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Advances in biological tracer techniques for hydrology and hydrogeology using bacteriophages

Optimization of the methods and investigation of the behavior of bacterial viruses in surface waters and in porous and fractured aquifers

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IMPRIMATUR POUR LA THÈSE

Utilisation de bactériophages comme traceurs en hydrologie et en hydrogéologie: Optimisation des méthodes et recherches sur le comportement des virus bactériens dans les eaux de surface et dans les aquifères poreux et fissurés

de M. Pierre Rossi

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Neuchâtel, le 14 mars 1995

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... L'âme du Monde se nourrit du bonheur des gens. Ou de leur malheur, de l'envie, de la jalousie. Accomplir sa Légende Personnelle est la seule et unique obligation des hommes. Tout n'est qu'une seule chose.
Et quand tu veux quelque chose, tout l'Univers conspire à te permettre de réaliser ton désir...

Paulo Coelho

Abstract:

This work was undertaken in the Laboratoire de Microbiologie of the University of Neuchâtel, in close collaboration with the Center of Hydrogeology (CHYN). This multi-disciplinary research project was instigated because of the growing need both for environmentally harmless hydrogeological investigation tools, and for new tracers that can be used in multitracing experiments.

This work treats of the use of bacteriophages as biological tracers in underground and surface waters. The size of bacteriophages is of the order of a few hundred nm. These viruses only attack specific bacteria. They are absolutely harmless for any other living organism. Previous experiments have shown the value of bacteriophages as tracers in fissured environments. The aims of the present work were:

- to select several bacteriophages particularly well adapted to the use as hydrogeological tracers;
- to optimize the production of these bacteriophages, and the analytical methods for detecting them;
- to study the behavior of these bacteriophages experimentally, under different physico-chemical conditions;
- to test the phages in karstic fissured environments and to extend their use to saturated porous environments and surface hydrology;
- to compare the migration of these phages to that of the best conventional hydrological tracers.

The first part of this work treats of the isolation and characterization of new bacteriophages. Over twenty bacteriophage/host bacterium (BHB) systems were studied. Seven bacteriophages were selected for the second part of the project. The selection was based on the physiological properties of the host bacteria and the physico-chemical characteristics of the bacteriophages. These seven phages exhibit an interesting variety of shapes and physical characteristics. They include three phages of strictly marine bacteria. None of these BHB systems are naturally found in aquifer waters.

The rest of this work includes the development of an analytical and enumeration technique. We chose to work with the double agar-layer technique, using Petri dishes. The growth media and the different steps of this technique were optimized for each BHB system. This increased the reliability and reproducibility of the technique, without adding to the cost or workload.

The behavior of the phages was studied in laboratory experiments. As this behavior is mostly determined by the inactivation and adsorption of the phages, we investigated the influence on these two phenomena of various parameters, either physical (temperature, pH, agitation), or chemical (ionic concentrations, presence of proteins, of sand or colloidal clay particles).
Our results show that in water, phages react very rapidly and massively to the presence of colloidal particles, even to very low concentrations. Agitation causes the viruses to be rapidly inactivated. Raising the temperature increases this inactivation. Colloidal clay particles (Montmorillonite and Attapulgite) as well as organic macromolecules efficiently protect the phages from inactivation. Only one phage was inactivated faster in the presence of mineral colloids.

Each phage reacted individually to the presence of these mineral colloids. However, no correlation could be established with the physico-chemical characteristics of the bacteriophages. The reactions can be classified into three distinct types, described by Grant et al. [1993]. According to the type of reaction of a phage, it is possible to qualitatively predict its behavior during a tracing experiment.

The tracing experiments performed in karstic fissured environments showed that the migration of bacteriophages is similar to that of the best fluorescent tracers (Uranine, Sulphorhodamine). The restitutions are generally high. The phages always reappear faster, which shows that they can be diluted more, before reaching their inferior detection limit.
The trials performed in a porous saturated environment, on the Wilerwald (CH) test site, showed that bacteriophages are also adapted to this type of conditions. Their speed of migration is higher than that of dissolved chemical compounds (Uranine, Naphtionate). Only one trial was performed in a river. It nevertheless showed that phages are also perfectly suitable to these types of tracings. By

the simultaneous injection of several bacteriophages at different points along the river, it was possible to scan its course and discover an infiltration zone.

This work confirms that phages are of undeniable value to hydrology as biological tracers. Their impact on the environment is practically nil and they can therefore be used also in delicate situations (springs used for drinking water, tracings in inhabited areas). This method is not limited to karstic aquifers anymore. Our modifications and adaptations of the technique make it suitable also for saturated porous environments and rivers. It advantages are numerous:

- The phages are non-pathogenic, non-toxic and invisible. So this method can also be used for studying drinking water (springs, reservoirs, etc).
- Each phage generally attacks only one bacterial species. By a careful selection of the BHB systems, any effect on the aquifers microflora can be avoided.
- No background noise exists in the aquifers, since they phages are not found there naturally. None will be generated or will persist over time, because of their short life span.
- The analysis of the samples is fast and cheap. Only a few milliliters of water are necessary for the enumeration of the phages. The detection level of the routine-analysis technique is about 1 phage per 2 ml of water. This sensitivity is comparable, and most often superior, to that of the best fluorescent tracers. If need be, the sensitivity can be lowered to 1 phage per 10 ml.
- It is also possible to differentiate and count a mixture of phages in a single sample. Theoretically, bacteriophages offer unlimited possibilities of multitracings.
- Ten to twenty liters of phage culture are necessary for one tracing experiment. Such an amount is easily transportable, even to inaccessible injection sites. It will contain 10^{14} - 10^{15} phages, which represents in all about 1 gram of protein and a few grams of mineral salts and of various organic substances (amino acids, growth medium components). The influence on the aquifer will be negligible, even if the flow is small.

Keywords: Bacteriophages, bacteria, dyes, Uranine, porous aquifer, karstic aquifer, rivers, tracing experiments, biological tracers, colloidal particles.

Contents:

Chapter 4: Comparative study of the behavior of bacteriophages in ground waters

Chapter 5: Tracing experiments

§ 1. Introduction

1.1 Quo vadis, hydrogeology?

Mead [1919] was the first to propose a serious definition for the term “hydrogeology”, even if the word itself had been coined by Lamarck as early as 1802. He defined hydrogeology as the study of the laws of occurrence and movement of subterranean waters [Davis and DeWiest, 1980]. Chow [1964] more simply states that hydrogeology studies the hydrological interactions and geological processes (erosion, dissolution and deposition). Since Mead's time, hydrogeology has evolved along three main lines of research:

- elaboration of the relations between geology and ground water occurrence
- development of mathematical equations describing water movement through unconsolidated sediments and fissured rocks
- study of the chemistry of ground water (hydrogeochemistry)

Recently, new fields of research have opened. They have sprung from man's "new" preoccupations and activities. The increasing need for drinking water means that a better knowledge of the conditions determining flow velocities and of the distribution of water basins has become necessary. The global pollution of our environment has now also reached the underground waters. Therefore, encouraged by an ever stricter legislation, new subjects of research have appeared concerning protection zones or self-depuration processes.

Today hydrology is no more restricted to the calculation of aquifer capacities and to the problem of finding new water resources. Freeze and Charry [1979] show that the evolution of this (young) science demands an opening towards other scientific fields: "*We perceive a trend in the study and the practice of ground water hydrogeology. We see a science that is emerging from its geological roots and its clearly practical applications into a full-fledged environmental science. We see a science that is becoming more interdisciplinary in nature and of greater importance in the affairs of man*."

While mathematics and chemistry have long formed an important part of hydrogeology, the use of physics, biology, and even law are very recent. Their contribution has multiplied the possible approaches. The multi-disciplinary side of hydrogeology is continuously developing and becoming richer. To the classical and direct ways of measuring (piezometry, flow rate measurements), indirect methods have been added, such as chemical, isotopic and biological tracers, geophysical methods, etc. In short, it is clear that if hydrogeology's first aims were to satisfy human needs, it now seeks gain knowledge about all the interactions between water, mankind and the environment.

1.2 Structure and flow in porous and fissured environments:

Materials that let water through at appreciable speeds (from a few meters to several thousand meters per year) form permeable geological formations. They are the sole origin of the underground water layers, also called aquifers. These materials are of different types: gravels, alluvium and sands, fissured limestone, volcanic rocks, etc.

A. Porous environments:

Underground flows in porous environments can also occur in loose rocks, mainly composed of quaternary deposits or fissured agglomerates. A distinction is often made between interstitial flows (loose rocks) and those through fissures that were described above. Exploitable water tables (i.e. those where the flow is relatively rapid), are generally found in gravel or sandy alluvial deposits.

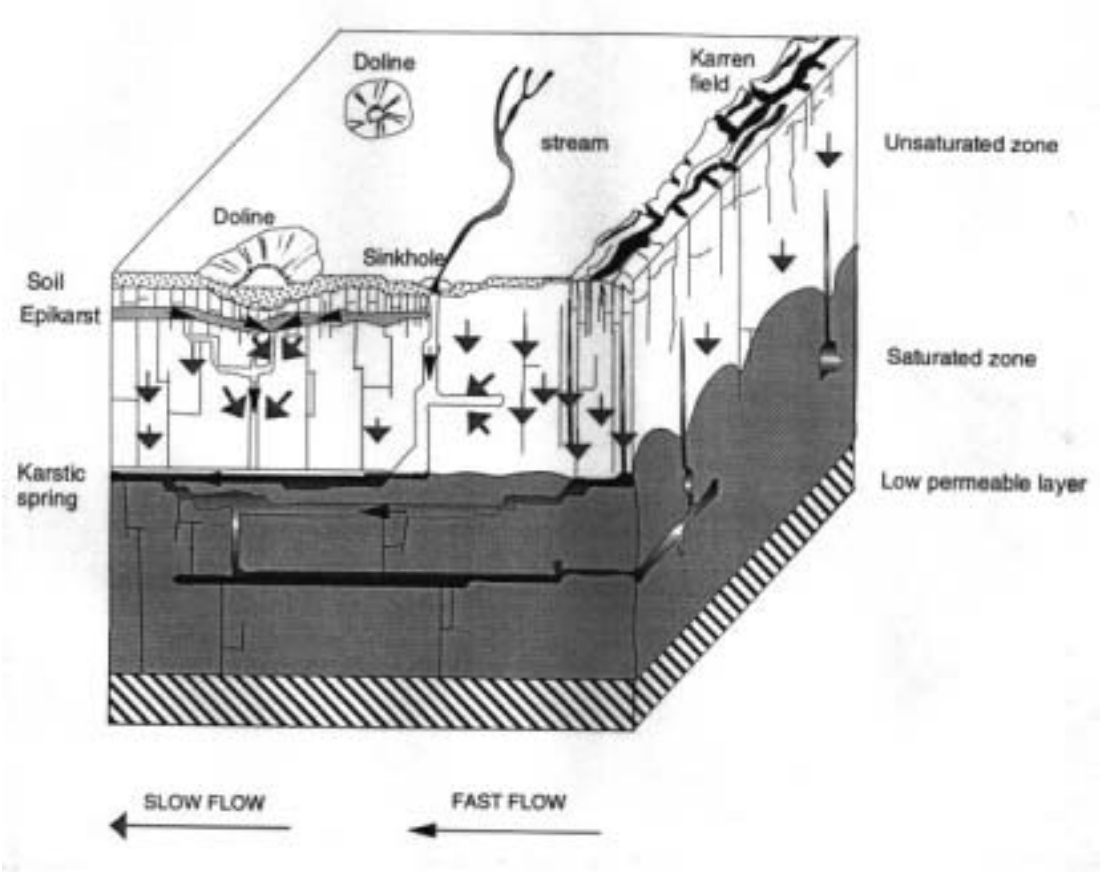
Fine grained rocks (silts and clays) are often as porous as sands and gravels and therefore contain large quantities of water. However, it remains trapped because the rocks' permeability is too low. The water will therefore drain very slowly, even if the hydraulic gradient is high.
If a porous environment is very permeable and the hydraulic gradient is high, the flow will change from linear to turbulent. This sometimes occurs in gravels free of sand or in loose conglomerates.

B. Karstic environments:

"Karst" is the name of a region in northern Slovenia. The characteristic contours of this region are mainly due to the underlying limestone formations. It is now used for all the areas presenting similar morphological characteristics [Meus, 1993].
The formation of a karstic aquifer is linked to dissolution. The chemical action of water enlarges pre-existing fissures in carbonated rocks (limestones and dolomites), or in rocks containing a large proportion of soluble elements (evaporites). This causes the flow in the enlarged fissures to increase and the particles transported by the waters start eroding the rocks. This combination of dissolution and erosion form what is called the *karstification process*. Figure 1.2.1 shows an example of karstic aquifer (conceptual model).

The flow in karstic aquifers is generally turbulent. It will be influenced by the distribution of permeabilities and inlets, as well as by the location of the outlets. A typical aquifer consists of a very permeable fast-draining network, with cracks over several meters wide. A network of much less fissured rocks is connected to this rapid network. This system will be much less permeable and have slower flows.

Figure 1.2.1: Conceptual model of karstic aquifer (low stand water level). Taken with permission from Doerfliger [1994].



1.3 An introduction to tracers

A tracer is any substance or particle (chemical or biological) that can be used to follow, either punctually or continuously, the movement of a water volume, either in an open environment (surface hydrology) or underground (porous or fissured environments). Tracers are used for the following tasks:

- *to define the limits of hydrogeological water basins;*
- *to determine flow velocities;*
- *to simulate pollutions (spills, contaminations or migrations);*
- *to calculate by dilution the flow of small streams.*

The requirements that make a substance a suitable tracer are few. They can be summarised as follows:

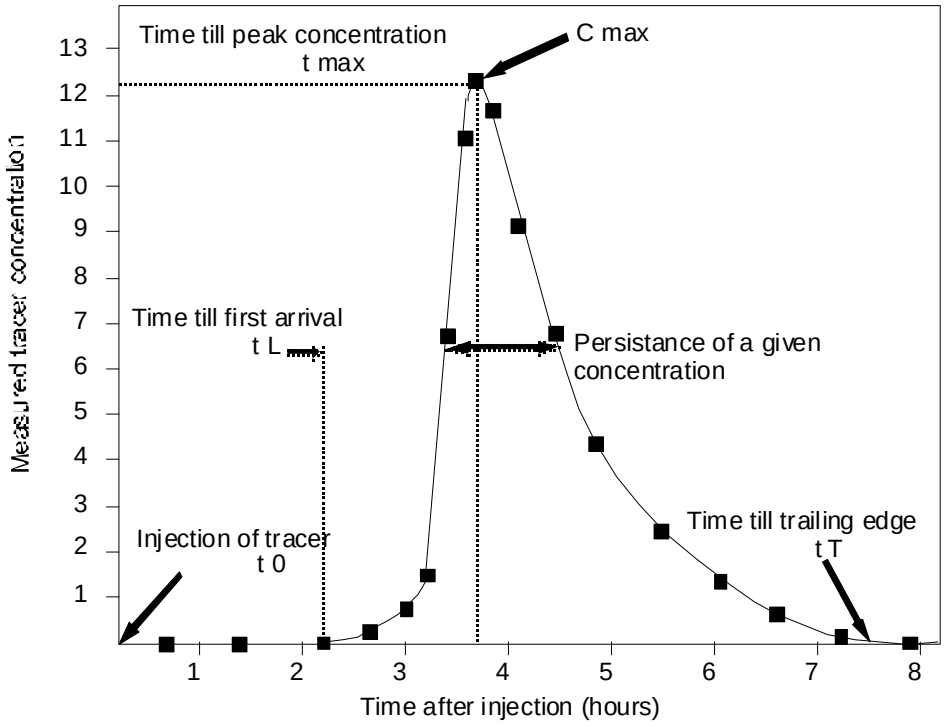
- *the tracer must be easy to inject into the aquifer;*
- *it must be easily identifiable in the samples taken at the resurgence.*

Many chemicals (organic and inorganic) and micro-organisms fulfil these basic requirements, but this short list actually fails to take several other important ones into account. Among them one could mention questions of solubility, the fact that the detection level must be as low as possible, or the possible interference with the media traced (adsorption, absorption, destruction and inactivation), etc. Other aspects to be considered are the tracer's ease of manipulation (when injecting and for analysis), its impact on the environment (toxicity, radioactivity, pathogenicity) and the cost, of the tracer or of the analyses. The number of tracers that are really usable is therefore limited. They can be divided into four categories: chemical, fluorescent, isotopic and biological tracers. The advantages, disadvantages and limitations of these substances and particles are discussed in the following pages.

Artificial tracers give much more than simple "all or nothing" information about the aquifer. The restitution curve of the tracer will allow the calculation of flow velocities and of the tracer’s transit time. This information about the aquifer structure and flows are obtained by studying the deformation of the entry signal (i.e. the injection of the tracer) during its underground progress (dilution, adsorption, etc.). The entry signal is generally punctual, since the injection only lasts a few minutes. the tracer is then transported by the water. At the outlet this punctual signal will, as we have already said, be transformed in a way depending on the aquifer’s characteristics. One could say that it acquires the aquifer’s typical fingerprint. In general the signal will now be dispersed over a certain amount of time. The amount of tracer in each sample can be represented on a two-dimensional graph, with the sampling time on the abscissa and the concentration of the tracer on the ordinate. The curve obtained is called the **restitution curve.** An ideal breakthrough curve is shown in figure 1.3.1

This restitution curve, if the outlet's flow is known, can also be used to calculate the amount of tracer that has travelled to this particular point. It is also possible to calculate the tracer’s dilution factor. This last parameter is particularly interesting when studying the transport of pollutants.

Figure 1.3.1: Ideal breakthrough curve (modified after Lepiller and Mondain, 1986).



t_0: Time zero, moment of injection.
t_{max}: Time till the arrival of the maximal concentration of the tracer C_{max}.
V_{max}: Maximal flow velocity, calculated using t_{max} (not shown).
t_L: Time till the first arrival of the tracer. Depends on the technique employed and on the detection method.
t_T: Disappearance of the tracer.

A. Chemical solutions:

Salt solutions, such as sodium chloride, are frequently used. These substances have many disadvantages. Big quantities must be injected (tens or hundreds of kilos for each injection) in order to obtain a detectable signal (measured as an increase in conductivity, or by using selective electrodes). The charged waters are heavier and tend to descend to the bottom of the aquifers.

One must also consider the strong interactions of certain ions with the immediate environment, their toxicity (either of the ion itself or of the quantities injected) and their possible biological uptake. The choice is therefore limited: Na^+, Li^+, K^+ (for the cations) and Cl^-, I^-, Br^- (for the anions). We will not comment here on the continued use by some institutions and private firms of ions toxic for the environment, such as Cu, Zn, Co, Cr, or Pb ! The ions can also be used along with organic chelating agents (EDTA, ethylene diamino tetraacetic acid).

B. Fluorescent tracers (organic dyes):

These fluorescent substances are without doubt the most used today. This has actually caused the appearance of a permanent background noise in some aquifers. Furthermore, some dyes are strongly suspected to be carcinogens mutagens or even teratogens [Smart, P.L., 1984]. The pathogenicity is rarely due to the dye itself. The impurities it contains are often more dangerous.

Photo-decomposition, biodegradation by micro-organisms (release of bromine atoms by eosin for example) and chemical reactions with other substances present in the aquifer (the chloration of waters as well) are all susceptible of resulting in degradation products dangerous for the environment [Smart, 1984]. Up till now only three tracers have been cleared of the charge of being carcinogenic or mutagenic hazards: fluorescein, Tinopal CBS-x and Rhodamine WT.

The most commonly used is sodium fluorescein (Uranine), because it is non- toxic in low concentrations (LD_{50} 1700 mg/kg) and is relatively inexpensive [Parriaux et al., 1988]. It has a characteristic bright yellowish-green colour in dilute concentrations. However fluorescein decomposes rapidly in the sunlight and is slightly sorbed by most solids [Davis et al., 1980].

C. Stable radio-isotopes natural and artificial:

Several naturally occurring stable isotopes have been used as ground water tracers: ^{18}O, ^{13}C and ^{2}H (deuterium). When two ^{2}H are bonded in a water molecule, they form "heavy water". It is particularly used to calculate flow velocities. However, the price of these tracers and of the analyses make their use prohibitive.

D. Radioactive tracers:

The literature of the years 1950 and 1960 contains many optimistic articles treating of the use of radioactivity, not only in hydrology, but in all the fields of science. However, since that time the increased awareness of radiation hazard has halted most field tests using radioactive tracers [Davis et al., 1980].
The tracers which have been most used are ^{3}H (tritium), ^{32}P, ^{51}Cr, ^{60}Co and (!) ^{198}Au. Actually, the most important applications of these tracers is the use of atmospheric radionucleides, natural or artificial (dispersed by atmospheric atomic explosions or nuclear research, industry and waste), to estimate the amount of time that water and ground water have been isolated from the atmosphere (e.g. to date the waters).

E. Biological tracers:

Both the lack of environmentally innocuous tracers and their general shortage encouraged hydrogeologists to increase the number available by the use of biological agents (spores, bacteria or viruses). These tracers form a separate class since they are not solutes, but form a suspension of microscopic particles (colloidal suspension). They have the advantage, at least for some of them, of mimicking the behaviour of colloidal particles in water, such as that of pathogenic bacteria and viruses.

The requirements for a good biological tracer are numerous. One must not forget that biological tracers are organisms, dead or alive, and that their environmental impact must be as small as possible. The characteristics listed below (Table 1.3.1), eight in all, are taken *in extenso* from Keswick et al. [1982]. They are equally valid for chemical, isotopic or fluorescent tracers if the words "non-pathogenic" under point 1. are replaced by "non-toxic".

Table 1.3.1

Properties desirable of a microbial tracer [from Keswick et al., 1982]

1. The tracer should be non-pathogenic (i.e. not capable of causing disease in man, animals or plants).
2. The tracer should not be present normally in ground water or at least be distinguishable readily from micro-organisms found in water and have stable markers for such identification.
3. The tracer should not affect the flow of ground water.
4. The tracer should be stable in the environment for suitable lengths of time.
5. A tracer should move with the flow of ground water and not be filtered, adsorbed or otherwise removed by the travel in ground water.
6. A tracer should be readily detected in low concentrations by inexpensive methods.
7. A tracer should not interact with other micro-organisms in ground water to produce changes in its or the ground-water organisms properties.
8. When it is desirable to use several tracers at one time, they should be readily distinguishable.

The first trials using biological tracers were made with **bacteria**. Bacterial cells have the advantages of being easy to grow in very large numbers and to assay. The size of a bacteria is of the order of one micron. They are usually distinguishable from the native flora by various markers: colors, genetic resistances, etc. It now also possible to envelop the bacteria in various polymers [Stormo and Crawford, 1992]. This makes it possible to radically change the size and surface characteristics (hydrophobicity, electrostatic charge) of the organisms treated, and thus modify their interactions with the other particles and surfaces of the aquifer. However, care should be taken in the choice of the micro-organisms since they could be potential pathogens. Some are capable of growth within the aquifer, thus producing erroneous results. They are large enough to be filtered out in certain soils and they can adsorb on to a variety of surfaces. In addition, even using a genetic marker like a resistance to an antibiotic, it is often difficult to distinguish test organisms from those naturally present, because markers can be transferred. It also seems really imprudent to use bacteria resistant to antibiotics, since the resistance may be transmitted to potential human, animal, or plant pathogens [Keswick et al, 1982].

Spores of the club moss *Lycopodium clavatum* have been used as tracers primarily by speleologists, to determine subterranean drainage characteristics in limestone karst regions [Smith and Atkinson, 1974; Aley and Fletcher, 1976]. The spores are dyed and injected. They are trapped with plankton nets at potential resurgencies [Gardner and Gray, 1976]. Because of the size of the spores (30 m diameter), the technique is considered of little value in porous aquifers. Furthermore the spores do not have the surface properties of pathogenic micro-organisms, like bacteria and viruses.

Some researchers have also used as tracers **viruses** that are pathogenic for animals or man [Scandura and Sobsey, 1981; Marti et al., 1979]. The injection of 10^{12}-10^{14} pathogenic viruses in an aquifer is ethically questionable, even more so when one knows that certain viruses may be preserved in saturated soils. They can remain active for very long, adsorbed on the soil particles [Bitton and Mitchell, 1974]. This type of experimentation is dubious to say the least, even if one uses attenuated viral strains, such as the Sabin strain of the poliomyelitis virus.

It is becoming more and more urgent to develop an inoffensive tracer that can harmlessly mimic the propagation of chemical substances and pathogenic viruses. Keswick [1980] writes: *"The myriad of chemicals that may be found in contaminated ground water make it difficult to study their fate, transport and health effects. Because of past, often indiscriminate, dumping of hazardous waste, the number of cases of serious ground water pollution is likely to increase in the near future."* In developing countries for example, nearly 60% of the population is exposed to sewage-polluted water and water-born infections, including viral diseases, account for 5-10 million deaths each year [Ramia, 1985; Havelaar, 1986].

1.4 The idea of bacteriophages:

The first article mentioning the use of bacteriophages dates back to 1972 [Winpenny et al.]. The authors used the lambda phage (*E. coli*), which showed excellent dispositions for tracings in polluted rivers. Thereafter, bacteriophages were used not only to study the behaviour of pathogenic viruses without endangering the environment, but also as true hydrogeological tracers.

Among all the possible biological tracers, bacteriophages certainly present the most advantages. Their small size puts them on the same scale as pathogenic viruses of eucaryotic organisms. Furthermore, contrary to their "cousins", they are non-pathogenic and non-toxic. By choosing an appropriate phage/host bacterium system one can avoid all risk of influencing the aquifer's micro-flora. Another advantage of these particular tracers is that they are totally invisible at whatever concentration, so they can be used for the study of underground waters passing through inhabited areas. Their last advantage consists in the specificity of every bacteriophage for its only host bacterium. This biological particularity means that several bacteriophages can be injected simultaneously (multi-tracing), without any problem of interference appearing when analysing the samples.

Following a primary test on the use of bacteriophages, the Laboratoire de Microbiologie performed several very encouraging trials in the jurassian karst (Switzerland), in association with the Centre d'Hydrogéologie [Aragno and Müller, 1982]. After that, a real collaboration developed, as this in-depth research on the use of bacteriophages as biological tracers in hydrogeology testifies.

1.5 Aims of this work:

This work aimed at establishing the necessary conditions for the use of bacteriophages as biological tracers in surface and underground waters. This means defining and perfecting the various techniques for the production, use and analysis of phages in water. Among those available, a series of tests will determine which phages have the greatest potential as tracers. Furthermore, the behavior of the viruses under the various physico-chemical and biological conditions reigning in the underground environments must be studied. This account has been divided into six separate chapters. They are not in chronological order, since the course this work followed was determined by the needs of the hydrogeologists and the contingencies linked to the perfecting of the various techniques and the isolation of new phages. The chapters have been divided as follows:

§2 - Choice of the bacteriophages and their physical and chemical characterization. Our work material is thus described. A first selection produced a collection of phages potentially usable for water tracings.

§3 - Perfecting of a numeration technique for the bacteriophages. No existing technique was fully satisfying. The method must not only be scientifically correct, but also economically sustainable.

§4 - Study of the behavior of bacteriophages in water. More precisely we should say "in waters", since this chapters treats of the phages' survival and possibilities of propagation in underground environments.

§5 - Migration experiments on columns. This chapter describes the results obtained with an experimental system for the study of the migration of phages through various substrates. Parameters such as water composition or the presence of organic and inorganic particles were followed.

§6 - Field trials. In this chapter are discussed the various tracing experiments we performed in the course of this research. Comparisons with the migration of chemical, isotopic and fluorescent tracers are also discussed.

Our aim was to equip hydrogeologists with a dependable and practical research tool, which would enable them to perform repeated tracing experiments in aquifers without harming the environment. These particular tracers are also well adapted for studying of the behavior of other biological colloidal particles, such as pathogenic viruses and bacteria.

§2. Isolation and characterization of bacteriophages:

2.1 Introduction:

Bacteriophages have adapted to the whole bacterial kingdom. The possibility of isolating at least one phage for every existing bacteria proves the ubiquity of these viruses. A great number still remain to be discovered. In 1987 Ackermann and DuBow counted no less than 2700 different tail-bearing phages (of all shapes and sizes) and over a hundred of other shapes (tailless, filamentous, ...). Table 2.1.1 presents the many habitats from which it is possible to isolate bacteriophages (modified after Ackermann and DuBow, 1987). The favourite environment of bacteriophages is of course the host bacterium itself. When a virus invades a bacterium, its genetic material may physically associate with the bacterial genome. In this latent form, the phage, then called a *prophage*, is not transcribed but multiplies passively with each bacterial division [Oguseitan et al, 1992]. This type of phage is called *temperate*. It will multiply inside its host cell (i.e. be transcribed) only following some particular external stimulation (such as UV-irradiation of the host cell).

Table 2.1.1
Habitats of bacteriophages [from Ackermann and DuBow, 1987]

Water	Surface water	lakes, ponds, streams, cisterns, drinking waters, hot springs, irrigation channels, salt ponds, lagoons
	Seawater	brack water, deep sea, coastal sediments, tidal ponds
	Sewage	raw or chlorinated, activated sludge
Soil	Types	arable, clay, compost, forest, moor, mud and silt, podzol rhizosphere, sand
Air		Droplets, dust particles
Plants	Organs	buds, leaves, nodule (leguminous plants), rotting fruits, roots, seeds
Animals	Species	healthy or diseased man, ass, bee, cattle, chicken, crab, earthworm, squirrels, horse, mouse, pig, rabbit, turkey, water buffalo, various fishes, mosquitoes, mussels
Food	Dairy products	raw and skim milk, butter, cheeses (Cheddar, cottage), cheese starter (Bel Paese, Emmenthal), yogurts
	Meat, fish	chicken, beef, salami, steaks, fish fillets and sauce
	Other	Lactic acid beverage, oysters, sake starter, wine

This ability to physically insert its DNA fragment into the bacterial genome is not common to all the members of the viral world. All the other phages, which do not share this capacity, are called *virulent* or *lytic*. Their intracellular multiplication occurs immediately after the infection of the host cell. The cycle may end with the cell bursting, as happens following an attack by phage T7. In other cases, such as f1, the cell subsists after the multiplication of the phages, which are liberated by extrusion. It is clear that only lytic phages are interesting as tracers.

Not only is the diversity of phage habitats great, but the numbers of viral particles found in some samples is sometimes quite impressive. Børsheim's [1993] comprehensive review of research on the ecology of viruses and bacteriophages since 1979 shows that concentrations of viral particles in sea and fresh water varies from 10^3 to 10^7 per ml [Bergh et al, 1989; Bratbak et al, 1990]. It may even reach $4{,}6 * 10^8$. This absolute record was measured far out at sea (Gulf Stream). Obviously an important part of these particles are viruses of Eucaryotic cells (algae, protozoa, fish etc). Counts of phage particles, as opposed to bacteriophages only, are done by electron microscopy.

Inactivated phages will therefore also be counted by this method. In sea water the biomass of viral particles is estimated at 0.27 mgC/l. That of bacteria reaches 2-40 mgC/l. The half-life of the viruses is about 1,3 - 2 hours [Børsheim, 1993]. This means that the turn-over of viral and bacterial populations is very rapid. The bacteriophages, though they form but a small fraction of the total biomass, certainly play an important role in the regulation of the various bacterial populations and in the exchanges of genetic material [Bratbak et al, 1990; Wommack et al, 1992].

2.1.2 Classical methods for isolating bacteriophages:

A great variety of methods exist for isolating and characterizing bacteriophages. This includes the different techniques for immediate analysis on Petri dishes, as well as those by concentration (filtration, filtration-elution, physico-chemical precipitations, etc). [Atmar et al, 1993; Seeley and Primrose, 1982; Purdy et al, 1984; Klieve and Swain, 1993]. The most used method however is the enrichment in liquid media. One can use either a bacterial strain isolated from the same environment [Moebus, 1980 and 1992; Hidaka, 1972] or a strain coming from a different one [Kauri, 1991; Kuhn, 1987; Pauling, 1982]. These enrichment methods were used successfully in the course of this work. Several media, solid (soils and composts), or liquid (sea water, sewage), were used.

2.1.3 Defining the bacteriophage-host bacterium system (BHB):

The characteristics that a hydrogeologist demands of a good microbial tracer are given under the eight points of table 1.1 presented in the general introduction. For the specific case of bacteriophages, one can then list an order priorities for selecting a BHB. First of all one must choose the host bacterium. This choice will condition all further work. Indeed, the host must possess certain essential biological characteristics:

- It must be easy to grow in liquid or solid media. This will therefore restrict the choice mainly to the aerobic, heterotrophic and mesophilic bacteria that grow rapidly on a simple medium. Mainly but not absolutely, as we shall see.

- Total absence from natural waters (karst, streams, porous environments). This second condition is necessary to avoid a too important background noise, which might interfere with the results of a tracing. If the host exists in the waters, phages of this species are sure to be present too.

- Non-pathogenicity of the bacterial strain. When producing phages in great quantities it is difficult, even with chloroform or cyanide, to totally eliminate the remaining bacteria. Some will inevitably be injected into the aquifers. One can therefore seriously question the ethics of performing tracing experiments using phages of the genera *Salmonella, Shigella* or other enteropathogens, chosen mainly for the ease with which they can be cultivated, or for the great resistance of the phages of these bacterial species [Bricelj and Sisko, 1992].

- No cross lysis reactions between the different phages and bacteria chosen, which would make multi-tracing experiments impossible.

To the necessary qualities of the host bacteria one must add a few for the phage itself. They

mostly concern its physical and natural properties:

- Resistance to chloroform. It was initially planned to sterilize the water samples to be analyzed with chloroform. As we shall see later on, this sterilization was abandoned, following the development of a new analysis technique for the water samples (see chapter 3).

- High multiplication rates when produced in liquid media. It is important to be able to obtain as big a quantity of phages as possible, so as to reduce the volume of medium that must be prepared and injected. The lowest acceptable concentration is about 10^9 phages/ml. The maximum concentrations reached are about $2* 10^{11}$ phages/ml.

- Appearance of the lysis plaque when using the double agar-layer technique. These areas must be clear and well-defined. This is a considerable advantage when many samples have to be counted.

This chapter presents the different techniques used, as well as the habitats prospected, during the search for bacteriophages and their isolation. When one tries to understand the overall behavior of phages in a liquid environment (their adsorption and inactivation), it is absolutely necessary to have as complete a knowledge as possible of the viral particle itself. Indeed, its behavior in water depends greatly on its charge, measured as its Zeta potential, and on the hydrophobicity of its capsid. The second part of this chapter therefore discusses the physico-chemical and structural characteristics of phages that were selected as potential candidates. The methods for producing large quantities of bacteriophages, the conservation of strains and the preparation of purified samples of phages for the next part of this research are also presented in this chapter.

2.2 Material and Methods

2.2.1 Isolation:

A. Isolation by enrichment in liquid media:
To isolate phages by enrichment from water or solid material the idea is to add a culture of the host bacterium to the sample. In this way the phages specific of this bacterium that are present in the sample will multiply. This multiplication is enough to allow detection of the phages in the sample, without further manipulation.

2 to 5g of material (compost, various types of soils) are placed in 50ml bottles with 20ml of growth medium (LB or SWB according to the bacterium), and 2ml of an exponentially-growing culture of the host bacterium. The bottle is placed under agitation (150rpm) at the bacterium's optimal growth temperature for 12 to 18 hours. The same procedure is applied for isolating phages from liquid samples. In this case one generally uses 20ml of the sample to which one adds 20ml of the growth medium and 2ml of the exponentially-growing culture. The contents of the bottles are then centrifuged (2500g, 20min). The supernatant is collected and 5% (v/v) of chloroform added. This is then energetically shaken for 2 minutes, and centrifuged once more (10'000g, 20 min). 1ml of the supernatant is then used for an analysis on Petri dishes in presence of the host bacterium, following the procedure described in chapter 3.

B. Isolating bacteriophages on Petri dishes:
This method can be used when a great number of phages are present in the water sample or adsorbed on solid material. The phages contained in a few grams of material are suspended in the growth medium: 5g of material (soil, compost) are placed in a sterile 50ml bottle with 20ml of the host bacterium's growth medium (LB, J&B, SWB). The bottle is shaken for 30min. The supernatant is centrifuged (10'000g, 30 min), and tested immediately on Petri dishes. In general it is not necessary to add chloroform to sterilize the supernatant. This procedure can also be used with water samples, as described in chapter 3.

C. Purification stages:
After both types of isolation procedures, two or three purification stages are still necessary. This is to be sure one has not isolated more than one phage, as happens very frequently. A lysis plaque is selected and cut out from one of the Petri dishes of the fist analysis. It is plunged directly into 20ml of growth medium with 2ml of an exponentially-growing culture of the host bacteria. The bottle is incubated under agitation overnight at the bacterium's optimal growth temperature. After the lysis of the bacteria, that is after about 18 hours, a sample of the culture is diluted (decimal dilutions) and tested on a double agar-layer Petri dish. A new lysis plaque is cut out and the procedure is repeated a second, and even a third time, ensuring only a single type of phage is finally isolated.

2.2.2 Preparation of purified stocks of bacteriophages for the physico-chemical and structural analyses:

A suspension of bacteriophages is centrifuged (10'000g, 20 min.). Chloroform is added in 1:1 proportions. The mixture is vigorously shaken for 5 minutes, and centrifuged again (5000g, 10 min.). The supernatant is collected and the operation is repeated. The supernatant is collected and filtered through a 0.025 m filter (cellulose ester, Millipore HA). The phages in the filtered suspension are then precipitated as described by Yamamoto et al. [1970], using polyethylene glycol, in the same way as for the extraction of genetic material presented in annex 2.2. The phages are resuspended as needed in 10mM NaCl, 10mM PBS buffer (pH = 7.4), or TE buffer (annex 2.1).

2.2.3 Physico-chemical and structural characterization of the bacteriophages:

A. Examination of the structure by electron microscopy:
The phages are revealed by negative staining with uranyl acetate [Lüther (ETHZ), pers. comm.]. The complete protocol for this procedure is described in annex 2.3. The composition of the buffers and other solutions is given in annex 2.4. The phages were observed with a Philips 201 TEM microscope, at 60 kW, and a maximum enlargement (on the 35 mm negative) of 17'100x.

B. Genome analysis:
The genetic material (double stranded DNA for all the phages studied) is extracted following a procedure derived from the extraction of DNA from phage lambda and M13, (personal adaptations of the protocol taken from "Molecular cloning", Sambrook et al., 2nd ed., 1989). The extraction protocol is presented in extenso in annex 2.2. The solutions and buffers needed for the extraction are given in annex 2.1. The phages are precipitated following the method of Yamamoto et al. [1970] using polyethylene glycol. The genetic material is then analysed by enzymatic digestion using the Taq, Hind III, Pvu I, and Bst restriction enzymes (Boehringer, Mannheim). The procedure is given in detail in annex 2.3. The digested material is separated on an agarose gel.

C. Analyse of the capsid's hydrophobicity:
The hydrophobicity of the phage capsid is measured by the SCAM method (Surface Contact Angle Measurement). A 10mM solution of NaCl containing $2*10^{10}$ to $1*10^{11}$ phages is prepared as described under point 2.2.2. It is filtered through a cellulose acetate filter (0.025μm, 2,5 cm diameter, USWP, Millipore) on a Millipore filtration system. The filter is fixed with double-sided scotch tape on a glass slide and air dried for at least 1 hour at room temperature. The angle measurements were done at the EAWAG (Dübendorf, Switz.), by Dr.Hauke Harms on a specially equipped stereoscope (Carl Zeiss Stereoscope, fitted with a goniometer eyepiece). One measures the angle formed by a droplet of distilled water (a few μl measured by a micro-syringe) lying on the filter and the surface of the filter itself. The result given is the average of at least ten measurements.

D. Zeta potential of the phage particle:
This is measured by following the migration of a charged particle in a cell under the influence of a strong electric field (Electrophoretic Mobility). The movement of the particle in the electric field will depend on its global electrostatic charge, which is determined by the pH of the environment and the hydrated ions linked to its capsid. The movement is translated into a potential called *Zeta potential*, measured in mV. When the total negative and positive charges of the capsid are equal, the potential equals 0mV. This corresponds to the phage's isoelectric point (pI). Generally the Zeta potential of viruses is negative at pH 7. The particle is then negatively charged.

A concentrated suspension of phages prepared according to point 2.2.2, is resuspended in 10mM PBS buffer (pH= 7.4). The potential is measured with a ZetaMaster apparatus (cell type ZEM010, Cross beam mode, $F(K_a)= 1.50$ (Smoluchowski)). The potential of the bacteriophages was generally measured at pH= 7.4. For phage H6/1 a few trials were made at a lower pH (around 6.2 and 4.2). Each value given is the arithmetical average of at least ten different measurements.

E. Mean size of the phage particles:
This was measured using a Malvern ZetaSizer 4 apparatus, using a purified and concentrated suspension obtained as under point 2.2.2, and resuspended in 10mM PBS buffer at pH= 7.5.

2.2.4 Production of bacteriophages in large volumes:

Very concentrated suspensions of phages can be obtained in two ways [Eisenstark, 1967]: by liquid culture (erlenmeyer and fermenter) and by culture in Petri dishes. In general higher concentrations are reached by this last method.

A. Production of bacteriophages in liquid media:
The phage and its host bacterium are placed in the latter's growth medium. Optimal growth is assured by constant agitation. The initial ratio of phages to host bacteria is important. Several experiments showed that a too high concentration of bacteria will exhaust the medium before the phages have had time to develop optimally. Too many phages means premature lysis of the cells. An empirical ratio between 1/2 and 1/20 was determined for each BHB. A example of the preparation of big volumes of bacteriophages, by cultivation in erlenmeyers is given in annex 2.4.

B. Production in Petri dishes:
The preparation of a stock of phages by this method closely resembles the double agar-layer titration (chapter 3). About 300 to 500 phages per Petri dish are required. If too few phages are added, the lysis will not be confluent (the lysis plaques do not touch) and the final number of phages will be low. If too many are added the bacterial population will be prematurely lysed, before it has reached its maximum development. After about 24 hours of incubation, 5 ml of PBS or Tris-HCl buffer (pH = 7.4) are poured onto the double-layer agar. The Petri dish is left at room temperature for 4 to 5 hours, during which time the phages will diffuse into the liquid phase. The supernatant is then collected. In this way one obtains small volumes of very concentrated phage suspensions, usually containing over $1* 10^{11}$ phages/ml. Another advantage is the fact that the phages are suspended in a solution free of bacterial cellular debris.

C. Production of bacteriophages in a fermenter:
Phage f1 was cultivated in a Biostat Braun fermenter equipped with a 10 liter culture vessel. This apparatus allows an optimal control of the agitation, the aeration, the temperature and the pH. A few milliliters of antifoaming agent A (Sigma) (or of olive oil) are necessary to inhibit the formation of foam (and the stoppage of filters!). Very high concentrations, of the order of 10^{10} phages/ml,can be obtained in this manner.

2.2.5 Conservation of the bacteriophages at 4° and –20°C:

Phages are generally kept in the liquid bacterial growth medium, the cells having been removed by centrifugation (10.000g, 20 min.) and filtration (0.25μm). They are kept in the dark at 4° C, in sterile 20 ml glass tubes. Experience showed that these stocks of phages decrease slowly and regularly. They are renewed every six to twelve months. The phages, purified and concentrated as under point 2.2.2.D, and intended for the analyses (genome, hydrophobicity, size and Zeta potential) are kept at -20 C, in 1.5 ml TE or SM buffer (Eppendorf tubes).

2.3 Results and discussion

2.3.1 Preamble:

Our first attempts at isolating bacteriophages were made using the bacterial strains *Bacillus subtilis, Bacillus megaterium* and *Streptococcus faecalis*. Phages T7 (*Escherichia coli B*) and f1 (*Escherichia coli K12*) were already available in our laboratory. Later on, the search for bacteriophage was extended to *Bacillus stearothermophilus* and *Pseudomonas fluorescens*. So it is only really at the very end that our search included marine phages of halophilic bacteria. The marine strains used were: *Vibrio harveyi, Vibrio fischerii, Vibrio natriegens, Pseudomonas nautica, Deleya pacifica, Bacillus marinus.* Finally, Professor Moebus (Anstalt Helgoland, Germany) very kindly gave us six bacteriophage-host bacteria systems that had been isolated from the Atlantic Ocean and the North Sea. The marine bacteria strains are called *H2, H3, H4, H6, H40 et H85*.

Table 2.3.1
List of bacteriophages and host bacteria

Bacteriophages	Host bacteria (ATCC; DSM number)
f1	*Escherichia coli K12* (ATCC 15766)
f2	idem
T4	*Escherichia coli B* (ATCC 11303)
T5	idem
T6	idem
T7	idem
H2/1	*H2*
H3/1	*H3*
H4/1	*H4* Bacterial strains not yet registered
H6/6	*H6*
H40/1	*H40*
H85/1	*H85*
Vh5a	*Vibrio harveyi* (ATCC 14126)
Vh5b	idem
Vn4b	*Vibrio natriegens* (ATCC 14046)
Vn5a	idem
CS5	*CS5* Bacterial strain not yet registered
Psf2	*Pseudomonas fluorescens* (ATCC 27663)
Psf3	idem
phiS1	idem
Bs3a	*Bacillus stearothermophilus* (DSM 22)
Bs3b	idem
Pm7a	*Bacillus megaterium* (DSM 32)
Pm6b	idem
Ps9b	*Bacillus subtilis* (DSM 10)
Ps6b	idem
Sfd3	*Streptococcus (Enterococcus) faecalis* (DSM 2570)
Sfd2	idem

DSM: German Collection of Microorganisms
ATCC: American Type Culture Collection

The total number of bacteriophages isolated in the course of this work amounts to over twenty. They are listed *in extenso* in table 2.3.1. Considering the time at our disposal and the conditions set out for the candidates in the introduction of this chapter (point 2.3), we then chose seven of phages for a more detailed study. These were T7, f1, Sfd3 and Psf2 as well as the marine phages

Vh5a, H40/1 et H6/1. We must add that no phage could be isolated from *Vibrio fischerii, Pseudomonas nautica* and *Deleya pacifica* with the enrichment methods that were used.

This sub-chapter presents all the characteristics that we determined for each of our seven candidates. The results are given in the form of "identity cards" in annex 2.6. Each includes an electron micrograph and details about the host bacteria (reference number, growth medium, incubation temperature, etc.).

2.3.2 Bacteriophages T7 and f1:

These two bacteriophages already existed in the laboratory's collection. They attack the same bacterial species (*E. coli*). The characteristics determined for these two bacteria are presented in table 2.3.2. These phages belong to two distinct morphological groups. Phage T7 is a small phage of the family Podoviridae [Ackerman and DuBow, 1987] with an icosahedral head and a short noncontractile tail. Its genetic material is double-stranded DNA. It attacks the strain *E. coli B* and is lytic. The sub-species of T7 that is in the laboratory's collection is specific of the female strain *E.coli* (F^-).

Phage f1 is a filamentous phage of the family Inoviridae, genus Inovirus [Ackermann and DuBow, 1987]. Phages f1, M13 and fd are very closely related: their genomes are organized identically (single stranded DNA), their particles are similar in size and shape, and their DNAs are highly homologous [Sambrook et al., 1989]. Phage M13 is used a lot in molecular biology for various genetic recombinations. Phage f1 can only adsorb onto the pili of bacteria belonging to the *E. coli K12* strain, that carry the genetical factor F^+ (f'). This is an episome coding for the pili proteins of *E. coli*. These pili are produced by the bacteria only above 34° C. The replication of the filamentous bacteriophage f1 occurs in harmony with that of the host bacterium, and the infected cells are therefore not destroyed, but continue to grow (albeit at one half to three quarters of their normal rate) while producing several hundred virus particles per cell per generation [Sambrook et al., 1989]. So as to favor the production of this phage, we selected an *E. coli* carrying a resistance to tetracycline coded on the episome F^+. In the presence of this antibiotic this bacteria will retain the information necessary to synthetise pili. In this way it is possible to optimize the production and detection of these phages on Petri dishes.

Table 2.3.2

Name	Family (genus)	Dimensions (head*tail length in nm)	Hydrophobicity Θa	Zeta Potential (mV)	Genetic material
T7	Podoviridae	60 * 17	ND	-31.7 pH 7.4	Double strand DNA
f1	Inovirus (Inoviridae)	100 * 6 Ø	85,7	-42,3 pH 7.4	Single strand DNA

2.3.3 Bacteriophages Sfd3 and Psf2:

These two bacteriophages were isolated from the water of the primary settling tank of the Neuchâtel waste-water treatment plant, using the enrichment method described in this chapter. Of the many phages isolated and that were present in great numbers in these waters only two, Psf2 (*Pseudomonas fluorescens*) and Sfd3 (*Streptococcus faecalis*), were finally selected. The first belongs to the family Siphoviridae (type B1 according to Ackermann et Eisenstark, 1974) following the ICTV taxonomy (International Committee on Taxonomy of Viruses). Its head is polyhedral (icosahedron) and its tail flexible and noncontractile. This phage requires minimal concentrations of 50 mM CaCl2 and 5mM MgSO4 to multiply in great quantities. Phage Sfd3 belongs to the same family (Siphoviridae), but is of type B3. Its head is an elongated

icosahedron, its tail is flexible and noncontractile. The characteristics of these two phages are summarized in table 2.3.3.

Table 2.3.3

Name	Family (genus)	Dimensions (head*tail length in nm)	Hydrophobicity Θa	Zeta potential (mV)	Genetic material
SFD3	Siphoviridae (type B3)	105-110/30 * 78-82	ND	ND	Double strand DNA
Psf2	Siphoviridae (type B1)	ND	34.2°	-36.0 pH 7.4	Double strand DNA

2.3.4 The marine bacteriophages:

Of all the bacterial strains used for isolating bacteriophages from marine environments, only *Vibrio harveyi* and *Vibrio natriegens* gave positive results. The phages were isolated from mussel extracts (*Mytilus edulis*) from the Atlantic coast, by enrichment in liquid cultures. Only the Vh5a-*Vibrio harveyi* BHB system was then finally retained for further use. This phage has extremely small lysis plaques (of less than 1mm in diameter). This is surely due to the very rapid growth of the host bacterium. The doubling time of *Vibrio harveyi* is among the shortest known, around 15 minutes. This phage also belongs to the family Siphoviridae, class B1, after Ackermann and Eisenstark [1974]. It has a noncontractile and flexible tail. The characteristics of the marine phages are presented in table 2.3.4.

Table 2.3.4

Name	Family (genus)	Dimensions (head*tail length in nm)	Hydrophobicity Θa	Zeta Potential (mV)	Genetic material
Vh5a	Siphoviridae (type B1)	90-100 * 45-50	ND	ND	Double strand DNA
H40/1	Siphoviridae (type B1)	82-85 * 39 -43	68,7°	-42.0	Double strand DNA
H6/1	Leviviridae (type E1)	30	90.6°	-50.1	ND

On the basis of the selection criteria used for all the other phages, we chose the bacteriophages H40/1 (*H40*) and H6/1 (*H6*) from the 6 BHB systems that professor Moebus very kindly gave us. These two phages were isolated during marine exploration campains in the Atlantic [Moebus, K., Hermann, F., 1987; Moebus, K., Nattkemper, H., 1989; Moebus, K., 1992]. H40/1 belongs to the Siphoviridae family. It has an icosahedral head and a flexible noncontractile tail. H6/1 is an icosahedron without a tail. It is classified in the Leviviridae family. The Zeta potential of this phage at pH= 7.4 is the lowest of all those we measured. At pH= 4.2 its potential is -19.3 mV.

2.3.5 Electrostatic charge and hydrophobicity of the viral particle:

According to Wan et al. [1994], the behavior of bacteria in water is basically determined by their hydrophobicity and their isoelectric point. More specifically, these two values make it possible to predict the behavior of the cells at air-water and water-substrate interfaces. We think that these two characteristics are equally fundamental for any research on the behaviour of bacteriophages and viruses in water. We will return to these aspects in chapter 4, when discussing inactivation and adsorption mechanisms.

