View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by RERO DOC Digital Library

Viral abundance and genome size distribution in the sediment and water column of marine and freshwater ecosystems

Manuela Filippini[1,2] & Mathias Middelboe[1]

[1]Marine Biological Laboratory, University of Copenhagen, Helsingør, Denmark; and [2]Department of Aquatic Ecology, Eawag: Swiss Federal Institute of Aquatic Science and Technology, and Institute of Integrative Biology (IBZ), ETH Zurich, Kastanienbaum, Switzerland

Correspondence: Mathias Middelboe, Marine Biological Laboratory, University of Copenhagen, Strandpromenaden 5, 3000 Helsingør, Denmark. Tel.: +45 35 32 19 91; fax: +45 35 32 19 51; e-mail: mmiddelboe@bi.ku.dk

Received 27 September 2006; revised 9 January 2007; accepted 15 January 2007.
First published online 28 March 2007.

DOI:10.1111/j.1574-6941.2007.00298.x

Editor: Patricia Sobecky

Keywords
pulsed field gel electrophoresis (PFGE); virus community structure; sediment; freshwater; marine; cross-system analysis.

Abstract

The size distribution of viral DNA in natural samples was investigated in a number of marine, brackish and freshwater environments by means of pulsed field gel electrophoresis (PFGE). The method was modified to work with both water and sediment samples, with an estimated detection limit for individual virus genome size groups of $1–2 \times 10^4$ virus-like particles (VLP) mL^{-1} water and $2–4 \times 10^5$ VLP cm^{-3} sediment in the original samples. Variations in the composition and distribution of dominant virus genome sizes were analyzed within and between different habitats that covered a range in viral density from 0.4×10^7 VLP mL^{-1} (sea water) to 300×10^7 VLP cm^{-3} (lake sediment). The PFGE community fingerprints showed a number of cross-system similarities in the genome size distribution with a general dominance of genomes in the 30–48, 50–70 and 145–200 kb size fractions, and with many of the specific genome sizes detected in all the investigated habitats. However, large differences in community fingerprints were also observed between the investigated sites, and some virus genome sizes were found only in specific biotopes (e.g. lake water), in specific ecosystems (e.g. a particular lake) or even in specific microhabitats (e.g. a particular sediment stratum).

Introduction

Viruses are ubiquitous and the most abundant biological entities in aquatic ecosystems, with abundances typically in the range of $10^7–10^8$ virus-like particles (VLP) mL^{-1} in pelagic environments and $10^8–10^9$ VLP cm^{-3} in sediments (e.g. Weinbauer, 2004; Suttle, 2005). Most of these viruses are believed to be bacteriophages, accounting for a significant part of prokaryotic mortality (e.g. Weinbauer, 2004; Suttle, 2005). Increasing evidence suggests that viruses play an important role in marine carbon cycling by mediating the transformation of matter and energy in microbial food webs through cell lysis (e.g. Wilhelm & Suttle, 1999; Riemann & Middelboe, 2002a), and potentially influence microbial diversity and community dynamics by the selective suppression of specific host populations (e.g. Thingstad & Lignell, 1997; Weinbauer & Rassoulzadegan, 2004).

Most studies of aquatic viruses have so far been carried out in pelagic marine environments. However, viruses are typically 10–100 fold more abundant in the sediment than in the water column (Hewson *et al.*, 2001; Danovaro & Serresi, 2002; Weinbauer, 2004), suggesting that they are important players also in benthic systems, yet we still have relatively limited and divergent observations on the role of viruses in sediments: While most marine studies have found high benthic viral activity and a significant impact of viruses on benthic bacterial mortality (e.g. Hewson & Fuhrman, 2003; Mei & Danovaro, 2004; Middelboe *et al.*, 2006), a number of studies from freshwater (e.g. Fischer *et al.*, 2003; Filippini *et al.*, 2006) and other inland aquatic systems (Bettarel *et al.*, 2006) have found evidence of a low impact of viruses on in the investigated sediments. These apparently contradicting observations may be due to systematic differences in the role of viruses for controlling bacterial mortality and biogeochemical cycling in different benthic environments, emphasizing the need for further investigations on benthic viruses.

Until recently, viruses have been treated as a 'black box' with only little available information about the composition and dynamics of environmental viral populations (e.g. Wichels *et al.*, 1998; Steward *et al.*, 2000). Such information about viral diversity and population dynamics on different temporal and spatial scales is important, not only to resolve the impact of viruses on bacterial community structure and vice versa but also to understand the long term evolution

© 2007 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

and spreading of viruses in the environment. Viruses constitute an enormous source of unexplored genetic information (Suttle, 2005) and we are still in the early phase of investigating the genetic composition, the spatial and temporal distribution of individual virus populations and the complex patterns of interactions between viruses and their hosts.

Different approaches have been employed in the study of virus diversity in aquatic systems. Examination of viral diversity based on morphological characteristics obtained by transmission electron microscopy has demonstrated a large morphological variation (Corpe & Jensen, 1996), with more than 80 different morphotypes observed in a single lake (Finlay & Maberly, 2000). The use of PCR-based methods to evaluate viral diversity has been restricted by the absence of a conservative region shared among all viruses. This approach has, however, been successfully applied in the study of specific virus subgroups, such as cyanophages, algal viruses and picorna-like viruses (e.g. Culley *et al.*, 2003). Most recently, metagenomic analyses of viral DNA from environmental samples have provided the first direct evidence of an enormous genetic diversity in marine viral communities – an estimated 3000–7000 viral types in 200 L of seawater (Breitbart *et al.*, 2002) and 10^4–10^6 viral types in one kg of sediment (Breitbart *et al.*, 2004a) – with the most abundant types comprising only 2–3% and 0.01–0.1%, respectively, of the total viral community.

Pulsed field gel electrophoresis (PFGE) is another method to analyze viral community composition (e.g. Steward & Azam, 1999). This method provides a viral community fingerprint based on the size distribution of dominant genomes after separation of the genomes on an agarose gel (Steward & Azam, 1999). Previous studies have demonstrated that both viral community composition and the relative intensity of individual bands can vary with season (e.g. Wommack *et al.*, 1999; Castberg *et al.*, 2001; Larsen *et al.*, 2001, 2004; Sandaa & Larsen, 2006) and location (e.g. Wommack *et al.*, 1999; Riemann & Middelboe, 2002b), illustrating that individual viral populations are dynamic parts of pelagic microbial communities. On the other hand, reports of only minor changes in the PFGE fingerprints across large temporal and spatial scales despite several-fold variations in general microbial abundance and activity (Riemann & Middelboe, 2002b; Auguet *et al.*, 2006) suggest that part of the dominant viral genome sizes may be rather persistent and perhaps permanently present in a given ecosystem.

This approach has primarily been used in pelagic marine waters (e.g. Wommack *et al.*, 1999; Diez *et al.*, 2000; Steward *et al.*, 2000; Fuhrman *et al.*, 2002; Riemann & Middelboe, 2002b; Jiang *et al.*, 2003, 2004; Sandaa *et al.*, 2003) and to a lesser extent in freshwaters (Auguet *et al.*, 2006), whereas its potential to describe benthic viral communities has not yet been explored. One reason is likely to be that the critical requirement for the clean preparation of viral DNA poses a major challenge to the use of PFGE with sediment communities. Moreover, the majority of the pelagic PFGE studies have been performed in marine environments, while a few studies have investigated changes in PFGE patterns along salinity gradients in estuarine systems (Riemann & Middelboe, 2002b; Auguet *et al.*, 2006). There exist, on the other hand, to our knowledge no comparative analyses of PFGE fingerprints between different environments, such as between water column and sediment communities, or between lake communities and marine communities. It is therefore not known to what extent specific ecosystems are characterized by a specific composition of dominant virus genome sizes or whether there is an overlap in genome sizes between environments.

The purpose of this study was therefore to (1) adapt and apply the PFGE method to benthic viruses, and (2) investigate patterns in viral genome size distribution obtained from pelagic and benthic samples in a given ecosystem, and from separate marine, brackish and freshwater environments.

Materials and methods

Study sites

Viral abundance, distribution and community structure was studied in four different ecosystems between October 2004 and March 2005.

Lake Frederiksborg Slotssø is a small (0.21 km^2) dimictic and eutrophic lake with high sedimentation rate (Markager *et al.*, 1994) and an average depth of 3.1 m. The annual primary production is *c.* 400 g C m^{-2} $year^{-1}$ and the concentration of dissolved organic carbon (DOC) ranges from 10 to 15 mg L^{-1} (Middelboe & Søndergaard, 1993). Sediment samples were collected at the deepest point (9 m) in the centre of the lake.

Lake Esrum (17.3 km^2) is the largest freshwater lake in Denmark (maximum depth: 22 m, mean depth: 12.3 m). It is meso-/eutrophic, with an annual primary production of 260 g C m^{-2} $year^{-1}$ (Søndergaard, 1991). Sediment samples were collected in the center of the lake at 19.5 m water depth.

