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SHORT COMMUNICATION

Viruses in granitic groundwater from 69 to 450 m depth of the Äspö hard rock laboratory, Sweden

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The objectives of this study were to determine if viruses exist in deep granitic groundwater and to analyse their abundance and morphological diversity. Fluorescent microscopy counts on 10 groundwater samples ranging from 69 to 450 m depth were in the range of 10^4–10^6 TNC ml^{-1} (TNC, total number of prokaryotic cells) and 10^5–10^7 VLP ml^{-1} (VLP, virus-like particles). A good positive correlation of VLP with TNC ($r = 0.91$, $P = 0.0003$) was found with an average VLP/TNC ratio of 12. Transmission electron microscopy revealed four distinct bacteriophage groups (polyhedral, tailed, filamentous and pleomorphic) with at least seven phage families of which some are known to be lytic. Our results suggest the presence of viruses in deep granitic groundwater up to 450 m depth. If they are active and lytic, they will constitute an important group of predators that might control the numbers of microorganisms in the analysed groundwater.
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Prokaryotes colonise the deep subsurface to a depth of at least 3.3 km (Amend and Teske, 2005; Lin *et al.*, 2006). Deep intraterrestrial microbial life is investigated to understand the diversity of life on Earth, the evolution and potential origin of life deep underground and the tolerances of intraterrestrial life to extreme environmental conditions (Fredrickson and Balkwill, 2006). Applied aspects, for example, the impact of microbial activity on deep intraterrestrial storage of spent nuclear fuel, are also important (Pedersen, 2002). To completely understand the ecology of microorganisms and their impact on the surrounding environment, consideration needs to be given to the smallest member of microbial communities, viruses. Groundwater samples were obtained in November 2006 from 10 boreholes along the Äspö HRL tunnel, ranging from 69 to 450 m depth. The samples were analysed for numbers of virus-like particles (VLP), total number of prokaryotic cells (TNC) and chloride. In addition, one sample from each borehole was observed with transmission electron microscopy (TEM) and the viral morphological diversity of the samples was registered. To our knowledge, this is the first investigation of viruses in a deep intraterrestrial, fractured hard rock environment.

Äspö HRL is located in the island of Äspö near Oskarshamn, Sweden, and comprises a 3.6-km long tunnel that spirals down from the surface to a depth of 460 m in a granitic bedrock (Pedersen, 2001). It is a deep research facility that investigates the geological storage of spent nuclear fuel (Pedersen, 2002). The sampled boreholes were collected under *in situ* borehole pressure, as described elsewhere (Pedersen, 2001). Samples used for determining abundance and TEM imaging were collected in four sterile 50-ml polypropylene tubes and immediately preserved with 0.02-μm-filtered 37% acid-free formaldehyde to a final concentration of 2%. Samples were stored at 4 °C until further analysis. Three of the samples were used for determining TNC and VLP; they were stained with SYBR Gold according to the methods of Noble and Fuhrman (1998) and Chen *et al.* (2001) and analysed using an epifluorescence microscope (Leica Microsystems AB, Kista, Sweden). At least 300 microbial and 400 viral particles were counted per filter in up to 30 fields, except in borehole KJ0052F03 at 447 m depth, where 10 ml of sample per filter resulted in approximately 150 VLP and TNC counted in 30 fields. Each field counted was 0.01 mm^2 in size. TEM (Philips 201 TEM operating at 60 kV) was used to image the viral particles in the fourth sample of

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every sample set. Samples were filtered through a 0.2 μm syringe filter and centrifuged (RC5B Plus Superspeed centrifuge) at 19 000 r.p.m. for 2 h. All of the water except for 20–50 μl was removed; 20 μl of sample was then transferred onto formvar- and carbon-coated copper grids for 25–30 min and then stained with 1% uranyl acetate for 60 s, after which excess sample was wicked from the grid using filter paper. Grids were stored in the dark until being viewed on the TEM.

A good positive correlation of VLP with TNC (Figure 1a) was found. The VLP/TNC ratios ranged from 1.1 to 18.0, with an average ratio of 12. The groundwater in the boreholes at 69 m was young, comprising a mixture of groundwater aged from months to years (Banwart *et al.*, 1994), and has been demonstrated to harbour significant microbial activity (Banwart *et al.*, 1996). The VLP abundance and TNC were among the highest in these boreholes (Figure 1), reflecting recent contact with shallow (0–5 m), microbiologically diverse and active groundwater (Banwart *et al.*, 1996). However, deep groundwater from 300 and 415 m showed similar numbers.

