Multiple fluorescence approaches to identify rapid changes in microbial indicators at karst springs

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Karst springs are globally important for drinking water supply but are often also exceptionally vulnerable to contamination. Such springs usually exhibit strong variation in microbial water quality in sharp response to rainfall events, thus, posing a health hazard to consumers of water supplied from these sources. The rapid detection of such changes is extremely important as well as being able to establish a link to the sources of such pollution, so that appropriate measures can be taken both in terms of immediate protection of human health and the management of karst aquifers. In this study, a fluorescence-based multi-parameter approach was trialed in order to evaluate which methods can be used to monitor rainfall-induced rapid changes in microbial water quality at karst springs, as well as determine whether such changes can be linked to sources of human effluent contamination. The results from three monitoring periods at two karst springs revealed marked responses to rainfall events for all of the microbial parameters measured. Total cell count (TCC) measurements using flow cytometry (FCM) showed very strong positive correlations with the more conventionally monitored faecal indicator bacteria (FIB) and total coliforms (TC), indicating that such a fluorescence-based and cultivation-independent technique can be very useful to indicate rapid changes in microbial water quality at karst springs. Furthermore, very strong positive correlations were also found between tryptophan-like fluorescence (TLF) measurements and concentrations of all monitored microbial parameters, again demonstrating that such a fluorescence-based approach can also be useful for detecting rapid changes in concentrations of traditional faecal indicators. Interestingly, it was found that fluorescent whitening compounds (FWCs) signals do not necessarily follow temporal variations of microbial indicators. However, the frequency of detection of positive FWCs signals may still reveal useful information about the overall magnitude of human wastewater effluent impacts on karst aquifer systems.

1. Introduction

Groundwater from karst aquifers (through springs and wells) is a major source of drinking water worldwide, estimated to supply around 9% of the global population (Stevanović, 2019). However, karst aquifers are often exceptionally vulnerable to contamination because of commonly thin soil coverage with a low field capacity above the highly weathered upper part of the karst system (epikarst), and/or the rapid infiltration into the groundwater system directly through discrete flowpaths (i.e. swallow holes also known as “ponors”, and closed depressions also known as “dolines”). Such pathways can connect directly into the highly conductive network of solutionally enlarged conduits, where turbulent flow conditions generally dominate, which finally discharge at springs (Thorn and Coxon, 1992; Vesper et al., 2001; White and White, 2005; Hillebrand et al., 2012; Gutiérrez and Gutiérrez, 2016). These exacerbate the threats to karst groundwater contamination through concentrated inputs of faecal point and non-point sources which have been regularly observed worldwide (Kaçaroglu, 1999; Heinz et al., 2009; Coxon, 2011). In rural and suburban areas, wastewater effluent from on-site domestic wastewater treatment systems (DWTSs) and agricultural sources are considered among the most significant threats to groundwater quality. Many incidences of waterborne diseases can be

Abbreviations: DWTSs, on-site domestic wastewater treatment systems; FCM, flow cytometry; FIB, faecal indicator bacteria; FWCs, fluorescent whitening compounds; TC, total coliforms; TCC, total cell counts; TLF, tryptophan-like fluorescence.

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associated with contamination of surface water and groundwater due to failing on-site domestic wastewater treatment systems (Fetter, 2001; WHO, 2003). Therefore, the potential impacts of domestic wastewater effluent contaminants, especially in cases of inadequate performance or failure of DWTSs (e.g. due to poor maintenance, design or siting), are of particular interest in the management of karst aquifers due to their high vulnerability (Katz et al., 2011).

Springs provide appropriate natural locations for monitoring pollutant concentration dynamics in karst aquifer systems as they provide an integrated picture of contaminant transport through a karst conduit network, compared to wells and boreholes which are not necessarily directly connected to the most transmissive parts of the aquifer (Geyer et al., 2007). Karst springs often have strong and rapid variations in microbial water quality in response to rainfall events (Prønke et al., 2007) posing a health hazard to consumers of water supplied from these sources (Katz et al., 2011). Hence, being able to detect such rapid changes in faecal bacteria concentrations during and/or after rain event conditions at karst springs used for water supply is critical, as well as being able to establish the sources of the pollution, so that appropriate measures can be taken both in terms of immediate protection of human health and the management of karst aquifers. Unfortunately, conventional and advanced microbiological methods (e.g. standard methods for culturing bacteria, microbial source tracking, etc.) are usually either very expensive, complex, labour-intensive and/or time-consuming (NRC US, 2004; Nemati et al., 2016; Savio et al., 2018 and more); such limitations, therefore, diminish their value in terms of rapid assessment of variations in microbial water quality and the potential public-health risks from waterborne diseases in karst terrains. Accordingly, the suitability of various surrogate techniques with easy-to-measure indicators (e.g. turbidity, DOC, TOC, etc.) of microbial water contamination in karst environments has been explored in the past (Thurman, 1985; Prønke et al., 2006, 2007; Allen et al., 2008; Heinz et al., 2009; Ender et al., 2017; Frank et al., 2018 and more).

