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ORIGINAL ARTICLE

Unexpected and novel putative viruses in the sediments of a deep-dark permanently anoxic freshwater habitat

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Morphological diversity, abundance and community structure of viruses were examined in the deep and anoxic sediments of the volcanic Lake Pavin (France). The sediment core, encompassing 130 years of sedimentation, was subsampled every centimeter. High viral abundances were recorded and correlated to prokaryotic densities. Abundances of viruses and prokaryotes decreased with the depth, contrasting the pattern of virus-to-prokaryote ratio. According to fingerprint analyses, the community structure of viruses, bacteria and archaea gradually changed, and communities of the surface (0–10 cm) could be discriminated from those of the intermediate (11–27 cm) and deep (28–40 cm) sediment layers. Viral morphotypes similar to virions of ubiquitous dsDNA viruses of bacteria were observed. Exceptional morphotypes, previously never reported in freshwater systems, were also detected. Some of these resembled dsDNA viruses of hyperthermophilic and hyperhalophilic archaea. Moreover, unusual types of spherical and cubic virus-like particles (VLPs) were observed. Infected prokaryotic cells were detected in the whole sediment core, and their vertical distribution correlated with both viral and prokaryotic abundances. Pleomorphic ellipsoid VLPs were visible in filamentous cells tentatively identified as representatives of the archaeal genus *Methanosaeta*, a major group of methane producers on earth.

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Introduction

Aquatic sediments, even most deep and ancient, harbor abundant communities of viruses and their prokaryotic hosts (Bird *et al.*, 2001; Middelboe *et al.*, 2011). Reported viral abundances in sediments are 10–1000 times higher than in the water column (Danovaro *et al.*, 2008a). However, in aquatic systems, viruses are mainly studied in the pelagic compartment and only very limited information is available on viruses in shallow marine and freshwater sediments (reviewed in Danovaro *et al.* (2008a)). Accordingly, the diversity of viriobenthos is nearly unknown (Breitbart *et al.*, 2004; Filippini and Middelboe, 2007; Leroy *et al.*, 2008; Helton and Wommack, 2009). The analyses by metagenomic sequencing (Breitbart *et al.*, 2004) and pulsed field gel electrophoresis (Filippini and Middelboe, 2007) revealed that 1 kg of near-shore marine sediment contains 10^4–10^6 viral types and that viral diversity fluctuates over space and time. These studies suggest (i) that the majority of sediment viruses does not originate from the upper water column (Fischer *et al.*, 2004), and (ii) that viruses are active components of benthic environments. Indeed, the dilution or viral decay assays indicate that viruses could control a large part of microbial activities in the sediments (Mei and Danovaro, 2004; Middelboe and Glud, 2006; Middelboe *et al.*, 2006; Danovaro *et al.*, 2008b; Corinaldesi *et al.*, 2010). However, only small numbers of visibly infected cells have been observed in both freshwater (Bettarel *et al.*, 2006; Filippini *et al.*, 2006; Säwström *et al.*, 2009) and marine benthic environments (Danovaro *et al.*, 2008b). These results have led to the concept of 'infectivity paradox' in which high viral abundance

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contrasts with low number of visibly infected prokaryotes in sediments (Bettarel *et al.*, 2006; Filippini *et al.*, 2006; Danovaro *et al.*, 2008a).

The statement that the viriobenthos is understudied is particularly true for the viral communities inhabiting deep, dark and permanently anoxic freshwater sediments. To our knowledge, no study has been conducted in these environments that are nevertheless known to have particular, highly diverse microbial communities. Moreover, microbial communities in these environments have metabolisms with potential roles exceeding the regional scale. For example, lacustrine anoxic sediments are the site of an intense methane production (responsible of 5–16% of natural methane emissions, Bastviken *et al.,* 2004) catalyzed by methanogenic archaea. However, interactions between archaea and viruses in deep anoxic freshwater sediments remain enigmatic.

The present study is an exploratory work aimed at the acquisition of first data on viral communities thriving in anoxic freshwater sediments along a gradient encompassing 130 years of sedimentation. We analyzed the sediments, at high vertical resolution, in order (i) to access to the diversity of viral morphotypes, (ii) to analyze the abundances and spatial distribution of viral and host communities and (iii) to determine the infectivity potential of viruses based on direct observations of virus-infected prokaryotes.

