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Highlights

- TLF/HLF more related to thermotolerant coliforms (TTCs) than other rapid approaches
- Relationships between TLF/HLF and TTCs are significant in the wet season
- TLF is significantly related to total bacterial counts, not TTCs, in the dry season
- TLF/HLF at any point in time relate to wet season TTCs, when TTCs are elevated
- In-situ TLF/HLF are more resilient faecal contamination risk indicators than TTCs
In-situ fluorescence spectroscopy is a more rapid and resilient indicator of faecal contamination risk in drinking water than faecal indicator organisms

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Abstract

Faecal indicator organisms (FIOs) are limited in their ability to protect public health from the microbial contamination of drinking water because of their transience and time required to deliver a result. We evaluated alternative rapid, and potentially more resilient, approaches against a benchmark FIO of Thermotolerant coliforms (TTCs) to characterise faecal contamination over 14 months at 40 groundwater sources in a Ugandan town. Rapid approaches included: in-situ tryptophan-like fluorescence (TLF), humic-like fluorescence (HLF), turbidity; sanitary inspections; and total bacterial cells by flow cytometry. TTCs varied widely in six sampling visits: a third of sources tested both positive and negative, 50% of sources had a range of at least 720 cfu/100 mL, and a two-day heavy rainfall event increased median TTCs five-fold. Using source medians, TLF was the best predictor in logistic regression models of TTCs ≥10 cfu/100 mL (AUC 0.88) and best correlated to TTC enumeration (ρ, 0.81), with HLF performing similarly. Relationships between TLF or HLF and TTCs were stronger in the wet season than the dry season, when TLF and HLF were instead more associated with total bacterial cells. Source rank-order between sampling rounds was considerably more consistent, according to cross-correlations, using TLF or HLF (min ρ, 0.81) than TTCs (min ρ,
Furthermore, dry season TLF and HLF cross-correlated more strongly ($\rho_s 0.68$) than dry season TTCs ($\rho_s 0.50$) with wet season TTCs, when TTCs were elevated. In-situ TLF or HLF are more rapid and resilient indicators of faecal contamination risk than TTCs.

Keywords: tryptophan-like fluorescence; humic-like fluorescence; coliforms; microbial contamination; groundwater; indicator

1. Introduction

Microbial contamination of drinking water remains a primary water quality concern in low-, middle-, and high-income countries (Hunter et al. 2010, WHO 2017b). The greatest public health risk relates to the consumption of drinking water contaminated with human and animal faeces to which at least two billion people are currently exposed worldwide (WHO 2019). Faecal contamination of drinking water sources has traditionally been assessed by overnight culturing of surrogate faecal indicator organisms (FIOs) to infer the potential presence of enteric pathogens. However, drinking water compliance monitoring using FIOs provides ineffective protection of public health (Stelma Jr and Wymer 2012, WHO 2017a) and waterborne outbreaks remain common, even in high-income countries (Collier et al. 2021). The main concerns relating to FIOs are that microbial contamination is highly variable temporally, which is not characterised by infrequent (e.g. quarterly/annual) FIO sampling in many circumstances (Hrudey and Hrudey 2004), and results are delivered after exposure has occurred. Furthermore, FIO analysis requires well-trained personnel, restricting the extent of nationally representative surveys, and because no result is provided in-situ at the source, communication of risks and behavioural change is also inhibited (UNICEF/WHO 2017).

To address some of these limitations with FIO monitoring, the World Health Organisation (WHO) recommends a risk-based management approach to ensure water safety (WHO 2017b). A risk-based approach often includes sanitary inspections of the source (Kelly et al. 2020) and operational monitoring of parameters that can be quantified rapidly to indicate changes in source water quality
Fluorescence spectroscopy is a rapid, reagentless technique used to characterise fluorescent natural organic matter (NOM) in water (Bieroza et al. 2009, Carstea et al. 2010, Fellman et al. 2010, Hudson et al. 2007). There is substantial evidence that natural waters contaminated with wastewater display enhanced fluorescent NOM (Baker 2001, Baker and Inverarity 2004, Carstea et al. 2016, Goldman et al. 2012, Lapworth et al. 2008, Reynolds and Ahmad 1997, Zhou et al. 2016). Of particular interest has been a fluorescence peak at an excitation ($\lambda_{ex}$)/emission ($\lambda_{em}$) wavelength pair of 280/350 nm, termed tryptophan-like fluorescence (TLF). TLF has long been considered an indicator of biological activity in water (Cammack et al. 2004, Elliott et al. 2006, Sorensen et al. 2020b) and occurs in high concentrations in human and animal wastes (Baker 2001, 2002). This latter observation led to the suggestion that fluorescence spectroscopy could be a useful early-warning indicator for the wastewater contamination of drinking water (Dalterio et al. 1986, Fox et al. 2017, Stedmon et al. 2011). Fluorescence can be quantified either instantaneously in-situ using portable sensors (Carstea et al. 2019) or in real-time during inline deployment at piped water sources (Sorensen et al. 2018a).

There is now growing evidence fluorescence spectroscopy is an instantaneous indicator of faecally contaminated drinking water, as determined by the relationship between TLF and the FIO thermotolerant (faecal) coliforms (TTCs) (Sorensen et al. 2015a, Sorensen et al. 2016, Sorensen et al. 2018b, Ward et al. 2020), or specifically Escherichia coli (Baker et al. 2015, Fox et al. 2017, Frank et al. 2017, Mendoza et al. 2020, Nowicki et al. 2019, Sorensen et al. 2018a). Furthermore, laboratory studies have shown that E. coli directly produce TLF and excrete compounds that fluoresce in the TLF region (Dalterio et al. 1986, Fox et al. 2017). In a collation of groundwater and surface data across four countries (n=564), a TLF threshold of 1.3 ppb dissolved tryptophan could classify TTC and E. coli presence-absence with false-negative and false-positive error rates of 4 and 15%, respectively.
(Sorensen et al. 2018b). There was also a very strong correlation ($\rho$, 0.80) between TLF intensity and TTC and *E. coli* enumeration. Importantly, there is also provisional evidence that TLF is a more resilient indicator of faecal contamination risk than TTCs in groundwater (Sorensen et al. 2015a). We have demonstrated that modelled faecal contamination risk using TLF remained perennially elevated in some contaminated sources, whilst risks suggested by TTCs were only seasonally elevated (Sorensen et al. 2015a).