Flow cytometry (FCM) is emerging as a very promising fluorescence-based and cultivation-independent technique in environmental microbiology as a result of the ability of flow cytometers to rapidly quantify bacteria and discriminate them from debris after staining the bacterial DNA with fluorescent dyes (Gatza et al., 2013; Prest et al., 2016). Safford and Bischel (2019) have identified and examined nearly 300 studies from the last two decades mainly focused on FCM applications in water treatment, distribution and reuse, but conclude that more research is needed in order to realise the full potential of FCM. Moreover, although extensive research has been carried out on the monitoring and assessment of drinking water quality (e.g. Hammes et al., 2008; De Roy et al., 2013; Fox et al., 2017) and the characterisation of microbial communities (e.g. Hammes and Egli, 2010; Van Nevel et al., 2013) using FCM, studies in karst aquifer systems are still rare (e.g. Sinreich et al., 2014; Besmer et al., 2017; Page et al., 2017; Vucinic et al., 2022). Nevertheless, on the basis of these previous studies in karst aquifer systems it seems clear that flow cytometric rapid counting of total cell counts (TCC) could be a very useful approach to track precipitation-induced contamination events at karst springs and thereby improve our understanding of microbial contamination dynamics in karst systems.

Fluorescence spectrometry has been used in the past for assessing surface water and groundwater quality and identifying human wastewater pollution impacts (Baker, 2002; Cumberland et al., 2012; Baker et al., 2015; Carstea et al., 2016; Nowicki et al., 2019; Ward et al., 2021 and more) since the intensity of fluorescence detected at different excitation-emission wavelengths can be used to identify pollution and/or the potential origin of compounds (Carstea et al., 2020). For example, dissolved humic substances in natural water are mainly derived from dead and decaying soil detritus, aquatic plants, animals and debris (Hongye, 1999), with fulvic substances derived predominantly from tree and plant residues (Chen et al., 2003). However, it should be noted that bacteria may also produce compounds that fluoresce at humic-like wavelengths (Fox et al., 2021). Aquatic protein-like fluorescence (which is less well understood than humic- and fulvic-like fluorescence) is linked to a mixture of amino acids, free or bound in proteins and/or other organic materials with similar fluorescence characteristics, and is generally described as an indicator of biologic activity (Fellman et al., 2010; Frank et al., 2018; Sorensen et al., 2020). It has been shown to be the dominant form of fluorescent dissolved organic matter in domestic on-site wastewater effluent (Dubber et al., 2021). Tryptophan-like fluorescence (TLF) is a term that describes fluorescence occurring from a range of compounds (generally aromatic and proteinaceous compounds) that share similar fluorescence properties to the amino acid tryptophan (Baker, 2002; Sorensen et al., 2018a). Notably, in controlled laboratory studies, E. coli cells have been proven to directly emit TLF as well as to excrete compounds that fluoresce in the TLF region (Dalterio et al., 1986, 1987; Darmel et al., 2013; Fox et al., 2017). Hence, tryptophan-like fluorescence has been evaluated (and in many cases used successfully) as an indicator of faecal contamination (e.g. thermotolerant (faecal) coliforms) in drinking water supplies and groundwater (Sorensen et al., 2015, 2016, 2018a, 2021; Ward et al., 2021 and more). More specifically, Frank et al. (2018) evaluated TLF as a part of their fluorescence-based multi-parameter approach conducted at alpine karst springs, while Sorensen et al. (2018b) conducted fluorescence-based multi-parameter approach at springs and boreholes in karst environments. Moreover, Frank et al. (2021) evaluated transport properties of dissolved tryptophan in karst and demonstrated that the transport properties of tryptophan are similar to those of uranine (conservative dye/tracer commonly used for conducting artificial tracer tests in karst aquifer systems).

Fluorescence-based approaches can also be used to target well-known source-specific chemical markers of human wastewater origin known as fluorescent whitening compounds (FWCs), and, therefore, help to link groundwater pollution with DWTS impacts in rural and suburban areas and/or to assess the magnitude of human wastewater impact on groundwater. Fluorescent whitening compounds (FWCs), also known as fluorescent whitening agents and optical brighteners, are added to common liquid and powder laundry detergents due to their ability to make fabrics appear brighter in colour and prevent yellow staining and fading over time (Poiger et al., 1998; Hart et al., 2007; Cao et al., 2009; Hagedorn and Weisberg, 2009; Dubber and Gill, 2017). The most commonly used FWCs in laundry detergents include the distyrylbiphenyl-types such as distyrylbiphenylsulfonate (DSBP) and stilbene-types such as the diaminostilbene (DAS 1) (Dubber and Gill, 2017). The use of washing detergents in modern households makes these compounds ubiquitous in human wastewater, therefore, they enter the environment through the discharge of domestic wastewater effluents (Poiger et al., 1998; Cao et al., 2009; Dubber and Gill, 2017; Fennell et al., 2021), especially from failing DWTSs (Maswabi, 2015). The specificity, solubility and low potential for biodegradability of FWCs in the environment have led to studies into their use as a tracer of wastewater contamination in surface waters and groundwater (Tran et al., 2019; Fennell et al., 2021).

