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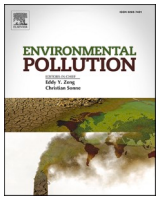

Back to the future: Comparing yeast as an outmoded artificial tracer for simulating microbial transport in karst aquifer systems to more modern approaches☆

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ABSTRACT

Bacterial contamination of karst groundwater is a major concern for public health. Artificial tracing studies are crucial for establishing links between locations where pollutants can rapidly reach the aquifer systems and subsequent receptors, as well as for enhanced understanding of pollutant transport. However, widely used solute artificial tracers do not always move through the subsurface in the same manner as particles and microorganisms, hence may not be ideal proxies for predicting movement of bacterial contaminants. This study evaluates whether a historically used microbial tracer (yeast) which is readily available, inexpensive, and environmentally friendly, but usually overlooked in modern karst hydrogeological studies due to challenges associated with its detection and quantification in the past, can reemerge as a valuable tracer using the latest technology for its detection. Two field-based studies on separate karst systems were carried out during low-flow conditions using a portable particle counter along with flow cytometry measurements to monitor the recovery of the yeast at the springs. Soluble fluorescent dyes were also injected simultaneously with the yeast for comparison of transport dynamics. On one tracer test, through a karst conduit of much higher velocities, the injected yeast and fluorescent dye arrived at the same time at the spring, in comparison to the tracer test on a conduit system with lower groundwater velocities in which the yeast particles were detected before the dye at the sampling site. Both a portable particle counter and flow cytometry successfully detected yeast during both tests, thereby demonstrating the applicability of this tracer with contemporary instrumentation. Even though no significant advantages of flow cytometry over the portable counter system can be reported on the basis of the presented results, this study has shown that flow cytometry can be successfully used to detect and quantify introduced microbial tracers in karst environments with extremely high precision.

1. Introduction

Groundwater tracing (typically employing artificial tracers) is an extremely important method used in karst hydrogeological studies (Smart, 1988; Kaess, 1998; Ward et al., 1998; Goldscheider and Drew, 2007; Benischke et al., 2007; Benischke, 2021). These tests, initially developed for the investigation of karst aquifer systems (and still most widely used in karst), have been conducted using a variety of artificial and natural tracers (e.g. inorganic ions, radionuclides, fluorescent dyes, fluorescent microspheres, various microorganisms and viruses, natural sediments, etc.) to trace groundwater flow in different hydrological and hydrogeological environments and are of great value across many applications (Wood and Ehrlich, 1978; Auckenthaler et al., 2002; Goldscheider et al., 2003, 2008; Goeppert and Goldscheider, 2008, 2019; Maurice et al., 2010; Schiperski et al., 2016; Ward et al., 2016; Bandy et al., 2018, 2019; Richter et al., 2021). For example, artificial tracer tests are used to establish hydraulic connections between a defined injection site (e.g. sinkhole, swallow hole (ponor), borehole) and a discrete outflow point (usually a spring), to estimate groundwater transit times (Cronin and Pedley, 2002; Lauber and Goldscheider, 2014; Margane et al., 2018; Schuler et al., 2020), to assess hydrogeologic behaviour of karst vadose zone (Poulain et al., 2015), to investigate conduit

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parameters (Geyer et al., 2007; Luhmann et al., 2012), as well as the transportation characteristics of potential contaminants (Cronin and Pedley, 2002; Flynn and Sinreich, 2010).