Materials and methods

Study site and sampling procedure

Lake Pavin (2°53′12″E, 45°) is located in the French Massif Central. Environmental characteristics of this lake were described elsewhere (Viollier *et al.*, 1997; Aeschbach-Hertig *et al.*, 2002). Briefly, Lake Pavin (altitude 1197 m) is a typical meromictic and dimictic mesotrophic crater mountain lake characterized by a maximum depth of 92 m and low surface (44 ha) and catchment (50 ha) areas. A feature of the physical structure of Lake Pavin is the existence of a chemocline (60 and 70 m depth), that separates the seasonally mixed mixolimnion from the permanently anoxic monimolimnion. The sedimentation rate is stable and low (3.7 mm per year, Schettler *et al.*, 2007). In the monimolimnion, sediments, permanently anoxic, are mainly of biogenic origin (20–70% of siliceous skeleton of diatoms, Schettler *et al.*, 2007), are iron-rich (2–25% of particulate iron, Schettler *et al.*, 2007) and have an organic carbon content of 4–9% solid (Schettler *et al.*, 2007).

Triplicate sediment cores were carried out using an Uwitec gravity corer (UWITEC devices, Mondsee, Austria) at the deepest place of the lake in December 2009. Sediment cores of 40 cm were sampled, representing approximately 130 years of sedimentation history of the lake (Schettler *et al.*, 2007). Subsamples were collected every centimeter (that is, from 1 to 40 cm) along the sediment cores. Identical depth subsamples (1 cm layer of sediment) from three independent cores were pooled, homogenized, flash frozen and kept at $-20\,^{\circ}C$ until further analyses. Sediment porosity was determined according to the following equation $V = (((\text{dry weight})/2.51) + (\text{wet weight} - \text{dry weight}))$, where $2.51\,g\,cm^{-3}$ represents the mean particle density (Avnimelech *et al.*, 2001). The average porosity was $95.6 \pm 0.7\%$ along the 40-cm sediment core.

Extraction of viruses and prokaryotes from sediment

Viruses and prokaryotes were extracted from sediment as recommended by Danovaro *et al.* (2001). Following thawing at room temperature, sediment aliquots were diluted with tetrasodium pyrophosphate solution (final concentration, 10 mM), shaked for 30 min, and sonicated three times (1 min each) in a water bath (Fisher Bioblock Scientific 88156, 320W, Illkirch, France). Larger particles were removed by centrifugation at $800 \times g$ for 1 min. Supernatant was divided in subsamples. A subsample was fixed with glutaraldehyde (1% final concentration) and stored at 4 °C before flow cytometry analyses and to the observation of the viral infection and of the morphological diversity of viruses by transmission electron microscopy (TEM). Viral DNA extraction was performed on the raw subsample (not fixed) within few hours.

Abundances of viruses and prokaryotes

Viral and prokaryote counts were performed within 48 h following extraction by flow cytometry using a FACSCalibur flow cytometer equipped with an air-cooled laser providing 15 mW at 488 nm with the standard filter set-up (Becton Dickinson, Franklin Lake, NJ, USA) as described in Marie *et al.* (1999); Brussaard (2004), and Duhamel and Jacquet (2006). Briefly, extracted samples were diluted in 0.02 μm filtered TE buffer and stained with SYBR Green 1 (10 000fold dilution of commercial stock, Molecular Probes, Eugene, OR, USA). Mixture was incubated for 5 min, heated for 10 min at 80 °C in the dark and cooled for 5 min before analysis. Populations of prokaryotes and viruses differing in fluorescence intensity were distinguished on plots of side scatter versus green fluorescence (530 nm wavelength, fluorescence channel 1 of the instrument). Flow cytometry list modes were analyzed using CellQuest Pro software (BD Biosciences, San Jose, CA, USA; version 4.0). Viral and prokaryotic abundances were expressed as virus-like particles (VLPs) or prokaryotes g^{-1} of dry sediment.

Concentration of viruses

Viral concentration and purification were performed using polyethylene glycol (PEG) as described in

Colombet *et al.* (2007). Briefly, following successive prefiltration steps, viruses contained in 5 ml of $<0.2\,\mu m$ size-fraction of extracted subsamples were submitted to PEG precipitation. Owing to the high number of VLPs in the raw natural samples ($>7\times10^9$ VLPs g^{-1} dry sediment), the step of preconcentration by ultrafiltration classically used in the PEG-protocol was not necessary. Polyethylene glycol 8000 (catalog no. 81268; Sigma-Aldrich, St Louis, MO, USA) together with NaCl were directly added to the natural samples at final concentrations of 10% and 0.6%, respectively, and incubated at 4 °C in the dark during 5 days. The white phase containing crystallized viruses was centrifuged at $16\,000\times g$ for 25 min at 4 °C, and resuspended in 0.02 μm filtered water. KCl of 1 M was then added, the mixture incubated on ice for 20 min and centrifuged ($12\,000\times g$, 10 min at 4 °C). The supernatant with clean concentrated viruses was either stocked at 4 °C or fixed with glutaraldehyde (1% final concentration) and stored at 4 °C before microscopic examination of viral phenotypes.

Extraction of prokaryotic and viral DNA

Prokaryotic DNA. Genomic DNA of prokaryotes was extracted from untreated subsamples of sediment kept at −20 °C, using the Ultra clean soil DNA extraction kit (MOBio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions.

