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

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Abstract

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

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

1. Introduction

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

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

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

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

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

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

2. Materials and methods

2.1. Study catchment

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

INSERT FIGURE 1 HERE

2.2. In situ hydrological monitoring

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

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

2.3. Water sample collection during rainfall events

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

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

2.4. Microbiological analysis

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

2.5. Autosampler versus grab sampling

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

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

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

2.7 Statistical analysis

All *E. coli* counts underwent $\log_{10}$ transformation prior to statistical analysis. To determine whether there was any difference in the CFUs reported using autosampler versus grab sampling methods we used the Altman-Bland graphical method coupled with a follow-up correlation and paired *t*-test (Altman & Bland, 1983). For analysis of die-off curves, different phases of cell population dynamics were identified from a visual inspection of the curves and categorised as: 1) slow die-off and 2) rapid die-off. Linear least squares regression was used to find the rate of change for replicates within each phase of population 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^{-1} (event 2; 15th June) and the
263 largest event reaching a maximum Q of 1.04 Ls^{-1} (event 1; 27th 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^{-1}), 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^{-1}, 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^{-1}. 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

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

INSERT FIGURE 2, 3 & 4 HERE

INSERT TABLE 1 HERE

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

3.2 Autosampler vs *Grab sampling*

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

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

INSERT FIGURE 5 and 6 HERE

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

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

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

INSERT FIGURE 7 HERE

INSERT TABLE 2 HERE

4. Discussion

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

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

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

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

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

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

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

4.2 Evaluating the role of autosamplers for microbial water quality assessment

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

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

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

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

Conclusion

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

Acknowledgements

This study was part-funded by the UK Department for Environment, Food and Rural Affairs (Defra) in association with project WQ0129 and by the University of Stirling. The authors would like to acknowledge the valuable and insightful contribution of the participating farmers

and are particularly grateful for land access permissions. Finally, the constructive comments from two anonymous referees helped to improve the overall quality of this manuscript.

