

DNA and fluorescein tracer tests to study the recharge, groundwater flowpath and hydraulic contact of aquifers in the Umbria-Marche limestone ridge (central Apennines, Italy)

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Abstract The purpose of this paper is to highlight the hydrogeological contact among aquifers in karst and fissured systems and study the different flowpaths present in said aquifers; given the complex hydrogeological and tectonics settings, detailed surveys in the Umbria-Marche limestone ridges and tracer field tests were used to achieve this goal. DNA and fluorescent tracers were injected into a sinkhole and thereafter recovered at different points, allowing both for the identification of contact among aquifers, as well as the main and secondary directions of groundwater. The tracer tests have shown that the majority of the groundwater flow is directed towards the axis of the tectonic structures, emerging in more depressed areas. In addition, they have confirmed the influence of karstification on the recharge, which allows for a more detailed characterisation of water circulation in the unsaturated zone of the Maiolica and the Calcare Massiccio limestone aquifers thus offering confirmation of the hydrogeological survey results, and providing additional information that was previously unknown. Furthermore, the DNA tracer provided good results in the field especially in the karst systems and fissured rocks, thus confirming its appropriacy for flowpath investigations and vulnerability analyses of springs. In particular, DNA is suitable for very large amounts of groundwater owing to its very low detection

limit. Further investigations and studies are, however, required to assess its validity also for the evaluation of hydrogeological parameters.

Keywords Carbonate rocks · Groundwater flowpath · Karst · Marche region · DNA tracer

Introduction

The investigated area located in the inner part of the Marche region (central Italy) and belonging to the Umbria-Marche limestone ridges in the central Apennines (Fig. 1) is characterised by several folds and overthrusts displaced by fault systems. Very complex geo-structural settings exist in the limestone ridges, according to Barchi et al. (1998), Boccaletti et al. (1990), Calamita and Deiana (1986), Calamita et al. (1991a, b), Scisciani (2009), and karst phenomena (although poorly studied) are also present, contributing to the difficulties in defining the hydrogeological setting and the relations among the different aquifers. Some studies (Boni et al. 1986, 2009; Nanni and Vivalda 2005; Tarragoni 2005) have assumed that hydraulic contact among the hydrogeological complexes is likely to exist; indeed the mixing of groundwater emerging from areas of overthrust fronts (behaving as close environments) has been seen. Moreover different flowpaths are presumed to exist in carbonatic aquifers based on the examination of the springs hydrograph (Nanni et al. 2006).

Although the groundwater chemistry in fractured rocks suggests that hydraulic contact among aquifers is likely and the mixing of different groundwater has also been observed, no realistic evidence for the identification of groundwater circulation exists yet. Isotopic studies performed in central Italy (Barbieri et al. 2002; Nanni et al.

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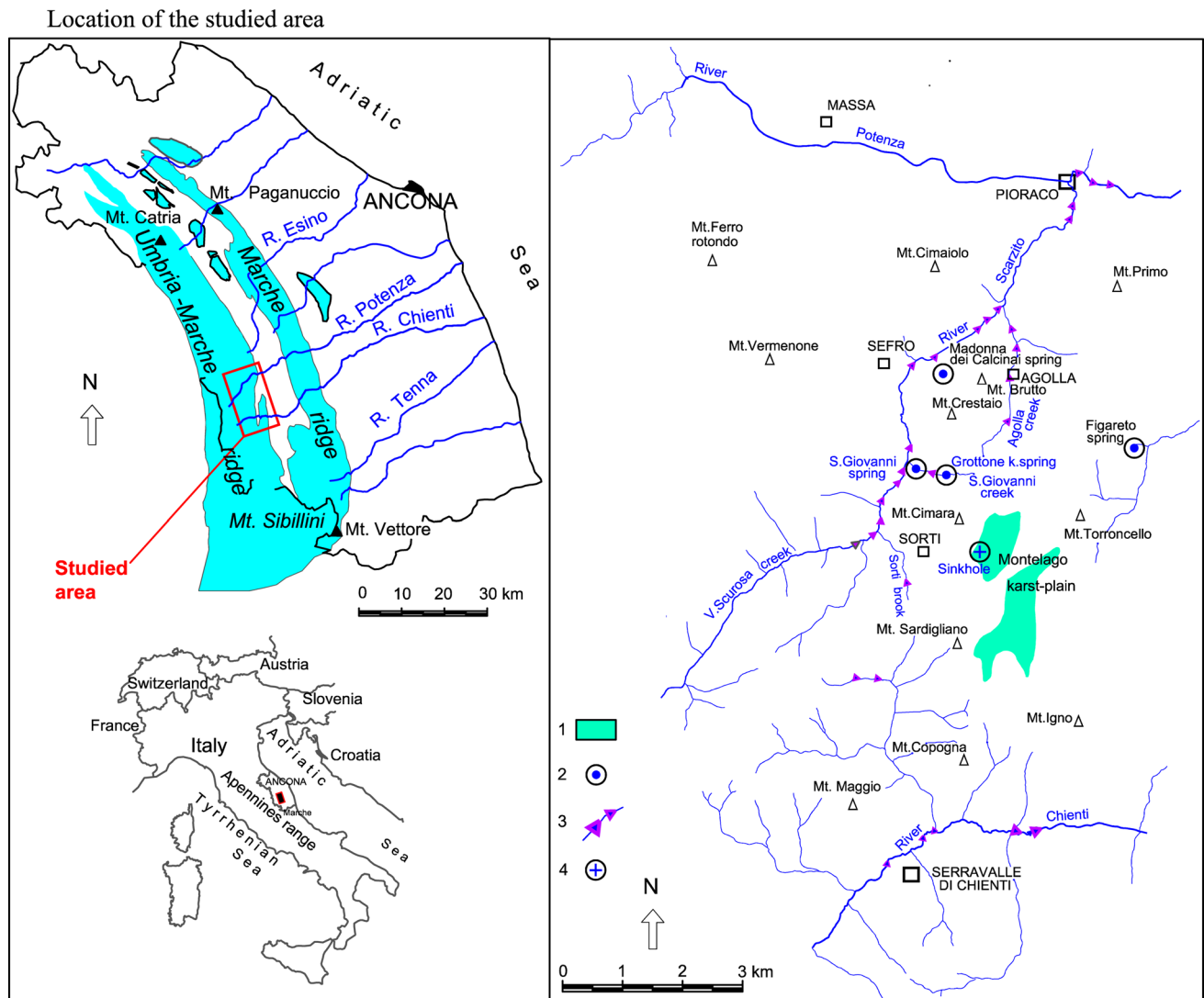


Fig. 1 Location of the investigated area. Main hydrographic features are reported on the right. 1 Montelago karst plains, 2 Spring, 3 Linear spring, 4 Montelago sinkhole

2013; Tarragoni 2006; Tazioli et al. 2007, 2012), have furthermore stressed the problems involved in the investigation of groundwater origin and groundwater flowpaths in different aquifers. In fact, while H_2O isotopic content suggests possible groundwater mixing, these data are often difficult to read: different hydrogeological complexes, in fact, often outcrop and are recharged at similar altitudes. Although these studies have contributed to our understanding of the groundwater circulation in the Umbria-Marche limestone ridges, further evidence is still required.

As regards the present study, a detailed geo-structural and hydrogeological field survey was performed recently in the area between the Potenza and the Chienti Rivers (Palpacelli 2013), emphasising the complex relations among aquifers and the groundwater circulation. Tracer methods were employed in the investigated area aimed at

demonstrating the actual hydraulic contacts among aquifers and studying the groundwater flowpaths and the recharge mechanism. This purpose was supported by the presence of a well-defined sinkhole (uncommon in the Umbria-Marche ridges) located in the Montelago karst plain (Fig. 1), connected to several karst conduits able to put different aquifers in contact with one another and activated only during the winter/spring seasons. The area selected for the investigation, particularly suitable for understanding the groundwater pathways and discovering connections among hydrogeological complexes, may be considered as an excellent pilot test area in the Apennines.

Owing to the considerable problems in the application of tracer techniques in areas characterised by fractured rocks and almost unknown karstification, a first series of tracer tests was performed investigating a wide region included

between the upper Chienti River and the Potenza River valleys (Fig. 1), using either a fluorescent dye or the DNA tracer previously tested by Aquilanti et al. (2013). A second series of tests was then made to identify different groundwater flowpaths and to study groundwater movement in the unsaturated zone in a smaller area.

