KARI KINNUNEN

TRACING WATER MOVEMENT BY MEANS OF ESCHERICHIA COLI BACTERIOPHAGES

Tiivistelmä

Escherichia coli bakteriofaagit merkkiaineena vesien kulkeutumistutkimuksissa

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Lopputiivistelmä

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TRACING WATER MOVEMENT BY MEANS OF ESCHERICHIA COLI BACTERIOPHAGES

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The author has developed a method which uses E. coli bacteriophages as tracers of water movements. The method has been tested in rivers, ground waters, municipal waste water treatment plants and a lake polluted by pulp and paper mill effluents. The phage tracer was found to suit rivers, and on certain conditions, the other water bodies referred to above. The method has several advantages: 1. It is harmless to the environment. 2. Great dilution ratio \(10^{-11} - 10^{-13}\) equals that of radioactive tracers and neutron activation analysis tracers, and considerably exceeds that of fluorescent dyes. 3. Several water fractions can be marked simultaneously. 4. The cost is relatively low. The most serious disadvantage is the delay in obtaining the results; water samples have to be analysed in laboratory and the result can not be enumerated until 4 to 6 hours after plating the samples.

Index words: Bacteriophages (quantitative analysis), bacteriophages (cultivation), biological tracers, water tracing, water velocity measurements.

1. INTRODUCTION

Water movements are studied mainly for two reasons:
- to learn the speed of travel of water
- when studying pollution to simulate the dispersion of waste waters.

Commonly, such tracers have been employed as fluorescent dyes (Smart & Laidlaw 1977), salt solutions (Knutsson 1970), radioactive isotopes (Smith 1972), and some elements sensitive to neutron activation analysis (Kuoppamäki & Murriinen 1976a). In some rare cases microbiological tracers have been used, such as bacteria (Pfuhl 1897, Abba et al. 1899, Robson 1956), yeasts (Gärtner 1915, Wimpenny et al. 1972), bacterial spores (Pike et al. 1969), and bacteriophages (Niemelä & Kinnunen 1968, Wimpenny et al. 1972). The present study introduces a method in which Escherichia coli bacteriophages are used as tracers in water movement studies. Phages can be used as tracers provided that
- it is possible to grow them in sufficiently dense suspensions to enable large quantities of water to be tagged with manageable amounts of the tracer suspension.
- they remain active long enough in the environment in which they are used so that observations of water movements can be made.
- their quantitative analysis is reliable.

In order to evaluate the applicability of the method, it has to be tested on natural systems.
Waterways considered suitable testing grounds include rivers, ground waters, waste water plants, and lakes. In addition, the method has to be compared with the tracing methods already in use to find out how it rates among these.

Various *E. coli* bacteriophages have been used as tracers. Of these, T7 phage and its host strain *E. coli* B11303 are ATCC type strains. The other three phages used and their host strains have been isolated from the River Vantaanjoki and have been named F46, F52, and F137.

The phage method has been studied mainly on the basis of the problem setting presented above.

2. MICROBIOLOGICAL TRACERS

The application of microbiological tracers in studying water movements is an old method. The first persons to employ it seem to have been Abba, Orlandi and Rondelli (Abba et al. 1899) at the University of Turin and the German scientist Pfuhl (1897).

The purpose of the studies of Abba and his co-workers was to find out, if the gathering network of drinking water for Turin was liable to be contaminated by bacteria filtered from the top soil. This was because the water supply in Turin depended on a network of tunnels constructed outside the town at the depth of 1.5—2 m. Groundwater from the surrounding limestone deposits gathered in them, and was led directly to the wells and the water distribution network of the town.

Abba et al. reasoned that the best way to study the problem was to let a pure culture of some bacteria absorb into the ground above the gathering network and beside it, and to observe its eventual appearance in the water of the network. The bacterium to be selected as a tracer had to fulfil the following conditions:

- it must not be pathogenic
- it was not to be detected in the tap water of Turin prior to the experiment
- the bacterium should be easy to grow, and it should be clearly distinguishable from other bacteria found in the tap water.

First Abba et al. experimented with *Bacillus prodigiosus* which forms a red pigment and which is identical with *Serratia marcescens* (Breed et al. 1948) as well as some strains of *Micrococcus* and *Sarcina lutea*. The last one formed a yellow pigment.

After preliminary tests they decided to use *B. prodigiosus*. The experiments were performed by letting c. 20 l of the *B. prodigiosus*-suspension absorb into an area of 40—50 m², after which the area was generously watered. Sampling from the gathering network was started simultaneously with the introduction of the bacteria.

From the samples, the presence of the bacteria used as tracers was analysed by means of a kind of enrichment method the results of which could be read within 2—3 days.

In the experiments in which the place of absorption was right above the gathering tunnel, bacteria were discernible as soon as 1—7 h after absorption.

Abba et al. performed some experiments in which the place of absorption was even 200 m from the gathering tunnel. They also used uranin side by side with the bacterial tracer. The results of these parallel experiments showed that *B. prodigiosus* was detected in the samples in half the time needed by uranin. Thus they concluded that bacteria are much better suited to marking experiments than dyes.

The difference mentioned above in the time of travel of the bacteria and the dye was obviously due to different sensitivity in analysis. At that time, colour was determined by the eye, so that the dye had to be relatively well concentrated before it became visible. With bacterium analysis, however, even very small concentrations could be detected.

In their final conclusions, Abba et al. suggested that even pathogenic bacteria could have access into the gathering tunnels of the water supply in Turin, and thus cause quite wide-spread epidemics.

Pfuhl (1897) carried out similar experiments near Strassbourg where he studied the ability of bacteria, and particularly those vibrios which cause cholera, to move in the groundwater of flint areas.

As the tracer bacterium he chose *Micrococcus prodigiosus*, at present also called *Sr. marcescens* (Breed et al. 1948) and a *Vibrio*, called by him
the "Luminous Vibrio", because of its bioluminescence.

The grounds on which the tracer bacteria were selected were identical with those of the team of Abba (Abba et al. 1899). One further reason for the selection of the "Luminous Vibrio" was the presumption of Pfuhl that in the groundwater it would behave like the cholera Vihrio. Side by side with the bacteria, Pfuhl used uranin which stained the marked water bright green. He had arranged his experiments simply and logically. He had two holes dug which reached beneath the groundwater level. Into one of these he added the tracers, and from the other he pumped groundwater. By making observations of the appearance of the tracers in the water being pumped, he was able to estimate the speed of the tracers travelling with water.

Uranin was analysed by the eye. The bacteria were analysed by enrichment, and thus the results could be read within 1–2 days.

The longest distance measured by Pfuhl was 12 m, which uranin travelled in 0.5 h, M. prodigiosus in 1 h, and the "Luminous Vibrio" in 2 h.

On the basis of the results obtained Pfuhl concluded that even the Vibrios that cause cholera may travel long distances in groundwaters. Thus cholera epidemics might spread through groundwaters.

The experiments referred to above were performed independently of each other.

Richards and Brincker (1907–1908) used an unidentified pigment forming bacterium as a tracer when studying a case of groundwater contamination near London. They suspected there might be cleavages in the ground containing limestone, along which the contaminated run-off waters might travel into a public well.

The longest distance they measured was c.3 200 m, which the bacteria used as tracers travelled in c. 80 h. Richards and Brincker were able to determine the concentration of the tracer bacteria quantitatively, and they detected almost perfect tracer curves.

Gärtner (1915) used the yeast Saccharomyces cerevisiae in similar studies.

In spite of the promising experiments referred to, the utilization of biological tracers did not become popular among scientists. There was a new interest in biological tracers in the 1950's when they were re-discovered in a way. Then, among others, Mayr (1953) and Maurin and Zöttl (1959) started to use the spores of Lycopodium clavatum when they studied groundwater flows in limestone regions. For example Buchtel et al. (1964) and Atkinson et al. (1973) have later used the same method.

The first experiments whose purpose was to trace the movements of waste waters by utilizing tracer bacteria were performed only in the middle of the 1950's, when Robson (1956) and Putman et al. (1956) used a mutant of Serratia indica produced by radiation. It was characterized by resistance to antibiotics and an abundant formation of red pigment. It was possible to make a quantitative analysis of it by determining the total colony count on a medium containing antibiotics and counting the red colonies growing on it. The formation of red colonies first took about 6 days, but later on Robson managed to produce a mutant that formed pigment after 2 days' incubation.

Robson performed his first experiments in nature on the south coast of England where he marked the water of a sewer running off to the sea by using a mutant of Sr. indica, 32P-radioactive isotope as well as floats.

On the basis of the results of the movements of 32P-isotope and Sr. indica he concluded that the marked waste water moved with the tidal currents independently of the wind blowing along the coast. The floats, on the other hand, gave a misleading idea of the waste water movements, as they were liable to the effect of wind.

Robson made the following conclusions when he estimated the applicability of the bacterium method and compared it to the utilization of the radioactive isotopes:

1. The greatest advantage of using radioactive tracers lies in the fact that they are quickly and easily analysed. The analysis can be performed under field conditions thus making it possible to observe the tracer movements continuously. Their utilization is restricted by the environmental risk of radioactivity, the half-life of the isotopes, and the expensive equipment needed for their analysis.

2. The use of bacteria is favoured by their complete harmlessness to the environment as well as their adaptability to long-term observation
of water flows. On the other hand, their usefulness is reduced by the slow and elaborate analysis. Robson obviously was not aware of the fact that Serratia had been used as a tracer before, as he made no reference to earlier studies. In some studies published later on, Robson has even been considered the original inventor of the method.

The next step in the development of the use of *Sr. indica* as a tracer concerns mainly the method. Baalsrud (1961) and Rippon et al. (1961) started to use a membrane filter method in the analysis, thus being able to study even relatively dilute samples. Baalsrud studied the water movements in a small lake, and Rippon et al. the effects of tide on the movements of waste waters discharged into a river. Later on, *Sr. indica* has been used at least by Ormerod (1964) in brackish water and Eiland (1966) in sea water.

In all the studies referred to above the tracer was injected instantaneously.

Pike and his team (1969) cultivated *Sr. indica* suspension in a chemostat which they installed at a waste water pumping station. The bacterial culture leaving the chemostat was discharged directly into waste water. By continuously marking the waste water they were able to trace the dispersion and the extent of the waste water area in the sea. In their studies they also employed $^{82}$Br-isotope and *B. subtilis* var. *niger* spores. The latter were added into the waste water instantaneously.

Pike et al. considered the Bacillus-spores relatively good tracers because they survived for a long time in sea water. In comparison, the strain of *Sr. indica* used in the study survived only some tens of hours in sea water exposed to sunlight. The quantitative determination of the Bacillus-spores was, however, quite troublesome as the samples had to be heated before cultivation.

Schubert (1971) applied a mutant of *Aeromonas hydrophila*, which was resistant to antibiotics, when studying the movements of surface runoff waters.

Wimpenny et al. (1972) made a comparative study of *Sr. marcescens*, *B. subtilis* var. *niger*-spores, *Hansenula* sp. and *Rhodotorula glutinis*-yeasts as well as a phage of *E. coli* K12 strain as to their adaptability as tracers of water movements in rivers. Immediately they had to give up the use of Bacillus-spores because their background concentration in the marked river was often high. The yeasts and *Sr. marcescens* gave satisfactory results in the river in question. However, Wimpenny's team arrived at the conclusion that phages are the most promising biological tracers.

Niemelä and Kinnunen (1968) were the first persons to use phages as tracers. The study of Wimpenny et al. had been performed independently of that by Niemelä and Kinnunen.

Later on, phages have been used by Statham (1974) in a river and Kawata et al. (1974) in a stabilization pond of a waste water purification plant.

The history of microbiological tracers shows that both the principle of their application and the organisms applied as tracers have, in a way, been discovered many times, as can be seen in Table 1.

Biological tracers have not yet gained popularity in water movement studies, even though certain microbes are used e.g. in aerobiology for exactly similar purposes (Sattar et al. 1972). This is quite obviously due to the fact that when waters are marked, such large volumes are concerned that impractically large quantities of the microbiological tracer would be needed to mark them. In addition, their analysis is so slow that insufficient attention can be paid to the water movement process itself.

For a microbiological tracer to be adopted universally it has to overcome at least the difficulties referred to above, and possess some characteristics which would make its application practical.

### 3. THE PROPERTIES OF PHAGES IN RESPECT TO THEIR APPLICATION AS TRACERS

#### 3.1 The structure of phages

Bacteriophages, or short phages, are bacterial viruses and incapable of independent metabolism. They can only multiply inside active bacterial cells which are sensitive to them. In general their size varies from 5 to 300 nm. Chemically they are composed mainly of two substances, the
Table 1. The development of the use of microbiological tracers. The table includes the studies which were first, or assumed to be the first, in which a certain biological tracer was employed.

