Use of particulate surrogates for assessing microbial mobility in subsurface ecosystems

Michael Sinreich · Raymond Flynn · Jakob Zopfi

Abstract Mass fluxes from the ground surface can play a vital role in influencing groundwater ecosystems. Rates of delivery may influence intact ecosystem composition, while fluxes of substances associated with anthropogenic activity may critically alter the functioning of associated microbial assemblages. Field-based tracing experiments offer a valuable means of understanding mass transport rates and mechanisms, particularly in complex heterogeneous epikarst systems overlying vulnerable fissured aquifers. A short-term tracer experiment monitoring solute and particle tracer concentrations after they passed through a 10-m-thick sequence of limestone, capped by a thin soil, revealed rapid travel times and variable attenuation rates for the substances employed. Results demonstrated that particle tracers have shorter average travel times and can reach the subsurface in higher concentrations and over shorter times than non-reactive solutes. High recovery rates for the bacterial tracer *Ralstonia eutropha* H16 contrasted strongly with those of similarly sized fluorescent polystyrene microspheres, highlighting the importance of physico-chemical surface characteristics of particle tracers. Complementary laboratory batch experiments examined the role played by organic and inorganic soil/rock surfaces on particle tracer attenuation. Findings suggest that biofilms may significantly promote transport of particulate material below ground, i.e., the delivery of allochthonous microorganisms to karst groundwater.

Keywords Karst · Vadose zone · Bacterial transport · Tracer tests · Switzerland

Introduction

Microorganisms are ubiquitous in the environment and hence natural assemblages can also populate groundwater ecosystems. Natural fluxes of matter reaching the subsurface provide important energy and nutrient sources for groundwater ecosystems, while also providing a means of microbial colonization (Goldscheider et al. 2006). Conversely, infiltrating anthropogenic dissolved and particulate contaminants released at the land surface may alter the natural composition of autochthonous groundwater assemblages. Such contamination may contain allochthonous pathogenic microorganisms, such as viruses, bacteria, and protozoa. These can have adverse effects on groundwater quality which in turn can impact human health (Macler and Merkle 2000). In areas supplied with karst groundwater, these pathogens may be responsible for water-borne disease outbreaks (Howard 2003). Interactions between autochthonous and allochthonous microorganisms in the subsurface remains poorly characterized and require further investigation to permit the effects of human activities on natural ecosystems in the deeper subsurface (below the soil zone) and on groundwater quality to be evaluated. Knowledge of mass-transport processes operating in the vadose zone overlying aquifers can provide critical information for determining rates of contaminant transfer to groundwater.

A substantial body of research investigating the fundamental processes controlling microbial transport has been carried out at the laboratory scale, often using batch studies and dynamic columns filled with porous media (Mills et al. 1994; Bengtsson and Lindqvist 1995; Flynn et al. 2004; Franklin and Mills 2005; Foppen et al.

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2007). Well-established theories exist for explaining and modeling particle transport through homogeneous porous layers (McDowell-Boyer et al. 1986; Murphy and Ginn 2000). Many of these have been further developed to account for biological processes such as motility, inactivation, and temporal changes in bacterial surface properties (Ginn et al. 2002). However, findings obtained under controlled experimental conditions are not always easy to extrapolate to the field. Discrepancies may arise from the intrinsic spatial and temporal variations encountered in rock and soil. This may cause critical processes such as preferential flow, which permit contaminants to by-pass deposits that may otherwise attenuate them (Foppen and Schijven 2006). Such processes have been demonstrated to be fundamental in influencing groundwater quality in highly heterogeneous karst aquifers, as indicated by the rapid occurrence of coliforms at karst springs following rainfall events in many studies (Personné et al. 1998; Auckenthaler et al. 2002; Shevenell and McCarthy 2002; Pronk et al. 2006).

Although pathogen contamination is an important issue in karst hydrogeology (Drew and Hötzl 1999), very few studies deal with microbial transport and attenuation processes in the subsurface. While bacteria have been demonstrated to be highly mobile in saturated karstified media (Mahler et al. 2000), the processes influencing attenuation that may arise in the overlying soil and epikarst layers remain poorly characterized. Heterogeneity and possible rapid recharge suggest karst environments may be susceptible to both natural and anthropogenic microbial input. Consequently, knowledge of the microbial mobility in the subsurface can be of importance in

better understanding associated ecosystem composition and functioning.

High levels of heterogeneity encountered in karstified rock require transport and attenuation processes be investigated in situ. Field-scale investigations provide a means of studying the significance of temporal and spatial variability in rock and soil properties on solute and particle mass transport in the subsurface (Harvey et al. 1993; Mailloux et al. 2003; Sinreich and Flynn 2006). Moreover, such tests are necessary to evaluate the significance of processes, examined at the laboratory scale, in natural settings. Figure 1 provides an overview of relevant processes that may operate in karst environments and how they affect microbial mobility. Those processes that determine the transport of pathogenic bacteria in the subsurface may be classified into three groups (Ginn et al. 2002; Foppen and Schijven 2006): hydraulic properties of the media, abiotic interaction with surfaces encountered, and biological processes specific to microorganisms, e.g., inactivation.

