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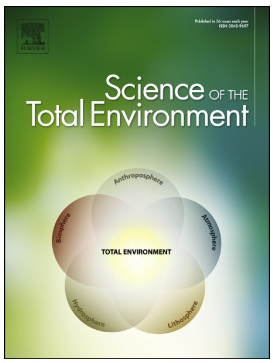

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PII: S0048-9697(20)34813-0

DOI: https://doi.org/10.1016/j.scitotenv.2020.141284

Reference: STOTEN 141284

To appear in: *Science of the Total Environment*

Received date: 23 May 2020

Revised date: 23 July 2020

Accepted date: 25 July 2020

Please cite this article as: J.S.T. Ward, D.J. Lapworth, D.S. Read, et al., Tryptophan-like fluorescence as a high-level screening tool for detecting microbial contamination in drinking water, *Science of the Total Environment* (2020), https://doi.org/10.1016/j.scitotenv.2020.141284

Tryptophan-like fluorescence as a high-level screening tool for detecting microbial contamination in drinking water

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Abstract

Regular monitoring of drinking water quality is vital to identify contamination of potable water supplies. Testing for microbial contamination is important to prevent transmission of waterborne disease, but establishing and maintaining a water quality monitoring programme requires sustained labour, consumables and resources. In low resource settings such as developing countries, this can prove difficult, but measuring microbial contamination is listed as a requirement of reaching the UN's Sustainable Development Goal 6 for water and sanitation. A nine-month water quality monitoring programme was conducted in rural Malawi to assess the suitability of tryptophan-like fluorescence (TLF), an emerging method for rapidly detecting microbial contamination, as a drinking water quality monitoring tool. TLF data was compared with thermotolerant coliforms (TTCs, *E. coli*) and inorganic hydrochemical parameters. A large (n = 235) temporal dataset was collected from five groundwater drinking water sources, with samples collected once or twice weekly depending on the season. The results show that TLF can indicate a broader contamination risk but is not as sensitive to short term variability when compared to other faecal indicators. This is likely due to a broad association of TLF with elevated DOC concentrations from a range of different sources. Elevated TLF may indicate preferential conditions for the persistence of TTCs and/or *E. coli*, but not necessarily a public health risk from microbial contamination. TLF is therefore a more precautionary risk indicator than microbial culturing techniques and could prove useful as a high-level screening tool for initial risk assessment. For widespread

use of TLF to be successful, standardisation of TLF values associated with different levels of risk is required, however, this study highlights the difficulties of equating TLF thresholds to TTCs or *E. coli* data because of the influence of DOC/HLF on the TLF signal.

Keywords: groundwater, water quality, faecal contamination, microbial risk assessment, drinking water, thermotolerant coliforms

1. Introduction

Understanding temporal variability of drinking water quality in low and middle income (LMI) countries is vital to protect human health from transmission of waterborne disease and harmful concentrations of organic and inorganic contaminants. In sub-Saharan Africa, millions of people rely on groundwater as a drinking water source, using hand pumped boreholes or shallow wells (Bonsor et al., 2011; WHO and UNICEF, 2020). In the dry season especially, groundwater provides a lifeline as alternative sources such as seasonal streams, ponds and rainwater harvesting systems inevitably dry up (Kelly et al., 2018; MacDonald et al., 2019). Natural filtration by the aquifer can reduce microbial contamination, which is extremely important where no engineered treatment is available (Macdonald et al., 2009). Hand-pumped boreholes in sub-Saharan Africa have been shown to have low levels of microbial contamination (Lapworth et al., 2020). However, this is dependent on local hydrogeological conditions, which can vary seasonally (Kostyla et al., 2015; Lapworth et al., 2020).

To address Sustainable Development Goal 6 (SDG 6), the WHO/UNICEF Joint Monitoring Programme (JMP) have adopted the WHO (2017) guidelines for assessing microbiological water quality; risk levels are defined by the number of *Escherichia coli (E. coli)* or thermotolerant coliform (TTC) bacteria cultured from a 100 mL water sample. These bacterial proxy indicators of faecal contamination are well established methods, however the protocols involved are not well suited to low resource environments and as a result water quality data in LMI countries is scarce (Adelena and MacDonald, 2008; Sorensen et al.,

2015a; Bain et al., 2012; Cumberland et al., 2012). With sufficient resources, advantages of culturing include the fact that proxy coliforms are relatively easy to culture and are present in high concentrations compared to pathogens, but are not necessarily pathogenic themselves (although, can contain pathogenic species) (Silva and Domingues, 2015; Paruch and Mæhlum, 2012). However, results are always retrospective due to an incubation period of 16 - 48 hours depending on the method used (Bridgeman et al., 2015; Aquagenx, 2019).

Fluorescence spectrometry has historically been used for assessing environmental quality of surface water such as identifying sewage inputs (Baker, 2002, Cumberland et al., 2012, Baker et al., 2015, Carstea et al., 2016). The intensity of fluorescence detected at different excitation-emission wavelengths is used to identify pollution (Carstea et al., 2020). Tryptophan-like fluorescence (TLF) describes fluorescence occurring from a range of compounds within the excitation-emission wavelengths associated with the fluorescence peak of the amino acid tryptophan (Baker, 2002). In groundwater, TLF has been used as a tracer of organic carbon (Lapworth et al., 2008), and more recently has been applied to assessing microbial contamination in drinking water sources (Sorensen et al., 2015a; Sorensen et al., 2015b; Sorensen et al., 2016; Sorensen et al., 2018a; Sorensen et al., 2018b; Nowicki et al., 2019).

These recent studies have used portable fluorescence sensors, obtaining rapid results, and have shown promising trends between TLF and TTCs and TLF and *E. coli*. Advantages of portable TLF in comparison to culturing methods include testing at the source, with instant results (negating the need for sample storage, transport and laboratory processing) and no requirement for consumables. These advantages are particularly beneficial in low resource settings. Capital costs are currently high and remain a key limitation of this method, but with long term use it could be cost effective. However, TLF and *E. coli* do not always show a strong correlation (Bridgeman et al., 2015), and further work is required to understand TLF in groundwater and for assessing drinking water quality (Carstea et al., 2020). The generic nature of fluorescence techniques, and the close proximity of the TLF peak with the humic-

like fluorescence (HLF) peak, could limit the use of TLF for detecting low levels of microbial contamination in some cases (Markechova et al., 2013; Bridgeman et al., 2015) Ward et al., 2020). Whilst TLF and HLF are broadly associated with microbial activity and allochthonous origin respectively (Baker et al., 2007), HLF has been shown to contribute to the TLF peak and HLF can also be produced from bacterial activity (Fox et al., 2017).The majority of published studies to date investigating the suitability of TLF for assessing faecal contamination in drinking water in LMI countries have comprised small datasets from short spot-sampling programmes (Sorensen et al., 2015a; Sorensen et al., 2015b; Sorensen et al., 2016; Sorensen et al., 2018a; Nowicki et al., 2019). One larger online dataset was collected in the UK by Sorensen et al. (2018b). Therefore, further research and higher frequency temporal datasets are required to evaluate TLF performance in more detail. This is crucial if TLF is to be used effectively as a drinking water quality assessment tool.

The aim of this study was to develop a better understanding of the suitability of TLF for detecting temporal variation of microbial contamination in groundwater-derived, untreated drinking water sources in a low resource setting. A large, detailed dataset of TLF, TTCs, *E. coli* and inorganic hydrochemical parameters was collected over a nine-month period in Malawi, a low income country. The study assessed changes in drinking water quality during i) the transition from the wet season to dry season; ii) the whole of the dry season, when groundwater is most relied on, and iii) the onset of the subsequent wet season.

This is the first study to investigate the temporal variability of TLF in such detail in a low resource setting, alongside other methods of detecting microbial contamination and inorganic water quality indicators. This has allowed comprehensive hydrochemical characterisation of different source types throughout the seasons. Regular monitoring of drinking water sources is essential to capture temporal changes in water quality and contamination risk.

2. Methods

2.1 Study Location

Lilongwe District is located in the Central Region of Malawi on the Central Region Plateau, also referred to as the Lilongwe Plain (Figure 1). The district comprises the urban area of Lilongwe City and the contrasting Lilongwe Rural. This study was conducted in Lilongwe Rural, which has a population of 1,600,000; this is the largest population of all districts and sub-districts in Malawi (Government of Malawi, 2018). An additional 990,000 people live in nearby Lilongwe City, the country's capital. Rural water supply is dominated by groundwater sources, principally a combination of hand-pumped boreholes and large diameter shallow wells (defined as 'improved sources' and 'unimproved' sources respectively (WHO and UNICEF, 2012). In the dry season, few or no surface water alternatives are available, therefore groundwater is a crucial resource. Pit latrines are the most common form of sanitation facility in the villages, many with no hand-washing facilities.

