6.4. RESULTS WITH PHAGES (M. BRICELJ)

6.4.1. Introduction

Phage P22H5 was first introduced in Greece (BRICELJ et al. 1986) in a combined tracing experiment on the Central and Eastern part of Peloponnesus. The phage of mouse typhoid bacterium was chosen because the phages of *Salmonella typhimurium* had been rarely encountered in surface waters (SEELEY & PRIMROSE 1982). In such manner, high background coliphage titres, usually encountered in natural polluted waters, that can interfere with tracer curve, could be avoided (ALTHAUS et al. 1986). Phage P22H5 is clear plaque mutant of transducing phage P22 (SMITH & LEVINE 1967) and produces very discernible clear plaques in the lawn of growing host bacteria. Besides this feature, P22H5 phage (Podoviridae) could be propagated to high titres in controlled growing conditions (BRICELJ 1994). During several tracing experiments in the Slovenian karst region (KRIVIC et al. 1987; KRIVIC et al. 1989; HABIČ et al. 1990) and in a tracing experiment in the Styrian karst near Graz (BEHRENS et al. 1992) in ten tracing experiments only once the background for salmonella phage was positive, but only in some samples. The phage P22H5 proved to be a better tracing agent in comparison to coliphage T7 in several deactivating experiments at the air-water interface (BRICELJ & ŠIŠKO 1992) and in recovery experiments of clay mineral adsorption tests (BRICELJ 1994).

6.4.2. Injection data

The phage tracer - P22H5 virulent mutant of host bacterium *Salmonella typhimurium* LT2 (TL474 w.t.) - was injected, subsequently three times at the location Zavrhovc - Otlica 88, which is a kilometre away from the Hubelj spring on the plateau below Črni Vrh.

In the first tracing experiment a hole with the diameter of 5 cm was drilled in the floor of the doline, 5.5 m deep into permeable strata. Before the injection, 1.5 m^3 of water was poured into the drilling hole, following with 16,500 ml of phage broth that were subsequently washed with additional 3.5 m^3 of water. The injection of phage tracer began at 14.25 on 14 October, 1993 and was continuing for 3 minutes. The total concentration of injected phage particles was 3.0×10^{15} pfu (= 1.84×10^{11} pfu /ml x 16,500 ml).

The second injection place for the phage tracer lied in other doline next to the place of the first injection. The injection of lithium and phage tracer took place on 16 April, 1994. A fissure at the bottom of the doline, was first washed with 3.5 m^3 of water, from 10.20 to 10.25 lithium chloride in quantity

of 110 l (30 kg) was poured into the fissure, followed by the washing of 1.0 m^3 of water. From 10.29 to 10.30 phage tracer in the quantity of 20,500 ml was poured and then washed with 3.0 m^3 of water. The total quantity of phage tracer was 3.75 x 10^{15} pfu (=1.83 x 10^{11} pfu/ml x 20,500 ml).

The place of the third injection of phage tracer was equal to the second one. On the 1 August, 1995 the phage tracer in quantity of 26,000 ml was poured into the same fissure, at the bottom of the doline. Injection began with the washing of the fissure with 3.5 m^3 of water at 10.21. Between 10.25 and 10.28 phage tracer was poured into fissure, followed by washing with additional 3.5 m^3 of water. The total quantity of phage tracer was 6.6. x 10^{15} pfu (= 2.52 x 10^{11} pfu/ml x 26,000 ml).

6.4.3. Results

The relevant parameters derived from all three experiments with the phage tracer P22H5 are summarised in the tables Tab. 6.17, 6.18 and 6.19.

In the first tracing experiments with phage tracer P22H5 the samples for the determination of phages were taken at sampling points Hubelj, Skuk, Gorenje Studenec, Lijak, Mrzlek and Hotešk. The phage tracer reappeared only in the Hubelj spring (Fig. 6.31). The first positive result was evaluated on October 16, 1993 at 07.02 as 0.6 pfu/ml. Maximum concentration 32.4 pfu/ml was determined in the sample from October 17, 1993 at 11.02. On November 4, 1993 there was the last positive result of 0.4 pfu ml at 23.00. The sampling was stopped on November 13 after several negative results.

