



Toward quantitative DNA tracer tests: development and validation of a novel capture device for groundwater flow characterization in karst and carbonate aquifers

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ABSTRACT

Synthetic DNA tracers represent an innovative solution to overcome the limitations of conventional hydrological tracers, particularly in complex and large-scale hydrogeological systems. However, prolonged tracer tests still rely on manual sampling, which often provides sparse temporal coverage, increasing the risk of missing concentration peaks and biasing breakthrough curve (BTC) interpretation. In addition, repeated site visits entail significant logistical effort and cost. To address these constraints, this study presents the development and validation of a novel passive device that selectively captures biotinylated synthetic DNA tracers, enabling time-integrated, semi-quantitative assessment of groundwater flow and pollutant migration. The device integrates streptavidin-functionalized superparamagnetic beads (1.0 μm ; binding capacity ~ 500 pmol/mg) encapsulated in 0.6% (w/v) agarose hydrogel pearls and housed in a sealed hybridization chamber. Laboratory column tests (1.65×10^{-4} L/s; hydraulic gradient 1.4%) and a field-scale experiment in a natural stream (~ 6 L/s; 200 m reach), used as an analog for fracture flow or conduit flow in karst systems, benchmarked DNA behavior against conventional tracers (KCl, NaCl, fluorescein). Quantification by qPCR showed 100.2% efficiency and $R^2 = 0.997$, with sensitivity < 10 DNA molecules per reaction. BTCs from manual sampling and the passive device closely matched conservative tracer responses. The passive device produced slightly broader BTCs, consistent with time-integrated capture and detection of delayed or retained fractions. This approach provides a reproducible method for tracing hydrological pathways in karst and carbonate aquifers, thereby facilitating broader application of DNA tracers in groundwater studies.

1. Introduction

In the last decades, there has been increasing concern about organic and inorganic pollutants, including pathogenic bacteria or viruses, synthetic hydrocarbons, radionucleotides, pesticides, ions, and even microplastics and perfluorinated alkylated substances (PFAS) (Lapworth et al., 2012; Lee et al., 2024; Singh et al., 2021; Viaroli et al., 2022; Xu et al., 2021), associated to diverse human activities, and worldwide released into the environment, with dramatic consequences on groundwater quality (Li et al., 2021; Schroeder et al., 2021; Srivastav, 2020; Tazioli et al., 2022). Accordingly, hundreds of the recent groundwater investigations have been focused on the occurrence and

migration of various pollutants in groundwater, and more specifically on their origin and release time (Butera et al., 2013; Lapworth et al., 2018; Nisi et al., 2014; Taussi et al., 2022). In this context, hydrological tracers have become undeniable tools to investigate surface and groundwater dynamics, flow paths, residence times, solutes transport, and contaminants migration (Fronzi et al., 2020; Jakóbczyk-Karpierz et al., 2020; Mammoliti et al., 2023; Yang et al., 2001).

Existing tracer methods consist of a range of conventional approaches widely used to investigate hydrological processes such as hydrological pathways, source contributions, and transport dynamics in surface and subsurface systems (Goldscheider et al., 2008; Mammoliti et al., 2022). These approaches include fluorescent dyes and

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conservative salts to assess hydraulic connectivity and flow system dynamics, stable and radioactive isotopes to characterize water mixing, residence times, and recharge processes, and atmospheric tracers such as chlorofluorocarbons (CFCs) and Tritium (^3H) to estimate groundwater age and travel times. Despite their long-standing and successful application, conventional tracers present several limitations, including background concentrations, chemical reactivity or sorption within the subsurface, incomplete recovery, and a limited number of distinguishable tracer formulations, which restrict their ability to resolve multiple, overlapping flow paths in complex hydrogeological settings (Bethke and Johnson, 2008; Benischke et al., 2014; Smart and Laidlaw, 1977).

The use of DNA tracers, synthetic oligonucleotides with unique sequences, has recently emerged as a promising frontier in hydrology and hydrogeology (Foppen, 2023; Zhang and Huang, 2022), enabling investigations at regional and watershed scales and offering new opportunities for addressing some of the most challenging applications in water source tracing, including the modelling of flow paths and the quantification of the relative contributions of multiple pollution sources to water quality at specific locations (Aquilanti et al., 2016; Georgakakos et al., 2019; Liao et al., 2018; Liao et al., 2025).

One of their primary advantages lies in the near absence of natural background interference and the virtually unlimited number of unique sequences that can be synthesized. This allows multiple tracers to be injected simultaneously or sequentially (either at different locations or at the same point at closely spaced time intervals) enabling the discrimination of superimposed paths that are difficult or impossible to resolve using conventional tracers (Pang et al., 2020). Downstream recovery of DNA tracers and their quantification through quantitative polymerase chain reaction (qPCR) provide high analytical sensitivity and specificity, allowing for accurate determination of hydrogeological connections and transport times between injection and sampling points (Botter et al., 2008; Goldscheider et al., 2008). Owing to their theoretically unlimited information capacity, DNA tracers have also been proposed as labels for waterborne contaminants or contaminant-bearing waters, including petroleum-derived compounds, pesticides, herbicides, and wastewater effluents (Sabir et al., 1999).

From a transport perspective, DNA tracers can be conceptualized as particulate markers that behave as effectively insoluble entities in water, making them particularly suitable for qualitative investigations of hydraulic connectivity and flow path identification (Leibundgut et al., 2009).

Moreover, ecotoxicological assessment of silica-encapsulated DNA tracers indicates a low environmental risk under realistic application scenarios. Standardized acute and chronic bioassays showed no acute toxicity to freshwater planktonic crustaceans at concentrations up to ~ 300 ppm (48 h) and no chronic effects on small freshwater zooplankton up to 12 ppm (7 d), nor on primary producers such as green algae up to 20 ppm (72 h), while adverse effects were observed only at substantially higher concentrations attributable to physical particle overloading rather than chemical toxicity (Koch et al., 2021). Owing to their extremely low detection limits, DNA-tagged silica tracers can be applied at minimal mass loadings, resulting in exposure levels well below identified effect thresholds, and can therefore be considered environmentally compatible for large-scale hydrological tracing applications.

However, despite these advantages, current applications of DNA tracers remain largely qualitative. In fact, conventional artificial tracers can be monitored *in situ* either through continuous fluorometric probes, which record tracer concentration as a function of time, or through the deployment of activated carbon traps, which fix the tracer and integrate its concentration over time. Both approaches provide robust breakthrough curves (BTCs) that can be used to derive quantitative or semi-quantitative information on the investigated aquifer. In contrast, DNA tracers can currently only be collected via manual sampling and analyzed *a posteriori* in the laboratory. This obliges the operator to remain on site throughout the entire test, performing manual sampling, and introduces a degree of subjectivity related to the sampling

procedure. Moreover, because the sampling schedule cannot be defined *a priori*, there is a significant risk of missing the tracer arrival peak or the end of the test.

To the best of the authors' knowledge, no passive capture devices able to recover synthetic DNA from water during tracer tests have been developed. Therefore, at present, a promising step toward the application of DNA tracers in hydrogeology for obtaining quantitative information on investigated aquifers, and for improving operational procedures during tracer tests, is represented by the possibility of immobilizing synthetic DNA molecules within specially designed capture devices. This approach leverages the principle used for conducting semi-quantitative tests with conventional tracers, for example, the use of activated charcoals to fix fluorometric tracers, which are later extracted in the laboratory and analyzed through analytical techniques (Fronzi et al., 2021).

For these purposes, this research focuses on the development and testing of a simple device for capturing DNA tracers, designed for application in karst and carbonate hydrogeological contexts. The development follows a multidisciplinary approach, carried out in several phases: i) design and preparation of the device for capturing biotinylated DNA tracers, ii) design and execution of a laboratory-scale tracer test, iii) design and execution of a watershed-scale tracer test, iv) refinement of the DNA extraction technique from the newly developed capturing devices and quantification of the eluted DNA using qPCR, and v) benchmarking the obtained BTCs with traditional hydrological tracers.

This study represents a significant advancement in the potential application of DNA tracers in hydrogeology, either as a complement or an alternative to traditional tracing methods.