Nivå Bay is a shallow (< 1 m), eutrophic coastal brackish area, which is characterized by high input of organic matter to the sediment, high benthic mineralization rates dominated by sulfate-reducing bacteria (Middelboe *et al.*, 2003), and benthic primary production dominated by diatoms and cyanobacteria.

Øresund is an estuarine sound characterized by a layer of marine bottom water originating from the North Sea, which is separated from the brackish surface layer by a pycnocline

© 2007 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

at 10–15 m depth. During winter, full mixing of the water column occurs occasionally, and in this study water samples were collected during such a mixing period. Mean annual primary production in Øresund is about 240 g C m^{-2} $year^{-1}$. Two stations in Øresund were investigated: a shallow station (10 m) which is exposed to the brackish surface water (only collection of sediment samples) and a deeper site (26 m) always situated below the pycnocline (collection of both water and sediment samples).

Sampling

At each sampling site, 20 sediment cores containing the upper 20 cm of sediment were collected (core diameter: 5.3 cm). Of these, 17 cores were used for the extraction of viruses for community analysis at different sediment depths; one core was used for determination of viral and bacterial depth distribution, total carbon content and sediment porosity, and two cores were used for measuring benthic oxygen distribution and consumption. Along with the sediment samples, 20 L of surface water were collected from each site. All samples were transported to the laboratory within 1–3 h after sampling and placed at *in situ* temperature.

Sediment characteristics

Sediment porosity (volume of water:volume of sediment) was calculated from the specific sediment density and the weight loss after drying at 105 °C for 24 h, and the total organic carbon content was determined as the weight loss after combustion at 450 °C for 24 h.

The type and size fractionation of the samples were determined by sieving through different mesh sizes according to Buchanan (1984). Twenty-five grams of oven-dried sediment was placed in a beaker with 250 mL of tap water, then 10 mL of aqueous sodium hexametaphosphate $(NaPO_3)_6$ (6.2 g L^{-1}) was added and the slurry stirred mechanically for 15 min. The sediment was left overnight, restirred the following morning for another 15 min and sieved through a series of nested sieves (mesh sizes from 1 mm to 62 μm). The different size fractions were dried at 100 °C and weighed.

Oxygen measurements

Oxygen microprofiles were measured in intact sediment cores with a Clark type microelectrode with a depth resolution of 100 μm. The signal of the electrode was detected by a pico ampermeter. Total oxygen consumption in the cores was measured according to Rasmussen & Jørgensen (1992) as the linear decrease in oxygen concentration after capping the submerged cores with a lid and insertion of a microsensor into the core water.

Vertical profiles of total viral and bacterial abundance

The sediment cores were cut into 1–2 cm slices (a total of 6–13 samples) to determine the vertical distribution of viruses and bacteria. The individual samples were manually homogenized and a subsample (*c.* 4 g) was weighed and transferred to a 50 mL centrifuge tube, fixed with 4 mL of virus free water and 1 mL of glutaraldehyde (3% final concentration) and stored in the dark at 5 °C for a maximum of 24 h. For extraction of bacteria and viruses, sodium pyrophosphate (10 mM final concentration) was added, and after 15 min the samples were sonicated in a sonication bath for 2 × 1 min before centrifugation for 5 min at 700 ***g*** (Glud & Middelboe, 2004). The supernatant was collected and the sediment washed twice with 2 mL virus-free water. The total extracted volume was pooled and a small volume of the virus-bacteria extract (50–100 μL) was filtered on to a 0.02-μm Anodisc filter (Whatman, Maidstone, UK) and stained with SYBR Gold (Chen *et al.*, 2001). On each slide, 200–600 bacteria and viruses were counted in 10–20 fields using epifluorescence microscopy at × 1250 magnification. Previous evaluations of the accuracy of this procedure from replicate samplings from the homogenized sediment (Middelboe *et al.*, 2003; Glud & Middelboe, 2004) have demonstrated that viral and bacterial abundance are determined with an average accuracy of 5–6%. In this study we have therefore applied this as a general accuracy of the enumeration procedure.

Concentration of viruses from water samples

To obtain a sufficiently high density of viruses for PFGE analyses, viruses from both water and sediment samples were concentrated prior to analysis. For the water samples, 20 L of water were filtered through first a GF/F filter (Whatman) and then a 0.2-μm capsule filter (Type CCS-020-C1HS; Advantec, Dublin, CA). Viruses were then concentrated to a final volume of 100–200 mL by tangential flow filtration using a 30 K Cartridge (PLTK Prep/Scale™-TFF 2.5 ft^2 Cartridge, 30 K regenerated cellulose membrane, Millipore, Bedford, MA). Further concentration was carried out in 15 mL centrifugal ultrafiltration devices (30 K Amicon Ultra, Millipore) to a final volume of 4 mL. Viruses were then pelletted by ultracentrifugation through a glycerol gradient (2 mL 5% glycerol on top of 2 mL 40% glycerol in a 5-mL tube) at 85.000 ***g*** for 98 min at 20 °C as described in Sambrook *et al.* (1989) (Beckman, Fullerton, CA, Optima LE 80 k Ultracentrifuge; SW 55 Ti Rotor). This purification step allowed only particles with a sedimentation coefficient of > 100 S to form a pellet, and has shown to reduce the interference from unknown polymeric material during electrophoresis (Riemann & Middelboe, 2002b). The supernatant was discarded, leaving *c.* 100 μL above the pellet. Hundred microliters of MSM (marine samples) or SM buffer (freshwater samples) (Sambrook *et al.*, 1989) and

© 2007 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

natrium azide (final concentration 0.1%) were added (MSM: 450 mM NaCl, 50 mM $MgSO_4$, 50 mM Tris, 0.01% gelatin at pH 8. SM: as MSM but with 50 mM NaCl), and the pellet was resuspended. Samples were stored at 4 °C until analysis.

Concentration of viruses from sediment samples

Viral community analysis was performed at three different sediment depths: surface sediment (0–1 cm), the subsequent layer (1–3 cm) and a deeper section (10–12 cm). To obtain enough viruses for the analysis, sediment layers from 17 cores (*c.* 1 kg of sediment in total) were pooled prior to the extraction of viruses.

Viruses were extracted from the samples as described for the vertical profile, but without the addition of glutaraldehyde. Briefly, 5–10 g of sediment was placed in 50-mL centrifuge tubes and 5 mL pyrophosphate (final concentration 10 mM) was added. Samples were shaken, incubated for 15 min and then sonicated in a sonication bath for 2 × 1 min before centrifugation for 5 min at 700 ***g*** (Glud & Middelboe, 2004). The supernatant was collected and the sediment washed twice with 2 mL virus-free water. The extract was then centrifuged at 15 000 *g* for 30 min to remove bacteria and the supernatant was GF/F filtered. As for the water samples, viruses in the extract were concentrated by centrifugal ultrafiltration devices (30 K Amicon Ultra, Millipore). The concentrate was again centrifuged (15 000 ***g*** for 30 min) before concentration of viruses by the glycerol gradient ultracentrifugation step (see above).

To further improve purification of the concentrated viruses, viral concentrates were run through a CsCl gradient following the glycerol gradient step. First, CsCl was added to samples to obtain a density of 1.15 g mL^{-1} and samples were then loaded on to a CsCl gradient of three densities (1.7, 1.5 and 1.3 g mL^{-1} in MSM or SM, respectively). The samples were ultracentrifuged at 48 000 ***g*** for 2 h at 4 °C (Sambrook *et al.*, 1989). Following centrifugation, the viruses were sampled with a needle from individual layers, transferred to separate Eppendorf tubes, and stored at 4 °C for further analysis of the viral community.

Pulsed field gel electrophoresis

Before electrophoresis, the purified viruses were further concentrated and purified using 0.5-mL centrifugation devices (30 K Microcon, Amicon Bioseparations, Millipore). During this procedure viruses were resuspended in Tris-EDTA (TE) buffer (10 mM Tris and 1 mM EDTA, pH 8.0). Viral genomes were released by heating samples to 70 °C for 10 min followed by cooling on ice. For electrophoresis, 20–25 μL virus sample containing DNA from 8×10^9 viruses for the water samples and 2×10^{10} viruses for the sediment were added to 4 μL of loading buffer and loaded on to a 1% agarose gel in 0.5 × Tris-Borate-EDTA (TBE) (10 × TBE: 890 mM Tris, 20 mM EDTA, 890 mM boric acid, pH 8.3). Molecular weight markers included a lambda DNA-PFGE ladder (Amersham, Hillerad, Denmark) and a high molecular weight DNA marker (8–48 kb; Invitrogen). The electrophoresis was performed in a CHEF-DR III system (Biorad, Hercules, CA) at 14 °C, 6 V cm^{-1} and a 120° angle for 22 h using 0.5 × TBE as running buffer, and switch times varying from 0.5–2 and 2–7 s. These conditions were based on numerous tests, which assessed sensitivity and reproducibility of procedures.