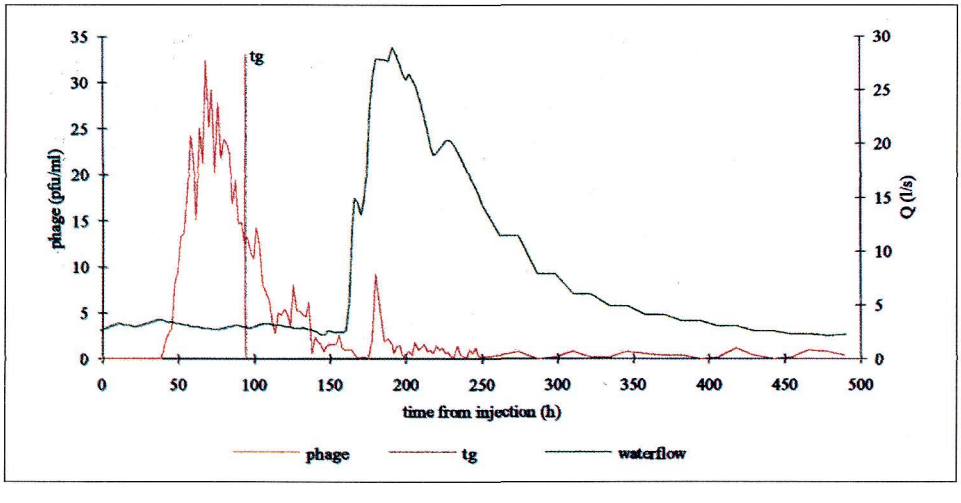

Fig. 6.31: The breakthrough curve of phage tracer P22H5 at the Hubelj spring of the first tracing experiment, in October 1993.

The recovery of tracer was 0.78 %. The gravity centre position of tracer curve t_g was calculated to 94.32 hours. The empirical formula $P_{trac} = a * t_{trac} * Q$ (P_{trac} = quantity of tracer needed in tracing experiment; a = deactivation factor; t_{trac} = time of tracer travel in sec; Q = waterflow in ml/s; Q was taken as average waterflow, including the data from the first appearance of tracer, to the last positive result) was used to calculate the needed quantity of phage tracer for a = 1, Q = 27.79 m3/s and t_{trac} = 94.7 h. The resultant quantity was 9.5 x 10^{12} pfu. The quantity injected was 3.0 x 10^{15} pfu, therefore the calculated inactivation factor is in the magnitude of 316.6. The real inactivation factor calculated from the recovery value 2.43 x 10^{13} was 128.0, that means 2.8 times lower than the calculated one.

Concerning the negative values of control samples, negative background of salmonella phage, sufficient quantity of phage tracer and flowthrough curve of the reappearing tracer, the conclusion could be, that the connection between drill hole in the doline at Zavrhovc and the Hubelj spring does exist.

In the second tracing experiment in spring 1994, the samples for the determination of phage were collected only in the springs of Gorenje Studenec, Skuk and Hubelj. The first positive result was evaluated after 54.30 hours on 18 April, 1994 as 0.5 pfu ml. The maximum value of 1.1 pfu ml 1 was determined on the same day, between 21.00 and 23.00 hours. The last positive result was determined on 25 April, 1994 found in the sample at 01.00 hour (Fig. 6.32).