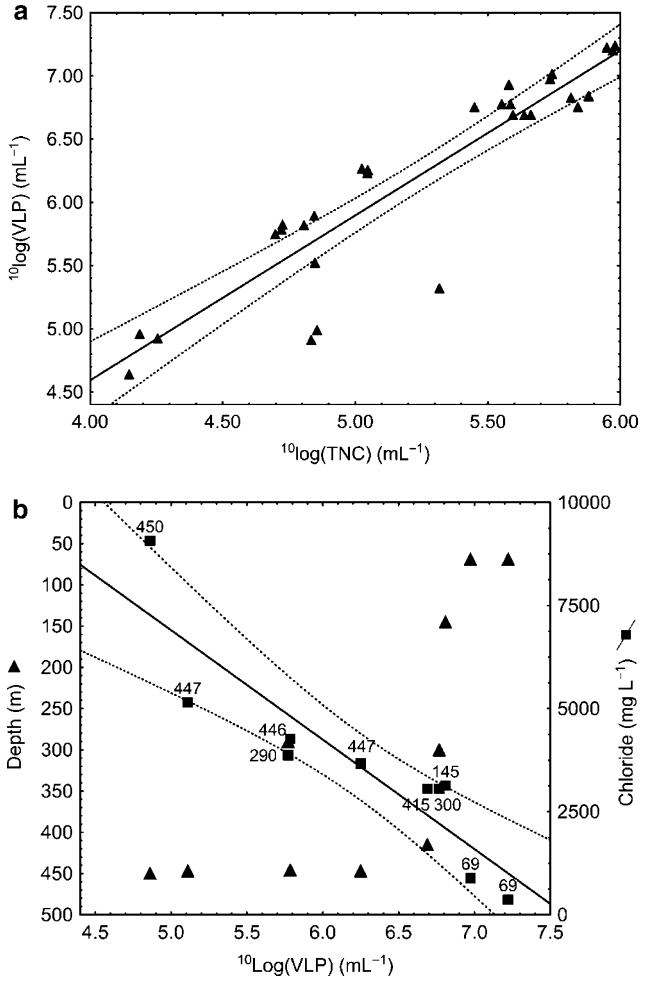

Figure 1 (**a**) The relation between the total numbers of cells (TNC) and the number of particles (VLP) in groundwater from 10 different boreholes distributed along the Äspö hard rock laboratory tunnel at depths from 69 down to 450 m. Three independent analyses were done for each borehole. Dashed lines show 95% confidence intervals. The least-squares regression line for VLP versus TNC is shown (^{10}log(VLP) = 1.30 × ^{10}log(VLP) − 0.62; $r = -0.91$, $P = 0.00001$, $n = 30$). (**b**) The relation between the average ($n = 3$) of ^{10}log(VLP), depth and amount of chloride in groundwater from 10 different boreholes distributed along the Äspö hard rock laboratory tunnel at depths from 69 down to 450 m. Numbers close to the symbols indicate sample depth. Dashed lines show 95% confidence intervals. The least-squares regression line for ^{10}log(VLP) versus chloride is shown (chloride = −2654 × ^{10}log(VLP) + 20 172; $r = -0.90$, $P = 0.0004$, $n = 10$).

The age and origin of groundwater surrounding the Äspö HRL tunnel have been studied and found to generally correlate with salinity (Laaksoharju *et al.*, 1999b). Typically, a large part of groundwater at a depth of 500 m is approximately 7000 years old; that is, it went underground at the end of the last Fennoscandian glaciation (Laaksoharju *et al.*, 1999a). The amount of chloride in the investigated Äspö groundwater, which represents salinity, was not well correlated with depth (Figure 1b), owing to the very heterogeneous character of aquifers in the rock. The VLP numbers showed a good exponential correlation with chloride (Figure 1b), as did TNC (not shown). There was no correlation of VLP, TNC or chloride with depth. The inverse exponential relationships between chloride and VLP and chloride and TNC may be due to electrostatic phenomena. A high ionic strength decreases the electrostatic double layer, which increases the chance that viruses and prokaryotes are trapped in the secondary attraction trough (Marshall, 1976) and the resulting attached virus–microbe ecosystems will not be revealed by groundwater samples. Alternatively, old saline groundwater that stands isolated (Laaksoharju *et al.*, 1999b) may be less favourable for microbial growth and viral activity, compared with more diluted groundwater. Future sampling and analyses of both biofilms and groundwater are required to fully understand the observed decrease of TNC and VLP in groundwater with high salinity.