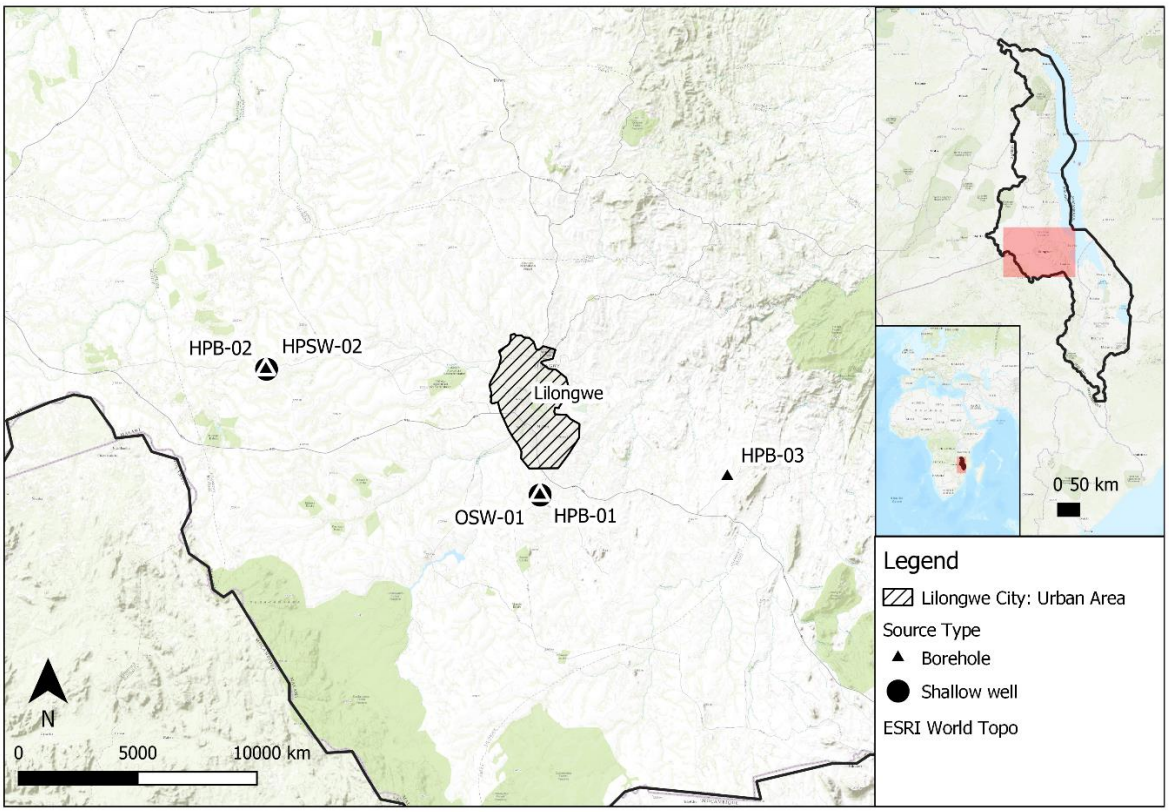


Figure 1: Study area: Lilongwe Rural, Malawi. Sources are coded by source type (HPB = hand-pumped borehole; HPSW = hand pumped shallow well; OSW = open shallow well) and location number (01, 02, 03). GPS co-ordinates for each site are listed in Supplementary Information.

Boreholes are typically drilled 30 - 50 m deep, with a narrow diameter (approximately 0.1 m), and draw from the weathered Precambrian - Lower Paleozoic crystalline basement complex (Smith-Carrington and Chilton, 1983; Wright, 1992). In the study area, the weathered zone is approximately 20 - 30 m thick, often resulting in productive boreholes (1.5 - 5 l/s) (Smith-Carrington and Chilton, 1983) that are capable of supporting hand pumps (yield requirement: 0.1-0.3 l/s) (MacDonald et al., 2012). Shallow wells are generally hand dug with a larger diameter (1 - 1.5 m), and draw from the shallow basement aquifer. The shallow basement aquifer is the most weathered and can be highly heterogeneous, characterised by clay, sand and laterite formations in some areas. Preferential flow pathways are likely to be present, depending on the local conditions.

Rainfall is seasonal, driven by the sub-tropical climate. Average annual rainfall is 734 mm and the wet season occurs from November to April (New, 1999). Local climate is influenced by altitude; the Lilongwe Plain is located at approximately 1050 m above sea level and therefore experiences moderate temperatures and rainfall in contrast to the hotter, semi-arid climate in low altitude areas on the shores of Lake Malawi (Upton et al., 2018). The average temperature range in Lilongwe varies from approximately 16 ^{o}C in July to 23.5 ^{o}C in November (World-Bank, 2019).

2.2 Experimental Design

Five groundwater sources were selected to intentionally include a range of water point construction types and microbial contamination observed in preliminary studies (Ward et al., 2020) (see Table 1 and Figure 2). The five sources comprised three Afridev hand pumped boreholes, one hand dug shallow well covered with an Afridev pump and one open hand dug shallow well (Figure 2). HPB-01 has a modification to the cement drainage apron that forms an informal soakaway pond within 5 m of the borehole. HPB-03 is situated 5 m from an aged pit latrine, abandoned for over seven years prior to this study. The study was conducted over nine months (April to December 2017). Sampling commenced at the end of the wet season

(April) and continued throughout the dry season and into the beginning of the following wet season (December). During the transition periods from wet to dry season and vice versa, sampling was undertaken twice weekly. During the middle of the dry season, sampling was undertaken once a week.

On average, 47 visits were made to each source (Table 1). On one occasion, sampling was cancelled at HPSW-02 and HPB-02 due to a funeral taking place in the village. HPB-03, was broken for a few weeks, therefore three sampling rounds were missed but an additional sampling round was completed immediately after the pump was fixed. Due to time and budget constraints, it was not appropriate to sample every parameter at each sampling visit. Sampling frequency and number of samples for each parameter is listed in Table 1. The total number of samples, across all sources and parameters is 2493.

Table 1: Source type and number of samples collected during the study. HPB = hand-pumped borehole; HPSW = hand pumped shallow well; OSW = open shallow well) and location number (01, 02, 03). TLF = tryptophan-like fluorescence; TTC = thermotolerant coliforms; HLF = humic-like fluorescence; DOC = dissolved organic carbon.

Source ID	***Source type***	***Method of water withdrawal***	***No. of visits***
HPB-01	Borehole (HPB)	Afridev pump	48
OSW-01	Open shallow well (OSW)	Rope and bucket	47
HPB-02	Borehole (HPB)	Afridev pump	47
HPSW-02	Pumped shallow well (HPSW)	Afridev pump	47
HPB-03	Borehole within 5m of old latrine (HPB)	Afridev pump	46
Total			**235**

Source ID	***TLF***	***TTC***	***E. coli***	***Turbidity***	***HLF***	***Temperature***	***pH***
HPB-01	48	48	35	48	11	48	48
OSW-01	47	47	47	47	9	47	47
HPB-02	47	47	34	47	9	47	47
HPSW-02	47	47	36	47	10	47	47
HPB-03	46	46	34	46	8	46	46
Total	**235**	**235**	**186**	**235**	**47**	**235**	**235**

Source ID	***Conductivity***	***Alkalinity***	***DOC***	***Sulphate***	***Nitrate***	***Chloride***	***Fluoride***
HPB-01	47	48	24	25	25	25	25
OSW-01	46	47	25	25	25	25	25

HPB-02	46	46	24	25	25	25	25
HPSW-02	46	46	25	25	25	25	25
HPB-03	45	46	24	25	25	25	25
Total	**230**	**233**	**122**	**125**	**125**	**125**	**125**

2.3 Groundwater Sampling

Prior to sampling, over 80 L of water was pumped or drawn from each source to ensure the sampling equipment was fully rinsed. All sources in this study were regularly used by the communities, which acted to purge the sources, and each source was sampled at approximately the same time of day on each visit. Samples were collected directly from the hand pump spout for boreholes and from the usual designated community sampling rope and bucket for shallow wells, to obtain a representative sample and avoid cross-contamination. Groundwater level was monitored manually using a dip meter at OSW-01 and at an additional OSW nearby to two of the other sources (HPB-02 and HPSW-02).