The effect of DNase and RNase treatment for reducing background fluorescence in the gels was investigated by incubating a subset of samples with DNase and RNase (final concentrations of 5 and 1 μg mL^{-1}, respectively) for 30 min at 37 °C prior to viral DNA release (Sambrook *et al.*, 1989).

Gels were stained with SYBR Gold (10 000 × dilution, Molecular Probes) in 0.5 × TBE for 1 h and then washed with distilled water for 30 min. To visualize the DNA bands, gels were illuminated on a UV transilluminator (BioRad) and pictures were taken with a digital camera at different exposure times and later edited with Adobe Photoshop. Photographs were analyzed using the software QUANTITY ONE® (Biorad) to determine the vertical intensity profile of individual lanes and the relative DNA distribution as the fraction of a given band intensity relative to the total intensity of the detected bands.

Results

Sediment characteristics

Sediment grain size varied considerably among the three marine/brackish sites (Øresund at 10 m depth, Øresund at 26 m and Nivå Bay) (Table 1). The shallow Øresund station consisted mainly of coarse sand (500–1000 μm), while the deeper Øresund station was characterized by silt and fine sand (< 250 μm) and Nivå Bay by sand fractions of intermediate size (250–500 μm) (Table 1). Both freshwater sites were dominated by soft and silty sediment (data not shown).

Sediment porosity increased from ~0.4 in the sandy sediment (Øresund 10 m) to ~0.5–0.6 in Nivå Bay and the deep Øresund station, and > 0.8 in the freshwater sediment. In general the porosity decreased slightly with sediment depth (Table 1).

Total organic carbon (TOC) content was much lower at the shallow Øresund station (< 0.3%) than at the other locations, where TOC contents ranged from ~1.5% in the deep Øresund station to > 3% at 10 cm sediment depths in Lake Frederiksborg Slotssø (Table 1).

Oxygen penetration depth ranged from 2.1 mm in Lake Frederiksborg Slotssø to 5.1 mm at the deep Øresund station (Table 1). At the shallow Øresund station, oxygen

© 2007 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

Table 1. Basic characteristics of the investigated benthic locations

Site	Sampling date	Water depth (m)	Salinity (‰)	Temp. (°C)	Porosity (vol:vol)	TOC* (%)	TOU† (mmol m^{-2} day^{-1})	O_2 penetration depth (mm)	Dominant grain size: Sand fraction‡ (%)	Dominant grain size: Silt fraction§ (%)
Frb. Slotssø	29 Nov 04	9	Fresh	3.5	0.78–0.94	1.56–3.43	80.6	2.08 (0.06)	ND	ND
Lake Esrum	13 Dec 04	19	Fresh	6	0.82–1.13	1.00–2.54	30.6	4.3 (0.55)	ND	ND
Nivå Bay	26 Oct 04	< 1	20	11	0.42–0.64	1.46–2.46	34.2	3.2 (0.22)	53 (medium) 24 (fine)	3.5
Øresund (10 m)	23 Jan 05	10	20	7	0.31–0.39	0.15–0.23	27.9	Nd	52 (coarse) 38 (medium)	0.9
Øresund (26 m)	12 Jan 05	26	31	8	0.47–0.62	1.36–1.67	14.7	5.12 (0.12)	42 (fine) 32 (very fine)	24

Values in parentheses represent ± 1 SE ($N = 5$) for O_2 penetration depth.
*Total organic carbon.
†Total oxygen consumption.
‡63–2000 μm (Coarse, 500–1000 μm; medium, 250–500 μm; fine: 150–250 μm; very fine, 63–150 μm).
§< 63 μm.
ND, not determined.

penetration was much higher (> 3 cm), but because of large sand particles it was impossible to measure accurately with the microelectrodes used. The benthic mineralization rate varied from a minimum of 14.7 mmol O_2 m^{-2} day^{-1} in the deep Øresund station to a maximum of 80.6 mmol O_2 m^{-2} day^{-1} in the eutrophic Lake Frederiksborg Slotssø, with intermediate rates of 28–34 mmol O_2 m^{-2} day^{-1} in Lake Esrum, Nivå Bay and the shallow Øresund station (Table 1).

Distribution of viruses and bacteria

Large differences in total viral and bacterial abundances were observed among locations. In general, viral and bacteria abundances were higher in freshwater than in the marine and brackish systems and at least 20-fold higher in sediments than in the overlying water column (Fig. 1 and Table 2). In the marine and brackish water samples, viral and bacterial abundance ranged from 0.4 to 1.2×10^7 VLP mL^{-1} and from 0.6 to 1.4×10^6 mL^{-1}, respectively. Viruses were 5–11 fold more abundant than bacteria (Table 2). In lake water, viral abundance ($3.8–15.7 \times 10^7$ VLP mL^{-1}) and the virus:bacteria ratio (23–40) were higher than in the marine and brackish water samples (Table 2).

In sediments, viral abundance varied by a factor of 100 among locations. Viral abundances in the sandy sediment at the shallow Øresund station (~2×10^7 VLP cm^{-3}) were 10–20 fold lower than at the deeper silty Øresund station and the eutrophic Nivå Bay (Fig. 1a), and two orders of magnitude lower than at the freshwater locations [$15–30 \times 10^8$ VLP cm^{-3} (Fig. 1a)]. Bacterial distribution showed a similar pattern with abundances, ranging from 0.8×10^7 cells cm^{-3} at the shallow Øresund station to $> 20 \times 10^7$ cells cm^{-3} in the surface sediment of Lake Esrum (Fig. 1b). In the sediment, the VBR showed no systematic differences between freshwater and marine locations and ranged from

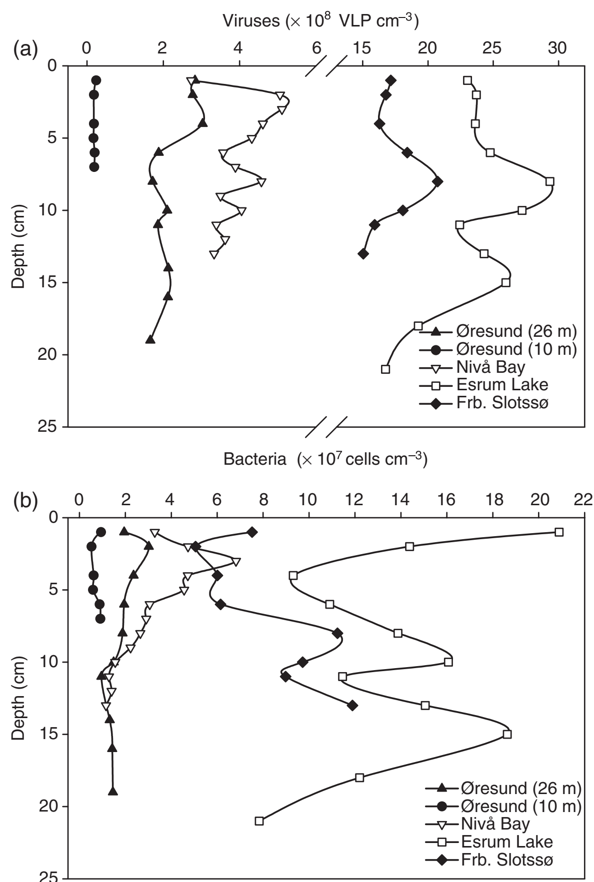

Fig. 1. Vertical distribution of viruses (a) and bacteria (b) in sediments at different locations: marine (Øresund), brackish (Nivå Bay) and freshwater (Lake Esrum and Lake Frederiksborg Slotssø).

8 to 33, whereas the shallow Øresund station (10 m) had relatively low virus densities and a VBR of only about 2.6.

At all locations, sediment subsurface peaks of viral abundance were associated with corresponding increases in

© 2007 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

Table 2. Average viral and bacterial abundance and virus-bacteria ratio (VBR) in the investigated pelagic environments

Ecosystem	Locations	Viruses (10^7 mL^{-1})	Bacteria (10^6 mL^{-1})	VBR
Marine	Øresund (mix)	0.73	0.68	10.7
Brackish	Nivå Bay	0.42	0.86	4.9
Freshwater	Lake Esrum	3.78	1.66	22.7
	Lake Frederiksborg Slotssø	15.71	3.97	39.6

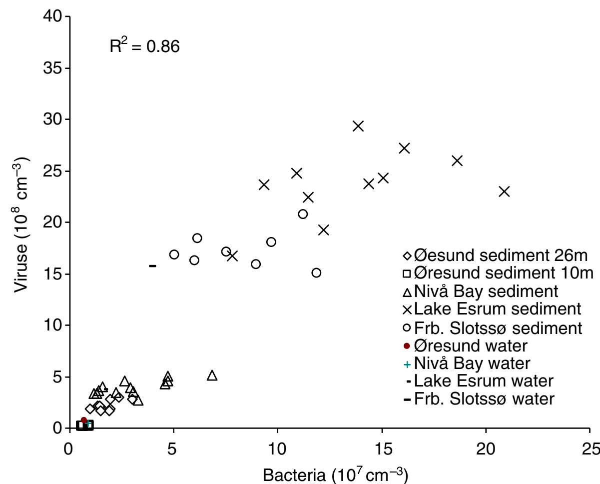

Fig. 2. Cross-system correlation between viral and bacterial abundance including data from all investigated pelagic and benthic environments. The line represents the linear regression.

bacterial abundance (Fig. 1a and b), and there was a clear overall positive correlation ($r^2 = 0.86$, $P < 0.01$, $n = 54$) between viral and bacterial abundance (Fig. 2).