The properties of these tracers have already been studied in previous works (Aquilanti et al. 2013; Tazioli and Palpacelli 2013), presented and discussed together with their suitability for hydrogeological purposes. Fluorescent tracers like Fluorescein, Rhodamine WT and Tinopal are well known and widely used in hydrogeological studies (see Davis et al. 1980; Hadi et al. 1997). DNA tracers, on the other hand, have only been applied in a few studies on both surface waters and groundwater qualitatively (Sabir et al. 1999, 2000; Sabir et al. 2001) and quantitatively (Foppen et al. 2011; Shama et al. 2012; Foppen et al. 2013). The DNA molecule is able to trace surface and groundwater flow, is detectable even at very low concentrations, no significant change in water density and viscosity can be observed and its use is not dangerous for the environment or human health (Aquilanti et al. 2013; Sabir et al. 1999). Nevertheless, DNA tracers have a low persistence in fields where microbial activity and low pH occur and, like other hydrogeological tracers do, they can be absorbed by clay minerals (Sabir et al. 2001). Furthermore, DNA (being a particle tracer, not soluble in water) shows advective transport behaviour, with very low dispersion; for this reason the method of moments (Maloszewski and Zuber 1992) cannot be successfully applied to this tracer.

The main aim of this paper is, therefore, to attain evidence of the hydrogeological contact among aquifers in karst and fissured systems and confirm the occurrence of different flowpaths within the aquifers by adding the results of tracer tests to the hydrogeological and geo-structural surveys.

Investigated area

The investigated area is located in the Umbria-Marche Apennines (central Italy), about 30 kms North of the Mt. Sibillini mountains (Fig. 1), included between the high valleys of the Chienti and the Potenza rivers. Elevations are from 370 (Potenza River) to 1433 m a.s.l. (Mt. Igno).

From SW to NE the zone is entirely incised by the Scarzito River, primary tributary of the Potenza River; the main tributaries of the Scarzito River are represented by small perennial streams, of which the most important are the Valle Scurosa creek, the Agolla creek and the S. Giovanni creek. The latter originates from a karst resurgence (Grottone karst spring, Fig. 1) and is continuously recharged by the S. Giovanni spring.

Near the middle between the Chienti and the Potenza River valleys, two different karst plains are located in the Montelago zone (the higher plain, at 918 m a.s.l., with an area of 1.1 km² and the lower plain, at 891 m a.s.l., having an area of 0.6 km²). These are connected by an ancient break in the rock, so that the water collected in the higher plain flows into the lower one and then infiltrates into the sinkhole, located in the middle.

Geological and hydrogeological features of the Umbria-Marche Apennines

In a wide sense, the area belongs to the Meso-Cenozoic limestone ridges of the Umbria-Marche central Apennines (Centamore et al. 1971; Centamore and Micarelli 1991), which can be categorised into two main ridges: the innermost ridge is the so-called Umbria-Marche ridge, whereas the Marche ridge is located more externally. The first one is characterised by less developed east-vergent anticlines, partly overlapped and enveloped by an important thrust plane which shifts them towards NE, above the Paleogenic and Miocenic formations belonging to the Acqualagna-Visso depression (Nanni et al. 2013).

In this ridge, the overthrust fronts separating the hydrogeological complexes are characterised by low hydraulic conductivity; so they usually act as aquicludes. In fact, in the anti-Apennines direction, they limit or block hydraulic contact among the aquifers. Groundwater circulation is therefore heavily conditioned by the geo-structural setting.

The Marche ridge, on the other hand, is made up of a macro anticline bordered by the Mt. Sibillini overthrust, which brings the Meso-Cenozoic carbonatic rocks onto the Tertiary sediments (Bally et al. 1986; Barchi 1991; Calamita and Deiana 1986; Calamita et al. 1991a, b). In this ridge a wide single hydrogeological catchment can be identified (although composed of different aquifers) bordered along the Apennines line by geological units with low permeability (Nanni et al. 2006). The two ridges join together in the South, generating the Mt. Sibillini range (Fig. 1).

Moreover, the geo-structural setting of the Umbria-Marche Apennines is characterised by an arched fold-and-thrust belt verging towards ENE (Barchi et al. 1998; Calamita et al. 1995, 1999; De Feyter et al. 1987; Mirabella et al. 2008; Pierantoni et al. 2005; Scisciani 2009), displaced by fault systems with N-S and E-W orientation.

The Meso-Cenozoic sequence is closed at the bottom by the Triassic Anhydrite (Anidriti di Burano Fm.) acting as aquiclude; the sequence (actually made by thick layers of calcareous rocks placed between thinner impervious strata of marls, calcareous marls and marly limestones) may occur as a complete series, a reduced series or a lacunose

series, depending on the location in the limestone ridges (Fig. 2a, b). It begins with the limestone of the Calcare Massiccio and the Corniola Fms., representing a single aquifer (called Calcare Massiccio aquifer from this point on), namely the basal aquifer of the regional flow on which the Jurassic Infra-Cretaceous marly limestone, marls and clayey marls (Jurassic aquiclude) of the complete and the reduced series stand at the top; this latter aquiclude separates the Calcare Massiccio aquifer from the upstanding limestone of the Maiolica aquifer. In some areas (where the lacunose series outcrops), the limestone of the Maiolica and the Calcare Massiccio form a single aquifer (Fig. 2, line B). The Meso-cenozoic sequence continues with the marls of the Marne a Fucoidi Fm., acting as an aquiclude and separating the Maiolica aquifer from the upper aquifer of the Scaglia (Scaglia Bianca, Scaglia Rossa Fms.). The sequence is closed on the top by the terrigenous Miocene Marche sequence given by marls and clayey marls of the Scaglia cinerea, Bisciario and Schlier Fms. (Paleocene-Miocene) acting as an aquiclude (Fig. 2). Miocene sediments are present on the Meso-Cenozoic sequence, outcropping in more external areas.

The Calcare Massiccio aquifer is the most important hydrogeological complex in the Umbria-Marche limestone ridges and represents the basal aquifer; groundwater circulation occurs in very well-developed fissures and karst conduits. In the Maiolica aquifer, karstification is more limited, while in the Scaglia aquifer groundwater flow mainly happens in fissures and only secondarily in karst conduits.

Different flowpaths are presumed to exist in the carbonatic aquifers as illustrated by the springs hydrographs (Nanni et al. 2006). A very fast one allowing rapid infiltration of rain water through the unsaturated zone up to the aquifer, and a deep and slow one, conversely connected to the basal flow of regional aquifers.

Further evidence of hydraulic contacts among different aquifers is given by the hydro-chemical facies of springs recharged by aquifers of the central Apennines, which is generally calcium-bicarbonate (Nanni and Vivalda 1999, 2005; Nanni et al. 2006). There are also springs, mostly arising in the Calcare Massiccio aquifer and secondarily in the Maiolica and Scaglia aquifers, with different salt contents. The Calcare Massiccio groundwater shows higher salinity, with enrichments in chloride, sodium and sulphate