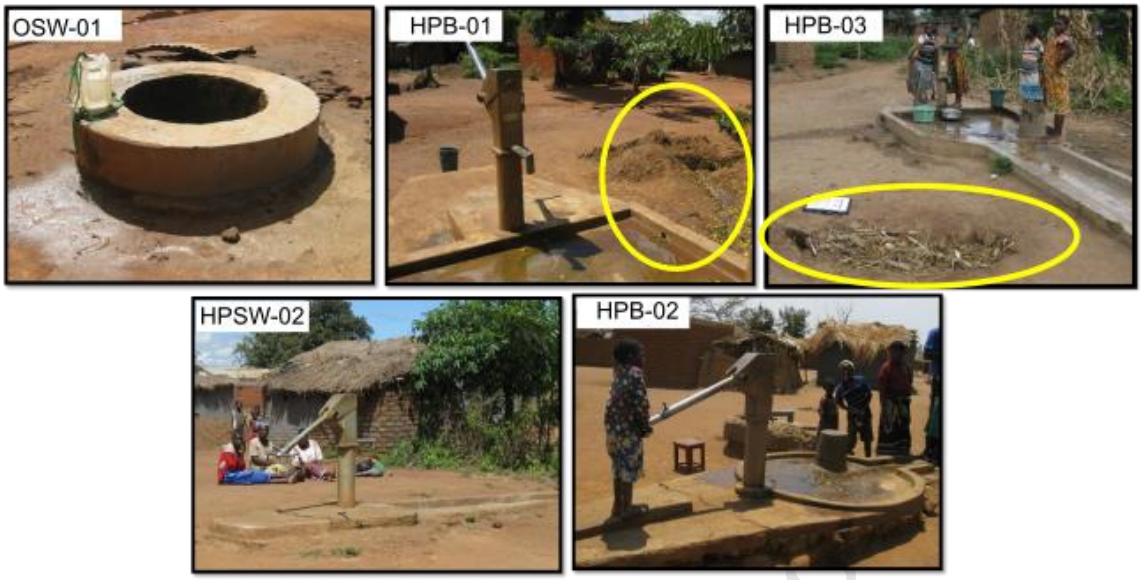


Figure 2: Drinking water sources sampled for this study: Top left: OSW-01 (open shallow well – location 01; centre: HPB-01 (borehole – location 01) with modifications to cement apron highlighted: right: HPB – 03 (borehole near abandoned latrine – location 03) with abandoned latrine pit highlighted; bottom left: HPSW-02 (hand pumped shallow well – location 02); right: HPB-02 (borehole – location 02).

TLF and humic-like fluorescence (HLF) were measured at the source and used according to manufacturer's protocol (Chelsea Technologies Group Limited, UK). The sensors are battery operated for ease of use in the field. Both sensors were immersed together in 5L of water in a bucket with a lid, placed in the shade to avoid UV light interference. Readings from both sensors are updated every few seconds and measurements were recorded once readings had stabilised. The sensors were both calibrated by the manufacturer prior to data collection and are designed to remain stable, therefore further calibration was not required during the study (Chelsea Technologies Group Ltd, 2016a; Chelsea Technologies Group Ltd, 2016b). The TLF probe measures fluorescence at the 280 +/- 15 nm excitation 360 +/- 27.5 nm emission wavelength. The HLF probe is set to the same excitation wavelength but has an emission wavelength of 450 +/- 27.5 nm, which enables the HLF sensor to capture any potential overlap between HLF and TLF (Ward et al., 2020). Both sensors record data in quinine sulphate units (QSU), which was converted to ppb for comparison with other datasets using the following equations: TLF_{ppb} = 2.1130TLFQSU; HLF_{ppb} = 1.3893H_{QSU}.

Turbidity, temperature, pH, conductivity and alkalinity were all measured at the source.

Laboratory analysis was undertaken to determine concentrations of chloride, nitrate, fluoride, sulphate and dissolved organic carbon (DOC). Anions were analysed by ion chromatography and cations by inductively coupled plasma mass spectroscopy. All DOC and inorganic analysis was undertaken in UKAS accredited laboratories in the UK, Further details can be found in Ward et al. (2020). Temperature, turbidity, pH, DOC and HLF all have potential to influence the TLF signal, so it was important to monitor changes in these variables alongside TLF (Khamis et al., 2015; Baker et al., 2007; Reynolds, 2003).

TTC counts were recorded using a plate counting method, see Ward et al. (2020) for further details. Three 0.25 L samples were collected from each source. For each of the boreholes, one sample from at least two different bottles was prepared and one sample from all three bottles was prepared for the shallow wells. This was to ensure greatest replication at the sources with consistently low/no TTC counts because these are the smallest risk classes with the greatest implications for drinking water quality assessment. The highest risk category for this method is defined as ≥ 1000 cfu/100mL.

Aquagenx Compartment Bag Test (CBT) kits were used to calculate *E. coli* concentrations with a statistical most probable number (MPN) method (Aquagenx, 2019). Samples were processed on site in accordance with manufacturer protocol. Incubation at a temperature above 25 ^{o}C for 48 hours was required. Results were recorded as the combination of compartments that had turned blue or remained yellow. Blue indicated presence of *E. coli*. Results were compared with the manufacturer-supplied table of each possible colour combination to record the associated MPN and risk category. The highest risk category for this method is defined as > 100 MPN/100mL.

2.4 Data analysis

Temporal trends for hydrochemical parameters were determined from time series graphs and quantified using descriptive statistics. The dataset was analysed for normality for each measured variable at each source. Only pH was normally distributed, therefore non-

parametric tests were selected for analysis. Significant correlations were identified using the Spearman's rank correlation coefficient (r^2) at the significance level of $p = 0.05$. The Kruskal-Wallis H-test was used to compare differences between sources and risk classes using mean ranks only, as the distribution of data varied between groups. Details of which specific groups were significantly different were identified using the post-hoc Dunn's test. No correction methods were used due to the small number of pair-wise comparisons and correction methods can be too conservative with a small number of groups. Significant differences were defined with $p \leq 0.05$. Statistical analysis was completed using R version 3.5.1.

3. Results

3.1 Negligible hydrochemical interference with TLF

Temperature and pH were stable throughout the study and are within ranges known to cause negligible interference with TLF (Reynolds, 2003; Baker et al., 2007; Khamis et al., 2015). Temperature has been shown to quench TLF signal, but this is greatest for high concentrations of TLF (e.g. 25-100 ppb), when the temperature range is large (5-35^{o}C) (Khamis et al., 2015; Nowicki et al., 2019). The mean TLF for this dataset is 1.9ppb (maximum: 7.4 ppb) and temperature range is 3.4 ^{o}C (25.3-21.9^{o}C). The interference of pH is negligible between pH 4.5-8 (Reynolds, 2003) and all samples in this dataset are within this range. For the data available HLF remained stable (equipment broke part way through the study). Turbidity remained below 50 NTU and therefore below levels that would cause concern regarding fluorescence signal attenuation, with the exception of only one data point (53.1 NTU recorded at OSW-01) (Baker et al., 2007; Khamis et al., 2015). DOC did not mirror TLF peaks and therefore indicates no interference with TLF peaks, although it is possible that at low concentrations, HLF may raise the TLF baseline (Ward et al., 2020). In addition, TLF observations are low (mean = 1.9; SD = 1.7 ppb) and hydrochemical influence has been shown to be smallest at low TLF values (Nowicki et al., 2019). Key descriptive

statistics are provided in the Supplementary Information Table S1 and time series graphs are provided in Figure S2.

3.2 Temporal variation of microbial water quality indicators

The temporal trend of TLF at each source is generally stable, with only occasional fluctuation (Figure 3). The data visually falls into two categories, defined by low and high TLF values. The higher TLF values are associated with HPB-03 (mean = 4.1 ppb) and OSW-01 (mean = 3.6 ppb). At HPB-03, TLF peaks in response to recommencement of pumping after the pump was fixed, which is then followed by a declining trend until the rains recommence. OSW-01 had more consistent TLF values. There were significant differences for TLF between sources in the two visually-defined categories (Kruskal-Wallis: χ^2 = 176, p = 0.001; Dunn's test: p = 0.001). There was no significant difference between the sources within each category except HPSW-02, which is significantly different to HPB-02 in the lower TLF group (Dunn's test: p = 0.04).