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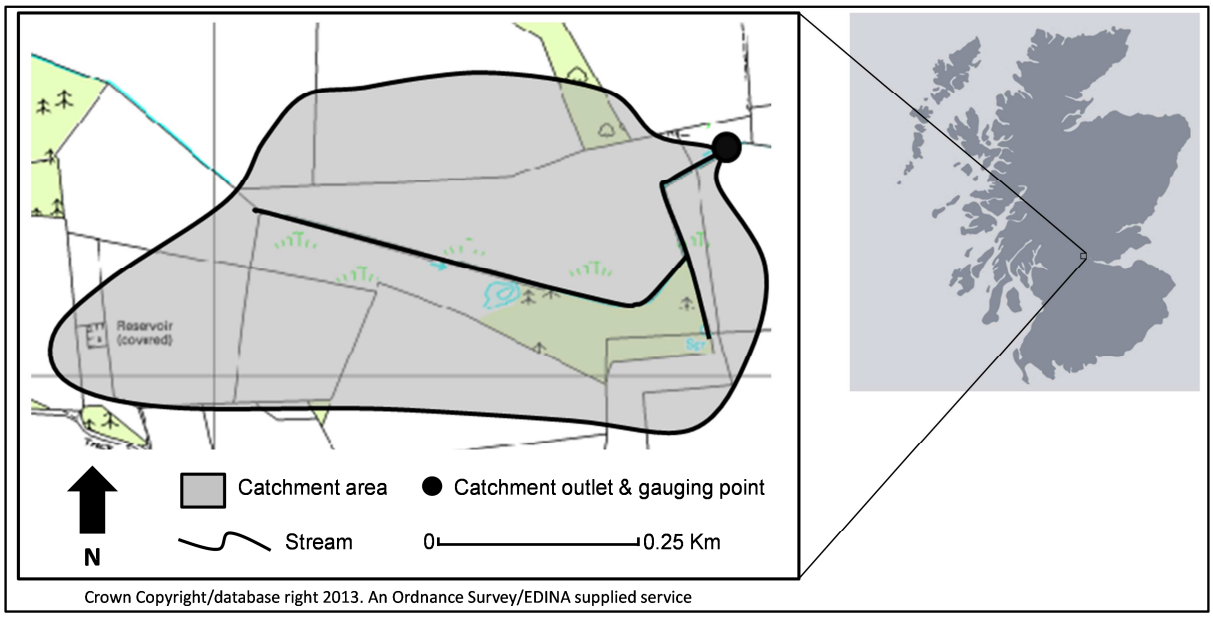
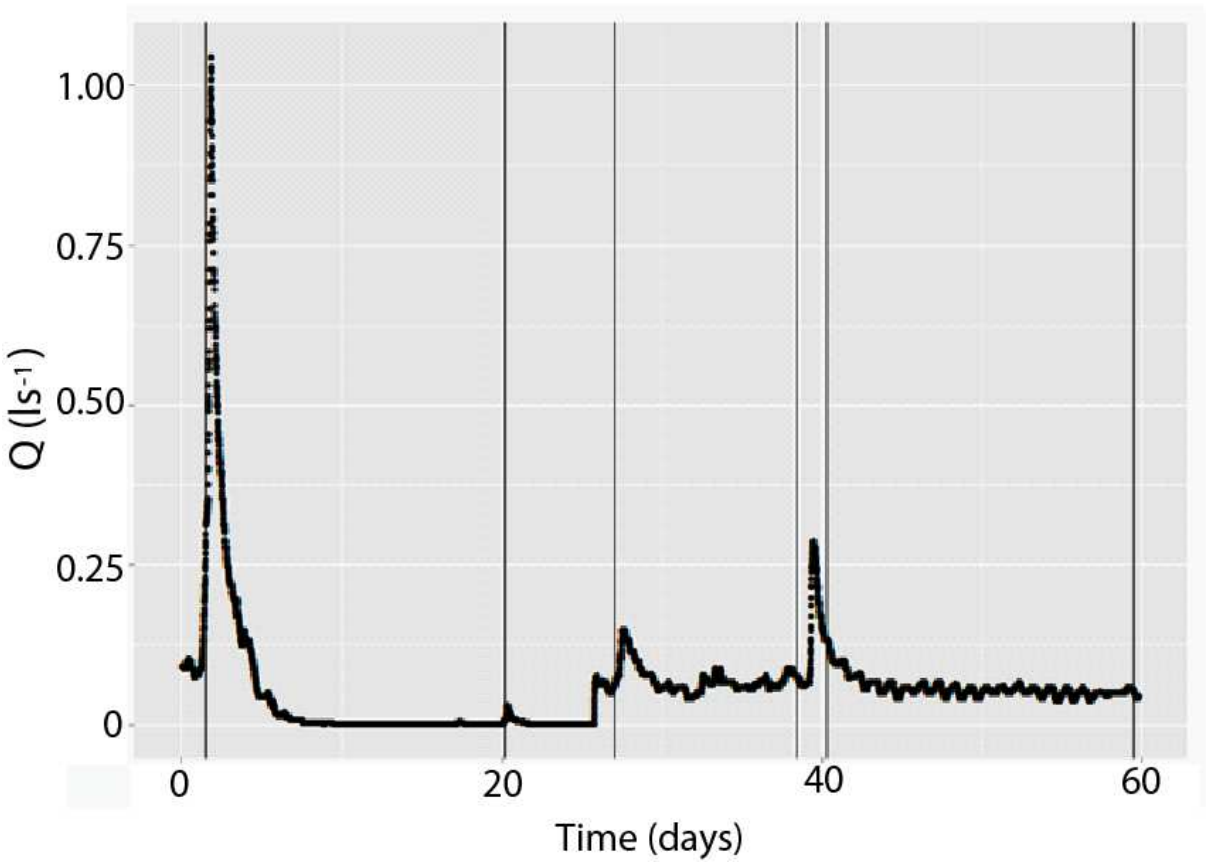