Method optimization

The sensitivity of the PFGE method was tested by running a concentration series of three viral isolates from Nivå Bay sediments, with genome sizes of 45, 50 and 90 kb, respectively. After staining with SYBR Gold, viral densities as low as $0.6–1.0 \times 10^6$ viruses per band were detected as clearly distinguishable bands, with no significant differences related to genome size (data not shown). Duplicate samples of a Nivå Bay concentrate in parallel lanes showed identical banding pattern and relative band intensities (Fig. 3).

The optimal switch time to discriminate PFGE bands in the genome size range of 20–50 kb was 0.5–2 s, whereas a longer switch time of 2–7 s provided better resolution of genomes > 50 kb, particularly in the 90–200 kb size class. A running time of 22 h and a voltage of 6 V cm^{-1} worked well for all samples tested.

For sediment samples, band resolution was considerably improved by including a CsCl gradient centrifugation step of viral concentrates following the standard glycerol concentration step (data not shown). Examination of three individual CsCl fractions showed identical genome size pattern; however the best resolution and highest intensity of bands was obtained in the heavier fraction (1.5–1.7 g mL^{-1}), which was therefore used in subsequent comparison between PFGE fingerprints from different locations. The use of enzymes (DNase and RNase) did not improve the quality of PFGE gels (data not shown).

Fingerprinting of virioplankton: comparison between locations

The PFGE fingerprints of different water samples could be divided into five dominant genome size classes corresponding to bands of (1) 12–17, (2) 24–29, (3) 30–48, (4) 50–70 and (5) > 90 kb (Fig. 4a). The most notable differences between marine and freshwater ecosystems were observed in the first and second size class, which were represented only in freshwater samples (Fig. 4a and b). Moreover, the number of bands in the 30–48 kb size fraction was higher in the lake (7–8) than in the marine (2–3) samples.

Samples from all locations contained bands in the 145–200 kb size fraction, although the signal in the coastal sample was very weak. In addition, the freshwater samples also showed bands in the > 200 kb fraction, particularly those from Lake Frederiksborg Slotssø where more genomes were present in that size fraction than in samples from any other location (Fig. 4). Discrimination of bands larger than 200 kb using the longer switch time-interval (7–20 s) revealed two clearly distinguishable bands of *c.* 290 and 360 kb in Lake Frederiksborg Slotssø and a number of bands in the 194–242 kb region in the marine samples (data not shown). Overall, the pattern of genome size distribution was similar across all locations with most of the genomes ranging between 33 and 60 kb (Fig. 4c). In general, between 10 and 15 clearly visible bands were detected, with 20 bands from Lake Frederiksborg Slotssø as an exception. This higher number in Lake Frederiksborg Slotssø samples was mainly due to bands in the 97 and 242 kb region.

Fingerprinting of benthic viral community: cross-system differences

PFGE bands of the sediment samples were clustered into four different genome size classes: (1) 12–19, (2) 30–48, (3) 50–70 and (4) 90–200 kb. These classes were found both in superficial sediment (top cm) and in the layer 10–12 cm below the surface (Fig. 5a and b). The overall pattern in genome size distribution was similar for all locations with most of the genomes ranging between 30 and 50 kb (Fig. 5c

© 2007 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

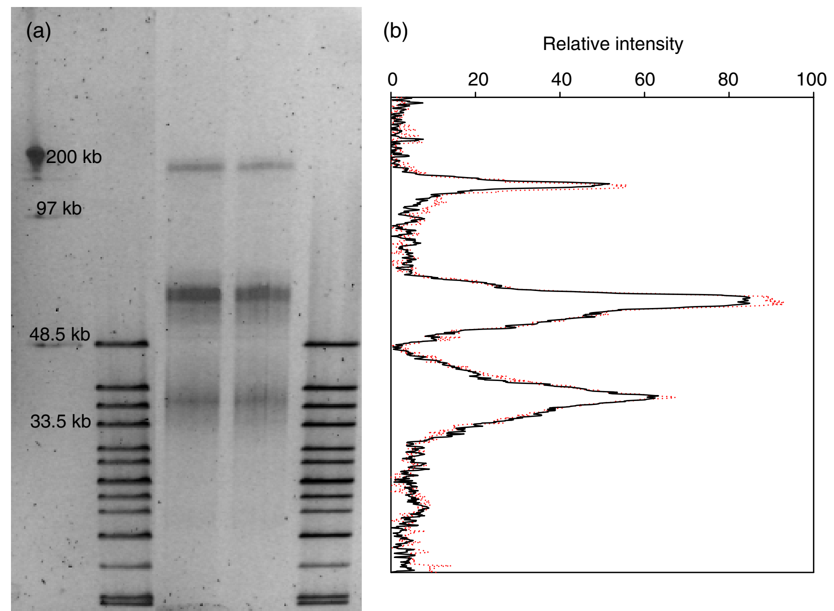

Fig. 3. (a) PFGE fingerprints of two identical samples run on parallel lanes. Gel parameters: 0.5–5 s, 20 h, 6 V cm^{-1} and 14 °C. (b) Vertical profile of the relative intensity of the two lanes.

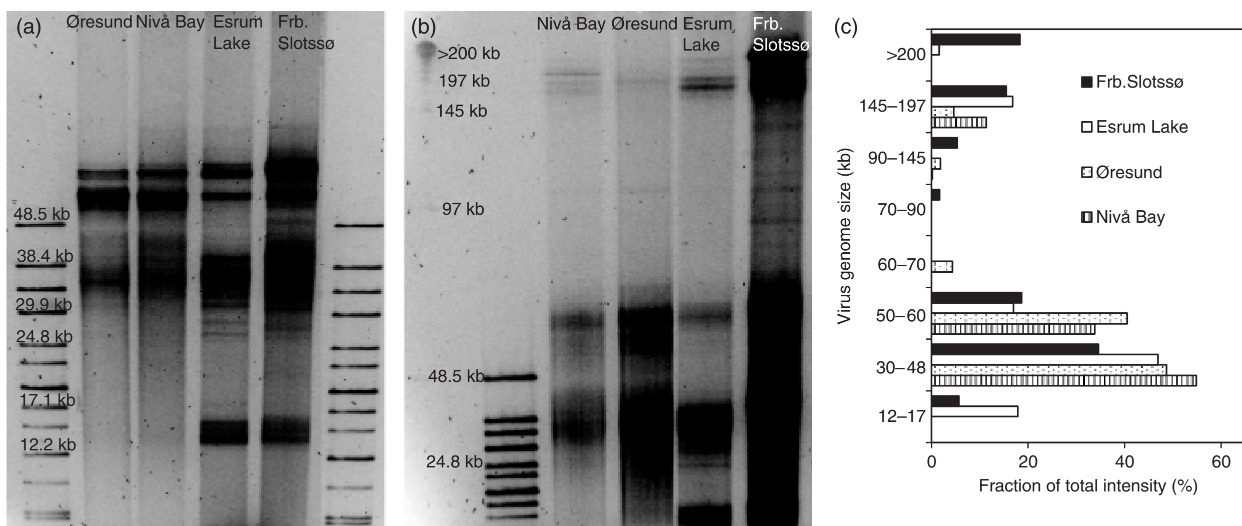

Fig. 4. Viral genome fingerprints of water samples collected from marine (Øresund), brackish (Nivå Bay) and freshwater (Lake Esrum and Lake Frederiksborg Slotssø) systems. (a) Switch time of 0.5–2 s to enhance resolution of viral genomes < 50 kb, (b) switch time of 2–7 s to improve separation of bands corresponding to high viral genome sizes (90–200 kb), and (c) relative distribution of band intensity in different genome size fractions in water samples as detected by PFGE.

and d). However, as for the water samples, there appeared to be a number of general differences between marine/brackish and freshwater sediments. The smallest size fraction (class 1) was only present in samples from lake sediment: Lake Esrum and Lake Frederiksborg Slotssø had well-defined bands at 17 and 15 kb, respectively, which were not found elsewhere. Moreover, the number and intensity of bands representing larger genome sizes (> 90 kb) were less pronounced in the freshwater sediment samples: this size fraction was absent in Lake Esrum, while only four bands were found in the surface sediment of Lake Frederiksborg Slotssø, which was reduced to one band in the deeper layer (Fig. 5a–d).