<table>
<thead>
<tr>
<th>References</th>
<th>Tracer</th>
<th>Incubation time</th>
<th>Type of water marked with the tracer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pfuhl (1897)</td>
<td><em>Micrococcus prodigiosus</em> <em>Serratia marcescens</em> &quot;Luminous Vibrio&quot;</td>
<td>c. 2 d</td>
<td>groundwater</td>
</tr>
<tr>
<td>Abba et al. (1899)</td>
<td><em>Bacillus prodigiosus</em> <em>Serratia marcescens</em></td>
<td>2–3 d</td>
<td>groundwater</td>
</tr>
<tr>
<td>Richards and Brinker (1907–08)</td>
<td>Unidentified bacteria producing yellow colonies</td>
<td>3 d</td>
<td>groundwater</td>
</tr>
<tr>
<td>Gärtner (1915)</td>
<td><em>Saccharomyces cerevisiae</em></td>
<td></td>
<td>groundwater</td>
</tr>
<tr>
<td>Mayr (1953)</td>
<td><em>Lycopodium clavatum</em> spores</td>
<td>Direct observation in microscope</td>
<td>groundwater</td>
</tr>
<tr>
<td>Robson (1956)</td>
<td>A mutant of <em>Serratia indica</em></td>
<td>2–6 d</td>
<td>waste water - sea</td>
</tr>
<tr>
<td>Niemelä and Kinnunen (1968)</td>
<td><em>Escherichia coli</em> phages</td>
<td>4–6 h</td>
<td>river</td>
</tr>
<tr>
<td>Pike et al. (1969)</td>
<td><em>Bacillus subtilis</em> var. <em>niger</em>-spores</td>
<td>18 h</td>
<td>waste water - sea</td>
</tr>
<tr>
<td>Schubert (1971)</td>
<td><em>Aeromonas hydrophila</em></td>
<td></td>
<td>runoff water</td>
</tr>
<tr>
<td>Wimpenny et al. (1972)</td>
<td><em>Hansenula sp Rhodotorula glutinis</em></td>
<td>-</td>
<td>river</td>
</tr>
</tbody>
</table>

protein of the sheath and nucleic acid inside the sheath that may be either DNA or RNA. They may contain negligible amounts of other substances as well, such as lipids and hydrocarbons (Csaky et al. 1950).

In all phage groups but one, the nucleic acid is inside a polyhedral capsid often called a head. Attached to the head there is often a helical protein formation or a tail (Fig. 1). The phage tail or tail fibers eventually occurring at the end of the tail act as organs that the phages use to adsorb to e.g. the cell walls of their host bacteria (Stanier et al. 1977).

### 3.2 Multiplication of phages

Multiplication or the so called lytic cycle of phages begins when a phage and the host bacterium which is sensitive to it come into contact with each other. The phage adsorbs to a specific receptor site on the bacterial cell surface, and injects the nucleic acid contained in it into the bacterium. The nucleic acid of the phage takes over the control mechanism of the metabolism of the host bacterium and makes it produce proteins and nucleic acid essential for the formation of new phages. After new phages have been formed, they burst out of the host bacterium breaking its wall. The released phages can infect new host bacteria and multiply inside these. Phages causing such lytic cycles are called virulent.

![Fig. 1. T-even phage components (Bradley 1967).](image-url)
Many phages can also have an alternative interaction with their host, the so called lysogeny i.e. after having injected their nucleic acid into the host bacterium, the phages do not multiply but instead the genome of the phage divides with the genome of the bacterium.

In this connection lysogeny will not be dealt with as it has little significance as to the tracer method.

3.3 Some factors affecting the survival of phages

For phages to be applied successfully as tracers on natural water systems or waste waters they have to remain active, i.e. to retain their ability to multiply for a sufficiently long time. In the following, some points affecting the survival of phages will be presented.

3.3.1 Temperature

The effect of temperature on animal viruses and phages has been studied relatively widely, because heating is the most common method of sterilization. The reactions of phages to various temperatures has been particularly well covered, because they are commonly used in simulating the behaviour of animal viruses, as the quantitative analysis of phages is much easier than that of animal viruses.

Thermal inactivation of phages is generally considered exponential (Krueger 1932, Chang et al. 1950, Pollard & Reaume 1951). This might not always be true, as the existence and formation of e.g. resistant mutants may cause deviations from exponentiality (Wilkowski et al. 1954).

There is significant variation between different viruses and phages as to their survival at different temperatures and environments (Metcalf & Stiles 1967). The results of various scientists show some variation, as well, and it is not sensible to make a comparison of the details as the experiments were quite differently conducted. In general it can be said that the rate of inactivation increases with rising temperature, the final inactivation taking place at 30–60°C (Jawetz et al. 1970).

Many environmental factors contribute to the thermal inactivation rate. Among such factors are e.g. pH (Pollard & Reaume 1951), the concentration of divalent ions (Foster et al. 1949), and chloride content (Adams 1948). The effect of the above-mentioned factors varies from phage to phage.

Temperature may be considered one of the main factors in the inactivation of phages and animal viruses. E.g. under marine conditions it is even more important than salinity (Shen et al. 1976).

On the basis of the information given in literature, it can be maintained that phages tolerate fairly well the temperatures occurring in Finnish natural waterways.

3.3.2 Radiation

Radiation also contributes to the inactivation of phages. Of various types of radiation, primarily the effect of short-wave, ionizing radiation on animal viruses and phages has been studied. In general it can be said that these types of radiation have a rather strong inactivating effect on phages (Lea & Salaman 1946, Stent 1958).

Relatively little attention has been paid to the effect of sunlight on the survival of phages, which is important from the point of view of applying phages as tracers. Carstens and his co-workers (1965) observed that direct sunlight had a fairly strong inactivating effect on Poliovirus I in the upper layers of waste water suspension.

Niemi (1976) found that sunlight inactivated T7 phage used as tracer. The author has studied the effect of sunlight on E. coli F52 phage used as tracer in Lake Saimaa.

In the experiment, the phages mixed with water from the site were kept both in unwrapped 100 ml bottles and in similar bottles wrapped in aluminium foil in situ at the depth of 1 m at temperatures +22°C and +4°C. To find out the effect of light the phage contents were analysed at certain intervals both in the dark bottles and the ones exposed to light.

The results obtained are presented in Fig. 2. As can be seen in Fig. 2, sunlight has a rather marked inactivating effect on the phages used in the experiment. Consequently, when per-
forming experiments with phages as tracers, care should be taken to avoid the times when the radiation of the sun is at its strongest, particularly as these coincide with the times when the inactivating effect of temperature is also at its greatest.

3.33 pH-value

Phages survive well in the pH-limits normally found in natural waters. Jawetz with co-workers (1970) suggested that phages and animal viruses generally remain infective if the pH-value is 5–9. Kerby and his team (1949) studied the pH-stability of T7 phage and came to the conclusion that T7 phage stays intact if the pH-value is between 5 and 10.

Sproul (1972) studied the inactivation of T2 phage and poliovirus 1 at high pH-values (Table 2) and concluded that some of them retain their activity even in an environment with pH-values over 11. With such high pH-values even a negligible rise accelerates the inactivation rate considerably. Metcalf and his co-workers (1974) suggested that most enteroviruses tolerate pH-values 10.5–11.0 if the contact lasts less than 15 min.

The experiments of the present writer in waste water treatment plants gave further evidence that phages may survive pH-values in excess of 11 (cf. 7.31).

The pH-value may impede the application of phages as tracers only in heavily polluted areas and in such waste water treatment plants where chemicals causing significant changes in pH-values are used.

![Log of PFU/ml vs. time](image)

Fig. 2. The effect of light on the survival of F52 phage in southern Lake Saimaa. ○ = 22°C/light, ▲ = 22°C/dark, ○ = 4°C/light, ▲ = 4°C/dark, PFU = plaque forming units.

3.34 Chemicals

Compared with bacteria, phages are highly resistant to various toxic materials. Mills (1955) discovered that only 13 among the 1,600 substances that he studied prevented the growth of Salmonella S13 while the phage remain active.

<table>
<thead>
<tr>
<th>Virus used</th>
<th>Contact time min</th>
<th>System</th>
<th>pH</th>
<th>Inactivation per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>T2 bacteriophage</td>
<td>90</td>
<td>Ca(OH)₂ and distilled water</td>
<td>10.5</td>
<td>90</td>
</tr>
<tr>
<td>Poliovirus Type 1 (Sabin)</td>
<td>90</td>
<td>Ca(OH)₂ and distilled water</td>
<td>11.2</td>
<td>No significant inactivation</td>
</tr>
<tr>
<td>Poliovirus Type 1 (Sabin)</td>
<td>90</td>
<td>Ca(OH)₂ and a precipitated and filtered secondary effluent</td>
<td>10.8</td>
<td>98.5</td>
</tr>
<tr>
<td>Poliovirus Type 1 (Sabin)</td>
<td>90</td>
<td>Ca(OH)₂ and distilled water</td>
<td>11.9</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NaOH and distilled water</td>
<td>11.9</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td></td>
<td>KOH and distilled water</td>
<td>12.2</td>
<td>90</td>
</tr>
</tbody>
</table>
Urea and urethan inactivate phages obviously due to the fact that they denature proteins and inactivate enzymes (Adams 1966). However, various phages differ greatly in their ability to tolerate urea (Burnet 1933). Urethan also affects the heat tolerance of phages. Foster and his co-workers (1949) observed that urethan increased the thermal inactivation rate in solutions over 0.05 M.

Many detergents inactivate phages even though the effect differs from phage to phage (Adams 1966). Generally speaking, phages tolerate detergents far better than bacteria do (Kalter et al. 1946).

Phages are not very sensitive to aqueous solutions of alcohols. Ethylalcohol has even been used in purifying phage solutions (Putnam et al. 1949).

Phages are remarkably resistant to such enzyme toxins as cyanide and fluoride. They also tolerate thymol and chloroform quite well. Any of the four substances mentioned above can be used to preserve phage lysates and to interrupt phage production (cf. point 5).

Strongly oxidizing substances such as peroxides, halogens, ozone, and permanganate, on the other hand, have a quickly inactivating effect on viruses (Adams 1966). Generally it can be said that even in respect to toxins, phages are extraordinarily stable under appropriate conditions (Adams 1966), and Finnish natural water systems may be considered to provide such conditions. In performing experiments with tracers on natural systems, toxins and chemicals may have a significant effect only in heavily polluted areas or when waste waters or their fractions are being marked.

3.35 Particles causing turbidity

Carlson with co-workers (1968) studied the adsorption of T2 phage and Poliovirus I on ordinary types of clay such as montmorillonite, kaolinite, and illite. The adsorption of the studied organisms on the clay particles was strictly proportional to the concentration of Na\(^+\), Ca\(^{2+}\), and Al\(^{3+}\)-ions. Also, a low pH-value favoured the adsorption of the viruses.

Divalent cations were more effective than the monovalent ones in stimulating the adsorption of viruses.

Under natural conditions, the adsorbing effect of clays depends, besides the factors presented above, on their surface exchange capacity, the size and shape of particles, and the mode of isomorphous substitution.

Carlson and his co-workers (1968) discovered also that the adsorption of viruses is a relatively quick reaction. 90% of the organisms they studied became adsorbed during the first five minutes and the remaining 10% during the next 15 minutes. According to their observations even proteins adsorbed on clay particles, which might have reduced the adsorption possibilities for viruses.

The adsorption of viruses on particles is a reversible reaction protecting viruses from the inactivating effect of the environment (Cookson & North 1967, Gerba & Schaiberger 1975, Stagg et al. 1977).

After adsorption on clay particles viruses leave the water phase attached to the sedimentating clay.

Adsorption is relatively permanent. For the viruses to detach from the particles causing turbidity, some changes have to occur in the physical-chemical conditions of the environment (Shaub et al. 1974).

As can be concluded from the above, the eventual adsorption of phages on clay particles may have some importance for the tracer method under discussion.

In experiments performed on natural systems, no difficulties caused by clay particles were noticed, not even in rivers that were fairly turbid with clay. The most turbid rivers studied were the River Vantaanjoki and the River Porvoonjoki whose average turbidity in 1972–1974 was 24.5 and 30.5 FTU respectively while the corresponding mean value for the whole country was 6.67 (National Board of Waters 1977).

3.36 Other organisms

Organisms occurring in water have a double effect on the survival of viruses.

Carlucci and Pramer (1960) observed that the number of infective phage particles decreased
more rapidly in unsterilized than in sterilized seawater. Obviously this was due to a group of microorganisms already existing in water or formed because of the additions of phages (Mitchell & Jannash 1969). Such a group may consist of e.g. proteolytic bacteria (Cliver & Hermann 1972, Hermann et al. 1974). Also some higher forms of microbes such as Protozoans (Möse et al. 1970), Ciliates and Nematodes (Chang 1970) may destroy viruses.

On the other hand, the adsorption of phages on the surface of living bacteria (even other than host bacteria) contributes to their survival (Bitton & Mitchell 1974). Thus viruses have been found to survive longer in water from e.g. domestic waste water treatment plants than in sea water (Metcalf & Stiles 1967).

The debris of bacteria has a similar protecting effect on phages, because the adsorption is reversible (Mitchell 1971).

3.4 The survival of phages in natural waters

Niemi (1976) tested how T7 phage, which was used in tracer experiments, survived in various Finnish natural waters. The experiments were performed in the laboratory simulating both summer and winter conditions. The waters used in the experiment are presented in Table 3.

Sampling sites were chosen so that they would represent conditions under which the phage method would be applied.