Transmission electron microscopy exposed a diverse suite of viral morphologies (Figure 2). In a total of 252 examined viruses, 4 different morphological groups were identified, including polyhedral, tailed, filamentous and pleomorphic shapes. At 69 m underground, 12 viral subgroups were represented (135 observations), compared with only 1 at 447 m (29 observations). Numbers of tailed viruses (Figures 2a, c and j–l) represented 43% of the viral morphotypes detected (110 observations), whereas numbers of polyhedral viruses (Figures 2b and m) represented 31% of the morphotypes (78 observations), except at 447 m where they represented 100% (29 observations). Of the tailed viruses, *Siphoviridae* (Figures 2a, j and k) were the most common, followed by *Podoviridae* (Figure 2l) and then *Myoviridae* (Figure 2c). Spherical capsids were more common than helical capsids in all the

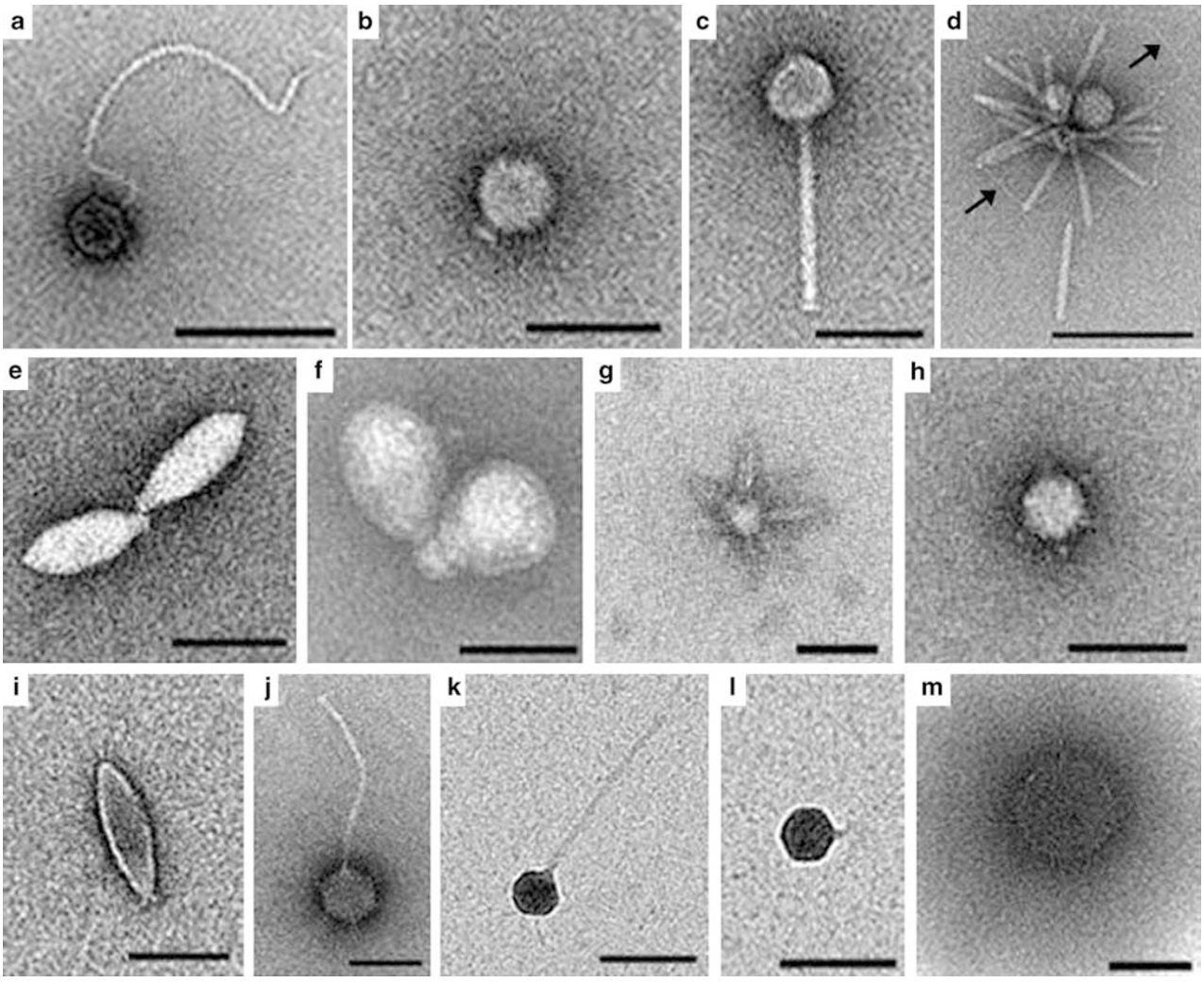

Figure 2 Transmission electron micrographs of viruses from Äspö hard rock laboratory groundwater. Viral morphotypes found near depths of 69 (**a**–**h**), 294 (**i**, **j**), 415 (**k**, **l**) and 447 m (**m**) are shown: (**a**) *Siphoviridae* (B1); (**b**) polyhedral virus with base plate; (**c**) *Myoviridae* (A1) with base plate and (**d**) *Inoviridae* connected by filaments around the outer ends (arrows). Two polyhedral viruses are also shown: (**e**) *Salterprovirus*; (**f**) *Guttaviridae*; (**g**) polyhedral virus with spike-like protrusions; (**h**) polyhedral virus (STIV-like); (**i**) *Fuselloviridae* with twinned tail; (**j**) *Siphoviridae* (B1) with curved tail; (**k**) *Siphoviridae* (B1) with straight tail; *Podoviridae* (C1) and (**m**) polyhedral virus. Scale bar is 125 nm, except in **a** and **d**, where it is 250 nm.

tailed viruses. Tail lengths and shapes also varied, along with the presence and/or absence of a base plate. Filaments were not found on any of the viruses examined. *Siphoviridae* and *Myoviridae* morphotypes are typically found infecting bacteria (Prangishvili *et al.*, 2006a), although tailed phages have also been commonly found infecting hosts in the archaeal domain, Euryarchaeota (Prangishvili *et al.