2. Materials and methods

2.1. Synthetic biotinylated DNA tracer

A 72-nucleotide single-stranded DNA (ssDNA) molecule (5'-ATC ACA TTC GAG GTG TCC ACT AGA TCC CGC GTT TTG TAC CAT CAG TCA TTG CGC AGG TCG GTT GGT GGT AAG-3') (Aquilanti et al., 2013), biotinylated at the 5' end, was used as a biotracer. It was synthesized, purified via High-Performance Liquid Chromatography (HPLC), and lyophilized by [biomers.net](https://www.biomers.net) GmbH (Ulm, Germany), with a yield of 36.9 nmol (836.2 μg). The lyophilized biotinylated DNA tracer was resuspended in 1 mL of molecular biology grade water (MBGW) (Chem-Lab nV, Zedelgem, Belgium), achieving a final concentration of 2.2×10^{16} DNA molecules/mL. The solution was stored at -20°C until use.

2.2. Preparation of the device for capturing biotinylated DNA tracer

The device used to capture biotinylated DNA tracers, schematically represented in Fig. 1, comprised a hybridization chamber containing commercially available superparamagnetic beads with a streptavidin monolayer covalently bound to the hydrophilic bead surface (Dynabeads™ MyOne™ Streptavidin C1, Thermo Fisher Scientific Inc., Milan, Italy), encapsulated within agarose hydrogel pearls permeable to water and the target DNA tracer molecule. The chamber was sealed at both ends with external macro-porous filters, such as mosquito netting with a pore size of 0.6–1 mm, to retain the encapsulated superparamagnetic beads while preventing the entry of debris or other obstructions, such as particulate matter or plant material.

According to the manufacturer, Dynabeads™ MyOne™ Streptavidin C1 contains 10 mg/mL of superparamagnetic beads (diameter of 1.0 μm), corresponding to approximately $7\text{--}10 \times 10^9$ beads/mL. The beads have a ssDNA binding capacity of approximately 500 pmol/mg.

The steps for preparing the device used to capture biotinylated DNA tracer are illustrated in Fig. 2. To prepare the superparamagnetic beads encapsulated in agarose hydrogel pearls, an aliquot of Dynabeads™ MyOne™ Streptavidin C1, selected based on the number of the capturing devices planned for the experiment, was resuspended and

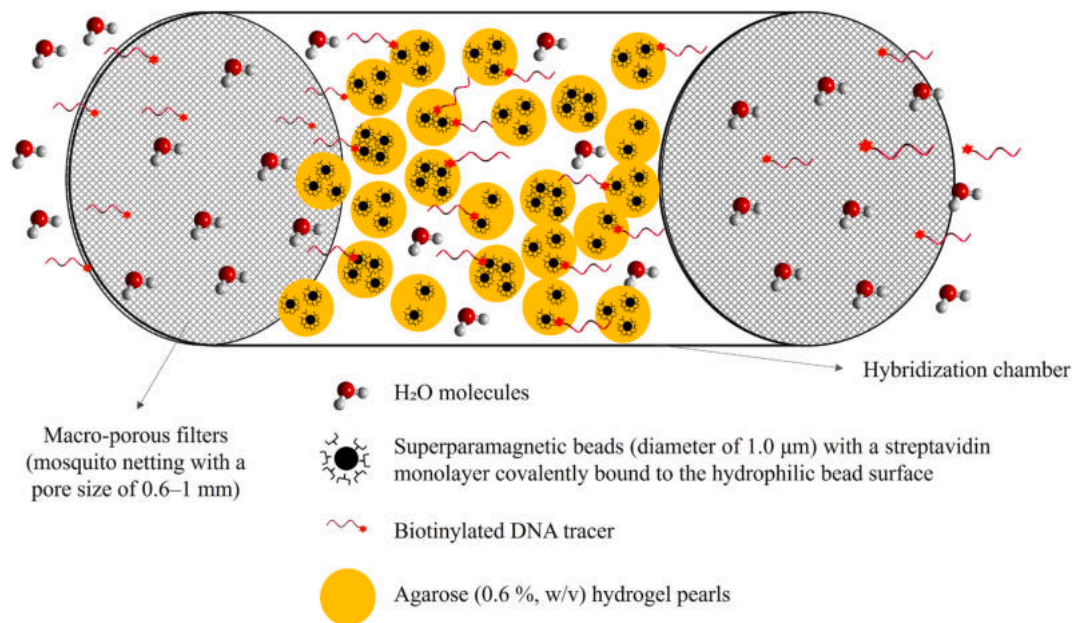


Fig. 1. Schematic representation of the device used for capturing biotinylated DNA tracer.

washed in 1X Binding and Washing (B&W) buffer (5 mM Tris-HCl, pH 7.5; 0.5 mM EDTA; 1 M NaCl) according to the manufacturer's protocol. The washed Dynabeads™ were then transferred to a dry bath (HB 120-C, Argolab, Carpi, Italy) and maintained at a constant temperature of 70 °C. In parallel, a 0.6% (w/v) agarose solution was prepared by dissolving agarose (SeaKem® LE Agarose, BMA, Rockland, ME, USA) in 1X B&W buffer and heating the solution in a microwave until fully dissolved. The freshly prepared hot agarose solution was added to the washed superparamagnetic beads at a 4:1 ratio. The mixture was vortexed to ensure even bead distribution and kept at 70 °C to prevent premature solidification (Fig. 2a). Using a micropipette, small agarose hydrogel pearls (5 μ L volume) containing Dynabeads™ were formed by dispensing droplets into chilled vegetable oil (4 °C), which immediately solidified upon contact with the cold oil. The pearls were then sequentially washed with 70% (v/v) ethanol and deionized water to remove any residual oil (Fig. 2b). Approximately 20 pearls were inserted into the hybridization chamber of each capture device, which was sealed at both ends with external filters. The prepared devices were submerged in water until used to prevent dehydration (Fig. 2c).

2.3. Column test and field investigations

The testing of the DNA-based tracer and the device for capturing biotinylated DNA tracer in the hydrogeological context was carried out at two spatial scales: a laboratory scale using column filtration tests, and at a single karst conduit scale through a field test in a small stream. The stream test was conducted within a carbonate geological context, representative of the Umbria–Marche Geological Succession (Mussi et al., 2017), whose hydrochemical facies are typical of fractured and karstified carbonate environments (Chemeri et al., 2025).

2.3.1. Column test

The column test was designed to simulate the transport of solutes (i. e., tracers) through saturated porous media under controlled laboratory conditions. Each test involved the permeation of a vertically oriented column, filled with compacted homogenous soil (e.g. medium sand), and housed within a rigid cylindrical tube made of Plexiglas (Tazioli and Palpacelli, 2013). The calcium carbonate composition of the filling material was deliberately chosen to replicate the hydrochemical conditions typical of carbonate environments, where, besides

interconnected fracture networks, well-developed karst conduits may occur. This choice also allowed for an experimental assessment of interactions between synthetic DNA tracers and a solid matrix representative of such settings. The column was weighed under unsaturated conditions and then was progressively saturated with water until the full saturation was achieved and weighed to obtain the pore volume value for the filling material (Table 1).

Once determined, the column was connected to a water reservoir positioned at a fixed elevation relative to the column by means of a PVC pipe. The use of a gravity-fed system, rather than a mechanically driven pump (e.g., peristaltic pump), was a deliberate choice aimed at more accurately replicating the hydraulic conditions typically encountered in natural aquifers. Moreover, the direction of flow was oriented upward (i. e., from the base to the top of the column) in order to prevent the entrapment of air bubbles, which could otherwise introduce artifacts into the flow regime and compromise the accuracy of the BTCs. Following saturation, the tracer experiment started with the instantaneous injection of a tracer solution at the inlet (bottom) of the column. The BTC was subsequently monitored by measuring its concentration in the effluent at the outlet of the column over time (Fig. 3). The injection methodology employed in the laboratory was purposely aligned with standard field-scale tracer test protocols to ensure methodological consistency and to facilitate the extrapolation of results (Fig. 4 a). The first test was conducted by injecting 40 mL of KCl 0.01% mass/volume ratio (instant method), and the BTC (expressed as a function of electric conductivity recorded every 2 s) at the column outlet was monitored thorough a Diver CTD Eijkelkamp (accuracy $\pm 1\%$ of electric conductivity measure, and resolution $\pm 0.1\%$ of electric conductivity measure). Then the mean electric conductivity values were computed for 1, 2, 3, 4, 4.5, 5, 5.5, 6, 7, and 8 pore volumes. The second test involved injecting 5 μ L of biotinylated DNA tracer suspended in 1 mL of MBGW through an instant release mechanism (Fig. 4 b). This amount corresponds to approximately 1.1×10^{14} DNA molecules. In this test the DNA capture devices positioned at the outlet of the column (see Fig. 3), as well as the water samples, were collected concomitantly at 1, 3, 4, 5, 6, 7, and 8 pore volumes to ensure BTC overlap between the performed tests. Following this experimental setting, the contact time for each trap spans from 1 to 2 pore volumes, while the water aliquot is directly sampled from the sample collection tank at the end of 1 or 2 pore volumes flow in the column. The flow conditions settled during the test of about 1.65 x