Niemi (1976) came to the following conclusions:
— All the studied factors (time, temperature of storage, and type of water) had a significant effect on the survival of phages.
— Survival was considerably better at +30°C than at +20°C.
— Phages survived better in clean tap and groundwaters than in other types of waters studied. They survived very well in the waste water of a pulp mill in winter.
— The good survival and the wide range of tolerance makes T7 phage a good tracer of water movements.

The survival of other phages used as tracers has been studied as well, particularly in connection with tracer experiments. The experiences obtained prove that, because of their good survival, phages are generally speaking well adapted as tracers of water movements provided that the inactivating effect of high temperature and sunlight on phages is taken into account when planning tracer experiments.

Table 3. Water samples used in T7 phage survival tests.

<table>
<thead>
<tr>
<th>Source</th>
<th>Description of water</th>
</tr>
</thead>
<tbody>
<tr>
<td>The river Vantaanjoki, Pitkäkoski</td>
<td>Polluted with domestic and industrial waste waters, turpid with clay particles</td>
</tr>
<tr>
<td>The river Tammerkoski, Ratianvuolle</td>
<td>Polluted with domestic and industrial waste waters</td>
</tr>
<tr>
<td>Serlachius Ltd. Lielahti</td>
<td>Waste water from a pulp mill</td>
</tr>
<tr>
<td>Lake Hakojärvi, Evo</td>
<td>Oligotrophic lake water with high concentration of humus</td>
</tr>
<tr>
<td>Brackish water, Katajaluoto</td>
<td>Brackish water, salt content 5 ppt</td>
</tr>
<tr>
<td>Drill well, Köyliö</td>
<td>Ground water</td>
</tr>
<tr>
<td>Helsinki City waterworks, Pitkäkoski</td>
<td>Mains water, dechlorinated</td>
</tr>
</tbody>
</table>

4. QUANTITATIVE DETERMINATION OF PHAGES

The quantitative determination methods can be divided into four groups as follows:
1. Plaque counting methods (Gratia 1936).
2. Determinations in liquid media, based on diluting the sample. Among these, there are the Dilution End-Point method (Bergstrand 1930) and the MPN method (Kott 1966).
3. Measuring the time of lysis (Krueger 1930).

Of these methods, the most commonly used is the agar-layer method for counting the plaques developed by Gratia. There are various modifications of it.
4.1 The principle of the agar-layer method

The method consists of mixing a suspension of host bacteria in a stage of active multiplication with a sample of phages in a liquid (c. 45 °C) soft-agar tube (concentration of agar 1.0 %). Without delay, the contents of the tube are plated on the top of solidified medium in a petri dish (concentration of agar 1.5 %) (Fig. 3).

The dish is incubated at 35—37°C for 3—24 h. If the sample contains infective phages, clear circles or plaques appear on the medium clouded by the bacterial growth (Fig. 4). Each plaque is considered to be formed by one phage.

4.1.1 Growing the inoculum of the host bacterium

The strain of the host bacterium is continuously kept young and pure by spreading it daily on the surface of the medium used. Growth of the inoculum of the host bacterium is always started with a single colony from which cells are inoculated aseptically into a bottle containing broth. The inoculum is grown at 35—37°C with or without a shaker. In a shaker, cultivation will be completed in c. 3—4 h, and without a shaker in c. 4—5 h. The suspension of the host bacterium is ready for cultivation when it is distinctly clouded. When using a tryptone-glucose-yeast-extract medium, the nephelometer reading is c. 10—20 units corresponding to c. 5·10^8—8·10^8 bacterial cells per ml depending on the bacterium used.

Fig. 4. Plaques caused by F52 phages on a petri dish.

4.12 Cultivation

- The medium used was the TGY-medium introduced by Sargeant and his co-workers (1968) with an addition of 0.25 mg MgSO₄.
- Soft-agar tubes are melted and put in a water bath at 45°C.
- 1 ml of the sample to be studied is pipetted into one of the test tubes. 0.1—1.0 ml of the suspension of the corresponding host bacterium is inoculated into the same tube.
- The tube is taken from the water bath, water drops are wiped off the surface, and the tube is shaken avoiding air bubbles.
- The contents of the tube are poured on the surface of the congealed medium and are spread as a thin film by moving the dish horizontally.
- The surface of the dish is allowed to solidify, and the dish is turned upside down.
- The dish is incubated at 35—37°C for 3—24 h. It is possible to make a preliminary count of the plaques after just 3 h incubation particularly if the only piece of information needed is to find out whether there are phages in the sample or not. The final count can be made after 4—6 h incubation when the growth of the plaques has practically ceased.
4.13 Plaque count

If the water sample is very polluted, i.e. it comes from e.g. a waste water treatment plant, the dishes have to be counted after 4—12 h incubation, before the microbes in the sample have had time to grow into disturbingly large colonies. With pure natural water this phenomenon will not occur. In general, the plaques are easy to count, because they usually have clearcut edges, even though they grow very close to one another.

When counting plaques usually the greatest difficulty is to distinguish them from air bubbles that may form during plating. A distinction can be made on the grounds that the border between an air bubble and bacterial growth is dark while it is light with plaques. Often there is a halo lighter than the bacterial growth around plaques which helps to distinguish plaques from air bubbles.

4.2 Some factors affecting the accuracy of the agar-layer method

In order to make the quantitative analysis of phages with the agar-layer method as reliable as possible, optimal conditions should be arranged for the growth of the host bacteria and the adsorption of phages. The cultivation procedure and conditions should also be kept the same.

4.21 The effect of the size of plaques

The size of plaques on a dish follows a typical distribution as presented in Fig. 5 (curve A).

In Fig. 5, the distribution of the sizes of plaques on two different dishes has been presented. On dish A (curve A, Fig. 5), there were plaques of normal size the commonest surface area of which with F52 phages was a little more than 2 mm² (diameter being c.1.5—1.7 mm). On dish B (curve B, Fig. 5) the commonest size of plaques was 0.1—0.2 mm² (with a diameter of 0.36—0.50 mm). The small size of the plaques was deliberately caused by plating on dish B only 1/4 (5ml) of the solid agar on the dish A. Equal amounts of phages were added to both dishes.

On dish A there were 204 plaques and on dish B 115 plaques. The small size of the plaques growing on dish B was obviously caused by the fact that the host bacteria ceased to grow because they ran out of nutrients. The growth of plaques came to an end thereby. As phages adsorb to bacteria at different rates (Paraschivesco 1942), the last phages to adsorb did not have time to achieve any plaques of detectable size by the time the growth of their host bacteria had ceased.

The experiments were repeated several times with different combinations of phages and bacteria, and invariably, the results fell in the same line. Thus it was considered essential to pay closer attention to the facts affecting the size of plaques.

4.211 Factors affecting the size of plaques

In the experiments performed, the following observations were made:
— the size of plaques is, up to a certain point, strictly proportional to the volume of medium on a petri dish. The diameter of plaques increases linearly with the volume of medium up to c. 30—35 ml, after which an increase in the volume of medium has no effect on the size of plaques (Fig. 6).
Fig. 6. The effect of the volume of medium on the size of F52 plaques on a petri dish 90 mm in diameter when the bacterial inoculum remains constant. A and B indicate results of different experiments.

— up to a limit, the size of plaques is inversely proportional to the number of cells in the bacterial inoculum (Fig. 7). The quantity of host bacteria has to be large enough so that their growth causes an even turbidity in the medium.
— up to a certain point, the size of plaques is proportional to the incubation time, as well (Fig. 8).

In general, the first plaques can be observed as soon as after 2–3 h incubation, when it becomes possible to determine whether there are phages in the cultivated sample or not.

Plaques do not grow noticeably any more after 7–8 h incubation. The result obtained agrees with the observations made by Gupta and Johari (1958).

According to Adams (1966) plaques cease to grow by the end of the logarithmic growth phase of the host bacterium.

If the host bacterium has been provided with good growth conditions, the plaque count can usually be made as soon as after c. 4–6 h incubation.

As can be noticed from the results presented above, the size of plaques is very much dependent
on the "well being" of the host bacteria. By arranging conditions favourable to the growth of the host bacteria i.e. sufficient nutrition, a long enough incubation time, proper incubation temperature, plaques can be expected to grow into detectable size.

According to literature, the agar-concentration of soft agar and the size of plaques are inversely proportional (Bronfenbrenner & Korb 1925, Werley & Monley 1964). The phenomenon is due to the fact that the more liquid the medium, the greater the diffusion rate of the phages. The number of phages released from one bacterial cell and the size of plaques are directly proportional (Fukuda 1928).

Even the size of phages has an effect on the size of plaques. The smaller the phage, the faster it is spread into the environment by diffusion, and the larger the plaque formed by it (Glynn & Bailey 1961).

4.22 The effect of the age of the bacterial inoculum on the number of plaques

On the basis of the experiments performed, it can be concluded that the age of the inoculum of the host bacteria had no effect on the number of plaques formed. 1-day-old suspensions of host bacteria were the oldest suspensions used.

Davison and his team (1964) observed that even with 3-day-old suspensions of host bacteria the efficiency of T7 phage would not be reduced.

If host bacteria cultures are used which are over 12 h old, the volume of the inoculum has to be small e.g. 0.1 ml, so that the large quantity of host bacteria shall not prevent the growth of plaques (cf. Fig. 7).

4.23 Factors affecting adsorption of phages

The importance of salts to the adsorption of phages has been studied relatively widely.

Divalent cations, such as Ba++, Ca++, Mg++, and Mn++ strongly promote the adsorption of phages.

Some monovalent cations also (K+, Li+, Na+, NH4+) have a stimulating effect on the adsorption of phages (Puck et al. 1951). Thus many media used in phage studied contain the above mentioned ions.

4.24 The importance of pipetting order

With the agar-plaque method there are two possible ways to perform pipetting, viz. either the bacterial inoculum is first pipetted into the soft-agar tubes in a water-bath and then the phage sample or the order is reversed.

When the effect of pipetting order on the number of plaques was studied, it was observed that with F52 phage, smaller numbers of plaques were obtained, with few exceptions, when the first-mentioned pipetting order was followed than if the sample was pipetted first and the host bacteria inoculum afterwards. The phenomenon was obviously due to the fact that the host of F52 phage did not tolerate the temperature of the water bath long enough, and thus a number of host bacteria were killed, and some phages adsorbed to dead cells of host bacteria.

However, the order in which pipetting was performed did not prove significant in cultivating a combination of E. coli B and phage T7.

Thus the safest way is to pipet first the phage sample and only after this the inoculum of host bacteria into soft-agar tubes. After pipetting, the contents of the tube have to be plated immediately on the surface of the medium in order to shorten the time the mixture of phages and bacteria has to stand in a water-bath.

4.25 Efficiency of plating (EOP)

When there is need to compare the quantitative determinations of phages made at different times, the efficiency of plating, (EOP), for each cultivation has to be known. This is because the properties of cultures of host bacterium may vary with different cultivations so that a standard solution of phages gives different results. This difference of results may be caused, among other things, by the fact that phages can be adsorbed even on dead bacteria and bacterial debris, in which case they no longer form plaques (Adams 1966). If cultures of host bacteria in widely different physiological stages are used for cultiva-
tion, the possibility of getting different results is increased.

To make the various cultivations comparable, the so called relative efficiency of plating (Ellis & Delbrück 1939) is used. For the determination of EOP, a standard solution of phages is prepared and kept in a refrigerator protected from light. This standard solution of phages is assumed to remain unchanged.

At each cultivation, a quantitative determination is made of the standard solution of phages, parallel to the ones made of the samples studied. At the end of the experiment, the cultivation with the highest phage titers (obtained from the standard solution of phages) is chosen as a reference. The results of other cultivations are related to it by multiplying the phage titers obtained from the samples with a correction coefficient *k* which can be obtained through the calculation presented in formula 1.

\[
k = \frac{\text{PFU ref.}}{\text{PFU cult.}}
\]

PFU ref. = the content of the standard solution of phages chosen as a reference
PFU cult. = the content of the standard solution of phages obtained at the cultivation to be correlated

The use of a correction coefficient is avoided when all samples are cultivated employing the same suspension of host bacterium. In tracer experiments on natural systems this is the common practice except in cases in which large numbers of samples make it impossible.

4.3 Suggestions for cultivating phages with the agar-layer method

The inoculum of the host bacteria should come from a young culture in an active stage of multiplication. Each plate is inoculated with equal amount of the inoculum (e.g. 1 ml). If dense cultures of host bacteria, already past the logarithmic stage of growth, have to be used, the volume of the inoculum has to be small (e.g. 0.1 ml) so that the growth of the host bacteria and that of plaques as well is not suffocated too early. If the results of various samples are to be comparable with each other, the same suspension of host bacteria must be used for cultivation. When this is not possible, the EOP determination should be employed to make results comparable.

A sample is always pipetted into a soft-agar tube prior to the inoculum of the host bacteria. The volume of the sample to be studied is usually 0.1−1.0 ml. The volume of the medium to be used should be large enough to allow sufficient nutrition for the host bacteria. When using petri dishes with a diameter of 90 mm, the recommended volume is c. 15−20 ml. Plaques can be counted after 4−6 h incubation.

5. PRODUCTION OF PHAGES

The principle of producing phages is simply as follows: a suspension of host bacteria is grown which is as dense as possible and in an exponential stage of multiplication. Phages are added to it before the exponential stage of multiplication is terminated as this provides the best possible conditions for the multiplication of phages. The culture of bacteria and phages is incubated long enough for lysis to occur.