Viral DNA. Concentrated VLP subsamples were filtrated onto 0.2 μm membrane filter (Amicon Ultrafree-MC, Millipore, Billerica, MA, USA) to remove any remaining cellular organisms, and treated with DNAse I (Invitrogen, Paisley, UK). Viral DNA was then released by thermal shock (2 min at 100 °C followed by 2 min on ice), which bursts viral capsids. Qiamp DNA mini kit (Qiagen, Hilden, Germany) was used to purify viral DNA. Possible bacterial contamination was checked by PCR amplification using the 16S rDNA primers 27f and 1492r as previously described (Lehours *et al.*, 2007).

Random amplification of polymorphic DNA-PCR (RAPD-PCR) fingerprinting of viral community

Primer OPA-9 (5′-GGGTAACGCC-3′) was used in all PCR reactions (Winget and Wommack, 2008). PCR mixtures (25 μl) contained 2.0 mM $MgCl_2$, 200 μM of each deoxyribonucleoside triphosphate, 4 μM of primer, 2.5 U of Taq DNA polymerase (Bioline, London, UK) and 20–40 ng of viral DNA. Reactions were carried out in a MJ Research PTC-200 thermocycler using the following program: a 3 min hot start at 94 °C; 33 cycles of 3 min at 35 °C, 1 min at 72 °C and 30 s at 94 °C; and a final extension for 10 min at 72 °C. Ten microliters of RAPD-PCR products were visualized by gel electrophoresis on a 2% Nusieve agarose gel (Tebu-Bio, Le-Perray, France) containing ethidium bromide ($0.5\,mg\,ml^{-1}$) and normalized with a 100-bp size marker (Invitrogen). Resulting band patterns were analyzed using GelCompar II (version 4.50; Applied Maths, Sint-Martens-Latem, Belgium). The similarity of RAPD-PCR banding patterns was determined using Dice's coefficient (presence versus absence of bands), and a dendrogram depicting banding pattern similarity was generated using the unweighted pair group method of average algorithms (UPGMA, Ward, 1963).

Analysis of prokaryotic structure by temporal temperature gel gradient electrophoresis

Bacterial and archaeal 16S rDNA genes were amplified using the set of primers 27f-GC/518r (Muyzer *et al.*, 1993) and 934f-GC/1386r, respectively (Skillman *et al.*, 2004). The PCR program used was a 15 min hot start at 95 °C, 30 cycles consisting of denaturation (1 min at 95 °C), annealing (1 min at 60 °C) and extension (1 min at 72 °C), with a final extension for 10 min at 72 °C. PCR products were quantified with the DNA quantification fluorescence assay kit (Sigma-Aldrich, St Louis, MO, USA). Each sample weighing 300 ng were electrophoresed through an 8% polyacrylamide gel (TAE 1.25×, urea 7 M, Temed 0.06%, ammonium persulfate 0.0625%) as previously described (Batisson *et al.*, 2007). Band patterns were analyzed using the GelCompare 4.6 software package (Applied Maths, Kortrijk, Belgium). A 1% band position tolerance (relative to total length of the gel) was applied in band assignment, which indicates the maximal shift allowed for two bands in different temperature gel gradient electrophoresis tracks to be considered as identical. Pairwise similarity matrices were calculated using the Jaccard's equation from presence/absence of data. Dendrograms were generated using UPGMA method.

TEM analyses

Phenotypic diversity of viruses. The shape and size of VLPs were analyzed by TEM from concentrated subsamples and from non-concentrated extracted benthic viruses in fixed samples (procedure described below in section 'viral infection of prokaryotes'). Concentrated VLPs were collected onto 400-mesh Cu electron microscopy grid supported with carbon-coated Formvar film (Pelanne Instruments, Toulouse, France). Fourty microliters of viral concentrate was diluted in 6 ml of 0.02 μm filtered distilled deionised water and centrifuged at $120\,000\times g$ for 2 h at 4 °C using a SW40Ti rotor in a Beckman LE-80K Ultracentrifuge (Brea, CA, USA). Each grid was stained at room temperature for 30 s with uranyl acetate (2% w/w), rinsed twice with 0.02 μm distilled water and dried on a filter paper. Grids were examined in a JEM 1200EX TEM (JEOL, Akishima, Japan) operated at 80 KV at a magnification of ×15 000–150 000. Individual morphotypes of VLPs were identified from overview.

Morphometric measures were done using the Axio Vision v 4.7.1.0 (2008) (Carl Zeiss, Oberkochen, Germany).

Observation of Methanosaeta concilii. A culture of the methanogenic species, *M. concilii* (strain Opfikon = DSM-2139, Patel and Sprott (1990)), was observed by TEM following the procedure described below.