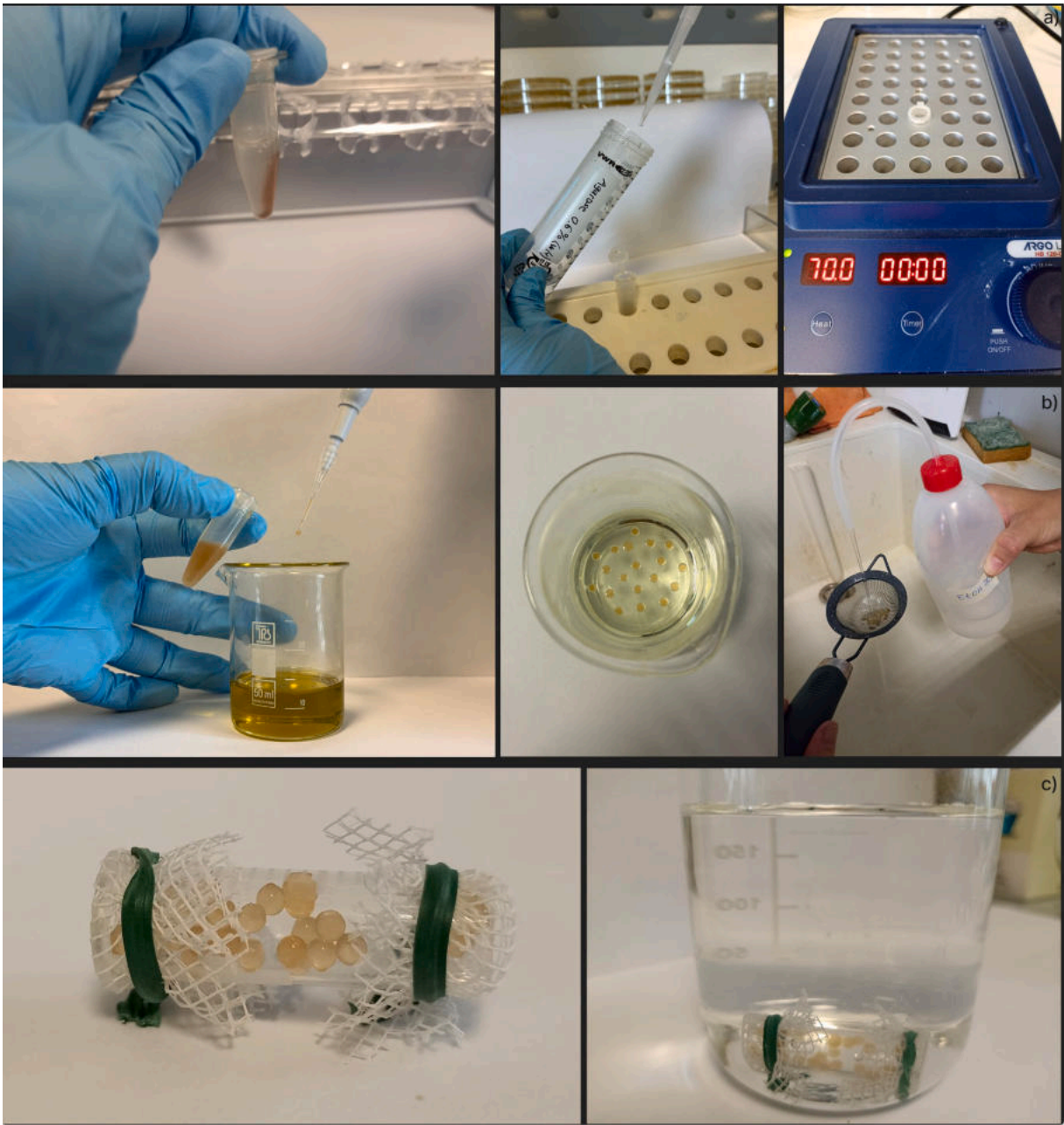


Fig. 2. Preparation of the device for capturing biotinylated DNA tracer: a) Washing of Dynabeads™ MyOne™ Streptavidin C1 using a magnetic stand and mixing with agarose solution (0.6%, w/v); b) Preparation of agarose hydrogel pearls containing Dynabeads™ by dispensing droplets into chilled vegetable oil, followed by washing in 70% (v/v) ethanol; c) Assembled capturing device composed of a hybridization chamber containing superparamagnetic Dynabeads™ encapsulated within agarose hydrogel pearls, sealed at both ends with external macro-porous filters (mosquito netting).

Table 1
Column characteristics used in the laboratory test.

Height	46 cm
External diameter	6 cm
Internal diameter	4.8 cm
Internal volume	832.40 cm ³
Geochemical nature of filling material	Calcium-carbonate
Granulometry of filling material	Medium sand
Soil bulk density (dry)	2.335 g/cm ³
Pore volume	514.9 cm ³
Porosity (φ)	61.86%

10⁻⁴ L/s, under an imposed hydraulic gradient of 1.4%, ensured a contact time between the capturing device and the water of about 55–60

min.

2.3.2. Field test

The field test was conducted *in situ* on a stream located in central Italy, whose geological and hydrogeological characteristics are detailed in Chemeri et al. 2025, Fronzi et al. 2022, and Fronzi et al. 2024. The hydrogeological and hydrochemical setting provides a relevant analog for a carbonate aquifer, allowing the simulation of flow conditions comparable to those occurring within a single conduit in karst environments, yet under semi-controlled field conditions, exploiting the stream water as a vector for tracers and controlling the experimental conditions in surface water. This experiment therefore represents an intermediate step between laboratory-scale assessments and fully natural aquifer-scale applications. The tracer tests involved the

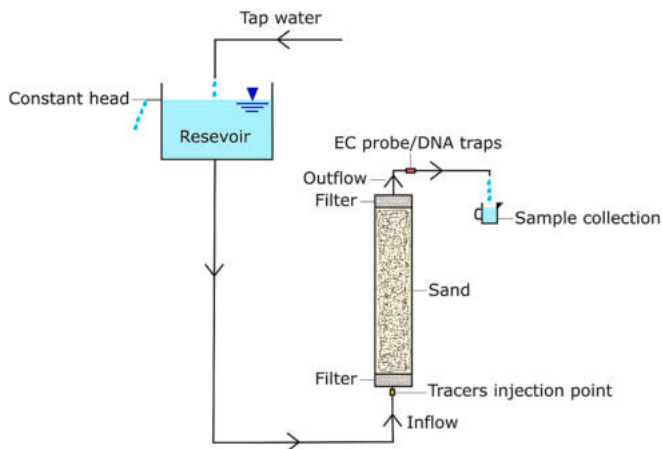


Fig. 3. Schematic layout of column test modified from Tazioli and Pacelli, 2013.

instantaneous injection of a saline tracer (NaCl), a fluorometric tracer (Fluorescein), and synthetic biotinylated DNA tracer. All three tests were carried out on the morning of October 25, 2024, under consistent stream flow conditions (~ 6 L/s) between 9:00 AM and 12:45 PM. Specifically, the first test was performed by injecting 150 g of NaCl (previously dried in an oven at 105°C). The BTC was recorded approximately 200 m downstream from the tracer injection point. Measurements were taken using a Diver CTD Eijkelpamp (accuracy $\pm 1\%$ of electric conductivity measure, and resolution $\pm 0.1\%$ of electric conductivity measure), recording every 1 min, which had been pre-calibrated to convert electrical conductivity into NaCl concentration expressed in g/L. Calibration involved reading three known concentrations: 0 g/L of NaCl (corresponding to site blank – stream water), 1 g/L (prepared by dissolving 1 g of NaCl in 1 L of stream water), and 3 g/L (prepared by dissolving 3 g of NaCl in 1 L of stream water). The same calibration procedure was applied to a manually operated WTW-type conductivity probe (accuracy $0.001\ \mu\text{S}/\text{cm}$ and resolution $\pm 0.5\%$ of electric conductivity measure), used as real-time benchmark to observe the salt tracer transit.