Very few measures of the electric charge of bacteriophages have in fact been made [Ackermann and DuBow, 1987]. The value of the isoelectric point (pI), that is the pH at which the number of positive and negative charges on the capsid of a phage are equal, has only been measured for a very few viruses. That of phage MS-2 (*E. coli*) was measured as pI= pH= 3.9 [Koya and Chaudhuri, 1977; Bales et al., 1992]. Enteropathogenic polioviruses of type 1 and 2 seem to have a much higher pI, around pH= 7. The pI of a virus can only be measured if the capsid is solid enough. The Zeta potential is easier to measure, even for delicate phages. It expresses the charge balance of the virus's capsid at a given pH. The potential equals 0 at the particle's pI.

The results we obtained agree with the values given by Ackermann and DuBow [1987]. The Zeta potentials of the phages suggest that their isoelectric points are situated between pI= 3.5 and pI= 4.5. This means that the bacteriophages are very negatively charged at the habitual pH values of underground waters. Only around pH= 4 will the electrostatic charges on the capsid cancel each other out, and the Zeta potential tend towards zero. The lowest potential was measured for phage H6/1 at -50 mV (PBS buffer 10mM, pH= 7.4). The Zeta potential of this phage is still worth -19.3 mV at a pH of 4.2. Its isolelectric point will therefore be largely below pH= 4.

The results given here are the average of at least ten successive measurements. It happens, though rarely, that one measures two potentials, like Moore et al. [1981] did for the poliovirus 1. This virus seems to have two isoelectric points, depending on which ionizable groups are exposed: a high-pI form, stable at a pH greater than 7.5 and a low one, stable below pH= 6. Near pH= 7, the population is distributed between both states. This same phenomenon was observed with phage T7. The potential values for this phage are -31.7mV for the main value and -18.6mV for the secondary value, which was measured only about once in every eight measurements. Both potentials mean a strongly negative charge at the normal pH values of underground waters.

The hydrophobicity (Θa) could unfortunately not be measured for each of the seven phages selected. It is also very difficult, even impossible to find any literature references on the subject, with which to compare our own data.

2.3.6 Purified stocks of bacteriophages for the analyses presented in this chapter:

The simplified preparation method described in this chapter seems to give good results. The measurements of particle size carried out in collaboration with the EAWAG showed that only a small amount of contaminants only remained in the viral suspension.

This simple method (chloroform, low-speed centrifugations and filtration-precipitation) is feasible also in small, simply equipped laboratories. It allows the rapid preparation of purified stocks of phages, avoiding the more complex steps of gradient ultracentrifugations. It cannot, however, be used with bacteriophages that are highly sensitive to chloroform.

2.4 Conclusions

Bacteriophages are present in all natural environments, sometimes in very high concentrations. They can be used for tracing underground and surface waters only if the background noise is extremely low, if possible completely absent. This an essential requirement to avoid considerably perturbing the results. This is why we had to choose bacteriophage/host bacteria systems that do not exist in the aquifers. Initially we selected more than twenty different BHBs. This number was then reduced to answer this requirement. Seven BHB couples remained for second part of this work.

The diversity among these seven couples is important, both for the bacteria and the viruses. The host-bacteria are heterotrophic mesophiles of very diverse origins. The strictly halophilic bacteria are the most promising among them, since they are totally absent from freshwater environments.

The phages of our collection present a great variety of shapes. Most of them belong to the family of tailed phages (Siphoviridae). The selection also contains a filamentous phage (f1), a short-tailed phage (T7) and a tailless phage (H6/1). Actual methods make it possible to characterize bacteriophages relatively quickly. Knowledge of their physico-chemical characteristics is an essential key to the study of their behavior in natural environments. Among the most important pieces of information one could mention the isoelectric point (pI), which reflects the electric charge of the virus. It will determine the way the phage will react with the other colloidal particles and the walls of the aquifer. The phages charge is negative at the pHs encountered in freshwater environments. The isoelectric points that were measured varied between 3.5 and 4.5.

This chapter also discussed the production of phages in big quantities. As we will show in chapter 6 of this work, 10 liters of suspension are generally needed for one tracing experiment. We are still restricted to a batch production in agitated vessels. The amounts of bacteria and phages necessary for this production are given by empirical relations. In this work, we did not optimize the production of phages in liquid media. However, after some trial and error, we obtained quantities of phages quite sufficient for our needs ($> 10^9$/ml).

It is not easy either to stock the phages. Many authors have tried storing them at temperatures below 0° C [Vieu, 1966]. Our experience showed that the seven phages selected can be stored over six months at 4° C in their culture medium.

§3. Detection and enumeration of bacteriophages: optimization of a detection and numeration technique using Petri dishes.

3.1.1 Introduction:

The main problem when studying bacteriophages is their extremely small size. It is generally comprised between a few tens and a few hundreds of nm. Furthermore, since they are parasites of bacterial cells, the methodology will be different from that used in microbiology. To visualize the viral particles it is necessary to use the sophisticated techniques of electron microscopy. But no behavior, no kinetics or reaction can be observed this way. So electron microscopy is in fact only rarely used, to enable us to picture the material with which we are working. Because of the size of viruses it is necessary to use indirect techniques of detection and numeration, using reactants or host-bacteria. These methods make it possible to obtain the necessary information about the evolution of the populations and the "physiological" state of the phages. The requirements that such a method must fulfil to be considered scientifically valid are numerous. We will only mention its precision and reproducibility. The financial aspect must also be taken into account: the method will not only be used in the laboratory, but also for the analysis of a great series of many water samples during tracing experiments. Below we have listed by order of importance the specifications required of such a method:

1. Very low detection limit (< 1 phage / ml);
2. High reproducibility;
3. The method must practical, as simple and rapid as possible, adapted for the serial analysis of a great many samples;
4. Low cost;
5. It must be possible to use in non-sterile conditions;

Some of these requirements are contradictory. Points 1 and 2 concern the analytical aspect. They conflict with the requirements 3 to 5 which concern the practical aspect of the method. So in this case the perfecting of the numeration technique also meant agreeing to compromise.

This introduction continues with a short description of the different techniques used today to count bacteriophages. We have grouped them in two categories. The first contains the techniques using molecular biology, immunology and electron microscopy. None of these methods requires the presence of the host-bacterium of the phage studied (in fact, one eliminates it most often by one or more purification stages). The second groups the microbiological detection techniques which all require the presence of the phage's host-bacterium as intermediary. So these techniques do not detect the phage's presence, but rather the lysis of the host-bacteria.

3.1.2 Detection and numeration methods independent of the host-bacterium:

These modern methods are the result of the technological development of biology. We will described the three techniques most frequently used today, starting with electron microscopy. This technique, perfected and brought up to date, can be used to detect viral populations in concentrations above 10^4 phages/ml [Luria et al, 1951; Ewert and Paynter, 1980; Bergh et al, 1989; Wommack et al, 1992; Bø rsheim, 1993]. It can therefore not be used for the systematic counting of very diluted populations of viruses. It is not possible either to determine the "physiological" state of the phages observed. Another disadvantage of this method is the fact that it detects all the viruses present in a sample. The phages one is specifically looking for may thus be lost among the sometimes considerable indigenous population of viruses [Bergh et al, 1989; Bratbak et al, 1990].

PCR (Polymerase Chain Reaction) [Erlich, 1989] is based on the selective amplification of genetic material. It is extremely sensitive for the detection of bacteriophages and viruses in general [Atmar et al, 1993]. Unfortunately, the quantification of the phages present in the sample is still too

complex for this method to be useful for the analysis of a great many samples. Using the host-bacterium is still a more specific way of detecting and counting a population of phages than the use of genetical probes. PCR is nevertheless a powerful detection tool of the presence/absence type. However the costs of this technique are also quite high (source material and enzymes).

The last technique in this category we will discuss is used in immunology. The phages are detected on dilution plaques by a reaction with specific antibodies (ELISA technique). With this very sensitive method it is possible to determine the various types of relations between the phage and its host-bacterium [Tortorello et al, 1991]. However, Tartera and Jofre [1987] have shown that this method is not as sensitive as Adams' one (see 3.1.3). Furthermore, the dilution plaques can only contain a couple of hundred l. This is a problem when detecting very low concentrations of bacteriophages during tracing experiments. The reactants and the antibodies must be specific for the phage used: no cross reaction must exist if possible. So the cost of such a method is quite high.

3.1.3 Detection and numeration using the host-bacterium:

In the methods described below the host-bacterium is indispensable to detect the bacteriophages. One measures in fact the lysis of bacterial cells. If the conditions are favorable, this will be marked by the liquid or solid culture becoming transparent. The most common method today is the one Adams described in 1959, using a double agar layer. This analysis is done on Petri dishes filled with a first layer of solid medium (1% agar). The bacteriophages and bacteria are poured onto this surface with a few milliliters of soft molten agar (0.6% agar) at 45-50° C. In this way, phages and bacteria are trapped in a medium soft enough to allow their diffusion, but solid enough to stop any movement of fluids. This allows the formation of lysis zones, also called lysis plaques.

Their appearance is due to a rapid succession of attacks on bacteria and production of phages. Each plaque originates by the presence of a single virus which invades a bacteria [Luria et al., 1950]. The plaques contrast with the rest of the Petri's surface, covered by a uniform bacterial carpet in the soft upper agar layer.

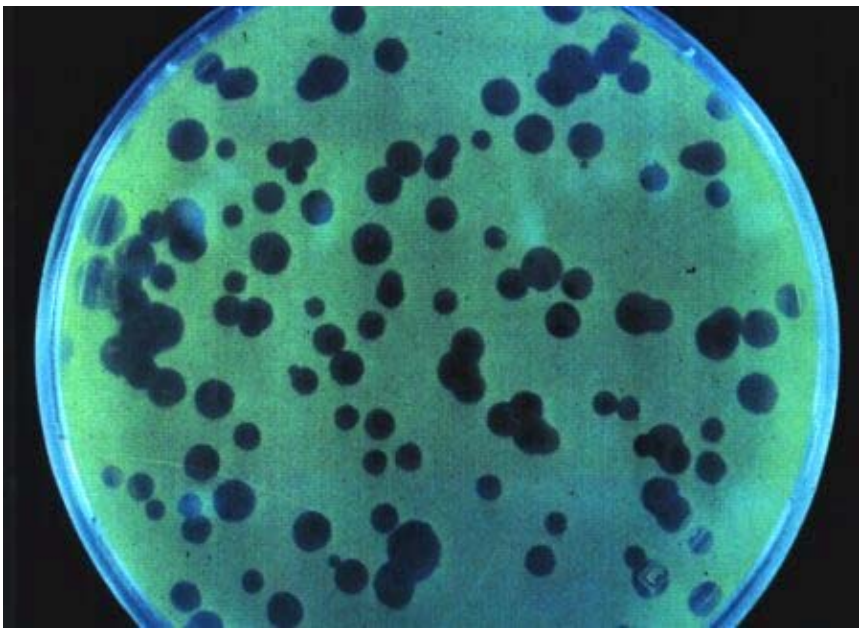
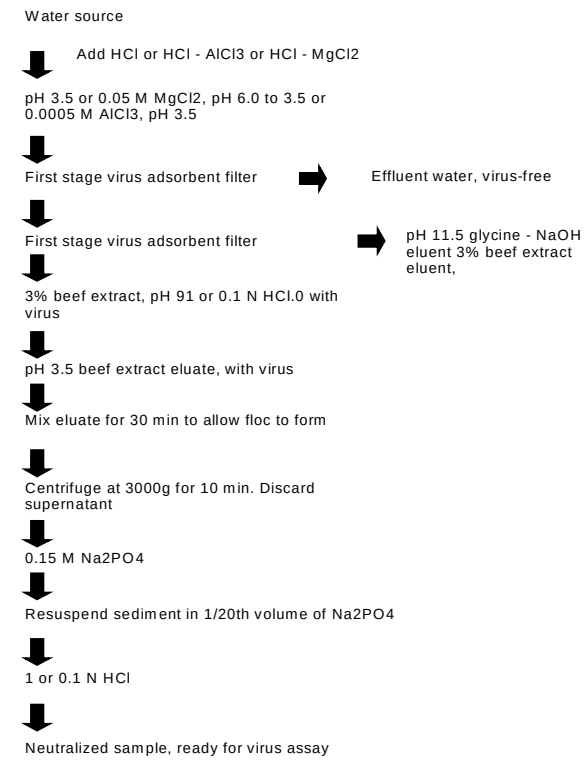


Photo 3.1.1: Lysis plaques of the phage phiS1 (*Pseudomonas fluorescens)* on a Petri dish.
This procedure has many advantages. Its application limits will be discussed further on in this

chapter, under point 3.1.4. According to the literature, it is the technique most commonly used by scientists all over the world. Efforts have been made to improve it by eliminating the first agar layer. Grabow and Coubrough [1986] treat samples of 100ml of water mixed with soft agar and distributed into 10 Petri dishes of 14 centimeters in diameter. This rather tedious method is neither more reproducible nor more efficient than the one developed by Adams. Kott [1966] developed a numeration method in liquid cultures. The samples are serially diluted (decimal dilutions) tubes. A culture that becomes clear after incubation signals that at least one bacteriophage was present in the tube at the beginning. Using the MPN technique (Most Probable Number), it is possible to determine the initial concentration of phages. This method cannot be used when numerous samples have to be treated. Besides, Cornax et al. [1990] have shown that the double agar layer technique gives better results than serial dilutions in tubes or microtiter plates [Tortorello et al., 1991]. The MPN methods tends to underestimate the number of bacteriophages present in a sample.

Many authors have used filtration-elution methods to detect and count viral particles in big volumes of water [Seeley and Primrose, 1979; Payment et al., 1988; Seeley et al., 1979; Logan et al., 1980; Gerba and Goyal, 1982; Goyal et al., 1980]. These procedures are very complex. A great many operations are required, first to wash the filters, then to adsorb and desorb the viruses, sometimes to concentrate them (chemically or physically) and finally to count them on Petri dishes. Figure 3.1.1 gives an example of the sequence of operations that are necessary to concentrate Enteroviruses [Goyal and Gerba, 1979].



Modified from Goyal and Gerba [1982]

Figure 3.1.1: Sequence of operations necessary to concentrate Enteroviruses from large volumes of water.

In our case it was impossible to imagine going through so many steps, because of the number of

samples we had to treat. It is also probable that only a small number of viruses are actually retained [Purdy et al., 1984], and that the cumulated losses at each step reach 10 to 90% of the initial number of viruses. Some authors use a pre-filter to eliminate suspended particles. In this way one also eliminates the viruses adsorbed on the particles [Schaub and Sorber, 1976].

Armon and Kott [1993] have developed a particularly ingenious method to detect phages in samples of 500ml of water. The detection limit seems to be of 1 phage per 500ml. The originality of this methods consists in plunging the bacterial carpet (the bacteria in soft agar on a holder) directly into the container holding the 500ml of water. The phage attack the bacteria and lysis plaques develop in the same way as in the double agar layer. A certain amount of time would be necessary to adapt this technique to the analysis of large series of samples. It remains a powerful means of presence/absence detection, without the numerous steps of the filtration-elution techniques.

The last method we will present is particularly original and deserves our attention. Purdy et al. [1984] detect bacteriophages by placing the host-bacteria directly in the water sample to be analyzed. Bivalent ions (Ca^{2+}, Mg^{2+}) are added to increase the efficiency of the method. After a period of latency permitting the definitive adsorption of the phages onto their host-bacterium, the sample is centrifuged. The pellet of cells is then diluted and analyzed by the double agar layer technique. This method has a definite advantage over all the others using the host-bacterium: the adsorption of the phages on the bacteria in a liquid environment. The possibilities of contact are far better than those existing in a gelled media, however soft it may be.

3.1.4 Optimization of the double agar layer technique:

This method is the one which best suits the requirements listed in the introduction to this chapter. In the other methods we presented here, one or the other step may seem interesting. However, often the equipment and reactants used are to expensive when a large number of samples must be analysed. The manipulations are often very tedious and time-consuming. It also seemed to us that the precision and reproducibility of these methods could be improved. The double agar-layer method as described by Adams [1959], can also be criticized:

- The phages and bacteria are placed in molten agar at 45-50° C, before being poured into the Petri dish. As we will show later on in this work (chapter 4), this rather high temperature may present some risks for the phages.
- It is impossible to completely empty the tube when pouring this thick mixture into the dish. Some phages will remain in the tube.
- The method was not optimized for the specific phages and bacteria used. The same method is applied to all the BHB systems.

We thought it necessary to perfect this procedure, so as to fulfil the requirements listed as closely as possible. We studied the literature thoroughly so as to obtain as much information as possible on the ways of improving the classical methodology. The results of our experiments are presented graphically. In general the abscissa gives the mean number of bacteriophages obtained by 3 plate counts. The term Pfu (Plaque forming unit) really indicates the number of lysis plaques that appeared and not the number of phages actually present in the sample when it was poured. All the phages will not form a lysis plaque. It is important to distinguish between these two notions and we will return to them later on in the chapter.

3.2 Material and methods:

The composition of the growth media, buffers (PH et SP) and synthetic spring water (SF, SF+) are given in the annex 3-1.

3.2.1 Numeration on Petri dishes: optimization of the double agar-layer technique [Adams,1959]:

All the manipulations of liquids are done using automatic pipettes with adjustable volumes (Socorex). The polypropylene tips are sterilized before use.

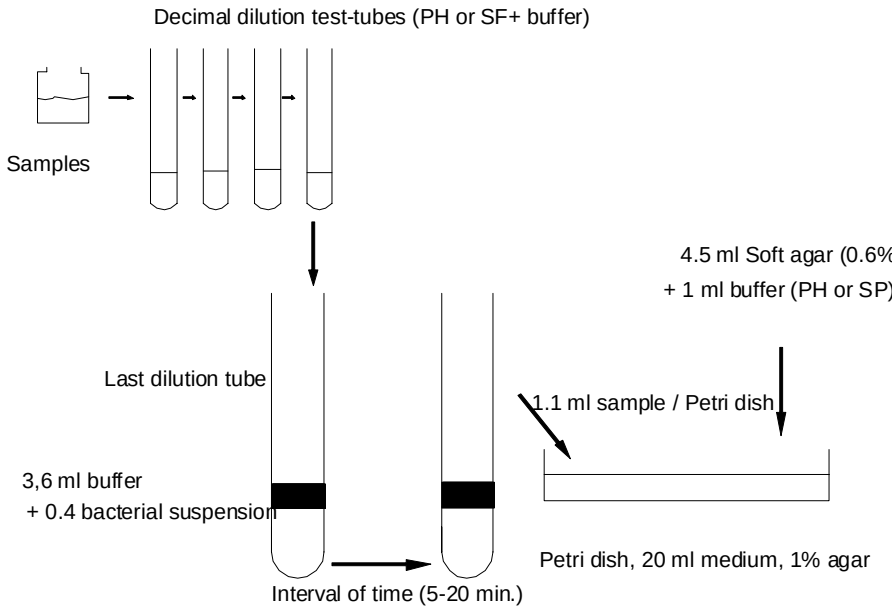


Figure 3.2: General method for detecting and enumerating bacteriophages on Petri dishes.

0.4 ml of the suspension containing the phages are added to 3,6ml of PH buffer in a glass test tube. Sometimes it is necessary to dilute the sample beforehand (decimal dilutions) in PH or SF+ buffer. The number of phages in the last tube must be comprised between 20 and 200 per ml. To the last tube are also added 400 l of an exponentially growing bacterial culture ($5*10^7$ - $1*10^8$ bacteria/ml). The tube is left to sit at room temperature for an interval of time permitting adsorption of the phages on the bacteria (5 to 20 minutes depending on the phage). 1100 l of the contents of the last test tube are poured onto a Petri dish already containing 20 ml of solid medium (agar 1%). 1 ml of PH buffer and 4 ml of soft medium (0.6% agar) at about 45° C (molten agar) are then added. This is then mixed by rotating the dishes gently and the left to cool, before being put to incubate at the optimal temperature (as determined in chapter 3.3). For certain phages (Psf2 and Sfd3), SF+ buffer is used for the contact interval and the dilutions (Synthetic Freshwater [Moore et al., 1983] with 20mM Tris-HCl pH 7.4, 50mM Ca^{2+}, 5 mM Mg^{2+}). A mixture of bivalent cations and colloidal clay (SP mixture) is added before pouring the soft agar.

All the analyses are normally repeated on three parallel Petri dishes. This number can be doubled or even reach 10 for certain specific analyses. The counting of the lysis plaques (PFU) is done a first time after 18 to 24 hours and a second time after about 48 hours. Each result is the mathematical average of the plaques counted on the three (or more) Petri dishes, multiplied by the inverse of the dilution factor. When no dilution is necessary the host-bacteria is directly added to 4 ml of the sample (10^0 dilution) in a sterile glass tube.

3.2.2 Analysis of the samples from a tracing experiment:

The analysis of the samples from a tracing experiment is done in two stages. The first is a simple presence/absence test. It also gives an estimate of the number of dilutions necessary for the second step. This consists of a precise numeration as described under point 3.2.1.

- 2 ml of the sample and 0.2 ml of bacterial culture are mixed in a sterile glass tube and left for 15 to 30 minutes at room temperature. This longer interval than the one under point 3.2.1 allows definitive contact between the phages and bacteria. The entire contents of the tube are then simply poured onto a Petri dish containing 20 ml of solid medium (1% agar). 4 ml of 0.6% soft medium are then added. This is then gently mixed, left to cool and incubated at the bacteria's optimal temperature.

If the result of this first step is positive (lysis plaques detected), a second analysis of the sample is performed, following the method presented under point 3.2.1.

3.3 Results and discussion:

The main modification we brought to the classical double agar-layer method consisted in giving the phages time to establish contact with their host bacterium in a liquid environment. The other modifications aimed at adapting the analysis to each specific BHB system according to the following parameters:

1. Optimal contact interval in liquid medium;
2. Optimal bacterial concentration in the Petri dish;
3. Choice of the solid growth medium;
4. Optimal growth temperature;
5. Mineral requirements: Ca^{2+}, Mg^{2+} and colloidal clay particles.

The contact interval allows the adsorption of the phage onto the bacteria [Luria, 1951] and the beginning of the infection. The infected bacteria is then trapped in the top agar layer of a Petri dish. This technique, used by Purdy et al. [1984] as a biological detection technique, here substantially improves our numeration technique. The developments particular to each of the five modifications listed above are presented in the following pages. The general method for numeration on Petri dishes is described in the "Material and methods" section. A comparison of the optimized method with the classical Adams' technique is presented at the end of point 3.3.4.

3.3.1 Variation of the number of Pfus according to the number of bacterial cells:

The graph 3.1 shows the Pfus obtained according to the number of bacterial cells added to each Petri dish. Fixed volumes of cells (50, 100, 200, 500 et 1000 µl) from a fresh exponentially growing culture are added to the last tube of a series of decimal dilutions. The number of cells in the culture for each system is given beside the phage name on graph 3.1. After the contact interval, (see following point 3.3.2) the phage / host bacteria mixture is poured onto a Petri dish and mixed with soft molten agar. After 20 and 44 hours of incubation the number of Pfus is read for each dish. Between 2 and 5 % of the plaques have not yet appeared after this time, depending on the BHB system. Each point on the graph is the average of the results of three Petri dishes.

The minimal number of cells is the quantity necessary to obtain a uniform carpet in the soft upper layer of agar. This number obviously depends on the growth rate and mobility of each strain. For the seven strains tested, it was determined to be at least $1* 10^7$ bacteria. When the number of cells in the upper layer is too high, the subsequent bacterial growth will mask the lysis plaques. The phage's multiplication is drowned by the proliferation of the bacteria. The Psf2/*P. fluorescens* system is a perfect example of this phenomenon. Graph 3.1 shows an noticeable decrease in the number of Pfus when the number of bacteria exceeds $1* 10^8$ bacteria (100µl) in the upper agar layer (top agar). Phage H6/1 is rather insensitive to variations of the cellular concentration, as are also T7 et H40/1. For this last two, the number of Pfus that appear stops increasing around $2* 10^8$ and $7* 10^7$ cells respectively. The increasing number of bacteria in the top layer only modifies the aspect of the lysis plaques, whose diameter and sharpness decrease. Counting becomes difficult and less precise around $8\text{-}9* 10^8$ bacteria. For the Sfd3/*S. faecalis* system, the equilibrium is more difficult to reach. It is situated in a narrow band between 2 and $3* 10^7$ bacteria. Vh5a is a special case, compared to what we observed with the other phages. It requires at least 2 to $3* 10^8$ bacteria for the maximum number of measurable Pfus to be reached. The spectacular increase in the number of Pfus that was observed is surely due to the small size of the lysis plaques when the bacterial carpet is diffuse. They simply go unnoticed during the counting. The growth temperature set for this BHB system is far superior to *Vibrio harveyi's* optimal growth temperature (see chapter 3.3.3). This is the only way we have found to slow down the bacteria's growth, without inhibiting the development of the bacteriophages. The optimal bacterial density for each phage-host bacterium system, that is the one allowing the appearance of a maximum number of Pfus, are listed in table 3.1 in chapter 3.3.4.

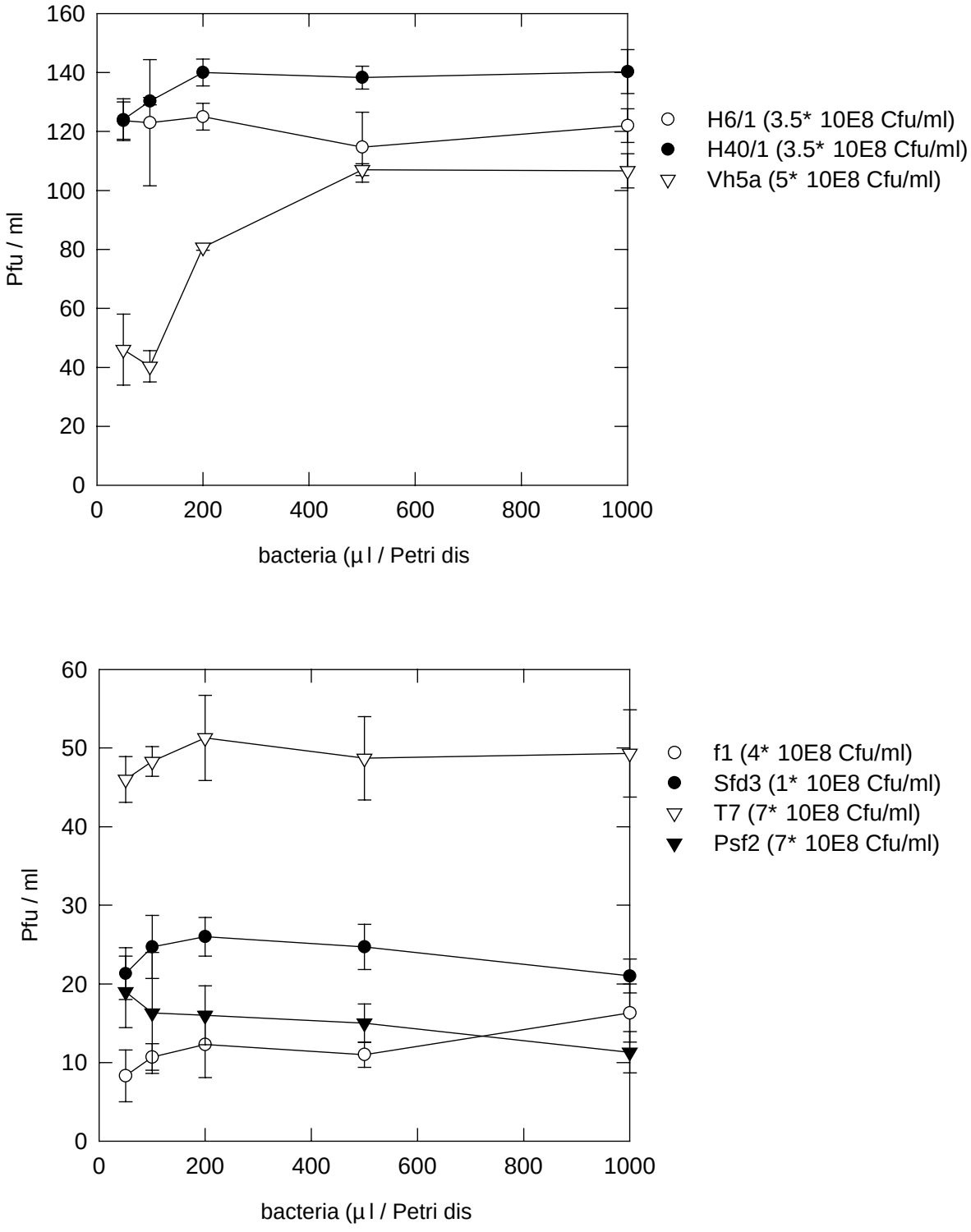


Figure 3.1: Variation of PFU numbers as a function of bacterial concentrations.

3.3.2 Determination of the optimal contact time (t_c) between bacteriophages and host bacteria in liquid medium:

Adams' method does not take into account the existence of a contact phase between a phage and its host bacterium. Considering the size of the particles concerned, the frequency of encounters will be mathematically lower in a solid environment, even a soft gel, than in a liquid one. This will even be accentuated if the bacteria are motile. When following Adams' methods, several parameters, such as the temperature, can inactivate the phage in the interval between the solidification of the agar and the phage's encounter with a bacterium. Allowing the contact to be established in a stable and controlled liquid environment will result in the phages adsorbing more rapidly and in greater quantities.

The graphs 3.2 show the different of behaviours observed for the BHB systems tested. The tests were performed twice, in phosphate buffer (PH), using the bacterial concentrations determined in 3.3.1. This high number of cells, of the order of 10^7 - 10^8 cells/ml, makes it possible to mathematically exclude the adsorption of more than one phage on a host cell. The number of Pfus and the optical density (OD) were measured at fixed intervals. One sees that the adsorption of the phages onto the host bacteria is extremely rapid. When the first measurement is made after only a few seconds (in fact, the time necessary to mix the contents of the vessel), over 80% of the total number of phages are already adsorbed onto the host bacteria.

The bacteriophages H6/1 and H40/1 are very rapidly adsorbed and their concentrations hardly changed during the 60 minutes of the experiment, even though the OD increases, indicating that the bacteria have multiplied in the buffer solution. This results confirms that obtained with these bacteria in chapter 3.3.1. The other systems tested (phages Sfd3, f1) gave similar results (data not shown).

T7 has a different behaviour. In a PH buffer solution the maximum number of Pfus is obtained after 15 to 20 minutes of contact. After about 30 minutes, the number of phages increases very rapidly, as does the OD.

The last type of behaviour that we observed is illustrated by phage Psf2. It resembles that of T7 in the sense that the number of phages increases greatly after 30 minutes. However the number of Pfus decreases between 20 and 30 minutes of incubation. The possible explanations of this behaviour are numerous: adsorption on bacterial debris, inactivation by proteolytic exo-enzymes, etc.). We did not try to determine the reason for this sudden drop.

The contact time can be determined directly on the graph for each of the seven BHB systems. One sees that an interval of 15 minutes at room temperature (22° C) and in PH buffer, seems suitable for all seven BHB systems (tolerance: +/- 1 minute). A contact interval of 30 minutes was used for the semi-quantitative detection of phages in tracing samples (step 1). This longer time ensures an optimal detection of the phages present in the sample, even in very low concentrations.

The phages Psf2 and Sfd3 are exceptions. Their requirements of inorganic compounds (bivalent ions and clay) are discussed in chapter 3.3.4. SF+ buffer and not PH was used (see annex 3.1 for composition). Furthermore, these two systems receive additional minerals and a colloidal clay suspension (SP solution) when incubated on Petri dishes.

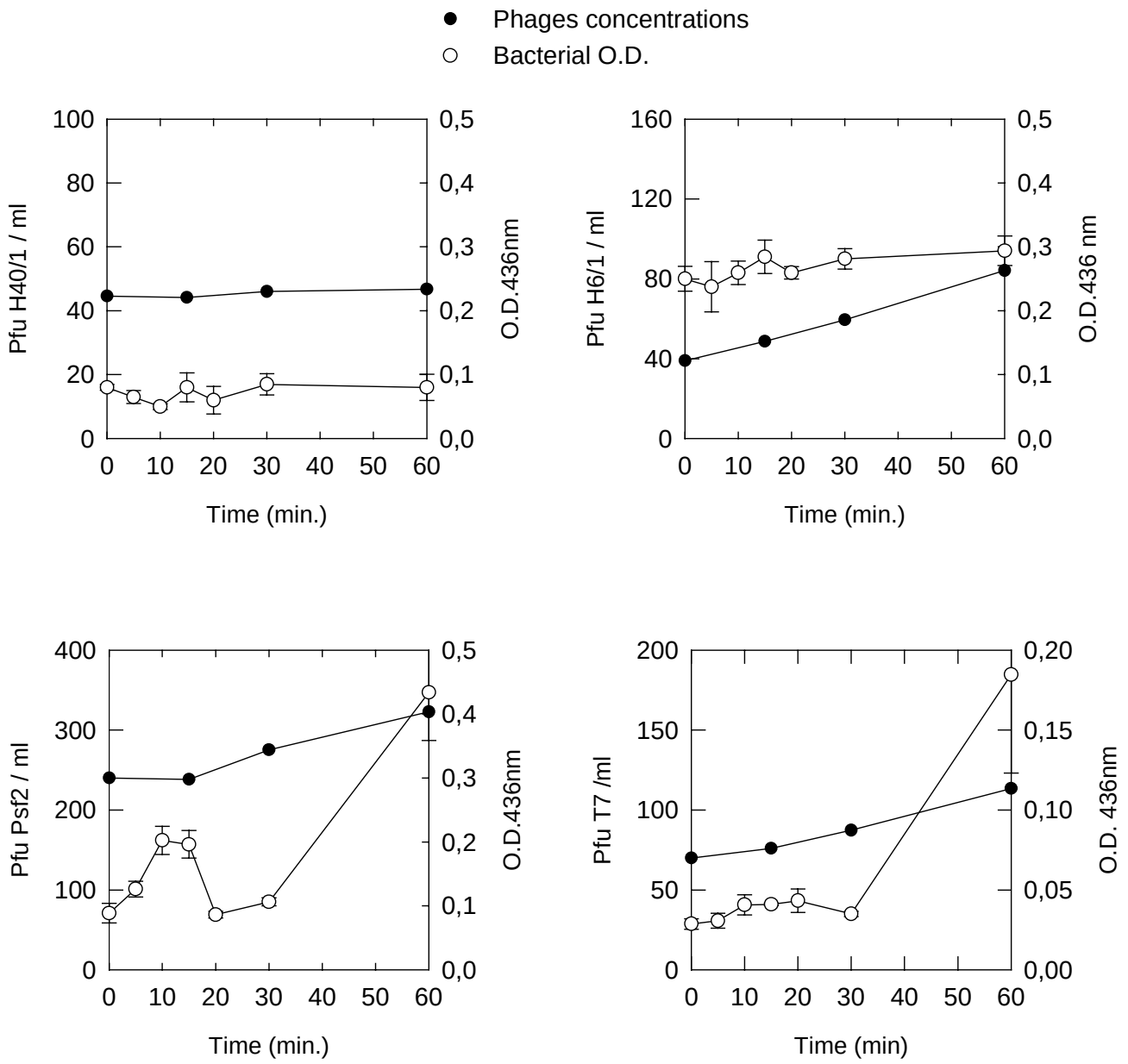


Figure 3.2: Contact time between phages and bacteria.

3.3.3 Influence of the incubation temperature on the number of Pfus. Selection of the growth media:

Graphs 3.3 and 3.4 shows the reaction of various BHB systems to changes in the incubation temperature of the Petri dishes. One sees that the fluctuations in the number of Pfus are in general quite important. T7/*E. coli* seems to be an exception. The number of Pfus remains constant over a wide temperature range. Only the appearance of the lysis plaques changes: their diameter increases with the temperature, going from 2-3mm at 14° C to over 15mm at 37° C, in LB growth medium (0.6% agar). This difference is so important that we fixed the incubation temperature at 22° C, corresponding about to the temperature of the laboratory (at least of a certain laboratory... [Marchiani, 1990]). The behavior of phages H6/1 and H40/1 is similar. The bacteria do not grow at 37° C, and no bacterial layer can be obtained in 24 hours. With the f1/*E. coli K12* system no plaques appear below 37° C. This seems quite normal, considering that this phages adsorb onto the bacterial pili, which are only formed above 34° C. There is therefore no attack below this temperature. Vh5a/*V. harveyi* is the most extreme case. The number of Pfus increases by almost 600% between 14 and 37° C. This latter temperature being above the bacterium optimal growth temperature. The size of the Pfus is also greater at that temperature. The lysis plaques are clearer and better defined. With this system, the temperature certainly slows the bacterial development, allowing the lysis plaques to appear more easily. Above 40° C however, the bacterial growth is inhibited. The optimal temperature was determined graphically by the highest number of Pfus obtained. For the T7/*E. coli B*, for example, it was determined on the basis of a compromise between three criteria:

1. Maximal number of Pfus;
2. Legibility of the lysis plaques (number and size);
3. Variability of the measurements.

An incubation temperature of 22° C was therefore chosen for this system. The comparison between the number of Pfus counted at 22 and at 28° C is not significant.

Table 3.3 shows the parameters chosen for all the phage-host bacteria systems.

Table 3-1:
Conditions required for the analysis on Petri dish of the different BHB systems

Phage	Host bacterium	Growth medium	Contact time (min)	Clay particles final conc. in (mg/ml) top Agar	Number of bacteria / Petri dishes	Incubation temperature	Dilution Buffer
f1	*E. coli K12*	LB	15	-	4-5* 10E8	37° C	PH
T7	*E. coli B*	LB	15	-	1-2* 10E8	22° C	PH
Sfd3	*S. faecalis*	LB	15	0.025 Ca-Montm	2-3* 10E7	27° C	SF+
Psf2	*P. fluorescens*	LB	15	0.025 Ca-Montm	6-7* 10E7	22° C	SF+
H40/1	*H40*	SWA	15	-	7-9* 10E7	22° C	PH
H6/1	*H6*	SWA	15	-	7-8* 10E7	22° C	PH
Vh5a	*V. harveyi*	SWA	none	-	3-4* 10E8	37° C	-

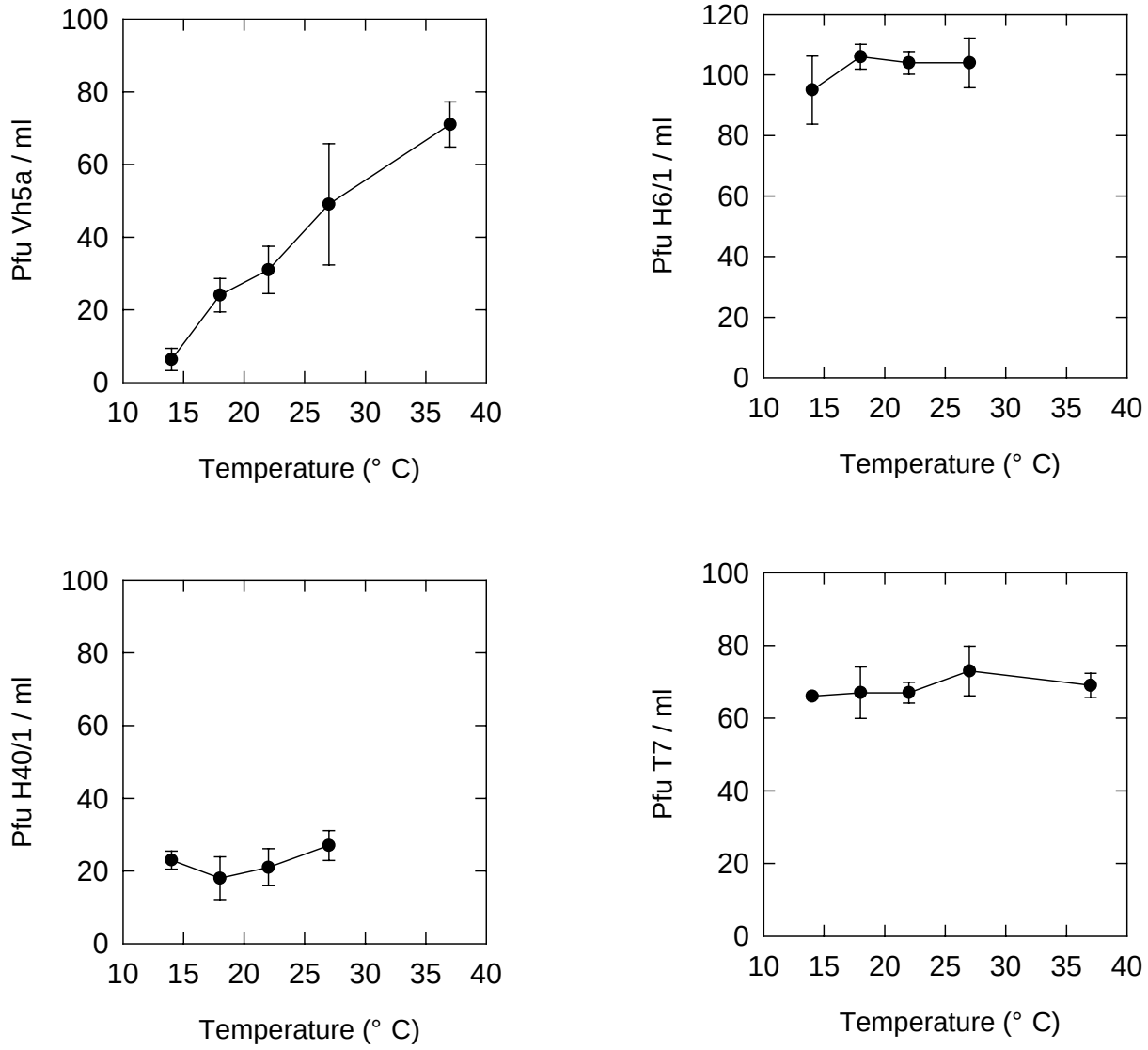


Figure3.3: Variations of the PFU numbers according to the incubation temperature

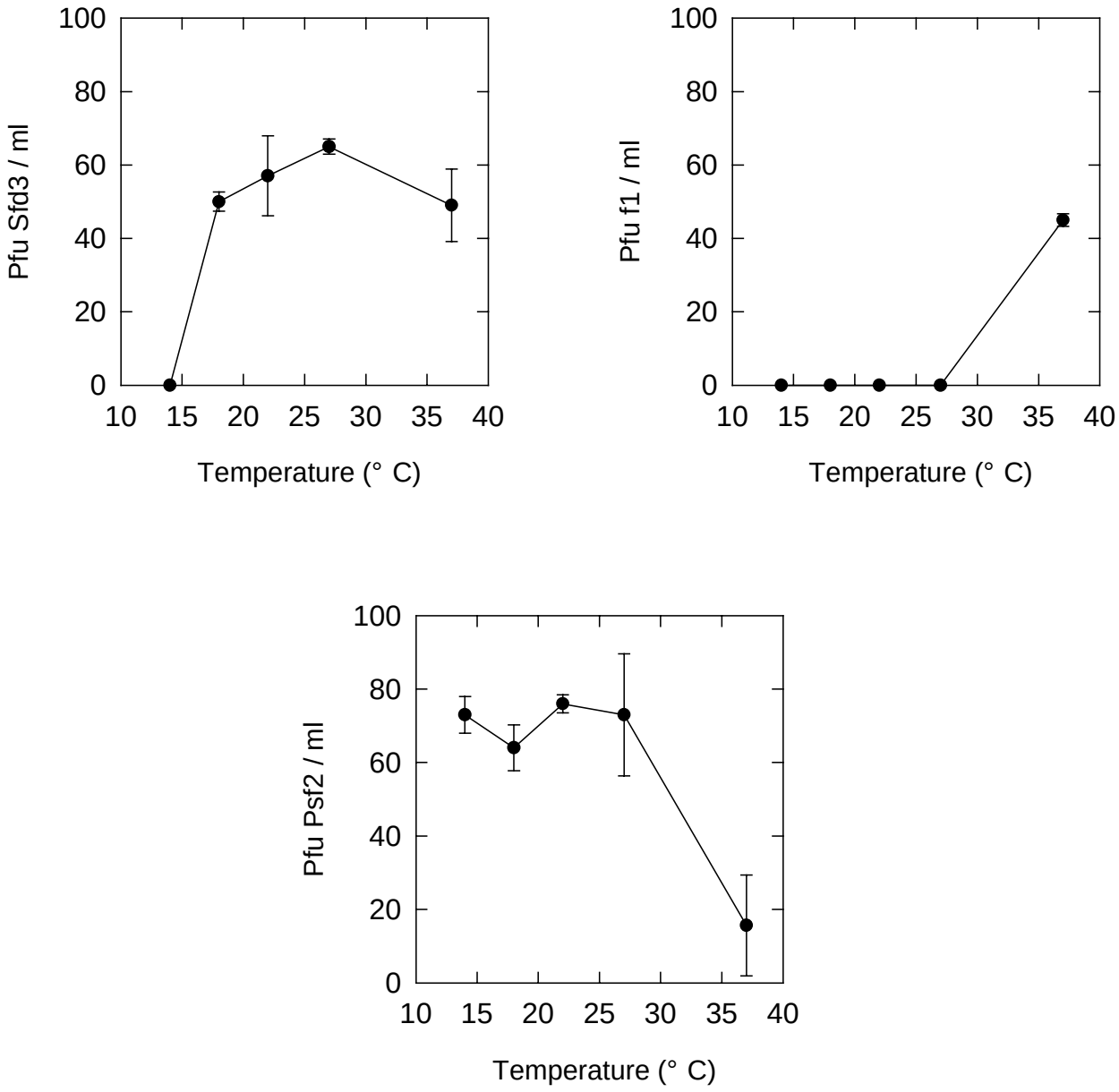


Figure 3.4: Variations of the PFU numbers according to the incubation temperature

3.3.4 Influence of the mineral and colloidal clay concentrations on the number of Pfus:

The following growth media were tested for the bacteria of "terrestrial"" origin: Nutrient agar, Nutrient Glucose agar, Luria Bertani agar, J&B agar. The marine bacteria were tested on Sea Water agar, Sea Water agar "light", DSM 246, 115, and 123 agar. The growth medium finally selected for the terrestrial bacteria was LB medium [Sambrook et al.], while Sea Water Broth agar "light" [Moebus, 1980] was chosen for the marines ones. The composition of these media is given in annex 3.2.

The number of Pfus appearing on a Petri dish depends on the composition of the medium, i.e. on the concentration of mineral salts. Bivalent ions (Ca^{2+}, Mg^{2+}), colloidal particles (Montmorillonite, Kaolinite and Attapulgite) and sometimes also organic substances (such as thiamine), can significantly increase the number of Pfus appearing on a Petri dish [Cornax et al., 1990; Havelaar and Hogeboom, 1983; Grabow and Coubrough, 1986; Lipson and Alsmadi, 1989].

We tested the behavior of the BHB systems in the presence of calcium and magnesium salts, and of two clay colloids, Ca-Montmorillonite (Texas) et Attapulgite (Florida). The preparation of the colloidal suspensions is described in annex 4.1. No organic substance was tested. Figures 3.5a and 3.5b summarize the results of these tests. The numbers 1 to 7 on the abscissa correspond to the following conditions:

1. Double agar-layer method, after Adams. Classical technique, adding bacteria and phages directly to the molten agar; immediate plating of this second layer; 1ml of PH buffer added;
2. Optimised method, described in diagram 3.1: plating following the conditions summarized in table 3.1, with contact time in PH buffer;
3. Same as 2, but using SF+ (see annex 3.3) for the contact time; 1ml of SF+ added for the plating, instead of PH buffer;
4. Same as 3, adding a colloidal Attapulgite suspension in SF+ before plating (final concentration in the top agar 0.025mg/ml);
5. Same as 3, adding a colloidal Ca-Montmorillonite suspension in SF+ before plating (final concentration in the top agar 0.030mg/ml);
6. Same as 3, adding a colloidal Ca-Montmorillonite suspension in SF+ before plating (final concentration in the top agar 0.070mg/ml);
7. Same as 4, adding a colloidal Attapulgite suspension in SF+ before plating (final concentration in the top agar 0.1mg/ml);

The results of N° 1 were arbitrarily set as 100%. The experiments were performed twice, using six Petri dishes each time. The error bars represent the variance (sigma n) of the means obtained when counting the 12 Petri dishes.

The different behaviour of each BHB system is noticeable at first glance. No general tendency seems to emerge. The only system seeming to have similar reactions are those of phages f1 and T7. It is quite probable (since both phages attack the same bacterial species, *E. coli*), that this test defines the ideal physico-chemical conditions for the attack of a particular bacterium and not those suiting a specific phage.

Method N° 2, the one allowing a contact phase, gives good results for 4 bacteriophages, H40/1, H6/1, f1 and T7. The number of Pfus increases by 20 to 50% compared to the classical method. Using SF+ and colloidal clay does not seem to be of any particular advantage, quite the opposite. Phage H6/1 reacts very strongly to the SF+ buffer: the number of Pfus falls drastically. Phage H40/, on the other hand, seems insensitive to the changes of conditions.

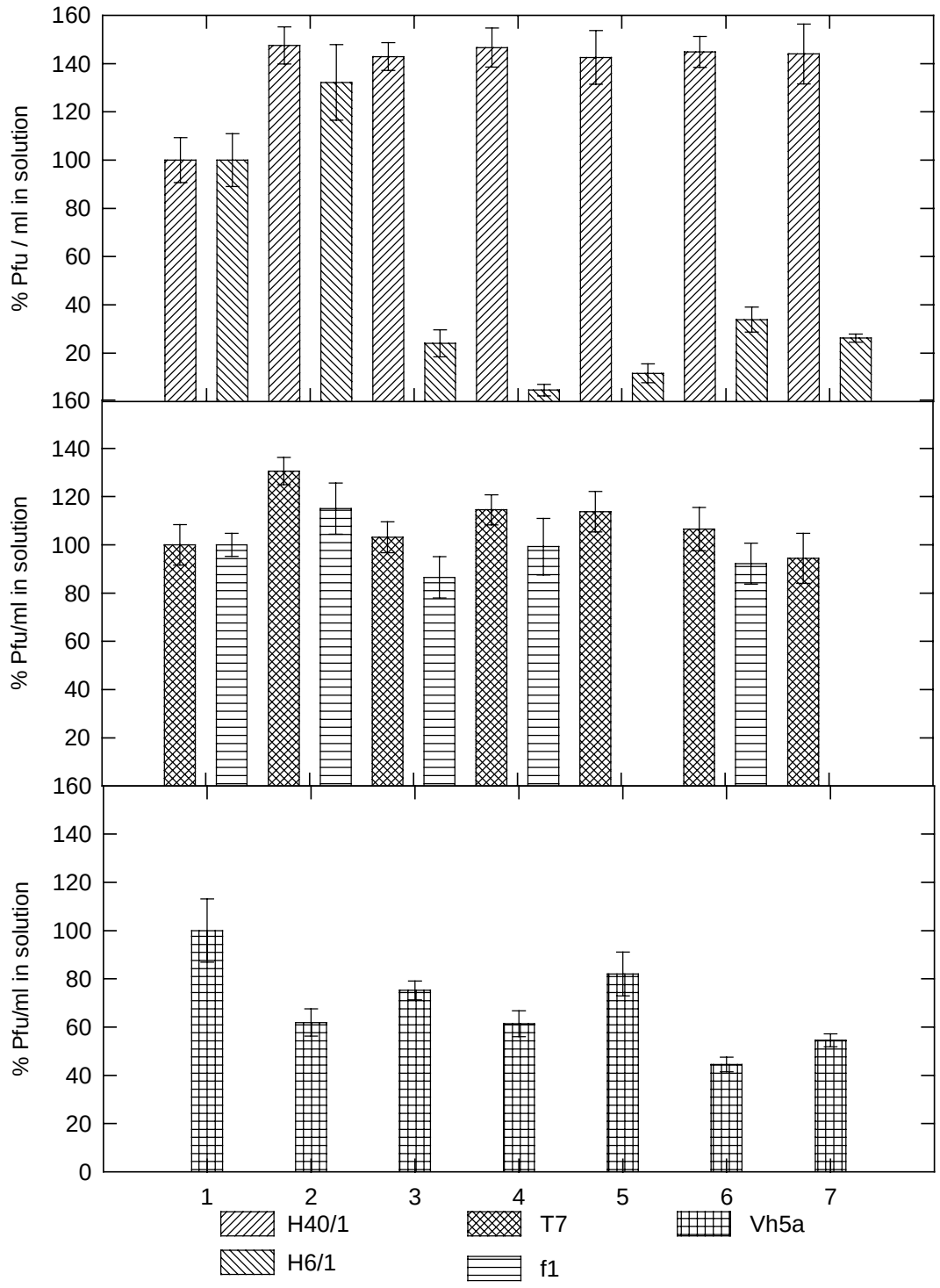


Figure 3.5a: Comparison of different plating methods.