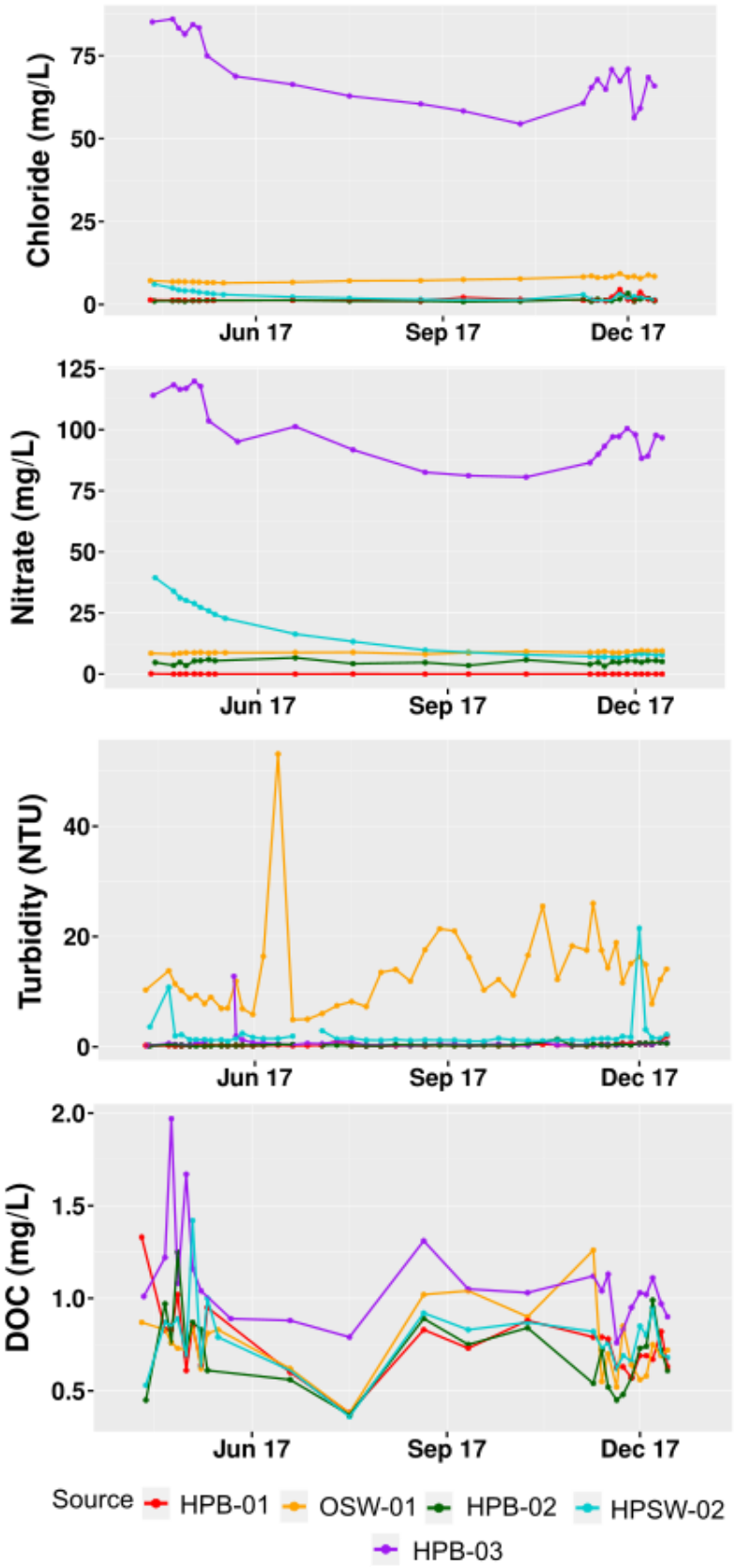


Figure 3: Temporal variation of microbial water quality measured using three different methods: A: Groundwater level (mbgL) and average monthly precipitation (mm) (Precipitation data source: World Bank, 2019); B: Tryptophan-like fluorescence (ppb); C: Thermotolerant coliforms (cfu/100 mL); D: E. coli. (cfu/100 mL).

TTC trends varied significantly between sources (range = 0 to 2250 cfu/100ml; Kruskal-Wallis: χ^2 = 141, p = 0.001). HPSW-02 and OSW-01, the shallow sources, showed the most variation throughout the study, with a trend of increasing TTC counts in the wet season. These two sources were significantly different to each other and all other sources (Dunn's test: p = 0.001). TTCs were only present intermittently (and only occurred in low concentrations) at HPB-02 and HPB-01, with the exception of a peak at HPB-01 in 2016. There were occasional seasonal breakthroughs (maximum = 121 cfu/100 mL) observed at HPB-03, however there was no significant difference in TTC trends between HPB-03, HPB-01 and HPB-02.

Similarly, there were significant differences between sources regarding *E. coli* (χ^2 = 127, p = 0.001). OSW-01 had consistently elevated *E. coli*, that was significantly higher than all other sources (Dunn's test: p = 0.001). HPB-03 and HPSW-02 showed seasonally elevated *E. coli* at the end of the wet season but not at the beginning of the subsequent wet season. There was no significant difference between these two sources, however HPSW-02 was significantly different to all other sources (Dunn's test: p = 0.001). There was only one occurrence of *E. coli* recorded at HPB-01, observed at the beginning of the wet season and HPB-02 was the only source at which *E. coli* is never recorded. There was no significant difference between HPB-03, HPB-01 and HPB-02.

3.3 Relationship between different indicators of microbial water quality

TLF correlated positively with other indicators of microbial contamination (Figure 4). Considering the dataset as a whole, there was a moderate positive correlation between TLF & TTCs (Spearman's Rank: r^2 = 0.45; p = 0.05). However, HPSW-02 was the only source to individually show a correlation (moderate, positive) between TTCs and TLF (Spearman's Rank: r^2 = 0.52; p = 0.05). This is probably due to little variation in TTCs at the other sources. There was a moderate positive correlation between TLF and *E. coli* across the whole dataset (Spearman's Rank: r^2 = 0.53; p = 0.05). Individually, however, only HPSW-02

and HPB-03 showed a correlation (both positive) between TLF & *E. coli* (Spearman's Rank: HPSW-02: r^2 = 0.56; p = 0.05; HPB-03: r^2 = 0.45; p = 0.05). HPB-01 and HPB-02 showed no correlation between TLF and any other microbial indicators; this is likely to be due to the stability of parameters at these sites. There was a very strong positive correlation between TTC and *E. coli* across the whole dataset (Spearman's Rank: r^2 = 0.90; p = 0.01). Individually, all sources except HPB-02 showed a strong or moderate positive correlation between TTCs and *E. coli* (Spearman's Rank: p = 0.01). HPB-02 did not show any variation for *E. coli*, which was recorded as 0 MPN/100mL consistently.