Some studies have also demonstrated strong relationships between humic-like fluorescence (HLF) ($\lambda_{em}$ of 400-480 nm) and *E. coli* either in the laboratory (Fox et al. 2017) or in groundwater (Frank et al. 2017, Sorensen et al. 2018a), although any relationship between FIOs and HLF is less well documented than FIOs and TLF. HLF has typically been considered of terrestrial origin in freshwater (Coble et al. 2014), but it is elevated in wastewater (Hur et al. 2010, Sihan et al. 2021) and can also be produced in-situ by bacteria, including *E. coli* (Fox et al. 2017, Kida et al. 2019).

In this study, we evaluate the utility of both TLF and HLF as instantaneous, in-situ indicators of faecal contamination risk in groundwater, the world’s largest store of freshwater and the primary source of drinking water for up to two billion people (Gleeson et al. 2010). We repeatedly sampled 40 groundwater sources in a community in Uganda across a period of fourteen months for TTCs and alternative rapid approaches that could be used to indicate faecal contamination. The rapid indicators included standard approaches of sanitary inspections, turbidity and electrical conductivity, alongside the more novel indicators of in-situ fluorescence spectroscopy and total (planktonic) bacterial cells (TBCs) by flow cytometry. We aim to demonstrate: (1) in-situ TLF and HLF are the superior rapid indicators of TTCs; and (2) the seasonal nature of the associations among TLF, HLF, TTCs, and TBCs.

2. **Methods**

2.1 **Study area**
Lukaya is a town in central Uganda around 100 km southwest of the capital city Kampala and close to the shores of Lake Victoria (Figure 1A). The town’s population was 24,000 in the last census (UBOS 2014), with a density of c. 640 inhabitants per km² within the built-up area that is growing at 3% per year (PDP 2017). To the east of town is the Lweera Swamp where commercial rice farming is practised (Figure 1B). The climate is humid with a mean annual rainfall of 890 mm (Nayebare et al. 2020) that is bimodal and focussed within the rainy seasons of March to May and September to November (Figure 1C).

The town predominantly sits on Precambrian basement rocks with aquifers developed within the weathered overburden and fractured bedrock with a shallow water table between 0.5 and 9 m below ground level (bgl). Groundwater is the primary source of water for the town, with the majority obtained from hand pumped wells and springs. Piped water is used by <1% of households (Nayebare 2021), which is obtained from a borehole operated by the National Water Sewerage Corporation (NWSC) to the south of the town (Figure 1B) (Nayebare et al. 2020).

The town possesses neither a sewer network nor a wastewater treatment facility. On-site sanitation facilities number around 2,100 and predominantly comprise partially lined pit latrines that are elevated because of the shallow water table. The pits are not emptied: when full, faecal matter is moved from one pit to another, or a new pit is dug. Many pits also have overflow outlets in case of inundation during the rainy seasons (Nayebare et al. 2020).

2.2 Hydrological monitoring

Tipping bucket rainfall gauges, Lambrecht meteo model 15189 (Lambrecht meteo GmbH, Germany), were installed in two locations (1 and 2, Figure 1B) and data were aggregated to daily sums. A rainfall timeseries for the study period was produced using the data from station 1, in the centre of Lukaya, unless records were absent or failed quality checks, in which case data were replaced using records from station 2. Groundwater levels in the weathered overburden were monitored in three
boreholes screened at the following depth intervals: 10.2-16.1, 11.4-17.3, and 23.5-29.4 m bgl. All boreholes are located within 20 m of each other at the surface (Figure 1B). Levels were monitored using Rugged TROLL 100 data loggers (In-Situ, USA).

2.3 Water sampling and analysis

2.3.1 Water sources and sampling rounds

An exhaustive survey of all water sources in the town was previously undertaken by Nayebare et al. (2020) and identified 56 shallow hand-dug wells equipped with hand pumps (shallow), 4 boreholes (deep) and 7 unprotected springs. The shallow sources vary between 3 and 8 m depth, and boreholes are at least 30 m deep, including the NWSC water supply well drilled to 61 m bgl (Nayebare et al. 2020). All shallow and deep sources are protected and considered *improved* water sources, and the springs are *unimproved* (WHO 2017c).

A stratified sampling approach was implemented to sample 40 of the water sources (Figure 1B). The selected sources included: three deep sources, with the fourth having no accessible sampling location, and five springs, with the other two springs being such gentle seepages that groundwater inputs were not visible and these springs were not heavily utilised by the community. Finally, 32 of the 56 shallow sources were selected to maximise the spatial spread of shallow sources across the town, whilst accounting for some sources which had become non-functional.

Sources were sampled in six rounds (R) across 14 months. R1 to 4 were undertaken in 2018 from late-April to late-May when monthly rainfall typically peaks (Figure 1C); each round was separated by six to nine days. R5 and 6 were undertaken in 2019 from mid- to late-June when monthly rainfall is close to its annual minimum and the rounds were separated by four days. In R5 and 6, four of the hand pumps on the shallow sources had become non-functional and only 36 sources were sampled. Note that rainfall had progressed through two wet and dry seasons between R4 and R5.

2.3.2 Water sampling and analysis
All shallow and deep water sources were unlocked and in frequent use by the community or owner throughout daylight hours. Nevertheless, all sources were allowed to flow for an additional minute before sampling to ensure all pipework was adequately flushed. All unprotected springs were sampled from the surface water channel as close to the point of groundwater discharge as possible.