Viral infection of prokaryotes. Fixed extracted benthic samples were diluted with 0.02 μm filtered distilled deionised water and harvested by ultracentrifugation at $70\,000 \times g$ for 20 min onto 400-mesh Cu grids using a SW40Ti rotor in a Beckman LE-80 K Ultracentrifuge. VLPs were then stained for 30 s with uranyl acetate (2% w/w) before examination at a magnification of $\times 10\,000$–$40\,000$ using the JEM 1 200 EX TEM (JEOL) operated at 80 kV to distinguish between viral-infected and -uninfected prokaryotes (Bettarel *et al.*, 2006). At least 300 prokaryotes cells were inspected per sample and a total number of about 15 000 prokaryotes were observed for the entire sediment core. Mean burst sizes (viruses per cell) were determined by averaging the number of viruses per infected cells.

Statistical analyses
Data were log transformed to satisfy the requirements of normality and homogeneity of variance, which are necessary for parametric analyses. Vertical variations of data sets were analyzed with a log-log linear regression. Simple relationships between variables were tested by Pearson correlation analysis. All statistical analyses were performed with Release 12 software for windows (Minitab, State College, PA, USA).

Results

Abundances and community structure of viruses and prokaryotes
The averaged viral and prokaryotic densities were $21.2 \pm 8.4 \times 10^9$ VLPs g^{-1} and $5.03 \pm 2.9 \times 10^9$ cells g^{-1} of dry sediment, respectively. These densities were highest in surface sediments and decreased significantly ($P<0.001$) with depth (Figures 1b and c). Both variables were correlated ($r=0.80$, $P<0.001$), with an average virus-to-prokaryote ratio of 5.0 ± 2.3. Virus-to-prokaryote ratio increased significantly ($P<0.001$) with the depth, with the highest values (>9 VLPs per prokaryote) recorded in the 30–35 cm deep sediment layer (Figure 1d).

The number of viral genome bands, determined by electrophoresis of RAPD-PCR products, varied between 7 and 13 (in average 9.1 ± 2.2) and the structure of viral communities showed differences across the depth (Figure 1f). Three clusters of bands were discriminated, representing the surface (0–10 cm), intermediate (11–27 cm) and deep (28–40 cm) sediment layers (Figure 1f). The similarity indices within each cluster ranged from 50 to 95%. These groupings of viral communities apparently matched with changes in the spatial community structure observed for both archaea and bacteria (Figures 1g and h).

Phenotypic diversity of viruses
The VLP morphotypes revealed by TEM were highly diverse and could be categorized in two broad groups: (A) 'typical freshwater viruses' (Sime-Ngando and Colombet, 2009), (B) morphotypes unusual for freshwater samples.

Group A included apparently non-enveloped VLPs with tailed morphology (Figures 2a–h), as well as nonenveloped, presumably icosahedral particles without tails (Figures 2h–l). Capsids and tails of these VLPs presented a large variety of sizes and forms, including remarkable siphovirus-like particles with remarkably long tails, up to 5 μm (Figure 2f), and giant polyhedral VLPs with capsid diameter up to 0.5 μm (Figure 2j).

Types of VLPs within group B exhibited morphotypes unexpected in the analyzed environment (Figure 3). Surprising was the observation of spindle-shaped (tailed or tailless) (Figures 3a–e) or rod-shaped (Figures 3f and g) particles that was similar to VLPs previously reported from geothermal and hypersaline environments (Bath *et al.*, 2006; Prangishvili, 2006; Sime-Ngando *et al.*, 2011). Four types of complex viral forms (Figures 3h–n) were also never reported from freshwater systems. One type of such particles, quasi-spherical, enveloped, carried in the 'lumen' an electron-dense polyhedral body (Figures 3i–l). The diameter of the particles was 220 ± 10 nm, their outer envelope was 35 ± 5 nm thick and the polyhedral body was 110 ± 10 nm in diameter. A second type of exceptional particles appeared as slightly pleomorphic spheres, about 30 nm in diameter, with an inner core of low density with a diameter of about 15 nm (Figure 3m). The third type of exceptional particles had a form of a prolate ellipsoid with axes of about 200 and 40 nm (Figure 3h). Finally, to the group B belonged quasi-cubic particles, with square faces measuring about 70×70 nm, with a central core of high density with a diameter of about 30 nm (Figure 3n).