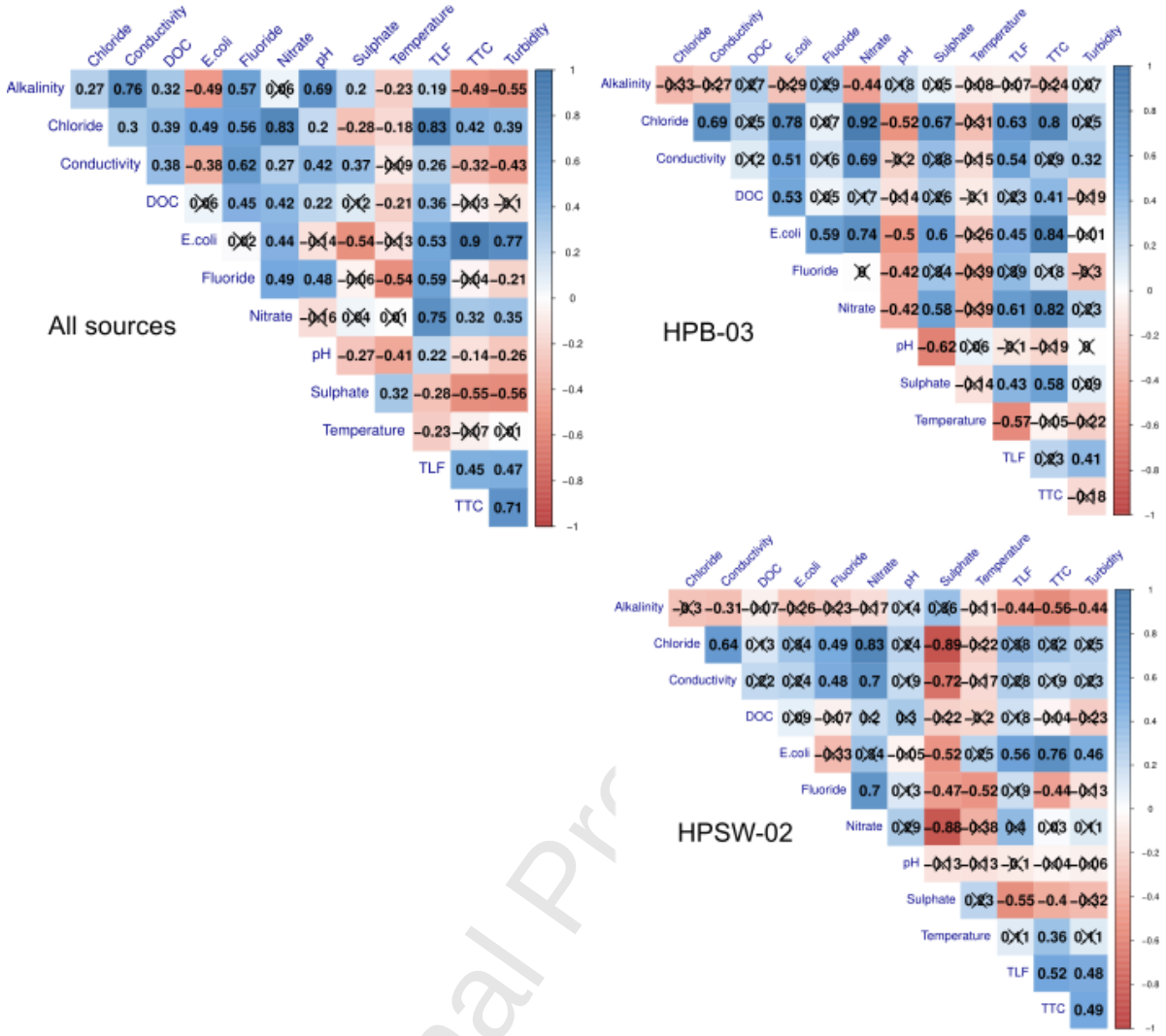


Figure 4: Correlation matrices for all sources (whole dataset), HPB-03 and HPSW-02. p = 0.05, insignificant correlations are crossed out. Dark blue: r^2 = 1; Dark red: r^2 = -1. Further correlation matrices for other sources are in Supplementary Information.

3.4 Temporal variation of inorganic hydrochemical parameters

The hydrochemistry at HPB-03 stands out from the other sources, with higher concentrations of several parameters recorded at this source. Chloride, nitrate, fluoride, conductivity, alkalinity and DOC were all consistently elevated at HPB-03 and were significantly different in comparison to other sources (Kruskal-Wallis: p = 0.001) (Figure 5 and Supplementary Information Figure S2). At HPB-03, nitrate and chloride showed a seasonal response, peaking in the wet seasons, with maximum concentrations of 119.2 mg/L and 86.1 mg/L respectively. Nitrate was consistently above 80 mg/L (exceeding the WHO (2017) guideline

of 50 mg/L), and chloride remained above 50 mg/L. DOC peaked at 1.97 mg/L, at HPB-03. This occurred at the end of the wet season, at the same time as peak in chloride and nitrate, but DOC returned to a similar level to other sources whereas chloride and nitrate remained elevated at HPB-03 throughout the study. The turbidity peak at HPB-03 was associated with the recommencing of pumping after fixing the borehole, otherwise it was consistently low.

Notable trends at other sources include a nitrate peak (33.9 mg/L) at the end of the wet season at HPSW-02 (Figure 5), however this was not observed at the beginning of the subsequent wet season. At all other sources, nitrate and chloride concentrations remained below 10 mg/L. Conductivity, pH and sulphate were stable at all sources. There was a steady increase in alkalinity observed at OSW-01 throughout the dry season (mean = 80.9; max = 122.2; min = 41.3; SD = 14.7 mg/L $HCO_{3;}$ Figure S2). Temporal trends for DOC were very similar for all sources, which is noteworthy given the differences in source type and fact that these sources are not in close proximity to each other. Regarding turbidity, all sources were significantly different to each other except HPB-02 and HPB-01 where turbidity was consistently low (Kruskal-Wallis: χ^2 = 179; p = 0.001; Dunn's test: p = 0.001). OSW-01 and HPSW-02 had the largest values and range. OSW-01 had consistently high turbidity, while at HPSW-02 turbidity increases were associated with the wet season.