Artificial tracers provide a useful means of investigating mass-transfer rates in highly heterogeneous systems, including karstified rock, where preferential flow paths can result in rates that are often orders of magnitude higher than those naturally encountered in porous media. Comparative tracer experiments, contrasting the responses of particulate tracers (acting as micro-biological surrogates) to non-reactive solutes, permit particle fate and transport in the subsurface to be investigated in further detail. However, due to incomplete characterization of a system, tracer-test results often provide limited conclusive data when viewed in isolation. On the other hand,
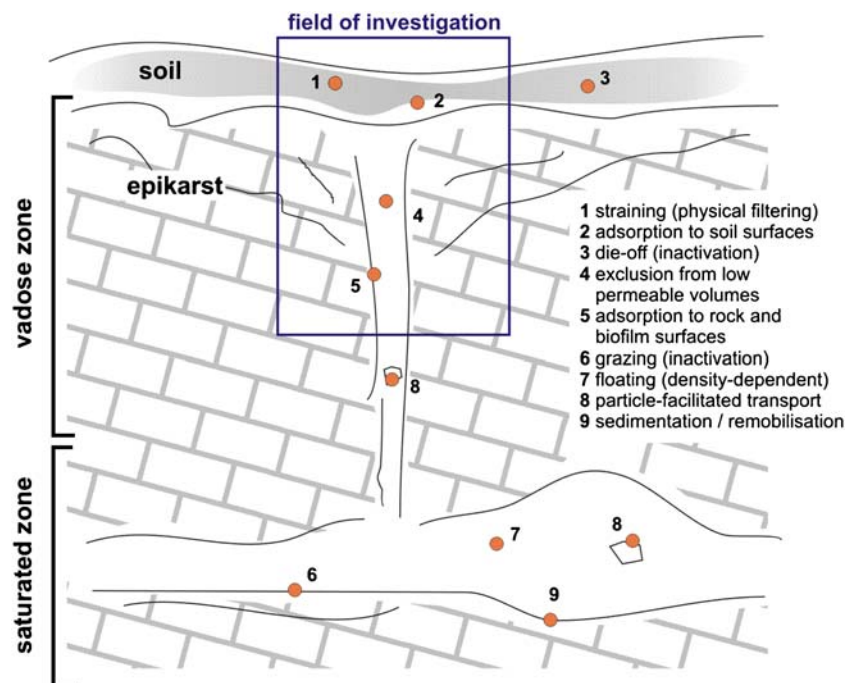

Fig. 1 Possible processes operating in the vadose and saturated zone of karst aquifers that may affect microorganism mobility

combining these results with those of controlled laboratory investigations can be very helpful in better explaining non-conservative tracer responses in the field and constraining potential attenuation mechanisms.

Several authors have identified the benefits of employing particulate tracers instead of solutes for assessing the risk of particulate contamination impacting groundwater quality in a range of hydrogeological environments (including karst), where fluorescent microspheres have been widely used as surrogates for bacteria (Harvey et al. 1989; Harvey and Harms 2002; Auckenthaler et al. 2002) and protozoa (Renken et al. 2005) of comparable size. However, although particle size is frequently the dominant criterion considered in microsphere selection, conclusive evidence of their suitability has rarely been demonstrated. Moreover, further questions concerning the utility of microspheres arise partly due to their inability to simulate biological processes, including inactivation, reproduction, motility, and temporal variations in surface properties arising in response to changing environmental conditions. The need to study these processes requires the use of living organisms. However, several prerequisites must be fulfilled to allow microorganisms to be employed as hydrological tracers in field studies without impacting the ecosystem or human health. Käss (1998) provided a detailed overview of these requirements (e.g., non-pathogenicity, limited natural background, detectability, persistence) and notes that few microorganisms satisfy most of the criteria listed.

Given these restrictions, the number of strains employed to date has been limited (Keswick et al. 1982; Hötzl et al. 1991; Harvey and Harms 2002; Ginn et al. 2002). Moreover, the continued use of a number of hitherto widely employed bacterial species has been drawn into question because of the potentially pathogenic properties of some strains. *Serratia marcescens* has been recognized as an agent for nosocomial infections in hospitals (Enciso-Moreno et al. 2004). Similarly, *E. coli* strains with genes for metal tolerance or antibiotic resistance cannot be used, as the introduction of antibiotic resistance genes into the environment needs to be avoided. Furthermore, the release of genetically modified organisms is under strict legal control and currently not allowed in most countries. These constraints highlight the need to find alternative bacterial tracers.

This paper presents an example of a tracer experiment completed through the vadose zone of a karstified limestone employing a non-reactive solute tracer, fluorescent microspheres, and a similarly sized bacterial strain, *Ralstonia eutropha* H16; the latter is used for the first time as microbial tracer in hydrological studies. The combined implementation of both laboratory-scale and field-scale experiments provides a potential approach for drawing more definitive conclusions on microbial transport processes operating in the subsurface. Comparison of bacterial tracer and microsphere tracer responses, with that of a conservative solute, provided a means of allowing the mobility of allochthonous microorganisms entering groundwater ecosystems to be better assessed while simultaneously permitting the degree of protection the vadose zone affords against infiltrating contaminants to be evaluated.

Materials

Bacterial tracer

This study uses the H16 strain of *Ralstonia eutropha* (*R. eutropha*), a naturally occurring Gram-negative β-*Proteobacteria*, as a microbial groundwater tracer. *R. eutropha* is a ubiquitous inhabitant of soil and freshwater environments, including groundwater. The H16 strain was isolated from freshwater spring sludge. It is a metabolically versatile organism that grows heterotrophically on a wide range of organic compounds, but also autotrophically using hydrogen and CO_2 as energy and carbon sources for biomass formation, respectively. Cells of *R. eutropha* are rod-shaped, motile, and typically measure 0.5×1.8–2.6 μm (Aragno and Schlegel 1992). In contrast to other species and strains, *R. eutropha* H16 has never been recognized as a human, animal, or plant pathogen (Pohlmann et al. 2006).