The main objectives of this study were to assess whether multiple different fluorescence approaches (total cell counts, tryptophan-like fluorescence, and fluorescent whitening compounds) can be used to identify rapid changes in microbial water quality at karst springs associated with rainfall events, and whether such variations in microbial indicators can be linked to contamination by DWTS effluent.

2. Materials and methods

2.1. Study sites and sampling

This study focuses on two karst springs in Ireland, which are herein named based on the closest locality or town: Elmvale spring and Ballindine spring (Fig. 1). Elmvale spring (see also Figs. S1 – S5 in Supporting Information) is one of the largest karst springs in the West of Ireland but has never been

L. Vucinic et al.
Journal of Contaminant Hydrology 254 (2023) 104129
used as a drinking water supply since the source is extremely vulnerable to contamination (Cronin and Deakin, 2000; GSI, 2020). The zone of contribution of Elmvale spring is 131.1 km² (EPA, 2020). Dinantian Pure Bedded Limestones underlie roughly 90% of this catchment (with overlying shallow well drained mineral soils, and/or areas with bedrock at or close to the surface (within 1 m of the surface) in almost 85% of the catchment), with some Namurian shales (overlain by peaty subsoils) and sandstones within the zone of contribution of the spring in the western part of the catchment (Cronin and Deakin, 2000; GSI, 2020). This spring appears at several outlets within the limestone formation with the vast majority of the groundwater emerging from a 3 m high limestone cliff over a distance of approximately 80 m (Drew, 1988). The aquifer has been described as conduit flow-dominated and some rapid groundwater velocities recorded in this area (between 10 and 110 m/h) suggest the presence of relatively sizeable conduits (Drew and Daly, 1993; GSI, 2020). Elmvale spring responds rapidly to rainfall even during the summer months, and appears to be fed significantly by diffuse input recharge (Drew, 1988), rather than by sinking streams. This catchment has a mixed land use, with open spaces with little or no vegetation, mixed forest, pastures/grasslands and arable land (CLC, 2018; EPA, 2020). The density of DWTSs in the zone of contribution to Elmvale spring is approx. 3 per km², however, a high percentage of on-site domestic wastewater treatment systems (10.5%) are located within 200 m of at least one karst feature (such as swallow hole, estavelle or turlough).

Fig. 1. The location of Elmvale and Ballindine karst springs on a simplified bedrock geology map of the island of Ireland (Geological Survey of Ireland 1:1000,000 scale map) (GSI, 2004; McNamara and Hennessy, 2010).
that can provide a pathway for the rapid transport of such effluent and associated contaminants into the karst aquifer system (these values were calculated using EPA Ireland (GeoDirectory) data for DWTSs and karst features data from GSI (2020), as explained in Section 2.4).

Ballindine spring (see also Figs. S6 – S10 in Supporting Information) is a locally significant karst spring that was used for the town’s (with the same name) water supply up to June 2010, at which point it was abandoned as a water source due to continual contamination incidences. This small agricultural (land use is almost entirely described as pastures/grasslands) catchment (3.4 km$^2$) (CLC, 2018; EPA, 2020; GSI, 2020) has no visible karst features at the surface that can provide direct transport of contaminants into the karst aquifer system (such as swallow holes and estavelles), however, it should be noted that some enclosed depressions have been recently mapped and reported by Schuler et al. (2021). Moreover, recent hydrological studies of the catchment have shown that under moderate recharge conditions the aquifer has a damped response to rainfall, but during higher sustained recharge conditions the spring becomes connected to a nearby river via a high level conduit, providing a much faster response to rainfall events (Schuler et al., 2021). The bedrock geology of this catchment consists of the Dinantian Pure Bedded Limestones (GSI, 2020) with mainly Carboniferous limestone tills and deep well drained mineral soils overlying the limestone formation (EPA, 2020). Hence, the groundwater vulnerability (which in Ireland is defined on the basis of the thickness and permeability of the subsoils overlying the bedrock aquifer, as explained in DoELG/EPA/GSI (1999) in detail) in the catchment is split between High, Moderate and Low with depth to bedrock being $>3$ m in 98% of the catchment (GSI, 2020). The density of DWTSs is very high at almost 13 DWTS per km$^2$ (calculated as explained in Section 2.4).

