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FEATURE ARTICLE

Viral abundance and activity in the deep sub-seafloor biosphere

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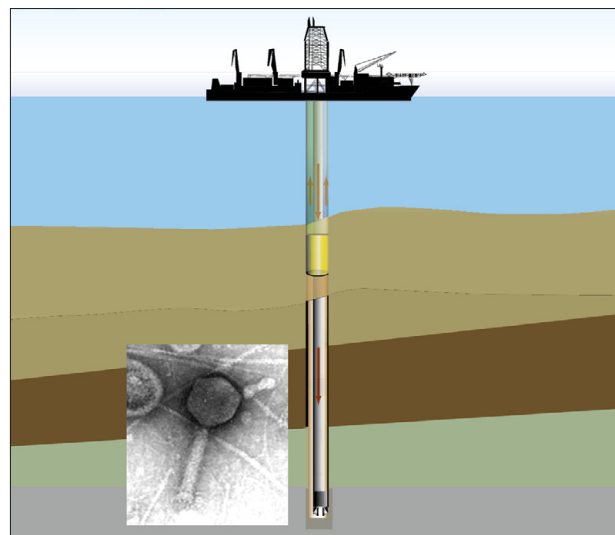
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ABSTRACT: Subsurface abundance and distribution of viruses and prokaryotes was determined along a depth profile, down to 96 m below seafloor (96 mbsf), at Challenger Mound from the Porcupine Seabight (IODP Expedition 307). Viral and prokaryotic abundance decreased exponentially with sediment depth from 1.0×10^8 viruses cm^{-3} and 3.8×10^6 cells cm^{-3} at 4 mbsf to 4.9×10^6 viruses cm^{-3} and 9.8×10^5 cells cm^{-3} at 96 mbsf. The age of the sediment ranges from ca. 0.5 million yr before present (Ma) at 4 mbsf to ca. 2 Ma at 96 mbsf. Assuming that the decline in viral abundance with depth reflects a gradual decay of the viral assemblage over time, the estimated decay rate of the viral community is $1.2 \times 10^{-6} \pm 0.3 \times 10^{-6}$ (SD) yr^{-1} , corresponding to a half-life of the viral community of 5.8×10^5 yr. Measurements of viral and prokaryotic change in abundance were performed in incubations of undiluted, but homogenized, sediment samples (13.3 and 79.8 mbsf) in anaerobic bags. Viral abundance decreased rapidly (decay rates of 0.010 ± 0.002 [SD] and 0.022 ± 0.018 [SD] h^{-1} , respectively) in the incubations, suggesting that homogenization exposed the viruses to degradation processes. We hypothesize that most of the deep subsurface viral communities inhabit a microenvironment where the viruses are protected against decay, and can therefore persist in undisturbed sediments for hundreds of thousands, perhaps even millions, of years.

KEY WORDS: Benthic viruses · Marine phages · Subsurface viruses · Viral decay · Sediment viruses · Viral ecology

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Advanced drilling technology allows sediment sampling several hundred meters into the deep subsurface biosphere. Inset: transmission electron micrograph of a benthic virus.

Graphic: Redrawn from an illustration by Greg Myers;
Photo: Mathias Middelboe

INTRODUCTION

The deep sub-seafloor, extending from a few decimetres below the sediment surface to several hundred meters into the sediment, constitutes a giant sub-surface biosphere, which harbours more than half of all prokaryotic cells on Earth (e.g. Whitman et al. 1998, Schippers et al. 2005, Jørgensen & Boetius 2007). Even in very old — ca. 16 million yr (Ma) — and deep — more

than 400 m below sea floor (mbsf) — sediments, a large fraction of the prokaryotes has been shown to be metabolically active with population turnover times of 0.25 to 22 yr (Schippers et al. 2005). Most of these cells have so far been resistant to cultivation, and little is known about the composition and diversity of this 'hidden majority' of *Bacteria* and *Archaea* (Whitman et al. 1998, Jørgensen & Boetius 2007).