The second test involved injecting 0.5 g of Fluorescein (Fig. 4 c) at the same injection point used for the NaCl test. The downstream passage of the fluorometric tracer (at the same monitoring point as the previous test) was tracked using a PME fluorometric probe (Cyclops-7 Logger detection limit $0.01\ \mu\text{g}/\text{L}$) which had been previously calibrated with a standard solution of $0\ \mu\text{g}/\text{L}$ (stream water) and $100\ \mu\text{g}/\text{L}$. Also, in this case the probe recorded Fluorescein concentration data every minute. The last *in situ* test involved the use of synthetic biotinylated DNA tracer, with the injection of $100\ \mu\text{L}$ of the tracer suspended in 2 mL of MBGW (Fig. 4 d) at the same injection point used in the previous tests yielding an injected mass equivalent to roughly 2.2×10^{15} DNA molecules. Approximately 200 m downstream from the injection point, both water samples and DNA capturing devices were collected. Each device was left in place for a contact time of 5 min before being replaced. The first capturing device was deployed 5 min before the DNA release into the stream, and the first water sample was also collected prior to the injection to determine the site blank.

2.3.3. Extraction of the biotinylated DNA tracer from the capturing devices

The collected water samples and DNA capturing devices, used in both the column and field tests, were transported to the laboratory and processed immediately. Upon arrival, the devices were opened by removing the external filters, and the agarose pearls were transferred into sterile, RNase- and DNase-free 2 mL polypropylene microtubes. To dissolve the agarose matrix and release the superparamagnetic Dynabeads™, $800\ \mu\text{L}$ of 1X B&W buffer was added to each tube, and the samples were heated in a dry bath at 100°C until the agarose pearls were

completely dissolved. Following dissolution, the tubes were placed on a magnetic stand (DynaMag™-2, Life Technologies AS, Oslo, Norway) for 3 min to allow bead separation. The beads were washed three times with 1X B&W buffer, with magnetic separation for 2 min between each wash to ensure thorough removal of residual agarose. After the final wash, the tubes were kept on the magnetic stand, and the remaining 1X B&W buffer was completely removed. The tubes were then taken off the magnetic stand, and the Dynabeads™ were resuspended in $100\ \mu\text{L}$ of MBGW. The samples were incubated overnight at 4°C to allow the elution of the biotinylated DNA tracer, which was initially bound to the Dynabeads™. Following incubation, the tubes were placed back on the magnetic stand to separate the Dynabeads™ from the eluate, which was carefully transferred to clean, sterile 1.5 mL RNase- and DNase-free polypropylene microtubes, ready for downstream analysis by qPCR.

2.4. qPCR for biotinylated DNA tracer detection

qPCR was used to detect and quantify the biotinylated DNA tracer captured by the devices and directly from the water samples. A standard curve was generated using tenfold serial dilutions of the biotinylated DNA tracer with a CFX Connect Real-Time System (Bio-Rad, Hercules, CA, USA), which automatically calculated the qPCR amplification efficiency (E) and the correlation coefficient (R^2) from the slope of the standard curve. The standard curve covered a range from 0.94 to $7.94\ \log(8.8\ \text{to}\ 8.8 \times 10^7)$ biotinylated DNA molecules per qPCR reaction, enabling the estimation of the qPCR detection limit. The qPCR mixture contained $4\ \mu\text{L}$ of biotinylated DNA tracer, $5\ \mu\text{L}$ of Type-it 2X HRM PCR Master Mix (Qiagen, Hilden, Germany), and the qPCR primers R1C (5'-ATCACATTCGAGGTGTCCAC-3') and L1C (5'-CTTACCACCAACCGACCT-3') (Aquilanti et al., 2013) at a concentration of $200\ \text{nM}$, with MBGW added to reach a final reaction volume of $10\ \mu\text{L}$. The qPCR thermal cycling conditions included an initial denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 10 s, primer annealing at 53°C for 15 s, and extension at 72°C for 30 s. Plate reads were performed at the end of each extension step. To confirm product specificity, a melt curve analysis was conducted, with the temperature gradually increasing from 60°C to 95°C at a rate of $0.5^\circ\text{C}/\text{s}$.

Absolute quantification of the biotinylated DNA tracer in the water samples or captured from the water by the devices was achieved by running the samples alongside a tenfold serial dilution of the biotinylated DNA tracer. Negative controls, consisting of samples prepared with MBGW instead of template DNA were incorporated in each run. The PCR experiments were carried out in triplicate and the number of DNA molecules per reaction was calculated automatically from the slopes of the standard curves. The results were expressed as the average value calculated from three technical replicates $\pm$ standard deviation.

3. Results

3.1. qPCR assay

The qPCR standard curve was generated using tenfold serial dilutions of the biotinylated DNA tracer, achieving an amplification efficiency of 100.2% with an R^2 value of 0.997 . This indicates highly consistent and reliable amplification across the dilution range, spanning from 0.94 to $7.94\ \log(8.8\ \text{to}\ 8.8 \times 10^7)$ biotinylated DNA molecules per reaction (Fig. 5a). The melting curve analysis revealed a single, sharp peak with a melting temperature (T_m) of 80°C across all dilution points (Fig. 5b). Negative controls showed no amplification signals above the established detection threshold.