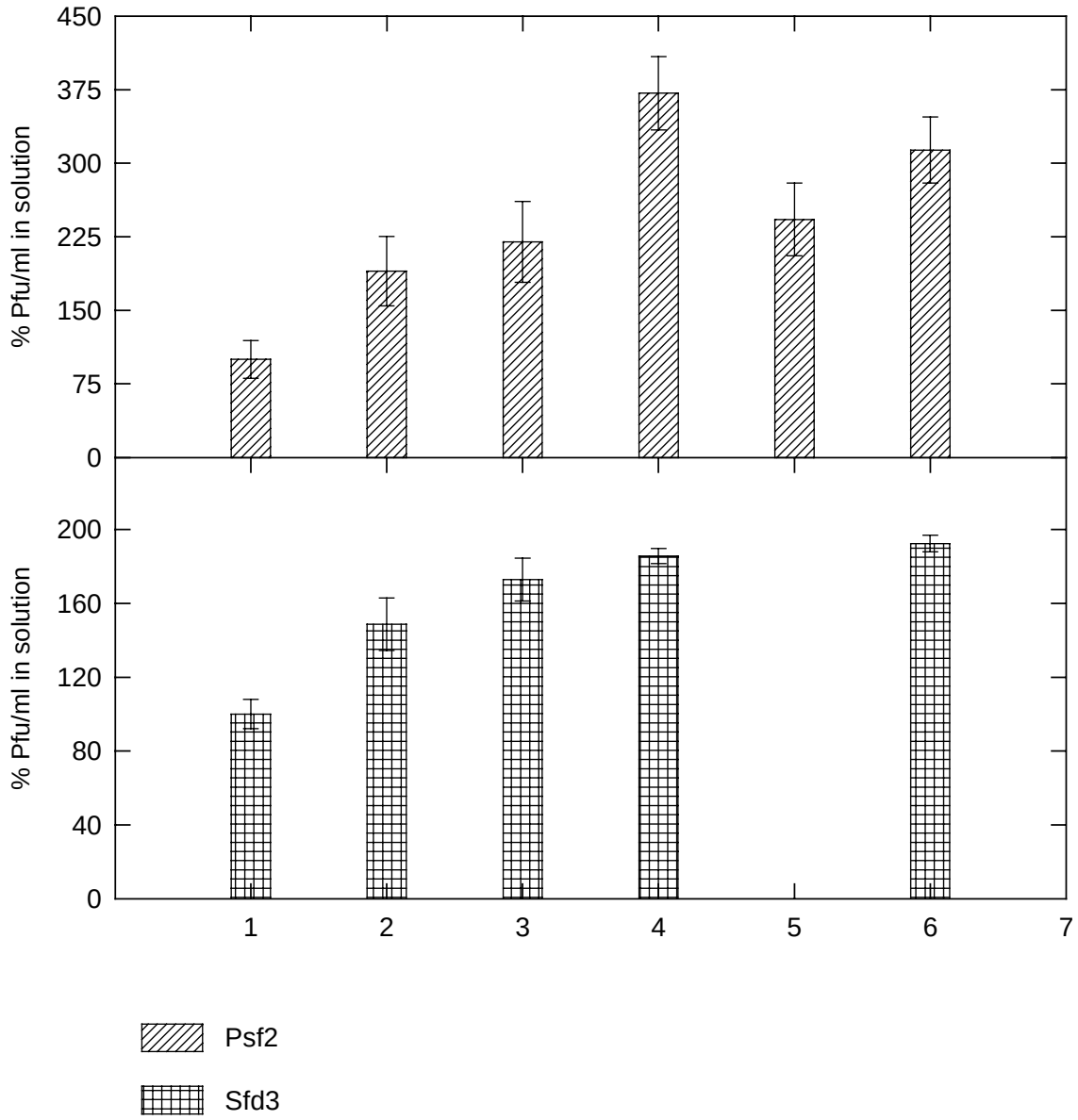


Figure 3.5b: Comparison of different plating methods.

The only bacteriophage whose number of Pfus does not increase with the adjunction of a contact phase is Vh5a. This does not mean however that the classical method suits this BHB system better. However it does certainly mean that we did not find the optimal conditions for detecting it on Petri dishes.

For Psf2 and Sfd3, the graph shows a big increase in the number of Pfus. We have already mentioned the important quantities of mineral salts that this phage requires for an optimal multiplication. The further adjunction of colloidal clay (Ca-Montmorillonite) seems to particularly favour its detection. The increase in the number of Pfus is of about 250%, compared to the numbers obtained using the classical method. This rate is far higher to that determined by Lipson and Alsmadi [1989] for the phage X-174 (*E. coli*), in the presence of a homo-ionic clay. These authors did not include a contact phase in their procedure. However, it is precisely this placing in contact of the bacteriophages and the bacteria in a liquid medium which is the important step for the detection of phages on Petri dishes.

3.4 Conclusions:

Luria et al. [1950] showed that relation between the number of phages counted on Petri dishes and the amount of phage material assayed is perfectly linear. This proves that each plaque stems from the action of one material particle. This does not necessarily mean that every phage particle present is counted as a plaque, but only that a constant fraction of the particles produces plaques. The phages detected by electron microscopy are more numerous than those appearing on a Petri dish through the disappearance of their host bacterium. The correspondence between these two counts is of the order of 40 to 100%. A number of hypotheses can be advanced to explained this situation. We could mention, like Luria et al., the possible formation of clumps, by the phages themselves, or on a fragment of bacterial cell. The photo of phage Sfd2 (chapter 2) illustrates this. This clumping could quite well only be temporary, and thus reversible. We tried to break these clumps in many different ways: by sonication or filtration, or by treating with chloroform or modifying the pH of the medium. However, no results were obtained.

A second hypothesis can be proposed. The phages counted by electron microscopy are not necessarily all virulent, even if no damage to the capsid is visible.
However, this linear relationship established in 1950 was gradually forgotten, while many new methods of isolation and numeration, sometimes extremely complex, were developed. This research was triggered by the discovery of very important populations of pathogenic viruses in the environment, more specifically in drinking waters. Numeration on Petri dishes is generally the very last step of these techniques and its optimization was never attempted. However, it is certainly by developing this last step that it would have been possible to increase the sensibility of the complex techniques described in the introduction to this chapter.

The results we obtained show that it is almost impossible to define physico-chemical conditions that would be optimal for the numeration of all bacteriophages. Each BHB system has its own particularities. Numeration therefore must inevitably pass by an optimization stage. This does not mean, however, that all the phages present will be detected. The aim of this optimization is to make it possible to count the maximum number of phages, regularly and reproducibly. It is therefore impossible to determine what fraction of the total is actually detected. We can only record that the variability of our measures is generally below +/- 5%. With our method, we therefore detect a constant proportion of the entire phage population.

The problem of phage detection is not resolved however. How can one spot a very small population of phages, if only a fraction of it will be detected ? The solution certainly requires a systematic research of all the parameters (contact time, organic and inorganic substances, ...) which influence the adsorption of the viral particle on its host bacteria. We have not exhausted all the possibilities, by far.

§4. Comparative study of the behavior of bacteriophages in ground waters

4.1 Introduction:

The study of the survival and propagation of pathogenic viruses in waters is a field of research in continuous expansion. This is a direct consequence of our increasing needs in drinking water. The consciousness about this problem of virus transmission dates from the beginning of the fifties. In 1957 Berg wrote: *"The increasing pollution of our natural waters and the fact that viruses are important agents of human diseases have prompted research of their fate in water."*

Some fluorescent tracers, such as uranin for example, are generally considered conservative. This means that they interact hardly or not at all with the saturated environment during their migration. Bacteriophages on the contrary are strongly influenced by the underground environment through which they travel. The two processes which determine their survival and migration in water are inactivation and adsorption. Many studies underline the importance of the environment's physico-chemical characteristics on the adsorption and inactivation of pathogenic viruses in soils. Moore et al. [1981] reviewed 34 different soils and their respective influence on the adsorption of polioviruses. According to these authors, there exists a strong correlation between the adsorption of the viruses and the presence of organic matter and also the number of negative charges carried by the substrate. Lipson and Stotzky [1983] showed a correlation between the adsorption of reoviruses on clays and the latter's cation exchange capacity. However, according to Yates et al. [1985], temperature is the only parameter that can be significantly correlated to the survival of viruses (poliovirus 1, echovirus 1 and bacteriophage MS_2). We could mention many other works [Bitton, 1975; Koya and Chaudhuri, 1977; Bixby and O'Brien, 1979; Seeley and Primrose, 1980; Lipson and Stotzky, 1983; Yates et al, 1985; Nasser et al., 1993]. They demonstrate the importance of research on phage inactivation and adsorption if one intends to study seriously their migration and persistence in the underground. They can adsorb either on suspended colloidal particles (organic and inorganic colloidal particles), or on any grain surfaces contained in the walls of the aquifer.

A. Inactivation:

The first process influencing the survival and propagation of phages and viruses is inactivation. The virus loses its capacity to infect its host-cell, and therefore its possibility to multiply. One also calls this *loss of virulence*. A virus can loose its virulence for several reasons (see also Babich and Stotzky [1980] on this subject):

- Following the alteration or destruction of the phage's capsid or the tail;
- When its specific receptors are destroyed, making it impossible for the phage to recognize its host;
- Due to the chemical or physical alteration of the phage's genetic material;
- Because of its bonding with complex organic macromolecules (humic acids), a generally reversible occurrence;

The presence of suspended material (organic or inorganic) also plays a role in the inactivation of viruses. This is where the second process intervenes: adsorption.

B. Adsorption:

Many works show that adsorption of the viruses on the colloidal particles present in the waters happens very rapidly and thus in great quantities. [Carlson et al., 1968; Bitton and Mitchell, 1974; Schaub and Sagik, 1975; Hurst et al., 1980; Gerba, 1984; Armon and Cabelli, 1988; Powelson et al., 1991]. The adsorption mechanisms between the viruses and the particles are governed by the laws of attraction and repulsion. They are due to electrostatic and Van der Waals forces and to

hydrophobic attractions [Smith et al., 1978; Babich and Stotzky, 1980; Liew and Gerba, 1980; Bales et al., 1991]. Adsorption on these particles does not inactivate all viruses. Moore et al. [1974] declare: *"Our data ... reaffirm and broaden the findings that certain viruses associated with suspended particulate are infective"*. Adsorption may give important advantages. The work of Schaub and Sagik [1975] show that adsorbed phages are protected from rapid inactivation. In that way they can be transported by water over longer distances.

Adsorption is not irreversible. The viruses inactivated by adsorption can recover their virulence when the physico-chemical conditions of the environment change. In laboratory experiments they were desorbed by modifying the pH, increasing the concentration of organic matter, or by decreasing the ionic concentration [Ostle and Holt, 1979]. The use of chaotropic agents or detergents is also an effective way of desorbing them [Armon and Cabelli, 1988].

4.1.1 The classical means of studying virus behavior:

The most common experimental approach for studying virus-surface interactions involves mixing a fluid suspension of viruses with a quantity of the solid material of interest in plastic centrifugation tubes. Concentrations of viruses present in the fluid fraction of this batch suspension are expressed in function of time, usually after centrifugation. By varying either the amount of virus or solid added to this suspension, different apparent-equilibrium distributions of viruses between the solid surface and the fluid phase can be obtained [Grant et al., 1993]. These steady-state concentrations are used to construct isotherms which typically fit with Langmuir or Freudlich equilibrium adsorption models [Burge and Enkiri, 1978; Moore et al., 1981; Lipson and Stotzky, 1983]. Both of these models assume that the distribution of adsorbate between adsorbed and unabsorbed states reaches an equilibrium, resulting from a dynamic balance between simple one-step adsorption and desorption. The two models differ in the way they treat the change in adsorption free energy as available adsorbate surface sites begin to saturate [Morel, 1983].

That isotherms are really adequate for studying the behavior of viruses during batch experiments is in fact questionable. The Langmuir and Freudlich isotherms imply that the concentration of viruses does not vary with time. In other words, this means that no viruses are inactivated during the experiment. These adsorption experiments are generally performed at ambient temperature and under constant agitation. The inactivation of viruses will vary during the experiments. The problem is *not* solved by postulating that the number of inactivated viruses is so low as to be negligeable. This hypothesis is certainly correct for a very short experiment or during the first minutes. However, we will show later on that over longer periods of time it is incorrect. It is therefore impossible to calculate the various fractions of the initial population (suspended, adsorbed, inactivated) correctly. It is also impossible to know if the viruses that have disappeared have been adsorbed on the solid material or have been inactivated. The information obtained from these isotherms is therefore quite restricted, since they do not indicate the evolution of the various fractions of the population during the experiment. Furthermore, as recalled by Grant et al. [1993]:*"Because virus inactivation is a continuous process batch experiments involving viruses are fundamentally non-equilibrium systems making application of equilibrium models to these data difficult to interpret"*. In effect, as we will show later on, our experiments rather show the establishment of a real adsorption-desorption dynamic equilibrium between the virus population and the colloidal particles in the suspension. Finally, and this drawback cannot be ignored, every author has his own method and fixes his own parameters. This makes it extremely difficult to compare the results of experiments performed by different authors.

4.1.2 Aims of this chapter:

The main objective was to compare the behavior of the various bacteriophages under different conditions. The experiments presented here do not go far enough to allow the development of a mathematical model describing all the aspects of inactivation and adsorption. Our aim was to review as completely as possible all the parameters that influence the survival and migration of bacteriophages in underground and surface waters. In the longer term this should make it possible to determine the suitability of each particular bacteriophage for its use as biological tracer in waters.

T7 bacteriophage is used as a model for developing the method. Its inactivation and adsorption at different pH, at varying concentrations of ions and organic matter, and the influence of clay particles, will be measured. The results will be then compared with those of the other phages (f1, Psf2, H6/1, H40/1, Sfd3).

4.2 Material and Methods:

4.2.1 Continuously agitated batch experiments:

A glass vessel (500ml Erlenmeyer flask) is generally used for these experiments. For some comparative experiments, different types of plastic vessels were used (polycarbonate, Teflon, polypropylene). The inner surface of the glass vessels were sometimes treated with 5ml Repel-Silane (LKB, Pharmacia), to make it hydrophobic. 4 ml of Tris-HCl buffer, pH 7.4, are poured into the flask and SF (Synthetic Freshwater), prepared after Moore et al. [1982] is added to 200ml. The pH is then adjusted to the desired value with NaOH or HCl 6N. When using colloidal clay particles, the desired volume of clay suspension is added to the Tris-HCl buffer, and then completed to 200ml with SF. The pH is adjusted as before. A second flask without clay is also prepared. Because the bacteriophage are often adsorbed very quickly, the first sample from this flask is used as reference for the rest of the experiment (the number of phages in this sample is arbitrarily set as 100%). The flask is placed in a constantly agitated water bath, set at a fixed temperature. The experiments are performed in duplicate, or triplicate if a confirmation of the results is necessary. For the experiments presented graphically in chapter 4, the experimental conditions were the following:

Temperature:20°C Agitation:180 mvmts/min. pH:7.4 - 7.6 (Tris Buffer 20mM) Medium:SF

In all the other cases, the particular conditions are given in each figure or in the commentary (point 4.3). The bacteriophages are prepared following the procedure described in chapter 2. About 2 to $4*10^5$ phages are added to the vessel, which is shaken manually before being placed in the water bath. A sample is taken from the second flask, containing only SF. The timer is then started and the samples are taken from the first vessel at regular intervals. All the samples are analyzed following the procedure described in chapter 3. For some experiments they are first centrifuged (3 minutes at about 6000rpm, Mini Centaur table centrifuge).

4.2.2 Batch experiments without agitation:

The Erlenmeyer flasks are placed in incubators at set temperatures. The samples are taken over a few days and analyzed as described in chapter 3. The solutions used in this chapter, the composition of SF and the procedure for preparing colloidal clay suspensions are given in annex 4.1.

4.2.3 Clays and organic material:

The agitated batch experiments were performed using variable concentrations of inorganic and organic material.

A. Clays:

		Surface area:	CEC:
Ca-Montmorillonite (Texas)	:	750 m^2/g	0.0013meq/m^2
Attapulgite (Florida)	:	125 m^2/g	ND
Kaolinite (Georgia)	:	15m^2/g	0.0037meq/m^2

Montmorillonite is a typical swelling smectite, a group of secondary trimorphic phyllosilicates derived from the weathering and modification of parent-layer silicates (micas). Kaolinite is a neogenetic dimorphic phyllosilicate, derived from end-products of tectosilicates such as feldspath [Piazzolla et al., 1993].

The colloidal suspensions were prepared on the basis of the method proposed by Babich and Stotzky [1980]. 4g of clay powder are placed in 400ml of SF. The mixture is shaken energetically for 30 minutes and then centrifuged (3 minutes, 160g). The supernatant is collected and kept at 4□C. Concentrations of particles in the supernatant are determined by filtration (ester of cellulose filters, 0.25□m, Millipore). The filters are dried under vacuum at 50□C for 24 hours and then weighed. 10 to 20 ml of the supernatant are filtered. The filters are dried again (same conditions) and weighed. The weight of particles on the filter (in mg per unit of volume) is given by the difference of weight between the filter before and after the filtration.

B. Organic material:

Humic acids (sodium humate ALDRICH), two proteins, lysozyme and bovine serum albumin (BSA) (MERCK), and a proteic tryptic hydrolysate resulting from the degradation of beef proteins (Tryptone, MERCK) were used. The isoelectric point of lysozyme is about pI=11; that of BSA is pI=4.9. Their molecular weights are 14,600 and 67,000, respectively.

4.3 Results and discussion:

4.3.1 Inactivation and adsorption kinetics of bacteriophages in fresh waters:

Figure 4.3.1 schematically illustrates the general behavior of bacteriophages in a liquid environment, at the water/solid interface (modified after Grant et al. [1993]):

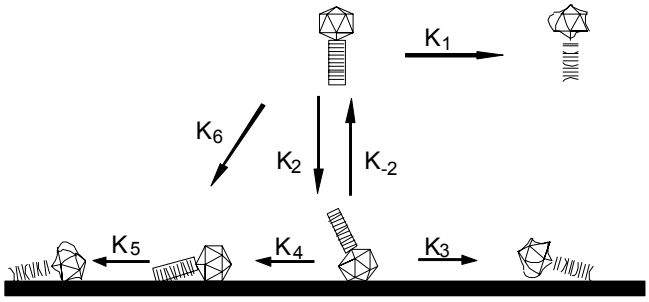


Figure 4.3.1 : theoretical model of the phages behavior.

In this figure a homogenous population of phages is subdivided into several fractions, described in figure 4.3.2:

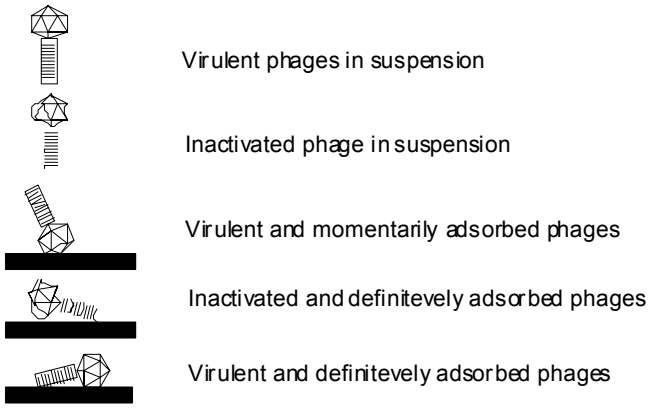


Figure 4.3.2: fractions of a homogeneous phage population

The possible interactions between a phage and an adsorbent substrate (mineral or organic) can be summarized by six reactions (K_1 to K_6). In the description of these reactions we will not discuss the virulence of the adsorbed phages. This point will be treated in section 4.3.4.

Reaction 1 (K1): the bacteriophages are inactivated in the liquid phase. For a homogeneous phage population this inactivation decreases exponentially obeying the following equation (1) [Stagg et al., 1977]:

$$(1) \qquad C(t) = C(0) * e^{-Kt}$$

where: $C_{(0)}$: Initial concentration of phages in the suspension
$C_{(t)}$: Concentration of phages in the suspension at time t
K : Inactivation constant (h^{-1} or min^{-1})

The results of the inactivation kinetics are presented in chapter 4.3.3. We analyzed the case of phage T7 specifically. This phage was used for developing the analytical methods and to define the standard inactivation kinetics. This phage was chosen because of number of publications

dedicated to it. The many references allowed a detailed comparative study to be made [Bitton and Mitchell, 1974; Niemi, 1976; Burge and Enkiri, 1978; Shields et al., 1986; Stotzky et al., 1981; Schiffenbauer and Stotzky, 1982 and 1983; Bricelj and Sisko, 1992].

The following reactions (**K_2 to K_6**), describe the process of adsorption. They are briefly described below. We will discuss them in detail in the section on adsorption mechanisms (4.3.4).

Reaction 2 (**K2**): the phages can adsorb temporarily onto the solid substrate. this reaction is reversible. The phage's desorption is describes as reaction -2 (**K-2**). This reversible adsorption can theoretically be followed by inactivation (reaction 3, **K3**). Temporary adsorption can, under certain conditions, be followed by a definitive adsorption (reaction 4, **K4**). **K4** may itself be followed by inactivation (reaction 5, **K5**). Finally, the bacteriophages can adsorb definitively directly from the liquid phase (reaction 6, **K6**).

4.3.2 Inactivation of bacteriophage T7:

A. Inactivation and shaking:

With our experimental conditions (180 back-and-forth mvts*min^{-1}, 20°C and SF medium, buffered at pH 7.4), T7's inactivation perfectly followed the first-order kinetics of equation (1). Graph no 4.1a shows two inactivation experiments. One sees that the phage's inactivation is rapid and complete. Loss of virulence reached 95% after 180 min. The regression on the experimental values was made using equation (1). Their error margin is +/-5%. The values of **K** obtained for these two experiments was 1.51*10^{-2}/min and 1.66*10^{-2}/min. The reproducibility of the experiment was good: the homology between the two curves is over 99%. A comparison with an experiment without agitation (fig. 4.2b) shows that this is precisely the cause of the very rapid inactivation of the bacteriophage. Without agitation, the loss of virulence after 180 minutes is not significant (less than 5%).

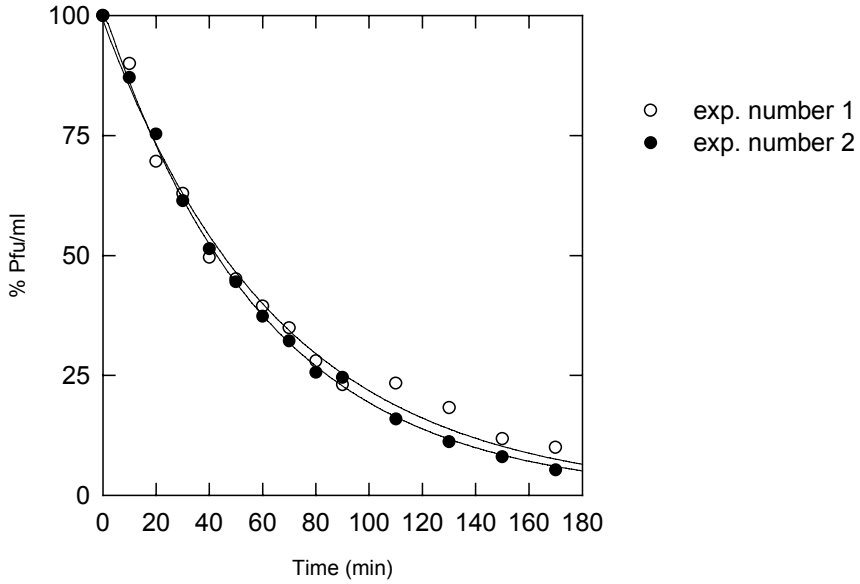


Figure 4.1a: Inactivation of phage T7 in agitated batch experiments

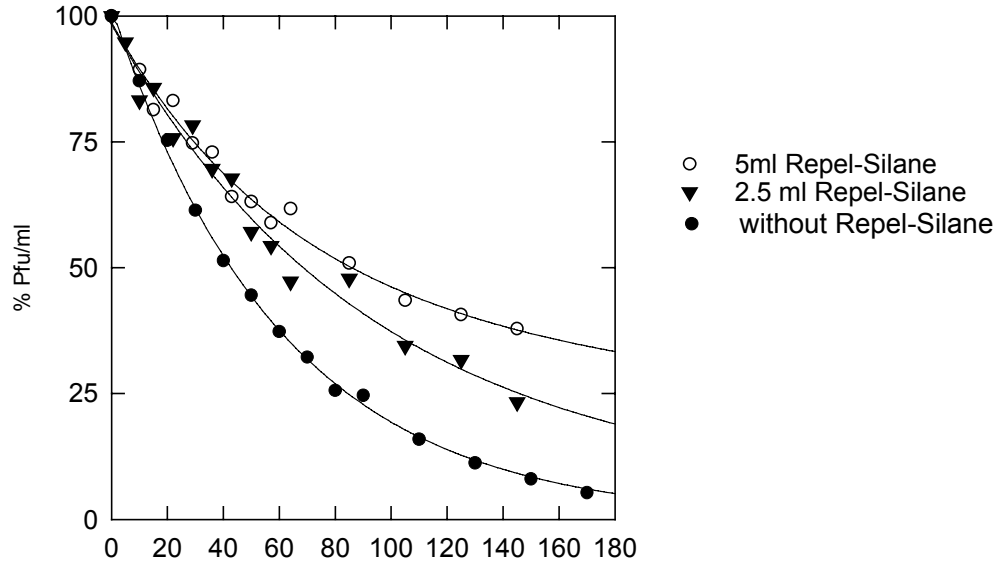


Figure 4.1b: Inactivation of phage T7 in agitated batch experiments. Silanised containers

Another experiment was performed to understand the response of T7 to agitation. The shaking was interrupted and then resumed (graph 4.2a). The population of phages remained stable during the minutes of interruption. When the agitation was resumed, the remaining population was inactivated followed the same exponential law.

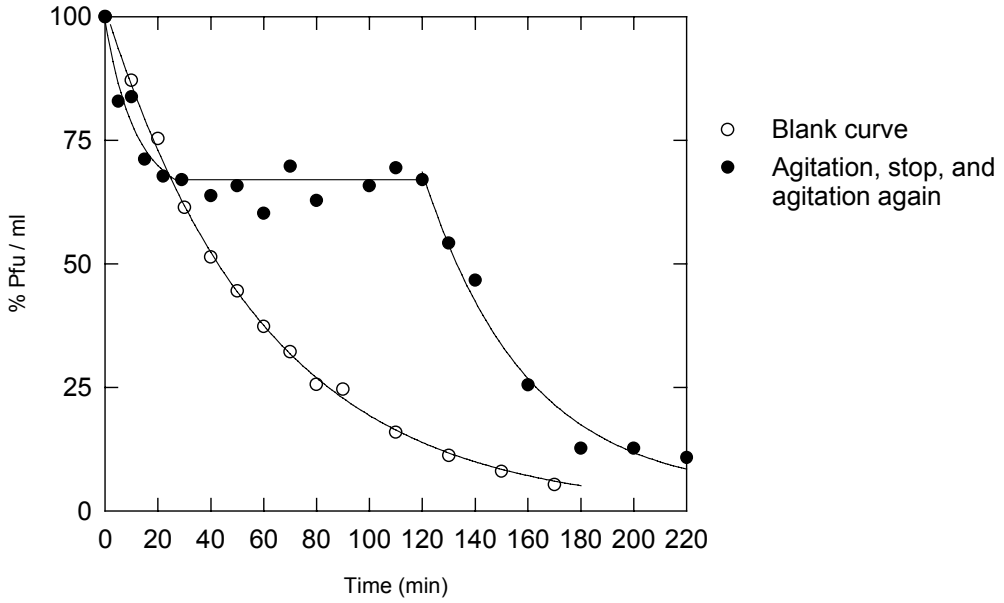


Figure 4.2a: Influence of agitation on the inactivation of phage T7. Container silanised.

Two plausible explanations can be given for this acceleration of inactivation by mechanical agitation. First the capsid may be destroyed due to the mechanical stress caused by the to and fro movements. The second hypothesis, proposed by Adams [1949] and taken up by Bricelj and Sisko [1992], states that the phages are inactivated by the surface tensions existing at the air/water interface. Shaking the liquid environment brings the phages to the surface. The surface of liquid in contact with air strongly attracts proteic molecules possessing hydrophobic domains [Wan et al., 1994]. At the air/water interface the surface tension is strong enough to cause the capsid to rupture. Further experiments (figures 4.3) shows that when these interfaces are saturated by organic material, the inactivation of the phages is slowed down considerably. This second assumption seems therefore to be correct.

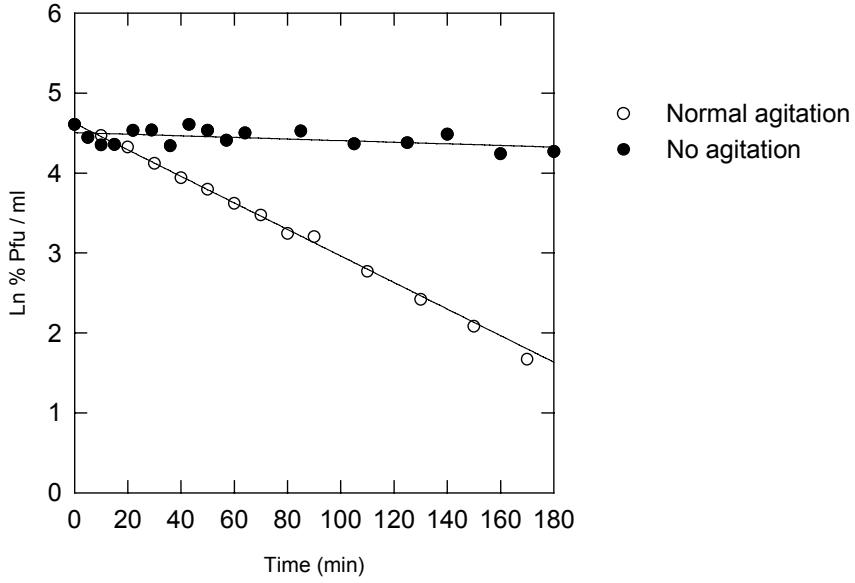


Figure 4.2b: Influence of agitation on the inactivation of phage T7

B. Inactivation and organic material:

To confirm the hypothesis discussed above, we followed the inactivation of phage T7 in the presence of two types of organic particles. First we used a protein (polypeptide) hydrolysed by trypsine (Tryptone, MERCK), at a concentration of 1 mg/ml. According to Stotzky [1986], the reduction of inactivation is due to the saturation of the air/water interfaces by the aminoacid chains. The phages are therefore physically excluded, and their capsids are no longer submitted to strong surface tensions. This protection is not total, however: still over 20% of the phages are inactivated after 180 min (figure 4.3).

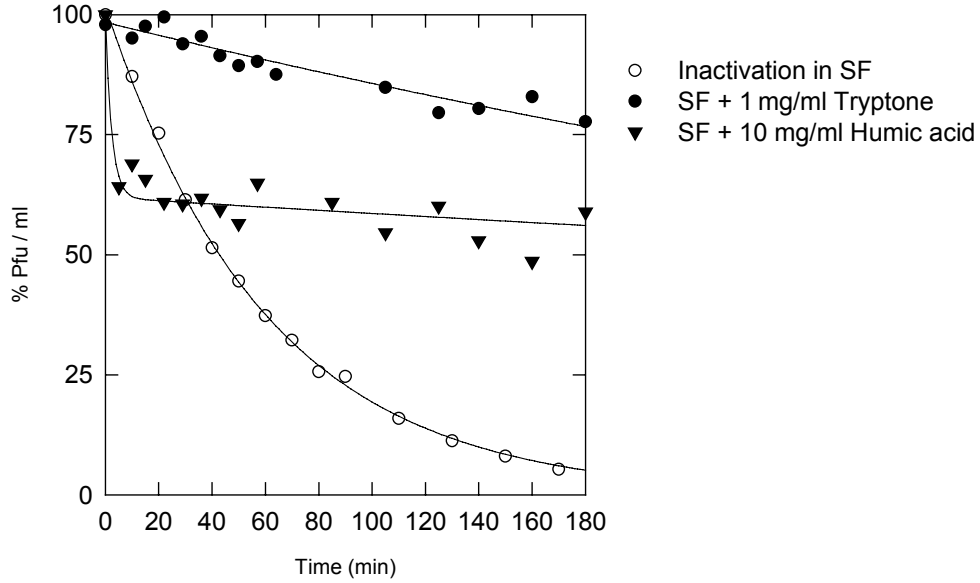


Figure 4.3: Influence of organic matter on the inactivation of phage T7

We then tried a soluble extract of humic acids (sodium humate, ALDRICH). At a high concentration (10mg/ml), these complex macromolecules strongly influence the phage's inactivation kinetics. Figure 4.3 shows a very interesting two-phase mechanism. The first part of the curve (less than 5 minutes) shows a very rapid decrease of the virulent population. Nearly 40% of the initial

population disappeared. The second phase is much slower, since after 170 minutes the population had decreased only by a further 10%. For the non-linear regression on these experimental values we used equation (2) (see section 4.3.4). To explain this phenomenon, one must first suppose the establishment of bonds between the complex humic molecules and the phages. These weak bonds (almost certainly H-bonds [pers. comm. from Prof. Senesi], could account for the adsorption of the viruses on organic macromolecules [Schaub and Sagik, 1975]. They could also cause the chelation of part of the phage population. The organic molecules cover or mask the recognition sites to the host bacteria. The chelation of phages might also results in the agglomeration of two or more viral particles. The second part of the kinetics depends on the saturation of the air/water interfaces by the organic molecules. This saturation strongly slows down the inactivation of the phages.

C. Inactivation and hydrophobicity:

So as to minimise the possible interactions between the bacteriophages and the electrostatic charges of the glass, we rendered the internal wall of the flask (500ml, borosilicate) hydrophobic, using Repel-silane. A comparison of figures 4.1a and 4.1b shows that this operation resulted in a slower inactivation of the phages. After 180 minutes, more than 30% of the initial quantity of phages was still active. It is interesting to note that the inactivation kinetics were not exponential anymore, but biphasic. A first rapid decrease was followed by a second, slower phase.

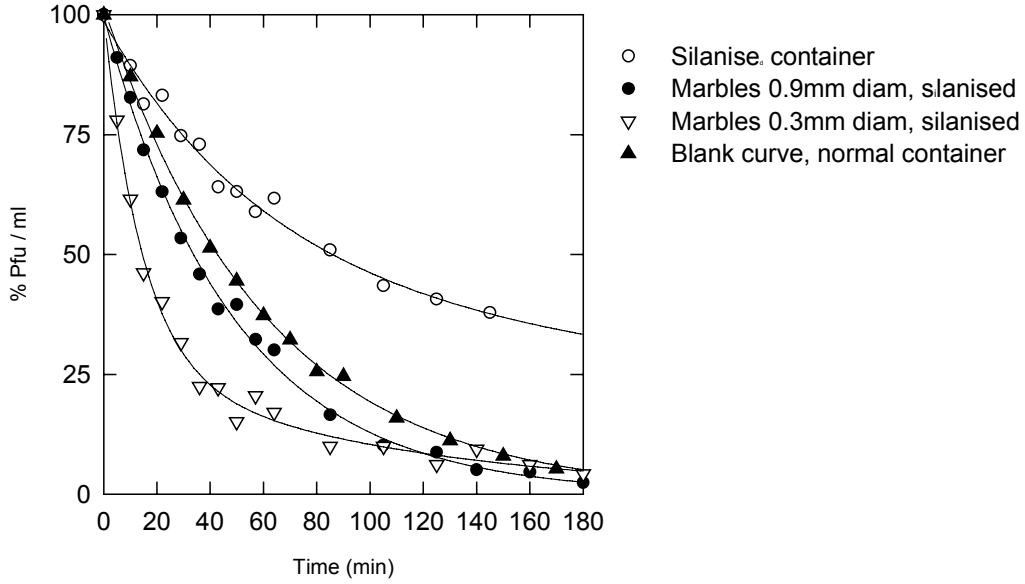


Figure 4.4: Influence of hydrophobicity on phage T7 inactivation

To understand the reason of this difference in behavior, the hydrophobic surface of the vessel was increased by adding glass beads of different diameters (92 beads of 0.9cm in diameter and 2136 beads of 0.25cm in diameter). The beads were silanised the same way as the flask. Graph 4.4 shows the results of these experiments. It is clear that the beads modified T7's behavior considerably. In the most extreme case (2136 beads representing a surface of about 420cm^2), the curve is clearly biphasic. The number of virulent phages in suspension fell rapidly to 20% after 40 minutes. From 40 to 180 minutes, 15% more virulent phages disappeared. The curve then intersects that of the inactivation experiment in an untreated flask. The same biphasic kinetics are apparent in the case of a silanised flask without beads (empty circles). It therefore appears that bacteriophages strongly interact with hydrophobic surfaces. The viruses probably adsorb reversibly onto these surfaces. This adsorption then offers them a slight protection against inactivation.

No other bacteriophage was tested. We nevertheless suppose that this interaction depends very much on the hydrophobicity of the phage's capsid. Wan et al. [1994] have shown the importance of hydrophobic bonds. These authors have shown that air/water (in effect hydrophobic/hydrophilic) interfaces strongly attract biological particles. Bales et al. [1991] also showed the important influence of hydrophobic surfaces on the behavior of bacteriophages in column experiments: *"The removal of [...] the bacteriophage MS-2 by attachment to silica beads had a strong, systematic dependence on the amount of hydrophobic surface on the beads."* Bales et al. [1993] add: *"Hydrophobic effects appear to be the important factor for adsorption of even relatively hydrophilic bio-colloids."* In trying to decrease the electrostatic bonds between the phages and the wall of the flask, we actually succeeded in doing exactly the opposite!

D. Inactivation and temperature:

The influence of temperature on the inactivation of phages and viruses has been closely studied. It is largely recognised that the inactivation of viruses increases with temperature. Yales et al. [185] gives temperature as the main cause for the inactivation of viruses in natural environments.
Figure 4.5 shows several inactivation experiments with phage T7, performed at different temperatures. They effectively show that the speed of inactivation increases with temperature. The shape of the curves does not correspond to an exponential decrease, but rather to a biphasic one. Several authors, of whom Adams [1959], have shown that some phage populations, like that of T7, are not homogenous. Several mutant types may appear (according to Adams, their proportions paradoxically remain constant over the generations). It is possible that these experiments reveal the presence of mutants resistant to higher temperatures. Logically the kinetics of this double population will be biphasic.

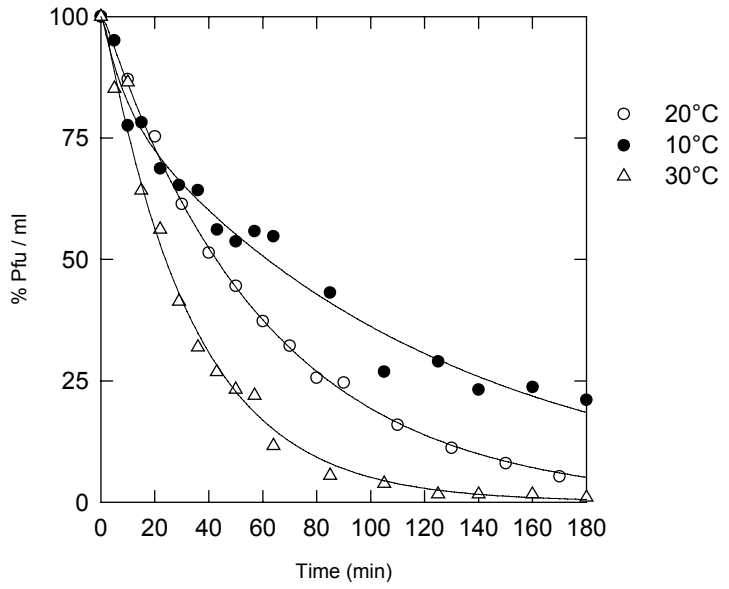


Figure 4.5: Influence of the temperature on the inactivation of phage T7

This explanation is confirmed by the works of Yin [1993]. He showed that mutant T7 phages appear inside single lysis plaques on Petri dishes. Non-essential genetic information is modified and/or lost. A population of phages is therefore composed not of a single type of bacteriophage, or even of two sub-types, but rather of a multitude of types with a different genetic inheritance. In our case, though the fact that a population is heterogeneous with respect to its resistance to temperature is interesting, it does not fundamentally influence the results of our work. We are interested in determining the effect of temperature on the population as a whole, and not in

isolating a single fraction of it, though it remains strangely constant over time [Adams, 1959]. It is obviously impossible for us to separate the various fractions before injecting them in an aquifer, so we must consider the population as a whole.

E. Inactivation and pH:

The graph 4.6 shows several experiments performed at different pHs (5, 7.4, 9) chosen because they represent the extremes that can be reached in an aquifer. The curves are practically identical (homology >95%). Changing the buffer (Potassium hydrogenophtalate instead of Tris), had practically no effect on the inactivation kinetics either. Adam [1959] obtained the same results, and according to him only extreme values (of the order of 2 or 11) will influence the bacteriophages.

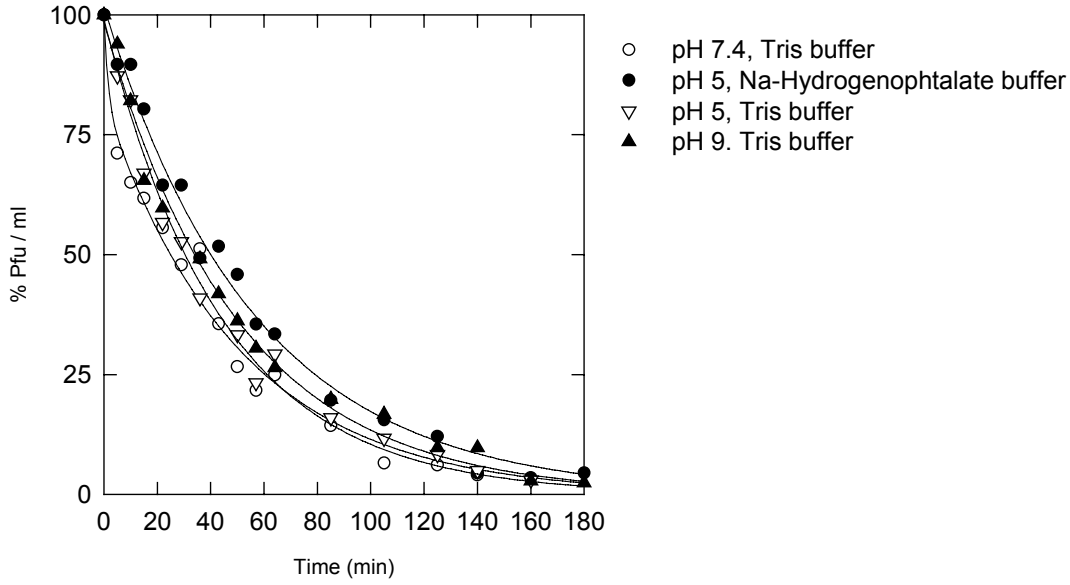


Figure 4.6: Effect of pH on the inactivation of phage T7

F. Inactivation and ionic strength:

The presence of Ca^{2+} (25mM) and Mg^{2+} (2.5mM) does not influence the inactivation of phage T7 in SF. The curve is practically identical to that obtained in SF alone (data not shown). The homology between the two curves exceeds 99%. One can therefore presume that the variations in ionic concentrations in the aquifers will have no effect on the inactivation of the phages.

4.3.3 Bacteriophage-adsorption processes: the theoretical model of Grant et al. [1993]:

A. Irreversible adsorption:

The first type of reaction described by the authors is an irreversible (e.g. definitive) adsorption mechanism of the virus onto the substrate. The adsorption is considered an accelerator of inactivation. The theoretical model and the expected experimental behavior are described in figure 4.3.3. The model predicts an exponential decrease of the virus population (full circles). This decrease is faster than the inactivation in the absence of substrate (empty circles). This case is called IA (*Irreversible Adsorption*). We never encountered this type of reaction in the course of our experiments.

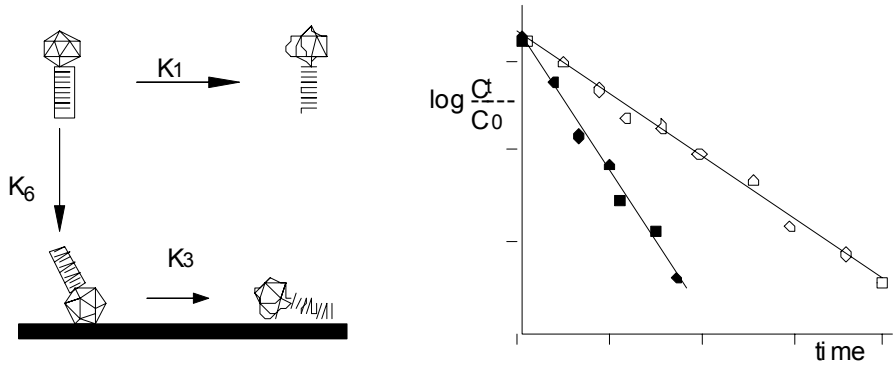


Figure 4.3.3: Irreversible adsorption (modified after Grant et al. 1993). Open circles: Inactivation reaction; Filled circles: Inactivation and adsorption reaction.

B. Reversible adsorption:

In the case of a reversible adsorption of the phage on a substrate, a dynamic equilibrium is established between the phage population and the adsorption site. According to Stotzky [1986]: *"Equilibrium adsorption is reversible, exhibits low specificity between the adsorbent and the adsorbate* (in our case the phages). *It may result in multilayer sorption and involves primarily physical forces such as London - Van der Waals interactions. Equilibrium is attained when the rate of adsorption equals the rates of desorption. As coverage of the adsorbent surface increases, it becomes increasingly difficult for adsorption to proceed, and mutual repulsion of the adsorbate molecules may result in a reduction in the bond strength and in lower values for the energy of adsorption".* This dynamic equilibrium is called *Quasi-Equilibrium Adsorption* (QEA) by Grant et al. The authors singled out three particular cases. Figure 4.3.4 shows the behaviors predicted by these three theoretical possibilities.

Case number 1: *Quasi-Equilibrium Adsorption* (QEA):

The stability of the viruses is not influenced at all by the presence of adsorbent material. Adsorption, if it occurs, is irreversible and is of no advantage to the phage for its survival. The model shows that first a variable fraction of the phages adsorbs onto the substrate (1st phase). Then, in a second phase, it shows that the inactivation rate of the adsorbed viruses is equal to that of those still free.

Case number 2: *Quasi-Equilibrium Adsorption and Reduced Inactivation* (QEARI):
The inactivation rate of the adsorbed viruses is smaller than that of the free floating ones. Adsorption onto suspended colloidal particles or solids protects the viruses from inactivation caused by the physico-chemical and biological conditions of the environment.

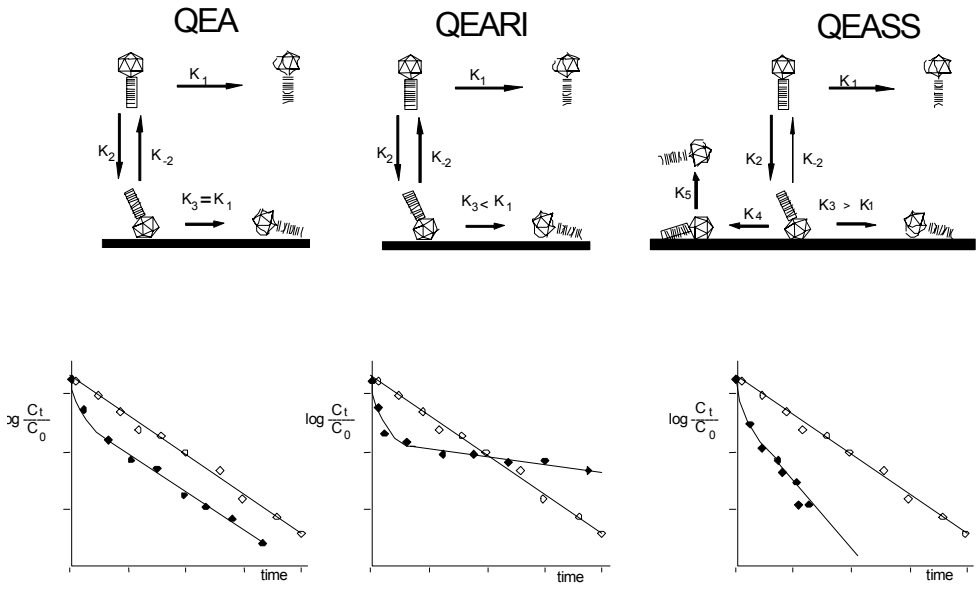


Figure 4.3.4: Quasi-equilibrium adsorption (modified after Grant et al. 1993). Open circles: Inactivation reaction; Filled circles: Inactivation and adsorption reaction.

Case number 3: *Quasi-Equilibrium Adsorption and Surface Sink* (QEASS):
In this case, the solid surface acts as a trap for the viruses. The inactivation rate of the adsorbed phage is higher than that of the free-floating phage. As we will show, only bacteriophage f1 shows this reaction model.

Grant's theoretical model was developed from the results of experiments on the reversible or definitive adsorption of viruses on sand particles. This model's main weakness is that it does not consider that the adsorbed fraction of viruses may remain virulent. Payment et al. [1987], however, showed that in natural habitats a very important part of either viruses (about 77%) or coliphages (about 65%) are associated with suspended particles but retain their virulence. This was also observed by other researchers [Lipson and Stotzky, 1985]. It is not clear how an adsorbed phage can remain virulent. Electron micrographs have shown that phages are generally adsorbed by their tail, which carries specific recognition sites [Stotzky et al., 1981 and 1986]. The presently accepted theory is that the phage is desorbed in the presence of its host bacteria. The affinity for the specific adsorption site is certainly much greater than that for the surface of a clay particle. However, this has not yet been proven.

Since Grant's model does not take the virulence of the adsorbed viruses into account, it will be correct only if one considers that the adsorbed phages have no possibility of contact with their host bacteria. This means that only the fraction of free-floating phages retains its capacity to multiply. In

our experiments with colloidal particles, this fraction was detected after centrifugation of the samples. The regressions on the experimental points of the inactivation kinetics were made using equation (1). For the adsorption experiments presented in this section, the empirical formula (2) was used:

$$(2) \quad C(t) = C01 * e^{-K1*t} + C02 * e^{-K2*t}$$

The use of this equation is much simpler than the three differential equations calculated by Grant, but also allows the calculation of the non-linear regression of biphasic adsorption kinetics. Just as with Grant's model, it makes it possible to graphically visualise and measure the evolution of the various fractions of a phage population.

4.3.4 Adsorption of the bacteriophage T7 on sand particles:

Our experiments with sand grains were performed in conditions where the number of adsorption sites is far greater than the number of phages (see graph 4.9). A dynamic equilibrium establishes itself when the flux of bacteriophages adsorbing onto the substrate becomes equal to that of desorbing bacteriophages. This does not mean that the whole system is in equilibrium. Because the suspension is agitated, inactivation processes still take place. In our experimental conditions, the sand particles present in the medium mean that the adsorption and desorption speeds ($\mathbf{k_2}$ and $\mathbf{k_{-2}}$) are far greater than the inactivation rates ($\mathbf{k_1}$, $\mathbf{k_3}$, $\mathbf{k_4}$ and $\mathbf{k_6}$). The establishment of the dynamic equilibrium is not instantaneous, however. The time necessary for it to become effective depends on the type and amount of particles in the suspension.

The characteristics of the sand used are given in annex 4.1. Buffered SF medium was used (pH = 7.49). Just as Grant et al. did, we centrifuged the samples before analysis. Therefore, only the evolution of the free and virulent phages was followed. The adsorbed fraction is eliminated with the adsorbing particle. Graph 4.7 shows the results of the first series of experiments. The adsorption of T7 was measured with increasing quantities of sand, washed and sifted at 2mm. One sees that the decrease in the concentration of free phages followed biphasic kinetics. The first stage was rapid and depended on the amount of sand present. The second was slower and seemed to be independent of the quantity of suspended particles.