Each source was sampled for a range of possible in-situ rapid indicators of faecal contamination. TLF and HLF were quantified using separate UviLux fluorimeters (Chelsea Technologies Limited, UK) targeting excitation-emission values of 280/360 ($\lambda_{ex}/\lambda_{em}$) and 280/450 nm ($\lambda_{ex}/\lambda_{em}$), respectively. Whilst the HLF $\lambda_{em}$ targeted the established peak, the $\lambda_{ex}$ was matched to that of TLF to monitor the extent of optical overlap between the two regions. The bandpass filters for $\lambda_{ex}$ and $\lambda_{em}$ were ± 15 and ± 27.5 nm, respectively, for both fluorimeters (Figure S1). The TLF fluorimeter was calibrated using eight standards (0, 1, 2, 5, 10, 50, and 100 ppb) of L-tryptophan dissolved in ultrapure water. The factory calibration was implemented for the HLF sensor, which expresses intensity in quinine sulphate units (QSU). This is a standardised unit relating the fluorescence intensity at $\lambda_{ex}$ 347.5 nm and $\lambda_{em}$ 450 nm from 1 ppb of quinine sulphate dissolved in 0.105M perchloric acid to direct calibration of the HLF sensor with pyrene tetrasulphonic acid in deionised water. The TLF ppb dissolved tryptophan data can be converted to QSU by division by 2.5037 or 2.3696 in rounds 1-4 and 5-6, respectively, to allow calculation of TLF:HLF ratios.

Fluorescence spectroscopy measurements were taken by submerging the fluorimeter in 150 mL of groundwater contained in a polypropylene beaker. Each measurement was taken in the dark by placing the beaker and fluorimeter within a covered stainless steel container. Given the sensitivity of the fluorimeters, all measurements were taken in duplicate, or repeated further to obtain reproducible data. Field repeatability ($\sigma$) of TLF and HLF measurements were calculated as 0.4 ppb and 0.1 QSU, respectively, across all data in R5 and 6. Specific electrical conductivity (SEC), pH and temperature were monitored using a multi-parameter Manta-2 sonde (Eureka Waterprobes, USA). Turbidity was measured using a DR/890 portable colorimeter (HACH, USA), including blank
correction with deionised water before each measurement, except during R4 when the Manta-2 was used. To account for absolute differences between the turbidimeters, the turbidity data were min-max normalised. Fluorescence data did not require linear correction for temperature quenching (Khamis et al. 2015), with only a range between 22.4-25.6°C. The pH of the samples was 4.6-6.7, hence pH would not appreciably have impacted the fluorescence (Reynolds 2003).
Figure 1 (A) Location of Lukaya within Uganda (TZ = Tanzania); (B) Sampling sources and hydrological monitoring in Lukaya mapped on Copernicus Sentinel data (2020), with the piped water source labelled NWSC; (C) Tukey boxplot, excluding outliers, of CRU monthly rainfall data (1900-2019) for grid cell 0.25 S, 31.75 E (Harris et al. 2020) indicating the timing of sampling rounds R1-6.
Sanitary risk inspections were undertaken at each source by the same assessor during sampling in R5 (WHO 2020). The surveys consisted of a list of nine yes-no questions: to identify sources of contamination observable at the surface, pathways for contaminants to the enter source, and breakdowns in barriers to contamination (Kelly et al. 2020). The questions differed for the shallow/deep sources and springs because of different potential pathways leading to contamination. The total number of positive responses to the questions equates to the sanitary risk score (SRS).

Flow cytometry analysis for total (planktonic) bacteria cells (TBCs) was conducted in the laboratory on preserved samples, but the analysis can also be undertaken rapidly and online at a water source (Safford and Bischel 2019). Samples (2 mL) were collected in 4.5 mL polypropylene cryovials (STARLAB, UK) that were pre-loaded with the preservative glutaraldehyde (Sigma-Aldrich, UK) and the surfactant Pluronic F68 (Gibco, USA) (Marie et al. 2014) at final concentrations of 1% and 0.01%, respectively. The samples were kept in a cool box for up to 8 h, then frozen at -18°C, defrosted overnight during transit to the UK in a cool box, and then analysed the following morning. Analysis was conducted using a BD Accuri C6 flow cytometer utilising a 488 nm solid state laser (Becton Dickinson UK Ltd., UK). Water samples (500 μL) were stained with a 1:50 v/v solution of SYBR Green I (Sigma-Aldrich, UK) to a final concentration of 1:10,000 v/v for 20 min in the dark at room temperature. Samples were run at a slow flow rate (14 mL/min, 10 mm core) for 5 min and a detection threshold of 1500 on channel FL1. A single manually drawn gate was created to discriminate bacterial cells from particulate background, and cells per mL were calculated using the total cell count in 5 min divided by the reported volume run in μL (Sorensen et al. 2018a).

Thermotolerant (faecal) coliforms (TTCs) were selected as the FIO of contamination. TTCs include the preferred FIO E. coli (WHO 2017b), in addition to other genera such as Klebsiella spp. that are less likely to originate from a faecal source (Leclerc et al. 2001). Nevertheless, TTCs are considered acceptable FIO alternatives to E. coli by the WHO (2017b), as the majority of TTCs comprise E. coli in most circumstances. Indeed, 99% of TTCs were confirmed as E. coli in shallow groundwater
contaminated by on-site sanitation in a similar climatological and hydrogeological setting in Kampala, Uganda (Howard et al. 2003). TTC samples were collected in sterile 250 mL polypropylene bottles and stored in a cool box (up to 8 h) before analysis. TTCs were isolated and enumerated using the membrane filtration method with Membrane Lauryl Sulphate Broth (MLSB, Oxoid Ltd, UK) as the selective medium (Sorensen et al. 2015a). Typically, 100 mL of the water sample was passed through a 0.45 µm cellulose nitrate filter (GE Whatman, UK). However, a smaller filtrate volume (1-50 mL) was used for a minority of samples to ensure colonies were not too numerous to count (TNTC), with the volume selected according to the corresponding TLF measurement and previous TTC analyses at the source. The filter was placed on an absorbent pad (Pall Gelman, Germany) saturated with MLSB broth in a plate and incubated at 44 °C for 18–24 h in a Paqualab® 50 (ELE International, UK). Plates were inspected within 15 mins of removal from the incubator and all cream to yellow colonies greater than 1 mm considered TTCs. Where plates were TNTC the analysis was repeated the following day using a smaller volume of the remaining sample that had been kept refrigerated at 4°C.