Viruses occur in high abundances (10^7 to 10^{10} viruses cm^{-3} ; e.g. Middelboe et al. 2006, Danovaro et al. 2008) in surface sediments and cause significant mortality of bacterial communities in these environments with large implications for bacterial activity and benthic nutrient cycling (e.g. Glud & Middelboe 2004, Middelboe & Glud 2006, Danovaro et al. 2008, Siem-Jørgensen et al. 2008). Production and abundance of viruses are closely correlated with benthic mineralization rates, and generally tend to decrease with sediment depth (Middelboe et al. 2003, 2006, Middelboe & Glud 2006). As most studies of viruses in sediments have been performed in the upper sediment layer (0 to 10 cm), little is known about the abundance, distribution and activity of viruses in the sub-seafloor biosphere.

To our knowledge, the only published study on the distribution of viruses in the deep biosphere is from an Ocean Drilling Project (ODP) sampling in Saanich Inlet, Canada. The results demonstrated high viral abundance ($>10^9$ viruses g dw^{-1}) down to >100 m into the sediment, but generally reflecting a decrease with depth (Bird et al. 2001). Saanich Inlet is an environment with relatively high sedimentation rates and the deep sediments are therefore relatively young ($<14\,000$ yr) compared with other ODP samplings. More recently, virus abundance and production were measured in the sediment at depths of 80 to 90 cm below the sediment surface in upwelling areas off Chile. Here, significant viral abundance (8×10^7 viruses cm^{-3}) and production (ca. 2×10^6 viruses $\text{cm}^{-3} \text{h}^{-1}$) were found, in spite of relatively low bacterial sulphate reduction rates at those depths (ca. $0.002 \mu\text{mol SO}_4 \text{cm}^{-3} \text{d}^{-1}$) (Middelboe & Glud 2006).

The presence of viruses in the deep biosphere raises questions regarding their origin, fate and impact on the microbial community and their activity in this environment: How can large communities of viruses and prokaryotes persist in a compact, nutrient-deprived environment with extremely low metabolic activity, limited mobility and without input of organic matter? Is there sufficient energy available to sustain viral production in such an environment, or are the viruses found there remnants from an active surface sediment that has been buried? Our current knowledge of the fate of viruses in the deep biosphere does not provide clear answers to these questions. In fact, we are far from understanding even the basic strategies for

microbial life, the persistence of prokaryotic and viral DNA and the interactions between microorganisms in the deep sub-seafloor biosphere. Exploration of these topics represents therefore an important challenge for future research.

The abundance of viruses in any environment is the net result of their production, decay and import/export processes. In sediments, viral production is primarily associated with the growth of *Bacteria* and *Archaea*, and viral particles are lost from the environment mainly by irreversible binding to particles and digestion by proteases and nucleases (e.g. Bales et al. 1991, England et al. 1998, Fischer et al. 2004). In surface sediments, the balance between production and decay varies with environmental factors, such as sedimentation events, fauna activity and temperature, and consequently, viral abundance may show temporal variations on both diurnal and seasonal scales (Fischer et al. 2003, Siem-Jørgensen et al. 2008). The relatively constant physical and chemical conditions at a given depth in the deep biosphere would imply that a given abundance of viruses reflects a long-term steady state between production and decay. However, the dynamics of the interactions between viruses and prokaryotes are unknown.

In the present study, we estimated the depth distribution of viral and bacterial abundance and the potential for stimulating their activity at selected depths in deep (4 to 96 mbsf) and old (ca. 2.5 Ma, Kano et al. 2007) sub-surface sediments. The results demonstrate the presence of ca. 5×10^6 viruses cm^{-3} in the old sediment collected at 96 mbsf and suggest that viruses may persist for hundreds of thousands of years in the deep biosphere.

MATERIALS AND METHODS

Study site and sample collection. The samples were collected from site U1317 during the Integrated Ocean Drilling Project (IODP) Expedition 307 on the eastern margin of the Porcupine Seabight. U1317 was located on Challenger Mound, a large cold-water coral bank, which extends 160 m above the seafloor and measures 0.55 km^2 at its base (Huvenne et al. 2009). On board the ship, the cores were sectioned into subsamples for pore-water chemistry with a minimum resolution of 10 cm. Sectioning and sealing of the samples was performed according to standard procedures for IODP sampling (Expedition 307 Scientists 2006), and the sealed samples for prokaryote and viral counts were stored at 4°C until further processing. Seven samples within a depth interval from 4 to 96.1 mbsf were allocated for analysis of viral and prokaryote abundance. In addition, incubations for viral and prokaryotic production were performed with samples from 13.3 and 79.8 mbsf.