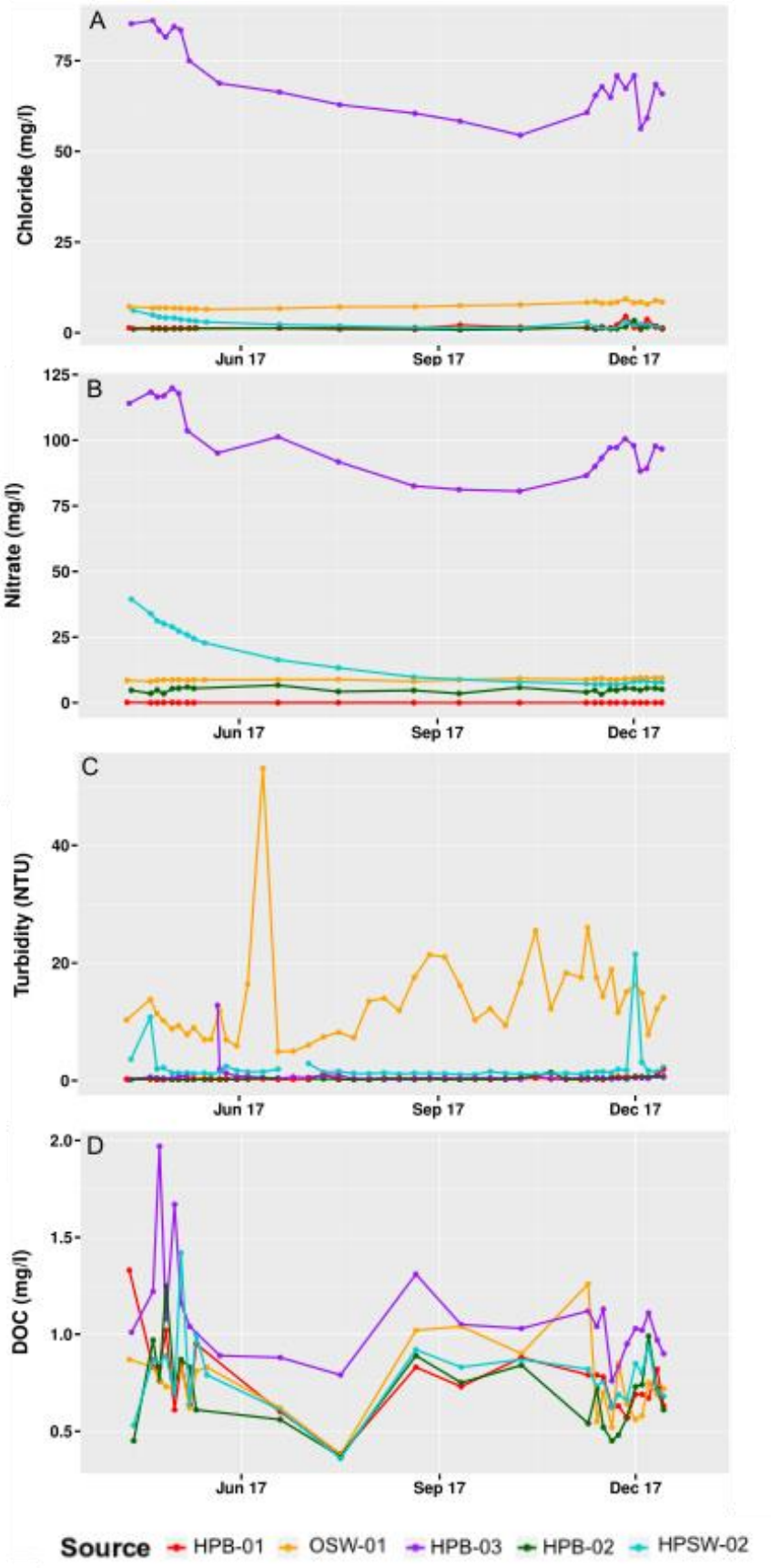


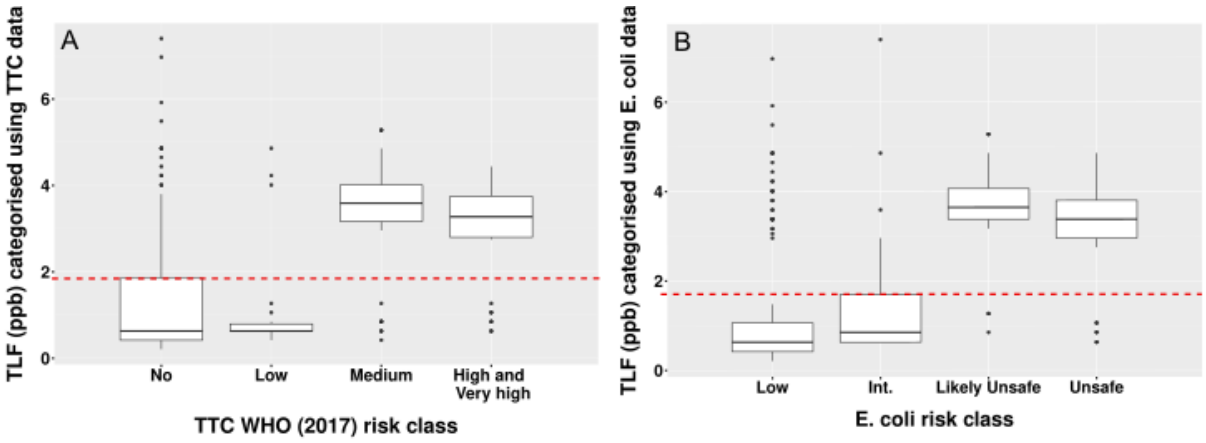
Figure 5: Temporal variation of inorganic water quality: A: Chloride (mg/L); B: Nitrate (mg/L); C: Turbidity (NTU); D; Dissolved Organic Carbon (DOC) (mg/L)

3.5 Relationship between microbial indicators and inorganic hydrochemical parameters

Across the whole dataset, TLF correlated strongly (positive) with chloride, nitrate and fluoride and moderately with turbidity (Spearman's Rank: chloride: r^2 = 0.83; nitrate: r^2 = 0.75; fluoride: r^2 = 0.59; turbidity: r^2 = 0.47; p = 0.05) (Figure 4). TTCs and *E. coli* were also strongly correlated with turbidity (Spearman's Rank: TTC r^2 = 0.71; p = 0.01; *E. coli* r^2 = 0.77; p = 0.05). In addition, TTCs had strong-moderate correlations with chloride, nitrate and sulphate (Spearman's Rank: chloride: r^2 = 0.42; nitrate: r^2 = 0.32; sulphate: r^2 = -0.55; p = 0.05) and *E. coli* correlated with chloride, nitrate and conductivity (Spearman's Rank: chloride: r^2 = 0.49; nitrate: r^2 = 0.44; conductivity: r^2 = -0.38; p = 0.05).

Considering sources individually, HPB-03 had the most correlations between microbial and inorganic parameters. At this source, TLF had a moderate positive correlation with conductivity, chloride, nitrate, turbidity and sulphate (Spearman's rank: conductivity: r^2 = 0.54; chloride: r^2 = 0.63; nitrate: r^2 = 0.61; turbidity: r^2 = 0.41; sulphate: r^2 = 0.43; p = 0.05). The TLF peak at HPB-03 also occurred at the same time as the peak in chloride, nitrate and DOC, if the effects of hand-pump repair are ignored. At HPB-03, TTCs had a strong-moderate positive correlation with chloride, nitrate, sulphate and DOC (Spearman's Rank: chloride: r^2 = 0.80; nitrate: r^2 = 0.82; sulphate: r^2 = 0.58; DOC: r^2 = 0.41; p = 0.05) and *E. coli* correlated with chloride, nitrate, sulphate, fluoride, DOC, conductivity and pH at HPB-03 (Spearman's Rank: chloride: r^2 = 0.78; nitrate: r^2 = 0.74; sulphate: r^2 = 0.60; fluoride: r^2 = 0.59; DOC: r^2 = 0.53; conductivity: r^2 = 0.51; pH: r^2 = -0.5; p = 0.05).

At other sources, there was a moderate positive correlation for TLF with turbidity and alkalinity at OSW-01 (Spearman's rank: turbidity: r^2 = 0.38; alkalinity: r^2 = 0.38; p = 0.05), but no correlations for TTCs and *E. coli* with inorganic parameters. TLF also correlated with turbidity, alkalinity and sulphate at HPSW-02 (Spearman's rank: turbidity: r^2 = 0.48; alkalinity: r^2 = -0.44; sulphate r^2 = -0.56; p = 0.05). In addition, at HPSW-02 there was correlation



between TTCs and alkalinity, fluoride and sulphate (Spearman's rank: alkalinity: r^2 = -0.44; fluoride: r^2 = -0.44; sulphate: r^2 = 0.40; p = 0.05). At HPB-01, TTCs correlated with chloride, sulphate and turbidity (Spearman's rank: alkalinity: r^2 = 0.49; sulphate: r^2 = -0.41; turbidity: r^2 = 0.38; p = 0.05), but there were no correlations for TLF with any inorganic parameters. There were no correlations between any microbial and inorganic parameters at HPB-02.

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3.6 TLF comparison with risk classes for TTCs and *E. coli*

TLF data was grouped into WHO (2017) TTC and *E. coli* risk classes, defined by the corresponding paired TTC and *E. coli* data (Figure 6). A summary of the WHO (2017) risk classes is provided in the supplementary information (Table S2). WHO (2017) risk classes are based on TTC/E*. coli* colony counts and comprise five categories ranging from No (1) risk (0 cfu/100mL) to Very High (5) risk (>1000 cfu/100mL). In Figure 6, the High (4) and Very High (5) risk classes have been combined due to a small number of samples in the Very High category (n = 7). This also makes it easier to compare directly with the *E. coli* data. *E. coli* risk classes range from Low risk (0 MPN/100mL; upper 95% confidence interval: 2.87 MPN/100mL) to Unsafe (> 100 MPN/100mL; upper 95% confidence interval: 9435.10 MPN/100mL).

Figure 6: A: TLF categorised by WHO risk classes using paired TTC data, red line shows 1.9 ppb TLF threshold calculated for this dataset; B: TLF categorised by E. coli risk class using E. coli data, red line shows 1.7 ppb TLF threshold calculated for this dataset (Table 2).

There were significant differences in the TLF dataset when grouped by both the TTC and *E. coli* data. (Kruskal-Wallis: $p = 0.001$). For both datasets, TLF can distinguish between the lowest two risk classes and the highest two classes (Dunn's test: $p = 0.001$) (Figure 6).

There was a high level of agreement (94%) between the TTC and *E. coli* data when comparing each of the four risk classes in Figure 6 (e.g. TTC No risk class and *E. coli* Low risk class). At sources HPB-01, OSW-01 and HPB-03 there was 100% agreement between the two datasets. At HPB-02 and HPSW-02 there was a 94% and 71% agreement respectively.

3.7 Defining TLF thresholds

TLF thresholds were defined for TTCs (≥ 10 cfu/100 mL; risk class: low) and *E. coli* (3.1 MPN/100mL, upper 95% confidence level: 11.36 MPN/100mL; risk class: intermediate) using the TLF value of the 75th percentile of the low and intermediate risk classes respectively. (Figure 6 – red dotted line). The TLF threshold is 1.9 ppb using TTC data and 1.7 using *E. coli* data; these are higher than other published thresholds (Nowicki et al., 2019, Sorensen et al., 2018a) (Table 2). The compliance rate is defined as true positives and negatives (i.e. above and below the threshold as would be expected from TTC data) and the error rate is defined as the total of false positives and false negatives. The performance of each source against these thresholds varies considerably; OSW-01, HPB-01 and HPB-02 have the highest threshold compliance rates for both TTC and *E. coli* thresholds defined for this dataset (96-100%). At HPSW-02, the compliance rate is slightly lower for TTCs (76%) but remains at 100% for *E. coli*. In contrast, at HPB-03 there is only a 16% compliance with the TLF threshold and a false positive rate of 84%. This is due to the consistently elevated TLF observed but only occasional TTCs and *E. coli* recorded. Compliance and error rates for this dataset when assessed against other published thresholds shows similar compliance rates even though the threshold values differ (Table 2).