Gels from samples taken at the Øresund shallow station (10 m) in 0–1 and 1–3 cm sediment depth showed genome size bands falling into the intermediate-size fractions (2 and 3) but single bands were difficult to distinguish (data not shown). This may have been because the number of viruses with the same genome size was below the detection limit of the method at this station.

© 2007 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

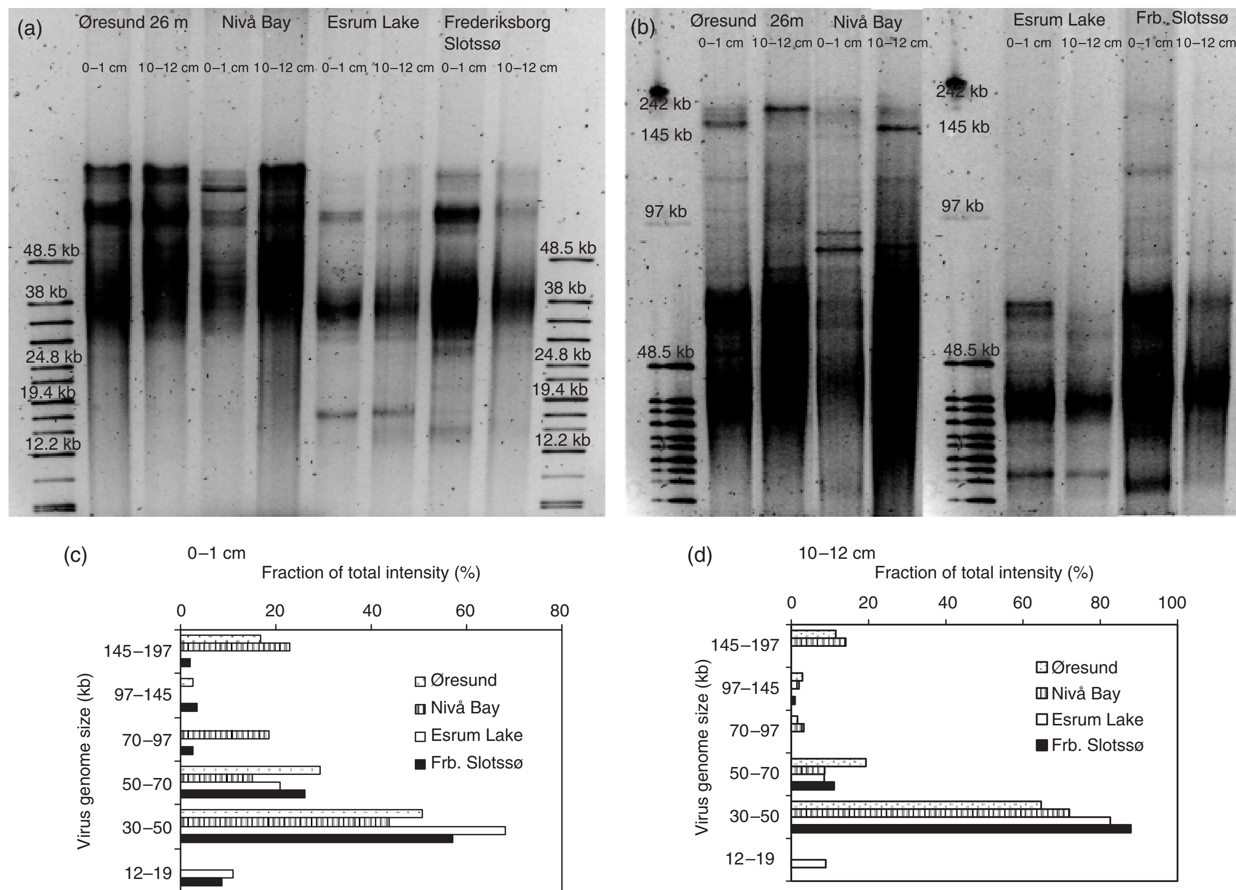

Fig. 5. Viral genome fingerprints of sediment samples taken at different locations: marine (Øresund), brackish (Nivå Bay) and freshwater (Lake Esrum and Lake Frederiksborg Slotssø) with switch times of 0.5–2 s (a) and 2–7 s (b). Relative distribution of band intensity in different genome size fractions as detected by PFGE in the surface (0–1 cm) sediment (c) and at 10–12 cm depth (d).

Changes in viral community structure with sediment depth

In general, changes in viral genome size distribution with sediment depth were less pronounced than differences between locations and were restricted to the large size fraction (genomes between 90 and 200 kb) (Fig. 5b). However, detailed analysis of vertical differences in Nivå Bay showed different intensities of individual bands in the three analyzed depths (Fig. 6). Furthermore, each depth was characterized by one or two specific bands (see arrows) in the large size fraction (Fig. 6). At the deep station of Øresund (26 m), the surface sediment (0–1 cm) contained five more bands between 90 and 200 kb than the deeper layers (1–3 cm and 10–12 cm), while at Nivå the deeper sediment had two bands more than the surface (Fig. 5a and b). At Nivå Bay and Lake Esrum the number of clearly visible bands was nearly the same in the first two sediment layers (13 and 12 bands, respectively) and then decreased in the deeper layer. In Øresund (26 m) and Lake Frederiksborg Slotssø there was a continuous decrease in band numbers with depth. The minimum number was always observed in the deeper sediment layer (between 6 and 11 bands).

Based on clearly discernable bands, a matrix of similarity values (Dice's correlation coefficients) was obtained using the UPGMA clustering algorithm (Quantity One, BioRad) program. For the water samples (Fig. 7a), Øresund locations branched out before Nivå, and the two lakes clustered together with similarity coefficients of 0.79. For the sediment communities (Fig. 7b), Nivå surface and deep sediment clustered together and showed low similarity to all other locations. At a lower level, samples from the freshwater communities of Lake Esrum and Lake Frederiksborg Slotssø also clustered together, with the exception of the surface sediment of Lake Esrum, which showed more similarity with the marine location of Øresund.

The relative intensity distribution on the gels showed that most of the viral DNA was distributed among the genome sizes smaller than 60 kb (44–89% of total intensity) and larger than 145 kb (5–34% of total intensity). The

© 2007 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

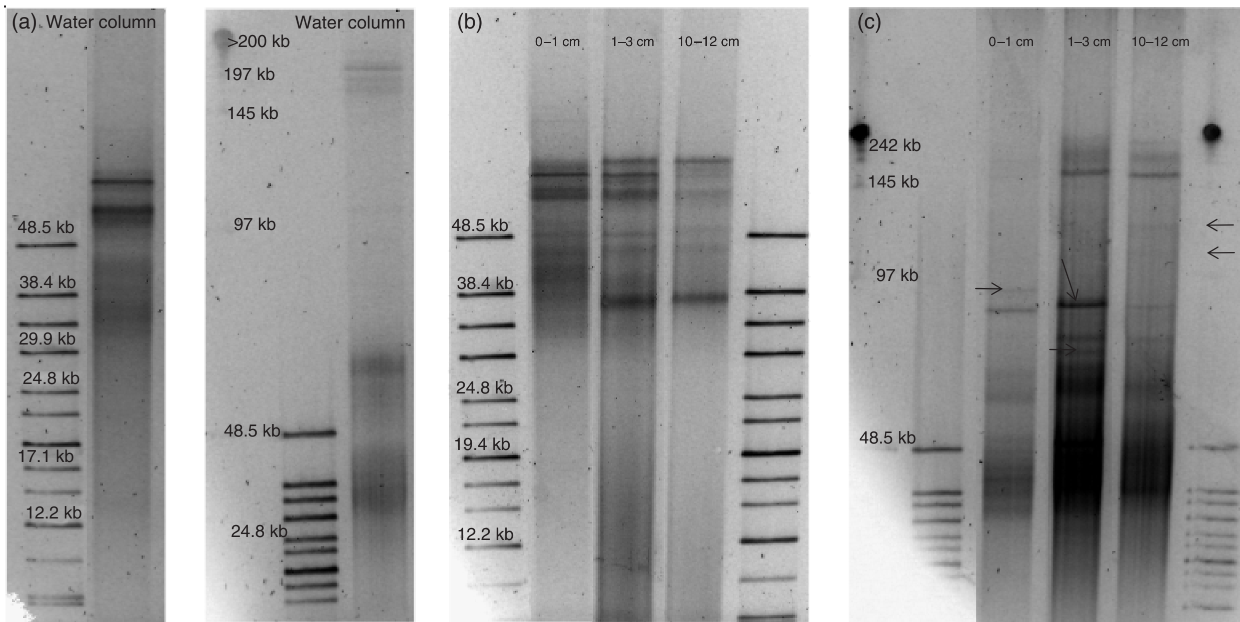

Fig. 6. Viral genome fingerprints of samples taken in the water column (a and b) and at three different sediment depths (surface 0–1, 1–3 and 10–12 cm) (c and d) from Nivå Bay. Gels were run using switch times of 0.5–2 s (a and c) and 2–7 s (b and d). Arrows indicate bands typical of a particular sediment layer.

intermediate-size fraction between 60 and 145 kb generally contained rather low intensity (< 7%) with the exception of Nivå Bay, where 19% of the total intensity was found within the 60–145 kb size fraction (Figs 4 and 5). As larger genomes would exhibit higher intensity per genome than smaller ones, the relative intensity was normalized to genome size by dividing the intensity of a given genome size fraction by the average size of the genome in that size interval. This calculation provided an estimate of the relative distribution of genomes in the different size fractions. The normalized distribution generally emphasized the dominance of small genomes and 59–95% of the normalized genome intensity was found in the < 50 kb fraction. In the freshwater environments the smallest genomes (< 19 kb) constituted as much as 23–52% of the total normalized intensity.