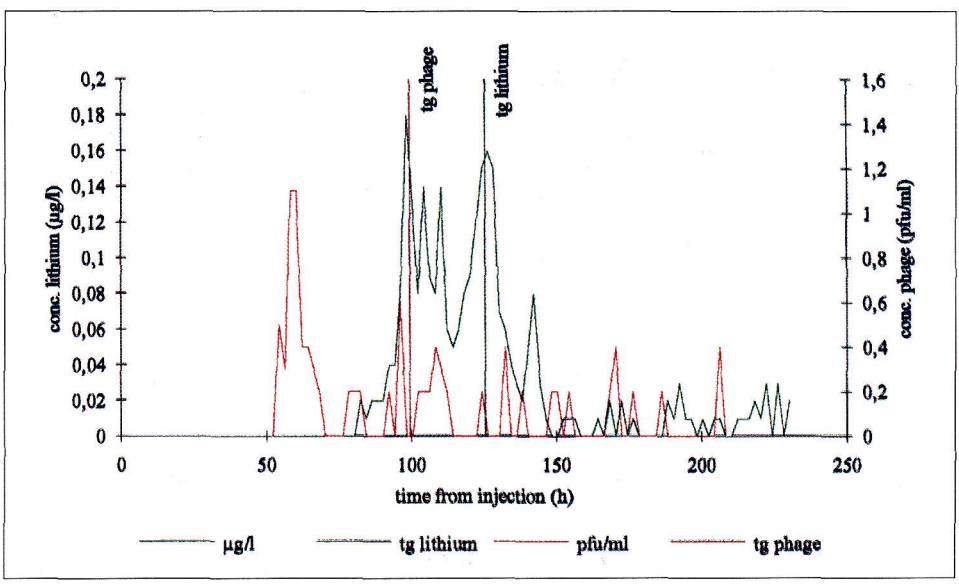

Fig. 6.32: The breakthrough curves of phage P22H5 and lithium tracer in the Hubelj spring during the tracer experiment, in April 1994.

The recovery of tracer was 0.012 %. The gravity centre position of tracer curve t_g was calculated to 99.61 hours. The resultant quantity for empirically calculated tracer quantity that we need in the case of a = 1, Q = 4.38 m^3/s and t_{trac} = 99.61 h was 1.57 x 10^{12} pfu. The injected quantity was 3.7 x 10^{15} pfu, so the calculated inactivation factor is in the magnitude of 2,354.6. The real inactivation factor calculated from recovery value 4.45 x 10^{11} is 8,314.6, that means about 3.53 times greater than the calculated one.

In the third tracing experiment, in the summer of 1995, the samples for the determination of phage were collected only in the spring of Hubelj. The first positive result was evaluated almost after one month, on August 29, 1995 at 00.00 hours as 0.5 pfu/ml. The maximum value of 1.0 pfu/ml was evaluated on the same day at 12.00 hours. The last positive result as 0.1 pfu ml determined on August 31 at 00.00 hours. The additional positive value was recovered from the sample taken on September 11 at 06.00. The value of the determined phage was 0.2 pfu/ml.

The recovery of tracer was 0.001 %. The gravity centre position of tracer curve t_g was calculated as 678.2 hours. The resultant quantity for empirically calculated tracer quantity in the case of a = 1, Q = 3.19 m^3/s and t = 678.2 h was 7.8 x 10^{12} pfu. The injected quantity was 6.6 x 10^{15} pfu, therefore the calculated inactivation factor is in the magnitude of 846.4. The real inactivation factor, calculated from the recovery value 4.62 x 10^{11} is 16.9 times greater than the calculated one.

Tab. 6.19: Measured and calculated data for the three tracing experiments with phage tracer P22H5, injected at the location Zavrhovc, a = deactivation factor calculated from injected and recovery values; av. precipit. = average precipitation in the month period before the injection of phage took place; av. Q inj. = average day's throughflow in the time of injection of tracer; max. con. Q = Q value at the peak concentration of tracer curve; recovery = calculated recovery of the phage tracer from injected and recovered quantity of phage tracer.