2.4 Statistical analysis and modelling

Rapid approaches to assess faecal contamination were tested against the benchmark FIO of TTCs using R v4.0.3 (R Core Team 2020) and base commands unless otherwise stated. Logistic regression models were developed for each rapid approach as a predictor of ≥10 cfu/100 mL TTCs. There were insufficient data (n=1) where TTCs <1 cfu/100 mL to develop models for TTC presence-absence. Model performance was assessed using the area under the receive operating curve (AUC) (Mandrekar 2010), which is a plot of the proportion of true positive results against the proportion of false positive results as the threshold of the predictor is varied. A perfect classifier has an AUC of 1 and a random classifier has a value of 0.5. Furthermore, we consider AUC values of 0.7 to 0.8, 0.8 to 0.9, and 0.9 and greater as acceptable, excellent, and outstanding, respectively (Hosmer Jr et al. 2013). Rank correlations between rapid approaches and TTCs were estimated using the non-
parametric Spearman’s rank ($\rho_s$) (Spearman 1904), given the non-Gaussian distribution of many of the variables. Coefficients of 0.80-1.00, 0.60-0.79, 0.40-0.59, 0.20-0.39, 0.00-0.19 were considered very strong, strong, moderate, weak, and very weak, respectively.

Multiple linear regression was applied to investigate what combination of rapid approaches was optimal for the prediction of TTC enumeration. A forward stepwise algorithm was used using 10-fold cross validation within the R package car (Fox and Weisberg 2018). One predictor is added to the model at a time to achieve the largest decrease in the root mean square error (RMSE), until no further reduction can be yielded. The normality of model residuals was evaluated using Q-Q plots. Initial models produced non-Gaussian residuals, in violation of the assumptions, so all variables with a skewness $>$1 were natural log transformed. An addition of 1 was made to TTCs to ensure the logarithm could be defined.

Differences in both rapid approaches and TTCs between sampling rounds were explored using the Friedman test in the R package PMCMR (Pohlert 2014), with post-hoc Nemenyi tests (Demšar 2006). The Friedman test is a non-parametric alternative to the repeated-measures ANOVA and tests the null hypothesis that at least one group does not belong to the same population. If the Friedman test is significant ($p < 0.05$), the subsequent multiple comparison Nemenyi tests report significant ($p < 0.05$) differences between each pair of groups, if their corresponding mean ranks differ by at least the critical difference (Demšar 2006, Pohlert 2014).

The comparative stability of rapid approaches and TTCs were evaluated by cross-correlating each variable with itself between sampling rounds. Additionally, TLF and HLF were cross-correlated with TTCs and TBCs across the sampling rounds to explore the seasonal nature of any associations. Spearman’s Rank was used because the variables were non-Gaussian and because we were most interested in the rank-order of the sources as an indicator of relative risk across the community.
Groundwater levels (GWLs) for the most complete record, BH ALP-3, were hindcasted by 24 days to contextualise groundwater conditions before and during R1 and 2 where GWL observations were not collected. Hindcasting was conducted using a forward model implementing the water table fluctuation method and assuming diffuse recharge from daily rainfall observations (Cuthbert et al. 2019). The model was parameterised using a linear rainfall-recharge relationship with a rainfall threshold of 10 mm, an exponential recession coefficient of $1.1 \times 10^{-3}$ day$^{-1}$ to a base of 1149 m asl, and a specific yield of 5%. The model effectively captures which rainfall events result in groundwater recharge, the timing of GWL responses, and the rate of recession, with an $r^2$ of 0.85 and RMSE of 0.23 m (Figure S2).

3. Results

3.1 Widespread prevalence and high variability of TTCs

All sources show evidence of at least intermittent faecal contamination, inferred through the presence of TTCs (Figure 2A&B). Fifty percent of the sources have median TTCs of at least 88 cfu/100 mL (Figure 2A), with a range in median counts between <1 and 5,101 cfu/100 mL. The shallow sources cover the entire range in median counts, with median TTCs at springs and deep sources are in the upper 50% and lower 53% of all sources, respectively (Figure 2A).

TTCs vary widely at each source with 50% of sources having a range of at least 720 cfu/100 mL (Figure 2B). The range in TTCs at a source is at least 8 cfu/100 mL and up to 34,000 cfu/100 mL, with all but two sources varying between risk categories, based upon the order of magnitude of TTCs, previously defined by WHO (1997). A third of the sources transit between testing negative and positive for TTCs, including the only source with a median count of <1 cfu/100 mL (Figure 3D). There is a tendency for spring sources to have greater ranges in TTCs, than the other types of source (Figure 3B).
Figure 2 Empirical cumulative distribution functions of (A) median TTCs and (B) range in TTCs for each water sources (n = 40).

### 3.2 TLF and HLF are superior rapid approaches to indicate TTCs using source medians

Median TLF is the only significant predictor of median TTCs $\geq 10$ cfu/100 mL according to logistic regression models ($\beta = 1.09$, p-value $= 0.042$) (Figure 3A; Table S1). The AUC using TLF as a classifier is 0.88, which is closest to the perfect classifier value of 1 than the random selector of 0.5 (Figure 3A) and considered “excellent”. An optimal TLF threshold of 2.2 ppb can be defined to classify TTCs $\geq 10$ cfu/100 mL, with associated false-negative and false-positive rates of 16% and 25%, respectively (Figure 3C).

The AUC when classifying median TTCs $\geq 10$ cfu/100 mL using HLF was 0.85 and considered “excellent”, with the logistic regression model being borderline significant ($\beta = 1.90$, p-value 0.059) (Figure 3A). A HLF threshold of 0.85 QSU can classify median TTCs of $\geq 10$ cfu/100 mL with identical
error rates to those as the proposed TLF thresholds. In fact, if the TLF ppb threshold is converted into QSU then they are almost equivalent to these HLF thresholds.

The AUC was “acceptable” for Sanitary risk scores (SRS) and median total bacterial cells (TBCs), and demonstrated that SEC and turbidity performed no better than a random classifier (Figure 3A). Considering only the shallow sources (n=32), SRS was a significant predictor ($\beta = 0.90$, p-value 0.038) with an “acceptable” AUC of 0.79. Only one individual sanitary inspection question, whether drainage was inadequate, was a significant predictor (p-value < 0.05) of median TTCs ≥10 cfu/100 mL for shallow and deep sources where sanitary inspection questions were identical. The AUC for inadequate drainage as a classifier was 0.75 and considered “acceptable” (Table S2).