In total, three continuous sampling campaigns targeted across rain events were conducted for the purpose of this study. Elmvale spring was continuously sampled on two occasions (EL1 in December 2018 and EL2 in January 2020), and Ballindine spring once (BD1 in January 2020). Samples were collected every hour for 24 h on all three occasions (EL1, EL2 and BD1) using ISCO portable automatic samplers (6712 model) with 24 × 1000 ml sterile polypropylene sampling bottles (Teledyne Technologies Inc., CA, USA). The sampling tube was automatically rinsed and purged several times before collecting each sample to avoid stagnation and cross contamination.

All samples collected during the three continuous sampling periods were analysed for total cell counts (TCC), total coliforms (TC) and faecal indicator bacteria (E. coli and enterococci), turbidity and presence of fluorescent whitening compounds (FWCs). In addition to these analyses, tryptophan-like fluorescence (TLF) measurements of compounds that share similar fluorescence properties to the amino acid tryptophan, as follows.

Samples for FWCs analysis were transferred from the automatic samplers directly into amber glass bottles (to protect samples from UV light) and kept in cool boxes during the transfer from the field to the laboratory, where they were stored in the fridge until analysis within 72 h. The analysis for FWCs was performed in the laboratory located in the School of Chemistry at Trinity College Dublin using a LS55 Fluorescent Spectrometer (Perkin Elmer, Massachusetts) and PMMA cuvettes with 10 mm optical path length. The emission wavelength was set at $\lambda_{em} = 436$ nm with a slit width of either 5 or 10 nm. The presence or absence of FWCs in karst spring samples was determined using the photodecay method recommended by Dubber and Gill (2017). The photodecay of the samples was measured in triplicate by recording the fluorescence signal after 0, 1, and 10-min of exposure to UV light. A dark box containing a sun lamp with 4 Philips Cleo 15 W UV tubes was used to control UV exposure. The sample cuvettes were placed into a LDPE holder, centrally positioned in front of the UV tubes at a height and distance of 16 and 5 cm respectively. Ventilation of the box was maintained throughout each exposure to minimise any heat accumulation. The ratio of the reduction after 1 min to the reduction after 10 min of UV exposure was determined and samples with a ratio (1/10 min) $>0.25$ were considered to contain FWCs (Dubber and Gill, 2017).

TLC analysis was performed using a portable UvIluX Fluorimeter ($\lambda_{ex} = 280$ nm, $\lambda_{em} = 360$ nm) (Chelsea Technologies Group Ltd., UK) by immersing probe in the groundwater sample (in the dark) as explained in Sorensen et al. (2015). The sensor utilises a UV light emitting diode (LED) light source and a miniature photomultiplier detector allowing a high level of sensitivity. The calibration of the instrument was done by the manufacturer before the sampling periods. Tryptophan-like fluorescence is reported in Quinine Sulfate Units (QSU).

2.4. Other analyses and data

Turbidity in karst groundwater samples was measured in each
collected sample with a HI9829 multiparameter instrument (Hanna Instruments, USA) which was calibrated according to the manufacturer’s instructions prior to each field sampling campaign. Rainfall data were obtained from the local Irish Meteorological Service (Met Eireann) stations, located at 7.2 km from Elmvale spring and 5.9 km from Ballindine spring respectively. For logistical reasons it was not possible to gauge either spring for flows. The densities of DWTSs in the zone of contribution of springs as well as the percentages of DWTSs in the catchments that are within 200 m of at least one karst feature (such as swallow hole, estavelle or turlough) were calculated using ArcGIS 10.7.1 with DWTSs layers obtained from the EPA Ireland (GeoDirectory) under the same conditions as explained in Gill and Mockler (2016), and karst features data from GSI (2020). Statistical analysis was carried out in R environment (R Core Team, 2020). A significance level of 0.05 is used throughout this study.

3. Results and discussion

3.1. First monitoring event at Elmvale spring

The results of the first continuous sampling at Elmvale spring in response to a rain event (EL1) in December 2018 (sampling started on the 4th December at 1 PM) are presented in Fig. 2.

It should be noted that prior to the start of the EL1 sampling, no rain had fallen for around 30 h. The targeted rain event started when the second sample was collected (one hour after the start of the EL1 auto-sampling), and, in total, 19.5 mm of rain fell during this 24-h sampling period. Consequently, at the beginning of the sampling events, values of TCC, TC, E. coli, ENT and turbidity were among the lowest measured during this 24-h period.

The analysed microbiological values as well as turbidity started rising within a few hours after rainfall began (concentrations slowly started to rise about 2 to 3 h after rainfall started), showing a rapid response to rainfall, as suggested by previous studies of the spring (i.e. Drew, 1988; Drew and Daly, 1993; Cronin and Deakin, 2000). This may be mainly due to the remobilization of sediments and microbial particles (both dead and live) from inside the karst network (i.e. autochthonous material) as opposed to representative of new contaminants washed through the system from surface sources by the rainfall event (i.e. allochthonous material - see Pronk et al. (2007)).