.	a	av. precipit.	av. $Q_{inj.}$	max.conc. Q	recovery
October 1993	128.0	15.4 mm	2.81 m^3/s	2.90 m^3/s	0.78 %
April 1994	8314.6	11.8 mm	6.33 m^3/s	9.21 m^3/s	0.012 %
August 1995	1.0 x 10	2.8 mm	0.51 m^3/s	9.50 m^3/s	0.007 %

*Tab. 6.17: The values of the time of appearance of phage tracer and its velocity in the spring of Hubelj for three tracing experiments. The distance between the injection point and the sampling point suits 1000 m for each tracing experiment: t_{min} = time elapsed from injection and the first appearance of tracer, t_{max} = time elapsed from injection and the maximal quantity of tracer; t_g = time calculated from following equation Σ ci*ti / Σ ci; v_{min} = velocity calculated with t_{min}; v_{max} = velocity calculated with t_{max}; v_{tg} = velocity calculated with t_g.*

	t_{min}	v_{min}	t_{max}	vmax	t_g	v_{tg}
October 1993	40.61 h	0.007 m/sec 592.2 m/day	68.62 h	0.004 m/sec 350.5 m/day	94.7 h	0.003 m/sec 253.9 m/day
April 1994	54.50 h	0.005 m/sec 442.9 m/day	59.30 h	0.005 m/sec 405.5 m/day	99.61 h	0.003 m/sec 241.4 m/day
August 1995	661.5 h	0.0004 m/sec 36.4 m/day	673.5 h	0.0004 m/sec 35.7 m/day	678.2 h	0.0004 m/sec 35.5 m/day

Tab. 6.18: Comparison of the determined parameters of the phage and the lithium (Chapter 6.5) breakthrough in the Hubelj during the second tracer experiment in April 1994. (explanation of the abbreviations, are given in Tab. 6.17).

Tracer.	t_{min}	v_{min}	t_{max}	vmax	t_g	v_{tg}
phage P22H5	50.5 h	0.0051 m/sec 442.9 m/day	59.5 h	0.0046 m/sec 405.5 m/day	99.61 h	0.0028 m/sec 241.4 m/day
lithium	82.5 h	0.005 m/sec 442.9 m/day	98.5 h	0.0028 m/sec 244.1 m/day	1265 h	0.0022 m/sec 190.1 m/day

6.4.4. Conclusions

The tracing experiments with phage P22H5 and other tracers on the karst plateau were performed in three different water level situations, low, high and medium. The first tracing experiment was performed in the medium level of water in the spring of Hubelj (compare Chapter 6.2.1). The medium day's water level in the time of injection, was 2.81 m^3/s. The average precipitation in the month period before the injection was 15.4 mm (Tab. 6.19). It is believed, that the underground passages under the permeable strata in the bottom of doline were partly saturated by water, after the steady raining, before the tracing experiment took place. In such conditions the bacteriophage tracer was injected to the permeable strata for the first time instead of directly into the water as was commonly done in preceding experiments in several karst locations (Tab. 6.20). The recovery of tracer in the first experiment was the highest comparison to the recovery values of the two subsequent tracer experiments (Tab. 6.17), although a part of phage tracer was adsorbed to the underground surfaces. This can obviously be seen in the breakthrough curve of phage tracer at the Hubelj spring (Fig. 6.31), where the second peak of phage tracer with 9.2 pfu/ml in 180.6 hours after the injections strictly follows the sudden augmentation of water throughflow at the Hubelj spring.