The *R. eutropha* cultures used for the present study were cultivated in an autotrophic liquid mineral medium for Knallgas bacteria (Aragno and Schlegel 1992) under an atmosphere of 80% H_2, 15% CO_2, and 5% O_2. After 4 days at 23°C, the culture reached an optical density (OD550) of about 1, corresponding to a cell density of ca. 10^9 cells/mL. This culture was either used directly in the field experiment or was diluted in filter-sterilized (0.45-μm pore size) spring water to about 1,000 cells/mL for the laboratory experiments.

Quantification of *R. eutropha* in water was done by adding 0.1–1 mL of sample water to 5 mL of sterile physiological water (9 g/L NaCl). After thorough mixing, samples were filtered through a sterile 0.2-μm membrane filter. The filter was subsequently placed on solidified mineral medium (1.5% agar) devoid of any organic substrates and incubated under the same atmosphere as described above. After 3–4 days at 23°C, hydrogenase-containing colonies (i.e., *R. eutropha*) were stained with 0.1% w/v triphenyl tetrazolium chloride (TTC) solution as described in detail by Aragno and Schlegel (1992) and then counted. This staining permits hydrogenase-containing colonies to be distinguished from those lacking the enzyme, which may sometimes grow on the mineral medium plates.

Microsphere tracer

Microspheres have been shown to be innocuous and easy-to-use tracers for studying particle transport and attenuation processes in karst systems; they are easy to apply and to subsequently detect (Käss 1998). Unimodal 1-μm carboxylated polystyrene microspheres (Polysciences) were selected to act as an abiotic particulate tracer simultaneously applied with *R. eutropha*. The use of microspheres with similar size and density to *R. eutropha*
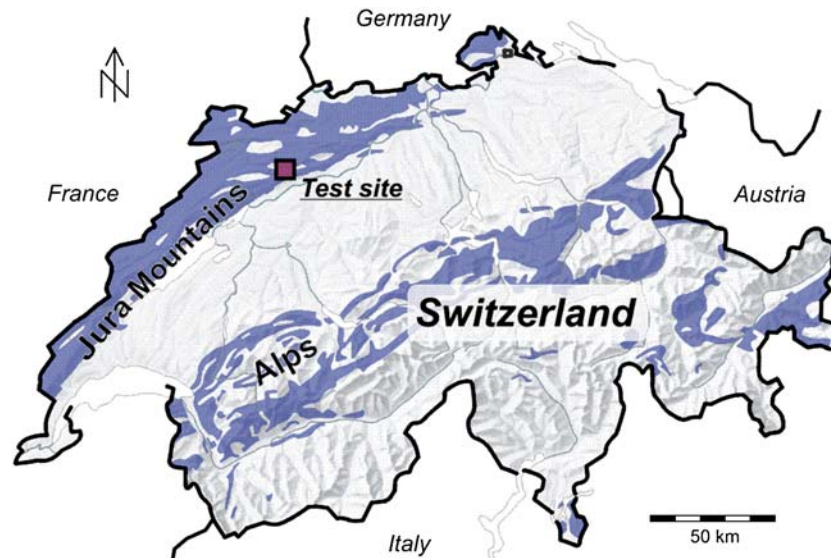
Fig. 2 Distribution of karstified rock in Switzerland (FOEN 2008) with test site location in the Swiss Jura Mountains

permitted the influence of abiotic physical properties on particle transport to be evaluated. Additionally, 3-μm microspheres of identical composition were employed in the field to encompass the size range of the rod-shaped bacterium (their field response demonstrated similar behavior to those of the 1-μm microspheres). For the purposes of this paper, only the 1-μm microspheres will be considered.

For the field experiment, 5 mL of 2.6% w/v fluorescent 1-μm microspheres were used. Quantification of field samples was completed by particle counting under a fluorescence microscope following brief agitation in an ultrasonic bath with identification conducted according to size, specific fluorescence, and shape. Tracer dilution or filtration enrichment permitted particle-tracer concentrations over three orders of magnitudes to be determined. Since tracer-output concentration in laboratory experiments can be better controlled than in field experiments, a less elaborate method may be employed for microsphere concentration assessment. Instead of counting individual microspheres, the overall fluorescence of a water sample was measured by means of fluorometry (Ward et al. 1997).

Methodology

Field tracing experiment
Field experiments were completed at the Gännsbrunnen test site in the Swiss Jura Mountains, Canton of Solothurn (Fig. 2). An artificial tunnel excavated approximately 10 m below ground surface provided access to the vadose zone of a fissured and moderately karstified Cretaceous limestone formation (Fig. 3). This site affords a rare opportunity to investigate mass transport through the epikarst under closely controlled and undisturbed conditions. The absence of fresh stress relief features in the tunnel suggests this near-surface excavation has not altered the natural hydraulic properties of the rock.

Experiments investigating the transport between land surface and the tunnel are thus believed to reflect conditions operating in the epikarst interface between soil and limestone rock, as described by Klimchouk (2004).