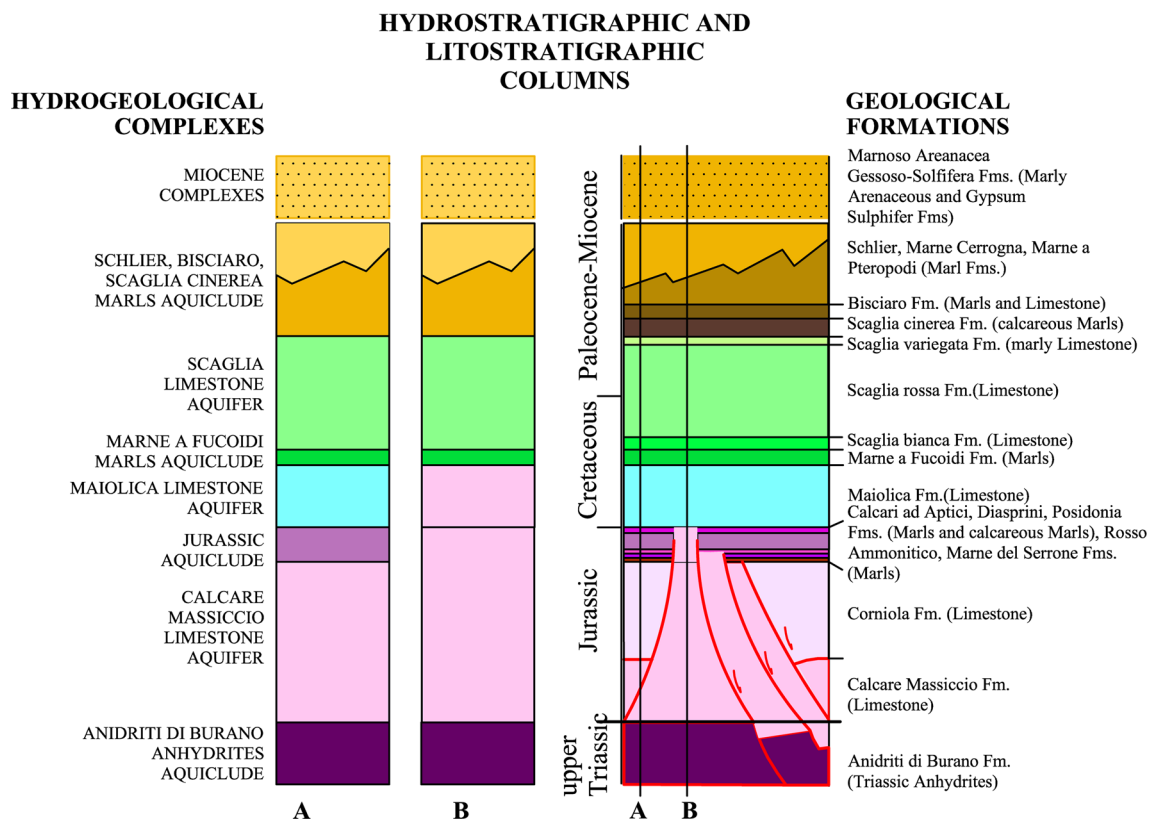


Fig. 2 Hydrostratigraphy of the Umbria-Marche and Marche Meso-Cenozoic ridges on the left: “A” is a synthetic visual model of the Umbria-Marche ridge, “B” of the Marche ridge (after Nanni et al.

2013, modified). On the right, schematic Umbria-Marche stratigraphic sequence: “A” is the complete series, “B” is the lacunose series

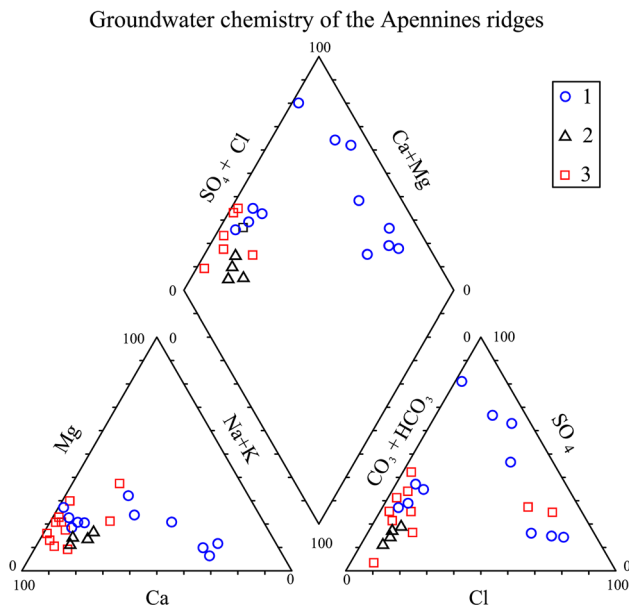


Fig. 3 Piper chart representing the groundwater chemistry of the Apennines ridges (adapted from Nanni et al. 2006). 1 Mineralised groundwater emerging from the central Apennines ridges, 2 Groundwater of the basal aquifer of the Apennines ridges, 3 Groundwater emerging from aquifers in the internal ridge (Umbria-Marche ridge)

(Fig. 3). Sometimes even groundwater hosted by the Maiolica and the Scaglia limestone aquifers may exhibit enrichments in these same elements, especially in fractured or faulted zones, thus supporting the hypothesis of hydraulic contact among the aquifers of the Umbria-Marche Apennines ridges. This continuity could appear through the anti-Apennines normal faults that release the hydrogeological complexes.

Geostructural, hydrogeological and karst features of the investigated area

The hydrogeological and geo-structural settings of the investigated area were recently studied by Palpacelli (2013) and illustrated in the hydrogeological map of Fig. 4a and in the longitudinal and cross hydrogeological sections of Fig. 4b. In this area (Fig. 1) several punctual (i.e., the S. Giovanni spring, utilised for drink-water supply) and linear springs (located in the Scarzito, Chienti, and Potenza Rivers) having rather high discharge rates are present, some of them recharging rivers and streams; increase of the discharge in several parts of the rivers is very frequent. This indicates the likely occurrence of hydraulic contact among different aquifers, made possible by the largely complicated geo-structural setting.

From the geo-structural survey, the following tectonic structures (from E to W) have been identified: the Mt. Primo anticline, the Agolla syncline, the Sefro anticline, the Mt. Sardigliano syncline, the Mt. Vermenone anticline

and the Laverino syncline. In this work these tectonic structures have been thought to be associated with the hydrogeological complexes and groundwater flow, due to the close relation between tectonics and hydrogeology.

In all the investigated area karstification is widespread with karst plains, sinkholes, caves and shafts (Galdenzi et al. 2008). The Calcare Massiccio, Maiolica and Scaglia limestone Fms. are subjected to a well-developed but poorly known karst phenomena that, as shown by spring hydrographs, plays an important role in the aquifers recharge (Nanni et al. 2006).

Caves, galleries and shafts are common especially in the Calcare Massiccio limestone, less frequent in the Scaglia rossa and Maiolica limestone Fms; the Montelago high plain (belonging to the Agolla tectonic structure) represents one of the most important karst plains in the Marche region with a well-developed epi-karstification; deep karstification is likely to be developed but is almost completely unknown.

The sinkhole (in the Maiolica Fm.) is represented by a morphologic depression, which in the late winter or early spring gives origin to a little lake (Fig. 5); as a consequence, surface water of meteoric origin infiltrates through the sinkhole only when the lake is less developed, hence allowing for the tracer tests to be performed only from January/February to May/June.