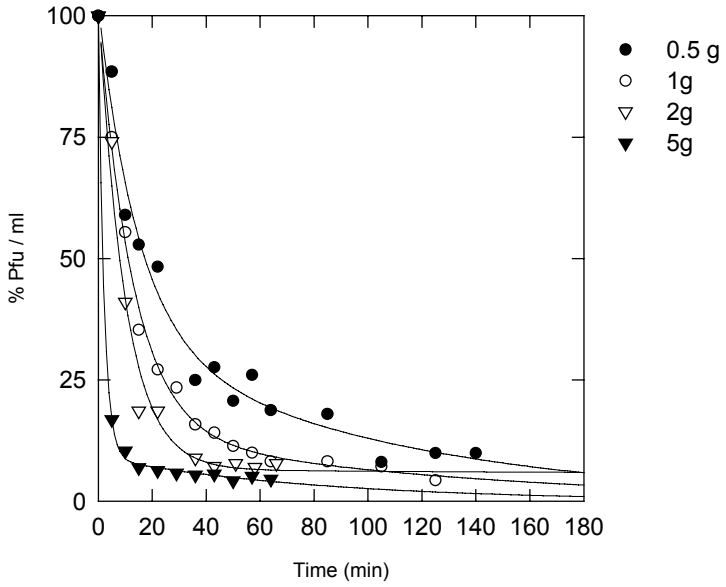


Figure 4.7: Influence of increasing amounts of sand on the adsorption of phage T7. Centrifuged samples.

At the beginning of the experiment, the rapid decrease in the number of Pfus corresponds to the

establishment of the dynamic equilibrium (QEA) between the phages and the sand's adsorption sites. The more sites available, the faster this equilibrium is reached. After, the amount of free phages decreases slightly. Adsorption seems to offer a slight protection against inactivation, as in the QEARI model. Graph 4.8a shows the results of the second series of experiments. Phage T7 was submitted to equal amounts (5g) of sand of varying diameters. The results again show a biphasic process. But this time, no significant difference can be seen between the experiments using sands of diameters below 0.2mm (figure 4.8.a, 0.2, 0.1mm and figure 4.8.b, 0.05mm). For the same amount of sand, decreasing particle size means increasing specific surfaces. The number of adsorption sites should therefore also increase. Paradoxically, a maximum of adsorption seems to be reached with a particle diameter of 0.2mm.

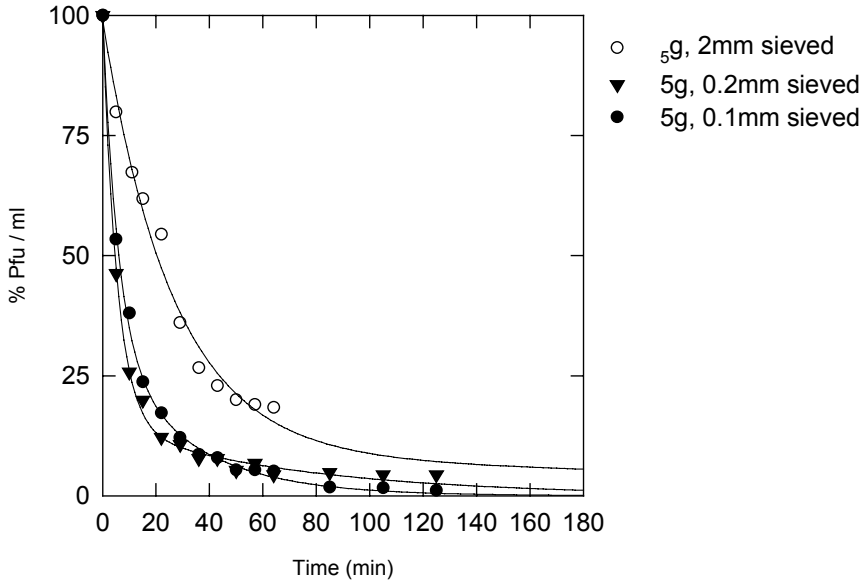


Figure 4.8a: Adsorption of phage T7 on sand particles. Centrifuged samples.

To explain this phenomenon, one must consider the methodology used. The shaking forms the sand grains in the bottom of the flask into a uniform layer. All the adsorption sites are not available simultaneously. Some will be masked, due to the position of the particles in the layer, making it impossible for the phages to bind onto them, despite the shaking. Furthermore, no the sifted fractions were not analyzed chemically. It is likely that the proportion of limestone and siliceous grains changes with the granulometry. The chemical composition of the fractions could also influence the kinetics.

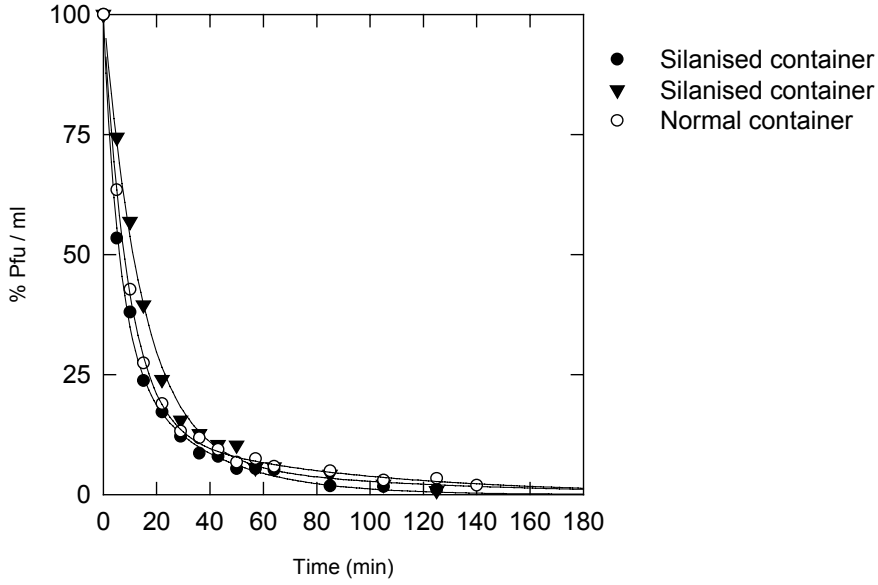


Figure 4.8b: Adsorption of phage T7 on 0.05mm sieved sand (5g). Centrifuged samples

Graphs 4.8b and 4.9 show some other experiments performed using the same sand. In the first instance, either an untreated borosilicate container or a silanised one (2ml Repel-silane, PHARMACIA) was used. The homology between the three curves is over 95%. This means that the reactions between the phages and the sand are much faster than those established with the hydrophobic surface of the flask. This confirms once more the hypothesis developed under 4.3.3C.

Graph 4.9 shows the results of an experiment were phages were injected twice into the same suspension. The kinetics of the second reaction are similar to that of the first. This confirms the fact that in these experiments the number of sites available is much higher than the number of phages in the suspension.

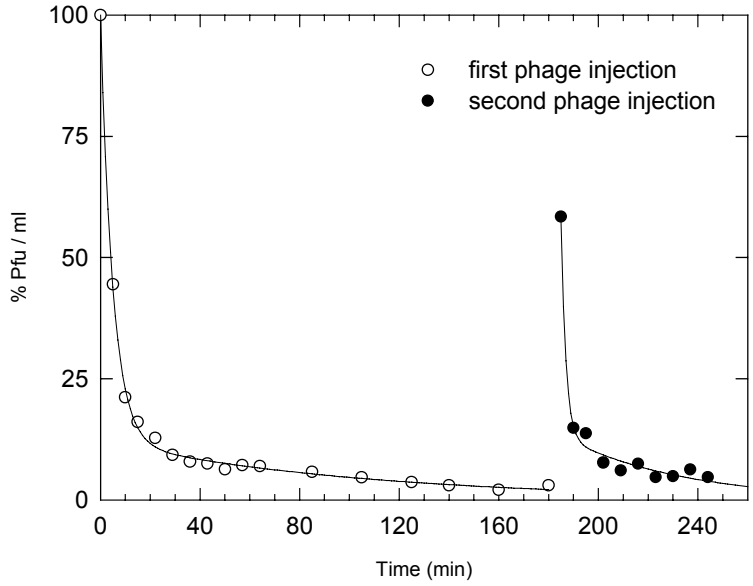


Figure 4.9: Adsorption of phage T7 on 2mm sieved sand (3g). Centrifuged samples.

As discussed under 4.34, it is not possible with this type of experiment to follow the evolution of the various fractions of a population of bacteriophages. All one obtains are the kinetics of the free and virulent fraction of phages. The evolution of the inactivated fraction or of the adsorbed and virulent one is not followed. Furthermore, though the sand was washed, fine particles are sometimes liberated after a few minutes agitation. The phages also adsorb onto this suspended particles, making further interpretation of results difficult. The disadvantages of studying adsorption kinetics on "heavy" particles are numerous. The use of sand particles was therefore restricted to a few experiments. This direction of research was then abandoned for that using suspended clay particles.

4.3.6 Adsorption of phage T7 onto particles of colloidal clay:

Because of their small size (around 0.2μm), suspended clay particles present many advantages over "heavy" ones such as sand. It is for instance possible to characterize the various fractions of a population of phages:

- The samples containing bacteriophages and suspended clay particles can be centrifuged. This makes it possible to identify the fraction of free and virulent phages (**FV**).

- Depending on the phage used, a variable fraction of the adsorbed phages remain virulent. Analysis of the non-centrifuged samples allows to detect all the virulent phages (**V**), both free and adsorbed.
- The difference between the two fractions described above (**FV - V**) corresponds to the fraction of adsorbed virulent phages (**AV**).
- The last fraction, that of inactivated phages (**I**), is obtained by subtracting **V** from the total number of phages present at the beginning of the experiment.

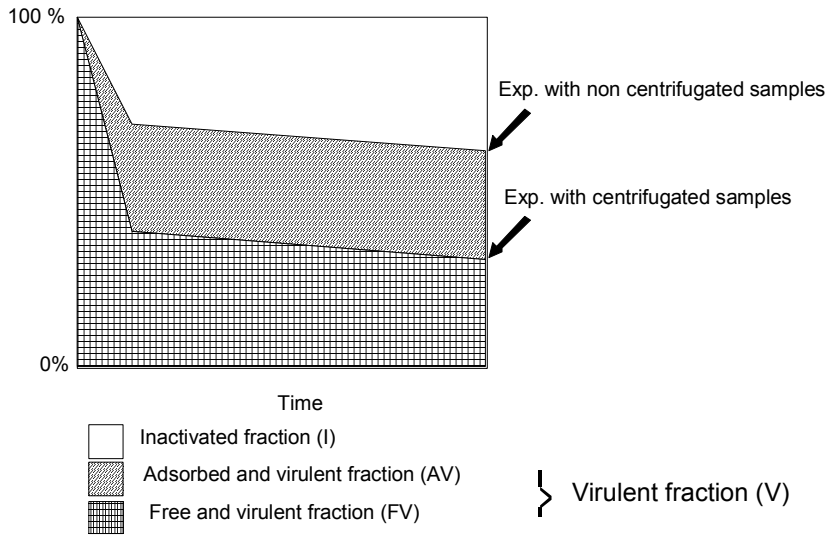


Figure 4.3.4: The various fractions of a phage population in the presence of suspended clay particles. Ideal case of QEARI-type kinetics, with phage concentrations given in percent.

For the experiments presented here, reference clays free of impurities were used. The phages were produced in the laboratory and also purified, just as were all the other compounds used. The experimental conditions are therefore very different from those of a natural aquifer. According to Stotzky, [1986] "*In situ, numerous organic and inorganic molecules, both monomers and polymers, may be simultaneously present in the solution. Competition for adsorption sites on the clays will occur not only among the molecules, but also between molecules and viruses*".

The adsorption of the bacteriophages onto the colloidal clay particles is very rapid. With concentrations varying between 0.025 and 0.050 mg/ml, a QEA dynamic equilibrium was generally reached after only 10 minutes. Shaking the samples, and thereby homogenizing the suspended particles, favors the equilibrium. Furthermore, agitation makes it possible to detect the decrease in inactivation by adsorption. Each experiment with phage T7 was performed at least twice. The homology of the kinetics of theses experiments was in general so important (>95%), that we were satisfied with two trials. However, when necessary, a third or even more trials were carried out.

A. Behavior of T7 in the presence of Ca-Montmorillonite:

The Montmorillonite used came from Texas and was obtained at the "Source Clay Minerals Repository" of the University of Missouri. It is homo-ionic in Ca^{2+} ions. The colloidal particles are suspended in SF medium as described in chapter 4.2. Quantities varying from 0.010 mg/ml to 0.100 mg/ml were used. One curve is obtained by a simple experiment, taking 17 samples (51 Petri dishes). Since most experiments were repeated, each of the figures, which contain 3 curves, are the result of at least 6 experiments (306 Petri dishes).

In graph 4.10a one sees that the dynamic equilibrium (QEA) is rapidly established (after 10

minutes, with 0.025 mg/ml of clay). Then, the number of phages decreases slightly, an inactivation due to the physico-chemical conditions of the environment, i.e. the agitation. With double the amount of particles (0.050 mg/ml), the QEA is established practically immediately (see graph 4.10b). In less than two minutes the proportion of virulent phages (**V**) falls to below 80% of the original number. The number of free and virulent phages (**FV**) falls to 55% of this number. The speed of this reaction is astonishing. Our method of analysis is not fast enough to follow variations over time intervals of a few seconds. Furthermore our empirical formula cannot be applied in this case. The results of this second series of experiments must be taken with reservations.

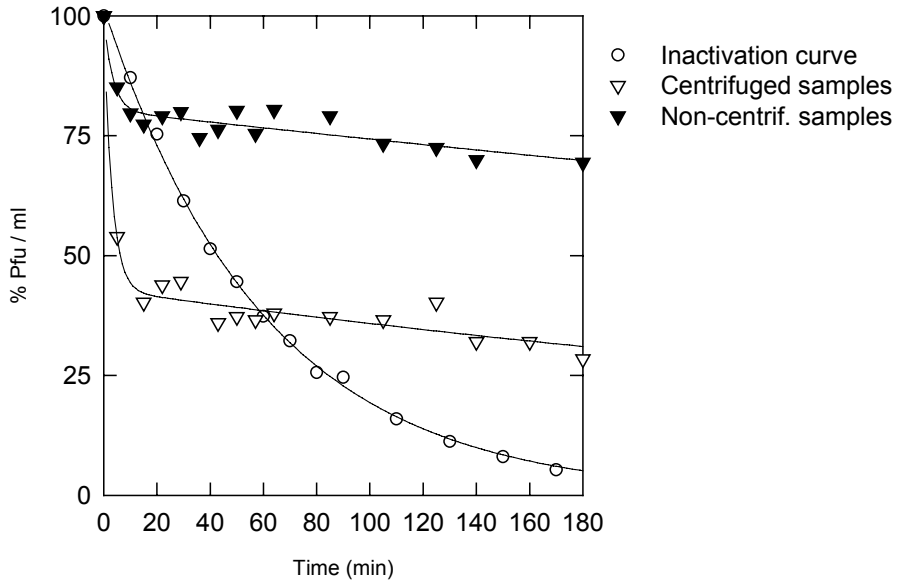


Figure 4.10a: Adsorption of phage T7 onto 0.025mg/ml of Ca-Montmorillonite

In graph 4.10a, the QEA is established after the same interval for centrifuged or non-centrifuged samples. The following decrease is identical, about 10% in 180 minutes. The fraction of adsorbed and virulent phages (**AV**) remains stable. The decrease of **V** is due to that of **FV**, the free and virulent phages. The decrease of **V** is much smaller than in the case of phages suspended in SF without clay (empty circles). The suspended particles offer considerable protection and the resulting kinetics are of QEARI type.

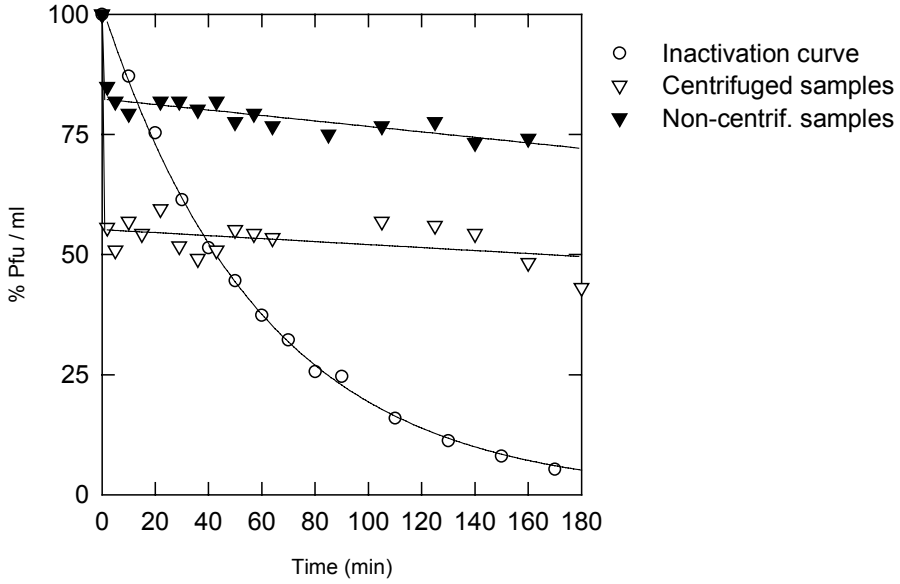


Figure 4.10b: Adsorption of phage T7 onto 0.050mg/ml of Ca-Montmorillonite

It is difficult to explain why the amount of virulent phages decreases rapidly before the establishment of the QEA. Further experiments (graphs 4.14a and 4.15a) show that when the medium is saturated with a protein whose pI is similar to T7's (BSA), not more than 90% of the initial number of phages are desorbed. Part of the phages must thus be inactivated during the establishment of the QEA, due to rapid adsorption-desorption phenomena. A very small fraction of the viral particles may also be definitively adsorbed.

The table below summarizes the different fractions of T7's population in agitated SF medium. These values were determined **graphically** on graphs 4.10a and 4.10b, using equation (2). According to our experience, the relative error margin can be estimated at +/-5%.

Ca-M (mg/ml)	Relative concentrations after 10 min.			Relative concentrations after 180 min.		
	FV phages	AV phages	I phages	FV phages	AV phages	I phages
0.025	44%	36%	20%	32%	38%	30%
0.050	(56%)	(26%)	(18%)	(49%)	(23%)	(28%)

Table 4.3.1: Relative concentrations of the various fractions of the population of T7 after 10 and 180 minutes. **FV**: free and virulent phages; **AV**: adsorbed and virulent phages; **I**: inactivates phages.

B. Behavior of phage T7 in the presence of Attapulgite:

This clay, also called Paligorskite, was also obtained from the "Source Clay Minerals Repository" of the University of Missouri. It comes from Florida. The colloidal particles are prepared as above (chapter 4.2). Figures 4.11a and b show the results of the experiments performed with Attapulgite. T7's behavior is totally different than with Montmorillonite. With almost the same quantity of material (0.032 mg/ml), far more phages adsorb than on the Montmorillonite particles. In graph 4.11a, more than 80% of the phages were adsorbed in less than 10 minutes. But despite this, an important fraction of the population remains virulent (80%). This is a typical case of QEARI.

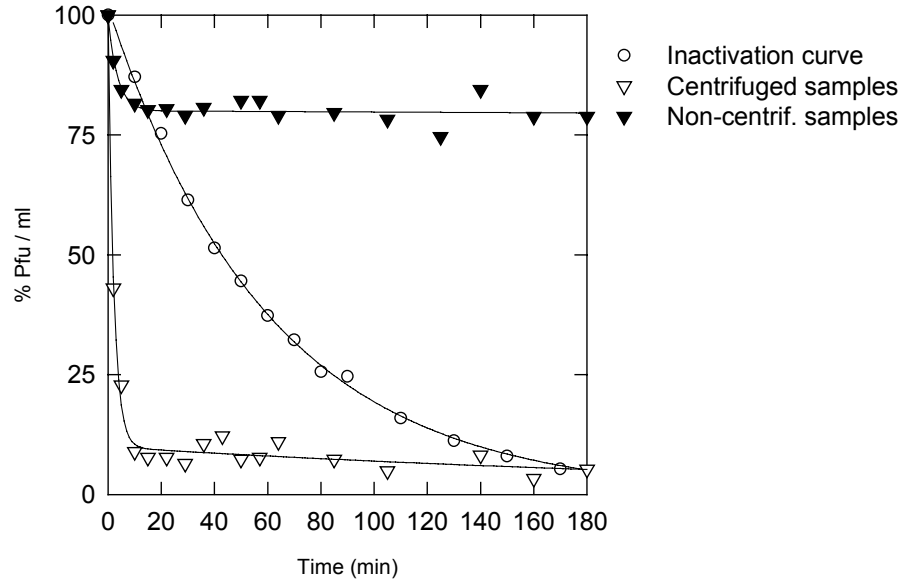


Figure 4.11a: Adsorption of phage T7 onto 0.032mg/ml of Attapulgite

With 0.032 mg/ml (graph 4.11a), QEA is established after only a few minutes. In the second phase the **V** and **FV** fractions have the same evolution. Their is practically no further inactivation, despite the agitation. The bacteriophage is better protected by the Attapulgite than by the Montmorillonite. Just as before however, the establishment of the QEA results in the inactivation of *circa* 20% of the initial population of phages.

In graph 4.11b, one sees that a concentration of clay ten times lower influences the speed at which the QEA is established. It takes about 80 minutes for the equilibrium to be reached. During this time the agitation causes an important part of the phage population to be inactivated. Once the QEA is reached, the Attapulgite offers a good protection, even at such a low concentration. It is remarkable that such a low concentration of colloidal particles can play such an important effect on the survival of a phage population.

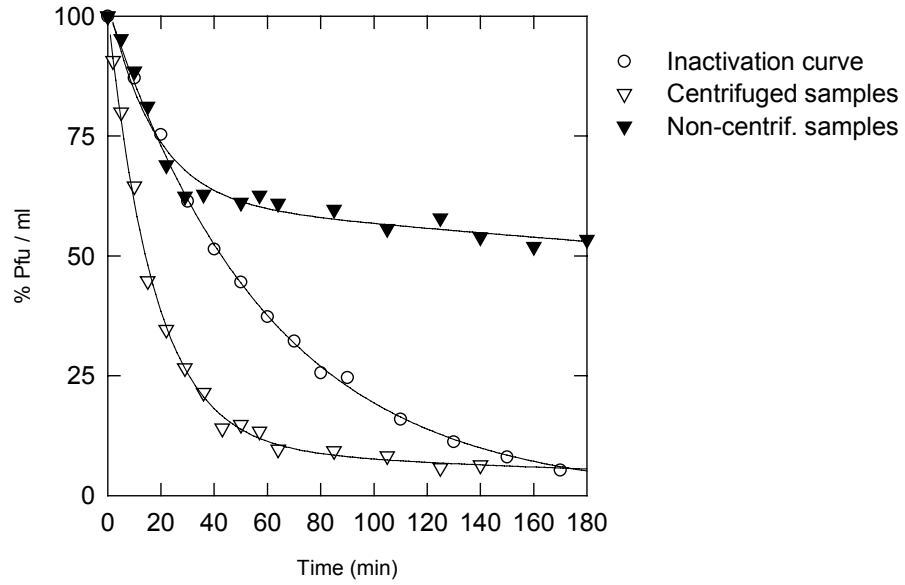


Figure 4.11b: Adsorption of phage T7 onto 0.0032mg/ml of Attapulgite

A (mg/ml)	Relative concentrations after 10 min.			Relative concentrations after 180 min.		
	FV phages	AV phages	I phages	FV phages	AV phages	I phages
0.032	12%	70%	18%	8%	72%	20%
0.0032	65%	22%	13%	8%	48%	54%

Table 4.3.2: see table 4.3.1 for details

C. Effect of certain physico-chemical parameters on the adsorption of phage T7: ionic concentrations and pH:

Bivalent cations play an important role in adsorption mechanisms. Graph 4.12 shows the results of a series of adsorption experiments, using 0.025 mg/ml of Montmorillonite colloidal particles in SF, with or without Mg^{2+} and Ca^{2+} (2.5 and 25 mM respectively).The normal kinetics of the centrifuged samples (full circles) corresponds to that of graph 4.10a. The **FV** fraction decreases rapidly to less than 45% till the QEA is established. This fraction then decreases slightly, till it corresponds to about 30% of the initial amount of phages, after 180 minutes. In the presence of bivalent cations, the same time interval is necessary for the QEA to establish itself. However fraction **FV** is

considerably reduced. Only about 5% of free and virulent phages remain after 10 minutes. The **V** fraction is not influenced: its kinetics are almost identical to those obtained without Mg^{2+} and Ca^{2+}. According to Stotzky [1986] *"When net negatively charged biological entities are involved* (as for T7 at pH=7.4)*, surface interactions between these entities and clay are usually greater when the valence of the exchangeable cation is higher or when its concentration increases. This enhancement is usually primarily the result of the reduction of the extent of the diffuse double layer (i.e. of the net negative charge) of the clays, which enables the clays and the biological entities to approach each other more closely"*.
Graph 4.12 shows that no loss of virulence occurs. The **AV** fraction increases because the adsorption sites are more accessible. The **FV** fraction decreases accordingly. Paradoxically, the QEA is not established faster. Only the ratio between the **FV** and **AV** fractions is strongly displaced by the increase of the ionic strength of the medium.

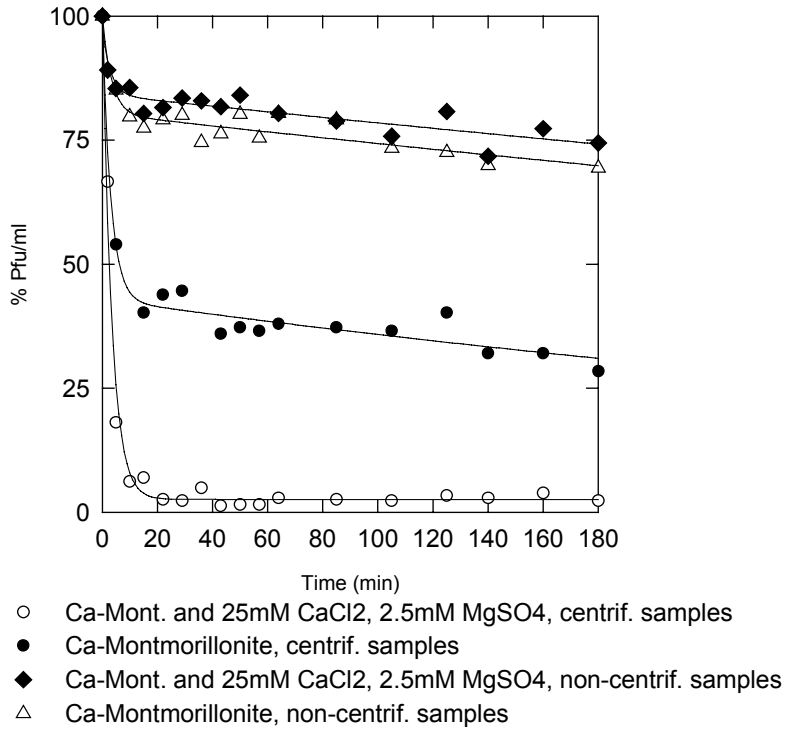


Figure 4.12: Influence of Ca and Mg cations on the adsorption of phage T7 onto Ca-Montmorillonite

The pH of the environment also plays an important role in adsorption reactions. The colloidal particles are amphoteric. Their net charge will depend on the concentration of H^+ ions in the environment. If the pH is below the isoelectric point of the virus, its capsid will be protonated and its net charge will be positive. This does not mean, however, that it possesses no negative charges. If the pH is higher than the phage's pI, the number of positive charges on its capsid will gradually decrease. At the pHs normally encountered in aquifers, the bacteriophages tested all have a net negative charge.

We determined the influence of pH on the adsorption of T7 onto Montmorillonite particles (0.025 mg/ml). Graph 4.13 shows the results of a series of experiments performed at pH 4 to 9. The buffers were used at concentrations of 25 mM (Tris for pH 9, 7.4 and 5.5; K hydrogenophtalate for pH 4). The samples were centrifuged. We therefore only measured the **FV** fraction of phages.

Contrary to what happened in SF medium only, the adsorption of T7 varies strongly depending on the pH. The kinetics show that the **FV** fraction decreases with the pH of the medium. At pH=4, close to T7's isoelectric point, the adsorption is maximal. No free phages remain after only 2

minutes! At this pH the phages certainly adsorb both onto the negatively charged and onto the positively charged sites of the clay particles. As the number of available sites is higher, adsorption is massive and very fast [Stotzky, 1986]. At pH = 5.5, the net charge of the viral particle is negative. However the proportion of free and virulent phages still decreases strongly. The speed at which the QEA is reached and the second phase of the kinetics are similar to those at pH = 7.4. The adsorption rate is lowest at this pH. At pH = 9, the adsorption is again a little higher. The second phase shows a slightly higher inactivation rate.

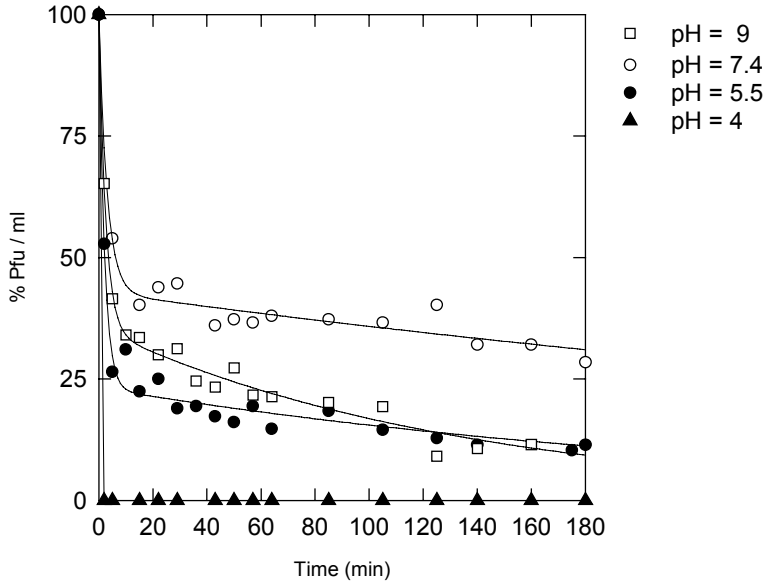


Figure 4.13: Influence of the pH on the adsorption of phage T7 onto Ca-Montmorillonite (0.025mg/ml). Centrifuged samples.

D. Influence of proteins of different pI on the adsorption of phage T7 onto colloidal clay particles:

The kinetics of these experiments are shown in graphs 4.14a and b, and 4.15a and b. The samples of all these experiments were centrifuged. We first tested lysozyme. This protein, MW = 14,600, has a pI of 11. It therefore has a strong positive charge at pHs around 7. The second protein we tested was BSA. Bigger in size (MW = 67,000), its isoelectric point is close to that of phage T7. We tested these two proteins at the same concentration, 1 mg/ml. They were added to the SF medium containing the phages and colloidal particles after 20 minutes, long enough for the QEA to have been reached.

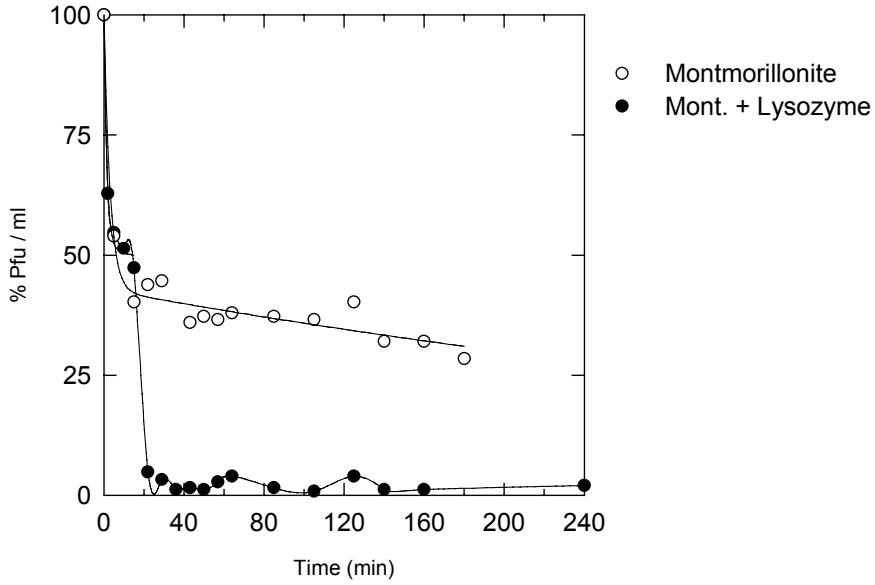


Figure 4.14a: Effect of lysozyme at 1mg/ml (added after 20 min. of experiment) on the adsorption behavior of phage T7 onto Ca-Montmorillonite (0.025mg/ml). Samples centrifuged.

With Montmorillonite, the adjunction of lysozyme causes bacteriophage adsorption to increase massively (figure 4.14a). In less than 5 minutes the **FV** fraction goes from 45% to less than 10%. The lysozyme's charge is opposite to that of T7. At high concentrations it will adsorb massively onto the negative sites of the clay, where it does not compete with T7's adsorption onto the positive sites. The overall net negativity of the clay particle is reduced, thereby facilitating the approach of the positively charged bacteriophages. The probability of interaction with the positive sites is greater. The phage therefore adsorbs massively and very fast.

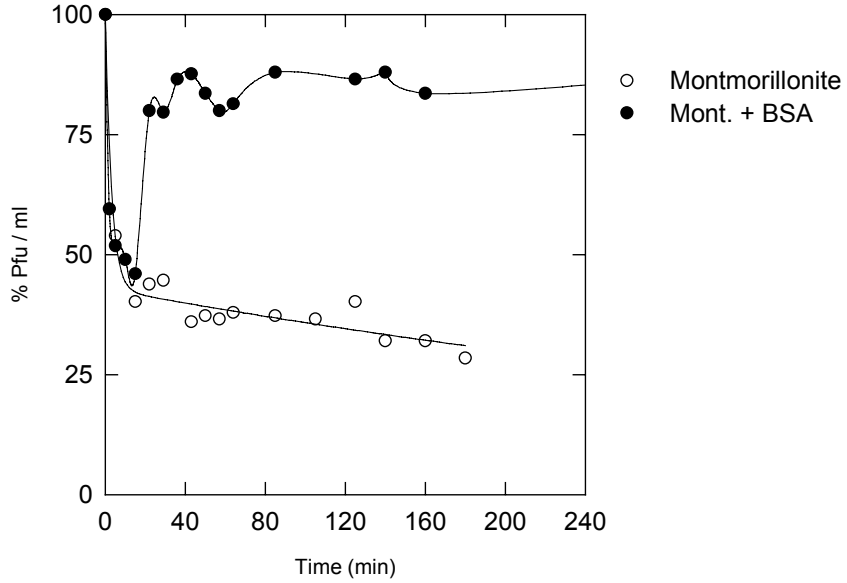


Figure 4.14b: Effect of BSA at 1mg/ml (added after 20 min. of experiment) on the adsorption behavior of phage T7 onto Ca-Montmorillonite (0.025mg/ml). Samples centrifuged.

In graph 4.15b we have the opposite case. BSA is negatively charged and competes with the phages for the same sites. The dynamic equilibrium established before between the phages and the clay particles is broken. The high concentration of protein causes the desorption of a very large part of the previously adsorbed phages. The **FV** fraction suddenly goes from about 45% to over 80%. All this happens extremely rapidly, in the course of a few minutes. The **FV** fraction of T7 then remains constant, despite the agitation. As in preceding experiments (graph 4.3), the protein saturates the hydrophilic/hydrophobic interfaces of the medium. The phages are excluded from them and therefore protected from inactivation.

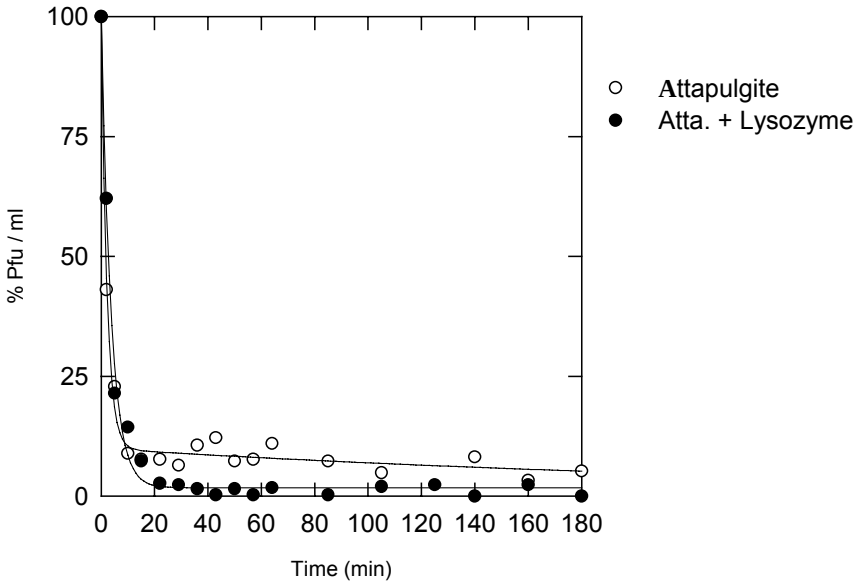


Figure 4.15a: Effect of lysozyme at 1mg/ml (added after 20 min. of experiment) on the adsorption behavior of phage T7 onto Attapulgite (0.030mg/ml). Samples centrifuged.

The same experiments were performed in the presence of Attapulgite (4.15a and b). With lysozyme, adsorption is increased. The very small **FV** is reduced even more, and stabilizes at a few percent. With BSA, a large number of phages are desorbed. **FV** increases to about 80% of the initial quantity, and then decreases slowly.

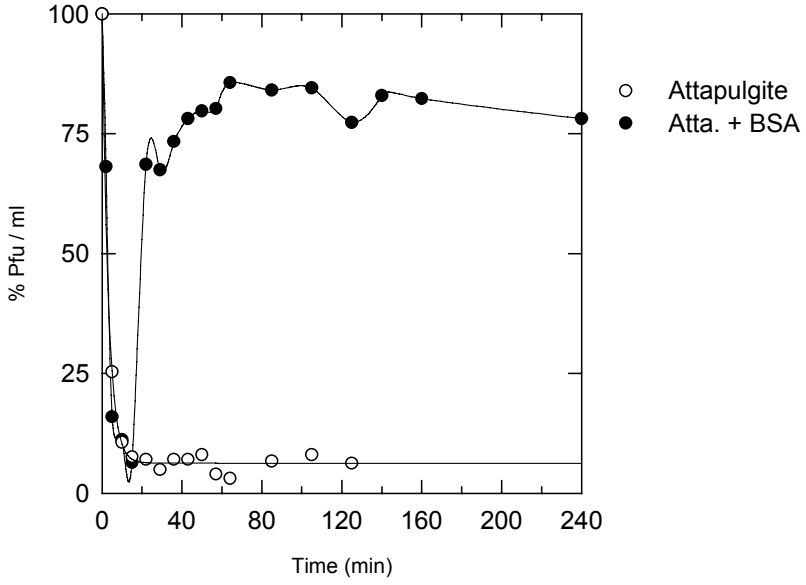
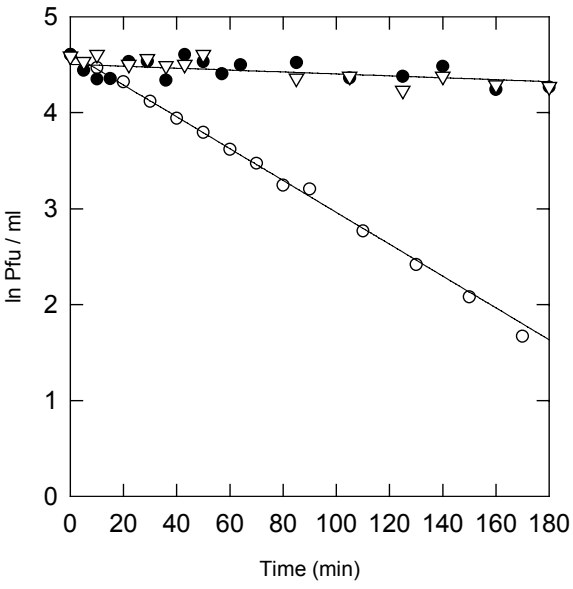


Figure 4.15b: Effec e adsorption behavior of phage T

E. Inactivation of p es:

Graph 4.15c shows vior of phage T7, after it has been tact with Ca-Montmorillonite (0.4 centrifuged (as described in sec ed and used for an inactivation e peated. The second time, the su 10 min., SS-34 Sorvall rotor). Th



§4. Comparative study o

Figure 4.15c: Phage T7 inactivation curves. Influence on a previous contact between phages and Ca-Montmorillonite. See text for details.

As is obvious on graph 4.15c, the inactivation rate of the phages put in contact with Ca-Montmorillonite is very small. It corresponds to that in the absence of agitation. Two explanations can be given for this phenomenon. First, one can suppose that the phages react with extremely fine clay particles which then were not precipitated by the centrifugation. These are certainly smaller than the phages [Stotzky, 1986]. These particles of unknown nature adsorb and reinforce the phage's proteic structure. At least, they prevent the phages accumulating at the hydrophilic/hydrophobic interfaces and blocks their degradation. The second hypothesis is based on the supposed heterogeneity of the phage's population. Yin and McCaskill [1993] showed that the mutants are formed inside single lysis plaque. Information non-essential for the multiplication on solid medium is modified and/or lost. A phage population therefore consists of a multitude of genetic variants. Most probably these variants also appear when the phage multiplies in a liquid medium.

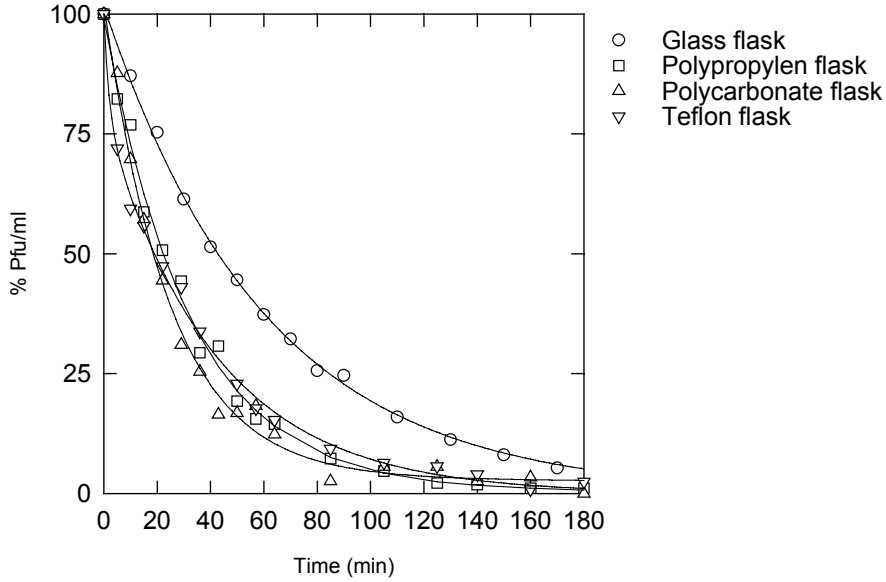


Figure 4.15d: Inactivation rates of phage T7 in different containers

Very often, however, a mutation is unfavorable. The constraints imposed on the tri-dimensional structure and reconnaissance sites are so great that it is unlikely that even a minor modification of the capsid appear. Several authors [among whom Adams, 1949] used this hypothesis to explain the biphasic shape of inactivation curves. They affirm that a phage population is composed of two sub-populations that have a different resistance to inactivation. A review of the techniques used by these authors [Grant et al., 1993], shows that they generally used plastic vessels for their experiments. Figure 4.15d shows inactivation experiments performed first in a glass vessel, than in three different plastic flasks (polypropylene, polycarbonate and Teflon). In the glass flask, the inactivation curve decreases exponentially. The three other curves, however, obviously do not. The weak interactions between the phages and the hydrophobic walls of the flask are enough to induce a biphasic inactivation, which is therefore not due to the existence of two sub-populations. The first explanation seems the most plausible. It seems also improbable that only two sub-populations coexist in a phage suspension. Variants certainly exist, whose physico-chemical characteristics have been modified by genetic mutations. But it is certainly not the case here for phage T7.

4.3.7 Inactivation and adsorption test for some bacteriophages:

A. Bacteriophage f1:

The inactivation kinetics of this phage are similar to those of T7. They follow the general law of exponential decrease (equation (1)). The inactivation rate, **K**, is equal to $1.28*10^{-2}$ [min^{-1}]. These kinetics are shown in figures 4.16a and b. The table below summarises the values of the various fractions of the population of f1 during adsorption experiments. These values were calculated graphically, using the regression equation (2).

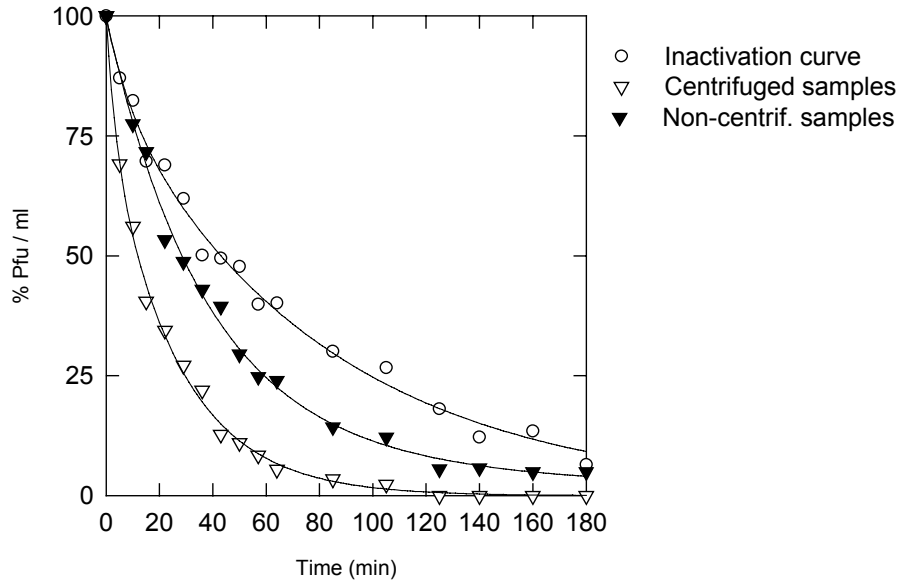


Figure 4.16a: Adsorption of phage f1 onto 0.0032mg/ml of Attapulgite particles

As one can see on graphs 4.16a and b, the presence of Attapulgite accelerates the inactivation of the phage. The virulent population decreases strongly, even with a very low concentration of clay (0.0032 mg/ml). In this case (graph 4.16a), the **V** fraction falls to 76% after 10 minutes and to only 5% after 180 minutes. After that time, there are no free and virulent phages in the suspension (fraction **FV**).

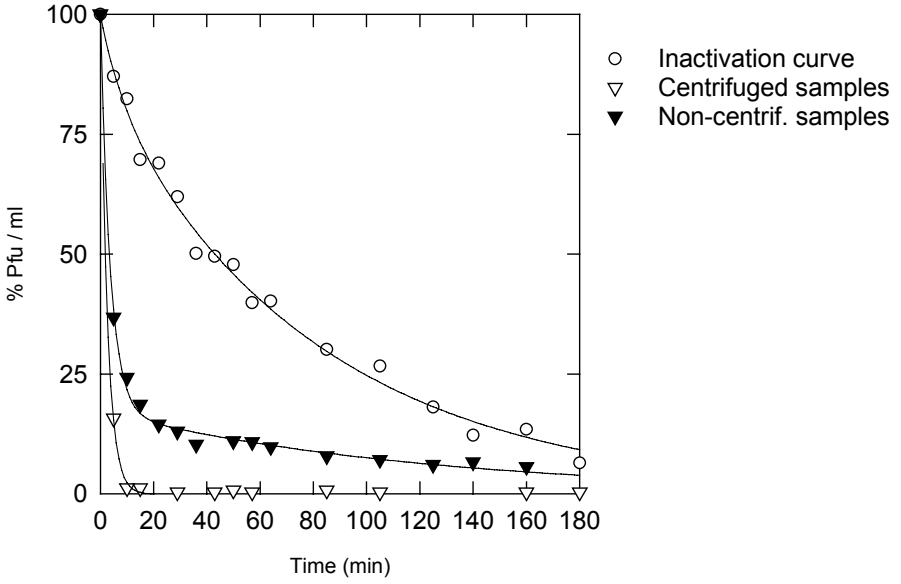


Figure 4.16b: Adsorption of phage f1 onto 0.032mg/ml of Attapulgite particles

Graph 4.16b illustrates experiments performed with ten times more colloidal clay particles. The QEA is reached much faster than before. But, as one can see in table 4.3.3, the various fractions of the population after 180 minutes are identical for both amounts of clay (0.0032 mg/ml and 0.032 mg/ml). Phage f1 adsorbs massively onto this type of particle. Unlike phage T7, most of the adsorbed phages lose their virulence. In the model proposed by Grant et al., this is a case of QEASS type: the inactivation of the adsorbed phage is stronger than that of the free one. This acceleration could be due to the phage's morphology.

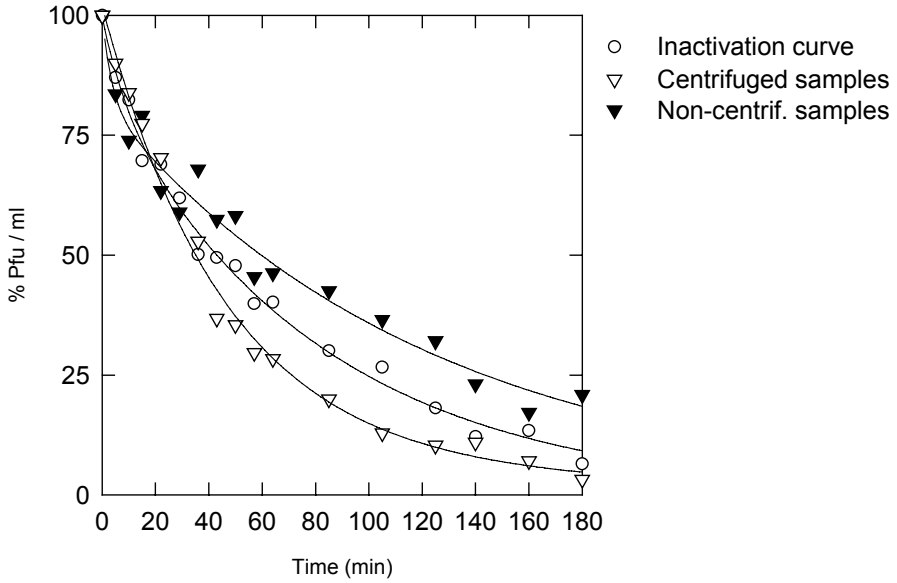


Figure 4.17: Adsorption of phage f1 onto 0.025 mg/ml of Ca-Montmorillonite particles

Phage f1 is the only filamentous virus that was selected. Its dimensions are unusual: its diameter is one hundred times smaller than its length. Unlike the polyhedral phages, this virus certainly adsorbs by its whole lenght onto the colloidal particles. The electrostatic bonding between the phage's capsid and the clay particle is certainly very strong. On the other hand, this phage reacts very little with Montmorillonite. Very few phages are adsorbed. The kinetics of the **V** fraction shows a slight protection, of the QEARI type.

Clay particles (mg/ml)	Relative concentrations after 10 min.			Relative concentrations after 180 min.		
	FV phages	AV phages	I phages	FV phages	AV phages	I phages
A: 0.032	1%	21%	78%	0%	5%	95%
A: 0.0032	52%	24%	24%	0%	5%	95%
M: 0.025	77%	8%	15%	5%	13%	82%

Table 4.3.3 : Results of phage f1 adsorption experiments on Montmorillonite (**M**) and Attapulgite (**A**) colloidal particles. **FV**: free (e.g. unadsorbed) and virulent fraction; **AV**: adsorbed and virulent fraction; **I**: inactivated fraction.

