Novel bacterial ratio for predicting faecal age

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Abstract This study presents an extension of ongoing research into the utility of the ratio of colonies isolated on membrane filters during the total coliform test using m-Endo broth media. Investigations into the relative shifts in concentrations of indicator bacterial populations over time, in laboratory-based survival studies conducted with filtered river water, were undertaken. Also, analysis of Kentucky River water quality data collected from the inlet of a local water treatment plant was carried out. Survival studies found that the ratio between the raw concentrations of atypical colonies (AC) and total coliform colonies (TC) was directly related to the amount of time coliform spiked river water had been held in open jars in the laboratory. The AC/TC ratio in the jars would rise from $<1$ at the time of coliform spiking to $>200$ within 4d. The rise in AC/TC ratio with time in river water was confirmed in the analysis of two years of Kentucky River water quality data where the average AC/TC ratio during months with high river flow (rain) was 3.37 and rose to an average of 27.58 during months with low flow. The average AC/TC ratio during high flow months compared to that of raw human sewage (3.9) and the ratio increased to values associated with animal impacted urban runoff (18.9) during low flow months.

Keywords Atypicals; bacteria; coliforms; indicators; ratios; water quality

Introduction
It is not enough to know that faecal coliforms (FC) are present in surface waters. Information about the most probable animal source and age of faecal materials is requisite to estimate the potential risk indicated and set standards for recreational contact. The objective of the study was to investigate how AC/TC ratios varied with age and relate these studies to historical data collected at the intake to the Kentucky American water treatment plant. During rain events, large influxes of AC, TC and FC can be present in surface waters. The levels of FC can exceed Kentucky’s recreational contact standard of 200CFU/100 mL. While TC and FC normally die off quickly after being flushed into the river by rain events, AC are more associated with the normal flora of the river and persist in relatively stable concentrations. This persistence of AC colonies can be an asset in determining the age of faecal matter when used in a bacterial ratio that captures relational meanings between the relative concentrations.

Materials and methods
Description of survival study water source and Kentucky River sample site
Surface water was collected for investigation from the Kentucky River below Lock Number 10 at the intake to a drinking water treatment facility. The Kentucky River is essentially a series of small lakes connected by the lock system and is heavily impacted by point and non-point sources of faecal contamination, often closing state beaches for recreational water contact due to high faecal coliform levels (>200 CFU/100 mL). For the historic river dataset the water was assayed as is at the water treatment plant laboratory without any pre-treatment. For the laboratory based survival study, a grab sample of the river water taken was on June 12th 2001 and treated to reduce turbidity and microbial populations by filtration through Whatman #1 paper filters (particle retention $>11$ $\mu$m) before use. The water from this day had a pH of 7.5, alkalinity of 79 mg/L as CaCO$_3$, total hardness of 152 mg/L.
as CaCO₃ and a turbidity of 16 NTU. A few days prior to the day the grab sample was taken, the river turbidity had risen from 6.3 NTU to a peak value of 77 NTU from a rainfall event, and had then fallen to 16 NTU.

Survival study sampling and sample analysis
Filtered, but still bioactive, Kentucky River water (3 L) was placed into three replicate sterile polypropylene jars. After samples (day = −1) were withdrawn to assay for initial bacterial concentrations in the three jars, the bioactive water within was spiked with 30 mL freshly grown, log-phase, tryptic soy broth-cultured *E. coli* CN13 (ATCC #700609) grown at 37°C overnight to levels of 10⁷ CFU/mL measured as faecal coliforms. The coliform spiked river water was shaken on an orbital table at low speed and sampled over a period of three weeks for TC and FC by standard membrane filter techniques (APHA, 1992) with care to record the numbers of atypical colonies (AC) that formed on the TC test. All membrane filter analyses had a minimum of three dilutions assayed with three duplicate samples per dilution run for each aliquot of bioactive water withdrawn. The survival jars were kept at a constant 20°C on the orbital table and samples were withdrawn for assay daily, except for weekends. When the TC and FC levels dropped to near detection limits, fresh cultured FC in broth were added directly into the water and bacterial levels monitored over time as before. Three bacterial spikes were accomplished over a 16d period (d1, 7 and 12). The results from the three jars were combined after statistical analysis and the average values used for calculations and graphics. Any microbial data points with zero or too numerous to count values were excluded from modeling and statistical analysis.

Historical Kentucky River water quality data
The Kentucky American water treatment plant monitors the intake Kentucky River water quality three times daily from Lock 10 for TC, AC and FC along with weather and river flow observations. An analysis of two years (1997, 1998) of assembled plant intake quality data was done to investigate relationships between the AC/TC ratio and average flow. The time span analysed was comprised of two years where rainfall was in excess of 45 inches (~115 cm) per year. Both years’ data were combined and then separated into two distinct categories of high and low monthly river flow conditions. Months with high flow conditions were defined as when the monthly average flow was greater than 2,000 ft³/s (~56 m³/s) and months with low flow conditions were when the monthly average flow was less than 2,000 ft³/s. Any microbial data points with values of zero or too numerous to count values were excluded from modeling and statistical analysis.