A hydrogeological topic of special concern for public health is the enhanced pathogen transport in the subsurface (via high velocity zones and preferential flow). Hence, improved approaches for the assessment of an increased risk of microbial contamination and transport in groundwater are needed (Flynn and Sinreich, 2010; Goeppert and Goldscheider, 2008; Bradford and Harvey, 2017; Vucinic et al., 2022, 2023). This is particularly true in the field of karst hydrogeology since karst aquifers are exceptionally vulnerable to contamination due to concentrated inputs from faecal point and non-point sources and their hydrological and hydrogeological characteristics, with discrete flow paths through conduit networks (Thorn and Coxon, 1992; Coxon and Drew, 1998; Vesper et al., 2001; White and White, 2005; Ford and Williams, 2007; Heinz et al., 2009; Coxon, 2011; Hartmann et al., 2014; Gutiérrez and Gutiérrez, 2016; Vucinic et al., 2022, 2023). As a result of such rapid contaminant transport with decreased attenuation potential, some of the most serious incidents of groundwater contamination worldwide have been reported in these terrains (Mull et al., 1988), a notable example occurring at Walkerton (Ontario, Canada) in May 2000 (O'Connor, 2002; Worthington and Smart, 2017). Unfortunately, solute tracers, the most commonly used artificial tracers, do not always behave and move through the subsurface in the same manner as particles and microorganisms. Numerous studies have reported faster travel times and lower recovery rates for particles compared to solutes (Rossi et al., 1998; Auckenthaler et al., 2002; Sinreich et al., 2009; Flynn and Sinreich, 2010; Goeppert et al., 2011; Goeppert and Goldscheider, 2008, 2019). Pore and size exclusion processes, which affect only particles, are a commonly accepted explanation for higher peak and mean flow velocities (Bradford et al., 2003) since microorganisms and particles cannot enter smaller pores or the pore space closer to walls where lower flow velocities occur (Goeppert and Goldscheider, 2019). Hence, the results of solute tracer tests are considered to be of limited value in predicting the movement of microorganisms in the subsurface (Kaess, 1998; Goldscheider et al., 2008; Ward et al., 2016). Nevertheless, tracer tests using various particle types and/or microorganisms (as surrogates for pathogens, colloidal and other natural particles, etc.) are still not a standard method used in karst hydrogeology (Schiperski et al., 2016; Goeppert and Goldscheider, 2019) even though the processes influencing the fate and transport of particulate contaminants in karst aquifer systems are only poorly understood (Flynn and Sinreich, 2010). It remains a challenge to find widely applicable, inexpensive, and readily available tracers that will accurately mimic microbial properties and behavior during transport in the subsurface (Kaess, 1998; Ward et al., 2016; Bandy et al., 2018, 2019; Goeppert and Goldscheider, 2019) but which also pose no potential threats to public health and/or the wider environment (Cronin and Pedley, 2002; Goldscheider et al., 2008). For example, *Serratia marcescens* bacteria were used as a tracer in the past but are now classified as pathogens and not in use anymore (Goeppert and Goldscheider, 2008). Such artificial tracers should also ideally be differentiable from microbes or particles already present in the groundwater (Cronin and Pedley, 2002) and should be easily detected at low concentrations with reasonable analytical costs by a method that is not highly time-consuming and/or complicated to perform (Reid, 1981; Kaess, 1998; Cronin and Pedley, 2002). Thus, in order to overcome these challenges and obtain better insight into microbial and particulate transport over time, a wide range of artificial tracers and tracing techniques are continuously being trialed and proposed. Advances in tracing methodologies and their application over time, as well as their associated problems and challenges, have been discussed in a recent review paper focused on tracing in karst aquifers (Benischke, 2021), and it seems clear that some of the original techniques and tracers used historically (e.g. colouring spores with fluorescent dyes) now play only a minor or no role in modern karst hydrogeology. The main reason for this is because the detection/identification of those tracers using traditional methods is labour-intensive and time consuming. Because of the problems associated with detection and quantification of such tracers, *Saccharomyces cerevisiae*, a unicellular microorganism commonly known as yeast, which is not naturally present in karst groundwater in any significant concentrations nor is associated with any known human health or environmental concerns, is usually overlooked as a tracer in the present times and is slowly becoming a "forgotten" tracer. However, this study presents a comparison of this type of yeast as an outmoded artificial tracer against two modern (and in some ways similar) techniques for detection of such microbial particles: flow cytometry (FCM) and particle counter system analysis. Portable particle counters are becoming a very important tool in the field of karst hydrogeology due to their mobility, ease of analysis and ability to detect particle tracers at extremely low levels (see Goeppert and Goldscheider, 2008, 2019). Flow cytometry, which is more sensitive, but a bit more complex, is now emerging as an important and routinely used fluorescence-based technique in environmental microbiology as a result of the ability of flow cytometers to rapidly and at low cost quantify bacteria and discriminate them from debris by staining the bacterial DNA with fluorescent dyes (Gatza et al., 2013; Prest et al., 2013; Safford and Bischel, 2019; Vucinic et al., 2022, 2023). However, flow cytometry is also a technique that can be used for conducting artificial tracer tests, although it does not appear to have been used directly in the field up to now. (For comparison of various techniques and methods, including flow cytometry, in a laboratory see DeFlaun et al., 2001). Therefore, two field-based artificial tracer tests have been designed to investigate whether readily available, inexpensive, and environmentally friendly tracers such as yeast can play a role in modern karst hydrogeology using the latest technology for their detection and quantification at karst springs, comparing FCM and a portable particle counter system. This study also examines whether FCM measurements of total cell counts (TCC), as a more sensitive, but at the same time a more complex, laborious and expensive, analytical approach, offer any significant advantages over portable particle counter measurements for conducting artificial tracer tests with microorganisms, especially in terms of detection and distinction of introduced microbial tracers in karst aquifer systems.

2. Materials and methods

2.1. Test sites

The two field sites used for the study (see Fig. 1 and Fig. S1 in Supporting Information) were on previously studied karst systems located in the west of Ireland. Thus, the connections in the karst subsurface between the swallow holes and karst springs had already been confirmed by tracing tests conducted in the past (GSI, 2023). These field sites were selected because they provide several key advantages for the investigations of such karst tracing concepts in karst environments, which are commonly followed by researchers (see Goeppert and Goldscheider, 2019): good accessibility, with relatively simple hydrogeological conditions and short distances between an active swallow hole and a karst spring, high flow velocities in the subsurface (in order to keep experiment durations as short as possible), and low background particle numbers with ideally relatively constant values during constant hydrological conditions. It should be noted that the swallow holes at both sites in this study into which the artificial tracers have been injected are known to have connections to other discharge points (particularly at higher flow rates in TS2) besides the springs that were monitored as a part of this investigation, as well as additional connections entering the network in the case of TS1 and so tracer recoveries were not estimated during this study.