The experimental protocol employed at the site consisted of simultaneously applying a non-reactive solute, microsphere, and bacterial tracers at the ground surface above the tunnel. Irrigation with tracer-free water over a 12-m² plot at rates of about 20 mm/h allowed steady-state flow conditions to be established, as validated by constant flow rates noted at monitoring points in the tunnel.
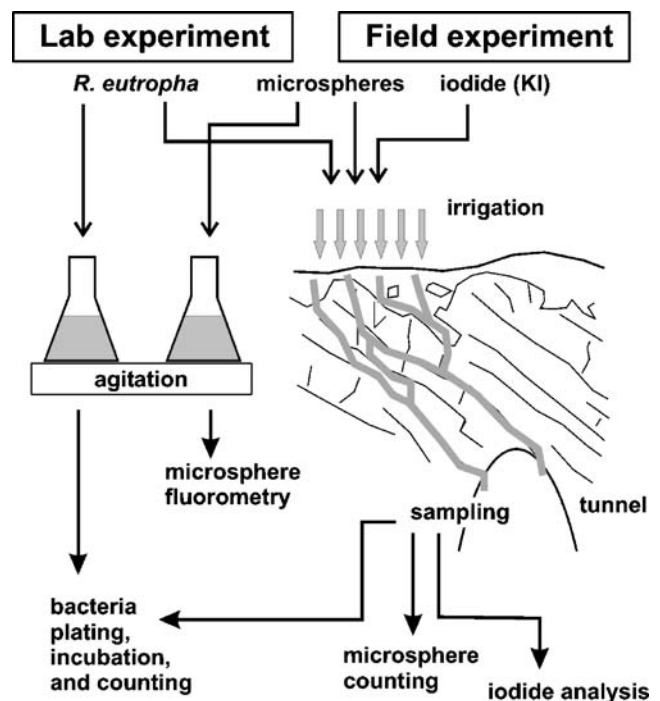

Fig. 3 Field and laboratory setups used in the experiments with tracer application and analyses procedures
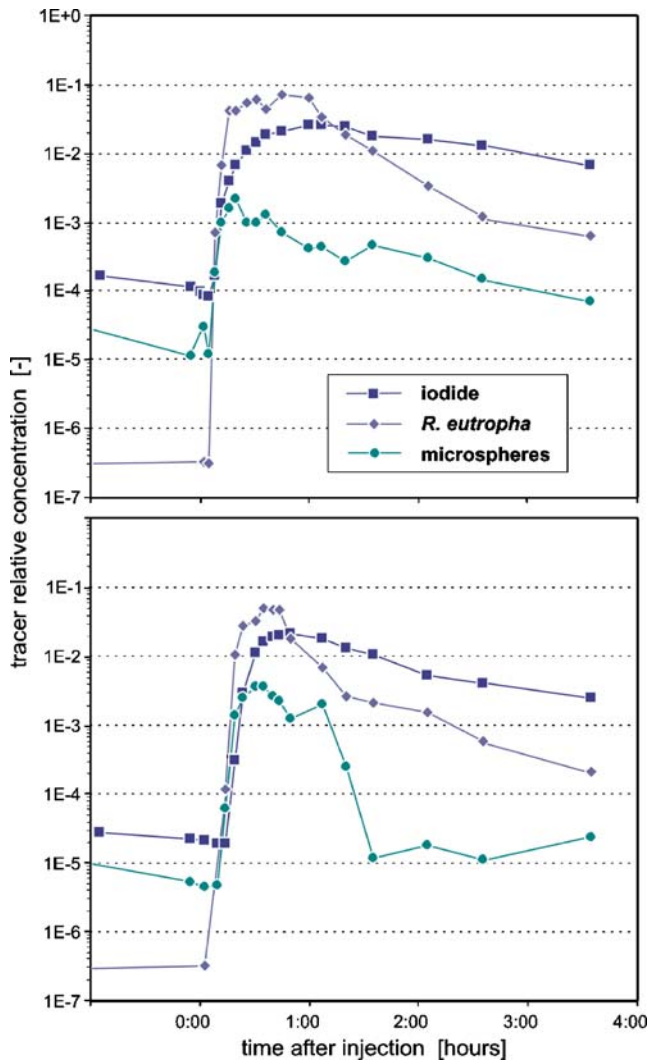
Fig. 4 In situ experimental breakthrough curves for *R. eutropha*, 1-μm microspheres, and iodide as conservative solute reference tracer at two different representative sampling points (values plotted relative to input concentration; estimated errors: iodide 5%, *R. eutropha* 20%, microspheres 10%)

lation points allowed tracer concentration variation with time to be monitored and breakthrough curves to be constructed following analyses (Fig. 4).

Laboratory-scale experiments

Batch experiments were conducted in order to investigate particle attenuation mechanisms in more detail, but under physico-chemical conditions similar to those encountered in the field experiment. More specifically, the tests permitted the temporal variation in suspended particle tracer concentrations to be evaluated upon exposure to different substrates (Fig. 3). Duplicate microscosm tests employed 200-mL conic flasks filled with 100 mL of water, which had hydrochemical characteristics close to the irrigation water employed for the field experiment (low mineralization and slightly basic pH, Table 1).

Two sets of batches investigated the attenuation capacity of the three different substrates (microcosms) possibly encountered by particle tracers in the field-based experiment. The first microcosm consisted of 5 g of organic-rich soil material (fraction < 2 mm) sampled from within 10 cm of the ground surface overlying the test site. A second microcosm contained 100 g of freshly crushed limestone gravel with a mean diameter of 5 mm, which had been autoclaved before application in order to inactivate biological matter (uncoated limestone). The third microcosm contained the same limestone gravel. However, prior to use, the gravel was placed in an underground karst stream for 1 year. Examination of the gravel after this time revealed that a visible biofilm had developed that coated the grain surfaces (coated limestone).

