Virus decomposition provides an important contribution to benthic deep-sea ecosystem functioning

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Virus decomposition provides an important contribution to benthic deep-sea ecosystem functioning

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Abstract

Viruses are key biological agents of prokaryotic mortality in the world oceans. This applies especially to deep-sea ecosystems, where nearly all of the prokaryotic C production is transformed into organic detritus. However, the extent to which the decomposition of viral particles (i.e., organic material of viral origin) influences the functioning of benthic deep-sea ecosystems remains completely unknown. Here we show by using various and independent approaches that in deep-sea sediments an important fraction of viruses, once they are released by cell lysis, undergo fast decomposition. Virus decomposition rates in deep-sea sediments are high even at abyssal depths, and these are primarily controlled by the extracellular enzymatic activities that hydrolyze the proteins of the viral capsids. We estimate that on a global scale the decomposition of benthic viruses releases ~ 37-50 megatonnes of C per year, and thus represents an important source of labile organic compounds in deep-sea ecosystems. Organic material released from decomposed viruses is equivalent to 3 ±1%, 6 ±2% and 12 ±3% of the input of photosynthetically produced C, N and P, supplied through sinking particles to bathyal/abyssal sediments. Our data indicate that the decomposition of viruses provides an important contribution to deep-sea ecosystem functioning that has remained neglected to date, and has an important role in the nutrient cycling within the largest ecosystem of the biosphere.

Significance statement

Viruses proliferate at the expenses of their hosts. After cell death the viruses released can infect other hosts or undergo decomposition processes. Here we show, for the first time, that in deep-sea ecosystems, the largest biome of the biosphere, approximately 25% of viruses released by lysed prokaryotic cells are decomposed at fast rates. Given the huge viral biomass of the ocean seafloor and the high rates of this process, we show that virus decomposition provides a major source of labile organic compounds able to sustain the microbial food webs and nutrient cycling at global scale. These findings provide new insights, so far completely neglected, for a better comprehension of the functioning of the global oceans.

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Introduction

Viruses are key agents of prokaryotic mortality in the global oceans and by killing their hosts they play an important role in the functioning of the marine food webs and carbon and nutrient (particularly N and P) cycling (1-5).

Deep-sea ecosystems cover *ca*. 65% of the Earth surface and have key roles in biomass production and biogeochemical cycles (6, 7). They thus provide essential 'goods and services' for the entire biosphere (7, 8). Except for hydrothermal vents and cold seeps, where chemoautotrophic production represents the main engine that sustains the ecosystem functioning, life in deep-sea ecosystems is dependent upon the input of organic matter produced by photosynthesis in the surface waters of the oceans (9, 10). The inputs of organic matter that reach the ocean floor sustain the metabolism of benthic food webs, which are largely dominated by prokaryotic biomass (11, 12). Previous studies carried out in deep-sea sediments worldwide have highlighted that nearly all of the prokaryotic C production is transformed into organic detritus by viral lysis, thus representing an additional important trophic resource for the metabolism of non-infected microbes (13). These findings have provided new clues to explain why benthic deep-sea ecosystems can sustain relatively high prokaryotic biomass and turnover despite their food limitations (7, 11, 12).

After cell lysis, the viruses released can infect either other hosts or can undergo decay and/or decomposition (2, 3). The presently available information suggests that sunlight, UV radiation, temperature, salinity, and different extracellular organic compounds (such as proteases and nucleases) can promote virus decomposition in oceanic waters (14-19). Virus decomposition can have important consequences both on the composition of viral assemblages and on the flow of energy and nutrients in aquatic ecosystems (2, 20). Indeed, virus decomposition provides labile organic compounds (i.e, proteins and nucleic acids) and the associated key elements (N, P) (21). This represents an important process for sustaining microbial food webs, especially in highly oligotrophic ecosystems that are characterized by very low inputs of organic material (22), such as

the deep-sea floor. While the impact of viral infections on the functioning of benthic deep-sea ecosystems has been recently recognized (13), the extent to which virus decomposition influences the functioning and biogeochemical processes of the deep-sea floor is completely unknown.

We conducted a study over a large spatial scale to investigate virus decomposition rates in benthic deep-sea ecosystems. To do so, 111 deep-sea sediment samples have been collected and three different approaches based on virus counts during time-course experiments were applied and compared. To assess the quantitative relevance of virus decomposition processes, the amounts of organic material released *in situ* from decomposed viral particles were compared with those released by viral lysis of benthic prokaryotic hosts or supplied from the water column through sinking particles. Additional metagenomic analyses based on 454 sequencing technology of DNA from intact viral particles and extracellular DNA (i.e. genetic material not associated to living organisms and/or intact viruses) were performed on deep-sea sediments. These have been carried out to identify the viruses which, once outside their hosts, can undergo decomposition processes of the proteins of the viral capsids, thus releasing viral genomes into the extracellular DNA pool.

Results and Discussion

Our data cover latitudes from 36° N to 79° N, and depths from ca. 200 to 5,600 m (Supplementary Figure S1), and include deep-sea sites spanning over a wide range of bottom-water temperatures and trophic conditions (Supplementary Table S1), typically encountered across benthic deep-sea ecosystems of the world oceans (7). Thus our results can reflect the range of variability potentially occurring in benthic deep-sea ecosystems on a global scale.

The virus decomposition rates determined in the present study range from 0.8 to 12.8 $\times 10^{12}$ viruses m^{-2} d^{-1} (on average for the complete dataset 4.89 $\pm 0.49 \times 10^{12}$ viruses m^{-2} d^{-1}) and are high at all depths, from the shelf-break down to the abyssal sediments (Figure 1).

Previous experimental studies provided evidence that decay rates of different viruses belonging to seven families in the absence of their host cells increase exponentially with increasing temperature

(23). Here we compared the decay rates among different oceanic regions displaying different temperature regimes and found that temperature *per se* does not represent the main factor influencing the decay rates of benthic deep-sea viruses (explaining only ca. 6% of the total variance, Supplementary Table S2). Indeed, viral decay rates in surface deep-sea sediments of the Arctic and Atlantic Oceans (on average 5.26 ±0.96 $\times 10^{12}$ and 6.43 ±1.26 $\times 10^{12}$ viruses m^{-2} d^{-1}, respectively) are similar to those of the Mediterranean Sea (4.04 ±0.57 $\times 10^{12}$ viruses m^{-2} d^{-1}), whose bottom water temperature at similar depths is more than 10°C higher. These findings are not dependent upon the seasonal variability of temperatures, since benthic deep-sea ecosystems are characterized by a constant temperature over time.

The decay constants of viral particles in the surface deep-sea sediments are consistent across the different methodological approaches (Supplementary Figure S2). Such decay constants vary from 0.13 to 1.27 d^{-1} (on average 0.56 ±0.05 d^{-1}) and they fall within the range of the few data that are available from other benthic ecosystems (range, 0.08-1.67 d^{-1}) (22, 24-26). These values, however, are much higher than those reported for deep-water masses at comparable depths (range, 0.026-0.089 d^{-1}) (27), suggesting that benthic deep-sea ecosystems are characterised by fast decomposition rates of viruses once they are released from their hosts.

The high virus decomposition rates are not artefacts due to adsorption to cells and/or detrital particles (Supplementary Figure S2), or to sample manipulation. Indeed, sample manipulation (due to sediment dilution or pressure conditions) does not affect significantly our measurements (Supplementary Figure S3). Similarly, previous studies reported no significant effects due to sediment dilution or hydrostatic pressure on viral production rates and the metabolism of prokaryotic hosts (in terms of prokaryotic heterotrophic C production) (13). Recent studies suggested that the presence of non-viral particles, such as extracellular vesicles and/or cell debris, can result in overestimates of viral counts by epifluorescence microscopy (28, 29). However, the lack of significant differences between epifluorescence microscopy and TEM determinations (Supplementary Figure S4) and the absence of amplifiable cellular rDNA within the viral fraction

assessed by qPCR (see Supplementary Methods) allow us to exclude artefacts in viral counts. At the same time, data obtained through laboratory experiments need to be further validated with *in situ* measurements, once an adequate technology, which at present is not available, will be developed. Viral dynamics in aquatic ecosystems are the result of the balance between the rates of viral production and decomposition (2, 22). This applies to benthic deep-sea ecosystems where the downward flux of viruses associated to settling particles is extremely low when compared to *in situ* production of viruses (13). In the current study, a significant relationship was observed between net viral production (hereafter defined as viral production) (22, 24) and virus decomposition rates (Figure 2A). Despite the high virus decomposition rates, on average, virus decomposition accounts for a quarter of the gross (i.e. total) viral production (*sensu* 22, 24) (as the sum of the net viral production and decomposition rates; range 8-48%; Figure 2B). These data provide the first evidence that a large portion of viruses produced by lytic infection is not decomposed, and can thus subsequently infect other hosts and/or can be used as a food source by grazers such as heterotrophic protozoa and deposit-feeding organisms (30, 31). In this regard, benthic deposit feeders, which represent the dominant component of metazoan assemblages in deep-sea sediments (6), can be responsible for the removal of an important fraction of viruses produced by lytic infection (31). Previous studies have suggested that the removal of viruses in the oceans can be promoted by an interplay of abiotic (e.g., sunlight, UV radiation, temperature) and biotic (e.g., extracellular enzymes, grazing) factors (14-19). However, the factors responsible for virus decomposition in marine sediments are still largely unknown (22). Virus decomposition rates in benthic deep-sea ecosystems investigated in the present study are primarily controlled by biotic factors (i.e., prokaryotic abundance and production, and extracellular enzymatic activities, primarily of proteases) rather than by abiotic factors (Supplementary Table S3). Our findings also indicate that virus decomposition rates in the different oceanic regions investigated are significantly enhanced by high proteolytic activities in the sediment (Figure 3). These results can be explained as the consequence of the degradation of viral capsids, which are made of proteins. The importance of

proteases in virus decomposition is confirmed by additional experiments in which the addition of protease inhibitors leads to the abolishment of viral decay when compared to untreated samples (Supplementary Figure S5).

Metagenomic analyses based on 454 sequencing indicate that the genetic material contained in the viromes (i.e. associated with intact viral particles) flows to the extracellular DNA pools following viral capsid degradation (Figure 4A-B). Indeed, the viral assemblage composition in the sediments investigated is similar (for more than 75%) to the genetic characterization of viral sequences contained within the extracellular DNA pools. However, the relative contribution of sequences belonging to the viral families identified within viromes and extracellular DNA pools is quite different. This suggests that different viral families undergo decomposition at different rates as already demonstrated from experimental studies conducted on a broad variety of viral strains (23). In particular, the extracellular DNA pools contain a minor fraction of viral sequences affiliated with *Inoviridae* and *Mimiviridae*, which are more represented in the viromes. Conversely, a large fraction of viral sequences within the extracellular DNA pools is affiliated with dsDNA lytic phages of the families *Myoviridae*, *Siphoviridae* and *Podoviridae* (accounting altogether for 31- 46% of the total viral sequences), which are represented to a minor extent in the viromes. The extracellular DNA pools contain also an important fraction of sequences of ssDNA viruses belonging to *Circoviridae* which infect metazoans (contributing to 7-15% of the total viral sequences) and dsDNA viruses belonging to *Phycodnaviridae*, which infect photosynthetic organisms (contributing to 14-15% of the total viral sequences). These results indicate that most of the viruses undergoing rapid decomposition rates in surface deep-sea sediments are lytic phages of prokaryotes.

To date, estimates of virus-mediated C release have been based on the release of organic detritus (cell debris) from lysed prokaryotic cells (1, 4, 13, 20, 26, 32). In particular, global estimates from deep-sea sediments worldwide have indicated that virus-induced prokaryotic mortality can release 0.37 to 0.63 Gt C yr^{-1} (13). However, estimates of the C flux due to viruses include both the fraction of cellular detritus that is released after prokaryotic lysis and the organic material that is derived

from the decomposition of the viral particles. In the present study, we estimate that decomposed viral particles cause the release of 0.3-0.4 mg C m^{-2} d^{-1}, and that on average, the C released by virus decomposition accounts for 8 ±2% of the C released by prokaryotes killed by viruses (i.e. amount of C contained into prokaryotic biomass which is released as debris through viral lysis; details for calculations in the Supplementary Methods; Figure 5A). Assuming that annually averaged *in situ* global rates are the same as rates we determined, the decomposition of viruses in surface deep-sea sediments of the world oceans can be responsible for the release of *ca*. 37-50 megatonnes of C per year. These estimates are largely unaffected by depth-related changes since water depth explains only ca. 7% of the variance of virus decomposition rates (Supplementary Table S2). Such amounts of C released by virus decomposition would be higher if we consider that the extracellular DNA pool contains not only viral DNA sequences produced *in situ*, but also viral genes of planktonic origin (i.e. *Phycodnaviridae*) which have been not considered in these estimates.

Primary production in ocean surface waters fuels the deep-sea ecosystems through particle export from the photic zone (9, 10). However, the amount of organic resources that reach the surface of deep-sea sediments through particle sinking from the water column is typically low (1-3% of the total C produced in the photic zone) (9), and these are mostly composed of compounds resistant to degradation (33), which might represent a limiting factor for benthic metabolism (7, 10). Viruses are composed of nucleic acids surrounded by a protein coat, and so their decomposition can represent a high quality food source to sustain microbial metabolism and promote the recycling of key elements in benthic deep-sea ecosystems (i.e., N and P). We found that the quantitative relevance of C that is released by virus decomposition to organic C produced in the photic zone, which is supplied through sinking particles to the deep-sea floor (based on values of the net photosynthetic primary production, Supplementary Table S1), increases significantly with increasing water depth (Figure 5B). Using conservative values of the elemental composition of organic particles sinking on the ocean floor (C:N:P = 106:16:1, i.e. the Redfield ratio), it can be estimated that on average, at depths >1,000 m, organic material from decomposed viruses is equivalent to 3 ±1%, 6 ±2% and 12 ±3% of

the organic C, N and P fluxes, respectively (see Supplementary Materials for calculations). As the organic compounds released by the decomposition of viral particles (i.e., proteins and nucleic acids) in surface deep-sea sediments have much faster degradation rates than the bulk of the organic matter sinking on the ocean floor (11, 33), the use of labile C from decomposed viruses by benthic prokaryotes contributes to rapid N and P cycling. In this regard, a significant relationship between prokaryotic heterotrophic C production and carbon released through virus decomposition was found (Figure 6), suggesting an interplay between virus decomposition and prokaryotic metabolism. The importance of virus-derived organic matter for the metabolism of deep-sea prokaryotes is confirmed by the results of a further experiment in which the addition of purified and inactivated viruses determines a significant increase of prokaryotic growth rates (Supplementary Figure S6). Given the global relevance of biogeochemical processes that occur in deep benthic ecosystems, our data indicate that virus decomposition is an important and so far completely neglected process, which should be included in future global models of C, N and P cycling. We conclude that the virus decomposition process leads to rapid cycling of the labile organic compounds, thus providing an important contribution to the functioning and biogeochemical processes of the largest biome of the biosphere.

Methods

Study areas. Sediment samples were collected from 37 deep-sea sites in the Arctic Ocean (Hausgarten region, *ca*.120 km west of Spitsbergen, an island of the Svalbard archipelago in Norway), in two areas of the NE Atlantic Ocean (offshore of north-east Ireland and west Iberia), and in three areas of the Mediterranean Sea (the Gulf of Lyon in the NW Mediterranean, and the South Tyrrhenian and Sicily channel in the Central Mediterranean). The areas investigated included only open-ocean sites and continental-margin systems, and excluded specific hot-spot ecosystems (i.e., deep-water coral sites, cold seeps, hydrothermal vents). This allowed comparisons of similar systems covering >96% of the deep-sea surface. At all of the sampling sites, the sediments were

collected using a multiple-corer (Maxicorer; i.d., 9.0 cm; depth penetration, >20 cm). Surface sediment samples (top 1 cm) from replicate sediment cores (n = 3) were collected from independent multiple-corer deployments at each deep-sea site and used for the laboratory analyses.

Viral abundance, production and decay. Viral abundances were determined by epifluorescence microscopy after extraction of benthic viruses from the sediments and their subsequent staining with SYBR Green I (34). Comparative analyses based on transmission electron microscopy were also carried out (13). The viral production in the deep-sea sediment samples was determined by the dilution technique (i.e. a dilution-based procedure) (35, 36) through the measurement of the increase in viral abundance over time (36), which occurs in the time interval between the beginning of the experiment and 6-12 h after (Supplementary Figure S7). This technique is the most widely used in benthic environments (thus enabling a comparison with available data) and it has the advantage of minimizing the impact of protozoa and/or fauna grazing on prokaryotes during the incubations (36). For the determination of viral decay rates, three different procedures were applied and compared (Supplementary Information for details). The first procedure is based on the dilution technique through the measurement of the decrease in viral abundance over time (22, 24), which occurs after the maximum increment of viral abundance due to prokaryotic lysis (i.e. in the time interval between 6-12 h and the end of the experiment, i.e., 24 h; Supplementary Figure S7). The second procedure is based on the analysis of the decrease in viral abundance over time after the inhibition of prokaryotic metabolism, to avoid the production of new viruses (25, 37). The third procedure is based on the analysis of the decrease in viral abundance over time after the removal of cells and sediment particles, to avoid the production of new viruses and particle adsorption (15, 27). To compare the data obtained from the different procedures utilized, we determined by a first order kinetic model the decay constants of the viruses (i.e. h^{-1}) (15, 25, 27). The decay constants of the viruses were obtained from the slope coefficients of the linear regression analyses of the decrease in the viral abundance (Ln transformed) versus time (15, 25, 27). This allows us also to make a proper comparison with the decay constants of the viral particles previously reported for different pelagic

and benthic ecosystems by using the same approach. The C content of decomposed viral particles was assessed on the basis of the mean size of the viruses which was identified in deep-sea sediments (Supplementary Information for details).

Effects of experimental conditions and proteases on viral decay rates. Possible artefacts on viral decay rates due to the manipulation of the sediment were tested by parallel incubation experiments carried out on intact sediments (i.e. not diluted/homogenized) and sediments previously diluted with virus-free seawater. Samples were then incubated at *in situ* temperature in the dark and analyzed for the determination of viral abundances over time. To test for differences in the estimates of viral decay rates due to pressure conditions, time-course incubation experiments of deep-sea sediment samples were conducted at both *in situ* and atmospheric (i.e., 0.1 MPa) pressure. Finally, to assess the role of proteases on viral decay rates, additional time-course incubation experiments of deep-sea samples supplied with a cocktail of protease inhibitors were carried out (Supplementary Information for details).

Metagenomic analyses of extracellular and viral DNA. To confirm that the viruses undergo decomposition, additional sediment subsamples that were collected during time-course experiments of viral decay were analyzed to search for viral sequences within the extracellular DNA pool (38), using 454 sequencing technology (Supplementary Table S4). Additional metagenomic analyses using 454 sequencing technology were also carried out on the DNA from intact viruses recovered from deep-sea sediments (39) just before the beginning of the time-course experiments (Supplementary Table S4). Details are reported in the Supplementary Information.

Prokaryotic abundance, biomass and C production. The prokaryotic abundance in the deep-sea sediments was determined from the same sediment samples used for the viral counts. The prokaryotic cells were extracted from the sediments according to standard procedures, stained with SYBR Green I, and counted under epifluorescence microscopy (13). For the determination of the prokaryotic biomass, the cell biovolume was converted into C content assuming 310 fg C μm^{-3} as a conversion factor (13). The prokaryotic heterotrophic C production in deep-sea sediments was

determined by [^{3}H]-leucine incorporation (up to 3 h of incubation, in the dark, at *in-situ* temperature). Prokaryotic abundance and C production have been also determined during time-course incubation experiments in which purified and inactivated viruses were added to deep-sea samples. Details are reported in the Supplementary Information.

Prokaryotes killed by viruses and burst size. The number of prokaryotes killed by viruses was determined by time-course experiments and then used to calculate the burst size (as the number of viruses released by cell lysis) (13) (Supplementary Information for details).

Extracellular proteolytic activity. The extracellular proteolytic activity in the sediments was determined by the analysis of the cleavage rates of the artificial fluorogenic substrate, L-leucine-4-methylcoumarinyl-7-amide (40) (Supplementary Information for details).

Environmental and trophic characteristics. The temperature and salinity of the bottom waters at each sampling site were determined by CTD. The trophic characteristics of the benthic deep-sea ecosystems were investigated through the analysis of the total phytopigment content in the sediment (used as a proxy for the input of organic matter produced through photosynthesis in the photic zone) (41), and by estimating the inputs of organic C reaching the seafloor through particle sinking. The inputs of organic C that reaches the seafloor through particle sinking were estimated using a widely applied algorithm based on the net photosynthetic primary production which was extracted from the ocean productivity database (http://www.science.oregonstate.edu/ocean.productivity/index.php) and referred to the same sampling periods when the sediments were collected, and the water column depth (42). This model takes into consideration the degradation processes occurring to organic particles during their sinking, thus allowing to estimate the amount of organic material which actually reaches the surface of the deep-sea floor.

Statistical analyses. To determine whether the viral decomposition rates are influenced by biotic and abiotic factors, we carried out multivariate multiple regression analyses. These were done with routine distance-based multivariate analysis for a linear model (DISTLM) forward (43). *P* values were obtained with 4,999 permutations of residuals under the reduced model (44).

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Author contributions

A.D. and R.D. planned the project; A.D. and C.C. performed the experimental work; A.D., C.C. and R.D. performed the data analysis; A.D., C.C. and R.D. wrote the manuscript.

Competing financial interests

The authors have no competing financial interests.

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Figure legends

Figure 1. Bathymetric patterns of virus decomposition rates in deep-sea sediments. Analyses were carried out on surface sediment samples collected by independent multi-corer deployments at each benthic deep-sea site in the Arctic Ocean (blue circles), Atlantic Ocean (light blue squares) and Mediterranean Sea (red triangles). Data are means ±standard deviations (n = 3).

Figure 2. Virus decomposition *vs.* virus production rates (A) and contribution of virus decomposition to gross (i.e. total) viral production *vs.* water depth (B). (A) The fitted curve shows the correlation between the log transformed virus decomposition and production rates ($Y = 0.652X + 4.035$; n = 105; $R^2 = 0.495$; $P < 0.01$). Data are referred to all time-course experiments carried out on sediment samples collected by independent multi-corer deployments (n=3) at each benthic deep-sea site. (B) Data of virus decomposition relative to the gross viral production rates (as the sum of the net viral production and decomposition rates; see details in the Supplementary Information) are means ±standard deviations (n = 3).

Figure 3. Relationship between virus decomposition rate and extracellular proteolytic activity. The color-coded fitted curves show the correlations for the Arctic Ocean ($Y = 2.199X + 10.22$; n = 21; $R^2 = 0.453$; $P < 0.01$), Atlantic Ocean ($Y = 0.538X + 12.09$; n = 15; $R^2 = 0.633$; $P < 0.01$), and Mediterranean Sea ($Y = 1.722X + 9.07$; n = 15; $R^2 = 0.721$; $P < 0.01$). Data are log transformed and originate from analyses carried out on sediment samples collected by independent multi-corer deployments (n = 3) in the different benthic deep-sea sites.

Figure 4. Contribution of viral sequences to the different viral families identified within (A) the DNA pools of intact viruses (i.e. viromes) and (B) in the extracellular DNA pools of the surface deep-sea sediments collected in the Arctic and Atlantic Oceans.

Figure 5. Quantitative relevance of C released by virus decomposition. (A) Relative contributions of C released by virus decomposition to C released by killed prokaryotes according to the depth of each benthic deep-sea site. Data are means ±standard deviations (n = 3). (B) Relative contributions of C released by virus decomposition to total organic C fluxes again according to

the depth of each benthic deep-sea site. The fitted curve shows the correlation between the contribution of C released by virus decomposition to total organic C fluxes *versus* water depth ($Y = 0.001X - 0.046$; $n = 20$; $R^2 = 0.435$; $P < 0.01$).

Figure 6. Relationship between C released by virus decomposition and prokaryotic heterotrophic C production. The equation of the fitting line is: $Y = 0.045X + 0.076$ ($n = 86$; $R^2 = 0.550$; $P < 0.01$).