The injection point for the first artificial tracer test TS1 conducted in County Galway was a swallow hole (SH1) known as the Castletown sink with a linear distance to the Kiltartan spring (KT) of 1117 m. The bedrock geology of this area consists of Dinantian Pure Bedded Limestones and conduit flow velocities in this area range from tens of m/h to

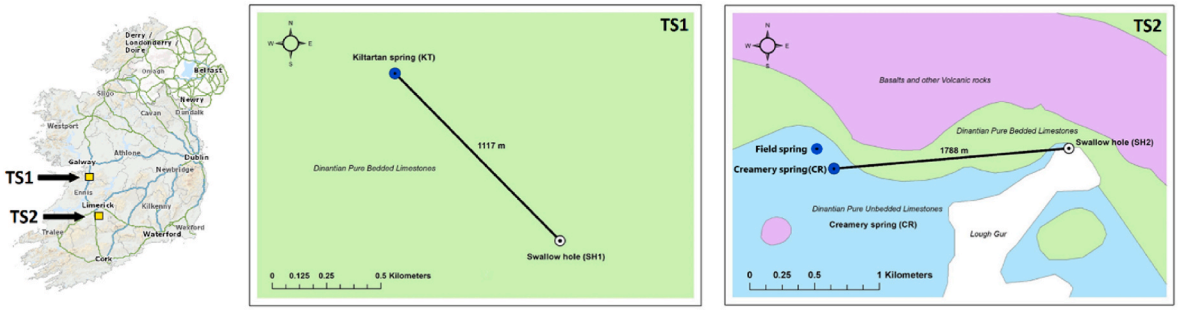

Fig. 1. The locations of field sites selected for two artificial tracer tests TS1 and TS2 (GSI, 2023).

1000 m/h (GSI, 2023). This conduit forms part of the extensive south Galway lowland karst network, which has a catchment of approximately 500 km^2, receiving allogenic runoff from three main rivers draining the Old Red Sandstone Slieve Aughty Hills. The lowland karst aquifer is fed by these sinking streams which disappear into underground fissures and conduits and then frequently reappear again in surface reaches or as turloughs in glacially modified depressions. The drainage takes the flows underground to the north-west to the Atlantic Ocean at Kinvara through a complex multi-level conduit system in this lowland network that has formed as a result of past glaciation cycles and their impact on karstification processes (Naughton et al., 2018). The karst system has been the subject of many studies over the past 20 years, most of which have been linked to the widespread groundwater-surface water interaction in the area (i.e. the turloughs), focusing on the hydraulics and modelling of the system (Gill et al., 2013a, 2013b) submarine groundwater discharge from the system (McCormack et al., 2014), groundwater flooding (Naughton et al., 2017; Morrissey et al., 2020, 2021), ecohydrology of the turloughs (Cunha Pereira et al., 2010; Bhatnagar et al., 2021) etc. The swallow hole chosen for the injection of the tracers takes flow which has come from one of the large rivers draining the Slieve Aughty mountains (representing about half of the catchment area). Between where tracer is injected (SH1) and the spring at Kiltartan, another major conduit is known to join the network, bringing flows from the other half of the catchment to the east.

The injection point for the second artificial tracer study TS2 was a swallow hole "Pollavaddra" (SH2), located on the edge of Lough Gur lake (lake water overflowing into SH2 swallow hole) in County Limerick with a linear distance to the Creamery spring (CR) of 1788 m. Lough Gur is a shallow, groundwater fed, eutrophic lake, and the link between SH2 and Creamery spring has been previously studied under high flow conditions in February 2017 (O'Connell et al., 2022) and low flow conditions in September 2014 (Langford and Gill, 2016). It should be noted that under high flow conditions, the karst conduits at higher elevations in the subsurface become active and join the Creamery spring as well as another smaller karst spring (named previously in the literature as "the field spring") located roughly 150 m to the north of the Creamery spring (O'Connell et al., 2022). This location was not monitored during the TS2, since the field spring was dry. The underlying geology of the Lough Gur area where the TS2 study was conducted consists of limestone formations (Dinantian Pure Bedded and Dinantian Pure Unbedded Limestones) and volcanic rock types (Ball, 2004; GSI, 2023; O'Connell et al., 2022). Hence, a multilayer aquifer system exists in this catchment, with an upper water level in volcanic bedrock to the E-NE of the lake, which is partially disconnected from a lower water table within the underlying limestone bedrock (Ball, 2004; O'Connell et al., 2022). Two tracer tests conducted prior to this study at this site showed conduit flow velocities of 18.58 m/h and 24 m/h (GSI, 2023).

2.2. Tracer tests, analytical methods, and data acquisition

The two tracer studies (TS1 and TS2) have been designed and conducted at the two different karst systems in order to investigate whether measurements of total cell counts (TCC) using flow cytometry (FCM) can provide any advantages in terms of detection and distinction of introduced microbial tracer from background particles and microbial concentrations over portable particle counter systems. An artificial tracer, *Saccharomyces cerevisiae* (Target Feeds Ltd., UK), a unicellular microorganism commonly known as yeast, has been selected because it is relatively inexpensive with no known negative environmental effects (Ward et al., 1998). Yeast has been used in groundwater tracer studies as far back as the early 1900s (Harvey, 1997), but modern applications seem to be very limited in number (Cronin and Pedley, 2002). Different yeast species can significantly vary in size (between 1 μm and around 40 μm) (Wood and Ehrlich, 1978; Gerba, 1984; Skilton and Wheeler, 1988), and this means this tracer alone can cover various sizes of the most known pathogenic microorganisms (Ward et al., 1998). This is a feature that makes it attractive for applications of this type in karst; however, it may also bring limitations in some non-karstic granular subsurface environments where pore spaces may limit transport (Gerba, 1984; Skilton and Wheeler, 1988; Cronin and Pedley, 2002). The *S. cerevisiae* cells used in this study are round to ovoid, 1–10 μm in diameter.