The living cells of host bacteria left in the culture are killed with chloroform, the addition being 1 % v/v.

5.1 Materials and methods

The chief principle in the production of phages to be used as tracers has been the one presented by Sargeant et al. (1968).

Biotec fermentor FL 110 with 7 l effective volume was used for production.

The principle medium used was a slightly modified tryptose-glucose-yeast-extract broth introduced by Sargeant et al. (1968) to which 0.25 g of MgSO4/l have been added. 2 g/l of glucose has been used instead of 1 g/l suggested by Sargeant. In addition, experiments have been made with Casaminoacid-glycerol broth (Fraser & Jerrel 1953) and PAB-broth (Adams 1966). Neither of these proved decidedly superior to the TGY-medium which consequently was used in the routine production of phages as well as
in quantitative determinations.

The temperature during production was 35—37°C.

Filtered pressurized air was used for aeration. The aim was to adjust the intensity of the aeration so that the volume of air flowing per minute through the culture would equal the volume of the culture (Elsworth et al. 1957).

Sufficient mixing to keep the culture homogeneous was aimed at. With Biotec's fermentors the rotation rate used was c. 800 rpm.

SAG 741 (Union Carbide Corporation, USA) was used as the antifoam agent.

pH was adjusted with 1 N or 2 N solution of NaOH, either manually or by means of an automatic pH-adjuster. The aim was to keep the pH-value of the culture neutral or a little below it.

The growth of the bacterial culture was observed with an EEL-nephelometer.

5.2 Cultivation of phages

A suspension of host bacteria at the logarithmic growth phase is inoculated into a fermentor, the temperature of the broth inside being 35—37°C.

The aeration, mixing, temperature and pH-adjustment of the culture are arranged as described above.

The growth of the culture is observed by means of a nephelometer from samples taken at 20—30 min intervals. With the help of the values obtained a growth-curve is drawn during the whole experiment to make it possible to follow the progress of the logarithmic growth phase (Fig. 9).

When the stage of exponential growth is estimated to approach an end, phages are added into the culture. The input ratio should be large enough to allow 1—4 infective phage particles to each bacterial cell (Adams 1966). Sargeant et al. (1968) employed phage-bacteria ratios that varied from 0.1 to 14.

In the production of phages to be used as tracers such phage-bacteria ratios have been used that fall within the limits presented above.

After the addition of phages, growing is continued for another 30 to 60 min after which 10 ml of chloroform per liter are added into the culture. Fermentation is continued without aeration for a further 15 to 20 min after which the culture is kept in a refrigerator.

Using the above-mentioned principles, suspensions of tracer-phages have been obtained the highest titers of which are in the order of $10^{11}$—$5\times10^{11}$ PFU/ml.

Phages can also be grown in ordinary erlenmayer-flasks without aeration. In this case the flask has to be shaken manually from time to time to promote the growth of bacteria. Otherwise the principle of growing is exactly the same. In this way it is possible to reach phage titers of c. $10^{10}$ PFU/ml.

Because in the tracer experiments there was no need to use more concentrated phage suspensions than those referred to above, no importance has been laid to produce these. According to literature, it is possible to produce phage suspensions with titres as high as $10^{12}$—$10^{13}$ PFU/ml (Sargeant & Yeo 1966).
Phages are kept in a refrigerator at about +4°C protected from light. Experiences prove that phages kept in a refrigerator for years without a considerable decrease in their number.

6. MARKING OF WATERS

When marking waters in order to find out the time and/or directions of travel the commonest procedure is as follows:

The tracer to be used is injected in a desired spot as a short impulse. At a certain distance the concentration of the tracer is analysed as a function of time. The results obtained (tracer-curve in Fig. 10) describe directly the mathematical distribution of the time of travel.

6.1 The principle of marking with phages

In marking waterways phages are used in principle as any tracer would. The basic principle of marking is the same, independent of the type of water.

In the following the principle of marking a river will be presented in detail.

Before marking the river a control sample is taken in order to determine the background concentration of the phages of the host to be used. 10 to 20 ml of the suspension of phages to be used as tracer is saved for the final analysis of the samples.

In marking rivers, the number of phages needed can be estimated by means of the following, entirely empirical formula (2):

\[ P_{\text{mark}} = a \cdot t_{\text{trav}} \cdot Q \]

where:
- \( P_{\text{mark}} \) (PFU) = the number of phages needed for marking
- \( a \) (PFU/ml) = coefficient with a value of 20 to 50
- \( t_{\text{trav}} \) (s) = estimated time of travel
- \( Q \) (ml/s) = streamflow in the river to be marked

If the background concentration of phages to which the host is sensitive is high or the conditions unfavourable for the survival of phages, higher values of the coefficient \( a \) should be used than that presented above.

After the above preliminary measures the tracer suspension is added at a point where it is readily mixed with water.

![Fig. 10. A typical time concentration curve obtained with tracer methods. The figure shows the results of two successive marking experiments with Rhodamin B. Vertical line \( t_g \) depicts the position of the time co-ordinate of the center of gravity of the tracer curve on the time axis which is considered to indicate the average time of travel of the marked water (Wright & Collings 1964).](image-url)
6.11 Sampling and storage of samples

Samples are usually taken at the same sampling site at fixed intervals. If the distance to marked is very long, and there are no previous estimates of the time of travel, it might prove useful for a start to take samples in different parts of the river. Thus it is possible to find out how far the tracer wave has reached after a particular time of travel. The result obtained helps to plan sampling to occur at fixed sampling sites so that the whole tracer wave is caught while unnecessary samples can be avoided.

The samples are usually kept in a dark refrigerator. If information is also wanted as to how phages survive under the conditions of the experiment, the sample bottles are kept at the temperature of the marked water and in the dark.

6.12 Analysis of samples

If the samples taken amount to e.g. several tens, it is practical to perform a preliminary analysis on undiluted samples. It helps to pick up the samples with the tracer as well as to estimate the dilution of samples that might be needed.

Final analysis is performed on the samples which, in the preliminary analysis, were found to contain the tracer. At the same time, employing the same suspension of host bacteria the titer of phages in the suspension used as tracer as well as that of phages in the control sample are analysed.

The purpose of the final analysis is to make all samples as comparable as possible with each other as regards cultivation.

It is impossible to attain perfect comparability as phages are unlikely to survive equally in the river and in the sampling bottles.

6.13 Analysis of results

The co-ordinate of the center of gravity on the time axis \( t_g \) is calculated from the tracer curve obtained in the final analysis according to formula (3):

\[
t_g = \frac{\sum_{i=1}^{n} c_i t_i}{\sum_{i=1}^{n} c_i}
\]

\( c_i \) = the concentrations of phages in sample \( i \)

\( t_i \) = the time elapsed from the beginning of the experiment to taking the sample \( i \)

The position of the center of gravity of the calculated tracer curve on the time axis is considered to represent the average travelling time of the tracer as well as that of the river to be marked.

7. TEST RESULTS OF THE PHAGE METHOD

The adaptability of the phage method to mark rivers, ground waters, waste water plants, and the waste water of wood industry moving in a lake along hypolimnion has been tested in 1968—1975.

All the tests have been performed in southern Finland.

7.1 Marking experiments in rivers

Marking experiments have been performed in the Rivers Kymijoki, Vantaanjoki and Porvoonjoki. The aims and methods of the experiments performed in the Rivers Kymijoki and Porvoonjoki (Kinnunen 1974) have a great resemblance, and therefore only the experiments in Kymijoki and Vantaanjoki will be presented here.

7.11 Dependence of water travel speed on river flow in the River Kymijoki

The dependence of the average water travel speed on the river flow in the segment of Kymijoki which extends from the main sewer of Kouvola to Myllykoski (Fig. 11) was studied. This piece of information was needed for a study on the movements of waste water.

The first marking experiment was carried out on 18th October 1968 (Niemelä & Kinnunen
depending on the sampling site.

Marking was carried out by injecting the suspension of phages in the middle of the river at the point where the main sewer of Kouvola is discharged into Kymijoki. The first marking experiment on 18th October 1968 was an exception as the tracer was added into the lower channel of the power plant at Keltti.

Phage F52 was used as tracer in the experiments performed on 18th October 1968 and 1st April 1975; in other experiments T7 was used.

The volume of the tracer suspension was 4000 to 5000 ml and the phage titer varied from \( \frac{5}{2} \cdot 10^{10} \) to \( \frac{5}{2} \cdot 10^{11} \) PFU/ml in different experiments.

![Fig. 11. Map of the River Kymijoki. 1=the power plant at Keltti, 2=the marking site at the point of discharge of main sewer of Kouvola, 3=sampling site at Myllykoski, 4=the power station at Myllykoski, 5=the Gulf of Finland.](image)

1968). At the time a distance of 17.2 km was measured from the power station of Keltti to Myllykoski. The phage wave was analysed at the discharge spot of the main sewer of Kouvola as well. The tracer waves were not caught completely as the timing of sampling was not quite successful, but the result was so promising that it became desirable to continue the experiments.

Later on, five marking experiments were performed in Kymijoki during a period from 15th May 1970 to 1st April 1975 (Kinnunen et al. 1974).

The discharge in Kymijoki varied from 258 to 574 m\(^3\)/s during the experiments.

The distance of travel was 8800 to 8760 m

![Fig. 12. The experiment in the River Kymijoki 15th to 16th May 1970. Streamflow 374 m\(^3\)/s. The volume of the phage suspension 4000 ml, phage titer \( \frac{5}{2} \cdot 10^{10} \) PFU/ml, time of travel 7.31 h.](image)
The average time of travel was calculated from the tracer curves as has been described above (cf. 6.13).

The tracer curves obtained in the experiments in the part of the river from the main sewer of Kouvola to Myllykoski are presented in Figures 12 to 16.

The results are combined in Table 4.

Table 4. Results of studies in the river Kymijoki.

<table>
<thead>
<tr>
<th>Date</th>
<th>Distance</th>
<th>Time of Travel</th>
<th>Velocity</th>
<th>Discharge</th>
</tr>
</thead>
<tbody>
<tr>
<td>15.–16.5. 1970</td>
<td>8 800</td>
<td>7.31</td>
<td>1 208</td>
<td>374</td>
</tr>
<tr>
<td>6.– 7.6. 1971</td>
<td>8 760</td>
<td>6.90</td>
<td>1 270</td>
<td>386</td>
</tr>
<tr>
<td>17.–18.8. 1971</td>
<td>8 670</td>
<td>10.44</td>
<td>839</td>
<td>258</td>
</tr>
<tr>
<td>7.– 8.9. 1971</td>
<td>8 760</td>
<td>9.62</td>
<td>911</td>
<td>283</td>
</tr>
<tr>
<td>1.4. 1975</td>
<td>8 760</td>
<td>4.68</td>
<td>1 872</td>
<td>574</td>
</tr>
</tbody>
</table>

On the basis of the results obtained the regression line of the velocity of travel on discharge was calculated (Fig. 17).

The part of Kymijoki studied is well regulated i.e. there are hydroelectric power plants both at Keltti, above the marking site, and below the sampling site at Myllykoski (Fig. 11). The regulation involves keeping the water level constant above the power plant of Myllykoski. Thus the area of the cross-section of the river bed in the measured river course is practically constant and only the slope of the water surface increases slightly with an increase in the discharge. During the studies on water movements the water level above the power plant of Myllykoski varied no more than 5 cm.

According to Chezy's formula the relationship between the discharge and the travelling velocity is linear, if the average cross-section of the river bed remains constant and only the slope of the
water level changes. Since the linear correlation between the discharge and the velocity of travel is excellent ($r=0.9997$), it is a positive proof of the reliability of the method.

7.12 Marking experiments performed in the River Vantaanjoki

In the water course of Vantaanjoki (Fig. 18) in 1974 a marking experiment was performed in which four different phages were employed simultaneously. The phages T7, F46, F52, and F137, had been selected because they did not affect the host strains of each other. Thus they could be determined quantitatively from the same water samples by using the suspensions of their respective host bacteria for cultivation.

The marking sites are seen in Fig. 18. The purpose was to time the marking so that the tracer waves would reach the sampling site at the Pitkäkoski pumping station at approximately the same time. The volume of tracer suspension varied from 1 000 to 3 000 ml and phage titers from $5.8 \times 10^9$ to $1.2 \times 10^{11}$ PFU/ml depending on the type of water to be marked. The marking was performed 19th to 21st January 1974 during a period of low water discharge.

The discharge of Vantaanjoki measured at Pitkäkoski

Fig. 15. The experiment in the River Kymijoki 7th to 8th September 1971. Streamflow 283 m$^3$/s. The volume of the phage suspension 5 000 ml, phage titer $1.00-10^{11}$ PFU/ml. Time of travel 9.62 h.

Fig. 16. The experiment in the River Kymijoki 1st April 1975. Streamflow 574 m$^3$/s. The volume of the phage suspension 5 000 ml, phage titer $1.95 \times 10^{11}$ PFU/ml. Time of travel 4.68 h.
was 3.9 m$^3$/s. During 1961–1970 the average discharge was 12.7 m$^3$/s and the average low water discharge 1.7 m$^3$/s (National Board of Waters 1976). Samples were taken at two-hour intervals by means of an automatic sampling device (North Hants Engineering Co Ltd, Mark 4).