Figure 1: Location and area of the study catchment



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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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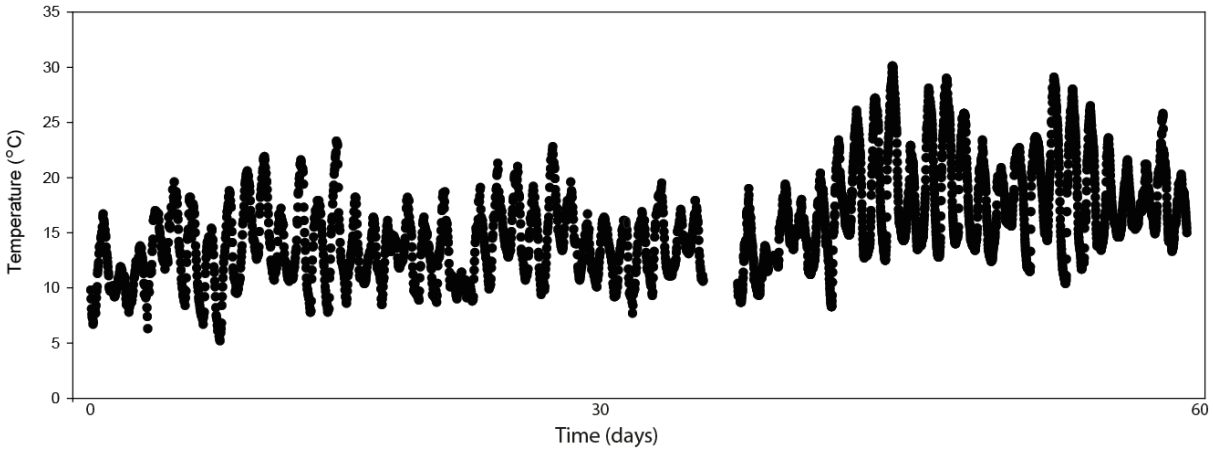
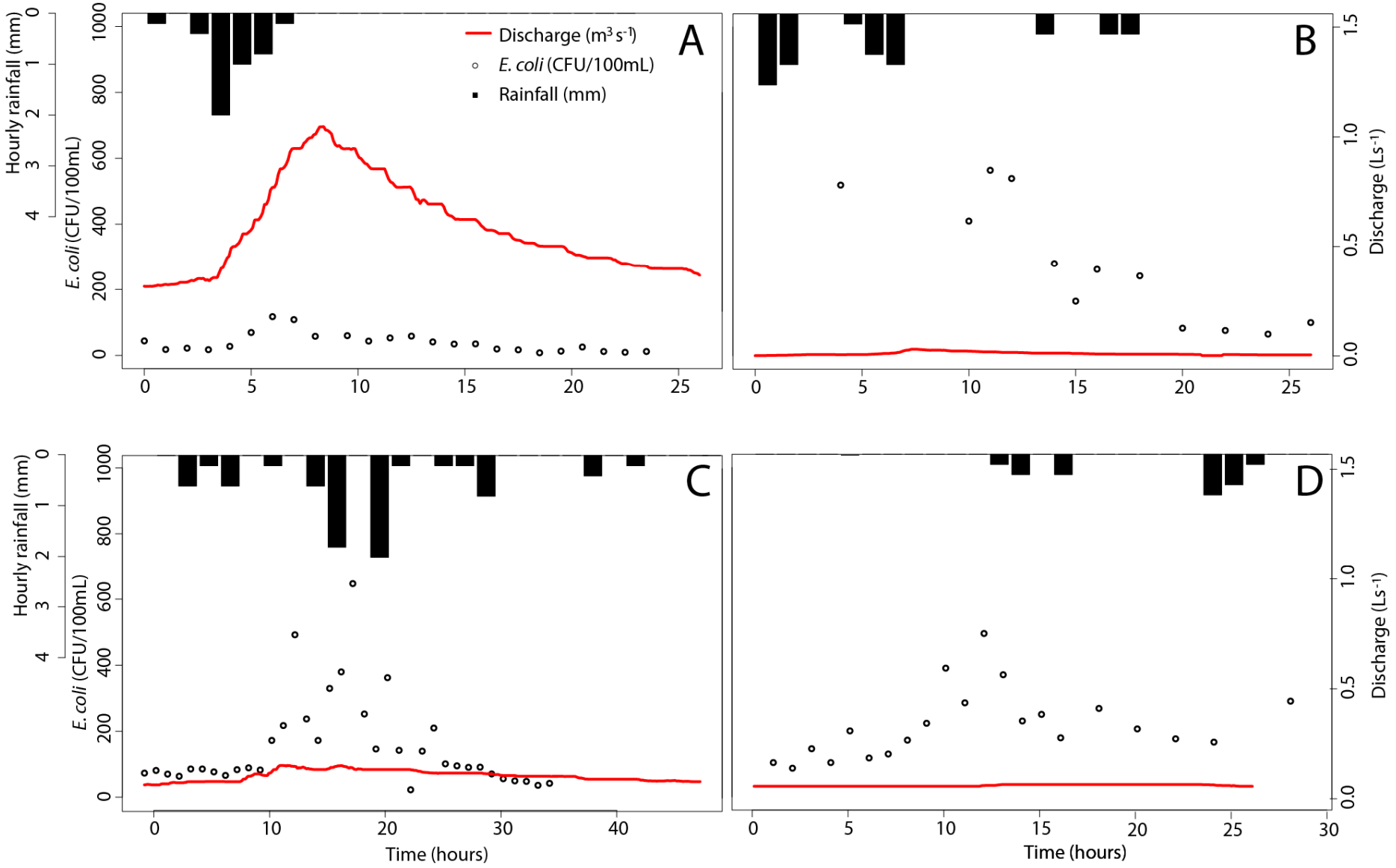