For the first artificial tracer study (TS1), conducted in early September 2018 during stable low-flow conditions, 12 kg of yeast was injected into the swallow hole together with 7 L of a 20% solution of rhodamine WT dye. While some authors such as Kaess (1998) do not recommend use of rhodamine WT for tracer tests due to potential toxicity of this dye, it should be noted that injected concentrations as a part of this test (i.e. low concentrations injected into a large karst aquifer system with high flow velocities) are not expected to pose any risk to aquatic freshwater life, as explained in Skjolding et al. (2021). For the second artificial tracer study (TS2), carried out at the end of October and the beginning of November 2018 (again, during low flow conditions due to a prolonged drought in Ireland in 2018), 75 kg of the same type of yeast was introduced into the swallow hole together with 3 kg of a conservative solute tracer uranine dye. For both TS1 and TS2 field experiments, yeast was mixed in a clean polyethylene barrel with water from the same catchments prior to the tracer injections. These artificial tracer concentrations were injected into the karst system via swallow holes within 10 min since the beginning of both tracer tests. It should be noted that the more conventional Rhodamine WT and uranine fluorescent dyes were also used as parallel soluble tracers during these investigations to provide additional confirmation/comparison against the yeast tracer with these two microbial/particulate tracer detection methods. Both fluorescent dyes were suitable for investigations reported in this study due to low detection limits and low cost.

In the case of the TS1 artificial tracer test, a GGUN-FL30 field fluorometer (Albillia Co., Switzerland) was used for on-site detection of the Rhodamine WT dye. For the TS2 artificial tracer test, when uranine dye was used, samples were collected at the spring with two ISCO portable samplers (6712 model) with 24 x 1000 ml polypropylene sampling bottles (Teledyne Technologies Inc., CA, USA). These samples were then transported to the laboratory of the Geological Survey of Ireland (GSI) in

Dublin where they were analysed with a Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). Peak excitation and emission for uranine are 490 and 516 nm respectively.

The samples collected with the ISCO portable samplers during TS1 and TS2 were also analysed using a BD Accuri C6® flow cytometer at the Flow Cytometry Facility (the School of Biochemistry and Immunology) at Trinity College Dublin Biomedical Sciences Institute in order to detect yeast tracers. Before the flow cytometry (FCM) analysis of total cell