Materials and methods

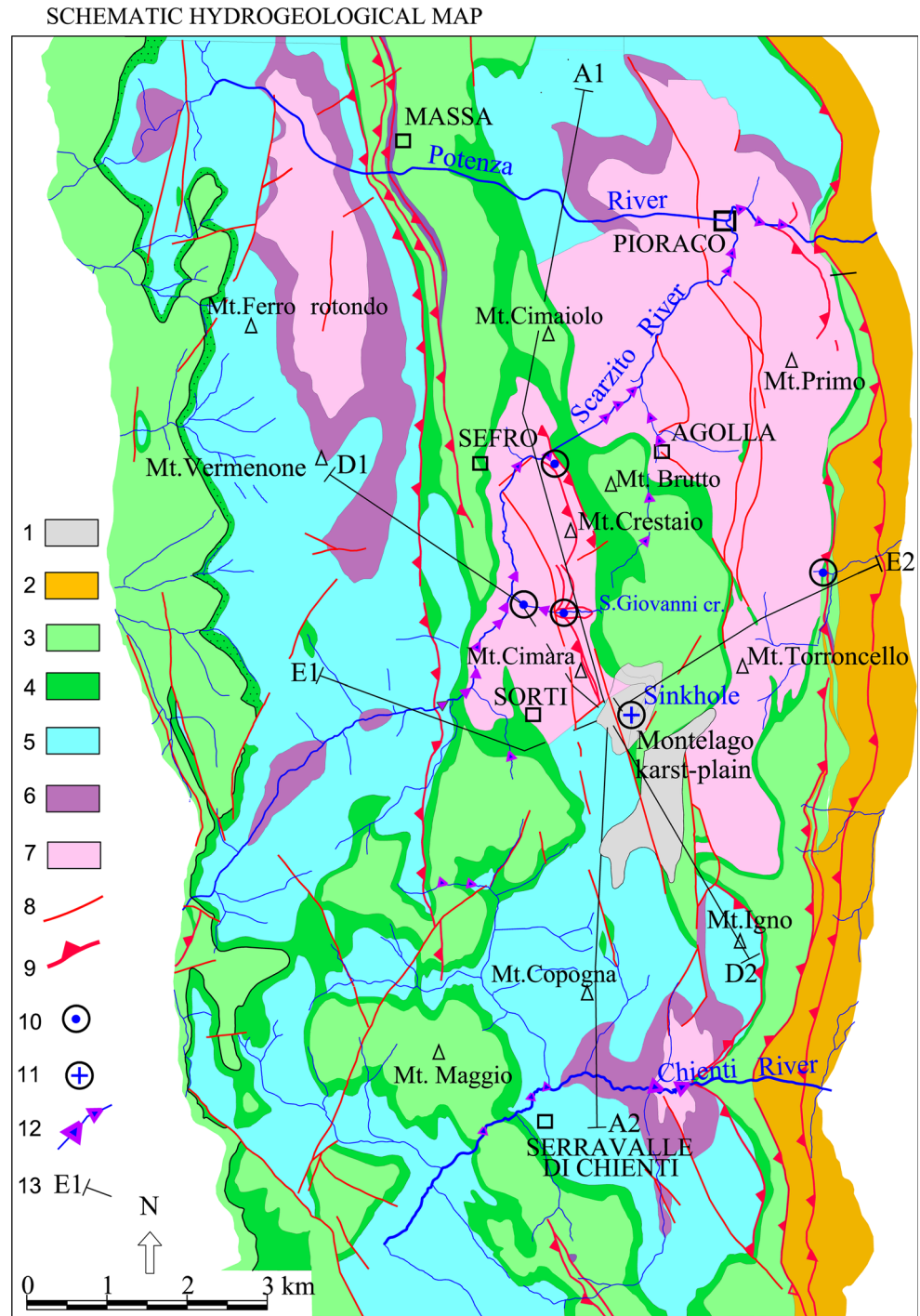
Tracer tests

Hydrogeological tracer tests base their success on a simple operational process: a known amount of tracer (expressed as mass or volume) is injected into a water point suspected of being connected to the groundwater network under investigation (i.e., sinkhole, stream or shaft). The dilution of a certain amount of tracer in the unknown volume of groundwater is, therefore, the key for the experimental practice. Tracer tests allow for the determination of the groundwater flow direction and hydrogeological parameters of aquifers simply by applying a mass balance to the quantity of the injected tracer and the aquifer volume.

A freeware software (QTRACER2) and a spreadsheet (MS Excel) were used to obtain the transport data of the tracer tests (tracer mass recovered, mean tracer transit time, flow velocity). QTRACER2 (EPA 2002) is a software based on the FORTRAN routine, and is reported to be particularly suitable for fluorescent dye BTCs analysis in karst areas (Field 2003).

However, the DNA tracer, being insoluble, travels in suspension like a particle/colloid tracer, therefore a semi-quantitative graphical method was chosen to estimate mean

Fig. 4 a Hydrogeological map of the area studied in the Umbria-Marche ridge between the Chienti and the Potenza Rivers. 1 Montelago karst plain, 2 Terrigenous aquiclude, 3 Scaglia aquifer, 4 Marne a Fucoidi aquiclude, 5 Maiolica aquifer, 6 Jurassic aquiclude, 7 Calcarea Massiccio aquifer, 8 Normal fault, 9 Overthrust, 10 Spring, 11 Sinkhole, 12 Linear spring, 13 Cross section line. **b** Hydrogeological cross and longitudinal sections of the investigated area. Section lines are reported in **a**. 1 Terrigenous aquiclude, 2 Scaglia limestone aquifer, 3 Marne a Fucoidi marly aquiclude, 4 Maiolica limestone aquifer (*a* unsaturated zone, *b* saturated zone), 5 Jurassic aquiclude, 6 Calcarea Massiccio limestone aquifer (*a* unsaturated zone, *b* saturated zone), 7 Anidriti di Burano (anhydrites) aquiclude (sealing the tectonic structures and acting as an aquiclude in the thrust areas, see Fig. 2), 8 Faults and thrust, 9 Jurassic faults



flow velocity and mean residence time instead of the QTracer2 software, while data interpretation was done with a spreadsheet (MS Excel). The mean residence time chosen is equal to the centroid time, corresponding to half of the tracer mass having passed; the mean flow velocity is equal to the distance from the injection to the measurement point divided by the mean residence time previously calculated. In karst aquifers a slight correction might be made to take

sinuosity into account (from 1 to 1.5 times the distance from the injection point, Field and Nash 1997). The mean residence time together with the mean flow velocity was used to identify different flowpaths in the aquifers. The total amount of recovered mass (for fluorescent tracers) was calculated by multiplying the integral of tracer concentration by the integral of the flow rate during the time of the tracer test. This provides an approximate indication of

Fig. 4 continued

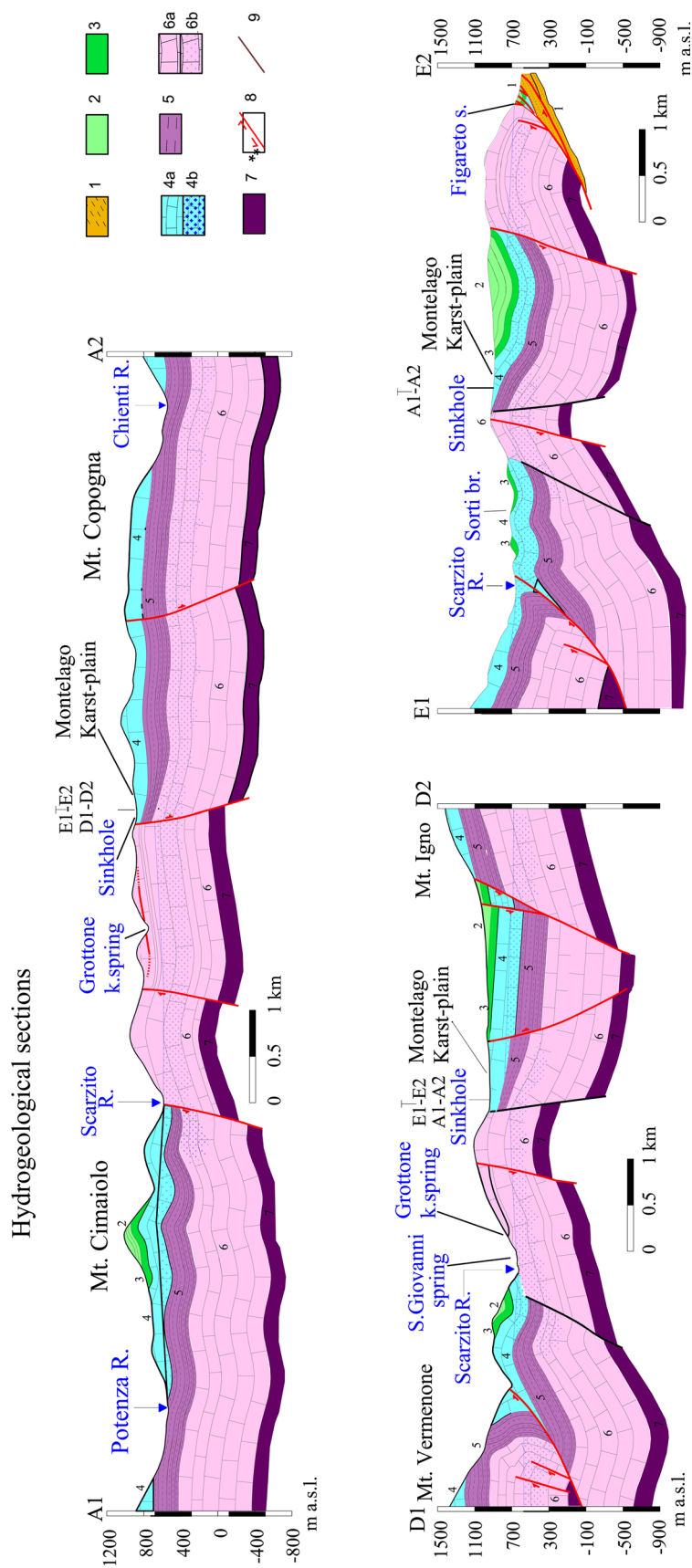




Fig. 5 Montelago karst plain landscapes with the sinkhole in different seasonal periods (on the *left* the end of the spring period, on the *right* the winter period)

the groundwater direction and flowpath velocity. QTRACER2 also allowed for aquifer volume estimation starting from the tracer mass recovery, thus giving an idea of the aquifer potential.