Discussion

Methodological considerations

Our results confirmed the conclusions from previous studies (e.g. Wommack *et al.*, 1999; Steward *et al.*, 2000; Riemann & Middelboe, 2002b) that PFGE represents a reproducible and sensitive method for analyzing viral community composition in natural water samples. In this study, we have further applied the technique to analyze benthic viral populations and present here for the first time data on PFGE fingerprints in marine and freshwater sediments. The sediment contained large amounts of humic and colloidal material that affected both DNA migration and background fluorescence in the gels. A number of modifications were applied to overcome these interferences, and a glycerol gradient centrifugation followed by CsCl density gradient centrifugation was found to reduce the background of nonviral material without significant loss of virus DNA, thus improving the resolution of viral genomes by PFGE considerably.

The sensitivity of the method has been reported to be *c.* 10^6 viruses band^{-1} (Wommack *et al.*, 1999), which was confirmed in the present study (data not shown). This sensitivity analysis allowed us to back-calculate the required concentration of a particular virus type in the original sample in order to obtain a detectable band on the gel. Accounting for the different concentration steps and assuming a 50% loss of VLP during handling, it was estimated that individual virus genome sizes present in densities higher than *c.* 10^4 VLP mL^{-1} sea water and 10^5 VLP g^{-1} sediment in the original samples were detectable by PFGE.

The PFGE method, thus, focuses on the dominant viral genome sizes and does not provide information about the total number of virus genotypes in a given sample. Recent estimates of total viral diversity in environmental samples by metagenomic approaches have indicated that the total number of viral genotypes was about 5000 in 200 L of sea water and between 10 000 and 1 million genotypes in 1 kg of sediment (Breitbart *et al.*, 2002; Breitbart *et al.*, 2004a, b; Edwards & Rohwer, 2005). In contrast to this large diversity

© 2007 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

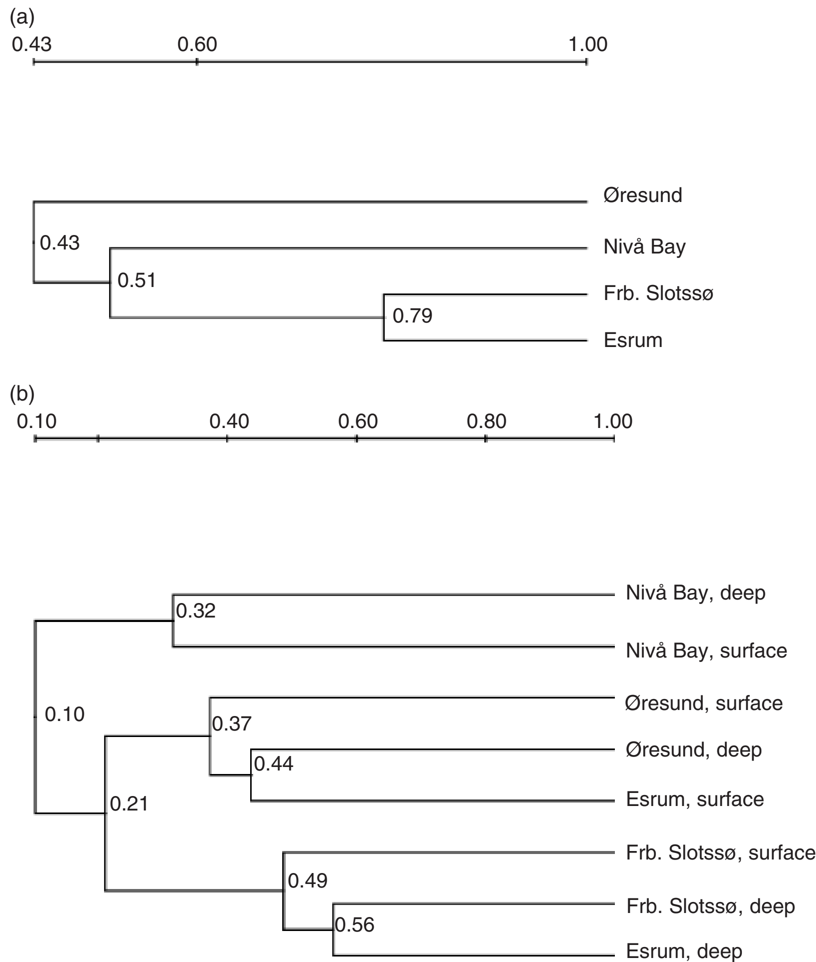

Fig. 7. Dendrogram based on a similarity matrix of virioplankton PFGE fingerprint banding patterns from all water samples (a) and two sediment layers (b). Surface = 0–1 cm and deep = 10–12 cm.

obtained from genomic analyses, virus community fingerprints by PFGE in water samples have typically identified 4–35 distinct bands (i.e. dsDNA genomes), in the size range from 12 to 600 kb, with the majority of DNA in the 26–69 kb size fraction (e.g. Steward *et al*., 2000; Riemann & Middelboe, 2002b; Larsen *et al*., 2004).

Model simulations of the viral community composition based on the number of sequences and their relative abundance revealed that the most dominant genotypes constituted *c.* 1% of the total community in sea water, and less than 0.1% in the sediment (Breitbart *et al*., 2002, 2004a). This suggests that most of the viral genotypes obtained by metagenomic analyses are present in densities below the detection limit of PFGE.

There are a number of limitations associated with the PFGE method that need to be taken into account when analyzing viral communities by PFGE (see overview in Steward *et al*., 2000). First, the assumption that single bands represent a single genotype is not always valid. In principle, each PFGE band may represent several viral genotypes; however, little is known about the genotypic diversity of viruses of similar genome size. In addition, despite our optimization of band resolution, many consecutive bands representing a continuum of individual genome sizes cannot always be resolved but produce a smear on the gel rather than distinct individual bands. Consequently, PFGE fingerprints cannot be used as an estimate of the diversity of dominant genotypes. Rather, as any fingerprint approach, the method provides a way to detect major differences in viral communities between environments or time-points, based on the distribution of dominant genome sizes, and the number of bands represents a minimum number of genotypes present in a given sample.

The samples were in all cases 0.2-μm filtered prior to concentrating viruses to avoid bacteria and other large particles interfering with the electrophoresis. Because some viruses, probably mostly algal viruses, may be larger than 0.2 μm (Bratbak *et al*., 1992; Brussaard, 2004), this filtration step might have introduced a bias in the obtained community composition towards the lower genome size viruses. Moreover, we have focused on the separation of DNA in the size range of 8–250 kb, where most viral genomes from

© 2007 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

environmental samples have been shown to fit in (e.g. Rasmussen & Jørgensen, 1992; Wommack *et al*., 1999; Larsen *et al*., 2001; Riemann & Middelboe, 2002b). This means that larger genomes (> 250 kb) may have been poorly separated by the applied electrophoresis conditions. On the other hand, there were no indications of bands in the size fraction > 250 kb, suggesting that the data are representative of the bacteriophage communities in the samples.

Viral and bacterial abundance

The abundances of viruses and bacteria in this study confirmed several general observations from other aquatic habitats: (1) higher abundances in freshwater than marine systems, (2) a decrease in abundances with water depth from shallow coastal regions towards deeper stations and (3) more bacteria and viruses in a given sediment volume than in the water column (e.g. Wommack & Colwell, 2000; Hewson *et al*., 2001; Weinbauer, 2004; Middelboe & Glud, 2006). Interestingly, the shallow Øresund station (10 m) that was characterized by coarse sand and low organic carbon content sustained extremely small populations of viruses and bacteria. Sandy sediments are characterized by strong physical forcing and advective pore water transport, which may limit the density of interstitial microbial populations, despite the relatively high benthic mineralization rate, which have previously been found to correlate positively with viral production (Glud & Middelboe, 2004; Middelboe & Glud, 2006). However, virtually nothing is known about viriobenthos abundance and activity in coastal sandy sediments.