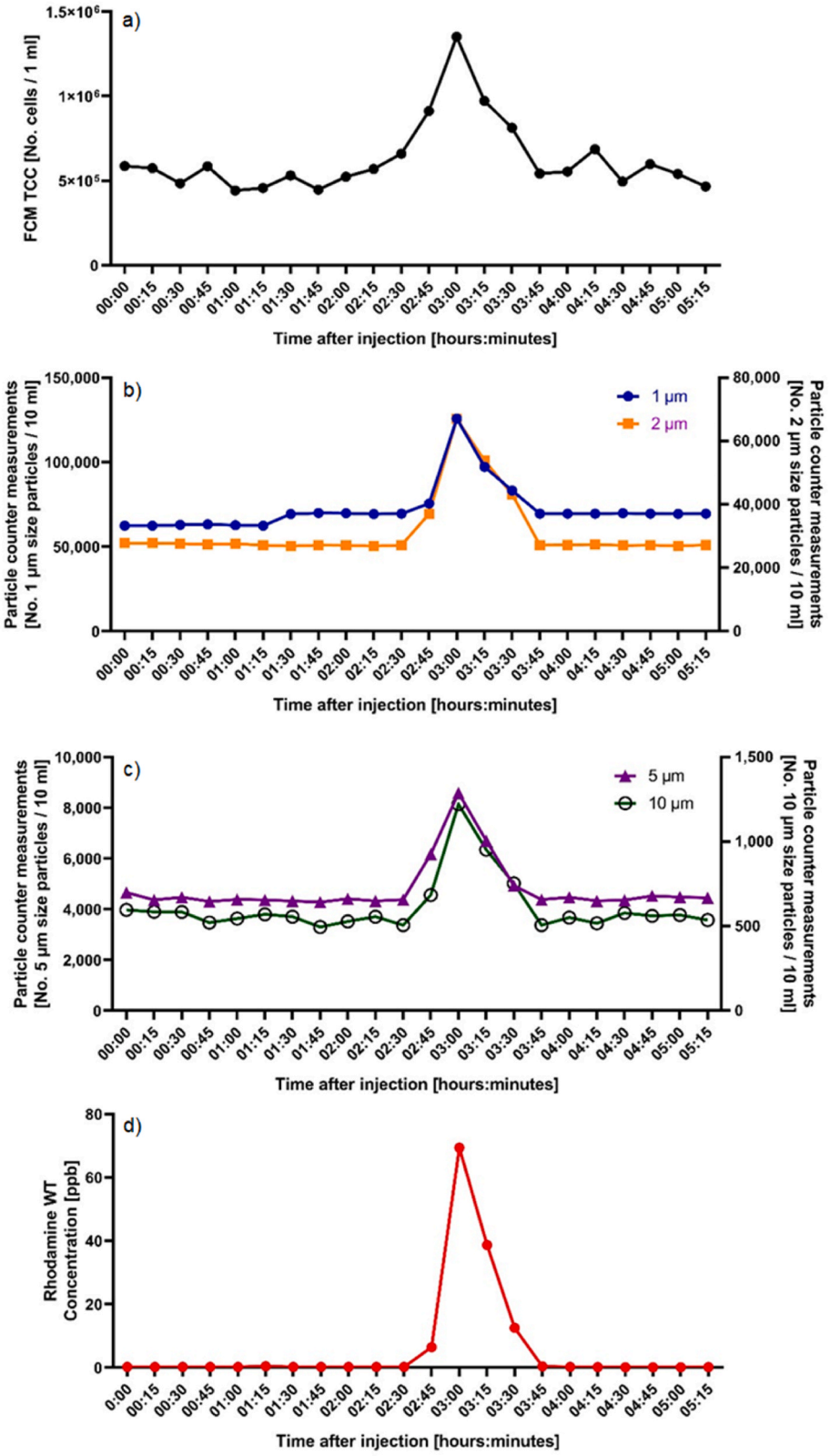

Fig. 2. Results of TS1 artificial tracer test: a) FCM TCC measurements; b) 1 µm and 2 µm particle counter measurements; c) 5 µm and 10 µm particle counter measurements; d) Rhodamine WT dye concentration measurements.

counts (TCC), samples were pre-treated and diluted (at least 1:10 dilution) in physiologic phosphate-buffered saline containing 0.2% Pluronic® F68 and 1 mmol/L EDTA which has been previously passed through a 0.22 μm syringe filter. The Eawag (Swiss Federal Institute of Aquatic Science and Technology) and BD Biosciences staining protocol (see Gatza et al., 2013) was followed for the next step, in order to stain all cells and enable discrimination of cells from debris during the FCM analysis, for each tube with 500 μL of pre-treated sample suspension. All tubes with individual samples were capped, vortexed for 30 s and incubated (37°C) in the dark for 10 min. All FCM measurements were performed with the constant (medium) flow rate of the instrument during data acquisition process in order to achieve comparable data.

Furthermore, the injected yeast particles during TS1 and TS2 were measured continuously on-site using a PCSS fluid lite portable particle counter (Markus Klotz GmbH, Bad Liebenzell, Germany), which counts particles in liquids, and can group particles into 16 size classes. However, for the purpose of this study only four particle-size classes were of interest and, therefore, monitored: 0.9–1.0 μm (hereinafter 1 μm), 1.1–2.0 μm (2 μm), 2.1–5.0 μm (5 μm), and 5.1–10.0 μm (10 μm) since the *S. cerevisiae* yeast cells are spherical to ovoid in shape with diameters generally within that range (predominantly between 1 and 7 μm) (Wood and Ehrlich, 1978; Gerba, 1984; Skilton and Wheeler, 1988; Cronin and Pedley, 2002). Rainfall data were obtained from local Irish Meteorological Service (Met Eireann) stations. Variations in natural background concentrations of fluorescence, particles and microorganisms at the springs were monitored from the beginning of TS1 and TS2 tracing experiments. Sampling and data acquisition intervals were set to 15-min intervals for TS1, while during TS2, two automatic samplers were turned on at the same time to take samples at different intervals (one automatic sampler to take sample every 3 h, and the other every 4 h). The particle counter was set to measure particle concentrations on every hour during the TS2.

3. Results and discussion

3.1. First artificial tracer test

The results of the TS1 tracer test in Fig. 2 clearly show that the yeast cells and rhodamine WT dye were first detected at the monitored Kiltartan spring (KT) at the same time (2 h and 45 min after injection). Both tracers also reached maximum peak concentrations at the same time (3 h after injection) and the last detection of these tracers was also synchronized (3 h and 30 min after injection). Therefore, the particulate yeast and soluble tracer apparently underwent the same transport processes within the turbulent flow conditions of the underground karst conduit. This was expected due to the relatively low injection quantity of tracers and high dilution since this is a large karst aquifer system with very large conduits and high flow velocities (average linear velocity for peak concentration on this occasion being 372 m/h), thus, tracers are likely to travel similarly on this route. It should be noted that the spring was monitored longer than reported in Fig. 2 (for another 13 h), but since no tracer concentrations were detected (that might have been indicative of remobilization or different transport pathways) these were curtailed from the graphs to allow better visualization of the breakthrough curve.

The breakthrough curve from this artificial tracer test has a regular shape with a single peak and a short tail. No precipitation events occurred during this tracer test or for roughly 4 days beforehand (and the test was conducted during low flow conditions), thus, natural background concentrations remained stable. There were no noticeable differences in the shapes of the breakthrough curve for the yeast tracer recorded by FCM or the portable particle counter (which also matched the solute tracer breakthrough curve). In fact, yeast cells across all the monitored sizes (1, 2, 5 and 10 μm) using particle counter followed the breakthrough curve almost identically, as revealed by a correlation matrix heatmap (Fig. 3).

