

Microbial ecology in deep granitic groundwater

– activity and impact of viruses

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It is not length of life, but depth of life.

Ralph Waldo Emerson

Abstract

The deep subsurface environments in granitic rock can be studied and sampled at the Äspö hard rock laboratory (HRL) close to Oskarshamn in Sweden. Here, the groundwater is anaerobic and total counts give numbers of 10^4 to 10^6 cells mL^{-1} . However, these measurements do not reveal if the cells are active and alive. Subsequently, an ATP assay measuring biovolume, has in this thesis been shown to be reliable for estimations of the viable number of cells.

Viruses are the most abundant biological agent on earth and microbial ecology in all environments is greatly affected by viruses. The world's viral populations in marine ecosystems are known to influence the mortality of bacteria and archaea and biogeochemical cycles, and to have great genetic diversity. At the Äspö HRL, 10^5 to 10^7 mL^{-1} viral like particles were found when samples down to a depth of 450 m were studied. Using transmission electron microscopy, the viral populations were shown to be morphological diverse and likely to infect both archaea and bacteria. From the viral populations lytic bacteriophages infecting the indigenous sulphate-reducing bacterium *Desulfovibrio aespoensis* were detected at four out of ten tested sites. Bacteriophages were isolated from the cultures and characterised as within the phage group *Podoviridae* with a genome size of 40,700 base pairs and to have a narrow host range. When the phage and host were grown in batch cultures, bacterial cells gained immunity towards the phages. In addition, bacteria belonging to the genus *Desulfovibrio* immune to the isolated lytic phages were isolated from subsurface samples. Temperate phages were found in four out of the ten bacterial isolates, and the presence of prophages in the bacterial genomes might be a protection for the cells towards lytic infection of a similar second phage.

Viruses are proposed as a factor controlling the number of micro-organisms in the environment and may allow a diversity of micro-organisms and viruses to co-exist in stable and nutrient limited groundwater systems. The found viruses are further suggested to sustain the activity of the cells in a similar way to how viral populations of marine environments are known to influence the cycling of nutrients via a viral shunt. Viruses are dependent upon the metabolism of their host to multiply. Hence, the viruses found in the deep subsurface imply active microbial communities. The activity of the microbial communities is further supported by ATP assay analysis data since the amount of ATP and the activity and cultivability of micro-organisms have been shown to correlate for subsurface samples. Sulphate-reducing bacteria producing sulphide are potential sources of corrosion on the copper surrounding the spent nuclear waste in the proposed Swedish KBS-3 storage model, and are known to live in the subsurface. Their numbers and the activity of sulphide producing populations are hence important factors for the storage.

Keywords: ATP, *Desulfovibrio*, groundwater, bacteriophage, *Podoviridae*, sulphate-reducing bacteria, viral ecology

Papers included

This thesis is based on the following papers, which will be referred to in the text by their roman numerals:

- I. **Eydal, H. S. C.** and Pedersen, K. 2007. Use of an ATP assay to determine viable microbial biomass in Fennoscandian Shield groundwater from depths of 3–1000 m. *Journal of Microbial Methods*, 70, 363-373.
- II. Kyle, J. E., **Eydal, H. S. C.**, Ferris, F. G. and Pedersen, K. 2008. Viruses in granitic groundwater from 69 to 450m depth of the Äspö hard rock laboratory, Sweden. *ISME Journal*, 2, 571-574.
- III. **Eydal, H. S. C.**, Jägevall, S., Hermansson, M., Pedersen, K. 2009. Bacteriophage lytic to *Desulfovibrio aespoensis* isolated from deep groundwater. Accepted for publication in the *ISME Journal* in May 2009.
- IV. **Eydal, H. S. C.** and Pedersen, K. 2009. Characterisation of bacteriophages of *Desulfovibrio* from deep granitic groundwater. Manuscript.

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Abbreviations

ATP	adenosine triphosphate
CRISPR	clustered regularly interspaced short palindromic repeats
DOC	dissolved organic carbon
ds	double stranded
ERIC-PCR	enterobacterial intergenomic repetitive consensus polymerase chain reaction
ICTV	International Committee on Taxonomy of Viruses
MPN	most probable number
phage	bacteriophage
PCR	polymerase chain reaction
QPCR	quantitative polymerase chain reaction
SKB	the Swedish Nuclear Fuel and Waste Management Company
SRB	sulphate-reducing bacteria
ss	single stranded
TEM	transmission electron microscopy
TNC	total number of cells
VLP	viral like particles
Äspö HRL	Äspö hard rock laboratory

Preface

The first observations of micro-organism in groundwater were made by Antonie van Leeuwenhoek in 1677 when he used primitive microscopes to study well water (van Leeuwenhoek, 1677). Today micro-organisms have been found below 3.3 km depth in South Africa (Moser *et al.*, 2005) and are known to dominate deep groundwater (Danielopol and Griebler, 2008). The microbial life in subsurface environments comprises a significant part of the life found on and inside our planet. The total number of cells in subsurface environments has been estimated to be 0.25 to 2.5×10^{30} (Whitman *et al.*, 1998), and the number is likely to exceed the numbers found in other parts of the biosphere. Accordingly, the amount of biomass is substantial, and by adding the content of carbon in the subsurface cells together the amount is approaching, or even comparable, to what is found in plants (Whitman *et al.*, 1998).

Not only is the deep biosphere full of life, the existence of living organisms in these environments has arisen the question of whether life can be found inside other planets (Chapelle *et al.*, 2002; Kerr, 2002) and the possibility of the subsurface as the location for the origin of life (Pedersen *et al.*, 2008). Interest has also been displayed in exploring the deep subsurface life for practical purposes such as extraction of oil and drinking water (Griebler and Lueders, 2009), and underground repositories for spent high-level radioactive nuclear waste (Pedersen, 2002).

Aim of thesis

The aim of the performed studies was to investigate the microbial ecology in deep groundwater in granitic rock with respect to:

- I. the possibility of using measurements of adenosine triphosphate (ATP) to estimate living biomass,
- II. demonstrate the existence of and enumerate viral particles in comparison to the number of cells, and study the viral morphology and diversity,
- III. cultivable bacteria and viruses and characterise lytic viral isolates,
- IV. the existence of inducible prophages in bacterial isolates,
- V. using the estimates of ATP and the known viruses to describe the ecology of subsurface microbial communities.

Introduction

Micro-organisms are found everywhere

All cellular life on our earth is believed to have one common ancestor. The organisms on earth have evolved since then and can, based on how they are related, be divided into the three domains of life *Bacteria*, *Archaea*, and *Eukarya*. Micro-organisms are generally single cellular, can be found within all the three domains and are known to inhabit most places on earth (Kerr, 2002). Microbes are known to exist at the height of kilometres in the atmosphere (Fulton, 1966) as well as in groundwater at depths of kilometres into metabasalt rock in a South African Gold mine (Chivian *et al.*, 2008; Lin *et al.*, 2006). In deep groundwater, life seems to exist where the basic requirements for life of liquid water, habitable space, and permissive temperatures are met (Moser *et al.*, 2005).

Viruses

Viruses have been found to infect all the three domains of life, each with its specific viruses (Comeau *et al.*, 2008). In fact, all cellular organisms are thought to be susceptible to at least one type of virus (Chibani-Chennoufi *et al.*, 2004). Hence, viruses in our environment have been shown to be important to take into account when studying microbial ecology. The world's viral populations are known to influence microbial mortality and biogeochemical cycles, and great genetic diversity is found among them in marine ecosystems (Fuhrman, 1999; Suttle, 2005).

What is a phage?

Bacteriophages were discovered twice; by Frederick W Twort in 1915 and by Félix d'Herelle 1917 (Kutter and Sulakvelidze, 2005). A bacteriophage (shortened phage) is a virus infecting bacteria. The word bacteriophage means 'eater of bacteria', and was named by D'Herelle based on the fact that he saw infectious agents lysing bacteria (Weinbauer, 2004). Viruses are absolute parasites; they have no machinery for generating energy and no ribosomes for making proteins (Kutter and Sulakvelidze, 2005). Hence, all viruses need a host for their replication.

A phage particle contains nucleic acid, DNA or RNA, which makes up its genome and the nucleic acid is enclosed in a protein coat called a capsid. The different types of viruses can be divided into groups based on morphology, type of nucleic acid in the genome (double or single stranded DNA or RNA), and their known hosts. Table 1 shows the main groups of viruses

infecting bacteria and archaea as described by the International Committee on Taxonomy of Viruses (ICTV) (van Regenmortel *et al.*, 2000). Further, classifications into taxa have been made based on the predicted phage proteome of genomes of isolated phages (Rohwer and Edwards, 2002), which showed similar groupings. The most commonly found viruses in environmental samples contain dsDNA (Weinbauer, 2004).

Viral life cycles

There are several viral life cycles of viruses infecting bacteria and archaea known, as shown in figure 1. The life cycles mentioned in the papers included in this thesis are the lytic and the lysogenic. Viruses in the lytic life cycle will use the host machinery to produce viral particles and lyse cells soon after the host has been infected. The following steps are included; adsorption to the host, separation of nucleic acid from the capsid, expression and replication of the nucleic acid, viral assembly, release and transmission (Weinbauer, 2004). Virulent phages can only multiply by means of the lytic cycle, while phages able to undergo lysogeny are known as temperate phages. During the lysogenic life cycle the virus will infect its host and incorporate its DNA into the host genome. The virus will remain in the genome as a prophage until the lytic life cycle is triggered.