Tracers

In this study, fluorescent dyes and a single stranded DNA tracer were selected and used to investigate the area.

Synthetic DNA tracer

The primary structure of single stranded DNA (ssDNA) molecules such those used as biotracers, consists of a linear sequence of nucleotides that are linked together by phosphodiester bonds; migration of ssDNA through subsurface environment is greatly affected by the chemical and physical properties of these biomolecules, including size and shape. In this study, a ssDNA molecule of 72

nucleotides, previously described by Aquilanti et al. (2013), was used as a biotracer. It was synthesised (synthesis scale 10.0 μmol), purified by High Performance Liquid Chromatography (HPLC), and delivered lyophilized by MWG-Biotech (MMedical, Milan, Italy), at a final yield of 254.4 nmol, corresponding to 5652 μg and approximately to 10^{17} DNA molecules. The DNA tracer was resuspended in 1 mL of molecular biology grade water (Eppendorf, Milan, Italy), reaching a final concentration of 10^{14} DNA molecules μL^{-1} and kept at -20°C until use.

Detection and quantification of the DNA tracer were performed by quantitative Polymerase Chain Reaction (qPCR, previously described by Aquilanti et al. 2013). qPCR assays were carried out in triplicate with gradient PCR (Mastercycler realplex, Eppendorf). Fluorescence was measured at 520 nm at the end of the annealing period of each cycle to monitor the progress of amplification.

Sodium fluorescein

Sodium fluorescein (SIGMA-ALDRICH, molecular formula $\text{C}_{20}\text{H}_{10}\text{Na}_2\text{O}_5$) is a fluorescent dye widely used in groundwater hydrology and hydrogeology (Davis et al. 1980; Goldscheider et al. 2008). Its high solubility, low cost, no toxicity, and low detection limit make it a suitable tracer for several scientific purposes. It has an emission wavelength of 515–525 nm and an excitation wavelength peak of 460 nm. Tracer detection was performed using a Perkin Elmer LS-2 filter fluorometer, which allows low concentrations of the tracer to be detected (until 10^{-4} mg l^{-1}). Tracer concentrations in background samples were analysed and subtracted from sample concentrations. Background values were always close to the instrument detection limit.

Field tests

Two series of tests were implemented in the entire area during the period of investigation (2011–2013). Fluorescent tracer and the DNA tracer were injected together into the sinkhole to compare the results, but only Fluorescein data were used to evaluate the transport parameter of flow.

A first series of tracer tests was performed in different flow conditions (with the water flow rate infiltrating the sinkhole ranging from 0.008 to 0.073 $\text{m}^3 \text{s}^{-1}$) to identify hydraulic contact among aquifers belonging to different hydrogeological complexes. 200 g of Fluorescein and 1 ml containing 10^{16} molecules of single stranded DNA tracer were mixed with groundwater and then injected into the Montelago sinkhole and recovered at several points of the entire investigated area in all directions by using manual sampling (fluorescent traps and discrete water sampling).

Furthermore, a second series of tests were then run—limited mainly to the Sefro and secondarily the Agolla

tectonic structures—using the same quantity of the fluorescent dye and the DNA tracer, aiming to estimate the features of groundwater and recharge and to define the pathways of groundwater flow. Automatic samplers were placed at two points along the expected groundwater direction. Discharge measurements of the surface water (Scarzito R., S. Giovanni creek and Agolla creek, Figs. 1, 4) and the surface water flow infiltrating the sinkhole were performed before, during, and at the end of the tracer tests to determine the flow rate to be used in the evaluation of the hydrogeological parameters. A current meter (SIAP ME-4001, sensitivity 0.05 m/s) was utilised to measure flow velocity at different points of a transversal section of flow. The transversal area was calculated by means of topographic surveys of the river sections. The flow rate was estimated using arithmetic computational methods (Tazioli 2011).

First series of tests

A first series of three tests were done in the area under investigation using Fluorescein (captured with fluorescent traps made of activated charcoal) and the DNA tracer (recovered in water samples collected at several points with manual sampling procedures). 10 packets of activated charcoal were positioned at different points around the investigated area, selected on the basis of the hydrogeological survey, both in the surface water (streams and rivers) and in groundwater (springs), at a linear distance from the injection ranging from 1300 to 6500 m. The traps in the Potenza and in the Chienti Rivers represent the furthest points. To limit the problem of false positives and negatives in the collected samples, three packets per monitoring point were placed and then analysed. Every 60 h each packet was replaced with a new one, to have a rough estimate of the tracer arrival time. In addition, water samples were collected every 36 h from the same points located in groundwater and surface water to allow for DNA tracer determination.

The first and third tests were performed during the recharge period (flow rate of water flowing into the sinkhole from about 0.02 to 0.07 m^3/s), the second test was completed at the end of the recharge period (flow rate of about 0.01 m^3/s), when the sinkhole was active. The tracer mass to be injected was estimated based on the maximum expected distance of tracer in the area. Sampling was performed until 39 days from the tracer injection.

Second series of tests

A second series of two tests were done in the area using Fluorescein and the DNA tracer; they were recovered in water samples collected by means of two automatic

samplers (ISCO 3700 °C and WTW PB-M-L, allowing sample collection time from a few minutes to several hours and powered by lead acid batteries). They were placed in the Grottone karst spring and in the S. Giovanni spring (Sefro hydrogeological system) during the tracer test and programmed to collect one sample per hour. After a week, sampling frequency was set to 3 h. Automatic samplers maintained the collected water samples in PE bottles (200 mL) to avoid tracer losses. The samples were then poured into glass vials (20 mL) weekly to allow fluorescence analysis and into Eppendorf 2 mL vials to perform laboratory DNA detection. The collected fluorescent tracer samples were stored in a dark place for at least a week to allow suspended sediments to settle. DNA tracer samples were maintained at $-20\text{ }^{\circ}\text{C}$.

In the first test (carried out at the end of spring 2012, when the flow rate of water infiltrating into the sinkhole was equal to $0.005\text{ m}^3\text{ s}^{-1}$) 120 samples were processed and analysed, while in the second one (carried out during spring 2013, when the flow rate was of $0.04\text{ m}^3\text{ s}^{-1}$) about 70 samples were collected and then processed.

Results and discussion

Aquifer connections (first series of tests)

The first series of tracer results show the connections among the different hydrogeological complexes. In the first and third tests (performed during the recharge period) all the fluorescent traps except traps n. 6 and n. 10 (Fig. 6a) provided more or less positive signals after 7–12 days. The vast majority of the tracer was indeed recovered at the Grottone karst spring, while about 10 % at the Figareto spring, the Valle Scurosa creek and the Madonna dei Calcinai spring; only a small amount of tracer reached the S. Giovanni spring in the recharge period. The flow velocity (calculated by Fluorescein tracer) ranges from a minimum of 100 to a maximum value of 3800 m/day (Table 1).

These data prove the connection between the Agolla hydrogeological system (in which the sinkhole is located, Fig. 6a) and the Sefro system (Grottone karst spring, S. Giovanni and Madonna dei Calcinai springs, linear springs in the Scarzito River); the Maiolica (of the Agolla system) and the Calcare Massiccio (of the Sefro system) aquifers are therefore in hydraulic continuity. Furthermore, a connection between the Agolla and the Mt Primo hydrogeological and tectonic systems (in which the Maiolica and the Calcare Massiccio form a single aquifer owing to the lack of the Jurassic series) is highlighted by these tests. Other less marked connections exist between the sinkhole and the western area (Fig. 6a).

In the second test (performed at the end of the recharge period) only traps n. 2, 4, 5, 7, 9 provided positive signals after 18–24 days, giving the directions depicted in Fig. 6b. The hydraulic contact among the Agolla, Sefro, and Mt Primo tectonic structures was confirmed; in fact whereas a higher amount of the tracer reached the Grottone karst spring (about one-third), about one-fourth was recovered in the Figareto, Madonna dei Calcinai and S. Giovanni springs (Table 1). Flow velocity is from 90 to 700 m/day.