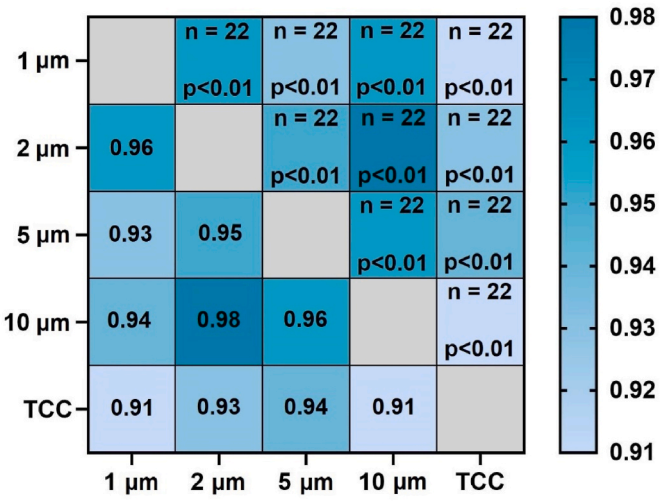

Fig. 3. Correlation matrix heatmap representing the Pearson's correlation (with n and p values) between particulate variables recorded with the portable particle counter (1, 2, 5 and 10 μm) and flow cytometry (TCC) during TS1.

Both FCM and particle counter measurements, therefore, easily detected yeast tracers that appeared in such concentrations that were easy to distinguish from the natural background variations under those flow conditions. For example, at 2 h and 45 min after tracer injection, when tracers were first detected at the spring, an increase of 71.2% (from the average natural background level) in TCC was observed, 153.7% increase in samples taken at 3 h after injection, when the maximum concentration was recorded, and 52.6% increase in TCC concentration at 3 h and 30 min after injection when the last and lowest detection of tracers occurred above background levels during this test. In terms of detection of yeast tracer using the particle counter, increases of 14.7% (1 μm), 35.5% (2 μm), 40.5% (5 μm) and 24.5% (10 μm) from the average natural background of particles were recorded at the time of first detection, 90.9% (1 μm), 144.9% (2 μm), 95.5% (5 μm) and 122.3% (10 μm) increases during the peak concentration, and 26.4% increases during the last and lowest detection.

Fig. 2 indicates that finer particles are more abundant than larger ones as natural background concentrations in this karst aquifer, as reported by other researchers in other karst systems (Pronk et al., 2007) and so, under stable flow conditions (as in this case), the larger particle sizes of tracers (if injected in sufficient quantities) should show a higher percentage increase. The results of TS1 test show that it is possible to detect yeast tracer with FCM TCC and particle counter measurements and clearly distinguish tracer concentrations from the natural background concentrations of other cells/particles during stable hydrological conditions (and associated low variability in the natural background), despite the fact that a relatively small concentration of yeast tracer (12 kg) has been injected into the large and very dynamic karst aquifer system. It should also be noted that, as reported in Section 2.1, another major conduit network connects into this conduit between where the tracers were injected and the Kiltartan spring. Modelling of this karst aquifer suggests that the flows at the swallow hole injection point were approximately 0.7 m^3/s during the time of the tracer study (Morrissey et al., 2020). It is known that flow cytometers can detect cells in extremely high resolution, however, despite that fact, no clear advantages over portable particle counter system can be reported from the results of artificial tracer test TS1 that can justify the additional labour, cost, and time associated with FCM analysis.

3.2. Second artificial tracer test

The TS2 tracer test was also conducted during low flow conditions, and it was started by the end of October when the swallow hole SH2

started receiving more water (in order to make injection of tracers easier). The results of TS2 (Fig. 4), during which a much larger concentration of yeast (75 kg) was injected together with a conservative tracer uranine, revealed significantly different dynamics compared to the first tracer test (TS1) on a different karst system. TS2 yielded multiple peaks and the breakthrough of yeast occurred before the conservative solute tracer. This has been confirmed both on the basis of FCM TCC and particle counter measurements.

It is not unusual to detect multiple peaks of tracers in karst (Goeppert and Goldscheider, 2019). Such breakthrough curves with multiple peaks are usually explained by multiple pathways (Maloszewski et al., 1992; Goeppert and Goldscheider, 2008, 2019), for example, individual flow paths with different flow velocities and dispersivities which converge close to the karst spring (Goeppert and Goldscheider, 2019). However, in this TS2 study, one explanation for the multiple peaks may be rainfall during the experiment and consequent remobilization of temporarily "trapped" tracers in some part of conduit system near the spring (i.e. due to extremely low flow it seems possible that a part of conduit system was acting as an immobile water region from where tracers were remobilized several times as a result of observed rainfall events). However, remobilization of tracers from different parts of the karst aquifer system could also be a likely explanation. As explained by Smart (1988) subsurface storage of tracers that can be remobilized during the rain events can occur in low-velocity zones of a trunk conduit or in fractures, sediment, discrete voids in the rock matrix or the arm of a branching conduit. Such observations during tracer tests have been reported in the past (for example, see Bandy et al., 2019). Another intriguing explanation might be that the karst network acts as a form of siphon at this location, which would cause fairly regular pulses of flow to be discharged at the spring from that part of the network (at approximately 30-h intervals in this case), as has been found in other karst systems (e.g. Bonacci and Bojanic, 1991). Although the spring appeared to be always discharging throughout TS2, measurement of flow was not possible and so potentially the spring receives flows from more than one part of the aquifer. The relatively complicated geology in the area with much lower permeability volcanic rocks underlying the limestone, as well as bands of low permeability clays known to intersect the limestone formations (O'Connell et al., 2022) could provide the hydraulic constraints required to form a siphon en route between the swallow hole and spring.