Second tracing experiment was performed after the melting of the snow, which gave high water with the average day's throughflow of 6.33 m^3/s in the time of the injection. The recovery of the phage tracer was lower than in the first tracing experiments because of the high dilution of phage tracer and possible dispersion of the phage tracer in highly saturated strata, away from the main flow. The difference in the values of deactivation factor in both experiments could be contributed to the dispersion of the phage tracer rather than to the enhanced adsorption. The effect of lithium chloride on phage tracer could not be omitted, but we think that because of the high dilution the effect of lithium tracer on phage is of less importance. Nevertheless, the velocity of phage tracer calculated from the centre of the gravity of tracer curve was a little bit lower than in the first tracing experiment (Tab. 6.17). The gravity values of tracer curves from the both mentioned tracing experiments have a difference about five hours (Tab. 6.17). The difference could be contributed to the different injection locations or different underground water conditions. Comparing the phage reappearance data of the second tracing experiment with the data of lithium that was injected in the same location simultaneously (Tab. 6.18 and Fig. 6.31), we can conclude that the phage tracer preceded the lithium for approximately one day (26.9 h). The first appearance of the phage tracer occurred on 18 April at 17.00 (54.5 hours after injection) and from the smoothed curve of lithium tracer we can conclude, that first appearance of lithium occurred on 19 April. at 17.00 (82.5 hours after the injection). The difference in recovery quantity of both tracers is very high,

comparing 1.69 % of lithium with 0.012 % of phage. This difference could be contributed to water level conditions and velocity of both tracers. The phage that was quicker in moving towards Hubelj area was probably more diluted on April, 18 when the through-flow augmented from 7.5 m sec (17 April) to 12.56 m sec than lithium that appeared on 19 April, when the throughflow at Hubelj lowered to 6.92 m sec.

Completely different conditions were in the time of the third tracing experiment, when the average precipitation in the monthly period before the injection was only 2.8 mm (Tab. 6.19). The phage tracer that was injected on 1st August and washed into the permeable strata with water, remained there immobile, or moved very slowly for nearly a period of a month. The high water level, which is indicated by the sudden augmentation of throughflow from 0.52 m^3/s (27 of August) to 13.03 m^3/s pushed the adsorbed and extremely slowly moving phages into the Hubelj spring., where the peak of tracer curve occurred on 29 August at 12.00. Recovery value of 0.001 % could be contributed partly to the deactivation of adsorption to different underground surfaces and partly to dilution of phage tracer because of the sudden augmentation of the ground water levels. Keeping in mind the time of the passage of phage tracer in first and second tracing experiment, that was 94.7 and 99.6 hours respectively and the day values of precipitation at Otlica in the time of reappearance of phage tracer; we can conclude that the main water which pushed the phages into the Hubelj spring came from other direction than from the background of Otlica, where the precipitation achieved the maximum values on 29 August with 98.5 mm in the time when the peak value of phage tracer in the Hubelj spring was already determined.

The recovery values for phage tracer P22H5 in both three experiments at Nanos plateau are for several magnitudes lower than in the preceding tracing experiments in different karst regions, where bacteriophage was injected directly into flowing waters (Tab. 6.20). Nevertheless, the results of all the three tracing experiments with the phage P22H5 on high karst plateau confirm, that the phage tracer could also be injected into permeable strata with additional washing, where the water flow doesn't occur. The best recovery values for phage tracer can be expected, when permeable strata are sufficiently saturated due to longer rainy periods.

Tab. 6.20: The centre of gravity values (t_g), and average velocity of phage tracer based on t_g value for several tracing experiments with P22H5 phage in karst environment. Recovery values for some tracing experiments are also included.

injection point	sampling point	distance [m]	t_g [h]	v_{tg} [m/day]	recovery [%]
Kapsia	Kiveri	39,000	233.0	4017	-
Smokavska vala	Rižana	3880	348.0	268	0.006
Hotičina	Rižana	12,450	482.0	620	4.5
Lurbach	Hammerbach	3000	53.4	1348	2.5
Kačji potok	Radeščica	19,200	511.4	901	3.0
Kačji potok	Obrh	20,000	704.6	681	-
Bajer	Krupa	6000	99.1	1453	-
Vrčice	Krupa	6000	156.8	818	-
Movražka vala	Ara	800	80.8	237	-
Movražka vala	Mlini	1000	94.6	165	3.4
Movražka vala	Sopot	1043	145.1	173	-
Zavrhovc	Hubelj	1002	94.7	255	0.78
Zavrhovc	Hubelj	1002	99.61	247	0.012
Zavrhovc	Hubelj	1002	678.2	36	0.007