Total coliforms (TC) first peaked around 8 h after rainfall began (as evident from the analysis of sample number 9), while TCC, E. coli and ENT (as well as turbidity) lagged one hour behind those of TC, peaking for the first time 9 h after rainfall began (sample 10). The concentrations of microbiological parameters and turbidity generally dropped a bit after the first peak (likely as a result of rainfall decrease for a few hours), and they all peaked for the second time together around the time when sample 17 was collected (hence, 16 h after the rainfall began) and when the highest concentrations of turbidity and microbiological parameters during the entire EL1 auto-sampling period were recorded.

A significant difference between the minimum and maximum values of microbiological and turbidity parameters was observed during the EL1 period. In fact, TCC values ranged from ~5.7 × 10^5 cells per ml (in sample 2) to a peak of ~1.08 × 10^6 cells per ml (in sample 17), coinciding with the peak concentrations in TC of 5231 MPN/100 ml as well as E. coli and enterococci (99 MPN/100 ml for E. coli and 11.8 MPN/100 ml for ENT). Turbidity varied between 2.3 and 8.2 NTU, with the highest turbidity value again recorded in sample 17, 16 h after the start of the rainfall.

The FWCs results from 24 samples collected during the EL1 sampling period show that 15 samples (~62.5%) have a mean photodecay signal reduction 1/10 min ratio of 0.67 was recorded in sample number 4 (3 h after rainfall began), while the lowest FWCs signal (0.16 ratio) was detected in two samples (sample numbers 9 and 26).

Pearson’s correlations between monitored parameters were also carried out (Fig. 3) which revealed no significant relationships were observed between temporal variations in FWCs signals and other monitored parameters during the EL1 period, except a negative correlation between FWCs signals and ENT concentrations (r = −0.48; p = 0.017). On the other hand, TCC concentrations during this sampling period correlated strongly with turbidity followed by FIB (E. coli and ENT) and then TC all with p < 0.01. Strong positive relationships between turbidity and E. coli, turbidity and ENT, and turbidity and TC (again all p < 0.01) were also observed. Strong positive and significant
relationships between TC and \(E. \ coli\), TC and ENT, and \(E. \ coli\) and ENT, as reported in Fig. 3, were also observed.

3.2. Second monitoring event at Elmvale spring

The results of the second continuous sampling at Elmvale spring during the rain event (EL2), conducted in January 2020 (sampling started on the 27th January at 7 PM), are presented in Fig. 4.

Several relatively short-lasting precipitation events were recorded within approx. 26 h prior to the start of the EL2 continuous hourly auto-sampling (6.2 mm of rain, of which, 3.2 mm of rain fell in about 6 h before the EL2 sampling). Hence, the EL2 auto-sampling period should be considered as sampling during the rain event which started before the first sample was taken. Nevertheless, during the EL2 sampling it was still possible to capture some temporal variation of monitored parameters considering that in total 7.2 mm of rain fell during the 24 h of EL2 sampling period (3.3 mm in short amount of time; between 18 and 21 h after the start of EL2 sampling).

The analysed microbiological values as well as tryptophan-like fluorescence (TLF) and turbidity started rising by the end of EL2 sampling period, after a more intense burst of consecutive rainfall, as shown in Fig. 4. Importantly, all analysed microbial parameters TCC, TC, \(E. \ coli\), ENT, as well as TLF and turbidity peaked at the same time during the EL2.

TCC concentrations during the EL2 sampling fluctuated between \(2.89 \times 10^5\) cells per ml (in sample 12) and a peak value of \(~6.34 \times 10^5\) cells per ml (in sample 22) which coincided with the highest TC (1960.8 MPN/100 ml), \(E. \ coli\) (70.8 MPN/100 ml) and ENT (17.4 MPN/100 ml) values. In contrast, no enterococci bacteria were found to be present in sample 8 or sample 11. Equally, Tryptophan-like fluorescence (TLF) values peaked at 13.3 QSU (in sample 22), as well as the highest turbidity values at 5.8 NTU.

The FWCs results from 24 samples collected during the EL2 sampling period show that whilst 21 samples (or around 87.5%) have a mean photodecay signal reduction ratio (1/10 min) equal to or higher than the threshold value of 0.25, only 16 of them also have the 95% LCI value higher than the threshold, and can thus be considered as positive for FWCs (with the remaining 5 samples considered as indicative for FWCs presence). Therefore, 66.6% of all samples collected during the EL2 sampling were positive for FWCs.