B. Bacteriophage Psf2:

Bacteriophage Psf2 (of *Pseudomonas fluorescens*) has similar adsorption kinetics on Attapulgite and Montmorillonite (figures 4.18a and b). In the first case (4.18a), adsorption is very small (less than 10%). The adsorbed phages are not protected from inactivation, since the kinetics of the **V** fraction are similar to the "blank" inactivation curve. This is a QEA-type mechanism. The dynamic equilibrium that is reached does not advantage or disadvantage the phages. The inactivation of the adsorbed phages is equal to that of the freely suspended ones.
With Attapulgite, the particles offer slightly better protection to the phages. This a borderline QEARI case. But again, very little bonding takes place between the phages and the colloidal particles.

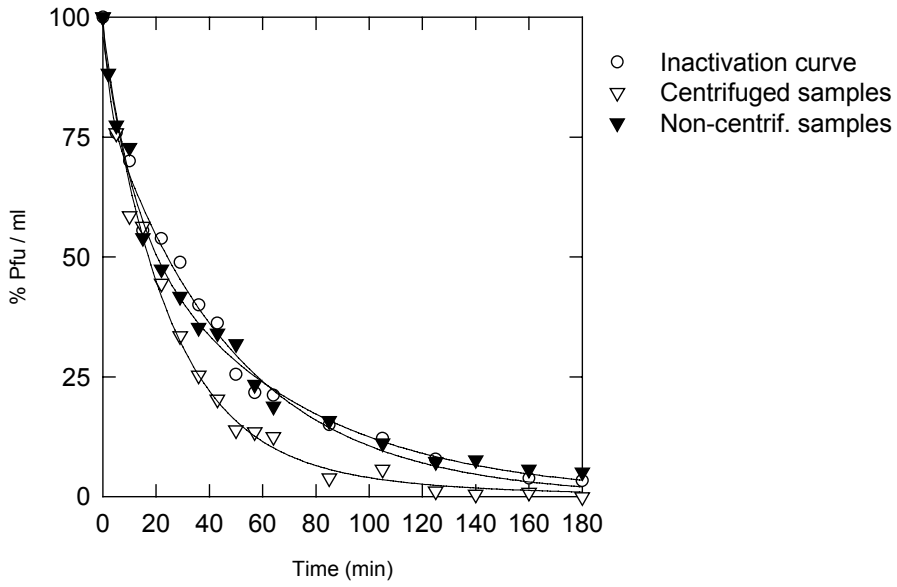


Figure 4.18a: Adsorption of phage PSF2 onto 0.025mg/ml Ca-Montmorillonite particles

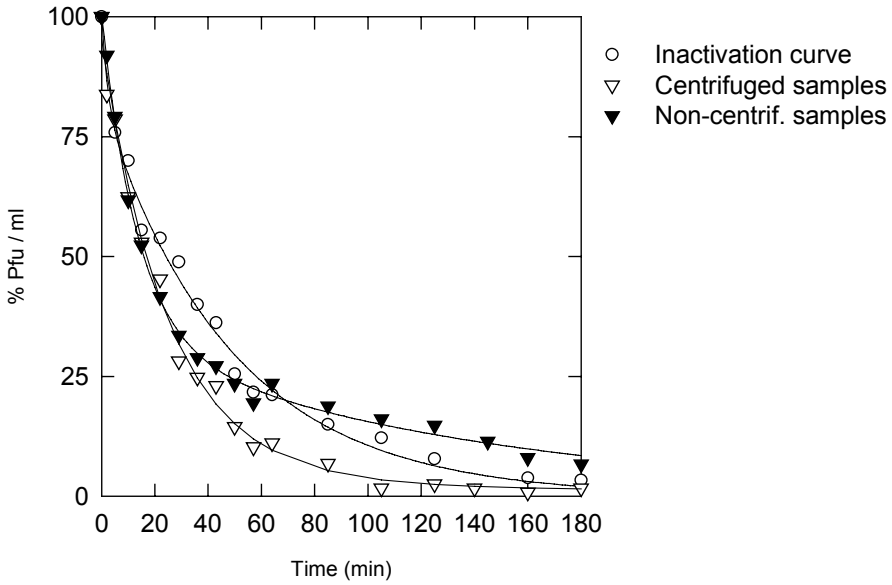


Figure 4.18b: Adsorption of phage PSF2 onto 0.032mg/ml Attapulgite particles

PSF2 phage inactivation kinetics are very similar to T7's. **K** is worth $1.59*10^{-2}$ [min]. Table 4.3.4 gives the values of the various fractions of phage Psf2's population after 10 and 180 minutes.

Clay particles (mg/ml)	Relative concentrations after 10 min.			Relative concentrations after 180 min.		
	FV phages	AV phages	I phages	FV phages	AV phages	I phages
M: 0.025	65%	2%	33%	1%	2%	97%
A: 0.032	62%	0%	48%	2%	6%	92%

Table 4.3.4: **A**: Attapulgite; **M**: Montmorillonite; **FV**: free (e.g. unadsorbed) and virulent fraction; **AV**: adsorbed and virulent fraction; **I**: inactivated fraction.

C. Bacteriophage Sfd3:

The results are presented in figures 4.19a and b for this phage's kinetics (**K** = $1.49*10^{-2}$ [min^{-1}]). In the presence of Montmorillonite (4.19a), the behavior of phage Sfd3 is of the QEARI type. The **V** fraction decreases strongly till the dynamic equilibrium is reached. The evolution of the **FV** fraction parallels that of **V**. Fraction **AV** remains constant throughout the experiment. **I** increases at the expense of **FV**. With Attapulgite, the adsorption is stronger. **FV** decreases rapidly and then stabilizes at a few percent. The **V** fraction follows a biphasic evolution of the QEARI type. As shown by the second part of the curve, the protection offered by the Attapulgite particles is almost identical to that of the Montmorillonite.

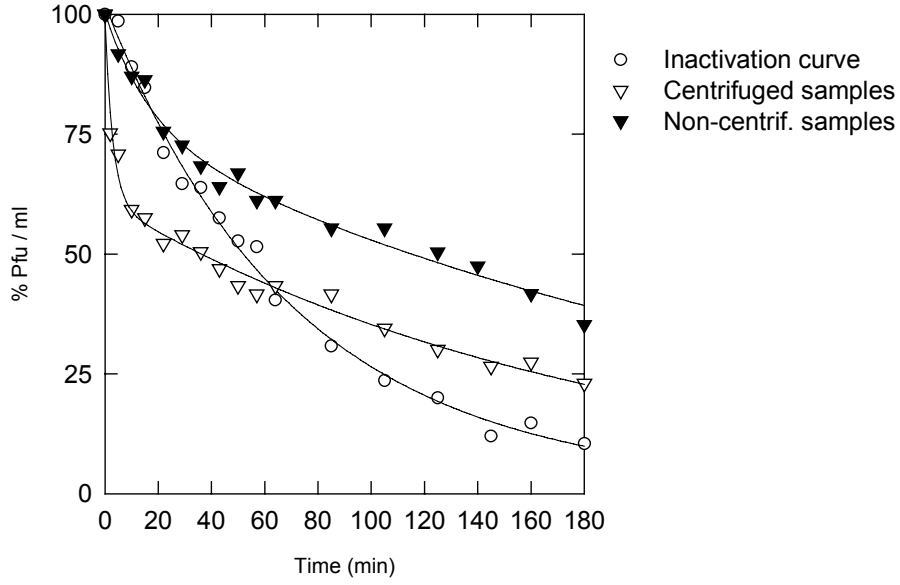


Figure 4.19a: Adsorption of phage SFD3 onto 0.025 mg/ml of Ca-Montmorillonite particles

Clay particles (mg/ml)	Relative concentrations after 10 min.			Relative concentrations after 180 min.		
	FV phages	AV phages	I phages	FV phages	AV phages	I phages
M: 0.025	60%	27%	13%	23%	17%	60%
A: 0.032	28%	37%	35%	3%	24%	73%

Table 4.3.5 **A**: Attapulgite; **M**: Montmorillonite; **FV**: free (e.g. unadsorbed) and virulent fraction; **AV**: adsorbed and virulent fraction; **I**: inactivated fraction.

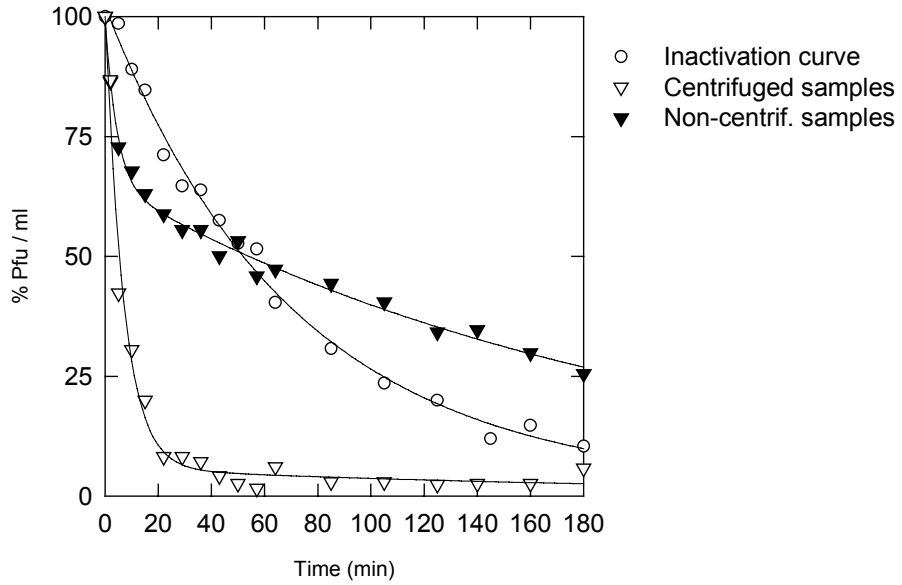


Figure 4.19b: Adsorption of phage SFD3 onto 0.030 mg/ml of Attapulgite particles

D. Bacteriophage H40/1:

The **K** value for this phage is very similar to that of the other phages examined: **K** = $1.21*10^{-2}$ [min^{-1}]. Graphs 4.20a and b show the adsorption kinetics of this marine phage on the two types of colloidal clay particles. With the Montmorillonite, the reaction is similar to that of phage Psf2. H40/1 only reacts very slightly to the presence of the clay particles. The kinetics of the centrifuged samples (FV fraction) are almost identical to the inactivation curve (homology >99.5%). The kinetics of the **V** fraction indicate a slight protection of the population. The **AV** fraction remains very small throughout the experiment. This is clearly a QEA-type mechanism.

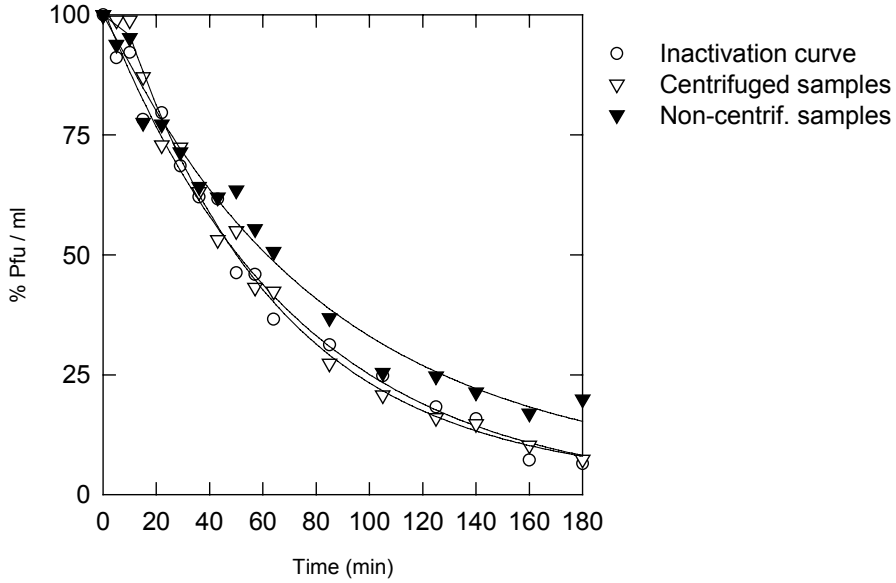


Figure 4.20a: Adsorption of phage H40/1 onto 0.025 mg/ml of Ca-Montmorillonite particles

The second series shows the phage's reaction to Attapulgite. It is much stronger: the phages adsorb massively and very fast. The curve is biphasic, of the QEARI type. The dynamic equilibrium is reached after 10 minutes. In the establishment of the equilibrium about 25% of the **V** fraction are lost. The **FV** fraction stabilises more slowly. These colloidal clay particles offer a strong protection to the phages. **V** only decreases by 12% between 10 and 180 minutes.

Clay particles (mg/ml)	Relative concentrations after 10 min.			Relative concentrations after 180 min.		
	FV phages	AV phages	I phages	FV phages	AV phages	I phages
M: 0.025	90%	6%	4%	15%	7%	85%
A: 0.032	66%	9%	25%	8%	55%	37%

Table 4.3.6: **A**: Attapulgite; **M**: Montmorillonite; **FV**: free (e.g. unadsorbed) and virulent fraction; **AV**: adsorbed and virulent fraction; **I**: inactivated fraction.

E. Bacteriophage H6/1:

The inactivation rate of H6/1 is the lowest of all the phages tested. **K** equals $6.92*10^{-4}$ [min^{-1}]. In 180 minutes, less than 20% of the phages were inactivated. Adding Montmorillonite (graph 4.21a) causes **V** to fall by about 10% in the first minutes. The rest of the kinetics is then similar to the inactivation curve, since the speed of inactivation is not influenced.

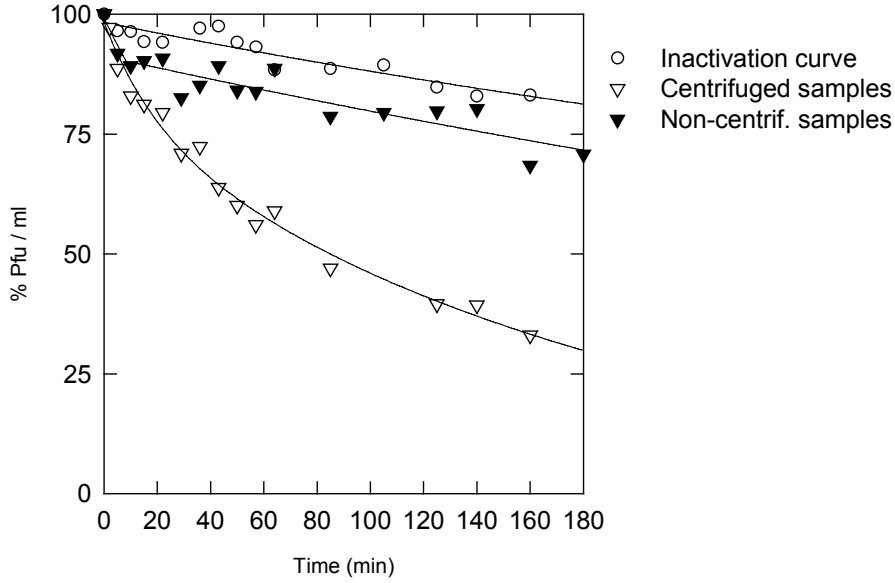


Figure 4.21a: Adsorption of phage H6/1 onto 0.025 mg/ml of Ca-Montmorillonite particles

According to Grant's model, the **V** fraction follows a QEA-type reaction. The bacteriophages only react very slightly with the adsorbent substrate. The **FV** fraction has a different behavior: it decreases more rapidly, without however influencing the **V** fraction. With Attapulgite (graph 4.21b), the adsorption kinetics also correspond to the QEA type. The **V** fraction of H6/1 decreases by about 20% in the first minutes of the experiment. The second part of the curve then parallels the inactivation curve. **FV** decreases more strongly than with Montmorillonite. After 60 minutes it reaches 5%, and then remains stable till the end. Just as for Sfd3, a small fraction of the phages remain free and do not adsorb onto the particles.

Clay particles (mg/ml)	Relative concentrations after 10 min.			Relative concentrations after 180 min.		
	FV phages	AV phages	I phages	FV phages	AV phages	I phages
M: 0.025	86%	2%	12%	30%	42%	28%
A: 0.032	52%	28%	20%	5%	48%	47%

Table 4.3.7: **A**: Attapulgite; **M**: Montmorillonite; **FV**: free (e.g. unadsorbed) and virulent fraction; **AV**: adsorbed and virulent fraction; **I**: inactivated fraction.

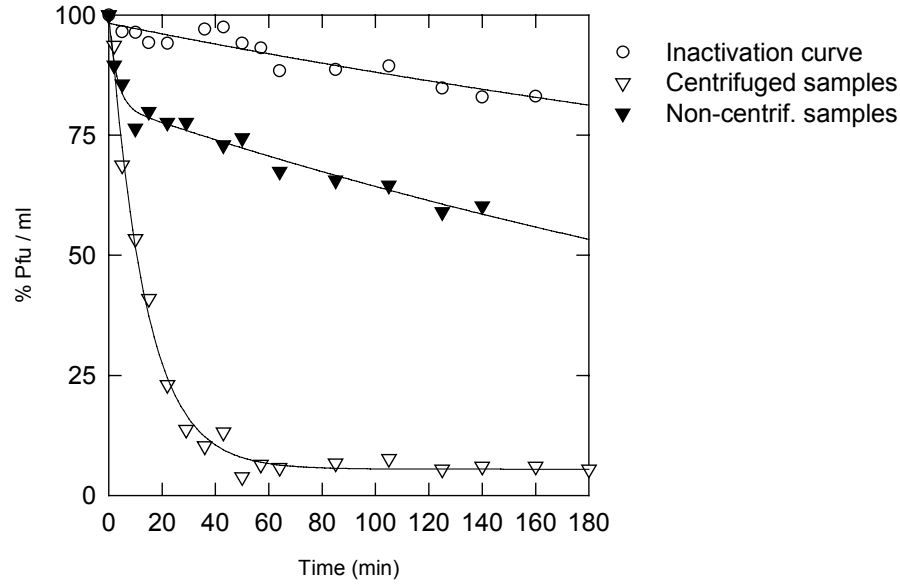


Figure 4.21b: Adsorption of phage H6/1 onto 0.032 mg/ml Attapulgite particles

4.3.8 Synthesis of results:

A. Inactivation:

If one excepts phage H6/1, the behavior of the five other bacteriophages is almost identical. After 180 minutes, between 2 and 10% of the initial virulent population (**V**) are left. Agitation, as we showed at the beginning of this chapter, is the reason of this brutal decrease. This decrease will also depend on physical factors, such as temperature. But it is independent of the pH and the ionic concentration. Furthermore, the inactivation of the phages will be strongly influenced by the presence of suspended material of organic (proteins, polypeptides and humic acids) or inorganic origin.

Bacteriophages	K [min-1]	V180 [Pfu/ml]
T7	1.59*10-2	5.8
f1	1.38*10-2	9.2
Psf2	1.59*10-2	2.1
Sfd3	1.49*10-2	9.9
H40/1	1.21*10-2	8.2
H6/1	6.92*10-4	81.3

Table 4.3.8: **K** values (inactivation rate) for each tested phages. **K**: inactivation rate, in minutes-1; **V180**: fraction of phages still virulent after 180 minutes under agitation.

The hydrophobicity values and the Zeta potential at pH 7.4 of these five phages are comprised between 34.2 (Psf2) and 85.7° (f1), and -31.7 mV (T7) and -42.3 mV (f1) respectively. These viruses also have very different shapes and sizes. Since the diversity among these phages is great, it is impossible to correlate their physical characteristics with their value of **K**.

Phage H6/1 is the most resistant to inactivation by agitation. The hydrophobicity value of this phage is also the highest (90.6°) and its Zeta potential at pH 7.4 is the lowest (-50.1 mV). Since we have only this one example, it is too early to establish a correlation between one or other of these values and the phage's behavior. A complete analysis of the phage proteins and of the disposition of the capsomers, as well as further experiments are necessary to reveal the factor(s) responsible for its inactivation speed.

B. Biological inactivation:

Works concerning the inactivation of viruses in sea water are recent. They show that an important part is due to proteolytic enzymes of bacterial origin [Toranzo and Barja, 1983]. Börsheim [1993] also showed that in this environment flagellated protozoa significantly contribute to the inactivation of bacteriophages. However, similar studies have shown that this inactivation by bacteria or protozoa does not occur in spring waters (fresh water) [Fujioka et al., 1980]. We performed several inactivation of experiments in SF medium at 12°C, using a strain of *Pseudomonas fluorescens* and the marine strains *H6* and *H40*. We also tried using cell-free extracts obtained by sonicating bacterial cells. No increase of inactivation was observed. On the contrary, the cellular debris slowed the viral inactivation down (data not shown)! No trial was made with protozoa. Our results confirms those obtained by Fujioka et al. [1980] in the fresh-water springs of Hawaii. These factors certainly have a significant influence in salt water or in fresh water highly contaminated by industrial or domestic waste. But when tracing in spring waters, which usually contain very few micro-organisms, their effect is negligible. No indication of it was found during our tracing experiments. This is why we did not treat this subject and will not take it into account in the following discussion.

Theoretically, phages can also be biologically inactivated by aggregation (clumping). This can happen when several phages adsorb onto an organic particle (generally a fragment of bacterial membrane) or between phages alone. It is still a little-known phenomenon. We detected it by electron micrographs of non-purified phage suspensions (see photo of phage Sfd3, in annex 2.4). Several trials to detect these clumps by Petri dish counts were unsuccessful (by treating with CHCl3, by sonication, by filtration, etc.). We therefore concluded that the aggregates formed by the phages when the host cell burst did not influence the general virulence of the population. We suppose these aggregates are only temporary, and decompose in the presence of the host bacteria, setting the virulent phages free. No effect of clumping on the survival of phages was measured.

C. Adsorption:

Table 4.3.9 summarizes the various physical characteristics of the bacteriophages studied and their type of reaction with the two mineral colloidal particles. Colloidal clays such as Montmorillonite and Attapulgite are often used too study the survival of phages in natural environments [Bitton and Mitchell, 1974; Roper and Marshall, 1978; Lipson and Alsmadi, 1989]. Montmorillonite particles are sometimes present in great quantities in the aquifers around Neuchâtel. However, no Attapulgite is found in the karstic aquifers of the Jura [Prof. Kübler, pers. comm.]. This clay was chosen only as a comparison with the studies presented in the literature [Babich and Stotzky, 1980].

As we showed in our numerous adsorption experiments, the reaction of the phages with the adsorbent particles are very rapid. They take place in a few minutes. Each virus exhibits a specific behavior in the presence of the different colloids. As Moore at al. [1975] also remarked: "*The results indicate that no one of the viruses tested can be used to describe a general pattern of adsorption*". If we cannot describe the general behavior of phages, we can classify their reaction according to three of the types described by Grant et al. [1993].

If one considers the results of the tracing experiments presented in chapter 6, some interesting parallels appear, between the phage's type of behavior in laboratory, and the one "*in aquifero*". We will return to this in the final conclusions of this work.

Phages	Behavior Ca-Mont.		V_{180} Ca-Mont.	Behavior A	V_{180} A	Θ_a	Zeta potential	Morphotype	Size [nm]
T7	QEARI	70%	QEARI	80%	ND		-37.7 mV	C1	60 * 11
f1	QEARI	18%	QEASS	5%	85.7°		-42.3 mV	F1	100 * 6∅
Psf2	QEA	3%	QEARI	8%	34.2°		-36.0 mV	B1	ND
Sfd3	QEARI	23%	QEARI	12%	ND		ND	B3	78-82/30 * 105-110
H40/1	QEA	18%	QEARI	63%	68.7°		-42.0 2mV	B1	82-85 * 39-42
H6/1	QEA	72%	QEA	53%	90.6°		-50.1 mV	E1	30∅

Table4.3.9: V_{180} Ca-Mont: virulent fraction of phages in % after 180 minutes in contact with 0.025 mg/ml of Ca-Montmorillonite colloidal particles. V_{180} A: idem, but contact with 0.030mg/ml Attapulgite particles. Θ_a: hydrophobicity of the capsid. QEA, QEARI and QEASS: see beginning of chapter 4.

Phage f1 is the worst candidate of our selection. Its restitution levels, when they exist, are low. This phage is the only one with a type QEASS behavior. Adsorbed onto a mineral colloid, it looses a lot of its virulence. This loss is certainly due to the phage's filamentous morphology. This behavior was also found for another filamentous phage, f2, by Moore et al. [1975]. Bacteriophages T7 and Sfd3 have QEARI-type behaviors. They react strongly with mineral colloids. An important fraction of the phages is inactivated in the first minutes of the experiment (1st phase). After that, the adsorbed bacteriophages are protected from a rapid inactivation. The best candidates of our selection are undoubtedly Psf2, H40/1, and in a lesser degree, H6/1. Their restitution levels are always very high. These three phages present QEA or QEARI-type reactions. They react only slightly with the mineral particles. Or, if the reaction is strong, a great proportion of the phages remain virulent.

4.4 Conclusions

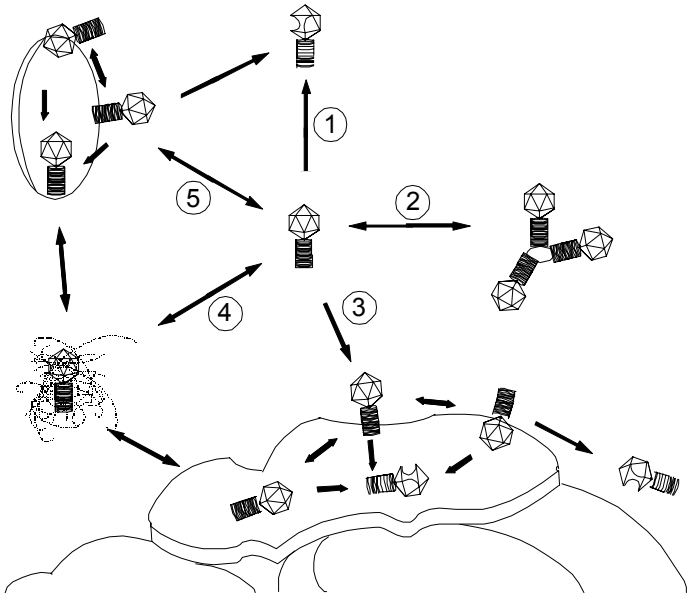
Our method of analysis of the behavior of bacteriophages in liquid medium makes rapid and reproducible inactivation and adsorption experiments possible. The inactivation experiments are performed in standard buffered medium, under constant agitation. The adsorption experiments are performed under the same conditions, in the presence of colloidal clay particles. With this material it is possible to follow the specific behavior of various fractions of a phage population with regard to a particular type of adsorbent. The inactivation kinetics show that five of the six phages studied react in an identical manner: more than 90% of the phages are inactivated after 180 minutes, following a law of exponential decrease. Phage H6/1 resists much better. This phage also has the highest Zeta potential and hydrophobicity values. However, the data in our possession is still too limited for it be possible to establish a link between these values and this phage behavior.

The adsorption experiments with phage T7 have shown that the virus adsorption sites are the positively charged sites of the clays. They certainly do not consist of interactions between the negative charges of the virus and the particle through a cationic bridge. The phage-particle bond is of an electrostatic nature [Stotzky, 1986], though H-bonds and hydrophobic interactions are also possible. As Grant et al. also showed, this bonding is not permanent. The viruses and the adsorption sites of the clay bond rapidly and establish a dynamic equilibrium, which will be strongly conditioned by certain physico-chemical parameters (pH, ionic concentrations) and by the presence of organic material. The same experiments performed with other phages show that each reacts individually (specifically) to a particular adsorbent. This different behavior can however be grouped into three categories, defined according to the theoretical model of Grant et al. Our experiments show that a phage can exhibit different reactions to according to the type of adsorbent.

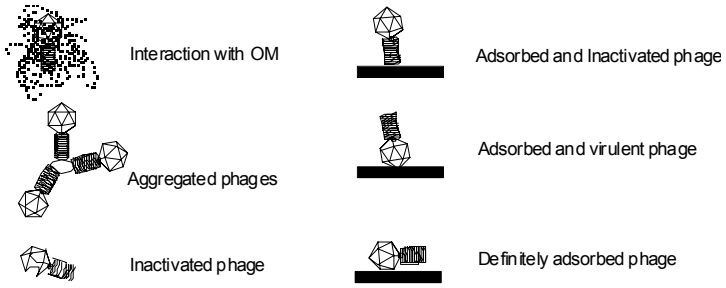
Following the selection performed in the preceding chapters (se chapter 2), we can now define the value of a bacteriophage as a potential tracer more precisely, depending on the results of a few tests of adsorption onto Montmorillonite particles. The phages that seem most interesting as biological tracers are those whose behavior is of the QEA type. The phages Psf2, H40/1 and H6/1 practically do not react with the mineral colloids tested. One should not forget, however, that laboratory experiments only give a very partial image of the behavior of bacteriophages in an aquifer. In a natural environment, one cannot dissociate inactivation and adsorption reactions, they are interdependent. In our experiments we use purified (clays and phages) and selected (organic compounds and proteins) components under standardized conditions. The experimental conditions are very far from those in a natural environment. Nothing allows us to assert that the sum of the influences of the various parameters tested in the laboratory allow the reconstitution of the general behavior of a virus in its natural environment. The results of our kinetic experiments are only aimed at estimating the capacities of our bacteriophages, in view of their possible selection as tracers. As our results have shown, we can at most predict the value of a virus as a biological tracer.

The behavior of viruses in aquifers is not only determined by physical factors. Temperature certainly does not play the major role that Yates et al. [1985] have assigned to it. This would only be the case in certain particular instances, such as transport in aquifers with very little suspended colloids. In general the presence of mineral and organic colloids influences the transport and survival of phages far more, than this one particular physical parameter. This shows that if one wants to study bacteriophage transport and survival in detail, one must follow not only the physical parameters of the aquifer, but also the type and quantity of suspended organic and mineral material. As we have just mentioned, each of the phages we tested reacted individually to the presence of mineral particles. This behavior does not seem to be linked to the physical characteristics measured (Zeta potential, hydrophobicity, shape and size). Other specific parameters must determine such a diversity of reactions (the composition of the capsid and the tail for example). Because of this it appears to us more and more that it is risky to simulate the

propagation of an eucaryotic virus with a bacteriophage, on the basis of what are still random suppositions. This confirms what Moore et al. [1975] stated: *"These data suggest that no one coliphage can acceptably model the behavior of the poliovirus in laboratory or field conditions*". We must at best satisfy ourselves with taking only as *example* the propagation and migration of the best bacteriophage available.



The above figure 4.4.1 summarizes the various types of reactions with which viruses in general may be confronted in aqueous environments. It does not claim to describe in detail all the possible interactions of the viruses with their environment.



We have shown only those which seem to us to most influence the behavior of viruses in aquifers. Not all the interactions symbolized by the arrows are described.

1. Inactivation: the phage loses its possibility of multiplication. By decomposing it enters the trophic chain.
2. Formation of aggregates: adsorption, we suppose is reversible, momentarily inactivates several bacteriophages.
3. Adsorption onto a fixed substrate: the phage leaves the mobile phase and adsorbs onto sedimented particles or onto the walls of the aquifer. This adsorption can be reversible or not, and can result in the temporary or definitive inactivation of the phage.
4. Interaction between a phage and organic macromolecules present in the water: this can inactivate the phage temporarily.
5. Adsorption onto colloidal particles: the adsorption is reversible, and the viruses generally remain virulent. The colloids can be organic or mineral. This adsorption keeps the phage in the dynamic aquatic phase. To repeat an expression dear to Prof. Kübler, it is similar to "the long-distance travel of a flea on the back of a dog".

§5.1 Comparative tracing experiments in a porous aquifer using bacteriophages and fluorescent dye on a test field located at Wilerwald (Switzerland) and simultaneously surveyed in detail on a local scale by Radio-Magneto-Tellury (12-240 kHz).

Rossi, P., A. De Carvalho-Dill, Müller, I., Aragno, M.

Environmental Geology (1994) 23: 192-200

Abstract:
This article presents an example of a tracing experiment using two bacteriophages, T7 and f1, and a fluorescent dye (naphthionate), in a saturated porous environment. The test field was equipped with an injection borehole and 22 sampling piezometers set in three concentric half-circles. The distribution of permeabilities and the thickness of the aquifer were indirectly determined by Radio-Magneto-Tellury (RMT 12-240 Khz). The results reveal a good correlation between the distribution of permeabilities obtained by RMT and the breakthrough curves and speed of migration of all three tracers. The restitution levels are far superior (by two to three orders of magnitude) in the more permeable zones, as opposed to those observed in the piezometers situated in less permeable areas.

The speed of migration of the biological tracers is much greater than that of the naphthionate. In the most extreme case, the T7 bacteriophage migrated about 3.15 times faster than the chemical solution. These results indicate that bacteriophages are able to travel considerable distances along permeable gravel channels. They may be used as biological tracers and as models for the migration of pathogenic viruses. The simultaneous use of tracing techniques and appropriate geophysical methods leads to a better knowledge of the hydrogeological parameters of the underground terrain. This combination allows for a better interpretation both of the speeds of migration and of the maximal concentrations of the tracers, and thus considerably increases the interpretability of hydrogeological impact studies.

5.1.1 Introduction

The transport and survival of pathogenic viruses in ground waters is an important issue. In the USA, over 50% of the diseases transmitted by water are caused by contaminated aquifers. Of these cases, 65% are due to enteric viruses (Yates et al. 1985). From 1971 to 1979, more than 50'000 people have been affected by a water-born virus (Craun 1984).

Virus propagation and survival in aquifers has been investigated in depth in the laboratory, in batch or column experiments. The influence of saturated and unsaturated media, of the presence of organic material and of various physical factors such as temperature and Ph have particularly been studied (Lance et al. 1982: Lance and Gerba 1984; Powelson et al. 1990, 1991; Straub et al. 1992). Trials in natural environments are less numerous, and knowledge of porosity, permeability distributions as well as boundary conditions is still insufficient to allow a precise relation between the propagation of viruses and local underground hydrological conditions to be defined. The use of bacteriophages as models for underground migration of pathogenic viruses started a few years ago (Goyal and Gerba 1979; Hurst et al. 1980). This tracing method is based on the apparent similitude of the proteic structures of the bacteriophages (bacterial viruses) and of animal and plant viruses. Bacteriophages, also called phages, are obligatory viruses of bacteria (bacteriophage means "which feeds on bacteria"). Like all viruses they are incapable of reproducing on their own. They invade a specific host, diverting its metabolism to their own end: self-replication inside their host cell.

Bacteriophages are constituted of a complex assembly of proteins enclosing the genetic information. It is intricately folded inside the phage's head, also called the capsid. Phages vary in

size from 45 to 230 nm. Their morphology is very diverse: there are phages with or without a tail, filamentous phages, and many others. Many also possess varied appendixes (Ackermann and DuBow 1987). The advantages of using phages for hydrological research are numerous:

- Their specificity towards their bacterial host is narrow. Several phages, each specific of a different bacterial species, can thus be used simultaneously during a tracing experiment and be detected individually.
- They are non-toxic and non-pathogenic for all organisms (human, animal and plant) other than their target bacterial species. 10^{15} phages weigh about one gram, so the organic pollution due to their introduction in an aquifer can be considered insignificant.
- Their persistence in the environment is limited to a few months. They do not accumulate, the background level in the environment remains negligible.
- The detection limit is low. In multiple serial analyses it is possible to detect one phage in two to five milliliters of water.

In karstic and fissured environments bacteriophages have been used as hydrogeological tracers in their own right since the 1970s (Fletcher and Myers 1974; Martin and Thomas 1974; Aragno and Müller 1982; Rossi et al. 1992). If many publications exist concerning bacteriophage migration in fissured environments, there are only a few treating of experiments conducted in areas of interstitial porosity (Schaub and Sorber 1977; Bales et al. 1989).

This multidisciplinary project is a study of the migration and survival of several bacteriophages in a saturated porous terrain, on a site specially equipped for studying the propagation of artificial tracers in the underground waters. Earlier experiments using different dye tracers had already revealed the existence of preferential channels at the Wilerwald test field (Sansoni et al. 1988). The tracers followed rather complicated pathways that were difficult to interpret. To try and understand the situation better a geophysical survey of the test field was made, using Radio-Magneto-Tellury (RMT). This method uses frequency ranges of 200, 70 and 20 Khz. It gives almost punctual values of the vertically-integrated electrical resistivity of the geological formations at different depths. This RMT method, calibrated on numerous borings in gravel and sand on the Swiss Plateau, allows an empirical relation to be established between the resistivity of the unconsolidated rock in saturated zones and its measured permeability. An image of the nature and thickness of the strata as well as of the distribution of permeabilities is thus obtained. The migration of tracers in the underground can then be interpreted in accordance with the spatial distribution of the hydrogeological parameters. The aims of this research were (i) to demonstrate the relation between the estimated permeabilities (established by the RMT-R method) and the behavior of the bacteriophages (ii) to judge of their utility for hydrogeology as biological tracers in saturated porous environments.

5.1.2 Material and methods

A. Principles of the Radio-Magneto-Telluric survey:

Materials deposited in deltas and flood plains, such as at Wilerwald test field, are characterized by numerous and rapid variations in their texture and mineral composition, from clean sand to silts and clays. The sand distribution is irregular, and frequently cross-bedded. The lateral patterns are irregular too, and there may be both long and narrow or broad stretches of irregular patterns. The best geophysical methods to explore these formations are those that do not homogenize the sediments at all (or do so only on a small scale), but also allow the detection of local heterogeneities to a great depth. The RMT electromagnetic method is perhaps the one best suited to explore these heterogeneous structures. Its good vertical selectivity and depth penetration, its high speed of performance and the light weight and small size of the apparatus used are the main advantages of RMT prospecting (Turberg et al. 1993, Mdaghri Aloui et al. 1993).

Radio waves generated by Very Low Frequency (VLF) and Low Frequency (LF) emitters produce a primary magnetic field. This alternating magnetic field (between 12-240 Khz) also induces electric currents in the ground. The RMT receiver picks up the intensity of the magnetic field (Hy) with a coil and at the same time measures the potential difference (Ex) produced between two ground electrodes placed five meters apart. Thus, the apparent resistivity (Rho_a) of the strata between the two electrodes can be obtained, given by the following equation:

$$(1) \qquad \rho_a = \left(\frac{Ex}{Hy}\right)^2 * \frac{1}{2\pi F \mu_0}$$

With: ρ_a (Rho_a), given in ohm meters (Ωm)
Ex (V/m), the electric component of the electromagnetic field (measured value)
Hy (A/m), the magnetic component of the electromagnetic field (measured value)
F, the emitter's frequency, given in Hertz, set between 12 and 240 Kilohertz
μ_0, the magnetic permeability of vacuum, given in Henry/meters ($4\pi \cdot 10^{-7}$)

The parameters measured for each of the frequencies used are the apparent resistivity of the rock and the phase shift between the electric and the magnetic component of the electromagnetic field. These are sufficient to calculate the thickness and the true resistivity of each layer, using 1-D Magnetotelluric Inversion (Fischer and Le Quang 1981). The depth D [m] of the sounding is given by the following equation:

$$(2) \qquad D = \sqrt{\frac{\rho a}{F}}$$

B. Geographical location and a brief hydrogeological description of the test field:

The Wilerwald test field lies in the Emme valley (fig. 5.1.1), 6 km south of Solothurn, on the edge of the forest which surrounds Wiler b. Utzensdorf (BE, Switzerland). The aquifer is composed of post-glacial sand and gravel and is covered by approximately 1,5 m of alluvial sediments (sandy silt) and humus. It is composed of 40-60% quartzite, 30-50% shales and calcareous sandstones and 5-15% crystalline rocks. The thickness of this formation is quite variable and overlays a very irregular Miocene surface of molassic marls (Ledermann 1978). Lying between the Miocene rocks and the Holocene gravels are lacustrine sediments of silt and clay very irregular in thickness that function as an aquitard. The groundwater table lies between 1.5 and 3.5 meters below the surface, with a gradient of about 0.4% in the northwest direction (Carvalho Dill 1993; Carvalho Dill and Müller 1992; Leibundgut et al. 1992). Two dates were chosen to represent the configuration of the water table during the experiment. The isolines reflecting the different responses to rainfalls, a consequence of the heterogeneity of the field, can be seen in fig. 5.1.2.

Fig. 5.1.1: Geographical situation of the test field of Wilerwald b. Utzensdorf (Switzerland)

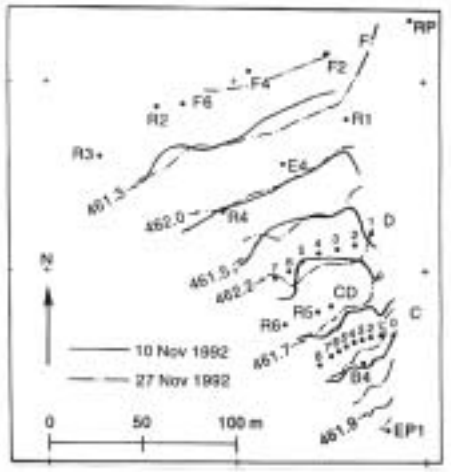


Fig. 5.1.2: Piezometric map of the Wilerwald test field (after Carvalho Dill and Müller, 1992). The altitude of the water table, in meters above sea-level, is shown by isolines: (——) on the day of injection, and (—·—·) after 17 days.

Piezometers are placed in concentric circles around the two injection wells B4 and EP1. Twenty-one observation holes set in three half-circles (C,D,F) and one injection well were bored on the site. They are 2.5 inches in diameter and descended through the entire thickness of the aquifer.

C. Injection method and tracers used:
Two bacteriophages were chosen for their different morphologies. The T7 phage has an icosaedral head and a short non-contractile tail (fam. Podoviridae). The f1 phage is filamentous (fam. Inoviridae). Naphtionate (sodium amino-4 naphtalenesulfonate-1, MW= 254.24) was chosen as the fluorescent tracer. This chemical is frequently used in hydrogeology. It gives a good image of the speed of propagation of chemical substances in an aquifer. The three tracers were injected simultaneously in piezometer B4, following the method given by Käss (1992 and 1993). Water is pumped from a depth of 0.5 meters below the water table. It is mixed with the different tracers aboveground and re-injected at a depth of 9 meters. The operation lasted 20 minutes. The concentration of the tracers throughout the piezometer's water column is thus rapidly homogenized.

D. Tracers:
Naphthionate: 1kg, diluted first in 10 l of water
Bacteriophage f1: $9.6 \cdot 10^{13}$ phages (total volume 960ml)
Bacteriophage T7: $8.9 \cdot 10^{13}$ phages (total volume 2280ml)

E. Sampling:
Water samples were taken with 10 m long weighted PVC tubes (int. diam. 1cm). Each piezometer was equipped with a separate tube to avoid contaminations. The tube was connected to a small vacuum pump and its weighted end lowered to four meters below ground. As soon as the water level in the tube reached the head of the piezometer, the tube was lowered regularly by hand till maximum depth. The pump was then turned off, the tube removed and emptied into a plastic (polypropylen) container. The whole operation was repeated, to rinse the apparatus and stir the water column before taking the sample. This is a rapid way of obtaining a homogeneous sample of the entire water column of a piezometer.

F. Phage analysis:
Analysis of the samples is done following a modified method of phage enumeration on Petri dishes (Adams 1959). The modification consists of a division of the analysis in two stages. The first, upon receiving the samples, involves a simple presence/absence test using one single Petri dish per sample. In the second stage, positive samples are diluted if necessary be and analyzed on three Petri dishes. This way of doing minimizes the analytical work necessary to treat the numerous samples of a multi-tracing experiment.

G. Naphthionate analysis:
The samples were analyzed for naphthionate by W. Käss (Umkirch, D.). This was done by fixed-wavelength fluorescence (λ_{Ex} = 322nm, λ_{Em} = 420nm). The lowest detection limit is 10^{1}ppb.

5.1.3 Results and Discussion:

A. Geophysical map of the Wilerwald site:

The variability of the quaternary formation mentioned in the literature is also apparent on the local scale of the Wilerwald test field. In order to map it, the field was divided into 11 profiles (fig. 5.1.3), totaling over 500 measuring points. Along each profile measures were made every 5 to 10 meters. Three frequencies were used: 183, 60 and 19Khz. The use of this frequency sounding allows the calculation of true resistivities and the local thickness of the underground layers by magnetotelluric inversion. The contour map (fig. 5.1.3) obtained from the 183 Khz apparent-resistivity data already gives a reasonably good picture the aquifer's structure. The higher Rho_a values correspond to permeabilities (k) over 10^{-3} m/s. On the fig.5.1.3 they take the form of a channel running first northwards, then north-westward and comprising piezometers C6 and C7, as well as D6 and D7. This paleochannel was formed during the last glacial retreat. It then probably formed a small affluent of the river Emme and was little by little filled up by coarser materials. The smallest values recorded by RMT are of the order of 100 Ωm. They correspond to permeabilities of 10^{-5}m/s to 10^{-4}m/s. These much less permeable zones of restricted dimensions comprise the piezometers C4, C5 and D5, D4 and D1.

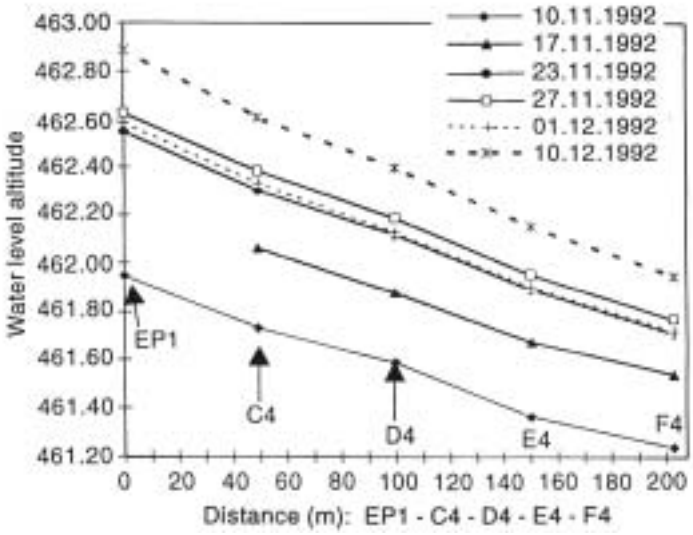


Fig.5.1.4: Longitudinal profile along what was once considered to be the main direction of water flow, showing the variability of the water level (almost one meter) during the tracer experiment (after Carvalho Dill, 1993).

B. Influence of the distribution of permeabilities on the speeds of migration and the breakthrough rates of the various tracers:

In addition to the geophysical map, fig. 5.1.3 presents four breakthrough curves for the tracers injected at B4 (C4, C6, D1, D7). These four curves are redrawn in detail in figures 5.1.5 to 5.1.8. These clearly show that the maximal concentrations reached can be very different inside a single row of piezometers. For row C, the maxima were reached most rapidly in the piezometer C6 (see also fig.5.1.6). The highest overall concentrations of tracers were measured in this piezometer: respectively 2.67· 10⁵ and 1.33· 10⁵ phages/ml for the phages T7 and f1, and 5220 µg/l for the naphthionate. The piezometer C4 only received a very small amount of all the tracers. This difference of several orders of magnitude can be explained by the very heterogeneous distribution of resistivities shown in fig.5.1.3. C4 is in the line of the current exactly facing the injection point B4, but situated in a zone of low permeability. Only a few meters away the influence of the high flow velocities in the paleochannel was considerable. The tracers were very rapidly and massively diverted northwestwards, towards the piezometers C5 and C6.

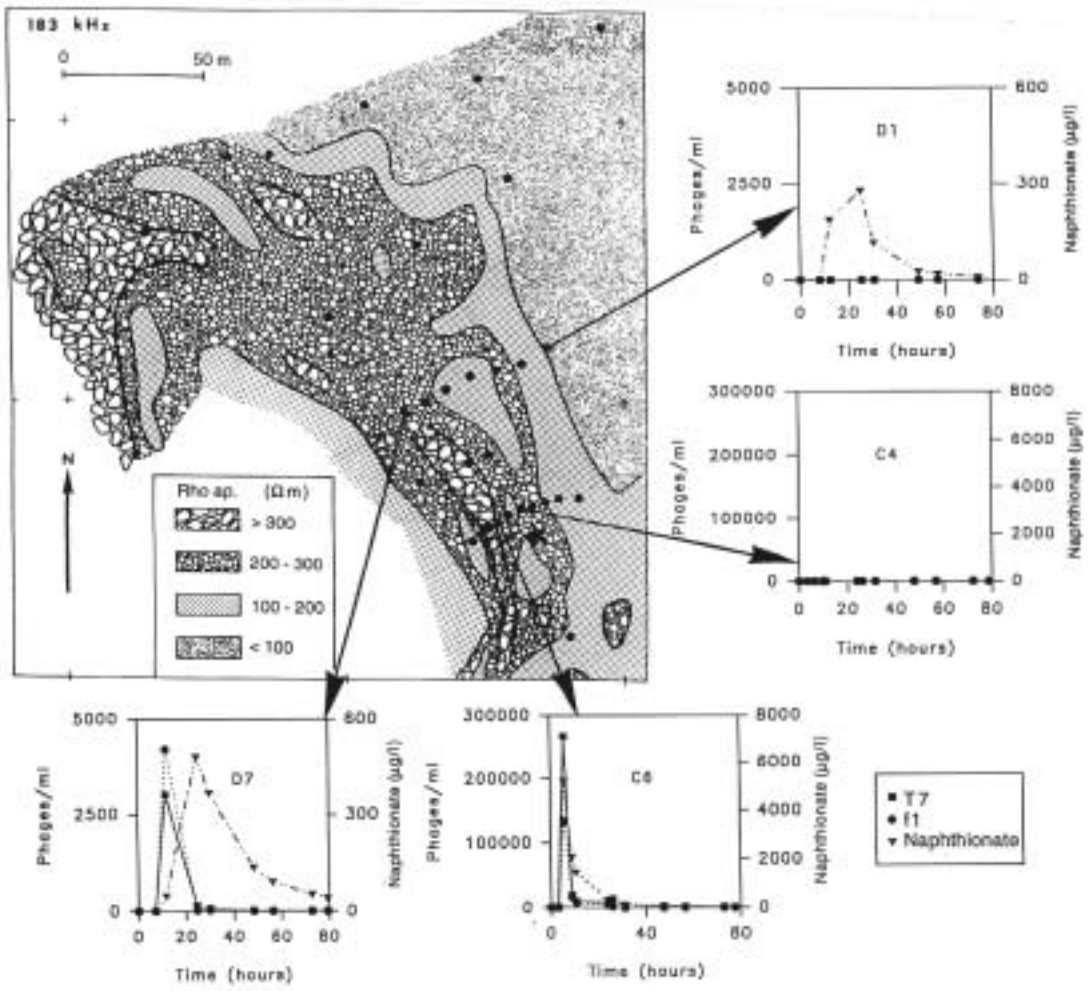


Fig. 5.1.3: Map of the Wilerwald test field showing the distribution of apparent resistivities determined by the RMT method and the breakthrough curves of the tracers for the piezometers C4, C6, D1 and D7 (after Carvalho Dill and Müller, 1992). (•): piezometers;(★): injection point B4.

This difference in the restitution rates is also very marked in the D row of piezometers (see also fig. 5.1.7 and 5.1.8). The highest concentrations of this series lying about seventy meters from the injection site were found in the samples taken from D7, situated on the highly permeable channel. Generally all the piezometers of this series lying in a zone of low permeability only received minimal concentrations of the tracers. In a same row of piezometers the differences between the maximal concentrations are better marked with the phages. For example, the D1 and D7 piezometers received respectively 3.5 and 3000 phages T7/ml, and 278 and 480 µg/l of naphthionate. It thus seems that the phages concentrations are much more sensitive to variations in permeability and they agree better than the naphthionate with the interpretation results of the RMT soundings.