Sediment characteristics. Sediment porosity and total organic matter content were measured at all the selected depths. The porosity was calculated from the density and the weight loss after drying at 100°C for 24 h, and showed a linear decrease with depth from 0.51 at 4 mbsf to 0.41 at 96.1 mbsf without significant correlation with viral ($r^2 = 0.41$, $p = 0.12$) or prokaryote ($r^2 = 0.28$, $p = 0.22$) abundance. Total organic matter content was measured as the weight loss after combustion at 450°C for 24 h (Buchanan 1984) and ranged from 1.4 to 2.6% dry weight with highest relative organic matter content in the deepest samples. This represents a standard procedure, but we cannot exclude contribution from carbonate compounds during combustion. Chemical data from pore-water analyses (Expedition 307 Scientists 2006) showed a linear increase in dissolved inorganic carbon (DIC) and ammonium concentrations with sediment depth from 2.5 mM and zero, respectively in surface samples to 18 mM DIC and 1.7 mM ammonium at 100 mbsf, whereas sulphate concentrations decreased along the same gradient from 28 mM to 10 mM. The temperature was relatively constant in the upper 100 mbsf, ranging between 11.0 and 13.5°C (Expedition 307 Scientists 2006).

Extraction and enumeration of prokaryotes and viruses. Sediment (4 g) was transferred to a sterile 50 ml Falcon tube and 4 ml of virus-free artificial seawater (34‰), 1 ml 0.02 µm-filtered glutaraldehyde (3% final concentration), and 1 ml of sodium pyrophosphate (10 mM final concentration) were added. After 15 min, the sample was sonicated 2 × 1 min on ice using a 60 W sonication probe (VibraCell™, Sonics & Materials) and then centrifuged for 5 min at 700 × *g*. The supernatant was collected and a further 2 ml virus-free (0.02 µm filtered) seawater was added to the sample followed by mixing and centrifugation (as above) (Danovaro & Middelboe 2010). The sample was washed 3 times using this procedure, and the total extracted volume (the supernatants) was pooled. For virus counts, 0.1 to 1 ml of extract was immediately filtered onto a 0.02 µm Anodisc filter (Whatman) and stained with SYBR Gold (Chen et al. 2001). For prokaryotes, a subsample of the extract (1 to 3 ml) was prestained with SYBR Gold for 5 min and subsequently filtered onto a 0.2 µm black polycarbonate filter (Poretics). Prokaryotes and viruses were counted in 20 to 30 fields using epifluorescence microscopy at 1250× magnification.

There are a number of extraction procedures for viruses and prokaryotes in sediments (e.g. Fischer et al. 2005, Danovaro & Middelboe 2010). In the present study, the sonicated sediment samples were initially processed through a centrifugation-based method with repeated steps of centrifugation and washes to reduce the background of sediment particles, which interferes

with the counting of viruses by microscopy. Such centrifugation steps may result in an underestimation of viral and prokaryote abundances, since even low centrifugation may remove viruses and prokaryotes from the water phase (Siem-Jørgensen et al. 2008). Alternatively, a simpler dilution method can be used, where the sonicated sediment is diluted with virus-free water to avoid centrifugation of the sample (Velji & Albright 1985, Siem-Jørgensen et al. 2008). The disadvantage of that method is that the higher background of organic and inorganic sediment particles may hide the fluorescently stained viruses and prokaryotes on the slide. The applicability of the different methods may therefore vary with the type of sediment and the density of viruses (Danovaro & Middelboe 2010). Following the initial determination of viral and prokaryote abundance, we tested the applicability of the 2 procedures in 4 sediment samples from a parallel IODP core, to evaluate whether the centrifugation procedure was applicable to these deep sub-seafloor samples.