The results obtained may suggest a primary flow direction from South (Montelago karst plain) to North and a secondary flow direction towards the East (Fig. 6a, b); they are ever present during the hydrologic year, but occur with variable groundwater velocity. In addition, during the recharge period other minor flow directions are present, which highlight the occurrence of contact among the sinkhole area and the linear springs in the Potenza River (on the North), the Valle Scurosa creek and the Sorti brook (on the West). This contact has been identified by means of the tracer tests and was not determined by the previous hydrogeological survey.

Groundwater flowpaths (second series of tests)

Tracer mass recovery, mean residence time and transport velocity for Fluorescein tracer are given in output by QTracer2 software programme and listed in Table 2. The results of the tracer tests are shown as a breakthrough curve (concentration vs time, Fig. 7). Total tracer mass recovery showed significantly variable results but was almost always higher than 50 %, thus providing a good enough test operation to determine the flow and transport parameters. The results of the tests regarding the Grottone karst spring and the S. Giovanni spring are presented hereafter. The first tracer test was performed at the end of the discharge period (May/June 2012), when the flow rate in the stream infiltrating the sinkhole was only $0.005\text{ m}^3/\text{s}$ (low flow condition), while the second test was during the recharge period (March/April 2013), with a moderate flow rate of $0.04\text{ m}^3/\text{s}$.

In both conditions, surface water infiltrates into the sinkhole and seeps through the unsaturated zone of the Agolla Maiolica aquifer towards the Calcare Massiccio of the Sefro hydrogeological system, in hydraulic continuity.

First test (May 2012)

At the end of the recharge period, water reaches the Grottone karst spring about 60 h after the injection (Fig. 7a); the DNA tracer precedes the fluorescent tracer peak slightly, this was also reported in previous studies (Ptak et al. 2004; Sabir et al. 1999; Aquilanti et al. 2013); other peaks, which are considerably smaller, are present both before and after the main peak. The mean tracer

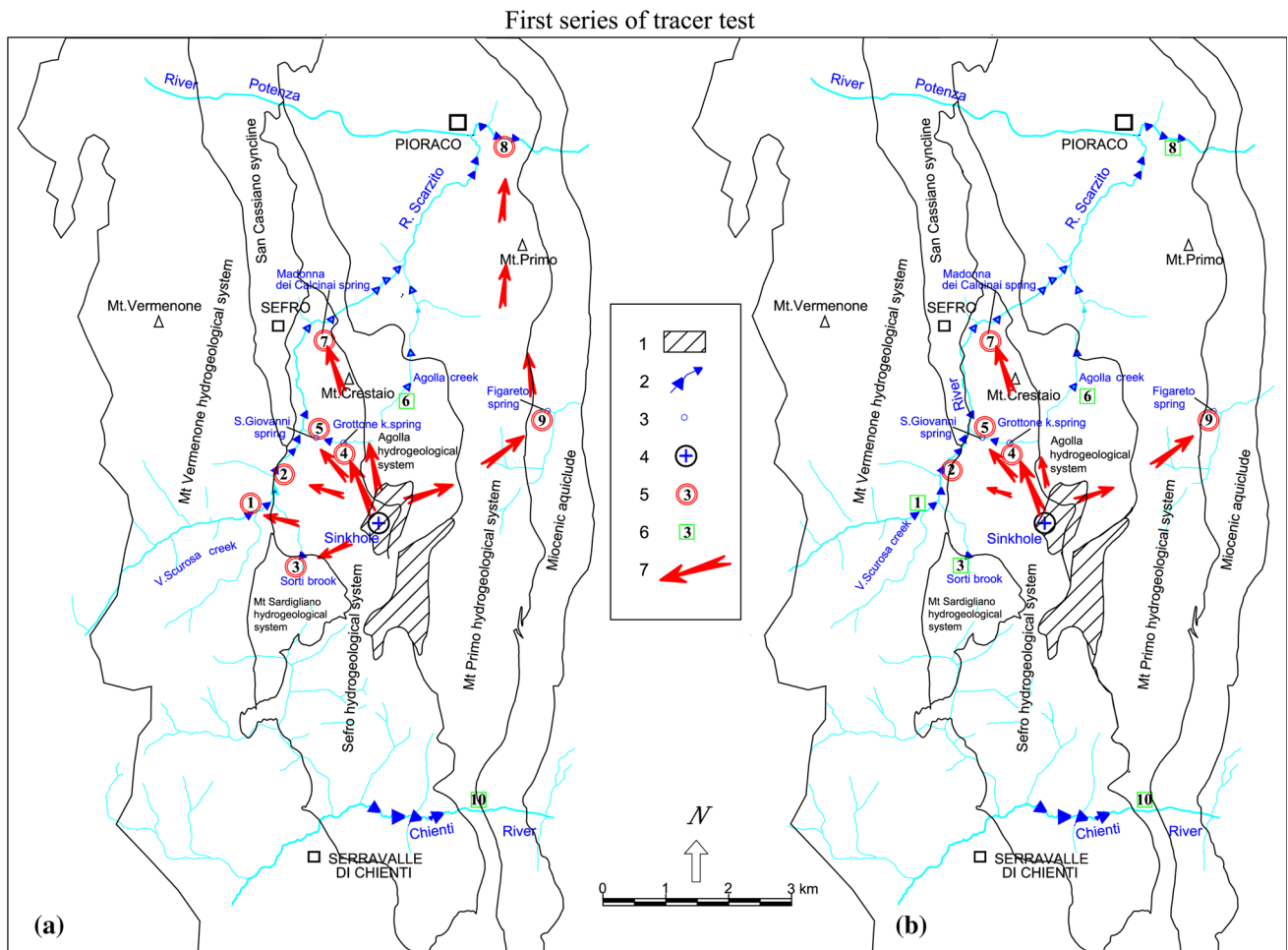


Fig. 6 Results of the first series of tracer tests: **a** 1st and 3rd tests during the recharge period, **b** 2nd test, at the end of the recharge period. Location of the test area; signals and groundwater flow directions are reported on the maps. 1 Montelago karst-plain, 2 Linear springs, 3 Spring, 4 Sinkhole, 5 Sampling point (positive signal), 6

Sampling point (negative signal), 7 Groundwater flow direction (Sampling points: 1. Valle Scurosa, 2. Scarzito Riv., 3. Sorti, 4. Grottone karst spring, 5. S. Giovanni spring, 6. Agolla creek, 7. Madonna dei Calcinai spring, 8. Potenza River, 9. Figareto spring, 10. Chienti River)

Table 1 Results of the first series of test (aquifer connection) with Fluorescein tracer, obtained by the output of the QTracer2 programme

Monitoring point	Distance from the injection (km)	% Of tracer recovered	
		1st/3rd Tests (%)	2nd Test (%)
Grottone k.spring	1.8	51–63	31
S.Giovanni spring	2.0	4–6	7
Figareto spring	3.5	10	11
M. dei Calcinai spring	3.4	9	7
Sorti brook	1.65	2	3
Agolla creek	2.5	0.4	<0.1
V.Scurosa creek	2.6	6	10
Scarzito lin.springs	2.4	0.9	–
Chienti River	5.6	<0.1	<0.1
Potenza River	6.9	–	6

Distance from the injection point are estimated on the basis of the expected flowpath. The 1st and 3rd tests were made during the recharge period (values are reported as a range), the 2nd test at the end of the recharge period

Table 2 Results of the second series of tests (groundwater flowpaths) with Fluorescein tracer, obtained by the output of the QTracer2 software

Monitoring point	Distance from the injection (km)	% Of tracer recovered		Mean tracer transit time (hours)		Mean tracer velocity (m/day) ^a	
		1st Test (%)	2nd Test (%)	1st Test	2nd Test	1st Test	2nd Test
Grottone k.spring	1.8	33.2	57.1	62.1	14.6	702 ± 194	3080 ± 428
S.Giovanni spring	2.0	6.9	5.9	417.8	391.2	114 ± 24	121 ± 27

1st test performed at the end of the recharge, 2nd test during the recharge period

^a The uncertainties are given in output by the QTracer2 software and concern the standard errors involved in the estimation procedure of the hydrogeological parameters