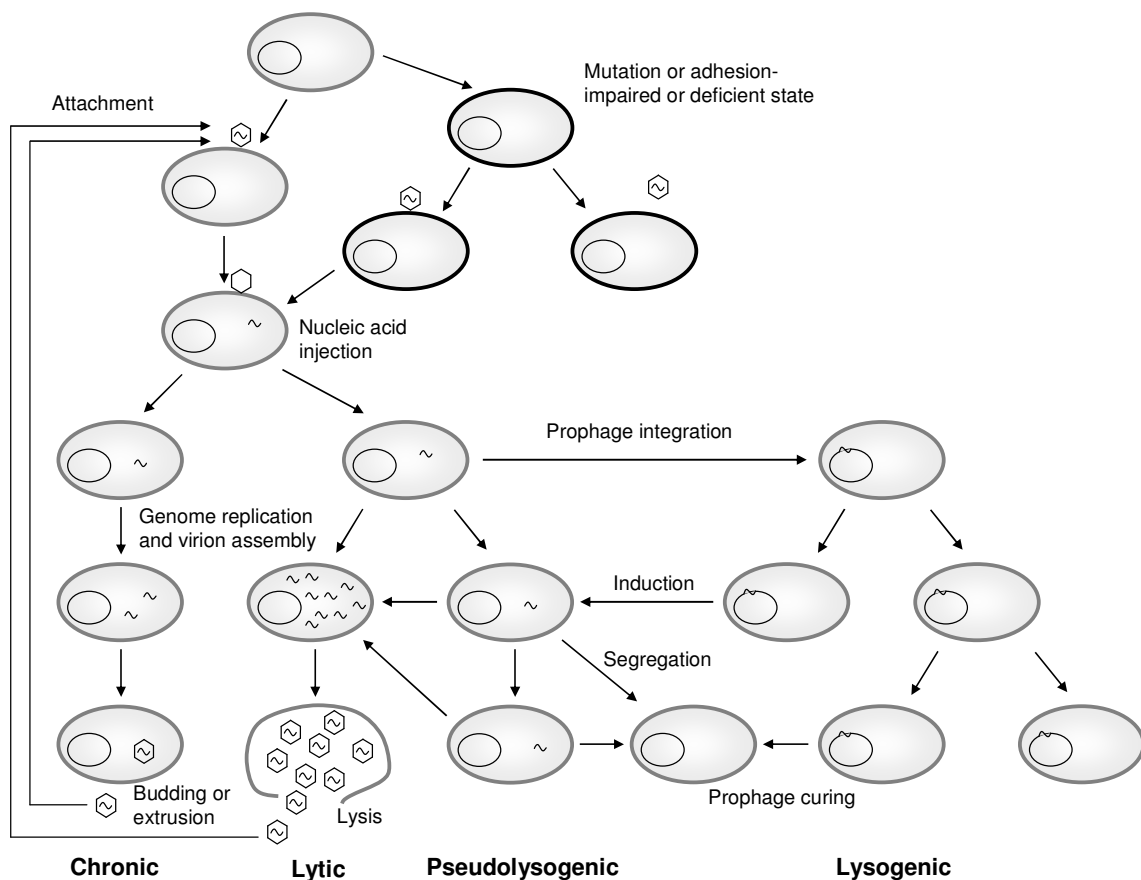

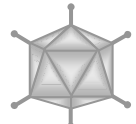
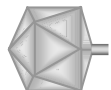
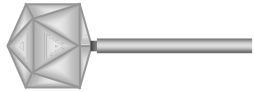













Figure 1 Model of viral life cycles based on a figure in (Weinbauer, 2004).

Table 1 The major viral groups known to infect Bacteria and Archaea. The family names are stated according to the 8th reports of the International Committee on Taxonomy of Viruses (van Regenmortel *et al.*, 2000). Viral families with a double stranded (ds) DNA genome are listed followed by single stranded (ss) DNA, dsRNA and ssRNA genomes. The known viral hosts as well as schematic drawings of the morphologies are shown.

Family	Nature of the genome	Host	Morphology
<i>Corticoviridae</i>	dsDNA	Bacteria	
<i>Tectiviridae</i>	dsDNA	Bacteria	
<i>Podoviridae</i>	dsDNA	Bacteria	
<i>Myoviridae</i>	dsDNA	Bacteria, Archaea	
<i>Siphoviridae</i>	dsDNA	Bacteria, Archaea	
<i>Fuselloviridae</i>	dsDNA	Archaea	
<i>Guttaviridae</i>	dsDNA	Archaea	
<i>Salterprovirus^a</i>	dsDNA	Archaea	
<i>Lipothrixviridae</i>	dsDNA	Archaea	
<i>Rudiviridae</i>	dsDNA	Archaea	
<i>Plasmaviridae</i>	dsDNA	Mycoplasma	

<i>Microviridae</i>	ssDNA	Bacteria, Spiroplasma	
<i>Inoviridae</i>	ssDNA	Bacteria, Mycoplasma	
<i>Cystoviridae</i>	dsRNA	Bacteria	
<i>Leviviridae</i>	ssRNA	Bacteria	

^a *Salterprovirus* is the genus; the family is unassigned.

Deep granitic groundwater

Deep granitic groundwater is anaerobic, i.e. the environment has no oxygen, and measurements of redox potential with E_h electrodes which give values of between -100 and -400 mV (Pedersen, 1997). Further, the environment is oligotrophic and hence contains low concentrations of dissolved organic carbon (DOC), as exemplified with groundwater from boreholes in Sweden in table 2. There is no light from the sun available, but carbon assimilated by phototrophic organisms does to some extent reach the subsurface groundwater (Chapelle *et al.*, 2002; Pedersen, 1997; Stevens and Mckinley, 1995). The environment conditions vary between sites, but can be very stable within different zones (Griebler and Lueders, 2009). Even though endolithic microbes are known to live between the crystals of porous rocks (Friedmann, 1982), the majority of the subsurface life in granitic rock is found in the groundwater in the fractures of the rock and in biofilms at the surfaces of the rock.

Äspö Hard Rock laboratory

Äspö Hard Rock laboratory (HRL) has been the main sampling site during the studies presented in this thesis. The Äspö HRL is situated on the east coast of Sweden, north of the town Oskarshamn and approximately 300 km south of Stockholm. The laboratory consists of a 3.6-km-long tunnel, which spirals down from the ground surface to a depth of 450 m into granitic rock (figure 2). It was built as a research laboratory and to demonstrate the potential for the geological disposal of nuclear waste (Pedersen, 2001). The granitic rock surrounding the tunnel is fractured and part of the Fennoscandian Shield and the fractures contain groundwater where different micro-organisms can live. The water can be accessed along the walls of the tunnel via boreholes intersecting the fractures. Geochemical data has been used to model the age and origin of the water surrounding the tunnel, which showed a correlation between age and salinity where older samples are more saline (Laaksoharju *et al.*, 1999). The water was heterogeneously distributed at different depths; water was dominated by meteoric water down to a depth of approximately 250 meters and water from depths of 250–600 m consisted of brackish–saline water with mixing proportions of current and ancient Baltic Sea water and meltwater from the last glaciation event approximately 10 000 years ago. Accordingly, at the more shallow sites sampled in the included studies, at 69 m, the water has been subsurface for months, while deeper

boreholes contain water which has been away from the surface for thousands of years. Table 2 shows the depth, Cl⁻ concentrations, and additional chemical data for a number of groundwater sites at the Äspö HRL. Sulphate reduction via microbial activities is later discussed in this thesis and sulphate which can support these activities is found in the water (table 2).



Figure 2 Schematic view of the field site Äspö hard rock laboratory, showing how the tunnel spirals down to a depth of approximately 450 m into the granitic rock. The artwork is provided by SKB (skb.se).

Table 2 Depth of boreholes and example of chemical characteristics for groundwater at Äspö HRL.

Borehole	Depth (m)	Cl ⁻ (mg L ⁻¹)	SO ₄ ²⁻ (mg L ⁻¹)	H ₂ S (mg L ⁻¹)	pH	DOC (mM)	Reference
KR0012B	68	567	43	n.d.	7.4	0.92	Kotelnikova and Pedersen, 1998
KR0012	68	840	113	n.d.	7.7	0.92	Pedersen, 2001
KR0015B	68	532	39	n.d.	7.2	1.5	Kotelnikova and Pedersen, 1998
KR0015	68	170	110	n.d.	7.5	1.5	Pedersen, 2001
SA1327B	179	3970	221	n.d.	7.4	0.54	Kotelnikova and Pedersen, 1998
KA3010A	400	5850	115	n.d.	7.5	0.21	Kotelnikova and Pedersen, 1998
KA3010/2 ^a	400	6590	335	n.d.	7.6	0.21	Pedersen, 2001
KA3110A	414	3860	106	n.d.	7.6	0.34	Kotelnikova and Pedersen, 1998
KR3110/1 ^a	414	3830	237	n.d.	7.6	0.34	Pedersen, 2001
KJ0050F01	448	9130	557	0.010	7.3	1.4 ^b	Hallbeck and Pedersen, 2008
KJ0052F01	450	9450	587	0.251	7.4	1.5 ^b	Hallbeck and Pedersen, 2008
KJ0052F03	448	7160	482	0.006	7.4	1.5 ^b	Hallbeck and Pedersen, 2008

n.d. not described

^a number with slash denotes sampled borehole section

^b the unit is mg L⁻¹

Micro-organisms and virus in the environment

This thesis focuses on cells and viruses found in subsurface groundwater environments in granitic rock. However, information about viral ecology in these environments is sparse, and the viral findings in these habitats are new. As a consequence of this, comparisons to research findings by others have been done to mainly marine environments, where more is known about the viral populations.

Numbers of and quantification of micro-organisms and viruses

The presence and numbers of cells and viruses can be investigated by estimating their abundances. This can be performed by counting the total number of cells (TNC) or the part which is able to grow on solid or in liquid media. Alternatively, the microbial populations can be enumerated indirectly by measuring the amount of microbe specific chemical compounds.

Total counts

When estimating the TNC living, dormant and dead cells are included. Counts can be performed by staining the cells with e.g. acridine orange followed by counting using epifluorescence microscopy (Hobbie *et al.*, 1977). For viruses, the first reports of high numbers in the sea were made using transmission electron microscopy (TEM) by e.g. Bergh *et al.* (1989). This method gives information about morphology, but has later been shown to underestimate the number of viral like particles (VLP) (Hara *et al.*, 1991; Noble and Fuhrman, 1998). Instead, staining with the dyes SYBR Green (Noble and Fuhrman, 1998) and SYBR Gold (Chen *et al.*, 2001) and counting of the TNC and number of VLP by visualisation using epifluorescence microscopy is now common (Weinbauer, 2004). Also dyes like DAPI has been used to count the TNC and the number of VLP (Hara *et al.*, 1991), and the number of stained VLP can alternatively be counted using flow cytometry (Chen *et al.*, 2001; Marie *et al.*, 1999).

