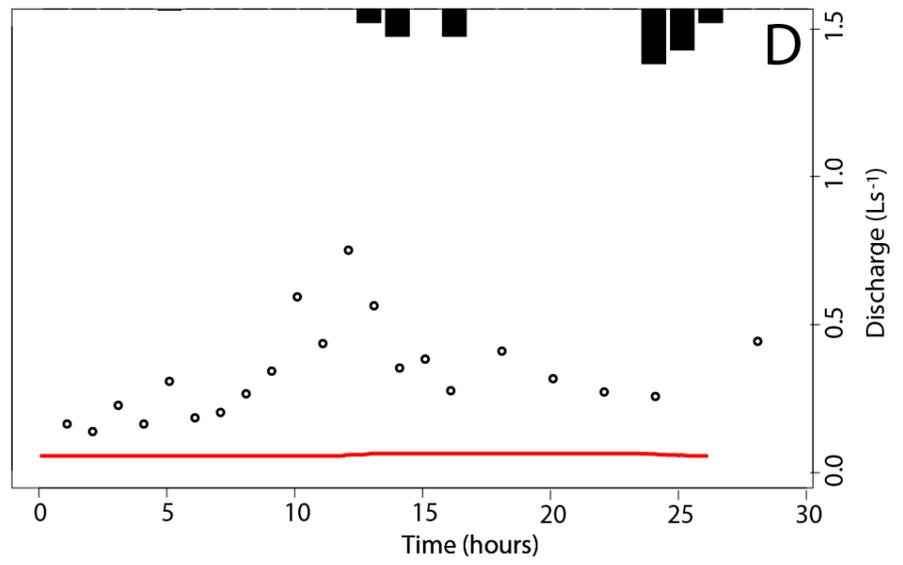
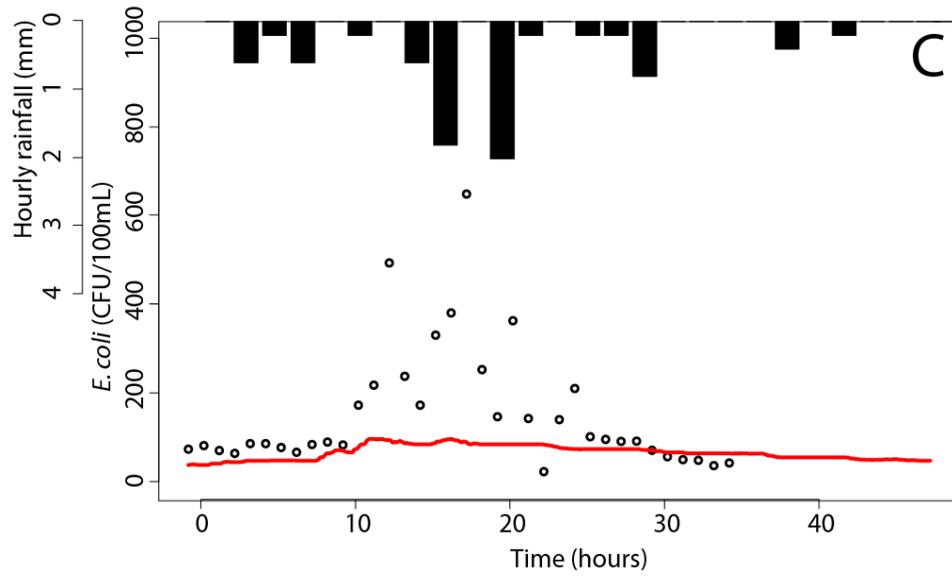
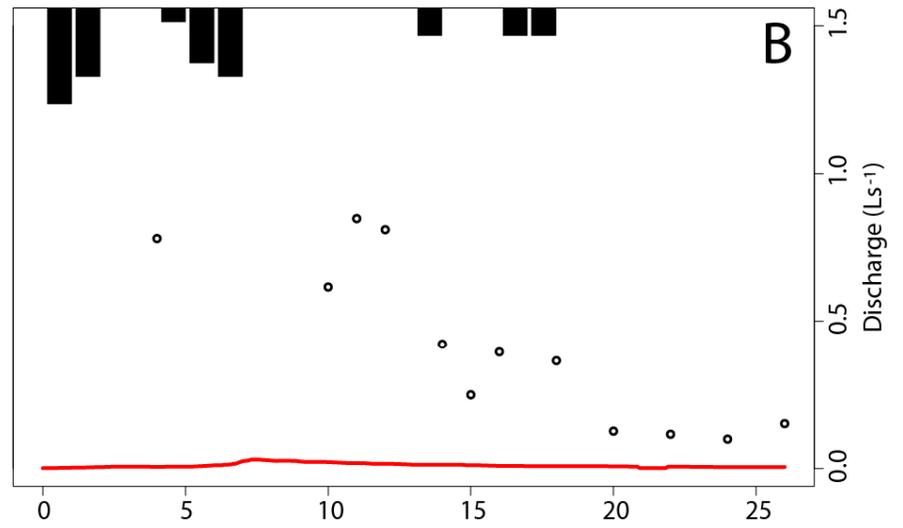
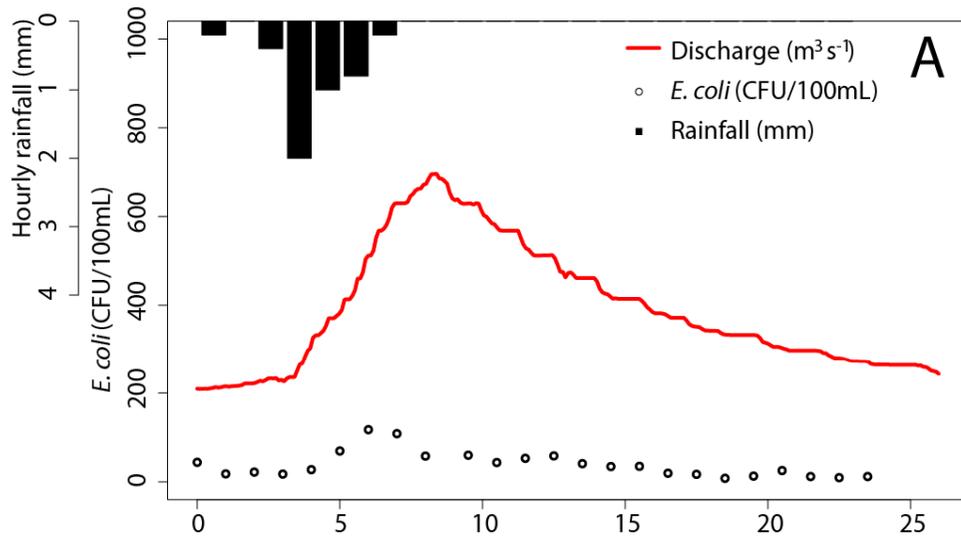
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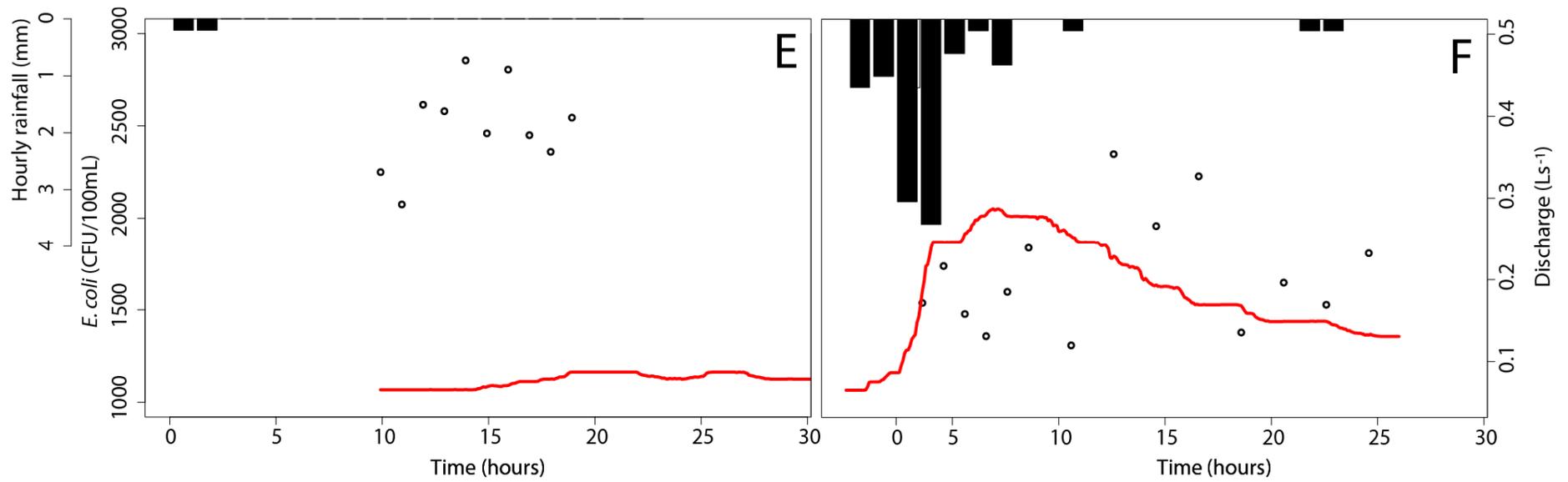
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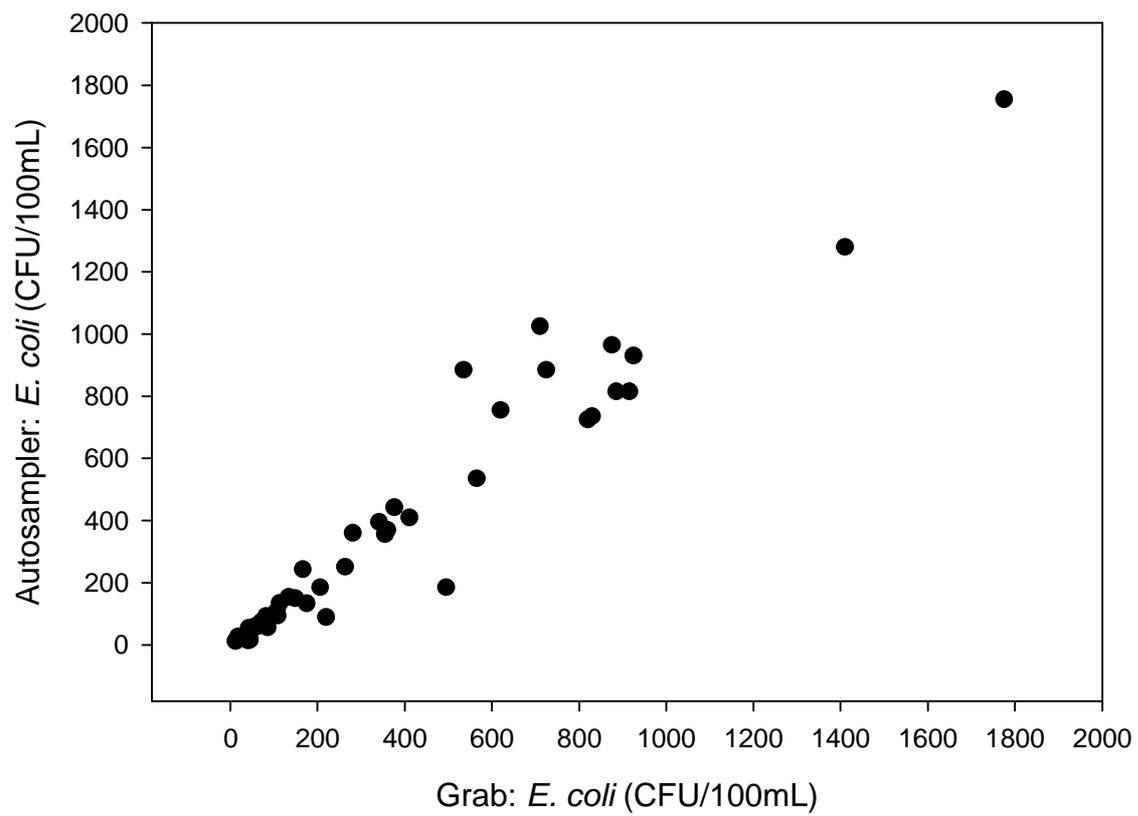
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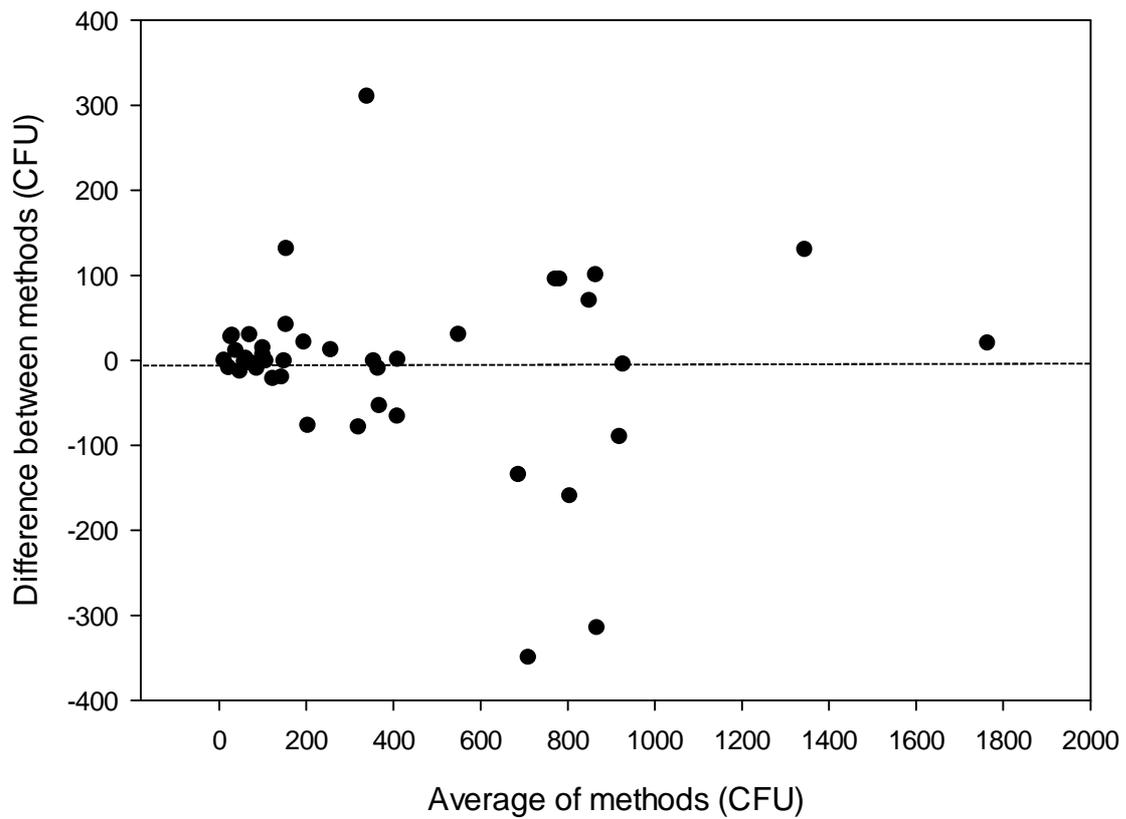




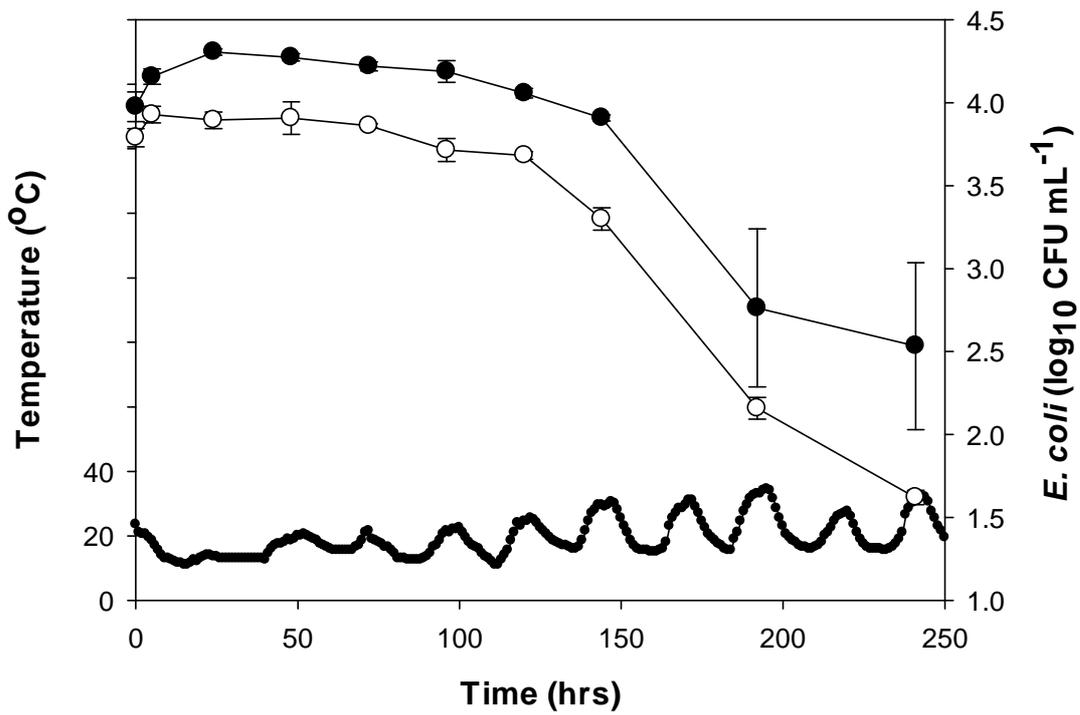
**Figure 4:** (a) to (d) show *E. coli* (circles) and Q (red line) during events 1, 2, 3, and 6, respectively; (e) and (f) show *E. coli* and Q for events 4 and 5, respectively. Note the differing scales for both *E. coli* and Q between plots (a) to (d) and (e) & (f).



**Figure 5.** Comparison of *E. coli* concentrations derived from autosampler and manual grab sampling.



**Figure 6.** Difference in CFUs determined using the grab and autosampler methods versus the average CFUs determined using both methods. Dashed line represents relative bias (mean of the differences across all paired samples; -5.9)



**Figure 7.** *E. coli* persistence over time under ambient (solid circles) and refrigerated (4°C; hollow circles) conditions. Ambient temperature fluctuations inside autosampler unit depicted by via black line)

**Table 1.** Summary characteristics for the six 'events' investigated.

Event	Date	Event duration (hours)	Peak Q (L s <sup>-1</sup> )	Peak <i>E. coli</i> concentration (CFU 100mL <sup>-1</sup> )	Peak <i>E. coli</i> instantaneous load (CFU s <sup>-1</sup> )	Antecedent rainfall (mm)		Range of turbidity (NTU; min-max)
						2 day rainfall	7 day rainfall	
1	27/05/2013	23.5	1.044	118	1232	9.2	9.2	1.35 - 1.82
2	15/06/2013	22.0	0.030	565	170	10.4	17.4	1.86 - 5.65
3	22/06/2013	47.0	0.149	650	969	8.0	8.6	1.72 - 68.92
4	03/07/2013	24.0	0.087	2855	2484	4.0	11.8	6.66 - 41.23
5	05/07/2013	29.0	0.287	2350	6744	8.4	18.0	19.29 - 131.60
6	24/07/2013	25.0	0.056	495	282	2.6	3.2	42.74 - 65.39

**Table 2:** Decline rate constants for *E. coli*, reflecting the two observed die-off phases of the *E. coli* population dynamics. The *p* value shows the results of a Mann-Whitney-Wilcoxon test investigating whether there were significant differences between the decline rates of each treatment at each phase.

Treatment temperature	Modelled linear rate constant	
	slow die-off (hr <sup>-1</sup> ) <sup>a</sup>	rapid die-off (hr <sup>-1</sup> ) <sup>a</sup>
Fluctuating ambient	-0.0037	-0.0143
Constant refrigerated	-0.0045	-0.0173
<i>p</i> value	>0.05	0.03