Flasks were spiked with either *R. eutropha* or microspheres, protected against evaporation by hermetically covering with parafilm, and placed onto rotary agitators. Experiments using microspheres were conducted at room temperature (about 21°C); bacterial studies were conducted at 12°C in order to provide environmental conditions similar to those encountered in the field and to avoid artefacts due to bacterial growth at elevated temperatures. Residual tracer suspension concentrations were sampled at increasing time intervals, starting immediately prior to analyses. A series of control experiments completed following the approach described above, but without soil or limestone, permitted the effects of the experimental apparatus on test results to be evaluated. All batch analyses were performed in triplicate.

R. eutropha and microspheres were mixed in a 20-L plastic watering can just prior to injection, together with iodide as the conservative solute reference tracer. Once steady-state flow conditions were reached, the tracer cocktail was evenly distributed over the surface plot, succeed by another 3.5-h period of tracer-free water irrigation.

Subsurface sampling started before tracer application and continued over the irrigation period at several percolation points in the underlying tunnel, two of which are presented in this paper. Samples were collected in 500-mL plastic bottles, from which 50 mL was decanted for both bacteria and microsphere analyses. Storage of bacteria samples in chilled dark cooling boxes prior to analyses, conducted within 24 h of collection, permitted *R. eutropha* in water to be determined with minimal risk of concentration changes due to rapid growth at elevated temperatures. Periodic sample collection from the perco-

Table 1 Water physico-chemical characteristics during field and laboratory experiments indicating comparable environmental conditions for both setups

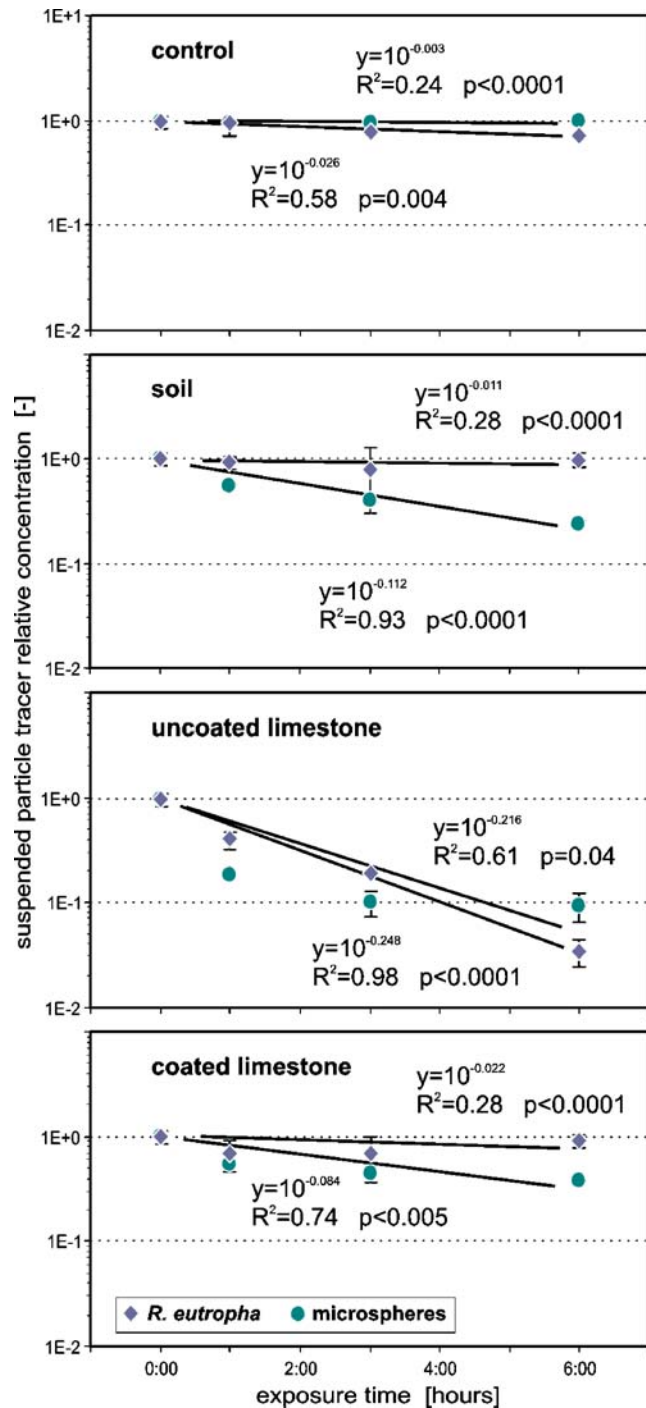|	Field experiment	laboratory tests
pH	8.0	7.9
Electrical conductivity	350 μS/cm	330 μS/cm
Temperature	9 °C	12 °C*/21 °C**
Duration	6 h	6 h

* bacteria; ** microspheres

Fig. 5 Kinetics of *R. eutropha* and microspheres reaction with different substrates during 6-h batch experiments (*symbols* are mean values derived from triplicate analyses; *error bars* indicate ± 1 standard deviation; *lines* represent regression curves starting at the initial relative concentration)

Results

In situ breakthrough curves

Analyses of water samples collected at percolation points in the tunnel prior to injection demonstrated all tracers to be present at low concentrations before tracer application.

These background values are orders of magnitudes lower than breakthrough concentrations and do not significantly affect the curves generated. Concentrations of iodide and microspheres recorded prior to injection reflect residues from previous *R. eutropha*-free tracer experiments at the site. *R. eutropha* concentrations in this phase of the experiment can be considered as the natural background levels of allochthonous hydrogenotrophic bacteria passing down from the overlying soil.

Figure 4 presents the breakthrough curves sets for iodide, *R. eutropha*, and the 1-µm fluorescent microspheres generated from the results of sample analyses collected in the tunnel at two representative percolation points. Particle-tracer breakthrough responses contrast strongly with those for the solute. However, comparable responses at both sampling points, as well as observations at other locations in the tunnel demonstrate the consistency of bacterial and microsphere analyses.

Tracer-breakthrough curves generated for *R. eutropha* show it first reaching the tunnel at concentrations above background levels approximately 10 min after injection. Levels subsequently rose to maximum concentrations at a number of sampling points at between 30 and 60 min following injection. Simultaneously injected conservative solute tracer displayed a more prolonged breakthrough curve response with a lower maximum relative concentration than *R. eutropha*. Furthermore, bacteria showed a higher recovery relative to iodide over the duration of the test.

Initial microsphere detection occurred at a similar time to *R. eutropha*. However, microsphere levels peaked earlier and had maxima 15 to 30 times lower than those of the bacteria. Both tracers had similar rates of decline, as reflected by the breakthrough curve tails in the later part of the experiment. Microsphere mass recovery is about 2% of that of *R. eutropha*. Concentrations observed at the end of the monitoring period at levels close to those noted prior to tracer injection indicate that breakthrough of the particulate tracers was almost complete. Once again, this contrasted to the high residual levels of iodide remaining in the system at this time.

Laboratory batch kinetics

Figure 5 presents batch experiment results for samples collected during the first 6 h of exposure to different microcosms. In order to quantify attenuation that both particle tracers experienced during this period, attenuation rates were calculated (Table 2) based on best-fit log-linear

Table 2 Attenuation-rate constants for the particle tracers during batch experiments deduced from regression curves in Fig. 5 (*errors* represent 1 standard deviation based on triplicate analyses)

	R. eutropha	Microspheres
Control	0.06 ± 0.01 h^{-1}	0.008 ± 0.002 h^{-1}
Soil	0.02 ± 0.03 h^{-1}	0.26 ± 0.02 h^{-1}
Uncoated limestone	0.57 ± 0.02 h^{-1}	0.50 ± 0.07 h^{-1}
Coated limestone	0.05 ± 0.03 h^{-1}	0.19 ± 0.02 h^{-1}

regression through origin (starting relative concentration set as 1), accounting for variability encountered in triplicate analyses. Such an approach assumes first-order kinetic processes, as mostly used for bacterial adsorption and desorption in porous media (Johnson et al. 1995; Foppen et al. 2008).

Laboratory experimental duration corresponds to the time frame for in situ sample collection at the site. No significant decline in suspended fluorescent microsphere concentrations were noted during the control experiment, suggesting that concentration variations observed in the presence of substrates in microcosm reflect the influence of the solid material added. However, variations in *R. eutropha* concentrations during this time in the control experiment cannot be fully explained by the analytical error. A slight decline in suspended concentrations due to adhesion to the apparatus or slow inactivation over the duration of the experiment cannot be excluded.

Batch experimental results for the soil and coated limestone microcosms indicate viable *R. eutropha* concentrations in suspension did not differ significantly from the experimental control (i.e., not more attenuated than for the experimental control, Table 2). This contrasts with the large difference in viable suspended *R. eutropha* concentrations observed with the uncoated limestone microcosm. Under these latter conditions, *R. eutropha* levels declined strongly over the 6-h period. As with *R. eutropha*, microsphere concentrations declined most rapidly in the uncoated limestone assay. However, in contrast to the bacteria, a decline in suspended microsphere concentration was also observed in the presence of soil and coated limestone. This attenuation process took place at a quasi-constant rate over the experimental time frame, which suggests first-order reaction kinetics.

The results obtained from laboratory batch experiments suggest that *R. eutropha* could be a suitable tracer under the conditions encountered during the field experiment. However, as shown by additional tests (not presented) conducted at longer time periods and/or at 30°C, *R. eutropha* has the capacity to grow on soil organic matter and aquifer material at higher ambient temperatures. These constraints may limit the applicability of *R. eutropha* as a groundwater tracer in other studies, i.e., when prolonged residence times and/or elevated temperatures are anticipated.

Discussion

Field-based tracing results obtained at the test site demonstrate that both *R. eutropha* and the microspheres can travel rapidly through the epikarst separating the irrigation point at the ground surface and the sampling points in the tunnel. In contrast, the solute tracer response shows that more of the iodide tracer still remained in the overlying vadose zone by the time almost all of the bacterial tracer had already been recovered. This response demonstrates that particles traveling through the epikarst may be delivered to the water table at faster rates and have

shorter overall residence times than non-reactive solutes derived from the same source. Such particle behavior, due to spatial variations in hydraulic conductivity and associated differences in pore aperture, has already been reported from porous aquifers (Toran and Palumbo 1992; Flynn 2003), and fractured media (Champ and Schroeter 1988; McKay et al. 2000).

Despite having similarly shaped breakthrough curves, the high rates of *R. eutropha* recovery contrast significantly with those of the microspheres and reflect the differing levels of attenuation experienced by each tracer. Contrasts in attenuation of microspheres and microbial tracers are also reported from studies in porous media (Lindqvist and Bengtsson 1995; Harvey et al. 1989). Complementary data from laboratory batch experiments indicate that inactivation/reproduction does not significantly affect bacterial concentrations over the time frame of the field experiment. Moreover, abiotic microspheres are not affected by this process. These points suggest that the particle attenuation observed reflects the influence of differential adsorption rates. Figure 6 shows that as the experiment proceeds, an increasing divergence in concentrations and recovery of both particulate tracers occurs. This reflects the influence of time-dependent kinetic processes that cause much higher attenuation of the microspheres relative to the bacteria. While the bacterial tracer may reflect predominantly advective-dispersive particle transport, the behavior of abiotic microspheres demonstrates higher rates of kinetic adsorption.

Understanding particle adsorption to fixed surfaces (filtration theory) conventionally views the process as consisting of two steps (Kretzschmar et al. 1999). These fundamental steps have been assumed to be also applicable to particle attenuation in fissured karstified rock (Flynn and Sinreich 2005). How often particles strike surfaces is expressed by collision frequency, while the probability that they might stick to these surfaces is expressed by the collision efficiency term (Elimelech et al. 1995). Collision efficiency is mainly a function of the physico-chemical surface properties of both the particles and the surfaces
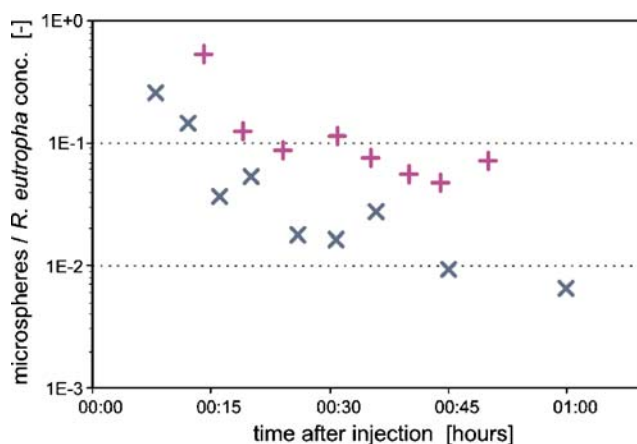

Fig. 6 Differences in particle-tracer responses indicating increasing divergence as the field experiment proceeds. Values are the ratio of microsphere and *R. eutropha* concentrations as obtained from Fig. 4 for the two sampling points

encountered, such as electrostatic charge and hydrophobicity. This in turn also depends on the chemistry of the water in the system. On the other hand, collision frequency is predominantly influenced by physical aspects of the system such as particle size and the aperture of pores, as well as the flow characteristics. Scheibe et al. (2007) concluded from field-scale experiments that adsorption rate is dependent on the hydraulic conductivity of the aquifer. Consequently, short travel times and the large fissure and conduit apertures encountered in many karst systems may provide limited attenuation by particle filtration and can permit bacteria to be transported over several kilometers, as observed by Mahler et al. (2000).

Filtration theory provides a well-established means of quantifying particle removal for porous media (Yao et al. 1971). According to this, both particle tracers used in the field experiment described in this article are anticipated to access similarly sized pores and to collide with fixed surfaces at comparable frequencies given their similar physical properties (size and density). Consequently, if the frequency of collision were the dominant factor in particle attenuation, similar particle responses could be anticipated for both tracers. Since this is not the case, differences in particle surface properties (and thus collision efficiency) are implicated as the dominant factor influencing the difference in behavior. This is consistent with results from Ward et al. (2001) who examined microsphere attenuation at distinct horizons in the vadose zone of a chalk limestone aquifer.

The material(s) responsible for the differential adsorption rates cannot be deduced based on breakthrough curve responses alone. However, laboratory batch experiments have provided significant additional information that may shed further light on the mechanisms generating the contrast in particle-tracer response (Fig. 7). Microcosm experimental results demonstrate that concentrations of both particles declined at comparable rates in the presence of uncoated limestone. This contrasts with the responses observed for other surfaces. The removal of microspheres in the presence of (biofilm-)coated limestone or organic-rich soil demonstrates the capacity of both materials to adsorb microspheres. In contrast, the quasi-constant concentrations of suspended *R. eutropha* indicate that neither biofilm nor soil should have played a significant role in its attenuation in the field test. This is in agreement with former studies that have shown that microorganism attachment to surfaces encountered in the subsurface may strongly depend on aquifer mineralogy (Scholl et al. 1990), and on coatings covering mineral surfaces, respectively (Mills et al. 1994).

The comparable attenuation rates of microspheres and *R. eutropha* in the uncoated limestone batch experiments suggest that both particles have similar degrees of adsorption to this surface in the absence of organic matter coatings. Consequently, both tracers would be anticipated to have similar responses if uncoated limestone were the dominant surface responsible for attenuation. The contrast in tracer responses observed in the field suggests that this is not the case and other surfaces must be invoked to
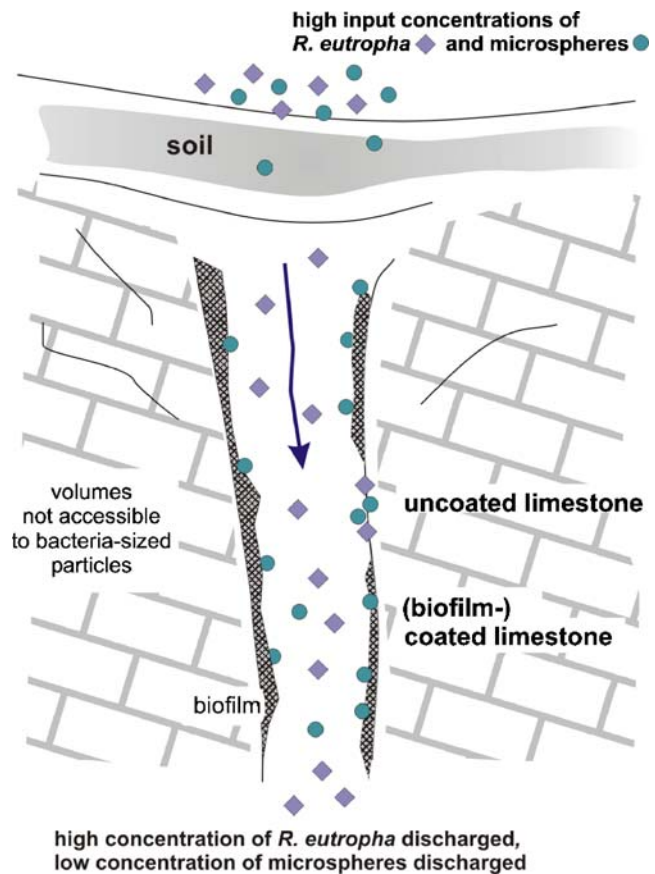

Fig. 7 Conceptual model for particle attenuation due to adsorption to fixed surfaces encountered in the vadose zone of the karst test site, established by means of coupled field and laboratory experimental results (see text for details)

explain the differing *R. eutropha* and microsphere responses.

Microbes are usually considered as negatively charged and most of them express strong to moderate cell-surface hydrophobicity (Murphy and Ginn 2000). The behavior of the particle tracers in the presence of uncoated limestone, and the absence of organic matter in the corresponding batch microcosm, suggests that the interactions between particles and the surfaces observed could be influenced by physical forces rather than hydrophobicity. However, in the case of soil and (biofilm-)coated limestone, the presence of organic matter suggests that hydrophobicity may also be important in the adsorption process. Consequently, hydrophobic adsorption is suspected to play a significant role generating the differences in microsphere and bacterial tracer responses at the field site, with microspheres being more hydrophobic than the bacteria (Lindqvist and Bengtsson 1995). Indeed, some studies indicate that biofilms may be hydrophobic (Knoll and Schreiber 1998; Goméz-Suárez et al. 2002), and that they may inhibit adsorption of some bacteria (Goméz-Suárez et al. 2002; Schaumann et al. 2007).

Moreover, the batch-test responses for the organic-rich soil overlying the test site suggest it might play a partial role in microsphere adsoprtion. However, experimental

data have shown that microsphere adsorption to this material is time-dependant. The limited thickness of this deposit and short residence time of tracer passing through the soil suggests that additional organic surfaces present at greater depth may be responsible for the degrees of microsphere attenuation observed. Experimental data have demonstrated that biofilm-coated limestone can effectively adsorb the microspheres employed in the field experiment, while having a negligible influence on suspended *R. eutropha* concentrations. The presence of comparable biofilms along flow paths employed by tracers in the epikarst is likely (Personné et al. 2004; Castegnier et al. 2006), and may thus explain the contrast in *R. eutropha* and fluorescent microsphere tracer responses.

Conclusions

A new non-pathogenic and easily cultivated microbial groundwater tracer, *Ralstonia eutropha* H16, hitherto untested in field experiments, was successfully applied to investigate mass transport through the vadose zone of a karst aquifer. The bacterium's intrinsic characteristics, assessed during complementary laboratory-scale batch experiments, and its response during short-term tracing experiments completed at a test site in the Swiss Jura Mountains show that *R. eutropha* may be effectively employed as a bacterial tracer in hydrological studies at field scale. Following such an approach is particularly useful since properties of many tracers and their inter-actions with the environment cannot always be inferred from the literature. However, the authors are aware of only one study where microsphere tracer employed to karst groundwater is compared to the response of a real microorganism in laboratory tests (Renken et al. 2005).

Solutes and particulate tracer responses may contrast significantly when being transported through the vadose karst zone. Particles may reach the water table faster, in higher concentrations, and over shorter periods of time than solutes. This confirms that non-reactive solute tracer breakthrough may not necessarily reflect microorganism responses. Furthermore, although both particulate tracers used in this study represent conditions encountered along the same flow paths, microsphere tracer application alone would not have demonstrated the potentially high micro-bial mobility in such heterogeneous environment, and particle size alone has proved an inadequate discriminant for understanding particle fate and transport at the site.

Coupled laboratory and in situ experimental results have permitted hypotheses concerning the mobility of dissolved and particulate matter in the vadose zone to be developed. Although microorganisms may interact with the materials overlying the water table during transport, they will not necessarily be highly attenuated. Conversely, particle properties promoting adsorption are capable of significantly reducing microbial mobility despite the presence of preferential flow paths in soils and carbonate rock. Biofilms present in the subsurface may alter rock surface properties and potentially play a significant role in controlling the fate and transport of allochthonous particles to groundwater. Biofilms may limit adsorption and even promote transfer of some potentially critical constituents, such as pathogens, to groundwater when they coat surfaces, which are otherwise capable of attenuation, such as limestone. Given the potentially ubiquitous nature of microorganisms in the environment, and the ability of many to form biofilms, this may be particularly significant in systems where residence times in the overlying deposits are short, such as in epikarst. As a corollary to these concepts, the use of well-characterized particle tracers may be employed to make deductions about subsurface physical and/or chemical conditions.

This study has highlighted the degree to which mass transport and attenuation depend strongly on the properties of the introduced particles and the physical and chemical conditions below ground. The findings demonstrate that potentially high fluxes of biotic and abiotic particulate matter can enter karst groundwater ecosystems during recharge events. Furthermore experimental results point out the elevated risk of potential impacts to groundwater quality resulting from anthropogenic activity in such environments. Insufficient data currently exist to permit conclusive theories concerning the behavior of solute and particle transport in heterogeneous karst environments and their influence on subsurface ecosystems to be developed. Coupled field and laboratory-based studies have the potential to make valuable contributions in this area.

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