The speed of migration of the tracer is also influenced by the distribution of heterogeneities within the aquifer. On short distances, in permeable porous environments such as from B4 to C6 (14,32m), the speeds of migration of the phages and the dye are identical. When the permeability decreases or the distance between the injection and sampling points increases, the phages move faster than the naphthionate. If both factors come into play, such as for the piezometer D1, the difference between the migration speeds of the phages and the chemical tracer becomes considerable. The $Tmax_{NAPH}/Tmax_{PHAGE}$ ratio, a good image of the relative migration rate of the

tracers, is globally very high for the piezometers of the D series (data not shown). This ratio shows that at about seventy meters from the injection site the phages have migrated 2 to 3.15 times faster than the fluorescent dye (see table 5.1.1). Furthermore, the ratios obtained are comparable to the permeability values given by the RMT soundings.

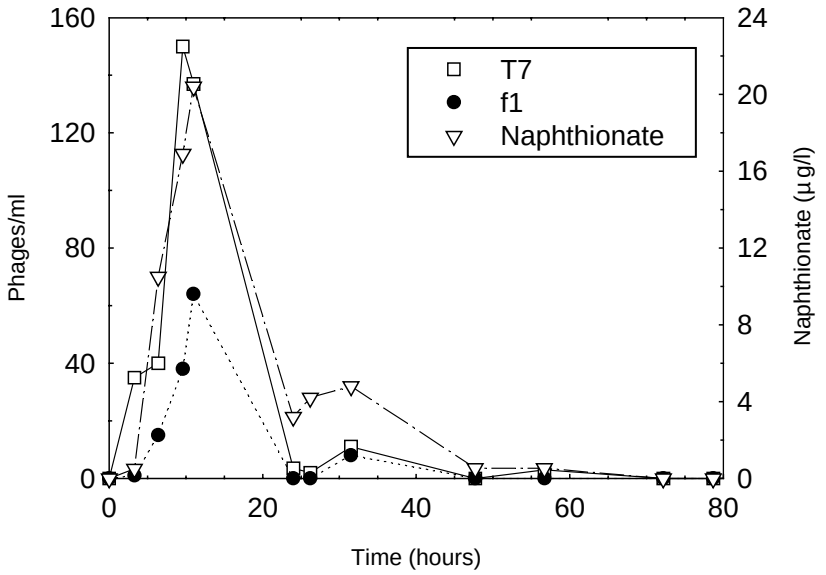


Figure 5.1.5: Breakthrough curves of phages and Naphtionate in borehole C4

However D1 presented relatively high concentrations of naphthionate, showing that the hydraulic situation at the moment of the experiment is another factor influencing solute transport. During the last experiment, daily observations of the piezometric level (Carvalho Dill 1993) showed the response of the aquifer to rainfall events. Piezometric observations are fundamental for the comprehension of solute transport. Higher amplitude variations, and correspondly delays in reaching the maximal level, seem to correspond with lower permeabilities and with where the aquifer is in confined or semiconfined situations.

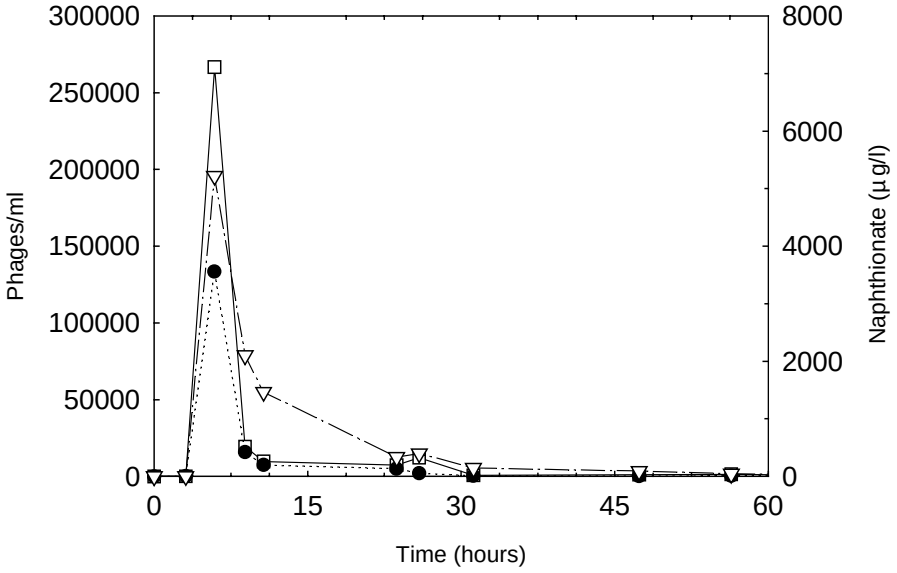


Figure 5.1.6: Breakthrough curves of phages and Naphtionate in borehole C6

On the other hand, it is clear that we are dealing with a complex phenomenon: fig. 5.1.2 shows depressions in the west part of the field at sites with higher permeabilities (paleochannel), but simultaneously at the northeast part of the field (less permeable and confined situation) temporary depressions caused by the delay in response are also created. These are important as well, conditioning the direction of flow and transport, explaining the unexpectedly high concentration of naphthionate at the piezometer D1. Fig. 5.1.4 shows the delta h of the observation wells situated on what was once considered to be the main direction of water flow, (Sansoni et al. 1987) showing the variability of the water level (almost one meter) during the tracer experiment.

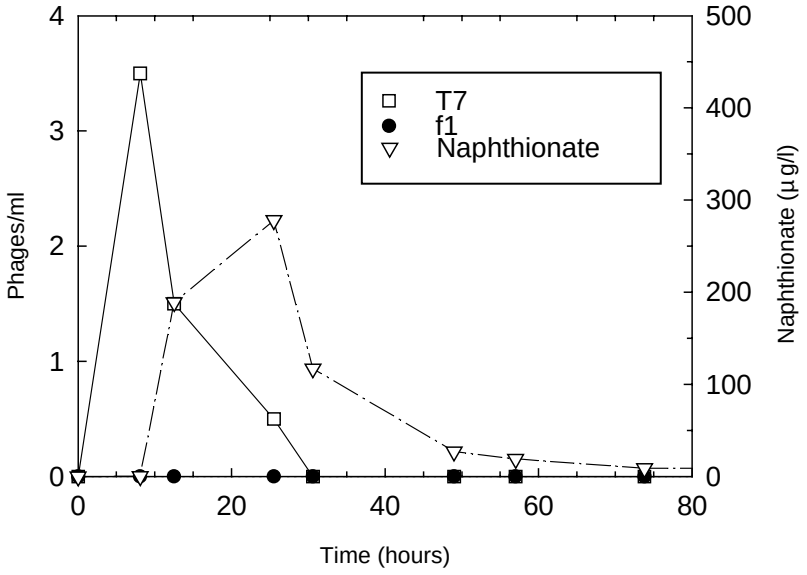


Figure 5.1.7: Breakthrough curves of phages and Naphtionate in borehole D1

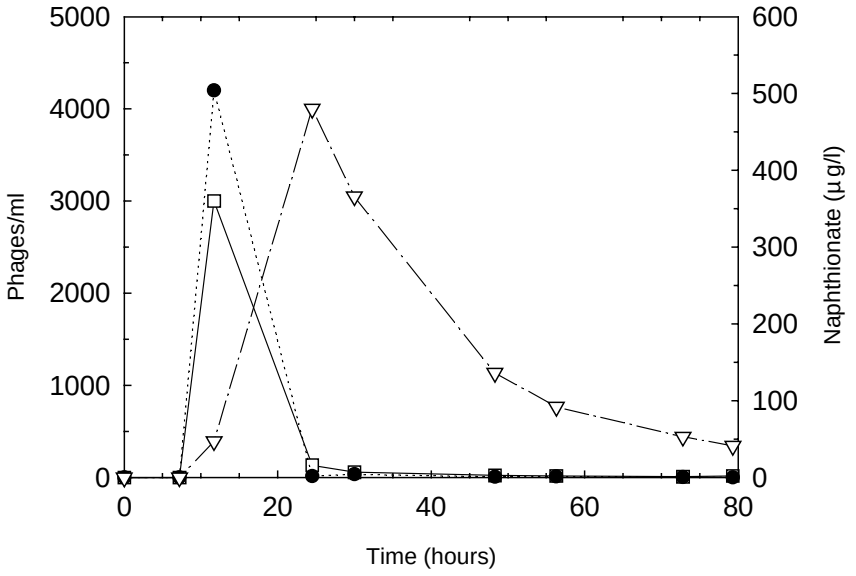


Figure 5.1.8: Breathrough curves of phages and Naphtionate in borehole D7

The speeds of migration of both the phages were identical for all the piezometers. The differences in phage morphology did not influence the rapidity with which these two phages traveled in the saturated porous environment. It seems however that it did strongly affect the restitution rates and the longest distance traveled. Phage T7 migrated in greater quantities than f1 in areas of low permeability. It was also recovered in the most distant piezometer that was reached (F4), covering a distance of 162.5m in 219 hours. The phage f1 migrated in greater quantities than T7 in permeable zones, as shown by the breakthrough curves of piezometer D7 (see fig. 5.1.8), but did not reappear in the piezometers situated in areas of low permeabilities.

Piezometer	Distance from B4	Phage T7		Phage f1		Naphthionate		Tmax ratios
		Cmax (Phages/ml)	Tmax (h)	Cmax (Phages/ml)	Tmax (h)	Cmax (µg/l)	Tmax (h)	$Tmax_{NAPH}/Tmax_{PHAGE}$
C4	11.70m	150	9.6	64	11	20.4	10.7	1.15
C6	14.32m	$2.67 \cdot 10^5$	5.9	$1.33 \cdot 10^5$	5.9	5220	5.9	1
D1	69.18m	9.5	8.1	0	-	278	25.5	3.15
D7	64.35m	3000	11.7	4200	11.7	480	24.5	2.09
E4	112.5m	22	243	1	243	N	N	N
F4	162.5m	1	219	0	-	N	N	N

Table 5.1.1: Summary of the maximal concentrations (Cmax) reached, their breakthrough times (Tmax) and of the distances traveled by the tracers. N: not determined. P/ml: Bacteriophages per ml. $Tmax_{NAPH}/Tmax_{PHAGE}$ ratio: ratio of breakthrough times of maximal concentrations.

C. Semi-quantitative interpretation of the tracer tests : modelling trials

RMT-R was used recently to obtain an image of the aquifer. This method is very accurate to locate lateral changes in resistivity (permeability) but does not give enough details of the vertical distribution of heterogeneities. The fig. 5.1.9 (after Carvalho Dill, 1993) shows a 3-D representation of the distribution of true resistivities on the site. These were calculated using 1-D Magneto Telluric Inversion (Fischer and Le Quang, 1984). Small block diagrams showing qualitative models of chosen punctual RMT soundings complete the figure. The three vertical lines correspond to the depths of penetration of the frequencies used. This will also depend on the resistivity of the strata, so the lengths of the lines differ for each sounding. The lateral discontinuity of the strata is also illustrated.

The study of the single drill-core CD provided a detailed punctual sedimentological description. Together with the indirect information given by the resistance to penetration registered during the piezometers installation, the existence of local irregular lenses of finer material was made clear. These lenses seem to be irregularly distributed, indicating that it is erroneous to speak of continuous layers mainly in fluvio-glacial sediments. Instead the aquifer is composed of different pockets, containing varying proportions of finer sediments and of paleochennals of virtually clean gravels. One observes situations where the aquifer seems to be confined or semi-confined, and others of free nappe. Different and constantly changing hydraulic gradients exist within the site. The hydraulic mechanisms are therefore still far from understood. However, analytical models have already tested (Leibundgut et al. 1992) based on a hypothetical unidirectional mean flow, restricted to a simple three layer situation, with a relatively small variation of the parameters. The solution of an analytical model can only be formulated after some simplifying assumptions, in general very

restrictive, have been made: it is usually necessary to postulate that the medium is homogeneous and isotropic, with a parallel flow of constant velocity, steady-state conditions and simple boundary conditions (infinite or semi-infinite media). A 3-D numerical model could be suitable, but it requires much more information about the structure and boundary conditions. Unfortunately not enough information about the vertical distribution of permeabilities was available. Besides, due to the narrow diameter of the observation wells, it was impossible to use other hydraulical techniques (such as flow-meters) which could have supplied useful information about the vertical distribution of permeabilities. It is always possible to create a model from a set of data. But a model should be valid, i.e, it should have the capacity to simulate the aquifer's behavior, and to answer a specify event. In a heterogeneous aquifer the above-written investigations are essential and must be done before any attempt at modeling is made.

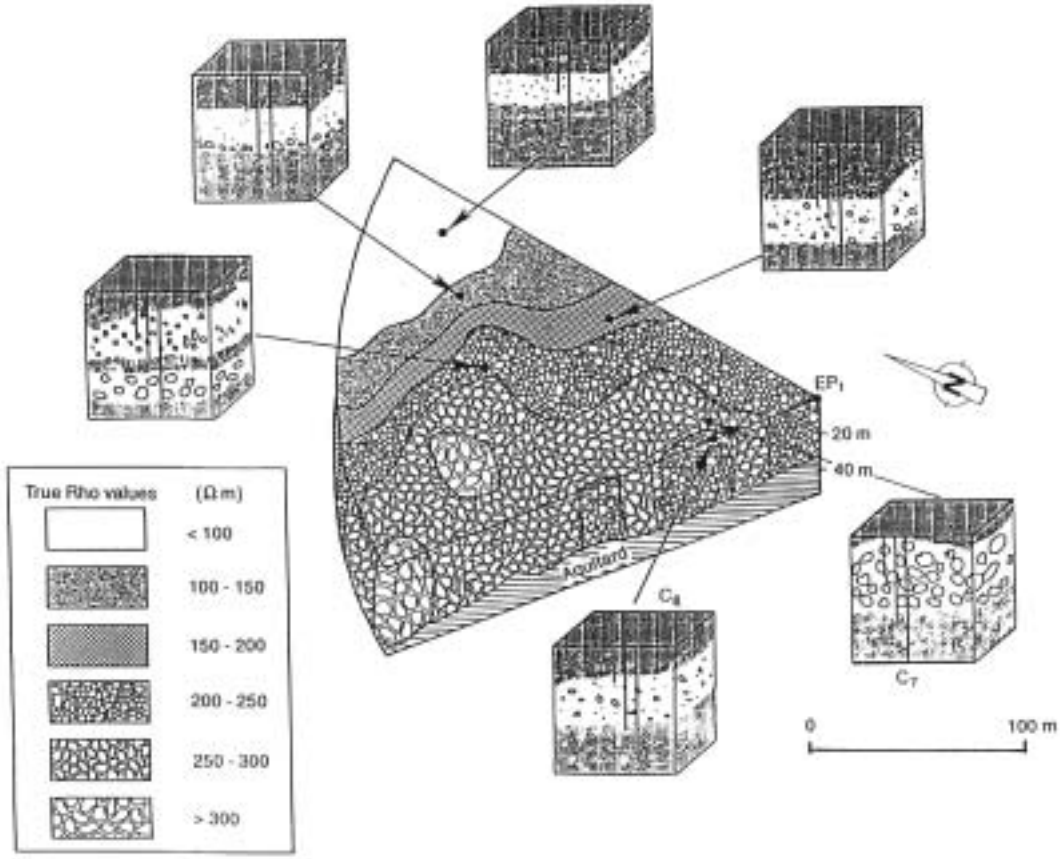


Fig. 5.1.9: 3-D representation of the distribution of true resistivities on the site calculated using 1-D Magneto Telluric Inversion. Small block diagrams show qualitative models of chosen punctual RMT soundings. The three vertical lines correspond to the depths of penetration of the frequencies used. For more details see text.

5.1.4 Conclusions

The radio-magneto-telluric method (RMT 12-240 kHz) offers new and very interesting possibilities for the analysis on a very local scale of the nature and thickness of geological formations. It also allows a better understanding of the migration of tracers in porous environments. The values obtained reveal the complexity of the environment: paleochannels appear, filled with coarse gravel, whose permeability is much higher than that of the adjoining regions. They therefore determine the

movements of the tracers. In the light of this geophysical evidence, it becomes clear that the tracers bypass the obstacles represented by pockets of lower permeability, and follow easier pathways through the more permeable areas. The breakthrough curves of the tracers will then be strongly influenced by the size and hierarchical organization of these channels. In the case of our experiment, the little permeable channel that cuts right through the test field would be practically undetectable by classical hydrogeological methods. However, its existence explains the massive and rapid drainage of the tracers from the injection point to the C6 piezometer and then towards D6 and D7.

T7 and f1, the two bacteriophages used in this trial, migrated faster than the naphthionate in all the piezometers tested, independently of the distribution of permeabilities. It is only the maximal concentrations, both of the biological and chemical tracers, that can be said to be strongly influenced by the heterogeneity of the environment. Bales et al. (1989) also noted the rapid migration of viruses and bacteriophages in a sandy aquifer. According to these authors, the concentration of viruses along the flow shows evidence of dispersion but not of retardation. Their observations show that viruses and phages migrate 1.6 to 1.9 times faster than conventional fluorescent tracers considered as conservative. Our results from Wilerwald testfield confirm those of these authors. The two bacteriophages T7 et f1, were injected simultaneously and migrated at the same speed, faster than the saline naphthionate solution. The ratio of the arrival times of the concentration maxima ($Tmax_{NAPH}/Tmax_{BACT}$) shows that the bacteriophages migrated up to 3.15 times faster than the conventional fluorescent tracer.

The tracing experiment on the Wilerwald test field reveals the interest of bacteriophages as biological tracers in environments of interstitial porosity. They could be used to simulate the propagation of pathogenic viruses in a porous aquifer and this may lead to a review of the currently accepted values for maximal propagation speeds. This trial also shows that using fluorescent tracers to define the size of protected areas around drinking-water pumping stations and sources is not always the best solution. The distribution of permeabilities should also be investigated by a geophysical method that does not homogenize the environment, so as to render possible the detection of privileged channels and underground flow pathways. Interpretation of tracer breakthrough curves will then become much easier. Furthermore, this type of investigation is absolutely necessary to determine the appropriate injection and sampling points.

Acknowledgements:

We are very grateful to W. Käss for his invaluable help during the injection of the tracers and for the numerous naphthionate analyses he performed for us. We would also like to thank Mrs M. Grob for preparing the culture media for the bacteriophages and treating the vast number of water samples that had to be analyzed. This research was financed by grant no. 20.36236.92. of the Swiss National Foundation for Scientific research (FNRS).

§5.2 Second tracing experiment in a porous environment on the Wilerwald test site:

5.2.1 Introduction

This experiment took place, as for the one presented in section 5.1, on the Wilerwald test site. The geographic situation of the site and its description are given in this same section. We might recall that the distribution of the permeabilities of this porous aquifer has been analyzed in detail (by RMT-R). This analysis revealed the existence of zones of preferential flow, relics of the last glacial thawing. The restitution of the tracers in the boreholes on the site is strongly conditioned by these paleochannels. This second experiment had two aims. First, to test the behavior of three new bacteriophages (H40/1, H6/1 et Psf2). The laboratory tests (see chapter 4) showed that these three phages were rather insensitive to the presence of mineral colloidal particles. It was therefore interesting to test them in a saturated porous environment. Alongside these three phages we tested phage T7, the bacteriophage that had showed the most interesting behavior during the first trial (highest restitution). The second aim of this particular experiment was to compare the results obtained by particulate biological tracers and the most efficient fluorescent tracer presently in use, uranine. Today this substance is considered a reference among tracers. Inert, non-toxic and cheap, uranine reacts very little with the environment through which it travels. It was therefore interesting to compare the migration speeds of these two types of tracers.

5.2.2 Material and methods:

A. Injection of the various tracers:

B.

Professor habil. W. Käss injected the tracers following the method described in section 6.2. In short, the injection was performed as follows: the tracers were injected in borehole B4, at 9:30 am on the 21st of May 1994. The viral suspensions and the uranine solution were poured into a plastic container. A 12 V pump (diam. 2 inches), drew water from a depth of 8 meters and flushed it into this container. This water and the tracers were then re-injected at a depth of 4 meters, using a Mariotte siphon. The injection/flushing system functioned for 15 minutes. It homogenised the tracers along practically the entire height of the borehole.

Tracers injected:	Uranine 350g		
	Bacteriophages:	H6/1:	$1.483 * 10^{13}$ Pfu (1000ml)
		H40/1:	$7.5 * 10^{14}$ Pfu (1000ml)
		T7:	$2.63 * 10^{13}$ Pfu (1000ml)
		Psf2:	$1.847 * 10^{13}$ Pfu (1000ml)

B. Water sampling and analysis:

In the first row, we sampled boreholes C4, C6 and C7. In the second row, only D1, D6 and D7 were sampled. The sampling method used was the one described in section 5.1. The bacteriophages were analysed according to the method described in chapter 3. The uranine analyses were performed by the CHYN (Hydrogeological Center, University of Neuchâtel) laboratories.

5.2.3 Results and discussion:

Figures 5.2.1 to 5.2.8 show the breakthrough curves of the various tracers in all the boreholes sampled. Tables 5.2.1 to 5.2.5 summarize the main parameters (speed, C_{max}, etc) measured for the five tracers.

A. Comparison between the bacteriophages:

The breakthrough curves in borehole C6 are identical for all the bacteriophages. C6 received the greatest concentrations of bacteriophages measured during this experiment. Phage H40/1 had the highest one with a C_{max} of 2.91* 10^{7} Pfu/ml. These concentration maxima (C_{max}) were reached very rapidly, after only two hours of migration. The curves are sharply defined and the dispersion is very reduced.

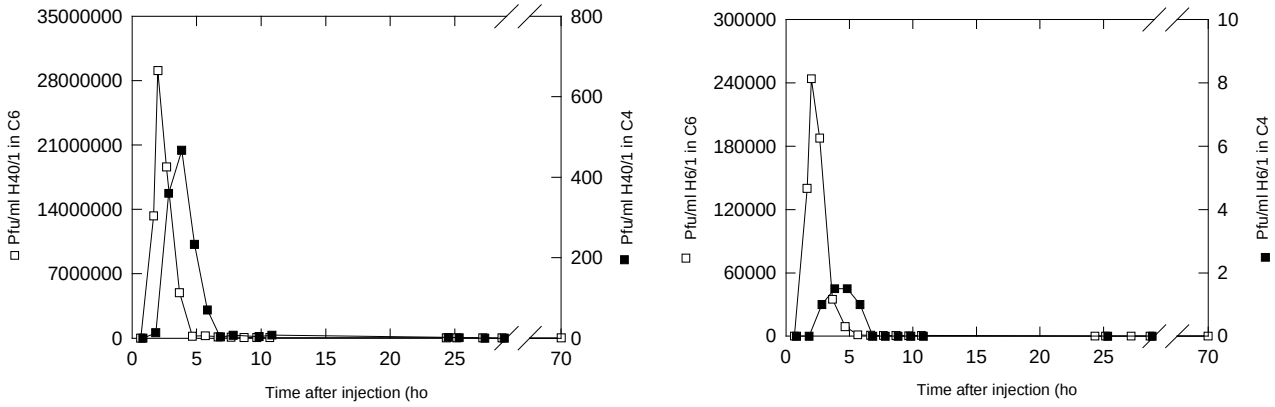


Figure 5.2.1: Breakthrough curves of phages H40/1 and H6/1 in boreholes C4and C6

The results of boreholes C4 and C7 are mixed. C4, directly facing B4, and in the line of flow, received very few phages. The situation is the same for C7, on the other side of the paleochannel. Except for phage H40/1, the restitutions in the two boreholes are low and of little significance. This confirms the results of the preceding experiment: the phages are deflected rapidly towards the channel. H40/1 is the exception to the rule. Important quantities of phages were detected in all the boreholes samples in lines C and D. Injected at ten times the concentration of the other phages, its restitutions are always 100 to 100 times superior to the others. In this saturated conditions, this phage is certainly our best tracer. Its low reactivity with the substrate traveled corresponds to what we observed during our adsorption tests on mineral colloidal particles (chapter 4).

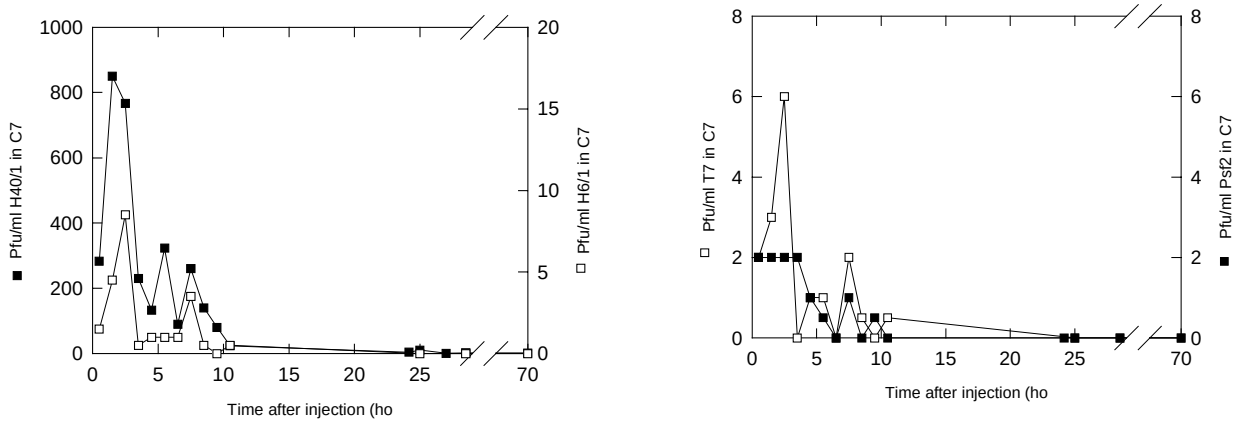


Figure 5.5.2: Breakthrough curves of phages H40/1, H6/1, T7 and Psf2 in borehole C7

The migration speeds (V_{max}) of all the phages for the D boreholes are almost equal. The T_{max} values concord almost perfectly for D7 and D6. The restitutions are much lower for borehole D1. Even if the results are somewhat concordant, they cannot be compared. Only the data obtained for phage H40/1 is significant. With a C_{max} of 248 Pfu/ml, this phage's restitution is 100 times higher than that of the other viruses. This concentration is also far higher than any we measured during the preceding experiment.

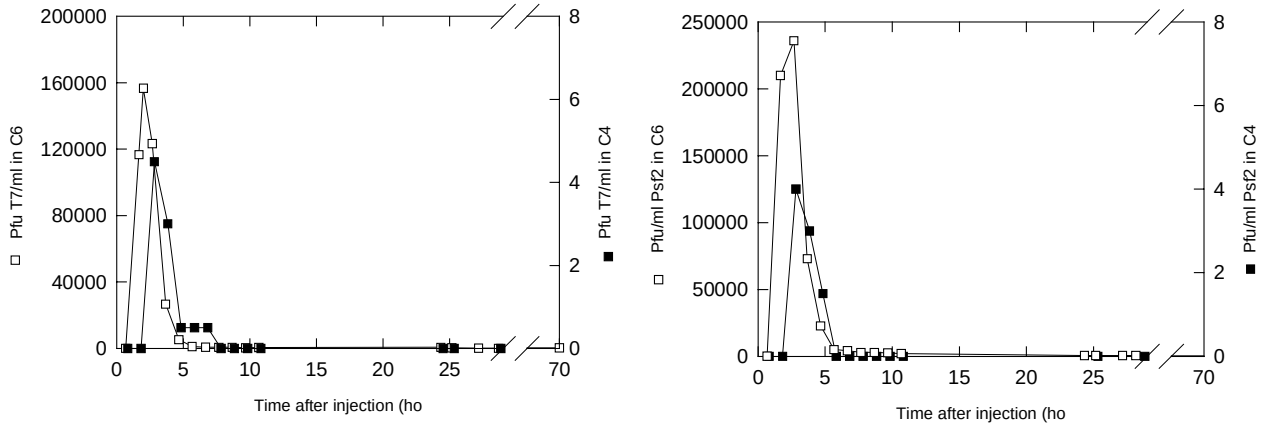


Figure 5.2.3: Breakthrough curves of phages T7 and Psf2 in boreholes C6 and C4

Tables 5.2.1 to 5.2.4: Parameters measured for the four bacteriophages during the second trial on the Wilerwald test site.

Table 5.2.1: Bacteriophage T7

Sampling points	1st app. [hours]	C_{max} [Pfu/ml]	T_{max} [hours]	V_{max} [m/h]	Dist. B4 [m]
C4	2.83	4.5	2.83	4.13	11.70
C6	< 0.67	$1.23 * 10^5$	2	> 7.16	14.32
C7	< 0.5	6	2.5	> 6.81	17.02
D1	7.33	1	8.33	8.3	69.18
D6	7.17	143	11.17	5.52	61.72
D7	6	307	9	7.15	64.35

Table 5.2.2: Bacteriophage Psf2

Sampling points	1st app. [hours]	C_{max} [Pfu/ml]	T_{max} [hours]	V_{max} [m/h]	Dist.	B4
C4	2.83	4	2.83	4.13	11.70	
C6	< 0.67	$2.36 * 10^5$	2	> 7.16	14.32	
C7	< 0.5	2	(0.5)	(> 34.03)	17.03	
D1	7.33	1.5	7.33	9.43	69.18	
D6	7.17	290	11.17	5.52	61.72	
D7	6	520	9	7.15	64.35	

Table 5.2.3: Bacteriophage H40/1

Sampling points	1st app. [hours]	C_{max} [Pfu/ml]	T_{max} [hours]	V_{max} [m/h]	Dist.	B4
C4	1.83	467	3.83	3.05	11.70	
C6	< 0.67	$2.91 * 10^{7}$	2	> 7.16	14.32	
C7	< 0.5	850	1.5	> 11.35	17.03	
D1	4.33	248	7.33	9.43	69.18	
D6	6.17	$6.33 * 10^{4}$	11.17	5.52	61.72	
D7	6	$1.31 * 10^{5}$	10	6.44	64.35	

Table 5.2.4: Bacteriophage H6/1

Sampling points	1st app. [hours]	C_{max} [Pfu/ml]	T_{max} [hours]	V_{max} [m/h]	Dist.	B4
C4	2.83	1.5	3.83	3.05	11.70	
C6	< 0.67	$2.44 * 10^{5}$	2	> 7.16	14.32	
C7	< 0.5	8.5	1.5	> 6.81	17.03	
D1	7.33	0.5	7.33	9.43	69.18	
D6	7.17	190	11.17	5.52	61.73	
D7	6	463	9	7.15	64.35	

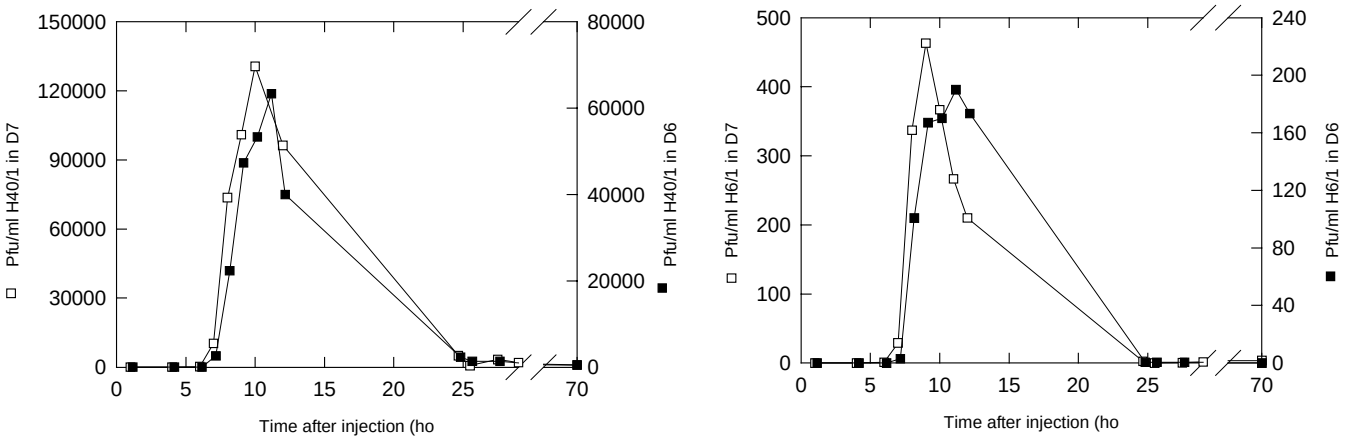
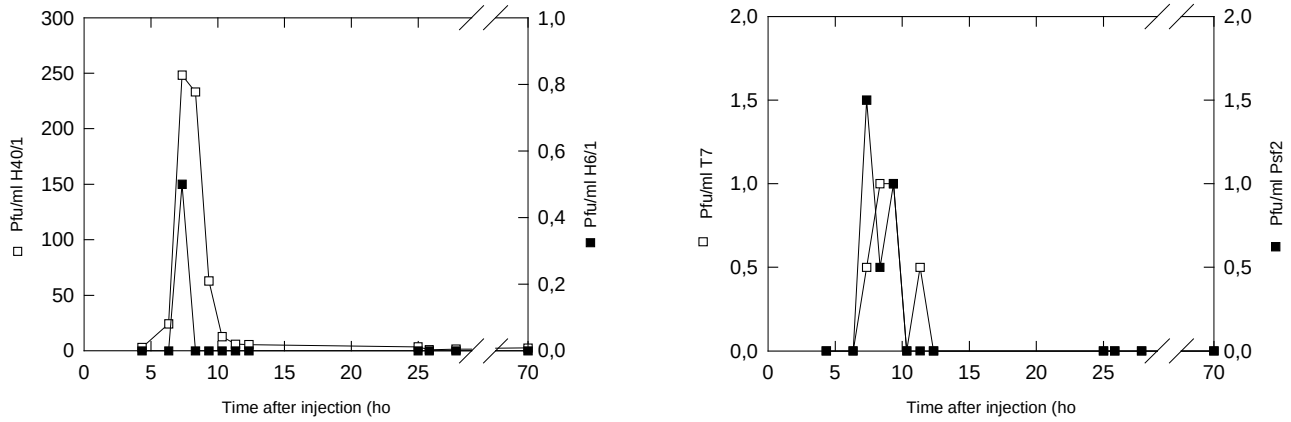


Figure 5.2.4: Breakthrough curves of bacteriophage H6/1 and H40/1 in boreholes D6 and D7

B. Comparison between the phages and the uranine:

A comparison of the T_{max} values shows that uranine exhibits a significant delay of two to three hours for C4, and of more than seven hours for C7. At C6, the borehole situated in the most permeable zone, this delay amounts only to about half-an-hour. The breakthrough curve of the uranine in borehole C6 (figure 5.2.7) is perfect and very similar to those of the phages. Its dispersal is nevertheless slightly greater.Figure 5.2.8 shows the breakthrough curves of the fluorescent tracer in the D series of boreholes. The peaks of these three curves are missing, so no comparison of the migration speeds can be made. However, the delay accumulated at the moment of the last sample taken on the first day (at T= 13 hours) is already considerable. This difference is important, especially if one considers that the distance between the injection point B4 and this series of boreholes is only of about 60 meters.



Breakthrough curves of phages H40/1, H6/1, T7 and Psf2 in borehole D1

Table 5.2.5: parameters measured for the uranine.

Table 5.2.5: Uranine

Sampling points	1st app. [hours]	C_{max} [10^{-9} g/ml]	T_{max} [hours]	V_{max} [m/h]	Dist. B4 [m]
C4	2.83	4.76	5.83	2.01	11.70
C6	1.67	3556	2.67	5.36	14.32
C7	9	0.99	9.5	1.79	17.03
D1	7.33	102	12.33	5.61	69.18
D6	8.17	51.1	12.17	5.07	61.72
D7	7	105	12	5.36	64.35

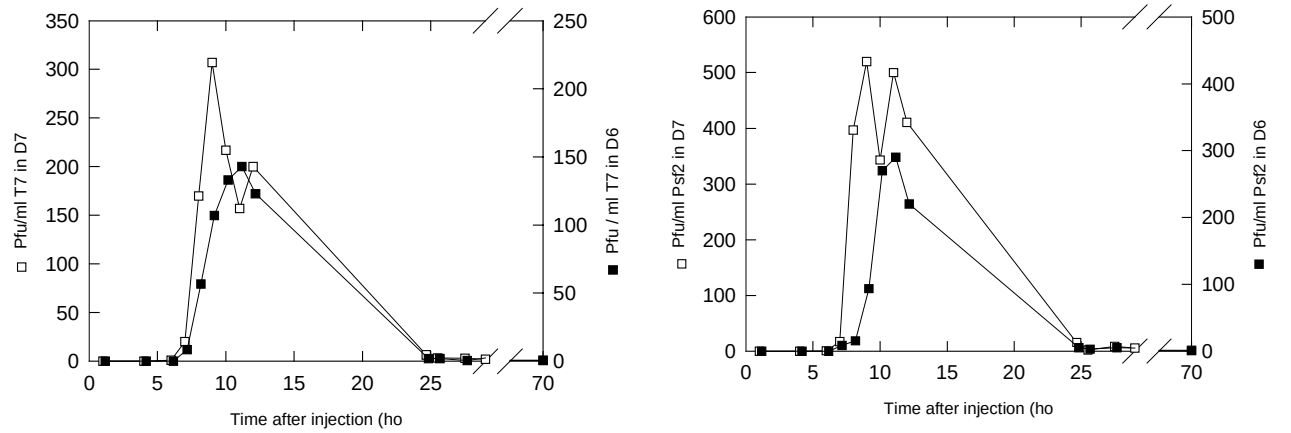


Figure 5.2.6: Breakthrough curves of phages T7 and Psf2 in boreholes D7 and D6

The dispersion of the chemical tracer is much greater than that of the phages. After 70 hours of migration, important amounts of uranine were still detected in the D series boreholes, when practically all the phages had already disappeared in these same samples. There is very little difference between the C_{max} values for uranine in the boreholes of the D series. This is not the case with the phages, where a difference of two orders of magnitude was recorded between D1 and D7. As we showed in the first experiment, the phages seem to be much more sensitive to differences in permeability than the fluorescent tracer. Their restitution is much lower in zones of low permeability, even if their speed of migration is high.

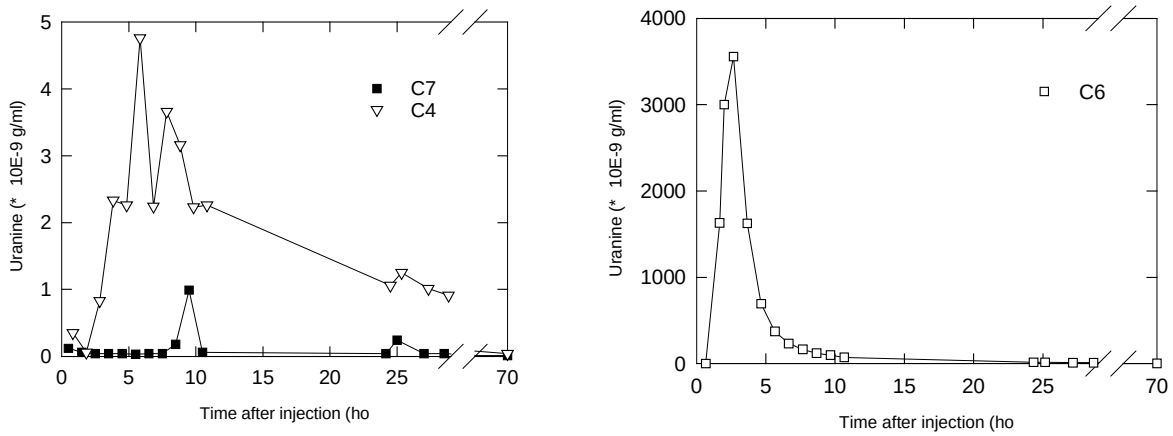


Figure 5.2.7: Breakthrough curves of uranine in boreholes C4, C6 and C7

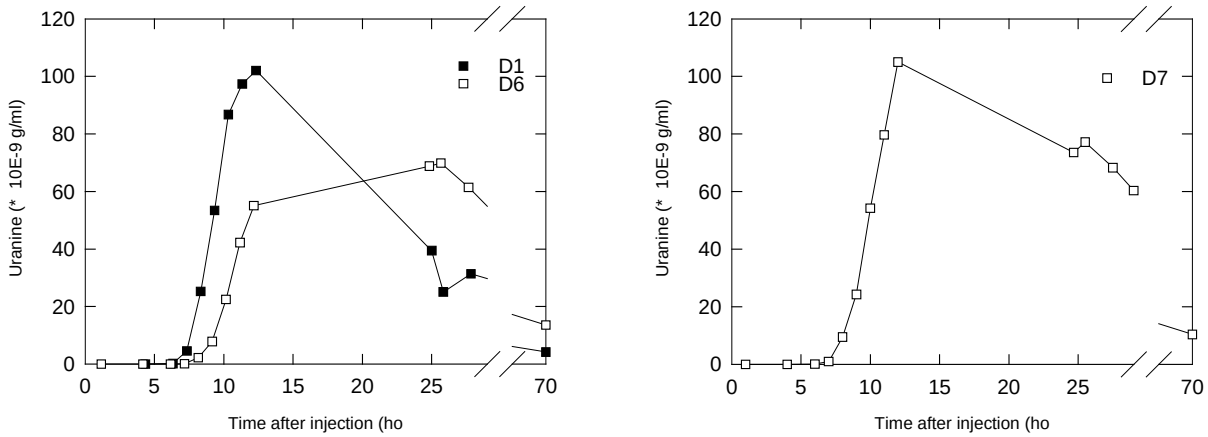


Figure 5.2.8: Breakthrough curves of uranine in boreholes D1, D6 and D7

5.2.4 Conclusions:

This second trial on the Wilerwald test site confirms the surprising results we had obtained with the injection of the first phages and the naphthionate. The bacteriophages migrate faster than a chemical solution. The difference between the migration speed of the phages and the naphthionate is considerable. This difference is also clearly marked with uranine. Unfortunately, the lack of information of the uranine breakthrough curves makes it impossible to quantify the delay precisely. Uranine is a reference tracer considered as non-reactive with the aquifer material. Should the delay accumulated with regard to the phages bring us to correct this view? Or should we search for another explanation to the rapid migration of the bacteriophages? It seems unlikely that the phages migrate faster than the water in a porous aquifer.

An additional force would be necessary to "propel" them ahead, faster than their environment. This force could be the hydrophilic/hydrophobic interactions to which they are subjected [prof. Matthess, pers. comm.). A more plausible explanation would however be that the interactions between the uranine and the saturated porous environment are stronger than suspected. If this is really the case, the interpretation of flow speeds determined using this substance must be questioned, and with them, the delimitation of the protected areas around water catchment plant. A new trial on the site is essential. This trial must be performed with the best bacteriophages (H40/1 and Psf2 for example) and uranine. An excellent way of determining flow speeds is to inject a few litters of Deuterium (heavy water) at the same time. Only in this way will we know if the phages migrate faster than the environment in which they are floating.

§ 5.3 Tracing experiment in the Neuchâtel karst (Lac des Taillères).

5.3.1 Introduction

Desor [1864] had already supposed that the spring waters of the Areuse river came from the precipitations and from the surface waters absorbed by the shallow holes in the Brevine valley. Schart [1904] performed four tracing trials, of which one at the water sink of the Moulin du Lac, that confirmed the Desor hypothesis. The water sink of the Moulin du Lac, also called water sink of the Lac des Taillères, is indeed located in the hydrogeological basin of the Areuse spring. It is the main outlet of the karstic aquifer that contains the synclinal of the Verrières. The catchment basin of the spring covers 127 km^2. From a geological point of view, the hydrogeological basin of the Areuse spring is situated in the folded mountains of the Jura. The aquifer is composed of karstified limestones of the superior Malm that have an average thickness of 300m. The aquiclude is formed by Argovian marls.

The goal of this experiment was not to determine the existing connection between the water sink of the Lac des Taillères and the Areuse spring, since this has already been confirmed several times [Müller and Zötl, 1981]. It consisted first in the examination of the quantitative aspect of the transit of the various tracers in this aquifer. Second, we wanted to establish a comparison between the propagation of the bacteriophages and the best fluorescent tracers to be found nowadays. This tracing experiment was performed within the frame of N. Doerfliger's Ph.D. work. Her work shows the quantitative characterization of various tracer flows in karstic aquifers.

5.3.2 Material and methods

A. Tracers and sampling:
The biological and fluorescent tracers were injected in the Neuchâtel Jura into the catchment basin of the Areuse spring (cf. Fig. 5.3.1), on the 5th of November 1993.
The first four tracers (phages and Sulphorhodamine G) were directly injected at the bottom of the collecting building at a depth of -12.5m. The fifth one, Uranine, was injected at the surface, immediately after the water sink of the Lac des Taillères.

- Phage f1 : $2.15 * 10^{15}$ pfu (8l of culture)
- Phage H40/1: $9.18 * 10^{15}$ pfu (10.5l of culture)
- Phage H6/1: $1.45 * 10^{15}$ pfu (10.5l of culture)
- Sulphorhodamine G extra: 8550g, dilution during the injection in 15'000l
- Uranine*: 5000g, dilution during the injection in 20'000l

*: Uranine certainly covered the first 12m in an unsaturated medium.

The flow at the Areuse spring was almost constant during the tracing trial. It is of about 1.18 m^3/s. No rain was registered during the test. The samples were taken by the means of an automatic sampler (ISCO). The time interval between two samples was of 4 hours.
Frost caused some difficulties. The samples could not be collected over a period of 24 hours, unfortunately when the concentrations (C_{max}) were highest. The next samples were collected by hand.

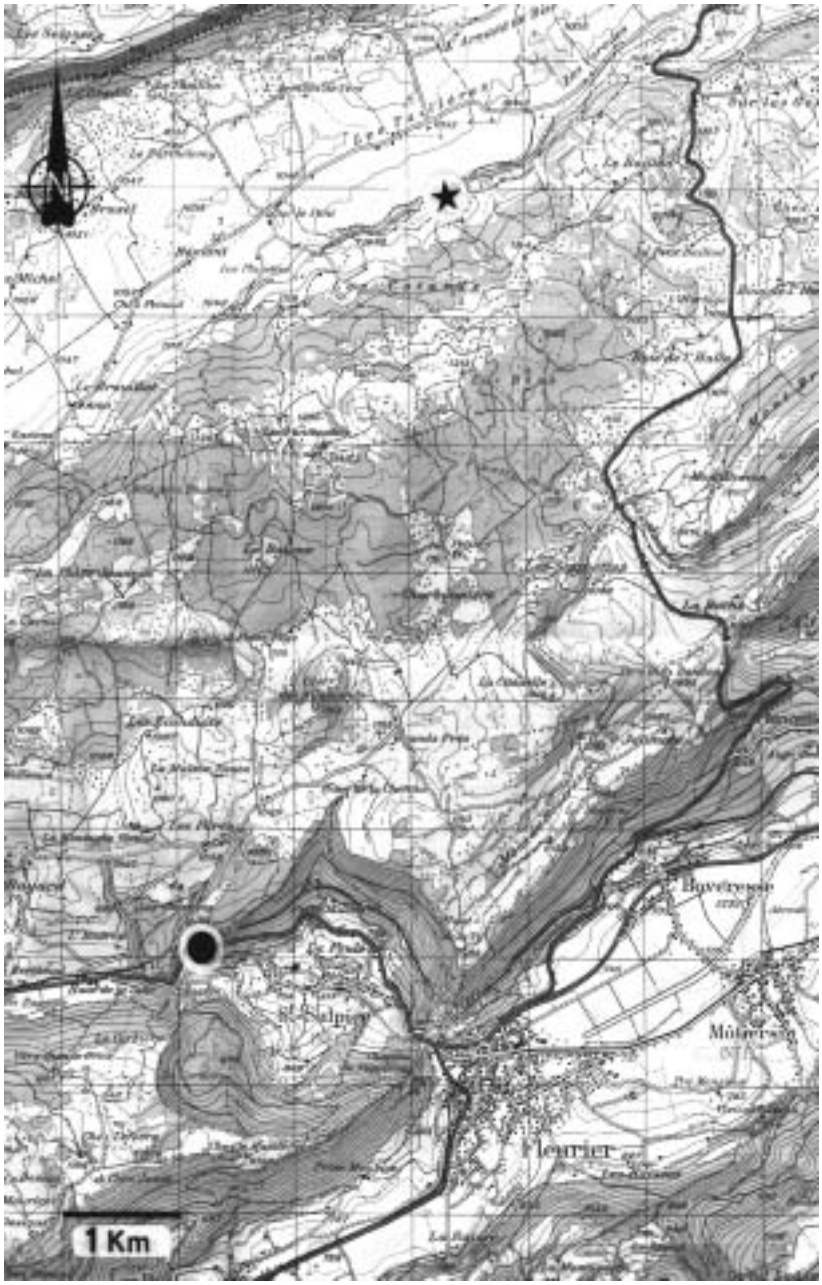


Figure 5.3.1: Situation of the aquifer of the Areuse spring, canton de Neuchâtel, Switzerland: (●) Areuse spring. (★) Injection point (Lac des Taillère water sink)

B. Analyses:

The samples were swiftly transported to the laboratory, sheltered from light in an ice box (at about 8-10° C). The phages analysis is described in chapter 3. The detection limit for this experiment is 1 pfu/2ml. Sulphorodamine G and Uranine analyses were performed at the Centre d'Hydrogéologie de l'Université de Neuchâtel. The detection limits for these fluorescent dyes are of $5^* 10^{10}$ g/l and $1^* 10^{10}$ g/l respectively.

5.3.3 Results and discussions:

Table 5.3.1 sums up the results of this test:

Tracers	Injected quantities	Covered distance	V^* (m/h)	Time 1st arrival	C_{max}	T_{max}	Restitution rates
f1	2.15* 10^{15} Pfu	6250 m	(24.9)	(251 h)	(7 pfu/ml)	344.5 h	0.15 %
H40/1	9.18* 10^{15} Pfu		25.7	243 h	7030 pfu/ml		21 %
H6/1	1.45* 10^{15} Pfu		23.1	271 h	53.5 pfu/ml		1 %
Sulpho. G	8550 g		22.1	283 h	170 * 10^9 g/l		80 %
Uranine	5000 g		22.1	283 h	43.3 * 10^9 g/l		40 %

N.B. V^*: Velocities calculated with the first arrival times. V_{max}, calculated with C_{max}, is about 18.1 m/h for all tracers.

The breakthrough curves of the tracers are presented in figures 5.3.2 and 5.3.3. These curves show important similarities. The general shape as well as the apparition time of C_{max} tally exactly. As for the phages in particular, the curves spread out over three scale units. Phage H40/1 is the one which reached the highest yield concentrations. With a C_{max} of 7030 pfu/ml, the global restitution is surprising. It is the highest quantity we have obtained during our tracing trial in karst. After about twelve days of migration in low water conditions, this phage showed a restitution of 21%. This quantity is comparable with the one obtained for fluorescent dyes. The C_{max} of H6/1 is lower than that of H40/1. Yet this phage presents a breakthrough curve very near the Uranine and H40/1 one for the first 450 hours. The tail of the Uranine curve is certainly due to a remobilisation of the tracer injected into the unsaturated surface medium. The dispersion of H6/1, and of the other phages in general, is distinctively weaker than that of the fluorescent dyes.

The lowest concentrations we measured are those of phage f1. Furthermore, the imprecision of this curve is great. A background level of 1-2 coliphages per ml was occasionally registered, indicating a fecal pollution of the aquifer. The imprecision resulting from this does not allow us to take the arrival time of this biological tracer into account. Moreover the restitution rate must be considered with suspicion too. It is extremely weak compared to the restitution rates of the other phages. Adsorption experiments performed in laboratory (chapter 4) show that this phage has a strong affinity for colloidal clay particles. The adsorbed phage easily looses its virulence.

During a low water period, the waters of a karstic aquifer charge themselves with mineral salts and colloidal particles [Prof. Kübler, CHYN, pers. comm.]. This, by itself, can certainly explain the weak restitution of f1. But it can also explain the variations shown by the two other phage breakthrough curves and not by the dyes curves.

Times of first appearance are different for the two different tracers categories. The detection limit for phages is of 1 phage per 2 ml of sample. Our analytical method allows us a dilution of the order of 10^{15} times. A simple calculation of the fluorescent dyes minimum concentration shows that the dilution possibilities are less than for the phages. The fluorescent tracer is thus certainly present in the first "phage positive" samples. But the classic detection method does not allow to reveal it. In fact these values do not reflect differences in velocity. The migration velocities, V_{max}, are equal for all tracers. The various times of first appearance represent a difference in the dilution possibilities of the tracers, and of course, in the detection limit of the analyses.

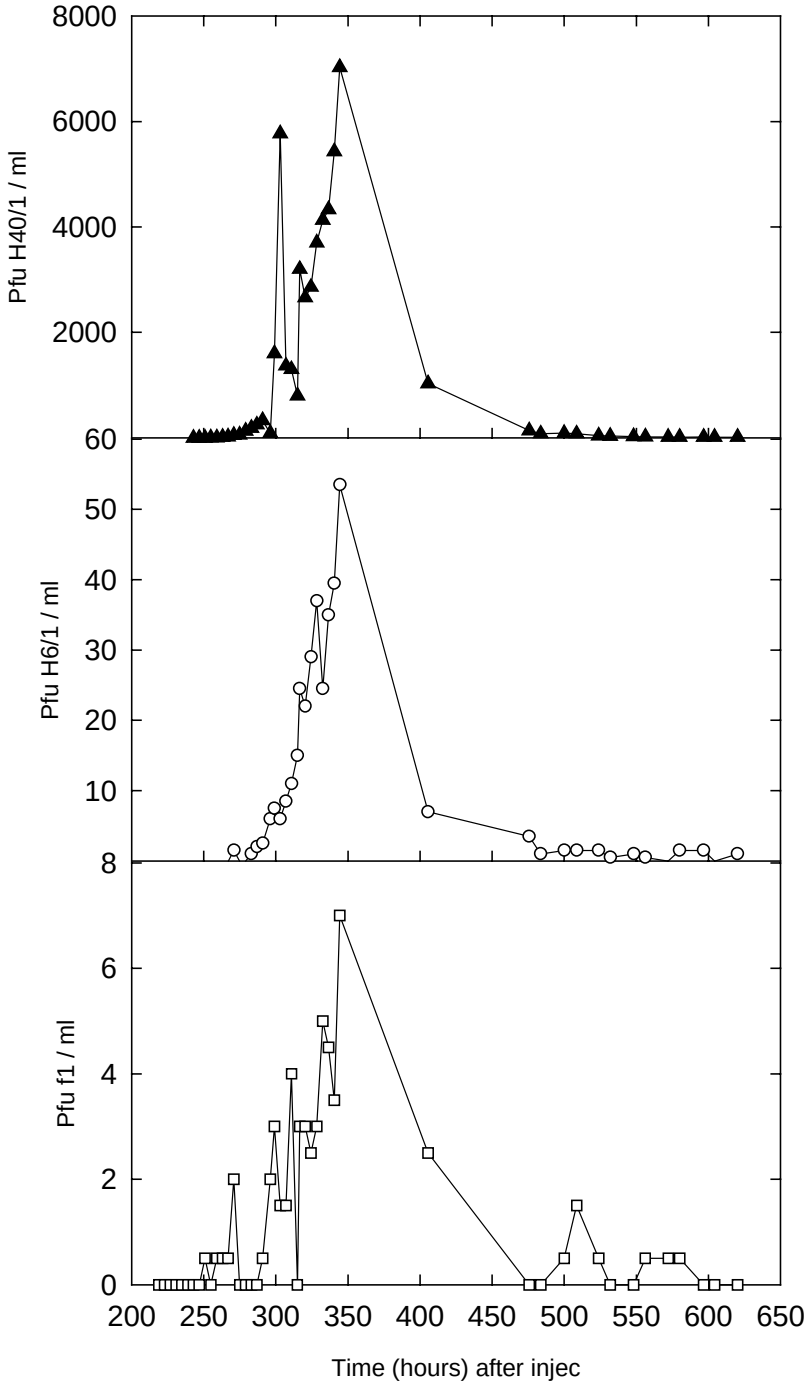


Figure 5.3.2.: Breakthrough curves of bacteriophages H40/1, H6/1 and f1 in the Areuse spring

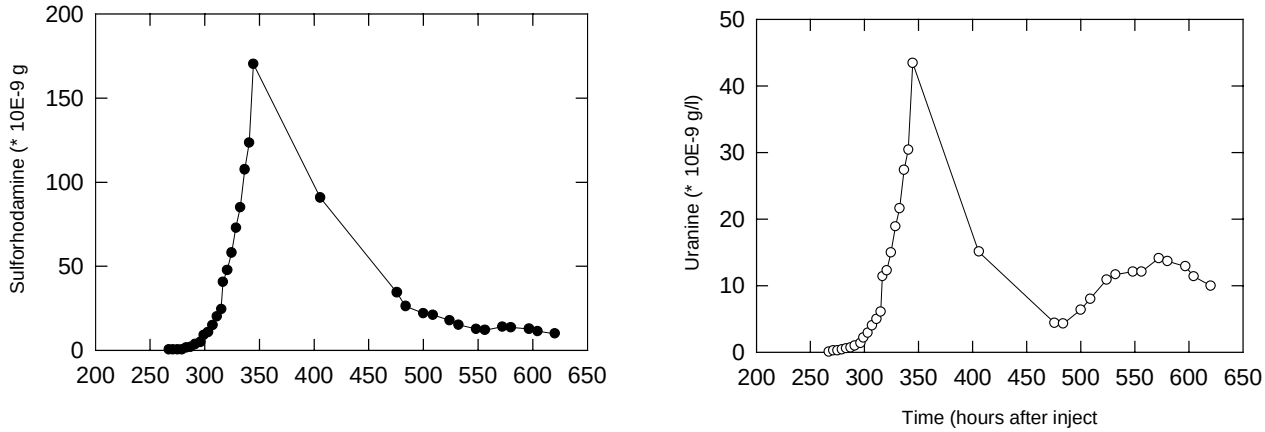


Figure 5.3.3: Breakthrough curves of uranine and sulphorhodamine G extra in the Areuse spring.

5.3.4 Conclusions

The freezing of the samples in the collecting machine did not influence the number of phages in suspension. When the breakthrough curves of the fluorescent tracers are considered, we can conclude that this slow and moderate freezing (about -3/-5 C) did not cause any damage to the bacteriophages.

The similarity that exists between the five tracers is striking. The yield of phage f1 is indeed insufficient to determine the transport parameters in this karst aquifer correctly. The marine phages on the contrary present breakthrough curves that are similar to those of the conventional fluorescent tracers, considered as conservative.

Thus, these marine phages are very promising for this type of tracing experiment. Our results show that it is perfectly possible to use them instead of the classic dyes. The migration velocities, determined using the times of arrival, are often used to estimate the migration velocities of polluting substances. The high velocities that were obtained by the phages question the validity of such estimates.

§ 5.4 Tracing in the karst of the Jura (Buix, Ajoie)

5.4.1. Introduction

This tracing took place within the frame of the 3rd cycle of hydrogeology (University of Neuchâtel) field camp. The alluvial plain of Buix is located in the tabular Jura in Ajoie (Switzerland). The Ajoie is a plateau of the superior Jurassic that progressively plunges towards the north beneath the Sundgau. The city of Porrentruy is situated at the center of this highly karstified plateau.

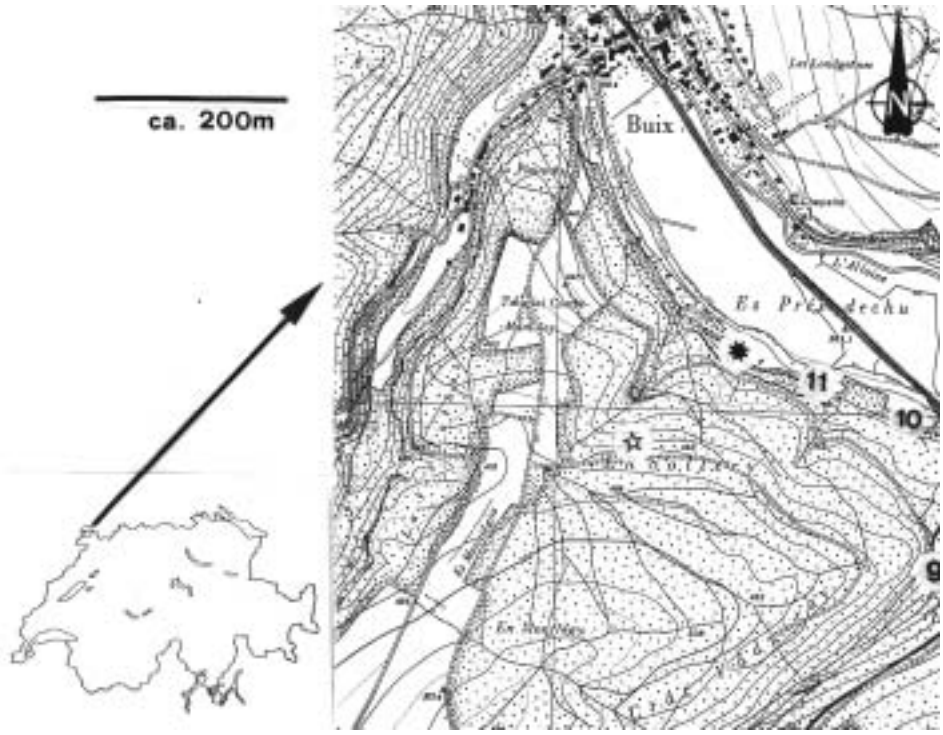


Figure 5.4.1.: Geographical situation of the studied field. (☆) injection site. (✶) spring 12 (Cô tais Hügli) (❍) other examined springs (number 9, 10 and 11)

Many studies have been carried out in this area. They were motivated by the frequent pollution of the springs supplying the village of Buix. This pollution is caused by two main factors:

- by the armoured vehicles exercising on the drill ground of Bure ploughing the soil and increasing the water's turbidity and
- by an organic pollution of the springs due to leaks in the wastewater pipes linking the water treatment plant of Bure to the Allaine river;

A geophysical survey, RMT-EM and RMT-R (cf. chapter 5.1), of the tracing area allowed to determine the geological situation of the underground traversed by the tracers. The geological model obtained from these results is presented in figure 5.4.4.

The goal of this study was to determine the direction of the water flow, from the northern border of the Bure's plateau on. This trial should circumscribe with an improved accuracy the various catchment basins of the multiple springs emerging around the base of the south-west flank of the plain. Furthermore it allows us to compare the biological particulate tracers (bacteriophages) and a fluorescent tracer (Sulphorhodamine G). Some of the results of this chapter are taken from the report written by Grasso and al. [1992].

5.4.2 Material and Methods

A. Injection, infiltration of the tracers and sampling

The following tracers were simultaneously injected at point A (cf fig. 5.4.1 and the photographs on next page) on the 27th of April 1993:

- Bacteriophage f1: $5.2 * 10^{15}$ pfu (8l of culture)
- Bacteriophage Vh5a: $7.8 * 10^{15}$ pfu (7.8l of culture)
- Sulphorhodamine G: 1 kg

The tracers were injected into a 1m deep pit, dug out by a power shovel. $4m^3$ of water were used to push the tracers during the injection. Because of the ground was well fissured, the tracers took only a few minutes to infiltrate. The pluviometry of the area was nil for the 10 days preceding the trial. The samples were taken at the spring number 12 by an automatic sampling machine (ISCO). A sample was taken every second hour on average. The temperature of the collecting spring chamber was constant, about 15° C.

B. Analyses

The samples were swiftly transported to the laboratory, sheltered from light in an ice box (temperature of about 8-10° C). The analysis of the phages is described in the chapter 3. The detection limit for these analyses is of 1 pfu / 2ml. The analyses of the Sulphorhodamine G were performed at the Center d'Hydrogéologie de l'Université de Neuchâtel. The detection limit for this tracer is of $5 * 10^{10}$ g/ml.

Injection site...

Injection of the Sulphorhodamine G...

Inside and outside the collecting chamber of the spring number 12

5.4.3 Results and discussion

The figure 5.4.2, as well as the figure 5.4.4 present the various breakthrough curves of the tracers that reappeared at the spring number 12 (Cô tais Hügli). No other tracer arrival was observed for the other springs emerging at the base of the S-W flank of the Buix plain. Some traces of Sulphorhodamine G were found in a fluocaptor set within the collector of the springs. This corresponds to the arrival of the dye into spring number 12. The distance between the injection point and the spring 12 is of about 300m. The first arrival was identical for all tracers. It happened 4 hours after the injection. The time intervals between each sampling (2 hours) are too wide in this case to detect differences in the arrival times. Thus the migration speeds are apparently identical. The dispersion of the tracer is weak. The C_{max} of the breakthrough curves are distinctly marked for the phages as well as for the dye. C_{max} is attained at T_{max} = 6 hours after the injection.

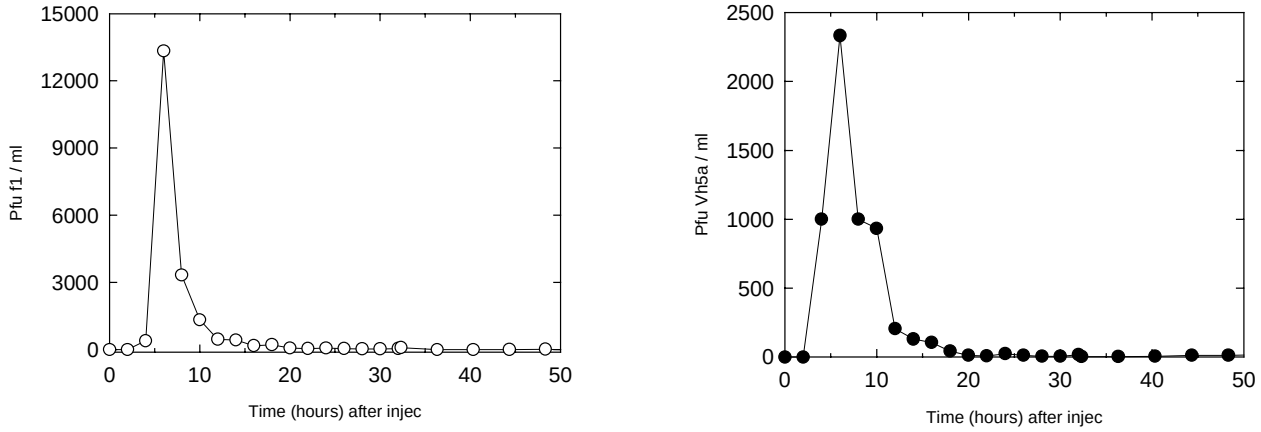
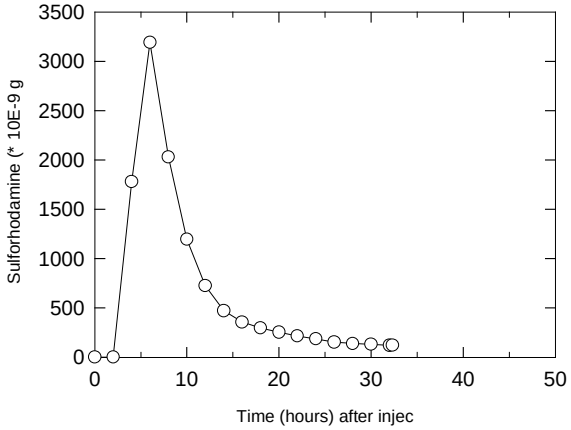


Figure 5.4.2: breakthrough curves of phages f1 and Vh5a in the spring no 12.

Figure 5.4.3: Breakthrough curve of the Sulphorhodamine G in the spring no 12



The search for bacteriophages in the samples taken from spring 9 (Favergeatte) shows a constant background level of 5-8 *E. coli* phages per ml. This background level shows that the pollution of this spring through fecal contamination is important. The chemical (analyses of the nitrates) and bacteriological (counting of enterobacterial colonies) analyses confirm this affirmation (data not shown). This contamination may come from two different sources. As previous works have shown, it may come from the wastewater that flows from the wastewater plant in Bure. But it may also result from the scattering of animal waste on the agricultural lands of the catchment basin. Spring 12 is not affected by these problems. Nitrate concentrations are weak as well.

Geophysical survey (data not shown)

The survey was directed by Prof. Imre Müller. The results are presented in the form of a W-E diagrammatic section of the S-W flank bordering the Buix plain (figure 5.4.5). This survey shows the presence of about 60m of undifferentiated Rauracian limestones. The base (aquiclude) is composed of Oxfordian marls. The thickness of the saturated aquifer in the limestones is very slight. According to the results of the survey, it is about 2-3m. The tracers injected in **A** therefore

traversed more than 50m of unsaturated fissured medium before they penetrated into the saturated aquifer. This migration in unsaturated conditions is certainly responsible for the very low breakthrough levels of the tracers in spring 12. The tracers were certainly trapped by the structure of the karstic network. A high adsorption on the surface of the calcareous walls of the fissures is also possible.

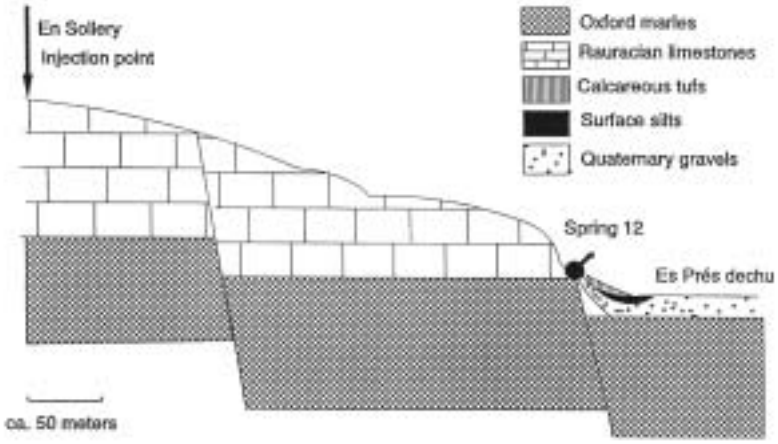


Fig. 5.4.4: W-E diagrammatic section of the studied area. Possible geological connections between The Buix plain and its S-W catchments. Modified following Burger [1975].

Table 5.4.1 sums up the result of this tracing experiment:

Tracers	Quantity injected	1st arrival	C_{max}	T_{max}	Migration speed	Tracers restitution
Phage f1	5.2* 10^{15} Pfu	4 hours	13'000 Pfu/ml	6 hours	50 m/h	0.03 %
Phage Vh5a	7.8* 10^{14} Pfu		2'300 Pfu/ml			0.05 %
Sulphorhodamine G	1 kg		3'200 * 10^{10} g/ml			7.4 %

5.4.4. Conclusions

The interpretation of the tracing experiment depends, as we have shown in chapter 5.1 (Wilerwald first trial), on the geological knowledge brought forth by geophysics. The results of the survey, completed by the chemistry of the water, show that the catchment basin of the spring 12 is limited. It covers mostly forest and very little of intensively cultivated land. The unsaturated medium is equally unfavorable to the migration of bacteriophages and of chemical tracers. The adsorption onto the walls as well as the trapping in the fissures considerably reduces the restitutions. In the case of this tracing experiment, the calculated breakthrough rates show that there are great losses for a 300m migration. For the phage, the loss is more than 99.9%. Nevertheless the breakthrough curves are clear, with peaks in the order of several thousands of viruses per ml. This demonstrated that these bacteriophages, f1 and Vh5a, may also be used in this highly unfavorable medium on short distances.

A few comparisons may be made between phages f1 and Vh5a. The ratio of C_{max} is the same as that of the injected concentrations. The breakthrough rates are almost identical. This short migration does not induce any significant difference between these two phages. A comparison with the classic fluorescent dye, Sulphorhodamine G, shows an obvious similarity of the breakthrough curves. But the restitution rate of the dye is higher. In this case, the kilogram of dye injected colored spring 12 in a very visible manner. Had the collectors of the springs been in service, this substance would have colored the consumption waters of the village of Buix. Bacteriophages would have gone totally unnoticed.

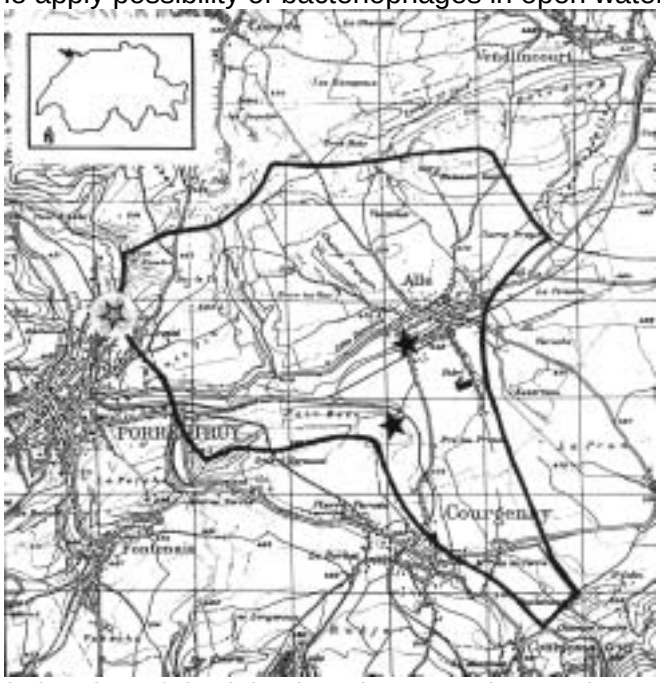
§5.5 Tracing in the karst of the Jura (Porrentruy, Switzerland)

5.5.1 Introduction:

The site called "Noir Bois" (Ajoie) was selected as a dumping ground for inert construction materials. The projected tracing trial had several hydrological goals (see map 5.5.1 and 5.5.2):

- To reveal a possible hydraulic link between the site selected for the dumping ground and the spring of Betteraz (Porrentruy);
- To reveal possible connections between the karstic flows of the "Noir Bois" area and the bed of the Allaine river;

Furthermore, this trial had different microbiological goals. Following the laboratory results, four different phages were injected. The different migration and restitution of the phages Pm7a (*Bacillus subtilis*), Sfd3 (*Streptococcus faecalis*), f1 (*Escherichia coli K12*) and Psf2 (*Pseudomonas fluorescens*) were surveyed. A fifth phage, Vh5a (*Vibrio harveyi*) was injected directly into the river Allaine. This marine BBH was used for the first time in a river. Used instead of a classic dye tracer, this phage will present the apply possibility of bacteriophages in open water courses.



Map 5.5.1: Geographical situation of the injection site. See also on the next page (map 5.5.2) the detailed map of the area surveyed, with the supposed migration paths of the tracers. (▬) Outline of the catchment basin. (☆) Betteraz spring. (★) Injection site.

5.5.2 Geological and hydrogeological context [following Adatte, 1993]:

The studied site is located in the catchment basin of the Betteraz spring that supplies Porrentruy with drinking water. It is located at the eastern extremity of the Banné anticlinal where it disappears beneath the Tertiary sandstone deposits present around Alle, in a depression formed by erosion around an area of dolines. Some outcrops of fine micritic limestone (superior Kimmeridgian) can be observed in the small bottom land at the base of the grave. Underneath these rocks, that are locally of about 50 meters thick and are often fractured and karstified, lies a layer of about 10m of "Scorpion shell marls" or "Banné marls". Karstified limestones of the superior Sequanian and of the inferior Kimmeridgian (more than 80 meters in thickness) are found there. A karstic drainage zone in the E-W direction is suspected in the "Noir Bois" sector. Furthermore the presence of N-S tectonic accidents limits the aquiclude of the "Banné marls" formation.

These marls separate the superior Kimmeridgian limestones from the superior Sequanian/inferior Kimmeridgian limestones, where the karstic Betteraz spring originates. An alluvial watertable is located in the sandy sediments deposited by the Allaine river downstream from the village of Alle. The piezometric measures showed that in high waters the alluvial watertable is here assimilable to a captive layer (piezometric head being superior to the aquifer's top layer). In low waters it has the characteristics of a free nape. The tracing trial was done during a period of average waters for the Allaine river. This corresponds to a flow of 180-200l/sec.

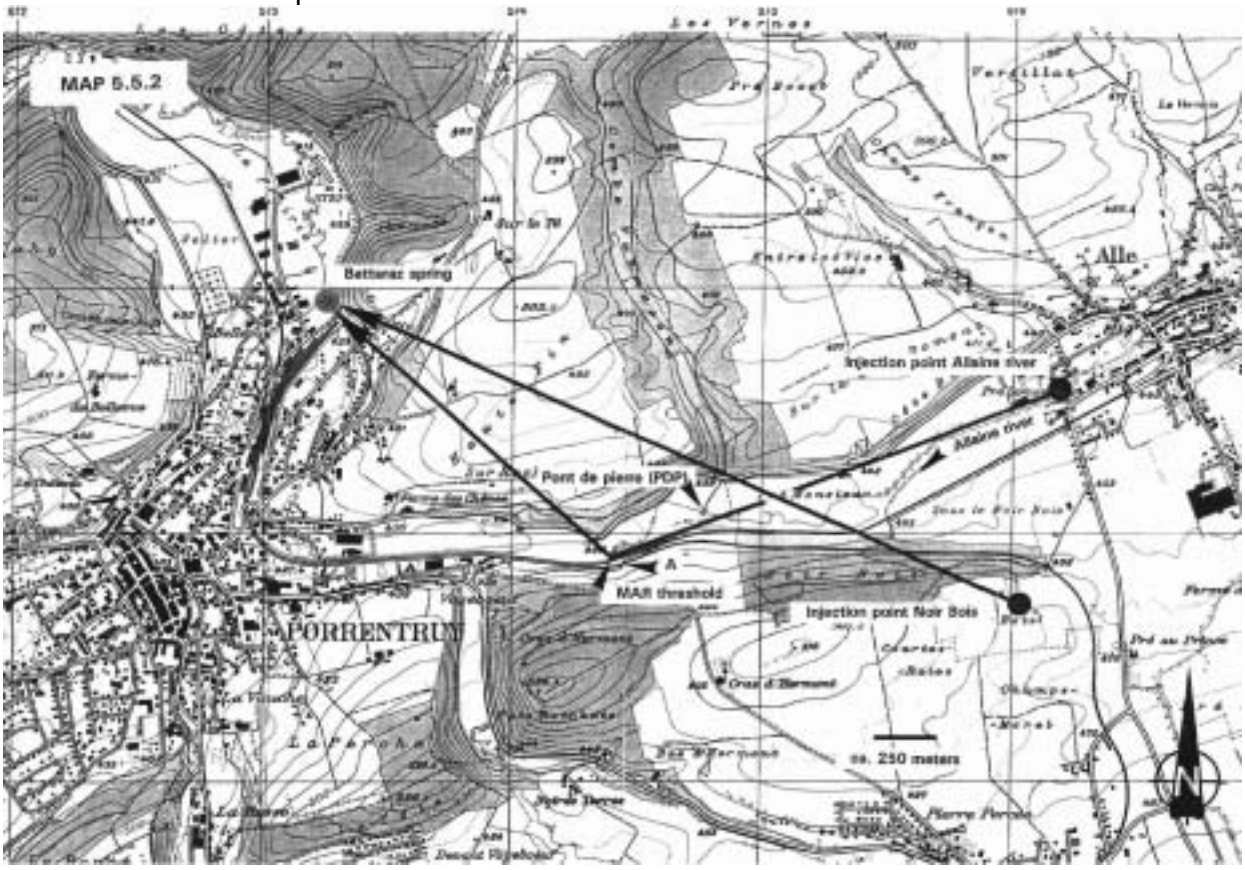


Figure 5.5.2: Detailed map of the area surveyed, with the supposed migration paths of the tracers.

5.5.3 Tracers and sampling points:

A. Tracers:

The tracers were injected on the 21th of June 1993 into a 2m deep excavation that brought the Kimmeridgian limestones to open air. A first infiltration trial was performed with $3m^3$ of water:

- Uranine: 3kg, diluted inside a tank with ca. 40 liters of water
- Phage Psf2: $2* 10^{12}$ pfu, (10'500 ml)
- Phage f1: $2.04* 10^{15}$ pfu (8'000 ml)
- Phage Sfd3: $3.24* 10^{13}$ pfu (10'500 ml)
- Phage Pm7a: $2.6* 10^{14}$ pfu (10'500 ml)

All the tracers were injected together with $6m^3$ of water. The infiltration was slow (ca. 0.8 l/sec.). The last tracer was directly injected into the Allaine river, at the end of the Alle village:

- Phage Vh5a: $1.29* 10^{14}$ pfu (10'500 ml)

B. Sampling:

The sampling points are located on map 5.5.2. Beside the Betteraz spring we sampled the river Allaine (MAR threshold) and a piezometer (point A) with an automatic samplers (ISCO).

5.5.4 Results and discussion on the trial:

The results of this tracing trial are summed up in tables 5.5.1 and 5.5.2. The breakthrough curves of the tracers are presented on the figures 5.5.1 to 5.5.3.

A. Betteraz spring:
The comparison of the phages shows that the best results are obtained with phage Psf2. It migrates swiftly and in great quantities even through the injected quantities are 10 to 100 times inferior to those of the other phages. The adsorption trials performed in the laboratory showed that this phage is adsorbed very little onto colloidal particles. The phage Sfd3 is on the contrary is massively adsorbed. This certainly suffices to explain the mediocre restitutions of this phage at the Betteraz spring. The restitution of phage Pm7a is poor. This is the only trial we made with this phage. The results of phage f1 are spoiled by a permanent background noise of 1 to 4 coliphages/ml. This indicates that the spring suffers an important organic pollution. So in this last case it is impossible to determine the arrival of this tracer correctly.

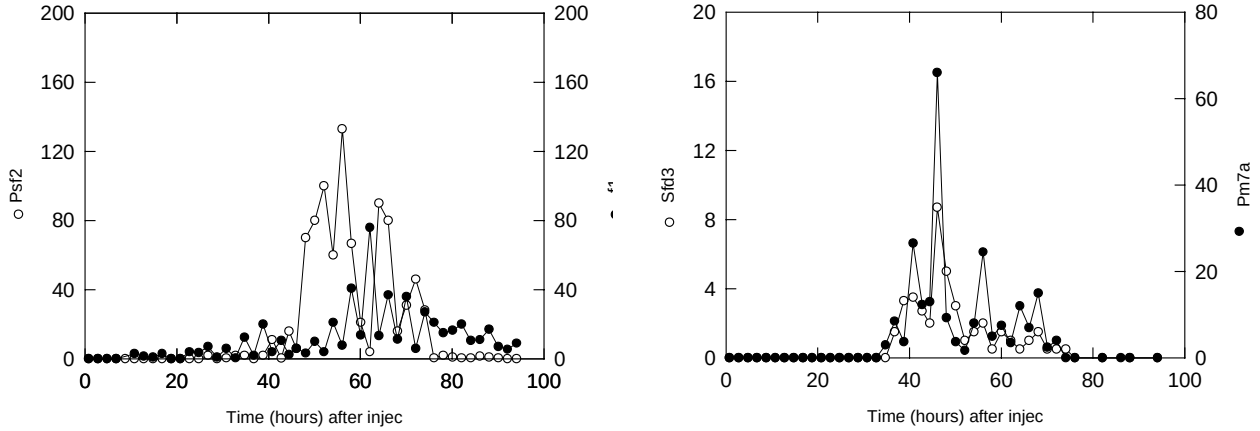


Figure 5.5.2: Breakthrough curves of the bacteriophages in the Betteraz spring (in PFU/ml).

For all phages the time of first arrival seem to be negatively correlated with the maximum measured concentrations ($\mathbf{C_{max}}$). Phage Psf2 has the highest migration velocity, and its $\mathbf{C_{max}}$ is the highest as well. This correlation may be attributed to the dilution phenomena undergone by the various tracers in the subsoil, Since the times of first arrival not only depend on the type of phage but also on the quantity injected. These time values must therefore be considered with precaution.

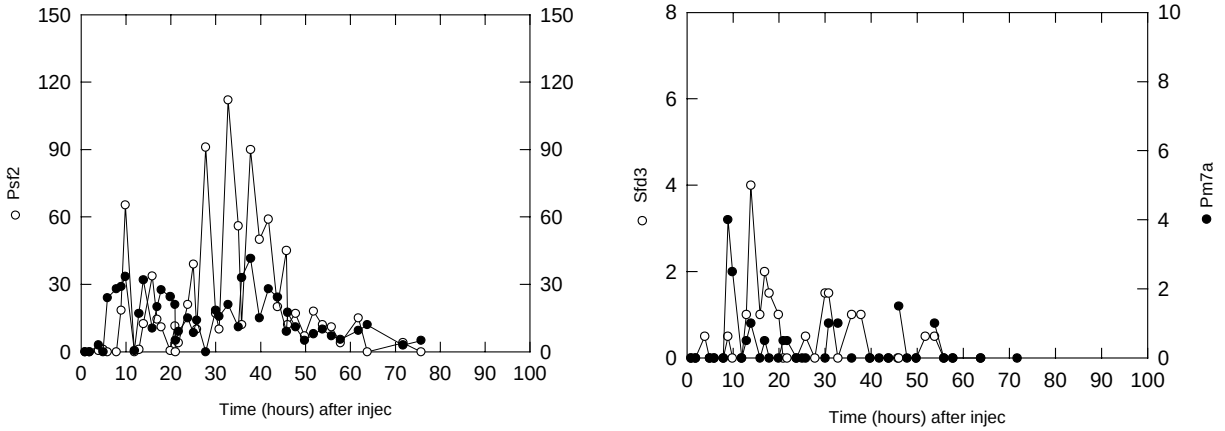


Figure 5.5.3: Breakthrough curves of bacteriophages in the Allaine river (in PFU / ml).

The breakthrough curves of the phages show many fluctuations. Errors during the sample analyze, a rising of the river water level a short time after the injection or else the breakdown of one of the sampling machines are not enough to explain these curves. Part of this behavior is certainly imposed by the beginning of their migration in the unsaturated section of the karst. The phages used in this trial seem to be sensitive to the conditions met during the passage through this medium. The fluorescent dye is relatively unsensitive to the adsorption/desorption phenomena.

The quantitative fluctuations of the recovered bacteriophages do therefore not allow a comparison of $\mathbf{C_{max}}$. This means that these values and those of $\mathbf{T_{max}}$ only give an indicative image and must be considered with precaution too. From what precedes we must admit that in this case the phages only render a qualitative image of the tracers' propagation in the underground. This tracing experiment nevertheless shows that the hydraulic link between the planned site for the dumping ground and the Betteraz spring is fast (94 m/h for the dye and 112 m/h for the phage Psf2). This fast link demonstrates that the tracers certainly proceed through the karst till the spring.

B. Allaine at the "Roches de Mars" (MAR) threshold:

The analysis of the water samples collected in the river by the automatic sampling machine did not reveal any significant concentration of Uranine. Only the presence of a fluocaptor installed at the "Pont de Pierre" (PDP) made it possible to show the hydrogeological connexion between the injection place and the Allaine river. On the other hand the presence of phages in the samples collected in the Allaine river by the same sampling machine proves without any doubt the exfiltration of the aquifer's tracers into the river. From what precedes we suppose that this exfiltration zone is located upstream from the "Pont de Pierre", showing an extremely fast migration. The best phage, Psf2, had a migration velocity of over 330 m/h. Its maximum concentrations were high as well.

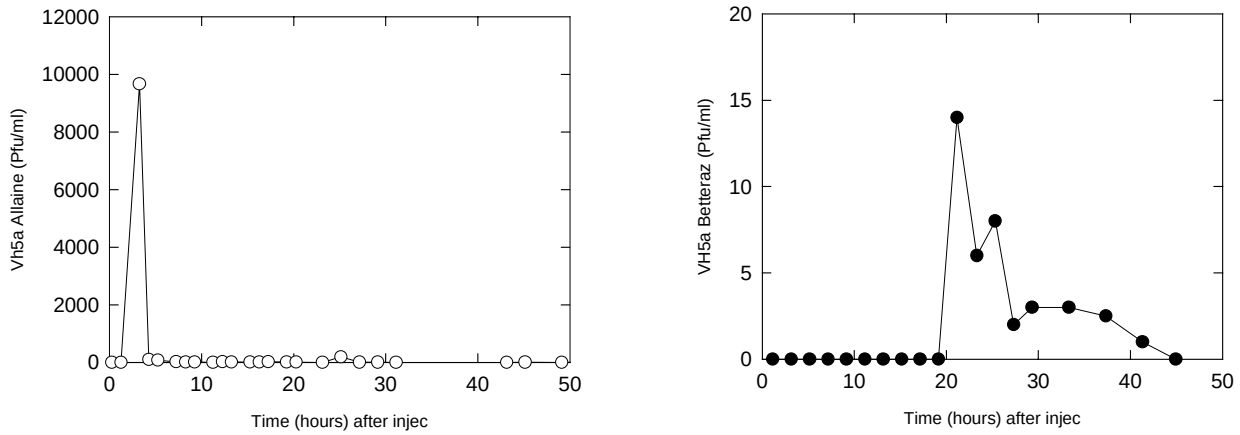


Figure 5.5.4: Breakthrough curves of bacteriophages Vh5a in the Betteraz spring and in the Allaine river (conc. in PFU/ml)

The injection of a fifth bacteriophage into the Allaine river on the same day demonstrates the presence of an infiltration of the river's waters into the aquifer towards the Betteraz spring. The phage Vh5a (a marine phage) was injected at the end of Alle. The figure 5.5.3 shows the breakthrough curves of this phage at both sampling points of the river. The breakthrough curves are clear and without ambiguity. They show low dispersion as well. The maximal concentrations obtained are very high. The results show the arrival of the first viruses at the Betteraz spring after 21 hours only ($\mathbf{V_{max}}$ = 164 m/h). The marine origin of this BBH makes it possible to detect it easily in a laboratory. No background noise disturbs the analyses. Furthermore, few indigenous bacteria are able to grow on salt agar medium. Because of the dilution phenomenon, the phage disappears from the aquifer in less than 100 hours. If needed a further tracing test could have been performed in very short time.

Table 5.5.1: Results of the tracing experiment for the Betteraz spring.

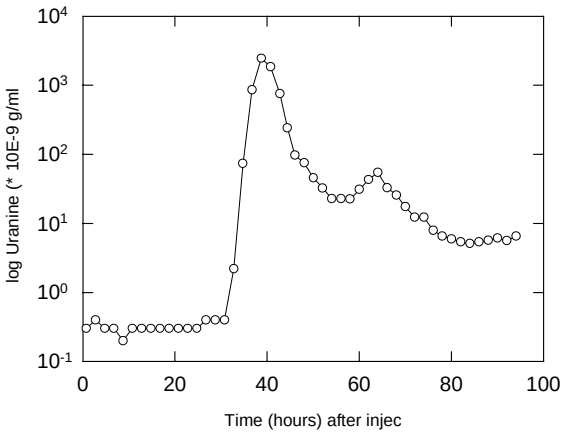
Tracers	1st arrival	V_{max} (m/h)	C_{max}	T_{max}
Uranine	32	94	2,53* 10^7 g/l	39
Phage Psf2	27	112	133 Pfu/ml	56
Phage f1	27	112	76 Pfu/ml	62
Phage Pm7a	35	87	66 Pfu/ml	46
Phage Sfd3	37	82	9 Pfu/ml	46
Phage Vh5a	21	164	14 Pfu/ml	21

Table 5.5.2: Results of the tracing experiment for the Allaine river, threshold MAR.

Tracers	1st arrival	V_{max} (m/h)	C_{max}	T_{max}
Uranine	traces**			
Phage Psf2	5	332	112 Pfu/ml	46
Phage f1	6	276	42 Pfu/ml	38*
Phage Pm7a	14	118	4 Pfu/ml	14
Phage Sfd3	9	184	4 Pfu/ml	14
Phage Vh5a	3.25	594	9667 Pfu/ml	3.25

*: Presence of a background noise; **:Revealed by a fluocaptor

Figure 5.5.5: Breakthrough curve of uranine in the Betteraz spring



C. Alluvial layer of the Allaine river:
No travel was revealed in the water samples taken from the alluvial layer. This fact can either be explained by too great a dilution of the tracers or by an extremely slow flowing of the water and the tracers into the alluvial aquifer. It is also possible that at this place the alluvial layer is neither in hydraulic connexion with the Allaine river between the exfiltration point of the tracers and the sampled point, nor in connexion with the karstic network.

5.5.5 Conclusions:

The tracing showed that the site planned for the Varin dumping ground is in a fast hydraulic connexion with the Allaine river as well as with the Betteraz spring. Considering the shape of the curves, the transfer velocities and the breakthroughs, it is likely that the propagation between the site for the dumping ground and the spring will mainly take place through karstic drains. Only a fraction of the tracers flows through the Allaine river. Because of various breakdowns and overstepping of the calibration curves no breakthrough rate could be calculated for the tracers at

the Betteraz spring. The results of the tracing are thus strictly qualitative. A fraction of the tracers had flowed into the Allaine river by exfiltration. The bacteriophages demonstrated this movement of a water portion towards the river. This transit could not have been revealed with uranine. The few traces that were found in a fluocaptor are not enough to prove this migration towards the river. Thus the phages complete the conventional dye tracer in a remarkable manner. The phage Vh5a used in the river demonstrates the possibilities of marine bacteriophages as tracers in surface hydrology. Its small size enables it to simulate the migration of colloidal particles of about 0.1 μm. Furthermore the use of this virus revealed the infiltration of a section of the watercourse.

Phages are thus a very handy hydrogeological tool for open media. The absence of coloration allows an injection into rivers that flow through inhabited areas without worrying the population. The comparison of the breakthrough curves obtained with the phages and uranine is difficult. The breakthrough curve of this last substance did not follow the fluctuations shown by the phages' curves. Without any doubt these very important fluctuations do not come from the laboratory analysis. It is possible that the presence of chemical organochlorinated substances (2-20 μg/l) in the spring waters produced an irregular degradation of the phages in the samples collected. Despite the qualitative character of the results, a comparison between the various phages remains possible. This comparison shows the indisputable value of phage Psf2. Injected in concentrations ten to hundred tines inferior to the other phages, it still presents the highest breakthrough levels.

§5.6 Delimitation of the infiltration zones of the Allaine river (Porrentruy, Ajoie) by the means of tracing experiments.

5.6.1 Introduction:

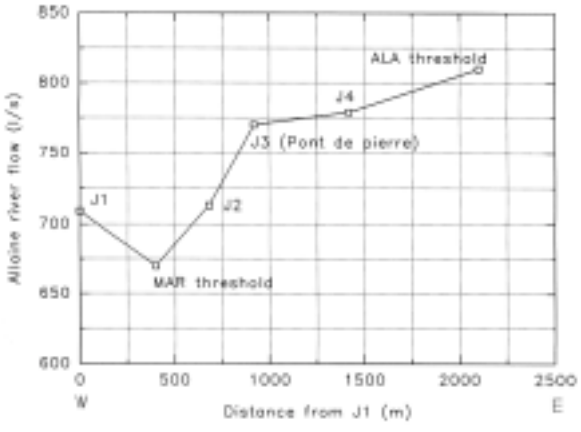
After the tracing of the "Noir Bois" (cf chapter 5.5) we prepared a trial to delimite the infiltration zones of the Allaine river. Other works [Monbaron, 1991] already revealed some losses of the Allaine river between Alle and Porrentruy (cf map 5.5.1). These previous tracing experiments shown that in low waters (flow= 150 to 200 l/s) an important fraction of the river infiltrates the karst towards the Betteraz spring. The preceding tracing trials gave transit times towards the spring of about 20 hours. The goals of our tracing experiment were as follows:

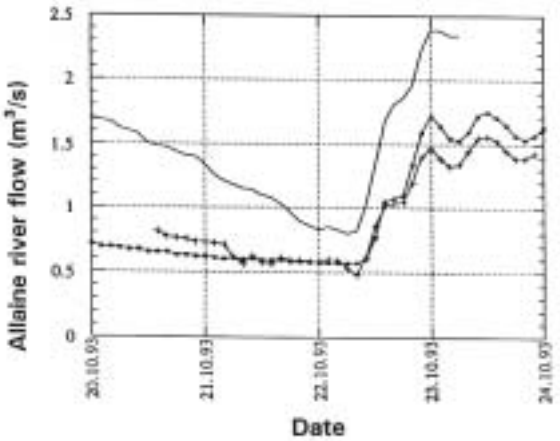
- To locate with the greatest possible precision the downstream limit of the Allaine infiltration zone;
- To determine the presence of one or more infiltration zones;
- To characterize and to quantify the behaviour of the zones under average waters conditions (600 to 800 l/s);
- To compare a conventional tracer, Uranine, with bacteriophages in an open medium;

The tracing trials were carried out along other series of measures on the field. The four injection points were determinated with the help of geophysical measures (RMT-R). Several Jens rod gaugings as well as automatic flow measures at the MAR and the ALA thresholds allowed us to follow the evolution of the river flow. A first trial was performed on the 20th of October 1993. The tracers migrated towards the Betteraz spring with such swiftness that we were caught completely off guard. Thus the first part of the breakthrough curves at the Betteraz spring was lost to us. This is why a second trial was performed a few months later (on the 12th of January 1994).

5.6.2 Revelation of the potential infiltration zones by means of flow measures and geophysical measures:

A. Flow measure of the river:
The hydrological conditions prevailing during the first trial are presented in table 5.6.1 as well as in figure 5.6.1. The flows at the sampling points PDP, MAR, ALA are presented in figure 5.6.2.





Figures 5.6.1 and 5.6.2: Flows of the river during the first injection (20.10.93). Modified from Adatte, 1993. Flow variation at the ALA (——), MAR (·····) and ECO (·····) thresholds. The river flow during the second trial is almost identical to the one observed during the first trial.

The injection was performed under unstable meteorological conditions after two days of dry weather. During the injection the river was in slight subsidence as shown in figure 5.6.2.

Measure points	Flow (m^3/s)
J1	0.709
J2	0.713
J3	0.770
J4	0.779
ALA	0.81
MAR	0.67

Table 5.6.1: Hydrological conditions during the first injection

B. Delimitation of the zone of loss by the means of geophysical methods [Adatte, 1993]:
The geophysical methods used (RMT-R) are indirect study means which allow among other things to quantify the rocks anisotrophy and therefore their fissuration. The sites studied revealed the presence of one preferential orientation zone of N-S phases between the ALA and Mar threshold in the Allaine plain, as well as a second one on the western flank of the Vaumacon combe. The infiltration of a part of the Allaine river waters could originate from this first highly fissurated zone. The zones in question are represented on map 5.6.1.

5.6.3 Nature of the tracers, injection and breakthrough points:

The injection points were determined on the basis of the results presented under point 5.6.2. They are presented on map 5.6.2 and summed up in table 5.6.2 below. The tracers we used are presented there as well. Table 5.6.2 below shows the tracer used in both tests and the corresponding injection points:

Tracers	Quantity	Injection Point
1st Trial		
Uranine	1 kg	IP_A
Phage H40/1	$9.03 * 10^{14}$ phages	IP_A
Phage H6/1	$6.93 * 10^{13}$ phages	IP_B
Phage Vh5a	$8.63 * 10^{12}$ phages	IP_C
Phage Psf2	$3.5 * 10^{12}$ phages	IP_D
2nd Trial		
Phage H40/1	$5.64 * 10^{15}$ phages	IP_A
Phage H6/1	$8.16 * 10^{13}$ phages	IP_B
Phage Psf2	$3.54 * 10^{12}$ phages	IP_E

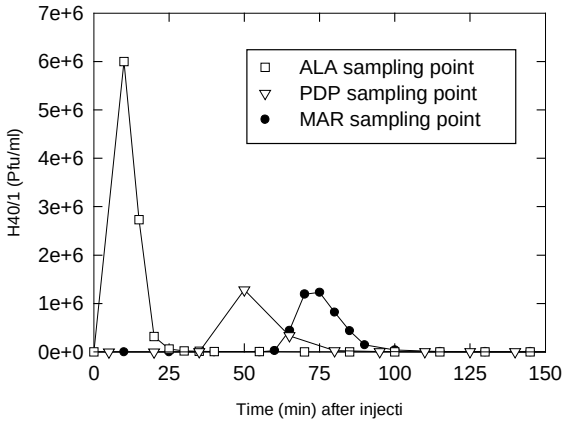
A. First tracing experiment (20.10.93):
Phage H40/1 and the Uranine were injected far upstream of the fracturation zone in order to cover all the supposed infiltration area. Phage H6/1 was injected just upstream of the same zone. The sites for the phages Psf2 and Vh5a were selected so as to reveal the downstream limit of the infiltration zone. These last two phages also allowed us to reveal a possible second infiltration zone. We equipped the river with samplers at three different points (ALA and MAR thresholds and 50 meters upstream from PDP= J3). The figures 5.6.3 (a,b and c) present the evolution of the breakthrough curves of H40/1, H6/1 and Uranine at these various points. The samplers were brought to the Betteraz spring some seven hours after the injection of the tracers. The breakthrough curves of this spring are presented in figures 5.6.4 and 5.6.5.
B. Second tracing experiment:

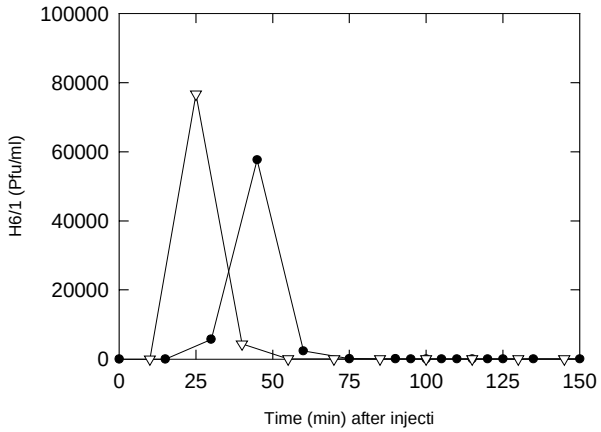
The injection points of the first two phages (H40/1 and H6/1) were identical. Phage Psf2 was injected upstream, just downstream of the fracturation zone revealed by the geophysical means (**IP_E**).

5.6.4 Results of the first injection:

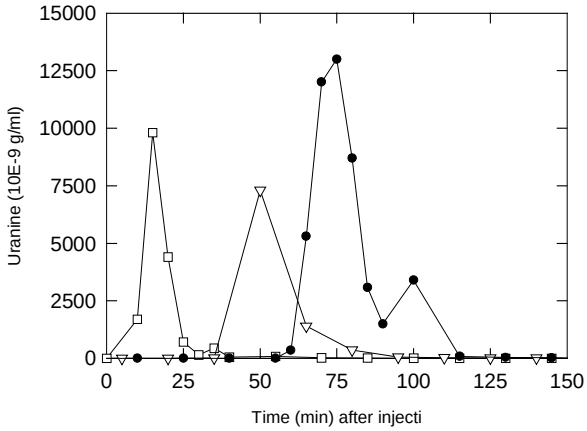
A. Results in the Allaine river:

The tables 5.6.3 to 5.6.5 sum up the results of this trial in the river. These results show that the microbiological tracers and fluorescent one travellled identically. The rises and falls in concentrations were very sudden, over a few minutes in fact. A comparison between the two types of tracers shows that in general the dispersion of the bacteriophages was by far less marked than the dispersion of the Uranine. The brevity of the tracers passage at the sampling points does not allow to obtain breakthrough curves that always include the concentration maxima. This imprecision is passed to the calculation of the tracers breakthrough rates (table 5.6.6). Nevertheless the calculation of these rates shows, at least in the case of the phages, that the losses registered are located within the fracturation zone revealed by the geophysical measures. The phages Psf2 and Vh5a were not analysed in the the samples collected upstream from their injection site.





Figures 5.6.3a,b and c (below): Breakthrough curves of phages H40/1, H6/6 and uranine in the river Allaine.



For the first threshold (ALA), i.e after 510m of migration, the times of first arrival of H40/1 and of the Uranine are equal. T_{max}, i.e the arrival time of C_{max}, the maximum concentration observed, is shorter for the phage. The dispersion of the virus breakthrough curve is lower as well.

Table 5.6.3: Results for **ALA** sampling point: distance from injection point **A**: 510m

Tracers	1st Arrival	V_{max} (m/h)	C_{max}	T_{max} (min)
Uranin	10 min	2040	9.8* 10^{7} g/ml	15
H40/1	10 min	3060	6* 10^{6} Pfu/ml	10
H6/1	-	-	-	-

Table 5.6.4: Results for **PDP** sampling point: distance from injection point **A**: 1220m; from **B** point: 450m.