Viral lysis of prokaryotes
A total of 15 000 individual prokaryotic cells were observed by TEM and among them 50 were visibly infected, that is, more than 5 intracellular VLPs could be recorded. The percentage of visibly infected cells ranged from undetectable to 1.05% at 13 cm depth, and decreased significantly with depth ($P<0.05$). Values $>0.9\%$ were recorded at 1, 6, 13, and 23 cm depth, but viral infection was clearly demonstrated in the whole sediment core (Figure 1e). Interestingly, the visibly infected cells was correlated to both viral ($r=0.5$, $P<0.01$) and prokaryotic abundances ($r=0.4$, $P<0.01$). Burst size

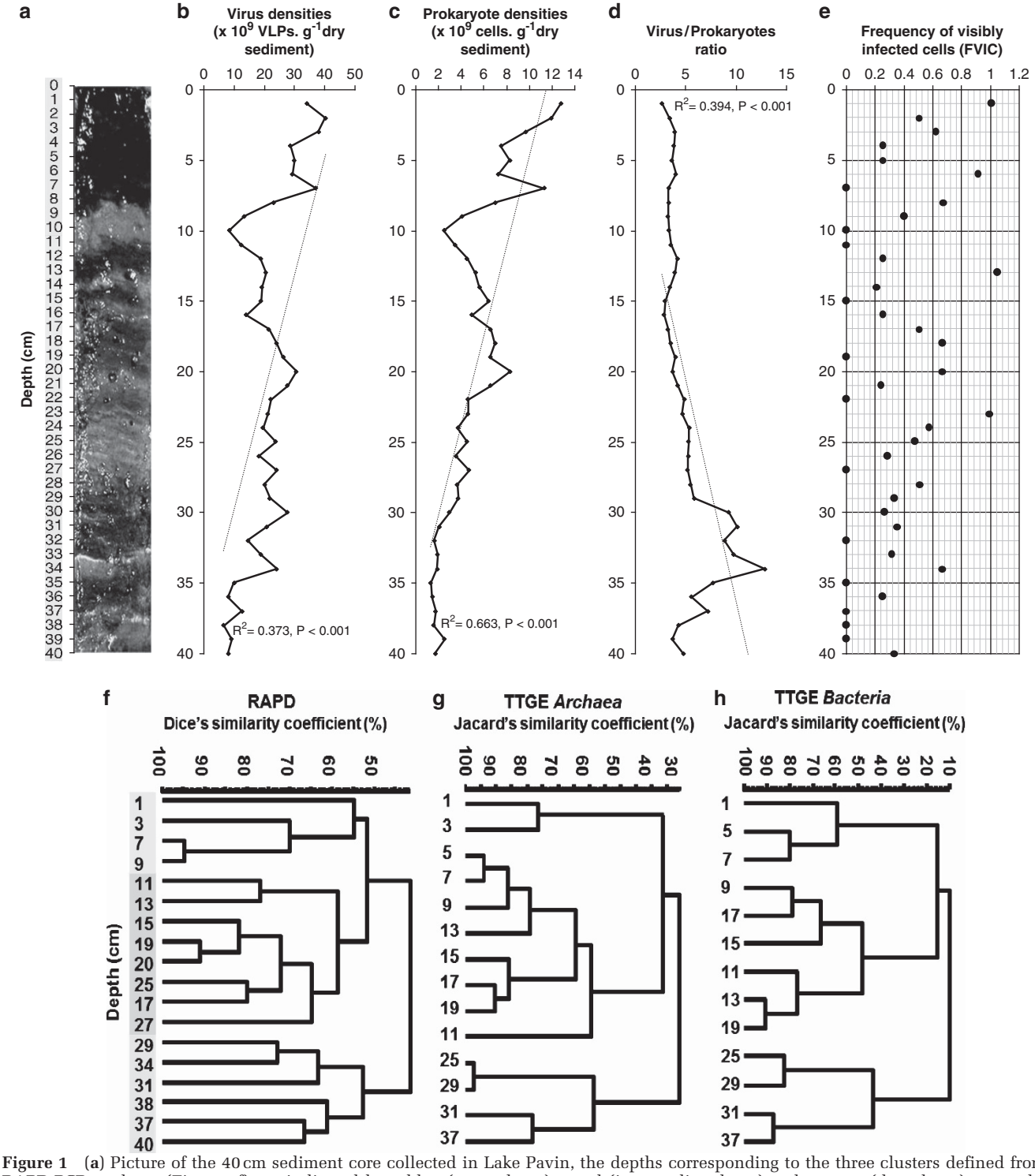

Figure 1 (**a**) Picture of the 40 cm sediment core collected in Lake Pavin, the depths corresponding to the three clusters defined from RAPD-PCR analyses (Figure 1f) are indicated by a blue (upper layer), a red (intermediate layer) and a green (deep layer) rectangle. (**b**) Vertical distribution of viral densities ($\times 10^9$ VLPs per gram of dry sediment), (**c**) Vertical distribution of prokaryotic densities ($\times 10^9$ cells per gram of dry sediment), (**d**) virus-to-prokaryotes ratio, (**e**) frequency (i.e., percentage) of visibly infected cells (%), (**f–h**) cluster analyses from RAPD-PCR and temporal temperature gel gradient electrophoresis data performed for viruses and 16S rDNA archaeal and bacterial gene fragments, respectively. Hierarchical cluster analyses were performed as indicated in the experimental procedures section.