3.2. Btcs benchmark between biotinylated DNA tracer and traditional hydrologic tracers

3.2.1. Column test

BTCs obtained for the column tests are reported in Fig. 6, both for

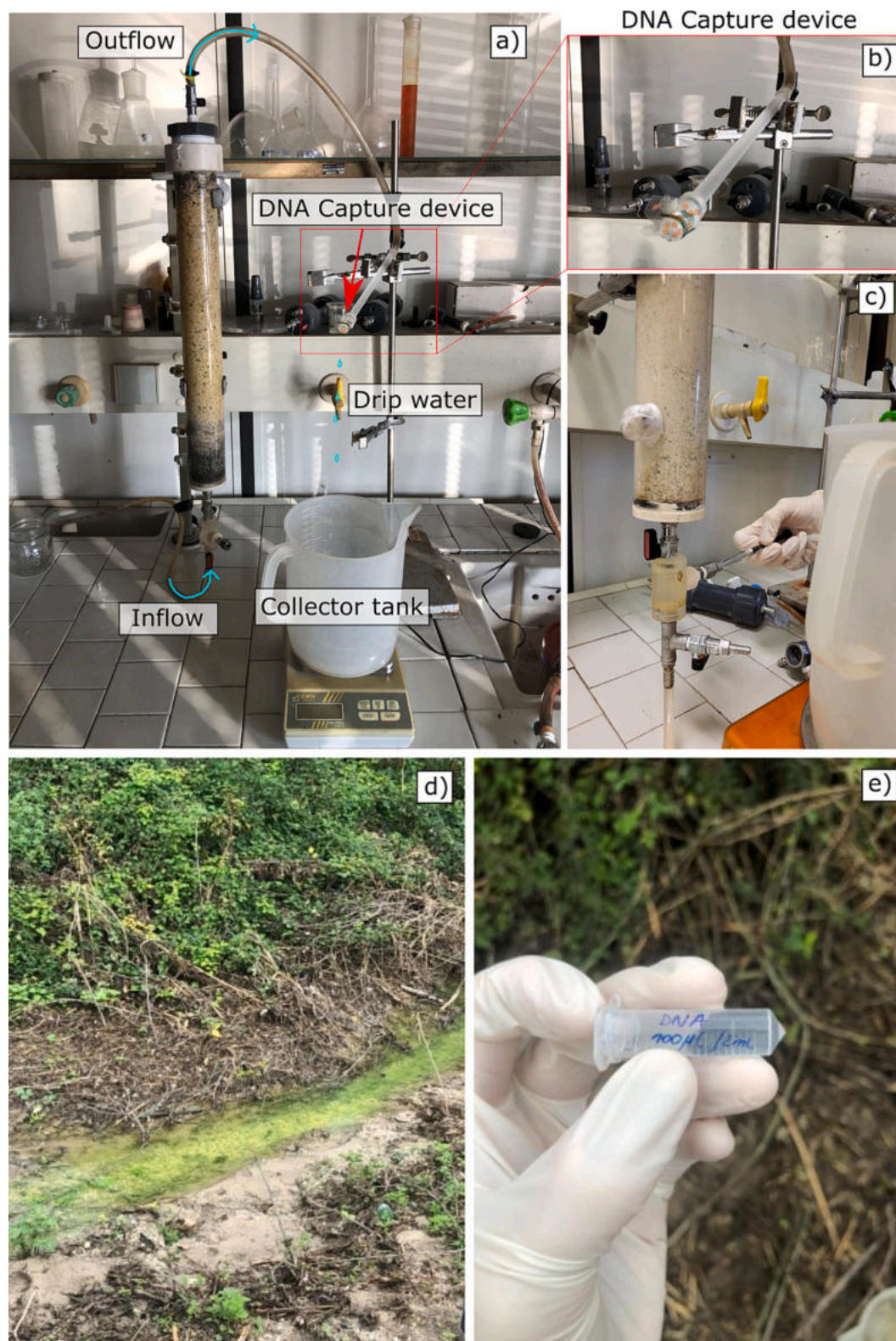


Fig. 4. Performance and assessment of hydrological tracer tests at the laboratory scale (a), with magnification of DNA capture device in line with the column outflow, injection of biotinylated DNA tracer during the column test (c); dye tracer test using Na-fluorescein performed at the catchment scale (d), and injection of biotinylated DNA tracer in the same stream (e).

electric conductivity and synthetic DNA. In both panels, electric conductivity (mS/cm) measured for the KCl injection thanks to the Diver CTD is plotted against pore volume and compared to DNA concentrations obtained through two different sampling methods: DNA capturing

devices (Fig. 6 a) and manual water sampling (Fig. 6 b).

In Fig. 6 a, the BTC for the KCl expressed as electric conductivity (yellow line) exhibits a sharp increase beginning between 1 and 2 pore volumes, peaking at ~ 3 pore volumes with a maximum conductivity of

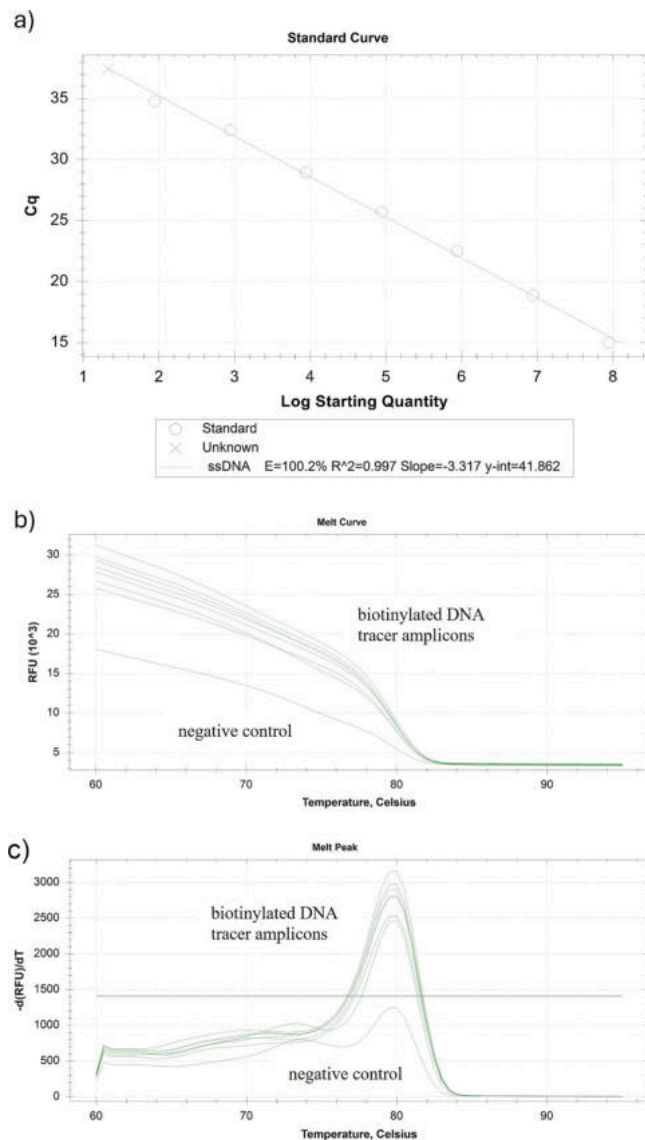


Fig. 5. qPCR standard parameters: a) Standard curve obtained by plotting the threshold cycle (Ct) values of amplification curves from tenfold serial dilutions of biotinylated DNA tracer; b) Melting curve analysis of qPCR amplification products, including the negative control, showing the specificity of the amplified products.

~ 0.64 mS/cm, gradually declining to baseline values around 8 pore volumes. The data from the DNA capturing devices (blue line) follow a similar trend, with the number of biotinylated DNA molecules per device also peaking at around 3 pore volumes, reaching approximately 2.1×10^6 molecules per capturing device. The curve corresponding to the DNA capturing devices is broader and less steep on the descending limb compared to the electric conductivity curve, suggesting a minimal, but not significant delayed release or retention effect. Indeed, the return to the baseline can be observed at about 7 pore volumes.

Fig. 6 b shows a comparison between electric conductivity and DNA concentrations obtained via manual water sampling. The electric conductivity curve (same of Fig. 6 a) again peaks at ~ 3 pore volumes, aligning closely with the peak of the DNA manual sample curve (purple line), which also shows a sharp increase and peak at approximately the same pore volume (~ 0.64 mS/cm and $\sim 3.87 \times 10^9$ molecules/mL, respectively). Unlike the DNA trap data in Fig. 6 a, the DNA signal in manual samples drops off slightly more steeply after the peak but returns to near baseline levels as well by 7 pore volumes.

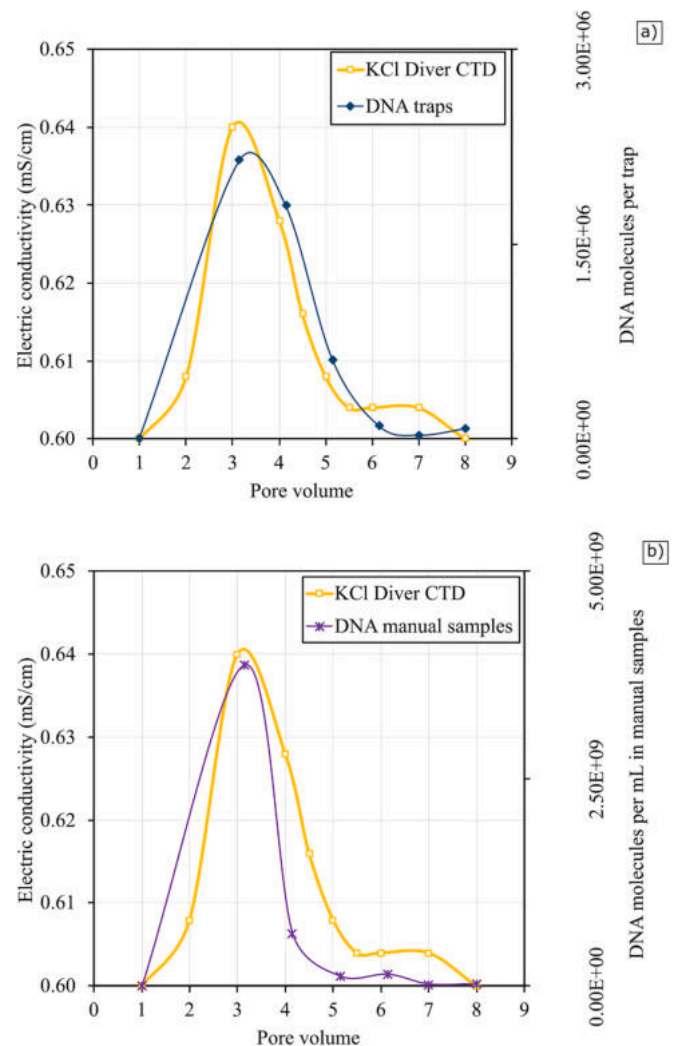


Fig. 6. Tracer's breakthrough curves for column test. a) Electric conductivity (mS/cm) for salt tracer (KCl) vs DNA molecules per trap, and b) Electric conductivity (mS/cm) for salt tracer (KCl) vs DNA molecules per mL in manual samples.