Tracers	1st Arrival	V_{max} (m/h)	C_{max}	T_{max} (min)
Uranin	35 min	1464	7.3* 10^{7} g/ml	50
H40/1	35 min	1464	1.3* 10^{6} Pfu/ml	50
H6/1	25 min	1079	7.7* 10^{4} Pfu/ml	25

Table 5.6.5: Results for **MAR** sampling point: distance from injection point **A**: 2171m; from **B** point: 1400m

Tracers	1st Arrival	V_{max} (m/h)	C_{max}	T_{max} (min)
Uranin	55 min	1736	1.3* 10^{6} g/ml	75
H40/1	55 min	1736	1.2* 10^{6} Pfu/ml	70
H6/1	30 min	2800	5.8* 10^{4} Pfu/ml	30

When the results of these three tables are considered, the concordance between the values of C_{max}, T_{max} and V_{max} of the phage H40/1 and the Uranine is almost perfect. Nevertheless figure 5.6.3 shows that the dye dispersion is clearly more important. The phages travel in a more concentrated manner and their passage is clearly swifter.

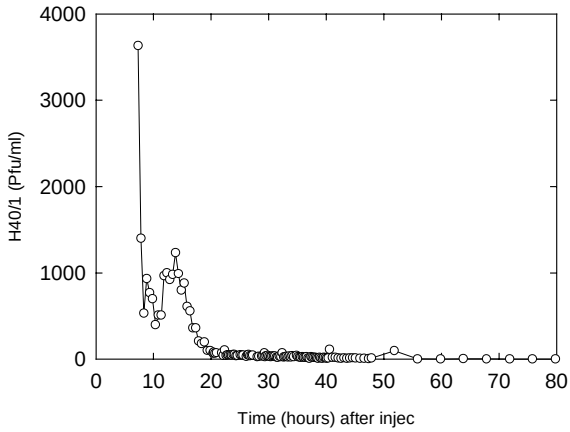
Table 5.6.6: Breakthrough rates of the three tracers in the Allaine river:

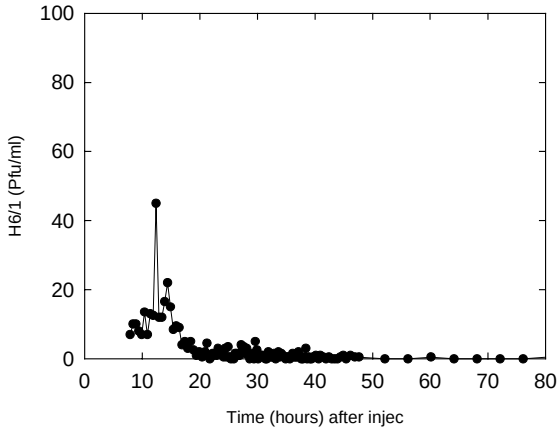
Sampling	River	Total	Restitution	Total H40/1	Restitution	Total H6/1	Restitution
ALA	810l/s	649g	64.9%	1* 10^{15}Pfu	110.7%	-	-
PDP	770l/s	646g	64.6%	1* 10^{15}Pfu	110.7%	5.4* 10^{13}Pfu	77.9%
MAR	630l/s	739g	73.9%	8* 10^{14}Pfu	85.3%	3.2* 10^{13}Pfu	46.2%

B. Results of the first experiment at the Betteraz spring:

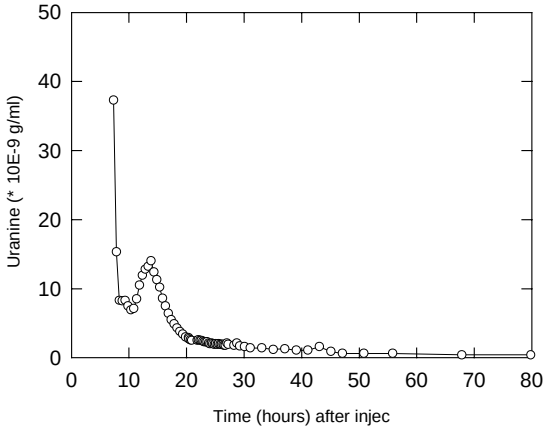
The gauging results show that the river losses are important under average waters (600-800 l/s) conditions. They are of the order of 140 l/s, which represents about 26% of the total flow. Bacteriophages Vh5a and Psf2 were not found again in the samples taken from this spring. We can therefore suppose that the upstream limit of the infiltration zone is defined by the injection point of the phage Vh5a (IP_C).

Only the marine phages H40/1 and H6/1 as well as the Uranine were found in the samples from the spring. The fissurated zone is thus certainly responsible for the river loss. Unfortunately the high transit velocities caught us off guard. The first sample collected, 7.20 hours after the injection, already contains high concentrations of the three tracers. The transit velocities are therefore much superior to those measured in low waters conditions. They are superior to 484 m/h.





Figures 5.6.4a,b and c (below): Breakthrough curves of phages H40/1, H6/6 and uranine in the Betteraz spring.



No calculation of the breakthrough rates is possible under these conditions. The peaks do not allow to specify the number of possible infiltration sites. Only the arrival of the three tracers is significant. Furthermore a comparison between the Uranine and H40/1 shows that these two tracers migrate in an almost perfectly similar manner. 72 hours after the multiple injection the concentrations of the three tracers reached values inferior to the detection limits.

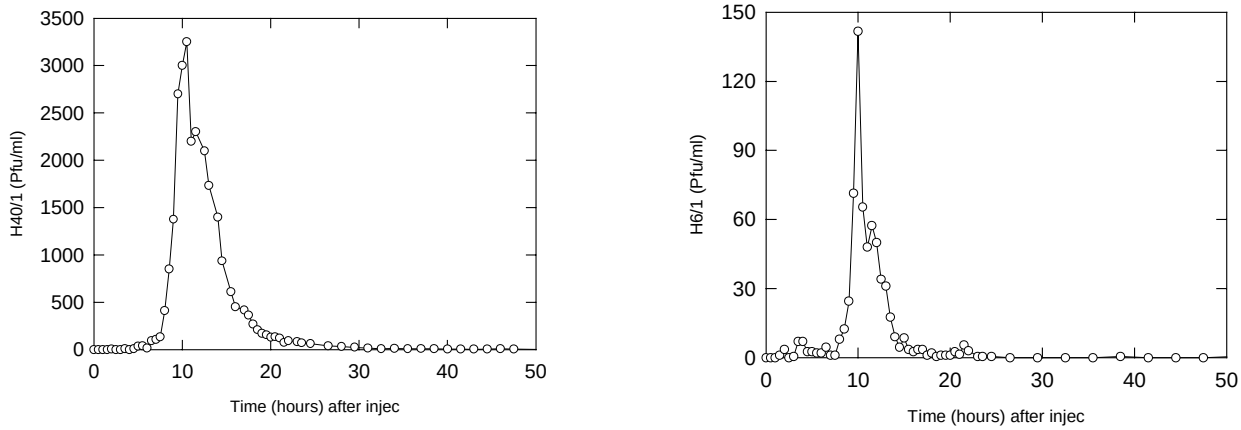
Table 5.6.7: Results for **Betteraz** spring: distance from injection point **A**: 3150m; from **B** point: 2760m

Tracers	1st Arrival (h)	V_{max} (m/h)	C_{max}	T_{max} (hours)
Uranin	< 7.2	> 434	3.7* 10^9 g/ml	7.25
H40/1	< 7.2	> 434	3633 Pfu/ml	7.25
H6/1	< 7.2	> 246	45 Pfu/ml	11.2

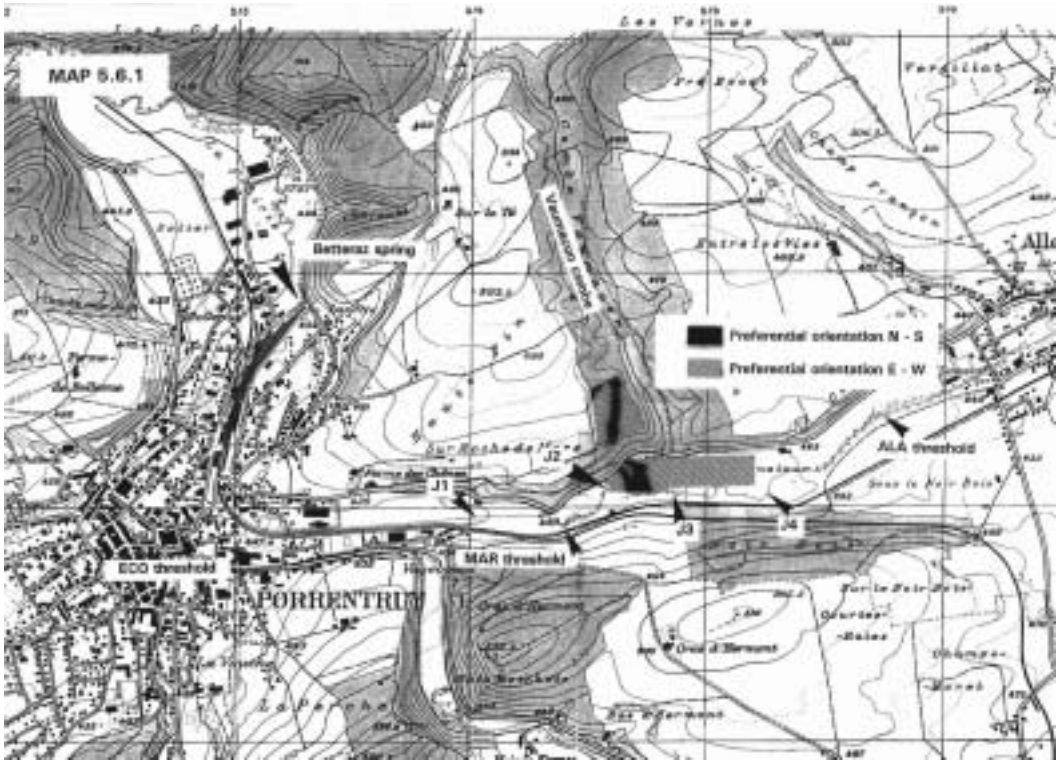
5.6.5 Results of he second tracing experiment:

The injection points of phages H40/1 and H6/1 were the same as those of the first trial. The injection site of phage Psf2 was placed further upstream (**IP_E** point). We could thus mark the downstream boundaries of the infiltration zone more accurately. The results are summed up in table 5.6.8. It was impossible to inject Uranine into the river for a second time - the phages definitely have the advantage of being discreet. Phage Psf2 did not appear in the Betteraz spring. The breakthrough curves of the two other phages are presented in figures 5.6.5. The breakthrough curves of the two phages are clear and present very high peak concentrations (C_{max}). These maxima are related to the quantities respectively injected. The first arrival time of the first phage is

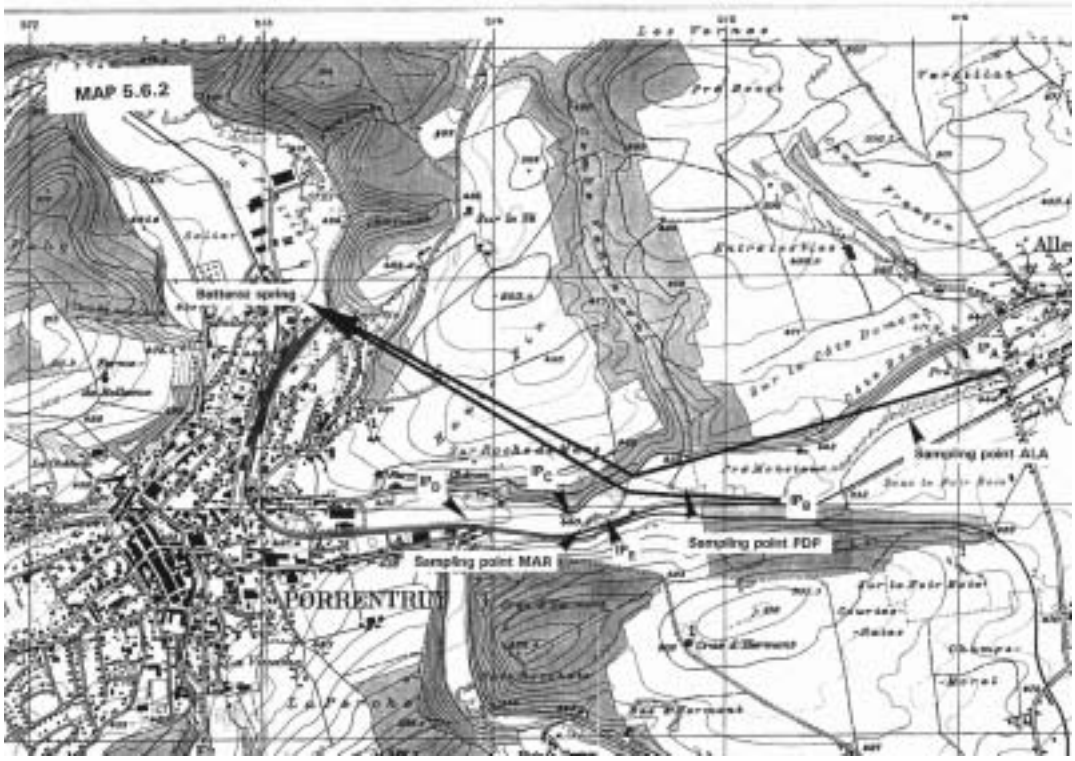
extremely swift, i.e. 3 to 3.5 hours after the injection. The migration velocities in the subsoil were very fast. They were of about 300 m/h. The dispersion of H6/1 was weaker than the dispersion of H40/1. This can be explained by the fact that the first tracer was injected nearer to the fissurated zone. The tracers dilution in the river was in consequence less marked during its passage through the infiltration zone. Yet again, no calculation of the breakthrough rates was possible. The automatic measures at the ALA and MAR measuring thresholds were stopped because of the risk of frost. Furthermore, the water levels registered at the Betteraz spring can not correlated with the taring curves. Therefore these few "incoveniences" due to the maintenance of the equipment did not allow us to determine the various proportions of phages that migrated into the river and towards the spring.



Figures 5.6.5a and b: Second trial. Breakthrough curves of phages H40/1 and H6/6 in the Betteraz spring.



Map 5.6.1 showing the locations of the physical measurements (water flow) and the results of the geophysical soundings.



Map 5.6.2 showing the injection and the sampling locations, as well as the possible flow path of the tracers.

5.6.6 Conclusions:

The utilization of a multidisciplinary approach allowed us to reveal an infiltration zone inside the bed of the Allaine river. The geophysical survey associated to the classic flow gauging revealed the fracturation zone. The two successive trials show that this zone is not warped and that it diverts more than one fourth of the river water.

The infiltrations revealed are clearly superior to those observed during low water periods. The transit velocities towards the spring are more important as well (about seven time faster). The trials we performed revealed the high vulnerability of the Betteraz spring. But this phenomenon can only be revealed by trials performed during average and high water periods. Only important flows make it possible to calculate maximum transit velocities.

The simultaneous utilization of several bacteriophages is an important advantage. The multitracing experiments at various points within intervals of a few minutes allowed for a scanning of the river without inducing any interference during the analysis. This trial confirms the value of phages in surface media. These viruses move about in a way much similar to classic fluorescent dye tracers, yet with a lower dispersion. The comparison with the Uranine is eloquent (at least for the end of the curves at our disposal). They perfectly replace the usual conventional tracer.

The sites of the Betteraz spring and of the Allaine river are equipped with important means that make it possible to record numerous physical parameters in a continuous manner. Sadly, it seems that the maintenance of this equipment is not satisfactory. Many breakdowns, as well as manipulation errors hindered the calculation of the flows. The breakthrough rates were thus often not obtainable.

§6. Final discussion and conclusions:

The use of bacteriophages as biological tracers answers the growing need for environmentally harmless investigation tools. These instruments are necessary, not only to acquire a better understanding of natural systems, but also to estimate the impact of human activities on these systems. The technique we have made available to hydrogeologists was developed through a multi-disciplinary research project. Its aim was not to find a replacement for conventional tracers, but to widen the choice and the possibilities of use.

In chapter 2 we presented an overview of the most recent work on the ecology of bacteriophages and viruses in water. This is a new and developing field. These works show that phages are present in all types of environments, sometimes in great numbers. Though nothing is known as yet about the origin of bacteriophages, it is clear that they are important agents of bacterial evolution [Bø rsheim, 1993].

In this project we studied more than twenty bacteriophage/host bacterium systems (BHB). This number was then reduced through several selections, which concerned not only the phages but also the host bacteria. The requirements concerning the host bacterium are just as important as those regarding the phage itself. There are three for each partner of the BHB system. First, the host bacteria must be totally absent in natural waterways. Their presence would generate a permanent background noise, that is, the natural presence of a small number of phages in every sample. This could seriously perturb the results of a tracing experiment. For example, samples taken in the effluent of a sewage treatment plant will contain a great number of enterobacteria, and, as a result, also of the phages of these species. Second, the host bacterium must be non-pathogenic. In this way one not only considerably simplifies the laboratory work, but also avoids contaminating the aquifer at each injection with organisms presenting an environmental hazard. Third, the growth rate of the host bacterium in a simple medium must be rapid. This last condition simplifies and accelerates the analyses. The three conditions for the selection of a phage are: the phage's resistance to chloroform, its maximum multiplication rate and the appearance of its lysis plaques.

The phages showed a remarkable morphological diversity. While most of them are tailed, one is filamentous (f1) and one tailless (H6/1). Their physico-chemical characteristics are also extremely varied. We selected seven BHB systems obeying all these requirements for the latter part of our work. Not just any phage can be used for the study of waterways. This selection is a very important step which will considerably influence the quality of the following work. It is the marine BHB systems that doubtless present the greatest advantages. They are totally absent from continental (inland) waterways. The analysis of these samples is done on a solid medium containing high salt concentrations (SWA). The growth of the indigenous, non halophilic, bacteria is strongly inhibited. This reduces the errors in the phage counts due to the interference of bacterial colonies. These marine BHB systems can be used for highly contaminated waters, as was the case during our river tracings (chapters 5.5 and 5.6).

In chapter 3, we showed that many different phage analysis and enumeration methods exist. It is important to select an efficient one. It must be possible to analyze many series of samples, rapidly and cheaply. The method must also be precise and reproducible. The most recent techniques, such as PCR, do not answer our needs. We chose an older method, using a double agar-layer in Petri dishes. However this technique, that Adams took up and developed, has several failings. We tried to improve it by adding an extra step before the plating on the Petri dish: that is a contact period in the liquid medium between the phage and its host. This step improves the adsorption of the phages onto the bacteria. In most cases it greatly improves the sensitivity and reproducibility of the analyses. We also adapted the method to the specificity of each BHB system. The use of individual media and buffers, or the adjunction of clay particles greatly increases the number of phages detected. The case of the Psf2/*P. fluorescens* system is a particularly striking example of

the success of these specific adaptations. Our modifications increased by 250% the number of phages detected, compared to results obtained with the classical technique (Adams 1959). Adapting the method to each BHB system is a crucial step towards obtaining reliable and reproducible results. Unfortunately, it is too often replaced by concentration and purification stages before the plating. However, it is on the Petri dish that the actual detection of the phage takes place. This last stage is the one to optimize before any other manipulation is considered.

The development of a reliable enumeration technique made it possible for us to continue our study of the behavior of bacteriophages in water. The observations were made under agitation. The kinetics of these experiments give lots of information on the behavior of viruses in water. The many influences of the environment, as well as of the suspended particles (organic and inorganic) can be observed and compared. The results of these experiments showed that, in a strongly agitated medium, an important part of the phage population is generally inactivated. The disappearance of the phages follows a perfect exponential law. We suppose that the phages are physically degraded when they enter in contact with the hydrophilic-hydrophobic interface of the medium, that is the water-air interface. Only one phage, H6/1, resisted this treatment for any amount of time. However, what we know as yet of its structure or characteristics does not explain this resistance. Apart from agitation, the only other physical parameter which strongly influences the speed of inactivation is the temperature. Its increase speeds up the inactivation. Grant et al [1993] present a complete theoretical and mathematical development of the adsorption of bacteriophages onto sand particles. If many similarities exist between their work and ours, the experimental methods, however, are quite different. After a few trials with sand particles, we chose to work with suspensions of colloidal clay under constant agitation. The reasons for this choice are listed below:

- The samples containing phages and colloidal clay can be centrifuged. In this way it is quite simple to determine the fractions of suspended, adsorbed or inactivated phages.
- The quantity of material necessary for these experiments is much smaller (a few mg/ml). The reaction kinetics are much faster, since the specific surface of the suspended material is very high.
- The influence of diffusion is reduced by the agitation.
- The agitation of the sample, as for inactivation experiments, accelerates the inactivation. This type of experiment only lasts a few hours, as opposed to the 5 to 6 days that a classical batch experiment takes.
- The physico-chemical parameters of the media can be modified at all times during an experiment, just as the relative concentrations of substrate and bacteriophages.

Depending on the types of colloid or phage, the adsorption will result in a rapid inactivation of the virus, or instead efficiently protect it from physical destruction. Our many experiments show that colloidal particles may considerably modify the behaviour of bacteriophages in water. Without reviewing them in detail, the results of the experiments of chapter 4 nevertheless show that every phage reacts differently. These experiments show that it is difficult to generalize the behaviour of a particular virus. It is in any case impossible to use randomly selected bacteriophages to model the reactions of vertebrate viruses with colloidal clays. At most, as Grant et al. [1993] did, one can identify different types of behaviors. Grant's theoretical model predicts that a dynamic equilibrium establishes itself between the adsorbed and freely suspended fractions of phages. The adsorption is not considered to be definitive anymore. The dynamic equilibrium is reached very rapidly. It depends on several physical and chemical factors, such as the pH of the medium, the relative concentrations of its constituents, the presence of organic matter, etc. Grant's theoretical model predicts three possible types of adsorption reactions. We observed all three types in the course of our experiments; they will be discussed further on. This therefore confirms Grant's hypotheses.

The adsorption and inactivation reactions of the bacteriophages in the laboratory were compared to the results of the first tracing experiments performed with these same phages. For this we used the experiments with Montmorillonite particles. It is clear however that the limited number of adsorption experiments restricts the validity of the comparison. It is only possible to predict the behavior of a particular phage in an aquifer. The adsorption experiments of the phages Psf2, H40/1 and H6/1 show that they react only slightly with colloidal Ca-Montmorillonite. Following Grant's model, their reaction with suspended particles are of the **QEA** and **QEARI** types. The **QEA** phages (H40/1 and Psf2) are hardly adsorbed at all and their inactivation, due to the agitation, is slowed down only slightly. With the **QEARI** type of phage (H6/1), losses are minimal. The rest of the experiment showed that the speed of inactivation was very close to that of the simple inactivation experiment. The tracing experiments presented in chapter 5 always show high restitution levels for these three phages. Based on the preceding discussion, we can suppose that they react very little with the suspended particles and other materials encountered in the course of their underground travel. So we can conclude that these three phages are usable in all conditions, in surface waters, in karst or in porous environments, even in the presence of high concentrations of colloids. These three phages are certainly the best ones we have for the study of underground and surface waterways.

Bacteriophages T7 and Sfd3 have **QEARI**-type behaviors. During the first minutes of the experiments, a variable proportion of these phages is inactivated or definitely adsorbed onto the substrate. The initial loss is more important than in the **QEA** types of reactions. After however, the inactivation is slowed down a lot. The restitutions of phage T7 were never as high as those of the three phages mentioned above. A higher proportion is lost during their underground migration. This phage must react more strongly with the walls and colloids of the aquifer. A lot are definitely adsorbed and disappear. So this phage can only be used in waters with few suspended colloids on over short distances. The last type of behavior we encountered, the **QEASS** type, is characteristic of phage f1. The presence of mineral colloids seems to accelerate its inactivation. This phage only gives good results over very short distances and in waters very little charged with colloids. This type of behaviour is much less satisfactory, and the use of this phage much more restricted.

As opposed to Yates et al. [1985], we do not think that the behavior of bacteriophages in water, and of all viruses in general, is mainly influenced by the temperature of the environment. Our many inactivation and adsorption experiments, and their confirmation by the tracing experiments, show that colloids play an essential role in the survival and propagation of phages in aquifers. Their presence, even at extremely low concentrations, will considerably modify the reactions of the bacteriophages. Furthermore, as shown by Corapcioglu and Jiang [...], "*colloidal particles or dissolved organic matter can act as carrier to enhance the transport of a contaminant in ground water, by reducing retardation effect*". The bacteriophages taken up by the floating colloids interact less with the structure of the aquifer. In this way they can be transported both massively and more rapidly.

Our tracing experiments with bacteriophages generally included the injection of a classical fluorescent tracer (Uranine or Sulphorhodamine). This simultaneous injection made it possible to draw a few interesting comparisons. In a karstic environment, as the trial in the Lac des Taillères (see chapter 5.3) showed, the migration of the two types of tracers (biological and chemical) is identical. The restitutions curves are very similar and the T_{max} identical. The only difference is in the first-arrival times: the phages generally appear faster than the fluorescent dyes. As discussed in chapter 5, this is probably due to the higher possibilities of dilution of the biological tracer. The detection limit for the bacteriophages is very low: one single phage in 2 ml of water already gives a positive signal. Because of this sensitivity, the restitution curves of the phages are often less regular than those of conventional tracers. With them, an analysis is positive only when a sample contains several thousand molecules per millilitre. The restitution signal is therefore somewhat smoothed by this abundance. A small fluctuation of the number of molecules will have only a very minor effect on the shape of the restitution curve.

Large differences between the two types of tracers appeared in the experiments in a porous environment. As shown in chapters 5.1 and 5.2, the phages migrate faster than the conventional tracers in saturated environments. The C_{max} of the phages are reached much faster than those of the conventional tracers. The delay can be very important. The trial we have planned for October 1994 should give us more information on this subject. We are planning to simultaneously inject several bacteriophages, Uranine and Deuterium on the Wilerwald test field. The injection of the isotopic tracer will make it possible to compare the real flow velocity of the waters with the migration speed of the two types of tracers. The use of bacteriophages in open environments is very promising. Besides going completely unnoticed, the dispersion of the bacteriophages was much smaller than that of Uranine. The time of C_{max} was identical for both types of tracers. As in the karstic aquifers, the first bacteriophages were detected before the dye. It is certainly in such open environments that phages are most susceptible of definitely replacing the chemical tracers. At the end of this thesis, we are able to offer a biological method which is perfectly adapted to the needs of hydrogeologists. This method will significantly contribute to the study of waterways in general, or the solution of problems related to the circumscription of catchment basins and to the protection of springs and collectors. It also offers possibilities for the study of reactions between different types of colloids. In short, the biological tracing method developed in the course of this work has the following advantages:

- The phages are non-pathogenic, non-toxic and invisible. So this method can also be used for studying drinking water (springs, reservoirs, etc).
- Each phage generally attacks only one bacterial species. By a careful selection of the BHB systems, any effect on the aquifers microflora can be avoided.
- No background noise exists in the aquifers, since they phages are not found there naturally. None will be generated or will persist over time, because of their short life span.
- The analysis of the samples is fast and cheap. Only a few milliliters of water are necessary for the enumeration of the phages. The detection level of the routine-analysis technique is about 1 phage per 2ml of water. This sensitivity is comparable, and most often superior, to that of the best fluorescent tracers. If need be, the sensitivity can be lowered to 1 phage per 10ml.
- It is also possible to differentiate and count a mixture of phages in a single sample. Theoretically, bacteriophages offer unlimited possibilities of multitracings.
- Ten to twenty liters of phage culture are necessary for one tracing experiment. Such an amount is easily transportable, even to inaccessible injection sites. It will contain 10^{14} - 10^{15} phages, which represents in all about 1 gram of protein and a few grams of mineral salts and of various organic substances (amino acids, growth medium components). The influence on the aquifer will be negligible, even if the flow is small.

The tracing experiments already completed have shown that several problems remained to be solved. The most urgent is certainly the conservation of the samples containing the phages. The use of bacteriophages resistant to inactivation make it possible to preserve them at 4 C for more than 24 hours, without any observable degradation. But this is still insufficient. However, the accidental freezing of some samples during the trial in the sources of the Areuse river indicated that a slow and moderate freezing of the samples (-2 to – 5° C) may offer a good solution to this problem.

Despite the many laboratory adsorption and inactivation experiments, we still know very little about what really happens inside the aquifer. This work has defined the different interactions between the phages and their environment better. However, as regards the behavior of phages in waters, we still have not progressed further than the conceptual model. So as to understand and better quantify the propagation and survival of viruses in water, future experiments must take into account the various parameters described in the preceding chapters, and particularly the presence of colloidal particles.

Further field trials must also be accompanied by the injection of one or more conventional tracers as references. These simultaneous injections will offer interesting comparisons between chemical and particulate tracers, but will also allow to define the limits of application of the biological method. The good results obtained with bacteriophages in all the environments tested, even in porous media considered to be difficult, show that these viruses are perfectly adapted to the study of underground or surface waterways. We are convinced that the future of this tracing technique with bacteriophages is promising, above all because it can significantly contribute to the protection of our environment.

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Annex 2.1 : Preparation of solutions and materiel for §2.

Preparation of the collodium solution:
0.5% of collodium in amyl-acetate. Place a few drops in a wide-necked flask containing distilled water and wait till the collodium forms a fine pellicle on the surface. Using tweezers, dip the copper screen into the water and slowly raise it horizontally to the surface, so as to deposit a light film on the top of it.

PBS buffer: (saline phosphate buffer)

8.0g NaCl purum
0.2g KCl
1.44g Na_2HPO_4
0.24g KH_2PO_4
ajust the pH to 7.4 with HCl conc.
ad 1000ml

PH buffer:	K_2HPO_4	0.86g
	KH_2PO_4	0.54g
	NaCl	5g
		ad 1000ml
	plus, autoclaved separately:	
	sol.10^{-4} M $CaCl_2$ * 2H2O	0.16ml
	sol.1 M $MgCl_2$ * 6H2O	2ml
SM buffer:	Tris-HCl pH 7.5	50mM
	NaCl	100mM
	$MgSO_4$	10mM
TBE 10x buffer:	Tris-base	108g (purum)
	boric acid	55g
	EDTA 0.5M pH 8.0	40 ml
TE buffer:	Tris-HCl pH 8.0	10 mM
	EDTA	0.1 mM

Annex 2.2 : DNA-extraction protocol:

This protocol is based on the one for extracting DNA from phages lambda and M13, after the methods described in "Molecular cloning", Sambrook (Maniatis) et al., 2nd ed., 1989. The phages were produced following the method for producing phages on Petri dishes, eluting them with a buffer.

Protocol:

1. Add 5ml of SM buffer onto the Petri dish. Incubate a couple of hours at room temperature, shaking them slightly from time to time..
   N.B.: To increase the production, a few drops of chloroform may be added to the buffer. This frees the phages still inside the cells.
2. Collect as much buffer as possible and place it in a centrifugation tube: 30ml Corex tubes, SS34 rotor, Sorvall RC-5B.
3. Wash the Petri dish again with 1ml SM buffer. Incline the dish so as to collect as much buffer as possible. Let is rest for 15 minutes.

4. Collect the rest of the buffer and add it to the preceding fraction.
   N.B.: A this stage the phages may be stored in a 5ml polypropylene tube, with 0.3ml chloroform. Store at 4° C.
5. Centrifuge at 10'000rpm, (about 12'000g) for 10 min.
6. Add to the supernatant: 10μg/ml DNAse I
   10μg/ml RNAse
   Incubate at room temperature for 30 minutes.
7. Add 5% (v/v) chloroform (about 250 l). Vortex. Centrifuge at 10'000 rpm, for 10 min. Place the supernatant at 4° C.
8. Collect the supernatant and add an equal volume of chloroform. Mix the two phases well with a Pasteur pipette. Centrifuge at 10'000 rpm, for 10min.
9. Collect the supernatant and add a half-volume of a 20% Polyethylene glycol (Peg 8000), 2.0 M NaCl solution (final conc. 10%). Place on ice and allow to precipitate for at least an hour.
   N.B.: For solutions containing fewer phages, first add NaCl 1 M (final conc.). Incubate for one hour at 4° C. Then add the PEG, and allow to precipitate for 1 hour.
10. Centrifuge 20 min at 10'000rpm. Discard the supernatant. Centrifuge again at 10'000rpm, for 5 min. Discard the supernatant.
    N.B.: PEG is a powerful inhibitor of restriction enzymes.
11. Resuspend the pellet of phages in 500μl de SM buffer.
    N.B.: Clean the walls of the tube with particular care, particularly if it is old, because the phages easily attach onto it. The phages can then be stored for several weeks at 4 C, without to much degradation occurring.
12. Add 5μl of a 10% SDS solution, 5μl 0.5 M EDTA. Incubate 10 minutes at 65° C.
13. Extract once with phenol-chloroform.
14. Extract twice with chloroform.
15. Add 1/10th volume sodium acetate 3M, pH 5.5, and two volumes ethanol 94% (ethanol at –20° C). Place à 30 minutes at -20 C.
16. Centrifuge the Eppendorf tubes for 5 minutes at room temperature. Wash the pellet with 75% ethanol. Dry gently turning the tubes upside down on paper. Eliminate the last droplet with an elongated Pasteur pipette. Avoid touching the pellet. Let dry.
17. Resuspend in 2 to 50 l TE buffer, depending on the size of the pellet.

Annex 2.3 : Electrophoresis of phage DNA on agarose gel:

Agarose is a polymer whose basic structure are a D-galactose and 3,6-anhydro-L-galactose molecule. The agarose obtainable is not pure. It contains polysaccharides, salts and proteins in variable concentrations, depending on its degree of purity. The reference used for the gels is phage lamda DNA digested by the Hind III restriction enzyme. Eight DNA fragments are obtained, varying between 23'130 bp and 125 bp in size. The DNA is visualized with ethydium bromide under UV light.

Gels and loading buffers:

TBE 10x: electrophoresis buffer, concentrated 10x

Tris base	108g
Boric acid	55g
EDTA 0.5 M pH 8.0	40ml

Adjust to 1000 ml with distilled water

BBP-XC: colorant and gel loading buffer

Glycerol 87%	2.7 ml	
TBE 10x		0.3 ml
EDTA 0.5 M pH 8.0		1 ml
Bromophenol blue		
Xylene cyanol		

N.B.: For the colorants dip a small dry spatula into the powder. What adheres to it is generally enough for 4-5 ml.

Agarose gel: Agarose type V (Sigma) 0.8% - 1.2% diluted in TBE **1x** buffer

N.B.: Clean the comb well and rinse with 70 alcohol before pouring the gel. For the "Wide mini-sub DNA electrophoresis" tank from Bio-Rad, the amounts are the following:

Gel: 80 to 100 ml (depending on the desired thickness)
TBE 1x electrophoresis buffer: 600 à 650 ml
5 l of a 10mg/ml solution of Ethydium bromide

Enzymatic digestion:

1) Digestion of the samples:

Sample:	5 µl
Buffer:	3 µl
H_2O:	21 µl
Enzyme (5-15 U):	1 µl
	30 µl

N.B.: 1 U (1 Unit) corresponds to the quantity of enzyme necessary to digest 1 µg of DNA in 1 hour, at 20° C.

2) Incubation : 1h30 to 2h00 at 37° C. N.B.: Check the specific temperature for each enzyme.

3) Then add 5 l of BBP-XC. Mix well and carefully fill the gel pockets.

4) Start the gel (4 Volt/cm) : 100-125 mA, 50 à 70 Volt.

N.B.: The DNA is negatively charge and migrates towards the positive pole!

Reference:

1) Preparation:

phage lambda DNA:	100 µ
10x Hind III buffer:	20 µl
H_2O :	77 µl
Hind III (20 U/ l):	3 µl
	200 µl

2) Digestion : 3h00 at 37° C.

N.B.: 2-3 µl are largely sufficient for one experiment. Add 6 µl of BBP-XC and load the gel.

Annex 2.4: Electron microscopy of bacteriophages:

Negative coloration with uranyl acetate after S. Lüther (ETHZ).

Prepare shortly before observation:
Cover a 400 mesh copper screen with a collodium film (annex 2.1). Make the collodium surface adhesive in oxygen plasma (optional step)

Fixation and coloration of the bacteriophages:

1) Place a drop of the phage suspension (as concentrated as possible) onto a Parafilm square.
2) Skim the screen (collodium side down) over the droplet. Incubate 5-20 minutes.
3) Prefix 5 minutes in a 0.5% solution of glutaraldehyde (in PBS buffer!).
4) Wash in the phages suspension buffer or in PBS buffer.
5) Colour in a 2% solution of uranyl acetate (in bidistilled water), for less than a minute.
6) Pump up the surplus of uranyl uniformly, using a filter paper.
7) Air dry for about 5 minutes.
8) Observe.

Annex 2.5: properties of bacteriophages:

Bacteriophage: *H6/1*
Family (genus): *Leviviridae (levivirus, E1 morphotype)*
Dimensions: *30nm*

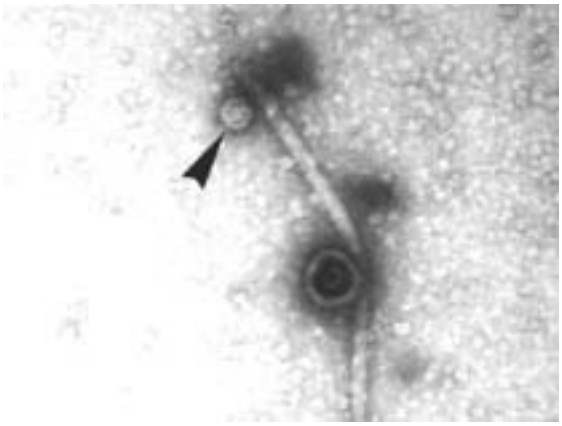


*Figure 2.6.1 : Phage H6/1 showing the E1 morphotype (Uranyl acetate, magnification ca. * 170'000). Phage seems to be adsorbed onto a bacterial flagella.*

Isolated from: *Atlantic Ocean*
Isolation method: *Enrichment procedure*
Hydrophobicity: Φ_a *= 90,6°*

Genetic material: *ND*

Host bacterium: *H6*
Bacterial requirements: *Sea Water agar*
Appearence of the plaques: *Clear, well contrasted, 2-3 mm diameter*
Zeta potential: *-19,3 mV pH=4,2; -50,1 mV pH=7.4*

Bacteriophage: *H40/1*
Family (genus): *Siphoviridae (B1 morphotype)*
Dimensions: *85-82 nm * 39-42 nm*

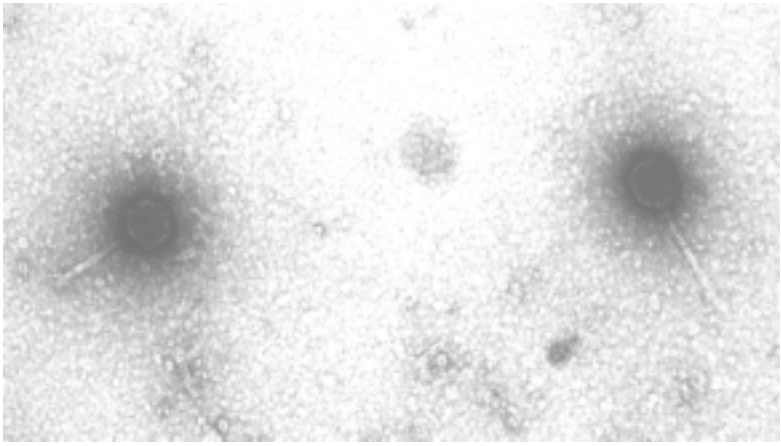


*Figure 2.6.2 : Phage H40/1, morphotype B1 (Uranyl acetate, magnification ca * 170'000).*

Isolated from: *Atlantic ocean*
Isolation method: *Enrichment procedure*
Hydrophobicity: Φ_a *= 68,7°*
Zeta potential: *-42,0 mV pH=7.4*
Genetic material: *Double strand DNA*

Host bacterium: *H40*
Bacterial requirements: *Sea Water agar*
Appearence of the plaques: *Clear, well contrasted, 4-5 mm diameter*

Bacteriophage: *Vh5a*
Family (genus): *Siphoviridae (morphotype B1)*
Dimensions: *95-100 nm * 40-50 nm*

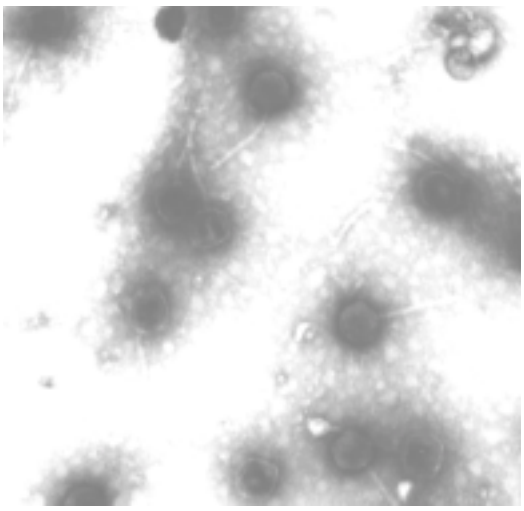


*Figure 2.6.3 : Phage Vh5a showing a typical B1 morphotype (Uranyl acetate, magnification ca * 170'000)*

Isolated from: *Mussels (Mytilus edulis)*
Isolation method: *Enrichment procedure*
Hydrophobicity: *ND*
Genetic material: *Double strand DNA*
Host bacterium: *Vibrio harveyi (ATCC 14126)*
Bacterial requirements: *Sea Water agar, 37°C*
Appearence of the plaques: *Tiny 1 mm diameter, clear, well contrasted*
Zeta potential: *ND*

Bacteriophage: *Sfd3*
Family (genus): *Siphoviridae (morphotype B3)*
Dimensions: *78-82 / 30 nm * 105-110 nm*

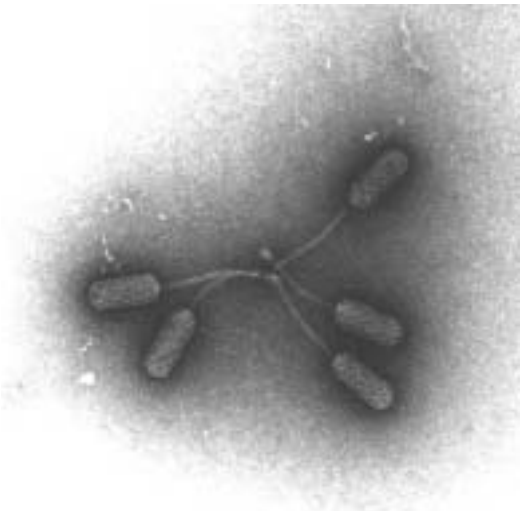


*Figure 2.6.4 : Phage Sfd3, typical B3 morphotype (Uranyl acetate, magnification ca * 170'000)*

Isolated from: *Water plant treatment*
Isolation method: *Enrichment procedure*
Hydrophobicity: *ND*
Genetic material: *Double strand DNA*
Host bacterium: *Streptococcus faecalis (DSM 2570; ATCC 29212)*
Bacterial requirements: -
Appearence of the plaques: *clear, well contrasted 2-3 mm diameter*
Zeta potential: *ND*

Bacteriophage: *Psf2*

Family (genus): *Siphoviridae (morphotype B1), no micrograph*
Dimensions: *ND*
Isolated from: *Water plant treatment*
Host bacterium: *Pseudomonas fluorescens (ATCC 27663)*
Isolation method: *Enrichment procedure*
Bacterial requirements: -
Hydrophobicity: Φ_a *= 34,2°*
Appearence of the plaques: *clear, well contrasted, large 4 - 5 mm diameter*
Zeta potential: *-36,0 mV pH = 7.4*
Genetic material: *Double strand DNA*
Special: *Phages require minimal concentrations of* Ca^{2+} *(50mM), and* Mg^{2+} *(5mM) for optimal burst size.*

Bacteriophage: *f1 (M13, fd)*

Family (genus): *Inoviridae (Inovirus, morphotype F1), no micrograph*
Dimensions: *100nm * 6 nm diam*
Isolated from: *ND*
Host bacterium: *Escherichia coli K12 (ATCC 15766)*
Isolation method: *ND*
Bacterial requirements: *minimal temp. 34°C*
Hydrophobicity: Φ_a *= 85,7°*
Appearence of the plaques: *clear, medium contrasted, 2 - 3mm diameter*
Zeta potential: *-42,3 mV pH = 7.4*
Genetic material: *Single strand DNA*
Special: *Optimised growth with TG1 strain: selection of the f' episome with tetracycline.*

Bacteriophage: *T7*

Family (genus): *Podoviridae (morphotype C1)*
Dimensions: *60nm * 17 nm*

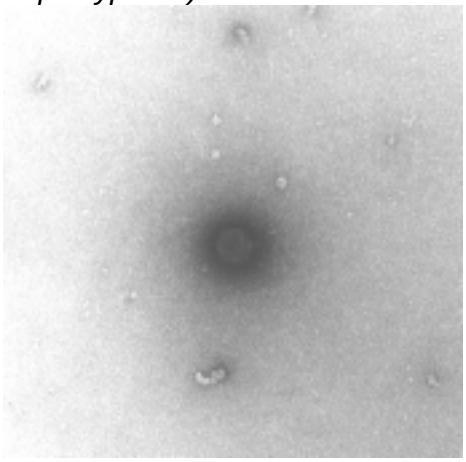
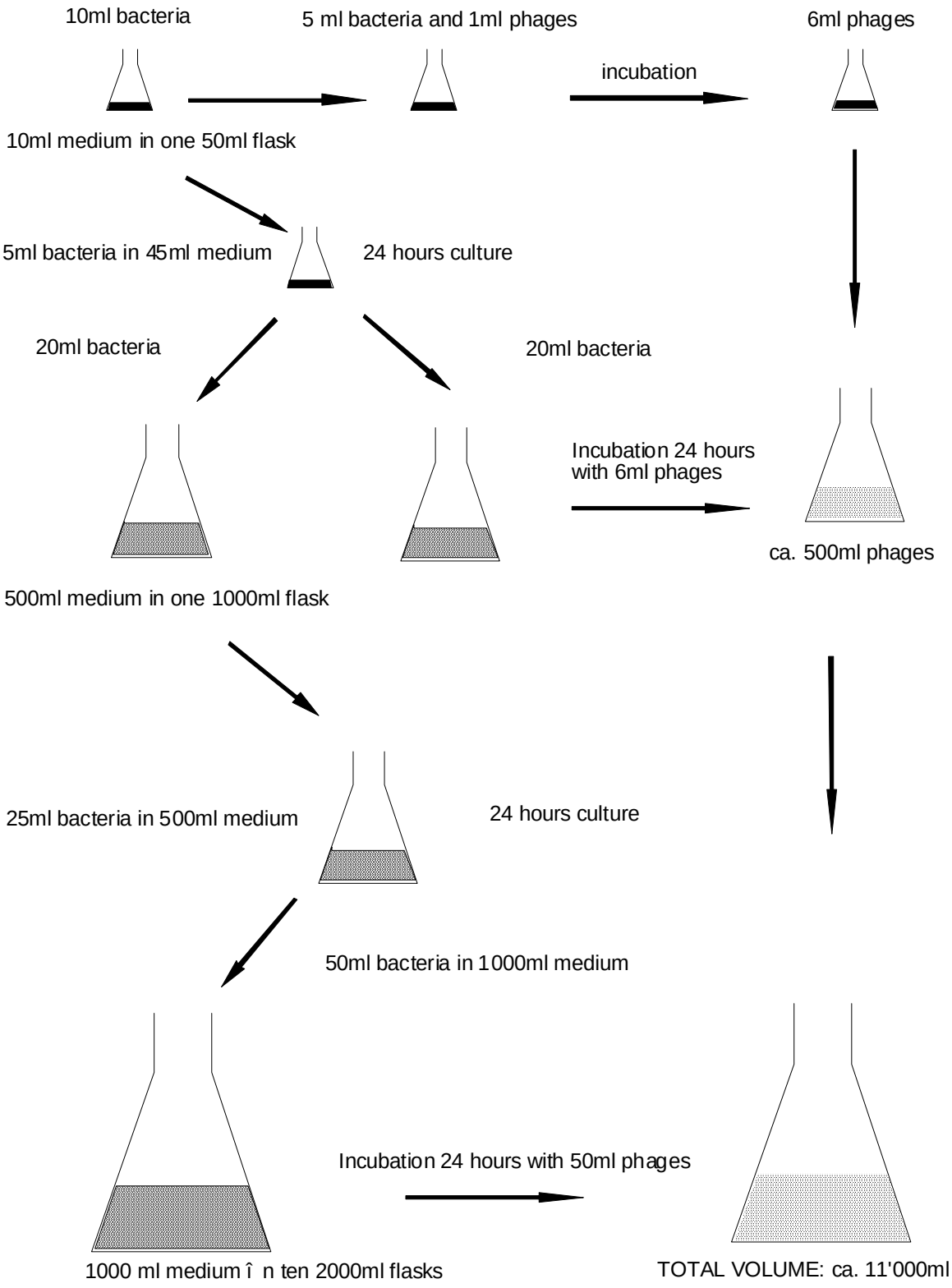


Figure 2.6.5 : C1 morphotype, showing the T7 short tail (Uranyl acetate, magnification ca 170'000)

Isolated from: *ND*
Host bacterium: *Escherichia coli B (ATCC 11303)*
Isolation method: *ND*
Bacterial requirements: -
Hydrophobicity: *ND*
Appearence of the plaques: *clear, well contrasted, large 4 - 5mm diameter*
Zeta potential: *-31,7 mV pH = 7.4*
Genetic material: *Double strand DNA*

Annex 2.6 :

Phage production in flasks. Exemple for the production of ca. 11 liters of phages suspension.



Annex 3.1 : Buffers and waters for the bacteriophages analysis:

PH buffer: See annex 2.1

Synthetic freshwater (SF): according to Moore et al. [1982]

KCl	8mg/l
$MgSO_4$* $7H_2O$	92mg/l
$CaCl_2$* $2H_2O$	147mg/l
$NaHCO_3$	105mg/l

SF+ buffer: add to 1000ml SF:

Tris-HCl 1M pH 7.4	20ml
$CaCl_2$ 1M	40ml
$MgSO_4$ 0.1M	40ml

Adjust to pH 7.4 with HCl or NaOH 6N

SP Buffer: add to 1000ml SF+:

Ca-Montmorillonite (Texas): 0.15mg/ml prepared as shown in chapter 4.2

Annex 3.2 : Growth media for host bacteria

Note: The concentration for the soft agar is always 6g/l agar. Media are autoclaved 20 minutes at 121° C for sterilization.

LB media: (Luria Bertani medium)

Tryptone	10g/l
Yeast extract	5g/l
NaCl	10g/l

Add 10g/l agar for the solid medium. Adjust to pH 7.2 before autoclaving

NGA medium: (Nutrient glucose agar)

Nutrient Broth (Difco)	8g/l
NaCl	3g/l
Glucose	8g/l
agar	10g/l

J&B medium:

Tryptone	20g/l
Yeast extract	3g/l
NaCl	8.5g/l
$CaCl_2$* $2H_2O$	1.47g/l
$FeCl_3$* $6H_2O$	7mg/l
$MgSO_4$* $7H_2O$	15mg/l
$MnCl_2$* $2H_2O$	1mg/l

Adjust to pH 7.4 before autoclaving

SWB and SWA media: (Sea Water Broth and Agar)

	Normal:	**Light**:
Marine salts for fish aquarium	24.75g/l	24.75g/l
Tryptone	5g/l	2.5g/l
Yeast extract	1g/l	0.5g/l

Use 10g/l agar for solid media.

DSM 246: (Sea Water Agar)

Beef extract	10g/l
Peptone	10g/l
Agar	20g/l
Sea water	750ml
Tap water	250ml

Dissolve beef extract and peptone by heating in water, adjust to pH 7.8, boil for 10 minutes. Readjust pH to 7.3. Add agar and autoclave.

Natural sea water is stored in the dark for at least three week to "age". If natural sea water is not available use artificial sea water:

Artificial Sea Water: (in distilled water)

NaCl	23.13g/l
KCl	0.77g/l
$CaCl_2$* $2H_2O$	1.60g/l
$MgCl_2$* $6H_2O$	4.80g/l
$MgSO_4$* $7H_2O$	3.50g/l
$NaHCO_3$	0.11g/l

DSM 115: *Vibrio natriegens* medium:

Peptone	5g/l
Meat extract	3g/l
NaCl	15g/l
agar	15g/l

DSM 123: (Marine Agar)

Add to 1000ml Artificial Sea Water:	
Peptone	5g/l
Yeast extract	1g/l
Agar	15g/l