ranged from 5 to 160 (in average 42.9 ± 33.1) VLPs per cell. Most of the infected cells were bacilliform, ranging from fat and short to thin and long rods (Supplementary Figure S1). Interestingly, we observed the occurrence of particular filamentous cells apparently infected by pleomorphic ellipsoid

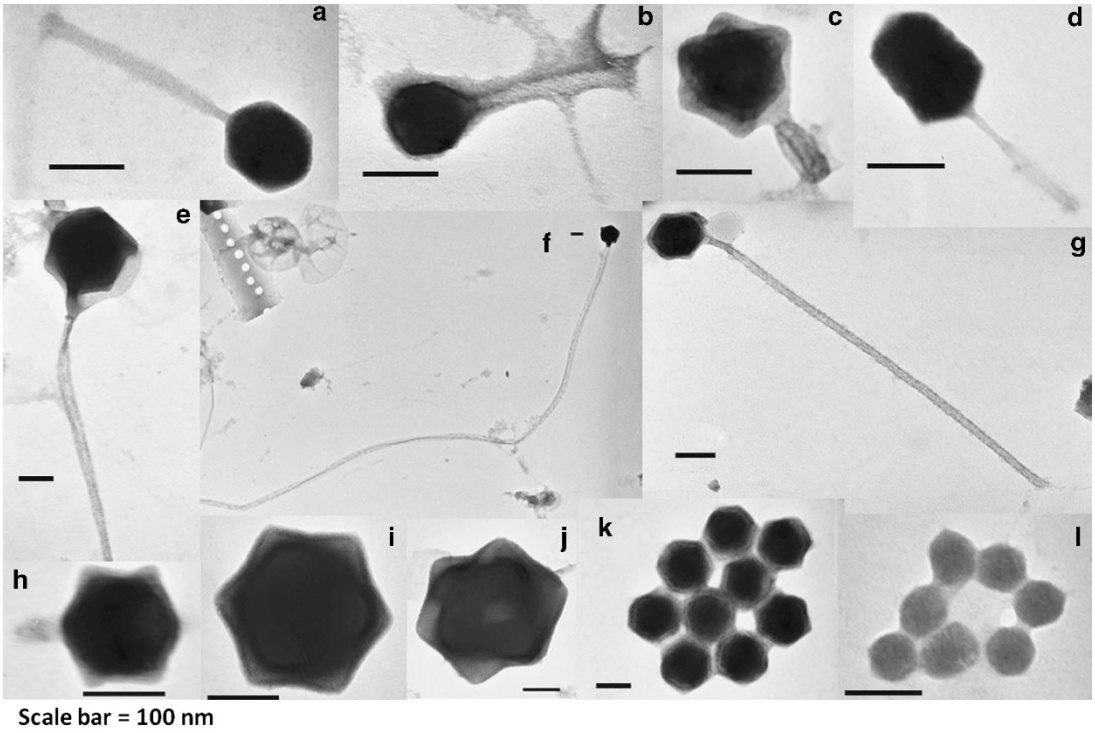

Figure 2 Transmission electron micrographs of typical freshwater viruses observed along the 40 cm sediment core from Lake Pavin. Magnification for each micrograph is indicated as follows: Myoviridae (**a** (× 120 000), **b** (× 50 000), **c** (× 150 000)), Siphoviridae (**d** (× 100 000), **e** (× 40 000), **f** (× 15 000), **g** (× 60 000)), Podoviridae (**h** (× 120 000)), tailless viruses (**i** (× 150 000), **j** and **k** (× 80 000), **l** (× 120 000)). Scale bar = 100 nm.

