Simulation of microbiological pollution in the unsaturated zone of karstified limestone aquifers – tracing with bacteriophages

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Received: June 30, 2005 **Accepted**: November 24, 2005

Abstract: The purpose of the research was to study the infiltration and migration of health-hazardous human viruses, such as enteroviruses, in the unsaturated zone of fractured and karstified rock, since these rocks present important aquifers in Slovenia. As a possible model for behavior of health-hazardous viruses, we used the salmonella bacteriophage P22H5. After injection, bacteriophages remain in the fractures (channels) and microfracture systems of the unsaturated zone and are rinsed by subsequent larger precipitation events even up to several months after the injection. The field experiments have shown different flow patterns depending on the fractured rock structure. In the research area some fast conduits (large fractures or faults) exist where water runs faster than in the total conductive part of the rock. On the other hand the tracer delay in microfracture system areas was observed.

Key words: pollutant transport, fractured and karstified rocks, bacteriophage, experimental field site, Sinji Vrh (Slovenia)

Introduction

The purpose of the research was to study the infiltration and migration of health-hazardous human viruses, such as enteroviruses, in the unsaturated zone of fractured and karstified rock, since these rocks present important aquifers in Slovenia. As a possible model for the behavior of health-hazardous viruses, salmonella bacteriophage P22H5 was used. Phages have served as useful models for the behavior of human enteric viruses in water treatment processes, groundwater viral transport, inactivation and attachment studies on various subsurfaces, because of their similarity to enteric viruses in structure, size, and resistance to inactivation (Hedberg & Osterholm, 1993; Harvey & Ryan, 2004). Better knowledge of the pollutant transport and persistence of tracer in environment enables us to determine vulnerability and define protected areas for drinking water resources.

The bacteriophage P22H5 is a virulent mutant that propagates in mouse typhoid fever bacteria *Salmonella typhimurium* and rarely occurs in waters (Seeley, 1982). From previous tracing experiments (Bricelj, 1986) it is well known that coliphages are a common constituent of faecally polluted waters and for this reason are not a suitable

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tracer, especially in the case of very high dilutions of the tracer, when a high or low background of coliphages may interfere with the tracer curve. The phage tracer P22H5 was injected at ten locations in 14 tracer experiments in running water and into the unsaturated zone in a karstic area where no background of phages for its host bacteria were present (BRICELJ, 2003).

The tracer experiment was carried out in the subsurface zone, since microbial activity is assumed to be most active in the upper parts of the unsaturated zone.

EXPERIMENTAL FIELD SITE SINJI VRH

A tracer experiment with bacteriophages was performed on the experimental field site Sinji Vrh (ČENČUR CURK, 1997), Slovenia. It is located in the unsaturated zone of fractured and karstified Jurassic limestone at the edge of the Trnovski Gozd plateau (mean altitude 900 m a.s.l.), which is an overthrust of carbonate rock over Eocene flysch (Fig. 1). The subvertical Avče fault with a Dinaric direction NW-SE and several parallel faults cross this territory. These faults are interwoven with numerous connecting faults extending in the general direction N-S.

Their intensity varies from open wide fractured zones to crushed and broken zones (JANEŽ, 1997). The groundwater horizon lies extremely deep and appears on the surface at the lowest point of the impermeable flysh border (Fig. 1) at altitudes between 219 and 235 m as the karstic Hubelj spring. Its catchment area is estimated to about 50 km^2 with an average annual precipitation of 2450 mm (TRČEK, 2003).

The experimental field site Sinji Vrh presents a 340 m long artificial research tunnel, 5 to 25 m below the surface (Fig. 1). An agro-meteorological station has been installed on the surface near the tunnel entrance, where precipitation, evaporation, air temperature, air moisture, solar radiation, wind speed and direction (both at two levels) are continuously measured. A tracer experiment area (Fig. 1) was chosen close to the tunnel entrance on the north-western part. The main dip direction of fractures is NNE-SSW with subvertical dip because of the location of the area within a crushed zone of Avče fault. In the broken zone the tunnel is supported by concrete (Fig. 1). The Jurassic limestone of the tracer experiment area is composed of 99 % calcite and has a south-westerly dip direction and a gentle dip (of 5° to 30°). The unsaturated fractured and karstified limestone has a negligible matrix porosity and very high fracture density with some greater conduits (VESELIČ & ČENČUR CURK, 2001).

The injection hole was drilled through the soil cover in order to avoid tracer retardation because of sorption. A special construction for collecting water penetrating through the rock was developed. The water seeping from the ceiling of the research tunnel is gathered in 1.5 m long segments (MP1 - MP10; Fig. 1) with a gathering surface of 2.2 m^2.

MATERIAL AND METHODS

Bacteriophage P22H5 and salmonella mouse typhoid fever bacteria NIB22 (LT2 w.t. strain) were obtained from Dr. Miklavž Grabnar, Department of Molecular Biology, Biotechnical Faculty, University of Ljubljana.

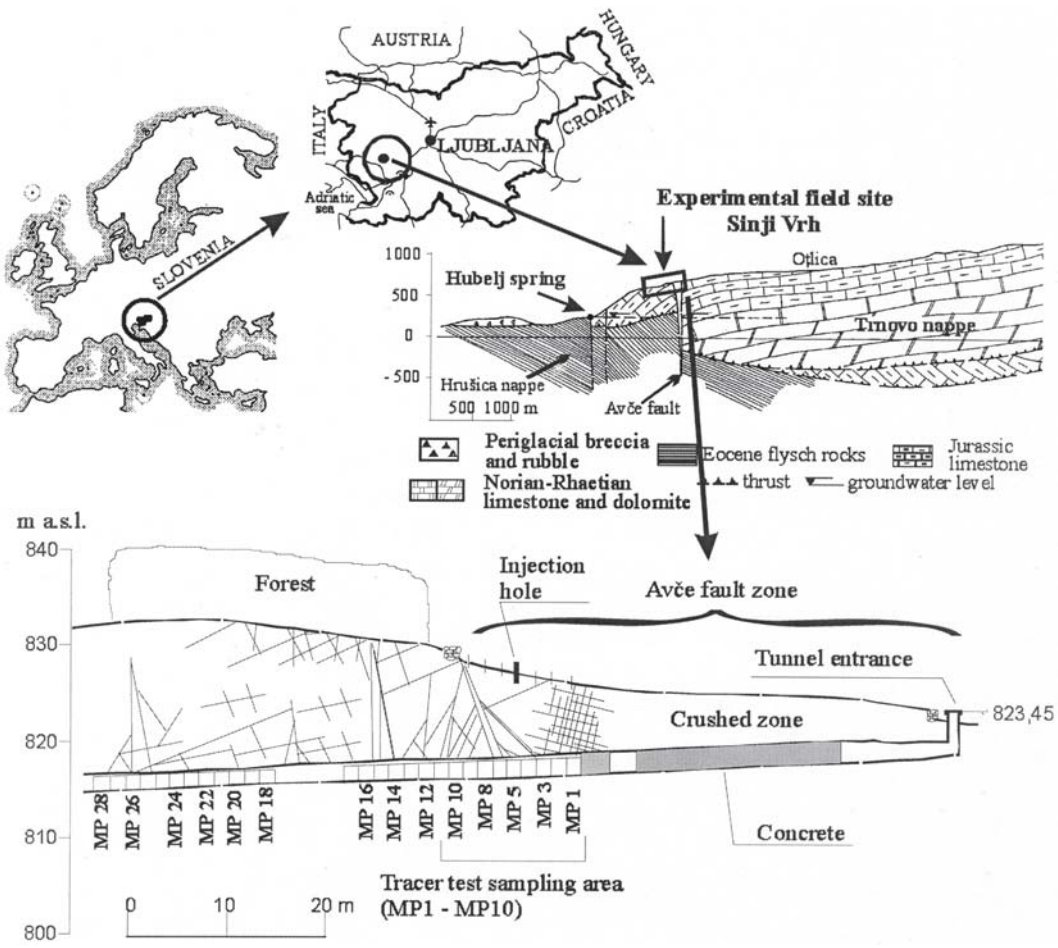

Figure 1. Location of the experimental field site Sinji Vrh (EFS Sinji Vrh) with geological cross section of Trnovo plateau (Janež, 1997; Veselič et al., 2001). Below: Longitudinal section of the EFS Sinji Vrh with tracer tests area and tracer test sampling points in the research tunnel: MP1 – MP10 (after Veselič and Čenčur Curk, 2001).

The propagation of phages to obtain crude bacteriophage lysates was done by the method described in dissertation thesis of Bricelj (1994). The nutrient media Brain Heart Infusion Broth and Nutrient Agar were from Biolife, Milano. Water samples and phage suspensions were tested for viable phages (plaque forming units - pfu) according to the agar layer method of Adams (1966), using host bacteria as the indicator strains.

On 29th September 2003 10.4 liters of tracing solution, composed of 11 tracers (salts, fluorescent dyes, deuterium, micro spheres and bacteriophage), was poured in 10 minutes into the new drilled borehole to the depth of 0.9 m. There were 1.2E+15 plaque forming units (pfu) of bacteriophage P22H5 as a part of tracer cocktail. It should be pointed out, that the phage concentration in injected tracing solution was calculated to

predicted concentration at measuring points from the microfracture system, since there is slower flow with higher dispersion of the tracer. Because of that an overdose is reached in fast pathways such as MP4 and MP5 (see structure on Figure 1 below).

RESULTS

The phage tracer appeared first at sampling point five (MP5) after 4.1 days with the peak value of 3.1 E+09 pfu/ml (Fig. 2, Table 1 and Table 2). One day later a positive result was obtained at MP4, the phage appeared with the peak value of 1.1 E+08 pfu/ml.

The tracer appeared at all sampling points within 22 days. Peak values occurred at the time of appearance at MP2 and MP8. At MP3 the tracer appeared after 8 days, but the peak value was not reached until 50 days. At the furthest sampling points from the injection hole (MP1, MP2 and MP9, MP10) the peak values were within the lowest pfu values (Fig. 2).

The first sampling campaign was completed in September 2004, after about one year (Fig. 2): at MP4 after 324 days and at MP5 after 347 days. At that time, there were still 4.2E+02 pfu/ml in MP4 and 9.8E+02 pfu/ml in MP5 in ml of water sample. Unfortunately the samples were not taken in October and November 2004, since at that time were some significant precipitation events. In winter there were no samples due to freezing of the ground and snow cover. The first water, seeping through the system, was obtained after snow melting at the end of March 2005. After 591 days (May 2005) of collecting the samples there