The tracer curves obtained are presented in Fig. 19.

Fig. 19 shows that the tracer waves reached Pitkäkoski overlapping and that they could still be distinguished from one another. The times and velocities of travel calculated from the tracer curves are presented in Table 5. In the same connection the estimated velocities of travel by Helsinki City Water Works for the same rivers and with the same discharge are presented.

The estimates of the Water Works are based on measurements performed with Rhodamin B dye (Table 5).

Since the estimates of the Water Works are based on measurements performed in segments of 2 to 3 km in which the travel of the largest concentration of Rhodamin B has been observed, it is most appropriate to compare the said values with the velocities of travel of phage peaks. All along, the time of travel estimated by the Water Works is higher. At its largest the difference is 49% in the River Tuusulanjoki, and at its lowest, only 8%, in the River Vantaanjoki, which may be considered rather insignificant. It is impossible
Table 5. The results of the experiments in the River Vantaanjoki. The velocity of travel was computed by means of the centers of gravity of the phage tracers.

<table>
<thead>
<tr>
<th>River</th>
<th>Phage</th>
<th>Distance</th>
<th>Time of travel</th>
<th>Velocity of travel</th>
<th>Time of travel</th>
<th>Time of travel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>m</td>
<td>h</td>
<td>m/h</td>
<td>h</td>
<td>h</td>
</tr>
<tr>
<td>Härkölänjoki</td>
<td>F137</td>
<td>46 300</td>
<td>111.8</td>
<td>414</td>
<td>106.2</td>
<td>-</td>
</tr>
<tr>
<td>Luhtajoki</td>
<td>F46</td>
<td>31 300</td>
<td>78.9</td>
<td>397</td>
<td>74.5</td>
<td>92</td>
</tr>
<tr>
<td>Vantaanjoki</td>
<td>F52</td>
<td>62 600</td>
<td>119.3</td>
<td>525</td>
<td>118.6</td>
<td>128</td>
</tr>
<tr>
<td>Tuusulanjoki</td>
<td>T7</td>
<td>24 200</td>
<td>70.9</td>
<td>341</td>
<td>52.5</td>
<td>78</td>
</tr>
</tbody>
</table>

to decide which results are superior as the absolute values for the velocities of travel are not known.

Instead, it is possible to compare the laboriousness of the methods. The measurements with Rhodamin B dye had to be performed in short segments of the river, partly because of the dilution of the dye and partly because of the environmental inconveniences caused by the colour of the tracer. In addition each river had to be marked separately. With the phage method the whole river system could be studied simultaneously in one single experiment.

One of the greatest advantages of the phage method is the possibility to mark several water sources simultaneously which has been referred to above. In theory similar studies can be performed using fluorescent dyes with adsorption maxima different from one another (Smart & Laidlaw 1977). Similarly it might be possible to combine e.g. dye tracers and radioactive isotopes.

7.2 Marking experiments in ground water

As far as is known, phages were first used in studies on ground water movements in 1971, when Kinnunen and Jokinen (1972) employed phage T7 in studying a polluted well. The distance of travel was 12 m. The ground of the area under study was rocky, and the marked ground water probably travelled along cleavages in the rock. Later on marking experiments were performed near Porvoo in the Saksalanniemi area where ground water is being pumped. In these experiments an attempt was made to find out the velocity of travel of water both under natural conditions (Kinnunen 1974) and on a model of an artificially recharged ground water plant.

Only the experiments on the model plant of Saksalanniemi will be dealt with in detail. Also Fletcher and Myers (1974) have used phage T7 to mark ground waters in karst ground.

7.2.1 Experiments on the model of the artificially recharged ground water plant of Saksalanniemi

Marking experiments were carried out on a model of the artificially recharged ground water plant of Saksalanniemi (Fig. 20) in which two phages T7 and F137, NaC1, Rhodamin B, and 82Br-radioactive isotopes were used as tracers under comparable conditions.

During the experiments the replenishing rate of water was 1 000 ml/min (Fig. 20).

7.2.11 Experiments with phage tracers

Two different phages were injected halfway the water column in the ground water pipe of the model (Fig. 20) on 10th November 1975.

The tracers used were:

- phage T7. The volume of the suspension was 1.5 ml, phage titer 5·10^9PFU/ml and the total number of phage particles 7.5·10^9PFU/ml.
- phage F137. The volume of the suspension was 1.0 ml, phage titer 9·10^10PFU/ml.

Samples were taken from tubes P2/2 and P3/2 as well as from the siphon (Fig. 20).

The results of the study are presented in Fig. 21 as well as in the summary table of the ground water studies (Table 6).

The results show that it was possible to meas-
Fig. 20. The model of the artificially recharged ground water plant of Saksalanniemi and the numbering of the ground water tubes.

measure the distance from the marking tube P1/2 to the first sampling tube P2/2 employing both phages. For phage F137 the time of travel was 18.0 hours and for phage T7 18.5 hours. The time of travel of the highest concentration observed was taken as the time of travel. Due to incomplete tracer curves the time co-ordinate of the center of gravity could not be determined.

In the results of the phage markings, attention is drawn above all to the different concentration levels of tracer curves of phages F137 and T7. In tube P2/2 the highest concentration of F137 was $3.5 \times 10^5$ PFU/ml. Considering the amounts of phages used for marking (F137 = $9 \times 10^9$ PFU and T7 = $7.5 \times 10^9$ PFU) the expected peak for T7 should have been c. $3 \times 10^4$ PFU/ml, while in fact it was only $10^2$ PFU/ml. However there was no difference in the survival of the phages.

The different concentrations of the peaks of the tracer curves are obviously caused by the remarkably quick adsorption of the phage T7 into the earth. This assumption is further supported by the fact that there were only occasional phages T7 in the next sampling tube P3/2. The movement of F137 through the whole model could be observed.

The yield of phages was counted from the tracer wave after it had travelled through the model. The yield was 0.7 % which means that during the experiment 99.3 % of the total amount of phage F137 used were absorbed in the model and 100 % of T7.

In some earlier experiments phage F52 was used which travelled through the earth-layer of the model at approximately the same speed as F137.

7.2.12 Experiments with $^{82}$Br-isotope

The experiments with isotopes were performed by the Isotope Laboratory of the State Technical Research Center. Marking experiments have been made on a model with both single-well and two-well methods (Kuusi 1973, Kuusi & Kuoppamäki 1974). Only one experiment involving two wells performed on 24th—30th October 1975 will be dealt with in this context as it can be considered...
In the experiment about 1 mCi (37 MBq) of $^82$Br-isotope in the form of potassium bromide was injected in the middle of the water column in tube P1/2. The travel of the tracer was observed by means of a continuously recording radiation detector immersed in water in tubes P2/2, P3/2, P4/2, and P5/2. The results were recorded on magnetic tape.

The results obtained are presented in Figures 21 and 22 as well as in Table 6.

7.213 Experiments with NaCl- and Rhodamin B-solutions

In the marking experiment of 20th November 1975, 5 000 ml of saturated NaCl-solution with 50 g of Rhodamin B dye dissolved in it were injected into tube P1/2. After marking, samples were taken from tubes P2/2 and P3/2.
Table 6. The results of marking experiments performed in the model of the artificially recharged ground water plant of Saksalanniemi.

<table>
<thead>
<tr>
<th>From tube to tube</th>
<th>Distance</th>
<th>Tracer</th>
<th>Time of travel of the highest concentration</th>
<th>Velocity of travel of the highest concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1/2—P2/2</td>
<td>1.85</td>
<td>F137</td>
<td>18.0</td>
<td>0.103</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T7</td>
<td>18.5</td>
<td>0.100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rhod. B</td>
<td>16.0</td>
<td>0.116</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NaCl</td>
<td>22.0</td>
<td>0.084</td>
</tr>
<tr>
<td></td>
<td></td>
<td>82Br</td>
<td>17.0</td>
<td>0.109</td>
</tr>
<tr>
<td>P1/2—P3/2</td>
<td>4.30</td>
<td>F137</td>
<td>41.0</td>
<td>0.105</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rhod. B</td>
<td>40.0</td>
<td>0.108</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NaCl</td>
<td>43.0</td>
<td>0.100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>82Br</td>
<td>31.0</td>
<td>0.139</td>
</tr>
<tr>
<td>P1/2—siphon</td>
<td>9.25</td>
<td>F137</td>
<td>74.0</td>
<td>0.125</td>
</tr>
<tr>
<td>P1/2—P5/2</td>
<td>8.75</td>
<td>82Br</td>
<td>70.0</td>
<td>0.125</td>
</tr>
</tbody>
</table>

From the samples the travel of NaCl was estimated by measuring conductivity and the travel of Rhodamin B by measuring the absorbance of the samples at 546 nm.

Oy Suunnittelukeskus Ltd was responsible for carrying out the experiment.

The results obtained are presented in Figures 22 and 23 as well as in Table 6.

---

Fig. 22. The travel of NaCl (v—v), Rhodamin B (o—o) and 82Br (—) in the model of the artificially recharged ground water plant of Saksalanniemi from the marking tube to tube 2/2. The concentration of NaCl is expressed as conductivity $10^2$ $\mu$S/cm, that of Rhodamin B as the absorbance reading of fluorometer measured at 546 nm, and that of 82Br-isotope as the $\log_{10}$ number of impulses per three-minute period.
7.214 Comparison of the results

As the different tracers were used only once under comparable conditions, the estimates of their respective velocities of travel cannot be compared statistically. Some conclusions, however, can be drawn of the observed tracer curves (Figures 22 and 23).

The tracer curves of Rhodamin B were the least definite. The tracer was also held up so effectively that it could not be detected any more in tube 4/2. NaCl-solution behaved similarly. Phage T7 was held up as early as during the stretch 2/2–2/3. Only phage F137 and 82Br-isotope could be observed practically through the whole artificially recharged ground water plant.

There were deviations in the observed times of travel of different tracers (Table 6). NaCl seemed to be the slowest of the used tracers. For the tracers which travelled through the model, phage F137 and 82Br-isotope, the same velocity of travel (0.125 m/h) was recorded. As phages were effectively held up in the model, they can only be recommended for these kinds of experiments when radioactive tracers are not available.

7.3 Marking experiments in domestic waste water treatment plants

Marking experiments on domestic waste waters were performed both in a chemical waste water treatment plant and in waste stabilization ponds.

7.31 Experiments in the chemical waste water treatment plant of Lammi Biological Station

Phages were first used to measure the detention of waste water in chemical treatment plant of Lammi (Fig. 24).

The tracers used were phages T7 and F52 as well as Lycopodium-spores stained with safranin and crystal violet (Mayr 1953). Marking was performed in the way presented in Table 7.

Starting from the moment of marking, samples were taken from the water leaving the plant. From time to time, samples were also taken from water coming out of the clarifier which lies after the lime reactor, from water coming from the precipitation tank and from the last clarifier. The samples were plated immediately to find out...
Table 7. Data on tracers used in the waste water treatment plant of Lammi Biological Station 8th November 1972.

<table>
<thead>
<tr>
<th>Marked water</th>
<th>Tracer</th>
<th>Amount of tracer</th>
<th>Number of particles in tracer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toilet waters</td>
<td>T7</td>
<td>500 ml</td>
<td>8.2 x 10^{12} PFU</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10^5</td>
</tr>
<tr>
<td></td>
<td>Red spores</td>
<td>2 g</td>
<td></td>
</tr>
<tr>
<td>Washwaters</td>
<td>F52</td>
<td>500 ml</td>
<td>1.2 x 10^{13} PFU</td>
</tr>
<tr>
<td></td>
<td>Green spores</td>
<td>2 g</td>
<td>10^5</td>
</tr>
</tbody>
</table>

the titer of phages.

Spores were analysed by filtering the samples through 0.45 μm pore size and observing them under the microscope. Lycopodium-spores were detected only occasionally, and therefore their analysis had to be given up while the study went on. Their negligible amount in the samples was obviously due to the small number of particles in the tracer. The high cost of spores made it unfeasible to use larger numbers of them.

The results of determinations of phages from the outlet water are presented in Fig. 25.

The amount of recovered phages was calculated from the results and the volume of water leaving the plant. For phage F52 it was c. 25 % of the phages used (washwater). For T7 the yield was only 0.023 % of the phages used (toilet water).

The time co-ordinates of the largest tracer titers and those of the center of gravity of the tracer wave were calculated theoretically assuming that the flow had been completely mixed (Table 8).

In the results attention is drawn above all to the poor yield of phages (T7) in toilet waters leaving the plant, which was only 0.023 % of the phages used for marking. The corresponding percentage for washwaters (F52) was 25 %.

The poor yield of phages in toilet waters was primarily due to the fact that these were first
Fig. 25. The titer of phages F52 (O—O) and T7 (△—△) in the outlet water of the treatment plant of Lammi.

Table 8. The ratios of the observed time of travel parameters with the theoretical values. 