3.2.2. Field test

BTCs for conventional tracers (NaCl and Fluorescein) and DNA are plotted to evaluate transport dynamics in the field test (Fig. 7). The upper panel (Fig. 7 a) presents the concentration profiles of conservative tracers (i.e., Fluorescein, and NaCl – measured by both a WTW-type conductivity probe and a Diver CTD), while the lower panel (Fig. 7 b) displays the DNA concentrations measured via manual sampling and DNA capture devices. In the upper panel, Fluorescein and NaCl concentrations exhibited nearly identical breakthrough patterns. All three tracers showed a rapid increase in concentration starting around 8 min post-injection, reaching their peak concentrations between 13 and 14 min. The fluorescein tracer peaked at approximately $160 \mu\text{g/L}$, while NaCl concentrations, as detected by the WTW-type conductivity probe and the Diver CTD, peaked at ~ 0.056 g/L. The descending limbs of all tracer curves followed an exponential decay, with concentration returning to baseline by approximately 50 min from the tracers' release. The close alignment of the NaCl and Fluorescein curves suggests consistent transport behavior for conservative tracers in the system. In the lower panel, DNA concentrations exhibited similar transport dynamics. DNA concentrations obtained through manual sampling (purple line) rose sharply, peaking around 15 min at $\sim 1.5 \times 10^8$ molecules/mL, followed by a rapid decline to near-background levels by 30 min. In contrast, the DNA trap signal (blue line) displayed a broader and

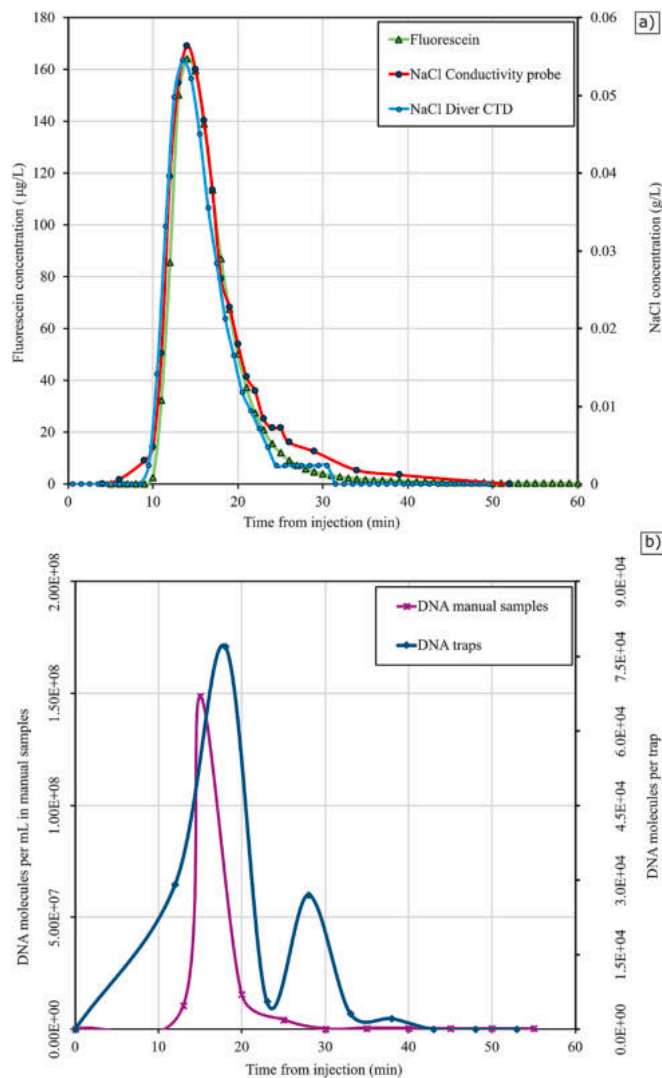


Fig. 7. Tracer's breakthrough curves for field test. a) tracer concentration (g/L) for NaCl measured through Diver CTD and NaCl Conductivity probe, and Fluorescein concentration (µg/L), vs b) DNA molecules per mL in manual samples and DNA molecules per trap.

bimodal distribution, with an initial peak at ~ 18 min reaching $\sim 7.8 \times 10^4$ molecules/trap and a secondary peak ($\sim 2.7 \times 10^4$) after 28 min from the injection. This delayed and more prolonged signal suggests that DNA trap collection integrates DNA over time, potentially capturing both free-floating and retained particles that are missed in instantaneous manual samples.

4. Discussion

This study introduces and validates a novel passive device designed for the selective capture of biotinylated synthetic DNA tracers, aimed at enabling semi-quantitative assessment of groundwater flow and pollutant migration. The device integrates streptavidin-functionalized superparamagnetic beads within an agarose hydrogel matrix (pearls), combining the high specificity of molecular streptavidin–biotin interaction with physical stability and practical field applicability. Streptavidin, a tetrameric protein derived from *Streptomyces avidinii*, binds biotin (vitamin B7) with extraordinary affinity, characterized by a dissociation constant (Kd) in the range of $\sim 10^{-15}$ M. This makes the streptavidin–biotin interaction one of the strongest known non-covalent biological bindings (Dundas et al., 2013; Green, 1975; Weber et al.,

1989). Moreover, this interaction is also extremely rapid and nearly irreversible under physiological conditions. Association rates have been reported in the range of 10^6 – 10^7 (L/mol)/s, allowing effective binding within seconds or even faster, especially when one partner, such as streptavidin, is immobilized on a solid support (Green, 1990). This interaction, widely employed in molecular biology, forms the chemical basis of the capture mechanism employed in our device.

The incorporation of streptavidin-coated superparamagnetic beads into the agarose hydrogel pearls, placed into a hybridization chamber sealed at both ends with external macro-porous filters, allowed for efficient and highly selective capture of the biotinylated DNA tracers under both laboratory and field-scale conditions. The agarose hydrogel served as a biocompatible and porous support that permitted the capture of the biotinylated DNA targets previously inoculated into an artificial/natural water flow, while the hybridization chamber retained the beads in place, ensuring stability and consistent exposure over time. The use of agarose hydrogel pearls as a matrix for encapsulating streptavidin-coated magnetic beads represents a key innovation in the design of the DNA tracer capture device. Agarose is a highly biocompatible, inert, and hydrophilic polysaccharide that forms a stable three-dimensional gel network when cooled below its gelling temperature (typically ~ 35 – 40 °C) (Narayanan et al., 2016). The semi-permeable nature of agarose enables the passage of target DNA molecules toward the immobilized streptavidin beads while effectively retaining the beads themselves and excluding larger particulates or debris present in environmental waters. Additionally, agarose provides an immobilization environment that does not interfere with the streptavidin–biotin interaction, which is known to be extremely rapid and stable (Green, 1990; Wong et al., 2017). Its thermal reversibility and non-toxicity make agarose ideal for field-deployable systems, allowing for simple, scalable production of uniformly sized pearls by thermal gelation in oil (Sahoo et al., 2016). In this study, the pearls demonstrated excellent mechanical stability, maintained permeability during laboratory and field experiments, and were easily dissolved post-recovery to release bound DNA for downstream qPCR analysis. These practical and functional advantages, such as ease of preparation, chemical inertness, and compatibility with molecular biology workflows, make agarose hydrogel an ideal medium for passive environmental DNA capture systems.

qPCR was employed to detect and quantify synthetic biotinylated DNA tracers both directly from water samples and the eluates obtained from the capture devices. The standard curve, generated using tenfold serial dilutions of the tracer, demonstrated excellent amplification efficiency and a high R^2 , indicating consistent and reliable amplification across a wide concentration range (8.8 to 8.8×10^7 biotinylated DNA molecules per reaction). The assay achieved a detection limit of less than 10 DNA molecules per reaction and maintained linearity across eight orders of magnitude, performance metrics that are consistent with or surpass those reported in previous studies (Aquilanti et al., 2013; Foppen et al., 2011). Melting curve analysis confirmed the specificity of the amplification, revealing a single sharp peak across all dilution points and indicating the absence of non-specific products or primer-dimer formation. Additionally, negative controls consistently showed no detectable amplification above the threshold, further confirming the specificity and robustness of the assay. These findings validate the qPCR method as a highly sensitive, precise, and reliable tool for the quantitative detection of biotinylated DNA tracers in environmental matrices.