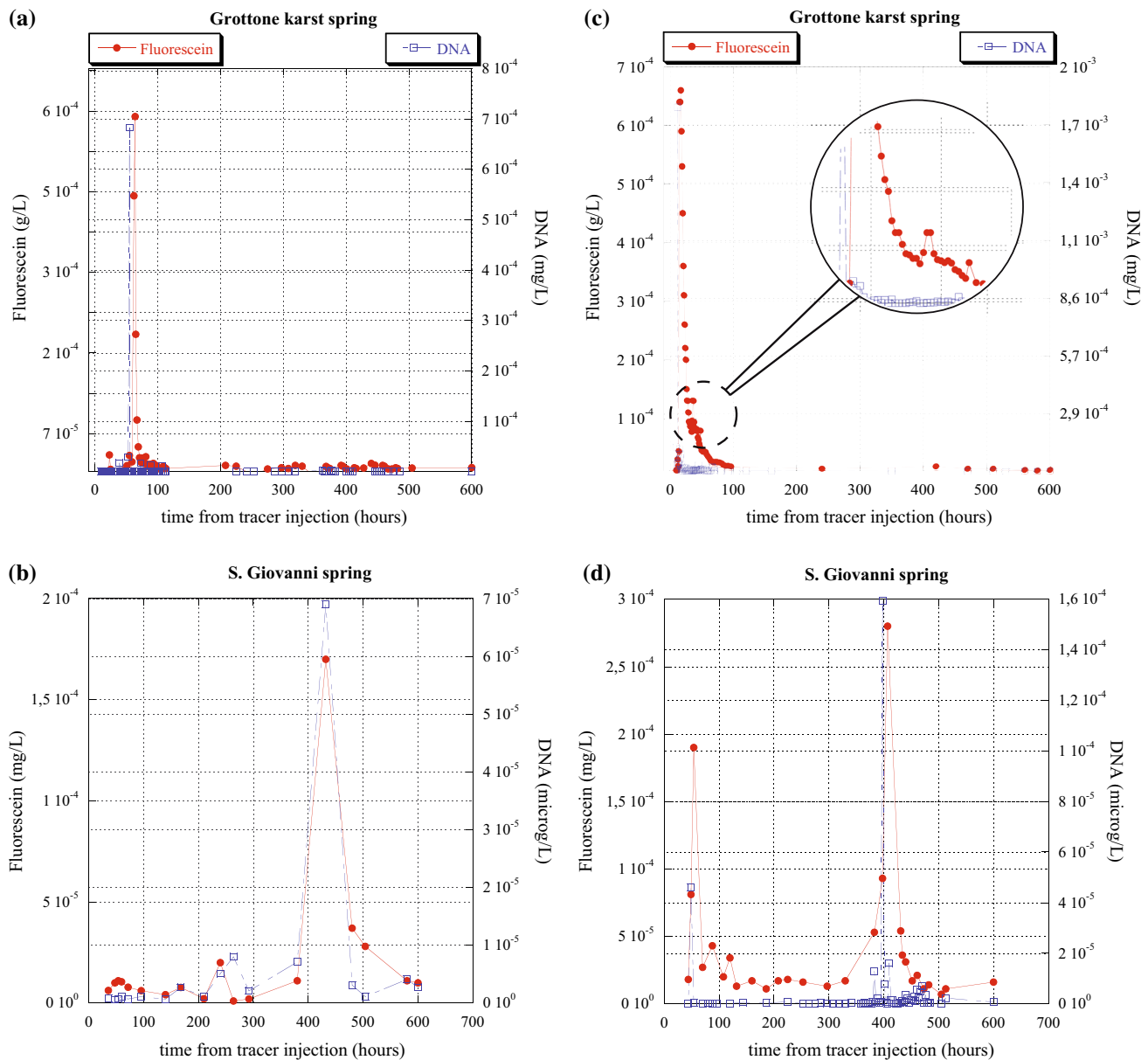


Fig. 7 Second series of tests. DNA and Fluorescein Breakthrough curve (tracer concentration versus time from tracer injection) concerning the Grottone karst spring and the S. Giovanni spring during the first test (a, b) and the second test (c, d recharge conditions)

transit time is equal to 62 h and the mean tracer velocity is around 700 m/day (Table 2). The DNA and fluorescent dye BTCs are comparable and show a similar behaviour. This period activates rather fast groundwater drainage, moving through a very shallow preferential pathway in the unsaturated and karstified zone of the Calcare Massiccio limestone. At the same time, deeper percolation occurs from the sinkhole, through the unsaturated zone of the Calcare Massiccio limestone of the Sefro anticline down to its saturated zone, thus reaching the S. Giovanni spring after about 410 h from tracer injection (Fig. 7b); the mean tracer velocity is roughly 100 m/day.

This graph and the difference in the mean tracer time between the S. Giovanni spring (partly recharged by the basal flow) and the Grottone karst spring highlight the occurrence of two flowpaths in the groundwater. In low flow conditions, only one-third of the tracer arrived at the Sefro anticline (Table 2).

Second test (April 2013)

During the recharge period, surface water infiltrates into the sinkhole and arrives at the Grottone karst spring (Fig. 7c) in a shorter lapse of time (mean tracer transit time equal to 11.9 h, tracer velocity just above 3000 m/day). Even the S. Giovanni spring (Fig. 7d) showed a first sign of tracer after only 48 h. In general the BTCs show main well-marked peaks and secondary peaks, although much smaller. This can be attributed to the very fast groundwater circulation in the upper part of the thick unsaturated zone of the Calcare Massiccio aquifer, occurring both in somewhat direct circuits (main peaks in the BTCs) and in the more complex and dense network of conduits (secondary peaks). This circulation is to be considered a peculiar feature of the entire seasonal rain period. In the Grottone karst spring the effect of this fast water flowpath is completed within about 100 h and no other peaks were observed; in the S. Giovanni spring, on the other hand, a further main peak can be observed on the right side of the BTC (Fig. 7d). The spring is reached by water through deep and slower percolation, and the tracer peak is after about 370 h, with a flow velocity a little higher than the first test (120 m/day). The DNA and Fluorescein BTCs are comparable.

General remarks on the tracer test results

The results obtained in the present work comply with a previous study (Aquilanti et al. 2013), when a tracer test was done during recharge conditions (flow rate equal to 0.07 m³/s), highlighting the existence of a very fast flowpath mainly occurring in karst conduits and fractures; the vast majority of the tracer moved towards the Sefro

hydrogeological-tectonic system very rapidly. The present results allow for the drawing of a first schematic model of groundwater circulation: from the unsaturated zone of the Maiolica limestone (Agolla syncline), the groundwater pathway reaches the Calcare Massiccio limestone of the Sefro anticline (Fig. 8). In general, surface waters infiltrating the Montelago sinkhole are split into different parts and follow different paths.

In particular, in the Sefro anticline specific conditions exist such that the development of a perched aquifer in the upper part of the unsaturated zone of the Calcare Massiccio limestone can be deduced. In this zone, rapid groundwater flow occurs both in large and small karst conduits (green arrows, Fig. 8), recharging the Grottone karst spring and the S. Giovanni spring (emerging at lower altitude) very quickly during the rainy season. A second pathway, represented by the blue arrows in Fig. 8, indicates the deep water percolation recharging the basal aquifer (Calcare Massiccio) and emerging at the S. Giovanni spring. The results of the first series of tests (see “[Aquifer connections \(first series of tests\)](#)”) also highlight the occurrence of a third pathway of groundwater flow directed towards the Mt Primo tectonic structure (green arrow on the right side of Fig. 8).

As a consequence of these findings, the S. Giovanni spring (utilised for drink-water supply) may be exposed to pollution phenomena especially during the recharge period, owing to the specific recharge features previously mentioned. In fact, in addition to the already known baseflow recharge, the spring also receives a contribution from a rapid and shallow flowpath occurring in karst conduits; the transit time of a hypothetical pollutant from the sinkhole area (where cattle and sheep are bred) to the spring would be very short. The pollution risk is hence very high in this area.