The strongest FWCs signal (0.45 mean ratio value) during the EL2 period was detected in sample 10, while the lowest FWCs signal (0.21 mean ratio value) was detected, interestingly, in sample 22 (where the highest concentration of microbial parameters, as well as TLF and turbidity were observed, as previously reported). The lowest FWC signals corresponding to the periods of highest microbial contamination during EL2 has several possible explanations. For example, Dubber and Gill (2017) found that higher organic matter content negatively affects the detectability of FWCs with this method, which could be a potential explanation (i.e. TLF concentrations were elevated in those two samples which could also indicate the potential presence of other compounds that could affect the detectability of FWCs).

Also, since positive FWCs signals were detected persistently in
samples taken before samples 21 and 22 (which were negative for FWCs) and positive samples were detected after (FWCs were detected in samples 23 and 24), it is possible that transport of FWCs through karst aquifer system was different than particulates (including microbial) transport. It is also possible that the microbial, turbidity and TLF peak during the EL2 could be attributed to the arrival of contaminants from the land surface (which are not of human wastewater effluent origin – e.g. agricultural) that entered the karst network (see Pronk et al., 2007) despite the fact that the detection frequency of positive FWCs signals during this 24-h sampling period (only three samples, or 12.5% of total samples, were neither positive nor indicative for FWCs presence) reveals a considerable impact of DWTSs effluent on groundwater quality.

A correlation matrix heatmap (Fig. 5) reveals that correlations between TCC and TC, TCC and *E. coli*, TCC and ENT, TCC and turbidity, TC and TLF, TC and turbidity, *E. coli* and TLF, *E. coli* and turbidity, ENT and TLF, TLF and turbidity and ENT and turbidity, all significant (*p* < 0.01) and positive. Correlations between TC and FIB, and *E. coli* and ENT, were again significant (*p* < 0.01) and positive during the second monitoring event at Elmvale spring. In terms of relationships between FWCs signals and other datasets, these relationships were mostly not significant, except between FWCs signals and TCC (*r* = 0.48; *p* < 0.05) and FWCs signals and TC (*r* = −0.41; *p* < 0.05) where moderate negative correlations were found.

### 3.3. Monitoring event at Ballindine spring

The results of the single continuous sampling at Ballindine spring during the rain event (BD1), conducted in January 2020 (sampling started on the 30th January at 7 PM), are presented in Fig. 6.

Before the BD1 continuous auto-sampling campaign, a very significant precipitation event occurred; 30.4 mm of rain fell within 96 h before the first sample was taken, however, only 3.5 mm of that rain fell within 48 h prior to the start of BD1 period. Thus, this potentially explains the elevated concentrations of microbial parameters as well as TLF in the beginning of the BD1 sampling period (which were slowly decreasing for several hours after the start of BD1 sampling), as shown in Fig. 6. During the 24 h of BD1 sampling, 7.7 mm of rain fell, mostly between the time of collection of samples number 5 and number 13 (therefore, approx. 8–9 h). Hence, the response in terms of microbial parameters and TLF captured by the end of BD1 sampling period, peaking at hour 23 would seem to be in response to that precipitation event, reflective of the more diffuse nature of the karst system in this catchment compared to Elmvale.

In terms of variation of monitored parameters during this sampling event, turbidity concentrations were low throughout the entire BD1 period (between 0.2 and 0.4 NTU) with very little variation, which contrasts against the turbidity responses seen in EL1 and EL2 at the other spring. However, other parameters, especially microbial and TLF compounds, showed much more variation. The maximum TCC concentration during this sampling period was ~1.01 × 10⁶ cells per ml (in sample 23) which coincided with the highest TC concentration of 1123.5 MPN/100 ml and maximum values of FIB of 78 MPN/100 ml of *E. coli* and 25.3

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Fig. 5. Correlation matrix heatmap representing the Pearson’s correlation between variables recorded during EL2 monitoring (*n* = 24). Asterisk indicates *p* < 0.05, and double asterisk indicates *p* < 0.01.

Fig. 6. Results from BD1 monitoring: a) precipitation and turbidity data, b) FCM total cell counts, c) total coliforms, d) *E. coli* and enterococci, e) FWCs analysis, and f) tryptophan-like fluorescence.
MPN/100 ml of ENT. In comparison, no ENT bacteria were present in sample number 10 which matched with when the lowest E. coli concentrations were recorded (9.4 MPN/100 ml). Regarding TLF measurements, these values ranged from 9.2 QSU (recorded in sample 11) to 14.3 QSU (measured in sample 23), coinciding with when the highest concentrations of monitored microbial parameters were found.

The FWCs results from 24 samples collected during the BD1 sampling period show that half of those samples (12 samples in total) have a mean photodecay signal reduction ratio (1/10 min) equal to or higher than the threshold value of 0.25, with 10 of those 12 samples considered as positive for FWCs since their 95% LCI values are also higher than 0.25. The remaining 2 samples with 95% LCI values below the threshold are being considered as indicative for FWCs.