Median TLF is very strongly correlated with median TTCs ($\rho = 0.81$, Figure 3D), being the most correlated rapid approach (Figure 3B). All types of water source follow the same rising trend (Figure 3D), with two notable outliers. One outlier is a shallow source, which has a median TLF of 65.9 ppb, more than three times the TLF intensity of any other source, although median TTCs are also high at 296 cfu/100 mL. The second outlier is a deep source with median TTCs of 89 cfu/100 mL, yet the lowest median TLF of 0.5 ppb, as well as being the only site with a zero SRS. Median HLF is similarly correlated with median TTCs ($\rho = 0.79$) as TLF, with identical outliers. Median TBCs correlate moderately with median TTCs, but other in-situ indicators are only weakly related to median TTCs (Figure 3B).

No other rapid approaches provide additive performance to $\ln$(TLF) for the prediction of median $\ln$(TTCs) using the stepwise forward linear regression algorithm. The linear regression model has an $r^2$ of 0.51 and p-value <0.001 (Equation 1). Omitting $\ln$(TLF), only $\ln$(HLF) is included by the algorithm and the model has an $r^2$ of 0.48 and p-value <0.001 (Equation 2). Natural log transforms of TTCs, TLF, and HLF were required in the linear regression models to ensure the model residuals were Gaussian (see Figure S3 for Q-Q plots).
\[ \ln(TTC + 1) = 1.66 \ln TLF + 2.19 \] \hspace{1cm} \text{Equation 1}

\[ \ln(TTC + 1) = 1.44 \ln HLF + 3.73 \] \hspace{1cm} \text{Equation 2}

Figure 3 (A) Area under curve (AUC) and significance of the logistic regression models for each in-situ parameter as a classifier of TTCs \( \geq 10 \text{ cfu/100 mL} \); (B) Spearman’s Rank correlation coefficients and significance for TTCs and each in-situ parameter; (C) False-negative (FNR) and false-positive (FPR) rates for TLF thresholds (0-10 ppb) as classifiers of TTCs \( \geq 10 \text{ cfu/100 mL} \) and an optimal TLF threshold highlighted with a dotted line; (D) Scatterplot of median TTCs and TLF for
3.3 Relationships between in-situ approaches and TTCs by sampling round

TTCs were significantly different between the wet season rounds of R1, R2 and R4 and the dry season rounds of R5 and R6 (Figure 4A & B). Median TTCs were higher during than wet season (up to 382 cfu/100mL, R1) than the dry season (as low as 13 cfu/100mL, R5) (Figure 4B). TTCs rapidly reduced in the absence of large rainfall events, for example, median TTCs reduced from 382 to 55 cfu/100 mL within 17-23 days between rounds R1 and R3. The two large successive daily rainfall events of 40 mm preceding round R4 resulted in substantial groundwater recharge, an almost five-fold increase in median TTCs to 262 cfu/100 mL, and increases in TTCs at 73% sources.

TLF shows a similar trend to TTCs across sampling rounds (Figure 4B). Significant differences exist between wet and dry season rounds, with median TLF being highest in round R1 and lowest in rounds R5 and R6. HLF and TBCs also show significant differences only between wet and dry season rounds, with both at minima in the dry season. Turbidity and SEC show the least variability by sampling round, with fewest significant differences between rounds.

TLF is generally the most strongly correlated in-situ approach with TTCs in each sampling round (Figure 4C). Positive correlations are very strong or strong during the wet season rounds R1-4, but only moderate or weak in dry season rounds R5 and R6, respectively (Figure 4D). The strongest coefficient is during round R4, following the two large successive rainfall events. Correlation coefficients between HLF and TTCs are similar or marginally lower, notably in round R4, than between TLF and TTCs, with significant correlations in all rounds (Figure 4C), apart from R6 where significance is borderline (p = 0.051). TLF and HLF are also better predictors of TTCs ≥10 cfu/100 mL in the wet season rounds (mean AUC 0.84 and 0.73, respectively) than dry season rounds (mean AUC 0.68 for both).
TBCs are intermittently significantly correlated with TTCs, with strong ($\rho_s 0.70$) and moderate ($\rho_s 0.56$) correlations during rounds R4 and R1, respectively. Other in-situ approaches are rarely significantly correlated within TTCs and coefficients are typically weak or very weak (Figure 4C). Only TBCs in the dry season and SRS in the wet season have an AUC $> 0.70$ for classifying TTCs $\geq 10$ cfu/100 mL (both mean seasonal AUC 0.71).

There are also notable associations between in-situ approaches. During rounds R5 and R6 there is an almost perfect positive correlation between TLF and HLF (mean $\rho_s 0.97$); $\rho_s$ remains very strong, but is lower in rounds R1-4 (mean $r^2 0.88$) (Figure S4). The TLF:HLF ratio is higher in rounds R1-4 (median 0.95) than R5-6 (median 0.70), with the percentage of samples having a ratio $>1$ also decreasing from 45 to 7%. The lower TLF:HLF ratio in R5-6 is a result of a greater reduction in TLF relative to HLF (Figure 4C). In rounds R5 and R6, when the relationships between TLF/HLF and TTCs weaken, TLF and HLF are both strongly positively correlated with TBCs (mean $\rho_s 0.62$).
Figure 4 (A) Relationship between groundwater levels (GWLs) and rainfall in Lukaya illustrating timing of all sampling rounds in grey; (B) Tukey boxplots of TTCs and in-situ approaches by sampling round with $\chi^2$ and significance of Friedman tests above each subplot and significant differences between rounds from post-hoc Nemenyi tests marked by ends of horizontal lines; (C) Spearman’s Rank correlation coefficients and significance between TTCs and each in-situ approach for
all sampling rounds; (D) Scatterplots of TTCs and TLF for each sampling round with corresponding Spearman’s Rank correlation coefficients shown. p-values of <0.05, <0.01 and <0.001 are denoted by ‘*’, ‘**’, and ‘***’, respectively.