The differences in shapes of breakthrough curve are largely due to differences in sampling/data acquisition frequencies (which need to be considered when comparing the yeast tracer results obtained from the FCM and particle counter system techniques and solute tracer fluorescence intensity measurements). However, they also reveal some clear differences in terms of transport between the yeast and conservative solute tracer, as well as some differences in transport between yeast cells of different sizes. Very strong positive correlations (Fig. 5) were observed also during TS2 test (although not as strong as those found during TS1) between particles of monitored sizes with the portable particle counter.

The first detection of yeast at the spring during TS2 was 69 h after injection according to the analysis of samples with the FCM and particle counter measurements, but uranine dye was not detected at that time. The next sample for the analysis of fluorescence intensity with a Cary Eclipse Fluorescence Spectrophotometer, however, collected by one of automatic samplers at 72 h after injection, did show the presence of uranine detected for the first time during TS2. Hence, solute breakthrough clearly occurred in between those two sampling times, 69 and 72 h after injection. The breakthrough of particulate and microbial tracers before the solute tracers, as observed during the TS2 tracing test, has commonly been reported in karst aquifer systems (Rossi et al., 1998; Auckenthaler et al., 2002; Goeppert and Goldscheider, 2008; Sinreich et al., 2009; Flynn and Sinreich, 2010; Schiperski et al., 2016). The highest concentration during the first peak was recorded at 76 h after injection for all tracers (corresponding to an average linear velocity of 23.5 m/h). The next sample for uranine and FCM analysis was taken at 78 h after injection and no tracers were detected at the time. The particle counter, however, detected yeast tracer also at 77 h after injection because measurements were taken every hour. The maximum concentration of all tracers during this artificial tracer test was observed during the second peak, 100 h after the injection of tracers (corresponding to an average linear velocity of 17.9 m/h). Uranine dye and yeast particles were detected in samples collected with automatic samplers at 99, 100, and 102 h after injection, while the particle counter also recorded yeast tracer at 101 h after injection. After the third peak, that started at some point between 130 and 131 h after injection and ended between 138 and 139 h after injection of tracers, concentrations of finer cells and particles in groundwater started to rise slowly but continuously until the fourth peak, most likely as a result of precipitation events. The fact that no uranine dye was detected between the third and fourth peak, nor similar rise in numbers of particles of other monitored sizes, may suggest that such rise is due to naturally present microorganisms and particles in the system (that could be potentially connected to the part of conduit network where tracers were not present). The fourth peak was detected between 165 (first detection by particle counter) and 169 h after injection (last detection during this peak with all instruments), followed by the fifth peak (that occurred between 174 and 176 h after injection) when only low concentrations of all tracers were detected. However, because of the rise in the naturally present microorganisms and particles due to rainfall events during the TS2, it's clear that it would be difficult to confirm the fifth peak using only portable particle counter to measure particle concentrations. It seems that the fifth peak was picked up better with the FCM TCC measurements due to the ability of the flow cytometer to measure concentrations of cells more precisely and discriminate microorganisms (including yeast tracer) from debris, despite the fact that natural concentrations gradually changed since the beginning of the TS2 tracing test as a result of precipitation events. However, the conservative solute tracer (uranine) provided the main evidence for this peak, which shows how hydrological conditions may affect these tests and that use of dyes in conjunction with particulate tracers may be needed to ensure that such low concentrations of the microbial or particulate tracers can be distinguished during less stable conditions. Despite the aforementioned variability of the natural background for the duration of TS2, successful discrimination between the natural background cells and particles and the injected yeast tracer has been possible with both techniques for all the other peaks.

4. Conclusions

Overall, these two tracer studies on different karst aquifers have shown how yeast can be used as an effective artificial tracer in conjunction with modern flow cytometry and particle counter technologies. Despite the fact that flow cytometry is a more sensitive technique that can detect cells in extremely high resolution while the portable particle counter cannot differentiate between cells and debris, no significant advantages in favour of flow cytometry can be reported on the basis of the presented results that can justify the additional labour, cost, and time associated with FCM analysis. However, in this study, flow cytometry data were obtained through automated sampling, lab-based sample preparation, and benchtop flow cytometer analysis. Thus, it is important to note that flow cytometers can also be deployed on site using additional automated equipment developed in recent years (see, for example, López-Gálvez et al., 2023). These technological advances, not available at the time when we carried these tracer tests, are making flow cytometry technology increasing attractive for in-situ data acquisition, since they can enhance the resolution of the datasets produced by the technique and reduce labour requirements associated with benchtop analysis. This provides opportunities for potential future studies where novel automated equipment developed specifically for flow cytometry can be further tested versus portable counter technology for conducting tracer tests in karst environments. Both of these technologies should provide a new lease of life for yeast as a karst groundwater tracer into