The tests showed that the dilution method yielded significantly higher values of both prokaryote and virus abundance compared to the procedure with pre-centrifugation (on average 10.9 ± 3.6 , SD, and 5.3 ± 3.4 times higher, respectively; Fig. 1). Thus, despite a high background noise from organic and inorganic particles on the filter, the dilution method was the most appropriate to apply to these sediments. Our viral and

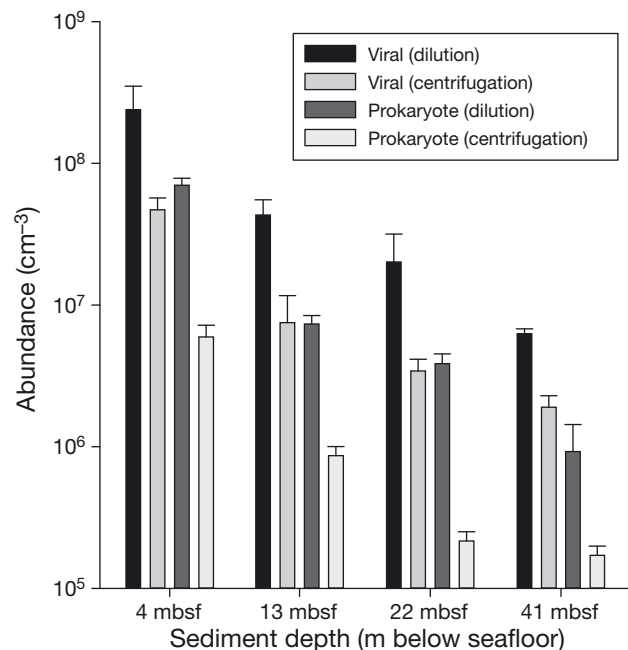


Fig. 1. Comparison of viral and prokaryote abundance in sediment samples obtained from 4 depths (4 m below seafloor [mbsf], 13 mbsf, 22 mbsf and 41 mbsf), using 2 different extraction methods (dilution and centrifugation). Error bars are \pm SD ($n = 2$)

prokaryote counts, which were based on the centrifugation method, have been corrected accordingly. It should be noted that decompression during sampling may have affected the viral and prokaryotic counts obtained; however, this was not investigated.

Viral and prokaryotic production in anaerobic Würgler bag incubations. Anaerobic Würgler bag incubations (e.g. Hansen et al. 2000, Glud & Middelboe 2004) were set up to investigate whether microbial growth in deep sediments could be stimulated by homogenization and elevated temperature (Wellsbury et al. 1997). Sealed samples from 13.3 and 79.8 mbsf were opened under sterile conditions and homogenized using sterilized tools. Each sample was distributed into 2 sterile, gas-tight Würgler bags (Hansen et al. 2000) and incubated anaerobically for 60 h at 20°C.

To avoid contamination, the Würgler bags were placed in a sterile plastic bag and placed inside a Laminar Air Flow (LAF) bench during incubation. After each sampling, the bags were transferred to a new, sterile plastic bag. Prior to and after each sampling the Würgler bags were sterilized on the outside with ethanol and all samplings took place in the LAF bench under sterile conditions. Bags were flushed with nitrogen (filtered through a 0.2 µm filter) following each sampling. Samples for virus and prokaryote abundance were collected at time zero and then every 6 to 12 h for 60 h (0, 6, 12, 24, 36, 48, 60 h).

RESULTS AND DISCUSSION

Depth distribution of viral and prokaryote abundance

The abundance of viruses decreased exponentially with depth from 1.0×10^8 viruses cm^{-3} at 4 mbsf to 4.9×10^6 viruses cm^{-3} at 96 mbsf ($r^2 = 0.996$, $p < 0.003$) (Fig. 2). The prokaryote abundance overall also approximated an exponential decline with depth and ranged from 3.8×10^7 cells cm^{-3} at 4 mbsf to 9.8×10^5 cells cm^{-3} at 96 mbsf ($r^2 = 0.994$, $p < 0.02$) (Fig. 2). The age constraints, geological structure and biogeochemistry of the sediments of the Challenger Mound have been described in previous publications from the IODP Expedition 307 (e.g. Kano et al. 2007, Webster et al. 2009). According to the strontium isotope stratigraphy, the sediment age ranges from 0.5 Ma at 4 m to 1.0 Ma at 20 mbsf. At 23.6 m a significant discontinuity from 1.0 to 1.7 Ma was discovered (horizontal line in Fig. 2) and below that the age of the sediment increased linearly to approximately 2 Ma at 100 mbsf (Kano et al. 2007). These sediments are therefore approximately 100-fold older than at the site for the previous deep biosphere viral quantification (Bird et al. 2001). The