<table>
<thead>
<tr>
<th></th>
<th>Washwaters (F52)</th>
<th>Toilet waters (T7)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Calculated</td>
<td>Observed</td>
</tr>
<tr>
<td>$t_p$</td>
<td>1.0</td>
<td>0.83</td>
</tr>
<tr>
<td>$t_g$</td>
<td>1.0</td>
<td>0.56</td>
</tr>
</tbody>
</table>

led into lime reactor (cf. Fig. 24) in which the pH of water was 11.0—11.5. Such a high pH-value inactivates phages rather efficiently (cf. point 3.33) which was observed in practice as well, because no phages used for marking were recovered in samples taken from water leaving the lime reactor. However, inactivation was reversible as there were several tens of PFU/ml of the phages T7 in water being discharged from precipitation tank (pH 9.0—9.5) and some hundreds of PFU/ml in water leaving the plant (pH c. 8). The inactivation and reactivation of phages must affect the form of the curve obtained to the extent that the result has to be considered rather unreliable.

With washwaters, conditions do not change nearly so radically as with toilet waters and the yield of 25% obtained can be considered fair.

The strong inactivation obviously has a greater effect on the point of the time co-ordinate of the
center of gravity of the tracer wave than the point of the time co-ordinate of the peak, as the phages which remained longer in the treatment plant were more effectively inactivated than those which stayed under unfavourable conditions for a shorter time. Thus conclusions should be made rather on the basis of the peak of the tracer curve than the time co-ordinate of the center of gravity.

From the results for washwaters it can be concluded (Table 8) that there is at least a minor short circuit in the treatment plant which partly may account for the poor function of the plant.

According to these experiences, it seems obvious that phages are not adapted to measurements of detention times in such treatment plants where there are marked changes in conditions within the plant which are unfavourable for phages. In cases like this it might be advisable to employ e.g. dyes or radioactive isotopes.

7.32 Experiments in the stabilization pond of Saimaanharju

A marking experiment was performed from 25th November to 23rd December 1975 in the stabilization pond of Saimaanharju (Fig. 26) situated in Taipalsaari parish.

1 000 ml of a suspension of phage F52 with a titer of 5.5·10^{10}PFU/ml were used as a tracer. The tracer was injected into the ice-covered pond at the opening of a waste water pipe on 25th November 1975. Samples were taken from the outlet water both with an automatic sampling device and manually. The results are presented in Fig. 27.

The time co-ordinate (t_g) of the center of gravity of the tracer curve was calculated to be 8.8 days. The effective volume of the basin can be calculated by means of formula (4) (Villemonte & Peclich 1962).

\[
\frac{V_{\text{eff}}}{V} = \frac{t_g}{T} < 1
\]

\( V_{\text{eff}} = \) effective volume
\( V = \) the calculated volume of the basin (17 000 m³)
\( t_g = \) the time co-ordinate of the center of gravity of the phage concentration curve (8.8 d)
\( T = \) theoretical detention time (85.5 d)

According to the calculations, the effective volume is 1 750 m³ which is only c. 10.3 % of the total volume of the pond. The study serves to prove that there is a considerable shortcut in the pond which might partly account for the deficient purification capacity of the pond.

The phage reduction in the pond was c. 90 % during the experiment.

Fig. 26. The stabilization pond of Saimaanharju. The area is 19 000 m², average depth 0.9 m, the volume 17 000 m³, the measured waste water discharge 200 m³/d and the theoretical detention 85.5 days.
Fig. 27. The titer of phage F52 in the outlet water of the stabilization pond of Saimaanharju.

7.4 Marking experiment in southern Lake Saimaa

The phage method was tested in tracing the movement of wood industry waste water in the lake hypolimnion of southern Lake Saimaa. The area is polluted chiefly by two pulp mills, Oy Kaukas Ltd and Oy Joutseno-Pulp Ltd (Fig. 28).

The waste waters of Oy Kaukas Ltd travel in summer in the epilimnion (through Small-Saimaa) into Main-Saimaa where they are flowing past both the east and the west side of Kätkytsaari island (Heinonen 1973). At this point the main flow of Saimaa is from north-west to south-east towards the River Vuoksi (Fig. 28).

The waste waters of Oy Joutseno-Pulp Ltd are discharged into the same mixing area to the east of Kätkytsaari island and further towards the Vuoksi (Fig. 28).

In winter the currents of waste waters are essentially different. The waste waters form a hypolimnic layer in the immediate vicinity of the discharge place and then travel relatively undisturbed along the bottom following its morphology. When discharged into Main-Saimaa, part of the waste waters of Oy Kaukas Ltd is mixed with the main flow travelling towards the Vuoksi, while another part continues its flow from this mixing area to the east of Kätkytsaari island following the profile of the bottom to the north-west against the direction of the main flow of the watercourse towards Lake Ilkonselkä (Heinonen 1973). The waste waters of Oy Joutseno-Pulp Ltd also flow mainly in the hypolimnion in winter (Heinonen & Kettunen 1975).

As the waste waters of Oy Kaukas Ltd and Oy Joutseno-Pulp Ltd greatly resemble one another, it is not possible to determine with any certainty which waste waters pollute Lake Ilkonselkä or if the waste waters of both factories travel that far.

To make a preliminary test of the movements of waste waters and to test the method, the waters of Oy Kaukas Ltd were marked before they were discharged into the mixing area mentioned above (Fig. 28).

On 12th April 1976 an observed waste water layer of approximately two meters' thickness was marked by injecting 37.2 l of phage F52 suspension into it 0.5 meters above the bottom. The
Fig. 28. The map of the testing area in southern Lake Saimaa (Heinonen 1973). In the map, the direction of the main flow is indicated with open arrows, the marking place to the south of Kätkytsaari island with (●), and the site of the dense sampling grid (Fig. 29) with a rectangle, the observed winter course of travel of the waste waters of Oy Kaukas Ltd with arrows and the course of the deep channel of Lake Saimaa with a dotted line.
density of the suspension was $5.3 \cdot 10^{10}$ PFU/ml.

Sampling was arranged in view of finding out first the main direction of the travel of the tracer. When this was discovered, the movements of the tracer in this direction were given undivided attention. The samples were plated in the field laboratory of the water bureau of the Kymi water district about 20 km from the area of study. The samples were transported there for analyses immediately after sampling.

At the first stage of the study it became apparent that the bulk of the tracer was travelling to the north and north-west along the deep-water channel. Similarly it was observed that only the waste water layer travelling along the bottom contained some tracer.

After the direction of the main flow was found out sampling was arranged in order to determine as accurately as possible the arrival of the tracer in some area which was important for the study. An area to the south-west of Kilpiänsaari island was chosen for this purpose, and a dense network of sampling sites was formed along the deep-water channel running through it. This area was chosen because if the tracer was found to travel this far against the current it would presumably continue unhindered until the basin of Ilkonselkä as there was no formation of the bottom to prevent it at that stage.

Samples were taken at the depth of 1 m from the bottom which meant about the middle of the observed waste water layer.

The network of sampling sites covered an area about 1100 m x 100 m (Fig. 29). The distance

![Fig. 29. The situation 72 h after injection. The dots mark the sampling sites and the figures above them the corresponding titers of phages PFU/ml.](image-url)
between the sampling sites was 100 m.

In Fig. 29 the 'tracer cloud' had almost reached the sampling network. Calculated according to the greatest titer observed, the speed of travel was c. 1300 m per day. The titer, 350 PFU/ml, observed to the north-west of the sampling network was either due to a fault in sampling or else the leading edge of the tracer wave travelled considerably faster than presented above.

After the situation in Fig. 29 (72 h) the sampling had to be discontinued owing to some technical difficulties, and all attempts to catch the tracer cloud were given up. When sampling was resumed the main interest was to make sure that the tracer went on travelling in the previously observed direction.

The next samples were not taken until 119 h and 141 h after the marking at four widely distributed sites. The last samples were taken as late as 216 h and 336 h after the beginning of the experiment near the Ilkonsaari islands.

The sampling points are presented in Fig. 30 and the results obtained in Table 9.

Table 9. The titers of phages in samples taken 119—336 hours after injection and the distances of sampling sites from the marking site.

<table>
<thead>
<tr>
<th>Station</th>
<th>Distance m</th>
<th>Time h</th>
<th>Titer PFU/ml</th>
<th>Time h</th>
<th>Titer PFU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>43</td>
<td>5 600</td>
<td>119</td>
<td>4</td>
<td>141</td>
<td>25</td>
</tr>
<tr>
<td>44</td>
<td>5 800</td>
<td>119</td>
<td>32</td>
<td>141</td>
<td>10</td>
</tr>
<tr>
<td>45</td>
<td>6 200</td>
<td>119</td>
<td>53</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>46</td>
<td>6 800</td>
<td>119</td>
<td>0</td>
<td>141</td>
<td>2</td>
</tr>
<tr>
<td>47</td>
<td>13 200</td>
<td>216</td>
<td>5</td>
<td>336</td>
<td>1</td>
</tr>
</tbody>
</table>

From the results presented in Table 9 the conclusion could be made that the tracer travelled in the direction suggested by previous results towards the basin in Lake Ilkonselkä.

On the basis of the study the following conclusions can be made among others:

Fig. 30. The position and numbering of sampling sites chosen 119—336 h after injection.
— Waste waters from Oy Kaukas Ltd travelled in winter in the hypolimnion against the main flow towards the basin of Lake Ilkonselkä, the speed of travel of the greatest titer observed being c. 1 300 m per day.

— Because the whole tracer cloud was not caught simultaneously and there was no exact information of the discharge, it was impossible to calculate what proportion the tracer (waste water) travelling against the main flow formed of the total volume of tracer (marked amount of waste water). As only occasional phages in remarkably low titers were discovered in the mixing area to the east of the marking site, the bulk of the marked waste water was supposed to travel against the main discharge.

8. DISCUSSION

The reliability of the quantitative determination of a tracer is of decisive importance to a tracer method.

The quantitative determination of phages was performed with the agar-layer method which is only semi-quantitative, in a way, because all the phages in the sample do not form plaques. Some phages may be adsorbed on dead bacteria or bacterial debris. Neither do phages form plaques if the growth period of the host bacteria after injection remains so short that there is no time for new phages to break out from the host cells of the bacteria and infect new host bacteria around them (Adams 1966). The method can be made sufficiently reliable providing that cultivation conditions are kept constant, and optimal conditions are provided for the growth of host bacteria and the adsorption of phages. It is important for the growth of the host bacteria that they have sufficient amounts of suitable nutrients and optimum temperature. In order to allow phages to grow into detectable size, it is advisable to provide the host bacteria with such conditions that the logarithmic growth period lasts as long as possible, as plaques cease to grow (Adams 1966) or, according to the experiences of the present writer, at least they grow considerably slower, when the host bacteria reach a stationary growth stage. This can best be ensured by using a small enough inoculum of the host bacteria with sufficient medium to grow on. On the other hand, the inoculum of the host bacteria shall be large enough to cause an even turbidity of the medium.

The quantitative determinations of various samples can be made comparable by using the same suspension of host bacteria for all cultivation. In cases when samples have to be cultivated on suspensions of host bacteria from different batches, the results are corrected with the EOP-coefficient. This must be used because the state and properties of the cultures of host bacteria may vary from one cultivation to another. Thus the efficiency of plating may be different. Considering the above-mentioned factors, the agar-layer method can be considered a sufficiently good quantitative method for the purpose being discussed.

When estimating tracer methods the dilution ratio of a tracer is of great importance. In literature the dilution ratios of tracers are given in a variety of ways which causes difficulties in comparing them. To make the thing clear two concepts are introduced i.e. the maximal dilution ratio and the practical dilution ratio. The former means the number of ml in which 1 g of tracer could be diluted to be still detected. Practical dilution ratio (g/ml) is obtained when the amount of tracer (g) is known which is needed to mark a certain volume of water (ml).

The maximal dilution ratio is only affected by the titer of the tracer or the number of impulses in the tracer per unit of volume as well as sensitivity of determination. The practical dilution ratio is affected, in addition to these, by the stability of tracer and the background concentration in the water to be marked. In Table 10 the maximal dilution ratios of some substances used as tracers are presented.

The maximal dilution ratios of phages are obtained straight from the titers of phage solutions. In this experiment the most concentrated phage solutions grown were c.5·10^11 PFU/ml. According to literature it is possible to grow phage solutions with titers in the order of 10^13 PFU/ml (Sargeant & Yeo 1966). Thus the maximal dilution ratio for phages is calculated to be c. 10^{-11} - 10^{-13} depending on concentration of the tracer. The maximal dilution ratio for phages is approximately in the same order as that for substances that are analysed with neutron activation analysis.
Table 10. The maximal dilution ratios of some tracers. Substances 1—3 have been analysed with neutron activation analysis, 4th with radiation detector, 5—9 with a fluorometer, 10th as conductivity and 11th with the agar layer method.

<table>
<thead>
<tr>
<th>Tracer</th>
<th>Maximal dilution</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Indium</td>
<td>$10^{-12}$</td>
<td>Kuoppamäki and Muurinen (1976a, b)</td>
</tr>
<tr>
<td>2. Europium</td>
<td>$10^{-13}$</td>
<td></td>
</tr>
<tr>
<td>3. Dyspropium</td>
<td>$10^{-12}$</td>
<td>Wimpenny (1977)</td>
</tr>
<tr>
<td>4. $^{82}$Br</td>
<td>$3\cdot 10^{-12}$</td>
<td>Smart and Laidlaw (1977)</td>
</tr>
<tr>
<td>5. Amino G acid</td>
<td>$2\cdot 10^{-9}$</td>
<td></td>
</tr>
<tr>
<td>6. Fluorescein</td>
<td>$3.5\cdot 10^{-9}$</td>
<td></td>
</tr>
<tr>
<td>7. Rhodamine WT</td>
<td>$7.7\cdot 10^{-10}$</td>
<td>Foxworthy et al. (1966)</td>
</tr>
<tr>
<td>9. Rhodamine B</td>
<td>$10^{-9} - 10^{-10}$</td>
<td></td>
</tr>
<tr>
<td>11. Phages</td>
<td>$10^{-11} - 10^{-13}$</td>
<td></td>
</tr>
</tbody>
</table>

and $^{82}$Br-isotope and a little higher than the ratio for fluorescent dyes.

The practical dilution ratio has of course a much greater importance than the maximal dilution ratio when comparing tracer methods.

As to phages, the background concentration affecting the practical dilution ratio has not usually been significant being in general c. $0-5$ PFU/ml. The only difficulties have been encountered in some experiments in waste water treatment plants where the background concentration may be considerable. This problem will be dealt with in detail in connection with the evaluation of experiments in treatment plants.

According to literature as well as the writer's own experiences phages are relatively resistant to the inactivating effects of the environment (cf. point 3.3) and thus, because of their good survival they are well adapted to be used as tracers in a variety of waters (Niemi 1976). It was observed that the survival of phages may cause trouble chiefly in the middle of the summer when the inactivating effect of light and temperature is at its highest as well as in treatment plants where the infectiveness of phages may be endangered by the use of chemicals.

The practical dilution ratio of phages can be illustrated with tracer experiments in rivers. It has proved in practise that there should be c. $20-50$ PFU/ml in the volume of water marked (cf. Formula 2). In the experiment in Kymijoki the average titer of the tracer was $8.3\cdot 10^{10}$PFU/ml, giving practical dilution ratio $1.7-4.2\cdot 10^{-9}$. If calculated from the highest value $10^{13}$PFU/ml found in literature the practical dilution ratio would be $2-5\cdot 10^{-11}$.

For Rhodamin B dye the practical dilution ratio in pure river waters is in the order of $10^{-8}$ (Koroleff & Virta 1962) when measured with Turner 110 fluorometer equipped with a flow-through cell. In waters containing humus it is apparently still lower as Rhodamin B is relatively easily adsorbed on humus particles (Feuerstein & Selleck 1963, Buchtela et al. 1964).

From the data presented by Kuoppamäki and Muurinen (1976 a and b) it can be calculated that in experiments performed in lakes the practical dilution ratio is in the order $10^{-11}-10^{-12}$ for substances like Indium, Europium and Dyspropium which are analysed with neutron activation analysis.

The practical dilution ratio of radioactive isotopes is affected by radiation protection regulations as well.

The practical dilution ratio of NaCl is very unfavourable as can be concluded from the low maximal dilution ratio (Table 10). According to Käss (1965) to mark the same volume of water 10 000 times more of NaCl is needed than fluorescein, which gives a good idea of the inferiority of NaCl as a tracer.

Table 11 is a summary of the practical dilution ratios of some tracers. As the ratios have been calculated from the studies of various writers they are comparable only as to the order of magnitude.

As can be noticed from the above, the practical dilution ratio of the phage method is rather high and in the same order of magnitude.
Table 11. The practical dilution ratios of some tracers. Analysis as in table 10.

<table>
<thead>
<tr>
<th>Tracer</th>
<th>Practical dilution</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indium</td>
<td>10–12</td>
<td>Kuoppamäki and Muurinen (1976a, b)</td>
</tr>
<tr>
<td>Europium</td>
<td>2.5–10–11</td>
<td>»</td>
</tr>
<tr>
<td>Dyspropium</td>
<td>3.5–10–11</td>
<td>»</td>
</tr>
<tr>
<td>Rhodamin B</td>
<td>10–8</td>
<td>Koroleff and Virta (1962)</td>
</tr>
<tr>
<td>NaCl</td>
<td>10–5</td>
<td>Käss (1965)</td>
</tr>
<tr>
<td>Phages</td>
<td>10–9–10–11</td>
<td></td>
</tr>
</tbody>
</table>

as that of the best tracers to be analysed with neutron activation analysis.

Compared with Rhodamin B dye the ratio is c. 10–100 fold, which means that the amount of the phage tracer is only 1/10–1/100 of that of Rhodamin B required for marking. In practice this has often become very obvious as it has been possible to mark considerably larger volumes of water with phages than with Rhodamin B dye e.g. in the River Kymijoki (7.11) and in the water course of the River Vantaanjoki (7.12). The great practical dilution ratio of the phage tracer is also evident in the fact that the amount of tracer needed for experiments has been rather small, in general only some liters. Owing to this, the tracer expenses are low provided that the required equipment which is in quite common use is available for growing the phages. Wimpenny (1977) has stated, that μ 2 phage is over a 1000 times more effective than 82Br-isotope when prices are compared.

The following conclusions can be made about the adaptability of the phage method to mark different types of waters:

Phages seemed to be very well adapted to studies on water movements in rivers. The linear dependence of travel speed on the streamflow of the River Kymijoki observed with the correlation coefficient 0.9997 can be taken as an evidence of the reliability of the method. The amounts of the tracer needed were also small, c. 4 000–5 000 ml, in spite of the streamflow which was large for Finnish conditions, 258–574 m³/s. Simultaneous marking of several rivers that was tested in the river course of the River Vantaanjoki was also quite successful. It is difficult to compare the results obtained with those obtained with Rhodamin B as the measurements have been performed at different times and in different ways. Because the measurement with Rhodamin B were performed in short stretches and the results combined to apply to the whole length of the river, while the whole length could be marked with phages at the same time, there is reason to consider the results obtained with phages more reliable than those obtained with Rhodamin B. The experiment with phages was also considerably less troublesome than the experiments with the dye. Also the fact that it was possible to mark the whole water system with phages at the same time gives a more reliable idea of the speed of travel in a given streamflow than if the measurements had to be timed at long intervals for each river separately during which the streamflows might change. This kind of simultaneous and relatively trouble-free use of several phages is one of the greatest advantages of the method. Similar experiments can be performed using e.g. dyes with different absorption maxima (Smart & Laidlaw 1977), Lycopodium-spores that have been coloured differently (Drew & Smith 1969), and combinations of radioactive isotopes (Abood et al. 1969). The poor practical dilution ratio, environmental harm etc. will limit the use of such tracers in general.

The tracer experiments in ground waters showed that phages are adsorbed rather effectively in sand in the artificially recharged ground water plant studied. This was expected as earlier experiments had proved that viruses in general are absorbed in sandy soil (Drewry & Eliassen 1968, Sobsey et al. 1975), particularly when the ground water is flowing slowly as was the case in the experimental situation. With an increase in the speed of flow the absorption of viruses is reduced and for example heavy rains may carry viruses into ground water (Duboise et al. 1974, Wellings et al. 1974, Duboise et al. 1976). The absorption rates varied from phage to phage.
Phage T7 was absorbed almost completely in as short a distance as 1.85 m of travel and it could no more be detected in outlet water (distance of travel 9.35 m). Phage 137 was absorbed 99.3 per cent in the artificially recharged ground water plant but due to its large initial concentration it could still be detected in the outlet water as a distinct tracer wave. Also Duboise et al. (1974) observed differences in the absorption of various phages into sandy soil.

In the artificially recharged ground water plant tracer experiments were performed also with NaCl, Rhodamin B and $^{82}$Br-isotope. Of these, Rhodamin B and NaCl proved to be undoubtedly the worst. The curves for NaCl were rather clear but the tracer was only observed as far as 4.30 m. The velocities of travel for NaCl were the slowest of all, which was obviously due to its strong adsorption into sand (Knutsson 1970).

The Rhodamin curves were also rather indefinite as can be seen in Figs. 22 and 23. This was due to the fact that Rhodamin B is readily adsorbed on grains of sand (Knutsson et al. 1963). It also could only be followed up to 4.30 m. $^{82}$Br-isotope whose activity could be determined with an automatic measurement in the ground water pipe proved the best tracer of those studied. The curves obtained with it were rather clear and easily read. For both tracers that travelled through the model of an artificially recharged ground water plant, phage F137 and $^{82}$Br-isotope, the velocity of travel was measured 0.125 m/h.

Thus it can be said that phage F137 gave as reliable a result as $^{82}$Br-isotope. It is to be suspected, however, that if the distance to be measured had been longer even phage F137 would have had time to be absorbed into soil and would have failed to produce a result. It is evident that radioactive isotopes which can be determined right on the field are very well adapted as tracers of ground waters because of their mobility and easy analysis. Phages should be considered only when the water to be marked travels along cleavages in rock (Kinnunen & Jokinen 1972) or in karst soil (Fletcher & Myers 1974) when they do not absorb into soil to a significant extent. In such cases the phage method provides good possibilities for e.g. pollution studies and marking various water sources simultaneously. Thus a situation like this could be compared to marking rivers with the difference of the "river" travelling underground.

Phages have been used as tracers in waste water treatment plants rather frequently. There they have been employed as model viruses to simulate the inactivating effect of various waste water treatment procedures on animal viruses (Malherbe & Strickland-Cholmley 1967, Cookson 1969, Manwaring et al. 1971, Amirhor & Engelbrecht 1974). On the other hand, strong opinions have been expressed that phages should not be employed for this purpose, as phages have been observed to behave rather differently from animal viruses in different treatment plants (Ranganathan et al. 1974, Moore et al. 1975). In the studies referred to above, only Malherbe and Strickland-Cholmley (1967) paid attention to the detention time of the treatment plant they studied. In general, it can be worked out if the phage reduction can be calculated.

In the present experiment phages were used to study the detention time in both a chemical waste water treatment plant and in waste stabilization ponds. Studies on detention times of plants to be performed with phages can be impeded mainly by two factors: the poor survival of phages in the water of plants as well as a possible large background concentration. Both these factors reduce the practical dilution rate of phage tracers.

In the experiment performed in a chemical waste water treatment plant (7.31) two phages and Lycopodium-spores were used at the same time. The analysis of the latter had to be given up because of the scarcity of the tracer. Phages were used to mark both toilet and washwaters. The time co-ordinates of the peak and the center of gravity of the tracer curve were compared with the corresponding theoretically calculated values (Table 8). The time co-ordinate of the center of gravity of the tracer curve for toilet waters was c. 80 % smaller than when calculated theoretically. As the toilet waters underwent lime precipitation with pH value of c. 11–11.5, the phages were inactivated quite quickly. Within this range even a small change in pH-value causes a large change in the speed of inactivation (Berg et al. 1968) and so has an effect on the shape of the tracer curve. Thus the tracer curve of toilet
waters must be considered undoubtedly unreliable.

For washwaters the time co-ordinate of the peak of the tracer curve was only 17% smaller than the theoretical estimate, while that of the center of gravity was as much as 44% smaller than the theoretical value. This also serves to prove that intense inactivation affects the form of the tracer curve by lowering the time co-ordinate of the center of gravity rather than that of the peak.

In the present writer's experience phages are not adapted to studies of detention times in such treatment plants where conditions vary greatly from one section of the plant to another. For such cases, radioactive isotopes such as $^{24}$Na and $^{82}$Br or substances to be analysed with neutron activation analysis e.g. Indium (Haimi et al. 1976) might be recommended.

In detention studies in stabilization ponds this problem does not occur, as chemical and physical qualities of the pond do not change drastically in different sections. Instead, the background concentration of phages may cause difficulties. According to literature (Voughn & Metcalf 1975) the dominating strain of phages may be different at different times. This phenomenon has appeared in some detention studies in stabilization ponds when the initial background concentration was 0 rising up to several hundreds of PFU/ml during the experiment. In platting, however, the plaques caused by the background phages can often be distinguished from the plaques caused by phages used as tracers on the bases of morphology. The safest way to avoid disturbances caused by the background is to employ an excess of the tracer to minimize the importance of the background concentration. This will elaborate the analysis of samples as long series of dilutions have to be made.

Microbial tracers have been used several times in water movement studies both in lakes and seas (Robson 1956, Baalsrud 1961, Ormerod 1964, Pike et al. 1969). Phages were first tested for this purpose in southern Lake Saimaa (7.4) with promising results. Because of their large practical dilution rate and their good survival phages seem to be well adapted to studies of the said kind. The tracer field was clearly observed and the travel of waste water against the main direction of flow in the water system could be proved. The farthest observation of the tracer, 13 000 m from the marking site after 336 hours had elapsed from the injection, can also be considered a remarkable achievement.

Similar experiments have been performed in Finland with e.g. Indium which is analysed with neutron absorption analysis (Kuoppamäki & Muurinen 1976a and b). As mentioned previously, the practical dilution ratios for both phages and Indium are roughly in the same order of magnitude. With phages, however, it is possible to observe the travel of the tracer more closely during the experiment as phages can be analysed in a rather unpretentious microbiological field laboratory, while the determination of Indium requires an activation reactor among other things.

The possibility of using several phages provides interesting applications under lake- and seawater conditions when there is need to trace the travel of waste waters coming from several sources in the area of one watercourse the flow conditions being the same for all waste waters.