3.4 Cross-correlations between in-situ approaches and TTCs across sampling rounds

There are very strong positive rank cross-correlations for both HLF and TLF between sampling rounds at the 36 sources, but rank cross-correlations are weaker and more varied for TTCs (Figure 5A, B, D). The source rank-order by HLF is most consistent with a mean $\rho_s$ of 0.91 ($\sigma$ 0.04) and, remarkably, a $\rho_s$ of 0.95 between rounds R1 and R6 (Figure 5B), separated by 14 months. The mean $\rho_s$ for TLF is 0.86, with consistently very strong correlations between all rounds ($\sigma$ 0.03). The rank-order of sources by TTCs is inconsistent, moderately correlated on average ($\rho_s$ mean 0.57, $\sigma$ 0.11), but with only a weak correlation between rounds R1 and R6 (Figure 5D). Bulk hydrochemistry rank-order of sources, as indicated by SEC, is also consistent between rounds ($\rho_s$ mean 0.90, $\sigma$ 0.07) (Figure 5D), but SEC is unrelated to TTCs (Figure 4C).

A survey of TLF or HLF across the community in either the dry or the wet season relates to TTCs during the wet season when TTCs are most elevated. Ranking the sources based on HLF intensity during any sampling round correlates well ($\rho_s$ mean 0.68, $\sigma$ 0.06, all p-values <0.001) with the rank-order of the sources by TTCs during the wet season rounds R1-4 (Figure 5F). TLF cross-correlates similarly to HLF with TTCs over the same time period ($\rho_s$ mean 0.67, $\sigma$ 0.09, 92% p-values <0.001), although some coefficients for round R1 with TTCs are weaker (Figure 5E). Note, because of the very strong rank-correlation between HLF and TLF in the dry season rounds (mean $\rho_s$ 0.97), both rank-correlate near-identically and strongly with TTCs during the wet season rounds. Importantly, dry season TLF and HLF (both mean $\rho_s$ 0.68, $\sigma$ 0.05, all p-values <0.001) both correlate more strongly than dry season TTCs (mean $\rho_s$ 0.50, $\sigma$ 0.10, 38% p-values <0.001) with wet season TTCs.
Figure 5 Cross-correlations between variables in each sampling round illustrated by Spearman’s Rank correlation coefficients for (A) TLF; (B) HLF; (C) SEC (D) TTCs; (E) TLF and TTCs; (F) HLF and TTCs (n = 36). p-values of <0.05, <0.01 and <0.001 are denoted by *, **, and $$$, respectively.
4. Discussion

4.1 In-situ TLF/HLF as rapid approaches to indicate faecal contamination

In our study, TLF/HLF are the superior rapid approaches to indicate faecal contamination of groundwater sources, as determined by TTCs. To set these results in a wider context, we re-analysed existing published datasets from contrasting hydrogeological settings following the same statistical approach (Figure 6). The datasets were collated from: i) boreholes drilled to a consistent depth in an alluvial aquifer in Bihar, India (n = 145) (Sorensen et al. 2016); and ii) boreholes (n = 50) and shallow hand-dug wells (n = 61) tapping either quartzite/dolomite or the overlying weathered saprolite/laterite, respectively, in Zambia (Sorensen et al. 2015a).

The re-analysis of these datasets demonstrates TLF is an effective significant predictor of the presence-absence of TTCs in a 100 mL sample in these other settings (Figure 6A). Logistic regression models using TLF are significant (p < 0.001) and the AUC is 0.89-0.94. SRS and turbidity perform no better than a random classifier in India; whilst both are significant predictors (p < 0.05) in boreholes in Zambia, their AUCs are much lower than TLF (Figure 6A). The shallow wells in Zambia were typically always contaminated with TTCs present in all but 5 of the 61 samples, so AUCs were not estimated.

TLF is the most correlated in-situ indicator of the number of TTCs in our study, and the re-analysis of other published data (Figure 6B). In India, there is a significant relationship between TLF and TTCs, but not between either SRS or turbidity and TTCs. In Zambia, TLF is strongly correlated with TTCs in boreholes, but only weak relationships exist between SRS or turbidity and TTCs. Correlation coefficients with TTCs also remain strongest for TLF, from the rapid approaches, in the shallow wells.

An online application of TLF in groundwater-derived public water supplies in the UK has also
demonstrated that TLF was better correlated (\(\rho_s 0.71\)) with \textit{E. coli} than online turbidity (\(\rho_s 0.48\)) (Sorensen et al. 2018a).

There are alternative groundwater studies that have presented evidence that TLF has been unrelated to FIOs in groundwater. Nevertheless, TLF has still served as an effective in-situ indicator of contamination deriving from faecal matter in these studies. For example, Sorensen \textit{et al.} (2020b) showed TLF was related to the density of on-site sanitation and associated nitrate but not TTCs beneath Dakar, Senegal. This study was also undertaken during the dry season and the results of our study suggests TLF/HLF relationships with FIOs are seasonal, and it is possible that during the wet season TLF/HLF could relate to FIOs in Dakar. Alternatively, the fluorophores in Dakar could relate to historic faecal contamination, as also observed at a source adjacent to an abandoned pit latrine in Malawi containing perennially high TLF, but sporadic and low TTC counts (Ward et al. 2021).

There remains inconsistent evidence regarding the use of turbidity and SEC (Buckerfield \textit{et al.} 2019, Jung \textit{et al.} 2014, Pronk \textit{et al.} 2006, Pronk \textit{et al.} 2009, Valenzuela \textit{et al.} 2009), or sanitary inspections (Bain \textit{et al.} 2014, Kelly \textit{et al.} 2020, Misati \textit{et al.} 2017) to determine faecal contamination risk in groundwater. Turbidity and SEC can derive from a variety of common sources, including the re-mobilisation of particles within the aquifer, and the relationship with faecal indicator bacteria in the literature is consequentially inconsistent (WHO 2017d). We consider that TLF/HLF are more appropriate indicators of variations in source water quality that relate to faecal contamination. A recent review by Kelly \textit{et al.} (2020) suggested it was inappropriate to use sanitary inspections as indicators of microbial water quality. They argued that microbial samples from the same source are highly varied, whereas a sanitary inspection serves as a “lasting condition of the water source”.

Other limitations of SRS are they only represent conditions local to the source, whereas rapid subsurface transport of enteric pathogens can occur over large distances in fracture flow aquifers (Worthington and Smart 2017), and it is not possible to assess failure of the sanitary seal subsurface. Nevertheless, sanitary inspections are undoubtedly invaluable irrespective of whether they are
indicative of microbial water quality, particularly as they provide information about potential risks and causes of contamination to inform interventions.