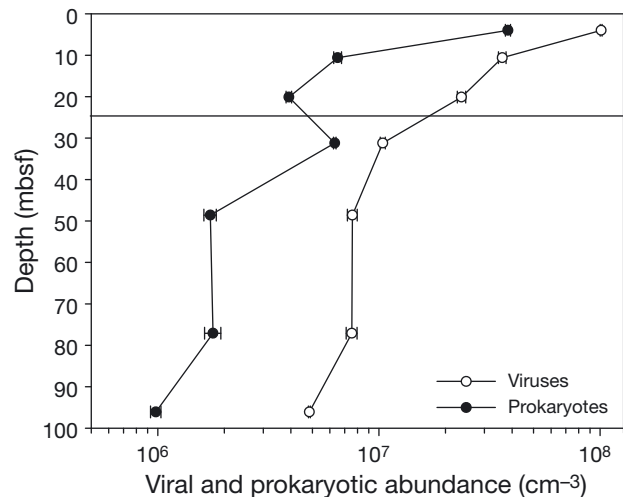


Fig. 2. Depth distribution and abundance of viruses and prokaryotes in the upper 96.1 m below seafloor (mbsf) of the deep sub-seafloor biosphere at the Challenger Mound. Error bars are \pm SD ($n = 3$). Horizontal line indicates a discontinuity at 23.6 m, where the estimated sediment age changes from in 1.0 to 1.7 million yr

present study thus represents the first attempt to quantify viral abundance in such extremely old sediments.

The abundance of viruses found in the present study (ca. 10^6 to 10^8 viruses cm^{-3}) was approximately 100-fold lower than that generally obtained from surface sediments (i.e. 10^8 to 10^{10} viruses cm^{-3} , Danovaro et al. 2008), and that found in the subsurface biosphere in Saanich Inlet (Bird et al. 2001). The virus:prokaryote (V:P) ratio ranged between 2 and 6 with no significant changes with depth ($p > 0.1$, ANOVA). This is similar to ratios obtained in the deep subsurface biosphere in Saanich Inlet (Bird et al. 2001) and in surface sediments (e.g. Siem-Jørgensen et al. 2008). The fact that the V:P ratio did not change systematically with sediment depth could be related either to similar decay rates for viruses and prokaryotes in the respective sediment layers or that relative prokaryotic mortality caused by virus infections is depth independent, or a combination of both.

Previous studies in surface sediments have demonstrated high virus production rates and a positive correlation between the activity of bacteria and the production of viruses (Glud & Middelboe 2004, Danovaro et al. 2008). At the same time, a number of studies have suggested that all benthic environments contain a high and relatively stable background level of viruses, even in environments of low activity or during periods with marginal input of organic material (e.g. Middelboe & Glud 2006, Siem-Jørgensen et al. 2008). For example, the viral abundance in a coastal sediment showed strong seasonal variation, both at the surface (0 to 0.5 cm) and in deeper (6 to 10 cm) sediment layers,

which was coupled with the input of organic matter from the water column. In both sediment layers, however, viral abundance never fell below ca. 4×10^8 and ca. 2×10^8 viruses cm^{-3} , respectively, even during periods with very low virus production (Siem-Jørgensen et al. 2008). In viral decay experiments, where viral production has been inhibited by for example KCN, there is usually a relatively fast decay of a fraction of the community, whereas part of the community is often resistant to decay within a time frame of days to weeks (e.g. Fischer et al. 2004). Based on such evidence, we hypothesize that there is a fraction of the viral community which is practically inert and protected from decay under any given set of conditions in marine sediments. This is in line with more recent molecular and metagenomic studies, which have suggested that only a small fraction of benthic viral communities is present in high abundance (Breitbart et al. 2004, Filippini & Middelboe 2007), whereas most of the viral gene pool consists of genomes occurring in small numbers, thus functioning as an inducible virus gene bank.