At the Nivå and deep Øresund stations, benthic viral and bacterial abundances were consistent with previous data from the same locations (Middelboe *et al*., 2003; Glud & Middelboe, 2004). Moreover, the relatively high abundances of bacteria and viruses in the investigated freshwater sediments corresponded to observations from other lakes (Maranger & Bird, 1996; Fischer *et al*., 2003; Filippini *et al*., 2006) and from two Australian rivers (Hewson *et al*., 2001). The observation of higher VBRs in lake samples than in marine samples in the present study may be the result of enhanced nutrient availability in freshwater systems and supported the observation of increased VBR along a trophic gradient in an estuarine environment (Hewson *et al*., 2001).

The large range in virus abundances in pelagic samples from $0.42–15.7 \times 10^7$ VLP mL^{-1} and in benthic samples from $0.2–30.0 \times 10^8$ VLP cm^{-3} emphasized that the selected marine, brackish and freshwater locations represented distinct environments providing extremely different habitat conditions for microorganisms. Interestingly, virus abundance was significantly correlated with bacterial abundance ($R^2 = 0.86$, $P < 0.01$) across this span of environments, suggesting a broad coupling between bacteria and viruses across ecosystem types.

Viral community composition in water and sediment samples

The dominant genome size classes (30–48, 50–70 and 145–200 kb) in both pelagic and benthic samples have been observed in most previous studies on viral genome size distribution in aquatic systems (e.g. Wommack *et al*., 1999; Steward *et al*., 2000; Fuhrman *et al*., 2002; Riemann & Middelboe, 2002b; Sandaa *et al*., 2003; Jiang *et al*., 2004; Auguet *et al*., 2006), and suggests that these genome sizes are generally dominant in aquatic ecosystems. The size pattern is in line with the fact that average genome sizes for different groups of cultivated bacteriophages, including the major taxonomic families *Siphoviridae*, *Podoviridae* and *Myoviridae*, fall within these size classes (Ackermann & DuBow, 1987; Steward *et al*., 2000). Moreover, T7-like podophages, which have genome sizes of ~40 kb, have been found widely distributed in aquatic and terrestrial environments (Breitbart *et al*., 2004b).

The water samples in this study gave rise to between 10 and 20 bands, with the highest abundance observed in the eutrophic Lake Frederiksborg Slotssø. This result is similar to data from Chesapeake Bay (7–16 bands Wommack *et al*., 1999), the coastal Pacific off Southern California (10–15 bands Fuhrman *et al*., 2002), diverse pelagic marine environments (8–16 bands Steward *et al*., 2000), and a range of Danish marine and brackish waters (15–20 bands Riemann & Middelboe, 2002b).

Besides the general observation that viral genome sizes fell into similar size classes in all the studied environments, the PFGE fingerprints from the different locations displayed interesting differences in specific banding patterns. Most striking was the specificity of low genome size bands (12–17 kb) to freshwater environments, where they represented dominant genotypes in both water and sediment samples (with different dominant low genome sizes in the two habitats). Presence of such low genome size viruses have previously been observed in samples of rumen and human feces (Breitbart *et al*., 2003) and from hypersaline ponds (Sandaa *et al*., 2003). However, the dominance of distinct low genome size bands has to our knowledge not been reported from other marine or brackish systems either, and the small genomes observed in the present study may represent genomes of specific lake viruses.

In the high genome size fraction (> 90 kb), we also identified differences between freshwater, marine and brackish environments. Lake water generally yielded more bands in the >145 kb fraction than sea water, whereas the brackish sediment contained several genomes in that size fraction, which were completely absent in lake sediment. Assuming that these relatively large genomes to some extent reflect algal viruses, these differences might be interpreted as differences in the amount and composition of algal

© 2007 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

populations in the systems. Both lakes have generally high pelagic algal biomass compared with the marine locations, whereas benthic algae were absent because of low light penetration and anoxic conditions prevailing in the bottom water layer. The Nivå Bay sediment, on the other hand, harbors active diatom and cyanobacterial communities, and also the coastal aphotic sediment is known to contain large abundances of living phytoplankton cells that may survive for several months in the sediment (Hansen & Josefson, 2001). Consequently, the similarity analysis showed that the freshwater (Lake Frederiksborg Slotssø and Lake Esrum), the brackish (Nivå Bay) and the marine (Øresund) environments branched out separately in the similarity index, with relatively low similarity between environments and even within individual sampling sites.

A comparison of the PFGE profiles from pelagic and benthic samples demonstrated that a number of bands, especially in the 48–60 kb size fraction, were observed on both environments. This may suggest that some of the benthic viruses may originate from pelagic viruses that fall out of the water column on sedimenting particles. Previous estimates of the input of pelagic viruses to the sediment indicated that this process could only account for a very small fraction of the daily viral production in the investigated sediment (Hewson & Fuhrman, 2003). Obviously, this depends both on the rate of sedimentation and the ability of viruses produced in the water column to maintain populations within the sediment, and still virtually nothing is known about the fate of pelagic viruses when they reach the sediment.

Although there was a large overlap in PFGE fingerprints from pelagic and benthic samples from the same environment as well as from different environments, each habitat also contained genomes that seemed to be specific for that particular environment. In the intermediate genome size fraction (70–100 kb), large differences were observed between sediment and water samples with a number of distinct bands occurring in the sediment, which were absent from the water samples. Even within a given sediment, small differences in 70–100 kb banding patterns were observed with depth, where specific bands were present in specific depths. This suggests that the some benthic viral populations are associated with specific stratifications in the microbial communities.

Consequently, even though some genome sizes were present in all the samples, the viral communities, as described by PFGE, varied considerably between different samples. It should be noted, however, that our samples taken at a single occasion are not fully representative of the investigated sites. Some of the observed differences between samples therefore might reflect temporal or spatial variation within a given location rather than actual differences between systems.

The total number of bands also decreased with sediment depth, probably reflecting that specific viral genome sizes decreased to below detection limit in the deeper sediments, which might be a benthic parallel to the decrease in band numbers with water depths from 5 to 500 m in the pelagic ocean (Fuhrman *et al*., 2002).

In conclusion, PFGE can be useful to obtain viral genome size fingerprints not only of pelagic water samples but also of sediment samples, and we present here for the first time a direct comparison of PFGE fingerprints from pelagic and benthic habitats within and between ecosystems. Data from marine, brackish and freshwater systems showed similarities in genome size distribution between a number of habitats covering a large range of microbial activities and abundances. However, PFGE analysis also indicated that certain genome sizes were found only in certain types of environments (e.g. lake water), in certain locations (e.g. a particular lake) or even in certain microhabitats (e.g. a particular sediment layer). The results suggest that some virus phenotypes are ubiquitous in aquatic systems and may be efficiently spread between environments, while the distribution of others may be limited to certain environments or conditions. However, more advanced genetic tools are necessary for providing further insight to the genotypic composition and spatial dynamics of natural viral communities.

Acknowledgements

We thank Benly True and Brian Green for help with collecting the marine samples and Anni Glud for preparation and help with oxygen microsensors. Mark O. Gessner provided valuable comments on the manuscript. The study was supported by the Danish Natural Sciences Research Council.

References

Ackermann HW & DuBow MS (1987) *Viruses of Prokaryotes: Natural Groups of Bacteriophages*. CRC Press, Inc., Boca Raton, FL.

Auguet JC, Montanié H & Lebaron P (2006) Structure of virioplankton in the Charente estuary (France): transmission electron microscopy *versus* pulsed field gel electrophoresis. *Microb Ecol* **51**: 197–208.

Bettarel Y, Bouvy M, Dumont C & Sime-Ngando T (2006) Virus-bacterium interactions in water and sediment of West African inland aquatic systems. *Appl Environ Microbiol* **72**: 5274–5258.

Bratbak G, Haslund OH, Heldal M, Næs A & Røeggen T (1992) Giant marine viruses? *Mar Ecol Prog Ser* **85**: 201–202.

Breitbart M, Salamon P, Andersen B, Mahaffy JM, Sagall AM, Mead D, Azam F & Rohwer F (2002) Genomic analysis of uncultured marine viral communities. *PNAS* **99**: 14250–14255.

© 2007 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

Breitbart M, Hewson I, Felts B, Mahaffy JM, Nulton J, Salamon P & Rohwer F (2003) Metagenomic analysis of an uncultured viral community from human feces. *J Bacteriol* **185**: 6220–6223.

Breitbart M, Felts B, Kelley S, Mahaffy J, Nulton J, Salamon P & Rohwer F (2004a) Diversity and population structure of a near-shore marine sediment viral community. *Proc R Soc Lond B* **271**: 565–574.

Breitbart M, Miyake JH & Rohwer F (2004b) Global distribution of nearly identical phage-encoded DNA sequences. *FEMS Microbiol Lett* **236**: 249–256.