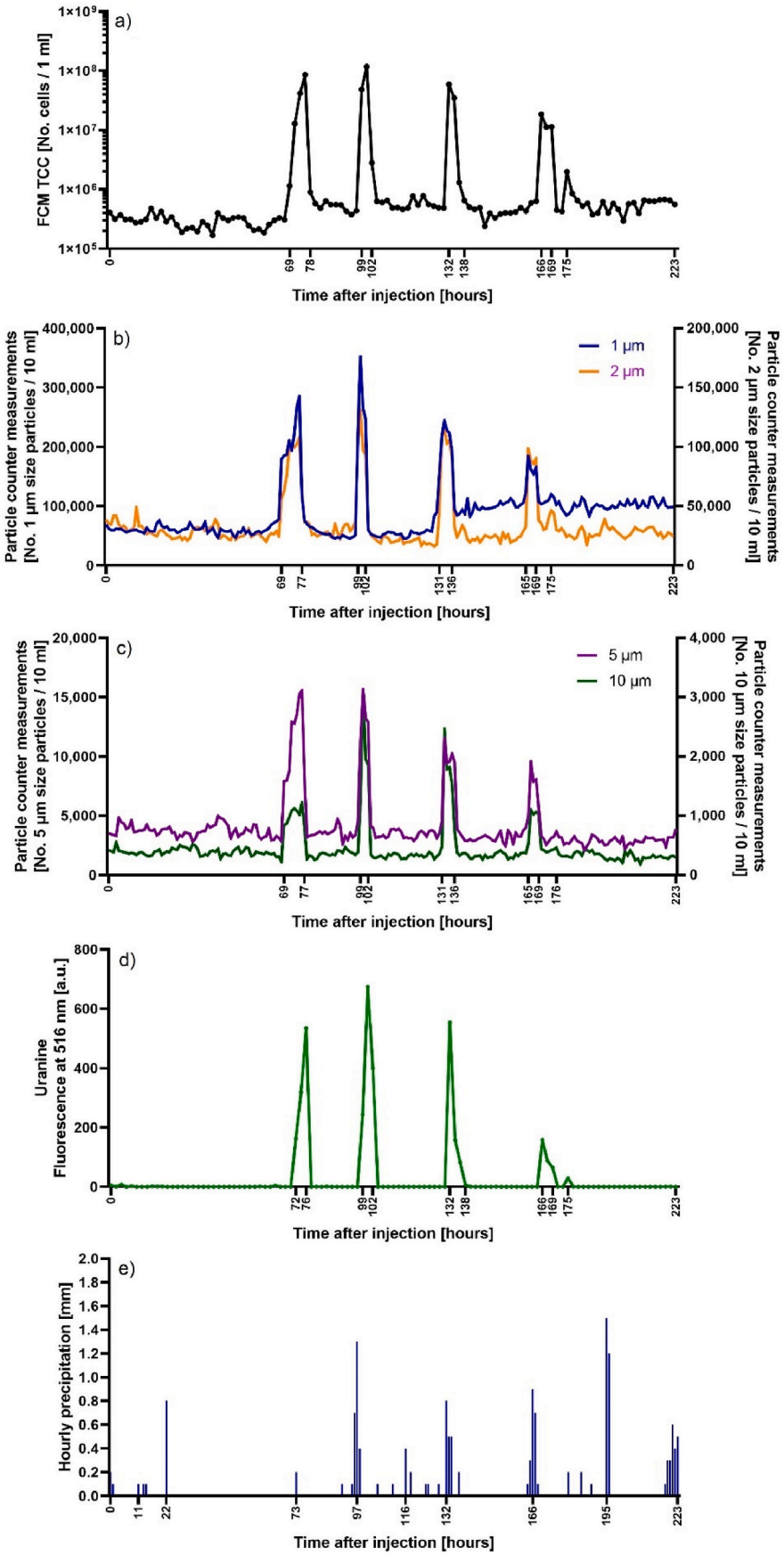

Fig. 4. Results of TS2 artificial tracer test: a) FCM TCC measurements presented using semi-log plot; b) 1 µm and 2 µm particle counter measurements; c) 5 µm and 10 µm particle counter measurements; d) Uranine dye concentration measurements; e) precipitation data.

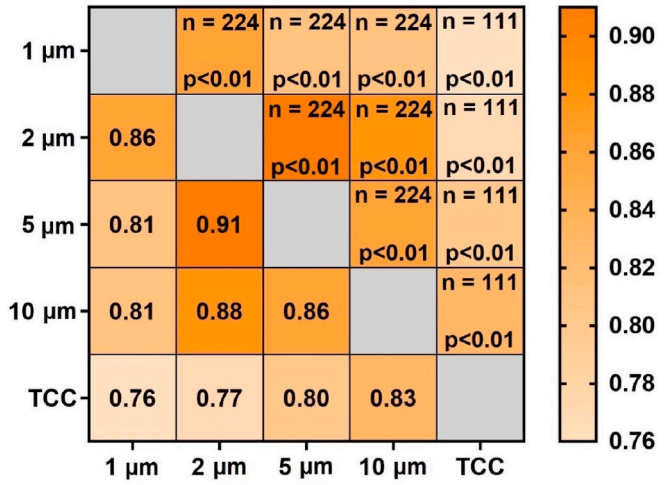

Fig. 5. Correlation matrix heatmap representing the Pearson's correlation (with n and p values) between particulate variables recorded with the portable particle counter (1, 2, 5 and 10 µm) and flow cytometry (TCC) during TS2.

the future, however, it should be noted that in different environmental (e.g. more complex karst systems with longer distances between swallow holes and springs and/or slower conduit flow velocities) and hydrological conditions (e.g. high flow conditions with multiple rainfall events during the tracer test) additional challenges could be expected (e.g. potential degradation of injected yeast tracers, significant changes in background microbial concentrations as a result of rainfall events, and similar).

CRediT authorship contribution statement

Luka Vucinic: Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **David O'Connell:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Catherine Coxon:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Conceptualization. **Laurence Gill:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

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/sbnfyf4kmk.1.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2024.123942.

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