In contrast to earlier studies (e.g. Middelboe & Glud 2006, Siem-Jørgensen et al. 2008), the applied incubation method did not allow an estimate of the potential for viral production (see Fig. 3). Hence, we do not have measurements of viral activity in the deep biosphere. The extremely low energy flux suggests that generation times for deeply buried prokaryotes range from years to thousands of years (Jørgensen & Boetius 2007). Consequently, it is unlikely that there is sufficient energy available to sustain high viral production in these environments. However, measurements of ^3H -thymidine (TdR) incorporation, as a measure of prokaryote activity, in the deep biosphere have shown high variability from 1 to 1000 $\text{fmol cm}^{-3} \text{d}^{-1}$ (Parkes et al. 2000, Fry et al. 2008, Webster et al. 2009). Such data suggest that prokaryote activity in some deep biosphere environments may be sufficiently high to support a measurable production of viruses. Assuming (1) a prokaryote cell production of approximately 1×10^{18} cells per mol of incorporated ^3H -TdR (e.g. Deming & Carpenter 2008), (2) that virus-induced mortality accounts for 6% of prokaryote production and (3) a virus burst size of 10 viruses per infection (Glud & Middelboe 2004), viral production in the deep biosphere would range between 6×10^2 and 6×10^5 viruses $\text{cm}^{-3} \text{d}^{-1}$. With an approximate average viral density of 1×10^7 viruses cm^{-3} , the potential range in turnover time of the viral community would be from 0.03 to 32 yr. This range is quite similar to an estimated range of turnover time of 0.25 to 22 yr for deep biosphere prokaryotes (Schippers et al. 2005). The assumptions behind these calculations (i.e. virus-induced mortality equal to 6% of prokaryote production and a virus burst size of 10) can be debated but seem reasonable. Virus-

mediated bacterial mortality and virus burst size are correlated with the growth rate of the bacteria (e.g. Middelboe 2000), and values for the deep sub-seafloor biosphere must be in the lower end of the published range for sediments (e.g. burst sizes from 10 to 20 viruses per infection and a viral mortality impact of 6 to 40% of bacterial production (Glud & Middelboe 2004).

In the present study, it is likely, therefore, that only a relatively small fraction of the total viral community of the investigated sediments is dynamically associated with prokaryote mortality and growth, whereas the majority of the community is an inactive reservoir of viruses with a long residence time. A background density of approximately 8×10^6 viruses cm^{-3} may very well represent a fraction of the viral community that is essentially non-degradable in this deep biosphere environment. On top of this, a smaller fraction of the viral assemblage may be more dynamic with faster rates of production and decay. According to Kano et al. (2007), there is a linear correlation between depth and age of the sediment from 0 to 20 m and again (below the unconformity at 23.6 mbsf) from 30 to 100 mbsf. Making the assumption that the decrease in viral abundance with depth reflects a constant exponential decay of the refractory background viral assemblage over time, the decay rate of this viral community is $1.2 \times 10^{-6} \pm 0.3 \times 10^{-6} \text{ yr}^{-1}$, corresponding to a half-life of the viral community of 5.8×10^5 yr. A similar calculation for the zone between 4 and 20 mbsf gives a decay rate of $1.7 \times 10^{-5} \pm 1.4 \times 10^{-5} \text{ yr}^{-1}$, and a half-life of 4.1×10^4 yr.

Consequently, we suggest that the majority of the viral particles observed in the deep biosphere represent extremely old viruses, which were produced several hundred thousand years ago in the upper 30 m of the sediment layer and subsequently became protected from decay and essentially permanently buried in the deep biosphere. However, this does not exclude some production and decay of viruses even in these very deep sediments, which occur at a time scale of months or even days, sustained by a small, but significant, prokaryote metabolism in the deep biosphere (e.g. Schippers et al. 2005).