Table 2: TLF thresholds and performance of individual source in this study, compared with other published thresholds

		TLF threshold (ppb)	Performance of sources in this dataset:	OSW-01	HPB-02	HPB-01	HPSW-02	HPB-03
TTC data – TLF (ppb) threshold for ≥ 10 cfu/100mL; risk class: low	Sorensen et al. (2018a)	1.3	Compliance	100%	98%	96%	74%	16%
			False positive rate	0%	2%	0%	0%	84%
			False negative rate	0%	0%	4%	26%	0%
	This dataset	1.9	Compliance	100%	100%	96%	74%	16%
			False positive rate	0%	0%	0%	0%	84%
			False negative rate	0%	0%	4%	26%	0%
***E. coli* data – TLF (ppb) threshold for 3.1 MPN/100mL, upper 95% confidence level: 11.36 MPN/100mL; risk class: intermediate**	Nowicki et al. (2019)	1.0	Compliance	96%	98%	94%	83%	38%
			False positive rate	4%	2%	6%	6%	62%
			False negative rate	0%	0%	0%	11%	0%
	This dataset	1.7	Compliance	96%	100%	100%	100%	38%
			False positive rate	4%	0%	0%	0%	62%
			False negative rate	0%	0%	0%	0%	0%

4. Discussion

4.1 TLF can indicate a broader contamination risk compared to other faecal indicators

TLF is less sensitive to rapid temporal changes in water quality compared to TTC and *E. coli* data because the TLF signal lasts longer in the groundwater system. This suggests that TLF is perhaps better at categorising sources as 'high' or 'low' risk rather than determining the absolute abundance of microbial contamination, and represents a more long-term assessment of the overall risk of the source. For example, TTC and *E. coli* values vary considerably for some sources throughout the study period, including the dry season (Figure 3). At HPSW-02, at the onset of the wet season TTC counts increase from an average of 3 cfu/100mL to 1000 cfu/100mL between consecutive sampling rounds (twice weekly); this

type of rapid change is not observed for TLF. With this degree of variability in TTC and *E. coli* data it is difficult to be confident that results from single spot sampling will give a representative assessment of the nature of risk from microbiological contamination. Although a frequent water sampling programme would be the ideal approach to water quality monitoring, this is difficult to achieve in low resource settings and spot sampling, providing only a 'snapshot' of water quality at a given time, is often undertaken instead (WHO and UNICEF, 2018). These findings are in general agreement with Nowicki et al. (2019), Fox et al. (2017) and Ward et al. (2020), who also conclude TLF is more suited to assessing high level risk from microbial contamination and microbial activity instead of enmeration.

Importantly, the two sources with the consistently higher TLF observations, OSW-01 and HPB-03, have different TTC and *E. coli* temporal profiles (Figure 3), and construction types (open shallow well and borehole) (Figure 2). The microbial water quality of OSW-01 is characterised by constant presence of TTCs and *E. coli*, with a clear seasonal increase in TTC concentration in the wet season. The seasonal signal is still present but not as clear in the *E. coli* data due to the definition of risk categories for this method (the highest risk class is equivalent to approximately 100 cfu/100mL rather than >1000 cfu/100mL). Turbidity is also high for a groundwater source, but not at a level of concern for TLF interference (Khamis et al., 2015). This water quality profile is synonymous with microbial contamination at an unprotected shallow groundwater source and the elevated TLF profile supports this conceptual model despite the lack of seasonal TLF variation.

In contrast, at HPB-03 a consistently elevated TLF profile (with no significant difference to the TLF profile at OSW-01) was observed despite few counts of TTCs or *E. coli*. Only short, occasional seasonal breakthroughs of TTCs and *E. coli* were recorded. TTCs were detected in 11 samples, grouped into four distinct periods; *E.coli* was detected in eight samples forming two groups, but *E. coli* was not sampled as frequently in the dry season. There was a TLF peak on recommencement of pumping after fixing the hand pump that coincided with a turbidity peak, presumably due to disturbance of sediment in the borehole, but no TTCs or

E. coli were recorded. The turbidity peak was too small to cause the TLF peak by interference (Khamis et al., 2015). Instead, the TLF peak is attributable to cross-contamination from unavoidable manual handling of the borehole parts during repair, before being placed back down the borehole.

The hydrogeochemical data for HPB-03 indicate contamination from the nearby pit latrine (approximately 5 m distance); chloride, nitrate and DOC are all elevated and the elevated TLF profile further supports this conceptual model. (Templeton et al., 2015, Graham and Polizzotto, 2013). This indicates hydraulic connectivity between the abandoned latrine and the borehole. Chloride and nitrate show an increase in the wet season, similar to TTCs and *E. coli*. Nitrate concentrations are consistently above the 50 mg/L WHO (2017) drinking water guideline, however, chloride concentrations remain below 250 mg/L, where a salty taste may be detected and could deter use as drinking water. Turbidity remains below 5 NTU, the recommended guideline for untreated drinking water, with the exception of recommencement of pumping at the borehole repair; therefore the water will remain visually acceptable. The abandoned latrine was not apparent at the time of borehole construction because the ground had been levelled and the housing removed. It was noted, from informal conversation with villagers, that this borehole had been specifically sited at the bottom of the hill because the borehole at the top ran dry in the dry season. Before HPB-03 was drilled, villagers would have had to travel much further to collect water. It is highly likely that this source is used for drinking water despite the contamination detected, because taste, appearance and distance to water point are influential factors for consumers (Gleitsmann et al., 2007). For the majority of sampling rounds at HPB-03, TTCs and *E. coli* indicated no/low risk of microbial contamination. However, the TLF data consistently highlight an increased contamination risk, albeit with a lack of specificity as to the cause. Supporting hydrochemical data and local knowledge were used to deduce the likely source of contamination as originating from the abandoned latrine in this case. This strongly indicates that the TLF signal may be able to identify sources of contamination from abandoned pit latrines, whereas

TTCs and *E.coli* do not, due to die-off. In this case, TLF is a more precautionary risk indicator than microbial culturing techniques.

4.2 TLF can be used for a high-level screening for microbiological contamination

TLF results from HPB-03 consistently failed (false positive rate of 84%) to identify TTC contamination when measured against the calculated threshold in this study for detecting faecal contamination (≥ 10 cfu/100mL) based on WHO (2017) guidelines. The false positive rate for TLF against the *E. coli*-defined threshold in this study was 62% (>100 MPN/100mL). These are much higher than error rates reported in other studies (18-20%) (Nowicki et al., 2019, Sorensen et al., 2018a) and highlights further the differences between what is actually being measured by TLF and culturing methods (TTCs and *E. coli*). Nowicki et al. (2019) stated that the thresholds defined in their study were not directly applicable outside the study area, however Sorensen et al. (2018a) combined several datasets including surface water with the purpose of defining a universal threshold. It is also important to note the different nature of the studies; this study is longitudinal in contrast to the snapshot survey design of the others.

The elevated TLF at HPB-03 is likely to be accurately highlighting the influence of the pit latrine on groundwater chemistry, driven by an elevated dissolved organic carbon, despite the lack of TTCs and *E. coli* cultured. Therefore, TLF data may not directly translate to a public health risk from faecal contamination at the time of sampling, as currently defined by TTCs and *E. coli* (WHO, 2017), but does still highlight the risk posed from the abandoned pit latrines, or other buried sources of organic carbon, which are not captured through other observations such as SRS. This study focussed on comparison of TLF with TTC and *E. coli* data, as two established methods of data collection. However, the TTC and *E. coli* methods themselves are not free from interferences and challenges with implementation (Nowicki et al., 2019). Culturing bacteria may not always be possible if the bacteria are in a non-viable state, which could contribute to a TLF signal but would not produce a colony count for the

TTC and *E. coli* methods (Sorensen et al., 2015a). The major advantage of TLF in comparison to culturing methods lies in its rapid, *in situ* results and no need for consumables (although an electricity supply is required to charge equipment), but users need to have a clear understanding that TLF data cannot be directly translated into public health risks from microbial contamination at an individual site.

However, there could be other health implications from the elevated inorganic hydrochemical parameters and the elevated TLF indicates favourable conditions for the persistence of coliforms and other potential pathogens if present. This illustrates how TLF can be of use for a 'high-level screening' survey of water quality, as suggested by Nowicki et al. (2019), where the frequency of data collection is sometimes more important than high accuracy of results for initial investigations.