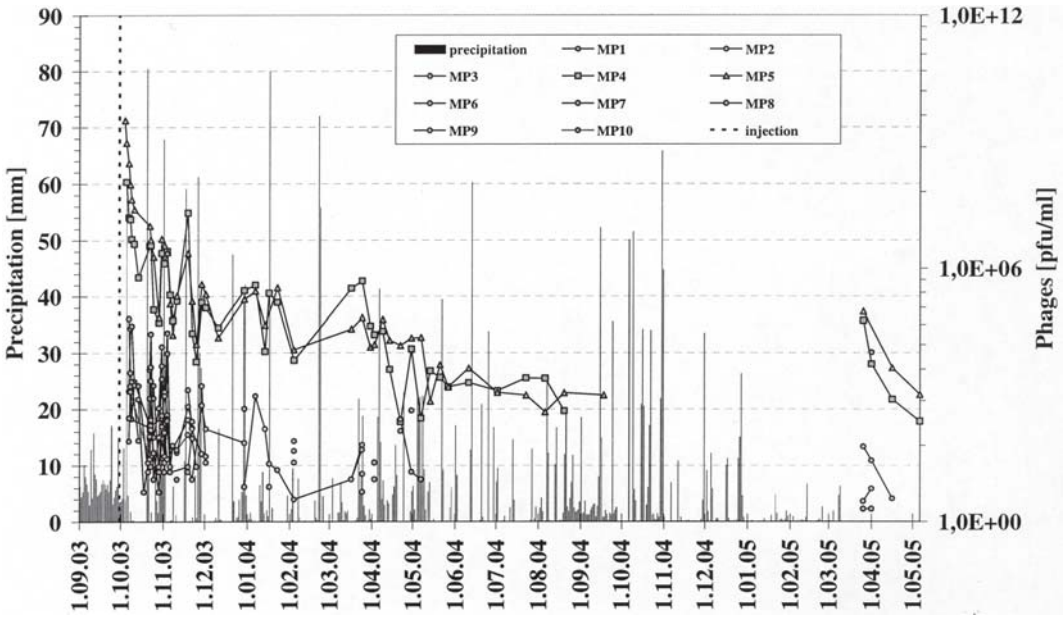

Figure 2. The presence of bacteriophage P22H5 at sampling points MP1- MP10. The concentrations in MP1 – M3 and MP6 – MP10 are grouped below and marked alike. They are depicted only for comparison with MP4 and MP5.

were positive results at MP4, MP5 and MP6 with the following concentrations: 9.5E+01, 6.2E+02 and 2.0E+00 pfu/ml, respectively (Fig. 2). The sampling of water is still going on, but with lower frequency. The recovery value was calculated only for MP4 and MP5 and was 0.95 % of the injected quantity at both sampling points (0.04 % for MP4 and 0.91 % for MP5).

The results of appearance of the phage tracer, the appearance of peak values and time of the last sample containing phage tracer at different sampling points are summarized in Table 1. Table 2 presents time sequence of the phage tracer appearance at sampling points.

Discussion

After the injection of bacteriophages, they remain in the fractures (channels) and microfracture systems of the unsaturated zone and are rinsed by subsequent larger precipitation events even up to year and a half after the injection. Some authors refer that the edges in subsurface structures could be one of the principal causes of charge heterogeneity. Such conditions could permit that negatively charged bacteriophages attach to otherwise repulsive surfaces, especially if the edges of crystals are oppositely charged (Bickmore et al., 2002; Flynn et al., 2004).

The results from Sinji Vrh have shown that the unsaturated zone in the fractured and karstified rocks plays an important role in pollution retardation and storage. The rinsing of pollutants to deeper parts of the karst aquifer depends on the saturation rate of the soil and the unsaturated zone (precipitation events). The field experiments have shown different flow patterns depending on the fractured rock

Table 1. Appearance and presence of the phage tracer at MP1 to MP10. The time is in days and quantity of phages in plaque forming units (pfu) in ml of sample.

sampling point	appearance of tracer	peak value	appearance of peak value	Last sample containing phages (last positive result)
	days	pfu/ml	days	days
MP1	7	2.1E+03	11	63
MP 2	7	2.9E+02	7	63
MP 3	22	5.7E+02	50	550
MP 4	5	1.1E+08	5	591
MP 5	4	3.1E+09	4	591
MP 6	8	4.3E+04	11	591
MP 7	13	1.4E+04	31	550
MP 8	7	6.5E+04	7	550
MP 9	22	1.7E+03	25	214
MP 10	8	4.9E+03	40	177

Table 2. Time sequence of the phage tracer appearance at measuring points.

days	measuring point
4	MP 5
5	MP 4
7	MP 2
7	MP 8
11	MP1
11	MP 6
25	MP 9
31	MP 7
40	MP 10
50	MP 3

structure. In the research area some fast conduits (large fractures or faults) exist where water runs faster than in the total conductive part of the rock, as in the case of MP4 and MP5. Tracer delay in microfracture system areas was also observed, especially at MP3, MP7, MP9 and MP10 (see Fig. 1 and 2). At these points the appearance of the peak value was delayed for 50, 31, 25 and 40 days respectively. A very low recovery rate is due to the dispersion of the tracer in directions where it could not be sampled and the decay of tracer, dependent upon removal mechanisms such as filtration, sedimentation and irreversible adsorption (SINTON ET AL., 1997).