Viral decay in Würgler bag incubations

The potential of stimulating the activity of prokaryotes and viruses in the deep biosphere was estimated by incubating subsamples of homogenized sediment from 13.3 and 79.8 mbsf at elevated temperature relative to *in situ*. The Würgler bag approach has previously been applied to estimate net production of benthic viruses and bacteria under anaerobic conditions by following changes in abundances of viruses and bacteria over time (Glud & Middelboe 2004, Middel-

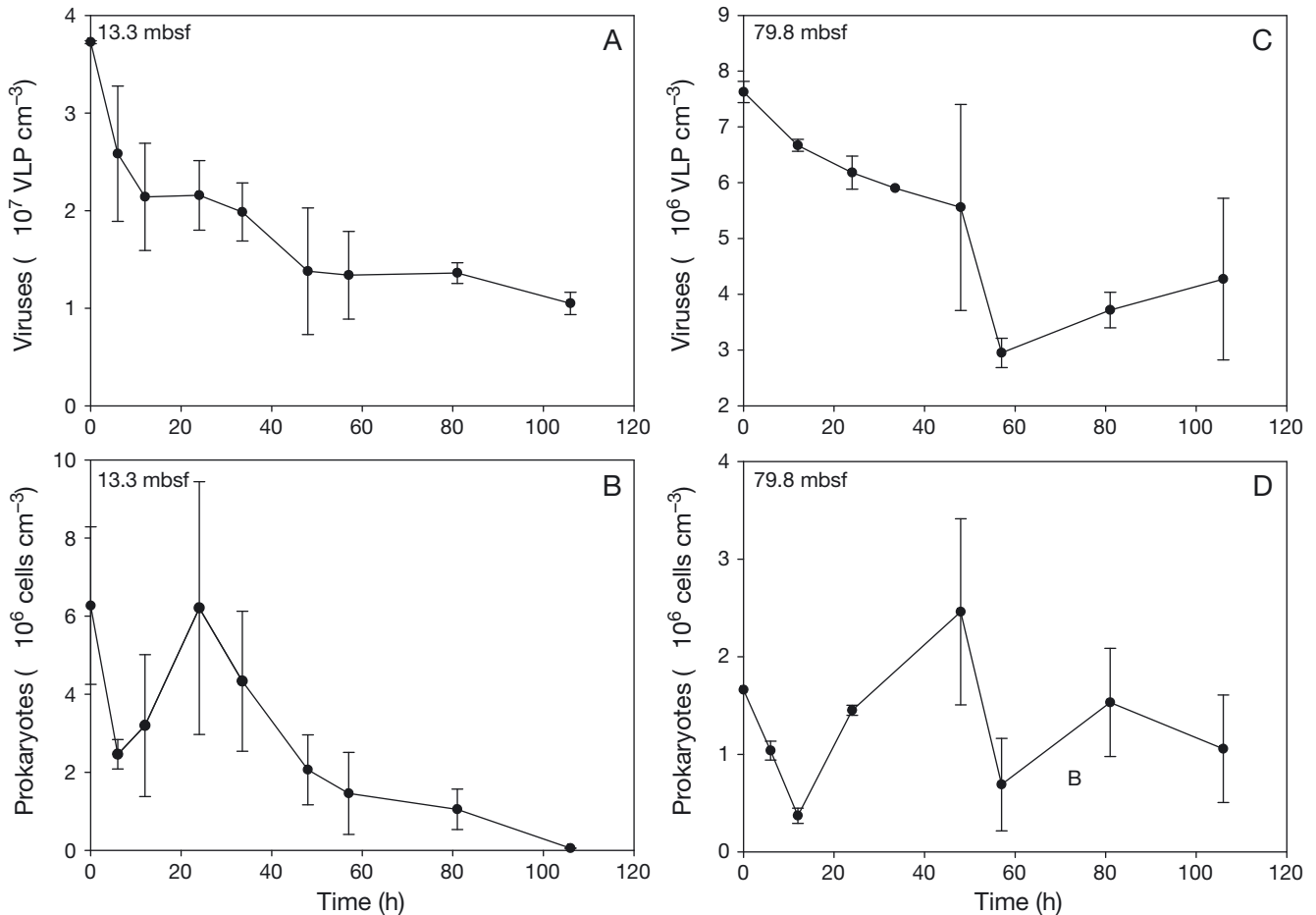


Fig. 3. Changes in the abundance of viruses and prokaryotes over time in duplicate anaerobic bag incubations with homogenized sediment collected from (A,B) 13.3 m below seafloor (mbsf) and (C,D) 79.8 mbsf. VLP: virus-like particles. Error bars are SD