There are potential gains to be made for using a rapid *in situ* TLF method in terms of ease of data collection and therefore the potential to increase sampling frequency, however, the results of this study suggest that TLF should not be used in isolation. It should ideally be used as a high-level screening tool in the initial stage of assessment, which could lead to further detailed investigations if necessary. This and other studies have found negligible influence on TLF from pH, turbidity and temperature in groundwater (Nowicki et al., 2019; Khamis et al., 2015; Sorensen et al., 2018a). However, the potential influence of DOC, as HLF, from either natural or anthropogenic sources, on TLF should be considered when screening for faecal contamination (Ward et al., 2020). If used as a rapid screening tool, when elevated TLF is observed further investigation is recommended. The nature of this second phase would be dependent on the potential contamination sources and contaminant pathways identified, as high organic carbon does not solely arise from pit latrines; other sources of contamination such as leachate from landfills or domestic waste dumps could have similar hydrochemical signals (Kamaruddin et al., 2014). Naturally high DOC can also occur in some geological settings such as buried peat and other organic rich horizons (McDonough et al., 2020). If on-site sanitation is thought to be the main cause, culturing

TTCs and/or *E. coli* would be an appropriate route to take to determine if there is likely to be a public health risk.

4.3 Is a universal TLF threshold based on WHO (2017) risk classes appropriate?

For widespread use of TLF to be successful, standardisation of TLF values associated with different levels of risk is required. Sorensen et al. (2018a) and Nowicki et al. (2019) have defined TLF thresholds for their datasets by comparing TLF observations with corresponding TTC and *E. coli* counts respectively. However, given that TLF cannot be assumed to imply a direct public health risk from faecal contamination, generic thresholds need to be used and developed with caution.

Sorensen et al. (2018a) and Nowicki et al. (2019) found no significant difference between TLF values for the two lowest risk classes for each method. This study found the same, which indicates that the current detection limit of TLF is currently approximately 10 cfu/100 mL for TTCs and 9.6 MPN/100mL for *E. coli*. For this study, it was not possible to define a threshold for higher levels of contamination as Sorensen et al. (2018a) and Nowicki et al. (2019) have done, because there was no significant difference between the higher risk categories in this data set. These findings are likely due to the limited association of TLF with culturing data discussed in section 4.2

The sources that perform best against thresholds defined in this study are the most stable in terms of TLF, TTCs and *E. coli* temporal variability (OSW-01, HPB-01 and HPB-02). At HPB-01 and HPB-02, TTCs and *E. coli* are consistently low/absent and TLF remains below the threshold. At OSW-01 the failure of TLF to capture the seasonal TTC and *E. coli* variation is not critical because the TLF value remains above the threshold consistently, therefore still accurately highlighting faecal contamination. For the TTC-defined TLF threshold, OSW-01 and HPB-02 have a 100% compliance rate and HPB-01 has a 96% compliance rate with no false positive errors and a false negative rate of 4%. In terms of public health risk, false negatives are of greatest concern, however these error rates are low. For the *E. coli*-defined

TLF threshold, OSW-08B has a 96% compliance rate while at HPB-01 and HPB-02 this is 100%.

At HPSW-02, TLF fails to capture the majority of contamination breakthrough at the onset of the wet season, in comparison to TTC- and *E. coli*-defined thresholds. This is due to the stability of TLF not reflecting the increase in TTCs and *E. coli* to >100 cfu/100mL and > 100 MPN/100ml respectively (Figure 3). This results in only 74% and 75% agreement of the results with the TLF threshold for TTCs and *E. coli* respectively (no false positive errors, but false negative rates of 26% and 25%). This level of false negative error is similar to that found by Nowicki et al. (2019) (26%) and more than reported by Sorensen et al. (2018a) (4%, but had false positive error rate of 18%). This strengthens the argument that TLF should not be used in isolation when investigating risks to public health, but instead as an initial screening tool. Any detection of TTCs or *E. coli* in a 100 mL sample breaches the WHO (2017) drinking water guidelines, and with TLF currently only able to detect ≥10 cfu/mL, this currently limits the use of TLF for assessing drinking water quality to high-level screening only. The unacceptably high error rates observed at HPB-03 have been discussed previously in Section 4.2.

The TTC-defined TLF threshold in this study is higher than by Sorensen et al. (2018a) and *E. coli*-defined TLF threshold is higher than that defined by Nowicki et al. (2019); this questions if a universal TLF threshold defined by TTCs or *E. coli* is appropriate (Table 2). Defining a threshold is important, but the more crucial aspect of developing TLF for widespread use is considering the accuracy of the threshold and assessing performance of TLF against the threshold. TLF thresholds defined in different studies do not differ greatly, however, error rates indicate a range in accuracy of performance. The consistent poor performance of one source (HPB-03) in relation to these thresholds highlights the difficulties of equating TLF thresholds to TTCs or *E. coli* data because of the influence of DOC/HLF on the TLF signal (Ward et al. 2020).

5. Conclusion

Monitoring drinking water quality, and microbial contamination in particular, is essential for progress towards Sustainable Development Goal 6. TLF offers a rapid assessment of water quality, as an early-warning indicator, but cannot be related directly to public health risk from faecal contamination and therefore usage is limited to high-level screening approaches. This study concludes:

1. TLF indicates a broader contamination risk than traditional faecal indicators; TLF is not as sensitive to short term variability. TTC and *E.coli* trends show high variability, whereas TLF remains more stable;
2. Elevated TLF indicates preferential conditions for the persistence of TTCs and/or *E. coli* if present, but not necessarily a public health risk from microbial contamination for a given sampling occasion. TLF is unable to detect large (e.g. 0-1000 cfu/100 mL) short term fluctuations in microbial contamination that are recorded by culturing methods, likely due to a broad association with elevated DOC concentrations. As such, TLF may be better suited than traditional faecal indicators for large-scale snap-shot surveys, for the purpose of high-level screening to assess potential risk of faecal contamination; TLF is a more stable and precautionary microbial risk indicator than culturing techniques;
3. If used as a rapid screening tool, when elevated TLF is observed further investigation is recommended. The nature of this second phase would be dependent on the potential contamination sources and contaminant pathways identified. TLF should not be used in isolation, or instead of culturing methods, for assessment of health risks from faecal coliforms at a specific time;
4. For widespread use of TLF to be successful, standardisation of TLF values associated with different levels of risk is required, however, this study highlights the difficulties of equating TLF thresholds to TTCs or *E. coli* data because of the

influence of DOC/HLF on the TLF signal.

Acknowledgements

This research was funded through the following sources: SCENARIO NERC Doctoral Training Partnership Grant number NE/L002566/1, through a REACH programme research grant funded by UK Aid from the UK Department for International Development (DFID) for the benefit of developing countries (Aries Code 201880) and NERC, Economic and Social Research Council (ESRC) and Department for International Development (DFID) through Unlocking the Potential of Groundwater for the Poor (UPGro) Consortium, Hidden Crisis Project, Grant number NE/M008606/1. DSR was supported by the Natural Environment Research Council award number NE/R000131/1 as part of the SUNRISE programme delivering National Capability. The views expressed and information contained in this paper are not necessarily those of or endorsed by DFID, which can accept no responsibility for such views or information or for any reliance placed on them. The authors would like to thank Grace Mwangulu for assistance with data collection, Michael Watts (BGS) and Heather Wickham (CEH) for processing the laboratory samples, Joseph Banda at the Lilongwe District Water office for assistance arranging site visits and Dixon Mfuyeni for fieldwork support. BGS authors publish with permission of the Executive Director (BGS-UKRI).

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Declaration of competing 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Jade Ward – Conceptualization; Data collection and curation; Formal analysis; Investigation; Methodology; Project administration; Validation; Visualization; Writing - original draft; Writing - review & editing

Daniel Lapworth – Conceptualization; Formal analysis; Methodology; Project administration; Resources; Supervision; Validation; Writing - review & editing

Daniel Read – Conceptualization; Formal analysis; Methodology; Project administration; Supervision; Writing - review & editing

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Sembeyawo Banda – Investigation; Project administration;

Maurice Monjerezi – Project administration; Writing - review & editing

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Alan MacDonald – Conceptualization; Methodology; Project administration; Resources; Supervision; Writing - review & editing

Graphical abstract

Highlights:

- First large temporal TLF water quality monitoring study in a low resource context
- TLF suitable as a high level screening tool for potential faecal contamination
- TLF can be more precautionary than culturing methods for detecting contamination
- TLF shows a more stable signal compared to other faecal indicators
- It may be inappropriate to define TLF thresholds based on TTC risk classes