The highest FWCs signal was detected in sample number 5 (0.47 mean ratio value) and the lowest in sample number 18 (0.15 mean ratio value). Because roughly 42% of all samples collected during the BD1 sampling period were positive for FWCs, this indicates a considerable impact of DWTSs effluent on groundwater quality at the Ballindine spring. Moreover, considering that sample 23 (with the highest microbial and TLF values during the BD1 monitoring) was also positive for FWCs, a link between the observed pollution peak and DWTSs effluent may be suggested (although, it should be noted that this does not mean that pollution peak can be attributed to only this pollution source).

Correlations between TCC and TC, TCC and ENT, TCC and TLF, TC and TLF, E. coli and TLF, and ENT and TLF were all significant ($p < 0.01$) and positive (Fig. 7). Also, correlations between TC and FIB, and E. coli and ENT were all significant ($p < 0.01$) and positive, as reported in the same correlation matrix heatmap. The relationships between recorded FWCs signals and other datasets were not significant, nor relationships between any monitored microbial parameter and turbidity, since turbidity variations during the BD1 period were negligible. Turbidity showed only a moderate positive correlation with TLF ($r = 0.42; p < 0.05$) mainly because TLF concentrations varied less than collected microbial data during this event at the Ballindine spring.

### 3.4. Discussion of the results

Both springs during these three continuous 24-h sampling periods showed marked responses to rainfall events for all of the microbial parameters measured. It was expected that Elmvale spring would show faster responses to rainfall events than Ballindine spring on the basis of differences between the two catchments. Ballindine catchment is a lot smaller in size but has thicker layer of soil and no swallow holes or estavelles yielding more diffuse recharge conditions. It also appears to be less karstified, with the exception of the high level conduit between a river and the spring that only activates under very high recharge conditions, as discussed in Schuler et al. (2021). This was indeed the case with the more conduit flow-dominated Elmvale spring exhibiting a 3 to 4 h lagged response compared to the 12 to 13 h lag at Ballindine spring during those winter periods when the trials were carried out.

#### 3.4.1. Evaluation of Total Cell Counts (TCC) with flow cytometry (FCM)

Since TLF was not measured during the EL1 period, the focus was on evaluating whether TCC measurements with FCM can be successfully used to predict precipitation-induced rapid changes in TC and FIB whilst using FWCs to try to source apportion the relative pollution impact from DWTS effluent. As reported earlier (in Section 3.1.), during the EL1 period, TCC showed a very strong positive correlations with E. coli and ENT and somewhat lower but still a very strong positive correlation with TC. Such significant and a very strong positive relationships between TCC and FIB as well as TC was further confirmed during the EL2 period (see Section 3.2.) when the correlation between TCC and ENT was similarly strong and positive as during the EL1 period, as well as the strong correlations between TCC and TC and TCC and E. coli. The statistical analysis of combined datasets from the EL1 and EL2 periods ($n = 48$), as shown in Fig. 8, also reveal strong positive correlations between TCC and other monitored microbiological data ($r = 0.87$ and $p < 0.01$ between TCC and TC; $r = 0.73$ and $p < 0.01$ between TCC and E. coli; $r = 0.51$ and $p < 0.01$ between TCC and ENT). Furthermore, correlations between TCC and TC and FIB at Ballindine spring during the single continuous sampling period (BD1), as reported in Section 3.3., also suggest significant and very strong positive relationships. Therefore, the results in this study further support some earlier findings (see, for example, Besmer et al., 2017) that TCC can be useful to track rapid changes in microbial water quality at karst springs as a result of rainfall events (despite the fact that TCC peaks are less sharp than peaks of the FIB, as expected). Additionally, findings from this study are important (given the need for more field-based research in terms of linking TCC and FIB concentrations in karst aquifer systems has been highlighted in previous studies – i.e. Page et al. (2017)) because they have demonstrated that TCC can be useful at karst springs such as Elmvale where it also appears that turbidity can be used as easy-to-measure indicator of microbial faecal pollution (as evident from EL1 and EL2 results and statistical analysis). However, at karst springs such as Ballindine with insignificant turbidity variations in comparison to variations in
3.4.2. Evaluation of tryptophan-like fluorescence (TLF)
On the basis of the EL2 and BD1 sampling periods, tryptophan-like fluorescence was also evaluated in terms of its potential to predict precipitation-induced rapid changes of monitored microbial parameters (TCC, TC and FIB). The results at both springs show that concentrations of compounds with TLF properties were changing in a similar way to microbial parameters and traditional faecal indicators in response to rain events with very strong positive correlations. Tryptophan-like fluorescence concentrations correlated slightly better with TC and FIB values in samples collected during the EL2 than with those found in samples collected during the BD1. Conversely, TCC concentrations during the BD1 period correlated better with TLF than TCC concentrations during the EL2 sampling period. Thus, on the basis of this analysis, it seems that TLF can be very useful at both springs, although, more useful at conduit flow-dominated Elmvalie spring considering the somewhat better correlation with traditional FIB. However, since temperature and/or turbidity (Khamis et al., 2015), pH (Reynolds, 2003), dissolved organic carbon (DOC) and humic-like fluorescence (HLF) all have potential to influence the TLF signal (Sorensen et al., 2020; Ward et al., 2021), and only turbidity was measured in this study, a more comprehensive approach needs to be taken in order to fully evaluate the potential benefits of TLF at these springs.