Recent research with bacteriophages MS2 (ZHUANG ET AL., 2003), PRD1 (BLANFORD ET AL., 2005) and T7 (FLYNN ET AL., 2004) is much more concerned with breakthrough percentage, recovery calculations of peak values of tracer curve, kinetics of virus surface inactivation and analytical models than with the longevity of phages in environment. Some notions on persistence of phages in the environment are referred by DEBORDE ET AL., 2003, for the phages MS2 and PRD1 in floodplain aquifer. The breakthrough curves of phages contained

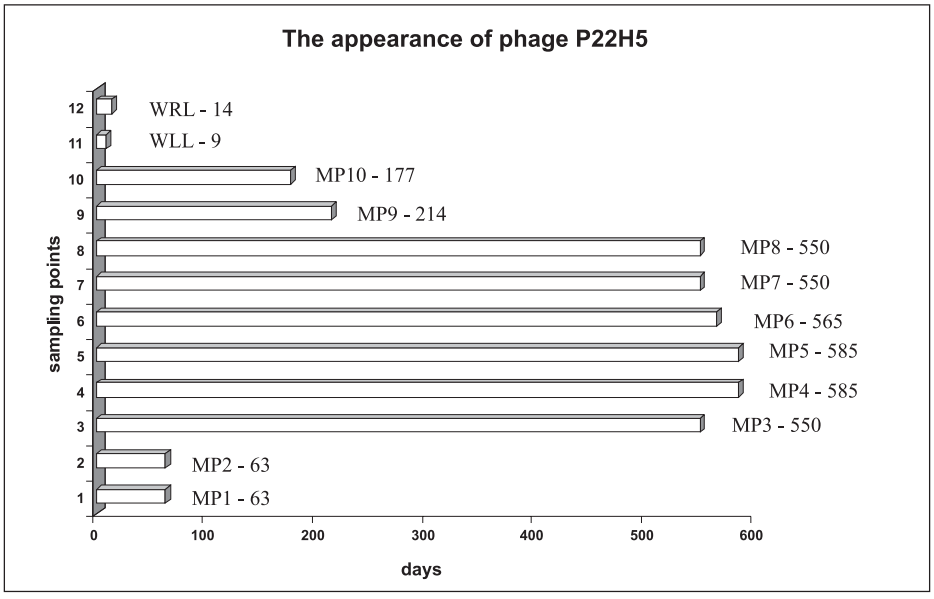

Figure 3. The presence of bacteriophage P22H5 at the sampling points MP1 – MP10 in Sinji Vrh. The sampling points WRL and WLD, represent the percolation of phage tracer through right (WRL) and left (WLL) soil lysimeter at Wagna experimental field near Graz.

long tails, so the slow releases of phages have been observed over a period of more than six months. There is need for further research of mechanisms of phage persistence in the environment and health-related significance of the mechanisms of such processes.

The results were compared (Fig. 2) with the results from tracer tests in soil and gravel at the Wagna lysimeter station (Austria). The phage tracer was very quickly eliminated from the water trickling through 1 m soil and 0.5 m gravel and positive results were concluded after nine or fourteen days respectively, in the left and right lysimeter (WLL and WRL). The results in the lysimeter are consistent with the findings of Van Elsas et al., 1991 and Powelson et al., 1991 and Van Cuyk et al., 2001.

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