Figure 6 (A) Area under curve (AUC) and significance of the logistic regression models for each in-situ rapid approach as a classifier of TTCs ≥1 cfu/100 mL and (B) Spearman’s Rank correlation coefficients and significance for in-situ rapid approaches against TTCs. Data are from previous studies in India (Sorensen et al. 2016), and Zambia split by source type: borehole (BH) and Wells (Shallow well) (Sorensen et al. 2015a). AUC is not shown for Zambia Wells because of only five from 61 samples where TTCs <1 cfu/100 mL p-values of <0.05, <0.01 and <0.001 are denoted by ‘*’, ‘**, and ‘***’, respectively.

4.2 TLF and HLF are more resilient indicators of faecal contamination risk than TTCs (thermotolerant coliforms)

TLF and HLF are more resilient faecal contamination indicators in groundwater than TTCs within our study. We highlight comparable observations in Zambia where TLF remained elevated in several shallow sources over a period of four months, whereas TTCs were only elevated in the wet season (Sorensen et al. 2015a); this dynamic was also recently suggested at five water sources in Malawi by Ward et al. (2021). Despite TLF remaining seasonally elevated in several Zambian sources, there was an overall trend towards higher median TLF and TTCs in the wet season (7.1 ppb and 48 cfu/100 mL) relative to the dry season (2.8 ppb and 2 cfu/100 mL). Re-analysis of the Zambia data demonstrates
that the relationship between TLF and TTCs is stronger in the wet ($\rho 0.82$) than the dry season ($\rho 0.67$). Moreover, there is also a stronger cross-correlation between dry season TLF and elevated wet season TTCs ($\rho 0.80$), than dry and wet season TTCs ($\rho 0.60$). In summary, both TLF and TTC vary seasonally in Uganda and Zambia, but ranking the sources within a community by faecal contamination risk using TLF is a more temporally robust approach than using TTCs.

Contrasting seasonal variations and relationships between TLF/HLF and TTCs suggest TLF/HLF differ from TTCs in one or more properties: (i) their source, (ii) their transport properties, and/or (iii) their persistence in the subsurface. The dominant source term for both types of faecal indicator is likely to be effluent from on-site sanitation in urban low-income settings, where present, except potentially where there are naturally high levels of sedimentary fluorescent NOM, or water is contaminated with fluorescent xenobiotic compounds, such as diesel (Carstea et al. 2010). Inputs from on-site sanitation are likely to be greatest during the wet season, particularly following large rainfall events, when latrines can be inundated and overflow (Nayebare et al. 2020), and accumulated faecal matter on the ground surface can be mobilised (Howard et al. 2003). There is also likely to be a continuous input function from on-site sanitation, as pit latrines and septic tanks, leak year-round.

The faecal indicators have different transport properties. Frank et al. (2021) demonstrated that dissolved tryptophan was comparable in transit time and recovery to the conservative dye tracer uranine over short distance, <2h tracer tests, with no evidence of retardation. Frank et al. also demonstrated similar recovery for a humic acid, although there was some evidence of retardation and the tracer peak was marginally delayed by five minutes, in comparison to uranine. It is unclear what proportion of TLF/HLF can be attributed to dissolved pure tryptophan or the humic acid used in any given setting, although TLF/HLF fluorophores are predominantly extracellular in groundwater (Sorensen et al. 2020a). Nevertheless, there will also be an element of sorption and desorption of dissolved OM between groundwater and the aquifer matrix and soils (Shen et al. 2015), particularly for more hydrophobic molecules, which may have a TLF/HLF component, as well as a minor
component contained within cells. TTCs can be transported more rapidly than solutes, notably in heterogeneous media such as weather crystalline rocks, but are subject to appreciable attenuation (Taylor et al. 2004). TTCs tend to accumulate and be transported laterally when flow velocities increase (WHO 2017b), such as during a rainfall event generating groundwater recharge. Therefore, TLF/HLF are likely to be more readily and continuously transported than TTCs in groundwater.

The persistence of TLF/HLF fluorophores and TTCs are likely to differ in groundwater. HLF is expected to be the most persistent indicator, demonstrating the strongest rank-order cross-correlation between sampling rounds. Furthermore, although HLF decreases in the dry season, there is a proportionally greater loss in TLF indicating either preferential breakdown or more efficient lateral transport of TLF fluorophores. HLF has been demonstrated to be more recalcitrant, resistant to breakdown, than TLF in surface water and wastewater (Cory and Kaplan 2012, Ignatev and Tuhkanen 2019) and a greater proportion of fluorophores are likely to be recalcitrant in groundwater where NOM is typically less bioavailable (Chapelle 2021, Shen et al. 2015). The refractory nature of some HLF fluorophores led Zheng et al. (2020) to suggest that HLF is an effective tracer of wastewater in groundwater. There is also potential for the in-situ production of TLF or HLF from NOM entering an aquifer system (Fox et al. 2017, Yang et al. 2020). Therefore, fluorophore persistence in the subsurface could be a result of the continuous recycling and microbial transformation of NOM arriving in the system as opposed to the accumulation of recalcitrant molecules (Benk et al. 2019, Roth et al. 2019). The dry season relationships between TLF/HLF and TBCs when faecal inputs are more limited, as also observed in Senegal (Sorensen et al. 2020b), suggest bacteria are using the NOM as a substrate and potentially generating fluorophores in-situ. Irrespective of the relative persistence of either TLF or HLF, either wavelength pair would provide a similar indicator of faecal contamination risk given their co-correlation in our study and the optical overlap between the peaks. TTCs are generally only considered indicative of recent contamination with die-off within 16-45 days (Taylor et al. 2004), in contrast to the more persistent fluorescence indicators.
The more efficient transport of TLF/HLF fluorophores and their greater persistence in the subsurface in comparison to TTCs could explain why dry season TLF/HLF relates to wet season TTCs. Firstly, more efficient transport could facilitate the perennial transport of TLF/HLF fluorophores from a faecal source to a water source, whereas TTCs are predominantly mobilised following rainfall in the wet season. It should also be reiterated that TLF/HLF does also respond to rainfall with higher intensity in the wet season, indicating higher seasonal risks. Secondly, faecal contamination events at a water source would remain detectable for a longer period by fluorescing more persistent TLF/HLF fluorophores than TTCs. If these events are focussed in the wet season, as observed here, then the proportion of TLF/HLF persisting into the dry season may relate to wet season TTCs, given the two types of indicator correlate very strongly after heavy rainfall (e.g. TLF, ρ, 0.83, R4).