The high specificity and sensitivity of the streptavidin–biotin-based system enabled us to create BTCs that were consistent with those of conventional tracers, both at laboratory scale using column filtration tests and in a field test in a small stream. In the laboratory-scale test, both the DNA capturing device and manual sampling methods produced BTCs that closely correspond with the conservative KCl tracer. The BTC derived from the DNA capture device was slightly broader, with a less steep descending limb compared to the KCl-based electrical conductivity curve. This reflects the time-integrative nature of the passive samplers, which continuously accumulated tracer molecules during deployment,

thereby providing a more representative measure of total tracer passage over the sampling interval. Accumulation occurs through diffusion of biotinylated ssDNA into the agarose matrix followed by high-affinity binding to streptavidin-coated magnetic beads, resulting in progressive retention of tracer molecules without relying on nonspecific adsorption. Because only a fraction of the flowing DNA interacts with accessible binding sites, the device integrates tracer passage over time rather than capturing instantaneous concentrations. In contrast, the DNA signal from manual water sampling declined more sharply after the peak, indicating lower dispersion and capturing a more instantaneous record of tracer concentration. These observations are consistent with the findings of Aquilanti et al. (2013), who reported that synthetic DNA tracers exhibit transport behavior like conservative tracers but with reduced dispersion. Similarly, Foppen et al. (2013) found that synthetic DNA injection BTCs closely matched those of conservative salt tracers and were recovered with high fidelity. However, despite the promising transport behavior observed in these studies, the application of synthetic DNA tracers remains operationally constrained by the sampling strategies currently available. In both laboratory and field experiments, the authors quantified DNA exclusively through discrete water sampling followed by a posteriori laboratory analysis, requiring either intensive manual sampling or the use of automatic samplers with limited temporal resolution. Unlike conventional tracers, which can be monitored continuously *in situ* using fluorometric probes or integrative devices such as activated carbon traps, DNA tracers lack an equivalent passive or time-integrated sampling approach. This limitation not only increases logistical effort and operator dependency but also introduces uncertainty in the reconstruction of breakthrough curves, with a non-negligible risk of missing peak concentrations or the late-time tail of tracer arrival. In this context, the development of passive DNA capture devices capable of fixing and integrating synthetic DNA over time represents a novel step toward fully exploiting the potential of DNA tracers for quantitative hydrogeological investigations, enabling more robust, reproducible, and operationally efficient tracer tests.

In our field test, the DNA tracer behavior also matched that of traditional tracers with slight variations arising from site-specific conditions, which exhibit greater complexity compared to fully controlled laboratory environments. The DNA detected by manual sampling corresponded temporally with that of both fluorescein and NaCl, reinforcing the conclusion that DNA tracers behave similarly to conservative tracers under hydrodynamic conditions typical of surface water transport (Zhang and Huang, 2022). Foppen et al. (2011), for example, found that synthetic DNA tracers co-injected with NaCl in Dutch streams had breakthrough times nearly identical to the salt tracer, suggesting no significant retardation. The BTCs from our capture devices, while slightly delayed and broader compared to manual sampling, captured the primary tracer peak. The secondary peak observed should be interpreted with caution, as further studies with additional repetitions are needed to determine whether it represents an experimental artifact. Relative to the laboratory experiment, the flow rate in the stream is considerably greater (1.65×10^{-4} L/s vs. 6 L/s, respectively) and, since the capture device does not completely span the stream cross-section, it may interact with water masses exhibiting different DNA concentrations during the test. The DNA capture device integrates DNA over time, potentially capturing both free-floating and retained particles that are missed in instantaneous manual samples. Such integration may be particularly valuable in hydrologically complex environments, such as karst systems, where flow velocities and residence times are heterogeneous (Rusjan et al., 2023; Zhang and Huang, 2022). As highlighted by Zhang and Huang (2022), one of the main challenges in tracer studies is resolving delayed transport through stagnant zones or preferential pathways. The passive device described here, by virtue of its accumulation mechanism, is particularly suited for these events. Its design therefore offers a promising alternative to traditional grab sampling, which can underestimate tracer mass recovery in transient storage conditions or during flow regime changes. It is important to clarify that

the field experiment presented in this study represents a simplified hydraulic configuration, analogous to a single fracture or conduit, rather than a fully developed groundwater system. The stream test was deliberately selected as an intermediate experimental scale between laboratory column experiments and real aquifer-scale applications, providing a controlled yet natural flow environment in which transport dynamics could be directly compared with conventional tracers. While this configuration is representative of conduit-dominated flow in karst or highly fractured carbonate aquifers, it does not capture the full complexity of groundwater systems characterized by multiple, interacting flow paths, diffuse recharge, and heterogeneous features. Encapsulated DNA tracers have been shown to exhibit transport behavior similar to conservative tracers in porous media, maintaining signal integrity after passage through both sand columns and unconsolidated aquifer material as demonstrated by Mikutis et al. (2018).

However, variations in flow velocity between preferential pathways and low-permeability zones could result in broader or multi-peaked breakthrough curves, increased tailing, and delayed tracer arrival associated with transient storage or matrix diffusion. Importantly, results from both the column and field experiments showed no evidence of specific retention or irreversible interaction between the synthetic DNA tracer and the calcium-carbonate material investigated in this study. While short ssDNA oligomers are known to adsorb onto mineral surfaces through electrostatic interactions, the extent of this process is strongly governed by hydrochemical conditions and sediment properties (Cleaves et al., 2011). Under the experimental conditions applied here, the close agreement between DNA breakthrough curves and those of conservative tracers (KCl, NaCl, fluorescein), together with the absence of systematic delays or measurable mass loss attributable to sorption, indicates that the biotinylated DNA behaved conservatively with respect to the carbonate matrix. This observation is consistent with previous studies reporting conservative or near-conservative transport of synthetic DNA tracers in a range of geochemical environments, including silicate-dominated sediments, fractured crystalline rocks, and surface-water systems (Foppen et al., 2011; Aquilanti et al., 2013; Pang et al., 2020).

Although DNA–mineral interactions have been reported under specific conditions (e.g., high ionic strength, presence of multivalent cations, or clay-rich materials), such effects were not observed in the present study, indicating that calcium-carbonate substrates typical of karst and fractured carbonate aquifers do not induce measurable DNA retention at the scale and conditions investigated. This is particularly relevant given that most artificial tracer tests in hydrogeology are conducted in carbonate and karst environments, where conduit-dominated flow and rapid transport prevail. Consequently, the experimental setting explored here encompasses a large proportion of real-world tracer test applications, supporting the broad applicability of the proposed passive DNA capture approach within the contexts most addressed by hydrogeological tracing studies.

Under such heterogeneous conditions, passive DNA capture devices may integrate tracer signals originating from fast-flow conduits as well as slowly exchanging domains, potentially enhancing the detection of delayed or retained tracer fractions that are often underestimated by discrete sampling. However, this same heterogeneity could also increase variability among capture devices deployed at different locations, emphasizing the need for spatially distributed sampling strategies in complex aquifers.

The application of this method to real groundwater catchments introduces several additional challenges that warrant careful consideration. At the aquifer scale, injected DNA tracers would experience significant dilution due to dispersion, mixing between flow paths, and large discharge volumes. However, owing to the extremely low detection limits of qPCR, the absolute mass of DNA required for large-scale applications remains relatively small compared to conventional tracers. Previous studies have demonstrated that DNA tracers can be detected reliably even after strong dilution. For example, Pang et al.

(2017) reported that only 36 μg of dsDNA spiked into effluent was detectable in a gravel aquifer more than 37 m down-gradient, still ~ 3 orders of magnitude above the qPCR detection limit. Nonetheless, determining the optimal injected mass remains site-specific and should account for expected dilution, travel times, system heterogeneity, and the number of monitored outlets.

A further challenge concerns sampling strategy in groundwater systems, which fundamentally differs from surface-water catchments. Unlike surface waters, where tracer recovery occurs at a single downstream outlet, groundwater systems may exhibit multiple discharge points (e.g., springs, wells,) and complex internal flow partitioning. In heterogeneous systems, different flow paths may respond at different timescales, requiring longer deployment durations or staggered retrieval of passive capture devices. In such contexts, the deployment of passive DNA capture devices would require a network-based approach, with traps positioned at strategically selected outlets or along suspected preferential flow paths. While this increases logistical complexity, it also represents a key advantage of DNA tracers, whose virtually unlimited sequence diversity allows simultaneous multi-source and multi-path investigations that are not feasible with traditional tracers.