boe & Glud 2006, Middelboe et al. 2006, Siem-Jørgensen et al. 2008). In the present study, however, viral abundance decreased over time in incubations of homogenized sediment sampled from 13.3 and 79.8 mbsf (Fig. 3).

In both sets of incubations, viral abundance decreased exponentially (Fig. 3A,C) with average decay rates of 0.010 ± 0.002 (SD, here and below) viruses h^{-1} ($r^2 = 0.84$, $p < 0.001$, ANOVA) and 0.022 ± 0.018 h^{-1} ($r^2 = 0.73$, $p < 0.02$, ANOVA) in the 13.3 and 79.8 mbsf incubations, respectively. Prokaryote abundance exhibited an initial decrease followed by an increase of $2.1 \times 10^5 \pm 0.3 \times 10^5$ cells $\text{cm}^{-3} \text{h}^{-1}$ ($p = 0.09$, ANOVA) and $5.6 \times 10^4 \pm 1.2 \times 10^4$ cells $\text{cm}^{-3} \text{h}^{-1}$ ($p = 0.13$, ANOVA) at 13.3 and 79.8 mbsf, respectively, and a subsequent decline (Fig. 3B,D). This indicated a stimulation of prokaryotic activity in the incubations; however, the increase in cell abundance was not statistically significant.

The suggestion that virus production in the deep biosphere could be stimulated by increased prokaryote activity is not supported by these experiments. On

the contrary, the perturbation of the sediment by homogenization resulted in relatively rapid decay of viruses. These results suggest that homogenization and incubation of the sediment exposes the viruses to degradation processes. Potentially, the exposure to proteases and nucleases in the sediment is the major cause of this rapid decay (Suttle & Chen 1992, Noble & Fuhrman 1997). These results support observations by Fischer et al. (2004) of a rapid viral decay in sediment samples associated with perturbations (e.g. slurring and homogenization) of the samples. The authors suggested that handling of the sediment prior to the experiments increased the encounter probability between free enzymes and viruses in the sample, thus increasing enzymatic degradation of the viruses (Fischer et al. 2004). In addition, sediment mixing may have caused release of viruses from particles. Consequently, we cannot rule out that part of the observed decrease in viral abundance may have been associated with re-sorbing of transiently released viruses during the incubations.

The presence of organic and inorganic particles has previously been shown to prolong persistence of viruses in marine environments (Mitchell & Jannasch 1969, Gerba & Schaiberger 1975). A protective effect of particles may be due either to a direct structural stabilization of particle-associated viruses or depend on adsorption of enzymes or other antagonistic factors, which promote inactivation of viruses (Gerba & Schaiberger 1975). For the deep sediments analyzed here, the homogenization represents a strong perturbation of an environment which has remained undisturbed for several thousands of years. It is most likely, therefore, that the observed decay of viruses during incubation is the result of sediment handling, and does not represent the actual viral decay in the samples. Consequently, we hypothesize that a fraction of any benthic viral assemblage inhabits a microenvironment where the viruses are not exposed to decay and can therefore persist in undisturbed deep biosphere sediments for hundreds of thousands, perhaps even millions, of years (i.e. an estimated decay rate of $1.2 \times 10^{-6} \pm 0.3 \cdot 10^{-6} \text{ yr}^{-1}$). This may imply that virus-degrading enzymes can also be inactivated for a corresponding period of time and become reactivated by the applied perturbations of the sediment. Alternatively, stimulation of microbial activity by homogenization (e.g. Thamdrup & Canfield 1996, Hansen et al. 2000) may have resulted in the production and release of extracellular enzymes, which may have contributed to viral decay.

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