3.4.3. Evaluation of fluorescent whitening compounds (FWCs)
On the basis of these sampling periods across three rainfall events, it seems that the use of FWCs signals as a specific way of fingerprinting domestic wastewater effluent at karst springs in parallel to monitoring FIB variations and other changes in microbial water quality parameters can reveal some interesting insights about the impact and pathways of human wastewater pollution, as well as more broadly the overall sources of contamination at karst springs following significant rainfall events. It is clear, in these two springs at least, that FWCs signals do not necessarily follow temporal variations of microbial parameters/indicators. The frequency of detection of positive FWCs signals (particularly during low flow conditions) seems to reveal better the magnitude of human wastewater effluent impacts rather than whether those samples are being recorded with respect to microbial pollution changes, since FWCs and particulates/bacteria might travel differently through the karst aquifer systems and/or be coming from different sources. The results show that positive FWCs signals were more frequently detected at Elmvalie spring during the two events (as well as the strongest FWC signals) than at Ballindine spring during the one monitored event, which could be due to the relatively high percentage of on-site domestic wastewater treatment systems located relatively near karst features that can provide rapid transport of such contaminants into the conduit-dominated karst aquifer system a (since there are no swallow holes or estavelles on the surface within Ballindine catchment).

The inverse relationships between FWC results and the E. coli and TCC results, suggesting that the FWC concentrations from human effluent seem to be diluted during high flow events, raises the question where the increases in microbial indicators with the high flows are coming from. These are obviously sources that are getting mobilized by the more saturated conditions on the catchments at these times. Some of the increase might be attributed to domestic wastewater treatment systems whereby more saturated conditions will mean shorter travel times (both through the soil and through the karst network) and therefore more chance of bacteria being intact by the time they reach the spring, but the other probable source is from agricultural activities in the catchments. Manure spreading is practised in the catchments and would have been allowed as per the Irish Good Agricultural Practice Regulations (Government of Ireland, 2017) up to beginning of November and it is conceivable that bacteria still surviving from landspreading might have been washed off the fields into the karst system by the EL1 sampling event. Equally, the closed season for manure and slurry spreading ends on the 15th January and so monitoring events EL2 and BD1 may well have been impacted by such agricultural sources of faecal microorganisms.

3.4.4. In-situ, real time monitoring of springs
Finally, this research has shown how such parameters can be used to gain real insights into contaminant transport in karst environments, which is particularly attractive as such techniques can be applied in-situ to provide real-time monitoring of spring water quality, which will be very beneficial for utility companies / consumers who rely on such water sources.

4. Conclusions
This study evaluated several different fluorescence-based techniques at two karst springs in Ireland over three monitoring periods. This research has shown that flow cytometry, as a fluorescence-based and cultivation-independent technique, can rapidly provide measurements of total cell counts that can be very useful to indicate rapid changes in faecal indicator bacteria and microbial water quality in general. Additionally, the results suggest that monitoring of tryptophan-like fluorescence concentrations at karst springs can also be very useful for detecting rapid changes in concentrations of traditional faecal indicators. Hence, direct in-situ, real time flow cytometric monitoring of total cell counts and tryptophan-like fluorescence concentrations hold great promise in karst environments. Moreover, it was found that detections of positive FWCs signals do not necessarily follow temporal variations of monitored microbial indicators; however, the frequency of detection of positive signals from such well-known indicators of human wastewater contamination may still reveal useful information about the magnitude of human wastewater effluent impacts on karst aquifer systems. Finally, this study confirms the importance of event-based monitoring of microbiological water quality at karst springs, given the resulting substantial increases in bacterial concentrations following rainfall events.

CRediT authorship contribution statement
Luka Vucinic: Writing – original draft, Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Software, Visualization, Project administration. David O’Connell: Writing – review & editing, Conceptualization, Funding acquisition, Supervision. Donata Dubber: Writing – review & editing, Methodology. Catherine Coxon: Writing – review & editing, Project administration, Supervision. Laurence Gill: Writing – review & editing, Conceptualization, Investigation, Supervision, Funding acquisition, Project administration.

Declaration of Competing Interest
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.

Data availability
Most of the data for this research are included in this paper. The full data sets can be found at this Mendeley Data link: doi: 10.17632/kvp8vdk4kt.1

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Appendix A. Supplementary data

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References