4.3 Remaining uncertainties, instrumentation improvements, and future work

There are a range of potential interferents with in-situ fluorescence measurements that are discussed in a review by Carstea et al. (2020) but these have not adversely impacted previous TLF-FIO studies (Sorensen et al. 2018b) or this study across a range of settings. Corrections for temperature, turbidity, and absorbance of light by the sample matrix (the inner-filtering effect) are not likely to be necessary in the majority of groundwater settings (Khamis et al. 2015, Sorensen et al. 2015a). Moreover, the next generation of commercially available portable fluorimeters are now capable of automatic corrections. pH does not have an appreciable impact on TLF/HLF between values of 5 and 8 (Reynolds 2003, Spencer et al. 2007), and groundwater outside this range is unlikely to be suitable for drinking. High concentrations of metal ions could quench fluorescence (Yang et al. 2018), which is most likely where water is contaminated by mining and industry. Certain water treatments, including chlorination, also quench fluorescence (Henderson et al. 2009) so the well owner or other informed individuals should be interviewed to assess if the water has been treated prior to testing, as would be undertaken before FIO sampling. Alternatively, a chlorine residual test could be performed.
There is the potential for TLF or HLF fluorophores to originate from contamination unrelated to faecal sources such as diesel and fuel derivatives, food waste, paper mills, and pesticides (Carstea et al. 2016). In these instances, a source displaying high TLF/HLF should still be considered a higher faecal contamination risk than one displaying low TLF/HLF, as there would be evidence that a pathway is present to a source of anthropogenic waste. Sedimentary fluorescent NOM contained within the aquifer could also potentially be problematic when comparing faecal contamination risks determined by TLF/HLF, particularly between study areas. In which case, deviation from baseline fluorescence intensity in uncontaminated sources would be more important than the absolute value for determining risk.

Relatively high upfront costs undoubtedly constrain widespread adoption of in-situ fluorescence spectroscopy. The present generation of single peak fluorimeters cost in the region of US$5000-7000, before considering accessories that can also cost a further US$2000-3000 (Sorensen et al. 2018b). However, there is substantial scope to reduce these costs through the development of lower-cost portable fluorimeters, engineered specifically to provide an in-situ indication of faecal contamination risk at a water source. Multiple researchers have developed prototype fluorimeters with various benefits over commercial alternatives (Bedell et al. 2020, Bridgeman et al. 2015, Simões et al. 2021), but field validation and a discussion of indicative costs are absent or limited. As part of our study, we successfully developed and demonstrated the efficacy of a lower-cost prototype portable multi-wavelength LED-based fluorimeter on duplicate samples in rounds R5 and R6 (Figure S5). The prototype provided comparable results to the UviLux sensors in both the laboratory (Table S5) and field. For example, the prototype derived HLF data from R5 and R6 both correlate strongly with TTCs in R4 (mean $\rho = 0.69$). Therefore, a low-cost, portable fluorimeter to indicate faecal contamination risk could be produced for a total component cost of $1100. Further details are provided in the supplementary information (S1). In addition to reducing costs, future development should investigate the production of low-cost sealed long-life containers of TLF/HLF standards. These containers would enable calibration checks, ideally annually, and negative controls to be performed.
by the end-user without return to the manufacturer or access to a well-equipped laboratory with reagents and high quality deionised water (Sorensen et al. 2018b).

It remains unclear how TLF/HLF relate to the presence of enteric pathogens or risks posed to human health. There is one published study showing a relationship between TLF and DNA markers of enteric pathogens, although this study is limited to 22 sources in one town (Sorensen et al. 2015b). Future work should explore the potential link between TLF/HLF and enteric pathogens using molecular approaches, as well as exploring the viability of pathogens where possible. Furthermore, studies should investigate if and how TLF/HLF could effectively be used for on-site risk communication to induce behavioural change in communities and reduce the disease burden relating to the consumption of faecally contaminated drinking water.

5. Conclusions

In-situ fluorescence spectroscopy provides an instantaneous assessment of water source quality that relates to faecal contamination risk determined by faecal indicator organisms (FIOs). Consequently, faecal contamination risks can be assessed immediately, including in real-time, and could be communicated on-site to consumers to reduce exposure to contamination, whilst confirmative regulatory FIO analysis is undertaken. Furthermore, in-situ fluorescence can extend FIO sampling programs because data can be collected rapidly by users who require minimal training; nor are there consumable costs for additional samples.

TLF and HLF are more resilient indicators of faecal contamination risk than FIOs. Both types of indicator respond to rainfall and contamination events, with the strongest relationships between the indicators observed in the wet season, notably immediately after heavy rainfall (e.g. TLF-TTC $\rho = 0.83$). However, ranking the sources across a community by risk using FIOs is more variable (cross-correlations $\rho = 0.34$–$0.72$) between sampling rounds, than using TLF or HLF (cross-correlations $\rho = 0.81$–$0.97$). This ranking of sources using TLF/HLF at any point in time relates to TTCs during the wet season,
when TTCs are significantly elevated and risks to human health would consequently also be expected to be greatest. Furthermore, the source rank-orders in the dry season using TLF/HLF cross-correlate more strongly (both mean $\rho_s$ 0.68) with wet season TTCs than dry season TTCs (mean $\rho_s$ 0.50). Therefore, the comparative faecal contamination risks between sources generated by a dry season survey of TLF/HLF would be more accurate than using highly transient FIOs to indicate the comparative risks occurring in the wet season when risks are elevated. This characteristic is advantageous given water quality surveys are infrequent for private water supplies globally, as well as across low-income countries. TLF/HLF provide a more repeatable and temporally robust approach than FIOs to ranking sources by faecal contamination risk across a community to strategise prioritisation of sources for drinking or interventions.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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