Consequently, the simple stream test presented here should not be interpreted as a direct analogue of an entire groundwater system, but rather as a proof-of-concept demonstrating the feasibility of passive DNA capture under flowing water conditions representative of a single conduit or fracture. The fundamental difference between surface-water systems with a single outlet and groundwater systems with distributed discharge points limits direct generalization. However, the results provide strong evidence that the passive capture approach can reproduce conservative tracer transport behavior and integrate tracer signals over time. In heterogeneous aquifers, this time-integrative property may be particularly valuable for identifying delayed transport, storage effects, and the superposition of multiple flow components.

Future work should therefore focus on full aquifer-scale deployments, testing the passive DNA capture system in real groundwater settings characterized by strong structural and hydraulic heterogeneity. Such studies will be essential to evaluate how heterogeneity influences tracer recovery, BTC shape, and capture efficiency, as well as to define optimal DNA injection masses, trap density, and deployment duration, ultimately enabling the translation of this methodology from controlled experimental settings to operational groundwater investigations.

At this stage, it is important to clarify that when using passive DNA capture devices, a conventional tracer recovery mass cannot be computed. The overall capture efficiency is influenced by both hydrodynamic and physical factors. The device allows the water flow to pass through freely, and only a small fraction of the DNA molecules in the flow are retained on the agarose pearls (0.6% w/v), with pore sizes ranging from approximately 100 to 300 nm (Narayanan et al., 2006). This pore size is sufficiently large for short ssDNA (72 nt) to diffuse, but likely not fast enough to allow uniform penetration during short deployments. As a result, most DNA molecules probably bind to the streptavidin-coated magnetic beads located near the outer surface of the agarose pearls, where diffusion pathways are shortest and contact with the flowing water is most direct. Consequently, the results obtained from the capture devices are expressed as the number of DNA molecules recovered per trap (Fig. 6 a and Fig. 7 b), rather than as concentration. The total amount of captured DNA molecules is substantially lower than the injected mass because the water flow continues downstream, carrying most of the DNA molecules beyond the monitoring point. This behavior is particularly evident in the field test, where the capture device, and the agarose pearls it contains, did not occupy the entire cross-section of the stream, allowing a portion of the tracer to bypass the device laterally. Even in the laboratory setup, where the entire flow passed through the device, the majority of DNA molecules traversed the pearls without being retained. Specifically, the number of DNA molecules recovered by the device was on the order of 10^6 compared to 10^{14} injected in the laboratory test, and approximately 10^4 compared to 10^{15}

injected in the field test, confirming that most of the DNA continued downstream of the monitoring point.

The focus of this study, therefore, is not on mass balance for the DNA capture device, but on the shape of the BTCs obtained from the passive capture method and their comparison with traditional tracers. The BTCs derived from the devices reproduce the same transport dynamics observed for conservative tracers, confirming both the conservative behavior of DNA and the device's capacity for time-integrated sampling.

By facilitating the passive, continuous capture of DNA tracers, our device opens new opportunities for studying water movement, especially for karst and carbonate aquifers, moving beyond the snapshot insights provided by traditional manual sampling (Pugliese et al., 2025). In fact, the use of DNA tracers can be implemented in hydrological and hydrogeological studies over traditional tracer methods by combining molecular specificity, high sensitivity, and operational simplicity. This improvement in DNA detection in water eliminates background noise and allows for unambiguous hydrologic tracer recognition. This step overcomes the inherent limitations of conventional tracers, which offer only a limited number of distinct signals (Foppen et al., 2011). In fact, in karst and highly fractured carbonate aquifers, recharge often occurs through discrete infiltration points (sinkholes, swallow-holes, fissures) which feed fast, preferential flow paths into the aquifer system (Bonacci, 2015; White, 2016). Conventional tracers (dyes, salts, isotopes) often become diluted or mixed in such heterogeneous systems, limiting the resolution of flow path discrimination and complicating the decomposition of multiple input pathways (Benischke, 2021). By contrast, the use of synthetic or natural DNA-based tracers, characterized by a virtually unlimited number of unique barcodes, permits high-resolution delineation of simultaneous flowlines from multiple infiltration sources. For example, artificial DNA tracers have been successfully used in fractured-reservoir settings to distinguish distinct flow paths and to recover tracer signals even at very low concentrations (Pugliese et al., 2025).

Operationally, our device is designed for ease of use and compatibility with standard laboratory workflows. It consists of an agarose hydrogel matrix embedded with streptavidin-coated superparamagnetic beads, which can be deployed directly into the flow without the need for pumps or mechanical disturbance. Also, DNA recovery is performed simply using a magnet, minimizing the risk of contamination or sample loss. This straightforward workflow enhances reproducibility and scalability, particularly for field applications.

Crucially, our system meets the growing demand for environmentally safe and low-impact tracer methods. It introduces no persistent pollutants and requires no external power source, making it ideally suited for remote or ecologically sensitive environments (Lapworth et al., 2012; Lee et al., 2024). The low cost of production further facilitates the simultaneous deployment of multiple distinct tracers across large or complex study areas.

By addressing the analytical and logistical challenges associated with conventional tracers and manual sampling, our novel DNA capture system offers a transformative tool for routine monitoring of groundwater and surface-water flow paths. Its deployment can significantly enhance hydrogeologic investigations and contribute to more effective water-resource management.

As regards the low number of DNA molecules collected per trap, future developments could aim to increase the amount of captured DNA by enhancing the contact surface area, for example, using smaller agarose pearls or a greater number of trapping beads. Nonetheless, as observed in previous traditional tracers' studies employing passive samplers, recovery percentages are inherently low because only a limited portion of the tracer flux is captured, making these approaches semi-quantitative by design (Fronzi et al., 2021). Moreover, the main limitations of this study must be acknowledged. At this stage, the use of the DNA capture device under varying geochemical conditions was not tested and should be the object of targeted *in-situ* testing. Future work could focus on identifying aquifers hosted within different geological formations, each characterized by distinct mineralogical compositions

and consequently varied hydrochemical properties of the circulating water and systematically evaluating the potential interactions with the geochemical environment on a case-by-case basis.

5. Conclusions

The application of DNA tracers in hydrogeological investigations represents a transformative step forward, integrating molecular biology with hydrological sciences to address the longstanding limitations of conventional tracers (Filippini et al., 2025). Traditional methods often struggle in large-scale or high-flow systems due to sensitivity to background concentrations and the need for high tracer quantities. DNA tracers, by contrast, offer exceptional specificity, environmental safety, and ultra-low detection limits, making them well-suited for complex environmental settings. This study's primary contribution is the development of a novel passive DNA capture device, demonstrating its application across laboratory and field-scale experiments. The device leverages streptavidin–biotin chemistry embedded in a robust agarose hydrogel matrix, combining high selectivity, mechanical stability, and compatibility with standard molecular workflows. It allows for easy deployment and retrieval without disturbing the flow system and enables, at the moment, semi-quantitative BTC analysis.

Importantly, the passive device enhances the advantages of DNA tracers by enabling time-integrated sampling, reducing the need for frequent manual collection, and capturing both rapid and delayed flow components. These features are particularly valuable in hydrologically heterogeneous environments such as karst systems or fractured aquifers, where conventional methods may underestimate transport complexity.

Its operational simplicity, low cost, and environmental safety make the system highly promising for routine field monitoring and large-scale tracer applications. By experimentally demonstrating the feasibility of coupling synthetic DNA tracers with a passive molecular capture system, this work contributes a novel methodological framework to the international hydrological community, opening new perspectives for tracing flow connectivity in heterogeneous aquifers while acknowledging that further research is required to optimize capture efficiency and assess tracer persistence under diverse hydrogeochemical conditions. With continued innovation and interdisciplinary collaboration across hydrology, molecular biology, materials science, and environmental engineering, DNA tracers are poised to become standardized and reliable tools for environmental diagnostics and water resource management.

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CRediT authorship contribution statement

Davide Fronzi: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Vesna Milanovic:** Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation. **Stefano Palpacelli:** Validation, Methodology, Investigation, Conceptualization. **Alberto Tazioli:** Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Lucia Aquilanti:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, 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

Data will be made available on request.

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