Added value of DNA tracer and open problems

A closer look at DNA tracer behaviour and other issues that are still open needs to be taken. DNA is a complex polymeric macromolecule, with a peculiar tridimensional structure, which greatly affects its chemical properties as well as its transport behaviour. Being long polymers, ssDNA tracers migrate through porous media and rock fissures through changes in their DNA configuration, which allows single molecules to reorientate in the flow direction, parallel to their length. New experiments are currently been run even in the porous media of the alluvial plains. These features imply that DNA is insoluble in groundwater and travels in suspension as a particle/colloidal tracer, thus characterised by an almost pure advective transport in fissured media. Therefore, it shows breakthrough curves characterised by more or less high pulses, rather than the

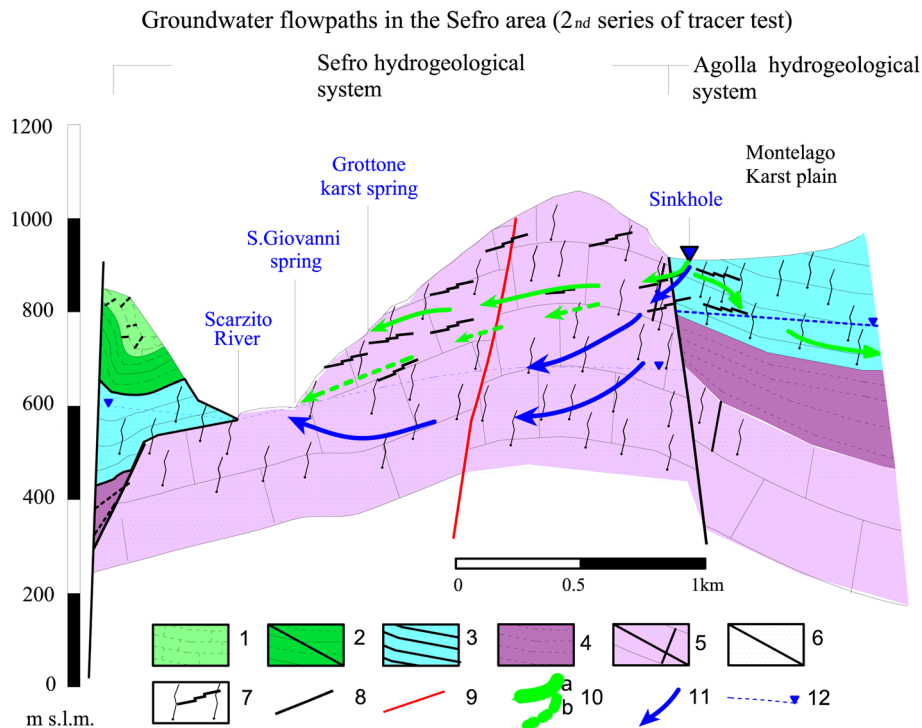


Fig. 8 Simplified scheme of the hydraulic contact between the limestones of the Maiolica (Agolla hydrogeological system) and the Calcare Massiccio (Sefro hydrogeological system). The interpretation is consistent with the tracer test results: a fast and shallow groundwater flowpath towards the Grottone and the S. Giovanni springs as well as deeper and slower percolation. The deep percolation recharges the basal aquifer of the Sefro anticline drained by the Scarzito River and the S. Giovanni spring. On the right side only the first part of the flowpath moving towards NorthEast (Mt

Primo anticline) is reported. 1 Scaglia limestone aquifer, 2 Marne a Fucoidi marly aquiclude, 3 Maiolica limestone aquifer, 4 Jurassic aquiclude, 5 Calcare Massiccio limestone aquifer, 6 Saturated zones, 7 Zone with karst conduits and fissures at high hydraulic conductivity, possible place of a perched aquifer, 8 Jurassic faults, 9 Faults, 10 Fast flowpath in the unsaturated zone of the aquifers: (10a) ever present, (10b) activated only in the recharge period, 11 Slow percolation recharging the basal aquifer by fissures and micro-karstification, 12 Mean water table

classical nearly Gaussian shape given by the hydrodispersive behaviour of traditional soluble tracers. The method of moments (Maloszewski and Zuber 1992) cannot be applied to DNA BTCs, making the hydrogeological parameters difficult to estimate and this is the first problem to be faced in future experiments, if DNA is also to be used for transport parameter determination. In addition, the aforementioned preceding concentration peak (as seen in “Groundwater flowpaths (second series of tests)” and Fig. 7, where a shift of 1–5 h can be observed) is a peculiar property of DNA (Sabir et al. 1999) and is related to its transport mechanism: the DNA tracer travels in suspension (not in solution) and its molecule (capable of re-orienting in the flow direction) is less retarded by diffusion and sorption phenomena than (soluble) traditional tracers. Furthermore, the shape and dimension of its molecules can play a role, creating a type of “size exclusion effect” showing selective pathways, as was reported by Ptak et al. (2004) and Tazioli and Palpacelli (2013). DNA molecules prefer large conduits of flow, in which they travel at higher velocity, especially in the limestone rocks. This kind of behaviour, however, can

be a valuable tool especially for contamination studies, where the DNA tracer can be tested in order to evaluate the confidence time of pollution (i.e., the minimum time necessary for a molecule of pollutant to be transferred from the contamination source to the tapped spring). This issue is worth considering further, together with its durability in the field (about 1 month), making DNA particularly useful in rather rapid circuits.

On the other hand, Fluorescein is soluble and completely dissolves in water, showing a more marked dispersive behaviour even in fissured media, as do the majority of traditional tracers.

Another value of the DNA tracer is related to its physical behaviour in water: it produces no significant change in water density and viscosity, is not visible to the unaided eye and its use is not dangerous for the environment (Aquilanti et al. 2013; Sabir et al. 1999). The latter is a key issue especially in very populated areas, where unnecessary alarms can distress citizens.

However, the real added value of DNA in these types of rocks is represented by its very low detection limit, some orders of magnitude higher than traditional tracers. In fact,

starting from an initial tracer mass of 10^{16} ssDNA molecules (injected into the sinkhole), the analytical procedures led to a detection limit of around 10^{-9} mg/L.

The Fluorescein detection limit is usually from 3×10^{-6} to 2×10^{-5} mg/L (depending on the instrument and the utilised procedure), therefore the DNA tracer determination is better even in recharge conditions, when the aquifer volume is very high and traditional tracers need to be used in enormous amounts to be equally detected, making DNA a valuable and engaging choice especially in karst and fissured media (usually characterised by high aquifer volumes).

Conclusions

Tracer techniques are crucial for the verification of hydraulic contact among aquifers and the study of groundwater flowpaths; besides the traditional fluorescent tracer, the DNA tracer used in this study provided good results in the field, yielding it as an excellent method for flowpath determination especially in karst systems.

The first series of tracer tests highlighted the hydraulic contact among aquifers belonging to different hydrogeological complexes, not always determinable by a traditional hydrogeological field survey. The Maiolica limestone of the Agolla syncline shows hydraulic continuity with the Calcare Massiccio limestone of the Sefro anticline. The main direction being from South (Montelago-Agolla) to North and to the North-West concerning the Sefro anticline, whereas secondary pathways, directed towards the Mt Primo anticline, were found in all periods of the hydrologic year. In addition, other secondary flowpaths were identified towards the West and South-West during the recharge period, when maximum velocity reached some kms per day.

The second series of tracer tests provided important elements necessary for the definition of the infiltration, percolation and water circulation in the unsaturated zone of the Maiolica limestone and the Calcare Massiccio limestone. From the sinkhole, the infiltrating water partly reaches the Grottone karst spring and the S. Giovanni spring through shallow conduits in a very short time and partly percolates deeper down to the baseflow, contributing to its recharge. As the S. Giovanni spring is tapped for drinking purposes, the results stress the importance of tracer test for assessing the vulnerability of the springs recharged by these aquifers.

In conclusion, the tracer tests have proven to be a reliable tool to provide evidence of connections between different aquifers; DNA tracer is detectable at very low concentrations thus allowing for more precise tracer determination. This is true especially when aquifer volumes are very high and traditional tracers cannot be

detected, unless an enormous mass of tracer is injected. Actually, due to its specific features, the DNA tracer is very suitable for vulnerability studies involving aquifers characterised by fast groundwater circulation and a high pollution risk.

Although the results obtained have considerably increased knowledge of the groundwater circulation in the Montelago area, supplementary investigations in a wider area of the central Apennines are to be scheduled to better define connections among different hydrogeological complexes and to outline the groundwater circulation at a regional scale.

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