To sum up the phage method the following notes can be made:

The greatest drawbacks of the method are:

- The determination of phages cannot be performed automatically in the field as with radioactive isotopes and fluorescent dyes and separate samples have to be taken.
- The analysis of phages takes more time than that of the tracers referred to above.

The greatest advantages of the phage method are:

- As far as is known at present phages are completely harmless to the environment. Neither do they cause any "visual inconvenience" as do e.g. dyes.
- Due to their great practical dilution ratio and their good survival small amounts of phages can be used for marking large volumes of water.
- With the help of phages several water fractions can easily be marked simultaneously and the tracers be analysed from the same samples.
- The tracer can be grown with standard equipment of a microbiological laboratory which makes the tracer easily attainable. Partly for this reason, the expenses of the production of the tracer are tolerable.
— The analysis of the tracer is quick compared with that of other biological tracers and those to be analysed with neutron activation analysis. The analysis can also be carried out with normal equipment found in any microbiological laboratory and is not so dependent on place as e.g. the analysis based on neutron absorption.

It can be deduced from the experiences obtained that phages are very well adapted to studies on water movements in rivers, and on some conditions presented above, even in ground waters and waste water treatment plants. The experiment in southern Lake Saimaa suggests that phages might be suitable tracers for waste water movements in lakes.

Considering the above, the phage method has realistic chances to compete with the tracer methods already in use.

9. ACKNOWLEDGEMENTS

First of all I wish to thank Professor Seppo Niemelä under whose direction the study has been performed and Mrs. Marja-Liisa Poikolainen who has participated in the water movement studies in rivers, treatment plants and ground waters, both in the field and in the laboratory.

Due to the large number of field experiments involved in the study, numerous persons mainly from the National Board of Waters have taken part in the practical work. Among them I want particularly to thank Mr. Ilpo Kettunen who carried out the arrangements for the experiment performed in southern Lake Saimaa and Mr. Matti Verta who has put at my disposal the results of the experiment he carried out in the stabilization pond of Saimaanharju.

The study has chiefly been financed by the Finnish Academy of Science. The National Board of Waters has shared in the cost by providing transportation and technical assistance as well as allowing the study to be published in its scientific series.

This publication will fulfil a part of the research obligation imposed on Finland by International Bank for Reconstruction and Development. Thus I express my deep gratitude to the Finnish Academy of Science and the National Board of Waters for supporting my study.

Finally I want to thank Mrs. Aino-Majia Niemelä for translating the study into English.

Helsinki, May 1978

Kari Kinnunen

LOPUTTIVISTELMÄ

Vesien kulkeutumistutkimuksia tehdään pääasiassa kahdesta syystä:
— Vesien käytännöllisen viipymän ja kulkeutumisnopeuden määrittämiseksi.
— Likaantumistutkimuksissa jättevesien leviämisen simulointimiseksi.

Merkkiaineina on yleensä käytetty fluoroosi-via väraineteita, suolaliuoksia, radioaktiivisia isotoppeja ja jälkiaktivointianalyysilla analysoitavia alkaineita. Vain joissakin harvoissa tapauksissa on käytetty mikrobiologisia merkkiaineita, kuten bakteereja, bakteeri-ittöitä, hiivoja ja faageja.

Tässä tutkimuksessa on esitetty kirjallisuus-katsaus käytetyistä mikrobiologisista merkkiainemenetelmissä sekä kirjoittajan kehitettyä menetelmää, jossa käytetään Escherichia coli-faageja merkkiaineena vesien kulkeutumistutkimuksissa.

Jotta faageja voitaisiin käyttää merkkiaineina:
— Niistä on voitava kasvattaa riittävän tiheitä suspensioita, jotta pystytään merkitsevän suuria vesimääriä kohtuullisilla merkkiaine-määriillä.
— Niiden tulee säilyä aktiivisina merkittävässä ympäristössä riittävän kauan, jotta kulkeutumishavainnot voitaisiin tehdä.
— Niiden kvantitatiivisen määrityksen tulee olla luotettava.

Menetelmän käyttökelpoisuuden arvioimiseksi sitä on testattava käytännön tutkimuksissa. Merkintäkohteenä tulevat tällöin kysymyksen lähinnä joet, pohjavedet, jättevedenpuhdistamot ja järvet. Menetelmää on pyrittävä myös vertaamaan jo käytössä oleviin merkkiainemenetelmiin, jotta voitaisiin arvioida faagimenetelmän arvoa niihin verrattuna.

Faagimenetelmää on tutkittu lähinnä edellä esitetyn ongelmanasettelun perusteella.

Tutkituista faageista on käytetty lyhenteitä...
T7, F46, F52 ja F137. Niistä ensiksi mainittu ja sen isäntäbakteerikanta E. coli B 11303 ovat tyypitettyjä ATCC-kantoja. Loput faagit ja niille herkät E. coli-kannat on eristetty Vantaanjoesta, eikä niitä ole tyypitetty tarkemmin.

Faagit ovat pienikokoisia n. 5—300 nm kokoisia bakteerien viruksia (kuva 1), jotka pystyvät lisääntymään vain niille herkkien, aktiivisessa lisääntymisvaiheessa olevien bakteerisolujen sisällä. Ne ovat yleisesti ottaen hyvin kestäviä ulkokuisia vaikutuksia vastaan.

Faagien inaktivoitumisnopeus kasvaa lämpötilan kohotessa. Jo näkyvä säteily (kuva 2) sekä korkea ja alhainen pH inaktivoivat niitä. Toisaalta faagit sietävät jopa pH 11:ta, jos kontaktiaika on vain joitakin minuutteja.

Faagit voivat adsorboitua myös sameutta aiheuttavien savipartikkeiden pinnoille ja inaktivoitua. Samoin muut mikro-organismit saattavat vaikuttaa faageihin inaktiivisesti. Sameutta aiheuttavia viruksilla ja muiden mikro-organismien vaikutus voi kuitenkin olla myös faageja suojaava, koska ne kestävät ympäristön inaktivoavaa vaikutusta paremmin adsorboituneina ja koska adsorptio on reversiibeli tapahtuma.

Yleistien voidaan kuitenkin sanoa, että faagit kestävät riittävän hyvin ympäristön inaktiivisesti vaikutusta, jotta niitä voidaan käyttää vesien merkkinäinehne, kunhan pitkäkaikaisissa merkintäkokeissa vältetään kesän kuumimmita aikoja, jolloin faageja inaktivoivat tekijät ovat voimakkaimmilta. Yleistä väiteä voidaan kuitenkin sanoa, että faagit kestävät riittävän hyvin ympäristön inaktiivisesti vaikutusta, jotta niitä voidaan käyttää vesien merkkinäinehne, kunhan pitkäkaikaisissa merkintäkokeissa vältetään kesän kuumimmita aikoja, jolloin faageja inaktivoivat tekijät ovat voimakkaimmilta. Yleistä väiteä voidaan kuitenkin sanoa, että faagit kestävät riittävän hyvin ympäristön inaktiivisesti vaikutusta, jotta niitä voidaan käyttää vesien merkkinäinehne, kunhan pitkäkaikaisissa merkintäkokeissa vältetään kesän kuumimmita aikoja, jolloin faageja inaktivoivat tekijät ovat voimakkaimmilta. Yleistä väiteä voidaan kuitenkin sanoa, että faagit kestävät riittävän hyvin ympäristön inaktiivisesti vaikutusta, jotta niitä voidaan käyttää vesien merkkinäinehne, kunhan pitkäkaikaisissa merkintäkokeissa vältetään kesän kuumimmita aikoja, jolloin faageja inaktivoivat tekijät ovat voimakkaimmilta. Yleistä väiteä voidaan kuitenkin sanoa, että faagit kestävät riittävän hyvin ympäristön inaktiivisesti vaikutusta, jotta niitä voidaan käyttää vesien merkkinäinehne, kunhan pitkäkaikaisissa merkintäkokeissa vältetään kesän kuumimmita aikoja, jolloin faageja inaktivoivat tekijät ovat voimakkaimmilta. Yleistä väiteä voidaan kuitenkin sanoa, että faagit kestävät riittävän hyvin ympäristön inaktiivisesti vaikutusta, jotta niitä voidaan käyttää vesien merkkinäinehne, kunhan pitkäkaikaisissa merkintäkokeissa vältetään kesän kuumimmita aikoja, jolloin faageja inaktivoivat tekijät ovat voimakkaimmilta. Yleistä väiteä voidaan kuitenkin sanoa, että faagit kestävät riittävän hyvin ympäristön inaktiivisesti vaikutusta, jotta niitä voidaan käyttää vesien merkkinäinehne, kunhan pitkäkaikaisissa merkintäkokeissa vältetään kesän kuumimmita aikoja, jolloin faageja inaktivoivat tekijät ovat voimakkaimmilta. Yleistä väiteä voidaan kuitenkin sanoa, että faagit kestävät riittävän hyvin ympäristön inaktiivisesti vaikutusta, jotta niitä voidaan käyttää vesien merkkinäinehne, kunhan pitkäkaikaisissa merkintäkokeissa vältetään kesän kuumimmita aikoja, jolloin faageja inaktivoivat tekijät ovat voimakkaimmilta. Yleistä väiteä voidaan kuitenkin sanoa, että faagit kestävät riittävän hyvin ympäristön inaktiivisesti vaikutusta, jotta niitä voidaan käyttää vesien merkkinäinehne, kunhan pitkäkaikaisissa merkintäkokeissa vältetään kesän kuumimmita aikoja, jolloin faageja inaktivoivat tekijät ovat voimakkaimmilta. Yleistä väiteä voidaan kuitenkin sanoa, että faagit kestävät riittävän hyvin ympäristön inaktiivisesti vaikutusta, jotta niitä voidaan käyttää vesien merkkinäinehne, kunhan pitkäkaikaisissa merkintäkokeissa vältetään kesän kuumimmita aikoja, jolloin faageja inaktivoivat tekijät ovat voimakkaimmilta. Yleistä väiteä voidaan kuitenkin sanoa, että faagit kestävät riittävän hyvin ympäristön inaktiivisesti vaikutust

Faagimenetelmän käyttännön sovelluksista on esitetty seuraavat tutkimukset:

Kymijoki-tutkimus (7.11), jossa selvitettiin virtaaman ja kulkeutumisnopeuden välinen riippuvuus 8,8 km matkalla Kouvolan pääviemärin purkupaikan alaspäin virtaaman vaihdellessa eri koekerroilla 258—574 m³/s. Tutkimuksessa havaittiin virtaaman ja kulkeutumisnopeuden välinen lineaarinen riippuvuus (kuva 17) korrelaatio kertoimen ollessa 0,9997. Koska edellä mainitu riippuvuus on tutkittuessa tapaamisessa myös teorian mukaan lineaarin, voidaan saatu tulosta pitää erinomaisena.

Vantaanjoki-tutkimus (7.12), jossa merkittiin samanaikaisesti sekä Vantaan että kolmen siihen laskevan joen vedet käyttäen eräitä faagimerkkiaineeita. Merkkiaineenalot voitiin erottaa toisistaan kvantitativisesti samoista näytteistä, koska käytetty faagit eivät vääntyneet vaikuttamaan toisten sa isäntäkantoihin. Tällainen useiden faagien samanaikainen, suhteellisen vaivaton käyttö, on eräs faagimenetelmän parhaimmasta puolesta.


Pesuvesien osalla merkkiaineykärän huipun vaikea koordinaatta oli vain 17 % pienempi kuin teoreettisesti laskettu. Painopisteen aikakoordinaatti
oli sen sijaan jo 44% teoreettista arvoa pienempi. Tästäkin näkyy, että voimakas inaktivoituminen vaikutaa merkkiainekäyräntä muotoon siten, että se pienentää enemmän painopisteiden kuin huipun aikakoordinaattia.

Saimanharjun lamikkopuhdistamalla suoritetussa kokeessa saatiin verraten selkeä merkkiainekäyrä lähdevästä vedestä (kuva 27) ja menelelliä näytti toimivan siellä mitteettomasti. Puhdistamokokeista saatujen kokemusten mukaan faagit eivät sovellu sellaisten jättevesipuhdistamoiden viipymien tutkimiseen, joissa ololauseet muuttuvat voimakkaasti puhdistamoni eri osissa. Tällaisiin tapauksiin soveltuvat ilmeisesti parhaiten radioaktiiviset isotoopit kuten 24Na ja 82Br tai jälliaaktivointianalyysillä analysoitavat aineet kuten indium.


Yhteenvetona faagimenetelmästä voidaan todeta seuraavaa. Menetelmän suurimmat heikouset ovat:

- Faagien määrittystä ei voida suorittaa automaattisesti suoraan kentällä kuten radioaktiivisten isotoopien ja fluoresoivien vääräineiden käsittelyssä ollen, vaan on otettava erillisiä näytteitä.
- Faagien analyysointi on hitaampaa kuin em. merkkiaineilla.

Faagimenetelmän suurimmat edut ovat:

- Faagit ovat nykyisen tietämystä varten hyödyllisiä ja fluoresoivien vääräineiden kyseessä ollen, vaan on otettava erillisiä näytteitä.
- Faagien analysointi on hitaampaa kuin em. merkkiainemateriaalilla.

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