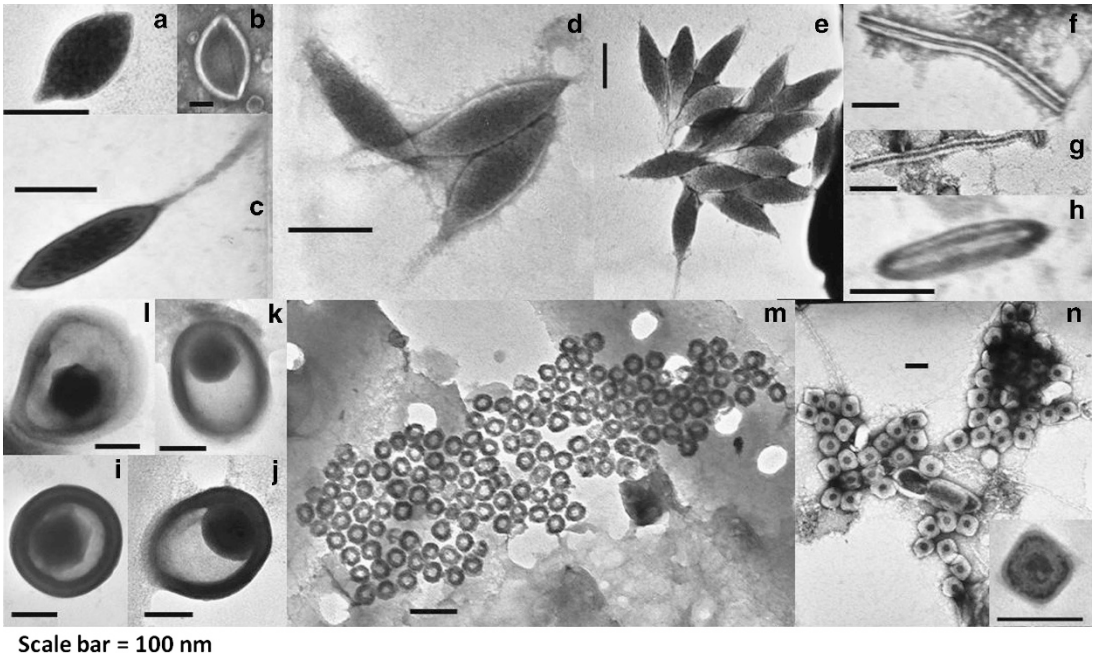

Figure 3 Transmission electron micrographs of unexpected and novel putative viruses observed along the 40 cm sediment core from Lake Pavin. Magnification for each micrograph is indicated as follows: **a**, c, **j**, appendix in **n** (× 150 000); **d**, **k** (× 120 000); **h**, **i**, **l** (× 100 000); **f** (× 80 000); **b**, **e** (× 60 000); **g**, **m**, **n** (× 40 000). Scale bar = 100 nm.

VLPs with an inner region of low density (Supplementary Figures S2a–c), superficially resembling VLPs shown in Figure 3h. The morphology of the filamentous cell appeared to be highly similar to that of the methanogenic archaeon *M. concilii* (Supplementary Figures S2c and d).

Discussion

The study of benthic viruses is still in its infancy, especially for freshwater ecosystems where only water–sediment interface was investigated so far (Filippini *et al.*, 2006). To our knowledge, our study provides the first data on the depth-related changes in viral abundance and community structure, as well as on morphological diversity and lytic activity of viruses in a freshwater subfloor habitat. Our discovery of unexpected and novel morphotypes of putative viruses in the analyzed habitat is a result with potentially far-reaching implications.

In the analyzed samples we detected VLPs morphologically highly similar to viruses infecting hyperthermophilic and extremely halophilic archaea. The observed spindle-shaped tailless particles (Figures 3a and b) resembled virions of members of the family Fuselloviridae and the unassigned genus *Salterprovirus,* whereas tailed spindle-shaped VLPs (Figures 3c–e) resembled members of the family Bicaudaviridae (Prangishvili *et al.*, 2006; Pina *et al.*, 2011). Such particles are abundant in extreme geothermal and hypersaline environments (Prangishvili, 2006; Sime-Ngando *et al.*, 2011) but, to our knowledge, were never observed in habitats at moderate temperature and salinity. Moreover, none of these virion morphotypes is known to be associated with bacteria or eukaryotes. Their occurrence in the samples could be linked to the presence of archaeal hosts. One more morphotype of hyperthermophilic archaeal viruses was represented by rod-shaped particles with central cavity (Figures 3f and g), characteristic of members of the family Rudiviridae (Prangishvili *et al.*, 2006; Pina *et al.*, 2011) and often observed in extreme geothermal environments (Prangishvili, 2006).

Along with typical archaeal virion morphotypes, VLPs with complex exceptional morphology were observed. One type of such particles, about 220 nm in diameter, carried an envelope and superficially resembled virions from the order Herpesvirales (King *et al.*, 2011), in that the outer envelope was separated from the inner, presumably icosahedral capsid (with a diameter about 110 nm) by an area with certain similarity to the tegument of herpesviruses (Figures 3i and j). Another type of the observed complex VLPs had a form of a prolate ellipsoid (Figure 3h) and an inner core of low density, and beared certain resemblance with the virions of the genus *Ichnovirus* of the family *P*olydnaviridae (King *et al.*, 2011). It is noteworthy that the complex viruses of the latter two types have never been reported to be associated with prokaryotic hosts. Two types of small particles were exceptional in their appearance and could be representing new viral families. One type of these particles, about 30 nm in diameter, were quasi-spherical and revealed a central core of low density (Figure 3m). Even more unusual were irregular cubic particles with central core of high density (Figure 3n). Their slight polymorphism could be either their natural property or an artefact of sample preparation.

Finally, particles with morphotypes known from dsDNA viruses of the bacteria were detected. These included particles resembling members of the families Myoviridae, Siphoviridae, and Podoviridae (Figures 2a–e). We also observed unusually long-tailed Siphoviridae (Figures 2f and g), typical for pelagic ecosystems and reported also in Baltic Sea sediments (Jakubowska-Deredas *et al.*, 2012). Nonenveloped polyhedral particles without tails, with the diameters ranging from 60 to 500 nm, could represent viruses from different families, for example, the smaller ones could be bacterial viruses from the familes Tectiviridae and Corticoviridae, whereas the larger ones, aquatic eukaryotic viruses from the families Iridoviridae and Phycodnaviridae (Figures 2h–l).

Our main findings clearly emphasize novel viral morphotypes, perhaps specific to deep-dark permanently anoxic freshwater habitats. Moreover, most of these original VLP morphotypes in the sediments had never been observed in the pelagic samples from Lake Pavin (Colombet and Sime-Ngando, 2008). It is, thus, likely that specific viral communities thrive in the deep-aged sediment of Lake Pavin where their dynamic nature was inferred by (i) the strong link between viral and prokaryote abundances, (ii) the fluctuating pattern in virus-to-prokaryote ratio and (iii) the depth-related structure in viral community recorded via the RAPD-PCR approach, that mimic those in archaeal and bacterial assemblages (Figure 1). Only one study has previously investigated the changes of benthic viral assemblages using RAPD-PCR (Helton and Wommack, 2009). These authors observed a huge change of virus assemblages with season in marine surface sediments.

A striking finding of the present study is the first direct evidence of viral lytic infection observed in aquatic sediments (Figure 1e; Supplementary Figure S1). These direct observations by TEM were never reported from marine and freshwater sediments (Bettarel *et al.*, 2006; Filippini *et al.*, 2006; Säwström *et al.*, 2009). This contrasts with viral infection rates inferred from indirect approaches where viral production is often calculated from changes in viral abundances before and after incubations (for example, dilution approach), primarily in contemporaneous marine sediments (Hewson and Fuhrman, 2003; Glud and Middelboe, 2004; Mei and Danovaro, 2004; Middelboe and Glud, 2006; Danovaro *et al.*, 2008b; Corinaldesi *et al.*, 2010).

Clearly, it appears that lytic viruses contribute to the regulation of prokaryotic communities in both recent and ancient aquatic sediments. Observation of spindle-shaped (Figure 3e) and unknown putative viruses (Figures 3m–n), under the form of aggregates typical of lytic burst events, suggested that both

bacterial and archaeal communities in the sediments may be affected by viral infection. Some of our micrographs also showed possible egress of viral particles (Supplementary Figure S1f–h). Furthermore, we observed a filamentous prokaryote infected with ellipsoid pleomorphic viruses (Supplementary Figure S2a–c). The infected hosts exhibited sheath and flat ends (Supplementary Figure S2c), similar to those typical of the methanogenic species *M. concilii* (Supplementary Figure S2d) (Patel and Sprott, 1990). Even if there is no certitude about the identity of the infected cell, its tentative affiliation to *Methanosaeta*, in addition to the morphological similarities, is supported by the data on the occurrence of members of this genus as a dominant archaeal group in the sediments of Lake Pavin (Lehours *et al.*, 2007; Biderre-Petit *et al.*, 2011). *Methanosaeta* genus is known to be widely distributed in different environments and is probably the predominant methane producer on earth (Smith and Ingram-Smith, 2007).

Concluding remarks

This study presents original transmission electron micrographs of a freshwater deep-aged sediment viral community in a site that offers a unique environment with low and stable sedimentation rate of mainly biogenic sediments, and that preserves records of the geologic past exceptionally well. Along with particles morphologically similar to virions of typical dsDNA or ssDNA viruses of bacteria, the viral community included particles with highly complex morphotypes. Some of the latter were similar to archaeal dsDNA viruses previously reported only in extreme geothermal and hypersaline environments. In addition, novel types of VLPs have been observed. The vertical distribution of viruses and prokaryotes along the sediment core were correlated and behaved trophodynamically, resulting in a dynamical virus-to-prokaryotes ratio. The diversity of viruses, archaea and bacteria exhibited three similar depth-related clusters, characteristic of surface, intermediate and deep sediment layers. Visibly infected prokaryotes were detected in the whole sediment core sampled. For the first time, we detected ellipsoid pleomorphic viruses in visibly infected filamentous prokaryotes tentatively identified as a member of the *Methanosaeta*, an archaeal genus species, which are omnipresent in freshwaters (Borrel *et al.*, 2011) and could be predominant methane producers on earth (Smith, Ingram-Smith, 2007). Even if few viruses of methanogens were isolated 2 decades ago (Wood *et al.*, 1989; Nölling *et al.*, 1993), their activity and incidence on the environmental control of those species that produce methane still remain a vast unexplored area of research (Rohwer and Thurber, 2009).

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