Figure 3: Air temperature profile of the study period


A
B
C
D
Discharge (m³ s⁻¹)
E. coli (CFU/100mL)
Rainfall (mm)
Hourly rainfall (mm)
E. coli (CFU/100mL)
Discharge (Ls⁻¹)
Time (hours)
Time (hours)

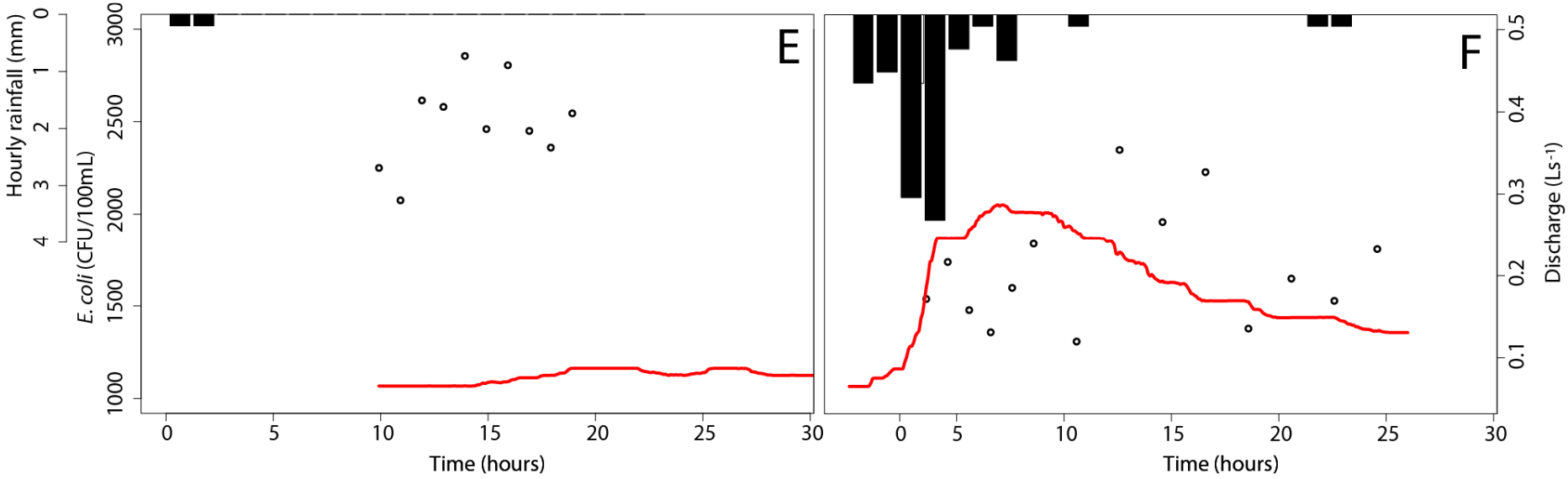


Figure 4: (a) to (d) show *E. coli (circles)* and Q (red line) during events 1, 2, 3, and 6, respectively; (e) and (f) show *E. coli* and Q for events 4 and 5, respectively. Note the differing scales for both *E. coli* and Q between plots (a) to (d) and (e) & (f).

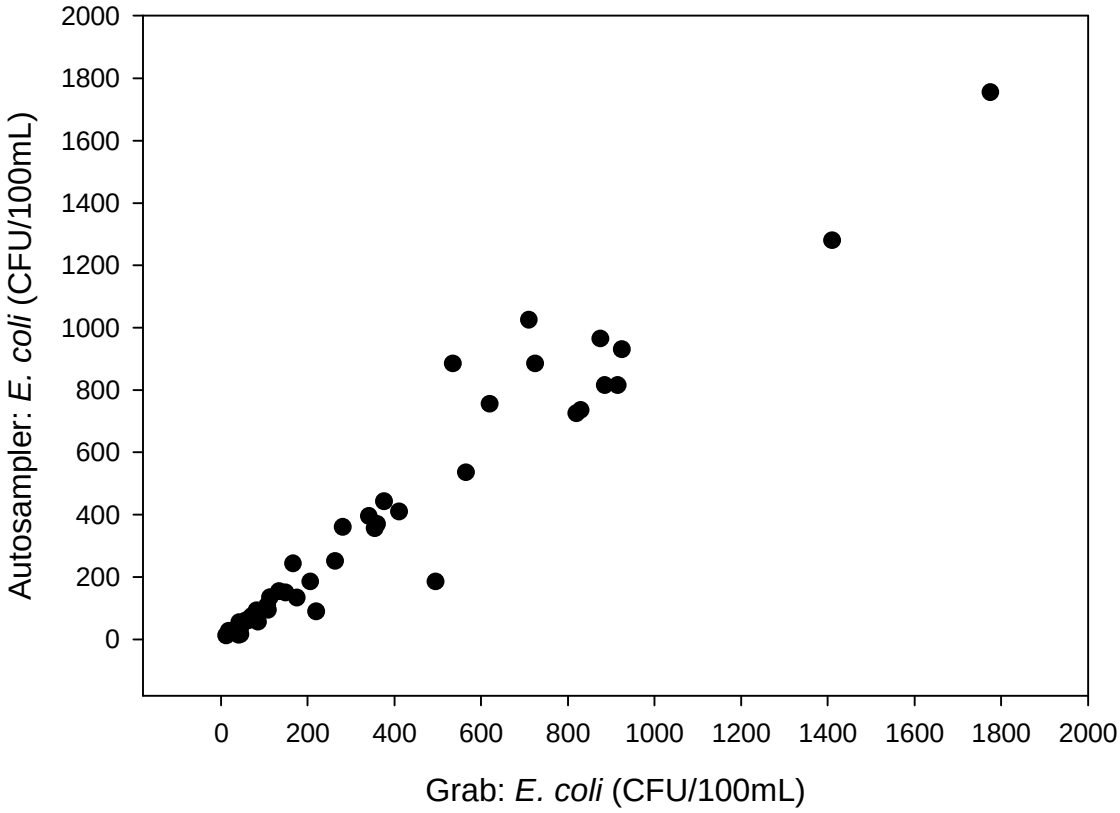


Figure 5. Comparison of *E. coli* concentrations derived from autosampler and manual grab sampling.

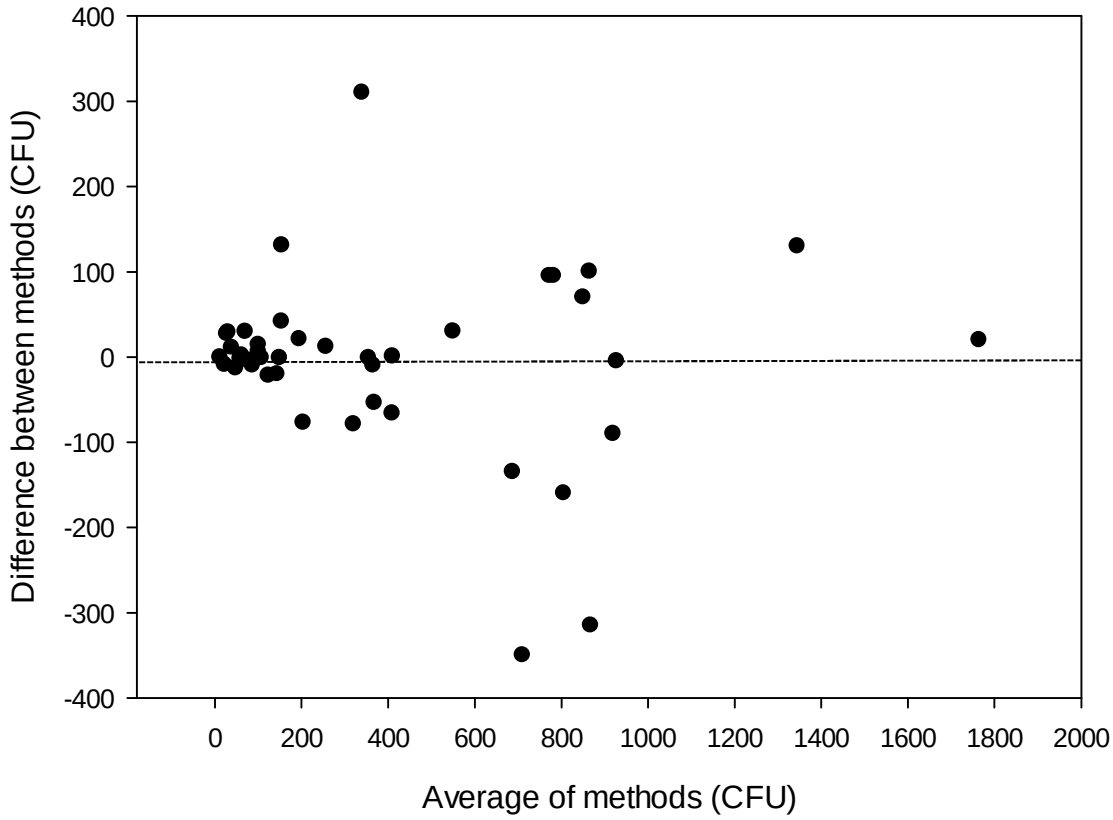


Figure 6. Difference in CFUs determined using the grab and autosampler methods versus the average CFUs determined using both methods. Dashed line represents relative bias (mean of the differences across all paired samples; -5.9)

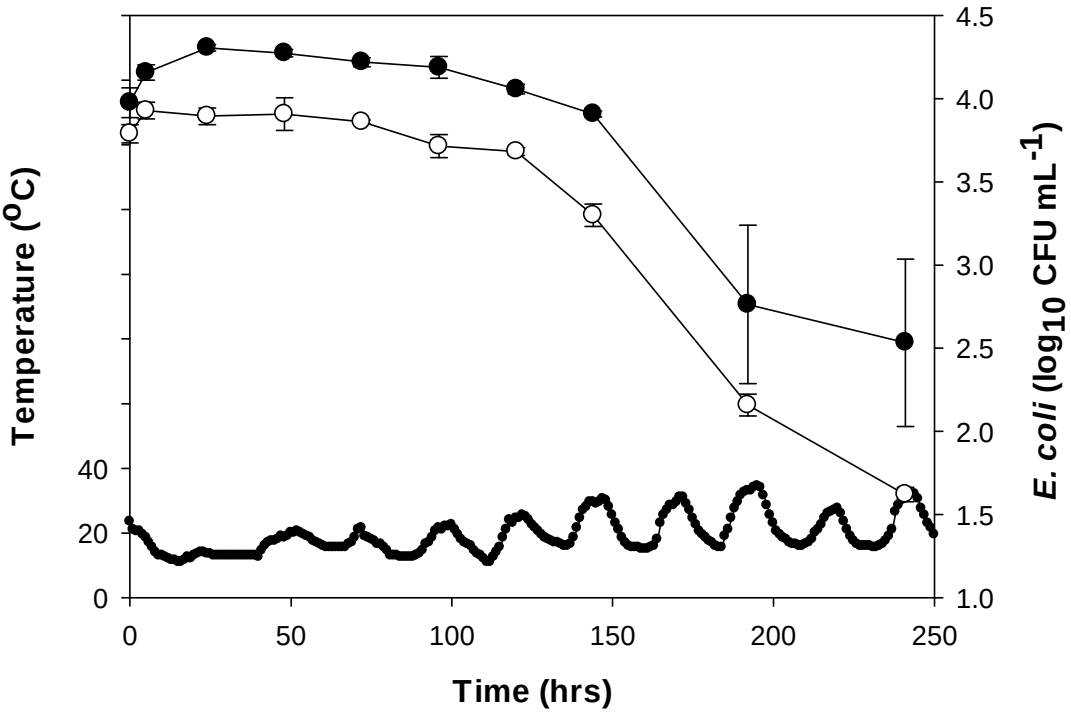


Figure 7. *E. coli* persistence over time under ambient (solid circles) and refrigerated (4°C; hollow circles) conditions. Ambient temperature fluctuations inside autosampler unit depicted by via black line)

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

Event	Date	Event duration (hours)	Peak Q ($L\ s^{-1}$)	Peak *E. coli* concentration ($CFU\ 100mL^{-1}$)	Peak *E. coli* instantaneous load ($CFU\ s^{-1}$)	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^{-1})[a]	**rapid die-off (hr^{-1})[a]**
Fluctuating ambient	-0.0037	-0.0143
Constant refrigerated	-0.0045	-0.0173
p value	>0.05	0.03