Results and discussion
Survival studies with filtered river water
Batch survival experiments involving Kentucky River water from summer flow conditions spiked with FC held in large polypropylene jars were used to investigate the changes in bacterial populations over time in nutrient enriched water. The initial microbial levels in the filtered river water for AC, TC and FC were 10¹.⁷, 10⁰.⁹⁶ and 10⁰.⁶ CFU/mL of filtered river water respectively before coliform spiking. The addition of the laboratory grown FC spike (a) drove levels of FC and TC to levels of 10⁷ CFU/mL, (b) added nothing immediately to the level of AC and (c) added some nutrient content from the broth growth media. The coliforms that had been added to the river water comprised the overwhelming majority of the TC and FC isolated from the spiked river water. The levels of AC rose after the addition of nutrients held in the broth that the coliforms had been cultured in. As can be seen in Figure 1, in the first seven days after initial bacterial addition, AC rose from 10¹.⁷ to 10⁶.⁶ CFU/mL before dropping to 10³.⁴ CFU/mL. The largest increase in AC levels was seen over the first
day after the spike. This rise in AC levels was noted after subsequent bacterial spikes (Figure 1) but, since the beginning levels of AC were higher than for the first spike, the difference was not as large. The levels of AC seemed relatively stable compared to the rapidly fluxing TC and FC concentrations.

TC and FC were correlated well in the days after the spike as would be expected ($R_{FC\cdot TC} = 0.86$). In the days following the initial spike, the associations between AC and TC or FC were not as significant but the TC and FC levels remained correlated (Figure 1). After the second spike had added large numbers of FC and TC to the river water, the ratio of AC/TC dropped to levels below 1 (0.0012) on the first day but soon began to rise to ratios over 200 within 4d after the spike (Table 1). The increase in the ratio was caused by the decay of TC while AC increased slightly in numbers but did not experience the decay seen for both coliform groups (Figure 1). Oxidase testing of a subset of the AC showed them to be predominantly oxidase positive (85%) and, therefore, presumed not to belong to the coliform group but to the groups identified as species of *Aeromonas*, *Salmonella*, *Pseudomonas* and *Vibrio* (Brion and Mao, 2000). In essence, the AC group was an environmental baseline, relative to the amount of nutrients added to the system, against which the true coliform levels could be related.

Previous work (Brion and Lingreddy, 1999; Brion and Mao, 2000) has shown the application of the AC/TC ratio for relative faecal age sorting of environmental runoff but those studies were not under tightly controlled laboratory conditions. This laboratory survival study confirmed the association between increasing AC/TC ratios and age of faecally-derived coliforms in surface waters.

**River database**

The three indicators (AC, TC, FC) developed distinct average concentrations during periods of high and low flow. Revealing its allochthonous nature, FC concentrations ($FCHigh\ Flow = 10^{1.74}$, $FCLow\ Flow = 10^{0.518}$ CFU/100 mL) fluctuated the most, in turn producing a statistically significant difference t-test of average FC concentrations.

![Figure 1](https://iwaponline.com/wst/article-pdf/47/3/45/423966/45.pdf)  
**Figure 1** Average values of indicator bacteria in survival study

**Table 1** AC/TC average ratio for survival experiment

<table>
<thead>
<tr>
<th>Day</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>7</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>12</th>
<th>13</th>
<th>14</th>
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<td>12</td>
<td>23</td>
<td>34</td>
<td>320</td>
<td>&gt;1</td>
<td>10</td>
<td>12</td>
<td>219</td>
<td>224</td>
<td>&gt;1</td>
<td>1</td>
<td>9</td>
<td>163</td>
</tr>
</tbody>
</table>
This difference seemed reasonable since an increase in flow would bring more runoff contaminated with faecal material into the surface water from point and non-point sources. Differences in the TC averages also varied with flow (TC_{High Flow} = 10^{2.95}, TC_{Low Flow} = 10^{2.17} CFU/100 mL) and were statistically different (P = <0.001). Being autochthonous, the concentrations of AC were quite stable under all conditions (AC_{High Flow} = 10^{3.33}, AC_{Low Flow} = 10^{3.28} CFU/100 mL) and did not show a statistical difference between average values for high or low flow months (P = 0.442). This stability in AC concentrations suggested that they might provide a true baseline against which to measure changes in the entire microbial community of the river.

Examining the AC/TC ratio during periods of high and low flow indicated a recurring distinctive pattern in the ratio (AC/TC_{High Flow} = 3.37, AC/TC_{Low Flow} = 27.58) that had a statistically significant difference (P = <0.001).

Figures 2 and 3 show the relationship between FC and the AC/TC ratio for different conditions of river flow. Wet periods with high flow produced AC/TC ratios of <10 that coincided with high concentrations of FC, while dry periods with low flow generally produced AC/TC ratios >10 coinciding with lower concentrations of FC (< recreational contact standard). Other watershed studies in our laboratory have shown the average AC/TC ratio for raw sewage to be 3.9 (n = 11) while the ratio for animal impacted agricultural land use associated runoff to be around 10.0 (n = 59) in a watershed that fed into the Kentucky River. The AC/TC ratio during high flow was <5 about 76% of the time and always <10 (Table 2).

Using the FC concentration of 200CFU/100 mL as a base line, FC concentrations did not surpass this level during periods of low flow and were found to violate this concentration during high flow periods. Table 2 summarises the probabilities of violating the FC base line and the chance of getting an AC/TC ratio >10 or <10 along with bacteria averages. As can be seen, the standard is not violated during low flow months with higher AC/TC ratios.

**Conclusions**

Flow and rainfall had a significant impact on the AC/TC ratio. This impact is related to the time that faecal contamination was present in the surface waters with increasing time increasing the AC/TC ratio. Hence, this ratio can be used as a relative measure of faecal age in surface waters impacted by runoff and point sources. When the AC/TC ratio was <10 in the Kentucky River there was a greater probability of encountering FC concentrations >200CFU/100 mL and much less of a chance when the AC/TC ratio was >10. Results suggested that AC from TC tests using the membrane filter method and m-Endo medium may
be used as supplemental indicators of the age of faecal matter for future watershed monitoring and regulatory requirements. AC values obtained from the TC test on environmental waters provided data against which to compare other indicators resulting in additional information about the age of faecal contamination and associated risk.

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References