Brussaard CPD (2004) Viral control of phytoplankton populations – a review. *J Eukariot Microbiol* **51**: 125–138.

Buchanan JB (1984) Sediment analysis (Holme O & McIntyre O, eds), pp. 41–65. *Methods for the study of marine benthos IBP Handbook N. 16*. Blackwell Scientific Publications, Oxford, UK.

Castberg T, Larsen A, Sandaa RA, Brussaard CPD, Egge JK, Heldal M, Thyrhaug R, van Hannen EJ & Bratbak G (2001) Microbial population dynamics and diversity during a bloom of the marine coccolithophorid *Emiliania huxleyi* (Haptophyta). *Mar Ecol Prog Ser* **221**: 39–46.

Chen F, Lu JR, Binder BJ, Liu YC & Hodson RE (2001) Application of digital image analysis and flow cytometry to enumerate marine viruses stained with SYBR GOLD. *Appl Environ Microbiol* **67**: 539–545.

Corpe WA & Jensen TE (1996) The diversity of bacteria, eukaryotic cells and viruses in an oligotrophic lake. *Appl Microbiol Biotechnol* **46**: 622–630.

Culley AI, Lang AS & Suttle CA (2003) High diversity of unknown picorna-like viruses in the sea. *Nature* **424**: 1054–1057.

Danovaro R & Serresi M (2002) Viral density and virus-to-bacterium ratio in deep sea sediment of the Eastern Mediterranean. *Appl Environ Microb* **66**: 1857–1861.

Diez B, Anton J, Guixa-Boixereu N, Pedros-Alio C & Rodriguez-Valera F (2000) Pulsed-field gel electrophoresis analysis of virus assemblages present in a hypersaline environment. *Internat Microbiol* **3**: 159–164.

Edwards RA & Rohwer F (2005) Viral metagenomics. *Nature Rev Microbiol* **3**: 504–510.

Filippini M, Buesing N, Bettarel Y, Sime-Ngando T & Gessner MO (2006) Infection paradox: high abundance but low impact of freshwater benthic viruses. *Appl Environm Microbiol* **72**: 4893–4898.

Finlay BJ & Maberly SC (2000) *Microbial Diversity in Priest Pot: A Productive Pond in the English Lake District*. Freshwater Biol Ass, Titus Wilson, Kendal, 73 pp.

Fischer UR, Wieltschnig C, Kirschner AKT & Velimirov B (2003) Does viral-induced lysis contribute significantly to bacterial mortality in the oxygenated sediment layer of shallow Oxbow lakes? *Appl Environ Microbiol* **69**: 5281–5289.

Fuhrman JA, Griffith JF & Schwalbach MS (2002) Prokaryotic and viral diversity patterns in marine plankton. *Ecol Res* **17**: 183–194.

Glud RN & Middelboe M (2004) Virus and bacteria dynamics of a coastal sediment: implications for benthic carbon cycling. *Limnol Oceanogr* **49**: 2073–2081.

Hansen JLS & Josefson AB (2001) Pools of chlorophyll and live planktonic diatoms in aphotic marine sediments. *Mar Biol* **139**: 289–299.

Hewson I & Fuhrman JA (2003) Viriobenthos production and virioplankton sorptive scavenging by suspended particles in coastal and pelagic waters. *Microbial Ecol* **46**: 337–347.

Hewson I, O'Neil JM, Fuhrman JA & Dennison WC (2001) Virus-like particle distribution and abundance in sediments and overlying waters along eutrophication gradients in two subtropical waters. *Limnol Oceanogr* **46**: 1734–1746.

Jiang S, Fu W, Chu W & Fuhrman JA (2003) The vertical distribution and diversity of marine bacteriophage at a station off Southern California. *Microb Ecol* **45**: 399–410.

Jiang S, Steward G, Jellison R, Chu W & Choi S (2004) Abundance, distribution, and diversity of viruses in alkaline, hypersaline, Mono Lake, California. *Microb Ecol* **47**: 9–17.

Larsen A, Castberg T, Sandaa RA, Brussaard CPD, Egge J, Heldal M, Paulino A, Thyrhaug R, van Hannen EJ & Bratbak G (2001) Population dynamics and diversity of phytoplankton, bacteria and viruses in a seawater enclosure. *Mar Ecol Prog Ser* **221**: 47–57.

Larsen A, Fonnes Flaten GA, Sandaa RA, Castberg T, Thyrhaug R, Erga SR, Jacquet S & Bratbak G (2004) Spring phytoplankton bloom dynamics in Norwegian coastal waters: microbial community succession and diversity. *Limnol Oceanogr* **49**: 180–190.

Maranger R & Bird DF (1996) High concentrations of viruses in the sediments of Lac Gilbert, Québec. *Microb Ecol* **31**: 141–151.

Markager S, Hansen B & Søndergaard M (1994) Pelagic carbon metabolism in a eutrophic lake during a clear-water phase. *J Plankton Res* **16**: 1247–1267.

Mei ML & Danovaro R (2004) Virus production and life strategies in aquatic sediments. *Limnol Oceanogr* **49**: 459–470.

Middelboe M & Glud RN (2006) Viral activity along a trophic gradient in continental margin sediments off central Chile. *Mar Biol Res* **2**: 41–51.

Middelboe M & Søndergaard M (1993) Bacterioplankton growth yield: seasonal variations and coupling to substrate lability and beta-glucosidase activity. *Appl Environ Microb* **59**: 3916–3921.

Middelboe M, Glud RN & Finster K (2003) Distribution of viruses and bacteria in relation to diagenetic activity in a estuarine sediment. *Limnol Oceanogr* **48**: 1447–1456.

Middelboe M, Glud RN, Wenzhöfer F, Oguri K & Kitazato H (2006) Spatial distribution and activity of viruses in the deep-sea sediments of Sagami Bay, Japan. *Deep-Sea Res I* **53**: 1–13.

Rasmussen H & Jørgensen BB (1992) Microelectrode studies of seasonal oxygen uptake in a coastal sediment: role of molecular diffusion. *Mar Ecol Prog Ser* **81**: 289–303.

Riemann L & Middelboe M (2002a) Viral lysis of bacterioplankton: implications for organic matter cycling and bacterial clonal composition. *Ophelia* **56**: 57–68.

© 2007 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

Riemann L & Middelboe M (2002b) Temporal and spatial stability of bacterial and viral community compositions in Danish coastal waters as depicted by DNA fingerprinting techniques. *Aquat Microb Ecol* **27**: 219–232.

Sambrook J, Fritsch EF & Maniatis T (1989) *Molecular Cloning*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sandaa RA & Larsen A (2006) Seasonal variations in virus–host populations in Norwegian coastal waters: focusing on the cyanophage community infecting marine *Synechococcus* spp. *Appl Environ Microbiol* **72**: 4610–4618.

Sandaa RA, Skjoldal EF & Bratbak G (2003) Virioplankton community structure along a salinity gradient in a solar saltern. *Extremophiles* **7**: 347–351.

Søndergaard M (1991) Phototrophic picoplankton in temperature lakes – seasonal abundance and importance along a trophic gradient. *Int Rev Ges Hydrobiol* **76**: 505–522.

Steward GF & Azam F (1999) Analysis of marine viral assemblages. (Bell CR, Brylinsky M & Johnson-Green P, eds), pp. 159–165. *Microbial Biosystems: New Frontiers Proceedings of the 8th International Symposium on Microbial Ecology*. Atlantic Canada Society for Microbial Ecology, Halifax.

Steward GF, Montiel JL & Azam F (2000) Genome size distributions indicate variability and similarities among marine viral assemblages from diverse environments. *Limnol Oceanogr* **45**: 1697–1706.

Suttle CA (2005) Viruses in the sea. *Nature* **437**: 356–361.

Thingstad TF & Lignell R (1997) Theoretical models for the control of bacterial growth rate, abundance, diversity and carbon demand. *Aquat Microb Ecol* **13**: 19–27.

Weinbauer MG (2004) Ecology of prokaryotic viruses. *FEMS Microb Rev* **28**: 127–181.

Weinbauer MG & Rassoulzadegan F (2004) Are viruses driving microbial diversification and diversity? *Environ Microbiol* **6**: 1–11.

Wichels A, Biel SS, Gelderblom HR, Brinkhoff T, Muyzer G & Schütt C (1998) Bacteriophage diversity in the North Sea. *Appl Environ Microbiol* **64**: 4128–4133.

Wilhelm SW & Suttle CA (1999) Viruses and nutrient cycles in the sea. *BioScience* **49**: 781–788.

Wommack KE & Colwell RR (2000) Virioplankton: viruses in aquatic ecosystems. *Microbiol Mol Biol Rev* **64**: 69–114.

Wommack KE, Ravel J, Hill RT, Chun J & Colwell RR (1999) Population dynamics of Chesapeake Bay virioplankton: total community analysis by pulsed-field gel electrophoresis. *Appl Environ Microbiol* **65**: 231–240.

© 2007 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved