

## ORIGINAL ARTICLE

# High virus-to-cell ratios indicate ongoing production of viruses in deep subsurface sediments

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**Marine sediments cover two-thirds of our planet and harbor huge numbers of living prokaryotes. Long-term survival of indigenous microorganisms within the deep subsurface is still enigmatic, as sources of organic carbon are vanishingly small. To better understand controlling factors of microbial life, we have analyzed viral abundance within a comprehensive set of globally distributed subsurface sediments. Phages were detected by electron microscopy in deep (320 m below seafloor), ancient (~14 Ma old) and the most oligotrophic subsurface sediments of the world's oceans (South Pacific Gyre (SPG)). The numbers of viruses ( $10^4$ – $10^9$  cm<sup>-3</sup>, counted by epifluorescence microscopy) generally decreased with sediment depth, but always exceeded the total cell counts. The enormous numbers of viruses indicate their impact as a controlling factor for prokaryotic mortality in the marine deep biosphere. The virus-to-cell ratios increased in deeper and more oligotrophic layers, exhibiting values of up to 225 in the deep subsurface of the SPG. High numbers of phages might be due to absorption onto the sediment matrix and a diminished degradation by exoenzymes. However, even in the oldest sediments, microbial communities are capable of maintaining viral populations, indicating an ongoing viral production and thus, viruses provide an independent indicator for microbial life in the marine deep biosphere.**

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## Introduction

The world's oceans harbor huge numbers of viruses. Viral lysis of cells is a driving force for shaping microbial community structures (Weinbauer and Rassoulzadegan, 2004). Furthermore, viruses cause a short-cut (viral shunt) in the carbon and nutrient cycling within the pelagic food web (Wilhelm and Suttle, 1999; Suttle, 2005). A high viral turnover and the associated release of organic carbon was previously shown for surficial deep-sea sediments (Danovaro *et al.*, 2008). For the marine deep biosphere, there are no comparable data available and the viral impact on microbial communities and the related carbon cycling in the marine deep biosphere is poorly constrained. The organisms within deep-sea sediments have to cope with severe substrate and nutrient limitation (Jørgensen and D'Hondt, 2006)

due to an increasing recalcitrance of organic matter with depth (Wellsbury *et al.*, 1997). Even though prokaryotes are prone to starvation (Jørgensen and D'Hondt, 2006), the majority of the cells were shown to be alive and metabolically active (Schippers *et al.*, 2005; Morono *et al.*, 2011). The available energy per cell was estimated to be close to the minimum requirement for cell maintenance. As a result, the carbon turnover of prokaryotic communities has been estimated between 10 and 1000 years (Hoehler and Jørgensen, 2013). Cell activity might only represent the restoration of structural cell damage, for example, amino-acid racemization and depurination. If cells proliferate, growth, however, cannot exceed the rate of prokaryotic mortality, as the community size is decreasing with depth (Hoehler and Jørgensen, 2013). Cells are capable to deal with this energy-limited life style (Roy *et al.*, 2012) but controlling factors for cell death in marine subsurface environments remain largely unidentified. The presence of inducible temperate phages in bacteria isolated from the deep subsurface (Engelhardt *et al.*, 2011; Engelhardt *et al.*, 2013) predict viral predation to control prokaryotic mortality.

Now we have analyzed the so far most comprehensive set of marine sediment samples for virus counts, covering a broad range of characteristics regarding total organic carbon (TOC) content,

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sedimentation rate, age and microbial activity (see Materials and methods section for detailed site description and Map Supplementary Figure S1). These comprise a highly active North Sea tidal-flat (Site Janssand) (Beck *et al.*, 2009), continental margin sites exhibiting high sedimentation rates, open ocean sites with lower sedimentation rates and the South Pacific Gyre (SPG), the most oligotrophic oceanic province. The latter was integrated in this study, as mid-ocean gyres represent 42% of the world's oceans (Kallmeyer *et al.*, 2012) and their sediments harbor far lower cell numbers ( $10^6$ – $10^3$  cells per  $\text{cm}^{-3}$ ) than continental margins ( $10^9$ – $10^5$  cells per  $\text{cm}^{-3}$ ) (Parkes *et al.*, 2000). We aimed for providing an independent indicator for microbial life in the marine subsurface as the presence of phages suggests active phage–host relationships.

## Materials and methods

### Site descriptions

Most sediment samples were collected during ODP/IODP cruises onboard the JOIDES Resolution and were stored at  $-80^\circ\text{C}$  before analysis (Supplementary Figure S1). Additional samples were obtained from the IODP core repository to complement the available sediment samples from ODP Leg 201. German tidal-flats were sampled by vibro coring and samples were prepared for cell and virus counting immediately after sediment recovery.

Tidal-flat sediments (Site Janssand) are constantly supplied with fresh organic material. Highly active microbial communities extend down to several meters below seafloor (mbsf) (Beck *et al.*, 2009, 2011). Three meter long cores were recovered, which is equivalent to  $\sim 5000$  years. Sediments from the continental slope of the Bering Sea (Site U1344, water depth 3172 meter), obtained during IODP Exp. 323, are located in the 'Green Belt' area. A relatively high deposition of organic matter is originating from the high primary production of  $170\text{ g C m}^{-2}\text{ y}^{-1}$  in the photic zone (Sambrotto *et al.*, 2008) and a high sedimentation rate of  $29$ – $89\text{ cm ky}^{-1}$  (Wehrmann *et al.*, 2011). Deep subsurface sediments from the Peru margin (sites 1227 and 1230) and the eastern equatorial Pacific (sites 1225 and 1226), recovered during ODP Leg 201, harbor a relatively high number of cells with low metabolic activity (D'Hondt *et al.*, 2004). Water depths ranged between 151 and 5086 m. Sediments of the SPG were sampled during IODP Exp. 329. This mid-ocean gyre is characterized by extremely low primary production (ChlA content  $\sim 0.01\text{ mg m}^{-3}$ ) leading to a low sedimentation rate and a TOC content of  $<0.2$ – $0.003\text{ wt\%}$  in sediments below the center of the gyre (D'Hondt *et al.*, 2009). The basement ages are between  $\sim 13.5$  and 100 Ma (D'Hondt *et al.*, 2011).

### Cell counts

Cell counts for sediments from Peru margin and slope sites (ODP Leg 201, Site 1227–1231) and the equatorial Pacific (Site 1225, 1226) provided for this study, were described previously (D'Hondt *et al.*, 2004). Preparations for cell counts for Bering Sea (Site U1344) and tidal-flat sediments (Site Janssand) were done accordingly, by applying SybrGreen I for cell staining (Noble and Fuhrman, 1998; Lunau *et al.*, 2005). Enumeration of cells for sediments obtained during IODP Exp. 329 (Site U1365–1369, U1371) were done onboard the JOIDES Resolution (D'Hondt *et al.*, 2011). Owing to the low cell abundances in sediments from IODP Exp. 329, a cell extraction protocol had to be used (Kallmeyer *et al.*, 2008). Extraction efficiency was tested against non-extracted samples and was found to be 60–80%.

### Extraction of viruses

Sediment samples for the extraction and enumeration of viruses were kept frozen at  $-80^\circ\text{C}$  after sediment recovery until further analysis. The preparation for virus counting was done immediately after thawing individual samples to minimize the loss of viruses that might occur in unfrozen and unfixed samples. The extraction of viruses was performed accordingly to the protocol by Danovaro *et al.* (2001), but was adapted to minimize the filter background that interfered with the virus counting. Briefly, individual subsamples of  $1\text{ cm}^3$  were added to a  $0.02\text{-}\mu\text{m}$  pre-filtered mixture of  $3.5\text{ ml ddH}_2\text{O}$  and  $1\text{ ml sodium-pyrophosphate (55 mM)}$ . Slurries were mixed at  $2000\text{ r.p.m.}$  by vortexing until the sediment was completely suspended, but at least for 15 min. Samples were sonicated for 3 min with interruption of 30 s each minute and vortexed at  $2000\text{ r.p.m.}$  during interruptions. After centrifugation at  $1000$ – $2000\times g$  for 2–10 min, supernatants were removed and  $4\text{ ml of Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, pH 7.8, 0.02-}\mu\text{m filtered)}$  was added to the remaining sediment. Slurries were shaken for 10 min after resuspension of sediment pellets followed by centrifugation as indicated above. Supernatants of both extraction steps were pooled and filtered through  $0.45\text{ }\mu\text{m}$  polycarbonate filters.

For SPG sites U1368 and U1369, a carbonate dissolution was performed prior to virus extraction to further reduce unspecific background signals for these sediments. In addition, sediment samples of SPG sites U1366 and U1367 were used for control counts to verify that the treatment with acetate buffer does not cause a significant loss of viruses during the extraction. Therefore, instead of  $\text{ddH}_2\text{O}$ ,  $3.5\text{ ml }0.02\text{-}\mu\text{m}$  pre-filtered sodium-acetate buffer ( $0.47\text{ M}$ ,  $\text{pH }4.7$ ) was added to  $1\text{ cm}^3$  of sediment samples. The sediment was homogenized by shaking at  $2000\text{ r.p.m.}$  Subsequently, the buffered slurries were gently shaken for 15–30 min. Further extraction procedure followed the protocol described above.

### Enumeration of viruses

For enumeration of viruses, 10  $\mu$ l to 6 ml of virus extracts were filtered onto 0.02  $\mu$ m Anodisc filters (13 mm, Whatman, Maidstone, UK). Filters were washed with Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, pH 7.8). Viruses were stained by applying SybrGold or SybrGreen I (Invitrogen, Carlsbad, CA, USA). Briefly, filters were mounted on a drop of 40  $\mu$ l 2  $\times$  SybrGold solution for 30 min in the dark. Remaining staining solution was removed and filters were mounted to a microscopic slide by using 4  $\mu$ l embedding solution (VectaShield (Vector Laboratories, Peterborough, UK): glycerol (1:1), 0.1% phenyldiaminoxalat). For SybrGreen I-staining, 3.5  $\mu$ l of a mounting solution (Lunau *et al.*, 2005) containing SybrGreen I (final concentration 1  $\times$ ) was mounted directly onto the filter. Between 50 and 400 viruses were counted in 10 to 300 randomly chosen fields per sample by using an Olympus BX51 microscope (Hamburg, Germany).

### Calculation of viral community half-lives in SPG sediments

To calculate viral community half-lives, semi-logarithmic plots of virus counts against the age of

SPG sediments were used. Sediment ages were determined by assuming a linear sedimentation rate, the sediment thickness and the basement age. For each site, a power-law function (a) was used to describe the decrease in virus counts with sediment age. First-order derivations (b) were used to calculate half-lives of viral communities (c) for sediments of an approximated age of 1 and 10 Ma and at the basement age of the respective site.

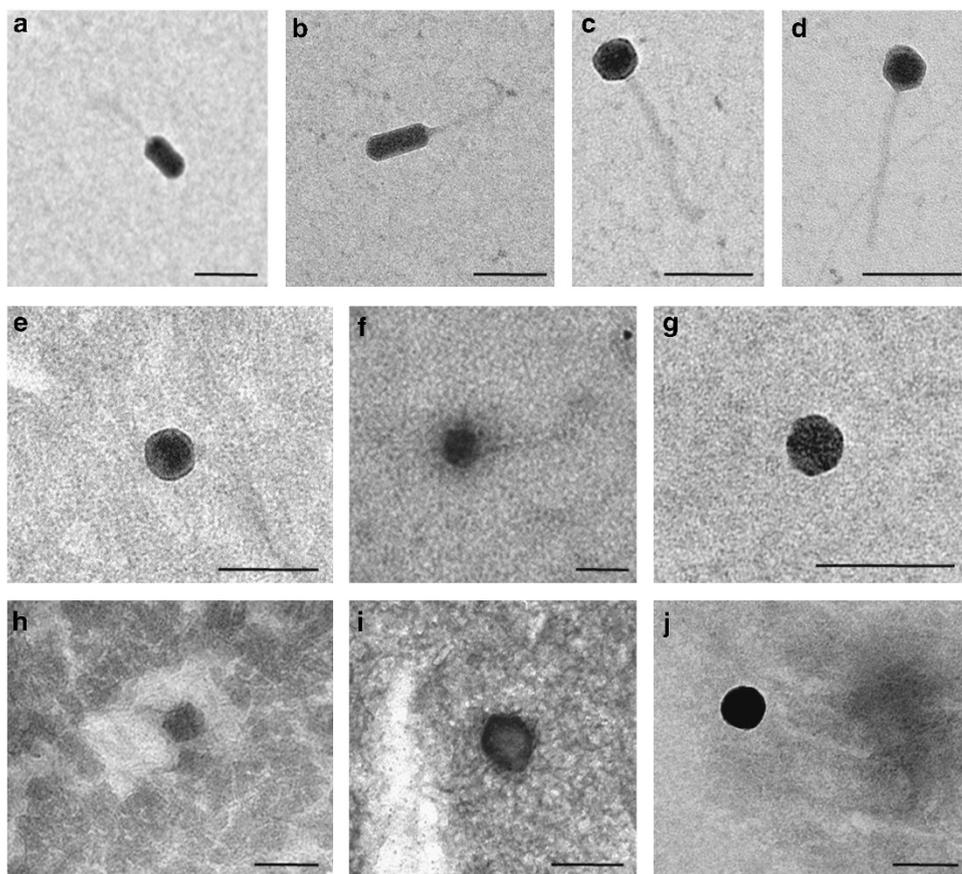
$$(a) f(x) = a * x^n$$

$$(b) f'(x) = a * n * x^{(n-1)}$$

$$(c) t_{1/2} = -\log(2)/f'(x)$$

### Transmission electron microscopy

Viruses from 4 to 6 ml of virus extracts from various sediment samples were attached to carbon-coated 200 mesh formvar copper grids (Plano, Wetzlar Germany) by centrifugation (swing-out rotor, SW-41, 90 000  $\times$  g, 90 min) Virus-like particles were examined with an EM 902 A Transmission electron microscope (Zeiss, Jena, Germany). A Proscan High Speed SSCCD camera system with iTEMfive software was used for image acquisition.



**Figure 1** Transmission electron microscopic images of bacteriophages from marine subsurface sediment. North Sea tidal-flat (Site Janssand) surface sediments (a) and 1 mbsf (b–d). SPG (IODP Exp. 329; Site U1368) 0.1 mbsf (e), 11.8 mbsf (f), 16.3 mbsf (g) and 0.1 mbsf (j). Equatorial Pacific (IODP Leg 201; Site 1225) 320 mbsf (h). Bering Sea (IODP Exp. 323; Site U1344) 288 mbsf (i). Bars, 100 nm.

## Results and discussion

### *Viruses outnumber prokaryotes in subsurface sediments*

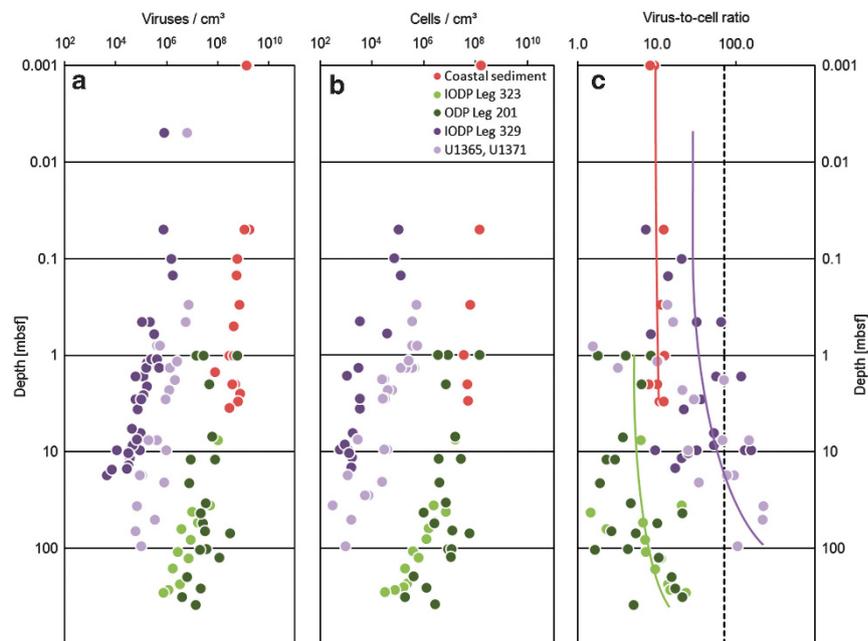
Viruses were present in all studied sediments and always outnumbered the total cell counts as examined by epifluorescence microscopy using SybrGreen I staining. Verification and the explicit identification of the particles as phages in sediments as deep as 320 mbsf and up to 14 Ma old was performed by transmission electron microscopy (Figure 1). The highest counts of cells and viruses were found in the tidal-flat sediment (Site Janssand, Figure 2), with cell numbers of  $> 10^8 \text{ cm}^{-3}$  and virus counts around  $10^9 \text{ cm}^{-3}$  for the upper 3 m, constituting a virus-to-cell ratio of about 10. In Peru margin and equatorial Pacific (ODP Leg 201, Sites 1225–1231) as well as in Bering Sea sediments (IODP Exp. 323, Site U1344), cell numbers decreased from  $10^8 \text{ cells cm}^{-3}$  in the upper sediment layers by three orders of magnitude over a depth interval of 320 mbsf. Viral counts in the upper sediment column were up to seven times higher, but decreased more slowly with depth, resulting in virus-to-cell ratios of up to 20 below 100 mbsf. Surface sediments of the central SPG (IODP Exp. 329, Sites U1366–1369) harbored only  $10^6 \text{ cells cm}^{-3}$ , and the counts strongly decreased by three to four orders of magnitude within the first meters below seafloor. At the same time, the viral counts decreased from about  $10^7 \text{ cm}^{-3}$  at the sediment surface by two to three orders of magnitude

only, resulting in increasing virus-to-cell ratios of up to 225.

### *Extraction efficiency of viruses in deep subsurface sediments*

The number of washing steps is crucial for the extraction efficiency of viruses from marine sediments (Danovaro *et al.*, 2001; Middelboe *et al.*, 2003; Siem-Jørgensen *et al.*, 2008). These studies consistently showed that the initial extraction recovers the majority of viruses. After one additional washing step, the extraction efficiency was about 60–80%. For our deep-subsurface sediments, each additional washing step increased the volume of the extract and the amount of co-extracted substances and small particles. Both resulted in a more intense background on the filters. In particular for deeper and older sediments, virus counts were close to the detection limit. A reduction of the filter background was essential to avoid viruses to be covered by particles. Accordingly, only a single washing step was conducted during the virus extraction which in turn might lead to an underestimation of virus counts. However, the virus-to-cell ratio and subsequent calculations are not effected as both, virus and cell extraction efficiencies are in the same range of 60–80%.

The additional reduction of background-staining by acidic dissolution of carbonates (Kallmeyer *et al.*, 2008) turned out to improve virus counting in



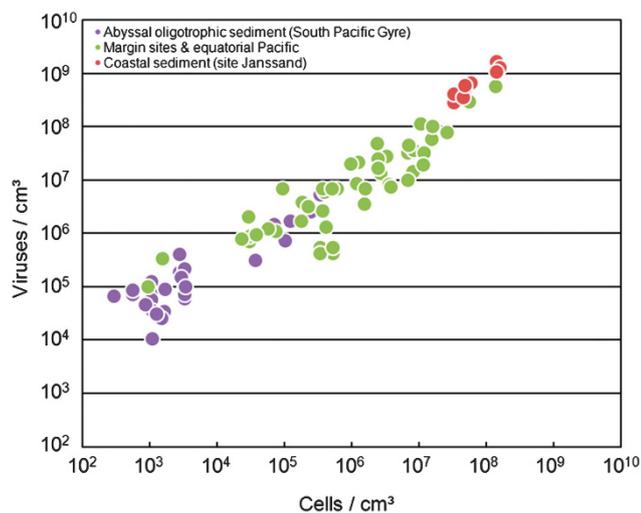
**Figure 2** Abundance of viruses and prokaryotes in marine subsurface sediments. The abundance of viruses (a) and cells (b) are shown for a German tidal-flat (Site Janssand; red), the continental margin of Peru and the equatorial Pacific (ODP Leg 201; dark green), a region of the Bering Sea with a high sedimentation rate (IODP Exp. 323; light green) and the central SPG (IODP Exp. 329; dark purple). Sites from the edge of the SPG are shown separately (Sites U1365 and U1371; light purple). (c) Virus-to-cell ratio for corresponding sediment layers. Dashed line indicates a virus-to-cell ratio of 70. Linear regression lines of virus-to-cell ratio versus sediment depth are given for the coastal area (red), continental margin areas and equatorial Pacific (green) and SPG (purple).

sediments with high carbonate content and low viral abundance. At control sites, a comparison of virus counts with and without carbonate dissolution showed no significant differences in the number of extracted viruses ( $1.56 \pm 0.71 \times 10^5$  and  $1.06 \pm 0.48 \times 10^5$ , *t*-test;  $P > 0.05$ ).

#### Ongoing viral production in subsurface sediments

Our data prove that deep-biosphere microbial communities are capable of maintaining viral communities of a respective size. Cell and viral counts correlate over seven orders of magnitude ( $r^2 = 0.941$ ,  $n = 89$ , Figure 3). At tidal flats (Beck *et al.*, 2009) and the deep seafloor, intense microbial activity supports high viral production (Danovaro *et al.*, 2008). Here, virus-to-cell ratios of about 10 throughout the upper few meters indicate a short-term balance of viral production and decay (Figure 2c) as frequently found in the water column (Weinbauer, 2004). Consistently higher virus-to-cell ratios at less active (Jørgensen and D'Hondt, 2006), deeper and old sediment layers, point toward a better preservation

of phages or a diminished viral decay. This might be caused by absorption of phage particles to the sediment matrix (Dowd *et al.*, 1998) and a reduced activity of exoenzymes (Fischer *et al.*, 2004). Semi-logarithmic plots of the viral counts against the sediment age can be fitted by a power-law function for the SPG sediments (Supplementary Figure S2). Not considering any viral production, first-order derivation was used to approximate half-lives of viral communities. At a sediment age of 1 Ma apparent half-lives of viral communities are between 0.5 and 1.6 Ma, for 10 Ma old sediments between 6.8 and 17.5 Ma and at basement age (13.5–100 Ma) (D'Hondt *et al.*, 2011) half-lives are between 13.9 and 97 Ma (Table 1). However, viral community half-lives are probably overestimated and compensated by viral production. In a recent study, Middelboe *et al.* (2011) addressed viral abundance and decay in sediments on the eastern margin of the Porcupine Seabight (IODP site U1317). The authors estimated half-lives of viral communities to be 41 000 years for the upper sediment column (4–20 mbsf) and 580 000 years for the sediments as deep as 100 mbsf. They suggest a very old viral background community to persist in these sediments. For the SPG, we estimated half-lives of viral communities to be at least 10-fold higher. As the persistence of virus particles for up to tens of millions of years is questionable, the extreme half-life times rather confirm a continuous viral production which apparently overlays viral decay.



**Figure 3** Relationship between virus and cell counts in a comprehensive set of globally distributed subsurface sediments. Regression equation for the whole data set is  $y = 271.8x^{0.768}$ ,  $R^2 = 0.941$ ,  $n = 89$ .

#### Viral biomass and the viral impact on subsurface carbon budget

After the recent recalculation of the global abundance of microbial cells in subseafloor sediments by Kallmeyer *et al.* (2012), the actual size of the deep biosphere is currently under debate (Hinrichs and Inagaki, 2012; Jørgensen, 2012; Kallmeyer *et al.*, 2012). Depending on the approach, for example, quantification by CARD-FISH, qPCR (Biddle *et al.*, 2006; Schippers *et al.*, 2005) or analysis of intact polar lipids (Lipp *et al.*, 2008), varying sizes of the bacterial and archaeal communities were determined. The quantification of

**Table 1** Apparent half-life of viral communities for sediments of distinct ages in the SPG

Site	Regression equation	Basement age (Ma)	Apparent half-life of viral communities (Ma)		
			At sediment age 1 Ma	At sediment age 10 Ma	At basement age
U1365	$y = 6.559 \times 10^{-0.0816}$ , $R^2 = 0.976$	100.0	0.56	6.79	81.90
U1366	$y = 5.144 \times 10^{-0.0421}$ , $R^2 = 0.795$	59.0	1.39	15.32	97.37
U1367	$y = 5.390 \times 10^{-0.0346}$ , $R^2 = 0.986$	33.5	1.61	17.48	61.06
U1368	$y = 5.292 \times 10^{-0.0654}$ , $R^2 = 0.941$	13.5	0.87	10.10	13.90
U1369	$y = 6.188 \times 10^{-0.0894}$ , $R^2 = 0.870$	58.0	0.54	6.69	45.37
U1371	$y = 6.251 \times 10^{-0.0431}$ , $R^2 = 0.857$	72.5	1.12	12.34	97.43

The calculation is based on virus counts versus sediment age. First-order derivations of fitted functions were used to calculate half-lives of viral communities in sediments of 1 and 10 Ma and at basement age (see Supplementary Figure S2).

viruses provides a novel and independent contribution to this debate. The high number of viruses supports the finding that cells are present and active within the deep biosphere.

Particularly in the oldest and extremely oligotrophic sediments, all quantification methods are close to their detection limits. Any variations in cell- and virus numbers, sizes and hence their carbon content would affect the virus-to-cell ratios and their biomass, respectively. In addition, the carbon content per cell encounters uncertainties that potentially change the global contribution of marine subsurface prokaryotic biomass (Hinrichs and Inagaki, 2012). However, by applying the so far most recent conversion factors of 0.2 fg per marine phage particle (Suttle, 2005) and 14 fg per prokaryotic cell (Kallmeyer *et al.*, 2012), the amount of viral carbon would exceed the cellular organic carbon at virus-to-cell ratios >70. Thus, for the extreme old and oligotrophic sediments of the SPG with a virus-to-cell ratio of up to 225, viral biomass would equal or probably even exceed the amount of prokaryotic biomass. Even with a lower virus-to-cell ratio of up to 20 (continental margin sites 1227, 1230, U1344) viral-bound organic carbon would still represent a proportion of up to 30% of the total biomass. Thus, phage particles might represent a considerable reservoir of buried biomass. If the microbial community is capable to utilize slowly decaying phage particles, for example, proteins, the pool of bioavailable carbon would substantially be changed.

We conclude that phages are highly abundant in the marine subsurface and consistently outnumber prokaryotic cells. Particularly, in older and more oligotrophic sediments, viral biomass might represent a notable amount of buried organic carbon. The relative increase of viruses with depth is likely due to viral production coupled to diminished decay rates. Active viral communities would have a strong impact on the bioavailable carbon within the marine deep subsurface.

## Conflict of Interest

The authors declare no conflict of interest.

## Acknowledgements

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## Author contributions

TE and BE developed the ideas and planned the project. TE performed the sample preparation, virus and cell counting

and data processing. JK provided additional cell counts. All authors (TE, BE, JK and HC) contributed to data interpretation and writing of the manuscript.

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