The deep subsurface at the Äspö HRL contains 10^4 to 10^6 TNC mL⁻¹ (Kotelnikova and Pedersen, 1998; Pedersen, 2001), and at other parts of the Fennoscandian Shield at Olkiluoto, Hästholmen, Kivetty and Romuvaara in Finland at 211-948 m depth the amount has been estimated to be 10^5 to 10^6 counts mL⁻¹ (Haveman *et al.*, 1999). The numbers of VLP in groundwater at Äspö HRL at depths of 69–455 were in **paper III** enumerated, by fluorescent microscopy counts, to be in the range of 10^5 to 10^7 mL⁻¹, which is comparable to what is found in many other aquatic environments. Reports from polar inland waters varies between 10^4 and

10^8 VLP counts mL^{-1} (Sawstrom *et al.*, 2008), the number of VLP in Californian hot springs has been enumerated to 10^5 to 10^7 mL^{-1} (Breitbart *et al.*, 2004b), freshwater environments typically contain 10^5 to 10^8 VLP mL^{-1} (Wilhelm and Matteson, 2008) and the range of numbers found in marine water is from less than 10^4 to 10^8 VLP mL^{-1} (Wommack and Colwell, 2000).

Viable numbers

Viable counts reveal the number of culturable micro-organisms under the particular growth conditions given. The number of heterotrophic micro-organisms in water-samples can be estimated on agar plates (Reasoner and Geldreich, 1985), but this method generally only includes a small part of the TNC. When studying three boreholes at Äspö HRL, less than 0.1% of the number estimated using total counts were successfully cultured on agar plates under aerobic and anaerobic conditions (Pedersen *et al.*, 1997). Further, in samples from 129 to 860 m depth from five boreholes in Simpevarp, Avrö, Sweden, a site situated a few kilometres away from Äspö HRL, 0.12 to 25 % of the total count was cultivable on agar plates under anaerobic conditions (Pedersen and Ekendahl, 1990). In groundwater samples from 240 m depth in the granitic rock in Canada 0.01 to 10% of the total count was possible to culture on agar plates when incubated under aerobic and anaerobic conditions (Jain *et al.*, 1997).

Viable counts can also be estimated using a most probable number (MPN) technique in liquid media. When this method has been applied on deep granitic groundwater different metabolic groups of anaerobic micro-organisms are cultivated separately, and it has been possible to cultivate up to 30 and 50% of the TNC in Olkiluoto (Pedersen *et al.*, 2008) and at Äspö HRL (Hallbeck and Pedersen, 2008), respectively. The metabolic groups included in these investigations were nitrate-reducing bacteria, iron-reducing bacteria, manganese-reducing bacteria, sulphate-reducing bacteria (SRB), acetogenic bacteria and methanogens.

To enumerate the viable number of viruses, a host is needed. Since only one host is used in each viable count, and viruses usually only infect a small range of host species, the numbers only represent a small proportion of the total number of viable viruses (Bergh *et al.*, 1989). Viable counts can be performed by a plaque assays in which plaque-forming units are counted on a lawn of host cells (Adams, 1959). Alternatively, viable numbers of viruses can be determined in liquid media using MPN assays (Anonymous, 1995).

Sulphate-reducing bacteria is an example of a metabolic group of which numbers can be estimated using the MPN method and SRB are often found at depth greater than approximately 100 m at Äspö HRL (Hallbeck and Pedersen, 2008; Pedersen, 1997; **paper III**). These anaerobic bacteria use sulphate as a terminal electron acceptor and thereby reduce the sulphate to sulphide. The sulphide can corrode the copper canisters proposed to be used for disposal of spent nuclear

waste (see the section on 'Impact on long-time storage of nuclear waste' below), which is one of the reasons to why SRB have been studied in granitic rock environments. The SRB *Desulfovibrio aespoeensis* has been isolated from the Äspö HRL and described (Motamedi and Pedersen, 1998), and been repeatedly enriched from Äspö HRL groundwater, identified by its 16S rRNA gene sequence (Fru and Athar, 2008; Pedersen *et al.*, 1996). Based on its abundance and the goal to gain knowledge about SRB in the deep groundwater it was chosen as a host to enumerate cultivable phages in **paper III**. Subsequently, when ten sites sampled using an MPN technique, between 0.2 and 80 infecting units mL⁻¹ groundwater were found at 342–450 m depth at four different depth locations. It is the first reports of viable numbers of phages infecting a member of the *Desulfovibrio* genus from environmental samples.

Cell components

Counts of the TNC do not reveal the activity of the counted cells and far from all cells from the environment can be cultivated. Hence, estimations of cell densities have been performed by measuring the amount of a cell component which is known to exist in all cells. The biomass and the cell component measured then preferably have to occur in stable ratios. Tranvik (1997) measured DNA content in aquatic environments for determination of biomass and in deep groundwater the viable microbial biomass has been measured by phospholipid fatty acid analysis (Moser *et al.*, 2005; Moser *et al.*, 2003; Sahl *et al.*, 2008).

Measurements of adenosine triphosphate (ATP) can also be used to estimate viable biomass, since ATP is an energy transport compound found in all living cells. The firefly luciferase bioluminescence method was first used by Holm-Hansen and Booth (1966) for estimations in seawater. Since then, modifications of the method have been developed such as using recombinant luciferase allowing ATP reagents with high luciferase activity (Lundin, 2000).

In **paper I**, the estimation of viable biomass using an ATP assay was applied. The method used was based on the results of Lundin *et al.* (1986) and was first used on pure aerobic batch cultures. In these cultures the ATP concentration was found to correlate with TNC and with the volume and metabolic status of the cells. Subsequently, groundwater samples with microbial populations from 3 to approximately 1000 m depth into the Fennoscandian Shield were tested, and the ATP concentrations were also here found to correlate to TNC counts. Some samples containing high concentrations of ATP were shown to have many cultivable cells. Hence, groundwater containing a high proportion of active cells will have higher concentrations of ATP than inactive sites and the ATP assay gives information about the metabolic state and biovolume of micro-organisms in deep groundwater. The ATP assay was found to be reliable for estimations of the total amount of viable biovolume with a detection range that covered all samples.

The ATP assay has subsequently been used to estimate viable biovolume in deep groundwater in Olkiluoto, Finland (Pedersen *et al.*, 2008), and at the Äspö HRL (Hallbeck and Pedersen, 2008). Here, a larger part of the microbial population was generally cultivable where higher ATP concentrations were detected, and a correlation was found between the amount of ATP and number of cultivable cells using an MPN method in Finland.

Isolation of phages

Each bacteria is thought to have at least one virus infecting it (Chibani-Chennoufi *et al.*, 2004), and the diversity of viruses in oceans have been shown to be high (Breitbart *et al.*, 2002; Comeau *et al.*, 2008). However, few viral isolates have been described, which in part is due to the fact that few of their hosts have been cultivated.

Lytic phages have been isolated from widely different environments, such as from Arctic sea ice (Borriss *et al.*, 2003), marine ecosystems (Borsheim, 1993; Sullivan *et al.*, 2003), the dairy industries (Sillankorva, 2008), and waste water (Oliveira *et al.*, 2009). In **paper III** the five phages HEy1-5 were for the first time isolated from deep groundwater using *D. aespoeensis* as a host. Isolation of lytic viruses can be done by exposing metabolically active cells to samples likely to contain infectious viruses. The presence of prophages in bacterial genomes can be tested by induction, i.e. inducing the bacteria to stress and thereby triggering the viral lytic cycle. To stress cells and induce temperate phages, UV or mitomycin C has commonly been used, both in environmental samples (Bongiorni *et al.*, 2005; Prigent *et al.*, 2005; Weinbauer and Suttle, 1999; Williamson *et al.*, 2008b), and with bacterial cultures of characterised isolates (e.g. **paper IV**; Handley *et al.*, 1973; Walker *et al.*, 2006; Williamson *et al.*, 2008a). In addition, stress of bacterial batch cultures in the form of low (Wang *et al.*, 2007) or high temperature (Bertani, 2004) and hydrogen peroxide (Weinbauer and Suttle, 1999) has been shown to induce temperate phages, and temperate phages have been seen in aged batch cultures of *Desulfovibrio* (**paper IV**; Clark *et al.*, 2006).

Phages infecting Desulfovibrio

Presence of, possibly inducible, prophages in isolated bacteria can be confirmed by determining the sequence of bacterial genome. The genome of *Desulfovibrio vulgaris* reveals the presence of three lysogenic bacteriophages and the remnant of a fourth phage genome (Heidelberg *et al.*, 2004). Induction of some of these prophages have been repeatedly reported; Handley *et al.* (1973) were, using TEM, able to visualize 53 nm wide tailed temperate phages after addition of mitomycin C to cultures, and Walker *et al.* (2006) were able to induce both 50 and 100 nm wide phages and transfer the phages to a closely related strain.

It is however known that not all prophages are induced by mitomycin C (Paul, 2008). Addition of mitomycin C to exponentially growing batch cultures of *D. aespoeensis*, *D. vulgaris* and ten isolates of *Desulfovibrio* from Äspö HRL was shown to have little or no effect on the production of VLP and the usage of mitomycin C as the sole way of inducing temperate phages was questioned (**paper IV**). In **paper IV**, the temperate phages were instead found in aged batch cultures of four out of ten bacterial isolates from the Äspö HRL.

Ultra violet light has also been used to induce temperate phages in *D. vulgaris* and *D. desulfuricans* (Seyedirashti *et al.*, 1991; Seyedirashti *et al.*, 1992). The phage induced in *D. desulfuricans* by Seyedirashti *et al.* (1991) had a genome size of at least 45,000 bp, which is different from the phage with a 13,000 bp sized genome able to mediate transduction in *D. desulfuricans* reported by Rapp and Wall (1987). The phage described by Rapp and Wall (1987) was not induced by mitomycin C. It had a 43 nm wide head, and a genome which produced a smear when running it on a gel after digestion with restriction enzymes, indicating that the DNA fragments were heterogeneous. Hence, the found phages might rather be prophage-like gene transfer agent instead of an ordinary phage as argued by Stanton (2007). The prophage-like gene transfer agents are not functional phages since they typically pack bacterial DNA fragments smaller than their own genome, rather than of their own genomes, and thereby mediate generalized transduction (Stanton, 2007).

Lytic phages infecting members of the genus *Desulfovibrio* has also been found. Kamimura and Araki (1989) isolated phages infecting *D. salexigens* from marine sediments and phage isolates using *D. aespoeensis* as a host, originating from MPN cultures of phages inoculated with deep groundwater, are described in **paper III**.

Characterisation of phages

Morphology

Certain characteristics are often used to describe both viruses studied in their natural habitats and the phages that have been isolated. Transmission electron microscopy can be used to visualise viral particles and study their morphology. The technique has been applied to describe the viral diversity found in many environments, e.g. in an oligotrophic lake (Corpe and Jensen, 1996), in hot springs (Rachel *et al.*, 2002), in the surface sand of the Sahara Desert (Prigent *et al.*, 2005), as well as at 69 to 450 m depth at the Äspö HRL (**paper II**).

Sketches of the main viral groups infecting bacteria and archaea are shown in table 1. In seawater, podoviruses (with short tails) or phages without tails are most common, but myoviruses (with contractile tails) and siphoviruses (with long and flexible tails) are most

commonly isolated (Suttle, 2005). At the Äspö HRL diverse morphological populations of viruses infecting both archaea and bacteria were observed in **paper II**. The morphology of viruses can give clues about their host range and viral replication - myoviruses are often lytic and have a broad host range, podoviruses are known to be lytic and have narrow host range, and siphoviruses have an intermediate host range and can often integrate into the host genome (Sullivan *et al.*, 2003; Suttle, 2005).

Latent period

The latent period is the time between adsorption of viral particles to cell lysis, where no extracellular or free phages are detected (Weinbauer, 2004). In isolated phage-host system the latent period can be studied under growth in batch cultures. Typical growth and how the number of cells in bacterial batch cultures changes over time has been drawn in figure 3a. If a lytic phage infecting the bacteria is added to the culture under exponential growth, as phage HEy2 added to a culture of *D. aespoeensis* in **paper III**, the number of bacterial cells will cease to increase (figure 3b). This is followed by a decrease in bacterial numbers and a viral burst of the cells. The latent period is then the time between when the exponential phase ends and the time for the viral burst.

Burst size

The burst size is the number of viral particles released during lysis of a cell, and is important for regulating population dynamics of cells and viruses, and epidemics of viruses (Parada *et al.*, 2006). The burst size of isolated phage-host systems can be estimated using one-step growth curves (Jiang *et al.*, 1998) and varies between species. When comparing 52 isolated marine phages, on average 185 phages were reported per lysed bacterial cell (Borsheim, 1993), which is comparable to the burst size of 170 described for the podovirus infecting *D. aespoeensis* in **paper III**.

In aquatic samples, the burst size is usually estimated by enumerating the number of viral particles inside cells using TEM, with the average burst sizes of 24 and 34 found in marine and freshwater environments respectively (Parada *et al.*, 2006). Even though burst size data show a large variability, data on bacterioplankton show that burst size increases with the cell size and the trophic status of the environment (Parada *et al.*, 2006; Weinbauer and Peduzzi, 1994).

Genome size

Since viral DNA content is known to be diverse, the size of viral genomes can be part of a characterisation of viral isolates or communities. Viral genomes in aquatic environments are known to vary in size and have been found to span a size range of 15 kb to >300 kb in aquatic environments (Filippini and Middelboe, 2007; Riemann and Middelboe, 2002; Steward *et al.*, 2000).

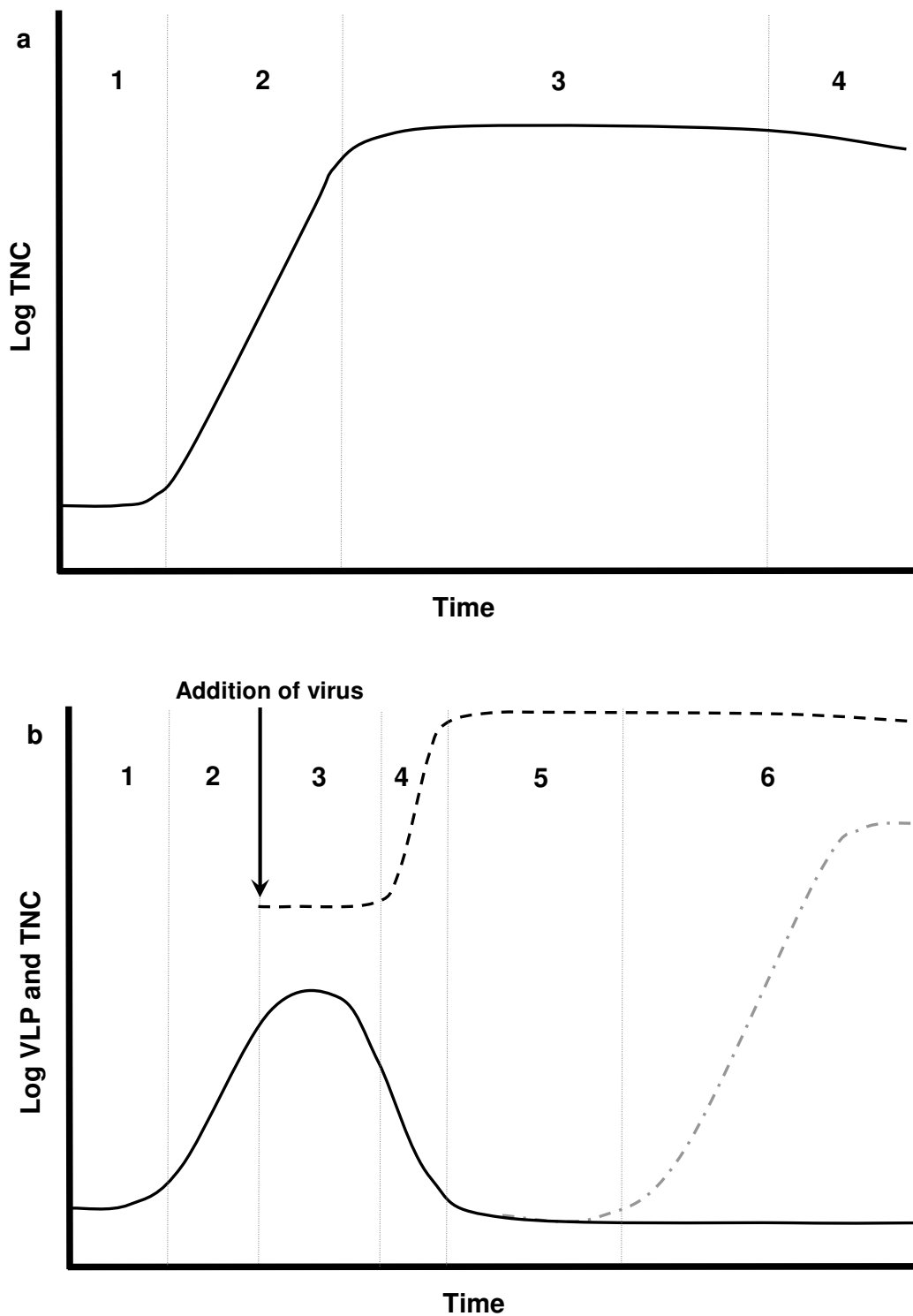


Figure 3 The total number of cells (TNC) followed over time **a)** during bacterial growth in a batch culture; 1 lag phase, 2 exponential phase, 3 stationary phase, and 4 death phase, and the TNC and the number of viral like particles (VLP) followed **b)** during bacterial growth in batch culture where virus has been added; 1 lag phase, 2 exponential phase, 3 latent period, 4 viral burst, 5 stationary phase, and 6 potential growth of immune cells.

For extraction of nucleic acid from viruses, the viral particles need to be purified from their host. This can be done by ultracentrifugation on a cesium chloride gradient as described by Casas and Rohwer (2007) and Sambrook and Russell (2001), and the DNA can then be further extracted. The size of DNA fragments can be estimated by running the DNA on an agarose gel and comparing it to sizes of DNA fragments of known lengths. However, for such comparisons, large DNA fragments like viral genomes need to be cut with restriction enzymes into smaller sizes as described by Helling *et al.* (1974). Restriction enzyme cleavage has also been used to compare and possibly differentiate between viral isolates, e.g. temperate phages in *D. vulgaris* and *D. desulfuricans* (Seyedirashti *et al.*, 1991), lytic phages infecting *D. aespoeensis* in **paper IV**, and isolates of marine temperate phages (Jiang *et al.*, 1998).

Different versions of pulsed field gel electrophoresis, as first described by Schwartz and Cantor (1984), are also used for separation and estimation of the sizes of large DNA fragments such as the genomes of viral isolates (e.g. Williamson *et al.*, 2008a). The method has further been used to finger-print temporal and spatial changes in viral communities in seawater (Riemann and Middelboe, 2002; Steward *et al.*, 2000), and water and sediment samples in marine and freshwater ecosystems (Filippini and Middelboe, 2007), as it determines the size distribution of virus-like DNA. When analysing the diversity of genome sizes using pulsed field gel electrophoresis, the total number of virus genotypes is not revealed, but rather the dominant viral genome sizes (Filippini and Middelboe, 2007) and the method gives the minimum diversity of viruses in the communities.

Methods based on comparison of whole genomes sizes of viruses have the advantage of not requiring prior knowledge about the genetic diversity and sequences of the investigated viruses, and avoids potential biases in PCR-based techniques. However, the amount of DNA required to get visible bands on an agarose gel might be more than what can be extracted from some samples.

Host range and bacterial susceptibility

The number of different hosts infected by a virus is called the host range and has, as well as the susceptibility to infection of viruses among bacteria, been shown to vary. The host range has been studied by e.g. Waterbury and Valois (1993) who isolated *Synechococcus* viruses where most of the *Synechococcus* populations were resistant to the viruses. Further, for 16 vibriophage-isolates the host range was shown to be unique of each isolate (Comeau *et al.*, 2006), and four temperate marine phages infecting *Shingomonas* or *Flavobacterium* were not shown to cross infect other host tested (Jiang *et al.*, 1998). The lytic phages infecting *D. aespoeensis* described in **paper III** had a narrow host range - bacterial isolates from the same sites as the phages and with up to 99.9 %

identical 16S rRNA gene sequence to the host were not infected. Lytic marine phages and their marine *Flavobacterium* hosts showed a large variability in phage host range and the bacterial strains susceptibility to infection (Holmfeldt *et al.*, 2007). Some of the bacteria were closely related when their 16S rRNA gene sequences were compared, and by using whole-genome PCR typing analysis the strains were divided into groups. All of the bacterial strains showed a unique combination of susceptibility against the isolated phages, and just as presented in **paper III**, the question of whether the bacterial isolate could be infected by the isolated phages was not determined by the similarities in the 16S rRNA sequence alone. To the contrary, Sullivan *et al.* (2003) described cyanophages with broad host ranges where the host range was related to how similar the 16S rRNA gene sequences of the different hosts were.

The host range of viruses is also known to be influenced by how the phages were isolated, as was demonstrated by Jensen *et al.* (1998), working mainly with samples from sewage sources, where higher frequency of viruses with a broader host range were found when two bacterial strains were used as hosts.

Identification and characterisation of cells and viruses using gene sequences

The media used to cultivate naturally occurring micro-organisms fail to mimic the conditions that particular organisms require for proliferation, and far from all of the diverse microbial life has been cultured (Amann *et al.*, 1995). Molecular techniques offer an opportunity to study the microbial communities, since cultivation is not always needed. The 16/18S rRNA gene sequence has been widely used to compare the microbial diversity within natural communities (Woese, 1987). For this the DNA in the microbial community is extracted, the 16/18S rRNA gene amplified using PCR with primers specific for the gene, and the amplified DNA fragments are cloned into a vector and sequenced. The sequences can then be compared between isolates or environments. Additionally, fingerprinting methods without sequencing can be used. Amplification of the 16S rRNA gene using PCR, followed by denaturing gradient gel electrophoresis to separate the amplified DNA fragments has been used to study the diversity of bacterial communities (Muyzer *et al.*, 1993) from Äspö HRL microbial ecosystems (Fru and Athar, 2008).

The genomes of viruses are diverse, and do not contain universal genetic markers, such as 16/18S rRNA genes (Rohwer and Edwards, 2002; Schoenfeld *et al.*, 2008; Zhong *et al.*, 2002). To study the genetic diversity of environmental samples, the sequences of genes within groups of viruses have instead been compared. Using this approach, T7-like podophages DNA polymerase sequences have been shown to occur in all environments investigated, including marine, freshwater, sediment, terrestrial, and well water (Breitbart *et al.*, 2004a). Further, the sequences of

a gene for viral capsid assembly protein, g20, in marine cyanophages has been shown to be diverse in marine environments (Zhong *et al.*, 2002), yet similar sequences can be found at different locations (Short and Suttle, 2005). However, since few viral sequences generally are known, there is a risk of the primers designed for the PCR reactions picking up sequences they were not meant to, which might then be difficult to identify (Short and Suttle, 2005).

PCR-based tools can also be used to fingerprint bacterial and viral genomes and differentiate between isolates without sequencing. Degenerated primers for random amplification of phage DNA have been applied to compare banding patterns on agarose gels of viral isolates (Comeau *et al.*, 2006; Comeau *et al.*, 2004). The random primers makes it possible to amplify also parts of the genome of the viral host, but care should be taken to assure that contamination of host DNA in viral isolates does not interfere with amplifications of the viral genomes (Comeau *et al.*, 2004). Debruijn (1992) used PCR to amplify repetitive DNA sequences, enterobacterial intergenomic repetitive consensus (ERIC) sequences, in bacterial soil isolates. This method was also used in **paper III** to differentiate *Desulfovibrio* isolates from the Äspö HRL, and showed a higher degree of variability between isolates than what the 16S rRNA gene sequences did.

The variation of a specific gene of interest, such as genes involved in metabolic functions, can also be tested. For sulphate-reducing micro-organisms the genes for dissimilatory sulphite reductase (*dsrAB*) and the alfa subunit of the adenosine-5'-phosphosulphate reductase (*apsA*), with functions in dissimilatory sulphate reduction, have been sequenced and compared to study microbial diversity (Ben-Dov *et al.*, 2008; Mogensen *et al.*, 2005; Wagner *et al.*, 1998). Further, quantitative PCR (QPCR) can be used to estimate the abundance or expression of the *dsrA* gene as in industrial wastewater where the method was used to follow bacterial abundance (Ben-Dov *et al.*, 2009). By enumerating the number of *apsA* genes in **paper IV**, the QPCR method was used to estimate how much bacterial DNA contributed to the total amount of extracted DNA from the viral isolates.

Sequenced viral genomes

The number of organisms where the whole genome has been sequenced is increasing with improving methods. Within the domain Bacteria for example the genome of *D. vulgaris* has been fully sequenced (Heidelberg *et al.*, 2004) and about 500 complete sequenced phage genomes are listed in the NCBI database. The phage sequences, as well as environmental samples, are dominated by dsDNA tailed phages (Comeau *et al.*, 2008). Lavigne *et al.* (2008) used BLAST-based tools to study the similarities and classify 55 fully sequenced bacteriophage genomes from NCBI and EBI databases, and were able to refine and confirm the ICTV phage classification.

Rohwer and Edwards (2002) used the known viral genomes of isolated phages to group phages into taxa based on the predicted phage proteome. One of the goals in **paper IV** was to sequence the genomes of the lytic phages infecting *D. aespoeensis*. This would hopefully further have confirmed the classification of the isolated phages as within the T7 group of *Podoviridae*.

Metagenomics

A metagenomic approach can be used to study the genetic material recovered directly from environmental samples. This method has been used to sequence viral DNA from small amount of DNA found in marine environments (Breitbart *et al.*, 2002), and viral sequences have been determined in samples from hot springs using a method for constructing libraries from picograms of DNA (Schoenfeld *et al.*, 2008). The metagenomic approach requires no cultivation, and gives substantial amounts of gene-sequences. However, the majority of the sequences retrieved from the metagenomic studies generally show no significant similarities to previously reported genes in GenBank and are not annotated, and the functions of the genes are hence unknown (Breitbart *et al.*, 2002; Schoenfeld *et al.*, 2008).

Microbial life in deep groundwater

The hydrogen driven biosphere

Many subsurface systems are thought to be limited by photosynthetic derived organic carbon (Chapelle *et al.*, 2002; Pedersen, 1997; Stevens and Mckinley, 1995). As nutrients are depleted, e.g. with increasing distance from the source, the micro-organisms should then become extinct (Stevens and Mckinley, 1995). However, cells are found at great depths in subsurface environments, e.g. bacteria and archaea at below 3.3 km depth in systems with 4 to 53 million-year-old meteoric water (Moser *et al.*, 2005), and microbial communities built on chemolithotrophic micro-organisms independent of photosynthetic primary production have been proposed to live in deep subsurface environments (Pedersen, 1997; Stevens and Mckinley, 1995).

Granite often contains natural radioactive elements, e.g. uranium, and as a consequence, radiolysis of water occurs, which can be a source of hydrogen (Kerr, 2002; Pedersen, 1997). This geologically produced H₂ can be used as energy to support the chemolithoautotrophic subsurface ecosystems founded upon biological methanogenesis or acetogenesis described as ‘the hydrogen driven biosphere’ by Pedersen (1997) and which is presented in figure 4. The organic carbon produced by the autotrophic organisms can subsequently be used by heterotrophic sulphate, iron, and manganese reducing micro-organisms. Autotrophic micro-organisms have been cultivated from Äspö HRL groundwater which support the hypothesis; acetogenes were found to dominate down to 112 meter and methanogens have been found deeper, down to 446 m (Kotelnikova and Pedersen, 1998). Autotrophic methanogens have also been found in deep basalt aquifers (Stevens and Mckinley, 1995) and in rocks of volcanic origin without recent surface-derived organic carbon (Chapelle *et al.*, 2002).

Viruses and indications of them

With the finding in the studies included in this thesis viruses can now be added to the microbial ecology of deep granitic groundwater. The viruses were approximately ten times more abundant than cells (**paper II**) and both virulent and temperate phages were found (**paper III and IV**). The amount of viruses shows that the viral populations at Äspö HRL are both abundant and propagating.

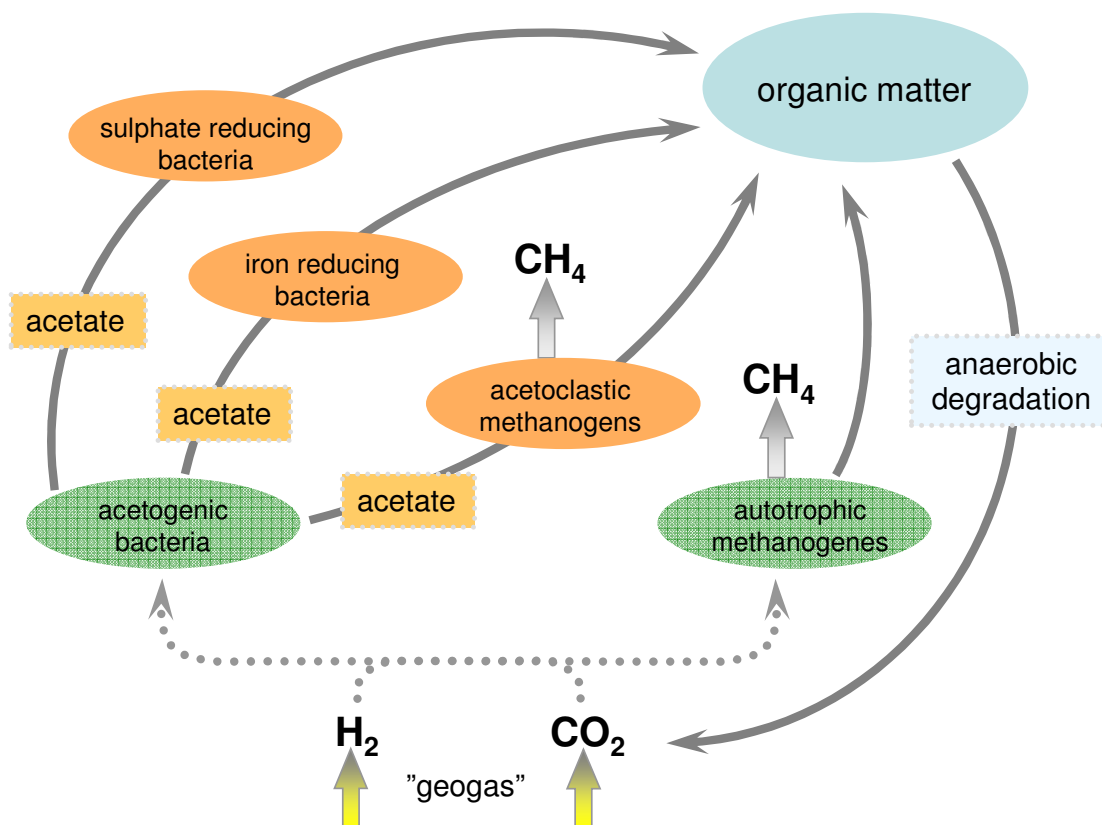


Figure 4 The deep hydrogen driven biosphere hypothesis is here illustrated by its carbon cycle after modifications from Pedersen (2000). Autotrophic micro-organisms able to use H₂ and CO₂ as energy and carbon sources produce acetate which can be used by other metabolic groups as a carbon source.

Another environment where archaea and bacteria are the dominating domains is hot springs. Here, the temperatures are above the upper limit of eukaryotic life, and phages are the only known predators of bacteria and archaea (Breitbart *et al.*, 2004b). The VLP in Californian hot springs have been calculated to be 0.07×10^6 to $7.0 \times 10^6 \text{ ml}^{-1}$, and phages were produced at a rate of 1.0×10^6 to $1.5 \times 10^6 \text{ ml}^{-1} \text{ day}^{-1}$ with turnover times of the phage communities of 1.2 to 2.2 days. Based on that phage communities were abundant, active and important components of the hot springs the authors proposed that the phages may also be major players in the microbial ecology of the deep hot biosphere (Breitbart *et al.*, 2004b). Further, using the results from the hot springs, 3.7×10^{29} cells and 3.7×10^{30} phages were calculated to be produced in this biosphere every year.

Even though no one probably ever searched for viruses deeper into the rock than what is presented in this thesis, there are indications of viruses from deeper depths. Using molecular methods, geochemistry data and isotopic signatures a thermophilic chemoautotrophic sulphate-reducing bacterium was found to dominate in groundwater at depths of 2.8 km in metabasalt

rock in a South African Gold mine (Lin *et al.*, 2006). Since the bacterium was so dominating it could be sequenced from the groundwater without prior cultivation using a metagenomic approach (Chivian *et al.*, 2008). The genome revealed genes which potentially allowed the bacterium to use H₂ as electron donor and SO₄²⁻ as a terminal acceptor, and fix carbon and nitrogen. These are important mechanisms to the micro-organisms in this deep environment to be able to live independently of photosynthesis. The genome was sequenced from groundwater filtered onto a 0.2 µm pore size filter, which meant that detection of possible viruses in the water was unachievable. However, there were indications of viral activity in the water since the bacterial genome contained genes used for viral defence. Additionally cells had genes for usage of heterotrophic carbon sources including recycling of dead cells, which could be a product from viral lysis of cells.

Diversity of subsurface life

Cultivations of micro-organisms have shown populations of nitrate-reducing bacteria, iron-reducing bacteria, manganese-reducing bacteria, SRB, acetogenic bacteria and methanogens in deep groundwater from Olkiluoto (Pedersen *et al.*, 2008) and at Äspö HRL (Hallbeck and Pedersen, 2008). Even though cultivation-independent techniques for analysis of microbial diversity have increased the amount of information gained about biodiversity (Amann *et al.*, 1995), and compared to more productive systems such as surface waters and terrestrial environments, microbial diversity in groundwater ecosystems have been shown to be low (Danielopol and Griebler, 2008).

Diverse microbial populations have been found in the subsurface (Amend and Teske, 2005) using both cultivation and molecular approaches when microbial populations in water in deep boreholes in South Africa (Moser *et al.*, 2003), Colorado (Sahl *et al.*, 2008), Finland (Haveman *et al.*, 1999; Pedersen *et al.*, 2008), Sweden (Hallbeck and Pedersen, 2008; Pedersen, 1997; Pedersen *et al.*, 1997), France (Basso *et al.*, 2009) and Japan (Hirayama *et al.*, 2005) have been described. Further, as shown in **paper III**, to detect micro-diversity within groups bacterial diversity needs to be described by supplementary methods to just analyzing the 16S rRNA gene sequence.

Viral impact on microbial ecology and modelling

Defence against viruses and the viral response

Cells in batch cultures of *D. aespoeensis* were shown to gain immunity to phage isolates HEy2 over time in **paper III** and a sketch of how the cells grew is shown in figure 3b. The HEy2 phages may have entered the lysogenic life cycle and the cells then have been protected against superinfection, i.e. infection of phage similar to the prophage. The type strain of *D. aespoeensis* did not contain inducible prophages, but other *Desulfovibrio* isolates from Äspö HRL have been shown to have temperate phages (**paper IV**).

Bacteria have developed defence mechanisms against phages and the cells in the above described batch cultures can have gained immunity to the phages through several potential mechanisms. Different defence mechanisms have been studied in *Lactococcus* species which are lactic acid bacteria important for the fermentation of dairy products (Dinsmore and Klaenhammer, 1995; Sturino and Klaenhammer, 2006). The known mechanism target various steps of the phage lifecycles; adsorption interference, preventing DNA injection, restriction and modification of incoming DNA, or are abortive infection systems. As the bacteria gain new ways to prevent phage infection there will be a selection for phages able to cope with the new barriers by e.g. loosing restrictions enzyme sites in the phage genomes over time (Dinsmore and Klaenhammer, 1995).

In recent years the clustered regularly interspaced short palindromic repeats (CRISPR) and *cas*-genes (CRISPR associated) have been shown to be involved in bacterial resistance to phages. Most bacteria and archaea contains CRISPR loci, which typically consists of several non-contiguous direct repeats separated by parts of variable sequences called spacers. Often found close to these loci are *cas*-genes (Barrangou *et al.*, 2007). Experiments using *Streptococcus thermophilus* strains and their viruses showed how CRISPR spacers provides resistance in bacteria by a type of nucleic acids-based immunity (Barrangou *et al.*, 2007). After the exposure to viruses, bacteria integrated new spacers which were obtained from phage genomic sequences and removal or addition of spacers altered the phage-resistance of the cells. The resistance specificity to the viruses was determined by the similarity between spacers and virus genome.

As expected, the viruses have been shown to have ways of coupling with the CRISPR spacers viral defence system. By analysing the genomic sequences of virus, bacteria and archaea from community genomic data from acidophilic biofilms, extensive recombination of viral

sequence in the genomes were found. The recombination was thought to be extensive enough to evade CRISPR spacers since this system seems to require an exact match between the spacer and the virus genome (Andersson and Banfield, 2008).

It can be concluded that there is a constant fight and co-evolution between phages and their hosts. Consequently, if the *D. aespoeensis* cells were not immune due to a prophage, the phage resistant *D. aespoeensis* clones which evolved over time in **paper III** are likely to meet approaches from the phages to overcome the resistance.

Diversification of micro-organisms and virus via the lytic life cycle

In a stable environment like the deep biosphere with limited nutrient sources a few bacterial populations would in theory be present at each time, limited by the number of different resources available (Thingstad, 2000; Weitz *et al.*, 2005). However, in **paper III** and in earlier investigation (Fru and Athar, 2008; Pedersen *et al.*, 1996a) a diverse population of SRB was described and viruses infecting the deep groundwater microbial communities might explain the observed richness in bacterial diversity. Models by Thingstad (2000) and Thingstad and Lignell (1997) describe how the bacterial population with different growth rates can coexist in steady state in aquatic systems due to viral control of the dominant bacteria species via mechanisms selectively 'killing the winner' (figure 5). Host-specific viruses prevent the best bacterial competitors from building up a large biomass via top-down control of bacterial diversity and the viral host specificity allows speciation within each functional group. The model further predicts that the viral abundance is controlled by the bacterial diversity and the dominating phage keeps changing.

Weitz *et al.* (2005) further modelled the co-evolution and arms race between bacteria and bacteriophage in a chemostat with one limiting resource, and were able to explain the generation and maintenance of diversity among bacteria and viruses. The model was based on that phage enter host cells via membrane-bound surface receptors which are often responsible for nutrient uptake. A selective pressure will exist for the bacteria to modify its receptor configuration to avoid phage infection and, the phage, in turn, to modify its tail fiber.

Both diverse viral and microbial populations are known to exist in the subsurface at the Äspö HRL (**papers II and III**; Hallbeck and Pedersen, 2008; Fru and Athar, 2008; Pedersen *et al.*, 1996a). The lytic viral populations in the oligotrophic and stable groundwater environment might sustain the diversity of both cells and viruses, as predicted by the models.

Models from analysing metagenomic data from marine samples has supported the 'killing the winner theory' and predicted it unlikely that the most abundant phage genotype to be the same at different time points (Hoffmann *et al.*, 2007). Analysing metagenomic data of viral and

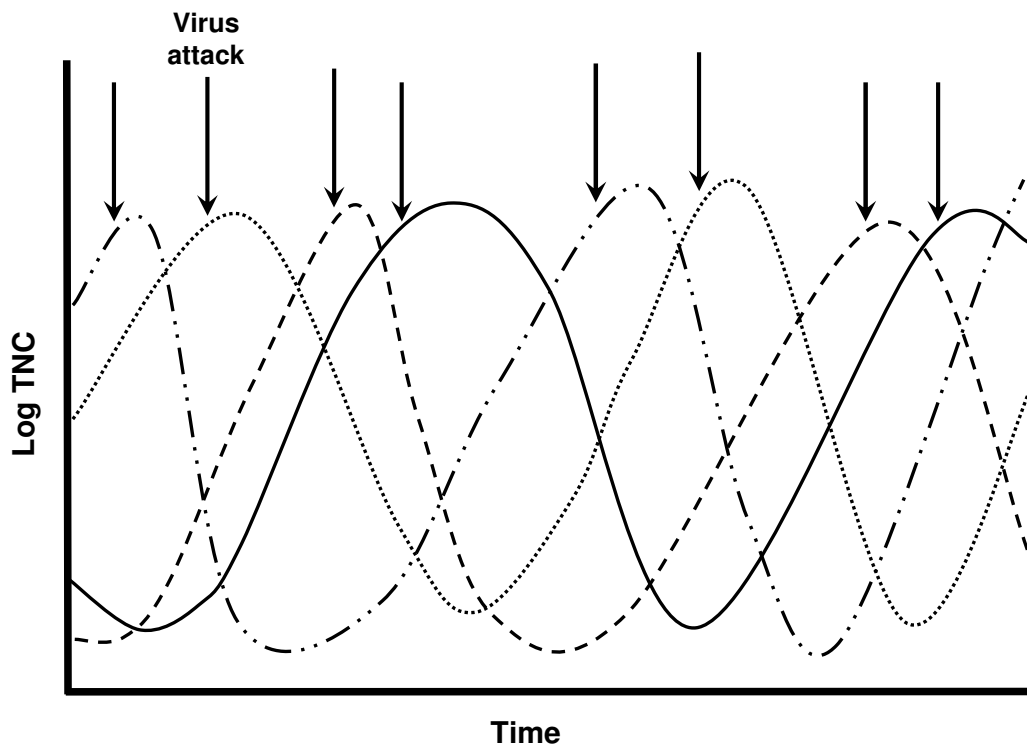


Figure 5 The viral populations are thought to support bacterial diversity by ‘killing the winner’ (Thingstad, 2000; Thingstad and Lignell, 1997). The black and patterned lines represent different bacterial populations which are thought to oscillate in numbers as the bacteria are attacked by specific viruses. Sometime after the bacterial populations have been lysed by viruses the bacterial populations gain immunity to the viruses, and then start to increase in biomass again before they are infected by modified or different viruses and subsequently decrease in numbers again.

bacterial origin from sludge bioreactors, clonal populations dominated in the samples were argued to be targets for the ‘killing the winner’ phage predation, and that the bacteria adapt to the local phage predation pressure (Kunin *et al.*, 2008).

The theory of viral populations structuring microbial communities has also been supported by experiments with enclosures with lake water (van Hannen *et al.*, 1999), seawater samples (Hennes *et al.*, 1995), and models systems with isolated marine bacteria and phages (Middelboe *et al.*, 2003b). Bouvier and del Giorgio (2007) incubated marine bacteria in sea water with a reduced number of VLP and found increased numbers of bacterial groups usually found in low concentrations, and hypothesised that rare marine groups may be more susceptible to viral-induced mortality and might be the faster growing species.

Lysogeny and microbial ecology

Temperate phages affect the bacterial diversity by increasing the horizontal gene transfer in microbial populations (Hendrix *et al.*, 1999). Horizontal gene transfer mediated by transduction

has been shown in *D. desulfuricans* (Rapp and Wall, 1987), and the temperate phages found in bacterial isolated from the Äspö HRL (**paper IV**) may also be a source of transferring genes between cells in the subsurface.

How common lysogeny is in environmental samples can be studied by inducing the lytic cycle in bacterial isolates. Chemical induction has revealed that about half of the marine isolates tested contained prophages (Paul, 2008). In six out of 20 bacterial isolates from soil, temperate phages were induced using UV or Mitomycin C and one isolate spontaneously produced phage particles. Hence, lysogeny was proposed to also be relatively common among soil bacteria (Williamson *et al.*, 2008a). In **paper IV** four out of ten *Desulfovibrio* isolates from the Äspö HRL grown in batch cultures were shown to contain temperate phages in late stationary phase, but chemical induction had little or no effect on their production. The extent of lysogeny can also be estimated analysing the genomes of bacteria; out of 113 genome sequences of marine bacteria, 64 contained prophages, and 21 of these were similar to gene transfer agents (Paul, 2008).

The production of lytic phages depends on the number of physical encounters between phages and host cells, while the production of lysogenic phages depends upon the number of cells containing prophages in the microbial communities, and the presence of an inducing agent (Weinbauer and Suttle, 1999). Oligotrophic conditions, like the ones found at Äspö HRL, have been suggested to drive viral life strategies towards the lysogenic life cycle rather than the lytic (Bongiorni *et al.*, 2005; Jiang and Paul, 1998; Weinbauer and Suttle, 1999). That is, lysogeny is thought to be the preferred viral life cycle when growth conditions for the host are unfavourable for rapid growth, and the phage can survive times of low host abundance as prophages. The metabolic activity of *Escherichia coli* has been shown to be down regulated by phage lambda (Chen *et al.*, 2005), and the same has been proposed to happen with marine bacteria containing prophages (Paul, 2008). The phages were suggested to increase the fitness of its host as it economizes the host energy usage by suppressing unneeded metabolic activities. Similarly, a high metabolic rate in the nutrient limited subsurface environments might not always be the most favourable way of living.

Also viral populations are thought to have exchanged genes (Hendrix *et al.*, 1999). The sequences of a conserved viral structural gene found in some cyanophages, has been shown to be diverse (Zhong *et al.*, 2002), but similar sequences are also found within different environments (Short and Suttle, 2005) implying that gene exchange occurs among phage communities from different environments. The similarity between DNA polymerase genes in podoviruses further supported this (Breitbart *et al.*, 2004a).

The activity of subsurface life

Due to the lack of high microbial biomass concentrations, intraterrestrial environments have been argued to be inhabited by inactive or extremely slowly metabolizing micro-organisms (Kerr, 2002). On the contrary, the microbial populations at Äspö HRL has been shown to be active using methods based on cultivation (Hallbeck and Pedersen, 2008; Kotelnikova and Pedersen, 1998; **paper III**) and cells have been shown to grow in biofilms (Pedersen and Ekendahl, 1990; Pedersen and Ekendahl, 1992). The viability of microbial communities has also been shown with data from deep granitic groundwater in Sweden, and Canada, which showed significant energy source assimilation (Jain *et al.*, 1997; Pedersen and Ekendahl, 1992), and results from ATP analysis proposed cells to be active (**paper I**).

Impact of viruses

Viral populations affects the modelling of ocean biology and biogeochemistry via the viral shunt, in which viral lysis kills infected cells, releases cell material, and makes nutrients available to other organisms (Danovaro *et al.*, 2008; Suttle, 2007; Wilhelm and Suttle, 1999). Carbon and nutrient transfer from a particulate to a dissolved form. Accordingly, Middelboe *et al.* (2003b) were using marine laboratory models to show how the viral lysis of cells of one bacterial species can increase the activity of another - the nutrients bound in cell material became available in an environment where growth of the second species was otherwise poorly supported.

Analysis of the phospholipid fatty acid content in granitic groundwater in Canada implied that the bacteria faced starvation stress (Jain *et al.*, 1997). However, under anaerobic conditions, a substantial amount, of up to 30 and 50%, of the micro-organisms in granitic groundwater can be cultivated in liquid media (Hallbeck and Pedersen, 2008; Pedersen *et al.*, 2008) showing that there is a potential for growth by the indigenous microbial populations once nutrients are provided. Such a chance to grow might arise partly from the nutrients released via viral lysis of cells. The lytic viruses in the deep subsurface are thought to effect the cycling of nutrients for the microbial population in deep granitic groundwater as shown in figure 6. Further, microbial populations in deep groundwater closed circulation systems at *in situ* conditions have been shown to grow to a certain number, where after the growth levelled out and started to decrease (Hallbeck and

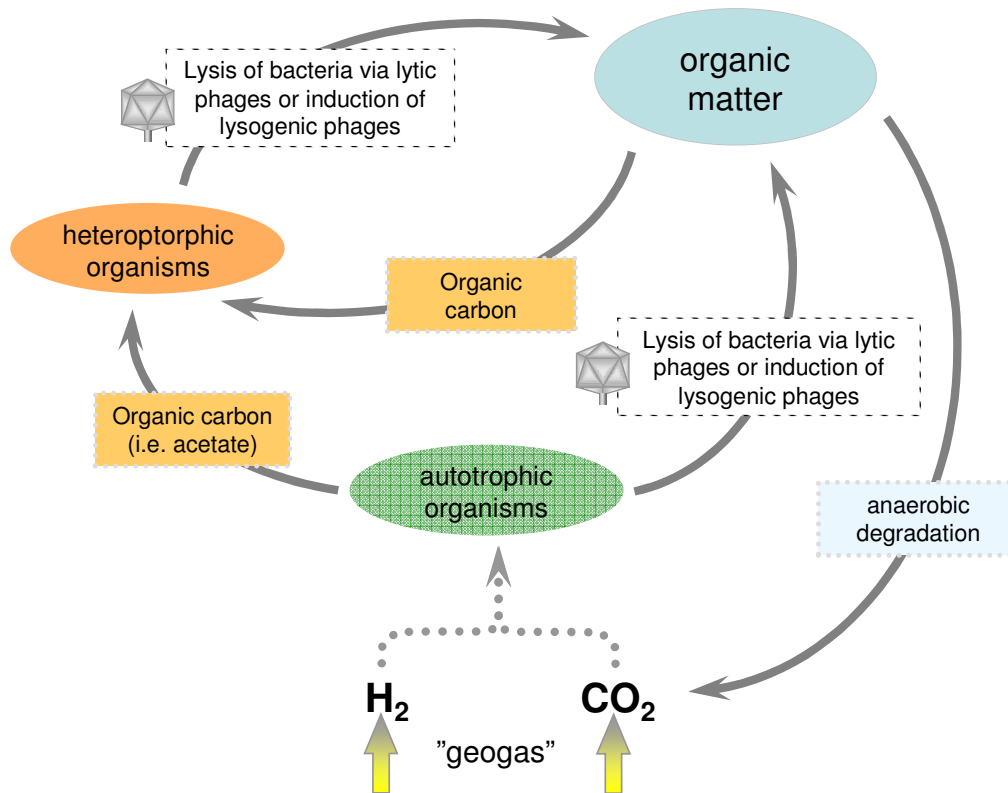


Figure 6 A model of how the viral populations mediate a viral shunt which affects the cycling of nutrients in the deep biosphere.

Pedersen, 2008). The microbial life in these circulation systems were likely limited by the amount of available nutrient and infection by viruses.

Deep-sea sediments are systems similar to the deep granitic groundwater in that there is no light available for photosynthesis. Here, the viral populations have been shown to control benthic bacterial and archaeal biomass by stimulating the metabolism of the cells (Danovaro *et al.*, 2008). Nutrient release caused by viruses was suggested to be associated with higher growth rates, i.e. the viral populations reduced the number of cells, but at the same time sustained the activity of the cells via a viral shunt in a system with severe organic resource limitations. In a similar way, Riemann *et al.* (2009) showed that the viral activity was important for the cycling of microbial carbon and phosphorus in the Öre Estuary, Sweden, particularly at the outer and more oligotrophic sites sampled.

The number of VLP has been further related to bacterial metabolism and turnover rates in samples from deep Mediterranean sediments (Danovaro *et al.*, 2002), bacterial activity in sediments from Nivå Bay in Denmark (Middelboe *et al.*, 2003a), to host cell numbers and activity in the Adriatic sea (Corinaldesi *et al.*, 2003), and when Middelboe *et al.* (2006) mixed and

incubated deep sea sediments from Sagami Bay in Japan with yeast extract the production of VLP was correlated to the bacterial metabolism.

The virus-to-bacterium ratio is frequently 10 to 1 in marine environments and the production and distribution of marine phages is determined by the density and productivity of their bacterial hosts (Chibani-Chennoufi *et al.*, 2004; Wommack and Colwell, 2000). The ratios found in marine systems was similar to what was reported for deep groundwater at the Äspö HRL in **paper II**, indicating metabolically active microbial communities in the subsurface.

Paper II reports on low virus to cells ratios in deeper boreholes compared to the more shallow ones. Schoenfeld *et al.* (2008) suggested that the lower virus to cell ratios they found in hot springs compared to moderate-temperature aquatic environments might be related to less lytic infections, since none of the cultured thermophilic crenarcheal viruses found in the system proliferate via lytic infection. Similarly, several morphologies which are known to be archaeal viruses were found at the Äspö HRL (**paper II**) and this part of the viruses may have a substantial effect on the ecology of archaeal populations without producing high numbers of viral particles through lysis of their host. Further, bacterial isolates from the deep groundwater containing temperate phages are described in **paper IV**. The part of the viral population surviving as prophages inside the host genomes can be an important part of the viral ecology even though they were not counted as VLP in the sampled water.

Additional support to micro-organisms being in a state of growth is the finding of active parasite-prey interactions of lytic viruses and the indigenous species *D. aespoeensis* (**paper III**). The mere findings of viruses in an environment are indicators of host activity since viruses only are known to replicate efficiently in living and preferably growing cells. Further, *Podoviruses*, which were isolated in **paper III**, are known to often have a narrow host range (Sullivan *et al.*, 2003; Suttle, 2005), which means that their specific host must be replicating and present in numbers able to sustain the viruses. Hence, the viruses have likely been isolated from dynamic microbial communities. Also, the lytic life strategy is thought to be more common when the host density is high (Chibani-Chennoufi *et al.*, 2004), in contrast to lysogeny, which has been shown to be more prevalent in isolates from oligotrophic marine environments (Bongiorni *et al.*, 2005; Jiang and Paul, 1998; Weinbauer and Suttle, 1999).

Also other nutrient poor systems have been shown to have active viral populations. Sawstrom *et al.* (2007) showed that a high frequency of bacterial cells, 26% on average, was visibly phage-infected in Antarctic and Arctic ultra-oligotrophic freshwater environments, and that the average bust size was low (4). The bacteria were found in low concentrations and were often slow growing, but the environments were still able to sustain constant virus populations.

Biofilms

In the papers included in this thesis, the microbial and viral populations of unattached micro-organisms were studied. However, the majority of the cells in groundwater is found in biofilms (Griebler and Lueders, 2009), and groundwater samples from Laxemar, an area a few kilometres away from the Äspö HRL, showed that attached communities were more metabolically active than unattached (Pedersen and Ekendahl, 1992). Viruses are known to interact with single and mixed species biofilms (Sutherland *et al.*, 2004), and are present in the biofilms in the deep granitic groundwater. The viral populations in deep subsurface ecosystems are thought to lyse cells, but how much the cells in the biofilms are affected by the viruses compared to the unattached cell is unknown. Riemann and Grossart (2008) used a model system of a marine phage-host pair, and showed how the addition of organic-matter-rich agarose beads enhanced the growth of bacteria and production of phages. The production of phages via the lytic cycle is dependent on the activity of the host, and since active microbial populations have been found in biofilms, the production of viral particles may be elevated in the biofilms compared to the free-living micro-organisms.

Impact on long-time storage of nuclear waste

Nuclear power is an effective way of producing energy, but has the disadvantage of leaving behind radioactive nuclear fuel which needs to be handled safely. Hence, a geological disposal of spent nuclear fuel has been proposed. In Sweden, the Swedish Nuclear Fuel and Waste Management Company (SKB) is taking care of the nuclear waste. They have developed a method for final disposal of the spent nuclear fuel which is based on three protective barriers (skb.se). The first barrier is an encapsulation in copper, the second is bentonite clay surrounding the copper canister, and the third protection is the granitic rock in which the waste is to be placed at a depth of approximately 500 m. A drawing of the Swedish KBS-3 storage model in deep granitic rock can be found in figure 7.

The effect of sulphate-reducing bacteria

Sulphate-reducing bacteria are ubiquitous in anoxic habitats (Muyzer and Stams 2008) and can be grown from water in granitic rock, at e.g. Äspö HRL (Hallbeck and Pedersen, 2008), in Finland (Pedersen *et al.*, 2008) and in Canada (Jain *et al.*, 1997). This type of bacteria have also been found in deep boreholes in South Africa, identified by their 16S rRNA gene sequence (Lin *et al.*, 2006), and phospholipid fatty acids associated with SRB were found in Colorado (Sahl *et al.*, 2008). Further, SRB have been detected, and can be cultured from, the bentonite clay used in the proposed model for the storage (Masurat *et al.*, 2008; Masurat *et al.*, 2009).

The SRB use sulphate as a terminal electron acceptor as they degrade organic compounds. Since SRB produce highly reactive, corrosive and toxic sulphide as they reduce sulphate they can cause serious problems for industries (Muyzer and Stams 2008). Sulphite produced by SRB can consequently corrode the copper surrounding the radioactive waste in the model in figure 7, and low numbers of SRB are preferred around the storage. The viral populations in the deep granitic groundwater are parasites on the cells, but to what extent it keeps the size populations low is unknown. Perhaps the viral shunt sustains active microbial communities in the groundwater via a viral shunt. The viral population would then reduce the number of SRB, but cycle the nutrients and make parts of the community more active.

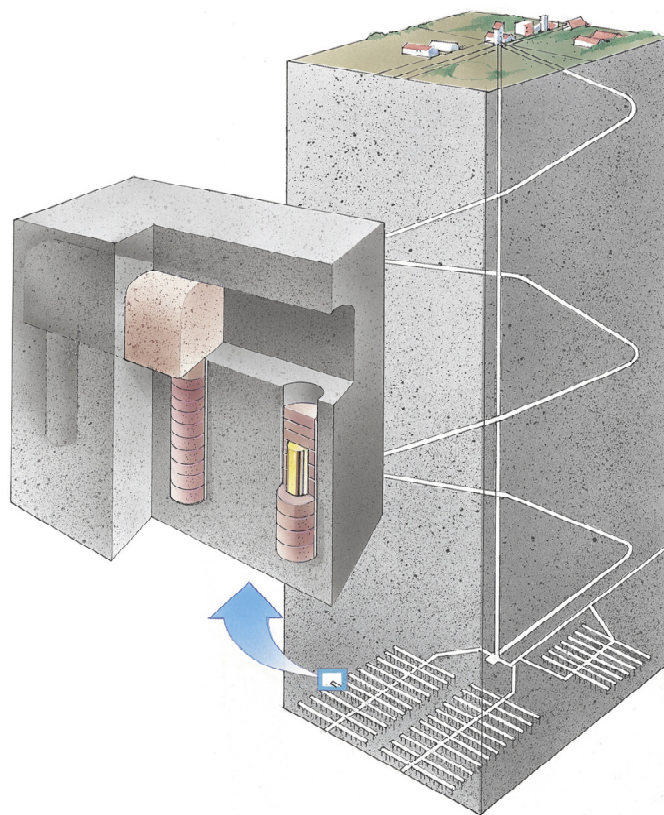


Figure 7 The model for long time storage of nuclear waste in deep granitic rock. The figure is from skb.se.

Concluding remarks

The aim of this thesis was to investigate the microbial ecology in deep groundwater with respect to activity and impact of viruses.

- I. The ATP assay used to estimate viable biomass in groundwater samples was shown to be reliable and also to reveal information about the metabolic status of the cells in tested samples. The microbial populations at different sites were likely to face different environmental conditions, as suggested by variations in the amount of ATP per cell.
- II. A diverse and abundant viral population was described and found to be ten times more abundant than microbial cells in samples from as deep as 450 m into the granitic rock groundwater. The total number of cells in subsurface environments has been estimated to be 0.25 to 2.5×10^{30} (Whitman *et al.*, 1998). Using these approximations, and if 5 to 15 times more viral like particles (VLP) than cells apply for the whole subsurface, a total number of 1.25 to 37.5×10^{30} VLP should exist in these ecosystems.
- III. Some of the viruses were found to be lytic to the indigenous sulphate-reducing bacterium *Desulfonivibrio aespoeensis*. The characteristics of the phages regarding burst size and other properties in subsurface environments is however unknown. Viral cultures have been shown to sustain their ability to infect their host for many years, but the loss of viral infectivity and the rate of degradation of viral particles in subsurface groundwater are factors still to be revealed. Further, the lytic phages described here were detected at several depth locations at the Äspö HRL using cultivation techniques and identifying these viruses by their gene sequences would in the future enable studies of viral diversity as well as their spread in the environment.
- IV. Sulphate-reducing bacteria were cultivated from water at the Äspö HRL, and sulphate reducing isolates were found to have temperate phages which were triggered in aged bacterial batch cultures in stationary growth phase. The temperate phages may alter the fitness of their host and promote genetic exchange via transduction.

- V. The subsurface viral populations are parasites on the micro-organisms and are probably involved in controlling the number of cells in the environment. The described viruses at the Äspö HRL were also likely sources of diversification of both cellular organisms and viruses via lytic infection of specific microbial populations.
- VI. The presence of lytic phages suggests that a viral shunt is involved in cycling of nutrients in the oligotrophic, deep groundwater environment, nutrients which can be a source of sustaining the activity of heterotrophic bacteria. The detected viable cells using the ATP assay, VLP abundances approximately ten times higher than cell number and lytic phages with a narrow host range imply living and active micro-organisms. It would be interesting to, in the future, study the activity and mortality of the micro-organisms in relation to viral numbers and production of viruses under *in situ* conditions.

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