*, 2006b). Filamentous *Inoviridae* were found at a depth of 69 m (21 observations), where they occurred radiating from a central point and attached to each other along their outer ends by thin filaments (Figure 2d). The pleomorphic viruses in the Äspö groundwater were represented by archaeal types (Figures 2e, f and i), most of which were fusiform archaeal viruses (Figure 2e and i) (12 observations), and *Guttaviridae* (Figure 2f) (2 observations). Abundance of archaeal viruses decreased with depth, as only *Fuselloviridae* viruses were reportedly found at or below 300 m. Of the archaeal viruses, *Salterprovirus* (Figure 2e) were the most abundant archaeal virus noted at 69 m (4 observations). Viral diversity was consequently large in the shallow samples and it decreased somewhat with increasing salinity.

Viruses are dependent on active and growing host microorganisms for their multiplication. The number of VLP has been demonstrated to be significantly related to bacterial turnover in samples from deep Mediterranean sediments (Danovaro *et al.*, 2002), to bacterial activity in sediments from Nivå Bay in Denmark (Middelboe *et al.*, 2003) and to the number of host cells in the Adriatic Sea aquatic system (Corinaldesi *et al.*, 2003). High VLP/TNC ratios of about 10, like those observed here (the average was 12), are consequently indicative of viruses actively infecting microorganisms that also must be metabolically active. This confirms earlier obtained

energy source assimilation data (Pedersen and Ekendahl, 1992) and recent ATP analysis data (Eydal and Pedersen, 2007), both of which suggested that the investigated microorganisms were in a state of growth. A predator–prey relationship may be present in deep groundwater that then contains active and growing microorganisms continuously predated by viruses to observed steady-state numbers in the range of 10^4–10^6 cells ml^{-1} (Pedersen, 2001), just as it is in many surface environments (Wiggins and Alexander, 1985).

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